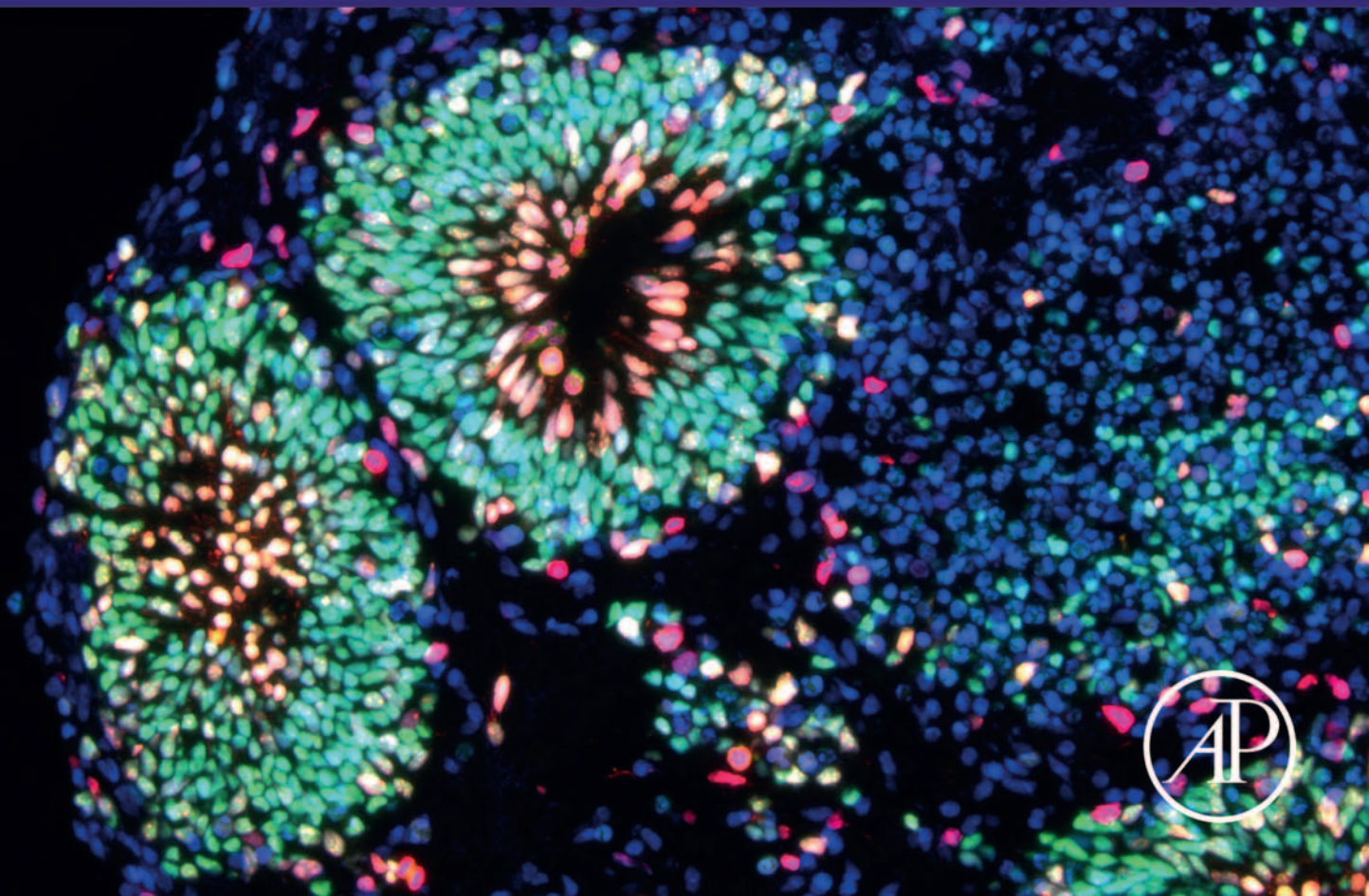
ADVANCES IN STEM CELL BIOLOGY

VOLUME 14

Current Progress in iPSC Disease Modeling

Edited by Alexander Birbrair



Current Progress in iPSC Disease Modeling, Volume 14

Advances in Stem Cell Biology

Series Editor
Alexander Birbrair

Advances in Stem Cell Biology

Current Progress in iPSC Disease Modeling, Volume 14

Edited by

Alexander Birbrair

Federal University of Minas Gerais
Department of Pathology
Belo Horizonte, Minas Gerais, Brazil

Columbia University Medical Center
Department of Radiology
New York, NY, United States

ACADEMIC PRESS

An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

Notices

Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

ISBN: 978-0-323-85765-9

Publisher: Stacy Masucci
Acquisitions Editor: Elizabeth Brown
Editorial Project Manager: Billie Jean Fernandez
Production Project Manager: Omer Mukthar
Cover Designer: Mark Rogers

Typeset by TNQ Technologies

This book is dedicated to my mother, Marina Sobolevsky, of blessed memory, who passed away during the creation of this volume. Professor of Mathematics at the State University of Ceará (UECE), she was loved by her colleagues and students, whom she inspired by her unique manner of teaching. All success in my career and personal life I owe to her.

My father Lev Birbrair and my beloved mom Marina Sobolevsky of blessed memory (July 28, 1959—June 3, 2020).

This page intentionally left blank

Contents

Contributors

Zeina R. Al Sayed
Université de Paris, INSERM, PARCC, Paris, France

Kelly Aromalaran
Masonic Medical Research Institute, Utica, NY, United States

Priti Azad
Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States

Laura Bernardini
Medical Genetics Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

Zhenyu Chen
Department of Biomedical Sciences, University of Illinois College of Medicine Rockford, Rockford, IL, United States; Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, United States

Saravanakkumar Chennappan
Masonic Medical Research Institute, Utica, NY, United States

Dennis O. Clegg
Program in Biomolecular Science and Engineering, University of California, Santa Barbara, CA, United States; Center for Stem Cell Biology and Engineering, Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA, United States

Marzia Corli
Université de Paris, INSERM, PARCC, Paris, France

Ila Dwivedi
Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States; Biomedical Sciences Graduate Program, University of California San Diego, San Diego, CA, United States

A. Gulhan Ercan-Sencicek
Masonic Medical Research Institute, Utica, NY, United States; Department of Neurosurgery, Program on Neurogenetics, Yale School of Medicine, Yale University, New Haven, CT, United States

Mohamed A. Faynus
Program in Biomolecular Science and Engineering, University of California, Santa Barbara, CA, United States; Center for Stem Cell Biology and Engineering, Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA, United States

Thomas A. Forbes
Murdoch Children's Research Institute, Parkville, VIC, Australia; Department of Paediatrics, The University of Melbourne, Parkville, VIC, Australia; Department of Nephrology, Royal Children's Hospital, Parkville, VIC, Australia

David H. Gutmann
Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States

Gabriel G. Haddad
Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States; The Rady Children's Hospital, San Diego, CA, United States

Takashi Hamazaki
Department of Pediatrics, Graduate School of Medicine, Osaka City University, Osaka, Japan

Kelly A. Hartigan
Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States

Taeka Hattori
Department of Pediatrics, Graduate School of Medicine, Osaka City University, Osaka, Japan; Donated Course of Disability Medicine and Regenerative Medicine, Graduate School of Medicine, Osaka City University, Osaka, Japan

Jean-Sébastien Hulot
Université de Paris, INSERM, PARCC, Paris, France; CIC1418 and DMU CARTE, AP-HP, Hôpital Européen Georges-Pompidou, Paris, France

Jaime Imitola
Division of Multiple Sclerosis and Translational Neuroimmunology, UConn Health Comprehensive MS Center, Department of Neurology, School of Medicine, University of Connecticut, Storrs, CT, United States

Katherine Julian
Penn State College of Medicine, Hershey, PA, United States

Maria Irene Kontaridis
Masonic Medical Research Institute, Utica, NY, United States; Department of Medicine, Division of Cardiology, Beth Israel Deaconess Medical Center, Boston, MA, United States; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, United States; Harvard Stem Cell Institute, Harvard University, Cambridge, MA, United States

Chiara Leoni
UOS Malattie Rare e Difetti Congeniti – Area Bambino, Dipartimento Scienze Salute Donna Bambino e Sanità Pubblica, Fondazione Policlinico Universitario A Gemelli – IRCCS, Rome, Italy

Xue-Jun Li
Department of Biomedical Sciences, University of Illinois College of Medicine Rockford, Rockford, IL, United States; Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, United States

Melissa H. Little
Murdoch Children's Research Institute, Parkville, VIC, Australia; Department of Paediatrics, The University of Melbourne, Parkville, VIC, Australia; Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, VIC, Australia

Nihar Masurkar
Université de Paris, INSERM, PARCC, Paris, France

Yongchao Mou
Department of Biomedical Sciences, University of Illinois College of Medicine Rockford, Rockford, IL, United States; Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, United States

Roberta Onesimo
UOS Malattie Rare e Difetti Congeniti – Area Bambino, Dipartimento Scienze Salute Donna Bambino e Sanità Pubblica, Fondazione Policlinico Universitario A Gemelli – IRCCS, Rome, Italy

Maria Pennuto
Department of Biomedical Sciences (DBS), University of Padova, Padova, Italy; Veneto Institute of Molecular Medicine (VIMM), Padova, Italy; Padova Neuroscience Center (PNC), Padova, Italy

Jessica Rosati
Cellular Reprogramming Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

Sarah V. Schurr
Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States

Haruo Shintaku
Department of Pediatrics, Graduate School of Medicine, Osaka City University, Osaka, Japan; Donated Course of Disability Medicine and Regenerative Medicine, Graduate School of Medicine, Osaka City University, Osaka, Japan

Laura Sireno
Department of Biomedical Sciences (DBS), University of Padova, Padova, Italy; Veneto Institute of Molecular Medicine (VIMM), Padova, Italy

Elisa Maria Turco
Cellular Reprogramming Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

Angelo Luigi Vescovi
Cellular Reprogramming Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy

Wei Wu
Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States

Hang Yao
Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States

Yang Yu
Clinical Stem Cell Research Center, Peking University Third Hospital, Beijing, China; Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China

Giuseppe Zampino
UOS Malattie Rare e Difetti Congeniti – Area Bambino, Dipartimento Scienze Salute Donna Bambino e Sanità Pubblica, Fondazione Policlinico Universitario A Gemelli – IRCCS, Rome, Italy

Helen Zhao
Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States

About the editor

Alexander Birbrair

Dr. Alexander Birbrair received his bachelor's biomedical degree from Santa Cruz State University in Brazil. He completed his PhD in Neuroscience, in the field of stem cell biology, at the Wake Forest School of Medicine under the mentorship of Osvaldo Delbono. Then, he joined as a postdoc in stem cell biology at Paul Frenette's laboratory at Albert Einstein School of Medicine in New York. In 2016, he was appointed faculty at Federal University of Minas Gerais in Brazil, where he started his own lab. His laboratory is interested in understanding how the cellular components of different tissues function and control disease progression. His group explores the roles of specific cell populations in the tissue microenvironment by using state-of-the-art techniques. His research is funded by the Serrapilheira Institute, CNPq, CAPES, and FAPEMIG. In 2018, Alexander was elected affiliate member of the Brazilian Academy of Sciences, in 2019, he was elected member of the Global Young Academy (GYA), and in 2021 he was elected affiliate member of The World Academy of Sciences (TWAS). He is the Founding Editor and Editor-in-Chief of *Current Tissue Microenvironment Reports*, and Associate Editor of *Molecular Biotechnology.* Alexander also serves in the editorial board of several other international journals: *Stem Cell Reviews and Reports*, *Stem Cell Research*, *Stem Cells and Development*, and *Histology and Histopathology.*

This page intentionally left blank

Preface

This book's initial title was "iPSCs: Recent Advances." Nevertheless, because of the ongoing strong interest in this theme, we were capable to collect more chapters than would fit in one single volume, covering induced pluripotent stem cells (iPSCs) biology from different perspectives. Therefore, the book was subdivided into several volumes.

This volume "*Current Progress in iPSC Disease Modeling*" offers contributions by known scientists and clinicians in the multidisciplinary areas of biological and medical research. The chapters bring up-to-date comprehensive overviews of current advances in the field. This book describes the use of induced pluripotent stem cells to model several diseases in vitro, enabling us to study the cellular and molecular mechanisms involved in different pathologies. Further insights into these mechanisms will have important implications for our understanding of disease appearance, development, and progression. The authors focus on the modern state-of-art methodologies and the leading-edge concepts in the field of stem cell biology. In recent years, remarkable progress has been made in the obtention of induced pluripotent stem cells and their differentiation into several cell types, tissues, and organs using state-of-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of several disorders. Thus, the present book is an attempt to describe the most recent developments in the area of iPSCs biology which is one of the rising hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the use of iPSCs for modeling multiple diseases. Eleven chapters written by experts in the field summarize the present knowledge about iPSC Disease Modeling.

David H. Gutmann and colleagues from the Washington University School of Medicine discuss iPSCs for modeling Neurofibromatosis Type 1. Katherine Julian and Jaime Imitola from the University of Connecticut School of Medicine describe iPSCs for modeling Multiple Sclerosis. Huiwen Zhao and Gabriel Haddad from the University of California San Diego compile our understanding of iPSCs for modeling Chronic Mountain Sickness. Maria Irene Kontaridis and colleagues from Masonic Medical Research Institute update us with what we know about iPSCs for modeling Noonan and Costello syndromes. Taeka Hattori and colleagues from Osaka City University Graduate School of Medicine summarize current knowledge on modeling MELAS/LEIGH overlap syndrome using iPSCs. Yang Yu from Peking University Third Hospital addresses the importance of iPSCs for modeling Polycystic Ovary Syndrome. Mohamed A. Faynus and Dennis O. Clegg from the University of California talk about the modeling of Inherited Retinal Dystrophies using iPSCs. Xue-Jun Li and colleagues from the University of Illinois at Chicago present the modeling of Hereditary Spastic Paraplegias using iPSCs. Angelo Luigi Vescovi and colleagues from the University of Padova give an overview of iPSCs for modeling Smith−Magenis syndrome. Jean-Sébastien Hulot and colleagues

from INSERM introduce what we know so far about iPSCs for modeling of Cardiac Arrhythmias. Finally, Thomas A Forbes and Melissa H Little from the University of Melbourne focus on iPSCs for modeling heritable kidney disease.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife Veranika Ushakova, and Ms. Billie Jean Fernandez and Ms. Elisabeth Brown from Elsevier, who helped at every step of the execution of this project.

Alexander Birbrair
Editor

CHAPTER

1 Human induced pluripotent stem cell modeling of neurofibromatosis type 1

Sarah V. Schurr*, **Kelly A. Hartigan***, **David H. Gutmann**

Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States

Chapter outline

Abstract

Neurofibromatosis type 1 (NF1) is a common monogenic disorder caused by germline mutations in a single copy of the *NF1* gene. While NF1 is inherited in an autosomal dominant fashion and is completely penetrant, patients heterogeneously exhibit a wide variety of clinical features, including neurodevelopmental delays and cognitive deficits, central and peripheral nervous system tumors, and musculoskeletal and pigmentary abnormalities. Although the genetic basis for NF1 is well

* These two authors contributed equally to this work.

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00008-4

established, the etiologies underlying many of the associated clinical features are not well understood. Moreover, the ability to provide accurate risk assessments is currently limited, and few preclinical models fully recapitulate their human counterparts. With the advent of human induced pluripotent stem cell (hiPSC) technology, it now becomes possible to develop humanized NF1 models to gain greater insights into the pathobiology of NF1, identify and evaluate new therapeutic agents, and discover potential risk factors. In this review, we describe the current state of NF1 hiPSC research and outline potential future directions.

Keywords: Astrocytes; Cancer; Human induced pluripotent stem cells; Microglia; Neurofibromatosis; Neurons; Organoids; Ras; Stem cells.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder affecting approximately 1 in 3000 individuals worldwide, caused by a mutation in a single copy of the *NF1* gene (Uusitalo et al., 2015). While 50% of children with NF1 have a parent with NF1, the *NF1* gene is subject to a remarkably high rate of mutation, such that 50% of NF1 cases arise from sporadic de novo mutations (Friedman, 1999). Additionally, despite being a monogenic disorder, NF1 is characterized by striking clinical heterogeneity, where affected individuals present with a wide variety of clinical features, mainly involving the nervous system (Fig. 1.1). In this regard, individuals with NF1 are at an increased risk for central and peripheral nervous system (CNS and PNS, respectively) tumors, most commonly low-grade gliomas (LGGs) of the optic pathway (optic pathway glioma—OPG) or brainstem (brainstem gliomas), and cutaneous and plexiform neurofibromas (cNF and pNF, respectively) arising in association with peripheral nerves (Gutmann et al., 2017). Other common clinical manifestations include pigmentary and musculoskeletal abnormalities. In addition, there are also higher rates of neurodevelopmental abnormalities, such as motor delays, attention deficit hyperactivity disorder, and autism spectrum disorder (ASD). Similarly, approximately 60%–80% of children with NF1 exhibit deficits in executive function, attention, memory, and visuospatial and visuoperceptual abilities (Hyman et al., 2005).

NF1 gene

Discovered in 1990, the *NF1* gene resides on chromosome 17q11.2, where it codes for the protein neurofibromin (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990; Collins et al., 1989; Gutmann et al., 1991; DeClue et al., 1991). Neurofibromin is a large molecule with three known isoforms, which arise from the alternative splicing of exons 11alt12, 30alt31, and 56alt57 (previously exons 9a, 23a, and 48a, respectively) (Danglot et al., 1995; Andersen et al., 1993; Cawthon et al., 1990; Anastasaki et al., 2017). Although neurofibromin is expressed in many tissues, isoform levels of the protein vary considerably by tissue and cell type, with the 11alt12 isoform being largely restricted to brain neurons and the 56alt57 isoform highly expressed in muscle (Gutmann et al, 1993, 1999).

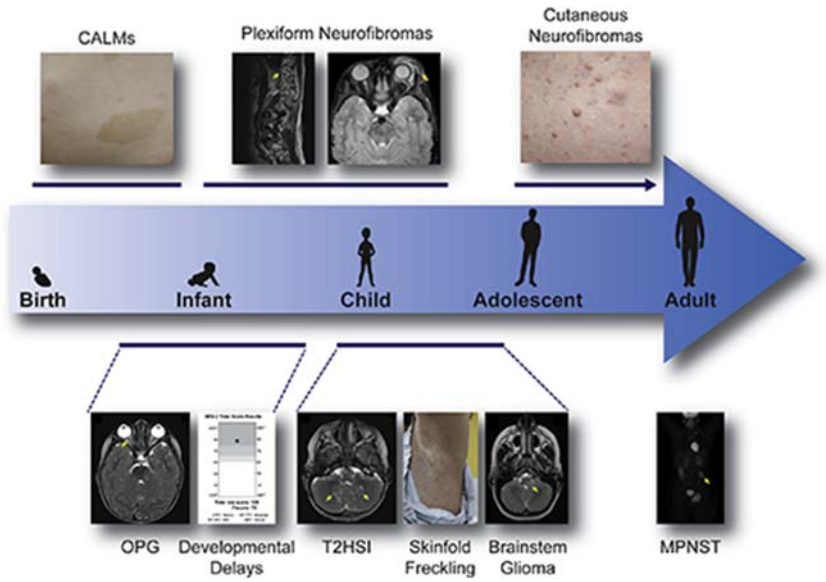


FIGURE 1.1 NF1 clinical features.

An overview of NF1 clinical features, with the typical ages of initial development. Yellow arrows denote noted pathology. *CALMs*, café-au-lait macules; *OPG*, optic pathway glioma; *T2HSI*, T2 high signal intensity lesions; *MPNST*, malignant peripheral nerve sheath tumor.

Most studies of neurofibromin function have focused on its ability to negatively regulate the Ras proto-oncogene (Ballester et al., 1990; Xu et al., 1990; DeClue et al., 1992; Basu et al., 1992) (Fig. 1.2). As a GTPase-activating protein (GAP), neurofibromin facilitates GTP hydrolysis, accelerating the conversion of Ras from its active, GTP-bound state to its inactive, GDP-bound state. The domain responsible for this catalytic activity is termed the GAP-related domain (GRD). As a negative Ras regulator, neurofibromin reduces signaling of Ras downstream effector molecules, including the Raf-MEK-ERK/MAPK and PI3K-AKT-mTOR pathways, to control cell proliferation and survival (Lau et al., 2000; Dasgupta et al., 2005; Johannessen et al., 2005). In addition, Ras-mediated regulation of cyclic AMP, important for axonal outgrowth, operates through atypical Protein Kinase C zeta signaling, likely by altering G protein-coupled receptor signaling (Anastasaki and Gutmann, 2014).

While the NF1-GRD has been well characterized, this domain comprises only 10% of the entire neurofibromin coding sequence, raising questions about the function of the rest of the protein. Additional domains of neurofibromin have been described (Fig. 1.2), including a cysteine-serine-rich domain (Tokuo et al., 2001), tubulin-binding domain (Bollag et al., 1993), syndecan-binding domains (Hsueh et al., 2001), Sec14 domain and pleckstrin homology domain (Welti et al., 2007),

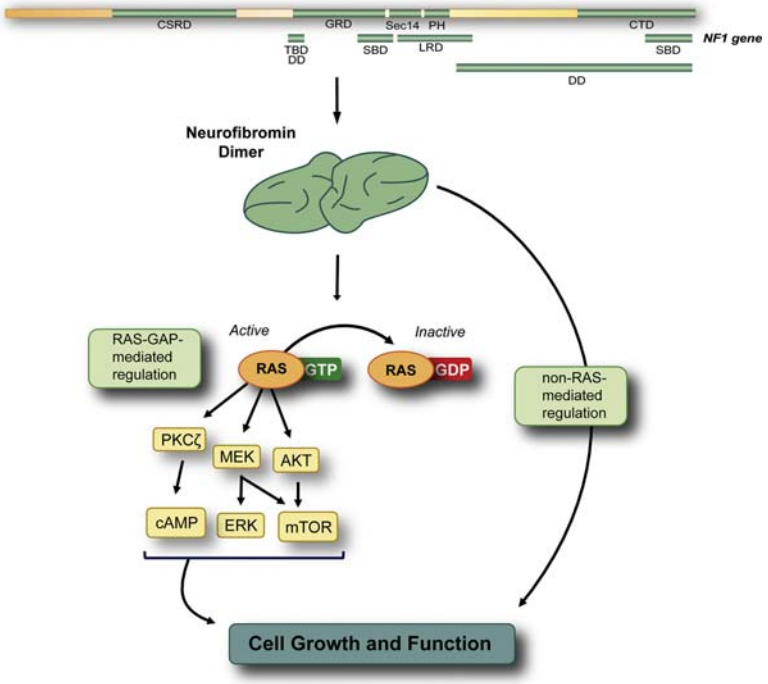


FIGURE 1.2 Neurofibromin signaling pathway.

The *NF1* gene codes for the protein neurofibromin with multiple annotated domains: a cysteine-serine-rich domain (CSRD), a tubulin-binding domain (TBD), a GTPase-activating protein-related domain (GRD), two syndecan-binding domains (SBD), a Sec14 domain and pleckstrin homology domain (Seck14/PH), and the carboxy-terminal domain (CTD). In addition, two regions believed to be associated with dimerization of neurofibromin are shown (Dimerization domain; DD). Neurofibromin regulates cell growth and function through several downstream signaling effectors, mainly by accelerating the conversion of active GTP-bound Ras to its inactive GDP-bound form. The presence of additional functional domains in the *NF1* gene suggests other non-Ras-mediated functions of neurofibromin.

leucine repeat domain (Izawa et al., 1996), and the carboxy-terminal domain (Tokuo et al., 2001) There have also been a number of reported potential neurofibromin-interacting proteins (Bollag et al., 1993; Patrakitkomjorn et al., 2008; Kweh et al., 2009; Wang et al., 2011). In addition, neurofibromin appears to function as a dimer, and requires localization to the plasma membrane. Dimerization is mediated by residues flanking the NF1-GRD (Sherekar et al., 2020), while membrane targeting is partially regulated by binding to the Spred1 protein (Stowe et al., 2012; Dunzendorfer-Matt et al., 2016).

Genotype-phenotype correlations

Whereas all mutations in the *NF1* gene are thought to impair neurofibromin function, studies from numerous groups have begun to reveal some degree of *NF1* mutational specificity. Most notably, individuals with c.2970-2972delAAT, c.5425C>T, and c.3112A>G germline *NF1* gene mutations do not develop cNFs or apparent pNFs (Upadhyaya et al., 2007; Pinna et al., 2015; Santoro et al., 2015; Rojnueangnit et al., 2015; Trevisson et al., 2019). Similarly, large genomic deletions, termed microdeletions, in which the *NF1* gene and a number of surrounding genes are deleted, are seen in individuals with NF1 exhibiting facial dysmorphism, marked cognitive delays, and an increased tumor burden (Pasmant et al., 2010; Kehrer-Sawatzki et al., 2017). Additional studies have identified correlations between the location of the *NF1* gene mutation and presentation of clinical features. Patients presenting with OPGs are more likely to harbor 5′ end *NF1* mutations (Sharif et al., 2011; Bolcekova et al., 2014; Anastasaki et al., 2017), while those with 3'end mutations have greater autism trait burden (Morris and Gutmann, 2018).

Roadblocks to treatment

Over the past three decades since the discovery of the *NF1* gene, there has been remarkable progress in understanding the etiologies for many NF1 clinical features. While promising treatments have been identified, the highly heterogeneous nature of this disorder continues to impose substantial barriers to improved care for affected individuals. In this regard, it is not currently possible to predict which clinical manifestations will arise in any given person or identify which promising therapy might be most effective for any particular individual.

Importantly, the development of accurate humanized models of NF1-related medical features remains a challenge. Generation of human LGG or neurofibroma patient xenograft tumor models has proven difficult, and the paucity of brain specimens from individuals with NF1 has limited our ability to understand the basis for cognitive and behavioral deficits in children. These limitations have resulted in a heavy reliance on mouse models of NF1 or the use of large animals, like minipigs (White et al., 2018; Isakson et al., 2018; Khanna et al., 2019; Uthoff et al., 2020), to define the molecular and cellular pathogenesis of NF1-related features, as well as to discover and evaluate potential therapies.

Nf1 genetically engineered mouse models

Mouse models have proven highly instructive for studying clinical phenotypes observed in NF1 patients. For example, *Nf1*+/− mice harboring only one functional *Nf1* allele have been used to study learning and memory (Silva et al., 1997), impulsivity (Lukkes et al., 2020), and autistic-like behaviors (Molosh et al., 2014). These studies have uncovered links between learning deficits and aberrant Ras signaling

(Costa et al., 2002), and have implicated increases in ERK/MAPK, a downstream effector of Ras, in some social behavioral deficits (Molosh et al., 2014). Furthermore, *Nf1*+/− mice with Cre-driven *Nf1* loss have also identified alterations in dopamine signaling as a contributor to attention and learning and memory deficits (Brown et al., 2010; Diggs-Andrews et al., 2013).

In addition, many research teams have generated *Nf1* knockout models of NF1-associated tumors, including OPGs (Bajenaru et al., 2003, 2005; Zhu et al., 2005), cNFs and pNFs (Le et al., 2009; Chen et al., 2019; Zhu et al., 2002; Wu et al., 2008), and malignant peripheral nerve sheath tumors (MPNSTs) (Cichowski et al., 1999; Wu et al., 2014; Hirbe et al., 2016). These models have been useful for identifying the cellular and molecular factors influencing tumor formation and progression, such as the role of the tumor microenvironment in OPGs (Daginakatte and Gutmann, 2007; Simmons et al., 2011; Pong et al., 2013; Solga et al., 2015; Guo et al., 2019, 2020) and pNFs (Yang et al., 2008; Liao et al., 2018). Moreover, these *Nf1* preclinical models were critical for the identification and evaluation of potential drug therapies including inhibitors of MEK (Jessen et al., 2013; Kaul et al., 2015), PI3K (Kaul et al., 2015), mTOR (Hegedus et al., 2008), and tyrosine kinases (Yang et al., 2008). These preclinical findings have helped lead to a number of clinical trials, including MEK inhibitor Selumetinib (NCT01362803) (Dombi et al., 2016; Gross et al., 2020) (NCT03871257), mTOR inhibitor Everolimus (NCT01158651), and tyrosine kinase inhibitor Imatinib mesylate (NCT01673009) (Robertson et al., 2012).

While *Nf1* genetically engineered mouse (GEM) have provided important insights relevant to disease pathobiology and clinical treatment, there are inherent differences between the murine and human nervous systems, which may impact on the clinical translation of preclinical findings. On a basic level, the murine brain is lissencephalic with a smooth outer surface, while the human brain contains intricate and extensive folding (gyrencephalic) (Sun and Hevner, 2014). Additionally, astrocytes and microglia, two CNS cell types implicated in NF1 pathogenesis, exhibit notable species-specific differences. Humans harbor astrocyte subtypes not seen in mice, and for subtypes present in both species, the human version is often characterized as larger and more complex (Oberheim et al., 2009). Furthermore, differential expression of a large number of genes in human astrocytes relative to their rodent counterparts has been reported (Zhang et al., 2016). Likewise, transcriptomic differences between human and rodent microglia have been reported, and human and rodent microglia have been shown to exhibit differential responses to certain drugs and stimuli in vitro (Hasselmann and Blurton-Jones, 2020; Smith and Dragunow, 2014). Finally, comparisons of gene expression profiles of neuronal and nonneuronal murine and human cells have revealed key differences in the expression of genes implicated in neuronal signaling (Hodge et al., 2019). For all of these reasons, it is important to complement rodent-based studies with human cellular and tissue modeling approaches.

Utility of human induced pluripotent stem cells for NF1 research

Human induced pluripotent stem cells (hiPSCs) offer a tractable experimental platform, as lines can be established directly from patient tissues, enabling the study of mutations in the context of their native genomes. In addition, the CRISPR/Cas9 genome editing system can be utilized to engineer specific mutations onto a uniform genomic background, allowing for the isolation of mutational effects (Israel et al., 2012; Paquet et al., 2016; Rehbach et al., 2020). Furthermore, the ability of hiPSCs to differentiate into nearly any cell type creates a powerful tool to study effects of *NF1* mutation on neurodevelopment, as well as to develop humanized tumor models (Fig. 1.3).

To this end, hiPSCs have been useful for modeling multiple complex neurogenic disorders, such as ASD (Deshpande et al., 2017; Gouder et al., 2019), Down syndrome (Ponroy Bally et al., 2020), microcephaly (Lancaster et al., 2013), and psychiatric disorders (schizophrenia and bipolar disorder) (Brennand et al., 2011;

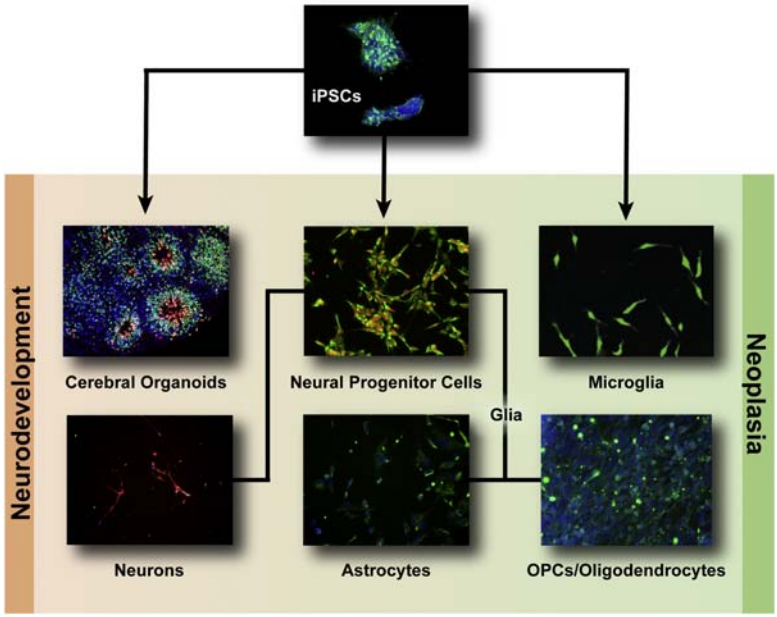


FIGURE 1.3 Human induced pluripotent stem cell differentiation and modeling.

Human induced pluripotent stem cells (hiPSCs) can be differentiated into many different cell types that contribute to NF1 pathobiology. In this respect, hiPSCs can be differentiated into microglia, neural progenitor cells, as well as mature astrocytes, oligodendrocytes, and neurons, as both two-dimensional and multicellular three-dimensional models (cerebral organoids). These cell types can be used to study both neurodevelopmental defects and neoplasia.

Mertens et al., 2015; Robicsek et al., 2013). In this review, we focus on the utility of hiPSC modeling to better understand the cellular and molecular etiologies underlying the pathogenesis of NF1-related clinical features.

Brain development and function

As healthy CNS cells are rarely available from individuals with NF1, the advent of hiPSCs and related technology provides unparalleled opportunities to study the impact of *NF1* gene mutations on human neurodevelopment.

Mutational specificity

Based on epidemiologic [population-based; (Trevisson et al., 2019; Morris and Gutmann, 2018; Anastasaki et al., 2017)] and mouse modeling studies (Guo et al., 2019; Li et al., 2016; Toonen et al., 2016), the specific germline *NF1* gene mutation may partially contribute to the diverse spectrum of cognitive deficits observed in children with NF1. With the advent of hiPSC technology, it is now possible to control for important variables, such as environmental factors and additional genomic variants, and evaluate the importance of different germline *NF1* gene mutations at the cellular and tissue levels. In this regard, using two-dimensional (2D) cultures of hiPSC-derived neuroglial cell populations, differences in the level of neurofibromin protein expression were detected, with some *NF1* mutations leading to minor reductions ($<25\%$) in neurofibromin expression and others with $>70\%$ reductions (Anastasaki et al., 2015). Similarly, these differences in neurofibromin were paralleled by similar differential reductions in dopamine levels. To control for genomic variation and sex, a series of germline *NF1* gene mutations were CRISPR/Cas9 engineered into a common hiPSC line, revealing similar differences in neural progenitor dopamine levels, as well as defects in cerebral organoid neuronal differentiation and apoptosis (Anastasaki et al., 2020).

Mutational effects can also be studied using hiPSC lines derived directly from patient samples, allowing for the investigation of mutation interactions and large-scale genome changes not easily engineered with current technology. This technique has been used to examine complex neurogenic diseases, such as ASD (Gouder et al., 2019) and Alzheimer's disease (Israel et al., 2012), and has been shown to be instructive for interrogating the cellular and tissue defects associated with 17q11.2 microdeletions (*NF1* total gene deletions) (Wegscheid et al., 2021).

Cell-autonomous effects

To date, there is a paucity of information available on the impact of *NF1* gene mutation on neuron function, which could be explored using a variety of different differentiation protocols (Engle et al., 2018; Pang et al., 2011; Yoo et al., 2011; Zhang et al., 2013). This is particularly important for understanding how *NF1* mutations

regulate neuron biology, including neurogenesis, synaptic plasticity, and response to injury. Moreover, it is not clear whether deregulated Ras signaling underlies all of the neuronal abnormalities reported to date. In this regard, all *NF1* mutations result in increased Ras activation, whereas differential effects on dopamine levels have been observed (Anastasaki and Gutmann, 2014). Similarly, *NF1* mutations differentially disrupt neurogenesis in cerebral organoids, but have near-identical levels of increased Ras activity (Anastasaki et al., 2020). Future use of 2D and three-dimensional (3D) models may elucidate the mechanisms underlying these differential effects, as well as define the role of the neuron-specific neurofibromin exon 11alt12 [previously exon 9a; (Anastasaki et al., 2017)] in brain function.

In addition to neurons, other cells in the brain, such as astrocytes, oligodendrocytes, and microglia, influence neuronal growth, differentiation, and maturation. As such, hiPSC-derived astrocytes harboring *NF1* mutations exhibit increased proliferation, both in 2D culture and in 3D cerebral organoids (Anastasaki et al., 2020). While not yet explored in human cells, mouse microglia with *Nf1* mutations exhibit increased proliferation and migration, whereas *Nf1*-mutant oligodendrocytes have aberrant myelination (Daginakatte and Gutmann, 2007; Mayes et al., 2013). Given the marked differences between rodent and human brain cells, hiPSCs could be harnessed for these analyses using a variety of methods (Abud et al., 2017; Goldman and Kuypers, 2015).

Modeling brain development and function

Intercellular interactions are integral to the regulation of cell growth, migration, and differentiation throughout neurodevelopment. While 2D cell cultures provide many insights into NF1 pathobiology, the advent of 3D models of brain development, termed cerebral organoids, allows for the investigation of neurodevelopmental defects in a relevant tissue context. Multiple protocols for establishing cerebral organoids specific to different brain regions have been developed, and each remarkably recapitulates aspects of early human neurodevelopment. At the transcriptome level, hiPSC-derived telencephalic organoid gene expression maps closely to the fetal neocortex from 8 to 16 weeks postconception, with approximately 80% of genes implicated in neocortex disease or evolution exhibiting similar expression patterns (Amiri et al., 2018; Camp et al., 2015). In addition, cerebral organoids faithfully mimic human ventricular zone organization and neural stem cell migration (Klaus et al., 2019; Li et al., 2017; Zhang et al., 2013). In this respect, defects in neuron migration have been implicated in the pathology of many neurodevelopmental disorders (Pan et al., 2019).

In addition to more accurately modeling human brain development, cerebral organoids form functional neural networks. By eight months in culture, brain organoids show spontaneous action potentials, as well as coordinated bursting activity, indicative of neural network formation (Quadrato et al., 2017). In addition, organoids representing different brain regions have been integrated into “assembloids” to construct cross-regional neural circuity (Bagley et al., 2017; Birey et al., 2017;

Xiang et al., 2017). Differences in neural circuit formation and functional connectivity between brain regions have been reported in many cognitive and developmental disorders, including NF1 (Apostolova et al., 2015; Baudou et al., 2019; Ibrahim et al., 2017). Whereas organoids may only model the most basic elements of neural circuity, they represent one method for studying human brain connectivity in vitro.

While serving as useful tools for studying early brain development, organoids lack vascularization and endocrine hormones, and often do not incorporate microglia. Engraftment of hiPSC-derived neurons and organoids into the murine brain can restore some of these missing interactions. As such, transplantation of hiPSC-derived neurons and organoids can partially restore neurologic function in murine models of stroke, Parkinson's disease, and Down syndrome (Maria et al., 2013; Xu et al., 2019; Yuan et al., 2013), where they continue to mature and become vascularized (Daviaud et al., 2018; Mansour et al., 2018).

Nervous system tumors

In addition to an increased incidence of neurodevelopmental disorders, an elevated risk of CNS and PNS tumor development is a defining feature of NF1. NF1 patients commonly develop LGGs, which arise primarily in the optic pathway and brainstem, as well as cNFs and pNFs which arise in association with peripheral nerves. While pNFs are benign tumors, they can undergo malignant transformation into MPNSTs, a cancer with a poor five-year overall survival rate (Kolberg et al., 2013). The ability to model these tumors is paramount to understanding and treating them effectively. Historically, preclinical modeling has been conducted in mice due to the limited lifespan and lack of availability of primary cell lines from NF1 patient biopsies. However, the advent of hiPSC technology makes the development of humanized tumor models possible.

Tumor modeling

Since it has been difficult to establish low-grade NF1 tumor models using patient-derived xenografts, hiPSCs could be employed to develop surrogate models. In addition to potentially establishing complementary preclinical platforms for drug discovery and testing, hiPSCs are ideally suited to define the cells of origin for NF1-associated nervous system tumors, where neural progenitor cells (NPCs), oligodendrocyte precursor cells (OPCs), and astrocytes have all been implicated in murine OPG development (Hegedus et al., 2007; Lee et al., 2012; Solga et al., 2014, 2017). Similarly, Schwann cell precursors (SCPs) have been identified as the potential cell of origin for neurofibromas (Zhu et al., 2002; Le et al., 2009; Chen et al., 2014, 2019). Using specific differentiation protocols, each of these cell types can be generated and examined [**NPCs** (Cheng et al., 2017), **OPCs** (Wang et al., 2013; Douvaras and Fossati, 2015), **astrocytes**

(Chandrasekaran et al., 2016), **SCPs** (Kim et al., 2017; Sugiyama-Nakagiri et al., 2016)] (Fig. 1.3). Moreover, other NF1 tumor-associated genomic alterations (e.g., *CDKN2A* loss, *TP53* mutation) could be engineered, in addition to *NF1* loss, to study tumor progression and malignant transformation.

Use of this technology has already been employed to establish hiPSC-based neurofibroma models. *NF1*-deficient hiPSCs derived from primary pNF patient cell lines and subsequently differentiated into stem cells (SCs) have been shown to form sphere-like structures mimicking pNF expression patterns of SC markers and exhibiting increased proliferation and loss of myelination capacity (Carrió et al., 2019). Work in several laboratories is underway to generate hiPSC models of NF1-associated neurofibromas and MPNSTs (Mo et al., 2021).

While hiPSC models of NF1-related brain tumors have not been reported to date, stem cell–based 3D cerebral organoid models of high-grade brain tumors have been described, including glioblastoma (GBM) (Ogawa et al., 2018; Bian et al., 2018) and medulloblastoma (Ballabio et al., 2020). These models faithfully replicate many of the key characteristics of GBM and medulloblastoma in vitro, as well as in vivo following implantation into immunodeficient mice, highlighting the feasibility of hiPSC-derived brain tumor models. Several laboratories, including our own, are also working to establish NF1-related LGG models from hiPSCs.

Tumor microenvironment

The promise and potential of hiPSCs lies not only in their ability to model tumors themselves but also in their capacity to recreate aspects of the tumor microenvironment, a crucial factor contributing to tumor pathogenesis. Neurons have received increasing recognition as key microenvironmental components in a broad range of tumors (Monje et al., 2020), including those relevant to NF1. The importance of nerves in pNF formation has been demonstrated in mouse models, where pNFs formed following implantation of *Nf1*-deficient SKPs into the sciatic nerve, but failed to form from intradermal/subcutaneous implantation (Le et al., 2009; Liao et al., 2016). The availability of numerous differentiation protocols for hiPSC-derived PNS neurons could prove useful in corroborating these findings (Guimarães et al., 2018; Muller et al., 2018). Furthermore, hiPSCs can also be used to probe the importance of neurons in NF1-related CNS tumors (Pan et al., 2021). Recently, hiPSC-derived CNS neurons CRISPR/Cas9 engineered to express *NF1* mutations were used to elucidate a mechanism by which neurons promote OPG growth through communication with immune cell intermediates (Guo et al., 2020).

In addition to neurons, microglia have been recognized as key microenvironmental players in NF1-related OPGs. Numerous studies in *Nf1* GEM and *Nf1* GEM-derived cell cultures have demonstrated the importance of microglia to OPG pathobiology, with recent work focusing on their role as one of the immune cell intermediates facilitating transmission of tumor supportive signaling from neurons to tumor cells (Daginakatte and Gutmann, 2007; Simmons et al., 2011; Pong et al., 2013; Solga et al., 2015; Guo et al., 2019, 2020). Although functionally

similar, murine and human microglia have important differences in transcriptomic profiles and in vitro responses to stimuli, suggesting that the impact of *NF1* mutation on microglia function needs to be studied in human cells (Smith and Dragunow, 2014; Hasselmann and Blurton-Jones, 2020). Using numerous optimized methods, it is now possible to examine hiPSC microglia function in 2D cultures, as components of cerebral organoids, and following transplantation into mice (Hasselmann and Blurton-Jones, 2020).

Similarly, hiPSC platforms can be utilized to model mast cells, a key cell type in the neurofibroma microenvironment. Mast cell infiltration is commonly observed in neurofibromas and is recapitulated nicely in mouse models (Zhu et al., 2002; Le et al., 2009; Chen et al., 2019). In vitro and in vivo studies have identified the mechanisms underlying mast cell recruitment to the tumor site (Yang et al., 2003) and have detailed mast cell interactions with other cell types implicated in neurofibromas (Yang et al., 2006). Furthermore, transplantation of *Nf1* heterozygous bone marrow−derived cells, likely representing a population of mast cells, has been shown to induce pNF formation in irradiated mice harboring *Nf1*-deficient Schwann cells (Yang et al., 2008). As pNFs have recently been shown to form in mice following experimental reduction in mast cell infiltration, further work is necessary to precisely detail their role in the pathogenesis of these peripheral nerve sheath tumors (Liao et al., 2018). Several hiPSC differentiation protocols for mast cells have been established (Igarashi et al., 2018; Kauts et al., 2018; Ikuno et al., 2019), and can be used as a humanized system to further studies of mast cells.

Other NF1 clinical features

In addition to neurodevelopmental delays and an increased tumor burden, patients with NF1 can present with a variety of other clinical features, including vision loss, peripheral nerve pain, and pigmentary and musculoskeletal abnormalities. As described above, hiPSC modeling may prove invaluable for further elucidating the mechanisms underlying NF1 pathology.

Vision loss arising from OPG development is observed in a subset of NF1 patients, with females more commonly requiring intervention (Diggs-Andrews et al., 2014). Retinal ganglion cell (RGC) death has been observed in mouse *Nf1*-OPG models (Diggs-Andrews et al., 2014; Kaul et al., 2015; Solga et al., 2017), and the intrinsic vulnerability of *Nf1*+/− RGCs has been demonstrated in vitro and in vivo (Brown et al., 2010). hiPSC RGCs (Teotia et al., 2017) and 3D hiPSC-based retinal preparations (Zhong et al., 2014; Parfitt et al., 2016), termed retinal cups, have been successfully generated and utilized for studies of glaucoma (Teotia et al., 2017) as well as Leber congenital amaurosis, a retinal disorder that often manifests in visual impairment (Parfitt et al., 2016). Application of such systems to NF1 could enable further investigation of the cellular and molecular mechanisms underlying vision loss and observed sexually dimorphic phenotypes.

Additionally, in a survey of NF1 patients, over half reported pain, highlighting the substantial pain burden, perhaps arising from dysfunctional sensory neurons (Kongkriangkai et al., 2019). To date, limited studies have focused on the role of sensory neurons in pain pathology. In mice, *Nf1*-mutant sensory neurons have greater excitability and an enhanced stimulus-evoked release of neuropeptides (Hingtgen et al., 2006; Wang et al., 2005). While hiPSC-derived sensory neurons have not yet been used for NF1 pain research to date, they have been increasingly useful in the study the PNS in other contexts (Clark et al., 2017; Meents et al., 2019; Muller et al., 2018).

NF1-related pigmentary abnormalities, such as café-au-lait macules (CALMs), and musculoskeletal abnormalities, such as skeletal dysplasia, scoliosis, osteopenia, and muscle weakness, also provide interesting avenues for hiPSC-based research (Arrington et al., 2013; Heervä et al., 2013; Schindeler and Little, 2008; Cornett et al., 2015). *NF1*-mutant human embryonic stem cell−derived melanocytes have been used to model aspects of hyperpigmentation and CALMs, while patient-derived dental pulp stem cells differentiated into chondrogenic and osteogenic lineages have been used to study NF1-related skeletal changes. Work in these model systems has indicated aberrant PKA-cAMP and ERK signaling is relevant to hyperpigmentation and CALMs (Allouche et al., 2015), whereas changes in cell proliferation, calcification, and extracellular matrix deposition may underlie NF1-related skeletal changes (Almeida et al., 2015, 2018). Such work underscores the viability of stem cell platforms for the study of NF1-related pigmentary and musculoskeletal clinical features. *NF1*-mutant patient-derived hiPSC melanocytes have been generated and reported to exhibit a senescent phenotype (Larribere et al., 2015). hiPSC differentiation protocols also exist for chondrogenic (Kawata et al., 2019) and osteogenic (Csobonyeiova et al., 2017) cell lineages. Furthermore, hiPSCs can be differentiated into myogenic progenitors (van der Wal et al., 2018) and used to model neuromuscular junctions (Lin et al., 2019) and skeletal muscles (Maffioletti et al., 2018), enabling further study of muscle formation and muscle weakness in NF1, which has only been modeled in mice to date (Kossler et al., 2011; Summers et al., 2018).

Future directions

While *Nf1* GEM models have served as the workhorses for studying pathobiology and evaluating promising drugs prior to human clinical trials, with the advent of hiPSC technology, it now becomes possible to complement and extend these studies. This is particularly important in light of the inherent differences between mouse and human tissues. As such, harmonization of murine and hiPSC models provides unique opportunities to define the etiologies for human disease, as well as discover and evaluate promising therapies for individuals affected with NF1 (Fig. 1.4).

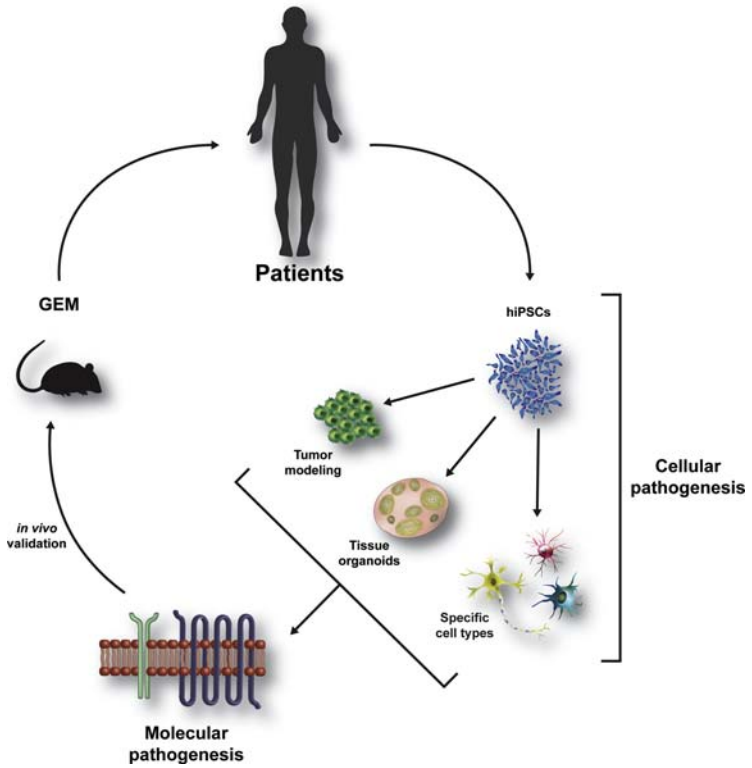


FIGURE 1.4 Integration between mouse models and human induced pluripotent stem cell.

Induced pluripotent stem cells derived from patients can be differentiated into individual cell types, used to generate three-dimensional (3D) organoids, or employed to create 3D tumor models. These human induced pluripotent stem cell platforms can be utilized to elucidate the molecular pathogenesis of various NF1 clinical features and to identify potential therapeutic targets. The efficacy of identified therapies can be further validated in vivo using mouse models. *GEM,* genetically engineered mouse models.

Acknowledgments

We thank the members of the Gutmann Laboratory for their advice and input during the writing of this review. We particularly appreciate the guidance provided by Michelle L. Wegscheid and graphic illustration assistance by Dr. Corina Anastasaki.

Funding

D.H.G. is funded by a Research Program Award grant from the National Institutes of Health (1-R35-NS07211-01). S.V.S is funded by an MSTP Training Grant (5T32GM007200).

References

Abud, E.M., Ramirez, R.N., Martinez, E.S., Healy, L.M., Nguyen, C.H.H., Newman, S.A., Yeromin, A.V., Scarfone, V.M., Marsh, S.E., Fimbres, C., Caraway, C.A., Fote, G.M., Madany, A.M., Agrawal, A., Kayed, R., Gylys, K.H., Cahalan, M.D., Cummings, B.J., Antel, J.P., Mortazavi, A., Carson, M.J., Poon, W.W., Blurton-Jones, M., 2017. IPSC-derived human microglia-like cells to study neurological diseases. Neuron 94 (2), 278−293. https://doi.org/10.1016/j.neuron.2017.03.042 e9.

Allouche, J., Bellon, N., Saidani, M., Stanchina-Chatrousse, L., Masson, Y., Patwardhan, A., Gilles-Marsens, F., Delevoye, C., Domingues, S., Nissan, X., Martinat, C., Lemaitre, G., Peschanski, M., Baldeschi, C., 2015. In vitro modeling of hyperpigmentation associated to neurofibromatosis type 1 using melanocytes derived from human embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. 112 (29), 9034−9039. https://doi.org/10.1073/pnas.1501032112.

Almeida, P.N., Barboza, D. do N., Luna, E.B., Correia, M.C. de M., Dias, R.B., Siquara de Sousa, A.C., Duarte, M.E.L., Rossi, M.I.D., Cunha, K.S., 2018. Increased extracellular matrix deposition during chondrogenic differentiation of dental pulp stem cells from individuals with neurofibromatosis type 1: an in vitro 2D and 3D study. Orphanet J. Rare Dis. 13. https://doi.org/10.1186/s13023-018-0843-1.

Almeida, P.N., Souza, G.T., de Souza, C.M., de Zanette, R.S.S., Maranduba, C.P., Rettore, J.V.P., de Santos, M.O., do Carmo, A.M.R., da Maranduba, C.M.C., de Silva, F.S., 2015. Proposing the use of dental pulp stem cells as a suitable biological model of neurofibromatosis type 1. Child Nerv. Syst. 31 (1), 7−13. https://doi.org/10.1007/s00381-014-2599-9.

Amiri, A., Coppola, G., Scuderi, S., Wu, F., Roychowdhury, T., Liu, F., Pochareddy, S., Shin, Y., Safi, A., Song, L., Zhu, Y., Sousa, A.M.M., Consortium†, T.P., Gerstein, M., Crawford, G.E., Sestan, N., Abyzov, A., Vaccarino, F.M., 2018. Transcriptome and epigenome landscape of human cortical development modeled in organoids. Science 362 (6420). https://doi.org/10.1126/science.aat6720.

Anastasaki, C., Gutmann, D.H., 2014. Neuronal NF1/RAS regulation of cyclic AMP requires atypical PKC activation. Hum. Mol. Genet. 23 (25), 6712−6721. https://doi.org/10.1093/hmg/ddu389.

Anastasaki, C., Le, L.Q., Kesterson, R.A., Gutmann, D.H., 2017a. Updated nomenclature for human and mouse neurofibromatosis type 1 genes. Neurol. Genet. 3 (4). https://doi.org/10.1212/NXG.0000000000000169.

Anastasaki, C., Morris, S.M., Gao, F., Gutmann, D.H., 2017b. Children with 5′-end NF1 gene mutations are more likely to have glioma. Neurol. Genet. 3 (5). https://doi.org/10.1212/NXG.0000000000000192.

Anastasaki, C., Wegscheid, M.L., Hartigan, K., Papke, J.B., Kopp, N.D., Chen, J., Cobb, O., Dougherty, J.D., Gutmann, D.H., 2020. Human iPSC-derived neurons and cerebral organoids establish differential effects of germline NF1 gene mutations. Stem Cell Rep. 14 (4), 541−550. https://doi.org/10.1016/j.stemcr.2020.03.007.

Anastasaki, C., Woo, A.S., Messiaen, L.M., Gutmann, D.H., 2015. Elucidating the impact of neurofibromatosis-1 germline mutations on neurofibromin function and dopamine-based learning. Hum. Mol. Genet. 24 (12), 3518−3528. https://doi.org/10.1093/hmg/ddv103.

Andersen, L.B., Ballester, R., Marchuk, D.A., Chang, E., Gutmann, D.H., Saulino, A.M., Camonis, J., Wigler, M., Collins, F.S., 1993. A conserved alternative splice in the von Recklinghausen neurofibromatosis (NF1) gene produces two neurofibromin isoforms, both of which have GTPase-activating protein activity. Mol. Cell Biol. 13 (1), 487–495.

Apostolova, I., Derlin, T., Salamon, J., Amthauer, H., Granström, S., Brenner, W., Mautner, V.-F., Buchert, R., 2015. Cerebral glucose metabolism in adults with neurofibromatosis type 1. Brain Res. 1625, 97–101. https://doi.org/10.1016/j.brainres.2015.08.025.

Arrington, D.K., Danehy, A.R., Peleggi, A., Proctor, M.R., Irons, M.B., Ullrich, N.J., 2013. Calvarial defects and skeletal dysplasia in patients with neurofibromatosis type 1: clinical article. J. Neurosurg. Pediatr. 11 (4), 410–416. https://doi.org/10.3171/2013.1.PEDS12409.

Bagley, J.A., Reumann, D., Bian, S., Lévi-Strauss, J., Knoblich, J.A., 2017. Fused cerebral organoids model interactions between brain regions. Nat. Method. 14 (7), 743–751. https://doi.org/10.1038/nmeth.4304.

Bajenaru, M.L., Garbow, J.R., Perry, A., Hernandez, M.R., Gutmann, D.H., 2005. Natural history of neurofibromatosis 1–associated optic nerve glioma in mice. Ann. Neurol. 57 (1), 119–127. https://doi.org/10.1002/ana.20337.

Bajenaru, M.L., Hernandez, M.R., Perry, A., Zhu, Y., Parada, L.F., Garbow, J.R., Gutmann, D.H., 2003. Optic nerve glioma in mice requires astrocyte Nf1 gene inactivation and Nf1 brain heterozygosity. Cancer Res. 63 (24), 8573–8577.

Ballabio, C., Anderle, M., Gianesello, M., Lago, C., Miele, E., Cardano, M., Aiello, G., Piazza, S., Caron, D., Gianno, F., Ciolfi, A., Pedace, L., Mastronuzzi, A., Tartaglia, M., Locatelli, F., Ferretti, E., Giangaspero, F., Tiberi, L., 2020. Modeling medulloblastoma in vivo and with human cerebellar organoids. Nat. Commun. 11 (1), 1–18. https://doi.org/10.1038/s41467-019-13989-3.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., Collins, F., 1990. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell 63 (4), 851–859. https://doi.org/10.1016/0092-8674(90)90151-4.

Basu, T.N., Gutmann, D.H., Fletcher, J.A., Glover, T.W., Collins, F.S., Downward, J., 1992. Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature 356 (6371), 713–715. https://doi.org/10.1038/356713a0.

Baudou, E., Nemmi, F., Biotteau, M., Maziero, S., Peran, P., Chaix, Y., 2019. Can the cognitive phenotype in neurofibromatosis type 1 (NF1) be explained by neuroimaging? A review. Front. Neurol. 10, 1373. https://doi.org/10.3389/fneur.2019.01373.

Bian, S., Repic, M., Guo, Z., Kavirayani, A., Burkard, T., Bagley, J.A., Krauditsch, C., Knoblich, J.A., 2018. Genetically engineered cerebral organoids model brain tumor formation. Nat. Method. 15 (8), 631–639. https://doi.org/10.1038/s41592-018-0070-7.

Birey, F., Andersen, J., Makinson, C.D., Islam, S., Wei, W., Huber, N., Fan, H.C., Metzler, K.R.C., Panagiotakos, G., Thom, N., O'Rourke, N.A., Steinmetz, L.M., Bernstein, J.A., Hallmayer, J., Huguenard, J.R., Paşca, S.P., 2017. Assembly of functionally integrated human forebrain spheroids. Nature 545 (7652), 54–59. https://doi.org/10.1038/nature22330.

Bolcekova, A., Nemethova, M., Zatkova, A., Hlinkova, K., Pozgayova, S., Hlavata, A., Kadasi, L., Durovcikova, D., Gerinec, A., Husakova, K., Pavlovicova, Z., Holobrada, M., Kovacs, L., Ilencikova, D., 2014. Clustering of mutations in the 5' tertile of the NF1 gene in Slovakia patients with optic pathway glioma. Neoplasma 60 (06), 655–665. https://doi.org/10.4149/neo_2013_084.

Bollag, G., McCormick, F., Clark, R., 1993. Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. EMBO J. 12 (5), 1923−1927. https://doi.org/10.1002/j.1460-2075.1993.tb05841.x.

Brennand, K.J., Simone, A., Jou, J., Gelboin-Burkhart, C., Tran, N., Sangar, S., Li, Y., Mu, Y., Chen, G., Yu, D., McCarthy, S., Sebat, J., Gage, F.H., 2011. Modelling schizophrenia using human induced pluripotent stem cells. Nature 473 (7346), 221−225. https://doi.org/10.1038/nature09915.

Brown, J.A., Emnett, R.J., White, C.R., Yuede, C.M., Conyers, S.B., O'Malley, K.L., Wozniak, D.F., Gutmann, D.H., 2010a. Reduced striatal dopamine underlies the attention system dysfunction in neurofibromatosis-1 mutant mice. Hum. Mol. Genet. 19 (22), 4515−4528. https://doi.org/10.1093/hmg/ddq382.

Brown, J.A., Gianino, S.M., Gutmann, D.H., 2010b. Defective cAMP generation underlies the sensitivity of CNS neurons to neurofibromatosis-1 heterozygosity. J. Neurosci. 30 (16), 5579−5589. https://doi.org/10.1523/JNEUROSCI.3994-09.2010.

Camp, J.G., Badsha, F., Florio, M., Kanton, S., Gerber, T., Wilsch-Bräuninger, M., Lewitus, E., Sykes, A., Hevers, W., Lancaster, M., Knoblich, J.A., Lachmann, R., Pääbo, S., Huttner, W.B., Treutlein, B., 2015. Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. Proc. Natl. Acad. Sci. U.S.A. 112 (51), 15672−15677. https://doi.org/10.1073/pnas.1520760112.

Carrió, M., Mazuelas, H., Richaud-Patin, Y., Gel, B., Terribas, E., Rosas, I., Jimenez-Delgado, S., Biayna, J., Vendredy, L., Blanco, I., Castellanos, E., Lázaro, C., Raya, Á., Serra, E., 2019. Reprogramming captures the genetic and tumorigenic properties of neurofibromatosis type 1 plexiform neurofibromas. Stem Cell Rep. 12 (2), 411−426. https://doi.org/10.1016/j.stemcr.2019.01.001.

Cawthon, R.M., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., White, R., 1990. A major segment of the neurofibromatosis type 1 gene: CDNA sequence, genomic structure, and point mutations. Cell 62 (1), 193−201. https://doi.org/10.1016/0092-8674(90)90253-B.

Chandrasekaran, A., Avci, H.X., Leist, M., Kobolák, J., Dinnyés, A., 2016. Astrocyte differentiation of human pluripotent stem cells: new tools for neurological disorder research. Front. Cell. Neurosci. 10. https://doi.org/10.3389/fncel.2016.00215.

Chen, Z., Liu, C., Patel, A.J., Liao, C.-P., Wang, Y., Le, L.Q., 2014. Cells of origin in the embryonic nerve roots for NF1-associated plexiform neurofibroma. Cancer Cell 26 (5), 695−706. https://doi.org/10.1016/j.ccell.2014.09.009.

Chen, Z., Mo, J., Brosseau, J.-P., Shipman, T., Wang, Y., Liao, C.-P., Cooper, J.M., Allaway, R.J., Gosline, S.J.C., Guinney, J., Carroll, T.J., Le, L.Q., 2019. Spatiotemporal loss of NF1 in Schwann cell lineage leads to different types of cutaneous neurofibroma susceptible to modification by the hippo pathway. Cancer Discov. 9 (1), 114−129. https://doi.org/10.1158/2159-8290.CD-18-0151.

Cheng, C., Fass, D.M., Folz-Donahue, K., MacDonald, M.E., Haggarty, S.J., 2017. Highly expandable human iPS cell-derived neural progenitor cells (NPC) and neurons for central nervous system disease modeling and high-throughput ccreening. Curr. Protoc. Hum. Genet. 92, 21.28.1−21.8.21. https://doi.org/10.1002/cphg.33.

Cichowski, K., Shih, T.S., Schmitt, E., Santiago, S., Reilly, K., McLaughlin, M.E., Bronson, R.T., Jacks, T., 1999. Mouse models of tumor development in neurofibromatosis type 1. Science 286 (5447), 2172−2176. https://doi.org/10.1126/science.286.5447.2172.

Clark, A.J., Kaller, M.S., Galino, J., Willison, H.J., Rinaldi, S., Bennett, D.L.H., 2017. Co-cultures with stem cell-derived human sensory neurons reveal regulators of peripheral myelination. Brain 140 (4), 898–913. https://doi.org/10.1093/brain/awx012.

Collins, F.S., O'Connell, P., Ponder, B.A.J., Seizinger, B.R., 1989. Progress towards identifying the neurofibromatosis (NF1) gene. Trends Genet. 5, 217–221. https://doi.org/10.1016/0168-9525(89)90085-1.

Cornett, K.M.D., North, K.N., Rose, K.J., Burns, J., 2015. Muscle weakness in children with neurofibromatosis type 1. Dev. Med. Child Neurol. 57 (8), 733–736. https://doi.org/10.1111/dmcn.12777.

Costa, R.M., Federov, N.B., Kogan, J.H., Murphy, G.G., Stern, J., Ohno, M., Kucherlapati, R., Jacks, T., Silva, A.J., 2002. Mechanism for the learning deficits in a mouse model of neurofibromatosis type 1. Nature 415 (6871), 526–530. https://doi.org/10.1038/nature711.

Csobonyeiova, M., Polak, S., Zamborsky, R., Danisovic, L., 2017. iPS cell technologies and their prospect for bone regeneration and disease modeling: a mini review. J. Adv. Res. 8 (4), 321–327. https://doi.org/10.1016/j.jare.2017.02.004.

Daginakatte, G.C., Gutmann, D.H., 2007. Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-deficient astrocyte and glioma growth. Hum. Mol. Genet. 16 (9), 1098–1112. https://doi.org/10.1093/hmg/ddm059.

Danglot, G., Régnler, V., Fauvet, D., Vassal, G., Kujas, M., Bernheim, A., 1995. Neurofibromatosis 1 (NF1) mRNAs expressed in the central nervous system are differentially spliced in the 5′ part of the gene. Hum. Mol. Genet. 4 (5), 915–920. https://doi.org/10.1093/hmg/4.5.915.

Dasgupta, B., Yi, Y., Chen, D.Y., Weber, J.D., Gutmann, D.H., 2005. Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1–associated human and mouse brain tumors. Cancer Res. 65 (7), 2755–2760. https://doi.org/10.1158/0008-5472.CAN-04-4058.

Daviaud, N., Friedel, R.H., Zou, H., 2018. Vascularization and engraftment of transplanted human cerebral organoids in mouse cortex. ENeuro 5 (6). https://doi.org/10.1523/ENEURO.0219-18.2018.

DeClue, J.E., Cohen, B.D., Lowy, D.R., 1991. Identification and characterization of the neurofibromatosis type 1 protein product. Proc. Natl. Acad. Sci. U.S.A. 88 (22), 9914–9918. https://doi.org/10.1073/pnas.88.22.9914.

DeClue, J.E., Papageorge, A.G., Fletcher, J.A., Diehl, S.R., Ratner, N., Vass, W.C., Lowy, D.R., 1992. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 69 (2), 265–273. https://doi.org/10.1016/0092-8674(92)90407-4.

Deshpande, A., Yadav, S., Dao, D.Q., Wu, Z.-Y., Hokanson, K.C., Cahill, M.K., Wiita, A.P., Jan, Y.-N., Ullian, E.M., Weiss, L.A., 2017. Cellular phenotypes in human iPSC-derived neurons from a genetic model of autism spectrum disorder. Cell Rep. 21 (10), 2678–2687. https://doi.org/10.1016/j.celrep.2017.11.037.

Diggs-Andrews, K.A., Brown, J.A., Gianino, S.M., Rubin, J.B., Wozniak, D.F., Gutmann, D.H., 2014. Sex is a major determinant of neuronal dysfunction in Neurofibromatosis Type 1. Ann. Neurol. 75 (2), 309–316. https://doi.org/10.1002/ana.24093.

Diggs-Andrews, K.A., Tokuda, K., Izumi, Y., Zorumski, C.F., Wozniak, D.F., Gutmann, D.H., 2013. Dopamine deficiency underlies learning deficits in neurofibromatosis-1 mice. Ann. Neurol. 73 (2), 309–315. https://doi.org/10.1002/ana.23793.

Dombi, E., Baldwin, A., Marcus, L.J., Fisher, M.J., Weiss, B., Kim, A., Whitcomb, P., Martin, S., Aschbacher-Smith, L.E., Rizvi, T.A., Wu, J., Ershler, R., Wolters, P., Therrien, J., Glod, J., Belasco, J.B., Schorry, E., Brofferio, A., Starosta, A.J., et al., 2016. Activity of Selumetinib in neurofibromatosis type 1—related plexiform neurofibromas. N. Engl. J. Med. 375 (26), 2550—2560. https://doi.org/10.1056/NEJMoa1605943.

Douvaras, P., Fossati, V., 2015. Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. Nat. Protoc. 10 (8), 1143—1154. https://doi.org/10.1038/nprot.2015.075.

Dunzendorfer-Matt, T., Mercado, E.L., Maly, K., McCormick, F., Scheffzek, K., 2016. The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation. Proc. Natl. Acad. Sci. U.S.A. 113 (27), 7497—7502. https://doi.org/10.1073/pnas.1607298113.

Engle, S.J., Blaha, L., Kleiman, R.J., 2018. Best practices for translational disease modeling using human iPSC-derived neurons. Neuron 100 (4), 783—797. https://doi.org/10.1016/j.neuron.2018.10.033.

Friedman, J.M., 1999. Epidemiology of neurofibromatosis type 1. Am. J. Med. Genet. 89 (1), 1—6. https://doi.org/10.1002/(SICI)1096-8628(19990326)89:1<1::AID-AJMG3>3.0.CO;2-8.

Goldman, S.A., Kuypers, N.J., 2015. How to make an oligodendrocyte. Development 142 (23), 3983—3995. https://doi.org/10.1242/dev.126409.

Gouder, L., Vitrac, A., Goubran-Botros, H., Danckaert, A., Tinevez, J.-Y., André-Leroux, G., Atanasova, E., Lemière, N., Biton, A., Leblond, C.S., Poulet, A., Boland, A., Deleuze, J.-F., Benchoua, A., Delorme, R., Bourgeron, T., Cloëz-Tayarani, I., 2019. Altered spinogenesis in iPSC-derived cortical neurons from patients with autism carrying de novo SHANK3 mutations. Sci. Rep. 9 (1), 1—11. https://doi.org/10.1038/s41598-018-36993-x.

Gross, A.M., Wolters, P.L., Dombi, E., Baldwin, A., Whitcomb, P., Fisher, M.J., Weiss, B., Kim, A., Bornhorst, M., Shah, A.C., Martin, S., Roderick, M.C., Pichard, D.C., Carbonell, A., Paul, S.M., Therrien, J., Kapustina, O., Heisey, K., Clapp, D.W., et al., 2020. Selumetinib in children with inoperable plexiform neurofibromas. N. Engl. J. Med. 382 (15), 1430—1442. https://doi.org/10.1056/NEJMoa1912735.

Guimarães, M.Z.P., De Vecchi, R., Vitória, G., Sochacki, J.K., Paulsen, B.S., Lima, I., Rodrigues da Silva, F., da Costa, R.F.M., Castro, N.G., Breton, L., Rehen, S.K., 2018. Generation of iPSC-derived human peripheral sensory neurons releasing substance P elicited by TRPV1 agonists. Front. Mol. Neurosci. 11. https://doi.org/10.3389/fnmol.2018.00277.

Guo, X., Pan, Y., Gutmann, D.H., 2019. Genetic and genomic alterations differentially dictate low-grade glioma growth through cancer stem cell—specific chemokine recruitment of T cells and microglia. Neuro Oncol. 21 (10), 1250—1262. https://doi.org/10.1093/neuonc/noz080.

Guo, X., Pan, Y., Xiong, M., Sanapala, S., Anastasaki, C., Cobb, O., Dahiya, S., Gutmann, D.H., 2020. Midkine activation of CD8 + T cells establishes a neuron—immune—cancer axis responsible for low-grade glioma growth. Nat. Commun. 11 (1), 1—15. https://doi.org/10.1038/s41467-020-15770-3.

Gutmann, D.H., Andersen, B., L., L. Cole, J., Swaroop, M., S. Collins, F., 1993. An alternatively-spliced mRNA in the carboxy terminus of the neurofibromatosis type 1 (NF1) gene is expressed in muscle. Hum. Mol. Genet. 2 (7), 989—992. https://doi.org/10.1093/hmg/2.7.989.

Gutmann, D.H., Ferner, R.E., Listernick, R.H., Korf, B.R., Wolters, P.L., Johnson, K.J., 2017. Neurofibromatosis type 1. Nat. Rev. Dis. Prim. 3 (1), 17004. https://doi.org/10.1038/nrdp.2017.4.

Gutmann, D.H., Wood, D.L., Collins, F.S., 1991. Identification of the neurofibromatosis type 1 gene product. Proc. Natl. Acad. Sci. U.S.A. 88 (21), 9658–9662.

Gutmann, D.H., Zhang, Y., Hirbe, A., 1999. Developmental regulation of a neuron-specific neurofibromatosis 1 isoform. Ann. Neurol. 46 (5), 777–782. https://doi.org/10.1002/1531-8249(199911)46:5<777::AID-ANA15>3.0.CO;2-H.

Hasselmann, J., Blurton-Jones, M., 2020. Human iPSC-derived microglia: a growing toolset to study the brain's innate immune cells. Glia 68 (4), 721–739. https://doi.org/10.1002/glia.23781.

Heervä, E., Leinonen, P., Kuorilehto, T., Peltonen, S., Pöyhönen, M., Väänänen, K., Peltonen, J., 2013. Neurofibromatosis 1-related osteopenia often progresses to osteoporosis in 12 years. Calcif. Tissue Int. 92 (1), 23–27. https://doi.org/10.1007/s00223-012-9661-y.

Hegedus, B., Banerjee, D., Yeh, T.-H., Rothermich, S., Perry, A., Rubin, J.B., Garbow, J.R., Gutmann, D.H., 2008. Preclinical cancer therapy in a mouse model of neurofibromatosis-1 optic glioma. Cancer Res. 68 (5), 1520–1528. https://doi.org/10.1158/0008-5472.CAN-07-5916.

Hegedus, B., Dasgupta, B., Shin, J.E., Emnett, R.J., Hart-Mahon, E.K., Elghazi, L., Bernal-Mizrachi, E., Gutmann, D.H., 2007. Neurofibromatosis-1 regulates neuronal and glial cell differentiation from neuroglial progenitors in vivo by both cAMP- and ras-dependent mechanisms. Cell Stem Cell 1 (4), 443–457. https://doi.org/10.1016/j.stem.2007.07.008.

Hingtgen, C.M., Roy, S.L., Clapp, D.W., 2006. Stimulus-evoked release of neuropeptides is enhanced in sensory neurons from mice with a heterozygous mutation of the Nf1 gene. Neuroscience 137 (2), 637–645. https://doi.org/10.1016/j.neuroscience.2005.09.030.

Hirbe, A.C., Dahiya, S., Friedmann-Morvinski, D., Verma, I.M., Clapp, D.W., Gutmann, D.H., 2016. Spatially- and temporally-controlled postnatal p53 knockdown cooperates with embryonic Schwann cell precursor Nf1 gene loss to promote malignant peripheral nerve sheath tumor formation. Oncotarget 7 (7), 7403–7414. https://doi.org/10.18632/oncotarget.7232.

Hodge, R.D., Bakken, T.E., Miller, J.A., Smith, K.A., Barkan, E.R., Graybuck, L.T., Close, J.L., Long, B., Johansen, N., Penn, O., Yao, Z., Eggermont, J., Höllt, T., Levi, B.P., Shehata, S.I., Aevermann, B., Beller, A., Bertagnolli, D., Brouner, K., et al., 2019. Conserved cell types with divergent features in human versus mouse cortex. Nature 573 (7772), 61–68. https://doi.org/10.1038/s41586-019-1506-7.

Hsueh, Y.P., Roberts, A.M., Volta, M., Sheng, M., Roberts, R.G., 2001. Bipartite interaction between neurofibromatosis type I protein (neurofibromin) and syndecan transmembrane heparan sulfate proteoglycans. J. Neurosci. 21 (11), 3764–3770.

Hyman, S.L., Shores, A., North, K.N., 2005. The nature and frequency of cognitive deficits in children with neurofibromatosis type 1. Neurology 65 (7), 1037–1044. https://doi.org/10.1212/01.wnl.0000179303.72345.ce.

Ibrahim, A.F.A., Montojo, C.A., Haut, K.M., Karlsgodt, K.H., Hansen, L., Congdon, E., Rosser, T., Bilder, R.M., Silva, A.J., Bearden, C.E., 2017. Spatial working memory in neurofibromatosis 1: altered neural activity and functional connectivity. NeuroImage. Clin. 15, 801–811. https://doi.org/10.1016/j.nicl.2017.06.032.

Igarashi, A., Ebihara, Y., Kumagai, T., Hirai, H., Nagata, K., Tsuji, K., 2018. Mast cells derived from human induced pluripotent stem cells are useful for allergen tests. Allergol. Int. 67 (2), 234−242. https://doi.org/10.1016/j.alit.2017.08.008.

Ikuno, T., Ito, S., Inoue, T., 2019. Human induced pluripotent stem cell-derived mast cells useful for *in vitro* mast cell activation assay exhibiting phenotypes and morphological characteristics of human mast cells. J. Toxicol. Sci. 44 (11), 789−797. https://doi.org/10.2131/jts.44.789.

Isakson, S.H., Rizzardi, A.E., Coutts, A.W., Carlson, D.F., Kirstein, M.N., Fisher, J., Vitte, J., Williams, K.B., Pluhar, G.E., Dahiya, S., Widemann, B.C., Dombi, E., Rizvi, T., Ratner, N., Messiaen, L., Stemmer-Rachamimov, A.O., Fahrenkrug, S.C., Gutmann, D.H., Giovannini, M., et al., 2018. Genetically engineered minipigs model the major clinical features of human neurofibromatosis type 1. Commun. Biol. 1. https://doi.org/10.1038/s42003-018-0163-y.

Israel, M.A., Yuan, S.H., Bardy, C., Reyna, S.M., Mu, Y., Herrera, C., Hefferan, M.P., Van Gorp, S., Nazor, K.L., Boscolo, F.S., Carson, C.T., Laurent, L.C., Marsala, M., Gage, F.H., Remes, A.M., Koo, E.H., Goldstein, L.S.B., 2012. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature 482 (7384), 216−220. https://doi.org/10.1038/nature10821.

Izawa, I., Tamaki, N., Saya, H., 1996. Phosphorylation of neurofibromatosis type 1 gene product (neurofibromin) by cAMP-dependent protein kinase. FEBS Lett. 382 (1−2), 53−59. https://doi.org/10.1016/0014-5793(96)00137-8.

Jessen, W.J., Miller, S.J., Jousma, E., Wu, J., Rizvi, T.A., Brundage, M.E., Eaves, D., Widemann, B., Kim, M.-O., Dombi, E., Sabo, J., Dudley, A.H., Niwa-Kawakita, M., Page, G.P., Giovannini, M., Aronow, B.J., Cripe, T.P., Ratner, N., 2013. MEK inhibition exhibits efficacy in human and mouse neurofibromatosis tumors. J. Clin. Invest. 123 (1), 340−347. https://doi.org/10.1172/JCI60578.

Johannessen, C.M., Reczek, E.E., James, M.F., Brems, H., Legius, E., Cichowski, K., 2005. The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc. Natl. Acad. Sci. U.S.A. 102 (24), 8573−8578. https://doi.org/10.1073/pnas.0503224102.

Kaul, A., Toonen, J.A., Cimino, P.J., Gianino, S.M., Gutmann, D.H., 2015a. Akt- or MEK-mediated mTOR inhibition suppresses Nf1 optic glioma growth. Neuro Oncol. 17 (6), 843−853. https://doi.org/10.1093/neuonc/nou329.

Kaul, A., Toonen, J.A., Gianino, S.M., Gutmann, D.H., 2015b. The impact of coexisting genetic mutations on murine optic glioma biology. Neuro Oncol. 17 (5), 670−677. https://doi.org/10.1093/neuonc/nou287.

Kauts, M.-L., De Leo, B., Rodríguez-Seoane, C., Ronn, R., Glykofrydis, F., Maglitto, A., Kaimakis, P., Basi, M., Taylor, H., Forrester, L., Wilkinson, A.C., Göttgens, B., Saunders, P., Dzierzak, E., 2018. Rapid mast cell generation from Gata2 reporter pluripotent stem cells. Stem Cell Rep. 11 (4), 1009−1020. https://doi.org/10.1016/j.stemcr.2018.08.007.

Kawata, M., Mori, D., Kanke, K., Hojo, H., Ohba, S., Chung, U., Yano, F., Masaki, H., Otsu, M., Nakauchi, H., Tanaka, S., Saito, T., 2019. Simple and robust differentiation of human pluripotent stem cells toward chondrocytes by two small-molecule compounds. Stem Cell Rep. 13 (3), 530−544. https://doi.org/10.1016/j.stemcr.2019.07.012.

Kehrer-Sawatzki, H., Mautner, V.-F., Cooper, D.N., 2017. Emerging genotype−phenotype relationships in patients with large NF1 deletions. Hum. Genet. 136 (4), 349−376. https://doi.org/10.1007/s00439-017-1766-y.

Khanna, R., Moutal, A., White, K.A., Chefdeville, A., Negrao de Assis, P., Cai, S., Swier, V.J., Bellampalli, S.S., Giunta, M.D., Darbro, B.W., Quelle, D.E., Sieren, J.C., Wallace, M.R., Rogers, C.S., Meyerholz, D.K., Weimer, J.M., 2019. Assessment of nociception and related quality-of-life measures in a porcine model of neurofibromatosis type 1. Pain 160 (11), 2473−2486. https://doi.org/10.1097/j.pain.0000000000001648.

Kim, H.-S., Lee, J., Lee, D.Y., Kim, Y.-D., Kim, J.Y., Lim, H.J., Lim, S., Cho, Y.S., 2017. Schwann cell precursors from human pluripotent stem cells as a potential therapeutic target for myelin repair. Stem Cell Rep. 8 (6), 1714−1726. https://doi.org/10.1016/j.stemcr.2017.04.011.

Klaus, J., Kanton, S., Kyrousi, C., Ayo-Martin, A.C., Di Giaimo, R., Riesenberg, S., O'Neill, A.C., Camp, J.G., Tocco, C., Santel, M., Rusha, E., Drukker, M., Schroeder, M., Götz, M., Robertson, S.P., Treutlein, B., Cappello, S., 2019. Altered neuronal migratory trajectories in human cerebral organoids derived from individuals with neuronal heterotopia. Nat. Med. 25 (4), 561−568. https://doi.org/10.1038/s41591-019-0371-0.

Kolberg, M., Høland, M., Ågesen, T.H., Brekke, H.R., Liestøl, K., Hall, K.S., Mertens, F., Picci, P., Smeland, S., Lothe, R.A., 2013. Survival meta-analyses for >1800 malignant peripheral nerve sheath tumor patients with and without neurofibromatosis type 1. Neuro Oncol. 15 (2), 135−147. https://doi.org/10.1093/neuonc/nos287.

Kongkriangkai, A.M., King, C., Martin, L.J., Wakefield, E., Prada, C.E., Kelly-Mancuso, G., Schorry, E.K., 2019. Substantial pain burden in frequency, intensity, interference and chronicity among children and adults with neurofibromatosis type 1 pain and NF1. Am. J. Med. Genet. 179 (4), 602−607. https://doi.org/10.1002/ajmg.a.61069.

Kossler, N., Stricker, S., Rödelsperger, C., Robinson, P.N., Kim, J., Dietrich, C., Osswald, M., Kühnisch, J., Stevenson, D.A., Braun, T., Mundlos, S., Kolanczyk, M., 2011. Neurofibromin (Nf1) is required for skeletal muscle development. Hum. Mol. Genet. 20 (14), 2697−2709. https://doi.org/10.1093/hmg/ddr149.

Kweh, F., Zheng, M., Kurenova, E., Wallace, M., Golubovskaya, V., Cance, W.G., 2009. Neurofibromin physically interacts with the N-terminal domain of focal adhesion kinase. Mol. Carcinog. 48 (11), 1005−1017. https://doi.org/10.1002/mc.20552.

Lancaster, M.A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., Knoblich, J.A., 2013. Cerebral organoids model human brain development and microcephaly. Nature 501 (7467), 373−379. https://doi.org/10.1038/nature12517.

Larribere, L., Wu, H., Novak, D., Galach, M., Bernhardt, M., Orouji, E., Weina, K., Knappe, N., Sachpekidis, C., Umansky, L., Beckhove, P., Umansky, V., Schepper, S.D., Kaufmann, D., Ballotti, R., Bertolotto, C., Utikal, J., 2015. NF1 loss induces senescence during human melanocyte differentiation in an iPSC-based model. Pigment Cell Melanoma Res. 28 (4), 407−416. https://doi.org/10.1111/pcmr.12369.

Lau, N., Feldkamp, M.M., Roncari, L., Loehr, A.H., Shannon, P., Gutmann, D.H., Guha, A., 2000. Loss of neurofibromin is associated with activation of RAS/MAPK and PI3-K/AKT signaling in a neurofibromatosis 1 astrocytoma. J. Neuropathol. Exp. Neurol. 59 (9), 759−767. https://doi.org/10.1093/jnen/59.9.759.

Le, L.Q., Shipman, T., Burns, D.K., Parada, L.F., 2009. Cell of origin and microenvironment contribution for NF1-associated dermal neurofibromas. Cell Stem Cell 4 (5), 453−463. https://doi.org/10.1016/j.stem.2009.03.017.

Lee, D.Y., Gianino, S.M., Gutmann, D.H., 2012. Innate neural stem cell heterogeneity determines the patterning of glioma formation in children. Cancer Cell 22 (1), 131–138. https://doi.org/10.1016/j.ccr.2012.05.036.

Li, K., Turner, A.N., Chen, M., Brosius, S.N., Schoeb, T.R., Messiaen, L.M., Bedwell, D.M., Zinn, K.R., Anastasaki, C., Gutmann, D.H., Korf, B.R., Kesterson, R.A., 2016. Mice with missense and nonsense NF1 mutations display divergent phenotypes compared with human neurofibromatosis type I. Dis. Model Mech 9 (7), 759–767. https://doi.org/10.1242/dmm.025783.

Li, Y., Muffat, J., Omer, A., Bosch, I., Lancaster, M.A., Sur, M., Gehrke, L., Knoblich, J.A., Jaenisch, R., 2017. Induction of 3xpansion and folding in human cerebral organoids. Cell Stem Cell 20 (3), 385–396. https://doi.org/10.1016/j.stem.2016.11.017 e3.

Liao, C.-P., Booker, R.C., Brosseau, J.-P., Chen, Z., Mo, J., Tchegnon, E., Wang, Y., Clapp, D.W., Le, L.Q., 2018. Contributions of inflammation and tumor microenvironment to neurofibroma tumorigenesis. J. Clin. Invest. 128 (7), 2848–2861. https://doi.org/10.1172/JCI99424.

Liao, C.-P., Pradhan, S., Chen, Z., Patel, A.J., Booker, R.C., Le, L.Q., 2016. The role of nerve microenvironment for neurofibroma development. Oncotarget 7 (38), 61500–61508. https://doi.org/10.18632/oncotarget.11133.

Lin, C.-Y., Yoshida, M., Li, L.-T., Ikenaka, A., Oshima, S., Nakagawa, K., Sakurai, H., Matsui, E., Nakahata, T., Saito, M.K., 2019. IPSC-derived functional human neuromuscular junctions model the pathophysiology of neuromuscular diseases. JCI Insight 4 (18). https://doi.org/10.1172/jci.insight.124299.

Lukkes, J.L., Drozd, H.P., Fitz, S.D., Molosh, A.I., Clapp, D.W., Shekhar, A., 2020. Guanfacine treatment improves ADHD phenotypes of impulsivity and hyperactivity in a neurofibromatosis type 1 mouse model. J. Neurodev. Disord. 12 (1), 2. https://doi.org/10.1186/s11689-019-9304-y.

Maffioletti, S.M., Sarcar, S., Henderson, A.B.H., Mannhardt, I., Pinton, L., Moyle, L.A., Steele-Stallard, H., Cappellari, O., Wells, K.E., Ferrari, G., Mitchell, J.S., Tyzack, G.E., Kotiadis, V.N., Khedr, M., Ragazzi, M., Wang, W., Duchen, M.R., Patani, R., Zammit, P.S., et al., 2018. Three-dimensional human iPSC-derived artificial skeletal muscles model muscular dystrophies and enable multilineage tissue engineering. Cell Rep. 23 (3), 899–908. https://doi.org/10.1016/j.celrep.2018.03.091.

Mansour, A.A., Gonçalves, J.T., Bloyd, C.W., Li, H., Fernandes, S., Quang, D., Johnston, S., Parylak, S.L., Jin, X., Gage, F.H., 2018. An in vivo model of functional and vascularized human brain organoids. Nat. Biotechnol. 36 (5), 432–441. https://doi.org/10.1038/nbt.4127.

Maria, S., Helle, B., Tristan, L., Gaynor, S., Arnar, A., Michele, M., Teresia, O., Oliver, C., Roger, S., Penelope, H., Ole, I., 2013. Improved cell therapy protocol for Parkinson's disease based on differentiation efficiency and safety of hESC-, hiPSC and non-human primate iPSC-derived DA neurons. Stem Cells 31 (8), 1548–1562. https://doi.org/10.1002/stem.1415.

Mayes, D.A., Rizvi, T.A., Titus-Mitchell, H., Oberst, R., Ciraolo, G.M., Vorhees, C.V., Robinson, A.P., Miller, S.D., Cancelas, J.A., Stemmer-Rachamimov, A.O., Ratner, N., 2013. Nf1 loss and Ras hyperactivation in oligodendrocytes induce NOS-driven defects in myelin and vasculature. Cell Rep. 4 (6), 1197–1212. https://doi.org/10.1016/j.celrep.2013.08.011.

Meents, J.E., Bressan, E., Sontag, S., Foerster, A., Hautvast, P., Rösseler, C., Hampl, M., Schüler, H., Goetzke, R., Le, T.K.C., Kleggetveit, I.P., Le Cann, K., Kerth, C., Rush, A.M., Rogers, M., Kohl, Z., Schmelz, M., Wagner, W., Jørum, E., et al., 2019. The role of Nav1.7 in human nociceptors: insights from human induced pluripotent stem cell−derived sensory neurons of erythromelalgia patients. Pain 160 (6), 1327−1341. https://doi.org/10.1097/j.pain.0000000000001511.

Mertens, J., Wang, Q.-W., Kim, Y., Yu, D.X., Pham, S., Yang, B., Zheng, Y., Diffenderfer, K.E., Zhang, J., Soltani, S., Eames, T., Schafer, S.T., Boyer, L., Marchetto, M.C., Nurnberger, J.I., Calabrese, J.R., Oedegaard, K.J., McCarthy, M.J., Zandi, P.P., et al., 2015. Differential responses to lithium in hyperexcitable neurons from patients with bipolar disorder. Nature 527 (7576), 95−99. https://doi.org/10.1038/nature15526.

Mo, J., Anastasaki, C., Chen, Z., Shipman, T., Papke, J., Yin, K., Gutmann, D.H., Le, L.Q., 2021. Humanized neurofibroma model from induced pluripotent stem cells delineates tumor pathogenesis and developmental origins. J. Clin. Invest. 131 (1). https://doi.org/10.1172/jci139807.

Molosh, A.I., Johnson, P.L., Spence, J.P., Arendt, D., Federici, L.M., Bernabe, C., Janasik, S.P., Segu, Z.M., Khanna, R., Goswami, C., Zhu, W., Park, S.-J., Li, L., Mechref, Y.S., Clapp, D.W., Shekhar, A., 2014. Social learning and amygdala disruptions in Nf1 mice are rescued by blocking p21-activated kinase. Nat. Neurosci. 17 (11), 1583−1590. https://doi.org/10.1038/nn.3822.

Monje, M., Borniger, J.C., D'Silva, N.J., Deneen, B., Dirks, P.B., Fattahi, F., Frenette, P.S., Garzia, L., Gutmann, D.H., Hanahan, D., Hervey-Jumper, S.L., Hondermarck, H., Hurov, J.B., Kepecs, A., Knox, S.M., Lloyd, A.C., Magnon, C., Saloman, J.L., Segal, R.A., et al., 2020. Roadmap for the emerging field of cancer neuroscience. Cell 181 (2), 219−222. https://doi.org/10.1016/j.cell.2020.03.034.

Morris, S.M., Gutmann, D.H., 2018. A genotype-phenotype correlation for quantitative autistic trait burden in neurofibromatosis 1. Neurology 90 (8), 377−379. https://doi.org/10.1212/WNL.0000000000005000.

Muller, Q., Beaudet, M.-J., De Serres-Bérard, T., Bellenfant, S., Flacher, V., Berthod, F., 2018. Development of an innervated tissue-engineered skin with human sensory neurons and Schwann cells differentiated from iPS cells. Acta Biomater. 82, 93−101. https://doi.org/10.1016/j.actbio.2018.10.011.

Oberheim, N.A., Takano, T., Han, X., He, W., Lin, J.H.C., Wang, F., Xu, Q., Wyatt, J.D., Pilcher, W., Ojemann, J.G., Ransom, B.R., Goldman, S.A., Nedergaard, M., 2009. Uniquely hominid features of adult human astrocytes. J. Neurosci. 29 (10), 3276−3287. https://doi.org/10.1523/JNEUROSCI.4707-08.2009.

Ogawa, J., Pao, G.M., Shokhirev, M.N., Verma, I.M., 2018. Glioblastoma model using human cerebral organoids. Cell Rep. 23 (4), 1220−1229. https://doi.org/10.1016/j.celrep.2018.03.105.

Pan, Y., Hysinger, J.D., Barron, T, Schindler, N.F., Cobb, O., Guo, X., Yalcin, B., Anastasaki, C., Mulinyawe, S.B., Ponnuswami, A., Scheaffer, S., Ma, Y., Chang, K.-C., Xia, X., Toonen, J.A., Lennon, J.J., Gibson, E.M., Huguenard, J., Liau, L.M., Goldberg, J.L., Monje, M., Gutmann, D.H., 2021. *NF1* mutation drives neuronal activity-dependent optic glioma initiation. Nature 594, 277−282.

Pan, Y.-H., Wu, N., Yuan, X.-B., 2019. Toward a better understanding of neuronal migration deficits in autism spectrum disorders. Front. Cell Dev. Biol. 7, 205. https://doi.org/10.3389/fcell.2019.00205.

Pang, Z.P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D.R., Yang, T.Q., Citri, A., Sebastiano, V., Marro, S., Südhof, T.C., Wernig, M., 2011. Induction of human neuronal cells by defined transcription factors. Nature 476 (7359), 220–223. https://doi.org/10.1038/nature10202.

Paquet, D., Kwart, D., Chen, A., Sproul, A., Jacob, S., Teo, S., Olsen, K.M., Gregg, A., Noggle, S., Tessier-Lavigne, M., 2016. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature 533 (7601), 125–129. https://doi.org/10.1038/nature17664.

Parfitt, D.A., Lane, A., Ramsden, C.M., Carr, A.-J.F., Munro, P.M., Jovanovic, K., Schwarz, N., Kanuga, N., Muthiah, M.N., Hull, S., Gallo, J.-M., da Cruz, L., Moore, A.T., Hardcastle, A.J., Coffey, P.J., Cheetham, M.E., 2016. Identification and correction of mechanisms underlying inherited blindness in human iPSC-derived optic cups. Cell Stem Cell 18 (6), 769–781. https://doi.org/10.1016/j.stem.2016.03.021.

Pasmant, E., Sabbagh, A., Spurlock, G., Laurendeau, I., Grillo, E., Hamel, M.-J., Martin, L., Barbarot, S., Leheup, B., Rodriguez, D., Lacombe, D., Dollfus, H., Pasquier, L., Isidor, B., Ferkal, S., Soulier, J., Sanson, M., Dieux-Coeslier, A., Bièche, I., et al., 2010. NF1 microdeletions in neurofibromatosis type 1: from genotype to phenotype. Hum. Mutat. 31 (6), E1506–E1518. https://doi.org/10.1002/humu.21271.

Patrakitkomjorn, S., Kobayashi, D., Morikawa, T., Wilson, M.M., Tsubota, N., Irie, A., Ozawa, T., Aoki, M., Arimura, N., Kaibuchi, K., Saya, H., Araki, N., 2008. Neurofibromatosis type 1 (NF1) tumor suppressor, neurofibromin, regulates the neuronal differentiation of PC12 cells via its associating protein, CRMP-2. J. Biol. Chem. 283 (14), 9399–9413. https://doi.org/10.1074/jbc.M708206200.

Pinna, V., Lanari, V., Daniele, P., Consoli, F., Agolini, E., Margiotti, K., Bottillo, I., Torrente, I., Bruselles, A., Fusilli, C., Ficcadenti, A., Bargiacchi, S., Trevisson, E., Forzan, M., Giustini, S., Leoni, C., Zampino, G., Cristina Digilio, M., Dallapiccola, B., et al., 2015. P.Arg1809Cys substitution in neurofibromin is associated with a distinctive NF1 phenotype without neurofibromas. Eur. J. Hum. Genet. 23 (8), 1068–1071. https://doi.org/10.1038/ejhg.2014.243.

Pong, W.W., Higer, S.B., Gianino, S.M., Emnett, R.J., Gutmann, D.H., 2013. Reduced microglial CX3CR1 expression delays neurofibromatosis-1 glioma formation. Ann. Neurol. 73 (2), 303–308. https://doi.org/10.1002/ana.23813.

Ponroy Bally, B., Farmer, W.T., Jones, E.V., Jessa, S., Kacerovsky, J.B., Mayran, A., Peng, H., Lefebvre, J.L., Drouin, J., Hayer, A., Ernst, C., Murai, K.K., 2020. Human iPSC-derived Down syndrome astrocytes display genome-wide perturbations in gene expression, an altered adhesion profile, and increased cellular dynamics. Hum. Mol. Genet. 29 (5), 785–802. https://doi.org/10.1093/hmg/ddaa003.

Quadrato, G., Nguyen, T., Macosko, E.Z., Sherwood, J.L., Min Yang, S., Berger, D.R., Maria, N., Scholvin, J., Goldman, M., Kinney, J.P., Boyden, E.S., Lichtman, J.W., Williams, Z.M., McCarroll, S.A., Arlotta, P., 2017. Cell diversity and network dynamics in photosensitive human brain organoids. Nature 545 (7652), 48–53. https://doi.org/10.1038/nature22047.

Rehbach, K., Fernando, M.B., Brennand, K.J., 2020. Integrating CRISPR engineering and hiPSC-derived 2D disease modeling systems. J. Neurosci. 40 (6), 1176–1185. https://doi.org/10.1523/JNEUROSCI.0518-19.2019.

Robertson, K.A., Nalepa, G., Yang, F.-C., Bowers, D.C., Ho, C.Y., Hutchins, G.D., Croop, J.M., Vik, T.A., Denne, S.C., Parada, L.F., Hingtgen, C.M., Walsh, L.E., Yu, M., Pradhan, K.R., Edwards-Brown, M.K., Cohen, M.D., Fletcher, J.W., Travers, J.B., Staser, K.W., et al., 2012. Imatinib mesylate for plexiform neurofibromas in patients with neurofibromatosis type 1: a phase 2 trial. Lancet Oncol. 13 (12), 1218−1224. https://doi.org/10.1016/S1470-2045(12)70414-X.

Robicsek, O., Karry, R., Petit, I., Salman-Kesner, N., Müller, F.-J., Klein, E., Aberdam, D., Ben-Shachar, D., 2013. Abnormal neuronal differentiation and mitochondrial dysfunction in hair follicle-derived induced pluripotent stem cells of schizophrenia patients. Mol. Psychiatr. 18 (10), 1067−1076. https://doi.org/10.1038/mp.2013.67.

Rojnueangnit, K., Xie, J., Gomes, A., Sharp, A., Callens, T., Chen, Y., Liu, Y., Cochran, M., Abbott, M., Atkin, J., Babovic-Vuksanovic, D., Barnett, C.P., Crenshaw, M., Bartholomew, D.W., Basel, L., Bellus, G., Ben-Shachar, S., Bialer, M.G., Bick, D., et al., 2015. High incidence of noonan syndrome features including short stature and pulmonic stenosis in patients carrying NF1 missense mutations affecting p.Arg1809: genotype−phenotype correlation. Hum. Mutat. 36 (11), 1052−1063. https://doi.org/10.1002/humu.22832.

Santoro, C., Maietta, A., Giugliano, T., Melis, D., Perrotta, S., Nigro, V., Piluso, G., 2015. Arg1809 substitution in neurofibromin: further evidence of a genotype−phenotype correlation in neurofibromatosis type 1. Eur. J. Hum. Genet. 23 (11), 1460−1461. https://doi.org/10.1038/ejhg.2015.93.

Schindeler, A., Little, D.G., 2008. Recent insights into bone development, homeostasis, and repair in type 1 neurofibromatosis (NF1). Bone 42 (4), 616−622. https://doi.org/10.1016/j.bone.2007.11.006.

Sharif, S., Upadhyaya, M., Ferner, R., Majounie, E., Shenton, A., Baser, M., Thakker, N., Evans, D.G., 2011. A molecular analysis of individuals with neurofibromatosis type 1 (NF1) and optic pathway gliomas (OPGs), and an assessment of genotype−phenotype correlations. J. Med. Genet. 48 (4), 256−260. https://doi.org/10.1136/jmg.2010.081760.

Sherekar, M., Han, S.-W., Ghirlando, R., Messing, S., Drew, M., Rabara, D., Waybright, T., Juneja, P., O'Neill, H., Stanley, C.B., Bhowmik, D., Ramanathan, A., Subramaniam, S., Nissley, D.V., Gillette, W., McCormick, F., Esposito, D., 2020. Biochemical and structural analyses reveal that the tumor suppressor neurofibromin (NF1) forms a high-affinity dimer. J. Biol. Chem. 295 (4), 1105−1119. https://doi.org/10.1074/jbc.RA119.010934.

Silva, A.J., Frankland, P.W., Marowitz, Z., Friedman, E., Lazlo, G., Cioffi, D., Jacks, T., Bourtchuladze, R., 1997. A mouse model for the learning and memory deficits associated with neurofibromatosis type I. Nat. Genet. 15 (3), 281−284. https://doi.org/10.1038/ng0397-281.

Simmons, G.W., Pong, W.W., Emnett, R.J., White, C.R., Gianino, S.M., Rodriguez, F.J., Gutmann, D.H., 2011. Neurofibromatosis-1 heterozygosity increases microglia in a spatially and temporally restricted pattern relevant to mouse optic glioma formation and growth. J. Neuropathol. Exp. Neurol. 70 (1), 51−62. https://doi.org/10.1097/NEN.0b013e3182032d37.

Smith, A.M., Dragunow, M., 2014. The human side of microglia. Trends Neurosci. 37 (3), 125−135. https://doi.org/10.1016/j.tins.2013.12.001.

Solga, A.C., Gianino, S.M., Gutmann, D.H., 2014. NG2-cells are not the cell of origin for murine neurofibromatosis-1 (Nf1) optic glioma. Oncogene 33 (3), 289−299. https://doi.org/10.1038/onc.2012.580.

Solga, A.C., Pong, W.W., Kim, K.-Y., Cimino, P.J., Toonen, J.A., Walker, J., Wylie, T., Magrini, V., Griffith, M., Griffith, O.L., Ly, A., Ellisman, M.H., Mardis, E.R., Gutmann, D.H., 2015. RNA sequencing of tumor-associated microglia reveals Ccl5 as a stromal chemokine critical for neurofibromatosis-1 glioma growth. Neoplasia 17 (10), 776−788. https://doi.org/10.1016/j.neo.2015.10.002.

Solga, A.C., Toonen, J.A., Pan, Y., Cimino, P.J., Ma, Y., Castillon, G.A., Gianino, S.M., Ellisman, M.H., Lee, D.Y., Gutmann, D.H., 2017. The cell of origin dictates the temporal course of neurofibromatosis-1 (Nf1) low-grade glioma formation. Oncotarget 8 (29), 47206−47215. https://doi.org/10.18632/oncotarget.17589.

Stowe, I.B., Mercado, E.L., Stowe, T.R., Bell, E.L., Oses-Prieto, J.A., Hernández, H., Burlingame, A.L., McCormick, F., 2012. A shared molecular mechanism underlies the human rasopathies Legius syndrome and Neurofibromatosis-1. Genes Dev. 26 (13), 1421−1426. https://doi.org/10.1101/gad.190876.112.

Sugiyama-Nakagiri, Y., Fujimura, T., Moriwaki, S., 2016. Induction of skin-derived precursor cells from human induced pluripotent stem cells. PLoS One 11 (12). https://doi.org/10.1371/journal.pone.0168451.

Summers, M.A., Rupasinghe, T., Vasiljevski, E.R., Evesson, F.J., Mikulec, K., Peacock, L., Quinlan, K.G.R., Cooper, S.T., Roessner, U., Stevenson, D.A., Little, D.G., Schindeler, A., 2018. Dietary intervention rescues myopathy associated with neurofibromatosis type 1. Hum. Mol. Genet. 27 (4), 577−588. https://doi.org/10.1093/hmg/ddx423.

Sun, T., Hevner, R.F., 2014. Growth and folding of the mammalian cerebral cortex: from molecules to malformations. Nat. Rev. Neurosci. 15 (4), 217−232. https://doi.org/10.1038/nrn3707.

Teotia, P., Hook, M.J.V., Wichman, C.S., Allingham, R.R., Hauser, M.A., Ahmad, I., 2017. Modeling glaucoma: retinal ganglion cells generated from induced pluripotent stem cells of patients with SIX6 risk allele show developmental abnormalities. Stem Cells 35 (11), 2239−2252. https://doi.org/10.1002/stem.2675.

Tokuo, H., Yunoue, S., Feng, L., Kimoto, M., Tsuji, H., Ono, T., Saya, H., Araki, N., 2001. Phosphorylation of neurofibromin by cAMP-dependent protein kinase is regulated via a cellular association of N G,N G-dimethylarginine dimethylaminohydrolase. FEBS Lett. 494 (1−2), 48−53. https://doi.org/10.1016/S0014-5793(01)02309-2.

Toonen, J.A., Anastasaki, C., Smithson, L.J., Gianino, S.M., Li, K., Kesterson, R.A., Gutmann, D.H., 2016. NF1 germline mutation differentially dictates optic glioma formation and growth in neurofibromatosis-1. Hum. Mol. Genet. 25 (9), 1703−1713. https://doi.org/10.1093/hmg/ddw039.

Trevisson, E., Morbidoni, V., Forzan, M., Daolio, C., Fumini, V., Parrozzani, R., Cassina, M., Midena, E., Salviati, L., Clementi, M., 2019. The Arg1038Gly missense variant in the NF1 gene causes a mild phenotype without neurofibromas. Mol. Genet. Genom. Med. 7 (5), e616. https://doi.org/10.1002/mgg3.616.

Upadhyaya, M., Huson, S.M., Davies, M., Thomas, N., Chuzhanova, N., Giovannini, S., Evans, D.G., Howard, E., Kerr, B., Griffiths, S., Consoli, C., Side, L., Adams, D., Pierpont, M., Hachen, R., Barnicoat, A., Li, H., Wallace, P., Van Biervliet, J.P., et al., 2007. An absence of cutaneous neurofibromas associated with a 3-bp inframe deletion in exon 17 of the NF1 gene (c.2970-2972 delAAT): evidence of a clinically significant NF1 genotype-phenotype correlation. Am. J. Hum. Genet. 80 (1), 140−151. https://doi.org/10.1086/510781.

Uthoff, J., Larson, J., Sato, T.S., Hammond, E., Schroeder, K.E., Rohret, F., Rogers, C.S., Quelle, D.E., Darbro, B.W., Khanna, R., Weimer, J.M., Meyerholz, D.K., Sieren, J.C., 2020. Longitudinal phenotype development in a minipig model of neurofibromatosis type 1. Sci. Rep. 10 (1), 5046. https://doi.org/10.1038/s41598-020-61251-4.

Uusitalo, E., Leppävirta, J., Koffert, A., Suominen, S., Vahtera, J., Vahlberg, T., Pöyhönen, M., Peltonen, J., Peltonen, S., 2015. Incidence and mortality of neurofibromatosis: a total population study in Finland. J. Invest. Dermatol. 135 (3), 904−906. https://doi.org/10.1038/jid.2014.465.

van der Wal, E., Herrero-Hernandez, P., Wan, R., Broeders, M., In't Groen, S.L.M., van Gestel, T.J.M., van IJcken, W.F.J., Cheung, T.H., van der Ploeg, A.T., Schaaf, G.J., Pijnappel, W.W.M.P., 2018. Large-scale expansion of human iPSC-derived skeletal muscle cells for disease modeling and cell-based therapeutic strategies. Stem Cell Rep. 10 (6), 1975−1990. https://doi.org/10.1016/j.stemcr.2018.04.002.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R., O'Connell, P., 1990. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62 (1), 187−192. https://doi.org/10.1016/0092-8674(90)90252-A.

Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., Al, E., 1990. Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. Science 249 (4965), 181−186. https://doi.org/10.1126/science.2134734.

Wang, H.-F., Shih, Y.-T., Chen, C.-Y., Chao, H.-W., Lee, M.-J., Hsueh, Y.-P., 2011. Valosin-containing protein and neurofibromin interact to regulate dendritic spine density. J. Clin. Invest. 121 (12), 4820−4837. https://doi.org/10.1172/JCI45677.

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., Maherali, N., Studer, L., Hochedlinger, K., Windrem, M., Goldman, S.A., 2013. Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. Cell Stem Cell 12 (2), 252−264. https://doi.org/10.1016/j.stem.2012.12.002.

Wang, Y., Nicol, G.D., Clapp, D.W., Hingtgen, C.M., 2005. Sensory neurons from Nf1 haploinsufficient mice exhibit increased excitability. J. Neurophysiol. 94 (6), 3670−3676. https://doi.org/10.1152/jn.00489.2005.

Wegscheid, M.L., Anastasaki, C., Hartigan, K.A., Cobb, O.M., Papke, J.B., Traber, J.N., Morris, S.M., Gutmann, D.H., 2021. Patient-derived iPSC-cerebral organoid modeling of the 17q11.2 microdeletion syndrome establishes CRLF3 as a critical regulator of neurogenesis. Cell Rep. 36 (1), 109315. https://doi.org/10.1016/j.celrep.2021.109315.

Welti, S., Fraterman, S., D'Angelo, I., Wilm, M., Scheffzek, K., 2007. The Sec14 homology module of neurofibromin binds cellular glycerophospholipids: mass spectrometry and structure of a lipid complex. J. Mol. Biol. 366 (2), 551−562. https://doi.org/10.1016/j.jmb.2006.11.055.

White, K.A., Swier, V.J., Cain, J.T., Kohlmeyer, J.L., Meyerholz, D.K., Tanas, M.R., Uthoff, J., Hammond, E., Li, H., Rohret, F.A., Goeken, A., Chan, C.-H., Leidinger, M.R., Umesalma, S., Wallace, M.R., Dodd, R.D., Panzer, K., Tang, A.H., Darbro, B.W., et al., 2018. A porcine model of neurofibromatosis type 1 that mimics the human disease. JCI Insight 3 (12). https://doi.org/10.1172/jci.insight.120402.

Wu, J., Patmore, D.M., Jousma, E., Eaves, D.W., Breving, K., Patel, A.V., Schwartz, E.B., Fuchs, J.R., Cripe, T.P., Stemmer-Rachamimov, A.O., Ratner, N., 2014. EGFR−STAT3 signaling promotes formation of malignant peripheral nerve sheath tumors. Oncogene 33 (2), 173−180. https://doi.org/10.1038/onc.2012.579.

Wu, J., Williams, J.P., Rizvi, T.A., Kordich, J.J., Witte, D., Meijer, D., Stemmer-Rachamimov, A.O., Cancelas, J.A., Ratner, N., 2008. Plexiform and dermal neurofibromas and pigmentation are caused by Nf1 loss in desert hedgehog expressing cells. Cancer Cell 13 (2), 105−116. https://doi.org/10.1016/j.ccr.2007.12.027.

Xiang, Y., Tanaka, Y., Patterson, B., Kang, Y.-J., Govindaiah, G., Roselaar, N., Cakir, B., Kim, K.-Y., Lombroso, A.P., Hwang, S.-M., Zhong, M., Stanley, E.G., Elefanty, A.G., Naegele, J.R., Lee, S.-H., Weissman, S.M., Park, I.-H., 2017. Fusion of regionally specified hPSC-derived organoids models human brain development and interneuron migration. Cell Stem Cell 21 (3), 383−398. https://doi.org/10.1016/j.stem.2017.07.007 e7.

Xu, G., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., Weiss, R., 1990. The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 62 (3), 599−608. https://doi.org/10.1016/0092-8674(90)90024-9.

Xu, R., Brawner, A.T., Li, S., Liu, J.-J., Kim, H., Xue, H., Pang, Z.P., Kim, W.-Y., Hart, R.P., Liu, Y., Jiang, P., 2019. OLIG2 drives abnormal neurodevelopmental phenotypes in human iPSC-based organoid and chimeric mouse models of down syndrome. Cell Stem Cell 24 (6), 908−926. https://doi.org/10.1016/j.stem.2019.04.014 e8.

Yang, F.-C., Chen, S., Clegg, T., Li, X., Morgan, T., Estwick, S.A., Yuan, J., Khalaf, W., Burgin, S., Travers, J., Parada, L.F., Ingram, D.A., Clapp, D.W., 2006. Nf1+/− mast cells induce neurofibroma like phenotypes through secreted TGF-β signaling. Hum. Mol. Genet. 15 (16), 2421−2437. https://doi.org/10.1093/hmg/ddl165.

Yang, F.-C., Ingram, D.A., Chen, S., Hingtgen, C.M., Ratner, N., Monk, K.R., Clegg, T., White, H., Mead, L., Wenning, M.J., Williams, D.A., Kapur, R., Atkinson, S.J., Clapp, D.W., 2003. Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for *Nf1+/−* mast cells. J. Clin. Invest. 112 (12), 1851−1861. https://doi.org/10.1172/JCI19195.

Yang, F.-C., Ingram, D.A., Chen, S., Zhu, Y., Yuan, J., Li, X., Yang, X., Knowles, S., Horn, W., Li, Y., Zhang, S., Yang, Y., Vakili, S.T., Yu, M., Burns, D., Robertson, K., Hutchins, G., Parada, L.F., Clapp, D.W., 2008. Nf1-Dependent tumors require a microenvironment containing Nf1+/−- and c-kit-Dependent bone marrow. Cell 135 (3), 437−448. https://doi.org/10.1016/j.cell.2008.08.041.

Yoo, A.S., Sun, A.X., Li, L., Shcheglovitov, A., Portmann, T., Li, Y., Lee-Messer, C., Dolmetsch, R.E., Tsien, R.W., Crabtree, G.R., 2011. MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476 (7359), 228−231. https://doi.org/10.1038/nature10323.

Yuan, T., Liao, W., Feng, N.-H., Lou, Y.-L., Niu, X., Zhang, A.-J., Wang, Y., Deng, Z.-F., 2013. Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurologic function in a rat model of middle cerebral artery occlusion. Stem Cell Res. Ther. 4 (3), 73. https://doi.org/10.1186/scrt224.

Zhang, Y., Sloan, S.A., Clarke, L.E., Caneda, C., Plaza, C.A., Blumenthal, P.D., Vogel, H., Steinberg, G.K., Edwards, M.S.B., Li, G., Duncan, J.A., Cheshier, S.H., Shuer, L.M., Chang, E.F., Grant, G.A., Gephart, M.G.H., Barres, B.A., 2016. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. Neuron 89 (1), 37–53. https://doi.org/10.1016/j.neuron.2015.11.013.

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., Xu, W., Yang, N., Danko, T., Chen, L., Wernig, M., Südhof, T.C., 2013. Rapid single-step induction of functional neurons from human pluripotent stem cells. Neuron 78 (5), 785–798. https://doi.org/10.1016/j.neuron.2013.05.029.

Zhong, X., Gutierrez, C., Xue, T., Hampton, C., Vergara, M.N., Cao, L.-H., Peters, A., Park, T.S., Zambidis, E.T., Meyer, J.S., Gamm, D.M., Yau, K.-W., Canto-Soler, M.V., 2014. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat. Commun. 5 (1), 4047. https://doi.org/10.1038/ncomms5047.

Zhu, Y., Ghosh, P., Charnay, P., Burns, D.K., Parada, L.F., 2002. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 296 (5569), 920–922. https://doi.org/10.1126/science.1068452.

Zhu, Y., Harada, T., Liu, L., Lush, M.E., Guignard, F., Harada, C., Burns, D.K., Bajenaru, M.L., Gutmann, D.H., Parada, L.F., 2005. Inactivation of NF1 in CNS causes increased glial progenitor proliferation and optic glioma formation. Development 132 (24), 5577–5588. https://doi.org/10.1242/dev.02162.

CHAPTER

2 Use of induced pluripotent stem cells to model inflammatory neurodegeneration and repair in multiple sclerosis

Katherine Julian[1], **Jaime Imitola**[2]

[1]*Penn State College of Medicine, Hershey, PA, United States;* [2]*Division of Multiple Sclerosis and Translational Neuroimmunology, UConn Health Comprehensive MS Center, Department of Neurology, School of Medicine, University of Connecticut, Storrs, CT, United States*

Chapter outline

Abstract

Multiple sclerosis (MS) is a neuroinflammatory condition characterized by demyelination and neurodegeneration. Currently, the pathogenesis of MS is not well understood. Animal experiments have previously been used to model the mechanisms behind CNS repair in MS, but interspecies differences pose limitations to studying the disease process in humans. Induced pluripotent stem cells (iPSCs) have more recently generated great enthusiasm as a mechanism to model neuroinflammatory conditions with findings that translate well to humans. Challenges of modeling MS with iPSCs include lack of known genetic variants in oligodendrocytes and neurons, limited supply of cell lines, and varying disease phenotypes present in MS patients. The purpose of this chapter is to outline the progress of using iPSCs to model MS, discuss the challenges within the field, and highlight future directions for modeling MS with iPSCs.

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00001-1

Keywords: Disease phenotype; Immunology; Multiple sclerosis; Neurodegeneration; Neuroinflammation; Neurological diseases; Neuron; Oligodendrocytes; Organoids; Personalized medicine; Pluripotency; Repair; Spinal cord; Stem cells; Translational research.

Introduction

Since the approval of the first disease-modifying therapy (DMT) for multiple sclerosis (MS) in 1993 (Derwenskus, 2011), substantial progress in identifying inflammatory regulators that underly MS pathogenesis has been made. Despite the current treatments available, there is no cure for MS and disease progression in many patients leads to debilitating brain and spinal cord atrophy. Animal studies have helped model many neuroinflammatory conditions, including MS, but the difference in interspecies molecular phenotypes results in significant limitations (Orack et al., 2015). To better model pathogenesis and repair in MS and identify potential treatments, researchers are expanding the use of diverse models and approaches to include patient-derived tissues and patient-derived cells. These models include induced pluripotent stem (iPSCs), which are somatic cells that are rendered pluripotent after the injection of pluripotent transcription factors.

Over the last 25 years, there has been great interest in stem cell research for the treatment of neurological diseases. Early stage clinical trials investigating the therapeutic potential of both neural and mesenchymal stem cells in MS are currently underway (Trounson and McDonald, 2015). Despite the enthusiasm surrounding stem cells, there has been slow progress in translating these findings to the bedside due to scientific hurdles, lack of reproducibility, unrealistic expectations from the public, and need for rigorous clinical trials. The result is that many patients with severe neurological conditions are left searching for cures from fraudulent stem cell clinics based on the hype surrounding stem cell research. Consequently, disseminating nonscientific approaches and unapproved unethical marketing of experimental stem cell therapies poses a significant risk to desperate patients (Julian et al., 2018).

Human-induced pluripotent stem cells (hiPSCs) perhaps provide the most significant potential for better understanding disease pathogenesis and identifying therapeutic targets in neurological diseases. Current experimental evidence shows the human brain has limited intrinsic capacity to exhibit repair and remyelination in MS (Yeunget al., 2019) by both endogenous stem cells or injected exogenous stem cells. In this chapter, we review the role of iPSC modeling in MS translational research and clinical applications, current challenges with iPSC modeling, and future directions within the field of neural stem cell (NSC) research.

Multiple sclerosis background

MS is a neuroinflammatory disease characterized by demyelination, neurodegeneration, and axonal loss (Orack et al., 2015). There are approximately one million people in the United States living with MS, and this disease tends to preferentially affect women and patients aged between 20 and 50 (Wallin et al., 2019). Current evidence suggests that disease prevalence is increasing as new medications increase the lifespan of MS patients (The prevalence, 2019). MS affects patients both physically and emotionally by causing early disability, gait alterations, depression, cognitive decline, and a loss of independence through job loss and financial ruin resulting from medical expenses.

To date, there have been three major disease courses identified for MS. The most common type of MS that affects 85% of patients is relapsing-remitting MS (RRMS), which is characterized by clearly defined attacks of new or worsening neurological symptoms followed by periods of remission. Primary progressive MS (PPMS) is defined as worsening neurologic function from the onset of symptoms without remission. PPMS affects both men and women and usually develops later in life. Lastly, secondary progressive MS (SPMS) is the late transition of an RRMS disease course into a progressive worsening of the clinical condition without periods of remission (Klineova and Lublin, 2018). The varying degree of SPMS onset depends on the initial lesion burden and localization of lesions, which is more severe if the patient exhibits a high lesion burden in the brainstem and the spinal cord (Lublin et al., 2014). Recently, it has been recognized that MS can show disease progression independent of relapses (University of California et al., 2019).

Despite increasing disease prevalence, the pathogenesis of MS is not well understood. Current evidence points to a disease with a complex etiology resulting from an interplay between genetics and environmental factors (Fig. 2.1). Genome-wide association studies over the past 10 years have linked MS to more than 240 genetic variants with a focus primarily on genes expressed on T, cells, B cells, and microglia (Cotsapas and Mitrovic, 2018). Environmental factors include vitamin D deficiency, smoking status, obesity, and exposure to Epstein−Barr virus (Ascherio, 2013). Current hypotheses about MS pathogenesis include the emergence of clones of autoreactive T cells and B cells, persistently activated microglia, the development of inflammatory follicles within the meninges, the intrinsic vulnerability of neurons and axons to injury, and the absence of myelin repair (Orack et al., 2015). This exuberant chronic inflammation compartmentalized to the brain results in both neuronal damage and neurodegeneration, but mechanisms underlying the lack of repair and neurodegeneration in MS are not well understood and under intense investigation.

Pharmacologic interventions that control MS disease progression are known as DMTs, which target the natural history of the disease by decreasing the number of relapses in patients. Randomized-controlled trials demonstrated that starting patients with a defined clinically isolated syndrome (CIS) on certain DMTs delays CIS progression to MS (Gajofatto and Benedetti, 2015). Recent evidence also suggests

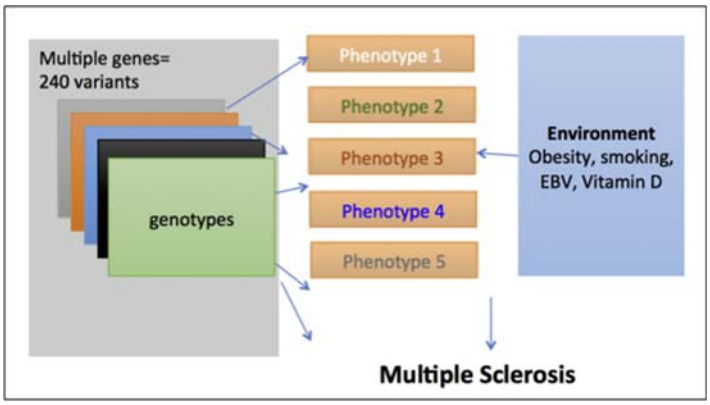


FIGURE 2.1

Multiple sclerosis (MS) is likely the result of an interplay between genetics and environmental factors. To date, over 200 genetic variants in MS have been identified. Certain environmental factors, such as smoking and vitamin D deficiency, also play a role in patient-specific phenotypes.

that starting patients on DMTs early in the disease course may improve MS prognosis by changing the individual trajectory of the patient's disease (Noyes and Weinstock-Guttman, 2013). Disease progression and patient outcomes depend on the burden of lesions at the onset, location of the patient's lesions, and ancestry. For example, patients of African-American ancestry typically show worsening disease progression (Cipriani and Klein, 2019). There is a high degree of clinical heterogeneity among patients; therefore, a better understanding of the disease process at the molecular level within the brain is needed to identify and improve current treatment options in a personalized fashion focusing on the drivers of MS progression.

Utility of animal models to study stem cells in multiple sclerosis

Initial experiments to model neuroinflammation in MS relied on animal models to explore the relationship between the immune system and NSCs. These studies have examined both endogenous stem cell capacity and exogenous stem cell potential. Human and animal brains contain reservoirs, or niches, of NSCs containing both stem cells and support cells located around the ventricle that establish a functional area for neurogenesis, oligodendrogenesis, and gliogenesis. One such niche, the dentate gyrus, gives rise to neurogenesis throughout development and in the adult brain in mice. Another niche, the subventricular zone (SVZ), is also prominent in mice brains and persists throughout prenatal human development. However, in humans,

the activity of both niches tends to disappear after birth, giving remnants with limited neurogenic potential in the adult human brain (Paredes et al., 2016).

In mice, the SVZ gives rise to neuroblasts that migrate to the olfactory bulb to replace dying neurons each day (Lim and Alvarez-Buylla, 2016). NSCs from the SVZ contribute to local myelin repair by differentiating into oligodendrocyte precursor cells that migrate to the site of injury and mature into myelinating oligodendrocytes (Tiane et al., 2019). The specialized niche of the SVZ allows for this process to occur normally. Interestingly, it has been noted that the SVZ is impaired in MS patients (Wang et al., 2008) and mice in the chronic stage of experimental autoimmune encephalomyelitis (EAE), which models acute and chronic stages of MS (Pluchino et al., 2008). Experiments have demonstrated that during the initial stages of the mouse models of MS and EAE, there is prominent activation of the SVZ stem cell niche, leading to initial repair and regeneration of oligodendrocytes, but these repair mechanisms are lost during the chronic phase of the disease (Starossom et al., 2011). The hypothesis was that microglia were involved in the responses of NSCs in the SVZ, as previous studies have suggested that microglia are involved in the responses of the dentate gyrus to inflammation (Ekdahl et al., 2009). To investigate this potential mechanism, microglia were isolated from the SVZ during the acute and chronic phases of EAE and analysis of gene expression among the differentially expressed genes was performed. It was discovered that the lectin ligand Chi3l3, once known as a marker for alternate activation of microglia and macrophages, also activates epidermal growth factor receptor and induces oligodendrogenesis. In animal models of MS, eradication of this protein led to progressive EAE (Starossom et al., 2019).

Several in vivo animal studies have also demonstrated that NSCs are activated by inflammation and respond by changing their proliferation, migration, differentiation, and secretory patterns. In rats, inflammation in the subgranular zone (SGZ) of the hippocampus, an area where neurogenesis occurs, resulted in microglia activation and subsequent lack of neuronal differentiation (Monje et al., 2003). The neurogenic dysfunction was specific to the SGZ niche itself, and blocking microglia activation subsequently restored neurogenesis. The impaired neuronal function was a reversible process fixed by removing the negative inflammatory influence with anti-inflammatory treatment (Monje et al., 2003).

More recent studies have combined the use of iPSCs and animal models to further explore the utility of NSCs during neuroinflammation. Initial experiments using neurospheres showed that disease severity in EAE was ameliorated by directly injecting NSCs into the ventricles or intravenously in the tails of mice (Pluchino et al., 2003). Subsequent studies confirmed that neurospheres act through mechanisms that are more anti-inflammatory than cell replacement in vivo to modulate the responses of T cells in EAE (Pluchino et al., 2005; Merzaban et al., 2015). For example, transplantation of mouse iPSC-NPCs into mice with EAE led to the in situ release of neuroprotective molecules such as leukemia inhibitory factor in addition to chemokines (Gresle et al., 2012). This transplantation resulted in the migration of iPSC-NPCs to the site of myelin damage and subsequent endogenous

myelin protection and improved functionality (Gresle et al., 2012). Because we cannot obtain patient stem cells directly from the human brain, studies use iPSC-derived NSCs to demonstrate feasibility as proof of principle. There are now several ongoing studies in phase I clinical trials in humans to demonstrate the safety of NSCs (Martino, 2020). Although animal studies have allowed clinicians and researchers to study the process and consequences of neuroinflammation, differences in interspecies molecular phenotypes pose limitations and generalizability to humans.

Modeling neurological diseases using stem cells

It is crucial to differentiate mature CNS cells (neurons, astrocytes, oligodendrocytes, and microglia) from stem cells characterized by long-term self-renewal and multilineage differentiation (Wang, 2019). Stem cells can be classified as embryonic, somatic, mesenchymal, and pluripotent stem cells (PSCs). PSCs expressly have provided the opportunity to generate diseased cells in a patient-specific setting due to their self-renewal capacity, potency, and capability to differentiate into derivatives of all three germ layers (Orack et al., 2015). Further, there are two categories of PSCs—embryonic stem cells and iPSCs, the latter of which can be generated through genetic reprogramming by transfection with key pluripotent factors (Oct3/4,SOX2, Klf4, c-myc). These specific transcription factors are associated with pluripotency into somatic cells such as skin fibroblasts or blood cells.

The generation of iPSCs from healthy and diseased donor tissue has provided excellent insight into the molecular mechanisms of various neurological diseases. The comparison of patient-derived cells to healthy control cells allows for the study of abnormal cell characteristics, or *disease phenotypes*, on both a molecular and functional cellular level (Hollingsworth et al., 2017). The collection of phenotypes from patients' iPSCs provides the opportunity to examine neurodegeneration in a unique environment at a personalized level. For instance, mutation-defined iPSCs have been generated to better understand pathogenesis and measure a response to treatment in several neurological syndromes, including Timothy syndrome and Rett syndrome. iPSCs have also been used to model Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis. Interestingly, iPSCs have been used to model both familial forms of AD with previously identified mutations (Yagi et al., 2011), in addition to nonfamilial conditions with sporadic mutations. These models have yielded disease phenotypes shared between patients with the familial disease with exact genetic mutations and sporadic disease. As the knowledge of iPSC phenotypes is expanded, there is a need to catalog and facilitate the association between genotypes and phenotypes. We have contributed with the first repository of phenotypes for all published iPSC models (Hollingsworth et al., 2017). This platform illustrates that previously unrelated genes show similar phenotypes, demonstrating the need to keep expanding iPSCs molecular and cellular phenotypes additional iPSCs-derived models of neurological diseases. For example,

it has recently been shown that astrocytes from patients that harbor NF-kB genetic variants are more pathogenic in vitro (Ponath et al., 2018). These findings provide optimism for modeling other neurodegenerative disorders like MS that do not clearly have a defined genetic mutation identified in neurons and oligodendrocytes (Israel et al., 2012).

Modeling multiple sclerosis with human-derived induced pluripotent stem cells

Recent advancements in genetics and the study of neurological diseases have led to the creation of iPSCs from patients with MS (Fig. 2.2) (Orack et al., 2015). These iPSCs have been used to generate various cell types, including neural progenitor cells (NPCs), mature neuron subtypes, astrocytes, and, more recently, oligodendrocytes. hiPSC-generated microglia have been produced in vitro, and human microglia progenitors have been studied in animal models (Xu et al., 2020). One of the critical arguments against the modeling of MS with patient-derived cells is that MS is a complex disease with multiple pathological phases. Therefore, no single model will recapitulate all the cellular and molecular dynamics from humans due to significant heterogeneity in the disease's pathological and clinical manifestations. The argument against MS can also be applied to complex neurological disorders like AD, yet there are studies modeling AD with patient-derived cells (Majolo et al., 2019). The use of iPSCs to model complex diseases contends that human cells may model several critical aspects of the disease process that cannot adequately be represented using animal experiments.

iPSCs specifically allow researchers to measure the effect of inflammation and neurodegeneration on these vulnerable cell lines. NPCs were modeled in PPMS using patient-derived iPSCs and were found to have inherent defects in neuroprotection and remyelination during active myelin injury in vivo. These authors also found a lack of oligodendrocyte progenitor cell (OPC) differentiation in vitro, indicating that NPCs in PPMS patients may contain inherent differences related to myelinating support (Nicaise et al., 2019). Further, the NPCs from each PPMS patient cell line varied in their responses to proremyelinating compounds, suggesting a range in patient-specific regenerative potential (Nicaise et al., 2017). This work used iPSCs from patients with PPMS to study cellular senescence pathways. It demonstrated an increase in SOX2/p16ink4a (+) cells in demyelinated lesions of PPMS, suggesting that the SOX2 in NPCs may upregulate cellular senescence. Interestingly, the senescence effects were found to be reversed by rapamycin, an mTOR inhibitor, which suggests the mTOR pathway as another contributor and potential target in MS treatment (Nicaise et al., 2019). Another study using iPSCs derived from patients with SPMS demonstrated an insufficient capacity to stimulate OPC in vitro migration to MS lesions, a necessary step before differentiation into mature oligodendrocytes can occur (Lopez-Caraballo et al., 2020).

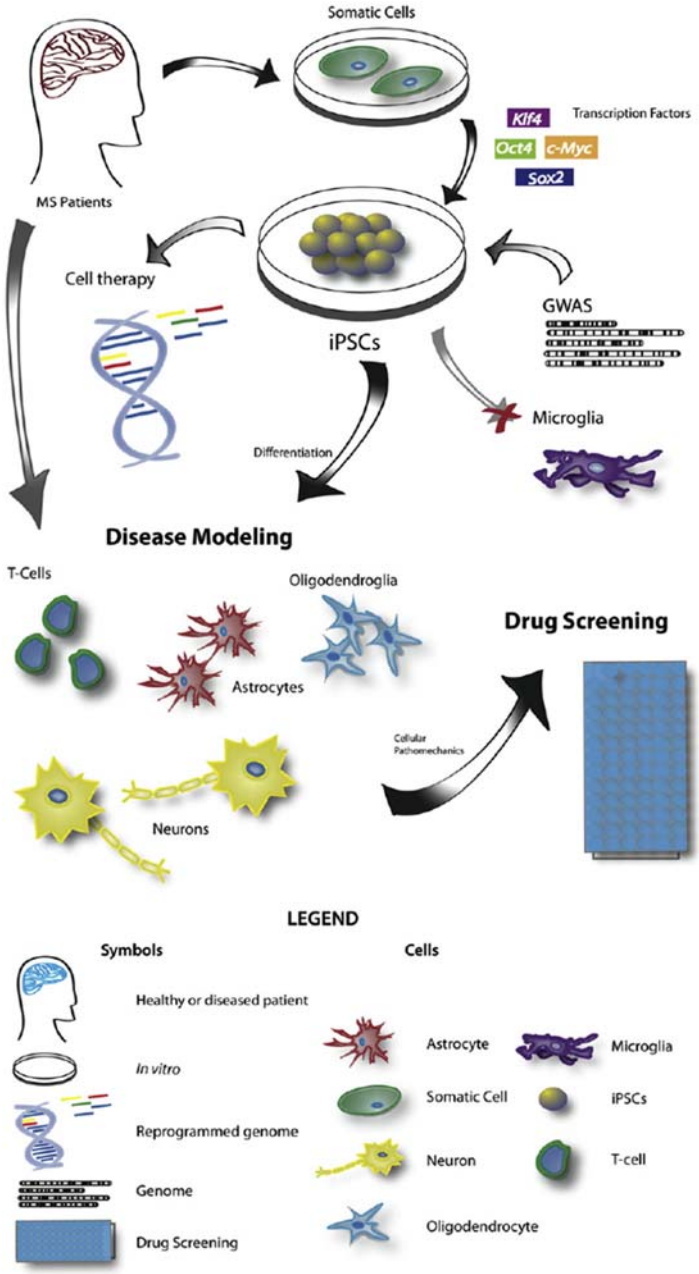


FIGURE 2.2

Human iPSC graphic schematically highlighting the usage and directives of iPSCs, especially pertaining to disease modeling and drug screening of MS using patient-derived cells. *GWAS*, genome-wide association study; *iPSC*, induced pluripotent stem cell; *MS*, multiple sclerosis.

Reproduced from Orack, J. C., et al., 2015. Concise review: modeling multiple sclerosis with stem cell biological platforms: toward functional validation of cellular and molecular phenotypes in inflammation-induced neurodegeneration. Stem Cell. Transl. Med. 4, 252–260.

Additional experiments generated iPSCs from both stable and progressive MS patients. They analyzed chronic exposure to low-dose interferon-gamma on the differentiation of these cell lines into oligodendrocyte-lineage cells. IFNg, a prominent cytokine in MS pathophysiology, possesses a unique ability to upregulate many inflammatory cytokines to augment an immune response. The work demonstrated that sustained exposure to low-dose IFNg resulted in impaired differentiation, especially in the preprogenitor stage, and redirection in lineage phenotype (Morales Pantoja et al., 2020). iPSCs have also illustrated that variants in NF-kB correlate with dysfunctional astrocyte response and increased inflammation (Ponath et al., 2018). This work highlights the importance of iPSCs to help model MS and support or refute current hypotheses surrounding disease pathogenesis.

Challenges in the field

Despite the progress made in modeling MS pathogenesis using iPSCs, researchers still face several key challenges. First, while the utility of modeling the response of nervous system cells to stressors has been discussed, the pathogenesis of MS is complex and no genetic variants in neurons and oligodendrocytes have been identified. Second, it is unknown how many cell lines from how many patients are needed to model biologically relevant MS phenotypes. We recently modeled microglia from several patients with neurological diseases, including MS, and demonstrated that patient-derived microglia-like cells harbor similar genetic susceptibility seen in microglia (Ryan et al., 2017). In addition, the sources of these cell lines, as well as genetically matched controls, are limited. It is currently unknown if donor selection for these cell lines will generate a strong phenotype in vitro. Individual donors may have genetic variants rather than somatic mutations, which can affect modeling the disease phenotype.

Another challenge involves obtaining cells needed to study the disease process in MS. These cells include neurons, oligodendrocytes, and astrocytes from patients affected by the disease. However, studying the disease process directly from human tissue has several limitations. First, the extraction of brain cells via biopsy is invasive and rarely justified. Second, certain neural cells, such as oligodendrocytes and neurons, are postmitotic and require precursors. In contrast to diseases defined by specific, known somatic mutations, MS is characterized by multiple susceptibility genes in immune cells and an interplay between cell-autonomous mechanisms in neural cells and immune dysfunction.

Future Directions

Despite the challenges faced by MS researchers and clinicians, there is ongoing research involving MS modeled with iPSCs. Critical unmet questions in the field

include better understanding the etiology of neurodegeneration in MS, the subsequent lack of repair, overall disease progression, and exploring what aspects of MS can be modeled in vitro. There is also a need to explore what aspects of MS can be modeled and studied through iPSCs and continue to identify both opportunities and limitations of iPSC modeling in MS. Several models of progression favor a multihit pathological cascade that worsens with increasing relapses, neuroaxonal dysfunction in normal-appearing white matter, and cortical lesions. In these events, there is sustained activation of microglia and astrocytes, and persistence of CD8+ T cells in lesions (Popescu et al., 2013).

Recently, three-dimensional (3D) models like cerebral organoids have been introduced as models of human brain development. These cultures are nonvascularized and face criticism; however, it is well known that mature neurons and oligodendrocytes can be generated in organoids. The utility of modeling demyelinating diseases with 3D human cortical organoids derived from hiPSCs has been further demonstrated by generating OPCs and mature oligodendrocytes from organoid models. These models aim to capture the complex microenvironment, cell−cell interactions, and development of the CNS that cannot be recreated using two-dimensional in vitro models (Madhavan et al., 2018; Marton et al., 2019).

Aside from modeling MS with iPSCs to better understand disease pathogenesis, these models should also be used to identify potential therapeutic targets in MS. While DMTs are available to patients with MS, disease progression is inevitable without a cure. One avenue to accomplish this identification is by using stem cells to model nervous system cells' response to stressors contributing to MS pathogenesis and identify neuroprotective compounds. This principle was previously demonstrated by finding that the centrally acting drug benztropine decreased a model of MS disease severity by stimulating progenitor differentiation, which subsequently resulted in increased remyelination and functional recovery (Deshmukh et al., 2013). Next, cellular phenotypes need to be improved to identify therapeutic targets. The phenotypes of synaptic plasticity, axonal growth, myelination, and the function and gene expression of neurons, astrocytes, and OPCs have been previously defined for various neurological conditions (Orack et al., 2015), but further work is needed to determine the MS-related phenotypes in vitro that are predictive of clinical success. Once the knowledge on cellular phenotypes in MS is improved, correlations between these phenotypes and similar phenotypes in neurological diseases with known genotypes can be made (Hollingsworth et al., 2017).

This work emphasizes the utility of patient-derived cells, like iPSCs, as powerful tools to investigate mechanisms of neurodegeneration (Hollingsworth et al., 2017). Compared to other neurological disorders, iPSCs are less frequently used to model MS, perhaps due to the traditional emphasis of studying immunopathogenesis rather than intrinsic neuronal and oligodendroglial mechanisms. As shown in recent research, there is a straightforward utility in using reductionist models to dissect the complexity of MS. It can be argued that these models may be more directly applicable to finding elusive CNS cell-autonomous defects independent of neuronal susceptibility in cortical MS lesions or oligodendrocyte heterogeneity in the human brain.

Conclusions

To advance efforts in understanding MS pathogenesis, new technologies to model MS with patient-derived cells are being deployed. Human 3D culture techniques are gaining traction for modeling unique aspects of the disease, as several labs are generating mini-brain organoids with oligodendrocytes. Additionally, patient-derived monocytes differentiated into microglia represent another robust modeling tool, especially since they conserve the genetic susceptibility of native microglia from MS. Advancing the treatments of MS, especially for progressive disease courses, relies on finding new molecules that avoid progression in MS and will require studying underlying mechanisms of disease pathogenesis within the CNS. Past translational success depended on the identification of fundamental steps of immunopathogenesis in T and B cells. Similar work is underway in investigating repair and neurodegenerative mechanisms in MS and focuses chiefly on neurons, oligodendrocytes, and endogenous stem cells. Combining emerging tools like organoids and iPSCs with novel pathophysiologic ideas will improve our ability to understand the basic principles of neurodegeneration and repair in MS to the extent that it is impossible with animal models.

References

Ascherio, A., 2013. Environmental factors in multiple sclerosis. Expert Rev. Neurother. 13, 3−9.

Cipriani, V.P., Klein, S., 2019. Clinical characteristics of multiple sclerosis in African-Americans. Curr. Neurol. Neurosci. Rep. 19, 87.

Cotsapas, C., Mitrovic, M., 2018. Genome-wide association studies of multiple sclerosis. Clin. Transl. Immunol. 7, e1018.

Derwenskus, J., 2011. Current disease-modifying treatment of multiple sclerosis. Mt. Sinai J. Med. 78, 161−175.

Deshmukh, V.A., et al., 2013. A regenerative approach to the treatment of multiple sclerosis. Nature 502, 327−332.

Ekdahl, C.T., Kokaia, Z., Lindvall, O., 2009. Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience 158, 1021−1029.

Gajofatto, A., Benedetti, M.D., 2015. Treatment strategies for multiple sclerosis: when to start, when to change, when to stop? World J. Clin. Cases 3, 545−555.

Gresle, M.M., et al., 2012. Leukemia inhibitory factor protects axons in experimental autoimmune encephalomyelitis via an oligodendrocyte-independent mechanism. PLoS One 7, e47379.

Hollingsworth, E.W., et al., 2017. iPhemap: an atlas of phenotype to genotype relationships of human iPSC models of neurological diseases. EMBO Mol. Med. 9, 1742−1762.

Israel, M.A., et al., 2012. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature 482, 216−220.

Julian, K., Yuhasz, N., Hollingsworth, E., Imitola, J., 2018. The "growing" reality of the neurological complications of global "stem cell tourism". Semin. Neurol. 38, 176−181.

Klineova, S., Lublin, F.D., 2018. Clinical course of multiple sclerosis. Cold Spring Harb. Perspect. Med. 8.

Lim, D.A., Alvarez-Buylla, A., 2016. The adult ventricular-subventricular zone (V-SVZ) and olfactory bulb (OB) neurogenesis. Cold Spring Harb. Perspect. Biol. 8.

Lopez-Caraballo, L., Martorell-Marugan, J., Carmona-Saez, P., Gonzalez-Munoz, E., 2020. iPS-derived early oligodendrocyte progenitor cells from SPMS patients reveal deficient in vitro cell migration stimulation. Cells 9.

Lublin, F.D., et al., 2014. Defining the clinical course of multiple sclerosis: the 2013 revisions. Neurology 83, 278–286.

Martino, G., IRCCS San Raffaele, 2020. Neural Stem Cell Transplantation in Multiple Sclerosis Patients. Clinical Trials.gov. Accessed February 1, 2021. https://clinicaltrials.gov/ct2/show/NCT03269071.

Madhavan, M., et al., 2018. Induction of myelinating oligodendrocytes in human cortical spheroids. Nat. Methods 15, 700–706.

Majolo, F., Marinowic, D.R., Machado, D.C., Da Costa, J.C., 2019. Important advances in Alzheimer's disease from the use of induced pluripotent stem cells. J. Biomed. Sci. 26, 15.

Marton, R.M., et al., 2019. Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. Nat. Neurosci. 22, 484–491.

Merzaban, J.S., et al., 2015. Cell surface glycan engineering of neural stem cells augments neurotropism and improves recovery in a murine model of multiple sclerosis. Glycobiology 25, 1392–1409.

Monje, M.L., Toda, H., Palmer, T.D., 2003. Inflammatory blockade restores adult hippocampal neurogenesis. Science 302, 1760–1765.

Morales Pantoja, I.E., et al., 2020. iPSCs from people with MS can differentiate into oligodendrocytes in a homeostatic but not an inflammatory milieu. PLoS One 15, e0233980.

Nicaise, A.M., et al., 2017. iPS-derived neural progenitor cells from PPMS patients reveal defect in myelin injury response. Exp. Neurol. 288, 114–121.

Nicaise, A.M., et al., 2019. Cellular senescence in progenitor cells contributes to diminished remyelination potential in progressive multiple sclerosis. Proc. Natl. Acad. Sci. U. S. A. 116, 9030–9039.

Noyes, K., Weinstock-Guttman, B., 2013. Impact of diagnosis and early treatment on the course of multiple sclerosis. Am. J. Manag. Care 19, s321–331.

Orack, J.C., et al., 2015. Concise review: modeling multiple sclerosis with stem cell biological platforms: toward functional validation of cellular and molecular phenotypes in inflammation-induced neurodegeneration. Stem Cell. Transl. Med. 4, 252–260.

Paredes, M.F., et al., 2016. Extensive migration of young neurons into the infant human frontal lobe. Science 354.

Pluchino, S., et al., 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 422, 688–694.

Pluchino, S., et al., 2005. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 436, 266–271.

Pluchino, S., et al., 2008. Persistent inflammation alters the function of the endogenous brain stem cell compartment. Brain 131, 2564–2578.

Ponath, G., et al., 2018. Enhanced astrocyte responses are driven by a genetic risk allele associated with multiple sclerosis. Nat. Commun. 9, 5337.

Popescu, B.F., Pirko, I., Lucchinetti, C.F., 2013. Pathology of multiple sclerosis: where do we stand? Continuum 19, 901–921.

Ryan, K.J., et al., 2017. A human microglia-like cellular model for assessing the effects of neurodegenerative disease gene variants. Sci. Transl. Med. 9.

Starossom, S.C., et al., 2019. Chi3l3 induces oligodendrogenesis in an experimental model of autoimmune neuroinflammation. Nat. Commun. 10, 217.

Starossom, S.C., Imitola, J., Wang, Y., Cao, L., Khoury, S.J., 2011. Subventricular zone microglia transcriptional networks. Brain Behav. Immun. 25, 991−999.

The prevalence of MS in the United States: a population-based estimate using health claims data. Neurology 93, 2019, 688.

Tiane, A., et al., 2019. From OPC to oligodendrocyte: an epigenetic journey. Cells 8.

Trounson, A., McDonald, C., 2015. Stem cell therapies in clinical trials: progress and challenges. Cell Stem Cell 17, 11−22.

University of California, S.F.M.S.E.T, et al., 2019. Silent progression in disease activity-free relapsing multiple sclerosis. Ann. Neurol. 85, 653−666.

Wallin, M.T., et al., 2019. The prevalence of MS in the United States: a population-based estimate using health claims data. Neurology 92, e1029−e1040.

Wang, Y., Imitola, J., Rasmussen, S., O'Connor, K.C., Khoury, S.J., 2008. Paradoxical dysregulation of the neural stem cell pathway sonic hedgehog-Gli1 in autoimmune encephalomyelitis and multiple sclerosis. Ann. Neurol. 64, 417−427.

Wang, X., 2019. Stem cells in tissues, organoids, and cancers. Cell. Mol. Life Sci. 76, 4043−4070.

Xu, R., et al., 2020. Human iPSC-derived mature microglia retain their identity and functionally integrate in the chimeric mouse brain. Nat. Commun. 11, 1577.

Yagi, T., et al., 2011. Modeling familial Alzheimer's disease with induced pluripotent stem cells. Hum. Mol. Genet. 20, 4530−4539.

Yeung, M.S.Y., et al., 2019. Dynamics of oligodendrocyte generation in multiple sclerosis. Nature 566, 538−542.

This page intentionally left blank

CHAPTER

Induced pluripotent stem cell technology to model chronic mountain sickness

3

Helen Zhao[1], **Priti Azad**[1], **Hang Yao**[1], **Wei Wu**[1], **Ila Dwivedi**[1,2], **Gabriel G. Haddad**[1,3]

[1]*Department of Pediatrics (Respiratory Medicine), San Diego, CA, United States;* [2]*Biomedical Sciences Graduate Program, University of California San Diego, San Diego, CA, United States;* [3]*The Rady Children's Hospital, San Diego, CA, United States*

Chapter outline

Abstract

Chronic Mountain Sickness (CMS) was first described by Carlos Monge in 1925 and potentially threatens more than 100 million highlanders during extended living at altitude above 2500 m. CMS is characterized by excessive erythrocytosis, severe hypoxemia and many neurologic manifestations. CMS patients often suffer from increased mortality and morbidity than others at high altitude due to the severe polycythemia, pulmonary hypertension, congestive heart failure and stroke. There are no ideal treatments available to prevent or cure CMS, except for phlebotomy and descent. Since (a) CMS is believed to be a maladaptation to hypoxia and (b) hypoxia is a common feature of many diseases at sea level including cardiovascular diseases and stroke, we believe that investigating the underlying mechanisms of maladaptation to hypoxia in CMS subjects may lead us to a better understanding of many hypoxia-related diseases as well. Over the past two decades, CMS research has shifted from physiology to genetics with the development of new technologies such as DNA and RNA sequencing. However, our understanding at the cellular level remains largely unexplored due to the lack of human samples and techniques. Induced pluripotent stem cell (iPSC) technology can overcome or avoid some of the obstacles from animal studies. Since then, it has been used by many laboratories to study a variety of human diseases including Sickle cell

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00010-2

Disease, Alzheimer's disease, Parkinson's disease and many more. We have recently applied this technology to CMS research. Combining iPSC technology and CMS disease-in-dish model allow us to investigate the cellular and molecular mechanisms of CMS pathobiology in vitro, which may ultimately shed light on developing therapeutic strategies in CMS as well as other hypoxia-related disorders. In this chapter, we focus primarily on trying to understand the pathophysiology and biology of CMS using iPSC-differentiated red blood cells, neurons, and astrocytes.

Keywords: Astrocytes; Chronic mountain sickness; Electrophysiology; Erythrocytosis; Hypoxia; Intracellular pH; Ionic homeostasis; iPSCs; Kir channel; Mitochondrial dysfunction; Na^+ channel; Neurons; Neuropathy; Red blood cells; SENP1.

Introduction

Chronic mountain sickness (CMS) was first described by Carlos Monge in 1925 and is more common in Andeans (up to 15%−20%) than Tibetan and Ethiopian populations. This disease potentially threatens more than 100 million highlanders during extended living at altitude above 2500 m (Monge, 1942, 1953; Monge and Whittembury, 1976). CMS is characterized by excessive erythrocytosis, severe hypoxemia, and many neurologic manifestations, and the severity of CMS is assessed by CMS score (Leon-Velarde et al., 2005). CMS patients often suffer from increased mortality and morbidity than others at high altitude due to the severe polycythemia, pulmonary hypertension, congestive heart failure, and stroke. There are no ideal treatments available to prevent or cure CMS, except for phlebotomy and descent to lower altitudes (Richalet et al., 2005; Rivera-Ch et al., 2007, 2008). Therefore, CMS has been classified as one of the global public health concerns (Leon-Velarde, 2003; Leon-Velarde et al., 2005; Villafuerte and Corante, 2016). Since (a) CMS is believed to be a maladaptation to hypoxia (Penaloza and Arias-Stella, 2007) and (b) hypoxia is a common feature of many diseases at sea level including cardiovascular diseases and stroke, we believe that investigating the underlying mechanisms of maladaptation to hypoxia in CMS subjects may lead us to a better understanding of many hypoxia-related diseases as well.

Over the past two decades, CMS research has shifted from physiology to genetics with the development of new technologies such as DNA and RNA sequencing. Through studies by our group as well as others, we believe that there is a genetic basis for this disease, and the candidate genes found in these studies play an important role in CMS pathology or hypoxia adaptation (Azad et al., 2016, 2017; Jha et al., 2016; Perkins et al., 2012; Ronen et al., 2014; Stobdan et al., 2015, 2017; Udpa et al., 2014; Zhao et al., 2015; Zhou and Haddad, 2013; Zhou et al., 2013). However, our understanding at the cellular level remains largely unexplored due to the lack of human samples and techniques. Although animal studies have improved our understanding of CMS pathobiology to a certain degree, the limitation

of using animals to study human diseases, especially in CMS projects, still exists. Induced pluripotent stem cell (iPSC) technology, first introduced by Dr. Yamanaka in 2007 (Takahashi et al., 2007, 2007b), can overcome or avoid some of the obstacles from animal studies. Since then, it has been used by many laboratories to study a variety of human diseases including sickle cell disease (Papapetrou, 2017), Alzheimer's disease (Kondo et al., 2013), Parkinson's disease (Hargus et al., 2010), autism spectrum disorder (Russo et al., 2019), Rett syndrome (Marchetto et al., 2010), long QT syndrome (Tiscornia et al., 2011), and many more.

We have recently applied this technology to CMS research. Combining iPSC technology and CMS disease-in-dish model allows us to investigate the cellular and molecular mechanisms of CMS pathobiology in vitro, which may ultimately shed light on developing therapeutic strategies in CMS as well as other hypoxia-related disorders. In this chapter, we focus primarily on trying to understand the pathophysiology and biology of CMS. Our laboratory has differentiated iPSCs derived from fibroblasts of CMS and non-CMS individuals into red blood cells, neurons, and astrocytes, in order to provide insight into the mechanisms underlying CMS disease (Fig. 3.1).

Induced pluripotent stem cell–derived in-vitro erythroid system to study hypoxia-induced excessive erythropoiesis in chronic mountain sickness patients

Excessive erythropoiesis (EE) in the Andean population is one of the critical traits of chronic mountain sickness (CMS). This excessive response has deleterious effects since a high hematocrit/hemoglobin increases blood viscosity and reduces blood flow to hypoxia-sensitive organs (brain and heart), often resulting in myocardial infarction and stroke in young adults (Villafuerte and Corante, 2016). Although HIF1α and its target erythropoietin (EPO) are considered major regulators of red blood cell (RBC) production, the role of EPO in EE is still inconclusive (Dainiak et al., 1989; Leon-Velarde et al., 2000). In addition, genetic approaches did not reveal evidence of association between candidate genes (e.g., EPOR, HIF1α, PHD1/2/3) polymorphisms and severe polycythemia (Haase, 2013; Mejia et al., 2005).

Genomic studies have yielded very valuable information (Azad et al., 2016, 2017; Stobdan et al., 2017; Zhou et al., 2013). For example, whole genome sequencing studies done in our laboratory have discovered a nu mber of candidate DNA selected regions containing genes that are differentially expressed in CMS and non-CMS subjects (Stobdan et al., 2017; Zhou et al., 2013). Approximately 1000 genes are potentially involved in high altitude adaptation to hypoxia in different human populations across the world (Azad et al., 2017). Many of these

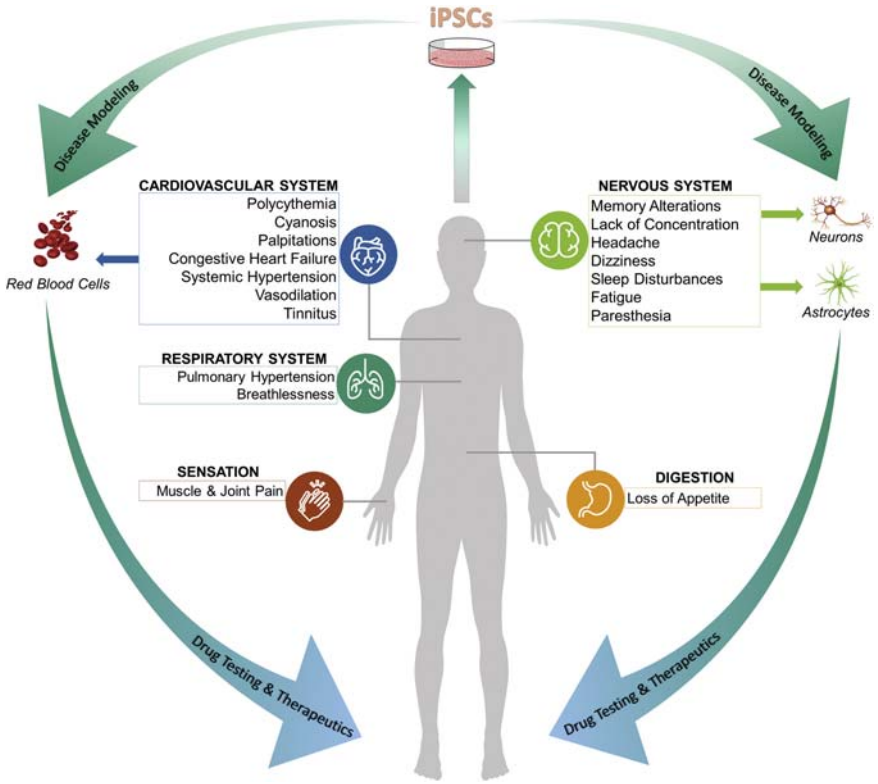


FIGURE 3.1 Modeling chronic mountain sickness (CMS) pathology-using induced pluripotent stem cell (iPSC) technology.

Colored boxes indicate the most common symptoms of CMS. iPSC-derived red blood cells, neurons, and astrocytes are the main focus of this chapter. The ultimate goal of disease-in-a-dish modeling is to reveal novel drug targets and develop therapeutic strategies.

candidate genes belong to various networks that are predicted to regulate biological processes and physiological functions during development. For example, interactions between the candidate genes that were obtained by analyzing three high altitude populations namely Andean, Ethiopian, and Tibetan highlander genomes have suggested that there is coordination in functions between various organs/tissues, such as the central nervous system, the hematological and the cardiovascular systems (Azad et al., 2017). The study by Lorenzo et al. shows how a missense mutation in the EGLN1 gene is functionally linked to erythropoiesis in the Tibetan population (Lorenzo et al., 2014). Similarly, our recent study by Azad et al. (Azad et al., 2016) has built a model of EE from the Andean population using induced pluripotent

stem cell (iPSCs). The differentiation of these iPS cells into RBC under normoxia and hypoxia allowed us to "replicate the EE phenotype in the dish" using cells derived from CMS and non-CMS subjects (Fig. 3.2). Interestingly, under hypoxia at the same stage, CMS cells showed a much higher level of CD235a (40%−80%) as compared to non-CMS as well as the normoxia group (Azad et al., 2016). Fig. 3.2 shows a substantial difference among the different groups in their response to hypoxia. Fig. 3.2 also shows the differential sensitivity of the three populations to graded hypoxia, with a large sensitivity to hypoxia in cells from CMS subjects. CMS cells also produced a significantly higher number of burst-forming units as compared to non-CMS (Azad et al., 2016), suggesting that the erythroid progenitors in the CMS subjects have a higher proliferative potential than the non-CMS subjects. Furthermore, we observed that, while all groups had similar rates of erythroid production in normoxia, CMS cells produced a higher proportion of erythroid cells, matured faster and proliferated more under hypoxia (Azad et al., 2016). We have functionally linked certain genes from our genomic studies (such as SENP1, a deSUMOylase), to the extreme erythrocytosis. In order to determine the role of SENP1 in the polycythemic response to hypoxia in CMS iPS cells, we tested whether SENP1 shRNA diminishes the exuberant response to hypoxia in CMS cells. In spite of the fact that we investigated the role of *only* SENP1, the reduction in the number of RBCs was dramatic (Azad et al., 2016). This iPSC technology has a tremendous potential to help us understand the mechanistic basis of disease. In addition, we were able to recently confirm our findings in iPSCs using native $CD34^+$ cells from the CMS and non-CMS Andean subjects (Azad et al., 2016; Bermudez et al., 2020). Both native $CD34^+$ and iPS cell systems provide different advantages: (a) $CD34^+$ cells provide a better approximation of the inherent capability of native hematopoietic stem cells; (b) iPSCs provide unlimited material for our investigation to understand further mechanisms.

Induced pluripotent stem cell-derived neural cells to study neuropathology in chronic mountain sickness

Besides excessive erythrocytosis, CMS patients also experience many neurological symptoms including headache, dizziness, tinnitus, sleep disturbance, and cognitive impairment (Villafuerte and Corante, 2016). Recently, Bao et al. reported that CMS patients have a blunted cerebral blood flow and exaggerated systemic oxidative-inflammatory stress with impaired cognition (Bailey et al., 2019; Bao et al., 2017). Reduced cerebral blood flow is correlated with increasing severity of CMS scores (Bao et al., 2017). Our laboratory has generated iPSCs from skin biopsies of non-CMS and CMS individuals, respectively (Zhao et al., 2015) and differentiated these into frontal cortex neurons to study the potential differences between CMS and non-CMS subjects. We were able to generate iPSCs, neuronal progenitor cells (NPCs), and neurons from both groups without apparent difference. The cells

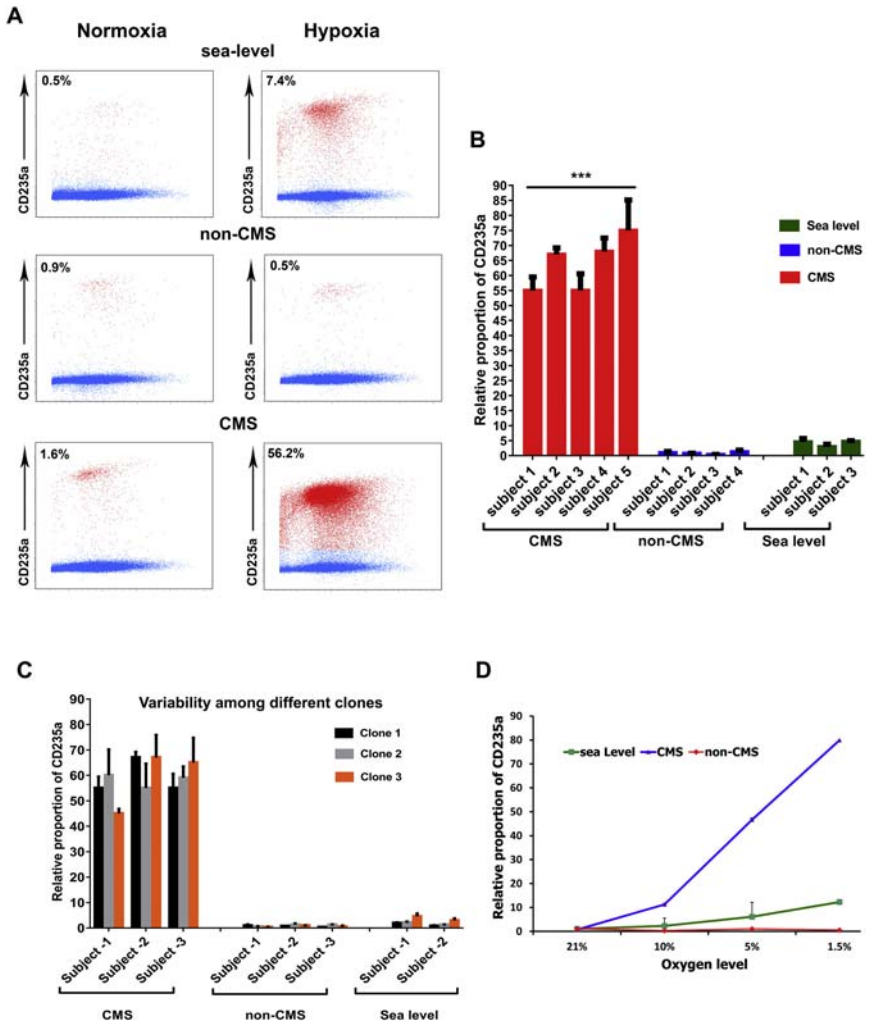


FIGURE 3.2 Erythropoietic response of sea level, non-chronic mountain sickness (CMS), and CMS cells under hypoxia: an exaggerated erythropoietic response in CMS samples.

(A) Cells were cultured as detailed in Azad et al. (2016) and flow cytometric analysis was performed at the EB stage at day 28 to assess the erythropoietic response as measured by CD235a (Glycophorin A) as a marker. The representative FACS plots of sea level, non-CMS and CMS in normoxia (left) and in hypoxia (the right). The dot plot represents the live cells as gated through propidium iodide (PI). $CD235a^{+}$ cells are shown in *red* (gray in print version) along the y-axis and $CD235a^{-}$ cells are shown in *blue* (dark gray in print version). The % in each figure represents the relative proportion of CD235a cells. There are drastic differences between CMS (bottom left) versus the non-CMS (middle) and sea level (top) samples under hypoxia. (B) The figure shows the hypoxic response of CMS

obtained from both populations had a similar morphology at all stages and a similar percentage of neural subtypes (Fig. 3.3A–H), suggesting that cells obtained from both groups have a similar reprogramming and differentiation ability in normoxia.

Global hypoxia is well known to lead to mitochondrial ultrastructural changes (Biswal et al., 2016), and mitochondria play an important role in hypoxia tolerance or adaptation in mammals (Chitra and Boopathy, 2014; Li et al., 2016; Perkins et al., 2012). Interestingly, CMS neurons exhibit altered mitochondrial morphology including a decreased mitochondrial volume density, length with less cristae membrane surface area (Fig. 3.3I–J). OPA1 is a mitochondrial fusion gene and plays an important role in mitochondrial biogenesis, cristae remodeling, and cell death. Under physiological conditions, a similar amount of long- and short-form OPA1 (L-OPA1 and S-OPA1, respectively) are formed (Wai and Langer, 2016), with L-OPA1 promoting fusion and S-OPA1 promoting fission. Under oxygen and glucose deprivation or hypoxia/ischemia, the loss of L-OPA1 and accumulation of S-OPA1 lead to the loss of cristae structures and mitochondrial fragmentation, which disturbs oxidative phosphorylation, reduces ATP production, and predisposes to excitotoxicity and cell death (Barbour and Turner, 2014; Baricault et al., 2007; Brooks et al., 2011; Duvezin-Caubet et al., 2006; Merkwirth et al., 2008; Song et al., 2007). We found a significantly increased S-OPA1 accumulation in CMS neurons (Fig. 3.3K) with decreased ATP levels, indicating an increased fission and energy disruption in CMS neurons. This is the first observation in CMS that would suggest a bioenergetic basis for their disease.

Even though CMS subjects exhibit abnormalities such as mitochondrial fragmentation and a decreased ATP level, there is no increased cell death observed in CMS neurons under normoxic conditions. In fact, CMS patients manifest the pathological phenotype only at high altitude but cease to have major symptoms after descent to low altitude. This observation led us to speculate that hypoxia is the major environmental factor that may uncover the predisposition in CMS patients to a bioenergetic disturbance and disease. With hypoxia exposure, we observed an increased

patients, non-CMS and sea level control subjects as measured by the relative proportion of CD235a after three weeks hypoxia (5% O_2). There is a remarkable significant difference between sea level, non-CMS and CMS under hypoxia. *** represents $P < .001$. (C) The figure depicts the interclonal variability among the subjects. Multiple clones were tested for CMS, non-CMS, and sea level subjects. The y-axis represents the relative proportion of CD235a under hypoxia for different clones. (D) The graph shows the dose response to various (1%, 10%, 5%, and 1.5%) O_2 levels (as measured by proportion of CD235a-y-axis) in CMS, sea level, and non-CMS cells. CMS cells show an exaggerated response under hypoxia.

Azad, P., Zhao, H. W., Cabrales, P. J., Ronen, R., Zhou, D., Poulsen, O., et al., 2016. Senp1 drives hypoxia-induced polycythemia via GATA1 and Bcl-xL in subjects with Monge's disease. J. Exp. Med. 213 (12), 2729–2744. https://doi.org/10.1084/jem.20151920

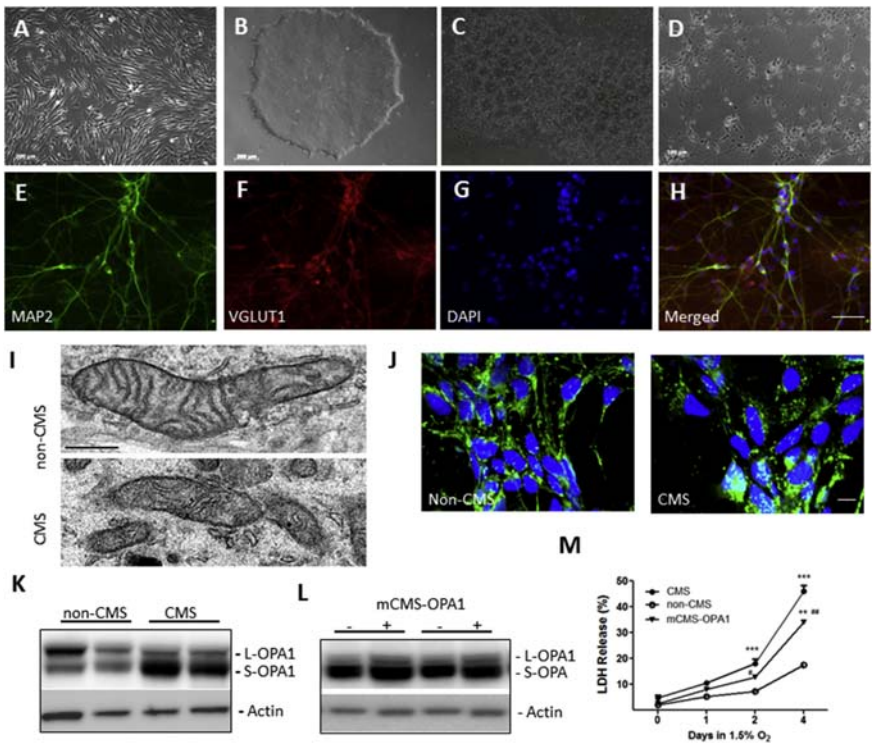


FIGURE 3.3 Altered mitochondrial function in induced pluripotent stem cell (iPSC)-derived chronic mountain sickness (CMS) neurons.

Generation of iPSC-derived neurons through (A) fibroblasts, (B) iPSCs, (C) rosette-containing neuronal progenitor cells, and (D) differentiated two-dimensional neurons. (E–H) Representative images of iPSCs-derived neurons expressing glutamatergic neuronal markers including MAP2 and VGLUT1, Scale bar = 50 μm. Representative (I) transmission electron microscope images and (J) fluorescence images of mitochondria labeled with MitoTracker Green FM from non-CMS and CMS subjects. The scale bar is 250 nm and 10 μm, respectively. (K) A representative blot of OPA1 expression in non-CMS and CMS neurons. (L) A representative blot of OPA1 expression in CMS and mCMS-OPA1 neurons. (M) Survival curve of mCMS-OPA1 neurons as compared to non-CMS and CMS neurons after 1.5% O_2 treatment for four days. ** indicates $P < .01$, *** indicates $P < .001$ as compared to non-CMS; # indicates $P < .05$, ## indicates $P < .01$ as compared to CMS neurons.

Reprinted from Zhao, H. W., Gu, X. Q., Chailangkarn, T., Perkins, G., Callacondo, D., Appenzeller, O., et al., 2015. Altered iPSC-derived neurons' sodium channel properties in subjects with Monge's disease. Neuroscience 288, 187–199. https://doi.org/10.1016/j.neuroscience.2014.12.039, Copyright (2015), with permission from Elsevier. Zhao, H.W., Perkins, G., Yao, H., Callacondo, D., Appenzeller, O., Ellisman, E., La Spada, A.R., Haddad, G.G., 2018. Mitochondrial dysfunction in iPSC-derived neurons of subjects with chronic mountain sickness. J. Appl. Physiol. (1985) 125(3):832–840. https://doi.org/10.1152/japplphysiol.00689.2017, Copyright (2018), Zhao et al. originally published in JAP.

LDH release and caspase 3 activation in CMS neurons. The increased susceptibility to hypoxic stress can be attenuated by OPA1 manipulation. By transfecting CMS neurons with retroviral pMSCV–OPA1 construct that increases L-OPA1 and the balance between L- and S-OPA1 (L-/S-OPA1 ratio), we found that the modified CMS-OPA1 (mCMS-OPA1) neurons have an increased ATP level, a decreased LDH release and caspase 3 activation than CMS cells without modification under hypoxia (Fig. 3.3L–M). These findings, for the first time, confirm that CMS neurons have altered mitochondrial dynamic and function at the cellular level. Even though the abnormalities in mitochondria do not result in significant cell death in CMS neurons, they render CMS neurons more susceptible to hypoxia.

Induced pluripotent stem cell–derived neural cells to study ionic homeostasis in chronic mountain sickness

The abnormal mitochondria morphology and decreased ATP level in CMS neurons suggest that mechanisms relying on the ATP level could be affected accordingly. ATP concentration is critical for the maintenance of ion homeostasis. Indeed, machinery such as ionic transporters and ion channels that maintain the ionic homeostasis in neural cells could be altered and potentially lead to cerebral edema in CMS patients (Bao et al., 2019; Bao et al., 2017).

Disturbances of ionic homeostasis in the brain can lead to many pathological conditions. The underlying mechanisms are related to major membrane proteins such as ion transporters and ion channels. Impairment of acid base transporters can cause the deregulation of intracellular pH (pHi) in neural cells and disturb ionic homeostasis in the brain, such as under hypoxia (Yao et al., 2001; Yao et al., 2003). The availability of human (normal or diseased) neurons and astrocytes derived from iPSCs provides tremendous opportunity for researchers to delineate the role of acid-base transporters in human brain hypoxia-related disorders such as CMS. High altitude residents have increased pHi in their brain cells, perhaps to help facilitate glycolysis, DNA synthesis, and cell cycle progression. Indeed, high altitude natives in Andes show acidic cerebral spinal fluid which is indicative of acidic extracellular pH in the brain of highlanders (Sorensen and Milledge, 1971). It is tempting to speculate that certain mechanisms promote acid extrusion in brain cells resulting in increased pHi but decreased extracellular pH in Andean high altitude natives. To understand the mechanisms underlying this pHi alteration in highlanders, we reprogrammed fibroblast obtained from CMS patients or non-CMS individuals of Andes and differentiated into astrocytes as shown in Fig. 3.4A–D. Using BCECF fluorescent dye, we investigated the pHi regulation in these astrocytes. The results showed that both CMS and non-CMS highlanders have alkalized pHi but CMS did not elevate their pHi to the level as non-CMS did which could be a sign of maladaptation of brain pHi regulation in CMS patients (Fig. 3.4E and F) (Yao et al., 2018). Further investigation revealed that sodium-hydrogen exchanger (NHE) is

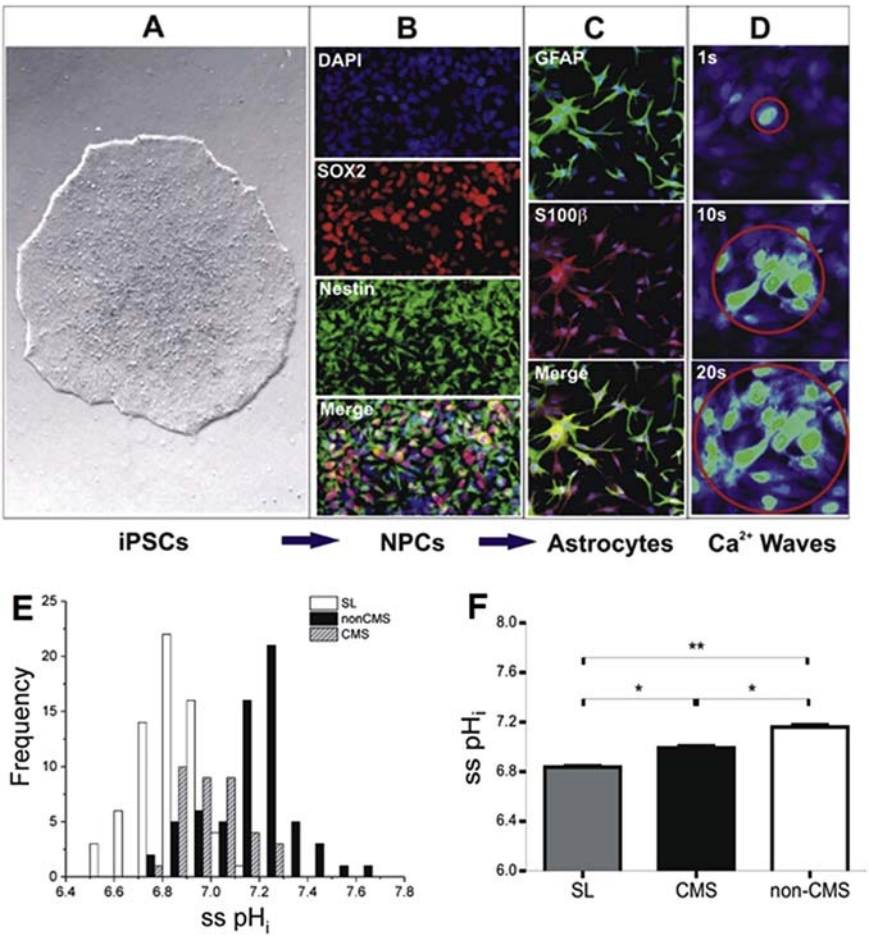


FIGURE 3.4 Generation of astrocytes from human Induced pluripotent stem cells (iPSCs) and its application in the study of steady state pHi in chronic mountain sickness (CMS) patients.

(A–C), iPSCs reprogrammed from a healthy subject were differentiated into neuronal progenitor cells and further into astrocytes. (D), Calcium waves recorded from the iPSC-derived astrocytes. Labels on the top left of each image denote the time after the mechanical stimulation on the first cell in the red circle. (E), Distribution of steady-state pHi in astrocytes derived from sea level, non-CMS, and CMS individuals (bin width: 0.033 pH unit). (F), Comparisons of steady-state pHi among astrocytes obtained from sea level, non-CMS and CMS groups. *: $P < .05$, **: $P < .01$ between each comparison.

Reprinted from Yao, H., Azad, P., Zhao, H. W., Wang, J., Poulsen, O., Freitas, B. C., et al., 2016. The Na+/HCO3− co-transporter is protective during ischemia in astrocytes. Neuroscience https://doi.org/10.1016/j.neuroscience.2016.09.050, Copyright (2016), with permission from Elsevier. Reprinted from Yao, H., Zhao, H., Wang, J., Haddad, G. G., 2018. Intracellular pH regulation in iPSCs-derived astrocytes from subjects with chronic mountain sickness. Neuroscience 375, 25–33. https://doi.org/10.1016/j.neuroscience.2018.02.008, Copyright (2018), with permission from Elsevier.

more active following acid loading in highlanders than in sea level controls which explained the increased steady-state pHi in brain cells of highlanders. Nevertheless, compared with non-CMS astrocytes, CMS cells have lowered pHi, most likely due to an enhanced acid loader activity, such as Na^+-dependent $Cl^-HCO_3^-$ exchanger. Indeed, hypoxia not only activates NHE but also HCO_3^--dependent acid loaders when CO_2/HCO_3^- is available in the in vitro hypoxia environment (Yao et al., 2003). Further efforts may focus on understanding how the acid loaders in astrocytes contribute to the brain pHi deregulation in CMS patients.

Another function of acid base transporters is to maintain ionic homeostasis in the nervous system. In order to move H^+ into/out of a cell, acid-base transporters simultaneously transport other ions, such as Na^+, Ca^{2+}, Cl^-, etc., which makes acid-base transporters an important contributor in many pathophysiological conditions, especially during hypoxia and ischemia (Bevensee and Boron, 2008; Yao and Haddad, 2004). Hence, malfunction of acid-base transporters can lead to impairment of brain tissue in CMS patients. Magnetic resonance imaging studies have shown that ischemic foci and edema occur in the CMS patients (Bao et al., 2017, 2019), suggesting that the high viscosity blood in CMS causes sporadic ischemic infarct in the brain. Using iPSC-derived astrocytes and an ischemic solution that simulates the ischemic infarct rim environment (Yao et al., 2007a, 2007b), we identified enhanced expression of NBCe1 mRNA and protein in human astrocytes under the ischemic condition. Functional studies demonstrated that ischemic solution (IS)-enhanced NBCe1 activities and the inhibition of NBCe1 with its selective blockers S0859 exacerbated the IS-induced astrocytes death, suggesting a significant protective role of NBCe1 in brain ischemia or CMS patients (Yao et al., 2016). Therefore, the impairment of NBCe1 function in the astrocytes during ischemia in highlanders, such as CMS, could lead to the formation of infarct (brain cell death).

Ion channels are another major class of membrane proteins that play critical roles in neural functions. The major neurologic symptoms like headache and migraine that CMS patients have can be caused by or associated with the abnormal electrophysiological functions in the brain. However, understanding the role of electrophysiology in CMS pathophysiology can be challenging due to the complexity of neurological symptoms and the absence of appropriate disease models for CMS. The iPSC model provides us a unique opportunity to study the electrophysiological functions in neural cells derived from CMS patients or non-CMS individuals. We performed whole-cell patch-clamp recordings to compare the neuronal properties between multiple CMS and non-CMS subjects (Zhao et al., 2015). Our results show that CMS neurons were less excitable than non-CMS neurons. The lowered neuronal excitability was caused by the decreased current densities of voltage-gated Na^+ channels and the depolarizing shift of the channel activation, while the passive neuronal properties such as the cell capacitance and resting membrane potential were not significantly changed in CMS subjects (Zhao et al., 2015). Western blot also confirmed that the altered electrical function of Na^+ channels is due to the decreased protein expression of the channels in the CMS neurons. In a more recent study, we showed that there was a reduction in the current densities of the

inwardly rectifying potassium (Kir) channels in CMS astrocytes as compared to non-CMS (Wu et al., 2018). The lowered Kir current densities are caused by the change of total number of Kir4.1 and Kir2.3 channels whereas single channel properties of Kir channels were not altered. iPSC-derived CMS astrocytes exhibited weakened K^+ uptake ability due to the downregulated activity of glial Kir channels (Chever et al., 2010; Djukic et al., 2007; Neusch et al., 2006; Wu et al., 2018). For the first time, these studies provide evidence of dysfunctions of ion channels and neuronal excitability in CMS neurons and astrocytes, indicating that CMS disease can manifest with changes in neural function at molecular and cellular level.

Despite successful application of a two-dimensional (2D) culture system for studying CMS, this approach has major limitations for modeling complex neurological disorders. The 2D iPSC model has limited cell-to-cell interactions and does not resemble the development and microstructure of the human brain in complex three-dimensional (3D) fashion. Recent advances in generating cerebral organoids allow a closer simulation of human cerebral cortex using the iPSC-derived, self-organized 3D human brain organoid (Lancaster and Knoblich, 2014; Pasca et al., 2015). Compared with the 2D culture system, the 3D cerebral organoids technology can closely simulate the key endogenous neurodevelopmental events with a cytoarchitecture that resembles the developing human brain (Pasca, 2018). The 3D human cerebral organoids recapitulate the trajectory of human brain development and maturation which are difficult to accomplish in other species. Indeed, using human 3D organoid model of hypoxic brain injury of prematurity, Pasca et al. identified specific defects in intermediate neural progenitor cells, a cortical cell type associated with the expansion of the human cerebral cortex, and showed that the impairment of neural progenitor cells are related to the unfolded protein response and changes. This finding was further validated using postmortem human brain tissue (Pasca et al., 2019). In addition, using a similar strategy, Daviaud, H et al. demonstrated that transient hypoxic injury resulted in immediate and prolonged apoptosis, with outer radial glial progenitors and neuroblasts/immature neurons suffering larger losses (Daviaud et al., 2019). In addition, recent studies have shown that brain organoids have a transcriptome profile that is close to that in the early human brain (Camp et al., 2015; Nascimento et al., 2019; Pasca et al., 2015; Trujillo et al., 2019). Therefore, iPSC-derived brain organoids represent an optimized approach for modeling neurological disorders such as CMS. The establishment of in vitro human iPSC-derived 3D neural cell culture systems not only helped our investigation of CMS but also many other hypoxia-related disorders, such as neonatal hypoxia injury.

The application of human iPSC-derived neural cells in disease modeling opened a new era for researchers to probe human disease−based studies. Since both 2D and 3D cultures of iPSC-derived neural cells demonstrated reliable functional and morphological properties similar to human neurons and astrocytes, comparisons of neural cells between control and patients will significantly advance our knowledge about etiology and pathophysiology of specific diseases such as CMS and hold promise for developing new therapeutic strategies.

Summary and future directions

Since its initial description in 1925, CMS has threatened over 100 million highlanders living at or above 2500 m worldwide. Consequently, efforts to understand this disease have become increasingly crucial. To date, the advent of new sequencing technologies has bolstered our understanding of the genetics of hypoxia tolerance and high altitude adaptation in humans and animal models. However, the complexity of this disease (i.e., its differential effect on different tissues and cell types) and the limited availability of human native tissue have made investigations at cellular and molecular levels challenging.

In recent years, we have pioneered the use of iPSC technology to examine the pathogenesis of CMS in various affected cell types. Since iPSCs are derived from subjects' somatic cells (i.e., peripheral blood mononuclear cells, fibroblasts), their use enables the examination of disease phenotypes in the context of patient genetic backgrounds. Our lab has focused on deriving and studying RBCs, neurons, and astrocytes, because the cardiovascular and nervous systems have proved particularly susceptible to hypoxia in CMS patients (Fig. 3.1). As a proof of concept, iPSC-derived RBCs from CMS patients exhibited a striking erythropoietic response to hypoxia in vitro (Fig. 3.2), demonstrating that subject- and tissue-specific iPSC-derived cells can help unravel the cellular and molecular basis of CMS. Individuals with CMS are also afflicted by pulmonary hypertension and systemic vascular dysfunction, which can be exacerbated by hypoxemia, hyperviscosity, and/or elevated hemoglobin. In such cases, patient-specific iPSCs can be used to derive endothelial cells in vitro in order to examine the origins of vascular differences between CMS and non-CMS. Peripheral neuropathies in CMS patients, such as mild demyelination (seen in burning feet-burning hand syndromes) (Thomas et al., 2000) and paresthesia, can also be studied by deriving sensory neurons from iPSCs (Guimaraes et al., 2018). IPSC-derived microglia allow us to further investigate the chronic inflammation that occurs due to increased systemic oxidative-inflammatory-nitrosative stress (Muffat et al., 2016). The accelerated rate of iPSC technology development will broaden potential avenues for the exploration of CMS.

Although we have investigated various affected cell types separately in vitro, our data demonstrate that CMS is a systemic disorder that affects multiple organ systems simultaneously. An important conclusion from these studies is that it is important not only to understand the symptoms of Monge's disease but also the maladaptive mechanisms that give rise to it and the adaptations that promote survival in non-CMS individuals. Such knowledge could inform remedies for the maladaptive responses to hypoxia in CMS afflicted individuals.

There are currently no treatments or therapies for CMS. Given its advantages, however, iPSC technology will support the translation of in vitro findings into

potential treatments for symptoms of CMS. There are a number of available drugs that target gene products reported by studies of high-altitude adaptation. For example, PPAR (alpha), EDNRB, EPAS1, and EGLN1, among others, can be targeted by existing drugs or compounds (Ronen et al., 2014). The consistent improvement of gene editing technologies like CRISPR enable target manipulation in vitro, not only confirming which genes or pathways are necessary for CMS pathology but also examining if the disease phenotypes can be rescued. New drugs that target pathways or gene products are crucial for CMS pathology can then be screened in patient-specific iPSC-derived cell cultures. Drugs or drug targets identified in the study of CMS may also be applicable for addressing the progression of other hypoxia-related diseases. Overall, iPSC technology offers an unprecedented opportunity to delve deeper into the cellular and molecular foundations of CMS and to explore novel avenues for treatment.

References

Azad, P., Zhao, H.W., Cabrales, P.J., Ronen, R., Zhou, D., Poulsen, O., et al., 2016. Senp1 drives hypoxia-induced polycythemia via GATA1 and Bcl-xL in subjects with Monge's disease. J. Exp. Med. 213 (12), 2729−2744. https://doi.org/10.1084/jem.20151920.

Azad, P., Stobdan, T., Zhou, D., Hartley, I., Akbari, A., Bafna, V., Haddad, G.G., 2017. High-altitude adaptation in humans: from genomics to integrative physiology. J. Mol. Med. 95 (12), 1269−1282. https://doi.org/10.1007/s00109-017-1584-7.

Bailey, D.M., Brugniaux, J.V., Filipponi, T., Marley, C.J., Stacey, B., Soria, R., et al., 2019. Exaggerated systemic oxidative-inflammatory-nitrosative stress in chronic mountain sickness is associated with cognitive decline and depression. J. Physiol. 597 (2), 611−629. https://doi.org/10.1113/JP276898.

Bao, H., Wang, D., Zhao, X., Wu, Y., Yin, G., Meng, L., et al., 2017. Cerebral edema in chronic mountain sickness: a new finding. Sci. Rep. 7, 43224. https://doi.org/10.1038/srep43224.

Bao, H., Li, R., He, M., Kang, D., Zhao, L., 2019. DTI study on brain structure and cognitive function in patients with chronic mountain sickness. Sci. Rep. 9 (1), 19334. https://doi.org/10.1038/s41598-019-55498-9.

Barbour, J.A., Turner, N., 2014. Mitochondrial stress signaling promotes cellular adaptations. Int. J. Cell Biol. 156020. https://doi.org/10.1155/2014/156020.

Baricault, L., Segui, B., Guegand, L., Olichon, A., Valette, A., Larminat, F., Lenaers, G., 2007. OPA1 cleavage depends on decreased mitochondrial ATP level and bivalent metals. Exp. Cell Res. 313 (17), 3800−3808. https://doi.org/10.1016/j.yexcr.2007.08.008.

Bermudez, D., Azad, P., Figueroa-Mujica, R., Vizcardo-Galindo, G., Corante, N., Guerra-Giraldez, C., et al., 2020. Increased hypoxic proliferative response and gene expression in erythroid progenitor cells of Andean highlanders with chronic mountain sickness. Am. J. Physiol. Regul. Integr. Comp. Physiol. 318 (1), R49−R56. https://doi.org/10.1152/ajpregu.00250.2019.

Bevensee, M.O., Boron, W.F., 2008. Effects of acute hypoxia on intracellular-pH regulation in astrocytes cultured from rat hippocampus. Brain Res. 1193, 143−152. https://doi.org/10.1016/j.brainres.2007.12.002.

Biswal, S., Sharma, D., Kumar, K., Nag, T.C., Barhwal, K., Hota, S.K., Kumar, B., 2016. Global hypoxia induced impairment in learning and spatial memory is associated with precocious hippocampal aging. Neurobiol. Learn. Mem. 133, 157–170. https://doi.org/10.1016/j.nlm.2016.05.011.

Brooks, C., Cho, S.G., Wang, C.Y., Yang, T., Dong, Z., 2011. Fragmented mitochondria are sensitized to Bax insertion and activation during apoptosis. Am. J. Physiol. Cell Physiol. 300 (3), C447–C455. https://doi.org/10.1152/ajpcell.00402.2010.

Camp, J.G., Badsha, F., Florio, M., Kanton, S., Gerber, T., Wilsch-Brauninger, M., et al., 2015. Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. Proc. Natl. Acad. Sci. U. S. A. 112 (51), 15672–15677. https://doi.org/10.1073/pnas.1520760112.

Chever, O., Djukic, B., McCarthy, K.D., Amzica, F., 2010. Implication of Kir4.1 channel in excess potassium clearance: an in vivo study on anesthetized glial-conditional Kir4.1 knock-out mice. J. Neurosci. 30 (47), 15769–15777. https://doi.org/10.1523/JNEUROSCI.2078-10.2010.

Chitra, L., Boopathy, R., 2014. Altered mitochondrial biogenesis and its fusion gene expression is involved in the high-altitude adaptation of rat lung. Respir. Physiol. Neurobiol. 192, 74–84. https://doi.org/10.1016/j.resp.2013.12.007.

Dainiak, N., Spielvogel, H., Sorba, S., Cudkowicz, L., 1989. Erythropoietin and the polycythemia of high-altitude dwellers. Adv. Exp. Med. Biol. 271, 17–21. https://doi.org/10.1007/978-1-4613-0623-8_3.

Daviaud, N., Chevalier, C., Friedel, R.H., Zou, H., 2019. Distinct vulnerability and resilience of human neuroprogenitor subtypes in cerebral organoid model of prenatal hypoxic injury. Front. Cell. Neurosci. 13, 336. https://doi.org/10.3389/fncel.2019.00336.

Djukic, B., Casper, K.B., Philpot, B.D., Chin, L.S., McCarthy, K.D., 2007. Conditional knock-out of Kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. J. Neurosci. 27 (42), 11354–11365. https://doi.org/10.1523/JNEUROSCI.0723-07.2007.

Duvezin-Caubet, S., Jagasia, R., Wagener, J., Hofmann, S., Trifunovic, A., Hansson, A., et al., 2006. Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. J. Biol. Chem. 281 (49), 37972–37979. https://doi.org/10.1074/jbc.M606059200.

Guimaraes, M.Z.P., De Vecchi, R., Vitoria, G., Sochacki, J.K., Paulsen, B.S., Lima, I., et al., 2018. Generation of iPSC-derived human peripheral sensory neurons releasing substance P elicited by TRPV1 agonists. Front. Mol. Neurosci. 11, 277. https://doi.org/10.3389/fnmol.2018.00277.

Haase, V.H., 2013. Regulation of erythropoiesis by hypoxia-inducible factors. Blood Rev. 27 (1), 41–53. https://doi.org/10.1016/j.blre.2012.12.003.

Hargus, G., Cooper, O., Deleidi, M., Levy, A., Lee, K., Marlow, E., et al., 2010. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proc. Natl. Acad. Sci. U. S. A. 107 (36), 15921–15926. https://doi.org/10.1073/pnas.1010209107.

Jha, A.R., Zhou, D., Brown, C.D., Kreitman, M., Haddad, G.G., White, K.P., 2016. Shared genetic signals of hypoxia adaptation in Drosophila and in high-altitude human populations. Mol. Biol. Evol. 33 (2), 501–517. https://doi.org/10.1093/molbev/msv248.

Kondo, T., Asai, M., Tsukita, K., Kutoku, Y., Ohsawa, Y., Sunada, Y., et al., 2013. Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular

Abeta and differential drug responsiveness. Cell Stem Cell 12 (4), 487–496. https://doi.org/10.1016/j.stem.2013.01.009.

Lancaster, M.A., Knoblich, J.A., 2014. Generation of cerebral organoids from human pluripotent stem cells. Nat. Protoc. 9 (10), 2329–2340. https://doi.org/10.1038/nprot.2014.158.

Leon-Velarde, F., Gamboa, A., Chuquiza, J.A., Esteba, W.A., Rivera-Chira, M., Monge, C.C., 2000. Hematological parameters in high altitude residents living at 4,355, 4,660, and 5,500 meters above sea level. High Alt. Med. Biol. 1 (2), 97–104. https://doi.org/10.1089/15270290050074233.

Leon-Velarde, F., Maggiorini, M., Reeves, J.T., Aldashev, A., Asmus, I., Bernardi, L., et al., 2005. Consensus statement on chronic and subacute high altitude diseases. High Alt. Med. Biol. 6 (2), 147–157. https://doi.org/10.1089/ham.2005.6.147.

Leon-Velarde, F., 2003. Pursuing international recognition of chronic mountain sickness. High Alt. Med. Biol. 4 (2), 256–259. https://doi.org/10.1089/152702903322022857.

Li, Q., Lin, K., Sun, H., Liu, S., Huang, K., Huang, X., et al., 2016. Mitochondrial haplogroup M9a1a1c1b is associated with hypoxic adaptation in the Tibetans. J. Hum. Genet. 61 (12), 1021–1026. https://doi.org/10.1038/jhg.2016.95.

Lorenzo, F.R., Huff, C., Myllymaki, M., Olenchock, B., Swierczek, S., Tashi, T., et al., 2014. A genetic mechanism for Tibetan high-altitude adaptation. Nat. Genet. 46 (9), 951–956. https://doi.org/10.1038/ng.3067.

Marchetto, M.C., Carromeu, C., Acab, A., Yu, D., Yeo, G.W., Mu, Y., et al., 2010. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell 143 (4), 527–539. https://doi.org/10.1016/j.cell.2010.10.016.

Mejia, O.M., Prchal, J.T., Leon-Velarde, F., Hurtado, A., Stockton, D.W., 2005. Genetic association analysis of chronic mountain sickness in an Andean high-altitude population. Haematologica 90 (1), 13–19.

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F.T., et al., 2008. Prohibitions control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes Dev. 22 (4), 476–488. https://doi.org/10.1101/gad.460708.

Monge, C.C., Whittembury, J., 1976. Chronic mountain sickness. Johns Hopkins Med. J. 139 (Suppl. L), 87–89.

Monge, C., 1942. Life in the Andes and chronic mountain sickness. Science 95 (2456), 79–84. https://doi.org/10.1126/science.95.2456.79.

Monge, C., 1953. Chronic mountain sickness in America. An Fac Med Lima 36 (4), 544–562.

Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., et al., 2016. Efficient derivation of microglia-like cells from human pluripotent stem cells. Nat. Med. 22 (11), 1358–1367. https://doi.org/10.1038/nm.4189.

Nascimento, J.M., Saia-Cereda, V.M., Sartore, R.C., da Costa, R.M., Schitine, C.S., Freitas, H.R., et al., 2019. Human cerebral organoids and fetal brain tissue share proteomic similarities. Front. Cell Dev. Biol. 7, 303. https://doi.org/10.3389/fcell.2019.00303.

Neusch, C., Papadopoulos, N., Muller, M., Maletzki, I., Winter, S.M., Hirrlinger, J., et al., 2006. Lack of the Kir4.1 channel subunit abolishes K+ buffering properties of astrocytes in the ventral respiratory group: impact on extracellular K+ regulation. J. Neurophysiol. 95 (3), 1843–1852. https://doi.org/10.1152/jn.00996.2005.

Papapetrou, E.P., 2017. Gene and cell therapy for beta-thalassemia and Sickle cell disease with induced pluripotent stem cells (iPSCs): the next frontier. Adv. Exp. Med. Biol. 1013, 219–240. https://doi.org/10.1007/978-1-4939-7299-9_9.

Pasca, A.M., Sloan, S.A., Clarke, L.E., Tian, Y., Makinson, C.D., Huber, N., et al., 2015. Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. Nat. Methods 12 (7), 671–678. https://doi.org/10.1038/nmeth.3415.

Pasca, A.M., Park, J.Y., Shin, H.W., Qi, Q., Revah, O., Krasnoff, R., et al., 2019. Human 3D cellular model of hypoxic brain injury of prematurity. Nat. Med. 25 (5), 784–791. https://doi.org/10.1038/s41591-019-0436-0.

Pasca, S.P., 2018. Building three-dimensional human brain organoids. Nat. Neurosci. https://doi.org/10.1038/s41593-018-0107-3.

Penaloza, D., Arias-Stella, J., 2007. The heart and pulmonary circulation at high altitudes: healthy highlanders and chronic mountain sickness. Circulation 115 (9), 1132–1146. https://doi.org/10.1161/CIRCULATIONAHA.106.624544.

Perkins, G., Hsiao, Y.H., Yin, S., Tjong, J., Tran, M.T., Lau, J., et al., 2012. Ultrastructural modifications in the mitochondria of hypoxia-adapted *Drosophila melanogaster*. PLoS One 7 (9), e45344. https://doi.org/10.1371/journal.pone.0045344.

Richalet, J.P., Rivera, M., Bouchet, P., Chirinos, E., Onnen, I., Petitjean, O., et al., 2005. Acetazolamide: a treatment for chronic mountain sickness. Am. J. Respir. Crit. Care Med. 172 (11), 1427–1433. https://doi.org/10.1164/rccm.200505-807OC.

Rivera-Ch, M., Leon-Velarde, F., Huicho, L., 2007. Treatment of chronic mountain sickness: critical reappraisal of an old problem. Respir. Physiol. Neurobiol. 158 (2–3), 251–265. https://doi.org/10.1016/j.resp.2007.05.003.

Rivera-Ch, M., Huicho, L., Bouchet, P., Richalet, J.P., Leon-Velarde, F., 2008. Effect of acetazolamide on ventilatory response in subjects with chronic mountain sickness. Respir. Physiol. Neurobiol. 162 (3), 184–189. https://doi.org/10.1016/j.resp.2008.06.010.

Ronen, R., Zhou, D., Bafna, V., Haddad, G.G., 2014. The genetic basis of chronic mountain sickness. Physiology 29 (6), 403–412. https://doi.org/10.1152/physiol.00008.2014.

Russo, F.B., Brito, A., de Freitas, A.M., Castanha, A., de Freitas, B.C., Beltrao-Braga, P.C.B., 2019. The use of iPSC technology for modeling Autism Spectrum Disorders. Neurobiol. Dis. 130, 104483. https://doi.org/10.1016/j.nbd.2019.104483.

Song, Z., Chen, H., Fiket, M., Alexander, C., Chan, D.C., 2007. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J. Cell Biol. 178 (5), 749–755. https://doi.org/10.1083/jcb.200704110.

Sorensen, S.C., Milledge, J.S., 1971. Cerebrospinal fluid acid-base composition at high altitude. J. Appl. Physiol. 31 (1), 28–30. https://doi.org/10.1152/jappl.1971.31.1.28.

Stobdan, T., Zhou, D., Ao-Ieong, E., Ortiz, D., Ronen, R., Hartley, I., et al., 2015. Endothelin receptor B, a candidate gene from human studies at high altitude, improves cardiac tolerance to hypoxia in genetically engineered heterozygote mice. Proc. Natl. Acad. Sci. U. S. A. 112 (33), 10425–10430. https://doi.org/10.1073/pnas.1507486112.

Stobdan, T., Akbari, A., Azad, P., Zhou, D., Poulsen, O., Appenzeller, O., et al., 2017. New insights into the genetic basis of Monge's disease and adaptation to high-altitude. Mol. Biol. Evol. 34 (12), 3154–3168. https://doi.org/10.1093/molbev/msx239.

Takahashi, K., Okita, K., Nakagawa, M., Yamanaka, S., 2007a. Induction of pluripotent stem cells from fibroblast cultures. Nat. Protoc. 2 (12), 3081–3089. https://doi.org/10.1038/nprot.2007.418.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007b. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861−872. https://doi.org/10.1016/j.cell.2007.11.019.

Thomas, P.K., King, R.H., Feng, S.F., Muddle, J.R., Workman, J.M., Gamboa, J., et al., 2000. Neurological manifestations in chronic mountain sickness: the burning feet-burning hands syndrome. J. Neurol. Neurosurg. Psychiatry 69 (4), 447−452. https://doi.org/10.1136/jnnp.69.4.447.

Tiscornia, G., Monserrat, N., Izpisua Belmonte, J.C., 2011. Modelling long QT syndrome with iPS cells: be still, my beating heart. Circ. Res. 108 (6), 648−649. https://doi.org/10.1161/RES.0b013e318216f0db.

Trujillo, C.A., Gao, R., Negraes, P.D., Gu, J., Buchanan, J., Preissl, S., et al., 2019. Complex oscillatory waves emerging from cortical organoids model early human brain network development. Cell Stem Cell 25 (4), 558−569 e557. https://doi.org/10.1016/j.stem.2019.08.002.

Udpa, N., Ronen, R., Zhou, D., Liang, J., Stobdan, T., Appenzeller, O., et al., 2014. Whole genome sequencing of Ethiopian highlanders reveals conserved hypoxia tolerance genes. Genome Biol. 15 (2), R36. https://doi.org/10.1186/gb-2014-15-2-r36.

Villafuerte, F.C., Corante, N., 2016. Chronic mountain sickness: clinical aspects, etiology, management, and treatment. High Alt. Med. Biol. 17 (2), 61−69. https://doi.org/10.1089/ham.2016.0031.

Wai, T., Langer, T., 2016. Mitochondrial dynamics and metabolic regulation. Trends Endocrinol. Metabol. 27 (2), 105−117. https://doi.org/10.1016/j.tem.2015.12.001.

Wu, W., Yao, H., Zhao, H.W., Wang, J., Haddad, G.G., 2018. Down-regulation of inwardly rectifying K(+) currents in astrocytes derived from patients with Monge's disease. Neuroscience 374, 70−79. https://doi.org/10.1016/j.neuroscience.2018.01.016.

Yao, H., Haddad, G.G., 2004. Calcium and pH homeostasis in neurons during hypoxia and ischemia. Cell Calcium 36 (3−4), 247−255. https://doi.org/10.1016/j.ceca.2004.02.013.

Yao, H., Gu, X.Q., Douglas, R.M., Haddad, G.G., 2001. Role of Na(+)/H(+) exchanger during O(2) deprivation in mouse CA1 neurons. Am. J. Physiol. Cell Physiol. 281 (4), C1205−C1210. https://doi.org/10.1152/ajpcell.2001.281.4.C1205.

Yao, H., Gu, X.Q., Haddad, G.G., 2003. The role of HCO3(-)-dependent mechanisms in pHi regulation during O2 deprivation. Neuroscience 117 (1), 29−35.

Yao, H., Shu, Y., Wang, J., Brinkman, B.C., Haddad, G.G., 2007. Factors influencing cell fate in the infarct rim. J. Neurochem. 100 (5), 1224−1233. https://doi.org/10.1111/j.1471-4159.2006.04299.x.

Yao, H., Sun, X., Gu, X., Wang, J., Haddad, G.G., 2007b. Cell death in an ischemic infarct rim model. J. Neurochem. 103 (4), 1644−1653. https://doi.org/10.1111/j.1471-4159.2007.04879.x.

Yao, H., Azad, P., Zhao, H.W., Wang, J., Poulsen, O., Freitas, B.C., et al., 2016. The Na+/HCO3- co-transporter is protective during ischemia in astrocytes. Neuroscience. https://doi.org/10.1016/j.neuroscience.2016.09.050.

Yao, H., Zhao, H., Wang, J., Haddad, G.G., 2018. Intracellular pH regulation in iPSCs-derived astrocytes from subjects with chronic mountain sickness. Neuroscience 375, 25−33. https://doi.org/10.1016/j.neuroscience.2018.02.008.

Zhao, H.W., Gu, X.Q., Chailangkarn, T., Perkins, G., Callacondo, D., Appenzeller, O., et al., 2015. Altered iPSC-derived neurons' sodium channel properties in subjects with Monge's disease. Neuroscience 288, 187–199. https://doi.org/10.1016/j.neuroscience.2014.12.039.

Zhou, D., Haddad, G.G., 2013. Genetic analysis of hypoxia tolerance and susceptibility in Drosophila and humans. Annu. Rev. Genom. Hum. Genet. 14, 25–43. https://doi.org/10.1146/annurev-genom-091212-153439.

Zhou, D., Udpa, N., Ronen, R., Stobdan, T., Liang, J., Appenzeller, O., et al., 2013. Whole-genome sequencing uncovers the genetic basis of chronic mountain sickness in Andean highlanders. Am. J. Hum. Genet. 93 (3), 452–462. https://doi.org/10.1016/j.ajhg.2013.07.011.

This page intentionally left blank

CHAPTER

4 Induced pluripotent stem cells for modeling Noonan, Noonan Syndrome with Multiple Lentigines, and Costello Syndromes

A. Gulhan Ercan-Sencicek[1,2], Saravanakkumar Chennappan[1], Kelly Aromalaran[1], Maria Irene Kontaridis[1,3,4,5]

[1]*Masonic Medical Research Institute, Utica, NY, United States;* [2]*Department of Neurosurgery, Program on Neurogenetics, Yale School of Medicine, Yale University, New Haven, CT, United States;* [3]*Department of Medicine, Division of Cardiology, Beth Israel Deaconess Medical Center, Boston, MA, United States;* [4]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, United States;* [5]*Harvard Stem Cell Institute, Harvard University, Cambridge, MA, United States*

Chapter outline

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00007-2

Abstract

Germline mutations in genes that reside along the canonical RAS-mitogen-activated protein kinase (MAPK) signaling pathway lead to disorders known as RASopathies, rare autosomal dominant diseases that include Noonan syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSMLs), and Costello Syndrome (CS). Clinical presentation of these disorders is similar, systemically affecting multiple tissues and organs including the heart and brain, yet distinct. However, while differential activation of RAS-MAPK and RAS-PI3K-AKT signaling has been attributed to the cause of NS, NSML, and CS, the nuances for how each of these pathways elicits distinct disease characteristics remains unclear. To begin to address these concerns and to elucidate the mechanisms of these disorders, animal model systems have been generated and utilized. However, these nonhuman model systems do not always effectively translate to human disease function and/or therapeutic efficacy in patients. To circumvent these issues, inducible pluripotent stem cells (iPSCs) along with clustered regularly interspaced short palindromic repeats associated protein 9 genome editing technology has recently been developed to study disease mechanisms associated with RASopathies. Here, we will discuss the current iPSC-related research as it pertains to NS, NSML, and CS, with particular focus on how this technology has advanced our understanding of RASopathies and the cardiac and neurodevelopmental defects associated with mutations in patients.

Keywords: AKT/mTOR; Cardiomyocytes; Costello Syndrome; CRISPR-Cas; Disease modeling; HRAS; iPSCs; Noonan syndrome; Noonan Syndrome with Multiple Lentigines; PTPN11/SHP2; RAF1; RAS-MAPK; RAS-PI3K-AKT; RASopathy; Stem cell technology.

Glossary of terms

ADHD	Attention deficit hyperactivity disorder
AKT	Protein Kinase B
ASD	Atrial septal defects
B-ALL	B-cell Acute Lymphoblastic Leukemia
BIX02189	Selective MEK5 and ERK5 inhibitor
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
CBL	*Casitas b-lineage lymphoma,* Proto-Oncogene
CBL0137	Metabolically stable curaxin that activates p53, inhibits NF-κB, functionally inactivates the facilitates chromatin transcription complex (FACT)
CHD	Congenital heart disease
CMs	Cardiomyocytes
CRSIPR-Cas9	Clustered regularly interspaced short palindromic repeats associated protein 9
CS	Costello Syndrome

DORV	Double-outlet right ventricle
EC	Endocardial cushion
EMT	Epithelial to mesenchymal transition
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGF4	Fibroblast growth factor 4
Gab1	GRB2 Associated Binding Protein 1
GAPs	GTPase activating proteins
GDP	Guanosine-5′-diphosphate
GEFs	Guanine nucleotide exchange factors
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOF	Gain-of-function
GSK-3b	Glycogen synthase kinase 3 beta
GTP	Guanosine-5′-triphosphate
GTPase	Guanosine triphosphate hydrolase
HCM	Hypertrophic cardiomyopathy
HRAS	Harvey rat sarcoma viral oncogene homolog, Proto-Oncogene, GTPase
iPSCs	inducible pluripotent stem cells
IRS-1	Insulin receptor substrate 1
JAK/STAT	The Janus kinase (JAK)-signal transducer and activator of transcription (STAT)
JMML	Juvenile Myelomonocytic Leukemia
JQ1	Thienotriazolodiazepine and a potent inhibitor of the BET family of bromo-domain proteins
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog, Proto-Oncogene, GTPase
LOF	Loss-of-Function
LZTR1	Leucine Zipper Like Transcription Regulator 1
MAPK	Mitogen-activated protein kinase
MEK	MAPK Kinase
miRs	Mirco-RNAs
MPD	Myeloproliferative disorder
MRAS	Muscle RAS oncogene homolog and R-RAS3
mTOR	The mammalian target of rapamycin
MYC	MYC Proto-Oncogene, BHLH Transcription Factor
NASEM	National Academies of Sciences, Engineering, and Medicine
NF-κB	Nuclear factor-κB
NFATc4	Nuclear Factor Of Activated T Cells 4
NPPA	Natriuretic Peptide A
NRAS	Neuroblastoma RAS Viral Oncogene Homolog, Proto-Oncogene, GTPase
NS	Noonan syndrome
NS-LAH	NS with loose anagen hair
NS/JMML	NS-associated JMML
NSML	Noonan Syndrome with Multiple Lentigines
$p90^{RSK}$	p90 ribosomal s6 kinase
PAM	Proto-spacer adjacent motif
PD98059	Non-ATP competitive MEK inhibitor

PI3Ks	Phosphoinositide 3-kinases
PPP1CB	Protein Phosphatase 1 Catalytic Subunit Beta
PTP	Protein tyrosine phosphatase
PTPN11	Protein tyrosine phosphatase non−receptor type 11 gene
PVS	Pulmonary valve stenosis
pY	Phosphotyrosyl
Pyr3	Transient receptor potential channel 3 (TRPC3) inhibitor
RAF1	Raf-1 Proto-Oncogene, Serine/Threonine Kinase
RIT1	Ras Like Without CAAX 1
RRAS	RAS related
RTKs	Receptor tyrosine kinases
scRNAseq	Single cell RNAseq
SHOC2	SHOC2 Leucine Rich Repeat Scaffold Protein
SHP2	Src homology region 2 (SH2)-containing protein tyrosine phosphatase 2
siRNA	Small interfering RNA
SNAI2	Snail Family Transcriptional Repressor 2
SOS	Son of sevenless, Ras/Rac Guanine Nucleotide Exchange Factor
TGF-β	Transforming growth factor beta
TP53	Tumor protein p53, also known as p53
TSC2	Tuberous sclerosis complex
U0126	Selective inhibitor of MAP kinase kinases, MEK1 and MEK2
VSD	Ventricular septal defects
Wnt3a	Wnt Family Member 3A

Preface

The causal mechanisms and potential treatment options available for patients with congenital disorders remain largely unknown, principally because of technological limitations and unclear determinations for best practices in therapeutic approaches for treating these patients. Now, human inducible pluripotent stem cells (iPSCs) provide hope for these patients and their families. Together with the development of clustered regularly interspaced short palindromic repeats associated protein 9 (CRISPR-Cas9) genome editing technology, iPSCs may not only facilitate identification of novel genes underlying human disorders, but could also provide an increased understanding of disease etiologies to facilitate novel, more effective, therapeutic interventions. Here, we will review the use of this novel technology as it pertains to RASopathy disorders, a group of autosomal-dominant developmental disorders that affect genes that reside along the canonical RAS-mitogen-activated protein kinase (MAPK) signaling pathway (hereby referred to as RAS-MAPK). Specifically, we will discuss the progress made using iPSCs and CRISPR-Cas9 in evaluating Noonan syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSMLs; formerly LEOPARD syndrome), and Costello Syndrome (CS). We will also discuss current applications, limitations, and future directions for modeling RASopathies using iPSC technology.

Induced pluripotent stem cells and CRISPR-Cas9

First generated by now Nobel laureate Shinya Yamanaka in 2006, iPSCs are derived from somatic cells that are genetically reprogrammed to an embryonic stem cell–like state through forced expression of genes and factors needed to maintain pluripotency (Takahashi and Yamanaka, 2006). Once in culture, iPSCs can then be redifferentiated into various cell types using a unique cocktail of growth factors specific for that cell lineage. The emergence of this new technology has allowed many laboratories, including our own, to generate patient-derived iPSCs to study the genetic basis of disease pathogenesis more effectively (Dimos et al., 2008; Higgins et al., 2019; Jaffre et al., 2019; Mulero-Navarro et al., 2015; Park et al., 2008; Sakai et al., 2018). Moreover, iPSC technology offers several advantages over other previously generated model systems. For example, although animal studies remain indisputably valuable in understanding the role of genetic defects, they are often not translatable to human disease. In addition, patient-specific iPSCs provide researchers the opportunity to delineate the effects of genetic alterations on specific cell types, to provide more functional, mechanistic, and therapeutic data for a particular disease of interest. Finally, iPSCs are an ideal model system for assessing therapeutic drug potency, efficacy, and specificity, without worrying about in vivo cytotoxic or off-target effects. For example, differentiating iPSCs from a patient's own cells to generate a cell type and patient-specific disease model can allow for low- or high-throughput pharmacological screening to identify the most suitable candidate drugs for treating, reversing, or curing a particular disease. This personalized medicine approach can also be more broadly applied to additional patients with the same genetic mutations, the molecular basis of which can be extrapolated and generally applied (Fig. 4.1).

In addition to the generation and differentiation of iPSCs, genome editing using CRIPSR-Cas9 has further revolutionized iPSC capabilities, allowing for both direct and indirect comparative understandings of specific genetic disease–causing mutations. This technology has the capability to alter the genomic sequence within iPSCs, to either create an isogenic control line from a patient's own iPSCs or to induce a disease-causing genetic mutation in the background of normal iPSCs, without needing to obtain the sample directly from the patient. Indeed, the greatest challenge for iPSC studies previously was in finding the most "appropriate" control for patient cells. Researchers needed to account for a patient's unique genetic background. They tried to do this by including several "normal" control subjects with the same gender, age, and ethnicity as the patient, but allelic variance and potential confounding contributions from genetic modifiers could not be excluded. Now, CRISPR-Cas9 technology directly modifies a patient's own genetic sequence to "correct" mutations, creating an isogenic control iPSC line, to determine the necessity and sufficiency of that mutation in causing a particular disease or phenotype. Conversely, when cells from patients with a particular genetic mutation are more difficult to obtain, or to ascertain the disease-causing capacity and potency of a specific mutation, researchers use CRISPR-Cas9 to "induce" a mutation in the background of a

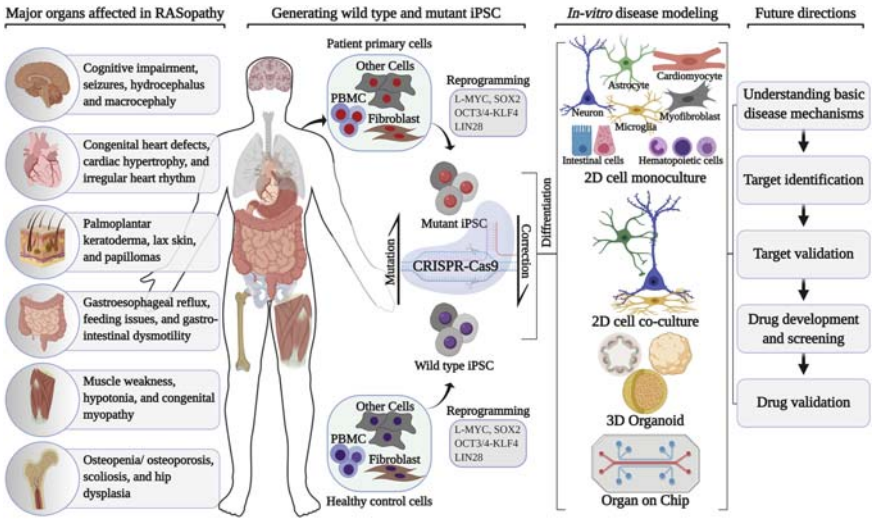


FIGURE 4.1 Overview of the use of iPSC technology for modeling Noonan syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSML), and Costello Syndrome (CS).

RASopathies are developmental disorders affecting several major organ systems listed on the left. Primary cells from a patient or a healthy control can be used to reprogram somatic cells through the introduction of Yamanaka factors (L-MYC, OCT3/4-KLF4, SOX2, and LIN28), generating embryonic pluripotent stem-like cells termed inducible pluripotent stem cells (iPSCs). Gene editing of human iPSCs using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 can also be used to correct genetic defects in diseased patient-derived iPSCs or introduce specific mutations in the background of normal patient-derived iPSCs. Using a variety of growth factors to stimulate these iPSCs, cells can be differentiated into any cell type for further analysis using either a monolayer (for a single cell type) or a co-culture cell system (for 2 or more cell types). To mimic *in-vivo* physiology, more advanced two-dimensional (2D) or three-dimensional (3D) organoid and organ-on-a-chip technologies can be used for disease modeling. As a tool, iPSCs can provide a valuable platform for basic molecular and functional studies, precision therapy and future drug development, screening and validation. *PAM*, proto-spacer adjacent motif.

normal (control) iPSC line (Fig. 4.1). In this regard, when compared to patient-derived iPSC lines, introducing mutations in the context of a normal background can also delineate potential contributions from genetic modifiers and allelic variants to a particular disease, including effects associated with gene dosage, disease penetrance, and phenotypic expressivity.

However, like all new technologies, limitations for iPSCs and CRISPR-Cas9 also exist. For example, an important limitation of iPSC-derived differentiated cells, particularly as it pertains to cardiomyocytes (CMs) or neurons, is that the cells remain in a fairly immature state, with embryonic or fetal-like electrophysiological,

calcium-handling, and/or metabolic signatures (Keung et al., 2014; Robertson et al., 2013). Therefore, while studying congenital or developmental disorders may be highly useful using this model system, studying adult disease physiology can be limiting, difficult, or even not possible in some lineages.

In the context of RASopathies, these technologies provide greater opportunities for understanding the precise mechanisms underlying congenital disease, particularly those associated with NS, NSML, and CS. Because there are currently no bona fide therapies for relieving the syndromic features associated with RASopathies, iPSCs may also help define new therapeutic targets and/or identify novel treatments for these patients. Finally, these technologies may demonstrate feasibility for potential future in utero genome editing strategies, although there are, of course, ethical considerations and boundary limitations that need to be, and have not yet been, carefully considered for this potential treatment approach. Here, we will discuss the research associated with RASopathies and iPSCs, and how it particularly pertains to the role of cardiac and neurodevelopmental diseases in these patients, as well as define the future directions of this technology in the study of rare diseases such as RASopathies.

RASopathies and signaling

While individually rare, collectively the RASopathies comprise one of the largest groups of congenital disorders worldwide, with a prevalence of ~1 in every 1000 live births (Rauen, 2013). These syndromes are characterized by a broad spectrum of functional and morphological abnormalities that include heart defects, short stature, neurocognitive impairment, craniofacial malformations, musculoskeletal, cutaneous lesions, ectodermal, and ocular abnormalities, hypotonia and a predisposition to developing benign and/or metastatic tumors (Aoki et al., 2008, 2016; Gripp et al., 2020; Rauen, 2013). However, the molecular etiology for each disorder is distinct, with differential modifiers potentially playing a role in the severity and expressivity of these characteristics (Gripp et al., 2019; Rauen, 2013).

How mutations that cause RASopathies elicit pathological responses remains to be fully elucidated. What is clear, however, is that RAS activation plays an integral part. RAS is a small guanosine triphosphate hydrolase (GTPase) that can switch between an active guanosine-5′-triphosphate (GTP) bound form and an inactive guanosine-5′-diphosphate (GDP) bound form in response to an incoming signal. Importantly, its activation initiates a cascade of downstream signaling that drives MAPK activation, as well as the activation of integral parallel signaling pathways, necessary to drive and maintain essential biological and cellular functions (Fig. 4.2).

The RAS-MAPK cascade is an intracellular *signaling pathway* that controls growth, proliferation, differentiation, and migration of cells (Lauriol et al., 2015; Tidyman and Rauen, 2009). Activation of Extracellular-Regulated Kinase (ERK) (also referred to herein as MAPK) is central for growth, development, and survival

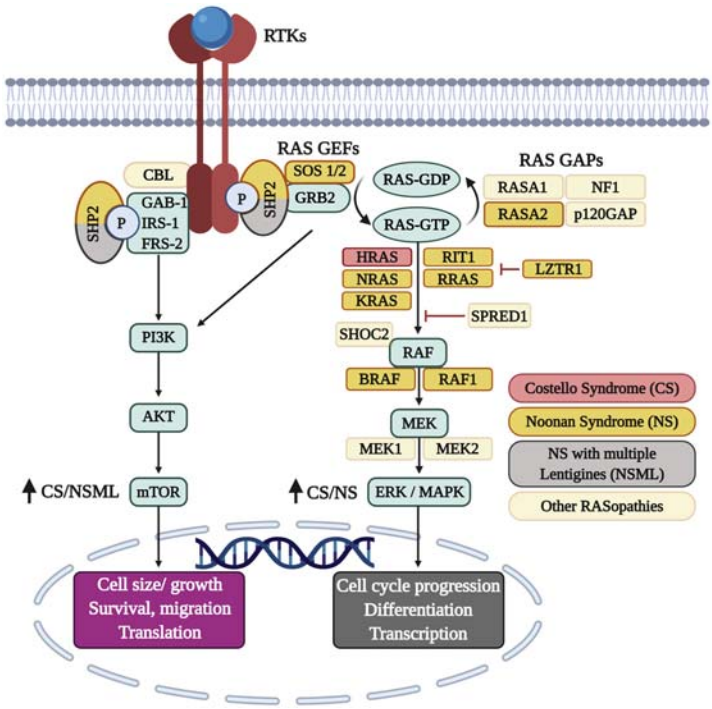


FIGURE 4.2 Mutations in genes residing along the canonical RAS-ERK/MAPK pathway are causal to the development of Noonan syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSML), and Costello Syndrome (CS), among other RASopathies.

Aberrant gene expression of genes encoding signaling proteins in the RAS-MAPK and RAS-PI3K-AKT pathway cause NS yellow, NSML gray, and CS red. While NS mutations lead to gain-of-function activity of ERK/MAPK, NSML mutations increase AKT activity. In contrast, activation of either/or ERK/MAPK and AKT can occur in a cell-type specific manner in CS.

of an organism. During development, several growth factors and mitogens, extracellular matrices, and cytokines engage the MAPK pathway to specify cellular proliferation, migration, and differentiation (Corson et al., 2003; Tanimura and Takeda, 2017). Preventing MAPK activation either by knocking down ERK2 or its immediate upstream kinases, MAPK Kinases 1/2 (MEK1/2), leads to embryonic lethality (Belanger et al., 2003; Giroux et al., 1999; Hatano et al., 2003; Saba-El-Leil et al., 2003, Fig. 4.2).

Apart from its vital role in embryogenesis, the MAPK pathway is also critical for various biological functions, such as memory formation, immune cell proliferation and maturation, and cellular, organ and organismal metabolic homeostasis (Fischer et al., 2005; Gehart et al., 2010; Gold, 2008; Medina and Viola, 2018; Papa et al., 2019). Dysregulation of the RAS-ERK/MAPK pathway, often due to gain-of-

function (GOF) mutations affecting the core kinases or loss-of-function (LOF) mutations for negative regulators of the pathway are paramount for the sustained cellular proliferation, growth and survival of cancer cells (Dhillon et al., 2007; Ryan et al., 2015). Though it appears linear and straightforward, the RAS-MAPK pathway is complex, with a myriad of regulatory layers and bifurcating signaling components that intricately modulate cellular activity, including at the transcriptional, posttranslational, and protein/protein levels. However, the intricacies of how these signaling networks crosstalk and their functional roles in mediating pathological changes in the context of specific mutations are not fully understood (Tajan et al., 2018a).

In addition, another critical signaling pathway modulated by RAS is the phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB or AKT) pathway (Buckles et al., 2017; Ong et al., 2001; Pacold et al., 2000; Suire et al., 2002). Parallel to MAPK, the PI3K-AKT pathway is critical for control and modulation of protein synthesis, survival, and apoptosis of cells (Castellano and Downward, 2011). Like MAPK, isoform specific activation of AKT and its cellular pathway components facilitates differential developmental outcomes. For instance, knocking out any of the three AKT isoforms manifests in distinct, but viable, physiological and pathological defects, but elimination of all three isoforms causes embryonic lethality, indicating the importance of this pathway in development (Chen et al., 2001; Cho et al., 2001; Dummler et al., 2006; Peng et al., 2003; Tschopp et al., 2005; Yang et al., 2005). Another critical function of PI3K-AKT signaling pertains to its effects on stem cell pluripotency, cell fate, and differentiation of cells, particularly during early development (Yu and Cui, 2016).

Crosstalk between the RAS-MAPK and the RAS-PI3K-AKT pathways influences multiple coordinated cellular functions that require feedback and feedforward loops (Mendoza et al., 2011; Saini et al., 2013). For instance, both ERK1/2 and $p90^{RSK1}$, a downstream kinase in the RAS-MAPK pathway, can phosphorylate and inhibit the activity of TSC2, a negative regulator of the AKT pathway, resulting in the activation of AKT (Arvisais et al., 2006; Winter et al., 2011). In addition, AKT itself can impair RAF activation through phosphorylation of its inhibitory domain at Ser259, and thus thereby can indirectly regulate activation of MAPK (Moelling et al., 2002). Taken together, disruption in any component of these intricate signaling pathway processes, all regulated by the upstream activity of RAS, can have significant and deleterious effects.

Differential activation of RAS-MAPK and RAS-PI3K-AKT signaling is reported in NS, NSML, and CS. While hyperactivation of RAS-MAPK is reported in NS, an increase in PI3K-AKT signaling is observed in NSML (Edouard et al., 2010; Ishida et al., 2011; Marin et al., 2011). To further complicate matters, cell type-specific increased activation of both RAS-MAPK or PI3K-AKT are observed in CS (Chen et al., 2009; Chennappan et al., 2017; Niihori et al., 2011; Oba et al., 2018; Rosenberger et al., 2009; Schreiber et al., 2017; Schuhmacher et al., 2008). How or why differential regulation occurs in associated disorders that disrupt similar downstream effectors remains unclear. Use of novel technologies in this regard

can have significant impact on understanding the regulation of these mutations and the pathways that are involved. Use of iPSCs in this regard will help provide insights into the molecular mechanism causal to the disease, and to the identification and development of more targeted, efficacious, and potent clinical therapies for RASopathies patients.

Noonan syndrome

NS (OMIM 163950), first described by Jacqueline Noonan 52 years ago, has an estimated prevalence of ~1:1000−1:2500 live births (Noonan, 1968; Roberts et al., 2013). Germline mutations in multiple genes that reside along the canonical RAS-MAPK signaling cascade have been identified as causal to NS, including *PTPN11* (Tartaglia et al., 2001), *SOS1* (Roberts et al., 2007; Tartaglia et al., 2007), *RAF1* (Pandit et al., 2007; Razzaque et al., 2007), *KRAS* (Schubbert et al., 2006), *RIT1* (Aoki et al., 2013), *NRAS* (Cirstea et al., 2010), *RRAS* (Niihori et al., 2011), *BRAF* (Sarkozy et al., 2009), *MRAS* (Higgins et al., 2017; Motta et al., 2020), *SOS2* and *LZTR1* (Johnston et al., 2018; Umeki et al., 2019; Yamamoto et al., 2015). In addition, mutations in *SHOC2* (Gripp et al., 2013) and *PPP1CB* (Gripp et al., 2016) are associated with an NS-like disorder termed NS with loose anagen hair (NS-LAH). Moreover, while the majority of NS mutations are autosomal dominant, homozygous recessive mutations in *LZTR1* have also been reported (Johnston et al., 2018). Importantly, most, if not all, of the mutations result in a GOF activity for the gene, leading to downstream hyperactivation of MAPK signaling (Fig. 4.1). Moreover, while a myriad of genes are causal to NS, the majority of NS cases (>50%) are caused by mutations in one single gene, *PTPN11*, encoding the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2).

Phenotypically, patients present with craniofacial abnormalities, short stature, neurocognitive disabilities, bleeding disorders, development of certain types of cancers, as well as cardiac defects, principally atrio/ventricular septal defects (VSDs), cardiac hypertrophy, and pulmonic stenosis (Romano et al., 2010). Of these phenotypic features, the most detrimental are the mutations that affect the heart, the brain, and cause cancer. Indeed, more than 80% of patients with NS have congenital heart disease, with pulmonary valve stenosis (PVS) being the most common cardiac structural anomaly (38.9%−63%) (Romano et al., 2010; Sharland et al., 1992; Sznajer et al., 2007). In addition, atrial septal defects (8%−25%), stenosis of the peripheral pulmonary arteries (~15%), hypertrophic cardiomyopathy (HCM) (9.5%−20%), coarctation of aorta (8.8%), mitral valve anomalies (5.8%), tetralogy of Fallot (4.4%), VSD (4.4%), patent ductus arteriosus (2.2%), and irregular heart rhythm have also been reported (Marino et al., 1999; Roberts et al., 2013). PVS and ASD are most associated with individuals who carry mutations in *PTPN11* (Sznajer et al., 2007). In contrast, HCM, a life-threatening condition that causes abnormal

thickening of heart muscle tissue and decreased cardiac function, is frequently associated with *RAF1* and *RIT1* mutations (Aoki et al., 2013; Marino et al., 1999; Razzaque et al., 2007; Sznajer et al., 2007).

While the majority of NS patients have only mild cognitive impairment (Pierpont, 2016), recent studies investigating the behavioral phenotypes in NS describes autism-like features (Garg et al., 2017; Watanabe et al., 2011) and impairment in social activity (Shaw et al., 2007). Indeed, evaluation of children and adults with NS show that 21% identify with significant autism-spectrum disorder characteristics (Adviento et al., 2014); however, the causal mechanisms remain unknown.

Finally, NS-associated mutations are associated with increased incidence in a number of cancers, including the development of breast cancer, lung cancer, neuroblastoma, B-cell acute lymphoblastic leukemia, and juvenile myelomonocytic leukemia (JMML). For example, somatic germline mutations in *PTPN11* have been identified as causal to NS-associated JMML (NS/JMML) (Strullu et al., 2014). As the clinical, functional, and mechanistic details associated with these phenotypic abnormalities remain to be fully elucidated, use of more humanized model systems, such as iPSCs, could help provide the interface needed to specifically target genes or pathways of interest, to more potently, more efficaciously treat RASopathies patients.

Disease modeling of Noonan syndrome

Animal models generated to study NS, including mouse, zebrafish, *Drosophila melanogaster* (fruit flies), and *Xenopus* have helped yield important biological insights into NS disease pathophysiology (Araki et al., 2004, 2009; Bonetti et al., 2014; Langdon et al., 2012; Nakagama et al., 2020; Oishi et al., 2006; Zheng et al., 2018). For example, mutant mice harboring NS-associated $PTPN11^{D61G/+}$, $PTPN11^{Q79R/+}$, $SOS1^{E846K/+}$, $KRAS^{V14I/+}$, $RIT1^{A57G/+}$, and $RAF1^{L613V/+}$ display phenotypic features also observed in NS patients, including short stature, craniofacial dysmorphia, growth retardation, and cardiac defects (Araki et al., 2004; Chen et al., 2010; Hernandez-Porras et al., 2014; Krenz et al., 2008; Nakamura et al., 2007; Takahara et al., 2019; Wu et al., 2011). With respect to the latter, these animal model systems have been instrumental in understanding some of the cardiac developmental and structural abnormalities associated with NS. For example, $PTPN11^{D61G/+}$ mice showed that both homozygous (100%) and heterozygous (~50%) expression of this mutation can lead to embryonic lethality. Of those heterozygous mice that survived, development of severe cardiac structural abnormalities, including VSD, double-outlet right ventricle (DORV), and enlarged outflow tract and atrioventricular valve primordia were observed (Araki et al., 2004). However, consistent with *PTPN11*-associated NS patients, no HCM was observed in

these mice (Araki et al., 2004). To understand the cardiac cell origin for the NS cardiac defects, Araki et al. generated inducible, lineage specific *PTPN11*$^{inD61Y/+}$ mice to examine the effects of NS-associated GOF activity in response to promotors that drive myocardial (αMHC-Cre), endocardial (Tie2-Cre), or neural crest (Wnt1-Cre) expression in the heart. Interestingly, endocardial-specific expression of mutant *PTPN11* alone was responsible for the enlarged cardiac cushions, the VSD, DORV, and the thinned myocardium. Endocardial cells line the heart and undergo epithelial to mesenchymal transition (EMT), contributing to the development of cushion mesenchyme; therefore, the enlarged cushions observed in NS mutant mice are likely attributed to increased endocardial activity of SHP2, causing an extension of EMT and leading to increased cellular proliferation of cushion mesenchyme. Unexpectedly, the *PTPN11* mutant myocardial-specific expressing mice had no obvious cardiac phenotype, suggesting that paracrine signals play an integral role in the development of cardiac muscle. In addition, a role for neural crest in response to GOF NS mutants was also determined; increased SHP2 activity led to ERK1/2 hyperphosphorylation in the pre- and postmigratory NCCs that resulted craniofacial abnormalities in mice (Nakamura et al., 2009b). Conversely, deletion of SHP2 in premigratory neural crest cells prevented migration of cells into the developing outflow tract, resulting in incomplete septation (Nakamura et al., 2009a).

Defects during looping morphogenesis can also result in the DORV phenotype (Ramsdell, 2005). Indeed, SHP2-D61G mRNA injected into zebrafish embryos reduced heart rates, as well as caused inverted looping or nonlooped hearts (Bonetti et al., 2014). This observation was also reported in *Xenopus*, where NS-associated mutant embryos presented with leftward heart displacement and left/right asymmetry due to impaired ciliogenesis and cilia function in the Kupffer's vesicle (Langdon et al., 2012), suggesting these phenotypes are linked to aberrant GOF RAS-MAPK signaling.

Similarly, transgenic mice expressing the *PTPN11*$^{Q79R/+}$ mutation on a cardiac specific developmental promoter (αMHC) developed morphological abnormalities in the interventricular groove of the developing heart and exhibited ventricular noncompaction without fibrosis (Araki et al., 2004; Nakamura et al., 2007). Here too, the enlarged endocardial cushions (ECs) in the atrioventricular canal and in the outflow tract of these mice were found to be a consequence of hyperactive RAS-MAPK signaling (Krenz et al., 2008). In contrast, *PTPN11*$^{Q79R/+}$ mice on a more adult-onset cardiac specific promoter (αMHC) showed no aberrant ventricular architecture in the heart, suggesting that the cardiac abnormalities associated with NS are mediated by disrupted RAS-MAPK signaling during embryonic development.

In addition to *PTPN11*, mouse models with other gene mutations also faithfully reproduce the cardiac phenotypes observed in patients. *KRAS*$^{V14I/+}$ mice displayed cardiac hyperplasia, without hypertension, and cardiac fibrosis (Hernandez-Porras et al., 2015). *RIT1*$^{A57G/+}$ and *RAF1*$^{L613V/+}$ mouse models also successfully recapitulated the HCM phenotype observed in NS patients (Takahara et al., 2019; Wu et al., 2011).

However, not all mutant animal models reproduce the clinical cardiac phenotypes. For example, *SOS1*$^{E846K/+}$ mice, which had increased RAS-MAPK, RAC, and STAT3 signaling, developed aortic stenosis, whereas, in patients, the primary cardiac phenotype is pulmonary stenosis. In addition, homozygous SOS1E846K/E846 mice proved not only to be viable but also manifested with additional cardiac phenotypes, including epicardial fibrosis and adipocyte infiltration to the heart, which had not previously been reported or observed (Chen et al., 2010). Studies in *Xenopus* showed that activating NS mutations in *PTPN11* (D61A and E76A) induced animal cap elongation, but showed minimal activation of the RAS-MAPK pathway (O'Reilly et al., 2000). In contrast, transgenic overexpression of the *PTPN11* N308D mutation in *Drosophila melanogaster* led to an increased activation of MAPK during wing vein formation (Oishi et al., 2006). Finally, morpholino-mediated knockdown of SHP2 in zebrafish resulted in cell movement defects as early as gastrulation, consistent with defects observed in SHP2-deleted mice (Yang et al., 2013), as well as impaired craniofacial and cardiac development (Bonetti et al., 2014; Jopling et al., 2007).

In addition to cardiac phenotypes, neurodevelopmental anomalies in RASopathies are rapidly gaining interest and are becoming important to the overall clinical phenotype (Gauthier et al., 2007; Ke et al., 2007). For example, genetic knockdown of *PTPN11* in mouse cultured cortical precursors or in the embryonic cortex blocks neurogenesis and enhances astrogenesis (Gauthier et al., 2007). Ectopic expression of wild type (WT) SHP2 or an NS-associated activating D61G mutation enhances neurogenesis, suggesting that SHP2 plays a pivotal role in determining neuronal cell fate (Gauthier et al., 2007). Moreover, neuronal activity in *PTPN11*$^{D61Y/+}$ mice also revealed aberrant signaling in parallel pathways, including that of PI3K/AKT/mTOR and JAK/STAT (Altmuller et al., 2017). Similarly, a recent study in *RAF1*^{L613V} mice revealed increased numbers of glial subtypes in the forebrain and increased numbers of astrocytes in the adult cortex and hippocampus (Holter et al., 2019).

Despite the inconsistencies of animal models, these models have helped clarify the significance of aberrant signaling pathways in NS. In this regard, several research groups have found that inhibiting RAS-MAPK signaling at the level of MEK or ERK can be beneficial in reducing or normalizing NS pathologies. *Specifically,* cushion explant outgrowth in *PTPN11*$^{Q79R/+}$ mice was reduced by treating mice with the MEK1 inhibitor U0126 (Krenz et al., 2008). Similarly, *KRAS*$^{V14I/+}$ mice treated with the same MEK inhibitor rescued the neonatal heart defects associated with that mutation (Hernandez-Porras et al., 2015). Prenatal treatment of *SOS1*$^{E846K/+}$ mice with another MEK inhibitor, PD0325901, reduced the embryonic lethality, penetrance of cardiac defects, and improved (but not normalized) the growth and skull shape abnormalities in these mice (Chen et al., 2010). Interestingly, postnatal inhibition of *RAF1*$^{L613V/+}$ mice with the same PD0325901 inhibitor also normalized the cardiac defects, growth deficiencies, and craniofacial abnormalities (Wu et al., 2011). In *PTPN11*$^{D61G/+}$ mice, however, only the growth retardation was partially rescued by the U0126 treatment, suggesting a potential parallel pathway

regulating this component of the NS phenotype (Tajan et al., 2018b). More recently, inhibitors of *PTPN11*, including the SHP099 drug, a potent allosteric inhibitor of phosphatase activity, have been used in mouse models to suppress tumor cell growth and proliferation (Chen et al., 2016), showing significant promise for further application in clinical trials.

Delineating Noonan syndrome–associated cardiac hypertrophy using induced pluripotent stem cell technology

As is frequently the case, the relevance of nonmammalian or nonhuman model systems to humans is not always clear, and the phenotypes that cannot be clearly delineated or understood in animal model systems remain an enigma. To circumvent these issues, researchers have started incorporating iPSC technology to study the human mechanisms associated with NS. To date, and so far, several groups, including ours, have modeled NS in iPSCs to study cardiac hypertrophy (Higgins et al., 2017; Jaffre et al., 2019; Sakai et al., 2018) and NS/JMML (Mulero-Navarro et al., 2015).

RAF1 is a critical gene along the RAS-MAPK signaling pathway. Although rare, more than 90% of all NS-associated *RAF1* mutations cause severe, and often lethal, HCM. Our lab generated iPSC-derived cardiomyocytes (iPSC-CMs) from a patient with a $RAF1^{S257L/+}$ mutation, as well from a CRISPR-Cas9 corrected isogenic control iPSC line. As a secondary confirmation of the effects of the *RAF1* mutation in cardiac pathology, we also generated iPSC-CMs from normal iPSCs and those garnered from inducing the *RAF1* mutation using CRISPR-Cas9 technology in the context of a normal background. Our results demonstrated that we could recapitulate the *RAF1* patient cardiac hypertrophy phenotype in both patient-derived and CRISPR-induced *RAF1* mutant iPSC-CM lines, as evidenced by increased cell size and myofiber disarray (Jaffre et al., 2019). However, we observed slightly lesser effects of cardiac hypertrophy in the normal iPSCs induced with the *RAF1* mutation than in the diseased patient-derived cells, suggesting genetic modifiers likely modify the severity and expressivity of the disease in NS-associated *RAF1* patients. Identification of these modifiers in this, as well as other RASopathies, will be crucial in fully understanding the mechanistic functions of these mutations in patients.

Nonetheless, using this model system, we were able to utilize pharmacological intervention to determine, for the first time, that two novel molecular signaling pathways modulate hypertrophy in *RAF1*: activation of MEK1/2 to modulate myofibrillar disarray and an increase in ERK5 to induce the enlargement of CMs. Interestingly, and perhaps surprisingly, ERK/MAPK was not involved in the development of hypertrophy in *RAF1* mutant iPSC-CMs. Specifically, myofiber disarray was rescued by treating cells with PD98059 or trametinib, blockers of *RAF1*-dependent activation of MEK1/2, but not with the MEK1/2 inhibitor U0126. Concomitantly, mutant iPSC-CMs treated with U0126 or BIX02189, inhibitors more potent for ERK5 signaling, significantly normalized the cell surface area. These

results suggest that combinatorial therapies using both MEK1/2 and ERK5 inhibitors could be used effectively to treat *RAF1*-associated cardiac hypertrophy. In addition, using RNA-sequencing (RNA-seq) technology, we revealed a number of downstream regulatory genes that are also dysregulated by aberrant MEK1/2 or ERK5 signaling in these patients; these too are likely causal to the hypertrophic phenotype and could serve as potential novel therapeutic targets for treating *RAF1*-associated HCM in the near future. Importantly, previous mouse models suggested that the hyperactivation of ERK1/2 was likely responsible for the HCM phenotype in *RAF1* patients; however, using the human iPSC-CM model system, we now reveal, for the first time, a novel aberrant signaling pathway and regulatory mechanism causal to the HCM phenotype in this RASopathy (Jaffre et al., 2019; Wu et al., 2011).

Similarly, Sakai et al. (2018), who generated iPSC-CMs from normal, idiopathic HCM or *RAF1*-associated NS patients, showed that the hypertrophic phenotype, as measured by increased cell size, could be recapitulated in both idiopathic and mutant *RAF1* patient-derived iPSC-CMs. In addition, concentration of diastolic intracellular calcium was also increased in these patient-derived iPSC-CMs, although this did not reach significance in the *RAF1* mutant cells. Like our study, Sakai et al. also tried using iPSC-CMs to identify potential drug candidates that could reverse the hypertrophic phenotype. Pyr3, a transient receptor potential channel 3 inhibitor, dose-dependently decreased the cell size area in both the idiopathic HCM and *RAF1* mutant iPSC-CMs. In addition, Pyr3 also dose-dependently decreased the diastolic intracellular calcium levels in both lines, but again, this did not reach significance in the *RAF1* mutant cells. These results collectively suggest that Pyr3, or its derivative, could potentially be used to treat patients with cardiac hypertrophy, irrespective of the disease etiology. Moreover, these data suggest that Pyr3 regulates both cell size and calcium homeostasis in iPSC-CMs (Sakai et al., 2018). Interestingly, these results also are important for another reason; Sakai et al. utilized healthy iPSC-CMs as controls for this study, derived from different genetic backgrounds, as opposed to using isogenic controls derived from the same patient's cells (Sakai et al., 2018). This is significant in that, despite this, significant disease relevance for the NS-associated *RAF1* patient iPSC-CMs could still be observed, suggesting that the *RAF1* mutations alone are both necessary and sufficient for the development of cardiac hypertrophy in RASopathies patients.

The RAS-related protein MRAS, also known as muscle RAS oncogene homolog and R-RAS3 (Kimmelman et al., 1997; Quilliam et al., 1999), functions as a signal transducer for a wide variety of signaling pathways, particularly those promoting growth and proliferation, such as the MAPK (Higgins et al., 2017). Importantly, *MRAS* was recently identified as a novel NS susceptibility gene. Most prominently expressed in brain and heart, NS-associated *MRAS* disease variants cause severe cardiac hypertrophy (Higgins et al., 2017). To sufficiently define the molecular mechanisms causal to *MRAS*-associated cardiac hypertrophy and to demonstrate the necessity and sufficiency of *MRAS* variants to cause this phenotype, iPSC-CMs were generated from two patients with the *MRAS* variant

p.Gly23Val-*MRAS* (Higgins et al., 2019). Both lines recapitulated the HCM phenotype observed in NS-associated *MRAS* patients. To validate the findings, CRISPR-Cas9 genome editing was used to correct the pathogenic variant in patient cells, creating an isogenic control, and the pathogenic variant was introduced into unrelated control cells, to ascertain that the p.Gly23Val-MRAS variant, in and of itself, was necessary and sufficient to elicit the hypertrophic phenotype in iPSC-CMs. Indeed, compared with controls, both patient and disease modeled iPSC-CMs were significantly larger and displayed abnormal changes in gene expression consistent with the development of pathological HCM, including increased expression of *NPPA* and *NPPB*, two genes involved in the reactivation of the fetal gene program indicative of pathological hypertrophy. Additionally, patient and disease-modeled iPSC-CMs displayed impaired calcium handling, including increased frequency of irregular calcium transients and changes in calcium handling kinetics (Higgins et al., 2019).

Understanding cancer causing Noonan syndrome mutations using induced pluripotent stem cells

JMML is one of several cancers commonly associated with RASopathies mutations. It is an aggressive and difficult-to-treat myelodysplastic and myeloproliferative neoplasm that presents in early childhood (Kratz et al., 2005; Martinelli et al., 2010; Niemeyer et al., 2010; Strullu et al., 2014) and is characterized by excessive proliferation of monocytic and granulocytic cells mediated by somatic GOF mutations in components of the RAS-MAPK signaling pathway (Yoshida et al., 2012). As this is a devastating disease with a low survival rate, iPSC technology offers researchers an opportunity to better understand the mechanism(s) causal to its development, as well as identify strategies to treat the disease more effectively in clinic.

As mentioned previously, >50% of NS patients and ~35% of JMML cases carry GOF *PTPN11* mutations. However, the molecular mechanisms for how or why these *PTPN11* mutations cause abnormal myelopoiesis remained unclear. SHP2 is a ubiquitously expressed nonreceptor protein-tyrosine phosphatase that contains two SH2 domains, a central PTP catalytic domain and a C-terminal tail with two tyrosine phosphorylation sites and a proline-rich motif (Tartaglia and Gelb, 2005). To become activated, a phosphotyrosyl (pY) peptide binds to its N-terminal SH2 domain, inducing a conformational change that unfolds the protein, allowing its substrates to bind to the catalytic site to render downstream pathway activation (Lauriol et al., 2015). SHP2 is required for RAS-MAPK pathway activation by most, if not all, RTKs as well as by cytokine receptors and integrins. In addition, depending on the receptor to which it binds, SHP2 can either positively or negatively drive RAS-PI3K-AKT pathway activation (Keilhack et al., 2005; Wu et al., 2001; Zhang et al., 2002). Therefore, SHP2 is a critical mediator of downstream signaling events that are essential for the control of cellular functions, including proliferation, differentiation, migration, cell cycle progression, and apoptosis of cells.

To better understand the role of GOF *PTPN11* mutations in causing NS/JMML, Mulero-Navarro et al. generated iPSC lines from healthy individuals or from patients with either *PTPN11* mutations causal to NS alone or *PTPN11* mutations causal to both NS and JMML, to evaluate the effects of these mutations in early oncogenesis, prior to the accumulation of secondary genomic alterations (Mulero-Navarro et al., 2015). They found that hematopoietic cells differentiated from iPSCs derived from patients harboring NS/JMML-causing *PTPN11* mutations could recapitulate the clinical oncogenic features of JMML, including the hypersensitivity to granulocyte-macrophage colony-stimulating factor and the hyper proliferation capacity of the myeloid cell population. Moreover, using transcriptome analysis, iPSC-derived NS/JMML myeloid cells showed increased STAT5 signaling and up-regulation of two specific microRNAs (miRs), miR-223 and miR-15a (Mulero-Navarro et al., 2015), suggesting a novel mechanism by which certain NS-associated mutations lead to onset of JMML. Indeed, these miRs were up-regulated in 11/19 JMML bone marrow mononuclear cells harboring *PTPN11* mutations (Mulero-Navarro et al., 2015). In an effort to validate the mechanism of regulation and to identify a potential therapeutic target, investigators reduced the expression of miR-223 in NS/JMML iPSCs and found that myelogenesis could be normalized. Taken together, this study inherently demonstrated how an iPSC model system could be used to study an inherited human cancer syndrome to identify novel molecular signatures and biomarkers that can be used therapeutically. Indeed, the two small miR inhibitors identified herein are currently being tested in clinical trials for a variety of cancers (Gordanpour et al., 2012; Yang et al., 2018).

Proteomics can also be a powerful tool when used in combination with iPSC technology. Most recently, Pearson et al. utilized this approach to delineate additional functional mechanisms associated with NS/JMML. In this case, the group generated iPSC-derived myeloid cells from NS patients with *PTPN11* mutations either known to be or not to be associated with increased incidence of JMML. Their proteomic assessments showed that JMML-associated *PTPN11* mutations induced TP53 and NF-κB signaling (Pearson et al., 2020). In addition, differential MYC gene expression was also identified as a significant mediator of NS/JMML (Mulero-Navarro et al., 2015). Taken together, iPSC technology was used to identify both the relevance of novel pathway mediators in disease, as well to demonstrate the therapeutic potential of specific and targeted inhibitors in treating NS or NS/JMML. In this regard, Pearson et al. identified two small molecular inhibitors, JQ1, a thienotriazolodiazepine and potent inhibitor of the BET family of bromodomain proteins, and CBL0137, a simultaneous inhibitor of NF-kB, Heat Shock Factor 1, and activator of p53, as preferential targeted therapies to treat NS-associated JMML (Pearson et al., 2020).

Noonan Syndrome with Multiple Lentigines

NSML, also known as LEOPARD syndrome, multiple lentigines syndrome, and cardiomyopathic lentiginosis, is a rare autosomal dominant disorder with an estimated

prevalence of 1:3500 (Bonetti et al., 2014). It was first described by Dr. Erwin P Zeisler MD and Dr. S. William Becker MD in 1936, and the first familial case was identified by I. Rosen, in twins, in 1942 (Martinez-Quintana and Rodriguez-Gonzalez, 2012). Presenting features of NSML include multiple lentigines (brown spots), ECG abnormalities, ocular hypertelorism, HCM, abnormal genitalia, retardation of growth, and sensorineural deafness (Digilio et al., 2006; Legius et al., 2002). As its features closely resemble those in NS, NSML can often be misdiagnosed in the clinic, especially early in life. Indeed, the presentation of multiple lentigines, perhaps the most uniquely distinguishing characteristic of NSML, does not manifest until ~5 years of age. Therefore, genetic validation of the mutations causal to this disorder is essential for proper diagnosis. In this regard, while mutations in multiple genes residing in the RAS-MAPK pathway have been attributed to causing NS, >85% of all cases of NSML are caused by germline heterozygous missense mutations in *PTPN11* (Carvajal-Vergara et al., 2010; Edouard et al., 2010; Lauriol and Kontaridis, 2011; Tartaglia et al., 2001).

Like NS, NSML patients develop cardiac abnormalities. Unlike NS, however, nearly all (over 90%) cases of NSML present with HCM, although valve anomalies similar to NS have also been observed (Limongelli et al., 2007). Since >50% of all patients with NS also have *PTPN11* mutations, how is it that *PTPN11* can cause both NS and NSML, similar, yet distinct, disorders? The answer to this conundrum could be explained by a series of highly significant biological and functional data. NS-causing *PTPN11* mutations are GOF, resulting in increased activation of RAS-MAPK signaling, whereas NSML-causing *PTPN11* mutations are LOF for SHP2 phosphatase activity (Hanna et al., 2006; Kontaridis et al., 2006). The reason for this difference lies in the location and function of each of the specific mutation causal to NS or NSML. Specifically, both NS and NSML mutations alter the SHP2 molecular switch to allow for a more open conformation of the phosphatase (Kontaridis et al., 2006). However, mutations causal to NS primarily reside in the N-terminal SH2 domains, thereby allowing for increased substrate accessibility and binding to the PTP catalytic cleft, while mutations causal to NSML affect conserved residues important for PTP catalysis, thereby decreasing catalytic activity despite the open conformation (Kontaridis et al., 2006, Fig. 4.3A).

Disease modeling of Noonan Syndrome with Multiple Lentigines

To determine the molecular mechanisms that underlie the pathophysiology of the NSML *PTPN11* mutations, a multitude of in vitro and in vivo studies have been conducted, some with conflicting results. First, several in vitro studies (Hanna et al., 2006; Kontaridis et al., 2006), as well as studies in zebrafish, demonstrably showed that NSML *PTPN11* mutations are LOF, with perhaps dominant negative activity on RAS-MAPK signaling. However, in *Drosophila,* NSML mutations had increased

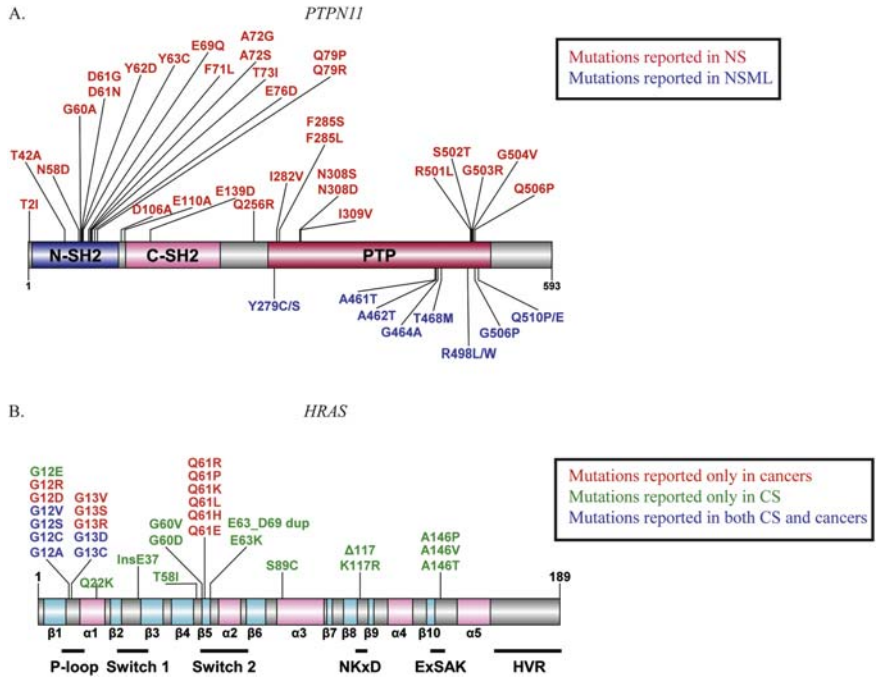


FIGURE 4.3 Mapping of mutations associated with Noonan syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSML), and Costello Syndrome (CS) to their protein domain.

(A) Schematic representation of germline *PTPN11* mutations on SHP2. The mutations associated with NS are colored in red, the mutations associated with NSML are colored in blue. Domains (based on NCBI Conserved Domain Database): N-terminal Src homology 2 (N-SH2) domain, C-terminal Src homology 2 (C-SH2) domain, Catalytic domain of tyrosine-protein phosphatase nonreceptor type 11 (PTP). (B) Schematic representation of germline *HRAS* mutations on HRAS. Secondary structural elements are shown as pink cylinders for α-helices and blue cylinder for β-sheets. Functional domains including P-loop, switch I, switch II, and hypervariable region (HVR) are highlighted at the bottom of the schematic. Mutations reported only in CS are colored in green, mutations reported only in cancer are colored in red, and mutations reported in both CS and cancer are colored in blue.

MAPK activity (Oishi et al., 2009). Moreover, these results were supported by an in vitro study using mouse embryonic fibroblasts, where NSML mutations were suggested to be GOF because of their open conformation, which potentially prolonged the binding of potential ligands, mediating increased substrate turnover, and, potentially, sustained RAS-MAPK activation (Yu et al., 2014). Therefore, in order to investigate these contradictory in vitro results and to more definitively understand the effects of NSML mutations in the context of a mammalian system, our lab generated the first NSML mouse model, using a recombineering approach to knock-in the

PTPN11 Y279C gene mutation (*PTPN11*$^{Y279C/+}$) (Marin et al., 2011). Our findings demonstrated that NSML-associated SHP2 activity was, in fact, decreased in vivo and that NSML mutations diminished the activation of MAPK signaling in response to agonist stimulation.

To make things more complicated, in order for SHP2 to confer its normal activity to the RAS-MAPK pathway, it must bind to upstream scaffolding adapters (or other Tyr(P) proteins) (Yu et al., 2013). Therefore, a catalytically inactive NSML mutant competes for binding with endogenous WT SHP2 (Kontaridis et al., 2006). Moreover, the preferentially "open" conformation of the NSML SHP2 mutants could enhance its binding and even prevent binding of other proteins that would otherwise differentially regulate/modulate downstream signaling (Stewart et al., 2010). Consistent with this notion, increased basal as well as IGF-1−induced association of SHP2 with IRS-1 and Gab1 were observed in NSML mice (Marin et al., 2011), suggesting that in addition to effects on catalytic activity, NSML mutants did, in fact, behave as dominant negatives. As well, the LOF effects of the NSML-associated SHP2 mutations could also prevent the dephosphorylation of receptors or scaffolding adapters to which it was bound, leading to downstream pathway hyperactivation (Edouard et al., 2010; Marin et al., 2011). In this regard, GOF activity of PI3K/AKT/mTOR signaling was demonstrated in hearts from NSML mice, which was found to be causal to the development of NSML-associated HCM (Lauriol and Kontaridis, 2011; Marin et al., 2011). Indeed, inhibition of this pathway using an mTOR inhibitor (rapamycin) both prevented and reversed this pathological phenotype, suggesting SHP2 is a critical regulator of this pathway in the modulation of CM size (Marin et al., 2011).

In addition, transgenic cardiac overexpression of another, even more severe, NSML mutant, Q510E, also displayed a severe cardiac phenotype, with increased CM size, heart-to-body weight ratios, interventricular septum thickness, CM disarray, and interstitial fibrosis (Krenz et al., 2008). Echocardiography revealed that the ventricular walls were thickened and contractile function was depressed. Hereto, AKT/mTOR signaling was hyperactivated and rapamycin treatment rescued the NSML-associated cardiac phenotype in vivo (Krenz et al., 2008). Using cultured neonatal rat CMs with adenoviral gene delivery and pharmacological inhibitors, it was similarly confirmed that NSML mutations mediate the hyper-activation of AKT/mTOR, as well as focal adhesion kinase (FAK), a protein with a role in cell motility and cell survival (Schramm et al., 2012). As well, primary and immortalized fibroblast cell lines from 2 patients with NSML, conferring mutations Y279C and T468M, showed hyperphosphorylation of PI3K/AKT, as well as GSK-3β, a parallel signaling effector of AKT (Edouard et al., 2010), confirming the necessity of this pathway in the development of HCM in NSML.

Finally, drawing parallels to other, more common forms of HCM, Clay et al. hypothesized that in addition to altered signaling pathways, changes in calcium homeostasis and/or sarcomeric mechanical properties could also play a role in NSML cardiac pathophysiology. Indeed, isolated primary CMs from transgenic CM-specific Q510E-SHP2 expressing mice showed increased calcium transient

amplitudes during excitation-contraction coupling, increased sarcoplasmic reticulum calcium content, and increased expression of sarco(endo)plasmic reticulum calcium-ATPase (Clay et al., 2015).

Like NS, causal mechanisms associated with the cardiac manifestations in NSML are developmental in origin. Moreover, the molecular mechanisms regulating cardiac development and morphogenesis may be dependent on crosstalk between multiple cell lineages, the aberrant regulation of which can induce the onset of cardiac phenotypes such as HCM. To investigate the direct role of NSML mutations in heart development, an inducible knock-in *PTPN11* mouse model (*PTPN11*$^{inY279C/+}$) was utilized to determine NSML-associated myocardial (αMHC-Cre), endocardial (Tie2-Cre) or neural crest (Wnt1-Cre) contributions in the heart (Lauriol et al., 2016). Surprisingly, only endocardial-specific expressing NSML mice were sufficient to induce the adult-onset cardiac hypertrophy (Lauriol et al., 2016; Schramm et al., 2012). In development, NSML mouse hearts showed diminished trabeculation and valvular hyperplasia, defects that could only be recapitulated in endocardial-specific, but not myocardial-specific or neural crest−specific NSML mice (Lauriol et al., 2016). These findings were validated in atrioventricular EC explants isolated from transgenic mice overexpressing the Q510E-SHP2 NSML mutation in endocardium, where cushions were found to be 80% larger than those of WT mice (Edwards et al., 2015). Similarly, explanted ECs from chick embryonic hearts induced with adenoviral expression of the Q510E-SHP2 mutation confirmed this NSML-associated increased valvular outgrowth (Edwards et al., 2015). Interestingly, unlike NS, the NSML-associated enlarged cushion phenotype is not a consequence of increased valve cell proliferation; rather, it is mediated by increased valve cell migration in ECs, possibly through aberrant regulation of the FAK/SRC/mTOR signaling axis (Edwards et al., 2015).

In addition to endocardium, other lineages also contribute to the NSML cardiac phenotype; VSDs, common features of NSML, are evident only in myocardial-specific NSML hearts (Lauriol et al., 2016). However, these myocardial-specific NSML developmental defects resolve before birth and mice never develop the adult-onset HCM, suggesting that the NSML-*PTPN11* mutations contribute to a developmental delay in myocardium, which is mediated by abnormal communication between endocardium and myocardium. Together, these data suggest that *PTPN11* plays a pivotal role in cardiac development and that NSML mutations contribute to both cell-autonomous and noncell-autonomous effects in the developing heart through regulation of pathways that control endocardial-to-myocardial crosstalk (Lauriol et al., 2016).

NSML mutations also affect neural crest development due to decreased RAS-MAPK signaling (Stewart et al., 2010). However, unlike NS, inducible neural-crest specific expressing NSML mice (Wnt1-Cre:*PTPN11*$^{inY279C/+}$) did not show any overt cardiac-associated defects or abnormalities during development (Lauriol et al., 2016), Despite this, neural crest clearly contributes to other NSML-associated *PTPN11* phenotypes, including the craniofacial abnormalities and development of multiple lentigines (Lauriol et al., 2016). The mutations promote

extended pluripotency, delaying differentiation and cell migration of neural crest cells. Concomitantly, this leads to an excess number of pigment cells, abnormalities in facial cartilage cells, and to the characteristic development of lentigines and craniofacial dysmorphia observed in NSML patients (Stewart et al., 2010). The importance of *PTPN11* in neural crest is further illustrated in a set of experiments in mice showing that deleted SHP2 specifically in neural crest leads to defective specification and migration of cells to the cranium, the heart, and to the central nervous system (Grossmann et al., 2009; Nakamura et al., 2009a).

Delineating Noonan Syndrome with Multiple Lentigines–associated cardiac hypertrophy using induced pluripotent stem cell technology

While NS and NSML have similar clinical phenotypes and predominantly affect the same gene (*PTPN11*), the biological function of the mutations and the molecular mechanisms leading to their disease etiology are uniquely different. The recent progress made in iPSCs suggests that this model system is ideal to study the differential regulation of NS versus NSML mutations.

To try to understand the importance of the AKT/GSK-3β pathway in NSML, investigators generated P19CL6 cells, pluripotent murine embryonic carcinoma cells, harboring the Q510E NSML mutation (Ishida et al., 2011). When differentiated into CMs, these mutant cells demonstrated increased AKT and GSK-3β phosphorylation, as compared to control cell lines. Moreover, this aberrant signaling activity led to reduced terminal differentiation of mutant CMs. For mutant cells that did terminally differentiate, however, CM size was apparently larger than WT, though a demonstrative correlation to HCM could not be made given the differentiation limitations of these cell lines (Ishida et al., 2011).

As the technology developed, another group generated iPSCs derived from fibroblasts isolated from NSML patients with a T468M mutation in *PTPN11*, and differentiated them into CMs to study the effects of NSML mutations on human heart function (Carvajal-Vergara et al., 2010). These iPSC-CMs showed a higher degree of sarcomeric organization and a preferential nuclear localization of NFATc4, an important regulator of cardiac hypertrophy, when compared with CMs derived from human embryonic stem cells or from iPSCs collected from a healthy sibling (Carvajal-Vergara et al., 2010). Unfortunately, more conventional markers of cardiac hypertrophy, including measures of increased protein synthesis and a reactivation of fetal genes, could not be observed in these NSML iPSC-CM lines. Phosphoproteomic microarray chip analysis of these cells, however, revealed that baseline epidermal growth factor receptor and MEK1 were both increased in mutant cells, as compared to controls. In addition, though baseline MAPK activity trended higher in NSML lines than in control groups, any potential difference in activity did not reach statistical significance. Moreover, there was

no observable hyperactivation of the pathway following fibroblast growth factor (FGF) stimulation (Carvajal-Vergara et al., 2010), suggesting an LOF activity of NSML mutants for RAS-MAPK signaling in response to agonist stimulation. Because the number of embryoid bodies in the control versus the NSML-derived cell lines in these experiments contained different CM ratios and a mixed population of cardiac cells (Carvajal-Vergara et al., 2010), potentially limiting the interpretation of the findings, a CM enrichment protocol was developed (Lin et al., 2012). Using this method, investigators could confirm that hypertrophy-related genes, including myosin heavy chain and phospholamban, were induced in NSML-derived CMs, as compared to controls, and to similar levels as those previously reported in NSML mice (Marin et al., 2011). Since a similar regulatory network appears to control cardiac hypertrophy in both human- and mouse-associated NSML, the data suggest that mechanisms identified in NSML mice could be extrapolated to human NSML as well. In addition, the enrichment of human iPSC-CMs could be used to identify new contributors to disease pathology. Indeed, increased TGF-β expression was identified as a novel contributor to NSML-associated hypertrophy (Lin et al., 2012).

As iPSC technology advances and purification and differentiation methods for these cells continues to improve, understanding and identifying novel mechanisms causal to NS and NSML will become more plausible. Clearly iPSC technology is a useful tool for studying these disease phenotypes and serves as a potentially significant drug screening tool, to better and more effectively treat patients with these disorders. To expedite this, it will be important for scientists to continue to share information, best practices, and resources. To this end, an iPSC line was recently generated from peripheral blood mononuclear cells isolated from a patient with NSML carrying the Q510P *PTPN11* gene mutation using a nonintegrating Sendai virus technique; these cells are now an available resource for the NSML research community (Li et al., 2019).

Costello Syndrome

The term Costello Syndrome (OMIM# 218040) was first used in 1991 to report a patient with a developmental phenotype similar to two others previously reported in the 1970s by Dr. Jack Costello (Costello, 1971, 1977; Der Kaloustian et al., 1991; Martin and Jones, 1991). CS is a rare autosomal dominant disorder with a prevalence of 1 in 300,000−1,250,000 live births. Most mutations occur de novo, but somatic mosaicism with typical CS phenotypes or transmission to offspring and recurrence in siblings have been reported (Girisha et al., 2010; Gripp et al., 2006; Sol-Church et al., 2009). Patients typically present with features common to other RASopthies, including craniofacial defects, coarse facial features, cardiac and skin abnormalities, postnatal growth retardation, gut abnormalities, and neurocognitive delay (Abe et al., 2012; Gripp and Lin, 2012). Uniquely, however, CS patients have high birth weights, with a subsequent failure to thrive, and develop severe

ectodermal anomalies (Costello, 1971, 1977; Der Kaloustian et al., 1991; Martin and Jones, 1991). In addition, these patients have increased predisposition to developing both benign and malignant tumors (Gripp, 2005).

Neurological malformations and delayed neurocognitive development are prevalent and perhaps more significant in CS than in other RASopathies. Macrocephaly, hydrocephalus, Chiari I malformation, syrinx, seizures, cerebral and cerebellar atrophy, leukomalacia, poor gray-white matter differentiation, smaller corpus callosum, deviation of the cerebellar tonsils, and demyelination of the basal tonsil are just some of the significant neurological malformations reported in CS (Gripp et al., 2019; Kang and Lee, 2019). In addition, and perhaps consequently, approximately 80% of individuals with CS have intellectual disabilities, most in the mild to moderate range. Common features manifested by the neurocognitive dysfunction in CS include speech and language deficiency, orthopedic impairment, delayed fine- and gross-motor skills, and impairment in adaptive functioning (Gripp et al., 2019).

The heart and muscle phenotypes in CS are not yet well characterized but are thought to be significantly involved in the overall CS syndromic etiology. General muscular weakness is common in CS, likely due to an excess in the number of neuromuscular spindles (Bolocan et al., 2014). In addition, cardiac abnormalities include pulmonic valve stenosis, septal defects, aortic dilation, HCM, and arrhythmias (Lin et al., 2011).

Clinically, the bone phenotypes observed in CS patients include spine curvature (scoliosis, as well as kyphosis), bilateral or unilateral hip dysplasia, anterior wall chest deformity, tight tendons and contractures of the elbow, wrist, hip, knee, ankle, and feet (Gripp et al., 2019; Leoni et al., 2014). Osteoporosis is also seen in older CS patients, with decreased bone mineral density evident from a young age, possibly due to increased bone resorption, which is also reported in other RASopathies (Leoni et al., 2014; Stevenson et al., 2011).

Failure to thrive in CS is, in part, attributed to the gastrointestinal defects associated with this disorder. Feeding and swallowing difficulties are potentiated by an enlargement of the tongue and by general oral hypersensitivity. Vomiting is common and is caused by pyloric stenosis or gastroesophageal reflux. Intestinal pseudoobstruction and chronic constipation are common and due to impaired gastrointestinal motility of the intestinal tract. In addition, malocclusion is also common, possibly disrupting proper eating abilities. Understandably, patients tend to be irritable and sleep deprived, adding to the level of the overall anxiety associated with this disorder (Gripp et al., 2019).

Dermatological manifestations of CS patients include loose skin around neck, hands, and feet, soft and thickened skin in palms of hand and soles of feet, and hyper pigmentation (Siegel et al., 2012). Perhaps most significantly, however, CS patients have a high predisposition to developing tumors. Benign tumors in CS patients include papillomas around the nose, mouth, and anus (Gripp, 2005). Malignant tumors include development of rhabdomyosarcomas, cancer in the connective tissue

of the muscle (Gripp et al., 2019; Sinico et al., 2011). CS patients also have a higher predisposition to developing solid tumors such as neuroblastoma and transitional cell carcinoma of the bladder (Gripp, 2005; Gripp et al., 2002).

Costello Syndrome and HRAS

In 2005, mutations in one gene, *HRAS*, which resides at the core of the canonical RAS-MAPK signaling cascade, was identified as causal to CS (Aoki et al., 2005; Gripp et al., 2007; Kerr et al., 2006). The mechanism of regulation of HRAS is similar to other RAS proteins. Briefly, in its GDP bound state, HRAS is inactive, preventing it from binding to its downstream effectors and activating downstream pathways. To activate HRAS, guanine nucleotide exchange factors exchange GDP for GTP. Conversely, GTPase activating proteins (GAPs) mediate the conversion of GTP back to GDP, restoring the inactive conformation and terminating downstream binding of HRAS to its effectors (Bos et al., 2007).

HRAS has been heavily studied with respect to its role as an oncogene, and somatic mutations in HRAS are known to cause cancers in prostate, salivary gland, skin, stomach, thyroid, urinary tract, and upper aerodigestive tissues (Prior et al., 2012). Since CS-associated HRAS mutations are germline, the molecular and functional signature of these mutations are distinct. For example, whereas somatic mutations in HRAS are concentrated to three hot spots, G12, G13, and Q61, CS-associated HRAS mutations are distributed throughout the protein (Fig. 4.2B) (Buhrman et al., 2010; Denayer et al., 2008; Gremer et al., 2010; Gripp et al., 2012, 2015; Hobbs et al., 2016; Kerr et al., 2006; Lorenz et al., 2013; Prior et al., 2012). However, like the oncogenic somatic mutations, the most common HRAS mutation in CS is a GOF mutation that renders active HRAS insensitive to GAP activity. This mutation, p.Gly12Ser, affects nearly 80% of all CS patients (Gripp and Lin, 2012; Kerr et al., 2006). Other, rarer GOF mutations are also identified in CS, including the p.Lys117Arg mutation, which alters GDP/GTP nucleotide affinity, and the p.Glu37dup mutation, which induces enhanced downstream effector binding to increase activity of the pathway (Denayer et al., 2008; Gremer et al., 2010).

Interestingly, and to make things more complicated, like *PTPN11*, both GOF and LOF CS-associated *HRAS* mutations exist. Specifically, two *HRAS* mutations, p.Gly60Asp and p.Ser89Cys, negatively regulate RAS activity and downstream signaling, leading to a much milder overall CS phenotype (Gripp et al., 2012, 2015). Moreover, selective hyper-activation of one effector pathway over another is also demonstrated with some CS-associated *HRAS* mutations. For instance, under steady-state conditions, *HRAS* p.Glu63_Asp69dup demonstrates enhanced binding to RAF1, leading to subsequent induction of MAPK activity, but shows impaired parallel binding to PI3K, decreasing downstream AKT/mTOR signaling (Lorenz et al., 2013).

Disease modeling of Costello Syndrome

Specific functional consequences of the different *HRAS* mutations associated with CS are not yet well understood. Most studies for CS-associated *HRAS* have utilized in vitro overexpression systems, which often mask the true pathophysiology underlying the disease. Because of that and the importance of demonstrating disease in more relevant biological systems, animal models for CS were created. To date, there are only three published papers modeling CS in murine models and one in zebrafish, each replicating some, but not all, of the features in human CS. The first CS model was developed by the Barbacid lab (CNIO, Spain), who generated an $HRAS^{G12V/+}$ knock-in mouse model. Generation of these mutant mice included an insertion of a β-gal-neomycin cassette fused to the 3′untranslated region of the *HRAS* knock-in gene locus in order to more effectively monitor *HRAS* expression in tissues. Both homozygous and heterozygous mice were viable on the 129Sv/j:C57BL/6J mixed background and successfully phenocopied at least some of features of CS, including the craniofacial malformations, malocclusion, neurocognitive defects, and the cardiac hypertrophy. However, the mice did not develop cancer during the observed time period for this study (1.5 years) (Schreiber et al., 2017; Schuhmacher et al., 2008; Viosca et al., 2009). Dermal, skeletal, and muscular phenotypes of CS were not characterized in this model system.

The second CS mouse model created by Chen et al. (2009), like the previous model is a $HRAS^{G12V/+}$ knock in mouse, but on a mixed 129Sv/j:C57BL/6J:FVB/N background. Interestingly, these mice displayed increased postnatal lethality in heterozygous mice and a complete postnatal lethality in homozygous mice. Necropsy did not reveal any gross or microscopic pathology of the heart muscle or lung or any evidence of bleeding in these neonates, leaving the cause of death in these mice largely unknown. However, as feeding difficulties are a well-recognized phenotype in CS, it is possible these mice failed to suckle after birth, leading to their early demise (Gripp et al., 2019). Surviving heterozygous mice developed craniofacial malformations and malocclusion, but interestingly, no cardiac abnormalities (Chen et al., 2009; Goodwin et al., 2014a). Importantly, this model developed the skin and gastric papilloma's observed in CS patients. Other CS phenotypes affecting bone, muscle, brain, and skin were not characterized. The difference in disease severity between the two mouse models is noteworthy but has not yet been fully elucidated. Likely, the mouse genetic background plays a significant role in modulating the severity of the CS phenotype. It may also be possible that the insertion of the β-gal-neomycin cassette in Schuhmacher et al. (2008) $HRAS^{G12V/+}$ mouse model prevents full or appropriate expression of the *HRAS* mutant allele (Chen et al., 2009; Schreiber et al., 2017), reducing the severity of the disease in these mice.

The third, and most recent, CS mouse model was generated by the Aoki lab and garners an $HRAS^{G12S/+}$ knock-in mutation in the C57BL/6J background (Tohoku University School of Medicine, Japan). These mice harbor the more clinically

relevant mutation, the p.Gly12Ser mutation, which is observed in >80% of CS patients. Heterozygous mice were born at the expected Mendelian ratios, but homozygous mice showed complete postnatal lethality of unknown cause. Hereto, mice phenocopied some of the features of CS, including facial dysmorphia, malocclusion, increased resting energy expenditure, and cardiac abnormalities (Oba et al., 2018). Other CS phenotypes involving brain, bone, skin, and muscle hereto were not evaluated. Thus, taken together, there are reported variations in the phenotypic spectrum for CS in developed murine models, variables that seem to be dependent on genetic background, genetic targeting strategy, and the specific *HRAS* mutation chosen for the study.

In addition to these mouse models, a zebrafish model stably expressing mutant $HRAS^{G12V/+}$ using the Tol2 transposase has also been generated. The mutant fish display reduced size, scoliosis, smaller heart, craniofacial dysmorphology, increased tumor incidence, and decreased life span (Santoriello et al., 2009). Interestingly, decreased proliferation and increased senescence are also observed in several tissues, including the adult progenitor compartments of the brain and heart. However, using a zebrafish model to delineate human disease pathophysiology poses major concerns. First, the genetic manipulation used to create this model is prone to insertion of multiple copies of the transgene, i.e., the mutant *HRAS*, which may confound results when compared to effects of endogenously expressing *HRAS* in CS patients. Second, and more importantly, this is not a mammalian model system, and, therefore, translatability of mechanism and function of the mutation in humans may be significantly different. Despite this, using the zebrafish model system is an invaluable tool to monitor some preliminary functionality of a disease mutation, particularly as it relates to early embryogenesis, which may not always be possible using mammalian model systems.

Interestingly, none of the described models showed a global hyperactivation of RAS-MAPK or RAS-AKT/mTOR signaling, as was expected from the in vitro oncogenic *HRAS* overexpression studies. Instead, only modest lineage and tissue-specific changes were observed. For example, all three CS mouse models showed increased RAS-MAPK signaling in the liver. On the other hand, the increased active GTP bound HRAS did not effectively translate to increased RAS-MAPK signaling in mouse embryonic fibroblasts, adult brain, or heart (Chen et al., 2009; Chennappan et al., 2017; Schuhmacher et al., 2008). However, later studies revealed that activation of the MAPK pathway was localized to the hippocampal region of the brain in CS mice (Schreiber et al., 2017; Viosca et al., 2009). Similarly, an increase in ERK activity was observed in CS mouse incisors, and inhibition of MAPK or AKT using pathway-targeted inhibitors normalized the hyper-proliferation of progenitor cells, thereby preventing the development of malocclusions (Goodwin et al., 2014b). Finally, studies in CS-derived mouse skin fibroblasts demonstrated increased RAS-AKT/mTOR signaling at baseline and in response to overnight serum starvation, an activation that was lost when cells were cultured in the presence of serum

(Chennappan et al., 2017). Taken together, it is clear that a better understanding of the molecular mechanisms involving HRAS signaling in CS are needed to determine the mechanistic and functional consequences of this disease in patients.

Costello Syndrome and induced pluripotent stem cells

Indeed, use of patient-derived iPSCs can now, for the first time, provide a humanized model system to better understand the functional and cellular consequences of specific *HRAS*-associated mutations causal to CS. Human iPSCs derived from patients with CS were first differentiated to astroglia. In vitro, these cells differentiated rapidly, were larger, exhibited hyperplasia, and displayed an abundance of extracellular matrix remodeling factors and proteoglycans. Moreover, CS-associated iPSC-derived astrocytes formed a complex morphology, with increased volume and branching, when grafted into mouse hippocampal organotypic slices, thereby mimicking the accelerated maturation observed in vitro. CS-associated iPSC-derived astrocytes also accelerated primary neurite outgrowth from purified rat postnatal retinal ganglion cells, in both in vitro co-culture and in indirect co-culture systems, suggesting increased astroglial to neuron signaling that is attributed to increased extracellular matrix and proteoglycan secretion. Indeed, decreasing the expression of SNAI2, a transcriptional factor downstream of *HRAS*, either by treating cells with a farnesyl transferase inhibitor that inactivates RAS or by knocking down its expression with an SNAI2 specific siRNA, reduced the secretion of extracellular matrix proteins and proteoglycans and normalized the signaling defects in CS-associated iPSC-derived astrocytes. Interestingly, the acute treatment with farnesyl transferase inhibitor had a pronounced effect on the expression of nondevelopmentally regulated transcription factors without altering those involved in normal astrocyte development, making it a viable therapeutic treatment for cognitive impairment and enhanced neuroplasticity in CS. Importantly too, these results could be recapitulated in mice with astrocyte-specific expression of CS mutant HRAS, where experience-independent accumulation of proteoglycans in the form of a perineuronal net was observed around the parvalbumin positive interneurons in the cortex, suggesting accelerated neuronal maturation, impaired cortical remodeling, and decreased learning (Krencik et al., 2015). In a follow-up study, investigators also evaluated the effect of CS-associated neuroectodermal differentiation. Here, CS-associated iPSC-derived neuroectoderm displayed an extended progenitor phase with increased production of cortical neurons and morphological deficits, effects also confirmed in a CS mouse model. Morphological analysis of mature neurons also revealed significantly altered neurite length and soma size in CS patients (Rooney et al., 2016).

By highlighting the synergy between mouse models and human iPSCs, mechanisms underlying cellular pathologies of CS can be better understood and therapeutic targets in the pathway can more easily be identified. For example, iPSCs have

helped determine that increased neuronal progenitor cell proliferation and cellular hypertrophy occur in CS, in both terminally differentiated neurons and astrocytes, suggesting that these attributes underlie the increased brain volume and overall cognitive deficiency in CS. However, limitations in studying CS using iPSC biology exist. For example, while the CS iPSC-derived neuronal models nicely reproduce the structural and morphological findings observed in vivo in *HRAS*$^{G12V/+}$ mice, the cells fail to properly differentiate, showing aspects of functional and electrophysiological immaturity (Rooney et al., 2016). However, these effects may be technical and could perhaps be easily overcome by use of high density astrocyte co-cultures, modified media culture systems, three dimensional (3D) brain organoids, or organ-on-a-chip technologies (Arlotta et al., 2017; Klapper et al., 2019; Lancaster et al., 2013; Pasca et al., 2015). In addition, use of genome editing technologies to "induce" specific mutations in *HRAS* could allow for more definitive assessments of various CS mutations, providing the opportunity to exponentially increase understanding of the complexities associated with CS pathophysiology.

Limitations and future direction of induced pluripotent stem cell model systems

So far, it has been demonstrated that iPSCs, together with CRISPR-Cas9 genome editing, can be powerful tools to study the differential molecular mechanisms of RASopathies, to identify novel targets in downstream signaling pathways, and to screen compounds that may be effective in treating human disease. Despite these advantages, however, use of in vitro model systems, do not always adequately predict disease etiology and demonstrate different functional mechanisms in culture as compared to whole organ systems. In this case, validation of findings using iPSC-derived differentiation systems should be made in animal models. In the future, it is likely that iPSC-derived single cell cultures will benefit from recent advances using 3D organoids model systems or organ-on-a-chip technologies, where multiple cell types can be studied at one time in the internal microenvironment where paracrine signals and co-contributing cellular functions can be assessed (Yin et al., 2016).

There are also other limitations to consider when using iPSCs to model disease. For example, several recent studies report that iPSCs preserve residual epigenetic memory of donor somatic cells and can lead to low quality iPSCs with limited clinical application (Tobin and Kim, 2012). In addition, the differentiation process does not always yield a pure population. For example, iPSC-differentiation could yield a nonuniform phenotype with a mixed population of cells (Moretti et al., 2010; Sharma et al., 2013). To overcome this problem, protocols have been focused on optimizing conditions to more effectively isolate and purify iPSC-derived differentiated cell lineages. In the case of CMs, FACS selection, using fluorescent dye labeling of mitochondria or surface expression of either the signal regulatory

protein alpha or an activated leukocyte cell adhesion molecule have been used (Dubois et al., 2011; Lin et al., 2012; Rust et al., 2009). There has also been success with >90% efficiency in isolating and enriching CMs from iPSC lines using lactate purification (Burridge et al., 2015; Lian et al., 2013). Additional limitations include batch effects, where the differentiation of iPSC clones often result in heterogeneity of the different cellular subtypes in a particular lineage; for example, in iPSC-CMs, cultures include a mixed population of atrial, nodal, and ventricular CMs (Sharma et al., 2013). One technique that has recently been utilized to help improve these limitations is single cell RNAseq (scRNAseq), where individual cells can be grouped into specific subpopulations based on their differential gene expression profile and used to predict the physiological and functional effects of the mutation or pathology (Chu et al., 2016; Wen and Tang, 2016).

Importantly, for some lineages such as CMs and neuronal cells, iPSC-derived cells do not fully mature, preventing a complete characterization of the adult phenotypes (Karbassi et al., 2020). For example, although brain organoids differentiated from iPSCs recapitulate many features of human brain development in vivo, current methods can only recapitulate development to the first trimester. Moreover, the lack of vascularization of this tissue limits the growth and the maturation of brain organoids due to an inadequate supply of key nutrients and oxygen. Indeed, 3D brain organoids also lack the presence of essential nonneural cells, such as microglia, endothelial cells, hematopoietic cells, and meninges, needed for the critical paracrine signals necessary for proper brain function. Finally, another serious challenge to organoid technology is that they are highly heterogeneous, with random positioning of brain regions within each organoid. Therefore, and to overcome some of these limitations, multiple iPSC lines and independent batches have to be differentiated for consistency and reproducibility of results (Qian et al., 2019). Moreover, hereto, scRNAseq can be used to help define the differentiation and maturation of excitable cells, such as neurons and CMs, to help more clearly define cell-lineage specific disease functionality. Ultimately, it will be this ability to isolate and characterize a pure, mature population of iPSC-derived cells that will make drug discovery, proteomic, genomic, and metabolomic studies more efficient in the future (Brodehl et al., 2019).

Lastly, an important consideration when using iPSCs is the contributing effect of allelic variance, altered gene expressivity, and modifier loci on disease severity from individual to individual. It is not often clear what cell line makes the best "control" for a diseased patient's iPSCs. Previously, diseased cells were compared to cells from a healthy donor, usually an unaffected sibling, the parents, age-matched, or sex-matched controls. However, assessing effects of a specific mutation in the context of a different genetic background made interpretation of any results difficult. To circumvent this problem, researchers combined iPSC-based disease modeling with CRISPR-Cas9 technology to "correct" mutations in a patient's own iPSCs, creating an isogenic control. However, the new challenge that arose in this case is the inability to distinguish the effect of the pathogenic mutation from other potential

disease-causing changes in a patient's background. In this case, researchers started using the CRISPR-Cas9 technology to also "introduce" a potential pathogenic mutation in the background of a healthy iPSC cell line, leading to isogenic mutated iPSC cell lines. However, though these isogenic mutated cells model the disease phenotype, the severity of the phenotype can sometimes be significantly less pronounced when compared to patient-derived iPSCs, suggesting that additional genetic modifications exist to influence the severity of a disease phenotype (Jaffre et al., 2019). Obviously, using multiple patient-derived iPSCs would be an ideal system to ascertain more definitively the contributions of a single point mutation in causing a disease. However, in RASopathies, which are rare, this can be a significant challenge. Thus, our next best strategy is to use both corrected and patient-derived iPSC lines to determine disease relevance and molecular/cellular analysis, as well as drug screening to prevent or reverse these diseases. In the future, we hope to use iPSCs to understand the functional role of these genetic modifiers in the context of disease heterogeneity in RASopathies. In addition, use of a patient's own cells, corrected for their mutations, may also prove useful in clinical applications, such as reparative and regenerative medicine.

Clearly, the direction for iPSCs is moving toward clinical application. However, cautionary steps in this regard must be taken. Like cancer cells, iPSCs have indefinite proliferation capacity, with epigenetic modifications and expression of oncogenic markers, such as c-MYC, which increases the efficiency of reprogramming. Therefore, iPSCs generated with c-MYC have a higher risk of tumorigenesis. Consequently, iPSCs might have genomic instability, which has to be checked regularly by karyotyping and next generation sequencing. Indeed, it is this consequential epigenomic and genomic instability that remains a major limitation for clinical application of iPSC-based cell therapies (Yoshihara et al., 2019). More recently, and to overcome this hurdle, multiple reprogramming approaches have now been developed to create iPSCs without the need for c-Myc or other oncogenes (l-Myc and Lin28) (Kamath et al., 2017).

As of today, some ethical concerns regarding potential off-target effects of Cas9 limits the use of this technology in a clinical setting. However, with the creation of advanced Cas9 variants with increased specificity, it is likely that this gene editing strategy will soon become a technological reality (Lee et al., 2018). Indeed, several studies have demonstrated successful in utero gene therapy in animal models in a variety of diseases, including hemophilia and lysosomal storage disorders, thus paving the path to clinical translation (Casal and Haskins, 2006). The correction of a monogenic mutation using CRISPR-Cas9 can be done ex-vivo on gametes or embryos before implantation, in utero, or even postnatally. The National Academy of Sciences, the National Academy of Medicine, and the National Academies of Sciences, Engineering, and Medicine recommend that "research on germline gene editing should continue, albeit cautiously, and only for therapeutic purposes to treat serious heritable diseases" (Kofler and Kraschel, 2018). Considering that genes

associated with RASopathies are fundamentally regulated during development, using genome editing tools in utero could prove beneficial, and perhaps even life-saving, to patients with these disorders.

RASopathies and induced pluripotent stem cells: where do we go from here?

As mentioned previously, RASopathies are a group of syndromic disorders that affect multiple organs and tissues. However, despite the overt systemic effects of the mutations that cause these disorders, research has only focused on modeling cardiac hypertrophy, JMML, and neuronal phenotypes. It will be invaluable to continue these studies but also to expand them in other equally devastating, aspects of RASopathies. One area currently being investigated in our own lab includes understanding the effects of the neurocognitive aspects of NS. These elements include delineating why some, but not all, NS patients develop cognitive and learning disabilities, attention deficit hyperactivity disorder, and autism (Adviento et al., 2014; Pierpont et al., 2018). Like iPSC-derived CMs or myeloid cells, use of iPSC-derived brain organoids will significantly advance our understanding of neuronal disease in NS for several reasons. First, it can be difficult to obtain biopsies from tissues such as the brain directly from NS patients; iPSCs can be generated from a simple blood draw. Second, brain function and disease can be modeled in vitro, through differentiation of iPSCs into human neuronal cells, allowing investigators to both recapitulate the clinical effects observed in patients, as well as understand the functional effects of disease-causing mutations.

In addition, RASopathies patients have severe gastrointestinal difficulties. For example, more than 75% of infants with NS present with mild to severe, often long-lasting, feeding difficulties, which include poor suckling, slow feeding, and recurrent vomiting (Roberts et al., 2013; Sharland et al., 1992). Interestingly, severe feeding problems in infancy are associated with a developmental delay in language and affect long-term educational achievement (Shaw et al., 2007). Similarly, severe feeding difficulties in early infancy lead to G-tube placement in CS patients (Gripp et al., 2002). In this regard, generation of intestinal 3D organoids will be advantageous in investigating the mechanisms underlying intestinal disease in RASopathies, along with helping identify potential therapies that can treat this problem in children. Indeed, intestinal organoids that mimic embryonic intestinal development have been differentiated from iPSCs in vitro using a combination of *Wnt3a* and *FGF4* for hindgut specification and *FGF4* alone to promote hindgut morphogenesis (McCracken et al., 2011; Spence et al., 2011), and they have already been used extensively for modeling a variety of diseases that affect the gastrointestinal tract (Mithal et al., 2020; Workman et al., 2017). Our lab is currently applying this technology to determine the functional mechanisms associated with gastrointestinal difficulties in NS and NSML.

Conclusions

In summary, the unlimited source of iPSCs, combined with CRISPR-Cas9 technology, will provide us an opportunity to more deeply understand the molecular mechanisms of diseases, such as RASopathies, in a humanized model system, improving our ability to achieve successful cell-based therapies that can translate to clinical trials. This technology will not only accelerate development of novel therapeutic interventions but will also serve as a personalized medicine approach to effectively treat patients with genetic disorders. This technology will undoubtedly address the genomic, metabolic, and pharmacogenomic diversity of RASopathy specific variants, studies that can be validated in mammalian systems and then quickly move to clinic. Further, combining reprogramming of iPSCs with 3D organoid cultures, organ-on-a-chip technology, and bioprinting holds additional promise for investigating and understanding tissue engineering and regenerative medicine in the near future. Indeed, methods to further translate these technologies are advancing every day and we hope that this technology will more readily yield findings to treat and improve the lives of RASopathies patients, perhaps yielding opportunities to also treat other, more common, congenital disorders with similar disease signaling mechanisms in the near future.

Acknowledgments

Figs. 4.1 and 4.2 were created using BioRender.com.

Funding resources

This work was supported by the National Institutes of Health (Grants R01-HL122238, R01-HL102368) and the Masonic Medical Research Institute to M.I.K.

References

Abe, Y., Aoki, Y., Kuriyama, S., Kawame, H., Okamoto, N., Kurosawa, K., Ohashi, H., Mizuno, S., Ogata, T., Kure, S., et al., 2012. Prevalence and clinical features of Costello syndrome and cardio-facio-cutaneous syndrome in Japan: findings from a nationwide epidemiological survey. Am. J. Med. Genet. 158A, 1083−1094.

Adviento, B., Corbin, I.L., Widjaja, F., Desachy, G., Enrique, N., Rosser, T., Risi, S., Marco, E.J., Hendren, R.L., Bearden, C.E., et al., 2014. Autism traits in the RASopathies. J. Med. Genet. 51, 10−20.

Altmuller, F., Pothula, S., Annamneedi, A., Nakhaei-Rad, S., Montenegro-Venegas, C., Pina-Fernandez, E., Marini, C., Santos, M., Schanze, D., Montag, D., et al., 2017. Aberrant neuronal activity-induced signaling and gene expression in a mouse model of RASopathy. PLoS Genet. 13, e1006684.

Aoki, Y., Niihori, T., Banjo, T., Okamoto, N., Mizuno, S., Kurosawa, K., Ogata, T., Takada, F., Yano, M., Ando, T., et al., 2013. Gain-of-function mutations in RIT1 cause Noonan syndrome, a RAS/MAPK pathway syndrome. Am. J. Hum. Genet. 93, 173−180.

Aoki, Y., Niihori, T., Inoue, S., Matsubara, Y., 2016. Recent advances in RASopathies. J. Hum. Genet. 61, 33−39.

Aoki, Y., Niihori, T., Kawame, H., Kurosawa, K., Ohashi, H., Tanaka, Y., Filocamo, M., Kato, K., Suzuki, Y., Kure, S., Matsubara, Y., 2005. Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nat. Genet. 37, 1038−1040.

Aoki, Y., Niihori, T., Narumi, Y., Kure, S., Matsubara, Y., 2008. The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. Hum. Mutat. 29, 992−1006.

Araki, T., Chan, G., Newbigging, S., Morikawa, L., Bronson, R.T., Neel, B.G., 2009. Noonan syndrome cardiac defects are caused by PTPN11 acting in endocardium to enhance endocardial-mesenchymal transformation. Proc. Natl. Acad. Sci. U. S. A. 106, 4736−4741.

Araki, T., Mohi, M.G., Ismat, F.A., Bronson, R.T., Williams, I.R., Kutok, J.L., Yang, W., Pao, L.I., Gilliland, D.G., Epstein, J.A., Neel, B.G., 2004. Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of Ptpn11 mutation. Nat. Med. 10, 849−857.

Arlotta, P., Quadrato, G., Sherwood, J.L., 2017. Long-term culture and electrophysiological characterization of human brain organoids. Protoc. Exch.

Arvisais, E.W., Romanelli, A., Hou, X., Davis, J.S., 2006. AKT-independent phosphorylation of TSC2 and activation of mTOR and ribosomal protein S6 kinase signaling by prostaglandin F2alpha. J. Biol. Chem. 281, 26904−26913.

Belanger, L.F., Roy, S., Tremblay, M., Brott, B., Steff, A.M., Mourad, W., Hugo, P., Erikson, R., Charron, J., 2003. Mek2 is dispensable for mouse growth and development. Mol. Cell Biol. 23, 4778−4787.

Bolocan, A., Quijano-Roy, S., Seferian, A.M., Baumann, C., Allamand, V., Richard, P., Estournet, B., Carlier, R., Cave, H., Gartioux, C., et al., 2014. Congenital muscular dystrophy phenotype with neuromuscular spindles excess in a 5-year-old girl caused by HRAS mutation. Neuromuscul. Disord. 24, 993−998.

Bonetti, M., Paardekooper Overman, J., Tessadori, F., Noel, E., Bakkers, J., den Hertog, J., 2014. Noonan and LEOPARD syndrome Shp2 variants induce heart displacement defects in zebrafish. Development 141, 1961−1970.

Bos, J.L., Rehmann, H., Wittinghofer, A., 2007. GEFs and GAPs: critical elements in the control of small G proteins. Cell 129, 865−877.

Brodehl, A., Ebbinghaus, H., Deutsch, M.A., Gummert, J., Gartner, A., Ratnavadivel, S., Milting, H., 2019. Human induced pluripotent stem-cell-derived cardiomyocytes as models for genetic cardiomyopathies. Int. J. Mol. Sci. 20.

Buckles, T.C., Ziemba, B.P., Masson, G.R., Williams, R.L., Falke, J.J., 2017. Single-molecule study reveals how receptor and ras synergistically activate PI3Kalpha and PIP3 signaling. Biophys. J. 113, 2396−2405.

Buhrman, G., Holzapfel, G., Fetics, S., Mattos, C., 2010. Allosteric modulation of Ras positions Q61 for a direct role in catalysis. Proc. Natl. Acad. Sci. U. S. A. 107, 4931−4936.

Burridge, P.W., Holmstrom, A., Wu, J.C., 2015. Chemically defined culture and cardiomyocyte differentiation of human pluripotent stem cells. Curr. Protoc. Hum. Genet. 87, 21.3.1−21.3.15.

Carvajal-Vergara, X., Sevilla, A., D'Souza, S.L., Ang, Y.S., Schaniel, C., Lee, D.F., Yang, L., Kaplan, A.D., Adler, E.D., Rozov, R., et al., 2010. Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. Nature 465, 808−812.

Casal, M., Haskins, M., 2006. Large animal models and gene therapy. Eur. J. Hum. Genet. 14, 266–272.

Castellano, E., Downward, J., 2011. RAS interaction with PI3K: more than just another effector pathway. Genes Cancer 2, 261–274.

Chen, P.C., Wakimoto, H., Conner, D., Araki, T., Yuan, T., Roberts, A., Seidman, C., Bronson, R., Neel, B., Seidman, J.G., Kucherlapati, R., 2010. Activation of multiple signaling pathways causes developmental defects in mice with a Noonan syndrome-associated Sos1 mutation. J. Clin. Invest. 120, 4353–4365.

Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., et al., 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. 15, 2203–2208.

Chen, X., Mitsutake, N., LaPerle, K., Akeno, N., Zanzonico, P., Longo, V.A., Mitsutake, S., Kimura, E.T., Geiger, H., Santos, E., et al., 2009. Endogenous expression of Hras(G12V) induces developmental defects and neoplasms with copy number imbalances of the oncogene. Proc. Natl. Acad. Sci. U. S. A. 106, 7979–7984.

Chen, Y.N., LaMarche, M.J., Chan, H.M., Fekkes, P., Garcia-Fortanet, J., Acker, M.G., Antonakos, B., Chen, C.H., Chen, Z., Cooke, V.G., et al., 2016. Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases. Nature 535, 148–152.

Chennappan, S., Schulze, T., Cirstea, I.C., 2017. Endogenous locus-driven H-Ras G12V expression induces senescence-like phenotype in primary fibroblasts of the Costello syndrome mouse model. Mol. Life 1.

Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw 3rd, E.B., Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., Birnbaum, M.J., 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292, 1728–1731.

Chu, L.F., Leng, N., Zhang, J., Hou, Z., Mamott, D., Vereide, D.T., Choi, J., Kendziorski, C., Stewart, R., Thomson, J.A., 2016. Single-cell RNA-seq reveals novel regulators of human embryonic stem cell differentiation to definitive endoderm. Genome Biol. 17, 173.

Cirstea, I.C., Kutsche, K., Dvorsky, R., Gremer, L., Carta, C., Horn, D., Roberts, A.E., Lepri, F., Merbitz-Zahradnik, T., Konig, R., et al., 2010. A restricted spectrum of NRAS mutations causes Noonan syndrome. Nat. Genet. 42, 27–29.

Clay, S.A., Domeier, T.L., Hanft, L.M., McDonald, K.S., Krenz, M., 2015. Elevated Ca^{2+} transients and increased myofibrillar power generation cause cardiac hypercontractility in a model of Noonan syndrome with multiple lentigines. Am. J. Physiol. Heart Circ. Physiol. 308, H1086–H1095.

Corson, L.B., Yamanaka, Y., Lai, K.M., Rossant, J., 2003. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development 130, 4527–4537.

Costello, J.M., 1971. A new syndrome. NZ Med. J. 74.

Costello, J.M., 1977. A new syndrome: mental subnormality and nasal papillomata. Aust. Paediatr. J. 13, 114–118.

Denayer, E., Parret, A., Chmara, M., Schubbert, S., Vogels, A., Devriendt, K., Frijns, J.P., Rybin, V., de Ravel, T.J., Shannon, K., et al., 2008. Mutation analysis in Costello syndrome: functional and structural characterization of the HRAS p.Lys117Arg mutation. Hum. Mutat. 29, 232–239.

Der Kaloustian, V.M., Moroz, B., McIntosh, N., Watters, A.K., Blaichrnan, S., 1991. Costello syndrome. Am. J. Med. Genet. 41, 69–73.

Dhillon, A.S., Hagan, S., Rath, O., Kolch, W., 2007. MAP kinase signalling pathways in cancer. Oncogene 26, 3279–3290.

Digilio, M.C., Sarkozy, A., de Zorzi, A., Pacileo, G., Limongelli, G., Mingarelli, R., Calabro, R., Marino, B., Dallapiccola, B., 2006. LEOPARD syndrome: clinical diagnosis in the first year of life. Am. J. Med. Genet. 140, 740–746.

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., et al., 2008. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321, 1218–1221.

Dubois, N.C., Craft, A.M., Sharma, P., Elliott, D.A., Stanley, E.G., Elefanty, A.G., Gramolini, A., Keller, G., 2011. SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. Nat. Biotechnol. 29, 1011–1018.

Dummler, B., Tschopp, O., Hynx, D., Yang, Z.Z., Dirnhofer, S., Hemmings, B.A., 2006. Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. Mol. Cell Biol. 26, 8042–8051.

Edouard, T., Combier, J.P., Nedelec, A., Bel-Vialar, S., Metrich, M., Conte-Auriol, F., Lyonnet, S., Parfait, B., Tauber, M., Salles, J.P., et al., 2010. Functional effects of PTPN11 (SHP2) mutations causing LEOPARD syndrome on epidermal growth factor-induced phosphoinositide 3-kinase/AKT/glycogen synthase kinase 3beta signaling. Mol. Cell Biol. 30, 2498–2507.

Edwards, M.A., Crombie, K., Schramm, C., Krenz, M., 2015. The Q510E mutation in Shp2 perturbs heart valve development by increasing cell migration. J. Appl. Physiol. (1985) 118, 124–131.

Fischer, A.M., Katayama, C.D., Pages, G., Pouyssegur, J., Hedrick, S.M., 2005. The role of erk1 and erk2 in multiple stages of T cell development. Immunity 23, 431–443.

Garg, S., Brooks, A., Burns, A., Burkitt-Wright, E., Kerr, B., Huson, S., Emsley, R., Green, J., 2017. Autism spectrum disorder and other neurobehavioural comorbidities in rare disorders of the Ras/MAPK pathway. Dev. Med. Child Neurol. 59, 544–549.

Gauthier, A.S., Furstoss, O., Araki, T., Chan, R., Neel, B.G., Kaplan, D.R., Miller, F.D., 2007. Control of CNS cell-fate decisions by SHP-2 and its dysregulation in Noonan syndrome. Neuron 54, 245–262.

Gehart, H., Kumpf, S., Ittner, A., Ricci, R., 2010. MAPK signalling in cellular metabolism: stress or wellness? EMBO Rep. 11, 834–840.

Girisha, K.M., Lewis, L.E., Phadke, S.R., Kutsche, K., 2010. Costello syndrome with severe cutis laxa and mosaic HRAS G12S mutation. Am. J. Med. Genet. 152A, 2861–2864.

Giroux, S., Tremblay, M., Bernard, D., Cadrin-Girard, J.F., Aubry, S., Larouche, L., Rousseau, S., Huot, J., Landry, J., Jeannotte, L., Charron, J., 1999. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr. Biol. 9, 369–372.

Gold, M.R., 2008. B cell development: important work for ERK. Immunity 28, 488–490.

Goodwin, A.F., Oberoi, S., Landan, M., Charles, C., Massie, J.C., Fairley, C., Rauen, K.A., Klein, O.D., 2014a. Craniofacial and dental development in Costello syndrome. Am. J. Med. Genet. 164A, 1425–1430.

Goodwin, A.F., Tidyman, W.E., Jheon, A.H., Sharir, A., Zheng, X., Charles, C., Fagin, J.A., McMahon, M., Diekwisch, T.G., Ganss, B., et al., 2014b. Abnormal Ras signaling in Costello syndrome (CS) negatively regulates enamel formation. Hum. Mol. Genet. 23, 682–692.

Gordanpour, A., Nam, R.K., Sugar, L., Seth, A., 2012. MicroRNAs in prostate cancer: from biomarkers to molecularly-based therapeutics. Prostate Cancer Prostatic Dis. 15, 314−319.

Gremer, L., De Luca, A., Merbitz-Zahradnik, T., Dallapiccola, B., Morlot, S., Tartaglia, M., Kutsche, K., Ahmadian, M.R., Rosenberger, G., 2010. Duplication of Glu37 in the switch I region of HRAS impairs effector/GAP binding and underlies Costello syndrome by promoting enhanced growth factor-dependent MAPK and AKT activation. Hum. Mol. Genet. 19, 790−802.

Gripp, K.W., 2005. Tumor predisposition in Costello syndrome. Am. J. Med. Genet. C Semin. Med. Genet. 137C, 72−77.

Gripp, K.W., Aldinger, K.A., Bennett, J.T., Baker, L., Tusi, J., Powell-Hamilton, N., Stabley, D., Sol-Church, K., Timms, A.E., Dobyns, W.B., 2016. A novel rasopathy caused by recurrent de novo missense mutations in PPP1CB closely resembles Noonan syndrome with loose anagen hair. Am. J. Med. Genet. 170, 2237−2247.

Gripp, K.W., Bifeld, E., Stabley, D.L., Hopkins, E., Meien, S., Vinette, K., Sol-Church, K., Rosenberger, G., 2012. A novel HRAS substitution (c.266C>G; p.S89C) resulting in decreased downstream signaling suggests a new dimension of RAS pathway dysregulation in human development. Am. J. Med. Genet. 158A, 2106−2118.

Gripp, K.W., Lin, A.E., 2012. Costello syndrome: a Ras/mitogen activated protein kinase pathway syndrome (rasopathy) resulting from HRAS germline mutations. Genet. Med. 14, 285−292.

Gripp, K.W., Lin, A.E., Nicholson, L., Allen, W., Cramer, A., Jones, K.L., Kutz, W., Peck, D., Rebolledo, M.A., Wheeler, P.G., et al., 2007. Further delineation of the phenotype resulting from BRAF or MEK1 germline mutations helps differentiate cardio-facio-cutaneous syndrome from Costello syndrome. Am. J. Med. Genet. 143A, 1472−1480.

Gripp, K.W., Morse, L.A., Axelrad, M., Chatfield, K.C., Chidekel, A., Dobyns, W., Doyle, D., Kerr, B., Lin, A.E., Schwartz, D.D., et al., 2019. Costello syndrome: clinical phenotype, genotype, and management guidelines. Am. J. Med. Genet. 179, 1725−1744.

Gripp, K.W., Schill, L., Schoyer, L., Stronach, B., Bennett, A.M., Blaser, S., Brown, A., Burdine, R., Burkitt-Wright, E., Castel, P., et al., 2020. The sixth international RASopathies symposium: precision medicine-from promise to practice. Am. J. Med. Genet. 182, 597−606.

Gripp, K.W., Scott Jr., C.I., Nicholson, L., McDonald-McGinn, D.M., Ozeran, J.D., Jones, M.C., Lin, A.E., Zackai, E.H., 2002. Five additional Costello syndrome patients with rhabdomyosarcoma: proposal for a tumor screening protocol. Am. J. Med. Genet. 108, 80−87.

Gripp, K.W., Sol-Church, K., Smpokou, P., Graham, G.E., Stevenson, D.A., Hanson, H., Viskochil, D.H., Baker, L.C., Russo, B., Gardner, N., et al., 2015. An attenuated phenotype of Costello syndrome in three unrelated individuals with a HRAS c.179G>A (p.Gly60Asp) mutation correlates with uncommon functional consequences. Am. J. Med. Genet. 167A, 2085−2097.

Gripp, K.W., Stabley, D.L., Nicholson, L., Hoffman, J.D., Sol-Church, K., 2006. Somatic mosaicism for an HRAS mutation causes Costello syndrome. Am. J. Med. Genet. 140, 2163−2169.

Gripp, K.W., Zand, D.J., Demmer, L., Anderson, C.E., Dobyns, W.B., Zackai, E.H., Denenberg, E., Jenny, K., Stabley, D.L., Sol-Church, K., 2013. Expanding the SHOC2 mutation associated phenotype of Noonan syndrome with loose anagen hair: structural brain anomalies and myelofibrosis. Am. J. Med. Genet. 161A, 2420−2430.

Grossmann, K.S., Wende, H., Paul, F.E., Cheret, C., Garratt, A.N., Zurborg, S., Feinberg, K., Besser, D., Schulz, H., Peles, E., et al., 2009. The tyrosine phosphatase Shp2 (PTPN11) directs Neuregulin-1/ErbB signaling throughout Schwann cell development. Proc. Natl. Acad. Sci. U. S. A. 106, 16704−16709.

Hanna, N., Montagner, A., Lee, W.H., Miteva, M., Vidal, M., Vidaud, M., Parfait, B., Raynal, P., 2006. Reduced phosphatase activity of SHP-2 in LEOPARD syndrome: consequences for PI3K binding on Gab1. FEBS Lett. 580, 2477−2482.

Hatano, N., Mori, Y., Oh-hora, M., Kosugi, A., Fujikawa, T., Nakai, N., Niwa, H., Miyazaki, J., Hamaoka, T., Ogata, M., 2003. Essential role for ERK2 mitogen-activated protein kinase in placental development. Gene Cell. 8, 847−856.

Hernandez-Porras, I., Fabbiano, S., Schuhmacher, A.J., Aicher, A., Canamero, M., Camara, J.A., Cusso, L., Desco, M., Heeschen, C., Mulero, F., et al., 2014. K-RasV14I recapitulates Noonan syndrome in mice. Proc. Natl. Acad. Sci. U. S. A. 111, 16395−16400.

Hernandez-Porras, I., Jimenez-Catalan, B., Schuhmacher, A.J., Guerra, C., 2015. The impact of the genetic background in the Noonan syndrome phenotype induced by K-Ras(V14I). Rare Dis. 3, e1045169.

Higgins, E.M., Bos, J.M., Dotzler, S.M., John Kim, C.S., Ackerman, M.J., 2019. MRAS variants cause cardiomyocyte hypertrophy in patient-specific induced pluripotent stem cell-derived cardiomyocytes: additional evidence for MRAS as a definitive noonan syndrome-susceptibility gene. Circ. Genom. Precis. Med. 12, e002648.

Higgins, E.M., Bos, J.M., Mason-Suares, H., Tester, D.J., Ackerman, J.P., MacRae, C.A., Sol-Church, K., Gripp, K.W., Urrutia, R., Ackerman, M.J., 2017. Elucidation of MRAS-mediated Noonan syndrome with cardiac hypertrophy. JCI Insight 2, e91225.

Hobbs, G.A., Der, C.J., Rossman, K.L., 2016. RAS isoforms and mutations in cancer at a glance. J. Cell Sci. 129, 1287−1292.

Holter, M.C., Hewitt, L.T., Koebele, S.V., Judd, J.M., Xing, L., Bimonte-Nelson, H.A., Conrad, C.D., Araki, T., Neel, B.G., Snider, W.D., Newbern, J.M., 2019. The Noonan syndrome-linked Raf1L613V mutation drives increased glial number in the mouse cortex and enhanced learning. PLoS Genet. 15, e1008108.

Ishida, H., Kogaki, S., Narita, J., Ichimori, H., Nawa, N., Okada, Y., Takahashi, K., Ozono, K., 2011. LEOPARD-type SHP2 mutant Gln510Glu attenuates cardiomyocyte differentiation and promotes cardiac hypertrophy via dysregulation of Akt/GSK-3beta/beta-catenin signaling. Am. J. Physiol. Heart Circ. Physiol. 301, H1531−H1539.

Jaffre, F., Miller, C.L., Schanzer, A., Evans, T., Roberts, A.E., Hahn, A., Kontaridis, M.I., 2019. Inducible pluripotent stem cell-derived cardiomyocytes reveal aberrant extracellular regulated kinase 5 and mitogen-activated protein kinase kinase 1/2 signaling concomitantly promote hypertrophic cardiomyopathy in RAF1-associated noonan syndrome. Circulation 140, 207−224.

Johnston, J.J., van der Smagt, J.J., Rosenfeld, J.A., Pagnamenta, A.T., Alswaid, A., Baker, E.H., Blair, E., Borck, G., Brinkmann, J., Craigen, W., et al., 2018. Autosomal recessive Noonan syndrome associated with biallelic LZTR1 variants. Genet. Med. 20, 1175−1185.

Jopling, C., van Geemen, D., den Hertog, J., 2007. Shp2 knockdown and Noonan/LEOPARD mutant Shp2-induced gastrulation defects. PLoS Genet. 3, e225.

Kamath, A., Ternes, S., McGowan, S., English, A., Mallampalli, R., Moy, A.B., 2017. Efficient method to create integration-free, virus-free, Myc and Lin28-free human induced pluripotent stem cells from adherent cells. Future Sci. OA 3, FSO211.

Kang, M., Lee, Y.S., 2019. The impact of RASopathy-associated mutations on CNS development in mice and humans. Mol. Brain 12, 96.

Karbassi, E., Fenix, A., Marchiano, S., Muraoka, N., Nakamura, K., Yang, X., Murry, C.E., 2020. Cardiomyocyte maturation: advances in knowledge and implications for regenerative medicine. Nat. Rev. Cardiol. 17, 341−359.

Ke, Y., Zhang, E.E., Hagihara, K., Wu, D., Pang, Y., Klein, R., Curran, T., Ranscht, B., Feng, G.S., 2007. Deletion of Shp2 in the brain leads to defective proliferation and differentiation in neural stem cells and early postnatal lethality. Mol. Cell Biol. 27, 6706−6717.

Keilhack, H., David, F.S., McGregor, M., Cantley, L.C., Neel, B.G., 2005. Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. J. Biol. Chem. 280, 30984−30993.

Kerr, B., Delrue, M.A., Sigaudy, S., Perveen, R., Marche, M., Burgelin, I., Stef, M., Tang, B., Eden, O.B., O'Sullivan, J., et al., 2006. Genotype-phenotype correlation in Costello syndrome: HRAS mutation analysis in 43 cases. J. Med. Genet. 43, 401−405.

Keung, W., Boheler, K.R., Li, R.A., 2014. Developmental cues for the maturation of metabolic, electrophysiological and calcium handling properties of human pluripotent stem cell-derived cardiomyocytes. Stem Cell Res. Ther. 5, 17.

Kimmelman, A., Tolkacheva, T., Lorenzi, M.V., Osada, M., Chan, A.M., 1997. Identification and characterization of R-ras3: a novel member of the RAS gene family with a non-ubiquitous pattern of tissue distribution. Oncogene 15, 2675−2685.

Klapper, S.D., Garg, P., Dagar, S., Lenk, K., Gottmann, K., Nieweg, K., 2019. Astrocyte lineage cells are essential for functional neuronal differentiation and synapse maturation in human iPSC-derived neural networks. Glia 67, 1893−1909.

Kofler, N., Kraschel, K.L., 2018. Treatment of heritable diseases using CRISPR: hopes, fears, and reality. Semin. Perinatol. 42, 515−521.

Kontaridis, M.I., Swanson, K.D., David, F.S., Barford, D., Neel, B.G., 2006. PTPN11 (Shp2) mutations in LEOPARD syndrome have dominant negative, not activating, effects. J. Biol. Chem. 281, 6785−6792.

Kratz, C.P., Niemeyer, C.M., Castleberry, R.P., Cetin, M., Bergstrasser, E., Emanuel, P.D., Hasle, H., Kardos, G., Klein, C., Kojima, S., et al., 2005. The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. Blood 106, 2183−2185.

Krencik, R., Hokanson, K.C., Narayan, A.R., Dvornik, J., Rooney, G.E., Rauen, K.A., Weiss, L.A., Rowitch, D.H., Ullian, E.M., 2015. Dysregulation of astrocyte extracellular signaling in Costello syndrome. Sci. Transl. Med. 7, 286ra266.

Krenz, M., Gulick, J., Osinska, H.E., Colbert, M.C., Molkentin, J.D., Robbins, J., 2008. Role of ERK1/2 signaling in congenital valve malformations in Noonan syndrome. Proc. Natl. Acad. Sci. U. S. A. 105, 18930−18935.

Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., Knoblich, J.A., 2013. Cerebral organoids model human brain development and microcephaly. Nature 501, 373−379.

Langdon, Y., Tandon, P., Paden, E., Duddy, J., Taylor, J.M., Conlon, F.L., 2012. SHP-2 acts via ROCK to regulate the cardiac actin cytoskeleton. Development 139, 948−957.

Lauriol, J., Cabrera, J.R., Roy, A., Keith, K., Hough, S.M., Damilano, F., Wang, B., Segarra, G.C., Flessa, M.E., Miller, L.E., et al., 2016. Developmental SHP2 dysfunction underlies cardiac hypertrophy in Noonan syndrome with multiple lentigines. J. Clin. Invest. 126, 2989−3005.

Lauriol, J., Jaffre, F., Kontaridis, M.I., 2015. The role of the protein tyrosine phosphatase SHP2 in cardiac development and disease. Semin. Cell Dev. Biol. 37, 73−81.

Lauriol, J., Kontaridis, M.I., 2011. PTPN11-associated mutations in the heart: has LEOPARD changed its RASpots? Trends Cardiovasc. Med. 21, 97−104.

Lee, J.K., Jeong, E., Lee, J., Jung, M., Shin, E., Kim, Y.H., Lee, K., Jung, I., Kim, D., Kim, S., Kim, J.S., 2018. Directed evolution of CRISPR-Cas9 to increase its specificity. Nat. Commun. 9, 3048.

Legius, E., Schrander-Stumpel, C., Schollen, E., Pulles-Heintzberger, C., Gewillig, M., Fryns, J.P., 2002. PTPN11 mutations in LEOPARD syndrome. J. Med. Genet. 39, 571−574.

Leoni, C., Stevenson, D.A., Martini, L., De Sanctis, R., Mascolo, G., Pantaleoni, F., De Santis, S., La Torraca, I., Persichilli, S., Caradonna, P., et al., 2014. Decreased bone mineral density in Costello syndrome. Mol. Genet. Metabol. 111, 41−45.

Li, R., Baskfield, A., Lin, Y., Beers, J., Zou, J., Liu, C., Jaffre, F., Roberts, A.E., Ottinger, E.A., Kontaridis, M.I., Zheng, W., 2019. Generation of an induced pluripotent stem cell line (TRNDi003-A) from a Noonan syndrome with multiple lentigines (NSML) patient carrying a p.Q510P mutation in the PTPN11 gene. Stem Cell Res. 34, 101374.

Lian, X., Zhang, J., Azarin, S.M., Zhu, K., Hazeltine, L.B., Bao, X., Hsiao, C., Kamp, T.J., Palecek, S.P., 2013. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. Nat. Protoc. 8, 162−175.

Limongelli, G., Pacileo, G., Marino, B., Digilio, M.C., Sarkozy, A., Elliott, P., Versacci, P., Calabro, P., De Zorzi, A., Di Salvo, G., et al., 2007. Prevalence and clinical significance of cardiovascular abnormalities in patients with the LEOPARD syndrome. Am. J. Cardiol. 100, 736−741.

Lin, A.E., Alexander, M.E., Colan, S.D., Kerr, B., Rauen, K.A., Noonan, J., Baffa, J., Hopkins, E., Sol-Church, K., Limongelli, G., et al., 2011. Clinical, pathological, and molecular analyses of cardiovascular abnormalities in Costello syndrome: a Ras/MAPK pathway syndrome. Am. J. Med. Genet. 155A, 486−507.

Lin, B., Kim, J., Li, Y., Pan, H., Carvajal-Vergara, X., Salama, G., Cheng, T., Li, Y., Lo, C.W., Yang, L., 2012. High-purity enrichment of functional cardiovascular cells from human iPS cells. Cardiovasc. Res. 95, 327−335.

Lorenz, S., Lissewski, C., Simsek-Kiper, P.O., Alanay, Y., Boduroglu, K., Zenker, M., Rosenberger, G., 2013. Functional analysis of a duplication (p.E63_D69dup) in the switch II region of HRAS: new aspects of the molecular pathogenesis underlying Costello syndrome. Hum. Mol. Genet. 22, 1643−1653.

Marin, T.M., Keith, K., Davies, B., Conner, D.A., Guha, P., Kalaitzidis, D., Wu, X., Lauriol, J., Wang, B., Bauer, M., et al., 2011. Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of LEOPARD syndrome-associated PTPN11 mutation. J. Clin. Invest. 121, 1026−1043.

Marino, B., Digilio, M.C., Toscano, A., Giannotti, A., Dallapiccola, B., 1999. Congenital heart diseases in children with Noonan syndrome: an expanded cardiac spectrum with high prevalence of atrioventricular canal. J. Pediatr. 135, 703−706.

Martin, R.A., Jones, K.L., 1991. Delineationofthe Costello syndrome. Am. J. Med. Genet. 41, 346−349.

Martinelli, S., De Luca, A., Stellacci, E., Rossi, C., Checquolo, S., Lepri, F., Caputo, V., Silvano, M., Buscherini, F., Consoli, F., et al., 2010. Heterozygous germline mutations in the CBL tumor-suppressor gene cause a Noonan syndrome-like phenotype. Am. J. Hum. Genet. 87, 250−257.

Martinez-Quintana, E., Rodriguez-Gonzalez, F., 2012. LEOPARD syndrome: clinical features and gene mutations. Mol. Syndromol. 3, 145−157.

McCracken, K.W., Howell, J.C., Wells, J.M., Spence, J.R., 2011. Generating human intestinal tissue from pluripotent stem cells in vitro. Nat. Protoc. 6, 1920−1928.

Medina, J.H., Viola, H., 2018. ERK1/2: a key cellular component for the formation, retrieval, reconsolidation and persistence of memory. Front. Mol. Neurosci. 11, 361.

Mendoza, M.C., Er, E.E., Blenis, J., 2011. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends Biochem. Sci. 36, 320−328.

Mithal, A., Capilla, A., Heinze, D., Berical, A., Villacorta-Martin, C., Vedaie, M., Jacob, A., Abo, K., Szymaniak, A., Peasley, M., et al., 2020. Generation of mesenchyme free intestinal organoids from human induced pluripotent stem cells. Nat. Commun. 11, 215.

Moelling, K., Schad, K., Bosse, M., Zimmermann, S., Schweneker, M., 2002. Regulation of Raf-Akt cross-talk. J. Biol. Chem. 277, 31099−31106.

Moretti, A., Bellin, M., Welling, A., Jung, C.B., Lam, J.T., Bott-Flugel, L., Dorn, T., Goedel, A., Hohnke, C., Hofmann, F., et al., 2010. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N. Engl. J. Med. 363, 1397−1409.

Motta, M., Sagi-Dain, L., Krumbach, O.H.F., Hahn, A., Peleg, A., German, A., Lissewski, C., Coppola, S., Pantaleoni, F., Kocherscheid, L., et al., 2020. Activating MRAS mutations cause Noonan syndrome associated with hypertrophic cardiomyopathy. Hum. Mol. Genet. 29 (11), 1772−1783.

Mulero-Navarro, S., Sevilla, A., Roman, A.C., Lee, D.F., D'Souza, S.L., Pardo, S., Riess, I., Su, J., Cohen, N., Schaniel, C., et al., 2015. Myeloid dysregulation in a human induced pluripotent stem cell model of PTPN11-associated juvenile myelomonocytic leukemia. Cell Rep. 13, 504−515.

Nakagama, Y., Takeda, N., Ogawa, S., Takeda, H., Furutani, Y., Nakanishi, T., Sato, T., Hirata, Y., Oka, A., Inuzuka, R., 2020. Noonan syndrome-associated biallelic LZTR1 mutations cause cardiac hypertrophy and vascular malformations in zebrafish. Mol. Genet. Genom. Med. 8, e1107.

Nakamura, T., Colbert, M., Krenz, M., Molkentin, J.D., Hahn, H.S., Dorn II, G.W., 2nd, Robbins, J., 2007. Mediating ERK 1/2 signaling rescues congenital heart defects in a mouse model of Noonan syndrome. J. Clin. Invest. 117, 2123−2132.

Nakamura, T., Gulick, J., Colbert, M.C., Robbins, J., 2009a. Protein tyrosine phosphatase activity in the neural crest is essential for normal heart and skull development. Proc. Natl. Acad. Sci. U. S. A. 106, 11270−11275.

Nakamura, T., Gulick, J., Pratt, R., Robbins, J., 2009b. Noonan syndrome is associated with enhanced pERK activity, the repression of which can prevent craniofacial malformations. Proc. Natl. Acad. Sci. U. S. A. 106, 15436−15441.

Niemeyer, C.M., Kang, M.W., Shin, D.H., Furlan, I., Erlacher, M., Bunin, N.J., Bunda, S., Finklestein, J.Z., Gorr, T.A., Mehta, P., et al., 2010. Germline CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. Nat. Genet. 42, 794−800.

Niihori, T., Aoki, Y., Okamoto, N., Kurosawa, K., Ohashi, H., Mizuno, S., Kawame, H., Inazawa, J., Ohura, T., Arai, H., et al., 2011. HRAS mutants identified in Costello syndrome patients can induce cellular senescence: possible implications for the pathogenesis of Costello syndrome. J. Hum. Genet. 56, 707−715.

Noonan, J.A., 1968. Hypertelorism with Turner phenotype. A new syndrome with associated congenital heart disease. Am. J. Dis. Child. 116, 373−380.

O'Reilly, A.M., Pluskey, S., Shoelson, S.E., Neel, B.G., 2000. Activated mutants of SHP-2 preferentially induce elongation of Xenopus animal caps. Mol. Cell Biol. 20, 299−311.

Oba, D., Inoue, S.I., Miyagawa-Tomita, S., Nakashima, Y., Niihori, T., Yamaguchi, S., Matsubara, Y., Aoki, Y., 2018. Mice with an oncogenic HRAS mutation are resistant to high-fat diet-induced obesity and exhibit impaired hepatic energy homeostasis. EBioMedicine 27, 138−150.

Oishi, K., Gaengel, K., Krishnamoorthy, S., Kamiya, K., Kim, I.K., Ying, H., Weber, U., Perkins, L.A., Tartaglia, M., Mlodzik, M., et al., 2006. Transgenic *Drosophila* models of Noonan syndrome causing PTPN11 gain-of-function mutations. Hum. Mol. Genet. 15, 543−553.

Oishi, K., Zhang, H., Gault, W.J., Wang, C.J., Tan, C.C., Kim, I.K., Ying, H., Rahman, T., Pica, N., Tartaglia, M., et al., 2009. Phosphatase-defective LEOPARD syndrome mutations in PTPN11 gene have gain-of-function effects during *Drosophila* development. Hum. Mol. Genet. 18, 193−201.

Ong, S.H., Hadari, Y.R., Gotoh, N., Guy, G.R., Schlessinger, J., Lax, I., 2001. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc. Natl. Acad. Sci. U. S. A. 98, 6074−6079.

Pacold, M.E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C.T., Walker, E.H., Hawkins, P.T., Stephens, L., Eccleston, J.F., Williams, R.L., 2000. Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. Cell 103, 931−943.

Pandit, B., Sarkozy, A., Pennacchio, L.A., Carta, C., Oishi, K., Martinelli, S., Pogna, E.A., Schackwitz, W., Ustaszewska, A., Landstrom, A., et al., 2007. Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat. Genet. 39, 1007−1012.

Papa, S., Choy, P.M., Bubici, C., 2019. The ERK and JNK pathways in the regulation of metabolic reprogramming. Oncogene 38, 2223−2240.

Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., Daley, G.Q., 2008. Disease-specific induced pluripotent stem cells. Cell 134, 877−886.

Pasca, A.M., Sloan, S.A., Clarke, L.E., Tian, Y., Makinson, C.D., Huber, N., Kim, C.H., Park, J.Y., O'Rourke, N.A., Nguyen, K.D., et al., 2015. Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. Nat. Methods 12, 671−678.

Pearson, S., Guo, B., Pierce, A., Azadbakht, N., Brazzatti, J.A., Patassini, S., Mulero-Navarro, S., Meyer, S., Flotho, C., Gelb, B.D., Whetton, A.D., 2020. Proteomic analysis of an induced pluripotent stem cell model reveals strategies to treat juvenile myelomonocytic leukemia. J. Proteome Res. 19, 194−203.

Peng, X.D., Xu, P.Z., Chen, M.L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararajan, D., Chen, W.S., Crawford, S.E., Coleman, K.G., Hay, N., 2003. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev. 17, 1352−1365.

Pierpont, E.I., 2016. Neuropsychological functioning in individuals with noonan syndrome: a systematic literature review with educational and treatment recommendations. J. Pediatr. Neuropsychol. 2, 14−33.

Pierpont, E.I., Hudock, R.L., Foy, A.M., Semrud-Clikeman, M., Pierpont, M.E., Berry, S.A., Shanley, R., Rubin, N., Sommer, K., Moertel, C.L., 2018. Social skills in children with RASopathies: a comparison of Noonan syndrome and neurofibromatosis type 1. J. Neurodev. Disord. 10, 21.

Prior, I.A., Lewis, P.D., Mattos, C., 2012. A comprehensive survey of Ras mutations in cancer. Canc. Res. 72, 2457–2467.

Qian, X., Song, H., Ming, G.L., 2019. Brain Organoids: Advances, Applications and Challenges, vol. 146. Development.

Quilliam, L.A., Castro, A.F., Rogers-Graham, K.S., Martin, C.B., Der, C.J., Bi, C., 1999. M-Ras/R-Ras3, a transforming ras protein regulated by Sos1, GRF1, and p120 Ras GTPase-activating protein, interacts with the putative Ras effector AF6. J. Biol. Chem. 274, 23850–23857.

Ramsdell, A.F., 2005. Left-right asymmetry and congenital cardiac defects: getting to the heart of the matter in vertebrate left-right axis determination. Dev. Biol. 288, 1–20.

Rauen, K.A., 2013. The RASopathies. Annu. Rev. Genom. Hum. Genet. 14, 355–369.

Razzaque, M.A., Nishizawa, T., Komoike, Y., Yagi, H., Furutani, M., Amo, R., Kamisago, M., Momma, K., Katayama, H., Nakagawa, M., et al., 2007. Germline gain-of-function mutations in RAF1 cause Noonan syndrome. Nat. Genet. 39, 1013–1017.

Roberts, A.E., Allanson, J.E., Tartaglia, M., Gelb, B.D., 2013. Noonan syndrome. Lancet 381, 333–342.

Roberts, A.E., Araki, T., Swanson, K.D., Montgomery, K.T., Schiripo, T.A., Joshi, V.A., Li, L., Yassin, Y., Tamburino, A.M., Neel, B.G., Kucherlapati, R.S., 2007. Germline gain-of-function mutations in SOS1 cause Noonan syndrome. Nat. Genet. 39, 70–74.

Robertson, C., Tran, D.D., George, S.C., 2013. Concise review: maturation phases of human pluripotent stem cell-derived cardiomyocytes. Stem Cell. 31, 829–837.

Romano, A.A., Allanson, J.E., Dahlgren, J., Gelb, B.D., Hall, B., Pierpont, M.E., Roberts, A.E., Robinson, W., Takemoto, C.M., Noonan, J.A., 2010. Noonan syndrome: clinical features, diagnosis, and management guidelines. Pediatrics 126, 746–759.

Rooney, G.E., Goodwin, A.F., Depeille, P., Sharir, A., Schofield, C.M., Yeh, E., Roose, J.P., Klein, O.D., Rauen, K.A., Weiss, L.A., Ullian, E.M., 2016. Human iPS cell-derived neurons uncover the impact of increased ras signaling in Costello syndrome. J. Neurosci. 36, 142–152.

Rosenberger, G., Meien, S., Kutsche, K., 2009. Oncogenic HRAS mutations cause prolonged PI3K signaling in response to epidermal growth factor in fibroblasts of patients with Costello syndrome. Hum. Mutat. 30, 352–362.

Rust, W., Balakrishnan, T., Zweigerdt, R., 2009. Cardiomyocyte enrichment from human embryonic stem cell cultures by selection of ALCAM surface expression. Regen. Med. 4, 225–237.

Ryan, M.B., Der, C.J., Wang-Gillam, A., Cox, A.D., 2015. Targeting RAS-mutant cancers: is ERK the key? Trends Cancer 1, 183–198.

Saba-El-Leil, M.K., Vella, F.D., Vernay, B., Voisin, L., Chen, L., Labrecque, N., Ang, S.L., Meloche, S., 2003. An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. EMBO Rep. 4, 964–968.

Saini, K.S., Loi, S., de Azambuja, E., Metzger-Filho, O., Saini, M.L., Ignatiadis, M., Dancey, J.E., Piccart-Gebhart, M.J., 2013. Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer. Canc. Treat Rev. 39, 935–946.

Sakai, T., Naito, A.T., Kuramoto, Y., Ito, M., Okada, K., Higo, T., Nakagawa, A., Shibamoto, M., Yamaguchi, T., Sumida, T., et al., 2018. Phenotypic screening using patient-derived induced pluripotent stem cells identified Pyr3 as a candidate compound for the treatment of infantile hypertrophic cardiomyopathy. Int. Heart J. 59, 1096–1105.

Santoriello, C., Deflorian, G., Pezzimenti, F., Kawakami, K., Lanfrancone, L., d'Adda di Fagagna, F., Mione, M., 2009. Expression of H-RASV12 in a zebrafish model of Costello syndrome causes cellular senescence in adult proliferating cells. Dis. Model Mech. 2, 56–67.

Sarkozy, A., Carta, C., Moretti, S., Zampino, G., Digilio, M.C., Pantaleoni, F., Scioletti, A.P., Esposito, G., Cordeddu, V., Lepri, F., et al., 2009. Germline BRAF mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. Hum. Mutat. 30, 695−702.

Schramm, C., Fine, D.M., Edwards, M.A., Reeb, A.N., Krenz, M., 2012. The PTPN11 loss-of-function mutation Q510E-Shp2 causes hypertrophic cardiomyopathy by dysregulating mTOR signaling. Am. J. Physiol. Heart Circ. Physiol. 302, H231−H243.

Schreiber, J., Grimbergen, L.A., Overwater, I., Vaart, T.V., Stedehouder, J., Schuhmacher, A.J., Guerra, C., Kushner, S.A., Jaarsma, D., Elgersma, Y., 2017. Mechanisms underlying cognitive deficits in a mouse model for Costello Syndrome are distinct from other RASopathy mouse models. Sci. Rep. 7, 1256.

Schubbert, S., Zenker, M., Rowe, S.L., Boll, S., Klein, C., Bollag, G., van der Burgt, I., Musante, L., Kalscheuer, V., Wehner, L.E., et al., 2006. Germline KRAS mutations cause Noonan syndrome. Nat. Genet. 38, 331−336.

Schuhmacher, A.J., Guerra, C., Sauzeau, V., Canamero, M., Bustelo, X.R., Barbacid, M., 2008. A mouse model for Costello syndrome reveals an Ang II-mediated hypertensive condition. J. Clin. Invest. 118, 2169−2179.

Sharland, M., Burch, M., McKenna, W.M., Paton, M.A., 1992. A clinical study of Noonan syndrome. Arch. Dis. Child. 67, 178−183.

Sharma, A., Wu, J.C., Wu, S.M., 2013. Induced pluripotent stem cell-derived cardiomyocytes for cardiovascular disease modeling and drug screening. Stem Cell Res. Ther. 4, 150.

Shaw, A.C., Kalidas, K., Crosby, A.H., Jeffery, S., Patton, M.A., 2007. The natural history of Noonan syndrome: a long-term follow-up study. Arch. Dis. Child. 92, 128−132.

Siegel, D.H., Mann, J.A., Krol, A.L., Rauen, K.A., 2012. Dermatological phenotype in Costello syndrome: consequences of Ras dysregulation in development. Br. J. Dermatol. 166, 601−607.

Sinico, M., Bassez, G., Touboul, C., Cave, H., Vergnaud, A., Zirah, C., Fleury-Feith, J., Gettler, S., Vojtek, A.M., Chevalier, N., et al., 2011. Excess of neuromuscular spindles in a fetus with Costello syndrome: a clinicopathological report. Pediatr. Dev. Pathol. 14, 218−223.

Sol-Church, K., Stabley, D.L., Demmer, L.A., Agbulos, A., Lin, A.E., Smoot, L., Nicholson, L., Gripp, K.W., 2009. Male-to-male transmission of Costello syndrome: G12S HRAS germline mutation inherited from a father with somatic mosaicism. Am. J. Med. Genet. 149A, 315−321.

Spence, J.R., Mayhew, C.N., Rankin, S.A., Kuhar, M.F., Vallance, J.E., Tolle, K., Hoskins, E.E., Kalinichenko, V.V., Wells, S.I., Zorn, A.M., et al., 2011. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470, 105−U120.

Stevenson, D.A., Schwarz, E.L., Carey, J.C., Viskochil, D.H., Hanson, H., Bauer, S., Weng, H.Y., Greene, T., Reinker, K., Swensen, J., et al., 2011. Bone resorption in syndromes of the Ras/MAPK pathway. Clin. Genet. 80, 566−573.

Stewart, R.A., Sanda, T., Widlund, H.R., Zhu, S., Swanson, K.D., Hurley, A.D., Bentires-Alj, M., Fisher, D.E., Kontaridis, M.I., Look, A.T., Neel, B.G., 2010. Phosphatase-dependent and -independent functions of Shp2 in neural crest cells underlie LEOPARD syndrome pathogenesis. Dev. Cell 18, 750−762.

Strullu, M., Caye, A., Lachenaud, J., Cassinat, B., Gazal, S., Fenneteau, O., Pouvreau, N., Pereira, S., Baumann, C., Contet, A., et al., 2014. Juvenile myelomonocytic leukaemia and Noonan syndrome. J. Med. Genet. 51, 689−697.

Suire, S., Hawkins, P., Stephens, L., 2002. Activation of phosphoinositide 3-kinase gamma by Ras. Curr. Biol. 12, 1068−1075.

Sznajer, Y., Keren, B., Baumann, C., Pereiraa, S., Alberti, C., Elion, J., Cave, H., Verloes, A., 2007. The spectrum of cardiac anomalies in Noonan syndrome as a result of mutations in the PTPN11 gene. Pediatrics 119, E1325−E1331.

Tajan, M., Paccoud, R., Branka, S., Edouard, T., Yart, A., 2018a. The RASopathy family: consequences of germline activation of the RAS/MAPK pathway. Endocr. Rev. 39, 676−700.

Tajan, M., Pernin-Grandjean, J., Beton, N., Gennero, I., Capilla, F., Neel, B.G., Araki, T., Valet, P., Tauber, M., Salles, J.P., et al., 2018b. Noonan syndrome-causing SHP2 mutants impair ERK-dependent chondrocyte differentiation during endochondral bone growth. Hum. Mol. Genet. 27, 2276−2289.

Takahara, S., Inoue, S.I., Miyagawa-Tomita, S., Matsuura, K., Nakashima, Y., Niihori, T., Matsubara, Y., Saiki, Y., Aoki, Y., 2019. New Noonan syndrome model mice with RIT1 mutation exhibit cardiac hypertrophy and susceptibility to beta-adrenergic stimulation-induced cardiac fibrosis. EBioMedicine 42, 43−53.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663−676.

Tanimura, S., Takeda, K., 2017. ERK signalling as a regulator of cell motility. J. Biochem. 162, 145−154.

Tartaglia, M., Gelb, B.D., 2005. Noonan syndrome and related disorders: genetics and pathogenesis. Annu. Rev. Genom. Hum. Genet. 6, 45−68.

Tartaglia, M., Mehler, E.L., Goldberg, R., Zampino, G., Brunner, H.G., Kremer, H., van der Burgt, I., Crosby, A.H., Ion, A., Jeffery, S., et al., 2001. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat. Genet. 29, 465−468.

Tartaglia, M., Pennacchio, L.A., Zhao, C., Yadav, K.K., Fodale, V., Sarkozy, A., Pandit, B., Oishi, K., Martinelli, S., Schackwitz, W., et al., 2007. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. Nat. Genet. 39, 75−79.

Tidyman, W.E., Rauen, K.A., 2009. The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation. Curr. Opin. Genet. Dev. 19, 230−236.

Tobin, S.C., Kim, K., 2012. Generating pluripotent stem cells: differential epigenetic changes during cellular reprogramming. FEBS Lett. 586, 2874−2881.

Tschopp, O., Yang, Z.Z., Brodbeck, D., Dummler, B.A., Hemmings-Mieszczak, M., Watanabe, T., Michaelis, T., Frahm, J., Hemmings, B.A., 2005. Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. Development 132, 2943−2954.

Umeki, I., Niihori, T., Abe, T., Kanno, S.I., Okamoto, N., Mizuno, S., Kurosawa, K., Nagasaki, K., Yoshida, M., Ohashi, H., et al., 2019. Delineation of LZTR1 mutation-positive patients with Noonan syndrome and identification of LZTR1 binding to RAF1-PPP1CB complexes. Hum. Genet. 138, 21−35.

Viosca, J., Schuhmacher, A.J., Guerra, C., Barco, A., 2009. Germline expression of H-Ras(G12V) causes neurological deficits associated to Costello syndrome. Gene Brain Behav. 8, 60−71.

Watanabe, Y., Yano, S., Niihori, T., Aoki, Y., Matsubara, Y., Yoshino, M., Matsuishi, T., 2011. A familial case of LEOPARD syndrome associated with a high-functioning autism spectrum disorder. Brain Dev. Jpn 33, 576−579.

Wen, L., Tang, F., 2016. Single-cell sequencing in stem cell biology. Genome Biol. 17, 71.

Winter, J.N., Jefferson, L.S., Kimball, S.R., 2011. ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling. Am. J. Physiol. Cell Physiol. 300, C1172−C1180.

Workman, M.J., Mahe, M.M., Trisno, S., Poling, H.M., Watson, C.L., Sundaram, N., Chang, C.F., Schiesser, J., Aubert, P., Stanley, E.G., et al., 2017. Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system. Nat. Med. 23, 49−59.

Wu, C.J., O'Rourke, D.M., Feng, G.S., Johnson, G.R., Wang, Q., Greene, M.I., 2001. The tyrosine phosphatase SHP-2 is required for mediating phosphatidylinositol 3-kinase/Akt activation by growth factors. Oncogene 20, 6018−6025.

Wu, X., Simpson, J., Hong, J.H., Kim, K.H., Thavarajah, N.K., Backx, P.H., Neel, B.G., Araki, T., 2011. MEK-ERK pathway modulation ameliorates disease phenotypes in a mouse model of Noonan syndrome associated with the Raf1(L613V) mutation. J. Clin. Invest. 121, 1009−1025.

Yamamoto, G.L., Aguena, M., Gos, M., Hung, C., Pilch, J., Fahiminiya, S., Abramowicz, A., Cristian, I., Buscarilli, M., Naslavsky, M.S., et al., 2015. Rare variants in SOS2 and LZTR1 are associated with Noonan syndrome. J. Med. Genet. 52, 413−421.

Yang, W., Wang, J., Moore, D.C., Liang, H., Dooner, M., Wu, Q., Terek, R., Chen, Q., Ehrlich, M.G., Quesenberry, P.J., Neel, B.G., 2013. Ptpn11 deletion in a novel progenitor causes metachondromatosis by inducing hedgehog signalling. Nature 499, 491−495.

Yang, Y., Jiang, Z., Ma, N., Wang, B., Liu, J., Zhang, L., Gu, L., 2018. MicroRNA-223 targeting STIM1 inhibits the biological behavior of breast cancer. Cell. Physiol. Biochem. 45, 856−866.

Yang, Z.Z., Tschopp, O., Di-Poi, N., Bruder, E., Baudry, A., Dummler, B., Wahli, W., Hemmings, B.A., 2005. Dosage-dependent effects of Akt1/protein kinase Balpha (PKBalpha) and Akt3/PKBgamma on thymus, skin, and cardiovascular and nervous system development in mice. Mol. Cell Biol. 25, 10407−10418.

Yin, X., Mead, B.E., Safaee, H., Langer, R., Karp, J.M., Levy, O., 2016. Engineering stem cell organoids. Cell Stem Cell 18, 25−38.

Yoshida, N., Doisaki, S., Kojima, S., 2012. Current management of juvenile myelomonocytic leukemia and the impact of RAS mutations. Paediatr. Drugs 14, 157−163.

Yoshihara, M., Oguchi, A., Murakawa, Y., 2019. Genomic instability of iPSCs and challenges in their clinical applications. Adv. Exp. Med. Biol. 1201, 23−47.

Yu, J.S., Cui, W., 2016. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Development 143, 3050−3060.

Yu, Z.H., Xu, J., Walls, C.D., Chen, L., Zhang, S., Zhang, R., Wu, L., Wang, L., Liu, S., Zhang, Z.Y., 2013. Structural and mechanistic insights into LEOPARD syndrome-associated SHP2 mutations. J. Biol. Chem. 288, 10472−10482.

Yu, Z.H., Zhang, R.Y., Walls, C.D., Chen, L., Zhang, S., Wu, L., Liu, S., Zhang, Z.Y., 2014. Molecular basis of gain-of-function LEOPARD syndrome-associated SHP2 mutations. Biochemistry 53, 4136−4151.

Zhang, S.Q., Tsiaras, W.G., Araki, T., Wen, G., Minichiello, L., Klein, R., Neel, B.G., 2002. Receptor-specific regulation of phosphatidylinositol 3′-kinase activation by the protein tyrosine phosphatase Shp2. Mol. Cell Biol. 22, 4062−4072.

Zheng, H., Yu, W.M., Waclaw, R.R., Kontaridis, M.I., Neel, B.G., Qu, C.K., 2018. Gain-of-function mutations in the gene encoding the tyrosine phosphatase SHP2 induce hydrocephalus in a catalytically dependent manner. Sci. Signal. 11.

CHAPTER

5 Modeling mitochondrial encephalopathy due to MELAS/Leigh overlap syndrome using induced pluripotent stem cells

Taeka Hattori[1,2], **Takashi Hamazaki**[1], **Haruo Shintaku**[1,2]

[1]*Department of Pediatrics, Graduate School of Medicine, Osaka City University, Osaka, Japan;*
[2]*Donated Course of Disability Medicine and Regenerative Medicine, Graduate School of Medicine, Osaka City University, Osaka, Japan*

Chapter outline

Abstract

Mitochondrial disease presents a variety of clinical manifestations such as encephalopathy, diabetes, and cardiomyopathy. Mitochondrial encephalopathy includes mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), Leigh syndrome (LS), and MELAS/Leigh overlap syndrome. Mitochondrial encephalopathy has been modeled to reveal pathogenesis, but this is also a challenging task. In this chapter, we discuss issues that must be considered for modeling mitochondrial encephalopathy using induced pluripotent stem cells (iPSCs). Based on the fact that iPSCs were established from the fibroblasts carrying a high proportion of random mitochondrial DNA (mtDNA) mutations, the mitochondrial function was not essential for reprogramming. Reprogramming efficiencies, however, differed from genotypes of mDNA mutations. Although the energy metabolism of iPSCs shifted from oxidative phosphorylation (OXPHOS) to anaerobic glycolysis, the metabolic signature of iPSCs was affected by a mutation

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00012-6

of mtDNA, which encoded a subunit of OXPHOS enzymes. Emerging genome editing technologies were also successfully applied to mtDNA and may provide further evidence of the pathophysiological mechanisms in mitochondrial diseases in vitro. Furthermore we discuss the usefulness of neuronal three-dimensional differentiation models using iPSCs. This will allow us to elucidate the pathogenesis of encephalopathy in the context of vascular angiopathy in MELAS/Leigh overlap syndrome.

Keywords: 3D; Heteroplasmy; iPSCs; Mitochondria; Mitochondrial encephalopathy; Mitochondrial gene editing; Modeling; mtDNA; Pathogenesis.

Mitochondrial disease

Mitochondria are the powerhouse of the cells and account for oxidative phosphorylation (OXPHOS). Mitochondria also play important roles and diverse cellular functions (e.g., proliferation, apoptosis, differentiation). Thus, mitochondrial dysfunctions are associated with a wide range of human diseases including Alzheimer's and Parkinson's disease. Mitochondrial diseases are inherited from mitochondrial disorders caused by either nuclear DNA mutation or mitochondrial DNA (mtDNA) mutation. In this chapter, we mainly discuss mitochondrial diseases caused by mtDNA mutations and how we can apply induced pluripotent stem cells (iPSCs) technology to elucidate disease pathogenesis and to further develop novel treatments.

When mitochondrial function is impaired, it fails to provide sufficient energy to the cells. The lack of energy leads to multiorgan dysfunction. The mtDNA mutations mainly disturb the OXPHOS of the electron transport chain due to respiratory complex defects. Cells with high energetic demands, such as neurons and myocytes, are more susceptible to the defective energy productions. The patients carrying the mtDNA mutation present a wide clinical spectrum: stroke-like episodes, dementia, epilepsy, lactic acidemia, myopathy, recurrent headaches, hearing impairment, diabetes, and short stature etc. Mitochondria contain the 37 genes. More than 250 types of point mutations, over 100 deletions (single deletions), and multiple deletions and duplication have been reported as disease-causing genetic abnormalities. Information on mtDNA mutations is available through the MITOMAP database (a Human Mitochondrial Genome Database: https://mitomap.org/MITOMAP).

Heteroplasmy and threshold effect

Certain types of mtDNA mutation affect specific organs. Moreover, the mutation load of mtDNA in each cell, which is called "heteroplasmy," contributes to the severity of the disease phenotype. Each organ or cell type has its own threshold to manifest a dysfunction. In order to demonstrate the phenotypic threshold effect, several experimental models have been reported. *In vitro*, enucleated cytoplasm can be fused with whole cells to create so-called cybrids, in which we will be able to manipulate different proportions of mutated mtDNA from 0% to 100%,

and to study the effects of a given "mutant load" on the activity of respiratory chain complexes, as well as other cellular functions (Rossignol et al., 2003). *In vivo*, Nakada et al. established a technique to produce "Mito-mice" harboring various degrees of mutant load (Nakada et al., 2001). The mito-mice were generated by introducing deletion mutant mtDNA (ΔmtDNA) from cultured cells into fertilized eggs of C57BL/6J (B6) strain mice (Nakada et al., 2004). In the case of mito-mice carrying more than 70% ΔmtDNA at birth, they showed mitochondrial respiration defects and consequently died around six months after birth. On the other hand, mito-mice carrying about 30%−50% ΔmtDNA at birth were healthy (Nakada et al., 2008). The threshold effect of heteroplasmy correlates with the onset and diversity of symptoms, but none of the mito-mice showed any neurological symptoms comparable to humans as described below. The pathogenesis of neurological symptoms of mitochondrial disease has yet to be elucidated.

Neurological manifestations

Neurological symptoms caused by mitochondrial dysfunction are classified into subgroups based on clinical manifestations as follows: mitochondrial encephalomyopathy with lactate acidosis and stroke-like episodes (MELAS), Leigh syndrome (LS), myoclonus epilepsy associated with ragged-red fibers (MERRF), etc. Furthermore, a growing number of patients failed to be categorized into the subgroups and were diagnosed as having overlap syndrome, such as MELAS/Leigh overlap syndrome (Crimi et al., 2003; Jun Matsui, 2014; Leng et al., 2015; Wang et al., 2010; Kori et al., 2019; La Morgia et al., 2014) or MEERF/MELAS overlap syndrome (Zeviani et al., 1993). Each patient of mitochondrial encephalopathy presented a variety of clinical manifestations. There was no correlation between phenotype and mitochondrial genotype in the patients with mitochondrial encephalopathy. The iPSCs are suitable for examining the molecular features of individual mitochondrial diseases. The iPSCs carrying heteroplasmic mtDNA mutations will enable demonstration of the patient-specific models in mitochondrial diseases (Hatakeyama and Goto, 2016) (Fig. 5.1).

Here, we describe obstacles to modeling mitochondrial diseases and what we learned from the use of iPSCs technology in mitochondrial studies. Lastly, we discuss emerging technologies that allow us to elucidate the pathogenesis of mitochondrial diseases from new dimensions and to develop novel treatment strategies.

Mitochondrial dysfunction and induced pluripotent stem cells

When we generate iPSCs from mitochondrial diseases, we need to undertake the following steps: reprogramming, maintenance, proliferation, and differentiation. In 2013, Folmes et al. first demonstrated that iPSCs were generated from patients with MELAS caused by mtDNA mutation (m.13513G > A) (Folmes et al., 2013).

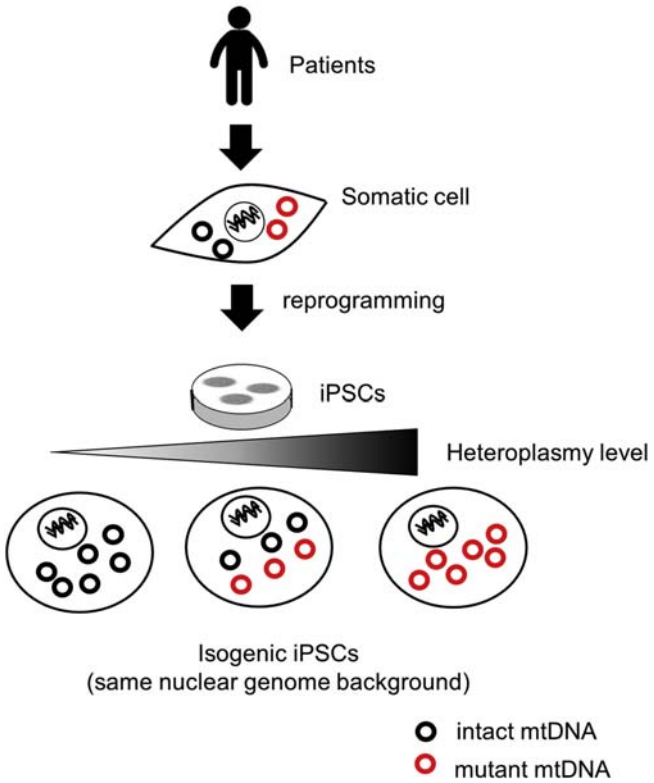


FIGURE 5.1 Demonstration of the patient-specific models in mitochondrial diseases.

Induced pluripotent stem cells (iPSCs) can be generated from patients with mitochondrial diseases caused by mitochondrial DNA (mtDNA) mutations. Each established iPSC clone from the same patient may contain different levels of heteroplasmic mtDNA mutation. Since the nuclear genomic background is identically "isogenic," it is ideal to evaluate the effect of mutation load of mtDNA on disease pathogenesis.

Thereafter several reports demonstrated the generation of iPSCs with other types of mtDNA mutations (Table 5.1). Based on these pioneering studies, we are beginning to understand how mitochondria dysfunctions affect the following steps: (1) reprogramming, (2) maintenance and proliferation of iPSCs, and (3) differentiation from iPSCs (Fig. 5.2C).

Role of mitochondria in reprogramming

Wahlestedt et al. generated iPSCs from somatic cells carrying random mtDNA mutations. They used the somatic cells harvested from $Polg^{tm1Lrsn}$ mice, which had the error-prone mtDNA polymerase. Interestingly, even if the cells had a high proportion of mtDNA mutations, iPSCs were established indicating that intact mitochondrial

Table 5.1 Induced pluripotent stem cells derived from the patients with mitochondrial encephalopathy.

Gene mutation	Gene expression	Clinical phenotype	Main findings of the study	References
m.13513G > A	*MT-ND5*	MELAS	Both the control induced pluripotent stem cells (iPSCs) and the iPSCs with mitochondrial DNA (mtDNA) mutation display spherically shaped mitochondria and reduced oxygen dependency. High load of mtDNA heteroplasmy interferes with cardiogenesis in patient-specific iPSCs	Folmes et al. (2013)
		LS	They succeeded in generating the iPSCs with genetically intact mtDNA by somatic cell nuclear transfer. These iPSCs showed normal oxidative phosphorylation (OXPHOS) upon differentiation into fibroblasts and skeletal muscle cells.	Ma et al. (2015)
		LS	Generation of the iPSCs from the patient with Leigh syndrome (LS) by using Sendai virus.	Galera et al. (2016)
m.3243A > G	*MT-TL1*	MELAS	Active down-regulation of complex I through mitophagy in iPSCs derived neurons with high mutant mtDNA load. The number of mitochondrial nucleoids is increased in iPSCs	Hämäläinen et al. (2013)

Continued

Table 5.1 Induced pluripotent stem cells derived from the patients with mitochondrial encephalopathy.—*cont'd*

Gene mutation	Gene expression	Clinical phenotype	Main findings of the study	References
			with high mutant mtDNA load. Upon neuronal differentiation, complex I specifically was sequestered in perinuclear PTEN-induced putative kinase 1 (PINK1) and Parkin-positive autophagosomes, suggesting active degradation through mitophagy.	
		MELAS	Generation of iPSC lines with different heteroplasmy levels. Enzymatic activity analysis for mitochondrial respiratory complexes in MELAS-iPSC–derived fibroblasts.	Kodaira et al. (2015)
		MELAS	They succeeded in generating the iPSCs with genetically intact mtDNA by somatic cell nuclear transfer. These iPSCs showed normal OXPHOS upon differentiation into fibroblasts and skeletal muscle cells.	Ma et al. (2015)
		MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)-iPSCs showed reduced efficiency to differentiate into endothelial cells. MELAS-iPSC–derived endothelial cells displayed dysregulated	Pek et al. (2019)

Table 5.1 Induced pluripotent stem cells derived from the patients with mitochondrial encephalopathy.—*cont'd*

Gene mutation	Gene expression	Clinical phenotype	Main findings of the study	References
			inflammatory responses. Antioxidant treatments were successful in improving endothelial functions.	
		MELAS	An increase of autophagy and mitophagy in MELAS-iPSCs.	Lin et al. (2019)
			Neuronal and cardiac maturation defects in the iPSCs line with a high proportion of m.3243A > G.	Yokota et al. (2017)
m.8993T > G	*MT-ATP6*	LS	They succeeded in generating the iPSCs with genetically intact mtDNA by somatic cell nuclear transfer. These iPSCs showed normal OXPHOS upon differentiation into fibroblasts and skeletal muscle cells.	Ma et al. (2015)
m.8344A > G	*MT-TK*	MERRF	Generation of the iPSC line from a patient with MERRF by using Sendai virus.	Wu et al., 2018
m.10191T > C	*MT-ND3*	MELAS/LS overlap syndrome	Metabolic signature of MELAS/Leigh overlap syndrome in the iPSC. The iPSCs with high mutant load mtDNA showed a distinct metabolic profile compared with control-iPSCs (pyruvate, malic acid, and lactic acid).	Hattori et al. (2016)

Continued

Table 5.1 Induced pluripotent stem cells derived from the patients with mitochondrial encephalopathy.—*cont'd*

Gene mutation	Gene expression	Clinical phenotype	Main findings of the study	References
m.5541C > T	*MT-TW*	MELAS	iPSC-derived neurons were detected due to serious mitochondrial dysfunction triggered by m.5541C > T. Significant loss of terminally differentiated iPSCs-derived neurons but not iPSC-derived skeletal muscle cells.	Hatakeyama et al. (2015)

function was not essential for reprogramming (Wahlestedt et al., 2014). Furthermore, the suppression of mitochondrial function improved reprogramming efficiency. In the presence of rotenone (complex I inhibitor), antimycin A or potassium cyanide (complex IV inhibitor), mitochondrial inhibition improved reprogramming efficiency and exhibited full differentiation potential (Son et al., 2013).

On the contrary, Yokota et al. reported that reprogramming efficiency decreased in fibroblasts carrying high proportions of mutant mtDNA (≥90% m.3243A > G encoded tRNA) (Yokota et al., 2015). Since m.3243A > G is located in the mitochondrial genome encoding tRNA for protein synthesis, a unique set of functional and structural changes of mitochondrial proteins may interfere in the reprogramming process.

Role of mitochondria in maintenance and proliferation of induced pluripotent stem cells

When the high degree of random mtDNA mutations was loaded by the error-prone mtDNA polymerase as described above, the proliferative ability of the iPSCs was compromised during the maintenance of pluripotency (Wahlestedt et al., 2014). In the case of the m.13513G > A mutation affecting the ND5 subunit of complex I, the proportion of heteroplasmy decreased from 50% at passage 3%−37% and then to 7% at passages 21 during the maintenance of iPSCs, respectively (Folmes et al., 2013). This result suggests that the m.13513G > A mutation of ND5 was unstable in the state of pluripotency, but it is unclear whether the ND5 function (complex 1 activity) is required for the maintenance of iPSCs. To support this notion, in our experiment of a mutation of ND3, which leads to a defect of complex I activity, the degree of heteroplasmy in iPSCs with the m.10191T > C mutation (ND3)

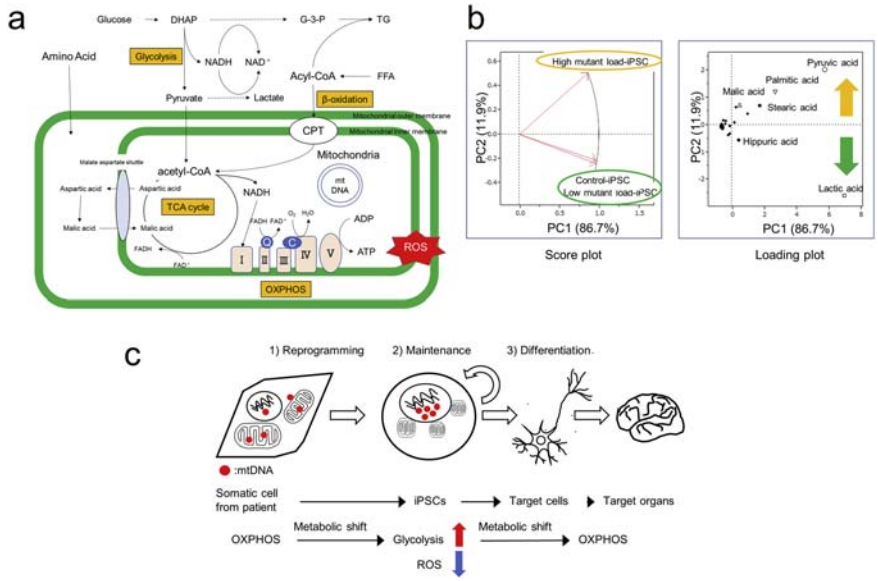


FIGURE 5.2 Mitochondrial dynamics in induced pluripotent stem cells (iPSCs) for disease modeling.

(A) Cellular metabolism: Various metabolic pathways exist in cytosol and mitochondria. (i.e., Glycolysis, TCA cycle, β-oxidation, and OXPHOS) The iPSCs state tended to lean toward increased glycolysis rather than oxidative phosphorylation (OXPHOS) in order to inhibit the production of ROS. Glycolysis, TCA cycle, and β-oxidation were activated by inhibiting OXPHOS. DHAP: Dihydroxyacetone Phosphate, G-3-P: Glyceraldehyde 3-phosphate, TG: triglyceride, FFA: free fatty acid, CPT: carnitine palmitoyl transferase, I, II, III, IV, V: respiratory complex, NAD: nicotinamide adenine dinucleotide, ROS: reactive oxygen species, mtDNA: mitochondrial DNA, Q: coenzyme Q, C: cytochrome C. (B) Principal component analysis (PCA) analysis data of gas chromatography/mass spectrometry (GC/MS) in iPSCs with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)/Leigh overlap syndrome. Score plot: The contributions of the top two principal components (PCs) to variance were 86.7% (PC1) and 11.9% (PC2). A principal component analysis of the metabolome analysis data clearly distinguished between high mutant load-iPSC and low mutant load-iPSC with mtDNA mutation on the second principal component axis. Loading plot: Metabolites that contributed to this distinction were pyruvic acid, palmitic acid, stearic acid, and malic acid (characteristic of high mutant load-iPSC) and lactic acid (characteristic of low mutant load-iPSC and control-iPSC). (C) Somatic cells have ellipsoid-shaped mitochondria and multicopies of mtDNA are distributed in mitochondrial cytosol. Upon reprogramming, the shape of mitochondria changes to spherical and mtDNA accumulates in the nucleus. Energy metabolisms also shifted from OXPHOS to glycolysis in iPSCs, which may have an advantage in reducing toxic ROS generation. Differentiation from iPSCs will revert these changes and recapitulate the disease progression at the cellular and organ levels.

remained unchanged even after extended passages (>60 passages), which was consistent with previous reports of iPSCs with m.3243A > G (Hämäläinen et al., 2013; Fujikura et al., 2012; Kodaira et al., 2015). The energy metabolism of iPSCs was shifted to anaerobic glycolysis from OXPHOS. It is suggested that the maintenance and proliferation in iPSCs was not affected by the mitochondrial deficiency with mtDNA mutation. The metabolic shift to glycolysis has the advantage of preventing the production of reactive oxygen species (ROS) from OXPHOS in the undifferentiated state of iPSCs (Armstrong et al., 2010; Prigione et al., 2010; Folmes et al., 2011; Panopoulos et al., 2012). However, the iPSCs with high mutant load mtDNA showed a distinct metabolic profile compared with control-iPSCs by gas chromatography coupled with mass spectrometry (GC-MS). The loading plot of principal component analysis revealed that the compounds contributing to the distinction were pyruvic acid, malic acid, palmitic acid, and stearic acid (characteristic of high mutant load-iPSC) and lactic acid (characteristic of control-iPSC and low mutant load-iPSC) (Hattori et al., 2016). These results suggest that m.10191T > C (ND3) influenced glucose metabolism, the tricarboxylic acid cycle, fatty acid synthesis, fatty acid β-oxidation, and a lactate-malate-aspartate shuttle in the iPSCs state (Fig. 5.2A and B).

More interestingly, during the maintenance of iPSCs, a large proportion of mtDNA fragments were observed in the nucleus and accumulated in the nucleus extrachromosomally (Schneider et al., 2014). Additionally, mitochondrial morphology in undifferentiated iPSCs displayed a spheroid shape with poor cristae structure and reduced mtDNA copies per cell (Folmes et al., 2011). These findings imply that mtDNA itself may play an additional role during the maintenance of iPSCs beyond wiring the energy metabolism in the mitochondria (Fig. 5.2C).

Role of mitochondria in differentiation from induced pluripotent stem cells

To recapitulate the disease-specific phenotype in vitro, iPSCs provide a huge advantage due to the ability of differentiation into various cell types. Mitochondrial diseases with mtDNA mutation present pathological changes in a wide variety of tissues and organs in humans.

Several research groups have attempted to induce differentiation into various cell types in vitro in order to elucidate the pathogenesis of mitochondrial disease caused by mtDNA mutation. MELAS is a common clinical phenotype of mitochondrial encephalopathy. Lactate acidosis and reversible stroke-like episodes are characteristic of the clinical features of MELAS. Patients with MELAS were reported to have atherosclerotic lesions in aortic tissues (Sazonova et al., 2015; Sazonova et al., 2009). Indeed, Pek et al. found proatherogenic and proinflammatory properties in endothelial cells differentiated from MELAS-iPSCs, which were carrying the m.3243A > G mutation (Pek et al., 2019). They speculated that the high level of ROS was the main culprit causing these properties in the endothelial cells. Furthermore, Lin et al. demonstrated that oxidative insults induced a marked increase of autophagy and broad activation of mitophagy, and led to compromised cell viability

in the MELAS-iPSCs with the m.3243A > G mutation (Lin et al., 2019). Regarding the pathological phenotype in neuronal lineage cells, Hämäläinen showed that complex I was actively degraded by an autophagy-mediated mechanism upon neuronal differentiation (Hämäläinen et al., 2013). In the case of MELAS-iPSCs with the m.5541C > T mutation, a significant loss of neurons was observed upon terminal neuronal differentiation and it was due to serious mitochondrial dysfunction (Hatakeyama et al., 2015). Yokota et al. established two isogenic iPSCs lines each carrying different proportions of a heteroplasmic m.3243A > G mutation from the same patient. The iPSCs with a high mutation load inhibited differentiation into mature neurons. High proportions of m.3243A > G showed inhibited cardiac lineage differentiation, as well (Yokota et al., 2017). These reports revealed that the mitochondrial function and mtDNA were involved in differentiation and maturation.

The mitochondrial genome only encodes 37 genes, and the vast majorities of proteins and molecules are provided from the environments created by their own nuclear genome. It is reasonable to consider that disease phenotypes of mtDNA mutations could be influenced by the nuclear DNA backgrounds of the patients (Hämäläinen et al., 2013; Fujikura et al., 2012; Kodaira et al., 2015). Direct manipulation of mtDNA would provide further evidence of the pathophysiological mechanisms in mitochondrial diseases in vitro. Recently, TALEN technology was successfully applied to iPSCs to manipulate mtDNA. The m.13513G > A and m.3243A > G heteroplasmy level in MELAS-iPSCs was decreased by the transduction of mitoTALENs (Yahata et al., 2017; Yang et al., 2018). The CRISPR-Cas9 system was initially considered to be challenging mainly due to inefficient delivery of guide RNA and Cas9 enzyme complexes into mitochondria. Hussain, however, succeeded in gene editing in the mtDNA by appending NADH-ubiquinone oxidoreductase chain 4 (ND4) targeting guide RNA to an RNA transport derived stem loop element (RP-loop) and expressing the Cas9 enzyme with the preceding mitochondrial localization sequence. They showed mitochondrial co-localization of RP-loop gRNA and a marked reduction of ND4 expression in the cells carrying a A11204G variant in their ND4 sequence coincidently decreasing the mtDNA levels (Hussain et al., 2021).

Future trends

The development of gene editing technology discussed above will allow us to investigate the effects of mitochondrial gene mutations on mitochondrial disease systematically by taking advantage of the iPSCs-platform. Another exciting trend using iPSCs would be the development of three-dimensional culture (Lancaster and Knoblich, 2014) (Fig. 5.3B), which allows us to recapitulate cellular interactions in the context of tissue or organ levels. As an instance of neuronal differentiation, conventional differentiation systems using two-dimensional cultures of iPSCs often evaluate the cell survival rate, morphological changes in the cells, and/or gene expression analysis, and these parameters do not always reflect the actual functions

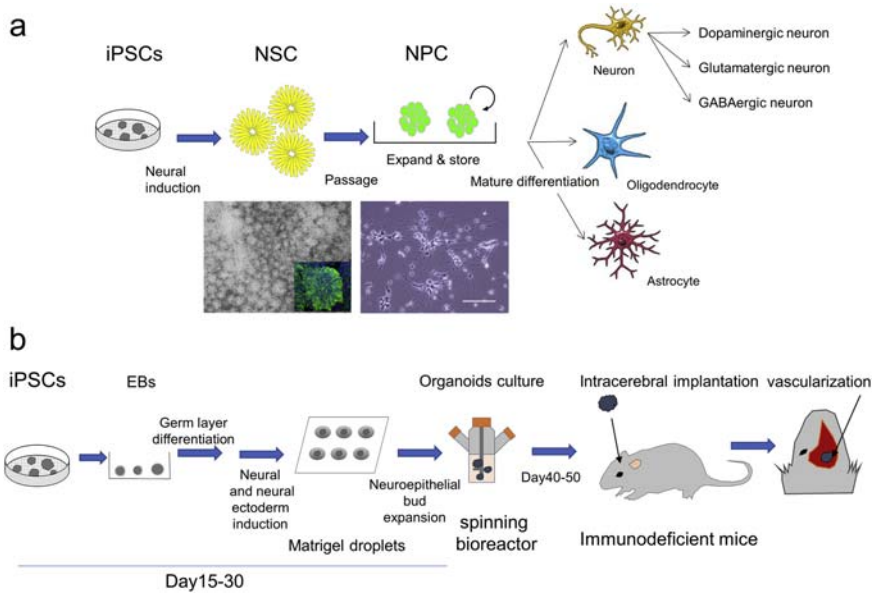


FIGURE 5.3 The development of neuronal culture derived from induced pluripotent stem cells (iPSCs).

(A) Neuronal differentiated two-dimensional culture. The type-specific neuronal differentiation. Various neuronal cells were derived from iPSCs. iPSCs were differentiated into NSC and NPC. NPC were maturely differentiated into neurons, astrocytes, and oligodendrocytes. Further neurons were differentiated into dopaminergic neuron, glutamatergic neuron, and GABAergic neuron. NSC: neuronal stem cells, NPC: neuronal progenitor cells. (B) Neuronal organoids three-dimensional culture. Organoid culture protocol begins the generation of embryoid bodies (EBs) from iPSCs. EBs are induced neural induction. Neuroectodermal tissues are transferred to droplets of Matrigel. Finally matrigel droplets are transferred to a spinning bioreactor. Additionally, organoids culture is implanted into the intracerebral membrane of immunodeficient mice. After vascularization, nutrition is supplied to the implanted organoids culture vascularly.

in the brain (Fig. 5.3A). Human iPSCs are now able to be differentiated into a large multicellular organoid-like structure that contains distinct layers of neuronal cells expressing characteristic markers of the human midbrain (Jo et al., 2016). The study of rotenone toxicity for the mitochondrial respiratory function in a human brain spheroid model has been reported. The results indicate that rotenone's toxic potency varies depending on the differentiation status and dopaminergic-neurons were more susceptible to the toxin than astrocytes and other neuronal cell types at noncytotoxic concentrations (1 μM) (Pamies et al., 2018). Dopaminergic and glutamatergic, and GABAergic neurons are mixed in the neuronal organoid culture. It has not yet been elucidated how the iPSCs carrying mtDNA mutations differentiate into the neuronal organoid-like structure (Fig. 5.3B).

According to clinical molecular imaging of patients with MELAS, we expect the pathological process involving vascular angiopathy, increase of anaerobic energy metabolism, increase of blood flow, angioedema, and accompanying cellular edema and neurological dysfunction due to increased oxidative stress (Ikawa et al., 2013; Yoneda et al., 1999; Ikawa et al., 2009). Mitochondrial neuropathology is suspected to be caused by various intertwining in the complex structure and function of the human brain. It makes the condition complicated and difficult to study in the pathology of mitochondrial disease. One limitation of the in vitro culture is the lack of physiological vascular circulations. Mansour et al. differentiated human embryonic stem cells into cerebral cortical organoids and implanted them into the brains of severe combined immunodeficient mice (Mansour et al., 2018) (Fig. 5.3B). This approach potentially enables us to study pathogenesis in the context of the native vascular environment and to reveal the interplay between neuron and vascular angiopathy (Inoue et al., 2000).

References

Armstrong, L., Tilgner, K., Saretzki, G., et al., 2010. Human induced pluripotent stem cell lines show stress defense mechanisms and mitochondrial regulation similar to those of human embryonic stem cells. Stem Cell. 28, 661−673.

Crimi, M., Galbiati, S., Moroni, I., et al., 2003. A missense mutation in the mitochondrial ND5 gene associated with a Leigh-MELAS overlap syndrome. Neurology 60, 1857−1861.

Folmes, C.D.L., Nelson, T.J., Martinez-Fernandez, A., et al., 2011. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metabol. 14, 264−271.

Folmes, C.D.L., Martinez-Fernandez, A., Perales-Clemente, E., et al., 2013. Disease-causing mitochondrial heteroplasmy segregated within induced pluripotent stem cell clones derived from a patient with MELAS. Stem Cell. 31, 1298−1308.

Fujikura, J., Nakao, K., Sone, M., et al., 2012. Induced pluripotent stem cells generated from diabetic patients with mitochondrial DNA A3243G mutation. Diabetologia 55, 1689−1698.

Galera, T., Zurita, F., González-Páramos, C., et al., 2016. Generation of a human iPSC line from a patient with Leigh syndrome. Stem Cell Res. 16, 63−66.

Hämäläinen, R.H., Manninen, T., Koivumäki, H., et al., 2013. Tissue- and cell-type−specific manifestations of heteroplasmic mtDNA 3243A>G mutation in human induced pluripotent stem cell-derived disease model. Proc. Natl. Acad. Sci. U. S. A. 110. E3622.

Hatakeyama, H., Goto, Y., 2016. Concise review: heteroplasmic mitochondrial DNA mutations and mitochondrial diseases: toward iPSC-based disease modeling, drug discovery, and regenerative therapeutics. Stem Cell. 34, 801−808.

Hatakeyama, H., Katayama, A., Komaki, H., et al., 2015. Molecular pathomechanisms and cell-type-specific disease phenotypes of MELAS caused by mutant mitochondrial tRNATrp. Acta Neuropathol. Commun. 3, 52.

Hattori, T., Hamazaki, T., Kudo, S., et al., 2016. Metabolic signature of MELAS/Leigh overlap syndrome in patient-specific induced pluripotent stem cells model. Osaka City Med. J. 62 (2), 69−76. Dec.

Hussain, S.-R.A., Yalvac, M.E., Khoo, B., et al., 2021. Adapting CRISPR/Cas9 system for targeting mitochondrial genome. Front Genet. 12, 627050. https://doi.org/10.3389/fgene.2021.627050.

Ikawa, M., Okazawa, H., Arakawa, K., et al., 2009. PET imaging of redox and energy states in stroke-like episodes of MELAS. Mitochondrion 9, 144−148.

Ikawa, M., Yoneda, M., Muramatsu, T., et al., 2013. Detection of preclinically latent hyperperfusion due to stroke-like episodes by arterial spin-labeling perfusion MRI in MELAS patients. Mitochondrion 13, 676−680.

Inoue, K., Nakada, K., Ogura, A., et al., 2000. Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. Nat. Genet. 26, 176−181.

Jo, J., Xiao, Y., Sun, A.X., et al., 2016. Midbrain-like organoids from human pluripotent stem cells contain functional dopaminergic and neuromelanin-producing neurons. Cell Stem Cell 19, 248−257.

Jun Matsui, Y.G., 2014. A case of mitochondrial myopathy, encephalopathy lactic acidosis and strokelike episode Leigh overlap syndrome. No Hattatsu 46, 363−366.

Kodaira, M., Hatakeyama, H., Yuasa, S., et al., 2015. Impaired respiratory function in MELAS-induced pluripotent stem cells with high heteroplasmy levels. FEBS Open Bio 5, 219−225.

Kori, A., Hori, I., Tanaka, T., et al., 2019. Transition from Leigh syndrome to MELAS syndrome in a patient with heteroplasmic MT-ND3 m.10158T>C. Brain Dev. 41, 803−807.

La Morgia, C., Caporali, L., Gandini, F., et al., 2014. Association of the mtDNA m.4171C>A/MT-ND1 mutation with both optic neuropathy and bilateral brainstem lesions. BMC Neurol. 14, 1−5.

Lancaster, M.A., Knoblich, J.A., 2014. Generation of cerebral organoids from human pluripotent stem cells. Nat. Protoc. 9, 2329−2340.

Leng, Y., Liu, Y., Fang, X., et al., 2015. The mitochondrial DNA 10197 G > A mutation causes MELAS/Leigh overlap syndrome presenting with acute auditory agnosia. Mitochondr.DNA 26 (2), 208−212. https://doi.org/10.3109/19401736.2014.905860.

Lin, D.-S., Huang, Y.-W., Ho, C.-S., et al., 2019. Oxidative insults and mitochondrial DNA mutation promote enhanced autophagy and mitophagy compromising cell viability in pluripotent cell model of mitochondrial disease. Cells 8, 65.

Ma, H., Folmes, C.D.L., Wu, J., et al., 2015. Metabolic rescue in pluripotent cells from patients with mtDNA disease. Nature 524, 234−238.

Mansour, A.A., Gonçalves, J.T., Bloyd, C.W., et al., 2018. An in vivo model of functional and vascularized human brain organoids. Nat. Biotechnol. 36, 432−441.

Nakada, K., Inoue, K., Hayashi, J.-I., 2001. Mito-mice: animal models for mitochondrial DNA-based diseases. Semin. Cell Dev. Biol. 12, 459−465.

Nakada, K., Sato, A., Sone, H., et al., 2004. Accumulation of pathogenic ΔmtDNA induced deafness but not diabetic phenotypes in mito-mice. Biochem. Biophys. Res. Commun. 323, 175−184.

Nakada, K., Sato, A., Hayashi, J.-I., 2008. Reverse genetic studies of mitochondrial DNA-based diseases using a mouse model. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 84, 155.

Pamies, D., Block, K., Lau, P., et al., 2018. Rotenone exerts developmental neurotoxicity in a human brain spheroid model. Toxicol. Appl. Pharmacol. 354, 101−114.

Panopoulos, A.D., Yanes, O., Ruiz, S., et al., 2012. The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. Cell Res. 22, 168−177.

Pek, N.M.Q., Phua, Q.H., Ho, B.X., et al., 2019. Mitochondrial 3243A > G mutation confers pro-atherogenic and pro-inflammatory properties in MELAS iPS derived endothelial cells. Cell Death Dis. 10, 802.

Prigione, A., Fauler, B., Lurz, R., et al., 2010. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. Stem Cell. 28, 721−733.

Rossignol, R., Faustin, B., Rocher, C., et al., 2003. Mitochondrial threshold effects. Biochem. J. 370, 751−762.

Sazonova, M., Budnikov, E., Khasanova, Z., et al., 2009. Studies of the human aortic intima by a direct quantitative assay of mutant alleles in the mitochondrial genome. Atherosclerosis 204, 184−190.

Sazonova, M.A., Sinyov, V.V., Barinova, V.A., et al., 2015. Mosaicism of mitochondrial genetic variation in atherosclerotic lesions of the human aorta. BioMed Res. Int. 2015.

Schneider, J.S., Cheng, X., Zhao, Q., et al., 2014. Reversible mitochondrial DNA accumulation in nuclei of pluripotent stem cells. Stem Cell. Dev. 23, 2712−2719.

Son, M.J., Jeong, B.R., Kwon, Y., et al., 2013. Interference with the mitochondrial bioenergetics fuels reprogramming to pluripotency via facilitation of the glycolytic transition. Int. J. Biochem. Cell Biol. 45, 2512−2518.

Wahlestedt, M., Ameur, A., Moraghebi, R., et al., 2014. Somatic cells with a heavy mitochondrial DNA mutational load render induced pluripotent stem cells with distinct differentiation defects. Stem Cell. 32, 1173−1182.

Wang, Z., Qi, X.K., Yao, S., et al., 2010. Phenotypic patterns of MELAS/LS overlap syndrome associated with m.13513G>A mutation, and neuropathological findings in one autopsy case. Neuropathology 30, 606−614.

Wu, Y.T., Hsu, Y.H., Huang, C.Y., et al., 2018. Generation of an induced pluripotent stem cell (iPSC) line from a 40-year-old patient with the A8344G mutation of mitochondrial DNA and MERRF (myoclonic epilepsy with ragged red fibers) syndrome. Stem Cell Res. 27, 10−14.

Yahata, N., Matsumoto, Y., Omi, M., et al., 2017. TALEN-mediated shift of mitochondrial DNA heteroplasmy in MELAS-iPSCs with m.13513G>A mutation. Sci. Rep. 7, 1−11.

Yang, Y., Wu, H., Kang, X., et al., 2018. Targeted elimination of mutant mitochondrial DNA in MELAS-iPSCs by mitoTALENs. Protein Cell 9, 283−297.

Yokota, M., Hatakeyama, H., Okabe, S., et al., 2015. Mitochondrial respiratory dysfunction caused by a heteroplasmic mitochondrial DNA mutation blocks cellular reprogramming. Hum. Mol. Genet. 24, 4698−4709.

Yokota, M., Hatakeyama, H., Ono, Y., et al., 2017. Mitochondrial respiratory dysfunction disturbs neuronal and cardiac lineage commitment of human iPSCs. Cell Death Dis. 8.

Yoneda, M., Maeda, M., Kimura, H., et al., 1999. Vasogenic edema on MELAS: a serial study with diffusion-weighted MR imaging. Neurology 49, 113−119.

Zeviani, M., Muntoni, F., Savarese, N., et al., 1993. A MERRF/MELAS overlap syndrome associated with a new point mutation in the mitochondrial DNA tRNA(Lys) gene. Eur. J. Hum. Genet. 1, 80−87.

This page intentionally left blank

CHAPTER

6 How induced pluripotent stem cells changed the research status of polycystic ovary syndrome

Yang Yu[1,2]

[1]*Clinical Stem Cell Research Center, Peking University Third Hospital, Beijing, China;* [2]*Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China*

Chapter outline

Abstract

As a hot topic in the frontier of medicine, stem cells can not only be applied in treatment of various diseases, but also provide reliable in vitro models for disease research. With the first step taken 40 years from now, the stem cell acquisition and isolation technologyhas developed relatively well. Nevertheless, limited by the sources of material as well as the ethical dilemma of using embryonic stem cells

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00009-6

(ESCs) and adult stem cells, scientists are now focusing on regeneratingthe infinite proliferation and multi-differential potential of those terminally differentiated somatic cells. The reprogramming technology mainly include somatic cell nuclear transfer technology (SCNT), cell fusion technology and induced pluripotent stem cell (iPSc) technology, and the last one outperformed the former two for its higher efficiency, simpler operation, higher cost performance, safety and higher precision. Polycystic ovary syndrome (PCOS) is featured by endocrine disorders and metabolic syndromes with complex clinical manifestations (persistent anovulation, insulin resistance, hyperandrogenemia and hyperinsulinemia) caused by hypothalamic-pituitary-ovarian axis dysfunction and a metabolic disorder. Despite thephysical suffering and the psychological burden of the patients, the etiology, diagnostic criteria and optimal treatment for PCOS are currently vague. Meanwhile, there still lacks animal model that can fully simulate all the characteristics of clinical PCOS patients at present. With the development of iPSC technology, the ability of continuous self-renewal and the potential of multidirectional differentiation of these cells have made them powerful tools for the research and development of disease mechanism. Up to date, progress has been madeon the establishment of PCOS-iPSCs cell models for maintaining the disease gene phenotype in vitro to further advance the exploration of the pathogenesis, pathogenic genes, and drug screening of PCOS.

Keywords: Cell model; Disease pathogenesis; Induced pluripotent stem cell; Polycystic ovary syndrome; Translational medicine.

Introduction to stem cells and embryonic stem cells

Stem cells are a generic term for a large group of cells with self-renewal and multipotential both in vitro and in vivo. In 1999, stem cells were named by Science magazine as one of the top 10 most important scientific fields in the 21st century. Stem cells can be divided into different groups for several different classification methods. According to their differentiation potential, they are generally divided into totipotent stem cells, pluripotent stem cells, and unipotent stem cells. The differentiation ability of these three groups of cells changes from strong to weak in turn, whereby the fertilized egg is a pluripotent stem cell with the highest differentiation potential that can differentiate into a complete individual. However, the other two types of cells can only differentiate into corresponding organs or tissues. According to the source, stem cells can be divided into embryonic stem cells (ESCs) and adult stem cells. Adult stem cells refers to a variety of pluripotent stem cells in the body, such as hematopoietic stem cells in the blood and neural stem cells (NSCs) in the brain.

In 1981, Evans (Martin, 1981) surgically removed the ovaries of mice at 2.5 days after fertilization and then interfered with the internal environment of the uterus with hormone injection to delay the implantation of the embryo. As a result, the first mouse ESC line was obtained. Subsequently, Axelord et al. (Axelrod, 1984) also obtained mouse ESC lines with the microdroplet culture method. Iannaccone and others (Iannaccone et al., 1994) isolated and cloned a rat ESC line that was positive

for SSEA-1 and AKP and can proliferate on the feeder cells of rat fetal fibroblasts and differentiate into various cell types in vivo. In addition, Vassilieva et al. (2000) established rat ES cell lines expressing SSEA-1 and OCT4, which were maintained by members of the interleukin-6 family of cytokines, but leukemia inhibitory factor (LIF) is the most critical factor for maintaining culture. Piedrahita and his colleagues (Piedrahita et al., 1990) isolated ESCs from porcine blastocyst inner cell mass (ICM) and found that porcine blastocyst ICM can attach and proliferate on a continuous cell line of murine embryonic fibroblasts (SIM mouse embryo-derived thioguanine and ouabain resistant fibroblast cell line, STOs) or porcine embryonic fibroblasts. However, the research team of Evans et al. (Notarianni et al., 1990) directly cultured 6−7 days old pig blastocysts on STO feeder cells, selected ICM cells and established the pig ES cell line through proliferation and passage culture. Saito et al. (1992) cultured bovine ICM in medium with LIF and isolated bovine ES cells. Moreover, Mitalipova et al. (2001) isolated bovine ES cells expressing SSEA-1, SSEA-3, SSEA-4, and c-kit receptors from dense morula. Handyside and others (Handyside et al., 1987) isolated ES-like cells from sheep blastocysts aged 7−8 days old fed sheep skin fibroblast cells and fetal fibroblast cells, but as a result, endoderm-like cells were obtained without ESCs. Rabbit ESCs were isolated from blastocysts by Graves et al. (Moreadith and Graves, 1992), and preliminary identification was performed to prove that they have the ability to proliferate in an undifferentiated state on feeder cells. After being passaged for more than one year, the rabbit ESCs still had normal karyotypes and were able to form embryoid bodies containing three germ layers. With the continuous progress of related research on ESCs, an increasing number of types of animal ESCs have been established, and research on human embryonic stem cells (hESCs) was originally explored by Prea and his colleagues (Pera et al., 1989) to isolate ESCs from human teratomas. Bongso et al. (1994) cultured prokaryotic embryos until the blastocyst stage on human fallopian tube epithelial cells until the blastocyst stage and obtained ESCs, but the cells died after only two passages of culture. In 1998, Thomson's research team (Thomson et al., 1998) first isolated the ICM from blastocyst embryos by immunological methods and finally established human ESC lines.

A large number of experiments conducted by scientists on the differentiation function of ESCs have shown that the obtained ESC lines cultured in vitro can differentiate into multiple cell types, such as myocardial cells (Wobus et al., 1997; Mummery et al., 2003; Huber et al., 2007), neurons (Wichterle et al., 2002; Li et al., 2008), and blood cells (Keller et al., 1993) (Fig. 6.1). Therefore, it is regarded as the "seed" cell for cell therapy and organ transplantation, with the promise of treating a variety of genetic and other treatment-resistant diseases.

It is well known that ESCs develop from the ICM of embryos to the blastocyst stage, which means that we must destroy the human embryo to establish human-derived ESCs (Fig. 6.1). Due to the particularity of this material, which involves legal and ethical issues (Robertson, 2001), the material selection has great limitations, which restricts the research and application of human ESCs. In addition, a fatal problem in the clinical application of ESCs is allotransplantation rejection.

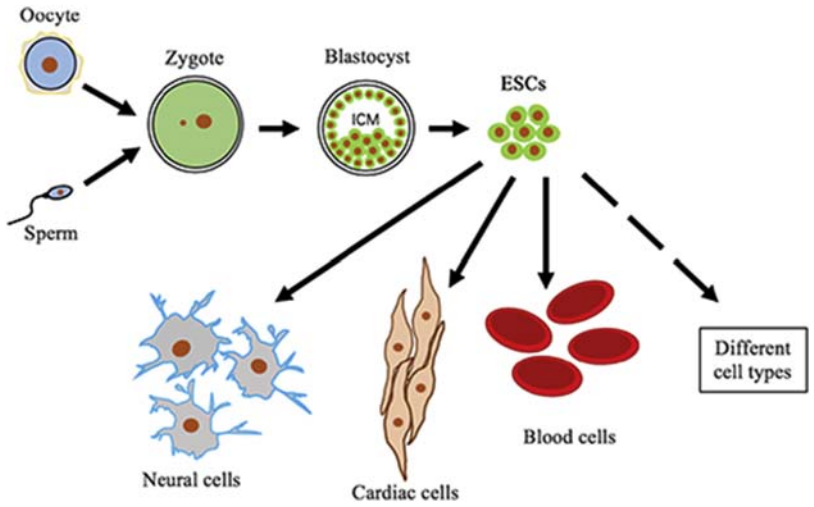


FIGURE 6.1

The source and differentiation potential of embryonic stem cells. Embryonic stem cells are derived from blastocyst inner cell mass (ICM) and can differentiate into various cell types of tissues and organs in vitro, such as neural cells, cardiac cells, and blood cells.

There are a small number of adult stem cells with limited differentiation potential in adult bone marrow, nerves, muscles, adipose tissue, and other tissues, which also solves the problem of immune rejection and the ethics of ESCs to some extent. It has become an important role in the development of regenerative medicine drugs. However, there are many unclear aspects in the process of differentiation of most adult stem cells. More importantly, it is difficult to separate and purify and cultivate adult stem cells in vitro. The number of obtained cells and the age of the donor are also limiting.

The development of somatic reprogramming

Although both ESCs and adult stem cells have value in vitro research and a wide range of application prospects, both of them have large limitations on the sources of materials. To solve this dilemma, scientists have tried to make terminally differentiated somatic cells "reverse growth" back to the original pluripotent state and regain the characteristics of infinite proliferation and multidifferential potential of ESCs. It is hoped that it can largely replace ESCs in scientific research and clinical applications.

The process of returning terminally differentiated somatic cells to an undifferentiated pluripotent state using a series of techniques is known as reprogramming. Since the 1950s, reprogramming methods and reprogramming mechanisms have been continuously explored, which has removed many obstacles for research and

development in the field of regenerative medicine. At present, there are three main somatic cell reprogramming technologies: somatic cell nuclear transfer (SCNT) technology, cell fusion technology, and induced pluripotent stem cells (iPSCs). These three technologies have different reprogramming methods and efficiencies, which can induce somatic cell reprogramming to the state of pluripotent stem cells, providing strong support and promotion for the development of biomedicine.

Nuclear transfer technique

SCNT technology in which somatic-derived nuclei are injected into enucleated oocytes by microinjection or other techniques to achieve the return of terminally differentiated somatic cells to an undifferentiated state can be used to establish pluripotent cell lines or to develop into complete individuals, which is what we commonly call cloning. In this process, the nucleus that has differentiated into a specific somatic cell is reactivated by some substances in the oocyte cytoplasm, and these biomolecules in the oocyte cytoplasm that enable the differentiated nucleus to regain totipotency are called reprogramming factors.

The technique was first developed in 1952. Two researchers, Briggs (Briggs and King, 1952) and King (King et al., 1996), injected the early follicular nucleus into the enucleated oocytes of the leopard frog, which eventually successfully developed into a normal tadpole. Another sensational research achievement of the cloned sheep Dolly (Wilmut et al., 1997) in 1997 was also a sign of the successful application of this technology in mammals. Subsequently, a team of researchers (Marx, 1981) produced three cloned mice using nuclear transfer technology. With a series of studies on this cloned mouse, many abnormal gene expression patterns during the embryonic period have been found, including telomere lengthening, obesity in adult individuals, immune system damage, susceptibility to tumors, premature death, and other physiological problems. These anomalies make scientists worry about the safety of nuclear transfer. Some scientists have tried to optimize these problems by chemically altering egg activation, adjusting the enucleation time, and inhibiting cytoplasmic division.

Although this technology is helpful in studying embryonic development and the pathogenesis of diseases in some aspects, it has a series of problems, such as low reprogramming efficiency, low survival rate of recombinant embryos, developmental defects in cloned animals, and even ethics. Moreover, only those who have professional micromanipulation skills can do so, which severely limits its development and application in the field of reprogramming. This also requires scientists to conduct research and develop new reprogramming techniques.

Cell fusion technique

Cell fusion technology is another reprogramming technology after nuclear transfer technology. It works by fusing somatic cells with various pluripotent embryonic cells to obtain hybrid cells with stem cell characteristics. These common embryonic

cells include ESCs, embryonic germ cells, and embryonic cancer cells. The methods of cell fusion are divided into spontaneous fusion and induced fusion in which spontaneous fusion refers to the spontaneous merger of two identical kinds of cells cultured. Induced fusion mainly means that the cells between different species must be treated with an inducer before they can fuse. Currently, there are mainly biological methods to induce fusion with the inactivated Sendai virus, chemical methods involving polyethylene glycol (PEG), and physical methods such as electrical pulse, vibration, centrifugation, and electrical stimulation to induce fusion. The most widely used is PEG because it is easy to obtain and convenient, and the fusion effect is stable.

As early as 1965, Harris and his research team (Harris, 1967) used Sendai virus to fuse HeLa cells with red blood cells and successfully activated red blood cells. In 2005, the fusion of somatic cells with hESCs resulted in the formation of pluripotent tetraploid cells (Sumer et al., 2010; Cowan et al., 2005; Yu et al., 2006) that expressed the pluripotent marker gene Oct3/4. It was found that those tetraploid cells were similar to ESCs with respect to DNA methylation levels and histone modification patterns.

Several studies have shown that ESCs contain important reprogramming factors that enable somatic cells to obtain pluripotency, but based on the analysis of the current research results, it is impossible to determine exactly whether these reprogramming factors are distributed in the nucleus or in the cytoplasm. It must be noted that the obtained heterozygous cells contain two sets of chromosomes. For cell and organ transplantation, the problem of immune rejection still exists. To solve this problem, Tada et al. (Matsumura and Tada, 2008) also tried to remove a whole set of chromosomes from tetraploid cells to restore the diploid state, but this would seriously affect the stability of the genome, so this method was not feasible. Since then, other scientists have also attempted to reprogram somatic cells with extracts from ESCs (Taranger et al., 2005). None of the above studies can effectively improve cell fusion technology, the fusion efficiency is still low, and there are also ethical problems. Therefore, the method of reprogramming by cell fusion has many limitations in the field of biomedicine.

Induced pluripotent stem cells

The various defects in SCNT technology and cell fusion technology limit their applications. Scientists are eager to find a new way to reprogram cells, which can be widely used in scientific research and clinical treatment. The emergence of iPSCs has caused a dramatic response in the scientific community. Its greatest advantage is that it cleverly and completely avoids the problems caused by the limitations of ethical issues. It can also overcome the defects inherent in the other two reprogramming methods, which sheds new light on the application of stem cells in the field of regenerative medicine.

This landmark contribution was discovered by Japanese scientists Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) in 2006. They screened out a set of

more effective transcription factors, namely, Oct4, Sox2, Klf4, and c-Myc, from 24 prepared candidate transcription factors. These four transcription factors are overexpressed in mouse embryonic fibroblast cells by retroviruses, which can reprogram cells from the terminally differentiated state into a pluripotent state. The identification results for iPSCs showed that they are very similar to ESCs in morphology, gene expression profile, protein expression profile, epigenetic pattern and in vivo and in vitro differentiation. Later, Yamanaka's research team (Takahashi et al., 2007) successfully reprogrammed human fibroblasts using OCT4, SOX2, C-MYC, and KLF4 in 2007 to obtain human induced pluripotent stem cells (hiPSCs). In the same year, Thompson's laboratory (Yu et al., 2007) adopted another combination of four transcription factors, OCT4, SOX2, NANOG, and LIN28, and achieved the acquisition of hiPSCs.

After the prelude to the reprogramming of human cells, a variety of somatic cells can be used for reprogramming experiments. Scientists have reprogrammed human mesenchymal stem cells (Wernig et al., 2008b), lung fibroblasts (Maherali et al., 2008), bone marrow mesenchymal cells (Park et al., 2008a), adipocyte stem cells (Sun et al., 2009), tumor cells (Lin et al., 2008), blood B lymphocytes (Seki et al., 2010), tooth germ cells (Tamaoki et al., 2010), and other cells of different types and different embryo layers to produce iPSCs. Many of the common types of human somatic cells that have been reported to be reprogrammed by transcription factors are shown in Table 6.1. However, different reprogramming donor cells have different degrees of difficulty in reprogramming, such as blood cells and adipose cells, which are relatively ideal and commonly used donor cells for iPSCs because it is easy to obtain a large number of cells.

Reprogramming factors are mainly introduced into somatic cells in the form of plasmids, proteins, mRNA, microRNAs, etc., through viral vectors, electric transfection, and liposome transfection. The safety and reprogramming efficiency of iPSCs are of concern to many researchers. Stadtfeld et al. (2008) chose adenovirus as the vector for the transfection method, which was a breakthrough in the process. This method causes the imported reprogramming factor to be highly expressed in a short time and does not involve permanent integration. In addition, the team also used transposons to successfully produce virus-free integrated, higher security iPSCs. Okita and his colleagues (Okita et al., 2008) used the characteristics of plasmid transfection to design a plasmid framework and perform cotransfection, which can eliminate the four integrated reprogramming factors to obtain iPSCs without viral genes. Some studies have shown that adding some small molecular compounds during reprogramming can improve the reprogramming efficiency of iPSCs. Shi et al. (2008) used the G9a histone methyltransferase inhibitor BIX-01,294 to improve the reprogramming rate of NSCs. Research by the Mikkelsen (Mikkelsen et al., 2008) team showed that 5-azacytidine can also improve the efficiency of reprogramming. Esteban et al. (2010) confirmed that valproic acid and vitamin C can greatly improve the reprogramming efficiency of iPSCs by approximately 500 times. In 2013, Hongkui Deng's research team (Hou et al., 2013) used chemical

Table 6.1 The main cell types and methods reported for human somatic cell reprogramming through transcription factors.

Cell type	Reprogramming factor	Delivery method	References
Fibroblasts	OCT3/4, SOX2, KLF4, c-MYC/OCT3/4, SOX2, KLF4/OCT4, SOX2, NANOG, LIN28	Retrovirus/PB transposition/ Lentivirus	Takahashi et al. (2007), Yu et al. (2007), Nakagawa et al. (2008), Meissner et al. (2007), Woltjen et al. (2009), Mali et al. (2008), Park et al. (2008b)
Keratinocytes	OCT4, SOX2, KLF4, c-MYC	Lentivirus	Aasen et al. (2008), Carey et al. (2009)
Blood cells	OCT4, SOX2/OCT4, SOX2, KLF4, C-MYC/ OCT4, SOX2, KLF4, C-MY, BCL-XL	Retrovirus/ Sendai virus	Giorgetti et al. (2010), Zaehres et al. (2010), Zhou et al. (2015), Ye and Wang (2018), Wen et al. (2016), Rim et al. (2016)
Papilla cells	OCT4/OCT4, KLF4/ OCT4, SOX2, KLF4, C-MYC/OCT4, SOX2, KLF4, c-MYC	Retrovirus/ Lentivirus	Tsai et al. (2010, 2011), Higgins et al. (2012), Muchkaeva et al. (2014)
Amniotic fluid-derived cells	OCT4, SOX2, KLF4, C-MYC/	Retrovirus/ Plasmid transfection	Li et al. (2009), Galende et al. (2010), Slamecka et al. (2016)
B lymphocytes	OCT4/OCT4, SOX2, KLF4, c-MYC	Cell fusion/ Sendai virus	Pereira et al. (2008), Bueno et al. (2016)
Neural stem cells	OCT4, KLF4/OCT4, c-MYC	Retrovirus	Kim et al. (2008)
Mesenchymal stromal/stem cells (MSCs)	OCT3/4, SOX2, KLF4/ LIN28, NANOG, OCT4, SOX2/c-MYC, KLF4, OCT4, SOX2	Retrovirus	Oda et al. (2010), Yan et al. (2010)
Urine-derived cells	OCT4, SOX2, KLF4, c-MYC/OCT4, GLIS1, KLF4, SOX2, L-MYC, miR-302/OCT4, SOX2, C-MYC, KLF4	Retrovirus/ Nucleofector Kit/Lentivirus	Wang et al. (2017b), Zhou et al. (2011, 2012, 2013)
Adipocyte stem cells	OCT4, SOX2, KLF4, c-MYC	Lentivirus/ Retrovirus	Sun et al. (2009), Sugii et al. (2011)

methods to induce the reprogramming of somatic cells and eliminated the dependence of previous reprogramming methods on genetic manipulation. They treated somatic cells with a combination of four small-molecule compounds to achieve somatic cell reprogramming, originally inducing the specialized mouse somatic cells into pluripotent cells that can redifferentiate into various tissues and organ types and

named them chemically induced pluripotent stem cells. The shorter time, higher efficiency, simpler operation, higher cost performance, safer method, and more precise outcome are the advantages of the reprogramming approach.

With continuous research and exploration by scientists, the technology of iPSCs has become increasingly mature and improved, and the iPSC pluripotency identification scheme is also important. Currently, it mainly includes the following six aspects: (1) pluripotent stem cell surface markers, such as SSEA-1 and OCT4, which are not expressed on the surface of differentiated cells; (2) the ability to induce differentiation in vitro; (3) teratoma experiments in nude mice; (4) embryo chimerism experiments; (5) germline mosaicism; and (6) embryonic developmental ability. The last three criteria determine whether iPSCs have the characteristics of ESCs from a scientific perspective. In addition, iPSCs and ESCs have similar DNA methylation patterns and histone modifications, which can also be identified at the epigenetic level.

Pathological features of polycystic ovary syndrome

Polycystic ovary syndrome (PCOS), also known as Stein-Leventhal syndrome, is a common reproductive dysfunction syndrome in women of childbearing age. First proposed by Stein and Leventhal in 1935 (Ozcan et al., 2012), the incidence is 5%−10% among women of childbearing age. PCOS is a reproductive disorder caused by hypothalamic-pituitary-ovarian axis dysfunction and a metabolic disorder with endocrine disorders and metabolic syndromes with complex clinical manifestations.

The pathophysiology and clinical manifestations of the disease are complex, heterogeneous, progressive, and markedly racially diverse. The main manifestations are persistent anovulation, insulin resistance (IR), hyperandrogenemia, and hyperinsulinemia. Patients are often characterized by thin menstrual discharge in small amounts and severe amenorrhea and infertility, accompanied by obesity, hairiness, bilateral polycystic enlargement, black acanthosis, and acne. It can cause serious diseases such as breast cancer, endometrial cancer, and cardiovascular and cerebrovascular diseases. Among them, menstrual disorders are the main symptoms of PCOS. The milder menstrual cycle is 35 days to six months, the menstrual flow is minimal, and severe patients may have amenorrhea. Additionally, there are also patients with irregular uterine bleeding, menstrual cycle, and menstrual volume without regularity. Among the relevant manifestations of high androgen-related clinical patients, varying degrees of hirsutism are one of the important manifestations of androgen elevation. Excessive androgen can cause excessive secretion of sebaceous glands and acne, and the patient's face, back, and shoulders can appear as acne, pimples, pustules, and nodules. It is often said that hyperandrogenemia refers to an increase in oil secretion, which causes patients to have too much oil on the head, face, chest, and back and enlarged pores. This is also the clinical manifestation of high androgen in PCOS patients. Obesity related to IR occurs in patients with

PCOS, and most of the obesity is in the abdomen. Severe IR can cause acanthosis nigricans, symmetrical brown pigmentation in the vulva, neck, and underarms, and skin thickening. The IR of patients with PCOS is related to obesity, and severe IR will cause acanthosis nigricans. Symmetrical brown pigmentation will appear in the vulva, neck, underarm, and other parts of the patient, resulting in skin thickening. Overweight patients with PCOS may develop obstructive sleep apnea, which occurs during sleep. However, patients with PCOS also have some psychological and emotional disorders, which may easily cause depression and anxiety and lack of confidence. Patients may have the above typical or partial symptoms, but infertility due to ovulation disorders is the most significant clinical manifestation of PCOS.

Strikingly, PCOS and polycystic ovary (PCO) are two different concepts. PCO is only a morphological disorder that can be caused by any type of disease with excessive androgen, showing polycystic changes in the ovary without clinical symptoms or changes in blood hormones.

Progress in polycystic ovary syndrome treatment and research

Research on polycystic ovary syndrome

PCOS is an endocrine and metabolic disorder that affects a woman's life. However, there are still many controversies and unresolved issues about the etiology, diagnostic criteria, and optimal treatment for PCOS. Therefore, PCOS has become a research hotspot, and the exploration of its pathogenic mechanism is still the top priority of scientists. At present, the exact cause of PCOS is still unclear, but according to the characteristics of family heredity and heterogeneity of patients with PCOS, research suggests that PCOS is related to heredity and environment (Jin and Xie, 2018).

Regarding the research on PCOS candidate genes, the current research results show that no obvious related genes have been found, and there are few reproducible conclusions, so a few specific genes related to PCOS have been found. Research has shown that CYP17α expression and enzyme activity are increased in patients with PCOS. CYP17α increases the phosphorylation level of serine residues after transcription, which may increase its 17,20-lyase activity, resulting in hyperandrogenemia. CYP11A encodes a cholesterol side chain lyase P450scc. Follicular membrane cells in patients with PCOS overexpress P450scc (Perez et al., 2008). CYT11A has nucleotide repeats at the translation initiation site. Gharani's team first reported that microsatellite polymorphisms are related to the pathogenesis of PCOS (Gharani et al., 1997). However, Gaasenbeek et al. (2004) verified the results of Gharani's research team with unreliable results after large-scale validation experiments. Subsequently, other research teams conducted research on a number of candidate genes, such as the aromatase gene CYP19, the sex hormone−binding globulin gene SHBG, the insulin gene, and the insulin receptor gene (Skrgatic et al., 2013), but failed to obtain a clear correlation with patients with PCOS.

However, the mechanism by which environmental factors affect the occurrence of PCOS has always been an open question. Epigenetics is the process of studying the genes' phenotype. It is a molecular phenomenon that can regulate gene expression without changing the DNA sequence.

Epigenetic modification is very important for mammalian development, and changes in environmental factors can visualize the epigenetic state, which may lead to the occurrence of various diseases. Special epigenetic mutations can be passed on to offspring. Abnormal epigenetic modifications are involved in the occurrence and inheritance of many diseases. In 2005, the study of LaVoie (2005) suggested that abnormal epigenetic modification may play a crucial role in the occurrence and inheritance of PCOS caused by high androgens. The PCOS epigenetic abnormality hypothesis provides a direction for studying the occurrence and inheritance of PCOS.

Epigenetics represents the new frontier of functional genomics for complex diseases. It is a potential molecular bridge linking the environment and genetic material. In recent years, many studies have also found that epigenetic changes are the underlying mechanism of PCOS (Xu et al., 2016). Studies on rhesus monkeys have shown that fetal exposure to high levels of androgens in utero determines the clinical manifestations of PCOS in adolescence. Studies in sheep have shown that excessive androgen exposure during the fetal period can affect early follicular activity, which may explain the typical follicular developmental changes shown by PCOS (De Leo et al., 2016). The role of epigenetics in PCOS cannot be underestimated. The current research in epigenetics is mainly reflected in the following four specific aspects:

Methylation

Through the DNA chip method, researchers found that some genes in the adipose tissue of women with PCOS showed corresponding modifications in some methylation sites (Fenichel et al., 2017). Other scientists conducted genome-wide DNA methylation analysis in PCOS ovarian tissue and found 7982 different methylated CpG sites, of which 59.8% were hypermethylated and 40.2% were hypomethylated. Another study found that the global methylation level of DNA in granulosa cells (GCs) of women with PCOS was lower than that of control women. In view of the different tissues collected in the two studies, the results are not contradictory (Pan et al., 2018). A research team reported that there were no significant differences between the DNA methyl groups of peripheral blood cells of patients with PCOS and the DNA methyl groups of control groups, but Shen and his colleagues found multiple different methylations in the peripheral blood of patients with PCOS.

Histone modification

Theoretically, most of the genetic variations found in PCOS are involved in the regulation of steroid formation, follicular maturation, or insulin signaling through the modified proteins they encode. These effects activate cells through the membrane receptors IR, epidermal growth factor receptor (EGFR), and luteinizing hormone

(LH)/human chorionic gonadotophin receptor (HCGR) and ultimately participate in the regulation of the gene expression of transcription factors. Studies have shown that the PPAR 12ala genotype increases the risk of PCOS (Seshadri Reddy et al., 2018). It was also found that changes in the expression of aquaporins may affect PCOS follicular development and follicular atresia (Rahimi et al., 2018). Through the study of the changes in FoxO3 expression and phosphorylation in human GCs, it was found that the activation and overexpression of FoxO3 in PCOS female GCs is associated with an increase in the level of apoptosis, suggesting that FoxO3 may be a candidate for PCOS female GC apoptosis (Wawrzkiewicz-Jalowiecka et al., 2017). Quantitative real-time polymerase chain reaction was used to observe the expression of BMAL1 in GCs of patients with PCOS, proving the effect of BMAL1 on the synthesis of estrogen in hGCs, and the positive feedback cycle of BMAL1-SIRT1-JNK was proposed, indicating that BMAL1 plays an important role in the development of PCOS (Mikaeili et al., 2016). The expression of APPL1 and INSR in PCOS was significantly reduced in GC. This finding indicates that APPL1 may be an important mediator of adiponectin and insulin signaling in GC and an important factor in the development of PCOS and IR (Zhang et al., 2016). It has been reported that heat shock protein 90B1 (HSP90B1) is highly expressed in ovarian cells of patients with PCOS and promotes the proliferation of GCs. Further research shows that overexpression of HSP90B1 in ovarian cells of patients with PCOS can reverse apoptosis. It can provide a new strategy for the treatment of PCOS and regulate the activity of HSP90B1 (Dehghan et al., 2016). There is evidence that during the maturation of PCOS oocytes, the mRNA expression patterns of angiopoietin-like protein 1 and angiopoietin-like protein 2 are disordered, which may be related to the developmental ability of abnormal oocytes (Li et al., 2016). Oxidative stress (OS) has been reported to be related to the pathogenesis of PCOS. In the presence of OS, normal human serum albumin is modified to form ischemia-modified albumin (IMA) with reduced metal binding capacity. Studies have found that elevated serum IMA levels reflect an increase in OS during the pathogenesis of PCOS (Liu et al., 2016b). Another analysis showed that IMA levels are related to serum-free testosterone levels in patients with PCOS. This correlation may affect the quality of oocytes and change the balance of key follicular fluid factors in the follicular microenvironment (Beyazit et al., 2016).

Genomic imprinting

The study found that 1 nM insulin significantly stimulated cell proliferation, induced apoptosis, and reduced telomerase activity in GCs of healthy women and patients with PCOS, but silencing INSR expression inhibited the role of insulin. In addition, insulin-induced GC cell apoptosis in patients with PCOS is significantly higher than that in healthy women (Song et al., 2018). By analyzing the genomes of a large number of patients with PCOS and the control group, it was found that 4 of the 10 SNP loci have significant alleles, three of which are closely related to the occurrence of PCOS (Chen et al., 2017). Studies have shown that the risk alleles carried by

THADA and DENND1A are associated with endocrine and metabolic disorders in patients with PCOS (Liu et al., 2016c). The ovaries isolated from FADS2 lack a mature corpus luteum, suggesting that FADS2 may play a role in follicular maturation and ovulation (Tian et al., 2016).

Long noncoding RNA and microRNA

The study found that the expression of long noncoding RNA (lncRNA) SRA in patients with PCOS was significantly higher than that in the control group, which was positively correlated with obesity. Therefore, the increase in lncRNA SRA may be an important mediator in the fat-related process of PCOS (Fu et al., 2018). It was also found that lncRNA HCG26 was upregulated in PCOS. HCG26 inhibits cell proliferation and cell cycle progression, and knocking out HCG26 can promote aromatase gene expression and estradiol (E2) production without affecting FSH receptor mRNA levels. This indicates that differentially expressed lncRNAs are involved in GC proliferation and steroid production, leading to the pathogenesis of PCOS (Liu et al., 2017). TERRAs are noncoding RNAs that are involved in the regulation of telomere length, telomerase activity, and heterochromatinization. In PCOS, the correlation between TERRA and TTE indicates that TERRA and TTE play an important role in the regulation of LTL in patients with PCOS (Wang et al., 2017a). Studies have shown that in cumulus cells of patients with PCOS, the expression level of lncRNAs is abnormal compared with that in normal women. This differential expression may lead to the occurrence of PCOS and affect the development of oocytes (Huang et al., 2016a).

All stages of follicular development can be regulated by miRNA activity (Naji et al., 2018). Previous studies have shown that important regulatory factors, such as microRNAs, play an increasingly important role in the development of PCOS (Liu et al., 2016a). Studies have shown that abnormally expressed mRNA is related to several specific signaling pathways, including IR, steroid hormone biosynthesis, the PPAR signaling pathway, cell adhesion molecules, and the AMPK signaling pathway (Fu et al., 2018). miR-33b-5p is overexpressed in the ovarian tissue of PCOS/IR rats and can target HMGA2 to inhibit glutamate during the development of PCOS. This finding suggests that increasing the expression of GLUT4 by inhibiting miR-33b-5p or increasing the levels of HMGA2 and SREBF1 may be a promising strategy for the treatment of PCOS (Yang et al., 2018). The study found that for every doubling of the likelihood of women suffering from PCOS, the expression of miR-23a will decrease by 0.01 times (Xiong et al., 2017). There is evidence that miRNA-509-3p can improve the secretion of E2 and inhibit the expression of MAP3K8, which helps to describe the pathogenesis of ovulation in PCOS, especially the regulation of E2 production (Huang et al., 2016b). It has also been shown that the increased expression levels of miR-21 and miR-146a suggest that low-level chronic inflammation and OS can be named as the main cause of PCOS (Salimi-Asl et al., 2016).

At present, epigenetic research on PCOS provides more convincing evidence to link epigenetic regulation with the pathogenesis of disease, but similar studies have obtained different results with different clinical research materials. The results obtained from the model require further verification, so practical research materials are more urgent for the study of PCOS. Overall, these new findings highlight the important role of epigenetic regulation in the pathogenesis of PCOS.

Treatments for polycystic ovary syndrome

Since the pathogenesis of PCOS has not been clarified, the current treatment is mainly aimed at clinical symptoms. The treatment objectives include correcting ovulation, inhibiting the effect of androgens on target tissues, and reducing IR. The main clinical treatment methods are as follows: (1) The caloric intake of patients is limited, which can reduce the storage of fat in the body, lower cholesterol, improve blood lipids, and improve the insulin sensitivity of patients to promote ovulation. Weight loss of 5%−10% in patients with PCOS can help reduce androgen, LH, and insulin levels and help regulate ovulation, thus increasing the likelihood of pregnancy (Zhang et al., 2018). Studies have shown that increased subcutaneous and visceral adipose tissue in the abdomen can lead to IR, inflammation, blood lipids, and cerebrovascular disease (Hernandez-Mijares et al., 2013). (2) Several different drugs directly or indirectly promote ovulation. For example, clomiphene citrate, a sterol, competitively combines with the hypothalamus to release the negative feedback of estrogen to the hypothalamus and indirectly promote ovulation. The aromatase inhibitor letrozole (Femara, Novartis) is approved for patients with hormone-responsive breast cancer, but it has also been studied to induce ovulation in PCOS. Treatment of patients with PCOS with letrozole can effectively increase the ovulation rate and promote endometrial development. The currently widely used drug for IR in patients with PCOS is the biguanide insulin sensitizer metformin, which can improve IR, regulate hormone levels, and promote ovulation. More specifically, for patients with PCOS infertility who have failed aromatase inhibitor ovulation promotion therapy or show resistance to the selective androgen receptor modulator clomiphene, gonadotropins are injected into the patients to directly promote ovulation. It is important to use low-dose propulsion drugs as much as possible; otherwise, it will cause excessive stimulation of the ovarian bursa, increase the rate of multiple birth, and decrease the pregnancy rate. (3) Surgical treatment refers to punching the ovarian surface and stroma through laparoscopic drilling and then destroying the ovarian stroma that produces androgens, aiming to regulate the expression of hormones to block the vicious cycle of hyperandrogenemia and increase the chance of pregnancy. Unfortunately, this surgical treatment may not be effective, and it can also lead to

complications such as pelvic adhesions and reduced ovarian function. The in vitro fertilization-embryo transfer technology is generally the last choice for patients with PCOS with failure to promote ovulation or other causes of infertility, but there may be too much follicle recruitment but too little maturation. Moreover, although surgical weight loss can achieve results in a short period, the risk is very high, and there are many surgical complications that are generally not recommended. (4) The combination of traditional Chinese medicine, Western medicine, acupuncture, surgery, and other different treatment methods can improve the symptoms of patients with PCOS with liver depression, kidney deficiency, spleen deficiency, blood stasis, phlegm, and dampness and help to improve the ovulation rate and pregnancy rate. Although the current treatment methods have certain effects, it is mainly through the control of ovarian secretion through drugs to adjust the synthesis and release of ovarian hormones. In fact, PCOS drugs need to be taken for a long time, there are certain side effects, and the symptoms of PCOS will stop after drug withdrawal occurs.

The clinical treatment of PCOS is mainly conservative treatment, and almost no surgical treatment is adopted. Therefore, it is difficult for scientific research to obtain clinical materials for PCOS. It is thus particularly important to find the ideal PCOS animal model for basic research of its etiology. Scientists have established various PCOS animal models using different methods to mimic the clinical characteristics of human PCOS, including nonhuman primate macaques, sheep, and rodent mice (Abbott et al., 1998; Eisner et al., 2002; Recabarren et al., 2005). There are two rat models that are more widely used at present. One modeling principle is to use letrozole as an aromatase inhibitor to block the conversion of androgens to estrogen, leading to a high androgen environment in the body and progressing to PCO (Maliqueo et al., 2013). Another modeling principle is that the levels of androstenedione and testosterone are increased through the increase in exogenous dehydroepiandrosterone (DHEA) levels, which shows the pathological characteristics of hyperandrogenemia in patients with PCOS. DHEA is an intermediate product of pregnenolone conversion to androstenedione via the A5 pathway (Rudnicka et al., 2010). Most of these animal models have some of the characteristics of PCOS, and some of them even have certain hormones and metabolic changes similar to those of patients with PCOS. However, for the time being, there is still no animal model that can fully simulate all the characteristics of clinical patients with PCOS. Moreover, the construction of animal models is not only time-consuming and laborious but also cannot truly simulate the true pathological conditions of patients with PCOS. Therefore, to study the pathogenesis of PCOS accurately, we need other experimental carriers that can support research in vitro. With the increasing application of iPSCs in disease research and treatment, there will be a new research platform opened by iPSCs for research on PCOS.

Application of induced pluripotent stem cells in polycystic ovary syndrome disease research

With the deepening of research on iPSCs of many species, including humans, rats, and rabbits, have been successfully generated. Induced pluripotent stem cells with the ability of continuous self-renewal and the potential of multidirectional differentiation have become a powerful tool for the research and development of the reprogramming mechanism and the pathogenesis of human diseases. The disease model established by iPSCs is conducive to further exploration of related diseases and provides a new research platform for the pathogenesis, pathophysiology research, and drug development of these diseases.

The application of induced pluripotent stem cells

Prior to the advent of iPSC technology, disease research and drug trials were mostly performed on animal models. Due to species differences, the experimental results obtained in animal models are less applicable to humans. The emergence of iPSCs has had a great influence on stem cell biology and regenerative medicine. With the pressing desire to treat diseases, research on the clinical application of iPSCs has been carried out rapidly.

Disease-specific iPSCs can differentiate into specific cell types, and these cells can reproduce the pathological phenotype of the disease, which is an ideal method for establishing a disease model. In 2008, Dimos et al. (2008) performed reprogramming experiments on epithelial cells in patients with familial amyotrophic lateral sclerosis (ALS), and the iPSCs obtained with the disease-specific characteristics could be induced to differentiate into donor cells of patient motor nerves in vitro, which also provide disease-specific cell sources for drug targets. This is the first time that the patient's somatic cells have been reprogrammed into disease-specific iPSCs. Then, in 2009, the Soldner research team (Soldner et al., 2009) induced iPSCs to differentiate into dopamine neurons in vitro to explore new and effective treatments for Parkinson's patients. Yagi and his colleagues (Yagi et al., 2012) established a good Alzheimer's disease (AD) model in 2012 by inducing iPSCs derived from patients with familial AD into neurons. At present, various iPSC disease models, such as neurological diseases, cardiovascular diseases, metabolic diseases, blood diseases, and immune system diseases, have been successfully established, including Huntington disease (Zhang et al., 2010; Niclis et al., 2013; Camnasio et al., 2012), Parkinson's disease (PD) (Hartfield et al., 2012; Byers et al., 2012; Roessler et al., 2013; Hargus et al., 2010), spinal muscular atrophy (Corti et al., 2012; Ebert et al., 2009), ALS (Egawa et al., 2012), AD (Young and Goldstein, 2012; Shi et al., 2012; Israel et al., 2012; Yagi et al., 2011), β-thalassemia (Raja et al., 2012; Fan et al., 2012; Papapetrou et al., 2011; Wang et al., 2009), and diabetes mellitus (Soejitno and Prayudi, 2011; Godfrey et al., 2012). The establishment of an

increasing number of disease models of iPSCs provides us with a true and reliable cellular carrier for better revealing the disease mechanism of diseases.

Disease-specific iPSCs can be used as a tool for screening drugs because they reproduce the development and pathophysiological characteristics of certain diseases in vitro and provide a new resource for drug research. Matsa et al. (2011) found that catecholamines can cause arrhythmias in cardiac muscle cells derived from type II long QT syndrome-specific iPSCs, and beta-blockers can reverse this effect. From the toxicological perspective, the toxic effects of certain drugs may not be consistent for humans and animals. Moreover, the application of different types of somatic cells differentiated from iPSCs for drug screening will result in more reliable evaluations of drug safety and effectiveness. In addition, drug evaluation using patient-specific iPSCs can provide individualized treatment.

There is an attractive application prospect in regenerative medicine for cell replacement therapy using differentiated cells from patient-specific iPSCs. In 2008, iPSCs were induced to differentiate into neural precursor cells and transplanted into the fetal rat brain, where these cells were able to differentiate into functional and active neurons and glial cells (Wernig et al., 2008a). The rat model of PD can improve its symptoms by transplanting dopaminergic neurons differentiated from iPSCs into the brain. In addition, type I diabetes-specific iPSCs can be induced to differentiate into pancreatic β cells (Maehr et al., 2009). American scientists have also successfully cultivated three-dimensional bone structures from human somatic cells through iPSC technology (de Peppo et al., 2013). Although it is still far from clinical application, iPSC technology provides a new and relatively easy-to-operate method for obtaining pluripotent stem cells for regenerative medicine, which will greatly promote the development of regenerative medicine.

The establishment of patient-specific disease-specific iPSCs overcomes the two major problems of ethics and immune rejection that cell therapy has been facing and greatly advances the process of patient-specific cell therapy. Moreover, the source of somatic cells for iPSCs is convenient. Many different types of somatic cells have been successfully induced as pluripotent cells. Among them, somatic cells that are commonly used and conveniently obtained include skin fibroblasts, peripheral blood mononuclear cells, and urine epithelial cells. The construction of a disease-specific model of iPSCs has opened up a new way to use iPSCs to study the pathogenesis of disease, drug screening, and cell therapy, showing its greater application value.

The induced pluripotent stem cell disease model of polycystic ovary syndrome

To more comprehensively understand the pathogenesis of polycystic ovaries and establish a targeted treatment plan, it is increasingly important to study the pathological mechanism of PCOS. At present, some pathological characteristics of patients

with PCOS are mainly simulated by constructing animal models for research, but animal disease models are different from humans. An increasing number of researchers believe that the materials of patients with PCOS are more realistic than the PCOS animal model simulated, and the obtained experimental data are more accurate.

To date, little is known about the pathological mechanism of PCOS. Some scholars believe that the pathological changes of PCOS begin at the embryonic stage. During the developmental stage of PCOS, genes have different spatiotemporal regulatory sites (Goodarzi, 2008; Wang et al., 2014a). There are ethical and legal limitations to the study of human embryos that have developed to different stages, which makes it impossible to study the pathology of PCOS from the perspective of dynamics and continuous cell and embryonic development. It is helpful to provide practical cell research vectors by constructing effective PCOS cell models because it can further advance the exploration of the pathogenesis, pathogenic genes, and drug screening of PCOS.

At present, scientists have mainly established two types of PCOS cell lines derived from patients with PCOS, one of which is the hESC line, which comes from the ICM isolated from the blastocyst stage embryos of patients with PCOS. This study was originally published by PengFen Li et al. (2012) in 2012 and was the first to establish the human ESC model of PCOS. Abnormal lipid metabolism is one of the important characteristics of PCOS. Subsequently, studies have shown that there are a large number of differentially expressed genes in adipocytes differentiated from PCOS-hESCs and non-PCOS-hESCs (Wang et al., 2014b). Although it is an hESC line from clinically discarded embryos of patients with PCOS, human embryo resources are still scarce. However, it is impossible to establish ES cell lines using assisted reproductive technology for every patient. Traditional treatment methods are mostly adopted by patients with PCOS, and surgical treatment is rarely used, so it is not easy to obtain embryo research resources for patients with PCOS. The incidence of patients with PCOS is higher among patients with anovulatory infertility, which means that these patients cannot establish their own ESClines, so the universality is poor for studying the heterogeneity of patient etiology with ESCs.

Another method to establish a PCOS disease model cell line is to use iPSC technology to obtain patient-specific iPSCs from patient-derived somatic cells in vitro. There is no need to obtain a patient's embryo, and it is only necessary to obtain a patient's somatic cells, which is convenient, inexpensive, and causes minimal injuries to the patient. Moreover, it is possible to build an iPSC line for each patient with PCOS, which is more targeted for research on the pathological mechanism and treatment. Some disease models of iPSC lines have been established (Peitz et al., 2013; Folmes et al., 2013; Hoekstra et al., 2012; Raya et al., 2009).

In 2016, Sheng Yang's research team (Yang et al., 2016) reprogrammed epithelial cells isolated from the urine of patients with PCOS, obtained iPSCs derived from

PCOS for the first time, and successfully constructed a PCOS-iPSC model of abnormal lipid metabolism. The obtained PCOS-iPSCs can differentiate into normal karyotype adipocytes. PCOS-iPSCs have a stronger ability to consume glucose during the differentiation and development of adipocytes in vitro compared with non-PCOS-iPSCs. It is known that the induced differentiation of adipocytes is of great significance for the study of lipid metabolism and obesity (Cuaranta-Monroy et al., 2014; Clynes, 2014).

Recently, the research team of Yang Yu (Min et al., 2018, 2019) performed reprogramming experiments on skin fibroblasts by lentiviral transfection to establish iPSC lines derived from patients with PCOS. Through the comprehensive transcription profile analysis of the RNA chip, some genes of PCOS-iPSCs were upregulated, while those related to cell communication, glucose transport and intake were downregulated compared with the control non-PCOS-iPSCs. These upregulated genes are mainly related to the tricarboxylic acid cycle, the metabolic process of the respiratory electron transfer chain, glycogen breakdown, and mitochondrial activity. Interestingly, the mitochondrial respiratory capacity and glycolytic function of PCOS-iPSCs decreased, but mitochondrial copy number and biogenesis increased. The research team also found that PCOS-iPSCs treated with metformin downregulated and restored some genes related to glucose metabolism. In addition, NSCs differentiated from PCOS-iPSCs showed decreased mitochondrial respiratory capacity. Overall, the research team used the PCOS-iPSC disease model to reveal the pathological characteristics of neuroendocrine abnormalities and mitochondrial respiratory dysfunction in PCOS.

In addition, the establishment of the PCOS-iPSC model can provide a practical cell research platform for the previously reported epigenetic modifications related to PCOS for verifying and discovering more PCOS-related epigenetic modifications. Since PCOS-iPSCs can be differentiated in vitro, this research material can also explore the changes in certain genes with genetic modification during differentiation into specific cell types. Therefore, it can better explore the pathogenic mechanism of PCOS in the field of epigenetic modification and provide a stronger scientific basis for the development of PCOS, making early diagnosis of PCOS and personalized treatment possible. Eventually, the environment and the controllable factors in lifestyle can be blocked in a targeted manner to effectively reduce the occurrence of PCOS.

The establishment of PCOS-iPSCs means that the disease gene phenotype of patients with PCOS can be maintained in vitro, making research more convenient, and increasingly more pathogenic mechanisms of PCOS will be gradually revealed. The PCOS-iPSC model is helpful in the screening and development of more targeted therapeutic drugs that can then better serve the clinical treatment of PCOS. Taking advantage of the characteristics of iPSCs for VIP establishment in patients, we can try to introduce cell therapy into the treatment of PCOS (Fig. 6.2). It is believed that the widespread application of the PCOS-iPSC disease model will bring new insights into the clinical treatment of PCOS.

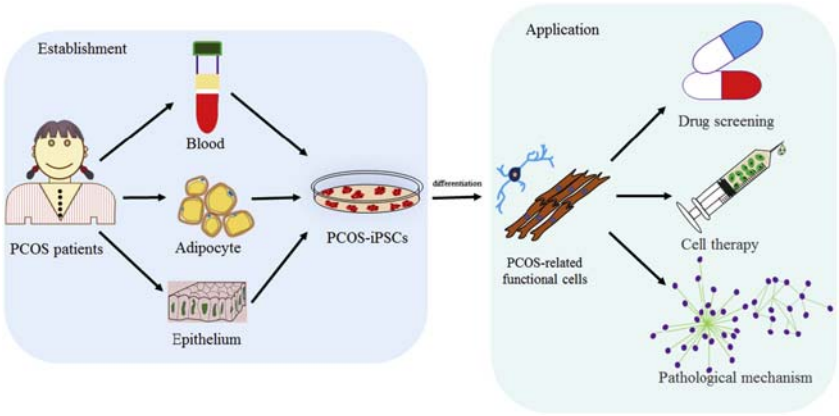


FIGURE 6.2

Establishment and application of the induced pluripotent stem cell (iPSC) line for the polycystic ovary syndrome (PCOS) disease model. Different types of somatic cells, such as blood cells, adipocytes, and epithelial cells, from patients with PCOS are used to establish PCOS-iPSC disease models. In vitro, the targeted differentiation of PCOS-iPSCs can be used in drug screening, cell therapy, and pathological mechanism research.

References

Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilic, J., Pekarik, V., Tiscornia, G., Edel, M., Boue, S., Izpisua Belmonte, J.C., 2008. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat. Biotechnol. 26 (11), 1276−1284.

Abbott, D.H., Dumesic, D.A., Eisner, J.R., Colman, R.J., Kemnitz, J.W., 1998. Insights into the development of polycystic ovary syndrome (PCOS) from studies of prenatally androgenized female rhesus monkeys. Trends Endocrinol. Metabol. 9 (2), 62−67.

Axelrod, H.R., 1984. Embryonic stem cell lines derived from blastocysts by a simplified technique. Dev. Biol. 101 (1), 225−228.

Beyazit, F., Yilmaz, N., Balci, O., Adam, M., Yaman, S.T., 2016. Evaluation of oxidative stress in women with polycystic ovarian syndrome as represented by serum ischemia modified albumin and its correlation with testosterone and insulin resistance. Intern. Med. 55 (17), 2359−2364.

Bongso, A., Fong, C.Y., Ng, S.C., Ratnam, S., 1994. Isolation and culture of inner cell mass cells from human blastocysts. Hum. Reprod. 9 (11), 2110−2117.

Briggs, R., King, T.J., 1952. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc. Natl. Acad. Sci. U. S. A. 38 (5), 455−463.

Bueno, C., Sardina, J.L., Di Stefano, B., Romero-Moya, D., Munoz-Lopez, A., Ariza, L., Chillon, M.C., Balanzategui, A., Castano, J., Herreros, A., Fraga, M.F., Fernandez, A., Granada, I., Quintana-Bustamante, O., Segovia, J.C., Nishimura, K., Ohtaka, M., Nakanishi, M., Graf, T., Menendez, P., 2016. Reprogramming human B cells into induced pluripotent stem cells and its enhancement by C/EBPalpha. Leukemia 30 (3), 674−682.

Byers, B., Lee, H.L., Reijo Pera, R., 2012. Modeling Parkinson's disease using induced pluripotent stem cells. Curr. Neurol. Neurosci. Rep. 12 (3), 237−242.

Camnasio, S., Delli Carri, A., Lombardo, A., Grad, I., Mariotti, C., Castucci, A., Rozell, B., Lo Riso, P., Castiglioni, V., Zuccato, C., Rochon, C., Takashima, Y., Diaferia, G., Biunno, I., Gellera, C., Jaconi, M., Smith, A., Hovatta, O., Naldini, L., Di Donato, S., Feki, A., Cattaneo, E., 2012. The first reported generation of several induced pluripotent stem cell lines from homozygous and heterozygous Huntington's disease patients demonstrates mutation related enhanced lysosomal activity. Neurobiol. Dis. 46 (1), 41−51.

Carey, B.W., Markoulaki, S., Hanna, J., Saha, K., Gao, Q., Mitalipova, M., Jaenisch, R., 2009. Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc. Natl. Acad. Sci. U. S. A. 106 (1), 157−162.

Chen, L., Hu, L.M., Wang, Y.F., Yang, H.Y., Huang, X.Y., Zhou, W., Sun, H.X., 2017. Genome-wide association study for SNPs associated with PCOS in human patients. Exp. Ther. Med. 14 (5), 4896−4900.

Clynes, M., 2014. Cell culture models for study of differentiated adipose cells. Stem Cell Res. Ther. 5 (6), 137.

Corti, S., Nizzardo, M., Simone, C., Falcone, M., Nardini, M., Ronchi, D., Donadoni, C., Salani, S., Riboldi, G., Magri, F., Menozzi, G., Bonaglia, C., Rizzo, F., Bresolin, N., Comi, G.P., 2012. Genetic correction of human induced pluripotent stem cells from patients with spinal muscular atrophy. Sci. Transl. Med. 4 (165), 165ra162.

Cowan, C.A., Atienza, J., Melton, D.A., Eggan, K., 2005. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309 (5739), 1369−1373.

Cuaranta-Monroy, I., Simandi, Z., Kolostyak, Z., Doan-Xuan, Q.M., Poliska, S., Horvath, A., Nagy, G., Bacso, Z., Nagy, L., 2014. Highly efficient differentiation of embryonic stem cells into adipocytes by ascorbic acid. Stem Cell Res. 13 (1), 88−97.

De Leo, V., Musacchio, M.C., Cappelli, V., Massaro, M.G., Morgante, G., Petraglia, F., 2016. Genetic, hormonal and metabolic aspects of PCOS: an update. Reprod. Biol. Endocrinol. 14 (1), 38.

de Peppo, G.M., Marcos-Campos, I., Kahler, D.J., Alsalman, D., Shang, L., Vunjak-Novakovic, G., Marolt, D., 2013. Engineering bone tissue substitutes from human induced pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A. 110 (21), 8680−8685.

Dehghan, R., Saidijam, M., Mehdizadeh, M., Shabab, N., Yavangi, M., Artimani, T., 2016. Evidence for decreased expression of APPL1 associated with reduced insulin and adiponectin receptors expression in PCOS patients. J. Endocrinol. Invest. 39 (9), 1075−1082.

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., Wichterle, H., Henderson, C.E., Eggan, K., 2008. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321 (5893), 1218−1221.

Ebert, A.D., Yu, J., Rose Jr., F.F., Mattis, V.B., Lorson, C.L., Thomson, J.A., Svendsen, C.N., 2009. Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 457 (7227), 277−280.

Egawa, N., Kitaoka, S., Tsukita, K., Naitoh, M., Takahashi, K., Yamamoto, T., Adachi, F., Kondo, T., Okita, K., Asaka, I., Aoi, T., Watanabe, A., Yamada, Y., Morizane, A., Takahashi, J., Ayaki, T., Ito, H., Yoshikawa, K., Yamawaki, S., Suzuki, S., Watanabe, D., Hioki, H., Kaneko, T., Makioka, K., Okamoto, K., Takuma, H., Tamaoka, A., Hasegawa, K., Nonaka, T., Hasegawa, M., Kawata, A., Yoshida, M.,

Nakahata, T., Takahashi, R., Marchetto, M.C., Gage, F.H., Yamanaka, S., Inoue, H., 2012. Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci. Transl. Med. 4 (145), 145ra104.

Eisner, J.R., Barnett, M.A., Dumesic, D.A., Abbott, D.H., 2002. Ovarian hyperandrogenism in adult female rhesus monkeys exposed to prenatal androgen excess. Fertil. Steril. 77 (1), 167−172.

Esteban, M.A., Wang, T., Qin, B., Yang, J., Qin, D., Cai, J., Li, W., Weng, Z., Chen, J., Ni, S., Chen, K., Li, Y., Liu, X., Xu, J., Zhang, S., Li, F., He, W., Labuda, K., Song, Y., Peterbauer, A., Wolbank, S., Redl, H., Zhong, M., Cai, D., Zeng, L., Pei, D., 2010. Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell Stem Cell 6 (1), 71−79.

Fan, Y., Luo, Y., Chen, X., Li, Q., Sun, X., 2012. Generation of human beta-thalassemia induced pluripotent stem cells from amniotic fluid cells using a single excisable lentiviral stem cell cassette. J. Reprod. Dev. 58 (4), 404−409.

Fenichel, P., Rougier, C., Hieronimus, S., Chevalier, N., 2017. Which origin for polycystic ovaries syndrome: genetic, environmental or both? Ann. Endocrinol. 78 (3), 176−185.

Folmes, C.D., Martinez-Fernandez, A., Perales-Clemente, E., Li, X., McDonald, A., Oglesbee, D., Hrstka, S.C., Perez-Terzic, C., Terzic, A., Nelson, T.J., 2013. Disease-causing mitochondrial heteroplasmy segregated within induced pluripotent stem cell clones derived from a patient with MELAS. Stem Cells 31 (7), 1298−1308.

Fu, L.L., Xu, Y., Li, D.D., Dai, X.W., Xu, X., Zhang, J.S., Ming, H., Zhang, X.Y., Zhang, G.Q., Ma, Y.L., Zheng, L.W., 2018. Expression profiles of mRNA and long noncoding RNA in the ovaries of letrozole-induced polycystic ovary syndrome rat model through deep sequencing. Gene 657, 19−29.

Gaasenbeek, M., Powell, B.L., Sovio, U., Haddad, L., Gharani, N., Bennett, A., Groves, C.J., Rush, K., Goh, M.J., Conway, G.S., Ruokonen, A., Martikainen, H., Pouta, A., Taponen, S., Hartikainen, A.L., Halford, S., Jarvelin, M.R., Franks, S., McCarthy, M.I., 2004. Large-scale analysis of the relationship between CYP11A promoter variation, polycystic ovarian syndrome, and serum testosterone. J. Clin. Endocrinol. Metab. 89 (5), 2408−2413.

Galende, E., Karakikes, I., Edelmann, L., Desnick, R.J., Kerenyi, T., Khoueiry, G., Lafferty, J., McGinn, J.T., Brodman, M., Fuster, V., Hajjar, R.J., Polgar, K., 2010. Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. Cell. Reprogr. 12 (2), 117−125.

Gharani, N., Waterworth, D.M., Batty, S., White, D., Gilling-Smith, C., Conway, G.S., McCarthy, M., Franks, S., Williamson, R., 1997. Association of the steroid synthesis gene CYP11a with polycystic ovary syndrome and hyperandrogenism. Hum. Mol. Genet. 6 (3), 397−402.

Giorgetti, A., Montserrat, N., Rodriguez-Piza, I., Azqueta, C., Veiga, A., Izpisua Belmonte, J.C., 2010. Generation of induced pluripotent stem cells from human cord blood cells with only two factors: Oct4 and Sox2. Nat. Protoc. 5 (4), 811−820.

Godfrey, K.J., Mathew, B., Bulman, J.C., Shah, O., Clement, S., Gallicano, G.I., 2012. Stem cell-based treatments for Type 1 diabetes mellitus: bone marrow, embryonic, hepatic, pancreatic and induced pluripotent stem cells. Diabet. Med. 29 (1), 14−23.

Goodarzi, M.O., 2008. Looking for polycystic ovary syndrome genes: rational and best strategy. Semin. Reprod. Med. 26 (1), 5−13.

Handyside, A., Hooper, M.L., Kaufman, M.H., Wilmut, I., 1987. Towards the isolation of embryonal stem cell lines from the sheep. Roux Arch. Dev. Biol. 196 (3), 185−190.

Hargus, G., Cooper, O., Deleidi, M., Levy, A., Lee, K., Marlow, E., Yow, A., Soldner, F., Hockemeyer, D., Hallett, P.J., Osborn, T., Jaenisch, R., Isacson, O., 2010. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proc. Natl. Acad. Sci. U. S. A. 107 (36), 15921−15926.

Harris, H., 1967. The reactivation of the red cell nucleus. J. Cell Sci. 2 (1), 23−32.

Hartfield, E.M., Fernandes, H.J., Vowles, J., Cowley, S.A., Wade-Martins, R., 2012. Cellular reprogramming: a new approach to modelling Parkinson's disease. Biochem. Soc. Trans. 40 (5), 1152−1157.

Hernandez-Mijares, A., Banuls, C., Gomez-Balaguer, M., Bergoglio, M., Victor, V.M., Rocha, M., 2013. Influence of obesity on atherogenic dyslipidemia in women with polycystic ovary syndrome. Eur. J. Clin. Invest. 43 (6), 549−556.

Higgins, C.A., Itoh, M., Inoue, K., Richardson, G.D., Jahoda, C.A., Christiano, A.M., 2012. Reprogramming of human hair follicle dermal papilla cells into induced pluripotent stem cells. J. Invest. Dermatol. 132 (6), 1725−1727.

Hoekstra, M., Mummery, C.L., Wilde, A.A., Bezzina, C.R., Verkerk, A.O., 2012. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. Front. Physiol. 3, 346.

Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., Liu, K., Ge, J., Xu, J., Zhang, Q., Zhao, Y., Deng, H., 2013. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341 (6146), 651−654.

Huang, X., Hao, C., Bao, H., Wang, M., Dai, H., 2016a. Aberrant expression of long noncoding RNAs in cumulus cells isolated from PCOS patients. J. Assist. Reprod. Genet. 33 (1), 111−121.

Huang, X., Liu, C., Hao, C., Tang, Q., Liu, R., Lin, S., Zhang, L., Yan, W., 2016b. Identification of altered microRNAs and mRNAs in the cumulus cells of PCOS patients: miRNA-509-3p promotes oestradiol secretion by targeting MAP3K8. Reproduction 151 (6), 643−655.

Huber, I., Itzhaki, I., Caspi, O., Arbel, G., Tzukerman, M., Gepstein, A., Habib, M., Yankelson, L., Kehat, I., Gepstein, L., 2007. Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. FASEB J. 21 (10), 2551−2563.

Iannaccone, P.M., Taborn, G.U., Garton, R.L., Caplice, M.D., Brenin, D.R., 1994. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. Dev. Biol. 163 (1), 288−292.

Israel, M.A., Yuan, S.H., Bardy, C., Reyna, S.M., Mu, Y., Herrera, C., Hefferan, M.P., Van Gorp, S., Nazor, K.L., Boscolo, F.S., Carson, C.T., Laurent, L.C., Marsala, M., Gage, F.H., Remes, A.M., Koo, E.H., Goldstein, L.S., 2012. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature 482 (7384), 216−220.

Jin, P., Xie, Y., 2018. Treatment strategies for women with polycystic ovary syndrome. Gynecol. Endocrinol. 34 (4), 272−277.

Keller, G., Kennedy, M., Papayannopoulou, T., Wiles, M.V., 1993. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol. Cell Biol. 13 (1), 473−486.

Kim, J.B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M., Scholer, H.R., 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454 (7204), 646−650.

King, W.A., Shepherd, D.L., Plante, L., Lavoir, M.C., Looney, C.R., Barnes, F.L., 1996. Nucleolar and mitochondrial morphology in bovine embryos reconstructed by nuclear transfer. Mol. Reprod. Dev. 44 (4), 499−506.

LaVoie, H.A., 2005. Epigenetic control of ovarian function: the emerging role of histone modifications. Mol. Cell. Endocrinol. 243 (1−2), 12−18.

Li, X.J., Hu, B.Y., Jones, S.A., Zhang, Y.S., Lavaute, T., Du, Z.W., Zhang, S.C., 2008. Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. Stem Cells 26 (4), 886−893.

Li, C., Zhou, J., Shi, G., Ma, Y., Yang, Y., Gu, J., Yu, H., Jin, S., Wei, Z., Chen, F., Jin, Y., 2009. Pluripotency can be rapidly and efficiently induced in human amniotic fluid-derived cells. Hum. Mol. Genet. 18 (22), 4340−4349.

Li, P.F., Wang, F., Kong, H.J., Zhao, F., Bai, A.H., Chen, X.M., Sun, Y.P., 2012. Establishment of polycystic ovary syndrome-derived human embryonic stem cell lines. Gynecol. Endocrinol. 28 (1), 25−28.

Li, L., Mo, H., Zhang, J., Zhou, Y., Peng, X., Luo, X., 2016. The role of heat shock protein 90B1 in patients with polycystic ovary syndrome. PLoS One 11 (4), e0152837.

Lin, S.L., Chang, D.C., Chang-Lin, S., Lin, C.H., Wu, D.T., Chen, D.T., Ying, S.Y., 2008. Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. RNA 14 (10), 2115−2124.

Liu, H.Y., Huang, Y.L., Liu, J.Q., Huang, Q., 2016a. Transcription factormicroRNA synergistic regulatory network revealing the mechanism of polycystic ovary syndrome. Mol. Med. Rep. 13 (5), 3920−3928.

Liu, Z., Liu, C., Hao, C., Xue, Q., Huang, X., Zhang, N., Bao, H., Qu, Q., 2016b. Aberrant expression of angiopoietin-like proteins 1 and 2 in cumulus cells is potentially associated with impaired oocyte developmental competence in polycystic ovary syndrome. Gynecol. Endocrinol. 32 (7), 557−561.

Liu, H., Zhao, H., Chen, Z.J., 2016c. Genome-wide association studies for polycystic ovary syndrome. Semin. Reprod. Med. 34 (4), 224−229.

Liu, Y.D., Li, Y., Feng, S.X., Ye, D.S., Chen, X., Zhou, X.Y., Chen, S.L., 2017. Long noncoding RNAs: potential regulators involved in the pathogenesis of polycystic ovary syndrome. Endocrinology 158 (11), 3890−3899.

Maehr, R., Chen, S., Snitow, M., Ludwig, T., Yagasaki, L., Goland, R., Leibel, R.L., Melton, D.A., 2009. Generation of pluripotent stem cells from patients with type 1 diabetes. Proc. Natl. Acad. Sci. U. S. A. 106 (37), 15768−15773.

Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., Hochedlinger, K., 2008. A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell 3 (3), 340−345.

Mali, P., Ye, Z., Hommond, H.H., Yu, X., Lin, J., Chen, G., Zou, J., Cheng, L., 2008. Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. Stem Cells 26 (8), 1998−2005.

Maliqueo, M., Sun, M., Johansson, J., Benrick, A., Labrie, F., Svensson, H., Lonn, M., Duleba, A.J., Stener-Victorin, E., 2013. Continuous administration of a P450 aromatase inhibitor induces polycystic ovary syndrome with a metabolic and endocrine phenotype in female rats at adult age. Endocrinology 154 (1), 434−445.

Martin, G.R., 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U. S. A. 78 (12), 7634−7638.

Marx, J.L., 1981. Three mice “cloned” in Switzerland. Science 211 (4480), 375−376.

Matsa, E., Rajamohan, D., Dick, E., Young, L., Mellor, I., Staniforth, A., Denning, C., 2011. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. Eur. Heart J. 32 (8), 952−962.

Matsumura, H., Tada, T., 2008. Cell fusion-mediated nuclear reprogramming of somatic cells. Reprod. Biomed. Online 16 (1), 51−56.

Meissner, A., Wernig, M., Jaenisch, R., 2007. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat. Biotechnol. 25 (10), 1177−1181.

Mikaeili, S., Rashidi, B.H., Safa, M., Najafi, A., Sobhani, A., Asadi, E., Abbasi, M., 2016. Altered FoxO3 expression and apoptosis in granulosa cells of women with polycystic ovary syndrome. Arch. Gynecol. Obstet. 294 (1), 185−192.

Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., Meissner, A., 2008. Dissecting direct reprogramming through integrative genomic analysis. Nature 454 (7200), 49−55.

Min, Z., Gao, Q., Zhen, X., Fan, Y., Tan, T., Li, R., Zhao, Y., Yu, Y., 2018. New insights into the genic and metabolic characteristics of induced pluripotent stem cells from polycystic ovary syndrome women. Stem Cell Res. Ther. 9 (1), 210.

Min, Z., Zhao, Y., Hang, J., Ren, Y., Tan, T., Fan, Y., Yu, Y., 2019. Neuroendocrine characteristics of induced pluripotent stem cells from polycystic ovary syndrome women. Protein Cell 10 (7), 526−532.

Mitalipova, M., Beyhan, Z., First, N.L., 2001. Pluripotency of bovine embryonic cell line derived from precompacting embryos. Cloning 3 (2), 59−67.

Moreadith, R.W., Graves, K.H., 1992. Derivation of pluripotential embryonic stem cells from the rabbit. Trans. Assoc. Am. Phys. 105, 197−203.

Muchkaeva, I.A., Dashinimaev, E.B., Artyuhov, A.S., Myagkova, E.P., Vorotelyak, E.A., Yegorov, Y.Y., Vishnyakova, K.S., Kravchenko, I.E., Chumakov, P.M., Terskikh, V.V., Vasiliev, A.V., 2014. Generation of iPS cells from human hair follice dermal papilla cells. Acta Naturae 6 (1), 45−53.

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A.B., Passier, R., Tertoolen, L., 2003. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107 (21), 2733−2740.

Naji, M., Nekoonam, S., Aleyasin, A., Arefian, E., Mahdian, R., Azizi, E., Shabani Nashtaei, M., Amidi, F., 2018. Expression of miR-15a, miR-145, and miR-182 in granulosa-lutein cells, follicular fluid, and serum of women with polycystic ovary syndrome (PCOS). Arch. Gynecol. Obstet. 297 (1), 221−231.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., Yamanaka, S., 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol. 26 (1), 101−106.

Niclis, J.C., Pinar, A., Haynes, J.M., Alsanie, W., Jenny, R., Dottori, M., Cram, D.S., 2013. Characterization of forebrain neurons derived from late-onset Huntington's disease human embryonic stem cell lines. Front. Cell. Neurosci. 7, 37.

Notarianni, E., Laurie, S., Moor, R.M., Evans, M.J., 1990. Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. J. Reprod. Fertil. Suppl. 41, 51−56.

Oda, Y., Yoshimura, Y., Ohnishi, H., Tadokoro, M., Katsube, Y., Sasao, M., Kubo, Y., Hattori, K., Saito, S., Horimoto, K., Yuba, S., Ohgushi, H., 2010. Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. J. Biol. Chem. 285 (38), 29270−29278.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., Yamanaka, S., 2008. Generation of mouse induced pluripotent stem cells without viral vectors. Science 322 (5903), 949−953.

Ozcan, A., Yucel, A., Noyan, V., Sagsoz, N., Caglayan, O., 2012. Total and lipid bound sialic acid levels in patients with polycystic ovary syndrome. J. Turk. Ger. Gynecol. Assoc. 13 (2), 79−84.

Pan, J.X., Tan, Y.J., Wang, F.F., Hou, N.N., Xiang, Y.Q., Zhang, J.Y., Liu, Y., Qu, F., Meng, Q., Xu, J., Sheng, J.Z., Huang, H.F., 2018. Aberrant expression and DNA methylation of lipid metabolism genes in PCOS: a new insight into its pathogenesis. Clin. Epigenet. 10, 6.

Papapetrou, E.P., Lee, G., Malani, N., Setty, M., Riviere, I., Tirunagari, L.M., Kadota, K., Roth, S.L., Giardina, P., Viale, A., Leslie, C., Bushman, F.D., Studer, L., Sadelain, M., 2011. Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. Nat. Biotechnol. 29 (1), 73−78.

Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., Daley, G.Q., 2008a. Disease-specific induced pluripotent stem cells. Cell 134 (5), 877−886.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., Daley, G.Q., 2008b. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451 (7175), 141−146.

Peitz, M., Jungverdorben, J., Brustle, O., 2013. Disease-specific iPS cell models in neuroscience. Curr. Mol. Med. 13 (5), 832−841.

Pera, M.F., Cooper, S., Mills, J., Parrington, J.M., 1989. Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. Differentiation 42 (1), 10−23.

Pereira, C.F., Terranova, R., Ryan, N.K., Santos, J., Morris, K.J., Cui, W., Merkenschlager, M., Fisher, A.G., 2008. Heterokaryon-based reprogramming of human B lymphocytes for pluripotency requires Oct4 but not Sox2. PLoS Genet. 4 (9), e1000170.

Perez, M.S., Cerrone, G.E., Benencia, H., Marquez, N., De Piano, E., Frechtel, G.D., 2008. Polymorphism in CYP11alpha and CYP17 genes and the etiology of hyperandrogenism in patients with polycystic ovary syndrome. Medicina (B Aires) 68 (2), 129−134.

Piedrahita, J.A., Anderson, G.B., Bondurant, R.H., 1990. Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. Theriogenology 34 (5), 865−877.

Rahimi, Z., Chamaie-Nejad, F., Saeidi, S., Rahimi, Z., Ebrahimi, A., Shakiba, E., Vaisi-Raygani, A., 2018. The association of PPARgamma Pro12Ala and C161T polymorphisms with polycystic ovary syndrome and their influence on lipid and lipoprotein profiles. Int. J. Fertil. Steril. 12 (2), 147−151.

Raja, J.V., Rachchh, M.A., Gokani, R.H., 2012. Recent advances in gene therapy for thalassemia. J. Pharm. BioAllied Sci. 4 (3), 194−201.

Raya, A., Rodriguez-Piza, I., Guenechea, G., Vassena, R., Navarro, S., Barrero, M.J., Consiglio, A., Castella, M., Rio, P., Sleep, E., Gonzalez, F., Tiscornia, G., Garreta, E., Aasen, T., Veiga, A., Verma, I.M., Surralles, J., Bueren, J., Izpisua Belmonte, J.C., 2009. Disease-corrected haematopoietic progenitors from *Fanconi anaemia* induced pluripotent stem cells. Nature 460 (7251), 53−59.

Recabarren, S.E., Padmanabhan, V., Codner, E., Lobos, A., Duran, C., Vidal, M., Foster, D.L., Sir-Petermann, T., 2005. Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. Am. J. Physiol. Endocrinol. Metab. 289 (5), E801−E806.

Rim, Y.A., Nam, Y., Ju, J.H., 2016. Induced pluripotent stem cell generation from blood cells using Sendai virus and centrifugation. J. Vis. Exp. 118.

Robertson, J.A., 2001. Human embryonic stem cell research: ethical and legal issues. Nat. Rev. Genet. 2 (1), 74−78.

Roessler, R., Boddeke, E., Copray, S., 2013. Induced pluripotent stem cell technology and direct conversion: new possibilities to study and treat Parkinson's disease. Stem Cell Rev Rep 9 (4), 505−513.

Rudnicka, E., Kunicki, M., Radowicki, S., 2010. Androgen and 17-hydroxyprogesterone concentrations in blood serum versus menstrual patterns in women with polycystic ovary syndrome (PCOS). Ginekol. Pol. 81 (10), 745−749.

Saito, S., Strelchenko, N., Niemann, H., 1992. Bovine embryonic stem cell-like cell lines cultured over several passages. Roux Arch. Dev. Biol. 201 (3), 134−141.

Salimi-Asl, M., Mozdarani, H., Kadivar, M., 2016. Up-regulation of miR-21 and 146a expression and increased DNA damage frequency in a mouse model of polycystic ovary syndrome (PCOS). Bioimpacts 6 (2), 85−91.

Seki, T., Yuasa, S., Oda, M., Egashira, T., Yae, K., Kusumoto, D., Nakata, H., Tohyama, S., Hashimoto, H., Kodaira, M., Okada, Y., Seimiya, H., Fusaki, N., Hasegawa, M., Fukuda, K., 2010. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 7 (1), 11−14.

Seshadri Reddy, V., Bukke, S., Munikumar, M., 2018. Elevated levels of the circulatory ischemia-modified albumin in patients with polycystic ovary syndrome: a meta-analysis. Gynecol. Endocrinol. 34 (10), 868−874.

Shi, Y., Li, X., Tong, L., Toukabri, R., Eustergerling, B., 2008. Decomposition of hexamethyldisilane on a hot tungsten filament and gas-phase reactions in a hot-wire chemical vapor deposition reactor. Phys. Chem. Chem. Phys. 10 (18), 2543−2551.

Shi, Y., Kirwan, P., Smith, J., MacLean, G., Orkin, S.H., Livesey, F.J., 2012. A human stem cell model of early Alzheimer's disease pathology in Down syndrome. Sci. Transl. Med. 4 (124), 124ra29.

Skrgatic, L., Baldani, D.P., Gersak, K., Cerne, J.Z., Ferk, P., Coric, M., 2013. Genetic polymorphisms of INS, INSR and IRS-1 genes are not associated with polycystic ovary syndrome in Croatian women. Coll. Antropol. 37 (1), 141−146.

Slamecka, J., Salimova, L., McClellan, S., van Kelle, M., Kehl, D., Laurini, J., Cinelli, P., Owen, L., Hoerstrup, S.P., Weber, B., 2016. Non-integrating episomal plasmid-based reprogramming of human amniotic fluid stem cells into induced pluripotent stem cells in chemically defined conditions. Cell Cycle 15 (2), 234−249.

Soejitno, A., Prayudi, P.K., 2011. The prospect of induced pluripotent stem cells for diabetes mellitus treatment. Ther. Adv. Endocrinol. Metab. 2 (5), 197−210.

Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G.W., Cook, E.G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., Isacson, O., Jaenisch, R., 2009. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell 136 (5), 964−977.

Song, W.J., Shi, X., Zhang, J., Chen, L., Fu, S.X., Ding, Y.L., 2018. Akt-mTOR signaling mediates abnormalities in the proliferation and apoptosis of ovarian granulosa cells in patients with polycystic ovary syndrome. Gynecol. Obstet. Invest. 83 (2), 124−132.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., Hochedlinger, K., 2008. Induced pluripotent stem cells generated without viral integration. Science 322 (5903), 945−949.

Sugii, S., Kida, Y., Berggren, W.T., Evans, R.M., 2011. Feeder-dependent and feeder-independent iPS cell derivation from human and mouse adipose stem cells. Nat. Protoc. 6 (3), 346−358.

Sumer, H., Jones, K.L., Liu, J., Heffernan, C., Tat, P.A., Upton, K.R., Verma, P.J., 2010. Reprogramming of somatic cells after fusion with induced pluripotent stem cells and nuclear transfer embryonic stem cells. Stem Cells Dev. 19 (2), 239−246.

Sun, N., Panetta, N.J., Gupta, D.M., Wilson, K.D., Lee, A., Jia, F., Hu, S., Cherry, A.M., Robbins, R.C., Longaker, M.T., Wu, J.C., 2009. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc. Natl. Acad. Sci. U. S. A. 106 (37), 15720−15725.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126 (4), 663−676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861−872.

Tamaoki, N., Takahashi, K., Tanaka, T., Ichisaka, T., Aoki, H., Takeda-Kawaguchi, T., Iida, K., Kunisada, T., Shibata, T., Yamanaka, S., Tezuka, K., 2010. Dental pulp cells for induced pluripotent stem cell banking. J. Dent. Res. 89 (8), 773−778.

Taranger, C.K., Noer, A., Sorensen, A.L., Hakelien, A.M., Boquest, A.C., Collas, P., 2005. Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Mol. Biol. Cell 16 (12), 5719−5735.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282 (5391), 1145−1147.

Tian, Y., Zhang, W., Zhao, S., Sun, Y., Bian, Y., Chen, T., Du, Y., Zhang, J., Wang, Z., Huang, T., Peng, Y., Yang, P., Zhao, H., Chen, Z.J., 2016. FADS1-FADS2 gene cluster confers risk to polycystic ovary syndrome. Sci. Rep. 6, 21195.

Tsai, S.Y., Clavel, C., Kim, S., Ang, Y.S., Grisanti, L., Lee, D.F., Kelley, K., Rendl, M., 2010. Oct4 and klf4 reprogram dermal papilla cells into induced pluripotent stem cells. Stem Cells 28 (2), 221−228.

Tsai, S.Y., Bouwman, B.A., Ang, Y.S., Kim, S.J., Lee, D.F., Lemischka, I.R., Rendl, M., 2011. Single transcription factor reprogramming of hair follicle dermal papilla cells to induced pluripotent stem cells. Stem Cells 29 (6), 964−971.

Vassilieva, S., Guan, K., Pich, U., Wobus, A.M., 2000. Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. Exp. Cell Res. 258 (2), 361−373.

Wang, Y., Jiang, Y., Liu, S., Sun, X., Gao, S., 2009. Generation of induced pluripotent stem cells from human beta-thalassemia fibroblast cells. Cell Res. 19 (9), 1120−1123.

Wang, X.X., Wei, J.Z., Jiao, J., Jiang, S.Y., Yu, D.H., Li, D., 2014a. Genome-wide DNA methylation and gene expression patterns provide insight into polycystic ovary syndrome development. Oncotarget 5 (16), 6603−6610.

Wang, F., Liu, W.W., Chen, X.M., Kong, H.J., Li, J., Sun, Y.P., 2014b. Differential genes in adipocytes induced from polycystic and non-polycystic ovary syndrome-derived human embryonic stem cells. Syst. Biol. Reprod. Med. 60 (3), 136–142.

Wang, C., Shen, F., Zhu, Y., Fang, Y., Lu, S., 2017a. Telomeric repeat-containing RNA (TERRA) related to polycystic ovary syndrome (PCOS). Clin. Endocrinol. 86 (4), 552–559.

Wang, L., Chen, Y., Guan, C., Zhao, Z., Li, Q., Yang, J., Mo, J., Wang, B., Wu, W., Yang, X., Song, L., Li, J., 2017b. Using low-risk factors to generate non-integrated human induced pluripotent stem cells from urine-derived cells. Stem Cell Res. Ther. 8 (1), 245.

Wawrzkiewicz-Jalowiecka, A., Kowalczyk, K., Pluta, D., Blukacz, L., Madej, P., 2017. The role of aquaporins in polycystic ovary syndrome – a way towards a novel drug target in PCOS. Med. Hypotheses 102, 23–27.

Wen, W., Zhang, J.P., Xu, J., Su, R.J., Neises, A., Ji, G.Z., Yuan, W., Cheng, T., Zhang, X.B., 2016. Enhanced generation of integration-free iPSCs from human adult peripheral blood mononuclear cells with an optimal combination of episomal vectors. Stem Cell Rep. 6 (6), 873–884.

Wernig, M., Zhao, J.P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O., Jaenisch, R., 2008a. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 105 (15), 5856–5861.

Wernig, M., Lengner, C.J., Hanna, J., Lodato, M.A., Steine, E., Foreman, R., Staerk, J., Markoulaki, S., Jaenisch, R., 2008b. A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat. Biotechnol. 26 (8), 916–924.

Wichterle, H., Lieberam, I., Porter, J.A., Jessell, T.M., 2002. Directed differentiation of embryonic stem cells into motor neurons. Cell 110 (3), 385–397.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H., 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385 (6619), 810–813.

Wobus, A.M., Kaomei, G., Shan, J., Wellner, M.C., Rohwedel, J., Ji, G., Fleischmann, B., Katus, H.A., Hescheler, J., Franz, W.M., 1997. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. J. Mol. Cell. Cardiol. 29 (6), 1525–1539.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H.K., Nagy, A., 2009. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458 (7239), 766–770.

Xiong, W., Lin, Y., Xu, L., Tamadon, A., Zou, S., Tian, F., Shao, R., Li, X., Feng, Y., 2017. Circulatory microRNA 23a and microRNA 23b and polycystic ovary syndrome (PCOS): the effects of body mass index and sex hormones in an Eastern Han Chinese population. J. Ovarian Res. 10 (1), 10.

Xu, J., Bao, X., Peng, Z., Wang, L., Du, L., Niu, W., Sun, Y., 2016. Comprehensive analysis of genome-wide DNA methylation across human polycystic ovary syndrome ovary granulosa cell. Oncotarget 7 (19), 27899–27909.

Yagi, T., Ito, D., Okada, Y., Akamatsu, W., Nihei, Y., Yoshizaki, T., Yamanaka, S., Okano, H., Suzuki, N., 2011. Modeling familial Alzheimer's disease with induced pluripotent stem cells. Hum. Mol. Genet. 20 (23), 4530–4539.

Yagi, T., Ito, D., Okada, Y., Akamatsu, W., Nihei, Y., Okano, H., Suzuki, N., 2012. Modeling familial Alzheimer's disease with induced pluripotent stem cells. Rinsho Shinkeigaku 52 (11), 1134–1136.

Yan, X., Qin, H., Qu, C., Tuan, R.S., Shi, S., Huang, G.T., 2010. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev. 19 (4), 469–480.

Yang, S., Ding, S., Jiang, X., Sun, B., Xu, Q., 2016. Establishment and adipocyte differentiation of polycystic ovary syndrome-derived induced pluripotent stem cells. Cell Prolif. 49 (3), 352–361.

Yang, Y., Jiang, H., Xiao, L., Yang, X., 2018. MicroRNA-33b-5p is overexpressed and inhibits GLUT4 by targeting HMGA2 in polycystic ovarian syndrome: an in vivo and in vitro study. Oncol. Rep. 39 (6), 3073–3085.

Ye, H., Wang, Q., 2018. Efficient generation of non-integration and feeder-free induced pluripotent stem cells from human peripheral blood cells by Sendai virus. Cell. Physiol. Biochem. 50 (4), 1318–1331.

Young, J.E., Goldstein, L.S., 2012. Alzheimer's disease in a dish: promises and challenges of human stem cell models. Hum. Mol. Genet. 21 (R1), R82–R89.

Yu, J., Vodyanik, M.A., He, P., Slukvin II, Thomson, J.A., 2006. Human embryonic stem cells reprogram myeloid precursors following cell-cell fusion. Stem Cells 24 (1), 168–176.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin II, Thomson, J.A., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318 (5858), 1917–1920.

Zaehres, H., Kogler, G., Arauzo-Bravo, M.J., Bleidissel, M., Santourlidis, S., Weinhold, S., Greber, B., Kim, J.B., Buchheiser, A., Liedtke, S., Eilken, H.M., Graffmann, N., Zhao, X., Meyer, J., Reinhardt, P., Burr, B., Waclawczyk, S., Ortmeier, C., Uhrberg, M., Scholer, H.R., Cantz, T., Wernet, P., 2010. Induction of pluripotency in human cord blood unrestricted somatic stem cells. Exp. Hematol. 38 (9), 809–818, 818 e1-2.

Zhang, N., An, M.C., Montoro, D., Ellerby, L.M., 2010. Characterization of human huntington's disease cell model from induced pluripotent stem cells. PLoS Curr. 2, RRN1193.

Zhang, J., Liu, J., Zhu, K., Hong, Y., Sun, Y., Zhao, X., Du, Y., Chen, Z.J., 2016. Effects of BMAL1-SIRT1-positive cycle on estrogen synthesis in human ovarian granulosa cells: an implicative role of BMAL1 in PCOS. Endocrine 53 (2), 574–584.

Zhang, H., Li, D., Li, R., Huo, Z., Qiao, J., 2018. Treatment of polycystic ovary syndrome with acupuncture. Acupunct. Med. 36 (4), 269.

Zhou, T., Benda, C., Duzinger, S., Huang, Y., Li, X., Li, Y., Guo, X., Cao, G., Chen, S., Hao, L., Chan, Y.C., Ng, K.M., Ho, J.C., Wieser, M., Wu, J., Redl, H., Tse, H.F., Grillari, J., Grillari-Voglauer, R., Pei, D., Esteban, M.A., 2011. Generation of induced pluripotent stem cells from urine. J. Am. Soc. Nephrol. 22 (7), 1221–1228.

Zhou, T., Benda, C., Dunzinger, S., Huang, Y., Ho, J.C., Yang, J., Wang, Y., Zhang, Y., Zhuang, Q., Li, Y., Bao, X., Tse, H.F., Grillari, J., Grillari-Voglauer, R., Pei, D., Esteban, M.A., 2012. Generation of human induced pluripotent stem cells from urine samples. Nat. Protoc. 7 (12), 2080–2089.

Zhou, J., Wang, X., Zhang, S., Gu, Y., Yu, L., Wu, J., Gao, T., Chen, F., 2013. Generation and characterization of human cryptorchid-specific induced pluripotent stem cells from urine. Stem Cells Dev. 22 (5), 717–725.

Zhou, H., Martinez, H., Sun, B., Li, A., Zimmer, M., Katsanis, N., Davis, E.E., Kurtzberg, J., Lipnick, S., Noggle, S., Rao, M., Chang, S., 2015. Rapid and efficient generation of transgene-free iPSC from a small volume of cryopreserved blood. Stem Cell Rev. Rep. 11 (4), 652–665.

CHAPTER

Modeling inherited retinal dystrophies using induced pluripotent stem cells

7

Mohamed A. Faynus[1,2], **Dennis O. Clegg**[1,2]

[1]*Program in Biomolecular Science and Engineering, University of California, Santa Barbara, CA, United States;* [2]*Center for Stem Cell Biology and Engineering, Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA, United States*

Chapter outline

Abstract

The eyes are tools that extract visual information from the surrounding environment. Acting like a digital camera, we use them daily to capture and convert light stimuli into images. This activity is highly regulated and is the direct responsibility of the numerous cell types of the retina. Strikingly, loss of any one of these retinal cells, whether a result of genetic complications or traumatic injury, culminates in

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00005-9

blindness. Therefore, it is understandable why diseases affecting the eyes are extremely debilitating and studied worldwide. With the advent of stem cell biology, scientists are now able to design retinal disease-specific models that recapitulate patient clinical phenotypes. In this review, we summarize some of the recent advances in modeling of inherited retinal dystrophies (IRDs) using patient-derived induced pluripotent stem cells (pd-iPSCs). Methods have been developed to direct the differentiation of iPSC into bonafide retinal cells in both two-dimensional adherent cultures and three-dimensional organoid systems. This novel approach to studying IRDs such as retinitis pigmentosa, leber congenital amaurosis, choroideremia, Bests disease, and Stargardt maculopathy has yielded valuable insights into the molecular and cellular basis of these currently untreatable maladies.

Keywords: Embryonic stem cells (ES); In vitro disease modeling; Induced pluripotent stem cells (iPSC); Inherited retinal dystrophy (IRD); Reprogramming; Retina; Retinal organoids; RPE; Stem cell biology; Three-dimensional culture.

Introduction

Retina: architecture and function

The mammalian retina is an intricate system that continues to fascinate researchers worldwide. Long ago scientists discovered the beautiful architectural layering of retinal circuitry (Cajal et al., 1894). This natural artistry is apparent through several studies depicting retina-specific cellular morphologies and live functional processing (Hoon et al., 2014; Gray et al., 2006; Yin et al., 2014; Ethan et al., 2017). The signal input/output processes throughout the eye, and the speed at which image processing occurs exemplify the degree of difference between primate retina and lower order organisms (Demb and Singer, 2015; Lobier et al., 2013).The neural circuitry in the retina—positioned and tuned to transfer light-stimulated electrical information to the brain—is superbly tight, sequential, and hierarchal (Demb and Singer, 2015). It is estimated that the neurome of the retina alone is composed of ~100 cell types including four types of rod and cone photoreceptors, 70 types of interneurons (bipolar, amacrine and horizontal), and 30 types of retinal ganglion cells (Fig. 7.1, Joshua and Richard, 2015).

Each cell type is confined to a specific layer within the retina, separated by dendritic rich fibril sections, this layering helps maintain synaptic connections between retinal ganglion and amacrine cells (inner plexiform) or photoreceptors and bipolar/horizontal cell populations (outer plexiform) (Demb and Singer, 2015; Joshua and Richard, 2015). The processing of visual information begins within the rod and cone photoreceptors, situated in the outer-nuclear layer, these light-sensitive pigment containing cells initiate the conversion of light energy to membrane potential changes which trigger synaptic release, commencing an electric cascade of events (Arshavsky and Burns, 2014). Photoreceptor synaptic processes end in the outer-plexiform where contact and subsequent synaptic

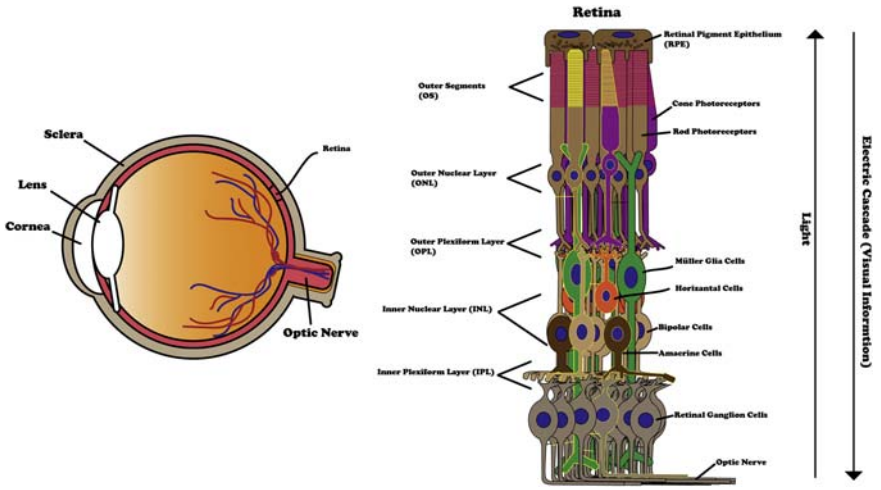


FIGURE 7.1 Schematic representation of the eye and retina.

The cell types of the retina include: retinal pigment epithelium (RPE), rod and cone photoreceptors; horizontal, bipolar, and amacrine cells; Müller glia and retinal ganglion cells. These cells and dendrites are restricted to specific layers such as the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), and inner plexiform layer (IPL). The retinal ganglion cells form into the optic nerve which direct electrical information to the brain for visual processing.

transmission is modulated by rod- and cone-specific bipolar cells and sent forth to the retinal ganglion cells which form into the optic nerve and direct information to the brain (Peichl, 2005; Masland, 2001; Singh and Tyagi, 2018).

Retinal degeneration: inherited retinal dystrophy

Charles Darwin called the eye "an organ … of exquisite perfection," and this is perhaps reflected in the exquisite interconnectedness of the retina. However, loss of cellular interactions in eye disease makes visual function less than perfect, and we lack therapies for many ocular conditions. Mutations affecting genes active in the eye can cause terminal blindness; indeed, a third of all known human diseases and syndromes are estimated to be eye related with the count continuing to grow (Pang, 2016). The inheritance of eye diseases can be monogenic or digenic and can follow Mendelian principles. Collectively referred to as inherited retinal dystrophies (IRDs), IRDs include a wide array of mutations and disease sequelae. To date there are >300 genes mapped to eye diseases with numerous causative mutations identified (Morrison et al., 1997). IRD mutations can affect different cells within the retina, and because of the interconnections between cells, many result in blindness. For example, mutations that are detrimental to RPE function, such as those in BEST, lead to a loss of support of photoreceptors, and

ultimate blindness. Many IRDs are linked directly to mutations that affect photoreceptor function or survival, and lead to retinitis pigmentosa (RP), the most common IRD (see below).

Current IRD treatment options are scant. While advances in stem cell biology have led to clinical trials for cellular therapeutics, induced pluripotent stem cells may also prove useful to model retinal disease in-vitro. This promising area of research has the potential to uncover unknown pathogenic mechanisms and test candidate therapeutics for the treatment of eye disease.

Stem cells

Embryonic and induced pluripotent stem cells

Stem cells are undecided, nonspecific cells that possess the capability to turn into specific cell types of an organism (Ramalho-santos and Willenbring, 2007; Pedro and Nance, 2011; Zakrewski et al., 2019). The totipotent fertilized zygote develops into an organism through a cascade of events that trigger germ layer−specific differentiation during embryogenesis (Christopher and Martin, 2018; Thomson et al., 1998). Pluripotent embryonic stem cells (ESCs), derived from the inner cell mass of five-day-old blastocysts, are capable of infinite division, an associated benefit of their elevated telomerase activity and high nuclear to cytoplasmic ratio (Thomson et al., 1998; Hiyama and Hiyama, 2007; Amit et al., 2000). ESCs have been shown to not only circumvent senescence through multiple passages but also simultaneously retain the ability to differentiate into virtually all cell types of the body (Thomson et al., 1998; Amit et al., 2000). Furthermore, Thomson et al. have isolated and cultured numerous ESC lines that maintain these requirements both in-vitro and in-vivo (Amit et al., 2000). The ethical considerations haunting ESC use and isolation have sparked an interest and exploration into different methods of obtaining pluripotent populations of cells (Martin, 1981; Wert and Mummery, 2003; King and Perrin, 2014). Breakthrough work of Yamanaka and colleagues demonstrated that expression of four transcription factors [octamer-binding transcription factor 4 (*Oct4*), sex-determining region Y box 2 (*Sox2*), cell cycle entry and proliferation regulator (*c-Myc*), and gut-enriched Krüppel-like factor (*Klf4*)] can reprogram murine somatic cells into a pluripotent state (Takahashi and Yamanaka, 2006; Huang et al., 2019). Expression of these embryonically active master transcription factors in differentiated somatic cells (which lack their expression) results in induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). This discovery was soon extended to human cells in 2007 by Yamanaka et al., and independently by Thomson and colleagues, who demonstrated reprogramming via a different cocktail of transcription factors (Oct4, Sox2, Lin28, and Nanog) (Takahashi et al., 2007; Junying et al., 2007).

Methods of obtaining induced pluripotent stem cell

iPSC reprogramming technology has developed rapidly during the past decade. Traditional reprogramming used retro or lentiviral methods to introduce the

Yamanaka/Thompson factors, or any combination of; however, increasing evidence has made clear the propensity of these methods in introducing random genetic insertions risking mutagenesis (Huang et al., 2019). Newer reprogramming methods use nonintegrating vectors such as adeno-associated and Sendai viruses or piggyBac transposons (Lowry and Plath, 2008; Fusaki et al., 2009; Knut et al., 2013). Of these, Sendai virus is frequently employed due to its high reprogramming efficiency and incapability of entering the nucleus (Huang et al., 2019). Reprogramming has been demonstrated using a variety of somatic cells, including dermal fibroblasts, peripheral mononuclear blood cells, epithelial cells shed in urine, keratinocytes, and several others, There are no concrete rules in selecting somatic cell source nor preferred starting material, although recent studies have implicated cell identity and its role in reprogramming efficiency and differentiation capability, selection is still at the discretion of the user, often based on availability and simplicity in processing operations (Marchetto et al., 2009; Hu et al., 2010).

Disease modeling

Clinical assessment of inherited retinal dystrophies

IRD assessment begins with a visit to a medical professional where clinical measurements are completed, cataloging retinal phenotypes and generating patient-specific profiles. Traditional retinal diagnostic tests include visual field evaluation, fundoscopic examination, optical coherence tomography (OCT), fundus autofluorescence, dark adaptation, and electroretinography (ERG) (Briang, 2017). The phenotypic summaries from several tests are compared to known disease references and a clinical diagnosis is formulated (Lori and Daiger, 1996). Because complications exist, particularly with respect to systemic diseases and genetic heterogeneity, this method of diagnosis is rarely proficient in providing an exact answer for further follow-up and treatment (Briang, 2017). To remedy this, several clinical laboratories utilize next generation sequencing to further conclude hereditary disorders (Moyra, 2017). There are polymerase chain reaction–based, single or multigene panels for candidate retinal dystrophy genes that are frequently employed by clinicians to assess a wide array of conditions (Briang, 2017; Hartman et al., 2019).

Inherited retinal dystrophy research using patient tissue or animal models

Studying IRDs in a research lab setting requires either patient samples, a costly and complicated endeavor, or separate animal models that recapitulate patient phenotypes, often rodents for simplicity and cost metrics (Shobi et al., 2015). Obtaining patient retinal tissue typically includes generating and maintaining strict institutional review board approvals, signed and maintained specific ethical considerations and informed consent (Monica et al., 2010). In the context of the eye, it is particularly difficult to obtain live samples from patient retinas.

First, there are complications in the surgical collection of retina-specific samples, isolating retinal biopsies is no simple feat (John and Chi-Chao, 2007). Second, patients are already under the stress of deteriorating vision, and further agitation may worsen the issue, leading to abrupt terminal loss of vision. Lastly, culturing in-vivo–derived tissues is difficult on a long-term basis (Seung and Hiroshi, 1988; Fronk and Vargis, 2016). Postmortem tissues, while useful in characterizing the end stage impact of a disease, are difficult to culture and do not represent the gradual degenerative phases of several retinal disorders (Luisa et al., 2013; Henry et al., 2004; Christine, 2005). In addition, animal models with IRDs do not usually capture the full spectrum of human phenotypes (Picaud et al., 1996). For example, rodents and lower order organisms do not contain a macula and thus lack this cone-rich central region that is often impacted by IRD mutations, such as those that underlie Stargardt's disease (Moshiri et al., 2019).

A limited understanding of the pathogenesis of IRDs has hampered the development of therapies. Recent studies suggest that patient-derived iPSC (pd-iPSC) may bridge the gap between clinical observations and preclinical models. pd-iPSC harboring IRD mutations are capable of differentiating into ocular cell types, thereby providing an alternative method of assessing pathognomonic phenotypes and permitting molecular and cellular analyses (Fig. 7.2, West et al., 2019;

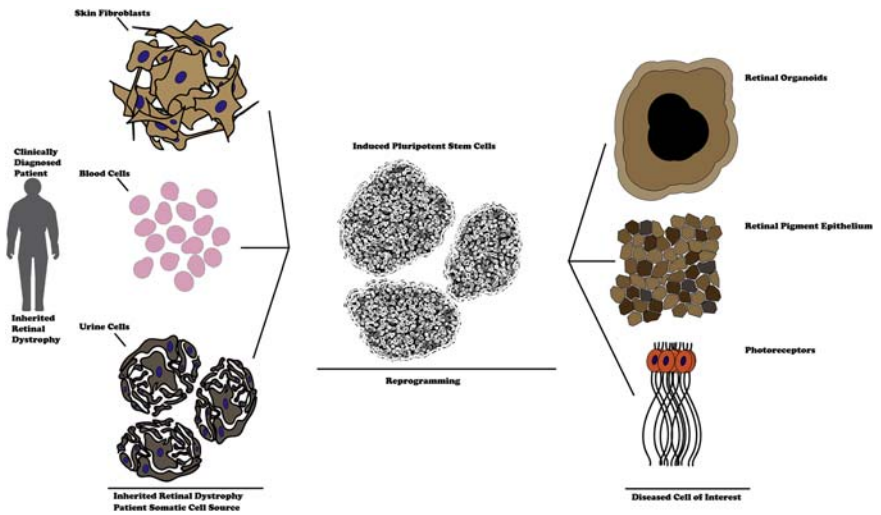


FIGURE 7.2 Schematic representation of stem cell–derived retinal models.

Patients are diagnosed with an inherited retinal dystrophy (IRD). Patient-derived induced pluripotent stem cells (pd-iPSC) are generated by reprogramming somatic cells such as skin fibroblasts, peripheral mononuclear blood cells, or urine cells through application of episomal, nonintegrating vectors expressing Yamanaka factors. pd-iPSC are differentiated into three-dimensional retinal organoids (ROs), adherent retinal pigment epithelium (RPE), and photoreceptors.

Karl et al., 2014; Ana et al., 2018; Castro et al., 2019). Furthermore, pd-iPSCs provide an avenue for study of disease progression throughout development (Kang and Ding, 2011). In the following section, we will summarize some of the methods used to generate specific retinal cells implicated in retinal diseases through in-vitro stem cell differentiation.

Pluripotent stem cell differentiation into retinal cell types

Retinal pigment epithelium

The RPE forms a cuboidal monolayer near the back of the retina. Responsible for transport of nutrients and water from the choroid, RPE maintains the health of adjacent, interdigitated photoreceptors (Strauss, 2005). During embryonic development, invagination of the optic cup forms two distinct cellular layers, one destined to mature into the RPE and the other into the neural retina (Heavner and Pevny, 2012; Mototsugu et al., 2011). In-vitro differentiation protocols harness an understanding of RPE embryonic development by mimicking in-vivo developmental cues in two-dimensional cultures (Hiroshi et al., 2001; Masatoshi et al., 2004; Irina et al., 2004; Bucholz et al., 2009). Several groups have shown that application of insulin growth factor (IGF), noggin, dickkopf-related protein 1 (Dkk1), activin A, and CHRI9902 to stem cell populations can effectively drive differentiation into an RPE fate with high efficiency (Bucholz et al., 2009; Maria et al., 2009; David et al., 2013; Lyndsay et al., 2015; Parul et al., 2017).

Neural retina: organoid systems

Sasai et al. have demonstrated the capability of in-vitro stem cell populations to form three-dimensional organoid systems that contain nearly all cells types of the retina (Mototsugu et al., 2011; Nakano et al., 2012). Traditional differentiation protocols utilize an adherent system in culturing and differentiating stem cells; however, these systems do not recapitulate in-vivo development, and the cells produced are usually at an immature state (Fumitaka et al., 2008, 2009). The retina forms through complex interactions, not only as a result of regulation of gene expression but also mechanosensitive responses (Fumitaka et al., 2009; Alina et al., 2016; Deepak et al., 2006; Jason et al., 2009). Many groups have since adopted an organoid-based system to study the development and function of the neural retina (Zhong et al., 2014; Capowski et al., 2019; Fligor et al., 2018). While there are several iterations of differentiation protocols, most loosely follow a similar timeline. This involves culture of undifferentiated stem cells in suspension, using low attachment conditions, which trigger aggregate formation of cells, so called embryoid bodies (Joshua and Serup, 2017). Next, these embryoid bodies are allowed to adhere to extracellular matrix surfaces, where they are pulsed with numerous supplements such as B27, N2, and bone morphogenetic protein 4 (BMP4). Areas rich in neural epithelial markers begin to form throughout the surface (Zhong et al., 2014; Capowski et al., 2019). These areas are harvested via microdissection, and aggregates are subsequently cultured in suspension for an extended period, upwards of

250 days. After extended culture, retinal organoids (ROs) resembling "golden cheerios" are observed, which contain layers of retinal cells, including light responsive photoreceptors (Zhong et al., 2014; Capowski et al., 2019; Wen-Li et al., 2018).

Neural retina: photoreceptors

To further our understanding of rods and cone photoreceptors, several groups have developed protocols that include differentiating pluripotent stem cells into retinal progenitor states and subsequently into photoreceptors (Fumitaka et al., 2008; Hanako et al., 2005; Xing et al., 2002; Shufend et al., 2015). Whereas the three-dimensional system provides mechanical cues that help formulate the outer and inner layering of the retina, two-dimensional photoreceptor differentiation protocols do not (Shufend et al., 2015). Nonetheless, Bernier et al. have shown that concurrent inhibition of BMP, TGFβ, and WNT promote stem cell differentiation into cone-specific photoreceptors with an $\sim$60%−80% efficiency. Through a combination of IGF and COCO, stem cell populations can be driven into a cone-specific state where differentiated cells could degrade cGMP upon exposure to light and self-organize into a sheet with inner and outer-segment formation (Shufend et al., 2015). Takahashi et al. demonstrate in-vitro generation of photoreceptors from human and monkey ES cells. Through serum free, define culture conditions, devoid of animal-derived substances, ES cells can be coaxed into photoreceptor lineages with a relative efficiency of $\sim$25%−35%. It is clear that directly differentiating photoreceptors in-vitro is difficult; however, obtaining a pure population of photoreceptors will drastically evolve the therapeutic range of treatments available to treat retinal dystrophies (Fumitaka et al., 2008).

Induced pluripotent stem cell disease models of inherited retinal dystrophies

Herein we hope to summarize some current disease models of IRDs that utilize patient-derived induced pluripotent stem cells. We will limit IRD review to single monogenic causative mutations that are inherited in an autosomal dominant (aD), autosomal recessive (aR), or x-linked (xL) pattern. We will consider stem cell−derived models that (1) utilize clinically diagnosed IRD patient-derived somatic cells, (2) reprogrammed into a pluripotent state generating iPSCs, and (3) subsequently differentiated pluripotent populations into suspected afflicted retinal cells types.

Retinitis pigmentosa

Perhaps one of the most recognized and common IRD, RP, affects between 1 and 3500 individuals world-wide, including simple, syndromic, and systemic forms of the disease (Stephen et al., 2008). The disease primarily affects rod photoreceptors and in late stages impacts cones and other retinal cell types (Stefano et al., 2011).

RP patients may experience issues with night vision due to loss of rods, loss of central vision and visual acuity (VA) due to cone death and in late stage cases complete blindness (Stephen et al., 2008). The complexity of the disease is apparent by the wide array of causative mutations and inheritance patterns. There are aD, aR, and xL forms of the disease, each with unique and in some cases overlapping impacted genes. Current estimates conclude >60 known mutations (https://sph.uth.edu/RETNET/sum-dis.htm#A-genes). The most common causes of adRP are mutations in genes encoding rhodopsin (RHO) and precursor messenger ribonucleic acid (mRNA) splicing factors PRPF6, PRPF8, PRPF31, with the latter estimated to cause ~10% of all dominant RP cases (Stephen et al., 2014). Other RP-affected genes are involved in multiple retinal functions including the phototransduction cascade, cilium processing and trafficking, ion transport, phagocytosis, and outer-segment renewal and structure. iPSC modeling of RP may provide clues as to the biomolecular impact of a mutation.

Lako et al. reprogrammed PRPF31 null iPSC from patient skin fibroblasts and generated photoreceptors and RPE. Patient-derived iPSC-RPE showed increased secretion of pigment epithelium-derived factor and vascular endothelial growth factor, two cytokines critically involved in maintaining retinal health. Furthermore, patient iPSC-RPE had reduced transepithelial resistance, structural abnormalities, and defective phagocytosis of outer segments. To characterize PRPF31 mutations in photoreceptors, ROs were examined. Patient-derived photoreceptors display morphological issues and an increase in apoptotic nuclei. Intriguingly, both RPE and photoreceptors display abnormal cilia, where PRPF31-mutated lines show decreased cilia length and incidence (Adriana et al., 2018). It remains unclear how a mutation in a splicing factor can give rise to these defects, however, this is but one study that demonstrates a mechanistic approach and a model system that utilizes patient-derived stem cells. Alternatively, Foltz et al. demonstrate that loss of PRPF8 does not impact RPE function, starkly opposed to murine models showing impaired phagocytosis, shedding light on the need to recapitulate retinal diseases in human models (Michael et al., 2014; Foltz et al., 2018).

Several groups have also discovered ciliopathic findings in RP-iPSC-RO and RPE with mutations in different genes (Brydon et al., 2019). Mutations in USH2A are known to cause aR-RP, commonly referred to as Usher's syndrome, but the pathogenic mechanisms are not well characterized. Chen et al. differentiated RO and RPE from patient-derived USH2A-iPSC. They demonstrated that early stage USH2A-RO exhibit abnormal cell morphologies, less laminin expression, improper protein localization, and differential transcriptional profiles. USH2A-iPSC-RPE also contained less RPE65, loss of melanin, improper ZO-1 localization, and display atrophic tendencies during culture (Guo et al., 2019). But not all USH2A mutations cause developmental defects. Stone et al. generated USH2A-RO and observed proper development of photoreceptor precursor cells but found exonification of intron 40 which presumably lead to elevated protein expression of GRP78 and GRP94, two proteins involved in the unfolded protein response and ER stress (Budd et al., 2013). Recently, Jin et al. questioned the pathogenicity of late-onset RP by generating PED6B-RO. PED6B encodes for the beta subunit of rod cGMP-phosphodiesterase

type 6, a protein critical in cGMP hydrolysis. PED6B-RO display diseased phenotypes in late stage organogenesis, abnormal photoreceptor morphology and location, and overall difficulty hydrolyzing cGMP. Furthermore, cGMP metabolic genes were upregulated in PED6B-RO suggesting impaired photoreceptor function (Table 7.1, Gao et al., 2020; Sanjurjo-Soriano et al., 2020; Schwarz et al., 2017; Jin et al., 2012; Yoshida et al., 2014; Ramsden et al., 2017; Megaw et al., 2017).

Leber congenital amaurosis

Leber congenital amaurosis (LCA) is a rare severe congenital monogenic IRD that manifests as early childhood blindness. With an estimated prevalence of 2 in 100,000 new-born infants, LCA is clinically diagnosed through combination of nystagmus, fundus changes, severe vision loss, and impaired or absent electrical activity of the retina observed through rod/cone responsive ERG. Additionally, infant patients tend to forcibly attempt stimulating vision by habitually rubbing eyes with knuckles or fingers, a response known as Franceschetti oculo-digital sign (Thompson et al., 2018; Koenekoop, 2004). There are at least 25 known causative mutations that result in LCA. Mutations in centrosomal protein of 290 kDa (*CEP290*), crumbs homolog 1 (*CRB1*), retinal guanylyl cyclase 1 (*GUCY2D*), and retinal pigment epithelium-specific factor 65 kDa (*RPE65*) represent the main body of mutations causing the disease (Koenekoop, 2004; Sharola et al., 2000). However, in roughly a third of patients with LCA the genetic causes are unknown (Chacon-camacho and Zenteno, 2015). The disease is typically inherited as an autosomal recessive trait although there are cases of aD inheritance. For example, mutations in cone-rod homeobox *CRX* can result in either aD or aR LCA (Robert et al., 2015).

Several of the implicated genes play a significant role in photoreceptor formation and function (Thompson et al., 2018; Koenekoop, 2004; Maria et al., 2018). Due to the congenital nature of the disease, early photoreceptor death and dysfunction are observed in LCA patients. The exact biochemical and molecular cause of this dysfunction is still unknown. With pd-iPSC, several groups have started to piece together model systems that recapitulate embryonic development of LCA patients with the goal of understanding what biochemical signaling is impaired and whether these issues can be remedied. Mutations in CEP290 are thought to cause issues in photoreceptor development and function. Cheetham et al. explored these hypotheses by generating iPSC from patients with mutations in CEP290, c.2991 + 1665A > G, and differentiating them into ROs and RPE. CEP290-LCA-RPE display no differences in RPE polarity and gene expression with no comparable difference in cilia-related proteins, Arl13B and pericentrin. Conversely, CEP290-LCA-RO had no detectable pericentrin, an essential component of the cilium, nor expression of CEP290 transcript. This suggests splicing defects are isolated to specific cells, in this case, photoreceptors, which may explain the lack of RPE phenotypes (David et al., 2016). Furthermore, aberrant splicing can be restored using antisense oligonucleotides (AONs). Since then, numerous groups have identified CEP290-associated ciliogenesis complications in LCA patient-derived RPE and RO (Hiroko et al., 2017; Kalyan et al., 2018). These data have led to the initiation of a clinical trial

Table 7.1 Retinitis pigmentosa stem cell–derived models.

Inherited retinal dystrophy	Mutation(s)	Initial cell source	Reprogramming method	Studied system(s)	Observation(s)	Publication
aR-RP: *Usher syndrome*	*USH2A c.2276G > T* *USH2A e.2299delG*	Skin fibroblasts	Sendai virus	*iPSC*	RP-iPSC mutations in USH2A can be rescued by CRISPR/Cas9 genome editing	Sanjurjo-Soriano et al. (2020)
aD-RP	*PRPF31* c.1115_1125del11 c.522_527 + 10del	Skin fibroblasts	Sendai virus	*RPE, RO*	RP-RPE and photoreceptors have structural and functional abnormalities including ciliogenesis complications	Adriana et al. (2018)
aD-RP	*PRPF31* c.1115_1125del11	Skin fibroblasts	Sendai virus	*RPE*	RP-RPE display functional issues in phagocytosis. Rescue of POS binding by AAV-PRPF31	Brydon et al. (2019)
xL-RP	*RP2* C.519C > T	Skin fibroblasts	Integration-free episomal vectors	*RO*	Reduced Kif7 at cilia tips in RP-RO	Schwarz et al. (2017)
aD-RP: *Usher syndrome*	*USH2A* c.8559-2A > G c.9127_9129delTCC	Urine cells	Sendai virus	*RPE, RO*	RP-RO and RP-RPE display defects in morphology and gene expression	Guo et al. (2019)
aD-RP	PRPF8 c.6901 C > T	Skin fibroblasts	Integration-free episomal vectors	*RPE*	RP-RPE do not display abnormal phenotypes	Foltz et al. (2018)
aR-RP: *Usher syndrome*	*USH2A* Arg4192His ABCA4 Thrl428Met	Skin fibroblasts	Sendai virus	*Retinal neurons*	Normal phenotype of retinal cells in-vitro and transplantation suggesting retinal degeneration postdevelopment	Budd et al. (2013)

Continued

Table 7.1 Retinitis pigmentosa stem cell–derived models.—*cont'd*

Inherited retinal dystrophy	Mutation(s)	Initial cell source	Reprogramming method	Studied system(s)	Observation(s)	Publication
RP	*RP1* c.2161_2 l62insC, *PRPH2* c.946T > G *RHO* c.562G > A *RP9* c.401>T	Skin fibroblasts	Retrovirus-mediated transfection with Oct3/4, Sox2, Klf4, and c-Myc	*Photoreceptors*	RP9 involved in oxidative stress pathways, RHO mutations associated with ER stress in patient-derived photoreceptors	Jin et al. (2012)
aD-RP	*RHO* E181K	Skin fibroblasts	Retrovirus-mediated transfection with Oct3/4, Sox2, Klf4, and c-Myc	*Photoreceptors*	Decreased survival of E181K RP rods correlates with increased ER stress and apoptotic markers	Yoshida et al. (2014)
RP	*MERTK* c.61+1G > A C.1951C > T	Skin fibroblasts	Retrovirus-mediated transfection with Sox2, Oct3/4, Klf4, L-Myc, and Lin28	*RPE*	Loss of MERTK in RP-RPE results in incapability of phagocytosing outer segments. Treatment with translational readthrough drugs restore expression of MERTK and junction	Ramsden et al. (2017)
RP	*RHO* c.562G > A	Skin fibroblasts	Sendai virus	*RPE, photoreceptors*	RP-RO rods display ER stress and gradual degeneration in vitro	Jin et al. (2012)
xL-RP	*RPGR* c.689-692del4	Skin fibroblasts	Integration-free episomal vectors	*RO*	RPGR functions with actin severing protein gelsolin. Gelsolin regulates disassembly of cilium in photoreceptors	Megaw et al. (2017)
aR-RP	*PED6B* c.694G > A	Primary peripheral blood mononuclear cells	Integration-free episomal vectors	*RO*	RP-RO have impaired cGMP hydrolysis, assessed by ELISA	Gao et al. (2020)

aD, *autosomal dominant;* aR, *autosomal recessive;* iPSC, *induced pluripotent stem cell;* RHO, *rhodopsin;* RO, *retinal organoid;* RPE, *retinal pigment epithelium;* xL, *x-linked.*

(NCT03140969) conducted by ProQr Therapeutics (Cideciyan et al., 2019). Wjinholds et al. demonstrate CRB1-associated LCA-RO lacking CRB1 result in issues with outer limiting membrane development and photoreceptor structure and location (Quinn et al., 2019). Pattnaik et al. consider the impact of potassium inwardly rectifying channel subfamily j member 13 (*KCNJ13*) mutations on LCA patient-derived RPE functionality. KCNJ13 encodes for Kir7.1, a tetrameric inward potassium ion channel exclusively located in the RPE of the retina. Lack of Kir7.1 in LCA-RPE results in issues with phagosome degradation and impaired rubidium ion (Rb+) currents, and introduction of Kir7.1 through gene augmentation restored LCA-RPE Rb + current activity (Table 7.2, Shahi et al., 2019; Li et al., 2019).

Choroideremia

Choroideremia (CHM) is a rare x-linked recessive retinal dystrophy that impacts roughly 1 in 50,000 patients. The disease clinically presents itself through early loss of night vision, which gradually progresses to severe peripheral vision impairment around the fourth decade of life (Vasiliki et al., 2013; Brambati et al., 2019). Late stage patients exhibit gradual shortening of inner and outer-segments, reduced thickness of outer nuclear layer, and depigmentation of RPE, typically culminating in areas of distinct chorioretinal atrophy (Vasiliki et al., 2013). CHM is caused by mutations in Rab escort protein I (*REP-1*), a ubiquitously expressed protein that manages fusion of unprenylated Rab proteins to associated binding partners and intracellular trafficking (Miguel et al., 1992; Jacobson et al., 2006; Preising and Ayuso, 2004). There are >280 known causative mutations that result in CHM phenotypes with frameshifts and nonsense mutations representing ~70% of cases (Brambati et al., 2019; Razek and Choroideremia, 2012). Using an iPSC approach, Kalatzis et al. demonstrated an under-prenylation of rab proteins in CHM-derived RPE (Nicolas et al., 2014). CHM-RPE lack REP1, but prenylation functions can be restored using AAV2/5-CAG-CHM. Similarly, Mills et al. observed lack of prenylation in CHM-RPE in addition to defects in phagocytic activity, phagolysosomal activation, and reduced Rab27a substrate prenylation (Duong et al., 2018, Table 7.3).

Bestrophinopathy (best disease)

Bestrophinopathies are a group of diseases that are caused by mutations in *Bestrophin* and result in retinal degeneration (Tingting et al., 2015). All four human paralogs of *Bestrophin* encode and function as calcium-activated anion channels. There is evidence that demonstrates *Bestrophin 2* (*BEST2*) mediates bicarbonate transport in colonic goblet cells, sweat glands, and nonpigmented epithelium (Kuai et al., 2010; Cui et al., 2012; Youwen et al., 2010; Golubinskaya et al., 2015). *Bestrophin 3* (*BEST3*) has been shown to play role in cellular protective services including: endoplasmic reticulum stress, oxidative stress, and inflammation (Wing-Kee et al., 2012; Lei et al., 2013; Wei et al., 2014; Adiv et al., 2018). *Bestrophin 1* (*BEST1*) is primarily expressed in RPE, an integral membrane protein, BEST1 functions as both an ion channel and regulator of intracellular calcium. There are >200 causative mutations

Table 7.2 Leber congenital amaurosis stem cell–derived models.

Inherited retinal dystrophy	Mutation(s)	Initial cell source	Reprogramming method	Studied system(s)	Observation(s)	Publication
LCA	*CEP 290* c.2991 + 1665A > G	Skin fibroblasts	Nonintegrating episomal vectors: Oct4, Sox2. Klf4, myc and shRNA to p53	*RPE, RO*	CEP290 was not detected at the connecting cilia, treatment with antisense morpholino (CEP290-M0) restored ciliogenesis in photoreceptors and RPE	David et al. (2016)
LCA	*CRB1* c.3122T > C c.I892A > G c.2843G > A	Skin fibroblasts	Lentiviral vectors	*RO*	CRB2 precedes CRB1 expression. CRB1 and CRB2 are located in photoreceptors and müller glia. Loss of CRB1 results in outer limiting membrane issues	Quinn et al. (2019)
LCA	*RPE65* c.200T > G c.430T > C	Urine	Nonintegrating episomal vectors	*RPE, RO*	LCA-RPE express lower levels of *RPE65* but otherwise were similar to control RPE.	Li et al. (2019)
LCA	*KCNJ13* c. 158G > A	Skin fibroblasts	Lentiviral vectors	*RPE*	LCA-RPE lack Kir7.1. LCA 16-RPE membranes were depolarized and had difficulty phagocytosing POS	Shahi et al. (2019)
LCA-type 10	*CEP290* c.2991 + 1665A > G	Skin fibroblasts	Nonintegrating episomal vectors	*RO*	LCA-RO exhibit reduced levels of *CEP290* mRNA. QR-110 antisense oligonucleotide restored wildtype *CEP290* mRNA	Kalyan et al. (2018)
LCA	*CEP 290* c.5668G > T c.1655A > G	Skin fibroblasts	Sendai virus	*RO*	LCA-RO display immature photoreceptor cilia.	Hiroko et al. (2017)

LCA, *leber congenital amaurosis;* mRNA, *messenger ribonucleic acid;* RO, *retinal organoid;* RPE, *retinal pigment epithelium.*

Table 7.3 Choroideremia stem cell–derived models.

Inherited retinal dystrophy	Mutation(s)	Initial cell source	Reprogramming method	Studied system(s)	Observation(s)	Publication
Choroideremia	*CHM*	Skin fibroblasts	Lentiviral vectors	*RPE*	CHM-RPE lack REP1 resulting in under-prenylation of rabs. AAV2/5- *CHM* restores biochemical phenotype	Nicolas et al. (2014)
Choroideremia	*CHM* c.1327_1328delAT exon 2–4 del Arg555STOP	Primary peripheral blood mononuclear cells	Sendai virus	*RPE*	CHM-RPE have reduced phagocytic activity and impaired intracellular trafficking and vesicular transport. AAV7m8-CHM can restore RPE function	Duong et al. (2018)

CHM, *choroideremia;* RPE, *retinal pigment epithelium.*

in *BEST1* that are associated with retinal degenerations. These collective bestrophinopathies are clinically classified as: Best vitelliform macular dystrophy (BVMD), adult-onset vitelliform macular dystrophy, autosomal recessive bestrophinopathy, and autosomal dominant vitreoretinochoroidopathy (Marmorstein et al., 2000). The most common form of the disease is *BVMD,* commonly referred to as Best disease, and presents itself through multiple stages with primary expression limited to the RPE (Adiv et al., 2018; Nachtigal et al., 2020). Patients initially display subtle abnormalities in RPE subsequently evolving into well-defined vitelliform lesions which worsen as the disease progresses. Patients tend to undergo an atrophic stage where choroidal neovascularization occurs and further formation of macular holes while concurrently experiencing a gradual loss of vision (Adiv et al., 2018). There are no current treatment options available for Best disease.

Several studies are underway utilizing patient-derived stem cells and the RPE generated (Table 7.4). Patient-derived Best diseased RPE provide an excellent tool to study and characterize pathogenic mechanisms. Weber et al. assessed Best1 diseased derived RPE harboring Best1 specific mutations and confirmed issues with anion transport and ER-associated degradation (Nachtigal et al., 2020). Gamm et al. observed buildup of autophagic material during long term photoreceptor outer segment feeding of Best1-RPE, suggesting complications in RPE degradation machinery. Additionally, Best1-RPE display stimulated calcium differences when compared to wildtype RPE and elevated oxidative stress (Ruchira et al., 2013). Several groups have arrived at similar conclusions: loss of Best1 in RPE results in Ca^{2+}-activated Cl-channel impairment (Li et al., 2017; Changyi et al., 2019; Cordes et al., 2020; Adiv et al., 2015). Interestingly this function can be rescued by restoring proper Best1 levels through viral-mediated supplementation, a promising gene therapy approach (Changyi et al., 2019; Lin et al., 2019).

Stargardt maculopathy

Stargardt maculopathy (STGD1) is a common autosomal recessive inherited macular dystrophy that is caused by mutations in ATP-binding cassette subfamily A member 4 (*ABCA4*). The disease roughly impacts 1 in 10,000 patients with >900 known causative mutations. ABCA4 is involved in active transport of retinoids across cell membranes. Lack of ABCA4 results in buildup of lipofuscin and toxic by-products in the RPE and photoreceptors, eventually causing abnormal cellular activity and cell death (Rahman et al., 2019; Kaoru et al., 2015; Rupert et al., 2016). Patients experience progressive bilateral central vision loss resulting in foveal sparing, distinct macular atrophy, and RPE abnormalities, including widespread buildup of lipofuscin in RPE—so called retinal flecks (Rahman et al., 2019). Because of the heterogeneity and wide array of disease-causing variants, patients exhibit varying degrees of the disease (Kaoru et al., 2015; Gerald et al., 1999). Typically, STGD1 patients experience mild reduced central vision with variable VA; however, because of macular involvement, VA tends to decrease as the patient progresses through life. The onset of the disease also directly affects disease severity, for example, early onset STGD1 results in greater vision loss (Preena et al., 2017). Albert et al. generated photoreceptor progenitors (PPs) from Stargardt patient iPSC (Table 7.5).

Table 7.4 Bestrophinopathies (BESTI) stem cell–derived models.

Inherited retinal dystrophy	Mutation(s)	Initial cell source	Reprogramming method	Studied system(s)	Observation(s)	Publication
BD-ADVIRC BD-ARB	16 patient unique mutations	Skin fibroblasts	Nonintegrating episomal vectors	*RPE*	Impaired BEST1-mediated anion transport in ARB-RPE. ADVIRC-RPE display increased anion permeability	Nachtigal et al. (2020)
BD-ADVIRC	D302A	Skin fibroblasts	Sendai virus	*RPE*	ADVIRC-RPE display reduced Cavl.3 channel activity	Cordes et al. (2020)
BD-ADVIRC	Patient 1: A146K Patient 2: N296H	Skin fibroblasts	Lentiviral vectors	*RPE*	ADVIRC-RPE display impaired phagosome degradation, calcium responses, oxidative stress, and endoplasmic reticulum calcium storage capabilities.	Ruchira et al. (2013)
BD-ADVIRC	Patient 1: A10T Patient 2: R218H Patient 3: L234P Patient 4: A243T Patient 5: Q293K Patient 6: D302A	Skin fibroblasts	Sendai virus	*RPE*	ADVIRC-RPE display impaired Ca^{2+}-dependent Cl channels. AAV-BEST1 restores function	Changyi et al. (2019)
BD-ADVIRC	Patient 1: P274R Patient 2: I201T	Skin fibroblasts	Sendai virus	*RPE*	BEST1 critically involved with Ca^{2+}-dependent Cl current. Function can be restored by Baculoviral-BEST1	Li et al. (2017)
BD-ADVIRC		Dental pulp	Retrovirus-mediated transduction with Oct3/4. Sox2. and Klf4	*RPE*	ADVIRC-RPE display reduced phagocytic capabilities and ZO-1 expression. Curcumin upregulated BEST1 and ZO-1 genes in ADVIRC –RPE	Lin et al. (2019)
BD-ADVIRC	R141H c.1098_1100+7del	Skin fibroblasts		*RPE*	ADVIRC-RPE display altered Ca^{2+}-dependent Cl channel activity	Adiv et al. (2015)

ADVIRC, *autosomal dominant vitreoretinochoroidopathy;* ARB, *autosomal recessive bestrophinopathy;* RPE, *retinal pigment epithelium.*

Table 7.5 Stargardt (STGDI) maculopathy stem cell–derived models.

Inherited retinal dystrophy	Mutation(s)	Initial cell source	Reprogramming method	Studied system(s)	Observation(s)	Publication
STGD1	*ABCA4* c.5461-10T- > C	Skin fibroblasts	Lentiviral vectors	*Photoreceptor progenitors* (*PPs*)	STGD1-PP ABCA4 *mRNA*, messenger ribonucleic acid (mRNA) lack exon 40 due to improper splicing	Riccardo et al. (2016)

STDG1-PP frequently resulted in exon 39 and/or exon 40 skipping resulting in truncated ABCA4. The authors suggest ABCA4 function may be impaired due to improper splicing, but further evidence must be provided to sufficiently defend this proposed mechanism of action (Riccardo et al., 2016).

Conclusion and caveats

iPSC disease models are proving useful in identifying and studying pathognomonic mechanisms of several IRDs. Although IRD-iPSC research is promising, there are complications and common pitfalls that should be addressed (Camille et al., 2015). Natural and mutational genetic heterogeneity is a difficult barrier to overcome when designing an appropriate study. How does one control for patient to patient genetic variability? These issues can be addressed using deep sequencing technologies, gender and age matched control groups. Indeed, an increasing number of studies have incorporated properly matched control cell lines that appropriately allow for statistical comparisons. Furthermore, research groups have started to consider protocol heterogeneity when generating retinal cells. For example, Gamm et al. assessed the reproducibility of ROs during development. The authors surmise that ROs should be characterized during specific stages rather than days in culture, a preventative measure to ensure results are conclusive and not due to RO-RO variability (Capowski et al., 2019). Despite this restriction, patient-derived ROs are increasingly becoming "star players" in the effort to characterize difficult to study IRDs. The "miniature eye in a dish" can effectively reflect a nearly intact retina and allow for functional studies, such as monitoring electrophysiological activities in response to stimuli. Furthermore, ROs can be used to study potential therapies such as gene augmentation, small molecules, and biologics (Castro et al., 2019). One can envision a future where researchers employ even more complex patient-derived iPSC ocular tissue constructs such as retinal organoids with RPE, RPE-choroid, or RO_RPE_choroid models. We believe that this future is not only possible but very likely to become the gold standard.

References

Adiv, J.A., Lori, B.A., Benjamin, G.J., Samuel, C.D., Kimberly, S.E., Resch, Zachary, T., Marmorstein, L.y., et al., 2015. Autosomal recessive bestrophinopathy is not associated with the loss of Bestrophin-1 anion channel function in a patient with a novel BEST1 mutation. Invest. Ophthalmol. Vis. Sci. 56, 4619−4630. https://doi.org/10.1167/iovs.15-16910.

Adiv, J.A., Karina, G.E., Justin, L.C., Ravi, K.C., Jose, P.S., Marmorstein, Y., et al., 2018. Bestrophin 1 and retinal disease. Prog. Retin. Eye Res. 58, 45−69. https://doi.org/10.1016/j.preteyeres.2017.01.006.Bestrophin.

Adriana, B., Lili, Z., Valeria, C., Basudha, B., Sina, M.-J., David, D., et al., 2018. Disrupted alternative splicing for genes implicated in splicing and ciliogenesis causes PRPF31 retinitis pigmentosa. Nat. Commun. 9, 4234. https://doi.org/10.1038/s41467-018-06448-y.

Alina, O., Jie, H., David, B.C., Larry, T.A., 2016. Tissue growth constrained by extracellular matrix drives invagination during optic cup morphogenesis. Biomech. Model. Mechanobiol. 15, 1405−1421. https://doi.org/10.1007/s10237-016-0771-8.

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C., Harris, C.P., Waknitz, M.A., et al., 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev. Biol. 227, 271−278. https://doi.org/10.1006/dbio.2000.9912.

Ana, C.A., Dunja, L., Jendelova, P., Erceg, S., 2018. Concise review: human induced pluripotent stem cell models of retinitis pigmentosa. Stem Cell. 36, 474−481. https://doi.org/10.1002/stem.2783.

Arshavsky, V.Y., Burns, M.E., 2014. Current understanding of signal amplification in phototransduction. Cell. Logist. 4, e29390.

Brambati, M., Borrelli, E., Sacconi, R., Bandello, F., Querques, G., 2019. Choroideremia: update on clinical features and emerging treatments. Clin. Ophthalmol. 13, 2225−2231.

Briang, H.P., 2017. Clinical progress in inherited retinal degenerations: gene therapy clinical trials and advances in genetic sequencing. Retina 37, 417−423. https://doi.org/10.1097/IAE.0000000000001341 (Clinical).

Brydon, E., Bronstein, R., Buskin, A., Lako, M., Pierce, E., Fernandez-Godino, R., 2019. AAV-mediated gene augmentation therapy restores critical functions in mutant PRPF31+/- iPSC-derived RPE cells. Mol. Ther. Methods Clin. Dev. 15, 392−402. https://doi.org/10.1016/j.omtm.2019.10.014.

Bucholz, D.E., Hikita, S.T., Rowland, T.J., Friedrich, A.M., Hinman, C.R., Johnson, L.V., et al., 2009. Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. Stem Cell. 27, 2427−2434. https://doi.org/10.1002/July.

Budd, T.A., Robert, M.F., Luan, S.M., Kristin, A., Mari, E.E., Emily, K., et al., 2013. Patient-specific iPSC-derived photoreceptor precursor cells as a means to investigate retinitis pigmentosa. Elife 2, e00824. https://doi.org/10.7554/eLife.00824.

Cajal, S.R., 1984. Die Retina der Wirbelthiere: Untersuchungen mit der Golgi-Cajalschen Chromsilbermethode und der Ehrlichschen Methylenblaufarbung. Wiesbaden: Bergmann.

Camille, Y., Connor, R.M., Amelia, L., Michael, P.B., Lyndon da, C., Peter, C.J., et al., 2015. Using stem cells to model diseases of the outer retina. Comput. Struct. Biotechnol. J. 13, 382−389. https://doi.org/10.1016/j.csbj.2015.05.001.

Capowski, E.E., Samimi, K., Mayerl, S.J., Phillips, M.J., Pinilla, I., Howden, S.E., et al., 2019. Reproducibility and Staging of 3D Human Retinal Organoids across Multiple Pluripotent Stem Cell Lines. https://doi.org/10.1242/dev.171686.

Castro, A.A., Rodriguez, F.J.R., Jendelova, P., Erceg, S., 2019. Deciphering retinal diseases through the generation of three-dimensional stem cell-derived organoids: concise review. Stem Cell. 37, 1496−1504. https://doi.org/10.1002/stem.3089.

Chacon-camacho, O.F., Zenteno, J.C., 2015. Review and update on the molecular basis of Leber congenital amaurosis. World J. Clin. Cases. 3, 112−124. https://doi.org/10.12998/wjcc.v3.i2.112.

Changyi, J., Li, Y., Kittredge, A., Hopiavuori, A., Ward, N., Yao, P., et al., 2019. Investigation and restoration of BEST1 activity in patient-derived RPEs with dominant mutations. Sci. Rep. 9. https://doi.org/10.1038/s41598-019-54892-7.

Christine, C.A., 2005. Imaging maculopathy in post-mortem human eyes. Vis. Res. 45, 3496−3503. https://doi.org/10.1016/j.visres.2005.07.038.

Christopher, B.L., Martin, P.F., 2018. Review capturing totipotent stem cells. Cell Stem Cell 22, 25−34. https://doi.org/10.1016/j.stem.2017.12.011.

Cideciyan, A., Jacobson, S., Drack, A., Ho, A., Charng, J., Garafalo, Roman, A., Alejandro Sumaroka, A., et al., 2019. Effect of an intravitreal antisense oligonucleotide on vision in Leber congenital amaurosis due to a photoreceptor cilium defect. Nat. Med. 25, 225−228. https://doi.org/10.1038/s41591-018-0295-0.

Cordes, M., Bucichowski, P., Alfaar, A.S., Tsang, S.H., Almedawar, S., Reichhart, N., et al., 2020. Inhibition of Ca^{2+} channel surface expression by mutant Bestrophin-1 in RPE cells. FASEB J. 34, 4055−4071. https://doi.org/10.1096/fj.201901202RR.

Cui, C.-Y., Victoria, C., Yulan, P., Marc, M., Adiv, J.A., Makoto, K., et al., 2012. Forkhead transcription factor FoxA1 regulates sweat secretion through Bestrophin 2 anion channel and Na-K-Cl cotransporter 1. Proc. Natl. Acad. Sci. U. S. A. 109, 1199−1203. https://doi.org/10.1073/pnas.1117213109.

David, B.E., Britney, P.O., Roxanne, C.H., Cassidy, H.R., Peter, C.J., Clegg, D.O., 2013. Rapid and efficient directed differentiation of human pluripotent stem cells into retinal pigmented epithelium. Stem Cells Transl. Med. 2, 384−393.

David, P.A., Amelia, L., Conor, R.M., Carr, Amanda-Jayne, Munro, P., Jovanovic, K., Schwarz, N., Naheed, K., et al., 2016. Identification and correction of mechanisms underlying inherited blindness in human iPSC- identification and correction of mechanisms underlying inherited blindness in human iPSC-derived optic cups. Cell Stem Cell 18, 769−781. https://doi.org/10.1016/j.stem.2016.03.021.

Deepak, L.A., Mike, K.O., Carol, W.B., Thomas, R.A., 2006. Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 103, 12769−12774.

Demb, J.B., Singer, J.H., 2015. Functional circuitry of the retina. Annu. Rev. Vis. Sci. 1, 263−291. https://doi.org/10.1146/annurev-vision-082114-035334.

Duong, T.T., Vasireddy, V., Ramachandran, P., Herrera, P.S., Leo, L., Merkel, C., et al., 2018. Use of induced pluripotent stem cell models to probe the pathogenesis of Choroideremia and to develop a potential treatment, 27, 140−150.

Ethan, R.A., Charles, G.E., Robin, S., Qiang, Y., Kenichi, S., Christina, S., et al., 2017. Imaging individual neurons in the retinal ganglion cell layer of the living eye. Proc. Natl. Acad. Sci. U. S. A. 114, 586−591. https://doi.org/10.1073/pnas.1613445114.

Fligor, C.M., Langer, K.B., Yuan, R., Akshayalakshmi, S., Shields, P.K., Edler, M.C., et al., 2018. Three-dimensional retinal organoids facilitate the investigation of retinal ganglion cell development, organization and neurite outgrowth from human pluripotent stem cells. Sci. Rep. 8, 14520. https://doi.org/10.1038/s41598-018-32871-8.

Foltz, L.P., Howden, S.E., Thomson, J.A., Clegg, D.O., 2018. Functional assessment of patient-derived retinal pigment epithelial cells edited by CRISPR/Cas9. Int. J. Mol. Sci. 19, 1−17. https://doi.org/10.3390/ijms19124127.

Fronk, A.H., Vargis, E., 2016. Methods for culturing retinal pigment epithelial cells: a review of current protocols and future recommendations. J. Tissue Eng. 7, 1−23. https://doi.org/10.1177/2041731416650838.

Fumitaka, O., Hanako, I., Michiko, M., Takafumi, W., Kiichi, W., Nagahisa, Y., et al., 2008. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat. Biotechnol. 26, 215−224. https://doi.org/10.1038/nbt1384.

Fumitaka, O., Zi-Bing, J., Yasuhiko, H., Hanako, I., Teruko, D., Kiichi, W., et al., 2009. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J. Cell Sci. 122, 3169−3179. https://doi.org/10.1242/jcs.050393.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., Hasegawa, M., 2009. Effcient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 85, 348−362. https://doi.org/10.2183/pjab.85.348.

Gao, M.-L., Lei, X.-L., Han, F., He, K.-W., Jin, S.-Q., Zhang, Y.-Y., et al., 2020. Patient-specific retinal organoids recapitulate disease features of late-onset retinitis pigmentosa. Front. Cell Dev. Biol. 8, 1−14. https://doi.org/10.3389/fcell.2020.00128.

Gerald, F.A., Edwin, S.M., Sandeep, G., Deborah, D.J., Heidi, H.L., Robin, H.R., 1999. Variation of clinical expression in patients with Stargardt dystrophy and sequence variations in the ABCR gene. Arch. Ophthalmol. 117, 504−510.

Golubinskaya, V., Elvin, J., Ebefors, K., Gustaffson, H., Mallard, C., Nystorm, J., et al., 2015. Bestrophin-3 is differently expressed in normal and injured mouse glomerular podocytes. Acta Physiol. 214, 481−496. https://doi.org/10.1111/apha.12516.

Gray, D.C., Merigan, W., Wolfing, J.I., Gee, B.P., Dubra, A., Twietmeyer, T.H., et al., 2006. In vivo fluorescence imaging of primate retinal ganglion cells and retinal pigment epithelial cells. Opt. Express 14, 7144−7158.

Guo, Y., Wang, P., Hongjie Ma, J., Cui, Z., Yu, Q., Liu, S., et al., 2019. Modeling retinitis pigmentosa: retinal organoids generated from the iPSCs of a patient with the USH2A mutation show early developmental abnormalities. Front. Cell. Neurosci. 13, 1−17. https://doi.org/10.3389/fncel.2019.00361.

Hanako, I., Fumitaka, O., Kiichi, W., Kenji, M., Tomoko, H., Hiroyuki, M., et al., 2005. Generation of Rx+/Pax6+neural retinal precursors from embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 102, 11331−11336.

Hartman, P., Beckman, K., Silverstein, K., Yohe, S., Schomaker, M., Henzler, C., et al., 2019. Next generation sequencing for clinical diagnostics: five year experience of an academic laboratory. Mol. Genet. Metab Rep. 19, 100464. https://doi.org/10.1016/j.ymgmr.2019.100464.

Heavner, W., Pevny, P., 2012. Eye development and retinogenesis. Cold Spring Harb. Perspect. Biol. 4, 1−17.

Henry, K., Boback, Z., Ivan, K.I., Young, M.J., Philip, S.H., 2004. Isolation of retinal progenitor cells from post-mortem human tissue and comparison with autologous brain progenitors. J. Neurosci. Res. 77, 334−343. https://doi.org/10.1002/jnr.20183.

Hiroko, S., Quanlong, L., Christine, I.-K., Nagashima, K., Milton, E.A., Semler, E., et al., 2017. In vitro modeling using ciliopathy-patient-derived cells reveals distinct cilia dysfunctions caused by article in vitro modeling using ciliopathy-patient-derived cells reveals distinct cilia dysfunctions caused by CEP290 mutations. Cell Rep. 20, 384−396. https://doi.org/10.1016/j.celrep.2017.06.045.

Hiroshi, K., Hirofumi, S., Kenji, M., Kiichi, W., Fumi, U., Hiroshi, I., et al., 2001. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proc. Natl. Acad. Sci. U. S. A. 99, 1580−1585.

Hiyama, E., Hiyama, K., 2007. Telomere and telomerase in stem cells. Br. J. Canc. 96, 1020−1024. https://doi.org/10.1038/sj.bjc.6603671.

Hoon, M., Okawa, H., Della Santina, L., Wong, R.O., 2014. Functional architecture of the retina: development and disease. Prog. Retin. Eye Res. 42, 44−84. https://doi.org/10.1016/j.preteyeres.2014.06.003.Functional.

Hu, Q., Friedrich, A.M., Johnson, L.V., Clegg, D.O., 2010. Memory in induced pluripotent stem cells: reprogrammed human retinal-pigmented epithelial cells show tendency for spontaneous redifferentiation. Stem Cell. 28, 1981−1991. https://doi.org/10.1002/stem.531.

Huang, C.-Y., Liu, C.-L., Chiu, Y.-T., Cheng, Y.-C., Nicholson, M.W., Hsieh, P.C., 2019. Human iPSC banking: barriers and opportunities. J. Biomed. Sci. 26, 87−101.

Irina, K., Jason, H., Kourous, R.A., Michael, W., Atala, A., Robert, L., 2004. Derivation and comparative assessment of retinal stem cells using transcriptomics. Clon Stem Cell 6, 217−245.

Jacobson, S.G., Cideciyan, A., Alexander, S., Tomas, A.S., Sharon, S.B., Windsor, E.A., Roman, J.A., Edwin, S.M., et al., 2006. Remodeling of the human retina in choroideremia: rab escort protein 1 (REP-1) mutations. Invest. Ophthalmol. Vis. Sci. 47, 3−10. https://doi.org/10.1167/iovs.06-0424.

Jason, M.S., Rebecca, S.L., Elizabeth, C.E., Lynda, W.S., Kyle, W.A., Erin, M.L., et al., 2009. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A. 106, 16698−16703.

Jin, Z.-B., Satoshi, O., Ping, X., Masayo, T., 2012. Integration-free induced pluripotent stem cells derived from retinitis pigmentosa patient for disease modeling. Stem Cells Transl. Med. 1, 503−509.

John, G.A., Chi-Chao, C., 2007. Biopsy techniques and yields in diagnosing primary intraocular lymphoma. Int. Ophthalmol. 27, 241−250.

Joshua, S.R., Richard, M.H., 2015. The types of retinal ganglion cells: current status and implications for neuronal classification. Annu. Rev. Neurosci. 8, 221−246. https://doi.org/10.1146/annurev-neuro-071714-034120.

Joshua, B.M., Serup, P., 2017. Properties of embryoid bodies. Wiley Interdiscip. Rev. Dev. Biol. 6, e259. https://doi.org/10.1002/wdev.259.

Junying, Y., Maxim, V.A., Kim, S.-O., Jennifer, A.-B., Frane, J.L., Shulan, T., Jeff, N., et al., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917−1920. https://doi.org/10.1097/01.ogx.0000305193.72586.39.

Kalyan, D., Monica, A., Amelia, L., Katarina, J., David, P.A., Iris, S., et al., 2018. Splice-modulating oligonucleotide QR-110 restores CEP290 mRNA and function in human c.2991+1655A>G LCA10 models. Mol. Ther. Nucleic Acids 12, 730−740. https://doi.org/10.1016/j.omtn.2018.07.010.

Kang, Z., Ding, S., 2011. Stem cells and eye development. N. Engl. J. Med. 365, 370−372. https://doi.org/10.1056/NEJMcibr1105280.Stem.

Kaoru, F., Jana, Z., Ravinder, C.K., Wright, G.A., Tsunoda, K., Yoko, O., et al., 2015. Clinical and molecular characteristics of childhood-onset Stargardt disease. Ophthalmology 122, 326−334. https://doi.org/10.1016/j.ophtha.2014.08.012.Clinical.

Karl, W.J., Maruotti, J., Donald, Z.J., 2014. Modeling retinal dystrophies using patient-derived induced pluripotent stem cells. Adv. Exp. Med. Biol. 801, 157−164. https://doi.org/10.1007/978-1-4614-3209-8.

King, N.M., Perrin, J., 2014. Ethical issues in stem cell research and therapy. Stem Cell Res. Ther. 5, 1−7.

Knut, W., Lacovos, M.P., Mohseni, P., Desai, R., Mileikovsky, M., Hämäläinen, R., et al., 2013. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458, 766−770. https://doi.org/10.1038/nature07863.piggyBac.

Koenekoop, R.K., 2004. An overview of leber congenital amaurosis: a model to understand human retinal development. Surv. Ophthalmol. 49, 379–398. https://doi.org/10.1016/j.survophthal.2004.04.003.

Kuai, Y., Rafael, L., Alan, M., Sherif, G., Criss, H.H., 2010. Bestrophin-2 mediates bicarbonate transport by goblet cells in mouse colon. J. Clin. Invest. 120, 1722–1735. https://doi.org/10.1172/JCI41129.

Lei, J., Yun, L., Ma, M.-M., Yong-Bo, T., Jia-Gou, Z., Yong-Yuan, G., 2013. Mitochondria dependent pathway is involved in the protective effect of Bestrophin-3 on hydrogen peroxide-induced apoptosis in basilar artery smooth muscle cells. Apoptosis 18, 556–565. https://doi.org/10.1007/s10495-013-0828-4.

Li, Y., Zhang, Y., Xu, Y., Kittredge, A., Ward, N., Shoudeng, C., et al., 2017. Patient-specific mutations impair BESTROPHIN1's essential role in mediating Ca^{2+} -dependent Cl- currents in human RPE. Elife 1–23.

Li, G., Gao, G., Wang, P., Song, X., Ping, X., Xie, B., et al., 2019. Generation and characterization of induced pluripotent stem cells and retinal organoids from a leber's congenital amaurosis patient with novel RPE65 mutations. Front. Mol. Neurosci. 12, 1–16. https://doi.org/10.3389/fnmol.2019.00212.

Lin, T.-C., Lin, Y.-Y., Hsu, C.-C., Yang, Y.-P., Yang, C.-H., Hwang, D.-K., et al., 2019. Nanomedicine-based curcumin approach improved ROS damage in best dystrophy-specific induced pluripotent stem cells. Cell Transplant. 28, 1345–1357. https://doi.org/10.1177/0963689719860130.

Lobier, M., Dubois, M., Valdois, S., 2013. The role of visual processing speed in reading speed development. PloS One 8, e58097. https://doi.org/10.1371/journal.pone.0058097.

Lori, S.S., Daiger, S.P., 1996. Inherited retinal degeneration: exceptional genetic and clinical heterogeneity. Mol. Med. Today 2, 380–386.

Lowry, W.E., Plath, K., 2008. The many ways to make an iPS cell. Nat. Biotechnol. 26, 1246–1248.

Luisa, M., Stefano, F., Pellegrino, C., Stella, G., Diego, P., Orsoni, J.G., et al., 2013. Human RNA integrity after postmortem retinal tissue recovery. Ophthalmic Genet. 34, 27–31. https://doi.org/10.3109/13816810.2012.720342.

Lyndsay, L.L., David, B.E., Vignesh, N.P., Stefan, L.E., Clegg, D.O., 2015. Canonical/b-Catenin Wnt pathway activation improves embryonic stem cells. Invest. Ophthalmol. Vis. Sci. 56, 1002–1013. https://doi.org/10.1167/iovs.14-15835.

Marchetto, M.C., Gene, Y.W., Kainohana, O., Marsala, M., Gage, F.H., Muotri, A.R., 2009. Transcriptional signature and memory retention of human-induced pluripotent stem cells. PloS One 4, e7076. https://doi.org/10.1371/journal.pone.0007076.

Maria, I., Ruslana, A., Alexey, O., Etti, B.-S., Itzhak, H., Yachimovich-Cohen, Nurit Khaner, H., et al., 2009. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. Cell Stem Cell 5, 396–408. https://doi.org/10.1016/j.stem.2009.07.002.

Maria, S.L., Scott, B.E., Artur, C.V., Wanda, P.L., Elizabeth, K.L., Edwin, S.M., Jacobson, G., Samuel, D.V.A., 2018. Expanded retinal disease spectrum associated with autosomal recessive mutations in GUCY2D. Am. J. Ophthalmol. 190, 58–68. https://doi.org/10.1016/j.ajo.2018.03.021.

Marmorstein, A.D., Marmorstein, L.Y., Mary, R., Wang, W.X., Joe, H.G., Bestrophin, K.P., 2000. The product of the Best vitelliform macular dystrophy gene (VMD2), localizes to the basolateral plasma membrane of the retinal pigment epithelium. Proc. Natl. Acad. Sci. U. S. A. 97, 12758–12763.

Martin, G.R., 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Develop. Biol. Proc. Natl. Acad. Sci. U. S. A. 78, 7634−7638.

Masatoshi, H., Yoshiki, S., Hiroshi, K., Kaori, A., Sotaro, O., Masaaki, K., et al., 2004. In vitro and in vivo characterization of pigment epithelial cells differentiated from primate embryonic stem cells. Invest. Ophthalmol. Vis. Sci. 45, 1020−1025. https://doi.org/10.1167/iovs.03-1034.

Masland, R.H., 2001. The fundamental plan of the retina. Nat. Neurosci. 4, 877−886.

Megaw, R., Abu-Arafeh, H., Jungnickel, M., Mellough, C., Gurniak, C., Witke, W., et al., 2017. Gelsolin dysfunction causes photoreceptor loss ininduced pluripotent cell and animal retinitispigmentosa models. Nat. Commun. 8, 1−9. https://doi.org/10.1038/s41467-017-00111-8.

Michael, F.H., Deborah, L.S., Maria, S.E., Bujakowska, Chatagnon, K., Johnathan, Battacharyaa, S.S., Eric, P.A., Emeline, N.F., 2014. Mutations in pre-mRNA processing factors 3, 8, and 31 cause dysfunction of the retinal pigment epithelium. Am. J. Pathol. 184, 2641−2652. https://doi.org/10.1016/j.ajpath.2014.06.026.

Miguel, S.C., Michael, B.S., Clive, S.A., Thomas, S.C., Joseph, G.L., 1992. Purification of component a of rab geranylgeranyl transferase: possible identity with the choroideremia gene product. Cell 70, 1049−1057.

Monica, A.J., Michelle, P.L.E., Gronowski, S.K., Ann, G.M., 2010. Human tissue ownership and use in research: what laboratorians and researchers should know. Clin. Chem. 56, 1675−1682. https://doi.org/10.1373/clinchem.2010.150672.

Morrison, S.J., Shah, N.M., Anderson, D.J., 1997. Regulatory mechanisms in stem cell biology. Cell 88, 287−298.

Moshiri, A., Chen, R., Kim, S., Harris, A.R., Li, Y., Muthuswamy, Raveendran Sarah, D., et al., 2019. A nonhuman primate model of inherited retinal disease. J. Clin. Invest. 129, 863−874.

Mototsugu, E., Nozomu, T., Hiroki, I., Masako, K., Eriko, S., Satoru, O., et al., 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472, 51−56. https://doi.org/10.1038/nature09941.

Moyra, S., 2017. DNA sequence analysis in clinical medicine, proceeding cautiously. Front. Mol. Biosci. 4, 1−14. https://doi.org/10.3389/fmolb.2017.00024.

Nachtigal, A.-L., Milenkovic, A., Brandl, C., Schulz, H.L., Duerr, L.M.J., Lang, G.E., Reiff, C., et al., 2020. Mutation-dependent pathomechanisms determine the phenotype in the bestrophinopathies. Int. J. Mol. Sci. 21, 1−20.

Nakano, T., Ando, S., Takata, N., Kawada, M., Muguruma, K., Sekiguchi, K., et al., 2012. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10, 771−785. https://doi.org/10.1016/j.stem.2012.05.009.

Nicolas, C., Marie, P.O., Lorenne, R., Fabienne, B., Valerie, D.L., Nicolas, N., et al., 2014. Proof of concept for AAV2/5-mediated gene therapy in iPSC- derived retinal pigment epithelium of a choroideremia patient. Mol. Ther. Methods Clin. Dev. 2, 14011. https://doi.org/10.1038/mtm.2014.11.

Pang, C.P., 2016. Molecular genomics of eye diseases. Asia-Pacif. J. Ophthalmol. 5, 225−226. https://doi.org/10.1097/APO.0000000000000221.

Parul, C., Heather, B., Alex, G., Beata, S., Irene, L., Juliette, S., et al., 2017. Directing differentiation of pluripotent stem cells toward retinal pigment epithelium lineage. Stem Cells Transl. Med. 6, 490−501.

Pedro, C.C., Nance, N.B., 2011. Biology of stem cells: an overview. Kidney Int. Suppl. 1, 63−67. https://doi.org/10.1038/kisup.2011.15.

Peichl, L., 2005. Diversity of mammalian photoreceptor properties: adaptations to habitat and lifestyle? Anat. Rec. Discov. Mol. Cell. Evol. Biol. 287, 1001−1012. https://doi.org/10.1002/ar.a.20262.

Picaud, S., Dalkara, D., Marazova, K., Goureau, O., Roska, B., José-Alain, S., 1996. The primate model for understanding and restoring vision. Proc. Natl. Acad. Sci. U. S. A. 116, 26280−26287. https://doi.org/10.1073/pnas.1902292116.

Preena, T., Rupert, S.W., Kaoru, F., Michel, M., 2017. Stargardt disease: clinical features, molecular genetics, animal models and therapeutic options. Br. J. Ophthalmol. 101, 25−30. https://doi.org/10.1136/bjophthalmol-2016-308823.

Preising, M.M., Ayuso, C., 2004. Rab escort protein 1 (REP1) in intracellular traffic: a functional and pathophysiological overview. Ophthalmic Genet. 25, 101−110. https://doi.org/10.1080/13816810490514333.

Quinn, P., Buck, T., Mulder, A., Ohonin, C., Alves, H., Vos, R., et al., 2019. Human iPSC-derived retinas recapitulate the fetal CRB1 CRB2 ComplexFormation and demonstrate that photoreceptors and muller glia are targetsof AAV5. Stem Cell Rep. 12, 906−919. https://doi.org/10.1016/j.stemcr.2019.03.002.

Rahman, N., Georgiou, M., Khan, K.N., Michaelides, M., 2019. Macular dystrophies: clinical and imaging features, molecular genetics and therapeutic options. Br. J. Ophthalmol. 104, 451−460. https://doi.org/10.1136/bjophthalmol-2019-315086.

Ramalho-santos, M., Willenbring, H., 2007. Commentary on the origin of the term "stem cell.". Cell Stem Cell 1, 35−38. https://doi.org/10.1016/j.stem.2007.05.013.

Ramsden, Conor Nommiste, B., Amelia, L.R., Carr, A.-J.F., Powner, M., Matthew, S.J.K., Li, C.L., Muthiah, M., Webster, A., et al., 2017. Rescue of the MERTK phagocytic defect in a human iPSC disease model using translational read- through inducing drugs. Sci. Rep. 7, 1−9. https://doi.org/10.1038/s41598-017-00142-7.

Razek, C.G., Traboulsi, E.I., 2012. Choroideremia: a review of general findings and pathogenesis. Ophthalmic Genet. 33, 57−65. https://doi.org/10.3109/13816810.2011.620056.

Riccardo, S., Nathalie, B.M., Miriam, B., Ingeborh van den, B.L., Elfride, D.B., Garanto, A., et al., 2016. Photoreceptor progenitor mRNA analysis reveals exon skipping resulting from the ABCA4 c.5461-10t/C mutation in Stargardt disease. Ophthalmology 123, 1375−1385. https://doi.org/10.1016/j.ophtha.2016.01.053.

Robert, S.A., Karthikeyan, B., Rosanne, K.B., Jenina, C.E., Alex, L.V., 2015. Autosomal-dominant leber congenital amaurosis caused by a heterozygous CRX mutation in a father and son. Ophthalmic Genet. 36, 156−159. https://doi.org/10.3109/13816810.2013.838273.

Ruchira, S., Wei, S., David, K., Jessica, M.M., Ziangrong, G., Molly, S.A., et al., 2013. iPS cell modeling of Best disease: insights into the pathophysiology of an inherited macular degeneration. Hum. Mol. Genet. 22, 593−607. https://doi.org/10.1093/hmg/dds469.

Rupert, S.W., Alex, H., Beatriz, M., Arthur, C.V., Jose-Alain, S., Janet, S.S., et al., 2016. The natural history of the progression of atrophy secondary to Stargardt disease (ProgStar) studies design and baseline characteristics: ProgStar report no. 1. Ophthalmology 123, 817−828. https://doi.org/10.1016/j.ophtha.2015.12.009.

Sanjurjo-Soriano, C., Nejla, E., Baux, D., Mamaeva, D., Hamel, C.P., Meunier, I., Roux, A.-F., Kalatzis, V., 2020. Genome editing in patient iPSCs corrects the most prevalent USH2A mutations and reveals intriguing mutant mRNA expression profiles. Mol. Ther. Methods Clin. Dev. 17, 156−173. https://doi.org/10.1016/j.omtm.2019.11.016.

Schwarz, N., Lane, A., Jovanovic, K., Parfitt, D.A., Aguila, M., Thompson, C.L., et al., 2017. Arl3 and RP2 regulate the trafficking of ciliary tip kinesins. Hum. Mol. Genet. 26, 2480−2492. https://doi.org/10.1093/hmg/ddx143.

Seung, K.U., Hiroshi, T., 1988. Tissue culture study of adult human retina neurons. Invest. Ophthalmol. Vis. Sci. 29, 1372−1379.

Shahi, P., Hermans, D., Sinha, D., Brar, S., Moulton, H., Stulo, S., et al., 2019. Gene augmentation and readthrough rescue channelopathy in an iPSC-RPE model of congenital blindness. Am. J. Hum. Genet. 104, 310−318. https://doi.org/10.1016/j.ajhg.2018.12.019.

Sharola, D., Eduardo, S., Ana, L.P., Ying, Y.L., Jun-Ming, Y., Colin, C.R., et al., 2000. Mutational analysis and clinical correlation in Leber congenital amaurosis. Ophthalmic Genet. 21, 135−150.

Shobi, V., Lazar, C.H., Chang, B., Paul, S.A., Eyal, B., Swaroop, A., 2015. Biology and therapy of inherited retinal degenerative disease: insights from mouse models. Dis. Model. Mech. 8, 109−129. https://doi.org/10.1242/dmm.017913.

Shufend, Z., Anthony, F., Mohamed, A., Nicolas, T., Andrea, B., Wadhwa, S., et al., 2015. Differentiation of human embryonic stem cells into cone photoreceptors through simultaneous inhibition of BMP, TGFβ and Wnt signaling. Development 142, 3294−3306. https://doi.org/10.1242/dev.125385.

Singh, M., Tyagi, S.C., 2018. Genes and genetics in eye diseases: a genomic medicine approach for investigating hereditary and inflammatory ocular disorders. Int. J. Ophthalmol. 11, 117−134. https://doi.org/10.18240/ijo.2018.01.20.

Stefano, F., Di, I.E., Vanessa, B., Diego, P., Sorrentinto, F.S., Parmeggiani, F., 2011. Retinitis pigmentosa: genes and disease mechanisms. Curr. Genom. 12, 238−249. https://doi.org/10.2174/138920211795860107.

Stephen, D.P., Sara, B.J., Lori, S.S., 2008. Perspective on genes and mutations causing retinitis pigmentosa. Arch. Ophthalmol. 125, 151−158. https://doi.org/10.1001/archopht.125.2.151.Perspective.

Stephen, D.P., Sara, B.J., Lori, S.S., 2014. Genes and mutations causing autosomal dominant retinitis pigmentosa. Cold Spring Harb. Perspect. Med. 5, 1−14.

Strauss, O., 2005. The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845−881. https://doi.org/10.1152/physrev.00021.2004.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663−676. https://doi.org/10.1016/j.cell.2006.07.024.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861−872. https://doi.org/10.1016/j.cell.2007.11.019.

Thompson, J., De Roach, J., McLaren, T., Lamey, T., 2018. A mini-review: leber congenital amaurosis: identification of disease-causing variants and personalised therapies. Adv. Exp. Med. Biol. 1074, 265−271.

Thomson, J.A., Itskovitz-eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., et al., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145−1147.

Tingting, Y., Sally, J., Yao, L., Stephen, T.H., 2015. BEST1: the best target for gene and cell therapies. Mol. Ther. 23, 1805−1809. https://doi.org/10.1038/mt.2015.177.

Vasiliki, K., Christian, H.P., Ian, M.M., 2013. Choroideremia: towards a therapy. Am. J. Ophthalmol. 156, 433−437. https://doi.org/10.1016/j.ajo.2013.05.009.

Wei, S., Zhen, Y., Ben, H., 2014. Bestrophin 3 ameliorates TNFα-induced inflammation by inhibiting NF-κB activation in endothelial cells. PloS One 9, e111093. https://doi.org/10.1371/journal.pone.0111093.

Wen-Li, D., Mei-Ling, G., Xin-Lan, L., Ji-Neng, L., Huan, Z., Kai-Wen, H., et al., 2018. Gene correction reverses ciliopathy and photoreceptor loss in iPSC-derived retinal organoids from retinitis pigmentosa patients. Stem Cell Rep. 10, 1267−1281. https://doi.org/10.1016/j.stemcr.2018.02.003.

Wert, G De, Mummery, C., 2003. Human embryonic stem cells: research, ethics and policy. Hum. Reprod. 18, 672−682. https://doi.org/10.1093/humrep/DEG143.

West, E.L., Joana, R., Ali, R.R., 2019. Development of stem cell therapies for retinal degeneration. Cold Spring Harb. Perspect. Biol. https://doi.org/10.1101/cshperspect.a035683.

Wing-Kee, L., Prabir, C.K., Eleni, R., Natascha, W.A., Frank, T., 2012. ERK1/2-dependent Bestrophin-3 expression prevents ER-stress-induced cell death in renal epithelial cells by reducing CHOP. Biochim. Biophys. Acta 1823, 1864−1876. https://doi.org/10.1016/j.bbamcr.2012.06.003.

Xing, Z., Jianuo, L., Iqbal, A., 2002. Differentiation of embryonic stem cells into retinal neurons. Biochem. Biophys. Res. Commun. 297, 177−184.

Yin, L., Masella, B., Dalkara, D., Zhang, J., Flannery, J.G., Schaffer, D.V., et al., 2014. Imaging light responses of foveal ganglion cells in the living macaque eye. J. Neurosci. 34, 6596−6605. https://doi.org/10.1523/JNEUROSCI.4438-13.2014.

Yoshida, T., Ozawa, Y., Suzuki, K., Ohyama, M., Akamatsu, W., Matsuzaki, Y., et al., 2014. The use of induced pluripotent stem cells to reveal pathogenic gene mutations and explore treatments for retinitis pigmentosa. Mol. Brain 7, 1−11.

Youwen, Z., Rajkumar, Patil, V., Marmorstein, A.D., 2010. Bestrophin 2 is expressed in human non-pigmented ciliary epithelium but not retinal pigment epithelium. Mol. Vis. 16, 200−206.

Zakrewski, W., Maciej, D., Szymonowicz, M., Zbigniew, R., 2019. Stem cells: past, present, and future. Stem Cell Res. Ther. 10, 1−22.

Zhong, X., Gutierrez, C., Xue, T., Hampton, C., Vergara, M.N., Cao, L., et al., 2014. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat. Commun. https://doi.org/10.1038/ncomms5047.

CHAPTER

Modeling hereditary spastic paraplegias using induced pluripotent stem cells

8

Yongchao Mou[1,2], **Zhenyu Chen**[1,2], **Xue-Jun Li**[1,2]

[1]*Department of Biomedical Sciences, University of Illinois College of Medicine Rockford, Rockford, IL, United States;* [2]*Department of Bioengineering, University of Illinois at Chicago, Chicago, IL, United States*

Chapter outline

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00006-0

Abstract

Hereditary spastic paraplegias (HSPs), a heterogeneous group of neurological disorders, are caused by axonal degeneration involving the long corticospinal tract which leads to the progressive spasticity and weakness of lower limbs. Over 80 different gene loci associated with HSP have been identified. Studying various HSP proteins reveals several common cellular themes in these debilitating diseases including vesicular trafficking, organelle shaping and morphogenesis, lipid/cholesterol metabolism, and endolysosomal and autophagic dysfunction. Though animal and cell models have been developed to study HSP, it remains a challenge to obtain patient-specific cortical neurons that are specifically affected and impaired in HSP. Recently, the discovery of induced pluripotent stem cells (iPSCs) provides a promising tool in neurological disease modeling due to their ability of self-renewal and capability of differentiation into various cell types. HSP patient iPSC-derived cortical projection neurons offer a unique source for HSP modeling to understand the molecular and cellular mechanisms, reveal neuronal pathological phenotypes, and test potential drugs. Here, we will introduce HSP and different common cellular themes, summarize in vitro HSP models using human pluripotent stem cells, and discuss the current challenges and future directions of HSP iPSC modeling.

Keywords: Axonal degeneration; Axonal transport; Cellular theme; Cortical projection neurons; Endolysosomal and autophagic dysfunction; Endoplasmic reticulum; Hereditary spastic paraplegias; In vitro modeling; iPSC; Lipid and cholesterol metabolism; Mitochondrial trafficking; Molecular genetics; Organelle morphogenesis; Organelle shaping; Vesicular transport.

Introduction

Hereditary spastic paraplegias (HSPs) are a large and heterogeneous group of inherited neurological disorders characterized by the prominent spasticity and progressive weakness of lower limbs (Blackstone et al., 2011; Crosby and Proukakis, 2002; Fink, 2006). Around two to five per 100,000 individuals worldwide are expected to be affected by HSPs based on the available data (Coutinho et al., 2013; Ruano et al., 2014; Shribman et al., 2019). Since the report of the first HSP gene, *SPAST* (Hazan et al., 1999), over 80 genetic loci that are associated with HSP have been identified. Different subtypes of HSP have a wide range of onset ages from early childhood to 70 years. HSPs are clinically diverse and are classified as "pure" forms that have spastic paraplegia as main symptom and "complicated" forms that have additional neurological abnormity such as ataxia, cognitive decline, peripheral neuropathy, and epilepsy. Based on the inheritance, HSPs are divided into autosomal dominant (AD), autosomal recessive (AR) subtypes, X-linked recessive, and sporadic cases (Blackstone, 2018; Shribman et al., 2019). Pathologically, the mutations of HSP genes cause axonal degeneration of cortical motor neurons that disrupts the signal transmission from these neurons to lower motor neurons and then muscles, leading to spasticity and weakness of low limb muscles. Currently, most treatments aim to alleviate symptoms, and there remains a lack of effective treatment to directly target axonal degeneration to slow, stop, and reverse this degeneration.

How human cortical motor neuron axons degenerate and why certain axons are specifically affected in HSP remain largely unclear. Animal models are common tools to elucidate molecular and cellular mechanisms underlying diseases. In HSP, animal models with HSP subtype-specific gene mutations are developed to model HSP and decipher the cause-effect relationship of gene mutations and the pathological phenotypes of various HSP subtypes. Various HSP animal models have been developed, including *Drosophila*, zebrafish, and mouse (Babin et al., 2014; Fassier et al., 2013; Wang and O'Kane, 2008). For example, SPG15 mice model with *Zfyve26* knockout show axonal degeneration and progressive loss of cortical motor neurons, as well as characteristic spastic paraplegia. Moreover, the elevated lysosomal enzymes are consistent with disruption of lysosomal compartment, implying the role of lysosome defects in SPG15 (Khundadze et al., 2013). In addition to animal models, cell lines and fibroblast cells derived from patients have been used to study the effects of perturbed HSP proteins. Different cellular themes have been reported to be associated with different HSP subtypes, including defects in organelles membrane remodeling and morphogenesis, disturbed axonal vesicular trafficking, imbalanced lipid/sterol homeostasis, and impaired endolysosomal system (Blackstone, 2018). Notably, it has been shown that cortical motor neurons in humans have differential functions compared to those in animals (Genc et al., 2019), bringing up the importance to use human neurons as additional models for neurological disorders. However, it remains a big challenge to obtain large quantities of patient-specific neurons to study the pathological changes in human neurons and to screen for therapeutic drugs, which partially contribute to the lack of effective treatment to slow, stop, or reverse axonal degeneration in HSP.

With the development of induced pluripotent stem cell (iPSC) technology (Takahashi et al., 2007; Yu et al., 2007), these stem cells have provided a unique source to generate different human neuronal subtypes to study various neurological diseases. Patient-specific iPSC models provide insights into understanding the cellular and molecular mechanisms, the underlying genetic factors, and the pathological phenotypes in HSP (Denton et al., 2016; Orack et al., 2015). Since the conditions for generating iPSCs and maintaining the self-renew of these cells are well-established, patient-specific iPSCs provide researchers an unlimited self-renewing cell source for differentiating into disease-relevant target neural cells, such as motor neurons in HSPs. More importantly, iPSCs maintain patient-specific genetic information and iPSC-derived neurons provide more precise patient models for high-throughput therapeutic screening. In this chapter, the applications of iPSCs in modeling HSP will be summarized, including cellular pathogenic themes in HSP, modeling different HSP subtypes using iPSCs, and the challenges and future directions.

Cellular pathogenic themes in hereditary spastic paraplegia

Understanding pathogenic mechanisms by which axons degenerate in HSP is one key to developing therapeutics for these debilitating diseases. As a highly

heterogeneous group of neurological disorders, HSPs present various cellular themes in different HSP types. Diverse molecular pathways causing cellular pathogenesis and neuronal degeneration are reported to be associated with different HSP proteins. An important unsolved question is how these functional diverse HSP proteins result in cortical axon degeneration and similar clinical symptoms. Several common cellular themes in HSP are discovered and highlighted the basic development and maintenance of neurons, such as vesicular trafficking, organelle shaping and morphogenesis, lipid/cholesterol metabolism, and endolysosomal and autophagic dysfunction (Fig. 8.1).

Membrane modeling and shaping

The impaired membrane modeling and shaping is a common cellular theme underlying HSPs. Several HSP proteins are strongly associated with the defects of organelles shaping and morphogenesis, such as endoplasmic reticulum (ER) and mitochondria.

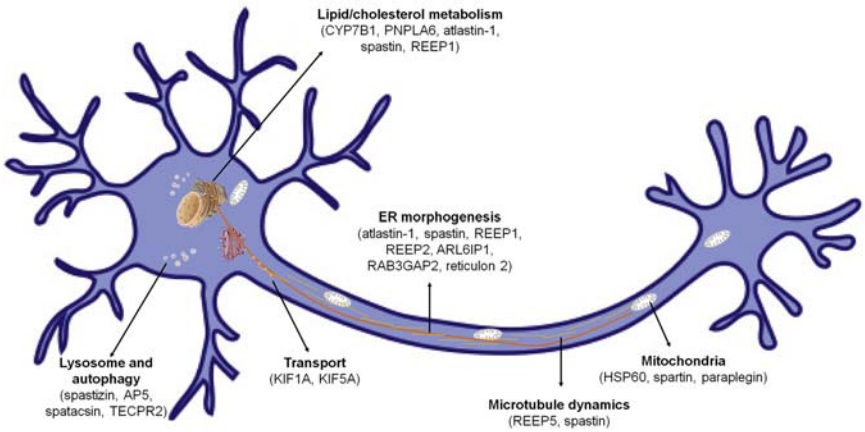


FIGURE 8.1 Schematic of common cellular themes affected in hereditary spastic paraplegia (HSPs).

The schematic figure shows major cellular themes affected in different HSP subtypes. Though different proteins are affected in different subtypes of HSP, HSP proteins can be clustered into several common cellular themes based on their functions, including lysosome and autophagy, lipid/cholesterol metabolism, transport, endoplasmic reticulum (ER) morphogenesis, microtubule dynamics, and mitochondria-associated pathways. *AP5*, adaptor related protein complex 5; *ARL6IP1*, ADP riboszylation factor like GTPase 6 interacting protein 1; *CYP7B1*, Cytochrome P450 family 7 subfamily B member 1; *HSP60*, heat shock protein 60; *KIF1A and KIF5A*, Kinesin family member 1A and member 5A; *PNPLA6*, patatin-like phospholipase domain containing 6; *RAB3GAP2*, RAB3 GTPase activating non-catalytic protein subunit 2; *REEP1*, receptor expression-enhancing protein 1; *REEP2*, receptor expression-enhancing protein 2; *REEP5*, receptor expression-enhancing protein 5; *TECPR2*, Tectonin beta-propeller repeat containing 2.

The mutation of atlastin-1 (*ATL1*, SPG3A), receptor expression-enhancing protein 1 (*REEP1*, SPG31), reticulon 2 (*RTN2*, SPG12), ADP ribozylation factor-like GTPase 6 interacting protein 1 (*ARL6IP1*, SPG61), RAB3 GTPase activating noncatalytic protein subunit 2 (*RAB3GAP2*, SPG69), and receptor expression-enhancing protein 2 (*REEP2*, SPG72) induced the impairment in ER morphogenesis, and several HSP proteins are associated with mitochondria, such as paraplegin (SPG7) and heat shock protein 60 (*HSP60*, SPG13) (Blackstone, 2018). In North America and Northern Europe, the mutations in genes encoding membrane modeling and shaping account up to 60% of HSP cases (Blackstone et al., 2011).

As a highly conserved continuous membrane system, the ER is an interconnected network of cylindrical tubules and cisternal sheets and extends throughout to the whole cell, including axons, dendrites, and soma in neurons (Shibata et al., 2006). In mature axons, most of the ER appears as smooth ER, which plays an important role in calcium homeostasis and signaling, local lipid synthesis, and communications with other organelles (Tidhar and Futerman, 2013; Vance, 2015; Yalcin et al., 2017). Meanwhile, the ER regulates various cellular functions through tightly contacting with other important organelles, such as ER-mitochondria, ER-lysosome, ER-lipid droplets (Phillips and Voeltz, 2016). A number of HSP proteins have been implicated in the regulation of ER morphogenesis and functions (Hu et al., 2011; Zhang and Hu, 2016). For example, SPG31 (*REEP1*), SPG72 (*REEP2*), and SPG12 (*RTN 2*) are directly associated with the defects in ER morphogenesis and function. These REEP/DP1/yop1 family and reticulon proteins are fundamental classes of proteins critical for peripheral ER tubules and sheets (Voeltz et al., 2006). The characteristic feature of reticulons and REEP proteins is that they share two long hydrophobic stretches separated by a hydrophilic sequence. The hydrophobic stretches preferentially reside in the ER membrane as a hairpin loop, which is strongly associated with generating and stabilizing the membrane curvature at ER tubules and sheet edges (Collins, 2006; Hu et al., 2008; Shibata et al., 2010; Voeltz et al., 2006). Another important ER morphogenesis regulator, atlastin GTPase family (atlastin 1-3) contain a hairpin membrane domain and mediate the ER tubular membrane fusion (Ulengin et al., 2015). Atlastin-1 is predominantly distributed in the vesicular tubular complexes and cis-Golgi cisternae in the brain, while atlastin-2 and atlastin-3 that are closely related to atlastin-1 are enriched in other tissues (Rismanchi et al., 2008). SPG3A is an AD HSP caused by the mutations of atlastin-1. The ER-shaping atlastin-1 cooperate with another protein, the lunapark, to fuse the ER tubular and maintain the ER network, which is stabilized by reticulons (Wang et al., 2016). Atlastin-1 mutant results in disturbed ER reticularization by disrupting the formation of three-way junctions in ER (Rismanchi et al., 2008). The ER morphology is also regulated by microtubules, such as one of the microtubules severing ATPase, spastin (SPG4). REEP1 and atlastin-1 interact with spastin to coordinate ER shaping and microtubule dynamics, suggesting the interplay of these processes in the pathogenesis of HSP (Park et al., 2010).

Mitochondrial abnormality is another common hallmark for HSP. SPG13 is an AD form of pure HSP and is caused by the mutations in mitochondria-residing

protein, chaperonin 60/heat shock protein 60 (Hansen et al., 2002). SPG7 is another mitochondria-mediated HSP (Casari et al., 1998). *SPG7* gene encodes paraplegin, a subunit of an ATPase associated with diverse cellular activities (AAA) protein in the inner mitochondrial membrane. The mutations of *SPG7* cause mitochondrial OXPHOS defects (Casari et al., 1998). A new HSP protein, ATPase family AAA-domain containing protein 3A (ATAD3A) is found to cause HSP by affecting the dynamics of mitochondrial network (Cooper et al., 2017). Two AR types of HSP, SPG15 and SPG48 are caused by perturbed spastizin and adaptor-related protein complex 5 subunit zeta 1 (AP5Z1) proteins that are involved in the endolysosomal system-associated complex. Interestingly, impaired mitochondrial dynamics and morphology were observed in SPG15 and SPG48 neurons, and mediated the axonal deficits of these neurons (Denton et al., 2018). These suggest that defects in dynamics of mitochondrial networks and functions contribute to the degeneration in HSP and targeting the abnormal mitochondrial dynamics provides a strategy to rescue HSP.

Vesicular transport along axons

Vesicular transport is a cellular activity for the movement of proteins, organelles, synaptic vesicles proteins, and lipids between different membrane-bound compartments in the cell. Effective transport of organelles and proteins along the long axons ensure neurons normally perform function and produce functional junctions with the target cells (Cai and Sheng, 2009). The axonal transport of subcellular cargos is classified into fast and slow transport with two moving directions, anterograde (from cell body to axonal terminal) and retrograde (from axonal terminal to cell body). Impaired axonal vesicular transport is implicated in the axonopathy of cortical motor neurons, and increased axonal swellings caused by accumulation of transported cargos due to impaired transport, is a characteristic pathological changes observed in multiple types of HSP (Kumar et al., 2015; Tarrade et al., 2006). Some HSP types are associated with the machinery of axonal transport, such as SPG10 and SPG30 that are caused by mutations of kinesin family member 5A (KIF5A) and kinesin family member 1A (KIF1A), respectively (Xia et al., 2003). Kinesin family are important motor proteins that regulate anterograde axonal transport of cargos in neurons (Cai and Sheng, 2009). In the *Drosophila* models with mutations in *KIF5* ortholog *Khc*, organelles were accumulated in axonal swellings of neurons (Hurd and Saxton, 1996). In some HSP subtypes, abnormal mitochondrial transport along axon was observed in degenerated neurons. The percentage of motile mitochondria and motile events per mitochondrion decreased in SPG3A and SPG4 iPSCs-derived cortical neurons (Denton et al., 2018; Zhu et al., 2014). In spartin (SPG20) mutated cells, spartin lost its mitochondrial localization and interaction with microtubules, leading to defective mitochondrial trafficking (Lu et al., 2006).

Synaptophysin is a major membrane protein in the transmitter-containing vesicles in neurons. The intracellular synaptophysin transport is found to be impaired in HSP neurons, including SPG4 mutant cells and SPG11 neurons (Leo et al., 2017; Perez-Branguli et al., 2014). SPG11 patients-derived neurons were cultured

in microfluid system and were found to have the decreased neurite complexity (Perez-Branguli et al., 2014). The spatacsin protein was found to be colocalized with vesicle markers presenting in synaptosomes. The axonal anterograde synaptophysin trafficking was reduced in SPG11 patient-derived forebrain neurons and spatacsin-silenced mouse cortical neurons, indicating impaired axonal transport is caused by the loss of spatacsin function (Perez-Branguli et al., 2014). Although the phenomenon of impaired axonal transport is widely observed in HSP neurons, detailed pathogenic mechanisms for axonal transport deficit and subsequent neuronal degeneration remain largely unknown.

Impairment in lipid/sterol metabolism

Lipid/sterol homeostasis plays an important role in regulating the cellular structure, cell signaling, and their contribution to the energy supply (Tracey et al., 2018). The abnormal lipid/sterol metabolism has been observed in various neurological diseases (Valenza et al., 2015; van der Kant et al., 2019). Emerging data indicate that defects in lipid/sterol metabolism appear another common cellular theme contributing to the degeneration of neurons in HSP (Klemm et al., 2013; Renvoise et al., 2012; Schule et al., 2010). Lipids have different classes, and alterations of all major lipid classes have been found to be implicated in the pathology of HSP, including synthesis and trafficking of cholesterol, phospholipid synthesis and degradation, sphingolipid metabolism, and fatty acid metabolism (Darios et al., 2020). Several HSP subtypes are directly caused by the mutations in lipid metabolism−associated proteins, such as *CYP7B1* (SPG5), patatin-like phospholipase domain containing 6 (*PNPLA6*, SPG39) (Song et al., 2013; Tsaousidou et al., 2008). Other HSP subtypes associated with lipid/sterol metabolism could be resulted from the damaged cellular organelles, such as ER, endolysosomal system, and mitochondria (Tesson et al., 2012).

Coinciding with the key role of ER in lipid/sterol metabolism, recent studies on ER-associated HSP reported defects in lipid droplets and lipid metabolism in SPG4, SPG3A, and SPG31 (Klemm et al., 2013; Papadopoulos et al., 2015; Renvoise et al., 2016). In SPG4, spastin M1 isoform is found to be translocated from the ER to pre- and mature lipid droplets and regulates the number and size of lipid droplets; downregulation of spastin using RNA interference reduced the fat stored in lipid droplets, implying the critical role of spastin in regulating lipid metabolism (Papadopoulos et al., 2015). In SPG3A, atlastin-1 is shown to have a conserved role in regulating lipid droplet size and numbers. The downregulation of atlastin-1 results in a reduction of lipid droplet sizes and triglycerides, and an increase of LD number in *Drosophila* fat bodies (Klemm et al., 2013). The most direct link between lipid defects and HSP comes from a recent study which observed lipid dystrophy and reduced numbers of lipid droplets in Reep1$^{-/-}$ mouse brain (Renvoise et al., 2016). In addition, SPG54, an early onset HSP subtype, is caused by mutations in *DDHD2* gene encoding DDHD2 that functions as lipases in vitro. Deletion of *DDHD2* caused the elevation of triacylglycerols in the central nervous system and the accumulations of lipid droplets in neurons (Inloes et al., 2014, 2018).

Therefore, emerging evidence suggests that lipids are critical for neural physiology, and further investigation is needed to elucidate what kinds of lipids are altered in the neurons and how lipid dysfunctions cause neurodegeneration in HSP.

Endolysosomal and autophagic dysfunction

Endolysosomal and autophagic pathways play important roles in maintaining cellular homeostasis by degrading plasma membrane components, extracellular macromolecules, intracellular macromolecules, and cellular fragments (van Weering and Scheper, 2019). The endolysosomal system mainly processes extracellular cargos when extracellular components enter cell, while the autophagic system mostly processes intracellular components. Lysosomes, an organelle containing hydrolytic enzymes, are critical for both pathways (Xu and Ren, 2015). Endolysosomes are the fusion product of endosome and lysosome, while autophagosomes are double-membraned vesicles that are formed during autophagy and then fused with lysosomes for degradation (Huotari and Helenius, 2011; Shibutani and Yoshimori, 2014). Dysfunction of lysosomes leads to incomplete degradation of stored components and accumulation of metabolic waste products, which is detrimental to neurons by causing high metabolic pressure, leading to aging and neurological diseases (Levine and Kroemer, 2008; Luo and Qin, 2019).

Endolysosomal and autophagic dysfunctions in neurons have been reported in various HSP subtypes in recent years, such as SPG11, SPG15, and SPG48 (Chang et al., 2014; Vantaggiato et al., 2013, 2019). SPG11, SPG15, and SPG48 are AR HSP caused by mutations in *SPG11*, *ZFYVE26*, and *AP5Z1* genes, which encode spatacsin, spastizin, and AP5Z1, respectively. Spastizin and spatacsin form a complex with AP5 and involve in the trafficking of endolysosomal compartments (Hirst et al., 2013). Loss of spastizin and spatacsin function depletes the production of new lysosomes by inhibiting autophagic lysosome reformation (Chang et al., 2014). Moreover, spastizin also acts at the intersection between endocytosis and autophagy to influence their fusion in patients' fibroblast cells, and lysosomal failure together with damaged autophagy were found in spastizin knockout mice (Vantaggiato et al., 2019; Varga et al., 2015). In SPG48 patients with different AP5Z1 mutations, accumulation of aberrant multilamellar storage components were observed in their fibroblast cells, indicating abnormal lysosomal degradation ability (Hirst et al., 2016). In an SPG48 mouse model, loss of AP5Z1 function led to a block of autophagic flux and accumulation of waste in elder mice neurons, further supporting the autophagic defects in the pathogenesis of SPG48 (Khundadze et al., 2019). In addition to these forms of HSP, impaired lysosomal degradation is also observed in SPG49. Loss of Tectonin beta-propeller repeat containing 2 (*TECPR2*, SPG49) led to reduced number of autophagosomes and the delivery of autophagosomes to lysosomes for degradation (Oz-Levi et al., 2012). Together, these studies imply the endolysosomal and autophagic dysfunction in HSP (Fig. 8.1), providing potential targets for therapeutic interventions.

Modeling different types of hereditary spastic paraplegia using induced pluripotent stem cells

Patient-specific iPSCs that have the ability to generate different types of neurons are used to model various neurological diseases, including HSP. Modeling HSP diseases in the dish using patient-specific iPSCs recapitulates the disease phenotypes of human motor neurons and further can be used to reveal the cellular themes and molecular pathways underlying different HSP subtypes. Importantly, the HSP models using iPSCs-derived neurons provide a unique platform for high-throughput drug screening of small molecule libraries to rescue axonal degeneration by targeting the specific defects of various cellular themes in different HSP subtypes. In this part, we will review recent different iPSC-based HSP models in revealing neuronal phenotypes and cellular themes and in testing the potential therapeutics for HSP.

Autosomal dominant hereditary spastic paraplegia

SPG4

SPG4 is the most common type of HSP accounting for up to 40%−67% of AD HSP cases (Kim et al., 2014). SPG4 is caused by mutations in the *SPAST* gene, which encodes a microtubule-severing protein, spastin. Spastin has two main isoforms in human: a full-length isoform, M1 (616 amino acids in length) and a slightly shorter isoform, M87 (530 amino acids). Comparing to M1, M87 lacks 86 amino acids on its N-terminal, which mediates the interaction with several HSP proteins located in ER, including atlastin-1, REEP-1, and reticulons (Park et al., 2010; Sanderson et al., 2006). Moreover, M87 isoform exhibits ubiquitous distribution, whereas M1 mainly distributed in adult spinal cord, where the nerve degeneration of corticospinal tract is occurred in SPG4 (Solowska et al., 2008). Though these data suggest that M1 may be a key mediator of the neuronal degeneration in SPG4, the roles of M1 and M87 spastin in neuronal development and motor function remain largely unknown. Knockdown of each isoform can cause different pathological phenotypes of motor neurons and defects in locomotion (Jardin et al., 2018), suggesting that both isoforms are involved in SPG4. Both M1 and M87 proteins have the microtubule binding and severing domains, and have shown the ability to severe microtubules. Moreover, spastin as a microtubule protein interacts with a complex for cargo degradation protein, endosomal sorting complex required for transport (ESCRT) to modulate the endosomal tubulation. Loss of spastin results in the defects of endosome tubulation and changes receptor sorting via endosomal tubular recycling compartments (Summerville et al., 2016), suggesting that multiple pathways are implicated in the pathogenesis of SPG4.

Since 2014, iPSC-based SPG4 models have been successfully established (Table 8.1) by reprogramming human skin fibroblast cells derived from SPG4 patients (Denton et al., 2014; Hauser et al., 2016a; Havlicek et al., 2014; Rehbach et al., 2019; Wali et al., 2020). The SPG4 iPSC-derived telencephalic glutamatergic neurons displayed increased axonal swellings and reduced axonal transport of

Table 8.1 Summary of generated induced pluripotent stem cells for hereditary spastic paraplegia modeling.

Inheritance characteristic	Hereditary spastic paraplegia type	Protein name	Phenotypes	Cellular themes	Tested drug or targets in induced pluripotent stem cell–based hereditary spastic paraplegia models	References
Autosomal dominant (AD)	SPG3A	Atlastin-1	Reduced axonal outgrowth, altered mitochondrial motility	Endoplasmic reticulum (ER) morphogenesis	A microtubule-destabilizing drug, vinblastine	Zhu et al. (2014)
AD	SPG4	Spastin	Significant increase in axonal swellings, decreased neurite length, drastically enlarged growth cones, characterized the SPAST mutation, accumulations of mitochondria in axons, and decreased mitochondrial transport	Vesicular transport along axons	Vinblastine, locked nucleic acid-anti-miR-33a, latrunculin B and GW3965	Denton et al. (2014), Hauser et al. (2016b), Nakazeki et al. (2019), Rehbach et al. (2019)
AD	SPG4	Spastin	Decreased M1 and M87 isoforms in SPG4 neuronal cells, decreased complexity of SPG4 neurites, imbalanced axonal transport with less retrograde transport, increased neurite swellings, abnormal microtubules	Microtubules, vesicular transport along axons	Overexpression of M1 or M87 spastin isoforms rescued the defects in SPG4 neurons	Havlicek et al. (2014)

AD	SPG4	Spastin	The failure of interaction between spastin and the endosomal sorting complex required for transport (ESCRT) protein IST1 at ER-endosome contacts caused the defects in sorting of mannose 6-phosphate receptor, disrupted lysosomal enzyme trafficking, and abnormal lysosomal morphology.	Endolysosomal dysfunction		Allison et al. (2017)
AD	SPG4	Spastin	Reduced stabilized microtubules, decreased peroxisome number and transport, increased axon swellings, more sensitive to the oxidative stress	Vesicle transport along axons	Tubulin-binding drugs epothilone D and noscapine	Wali et al. (2020)
AD	ATAD3A	ATPase family AAA-domain containing protein 3A	Altered dynamics of the mitochondrial network and increased lysosomes	Mitochondrial dynamics		Cooper et al. (2017)
Autosomal recessive (AR)	SPG5	CYP7B1	Generation and characterization of induced pluripotent stem cells (iPSCs) from SPG5 patient's fibroblasts; elevated levels of oxysterols, a significant reduction in total neurite length as well as branching points	Cholesterol metabolism		Hoflinger et al. (2016), Schols et al. (2017)

Continued

Table 8.1 Summary of generated induced pluripotent stem cells for hereditary spastic paraplegia modeling.—*cont'd*

Inheritance characteristic	Hereditary spastic paraplegia type	Protein name	Phenotypes	Cellular themes	Tested drug or targets in induced pluripotent stem cell–based hereditary spastic paraplegia models	References
AR	SPG11	Spatacsin	Reduced proliferation and neurogenesis in SPG11-cortical neural progenitor cells (NPCs); widespread transcriptional alterations in neurodevelopmental pathways, including cell-cycle, neurogenesis, cortical development pathways, and autophagic deficits; impaired GSK3β-signaling pathway	Autophagy	GSK3 inhibitor, CHIR99021	Mishra et al. (2016)
AR	SPG11	Spatacsin	Two-dimensional: Shorter and less complex neurites, increased cell death; Three-dimensional: An increased rate of asymmetric divisions of SPG11 neural progenitor cells, smaller cerebral organoids with larger ventricles as well as thinner germinal wall	Lysosomal autophagy, neural progenitor defects	Tideglusib	Perez-Branguli et al. (2019), Pozner et al. (2018)
AR	SPG11	Spatacsin	Accumulation of gangliosides, increased neuronal death, and inhibited lysosome membrane recycling	Lysosomal dysfunction		Boutry et al. (2018)

AR	SPG15	Spastizin	Reduced axonal outgrowth, decreased mitochondrial density, increased axonal swellings, mitochondrial dysfunction, and increased apoptosis	Impaired mitochondrial dynamics; lysosome autophagy	Mdivi-1 and Drp-1 shRNA	Denton et al. (2018)
AR	SPG47	AP-4B1	Generation and characterization of iPSCs; reduced neurite outgrowth and branching, reduced autophagy marker, LC-3II	Vesicles transport and autophagy		Teinert et al. (2019), Behne et al. (2020)
AR	SPG48	AP5Z1	Reduced axonal outgrowth, decreased mitochondrial density, increased axonal swellings, mitochondrial dysfunction, and increased apoptosis	Impaired mitochodniral dynamics; autophagy	Mdivi-1 and Drp-1 shRNA	Denton et al. (2018)
AR	SPG57	Tropomyosin receptor kinase fused gene (TFG)	Impaired secretory protein trafficking and elevated ER stress in transduced iPSC-derived cells. The TFG mutation results in a defect in axon fasciculation and in L1CAM accumulation at the surface of axons in iPSCs-derived neurons	Vesicle transport from ER to Golgi apparatus		Slosarek et al. (2018)
AR	SPG76	Calpain-1	Generation and characterization of iPSCs from SPG76 patient's fibroblasts.	Synaptic plasticity		Lu et al. (2019)

mitochondria (Denton et al., 2014), recapitulating disease-specific phenotypes. Forebrain neurons derived from spastin knockdown human embryonic stem cells also exhibit the similar axonal swellings, suggesting the cause-effect relationship between the axonal defects and loss of spastin function. These genetic modified human pluripotent stem cells (hPSCs) offer additional human models to study HSP. The accumulated axonal swelling can be rescued by a one day treatment of a microtubule-destabilizing drug (Denton et al., 2014), suggesting the usefulness of these neurons for screening therapeutic agents in the future. Using SPG4 patients-specific neurons, researchers showed that M1 and M87 spastin isoforms were decreased in SPG4 neuronal cells. The complexity of neurites and retrograde axonal transport were significantly reduced in SPG4 neurons. Interestingly, the axonal degeneration including decreased neurite length, branching and numbers, as well as increased axonal swellings can be rescued by overexpression of M1 or M87 spastin isoforms, implying the role of both isoforms in SPG4 (Havlicek et al., 2014). Importantly, the impaired axonal outgrowth and swellings have been shown to be cell-type−specific. SPG4 cortical neurons, but not GABAergic forebrain neurons, exhibited axonal defects that were rescued by a liver X receptor agonist, GW3965 (Rehbach et al., 2019). Interestingly, spastin was also found to be interacted with the ESCRT protein IST1 at ER-endosome contacts to drive endosomal tubule fission. The mutation of SPG4 caused the increased percentage of neurons with large lysosomes and the enrichment of lysosomes in axonal swellings of SPG4 iPSCs-derived neuronal cells (Allison et al., 2017). Patient iPSC-derived neuron axons have been shown to be vulnerable to oxidative stress, which is mediated by impaired axonal transport (Wali et al., 2020). In another study, Nakazeki et al. found that *SPAST* can be directly regulated by microRNA (miR)-33. Downregulation of miR-33a, the major form of *miR-33* in SPG4 iPSCs-derived cortical neurons can ameliorate the pathological phenotypes, reduced neurite length (Nakazeki et al., 2019).

SPG3A

As an AD HSP type, SPG3A accounts for approximately 10%−15% of all AD HSP cases and is the most common early onset form of HSP (Fink et al., 1996). SPG3A is caused by mutations in the *ATL1* (14q22.1) gene encoding atlastin-1, which is necessary for the formation of ER three-way junctions (Zhou et al., 2019). Currently, over 60 different *ATL1* gene mutations were described, including missense, small deletion, small insertion, splice site mutations, and whole exon deletions (Zhao and Liu, 2017). Atlastin-1 protein is a member of the dynamin-related GTPase superfamily. Atlastin-1, a 558 amino acid 64-kDa protein, is composed of three domains, a GTPase domain at N-terminus, a middle domain serving an unknown function, and two transmembrane domains at C-terminus (Zhu et al., 2003). It is highly enriched in the brain, especially in the lamina V pyramidal neurons (i.e., cortical motor neurons), the neuron type that is affected in SPG3A patients (Zhu et al., 2003). Atlastin-1 predominantly distributes in the cis-Golgi and ER tubular network and is required for Golgi and ER morphogenesis (Hu et al., 2009; Rismanchi et al., 2008; Zhu et al., 2003). Mutations in atlastin-1 with reduced GTPase activity

impaired ER networks in soma and dendrite branch points (Liu et al., 2019). Perturbed atlastin-1 also caused the substantial upregulation of the bone morphogenetic protein (BMP) signaling pathway through disrupting the BMP receptor trafficking (Fassier et al., 2010; Zhao and Hedera, 2013). In addition to impaired ER and BMP pathways, a recent study reveals the conservative role of atlastin-1 in regulating the size of lipid droplets, implying aberrant lipid metabolism in SPG3A (Klemm et al., 2013). However, how these pathogenic mechanisms interact with each other and result in axonal degeneration of cortical neurons in SPG3A await further investigation.

Using the episomal method, we successfully generated the first SPG3A iPSC lines (Table 8.1) in collaboration with Dr. Craig Blackstone (Zhu et al., 2003). These iPSCs were generated from a young girl's skin fibroblasts who has p.Pro342Ser mutations in atlastin-1, and were then differentiated into telencephalic glutamatergic neurons (Fig. 8.2). These patient iPSC-derived cortical projections neurons (PNs) exhibited reduced axonal length and branching. This is coinciding with a previous study that the reduction of atlastin-1 in rat cortical neurons caused the significant decrease of axonal length (Zhu et al., 2003). Interestingly, treatment of SPG3A neurons with microtubule-targeting drugs including vinblastine can partially rescue axonal outgrowth deficit, implying aberrant microtubules in SPG3A (Zhu et al., 2014). The protective effect of vinblastine is consistent with a previous study in which the abnormal neuromuscular junction of atlastin-1 mutant flies was partially reversed by the vinblastine (Lee et al., 2009). A significant advantage of iPSC-based model is that these cells provide a system to examine axonal and neuronal functions in live human neurons. By combining mitochondrial labeling and live cell imaging, we examined fast axonal transport of mitochondria and revealed a significant reduction in the percentage of motile mitochondria and the number of motile mitochondrial events (Fig. 8.2), confirming axonal transport defects in SPG3A neurons in live cultures. Since impaired axonal transport occurs at early stages of disease progression, this pathological change offers a target for early intervention to rescue axonal degeneration.

Autosomal recessive hereditary spastic paraplegia

SPG5

SPG5, an AR type of HSP, is caused by mutations in *CYP7B1* encoding the oxysterol 7-α-hydroxylase which is involved in degrading cholesterol into primary bile acids (Nan et al., 2019; Stiles et al., 2009). The degradation of cholesterol has two pathways, one is the "classic pathway," in which the degradation is initiated via 7α-hydroxylation of cholesterol by CYP7A1. The other pathway is the "acid pathway" during which cholesterols are first oxidized to 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC), and then these oxysterols (25-OHC and 27-OHC) were 7α-hydroxylated by CYP7B1 (Tsaousidou et al., 2008). Due to the loss function of CYP7B1, 25-OHC, 27-OHC, and 3β-hydroxy-5-cholestenoic acid (3β-HCA, oxidized product of 27-OHC) have been shown to

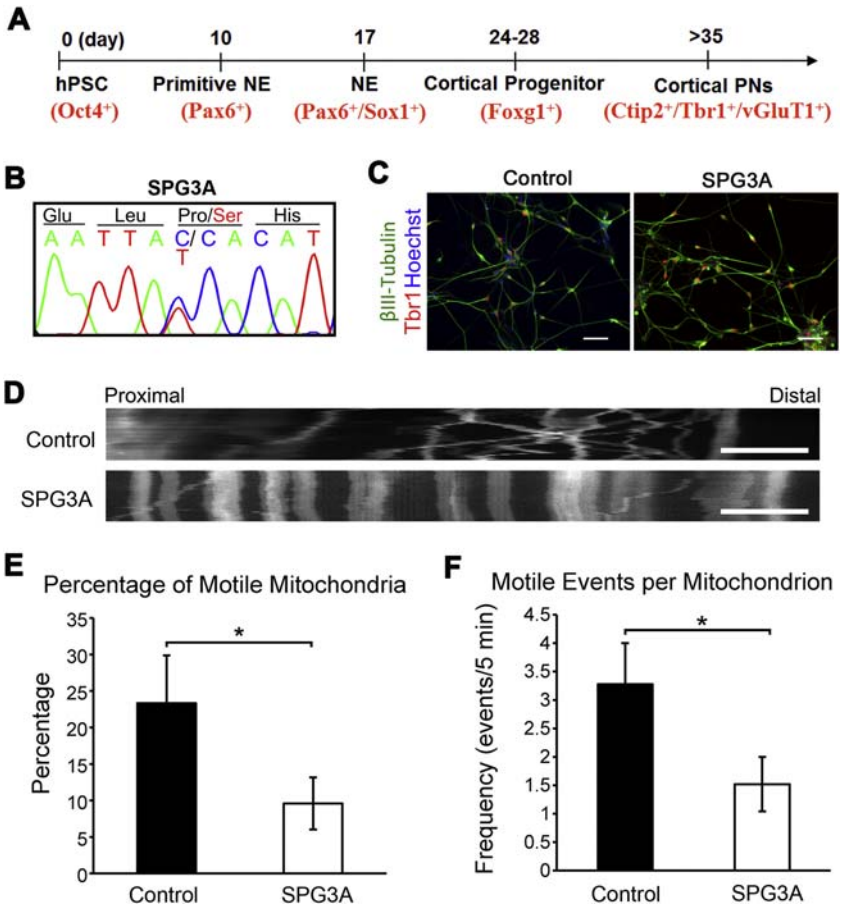


FIGURE 8.2 Modeling SPG3A with patient-specific induced pluripotent stem cells (iPSCs).

(A) Schematic procedure of cortical projection neuron (PN, glutamatergic neuron) differentiation from human pluripotent stem cells (hPSCs). NE, neuroepithelial cells. (B) Sequencing confirmed the presence of a heterozygous p.Pro342Ser mutation in SPG3A iPSC-derived neural cells. (C) Both control and SPG3A iPSCs efficiently generated $Tbr1^{+}$ glutamatergic neurons. Bars: 50 μm. (D) Representative time versus position kymographs showing mitochondrial transport in control and SPG3A axons. Bars: 10 μm. Quantification revealed reduced motile mitochondria (E) and motile event (F) in SPG3A cortical PNs.

(B–F) are adapted from our previous publication by Oxford University Press Zhu, P.P., Denton, K.R., Pierson, T.M., Li, X.J., Blackstone, C., 2014. Pharmacologic rescue of axon growth defects in a human iPSC model of hereditary spastic paraplegia SPG3A. Hum. Mol. Genet. 23, 5638–5648.

be accumulated in the plasma and cerebrospinal fluid of SPG5 patients (Bjorkhem et al., 2010; Schule et al., 2010).

SPG5 iPSCs lines were generated from several patient's skin fibroblasts, including one patient with a homozygous mutation p.Y275X in CYP7B1 and one patient carrying a homozygous mutation R486C in CYP7B1 (Hauser et al., 2016b; Hoflinger et al., 2016). The oxysterol concentrations in SPG5 are investigated using the SPG5 iPSC-derived neurons, and increased oxysterols (25-OHC, 27-OHC, and 3β-HCA) were confirmed in these neurons. Moreover, the impaired metabolic activity is correlated with the viability of SPG5 iPSC-derived cortical neurons, implying the role of lipid dysfunction in SPG5 (Schols et al., 2017). Cholesterol-lowering drugs, simvastatin, ezetimibe, chenodeoxycholic acid, atorvastatin, and resveratrol are evaluated in the SPG5 patients and showed a dramatic reduction of serum biomarker, oxysterols, although some patients had no tolerance to some drugs because of the side effects (Marelli et al., 2018; Mignarri et al., 2015; Schols et al., 2017). Further studies are needed to evaluate clinical outcomes, neurological function, side effects, and long-term effects. Recently, the administration of CYP7B1 mRNA in SPG5 mouse model was proved to be safe and led to a significant degradation of oxysterols in liver, serum, and brain (Hauser et al., 2019). With the help of iPSCs-derived neurons as a unique tool for the drug screening and evaluation, more effective strategies including drug or molecular manipulation can be screened and tested using these patient iPSC-derived cells.

SPG11, SPG15, and SPG48

SPG11 and SPG15 are the most common AR forms of HSP, accounting for ~25% of the AR HSP. Both forms present with cognitive impairment, dementia, ataxia, thinning of the corpus callosum, and lower limb spasticity (Hanein et al., 2008; Stevanin et al., 2007). SPG11 and SPG15 proteins, spatacsin and spastizin, are direct binding partners, interacting with endosome-relative small GTP-binding proteins RAB5A and RAB11 in endocytes and autophages to regulate cellular recycling system (Hirst et al., 2013; Murmu et al., 2011). These two proteins can also bind to the AP5, the subunits of which are mutated in the complex SPG48 patients (Hirst et al., 2013). SPG48 patients exhibit similar clinic symptoms as SPG11 and SPG15, and all three forms can have early onset Parkinsonism which has shown improvement following dopaminergic therapy (Guidubaldi et al., 2011; Schicks et al., 2011). Studies on these HSP proteins suggest that endolysosomal and autophagic dysfunctions are one characteristic pathogenic change and a major player in the pathogenesis of HSP (Vantaggiato et al., 2013, 2019). In addition, loss of spatacsin has been shown to alter lipid clearance in lysosomes, damage lysosomal recycling ability and then impair cholesterol trafficking and calcium levels maintenance (Boutry et al., 2018, 2019; Branchu et al., 2017). These data suggest the interaction of endolysosomal dysfunction and other pathogenic mechanisms, though further studies are needed to elucidate the detailed mechanisms of axonal degeneration in these HSPs.

Winner's group have successfully established SPG11 patient-specific iPSC lines (Table 8.1) by reprogramming fibroblasts of two SPG11 patients with compound

heterozygous mutations of spatacsin (Perez-Branguli et al., 2014). Spatacsin is expressed in iPSC-derived neurons as well as in mouse cortical neurons, which is enriched in axons and dendrites. Reduced neurite length and complexity were found in these SPG11 neurons with abnormal spatacsin function compared with control iPSC-derived neurons. These SPG11 neurons have significant reduction of synaptic vesicle trafficking in anterograde direction, leading to impaired axonal transport. One possible mechanism of axonal defects in SPG11 is abnormal cell-cycle regulation indicated by the decreased proliferation ability of neural progenitor cells (Mishra et al., 2016). Using three-dimensional (3D) organoids culture, it has been further revealed that high portion of neural progenitors undergo asymmetric divisions thereafter resulting in premature neurogenesis (Perez-Branguli et al., 2019). Dysregulation of GSK3β/β-Catenin pathway in SPG11 neural precursors was the major reason for the significant reduction of neural cell population in both monolayer and organoids culture systems. Moreover, regulating GSK3 pathway by tideglusib, an FDA-approved GSK3 inhibitor, could rescue the decreased proliferation of SPG11 neural precursors, implying the GSK3β/β-Catenin dysregulation in SPG11 (Mishra et al., 2016; Perez-Branguli et al., 2019; Pozner et al., 2018).

Recently, SPG15 and SPG48 iPSCs cell lines were successfully established from patients' dermal fibroblasts and then differentiated into telencephalic glutamatergic neurons (i.e., cortical PNs), major cell types affected in HSP, in Li's group (Denton et al., 2018). Disease-relevant axonal defects including reduced neurite outgrowth and increased axonal swellings were observed in SPG15 and SPG48 cortical PNs. Notably, the axonal outgrowth deficit is also present in midbrain dopaminergic neurons (the cell type affected and responsible for the Parkinsonism in SPG15 and SPG48) but not in spinal neurons (cell types not affected in HSP), recapitulating the cell-type−specific defects. Further investigations of SPG15 and SPG48 iPSC-derived cortical PNs showed altered mitochondrial morphology together with a reduced mitochondrial membrane potential, revealing mitochondrial dysfunction in these neurons (Denton et al., 2018). Mitochondria are highly dynamic organelles undergoing continuous fission and fusion to maintain normal morphology and function. Interestingly, inhibition of a mitochondrial fission mediator, dynamin-related protein (Drp1), both pharmacologically (using DRP1 inhibitor mdivi-1) and genetically (using lentiviruses containing Drp1 shRNA) could mitigate abnormal mitochondrial morphology and rescue axonal outgrowth deficit in SPG15 and SPG48 neurons (Denton et al., 2018). These data suggest that impaired mitochondrial dynamics underlie axonal defects in these HSPs, bringing up the importance to study interactions of multiple pathogenic processes to identify new targets for rescuing axonal degeneration in the future. In addition, given that pluripotent stem cells have the ability to generate different neuronal subtypes, these patient-specific iPSCs provide unique sources to generate various neuron types to study why certain type(s) of neurons are specifically affected. This is a fundamental unanswered question in neurodegenerative diseases.

Other types of hereditary spastic paraplegia

Human iPSCs for some other HSP subtypes were successfully established from the patients (Table 8.1), allowing researchers to study the HSP using these stem cells−derived neurons (Behne et al., 2020; Lu et al., 2019; Teinert et al., 2019; Wang et al., 2020).

Behne et al. established six iPSC lines from HSP patients with deficiency of the adaptor protein complex 4 (*AP-4*, for SPG47). Neurite outgrowth and branching of these SPG47 iPSCs-derived cortical neurons were reduced. AP-4 subunit levels are reduced and the autophagy protein, autophagy related 9A (ATG9A) was accumulated in the trans-Golgi network in SPG47 iPSC-derived cortical neurons (Behne et al., 2020). A recent study identified a dominantly inherited heterozygous mutation in *ATAD3A*, and this mutation was maintained in the corresponding iPSC-derived neurons (Cooper et al., 2017). In addition, iPSC lines were generated from patients with mutations in tropomyosin receptor kinase fused gene (*TFG*, SPG57) and calpain-1 (*CAPN1*, SPG76), providing new tools to study various types of HSP (Lu et al., 2019; Slosarek et al., 2018).

Challenges and future directions

Since the discovery of iPSC technology, patient-specific iPSCs have been successfully established from different subtypes of HSP patients, providing valuable systems to study pathogenic mechanisms and explore treatments for these debilitating diseases. Though the recapitulation of disease-relevant phenotypes has been observed in iPSC-derived neurons in vitro, challenges remain including the heterogeneity and functional maturation of these iPSC-derived neurons, the recapitulation of synaptic and circuitry defects, the interplay of multiple pathogenic pathways, and the identification of therapeutic agents using these models.

Heterogeneity and functional maturation of induced pluripotent stem cell–derived neurons

The direct differentiation and enrichment of neuronal subtype holds the key to apply these cells for neurodegenerative diseases in which certain type of neurons specifically degenerate. By applying morphogens that are critical for particular type of neurons, various neuronal subtypes have been specified from hPSCs (Chamberlain et al., 2008; Tao and Zhang, 2016). The generation of regional progenitors is highly efficient (e.g., over 90% spinal progenitors) and subsequently, particular type of neurons can be efficiently generated (Li et al., 2008, 2009). In addition to the target type of neurons, neural progenitors and other neuron types may also be present given that morphogens specify different types of neurons based on the gradient concentration of these morphogens (Briscoe and Ericson, 2001; Jessell, 2000). Another reason for generating a heterogeneous population of neurons is that neural progenitors can give rise to different type of neural cells based on timing (Toma et al., 2016). Therefore, to enrich neurons, compound E, a γ-secretase inhibitor is used to force cell-cycle exit from the progenitor stage, resulting in a synchronized postmitotic neuronal population (Du et al., 2015; Yang et al., 2008). Another common strategy for enrichment is to sort the particular neuron type using fluorescence activated cell sorting or magnetic sorting based on the expression of surface marker or the reporter (Arias-Fuenzalida et al., 2017; Paik et al., 2018; Toli et al., 2015).

It has been shown that iPSC-derived neurons resemble fetal neurons in some aspects, bringing up the question about the functional maturation of these cells. Electrophysiological analyses recorded repetitive action potential and synaptic current of hPSC-derived neurons (Bardy et al., 2016; Li et al., 2005; Liu et al., 2013), suggesting the functionality of hPSC-derived neurons. Moreover, neurons at long-term culture exhibit more mature properties, partially due to the generation of glial cells that promote the synaptic maturations of neurons in long-term cultures. Recently, different types of astroglial cells have been successfully generated from hPSCs (Krencik and Zhang, 2011; Li et al., 2018; Tchieu et al., 2019), allowing the co-culturing of glial cells with neurons to further improve their functionality. Though cells can be cultured months even a couple years in cultures, how to maintain the optimal survival and function for long-term cultures remains a challenge. One alternative is to transplant the iPSC-derived neural cells into animal (e.g., mouse brain) to examine their long-term changes, which also provides a unique system to study the maturation and disease phenotypes of iPSC-derived neurons in an in vivo environment.

Recapitulation of synaptic and circuitry defects

A major challenge in modeling human neurological diseases using iPSCs is to recapitulate synaptic and circuitry defects in cultures. The current iPSC-based HSP models are mainly the two-dimensional cultures of iPSCs-derived cortical neurons. One approach to increase synaptic connectivity is to build 3D models (Brown et al., 2018). In 2013, the establishment of 3D cerebral organoids by combining the 3D culture and bioreactor led to the successful model of microcephaly, a disease characterized by impaired cortical development (Lancaster et al., 2013). Studies also showed that the 3D model of Alzheimer's disease can mimic characteristic disease phenotypes comparing to regular two-dimensional model (Choi et al., 2016). For HSP models, 3D cerebral organoids derived from SPG11 iPSCs were smaller with larger ventricles and thinner germinal wall compared to those from control iPSCs (Perez-Branguli et al., 2019). Though the variation between organoids is a major concern, recent studies used enriched progenitors and showed the generation of more consistent brain organoids from iPSCs (Velasco et al., 2019). Furthermore, by combining with bioengineering technology, it is possible to culture different 3D organoids to build co-culture models. This is particularly useful for studying the interaction between different types of cells, for example, the synaptic connections between cortical motor neurons and their targets, spinal motor neurons. Therefore, 3D organoids models, especially combining with bioengineering and gene editing technology, will provide valuable paradigms to illustrate pathogenic mechanisms underlying various HSP subtypes.

Interplay of multiple pathogenic mechanisms

Given that more than 80 genetic loci are associated with HSP, an intriguing question is how the mutations of functional divergent HSP genes result in similar symptoms

in HSP. Emerging evidence identified several common pathological themes in HSP involving ER morphogenesis, membrane trafficking, vesicle transport, lipid and sterol metabolism, and autophagic and lysosomal defects as we described in the above sections. Multiple subcellular organelles such as ER, lysosome, microtubule, endosome, and mitochondria are affected in these processes (Blackstone, 2018; Blackstone et al., 2011; Crosby and Proukakis, 2002; Fink, 2006). Interestingly, these pathogenic processes can interact with each other, leading to axonal and neuronal degeneration. For example, abnormal ER morphogenesis results in lipid dysfunction since ER is the factory of lipid metabolism (Eastman et al., 2009; Falk et al., 2014; Klemm et al., 2013). Mitochondrial and lipid defects are also observed in recessive forms of HSP caused by autophagic and lysosome defects (e.g., SPG11, SPG15, and SPG48) (Boutry et al., 2019; Branchu et al., 2017; Denton et al., 2018). These findings not only imply multiple pathological changes in the pathogenesis of HSP, but also highlight the importance to dissect the interplay between these themes to identify common and critical therapeutic targets for HSP.

Identification of therapeutic approaches

An important application of iPSC-derived neurons is the utilization of these patient-derived nerve cells for drug screening to identify therapeutic agents. It has been shown that human cells may have very different responses to drugs compared to other species, and less than 10% of candidate drugs have been shown to be clinically effective during clinical trials (Kola and Landis, 2004). Using iPSC-based models of HSP, studies have tested some compounds including vinblastine, Midvi-1, CHIR99021, and tideglusib (Denton et al., 2016, 2018; Mishra et al., 2016; Pozner et al., 2018). As an example, microtubule-targeting drugs showed protective effects in rescuing axonal defects in SPG3A and SPG4 iPSC-derived neurons (Denton et al., 2014; Zhu et al., 2014). These studies provide proof-of-concept evidence of using iPSC-derived neurons to test drugs. To improve the throughput, reporter lines that target critical biochemical or functional phenotypes can be established using CRISPR/cas9-mediated gene targeting. In addition to high-throughput report lines, high-content screening platforms provide a unique system to examine morphological phenotypes for identifying therapeutic drugs (Rehbach et al., 2019).

Though iPSC-derived neurons offer a unique source for cell therapy, there are challenges in applying these cells for replacing the degenerate nerve cells in HSP patients. After transplantation, a large number of neural cells especially postmitotic neurons could die, and it is important to promote the survival of nerves cells after transplantation by optimizing the conditions and stages of transplanted cells (Perrier and Studer, 2003; Stoddard-Bennett and Reijo Pera, 2019; Zhang et al., 2008). Another major challenge is the integration of transplanted cells into the host circuitry. HSPs are characterized by the degeneration of cortical motor neurons that project long axons and connect with their targets, lower motor neurons in spinal cord. Better understanding the mechanisms underlying the axonal projection of

grafted neurons and the integration of these neurons into the circuitry will be valuable for the development of therapies to rescue axonal defects in HSP and other diseases involving axonopathy.

Acknowledgments

This study is supported by the Spastic Paraplegia Foundation, the National Institute of Health (R21NS109837 and R01NS118066), and the Blazer Foundation.

References

Allison, R., Edgar, J.R., Pearson, G., Rizo, T., Newton, T., Gunther, S., Berner, F., Hague, J., Connell, J.W., Winkler, J., et al., 2017. Defects in ER-endosome contacts impact lysosome function in hereditary spastic paraplegia. J. Cell Biol. 216, 1337−1355.

Arias-Fuenzalida, J., Jarazo, J., Qing, X., Walter, J., Gomez-Giro, G., Nickels, S.L., Zaehres, H., Scholer, H.R., Schwamborn, J.C., 2017. FACS-assisted CRISPR-cas9 genome editing facilitates Parkinson's disease modeling. Stem Cell Rep. 9, 1423−1431.

Babin, P.J., Goizet, C., Raldua, D., 2014. Zebrafish models of human motor neuron diseases: advantages and limitations. Prog. Neurobiol. 118, 36−58.

Bardy, C., van den Hurk, M., Kakaradov, B., Erwin, J.A., Jaeger, B.N., Hernandez, R.V., Eames, T., Paucar, A.A., Gorris, M., Marchand, C., et al., 2016. Predicting the functional states of human iPSC-derived neurons with single-cell RNA-seq and electrophysiology. Mol. Psychiatr. 21, 1573−1588.

Behne, R., Teinert, J., Wimmer, M., D'Amore, A., Davies, A.K., Scarrott, J.M., Eberhardt, K., Brechmann, B., Chen, I.P., Buttermore, E.D., et al., 2020. Adaptor protein complex 4 deficiency: a paradigm of childhood-onset hereditary spastic paraplegia caused by defective protein trafficking. Hum. Mol. Genet. 29, 320−334.

Bjorkhem, I., Leoni, V., Meaney, S., 2010. Genetic connections between neurological disorders and cholesterol metabolism. J. Lipid Res. 51, 2489−2503.

Blackstone, C., 2018. Converging cellular themes for the hereditary spastic paraplegias. Curr. Opin. Neurobiol. 51, 139−146.

Blackstone, C., O'Kane, C.J., Reid, E., 2011. Hereditary spastic paraplegias: membrane traffic and the motor pathway. Nat. Rev. Neurosci. 12, 31−42.

Boutry, M., Branchu, J., Lustremant, C., Pujol, C., Pernelle, J., Matusiak, R., Seyer, A., Poirel, M., Chu-Van, E., Pierga, A., et al., 2018. Inhibition of lysosome membrane recycling causes accumulation of gangliosides that contribute to neurodegeneration. Cell Rep. 23, 3813−3826.

Boutry, M., Pierga, A., Matusiak, R., Branchu, J., Houllegatte, M., Ibrahim, Y., Balse, E., El Hachimi, K.-H., Brice, A., Stevanin, G., et al., 2019. Loss of spatacsin impairs cholesterol trafficking and calcium homeostasis. Commun. Biol. 2, 380-380.

Branchu, J., Boutry, M., Sourd, L., Depp, M., Leone, C., Corriger, A., Vallucci, M., Esteves, T., Matusiak, R., Dumont, M., et al., 2017. Loss of spatacsin function alters lysosomal lipid clearance leading to upper and lower motor neuron degeneration. Neurobiol. Dis. 102, 21−37.

Briscoe, J., Ericson, J., 2001. Specification of neuronal fates in the ventral neural tube. Curr. Opin. Neurobiol. 11, 43−49.

Brown, J., Quadrato, G., Arlotta, P., 2018. Studying the brain in a dish: 3D cell culture models of human brain development and disease. Curr. Top. Dev. Biol. 129, 99−122.

Cai, Q., Sheng, Z.H., 2009. Molecular motors and synaptic assembly. Neuroscientist 15, 78−89.

Casari, G., De Fusco, M., Ciarmatori, S., Zeviani, M., Mora, M., Fernandez, P., De Michele, G., Filla, A., Cocozza, S., Marconi, R., et al., 1998. Spastic paraplegia and OXPHOS impairment caused by mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease. Cell 93, 973−983.

Chamberlain, S.J., Li, X.J., Lalande, M., 2008. Induced pluripotent stem (iPS) cells as in vitro models of human neurogenetic disorders. Neurogenetics 9, 227−235.

Chang, J., Lee, S., Blackstone, C., 2014. Spastic paraplegia proteins spastizin and spatacsin mediate autophagic lysosome reformation. J. Clin. Invest. 124, 5249−5262.

Choi, S.H., Kim, Y.H., Quinti, L., Tanzi, R.E., Kim, D.Y., 2016. 3D culture models of Alzheimer's disease: a road map to a "cure-in-a-dish". Mol. Neurodegener. 11, 75.

Collins, R.N., 2006. How the ER stays in shape. Cell 124, 464−466.

Cooper, H.M., Yang, Y., Ylikallio, E., Khairullin, R., Woldegebriel, R., Lin, K.L., Euro, L., Palin, E., Wolf, A., Trokovic, R., et al., 2017. ATPase-deficient mitochondrial inner membrane protein ATAD3A disturbs mitochondrial dynamics in dominant hereditary spastic paraplegia. Hum. Mol. Genet. 26, 1432−1443.

Coutinho, P., Ruano, L., Loureiro, J.L., Cruz, V.T., Barros, J., Tuna, A., Barbot, C., Guimaraes, J., Alonso, I., Silveira, I., et al., 2013. Hereditary ataxia and spastic paraplegia in Portugal: a population-based prevalence study. JAMA Neurol. 70, 746−755.

Crosby, A.H., Proukakis, C., 2002. Is the transportation highway the right road for hereditary spastic paraplegia? Am. J. Hum. Genet. 71, 1009−1016.

Darios, F., Mochel, F., Stevanin, G., 2020. Lipids in the physiopathology of hereditary spastic paraplegias. Front. Neurosci. 14, 74.

Denton, K., Mou, Y., Xu, C.C., Shah, D., Chang, J., Blackstone, C., Li, X.J., 2018. Impaired mitochondrial dynamics underlie axonal defects in hereditary spastic paraplegias. Hum. Mol. Genet. 27, 2517−2530.

Denton, K.R., Lei, L., Grenier, J., Rodionov, V., Blackstone, C., Li, X.J., 2014. Loss of spastin function results in disease-specific axonal defects in human pluripotent stem cell-based models of hereditary spastic paraplegia. Stem Cell. 32, 414−423.

Denton, K.R., Xu, C., Shah, H., Li, X.J., 2016. Modeling axonal defects in hereditary spastic paraplegia with human pluripotent stem cells. Front. Biol. 11, 339−354.

Du, Z.W., Chen, H., Liu, H., Lu, J., Qian, K., Huang, C.L., Zhong, X., Fan, F., Zhang, S.C., 2015. Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. Nat. Commun. 6, 6626.

Eastman, S.W., Yassaee, M., Bieniasz, P.D., 2009. A role for ubiquitin ligases and Spartin/SPG20 in lipid droplet turnover. J. Cell Biol. 184, 881−894.

Falk, J., Rohde, M., Bekhite, M.M., Neugebauer, S., Hemmerich, P., Kiehntopf, M., Deufel, T., Hubner, C.A., Beetz, C., 2014. Functional mutation analysis provides evidence for a role of REEP1 in lipid droplet biology. Hum. Mutat. 35, 497−504.

Fassier, C., Hutt, J.A., Scholpp, S., Lumsden, A., Giros, B., Nothias, F., Schneider-Maunoury, S., Houart, C., Hazan, J., 2010. Zebrafish atlastin controls motility and spinal motor axon architecture via inhibition of the BMP pathway. Nat. Neurosci. 13, 1380−1387.

Fassier, C., Tarrade, A., Peris, L., Courageot, S., Mailly, P., Dalard, C., Delga, S., Roblot, N., Lefevre, J., Job, D., et al., 2013. Microtubule-targeting drugs rescue axonal swellings in cortical neurons from spastin knockout mice. Dis. Model Mech. 6, 72−83.

Fink, J.K., 2006. Hereditary spastic paraplegia. Curr. Neurol. Neurosci. Rep. 6, 65−76.

Fink, J.K., Heiman-Patterson, T., Bird, T., Cambi, F., Dube, M.P., Figlewicz, D.A., Haines, J.L., Hentati, A., Pericak-Vance, M.A., Raskind, W., et al., 1996. Hereditary spastic paraplegia: advances in genetic research. Hereditary Spastic Paraplegia Working group. Neurology 46, 1507−1514.

Genc, B., Gozutok, O., Ozdinler, P.H., 2019. Complexity of generating mouse models to study the upper motor neurons: let us shift focus from mice to neurons. Int. J. Mol. Sci. 20, 3848.

Guidubaldi, A., Piano, C., Santorelli, F.M., Silvestri, G., Petracca, M., Tessa, A., Bentivoglio, A.R., 2011. Novel mutations in SPG11 cause hereditary spastic paraplegia associated with early-onset levodopa-responsive Parkinsonism. Mov. Disord. 26, 553−556.

Hanein, S., Martin, E., Boukhris, A., Byrne, P., Goizet, C., Hamri, A., Benomar, A., Lossos, A., Denora, P., Fernandez, J., et al., 2008. Identification of the SPG15 gene, encoding spastizin, as a frequent cause of complicated autosomal-recessive spastic paraplegia, including Kjellin syndrome. Am. J. Hum. Genet. 82, 992−1002.

Hansen, J.J., Durr, A., Cournu-Rebeix, I., Georgopoulos, C., Ang, D., Nielsen, M.N., Davoine, C.S., Brice, A., Fontaine, B., Gregersen, N., et al., 2002. Hereditary spastic paraplegia SPG13 is associated with a mutation in the gene encoding the mitochondrial chaperonin Hsp60. Am. J. Hum. Genet. 70, 1328−1332.

Hauser, S., Erzler, M., Theurer, Y., Schuster, S., Schule, R., Schols, L., 2016a. Establishment of SPAST mutant induced pluripotent stem cells (iPSCs) from a hereditary spastic paraplegia (HSP) patient. Stem Cell Res. 17, 485−488.

Hauser, S., Hoflinger, P., Theurer, Y., Rattay, T.W., Schols, L., 2016b. Generation of induced pluripotent stem cells (iPSCs) from a hereditary spastic paraplegia patient carrying a homozygous Y275X mutation in CYP7B1 (SPG5). Stem Cell Res. 17, 437−440.

Hauser, S., Poenisch, M., Schelling, Y., Hoflinger, P., Schuster, S., Teegler, A., Betten, R., Gustafsson, J.A., Hubener-Schmid, J., Schlake, T., et al., 2019. mRNA as a novel treatment strategy for hereditary spastic paraplegia type 5. Mol. Ther. Methods Clin. Dev. 15, 359−370.

Havlicek, S., Kohl, Z., Mishra, H.K., Prots, I., Eberhardt, E., Denguir, N., Wend, H., Plotz, S., Boyer, L., Marchetto, M.C., et al., 2014. Gene dosage-dependent rescue of HSP neurite defects in SPG4 patients' neurons. Hum. Mol. Genet. 23, 2527−2541.

Hazan, J., Fonknechten, N., Mavel, D., Paternotte, C., Samson, D., Artiguenave, F., Davoine, C.S., Cruaud, C., Durr, A., Wincker, P., et al., 1999. Spastin, a new AAA protein, is altered in the most frequent form of autosomal dominant spastic paraplegia. Nat. Genet. 23, 296−303.

Hirst, J., Borner, G.H., Edgar, J., Hein, M.Y., Mann, M., Buchholz, F., Antrobus, R., Robinson, M.S., 2013. Interaction between AP-5 and the hereditary spastic paraplegia proteins SPG11 and SPG15. Mol. Biol. Cell 24, 2558−2569.

Hirst, J., Madeo, M., Smets, K., Edgar, J.R., Schols, L., Li, J., Yarrow, A., Deconinck, T., Baets, J., Van Aken, E., et al., 2016. Complicated spastic paraplegia in patients with AP5Z1 mutations (SPG48). Neurol. Genet. 2 e98-e98.

Hoflinger, P., Theurer, Y., Schule, R., Schols, L., Hauser, S., 2016. Generation of induced pluripotent stem cells (iPSCs) from a hereditary spastic paraplegia patient carrying a homozygous R486C mutation in CYP7B1 (SPG5). Stem Cell Res. 17, 422−425.

Hu, J., Prinz, W.A., Rapoport, T.A., 2011. Weaving the web of ER tubules. Cell 147, 1226−1231.

Hu, J., Shibata, Y., Voss, C., Shemesh, T., Li, Z., Coughlin, M., Kozlov, M.M., Rapoport, T.A., Prinz, W.A., 2008. Membrane proteins of the endoplasmic reticulum induce high-curvature tubules. Science 319, 1247−1250.

Hu, J., Shibata, Y., Zhu, P.P., Voss, C., Rismanchi, N., Prinz, W.A., Rapoport, T.A., Blackstone, C., 2009. A class of dynamin-like GTPases involved in the generation of the tubular ER network. Cell 138, 549−561.

Huotari, J., Helenius, A., 2011. Endosome maturation. EMBO J. 30, 3481−3500.

Hurd, D.D., Saxton, W.M., 1996. Kinesin mutations cause motor neuron disease phenotypes by disrupting fast axonal transport in *Drosophila*. Genetics 144, 1075−1085.

Inloes, J.M., Hsu, K.L., Dix, M.M., Viader, A., Masuda, K., Takei, T., Wood, M.R., Cravatt, B.F., 2014. The hereditary spastic paraplegia-related enzyme DDHD2 is a principal brain triglyceride lipase. Proc. Natl. Acad. Sci. U. S. A. 111, 14924−14929.

Inloes, J.M., Kiosses, W.B., Wang, H., Walther, T.C., Farese Jr., R.V., Cravatt, B.F., 2018. Functional contribution of the spastic paraplegia-related triglyceride hydrolase DDHD2 to the formation and content of lipid droplets. Biochemistry 57, 827−838.

Jardin, N., Giudicelli, F., Ten Martin, D., Vitrac, A., De Gois, S., Allison, R., Houart, C., Reid, E., Hazan, J., Fassier, C., 2018. BMP- and neuropilin 1-mediated motor axon navigation relies on spastin alternative translation. Development 145, dev162701.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. 1, 20−29.

Khundadze, M., Kollmann, K., Koch, N., Biskup, C., Nietzsche, S., Zimmer, G., Hennings, J.C., Huebner, A.K., Symmank, J., Jahic, A., et al., 2013. A hereditary spastic paraplegia mouse model supports a role of ZFYVE26/SPASTIZIN for the endolysosomal system. PLoS Genet. 9, e1003988.

Khundadze, M., Ribaudo, F., Hussain, A., Rosentreter, J., Nietzsche, S., Thelen, M., Winter, D., Hoffmann, B., Afzal, M.A., Hermann, T., et al., 2019. A mouse model for SPG48 reveals a block of autophagic flux upon disruption of adaptor protein complex five. Neurobiol. Dis. 127, 419−431.

Kim, T.H., Lee, J.H., Park, Y.E., Shin, J.H., Nam, T.S., Kim, H.S., Jang, H.J., Semenov, A., Kim, S.J., Kim, D.S., 2014. Mutation analysis of SPAST, ATL1, and REEP1 in Korean patients with hereditary spastic paraplegia. J. Clin. Neurol. 10, 257−261.

Klemm, R.W., Norton, J.P., Cole, R.A., Li, C.S., Park, S.H., Crane, M.M., Li, L., Jin, D., Boye-Doe, A., Liu, T.Y., et al., 2013. A conserved role for atlastin GTPases in regulating lipid droplet size. Cell Rep. 3, 1465−1475.

Kola, I., Landis, J., 2004. Can the pharmaceutical industry reduce attrition rates? Nat. Rev. Drug Discov. 3, 711−715.

Krencik, R., Zhang, S.C., 2011. Directed differentiation of functional astroglial subtypes from human pluripotent stem cells. Nat. Protoc. 6, 1710−1717.

Kumar, K.R., Blair, N.F., Sue, C.M., 2015. An update on the hereditary spastic paraplegias: new genes and new disease models. Mov. Disord. Clin. Pract. 2, 213−223.

Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., Knoblich, J.A., 2013. Cerebral organoids model human brain development and microcephaly. Nature 501, 373−379.

Lee, M., Paik, S.K., Lee, M.J., Kim, Y.J., Kim, S., Nahm, M., Oh, S.J., Kim, H.M., Yim, J., Lee, C.J., et al., 2009. *Drosophila* Atlastin regulates the stability of muscle microtubules and is required for synapse development. Dev. Biol. 330, 250−262.

Leo, L., Weissmann, C., Burns, M., Kang, M., Song, Y., Qiang, L., Brady, S.T., Baas, P.W., Morfini, G., 2017. Mutant spastin proteins promote deficits in axonal transport through an isoform-specific mechanism involving casein kinase 2 activation. Hum. Mol. Genet. 26, 2321−2334.

Levine, B., Kroemer, G., 2008. Autophagy in the pathogenesis of disease. Cell 132, 27−42.

Li, X., Tao, Y., Bradley, R., Du, Z., Kong, L., Dong, Y., Jones, J., Yan, Y., Harder, C.R.K., Friedman, L.M., et al., 2018. Fast generation of functional subtype astrocytes from human pluripotent stem cells. Stem Cell Rep. 11, 998−1008.

Li, X.J., Du, Z.W., Zarnowska, E.D., Pankratz, M., Hansen, L.O., Pearce, R.A., Zhang, S.C., 2005. Specification of motoneurons from human embryonic stem cells. Nat. Biotechnol. 23, 215−221.

Li, X.J., Hu, B.Y., Jones, S.A., Zhang, Y.S., Lavaute, T., Du, Z.W., Zhang, S.C., 2008. Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. Stem Cell. 26, 886−893.

Li, X.J., Zhang, X., Johnson, M.A., Wang, Z.B., Lavaute, T., Zhang, S.C., 2009. Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. Development 136, 4055−4063.

Liu, X., Guo, X., Niu, L., Li, X., Sun, F., Hu, J., Wang, X., Shen, K., 2019. Atlastin-1 regulates morphology and function of endoplasmic reticulum in dendrites. Nat. Commun. 10, 568.

Liu, Y., Liu, H., Sauvey, C., Yao, L., Zarnowska, E.D., Zhang, S.C., 2013. Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. Nat. Protoc. 8, 1670−1679.

Lu, J., Rashid, F., Byrne, P.C., 2006. The hereditary spastic paraplegia protein spartin localises to mitochondria. J. Neurochem. 98, 1908−1919.

Lu, Y.Q., Dong, E.L., Yang, W.Q., Lai, L.L., Lin, X.H., Ma, L.X., Chen, W.J., Wang, N., Lin, X., 2019. Generation of an integration-free induced pluripotent stem cell line, FJMUi001-A, from a hereditary spastic paraplegia patient carrying compound heterozygous p.P498L and p.R618W mutations in CAPN1 (SPG76). Stem Cell Res. 34, 101354.

Luo, L., Qin, Z.H., 2019. Autophagy, aging, and longevity. Adv. Exp. Med. Biol. 1206, 509−525.

Marelli, C., Lamari, F., Rainteau, D., Lafourcade, A., Banneau, G., Humbert, L., Monin, M.L., Petit, E., Debs, R., Castelnovo, G., et al., 2018. Plasma oxysterols: biomarkers for diagnosis and treatment in spastic paraplegia type 5. Brain 141, 72−84.

Mignarri, A., Malandrini, A., Del Puppo, M., Magni, A., Monti, L., Ginanneschi, F., Tessa, A., Santorelli, F.M., Federico, A., Dotti, M.T., 2015. Treatment of SPG5 with cholesterol-lowering drugs. J. Neurol. 262, 2783−2785.

Mishra, H.K., Prots, I., Havlicek, S., Kohl, Z., Perez-Branguli, F., Boerstler, T., Anneser, L., Minakaki, G., Wend, H., Hampl, M., et al., 2016. GSK3ss-dependent dysregulation of neurodevelopment in SPG11-patient induced pluripotent stem cell model. Ann. Neurol. 79, 826−840.

Murmu, R.P., Martin, E., Rastetter, A., Esteves, T., Muriel, M.P., El Hachimi, K.H., Denora, P.S., Dauphin, A., Fernandez, J.C., Duyckaerts, C., et al., 2011. Cellular distribution and subcellular localization of spatacsin and spastizin, two proteins involved in hereditary spastic paraplegia. Mol. Cell. Neurosci. 47, 191−202.

Nakazeki, F., Tsuge, I., Horie, T., Imamura, K., Tsukita, K., Hotta, A., Baba, O., Kuwabara, Y., Nishino, T., Nakao, T., et al., 2019. MiR-33a is a therapeutic target in SPG4-related hereditary spastic paraplegia human neurons. Clin. Sci. (Lond.) 133, 583−595.

Nan, H., Shimozono, K., Ichinose, Y., Tsuchiya, M., Koh, K., Hiraide, M., Takiyama, Y., 2019. Exome sequencing reveals a novel homozygous frameshift mutation in the CYP7B1 gene in a Japanese patient with SPG5. Intern. Med. 58, 719−722.

Orack, J.C., Deleidi, M., Pitt, D., Mahajan, K., Nicholas, J.A., Boster, A.L., Racke, M.K., Comabella, M., Watanabe, F., Imitola, J., 2015. Concise review: modeling multiple sclerosis with stem cell biological platforms: toward functional validation of cellular and molecular phenotypes in inflammation-induced neurodegeneration. Stem Cells Transl. Med. 4, 252−260.

Oz-Levi, D., Ben-Zeev, B., Ruzzo, E.K., Hitomi, Y., Gelman, A., Pelak, K., Anikster, Y., Reznik-Wolf, H., Bar-Joseph, I., Olender, T., et al., 2012. Mutation in TECPR2 reveals a role for autophagy in hereditary spastic paraparesis. Am. J. Hum. Genet. 91, 1065−1072.

Paik, E.J., O'Neil, A.L., Ng, S.Y., Sun, C., Rubin, L.L., 2018. Using intracellular markers to identify a novel set of surface markers for live cell purification from a heterogeneous hIPSC culture. Sci. Rep. 8, 804.

Papadopoulos, C., Orso, G., Mancuso, G., Herholz, M., Gumeni, S., Tadepalle, N., Jungst, C., Tzschichholz, A., Schauss, A., Honing, S., et al., 2015. Spastin binds to lipid droplets and affects lipid metabolism. PLoS Genet. 11, e1005149.

Park, S.H., Zhu, P.P., Parker, R.L., Blackstone, C., 2010. Hereditary spastic paraplegia proteins REEP1, spastin, and atlastin-1 coordinate microtubule interactions with the tubular ER network. J. Clin. Invest. 120, 1097−1110.

Perez-Branguli, F., Buchsbaum, I.Y., Pozner, T., Regensburger, M., Fan, W., Schray, A., Borstler, T., Mishra, H., Graf, D., Kohl, Z., et al., 2019. Human SPG11 cerebral organoids reveal cortical neurogenesis impairment. Hum. Mol. Genet. 28, 961−971.

Perez-Branguli, F., Mishra, H.K., Prots, I., Havlicek, S., Kohl, Z., Saul, D., Rummel, C., Dorca-Arevalo, J., Regensburger, M., Graef, D., et al., 2014. Dysfunction of spatacsin leads to axonal pathology in SPG11-linked hereditary spastic paraplegia. Hum. Mol. Genet. 23, 4859−4874.

Perrier, A.L., Studer, L., 2003. Making and repairing the mammalian brain–in vitro production of dopaminergic neurons. Semin. Cell Dev. Biol. 14, 181−189.

Phillips, M.J., Voeltz, G.K., 2016. Structure and function of ER membrane contact sites with other organelles. Nat. Rev. Mol. Cell Biol. 17, 69−82.

Pozner, T., Schray, A., Regensburger, M., Lie, D.C., Schlotzer-Schrehardt, U., Winkler, J., Turan, S., Winner, B., 2018. Tideglusib rescues neurite pathology of SPG11 iPSC derived cortical neurons. Front. Neurosci. 12, 914.

Rehbach, K., Kesavan, J., Hauser, S., Ritzenhofen, S., Jungverdorben, J., Schule, R., Schols, L., Peitz, M., Brustle, O., 2019. Multiparametric rapid screening of neuronal process pathology for drug target identification in HSP patient-specific neurons. Sci. Rep. 9, 9615.

Renvoise, B., Malone, B., Falgairolle, M., Munasinghe, J., Stadler, J., Sibilla, C., Park, S.H., Blackstone, C., 2016. Reep1 null mice reveal a converging role for hereditary spastic paraplegia proteins in lipid droplet regulation. Hum. Mol. Genet. 25, 5111−5125.

Renvoise, B., Stadler, J., Singh, R., Bakowska, J.C., Blackstone, C., 2012. Spg20-/- mice reveal multimodal functions for Troyer syndrome protein spartin in lipid droplet maintenance, cytokinesis and BMP signaling. Hum. Mol. Genet. 21, 3604−3618.

Rismanchi, N., Soderblom, C., Stadler, J., Zhu, P.P., Blackstone, C., 2008. Atlastin GTPases are required for Golgi apparatus and ER morphogenesis. Hum. Mol. Genet. 17, 1591−1604.

Ruano, L., Melo, C., Silva, M.C., Coutinho, P., 2014. The global epidemiology of hereditary ataxia and spastic paraplegia: a systematic review of prevalence studies. Neuroepidemiology 42, 174–183.

Sanderson, C.M., Connell, J.W., Edwards, T.L., Bright, N.A., Duley, S., Thompson, A., Luzio, J.P., Reid, E., 2006. Spastin and atlastin, two proteins mutated in autosomal-dominant hereditary spastic paraplegia, are binding partners. Hum. Mol. Genet. 15, 307–318.

Schicks, J., Synofzik, M., Petursson, H., Huttenlocher, J., Reimold, M., Schols, L., Bauer, P., 2011. Atypical juvenile parkinsonism in a consanguineous SPG15 family. Mov. Disord. 26, 564–566.

Schols, L., Rattay, T.W., Martus, P., Meisner, C., Baets, J., Fischer, I., Jagle, C., Fraidakis, M.J., Martinuzzi, A., Saute, J.A., et al., 2017. Hereditary spastic paraplegia type 5: natural history, biomarkers and a randomized controlled trial. Brain 140, 3112–3127.

Schule, R., Siddique, T., Deng, H.X., Yang, Y., Donkervoort, S., Hansson, M., Madrid, R.E., Siddique, N., Schols, L., Bjorkhem, I., 2010. Marked accumulation of 27-hydroxycholesterol in SPG5 patients with hereditary spastic paresis. J. Lipid Res. 51, 819–823.

Shibata, Y., Shemesh, T., Prinz, W.A., Palazzo, A.F., Kozlov, M.M., Rapoport, T.A., 2010. Mechanisms determining the morphology of the peripheral ER. Cell 143, 774–788.

Shibata, Y., Voeltz, G.K., Rapoport, T.A., 2006. Rough sheets and smooth tubules. Cell 126, 435–439.

Shibutani, S.T., Yoshimori, T., 2014. A current perspective of autophagosome biogenesis. Cell Res. 24, 58–68.

Shribman, S., Reid, E., Crosby, A.H., Houlden, H., Warner, T.T., 2019. Hereditary spastic paraplegia: from diagnosis to emerging therapeutic approaches. Lancet Neurol. 18, 1136–1146.

Slosarek, E.L., Schuh, A.L., Pustova, I., Johnson, A., Bird, J., Johnson, M., Frankel, E.B., Bhattacharya, N., Hanna, M.G., Burke, J.E., et al., 2018. Pathogenic TFG mutations underlying hereditary spastic paraplegia impair secretory protein trafficking and axon fasciculation. Cell Rep. 24, 2248–2260.

Solowska, J.M., Morfini, G., Falnikar, A., Himes, B.T., Brady, S.T., Huang, D., Baas, P.W., 2008. Quantitative and functional analyses of spastin in the nervous system: implications for hereditary spastic paraplegia. J. Neurosci. 28, 2147–2157.

Song, Y., Wang, M., Mao, F., Shao, M., Zhao, B., Song, Z., Shao, C., Gong, Y., 2013. Knockdown of Pnpla6 protein results in motor neuron defects in zebrafish. Dis. Model Mech. 6, 404–413.

Stevanin, G., Santorelli, F.M., Azzedine, H., Coutinho, P., Chomilier, J., Denora, P.S., Martin, E., Ouvrard-Hernandez, A.M., Tessa, A., Bouslam, N., et al., 2007. Mutations in SPG11, encoding spatacsin, are a major cause of spastic paraplegia with thin corpus callosum. Nat. Genet. 39, 366–372.

Stiles, A.R., McDonald, J.G., Bauman, D.R., Russell, D.W., 2009. CYP7B1: one cytochrome P450, two human genetic diseases, and multiple physiological functions. J. Biol. Chem. 284, 28485–28489.

Stoddard-Bennett, T., Reijo Pera, R., 2019. Treatment of Parkinson's disease through personalized medicine and induced pluripotent stem cells. Cells 8, 26.

Summerville, J.B., Faust, J.F., Fan, E., Pendin, D., Daga, A., Formella, J., Stern, M., McNew, J.A., 2016. The effects of ER morphology on synaptic structure and function in *Drosophila melanogaster*. J. Cell Sci. 129, 1635–1648.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861−872.

Tao, Y., Zhang, S.C., 2016. Neural subtype specification from human pluripotent stem cells. Cell Stem Cell 19, 573−586.

Tarrade, A., Fassier, C., Courageot, S., Charvin, D., Vitte, J., Peris, L., Thorel, A., Mouisel, E., Fonknechten, N., Roblot, N., et al., 2006. A mutation of spastin is responsible for swellings and impairment of transport in a region of axon characterized by changes in microtubule composition. Hum. Mol. Genet. 15, 3544−3558.

Tchieu, J., Calder, E.L., Guttikonda, S.R., Gutzwiller, E.M., Aromolaran, K.A., Steinbeck, J.A., Goldstein, P.A., Studer, L., 2019. NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells. Nat. Biotechnol. 37, 267−275.

Teinert, J., Behne, R., D'Amore, A., Wimmer, M., Dwyer, S., Chen, T., Buttermore, E.D., Chen, I.P., Sahin, M., Ebrahimi-Fakhari, D., 2019. Generation and characterization of six human induced pluripotent stem cell lines (iPSC) from three families with AP4B1-associated hereditary spastic paraplegia (SPG47). Stem Cell Res. 40, 101575.

Tesson, C., Nawara, M., Salih, M.A., Rossignol, R., Zaki, M.S., Al Balwi, M., Schule, R., Mignot, C., Obre, E., Bouhouche, A., et al., 2012. Alteration of fatty-acid-metabolizing enzymes affects mitochondrial form and function in hereditary spastic paraplegia. Am. J. Hum. Genet. 91, 1051−1064.

Tidhar, R., Futerman, A.H., 2013. The complexity of sphingolipid biosynthesis in the endoplasmic reticulum. Biochim. Biophys. Acta 1833, 2511−2518.

Toli, D., Buttigieg, D., Blanchard, S., Lemonnier, T., Lamotte d'Incamps, B., Bellouze, S., Baillat, G., Bohl, D., Haase, G., 2015. Modeling amyotrophic lateral sclerosis in pure human iPSc-derived motor neurons isolated by a novel FACS double selection technique. Neurobiol. Dis. 82, 269−280.

Toma, K., Wang, T.C., Hanashima, C., 2016. Encoding and decoding time in neural development. Dev. Growth Differ. 58, 59−72.

Tracey, T.J., Steyn, F.J., Wolvetang, E.J., Ngo, S.T., 2018. Neuronal lipid metabolism: multiple pathways driving functional outcomes in health and disease. Front. Mol. Neurosci. 11, 10.

Tsaousidou, M.K., Ouahchi, K., Warner, T.T., Yang, Y., Simpson, M.A., Laing, N.G., Wilkinson, P.A., Madrid, R.E., Patel, H., Hentati, F., et al., 2008. Sequence alterations within CYP7B1 implicate defective cholesterol homeostasis in motor-neuron degeneration. Am. J. Hum. Genet. 82, 510−515.

Ulengin, I., Park, J.J., Lee, T.H., 2015. ER network formation and membrane fusion by atlastin1/SPG3A disease variants. Mol. Biol. Cell 26, 1616−1628.

Valenza, M., Marullo, M., Di Paolo, E., Cesana, E., Zuccato, C., Biella, G., Cattaneo, E., 2015. Disruption of astrocyte-neuron cholesterol cross talk affects neuronal function in Huntington's disease. Cell Death Differ. 22, 690−702.

van der Kant, R., Langness, V.F., Herrera, C.M., Williams, D.A., Fong, L.K., Leestemaker, Y., Steenvoorden, E., Rynearson, K.D., Brouwers, J.F., Helms, J.B., et al., 2019. Cholesterol metabolism is a druggable axis that independently regulates tau and amyloid-beta in iPSC-derived Alzheimer's disease neurons. Cell Stem Cell 24, 363−375.

van Weering, J.R.T., Scheper, W., 2019. Endolysosome and autolysosome dysfunction in Alzheimer's disease: where intracellular and extracellular meet. CNS Drugs 33, 639−648.

Vance, J.E., 2015. Phospholipid synthesis and transport in mammalian cells. Traffic 16, 1−18.

Vantaggiato, C., Crimella, C., Airoldi, G., Polishchuk, R., Bonato, S., Brighina, E., Scarlato, M., Musumeci, O., Toscano, A., Martinuzzi, A., et al., 2013. Defective autophagy in spastizin mutated patients with hereditary spastic paraparesis type 15. Brain 136, 3119−3139.

Vantaggiato, C., Panzeri, E., Castelli, M., Citterio, A., Arnoldi, A., Santorelli, F.M., Liguori, R., Scarlato, M., Musumeci, O., Toscano, A., et al., 2019. ZFYVE26/SPASTIZIN and SPG11/SPATACSIN mutations in hereditary spastic paraplegia types AR-SPG15 and AR-SPG11 have different effects on autophagy and endocytosis. Autophagy 15, 34−57.

Varga, R.E., Khundadze, M., Damme, M., Nietzsche, S., Hoffmann, B., Stauber, T., Koch, N., Hennings, J.C., Franzka, P., Huebner, A.K., et al., 2015. In vivo evidence for lysosome depletion and impaired autophagic clearance in hereditary spastic paraplegia type SPG11. PLoS Genet. 11, e1005454.

Velasco, S., Kedaigle, A.J., Simmons, S.K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., Regev, A., et al., 2019. Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. Nature 570, 523−527.

Voeltz, G.K., Prinz, W.A., Shibata, Y., Rist, J.M., Rapoport, T.A., 2006. A class of membrane proteins shaping the tubular endoplasmic reticulum. Cell 124, 573−586.

Wali, G., Liyanage, E., Blair, N.F., Sutharsan, R., Park, J.S., Mackay-Sim, A., Sue, C.M., 2020. Oxidative stress-induced axon fragmentation is a consequence of reduced axonal transport in hereditary spastic paraplegia SPAST patient neurons. Front. Neurosci. 14, 401.

Wang, S., Tukachinsky, H., Romano, F.B., Rapoport, T.A., 2016. Cooperation of the ER-shaping proteins atlastin, lunapark, and reticulons to generate a tubular membrane network. Elife 5, e18605.

Wang, X., Ma, Y., Duan, R., Liu, X., Zhang, H., Ma, J., Liu, Y., Sun, W., Liu, Q., 2020. Generation of a human induced pluripotent stem cell line (SDUBMSi001-A) from a hereditary spastic paraplegia patient carrying kif1a c.773C>T missense mutation. Stem Cell Res. 43, 101727.

Wang, X., O'Kane, C.J., 2008. Hereditary spastic paraplegia genes in *Drosophila*: dissecting their roles in axonal degeneration and intracellular traffic. SEB Exp. Biol. Ser. 60, 161−182.

Xia, C.H., Roberts, E.A., Her, L.S., Liu, X., Williams, D.S., Cleveland, D.W., Goldstein, L.S., 2003. Abnormal neurofilament transport caused by targeted disruption of neuronal kinesin heavy chain KIF5A. J. Cell Biol. 161, 55−66.

Xu, H., Ren, D., 2015. Lysosomal physiology. Annu. Rev. Physiol. 77, 57−80.

Yalcin, B., Zhao, L., Stofanko, M., O'Sullivan, N.C., Kang, Z.H., Roost, A., Thomas, M.R., Zaessinger, S., Blard, O., Patto, A.L., et al., 2017. Modeling of axonal endoplasmic reticulum network by spastic paraplegia proteins. Elife 6, e23882.

Yang, T., Arslanova, D., Gu, Y., Augelli-Szafran, C., Xia, W., 2008. Quantification of gamma-secretase modulation differentiates inhibitor compound selectivity between two substrates Notch and amyloid precursor protein. Mol. Brain 1, 15.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917−1920.

Zhang, H., Hu, J., 2016. Shaping the endoplasmic reticulum into a social network. Trends Cell Biol. 26, 934−943.

Zhang, S.C., Li, X.J., Johnson, M.A., Pankratz, M.T., 2008. Human embryonic stem cells for brain repair? Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 87−99.

Zhao, G.H., Liu, X.M., 2017. Clinical features and genotype-phenotype correlation analysis in patients with ATL1 mutations: a literature reanalysis. Transl. Neurodegener. 6, 9.

Zhao, J., Hedera, P., 2013. Hereditary spastic paraplegia-causing mutations in atlastin-1 interfere with BMPRII trafficking. Mol. Cell. Neurosci. 52, 87−96.

Zhou, X., He, Y., Huang, X., Guo, Y., Li, D., Hu, J., 2019. Reciprocal regulation between lunapark and atlastin facilitates ER three-way junction formation. Protein Cell 10, 510−525.

Zhu, P.P., Denton, K.R., Pierson, T.M., Li, X.J., Blackstone, C., 2014. Pharmacologic rescue of axon growth defects in a human iPSC model of hereditary spastic paraplegia SPG3A. Hum. Mol. Genet. 23, 5638−5648.

Zhu, P.P., Patterson, A., Lavoie, B., Stadler, J., Shoeb, M., Patel, R., Blackstone, C., 2003. Cellular localization, oligomerization, and membrane association of the hereditary spastic paraplegia 3A (SPG3A) protein atlastin. J. Biol. Chem. 278, 49063−49071.

This page intentionally left blank

CHAPTER

Induced pluripotent stem cells for modeling Smith–Magenis syndrome

9

Maria Pennuto[1,2,3], Elisa Maria Turco[4], Laura Sireno[1,2], Laura Bernardini[5], Roberta Onesimo[6], Chiara Leoni[6], Giuseppe Zampino[6], Jessica Rosati[4], Angelo Luigi Vescovi[4]

[1]*Department of Biomedical Sciences (DBS), University of Padova, Padova, Italy;* [2]*Veneto Institute of Molecular Medicine (VIMM), Padova, Italy;* [3]*Padova Neuroscience Center (PNC), Padova, Italy;* [4]*Cellular Reprogramming Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy;* [5]*Medical Genetics Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy;* [6]*UOS Malattie Rare e Difetti Congeniti – Area Bambino, Dipartimento Scienze Salute Donna Bambino e Sanità Pubblica, Fondazione Policlinico Universitario A Gemelli – IRCCS, Rome, Italy*

Chapter outline

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00013-8

Abstract

Smith–Magenis syndrome (SMS) is a genetic neurodevelopmental disease characterized by neurological, psychiatric, anatomical, and motor symptoms. The disease is caused by deletions on chromosome 17p11.2, which may lead to the loss of up to 95 genes, depending on the length of the chromosomal rearrangement. One of these genes is retinoic acid-induced 1 (*RAI1*). Evidence that loss of *RAI1* is responsible for several clinical manifestations of SMS came with the identification of patients carrying point mutations in this gene and presenting a phenotype overlapping with SMS. Rather, disease severity and some clinical presentations are associated with loss of genes other than *RAI1*. SMS patients are typically heterozygous for the mutation (*RAI1* mutations and chromosomal deletions), indicating that loss of one functional allele of *RAI1* is sufficient to cause disease. Interestingly, duplications of the same chromosomal region cause another neurodevelopmental disease with similar clinical manifestations, thus indicating that *RAI1* (and possibly other genes nearby) are dosage-sensitive genes. RAI1 is a polyglutamine- and polyserine-containing factor induced by retinoic acid and with nuclear localization. However, its function in physiological conditions and how its haploinsufficiency causes SMS is not known. Here, we will review several aspects related to SMS, from diagnosis to clinical presentation. Moreover, we analyze in detail what is known about the *RAI1* isoforms, expression pattern, native function, and the impact of different types of mutations (deletions, frameshift mutations that generate premature stop codons, and missense mutations that result in the production of the full-length protein). Finally, we review the animal and cell models available to date, focusing on those based on the immortalized pluripotent technology. These patient-derived cells allow replicate in a dish an otherwise irreproducible condition, which takes into account the genetic variability of patients and that may then complement mechanistic studies performed in mouse models of SMS.

Keywords: Brain abnormalities; Chromosome 17; Cognitive impairment; Copy number variation (CNV); Haploinsufficiency; Induced pluripotent stem cell (iPSC); Melatonin; Neural stem cells; Neurodevelopmental disease; Neuropsychiatric disease; Potocki–Lupski syndrome (PTLS); Retinoic acid-induced 1 (RAI1); Self-injury; Sleep disorder; Smith–Magenis syndrome (SMS).

Introduction

Smith–Magenis syndrome (SMS) is a complex congenital anomaly/mental retardation syndrome with physical and neurobehavioural symptoms. SMS is a rare disease,

which affects fewer than 200,000 people worldwide, with an incidence at birth ranging between 1:15,000 and 1:25,000. The majority (about 90%) of SMS patients carries a ~3.7 Mb interstitial deletion of chromosome 17p11.2, spanning about 95 genes, including retinoic acid-induced 1 (*RAI1*). A limited number of patients (about 10%) have missense, insertion, deletion, and nonsense mutations in the *RAI1* gene, leading to frameshifts and generation of premature stop codons that result in the formation of truncated RAI1. All of the *RAI1* gene mutations known to date are localized within exon 3, which codes for about 95% of RAI1 (Smith et al., 2005; Falco et al., 2017). Most mutations are de novo mutations, with no correlation to specific racial or ethnic groups. Although this genetic condition was described for the first time around 40 years ago by the geneticist Ann C. M. Smith and the pediatrician/geneticist R. Ellen Magenis, approximately only 500 research and review papers on SMS have been published to date, which can be divided into three main groups according to the approach undertaken and the topics covered. Two of the groups account for the vast majority of the publications. The first group focuses on the genetic causes of the disease and the characterization of the genes included in the deletions, while the second one is represented by clinical articles describing the morphological and behavioral characteristics of the patients. A third and smaller group of publications regards mechanistic aspects explored in *in vitro* models and genetically modified mice with haploinsufficiency and *RAI1* gene knockout. In this chapter, we will review the literature related to SMS, focusing on the following aspects: (1) the genetics of SMS and the genetic tests available for diagnosing the disease; (2) its clinical presentation and the therapies and drugs currently used to alleviate symptoms; (3) the *RAI1* gene and its protein products; (4) the recent development of SMS patient-derived model systems based on the use of induced pluripotent stem cells (iPSCs) and mice for the purpose of carrying out in-depth studies on the molecular mechanisms underpinning this disorder. Novel lines of research on disease pathogenesis combined with preclinical studies may lead to the development of strategies for improving symptoms, with the final goal of identifying new targets for therapy development.

The genetics of Smith–Magenis syndrome

SMS (MIM 182290) is linked to a 3.9 Mb deletion in the 17p11.2 chromosomal band, which encompasses 95 genes, including the *RAI1* gene (MIM 607642): del(17)(p11.2). In this region, which represents an unstable genomic segment prone to chromosomal rearrangements, such as microdeletions and/or microduplications, three complex and highly homologous low copy repeats (LCRs) have been mapped, with a sequence identity of about 98%, that flank or lie within the deleted region, termed "distal" (~176 kb), "middle," and "proximal" (~256 kb) SMS repeats (SMS-REPs), respectively (Chen et al., 1997; Bi et al., 2003). This genomic architecture indicates that the primary cause of the SMS deletion is a nonallelic homologous recombination (NAHR) between similar LCRs, which gives rise to two

reciprocal products: a microdeletion associated with SMS, and a microduplication associated with a distinct genomic disorder, namely Potocki–Lupski Syndrome (MIM 610883) (Fig. 9.1). Indeed, NAHR is an ectopic recombination event between paralogous LCRs (DP-LCR), leading to recurrent genomic deletions and reciprocal duplications/inversions (Stankiewicz and Lupski, 2002; Dittwald et al., 2013). In particular, the directly oriented distal and proximal SMS-REPs share four homologous segments (sequence blocks A, B, C, and D), with a total size of ~170 kb, which are involved in the recurrent NAHR occurring on this chromosomal segment (Bi et al., 2003). The remaining ~20% of deletions can be larger or smaller, with a minimum deletion region of approximately 650 kb (Stankiewicz and Lupski, 2002; Slager et al., 2003; Schoumans et al., 2005). These deletions are likely the result of homologous recombination events between the middle and distal SMS-REPS and other LCRs mapping within this region. On the other hand, about 8% of atypical SMS deletions originate from a nonhomologous end joining (NHEJ) event, a phenomenon implicated in double-strand break repair during DNA replication, which generates rearrangements of unique size and with unique breakpoints (Shaw and Lupski, 2005).

Although a limited number of SMS patients carry mutations in the *RAI1* gene, the phenotypic similarity between these patients and those carrying the deletion has allowed researchers to establish that *RAI1* loss of function is responsible for several aspects of SMS symptoms. Mutations in this gene were first described in 2003, when frameshift mutations leading to the production of truncated protein products were found in blood samples taken from three individuals with a clinical diagnosis of SMS, but who were not carrying deletions. These were then sequenced for a series of genes localized within the SMS del(17)(p11.2) critical interval (Slager et al., 2003). Thereafter, at least 30 distinct mutations in *RAI1* were linked to SMS, most of which are frameshifts, while a few are missense mutations. It has been predicted that most RAI1 mutations are likely to hamper gene expression and alter normal protein structure/function, ultimately leading to *RAI1* haploinsufficiency (Bi et al., 2004; Girirajan, 2005; Truong et al., 2010; Vilboux et al., 2011; Dubourg et al., 2014; Acquaviva et al., 2017).

The genetics of SMS highlights that SMS is associated with a haploinsufficiency of the dosage-sensitive *RAI1* gene. Many neurodevelopmental and neurobehavioral profiles are observed in both *RAI1*-mutated patients and SMS-deleted subjects, whereas multiorgan impairments are typical only of the latter (Girirajan et al., 2006). Therefore, the more complex phenotypes associated with the 17p11.2 deletions may be ascribed to the contribution of the genes mapping to this genomic region. In some of these cases, the deletion could unmask recessive mutations in the remaining allele, leading to phenotypic expression of other diseases (Girirajan et al., 2006; Edelman et al., 2007). In particular, mutations in *MYO15A*, causative of a nonsyndromic deafness form (DFNB3;OMIM #600316), have been detected in SMS patients showing sensorineural hearing loss (Liburd et al., 2001). Moreover, a second hit mutation of the wild type allele of *FLCN* has been related to renal tumors, which are occasionally found in SMS patients (Dardour et al., 2016). Short stature is among

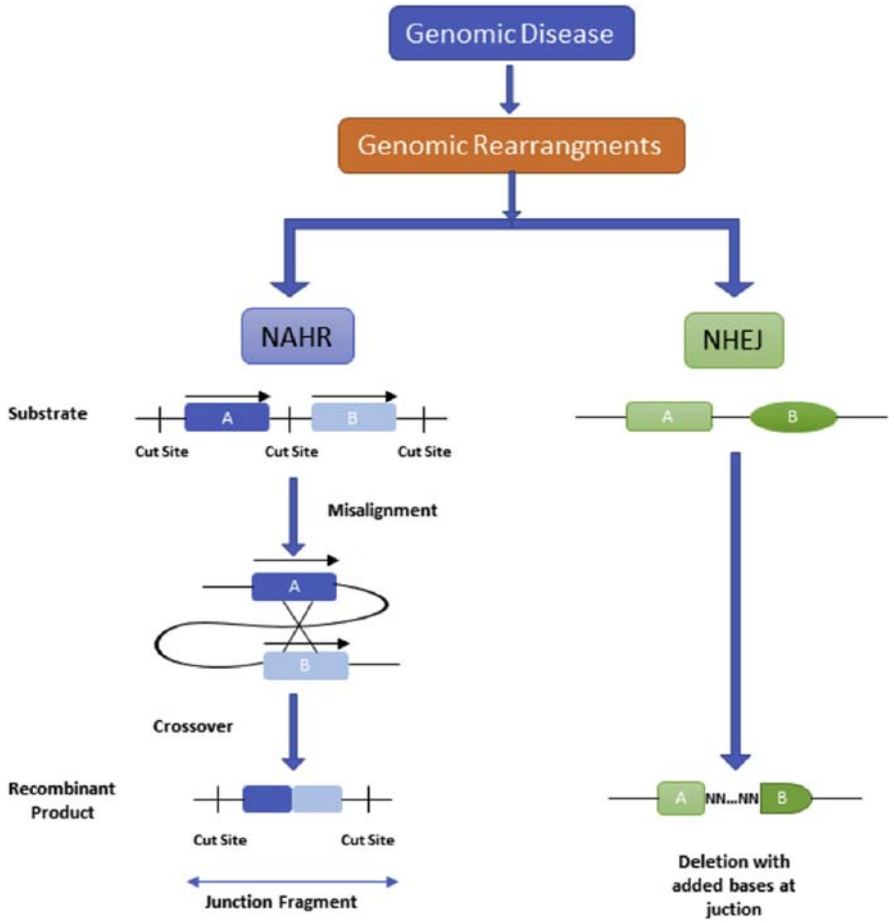


FIGURE 9.1

Generation of deletion rearrangement by nonallelic homologous recombination (NAHR) and nonhomologous end joining (NHEJ). The substrates and products of recombination are shown. NAHR (left), utilizes two nonallelic LCRs (A and B) as substrates for recombination. The LCRs are depicted as *blue rectangles*, due to high homology, but are different shades of blue, signifying the few cis-morphisms, or paralogous sequence variants, that distinguish them. LCRs A and B, oriented in the same direction (shown by *arrows*), misalign, and subsequent homologous recombination results in a deletion with a single recombinant LCR, shown as a *two-tone blue rectangle*. Restriction enzyme consensus sequences (cut sites) are depicted as vertical lines on either side of the recombinant LCR, with deletion of the consensus sequence between the two substrate LCRs. Digestion using this enzyme results in the isolation of a recombination-specific junction fragment, shown below. NHEJ (right), utilizes two nonhomologous sequences [*red rectangle* (A) and *green oval* (B)] as substrates for recombination. The two sequences are joined via NHEJ, with deletion of the intervening fragment. Additional bases (NN…NN) are added at the deletion junction.

Adapted from Shaw, C.J., Lupski, J.R., 2004. Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. Hum. Mol. Genet. 13 Spec No 1:R57-64. https://doi.org/10.1093/hmg/ddh073. PMID: 14764619.

the distinctive and more recurrent clinical characteristics restricted to patients carrying the deletion, and it has been suggested that the region included between *SREBF1* and *SHMT1* genes includes at least one dosage-sensitive gene responsible for this clinical feature (Falco et al., 2017; Edelman et al., 2007).

Diagnostic test

Although extensive efforts have been put into the development of effective tests for early diagnosis of SMS, this goal has not yet been reached. Larger deletions (>5−10 Mb) can be occasionally detected by a routine G-banded standard karyotype analysis. Another preliminary diagnostic analysis, which has been widely used for testing probands with clinical features of SMS, is fluorescent in situ hybridization analysis, which uses a nucleotide probe, spanning the *RAI1* locus, to detect 17p11.2 deletions encompassing the critical region on metaphases from peripheral blood (Fig. 9.2A; Vlangos et al., 2005). This kind of diagnostic approach can highlight potential complex familial chromosome rearrangements leading to del(17)(p11.2), although these are rare in the case of SMS deletions (Gamba et al., 2011). More recently, microarray platforms have been developed to perform a genome-wide microdeletion/microduplication [copy number variation (CNV)] analysis, known as chromosome microarray analysis (CMA), used to detect the 17p11.2 deletion and simultaneously determine the exact size and location of breakpoints (Miller et al., 2010; Fig. 9.2B and C). This is currently the test that is recommended first for all clinical conditions characterized by neurodevelopmental disorders and/or congenital defects and/or dysmorphisms (Martin et al., 2006). This genome-wide approach can also distinguish different CNVs associated with SMS-like phenotypes, other than del(17)(p11.2). In the case of a negative result from CMA, a sequence analysis of *RAI1* gene, in particular of exon 3, can be performed (Bi et al., 2004; Girirajan, 2005; Truong et al., 2010; Vilboux et al., 2011; Dubourg et al., 2014; Acquaviva et al., 2017). This analysis can be carried out by Sanger sequencing technique or by next generation sequencing, especially when *RAI1* is introduced in multigene panels, allowing patients with milder clinical phenotypes to be diagnosed with SMS as well.

Clinical presentation of Smith−Magenis syndrome

Disease onset, diagnosis, and symptomatology

SMS newborns are generally born at term and display normal growth up to the first month, followed by a period of decelerated weight gain that typically ensues with associated feeding difficulties and gastroesophageal reflux (Smith et al., 2005; Falco et al., 2017). At 18 months of age, a marked failure to thrive becomes evident. However, SMS will typically not be suspected until several of the behavioral and sleep abnormalities that are unique to this condition become more apparent with age (Martin et al., 2006). Unfortunately, this failure/delay in diagnosis at birth or in early infancy puts off essential therapies for preventing the onset of further problems, in

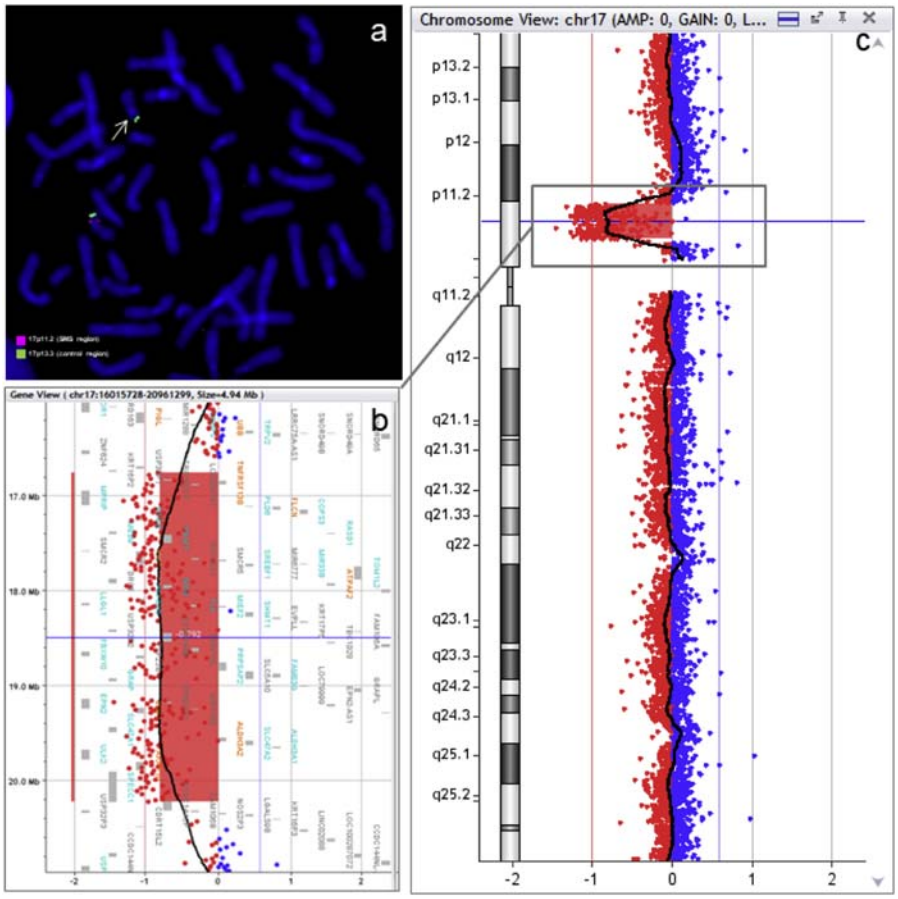


FIGURE 9.2

Fluorescent in situ hybridization (FISH) with an Smith–Magenis syndrome (SMS) locus-specific probe including RAI1 showing the (A) absence of the red signal on one chromosome 17 (*arrow*); a green-labeled probe mapping to the subtelomeric region 17p13.3 was used as a control; (B) and (C).

particular, neuropsychological symptoms. Even later, this neurodevelopmental disorder is underdiagnosed, as it is often confused with other pathologies, such as Down, Angelman, and Prader Willi syndromes, which display similar dysmorphisms or behavioral patterns. Moreover, the presence of speech delay as well as repetitive and obsessive behavior often lead physicians to diagnose autism spectrum disorder in comorbidity with SMS, which implicates that *RAI1* can be considered a candidate gene for susceptibility to nonsyndromic autism spectrum disorder (Burns et al., 2010). It is thus extremely important to take into account autistic spectrum disorder in differential diagnosis of SMS (Wilde et al., 2013; Wilde and Oliver, 2017).

Clinical differences in symptoms among patients may be influenced by the genetic variations mentioned above. The heterogeneity of clinical presentation reflects the genetic complexity of the disease, which can result from either different types of mutations in the *RAI1* gene, e.g., frameshift versus point mutations, or deletions of chromosome 17p11.2, leading to a spectrum of symptoms whose severity correlates with the length of the chromosomal deletion. Cognitive impairment and aberrant somatic features, such as short stature, hearing loss, and congenital heart defects, are less severe and have a lower incidence in patients with RAI1 variants (Shayota and Elsea, 2019), while SMS patients carrying smaller deletions exhibit flattened heads, dental and eye irregularities, head banging, and hyperactive behavior (Girirajan et al., 2006). On the other hand, extensive 17p11.2 deletions have greater effects on phenotypic appearance, and their length is highly correlated to the extent of multiorgan involvement, indicating that genes other than *RAI1* are also implicated in the pleiotropic manifestations of such SMS patients and in the severity of phenotype (Falco et al., 2017; Truong et al., 2010; Girirajan et al., 2006; Dardour et al., 2016; Huang et al., 2016). Almost all SMS patients, however, show behavioral and neurodevelopmental impairments, such as intellectual disability, auto- and heteroaggressivity, and alterations in the sleep-awake cycle, independently of the genetic disease etiology in affected individuals (Falco et al., 2017). This is most likely due to the fact that the *RAI1* gene plays, in all cases, a pivotal role in disease pathogenesis.

Brain structural abnormalities

Brain magnetic resonance imaging of SMS patients has revealed several structural abnormalities. Patients present with a pronounced enlargement of the cisterna magna, often accompanied by the agenesis of cerebellum. Some patients may either experience epileptic seizures or show electroencephalographic signs of epilepsy without clinical correlation. Maya et al. (2014) detected a significant decrease in gray matter with bilateral perfusion into the insula and lenticular nucleus, without any pathological sign in white matter structure. A deletion of the 17p11.2 region associated with bilateral periventricular nodular heterotopia has been described in two SMS individuals (Capra et al., 2014) Table 9.1.

Behavioral, neurodevelopmental, and cognitive aspects

Children affected by SMS show severe behavioral and cognitive impairments, with a devastating effect on the quality of life Table 9.1. The behavioral phenotype of SMS includes maladaptive/self-injurious, aggressive, stereotypic, and food-seeking behaviors (Shayota and Elsea, 2019). Auto- and heteroaggressiveness are present in 97% of diagnosed cases, which become evident only after 18 months of age, becoming more severe as the patient matures. Prior to this age, newborns carrying this disorder, according to Gropman and collaborators (Wolters et al., 2009) sleep more hours than average, are generally calm, are quite sociable and have an age-adequate disposition. The earliest clinical symptoms are hypotonia and certain feeding difficulties, which are visible at birth, and only later do they show a

Table 9.1 Smith–Magenis clinical findings.

Smith–Magenis syndrome clinical findings	
Behavioral aspects	
Maladaptive behaviors	Attention seeking, impulsivity, aggression, disobedience, frequent outbursts, hyperactivity, and attention deficits
Self-injurious behaviors	Wrist biting, skin picking, head banging, insertion of objects into bodily orifices, pulling out of nails, and insensitivity to pain
Sleep–wake cycle disfunction	Difficulty falling asleep, diminished rapid eye movement sleep, reduced total sleep and shortened sleep cycles, and early morning awakenings
Stereotypical behaviors	Self-hugging with excitement, autistic-like behaviors, spasmodic upper body squeeze, bruxism, and mouthing of objects
Eating disorders	Feeding difficulties, early onset obesity, impaired satiety, and hypercholesterolemia
Neurological and cognitive aspects	
Central nervous system aspects	Enlarged ventricles, decreased gray matter in the insula and lenticular nucleus, malformed brain stem, and underdeveloped cerebellar vermis
Motor aspects	Hyporeflexia, sensory integration problems, infantile hypotonia, and delay fine motor skills
Cognitive aspects	Delayed speech, mild to moderate intellectual disability, and short-term memory impairment
Developmental anomalies	
Craniofacial features	Brachycephaly, midface hypoplasia, micrognathia, prognathism, and tented upper lip
Skeletal and hearing system impairment	Short stature, brachydactyly, vertebral anomalies, and scoliosis Hearing loss, deep hoarse voice, and chronic ear infections
Dermatological impacts	Dermatofibromas, skin lesions that are secondary to self-injury, and acral pachydermia
Congenital defects	
Cardiological defects	Cardiovascular defects (ventricular septal defect (VSD), atrial septal defect (ASD), tetralogy of Fallot), anomalous pulmonary venous return, and tricuspid and mitral valve stenoses or regurgitation
Kidney abnormalities	Enlarged and ectopic kidneys, renal and urologic anomalies, hydroureters, hypertrophic kidney, and ureterovesicular obstruction
Hand development	Brachydactyly, fifth finger clinodactyly and syndactyly, and fetal finger pads
Dental irregularities	Tooth root dilacerations, tooth agenesis, and taurodontism

Adapted from Chen, L., Mullegama, S. V., Alaimo, J. T., Elsea, S. H., 2015. Smith-Magenis syndrome and its circadian influence on development, behavior, and obesity – own experience. Dev. Period Med. 19, 149–156. We extensively modified the table so it is not adaped from Chen et al.

nonstable gait and, in many cases, hyporeflexia (Smith et al., 2005; Falco et al., 2017; Dardour et al., 2016; Hildenbrand and Smith, 2012). Consequently, SMS patients are diagnosed with a neurodevelopmental disorder only when stereotypical behaviors, episodes of aggressiveness and motor-cognitive impairment become more evident (Laje et al., 2010; Huisman et al., 2018). Martin et al. (2006) also associated disinhibition with the onset of aggressive behavior. The behavioral dysfunction and stereotypic behaviors in these patients include marked distractibility, as well as repetitive actions, such as body rocking, spinning/twirling, nail-yanking, inserting objects into their mouths and other body orifices, teeth grinding, compulsive finger-licking and page flipping, ("lick-and-flip") and "self-hugging," an action they tend to perform when pleased (Laje et al., 2010; Huisman et al., 2018). Comparison between patients affected by SMS and patients showing an idiopathic autism spectrum disorder revealed that the tendency to engage into impulsive, aggressive and/or destructive behavior, including property destruction, seems to have a higher frequency in SMS patients (Shayota and Elsea, 2019).

The prevalence of behavioral difficulties in subjects with large deletions, who show more severe intellectual disability and limited mobility (Girirajan et al., 2006), is generally lower than in patients with smaller deletions and in patients with RAI1 mutations. In a comparative study between 15 SMS patients and 15 age- and sex-matched control subjects, it was found that a high level of cognitive function was inversely correlated with the severity of self-injurious behavior (Osório et al., 2012). This kind of behavior may result from a sense of frustration, due to their lack of communication skills, or to an attention-seeking attitude, whereas aggressive behavior directed toward others is generally more affected by external contingencies (attention, escape, and access to food) (Wilde et al., 2013; Laje et al., 2010; Huisman et al., 2018; Gnanavel, 2014). Moreover, several patients exhibit symptoms of peripheral neuropathy, such as low tendon reflexes and a decreased sensitivity to pain, two clinical characteristics that may have an impact on the severity of behavioral issues, especially those associated with aggressiveness, due to their significantly higher pain threshold. Because SMS patients have decreased sensitivity to pain, severe medical conditions may be undiagnosed, thus exacerbating behavioral disturbances (Poisson et al., 2015). Maladaptive behaviors typically worsen in adolescents and adults (Shayota and Elsea, 2019), declining significantly however among aging adults, even without specific management strategies (Nag and Nærland, 2020).

Intellective disability tends to vary from moderate to severe. The attention problems and hyperactive behavior of these children do not affect their capacity to integrate information. There is an overt delay in language skills, with greater impact on expressive language than on receptive language, which in turn is more impacted than mathematical reasoning and calculations skills. However, although patients suffering from SMS may show skills in specific fields, such as computer use, and present with extremely high attention to detail and long-term memory, these patients may not be self-sufficient and may need assistance at school and at home. No age-related cognitive decline has been reported to date (Wilde and Oliver, 2017).

The therapeutic approach for the behavioral/intellective disabilities typical of these patients should focus on improving communication skills and on identifying and treating attention deficit/hyperactivity, aggressiveness, and anxiety. The complexity of all these aspects requires a multidisciplinary team, including geneticists, psychiatrists, neuropaediatricians/neurologists, somnologists, developmental and behavioral paediatricians, and speech and language therapists (Smith et al., 2005; Falco et al., 2017; Wilde et al., 2013; Wilde and Oliver, 2017). The use of psychotropic medication is often required as well for the management of inattention and hyperactivity, although no single regimen has yet shown consistent and long-term efficacy in SMS patients.

Sleepwake cycle dysfunction

Sleep disorder is one of the most prominent symptoms of SMS and is present in 88% of patients carrying either *RAI1* mutations or chromosomal deletions Table 9.1. Patients show difficulty falling asleep, frequent nocturnal awakenings, reduced or absent rapid eye movement sleep, early sleep offset, and daytime sleepiness with a need for daytime naps (Nag and Nærland, 2020). The etiology of this phenomenon is still unclear. The observation that SMS individuals have elevated melatonin concentration in the daytime, in contrast to very low excretion at night, suggested that melatonin secretion might undergo an inversion process in SMS patients. However, some patients display normal melatonin secretion, despite suffering from sleep disturbances (Chik et al., 2010). This dysfunction could stem from various causes, ranging from an impairment in melanopsin-based photoreception, to a deficit in rod function, to a more highly activated circadian response to light. RAI1 deregulation may also play a role in circadian regulation, without, however, having a significant impact on melatonin secretion (Barboni et al., 2018). If this were the case, inverted melatonin secretion may be secondary to a deregulated molecular circadian network, thus influencing sleep patterns.

Because sleep deprivation has a huge impact on patients' quality of life, clinicians tend to treat it via exogenous administration of melatonin supplement. However, after repeated doses, tolerance to this substance is frequently reported (Dosier et al., 2017; Van Thillo et al., 2010; Carpizo et al., 2006). Another therapeutic approach is melatonin supplementation combined with a beta-receptor antagonist. Beta-blockers are given in the morning to halt the abnormal secretion of melatonin occurring at daytime, in combination with a melatonin supplement administered at night to regularize the sleep cycle. This strategy has been shown to be effective in improving patients' quality of life.

Eating disorders, auxologic and nutritional issues

SMS patients have significant pathological food-related behaviors. Interestingly, 90% of SMS individuals exhibit truncal obesity after 10 years of age, or reach the 90th percentile for weight prior to or during adolescence, which persists throughout their life, although newborns initially eat adequately and do not have weight issues (Burns et al., 2010; Chen et al., 2015; Alaimo et al., 2015; Alaimo et al., 2014) Table 9.1.

In SMS patients, there is an increased prevalence of hypercholesterolemia and hyperphagia. Despite the prevalence of compulsive behavior in individuals with SMS, their obesity is not always concurrent with compulsive eating. However, food-related obsessive-compulsive disorder analogous to the hyperphagia typically observed in Prader Willi patients is very common among SMS patients (Alaimo et al., 2015). A highly inclusive therapeutic approach, targeting different aspects of disease manifestations, from circadian rhythm to development, metabolism and behavior, as proposed by Chen and collaborators, is now generally approved by the medical community. An integrated treatment of all dysfunctions characterizing the clinical profile of SMS patients, using an individualized itinerary, may contribute to attenuate symptoms, prevent or slow progression, and improve the quality of life for patients and their relatives (Vilboux et al., 2011).

Craniofacial features, skeletal and hearing system impairment

A distinctive facies is common to both 17p11.2-deleted and *RAI1*-mutated patients, thus implying that RAI1 is involved in craniofacial development Table 9.1. SMS children have a bulging forehead, short midface and a broad, squared facial structure, characterized by brachycephaly and microcephaly, low or posteriorly rotated ears, frontal bossing, and a depressed nasal bridge. In addition, they generally have fair-colored hair, a hoarse voice, vocal cord nodules, synophrys, upslanting palpebral fissures, epicanthal folds, and hypertelorism (Huang et al., 2018). SMS can also be accompanied by abnormalities affecting the skeletal system. Scoliosis has an incidence of 30% in SMS, which is significantly higher (2%) with respect to the general population. Its etiopathogenesis is still unclear, although a link between retinoic acid (RA) and spine morphogenesis has been implicated (Spilsbury and Mohanty, 2003; Li et al., 2015). The craniofacial dysmorphology may provoke both conductive and neurosensorial alterations in the auditory system, leading to hearing loss, which represents a frequent, yet generally mild, clinical feature of SMS patients (Elsea and Girirajan, 2008; Smith et al., 1998). This clinical symptom results from alterations in the eustachian tube and shallow nasopharynx (Bi et al., 2003; Girirajan et al., 2006; Gamba et al., 2011; Huisman et al., 2018; Di Cicco et al., 2001; Brendal et al., 2017; Smith et al., 1986; Allanson et al., 1999). SMS patients show recurrence of otitis media and suffer from hyperacusis, a phenomenon also observed in patients with autistic spectrum disorder (Rubenstein et al., 1996). It is interesting to notice that 37,5% of patients carrying the *RAI1* mutation experience hearing loss in one or both ears, while the frequency rises to 74,4% in patients carrying 17p11.2 microdeletions, suggesting that *RAI1* is involved in the formation of the organ of Corti, while the high frequency of hearing loss, associated with the deletion, could be caused by haploinsufficiency of other genes (Falco et al., 2017; Brendal et al., 2017), such as MYO15A and DFNB3, whose loss causes nonsyndromic deafness (Zhang et al., 2019). Hearing evaluations and initiation of language/speech therapy and assistive communication should be undertaken as early as possible (Shayota and Elsea, 2019; Poisson et al., 2015).

Dermatological symptoms

Many patients are characterized by skin lesions that are secondary to self-injury, such as skin abrasions, bites, bruises and/or dystrophic lesions Table 9.1. Dermatofibromas are frequent in older patients. Xerosis is present in the vast majority of patients, in association with extensive keratosis pilaris. Acral pachydermia, fissured plantar keratoderma, eyelash trichomegaly, heavy brows, and folliculitis on the back are also common. Dry skin is more frequent among SMS patients with chromosomal deletions than in those with *RAI1* mutations, suggesting that the genes involved in the deletion might play a regulatory role in normal skin hydration (Guérin-Moreau et al., 2015).

Congenital defects

Cardiological defects have an estimated frequency of 37% and include atrial septal defects, ventricular septal defects, tetralogy of Fallot, anomalous pulmonary venous return, tricuspid and mitral valve stenoses or regurgitation, mitral valve prolapse, subvalvular aortic stenosis, and supravalvular pulmonary stenosis (Myers and Challman, 2004) Table 9.1.

Kidney abnormalities (renal dysplasia, ureterovesicular obstruction, hydroureters, hypertrophic kidney with malposition, ectasia of the renal pelvis, and vesicoureteral reflux) are found in 20% of patients Table 9.1. It is possible that loss of one or more genes in the 17p11.2 region affects the process that initiates ureteric bud formation and metanephric blastema differentiation, which ultimately leads to normal nephron development (Dardour et al., 2016). Renal and urologic anomalies have been reported in patients with atypically small deletions, but not in those with *RAI1* mutations, suggesting that loss of *RAI1* is not responsible for this phenotype. Concerning hand development, SMS patients have inward bending fingers often affected by brachydactyly, fifth finger clinodactyly, and syndactyly. Fetal finger pads may be present as well. Some patients present with dental irregularities: tooth root dilacerations, tooth agenesis, and taurodontism, a phenomenon leading to a massive development of the pulp chamber, jeopardizing root formation (Tomona et al., 2006). Some patients display hypogammaglobulinemia, due to a de novo 3.8-Mb deletion (breakpoints, chr17: 16,660,721-20,417,975), sometimes resulting in haploinsufficiency for transmembrane activator and CAML interactor. A number of SMS individuals with heterozygous 17p.11.2 deletions develop pneumonia and gastroenteritis, but not autoimmune, allergic, or malignant diseases.

Retinoic acid-induced 1: from gene to protein

The Retinoic acid-induced 1 gene

Mouse *RAI1* gene was identified in a molecular screening performed to map the genes whose expression is induced during neuronal differentiation in response to RA treatment (Imai et al., 1994, 1995). RA treatment resulted in a marked and sustained expression of a clone, namely GT1, which was then named *RAI1*. RA is the bioactive metabolite of vitamin A (Zieger and Schubert, 2017). Normally, binding of

RA to the retinoic acid receptor (RAR) leads to a conformational change, which results in the dissociation from transcription co-repressors and the recruitment of co-activators, leading to the formation of functional complexes that bind to the RA response elements on the *RAI1* promoter. The human *RAI1* gene shares 80% homology with the mouse gene, indicating that *RAI1* is highly conserved throughout mammalian evolution (Toulouse et al., 2003). The human *RAI1* gene spans over 120 Kb within chromosome 17p11.2 and is composed of 6 exons (Fig. 9.3).

The *RAI1* gene primary transcript consists of an ~8.5-Kb mRNA with a long 3′ untranslated region. The translation start site is localized in exon 3, which codes for most of the protein. There are four RAI1 isoforms produced by alternative splicing of the main mRNA transcript (Fig. 9.3) (UniProtKB/Swiss-Prot ID: Q7Z5J4-1, Q7Z5J4-2, Q7Z5J4-3, Q7Z5J4-4). Isoform 1 corresponds to the full-length protein (1906 amino acids), with a predicted molecular weight of 203 kDa, and an effective molecular weight in SDS-PAGE of more than 250 kDa, possibly due to posttranslational modifications (PTMs). Isoform 2 (1862 amino acids) is very similar to isoform 1, whereas isoform 3 (1640 amino acids) lacks part of the carboxy-terminal domain (residues 1641−1906). Isoform 4 (966 amino acids) is the shortest isoform, containing only the first half of RAI1 protein (residues 1−967) (Carmona-Mora and Walz, 2010).

Rai1 expression and subcellular localization

The *RAI1* primary transcript has been detected in all tissues, with higher expression in the heart and brain. In one study, transcript levels were shown to increase during development, peaking around the first week of age, to then slightly decline and remain higher in adulthood compared to developmental stages (Klionsky et al., 2016). Protein expression analysis performed in mice and humans showed that RAI1 is widely expressed in many tissues, at particularly high levels in neuronal tissues during the early stages of neurodevelopment, where it may have a crucial role in cognitive and motor skills (Huang et al., 2016; Toulouse et al., 2003; Bi et al., 2005; Potocki et al., 2007; Fragoso et al., 2015). In mouse embryos, RAI1 was expressed in craniofacial components at 10.5 days postconception (dpc), acquiring a more widespread expression around 12.5 dpc in epithelial cells of olfactory pit and nasal process, endoderm of branchial arches, apical ectoderm ridge, gut, and thyroid primordium. At the postnatal age, high levels of expression of RAI1 were detected in hippocampus (CA1, CA2, CA3, and dentate gyrus) and cerebellum. RAI1 is also expressed in the striatum, cortex, nucleus accumbens, ependyma, and choroid plexus. Interestingly, RAI1 is enriched in postmitotic neurons (Huang et al., 2016). In peripheral tissues, RAI1 is expressed in the thymus, lung bronchioles, kidney tubules and genital organs, such as testis, epididymis, and ovaries. In *postmortem* human brains, *RAI1* was highly expressed in hippocampal neurons and in the occipital cortex neurons, but not in glia (Fragoso et al., 2015). In the cerebellum, the second region of highest expression, RAI1 was detected in Purkinje cells, but not granule cells. There were also differences in RAI1 expression within different neuronal populations. At the subcellular level, RAI1 mainly localizes to the nucleus

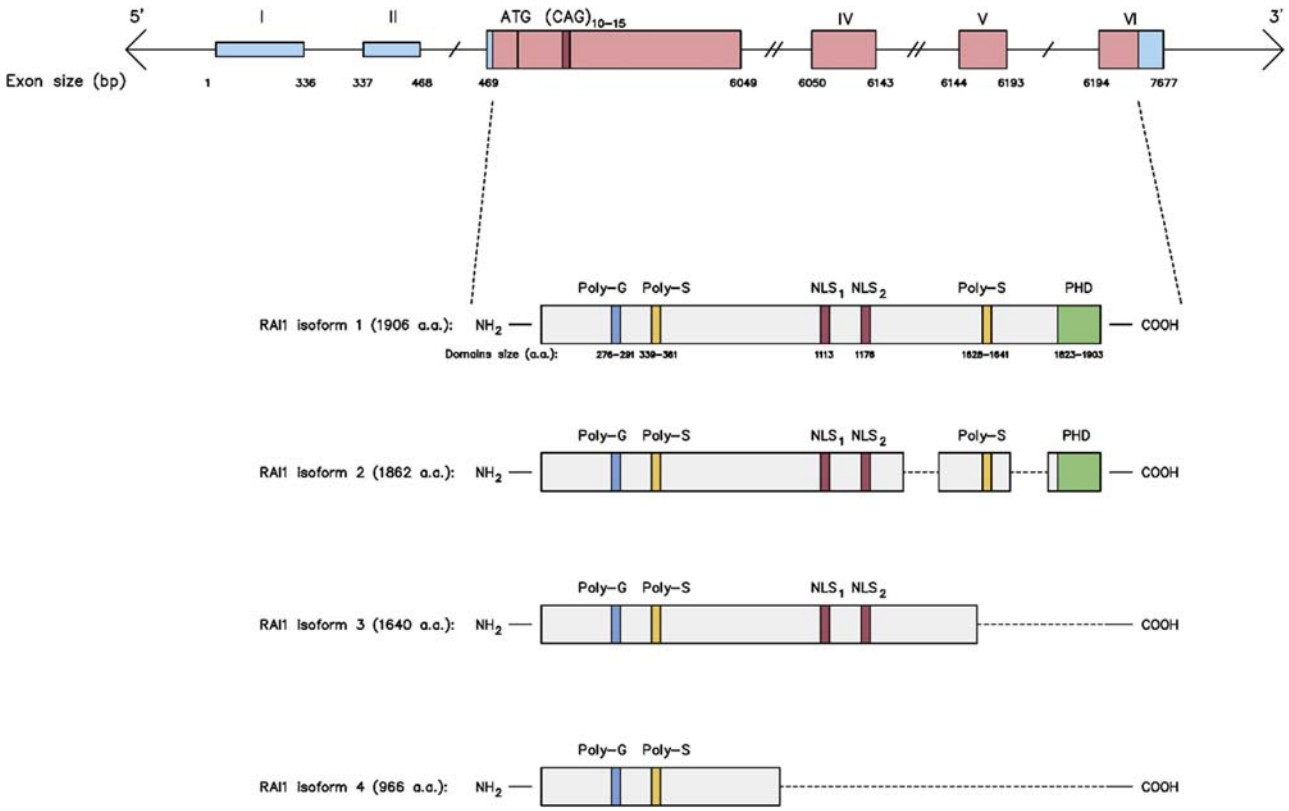


FIGURE 9.3

Retinoic acid-induced 1 gene structure and protein isoforms.

and cytoplasm (Fragoso et al., 2015). It has been hypothesized that nuclear RAI1 regulates gene expression, whereas peripheral (dendritic/axonal) RAI1 functions are unexplored. Comparative analyses of the expression of all RAI1 isoforms with predicted different subcellular localizations may provide information on the nuclear and peripheral RAI1 functions in neurons and nonneuronal cells.

Correlation between structure and function

Although no tertiary structure is available for RAI1, many annotations of structural and functional properties of the protein have been deduced from its primary structure, utilizing sequential and structural similarities for identification. Human RAI1 amino acidic sequence analysis revealed the presence of two main functional domains: the amino-terminal domain (NTD, amino acids 1−1034), and the carboxy-terminal domain (CTD, amino acids 1035−1906) (Bi et al., 2004; Darvekar et al., 2012; Fig. 9.5). Within the NTD, RAI1 has a polymorphic microsatellite exonic $(CAG)_n$ tandem repeat encoding a polyglutamine (polyQ) tract (amino acids 278−291 for a polyQ tract of 14 residues) (Fig. 9.4, highlighted in yellow).

In the normal population, RAI1 CAG repeat ranges from 10 to 16 triplets, with an average length of 10−15 repeats (Seranski et al., 2001). A patient with a CAG repeat length of 18 triplets was reported to have severe symptoms (Bi et al., 2006). The number of polymorphic CAG repeats at the N-terminus of the RAI1 was associated with phenotype severity and neuroleptic response in schizophrenic patients (Joober et al., 1999). Neuroleptic responders presented shorter repeats compared with non-responders, supporting a role for RAI1 in neurodevelopment disorders other than SMS. In addition, in the analysis of 173 nonoverlapping CNV regions, *RAI1* was identified as the most significant gene for susceptibility to nonsyndromic autism spectrum disorders (van der Zwaag et al., 2009). Although the function of CAG tandem repeats is not known, it is likely to mediate protein-protein interactions. There are several proteins containing polyQ tracts expressed in the nucleus and cytosol. Several of these proteins are involved in the regulation of gene expression. Importantly, the expansion of CAG tandem repeats in nine unrelated genes is responsible for a subfamily of neurodegenerative diseases, namely polyQ diseases (Pennuto and Sambataro, 2010). In most polyQ diseases, there is a fine correlation between the size of the CAG repeat and the onset and progression of disease, with subjects carrying longer repeats showing an earlier onset and a faster progression to death compared to those carrying shorter repeats, although these factors also appear to be affected by genetic modifiers. The length of the CAG tract of *RAI1* has been shown to significantly and positively correlate with age at the onset of SCA2 disease, providing a genetic link between RAI1 dysfunction and SCA2 pathogenesis (Hayes, 2000).

Within the NTD, in addition to the polyQ-encoding microsatellite CAG repeat, RAI1 has two proline-rich motifs (amino acids ^{124}PQPPPPQPQPLP^{135} and ^{174}QQPPPPQQP^{182}, where P is proline), which resemble those present in the NTD of huntingtin, and that have been implicated in HD pathogenesis and neurodegenerative processes (Fig. 9.4 boxed). These motifs lie within a large portion of the

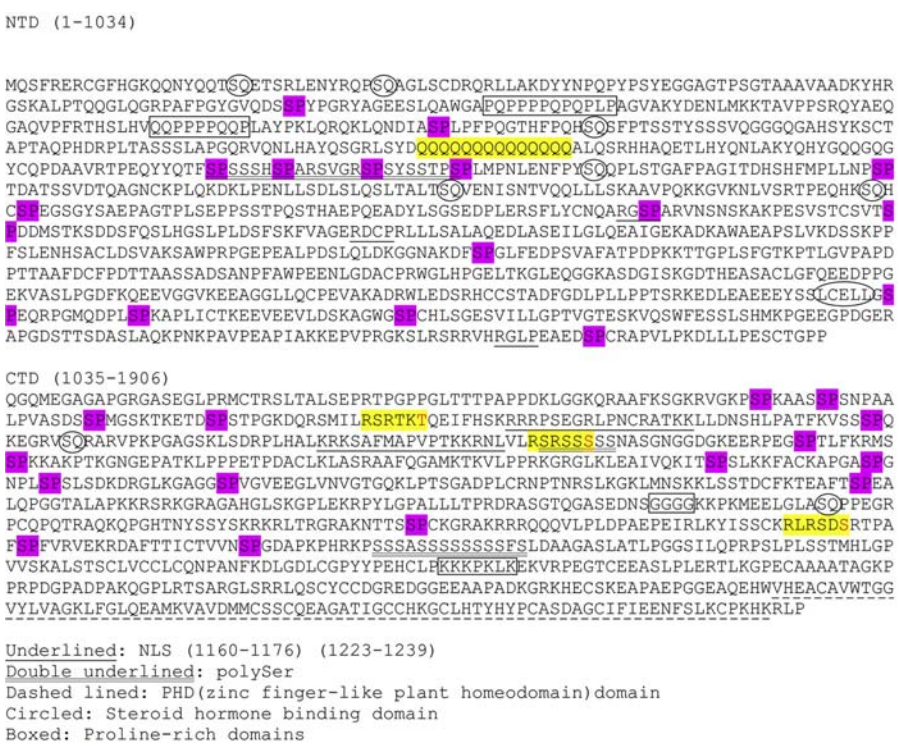


FIGURE 9.4

RAI1 structure. *Underlined*: Nuclear localization signal (NLS) (1160–1176) (1223–1239); *Double underlined*: polySer; *Dashed underlined*: PHD (zinc finger-like plant homeodomain) domain; *Circled*: Steroid hormone binding domain; *Boxed*: Proline-rich domains.

NTD that is rich in glutamine, proline, and serine, which resemble those found in hydroxyproline-rich glycoproteins (HRGPs) expressed in plants. RAI1 has a steroid hormone binding motif (^{874}LCELL^{878}, consensus sequence LxxLL, where L is leucine, C cysteine, E glutamate, and x any amino acid), whose role is unknown (Fig. 9.4, circled) (Heery et al., 1997). RAI1 has two polyserine (polyS) tracts, one located in the NTD (amino acids 339–361) and the other in the CTD (amino acids 1628–1641) (Fig. 9.4 double underlined). Moreover, there is another serine-rich region encompassing amino acids 1243–1249. Interestingly, polyS tracts are also present in atrophin-1. Microsatellite repeats, which are abundant in the genomes of all organisms, are unstable and subject to recombination (shrinkage and expansions). PolyQ and polyP stretches are predominantly found in proteins that regulate transcription, i.e., transcription factors and co-factors (Gerber et al., 1994). Importantly, the capacity for transactivation of general transcription factors is influenced by the length of these repeats: the longer the repeats, the stronger the transactivation,

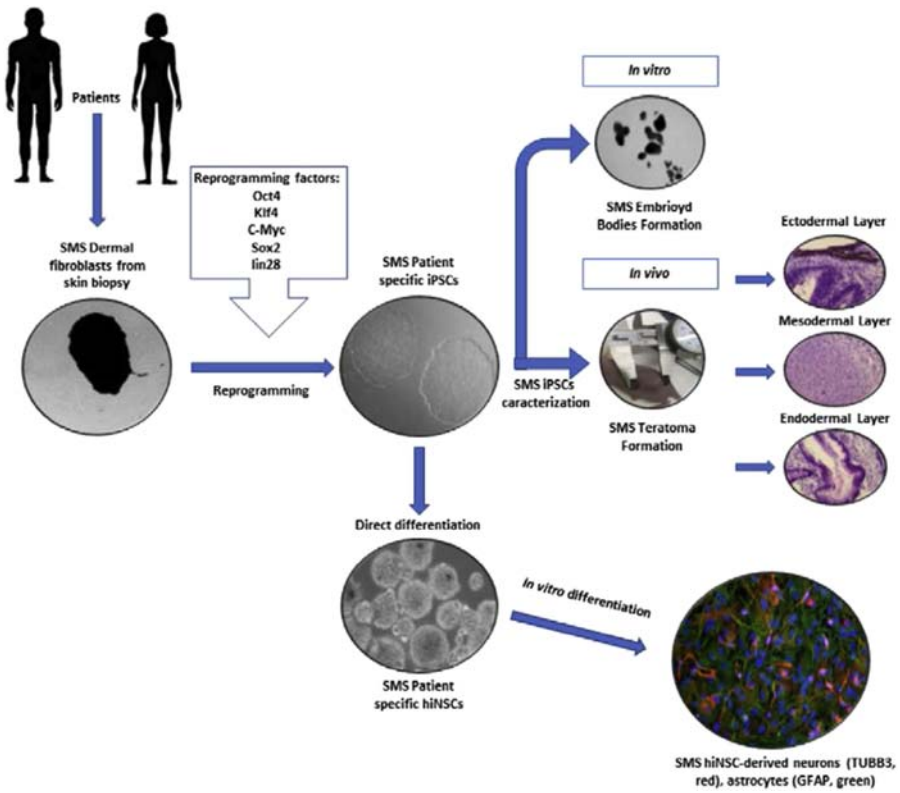


FIGURE 9.5

Induced pluripotent stem cells (iPSCs) line production workflow in cells obtained from the biopsy of a Smith–Magenis patient, carrying the novel mutation NM_030665.3: c.1194delC. Smith–Magenis syndrome (SMS) fibroblasts were reprogrammed into iPSCs, using nonintegrative episomal vectors containing the reprogramming factors OCT4, SOX2, L-MYC, KLF4, LIN28, and shp53. Clones showing embryonic stem (ES)-like morphology, flat and uniform, were characterized. Pluripotency capacity of the SMS iPSCs was tested by differentiating the iPSC both in vitro through embryoid bodies assay and in vivo through a teratoma assay. All three germ layers: ectodermal, mesodermal, and endodermal, were present in the teratoma, as demonstrated by immune-histochemical analysis. SMS iPSCs were successfully differentiated into human induced neural stem cell (hiNSC) and then differentiated into hiNSC-derived neural population, composed of neurons and astrocytes.

with the polyQ size of 10 to 30 repeats and polyP of 10 repeats having the highest activity. PolyQ and polyP repeats were shown to affect transactivation, whereas the role of polyS tracts is less obvious, as the latter did not modify transactivation in an in vitro transcriptional assay (Atanesyan et al., 2012).

In the CTD, RAI1 has two bipartite nuclear localization signals (NLSs) spanning amino acids 1160–1177 and 1223–1240 (numbered according to UNIPROT entry) (Fig. 9.4, underlined), and a zinc finger-like plant homeodomain (PHD)/domain spanning amino acids 1825–1903 (Fig. 9.4 dashed line) (Bi et al., 2006). The canonical PHD domain, formed by Cys4-His-Cys3 motif, is present in hundreds of nuclear proteins involved in chromatin remodeling and gene expression regulation (Aasland, 1995). Its sequence is homologous to that of the Trithorax family of nuclear proteins (Bi et al., 2006) and to the PHD motif of the transcriptional co-activator stromelysin-1 PDGF-responsive element-binding protein (SPBP), also known as transcriptional co-factor 20 (TCF20) (Darvekar et al., 2013). Another homologous domain in RAI1 and TCF20 is a nucleosome-binding domain (Darvekar et al., 2012).

Very little is known about PTMs of RAI1. The fact that the NTD region is rich in glutamine, proline, and serine residues suggests that RAI1 may undergo proline hydroxylation, glycosylation, and cross-linking, similar to other proteins with analogous domains, such as huntingtin and HRGPs (Kieliszewski and Lamport, 1994). It is very likely that RAI1 is phosphorylated by several cellular kinases. RAI1 has several serine residues followed by a proline residue (SP sites) that may be modified by cyclin-dependent kinases. Moreover, RAI1 has three consensus sites composed of RXRXXS/T (where R is arginine and T threonine), which are recognized by protein kinase A and protein kinase B. The higher effective molecular weight of RAI1 in SDS-PAGE suggests that the protein undergoes different PTMs, which most likely depend on cell context.

Rai1 function

What is the native function of RAI1? RAI1 structure, subcellular localization, and protein–protein interaction pattern suggest that RAI1 acts a regulator of gene transcription. RAI1 can regulate transcription through chromatin remodeling by interacting with chromatin remodelers as well as with components of the basic transcriptional machinery. Its PHD motif is probably important for performing a role as "histone reader" (Aasland, 1995). RAI1 does not have a canonical DNA-binding domain. Rather, RAI1 binds to nucleosomes and works as a co-factor of transcription (Darvekar et al., 2012, 2013). A genome-wide gene expression study performed on nonneuronal cells by silencing RAI1 expression, to mimic its haploinsufficiency, showed that the top dysregulated (activated and repressed) genes are involved in neuronal survival, differentiation, and growth, neurobehavioural functions, circadian activity, cell cycle regulation, lipid and glucose metabolism, neural crest migration and development of derivative cartilage. Dysregulated expression of these genes may underlie important clinical presentations of SMS, such as sleep disorder (Williams et al., 2012), facial dysmorphism (Girirajan, 2005), while downregulation of brain-derived neurotrophic factor may explain abnormalities in feeding behavior and obesity in the absence of signs of metabolic syndrome (Burns et al., 2010; Han, 2016). Interestingly, all *RAI1*-regulated genes are associated not only with the symptoms of SMS patients but also with those of other developmental disorders, such as Down syndrome, Prader–Willi syndrome, and fragile X

syndrome, suggesting *RAI1* involvement in these pathological conditions as well. Consistent with this idea, the most down-regulated gene upon RAI1 silencing was survival of motor neuron 1 (*SMN1*), whose deletion causes spinal muscular atrophy (SMA) (Kolb, 2011), raising an intriguing question as to whether RAI1 is implicated in the regulation of expression of the *SMN1* gene with key functions in the motor system and peripheral tissues as well as in the pathogenesis of SMA.

Recently, an in-depth analysis of gene expression was carried out in knock-in mice expressing Flag/Myc-tagged RAI1 (Huang et al., 2016). Chromatin-immunoprecipitation analysis followed by deep-sequencing (Chip-seq) revealed that RAI1 is present in the following genomic regions: CpG islands, 5′-UTR, active promoters (in proximity of transcription start sites) and enhancers. Bioinformatics analysis showed that the RAI1 binding sites were similar to those recognized by a zinc-finger transcription factor, ZNF711, implicated in X-linked mental retardation (Tarpey et al., 2009), further suggesting a common and overlapping pathogenetic pathway in SMS and X-linked mental retardation. RNA-seq was then performed in mice with tissue-specific (cortex, striatum, and hypothalamus) RAI1 haploinsufficiency or knockout to further investigate how RAI1 regulates transcription across developmental stages (Huang et al., 2016). RNA-seq data obtained from the cortex, striatum, and hypothalamus of 3-, 8-, and 12-week-old mice showed that loss of *RAI1* alters the expression of several genes. RAI1 ablation led to transcriptional dysregulation of genes involved in cell adhesion, such as cadherins and protocadherins, which are involved in tissue morphogenesis, formation of neuronal circuits, modulation of synaptic transmission, and the generation of specific synaptic connections in adults. Other dysregulated genes, such as ALCAM, SLITRK1, SLITRK3, SLITRK6, DMD, CNTN4, TBR1, EPHA7, and others, are involved in neuronal development and neurotransmitter signaling. Particularly interesting are HTR2A and HTR2C, which code for metabotropic G-protein coupled receptor for 5-hydroxytryptamine (serotonin). HTR2C plays a role in the regulation of appetite, eating behavior and responses to anxiogenic stimuli and stress, all of which are key clinical aspects of SMS. Moreover, *RAI1* loss of function altered the expression of genes encoding metal/ion binding proteins, including MCTP1, essential for the stabilization of normal baseline neurotransmitter release and induction and long-term maintenance of presynaptic homeostatic plasticity, and SLC8A1, which contributes to the regulation of cytoplasmic Ca^{2+} levels and Ca^{2+}-dependent cellular processes. After binding acetylcholine, the AChR undergoes a conformation change that leads to the opening of an ion-conducting channel across the plasma membrane. Interestingly, RAI1 also regulates genes involved in obesity, such as POMC, in chromatin remodeling, such as SIRT2, in genome-wide de novo methylation, such as DNMT3A, and in maintenance of myelin structure, such as MAG and PLP1. These genes were all upregulated in *RAI1* knockout mice.

Model systems of Smith–Magenis syndrome

Animal models of Smith–Magenis syndrome

The first SMS mouse model carrying the deletion of the mouse chromosome region (32 to 34 cM of chromosome 11) syntenic to the SMS critical genomic interval (chromosome 17p11.2) was generated in 2003 (Walz et al., 2003). Heterozygous mice showed craniofacial abnormalities, seizures and abnormal EEGs, obesity, and reduced male fertility. The deletion was lethal in the homozygous state. In 2005, Bi and colleagues generated a RAI1 null allele (RAI1+/−) in mice by inserting a lacZ reporter gene encoding β-galactosidase into the RAI1 gene (Bi et al., 2005). These mice showed obesity and craniofacial abnormalities, indicating that RAI1 haploinsufficiency recapitulates these features of SMS. Almost all homozygous null mice (RAI1−/−) were embryonic lethal, and the few surviving mice showed severe postnatal growth retardation, craniofacial and skeletal abnormalities, motor dysfunction and learning impairments. In 2014, Alaimo and colleagues bred $RAI1^{+/-}$ mice with different congenic backgrounds to generate a mixed background, an approach that is used to make the phenotype slightly milder and extend mouse survival for phenotypic characterization (Alaimo et al., 2014) SMS modeling mice were more sensitive to high-fat and high-carbohydrate diets, notably in the absence of glucose insensitivity and insulin resistance. These findings support the idea that a low-calorie diet may have therapeutic potential to delay obesity and ameliorate some aspects of disease. In 2016, Huang and colleagues generated knock-in mice expressing FLAG and Myc peptides fused to the carboxy-terminus of endogenous *RAI1* (Huang et al., 2016). Using this approach, RAI1 function can be investigated by taking advantage of the presence of Flag/Myc tags, which allows researchers to purify the protein for further characterization of the protein–protein interaction map and for other biochemical and molecular biology analyses, all performed in the absence of overexpression, which is a key point for dosage-sensitive genes, such as RAI1. Conditional deletion of *RAI1* in the nervous system triggered several SMS-like symptoms, including deficits in body weight homeostasis, motor skills, and associative learning and memory, suggesting that loss of *RAI1* function in the central nervous system in mice is sufficient to recapitulate these disease manifestations. Moreover, knockout of *RAI1* in GABAergic inhibitory neurons, as well as in cortical and hippocampal excitatory neurons and glia, showed that loss of *RAI1* from specific cell populations recapitulated SMS-like phenotypes (motor function, learning, and food intake), suggesting that *RAI1* has specific roles in certain cell types. Overall, the development of animal models for the conditional ablation of RAI1 has provided a means to establish what phenotypes derive from RAI1 loss of function in SMS, and for a better fundamental understanding of RAI1 function in the central nervous system, and possibly in peripheral tissues as well, throughout development and adulthood.

Cellular models: Smith–Magenis syndrome patient-derived primary cell lines

RAI1 is a dosage-sensitive gene, thus overexpression and haploinsufficiency of this protein are equally deleterious. Two studies were published in 2010 and 2018, using cell lines to overexpress the various regions of RAI1 in order to determine its localization in the nucleus and comprehend its function as transcription factor (Carmona-Mora et al., 2010; Abad et al., 2018). However, to investigate disease mechanisms more thoroughly, it is also important to develop cell models reproducing the disease pathophysiology in the absence of overexpression/downregulation of the RAI1 protein. Another approach that has been taken, which we have also chosen to adopt in our laboratories, is the use of primary fibroblast cells obtained from the skin biopsies of SMS patients. Using these cells, Williams et al. (2012) demonstrated that transcriptional regulation of the molecular clock genes is disrupted, indicating a deregulation of the circadian cycle, which is one of the main symptoms present in SMS patients. Based on these findings, which suggest that RAI1 plays an important role in maintaining circadian rhythmicity, reflecting clinical symptomatology, studies on cellular behavior are now being carried out on SMS fibroblast lines, which may allow us to delineate the pathways with which circadian rhythms impact development, sleep, and behavior. We are conducting proteomic, transcriptomic, and metabolomic analyses on primary fibroblasts obtained from two SMS patients, respectively, carrying a single nucleotide deletion and a deletion of 65 genes on chromosome 17. The use of primary fibroblasts derived from patients may ultimately help to clarify the deregulated molecular mechanisms underlying disease pathogenesis, and may thus pave the way to identification of novel therapeutic targets for SMS.

Induced pluripotent stem cells for modeling Smith–Magenis syndrome

Considering the neurological symptoms of SMS patients, it is critical to carry out studies on the cellular phenotypes relevant for the disease, i.e., neural cells, derived from patients. iPSC lines can be generated by reprogramming somatic cells and can be differentiated into any cell type, including neurons and glial cells, offering an opportunity to study the disease in cells from the precise genetic background of the patient. Moreover, they have a unique capacity for tracking the neurodevelopmental profile of patient cell differentiation. Proteomic, transcriptomic, and functional analyses on iPSCs can give us valuable insight into both causes and consequences of the disease.

Studying complex disorders, such as SMS, using patient-derived iPSCs has two significant advantages: (1) working with iPSC lines generated from numerous SMS patients allows us to investigate disease mechanisms in a system that reflects the variable penetrance and expressivity typical of the patients' symptomatology; (2) studying individual cell lines, each with a distinct genetic background in which the disease has developed, allows us to observe the deregulated mechanisms common to the disease despite phenotypic variability.

Today, iPSCs are being used in studies on almost all known diseases. However, despite the recent boom in iPSCs production, currently very little experimentation on producing iPS specifically for SMS has been reported. Recently, we generated iPSCs from an SMS patient with a single mutation in *RAI1* gene, thus demonstrating that iPSCs carrying such a mutation can be produced, have all of the characteristics of pluripotency and produce embryoid bodies in vitro and teratoma in vivo (Fig. 9.5; Altieri et al., 2018). In the future, iPSCs will be used more and more frequently in studies on SMS, as further research on this disease and on studying strategies for improving its symptoms is urgently needed, with the final objective of identifying a specific mechanism and therefore a therapy.

Organoids and neurospheres

Neurospheres can be obtained from SMS patient-derived iPSCs, which differentiate spontaneously into three neural lineages: astrocytes, oligodendrocytes, and neurons (Rosati et al., in preparation). These three cell lines display synchronous, mutually beneficial growth and interaction, without external stimuli, such as the utilization of inducer molecules. Observation of the pathological neural phenotype in *in vitro* cells will allow us to perform biomolecular, biochemical, and immunocytochemical analyses, whose results will be associated with the clinical phenotypes. These cells will be used to study impairments in the nervous system, considering that the main problematics of these patients involve severe behavioral and cognitive disruption (Fig. 9.5).

Concluding remarks

The underlying molecular and cellular pathological processes of SMS are still obscure or only partially understood, thus highlighting that an enormous amount of research needs to be carried out in order to fully comprehend the pathogenesis of SMS and to develop new and effective therapeutic strategies for SMS patients. Investigation of RAI1 function in cell and animal models suggests that RAI1 regulates gene expression. However, several questions remain to be addressed: Is RAI1 function regulated by neuronal activity? Is regulation of gene expression the only native function of the protein? Is RAI1 involved in other neuronal-specific functions, such as synaptic function, axonal transport, and others? An analysis of RAI1 subcellular localization and function in different types of neurons, such as hippocampal, cortical, striatal, and hypothalamic neurons, may provide information on protein function, which could lead to a better understanding of the pleiotropic clinical presentation of SMS patients. Another important aspect that has not yet been explored is the role of different RAI1 isoforms in a physiological context. Moreover, very little is known on how the mutations leading to the generation of truncated RAI1, lacking the NLSs and CTD, cause disease. Mutations of RAI1 that localize in the NTD and result in the generation of premature stop codons may lead to disease through at least two main mechanisms. Transcripts with premature stop codons are usually unstable

and have a short half-life, due to degradation through a process known as nonsense-mediated decay (Cheng and Maquat, 1993). On the other hand, if the transcript with a premature stop codon is translated, even at very low level, it may lead to the production of truncated proteins lacking the NLSs and CTD, which localize to the cytosol, thus acquiring new functions, for example, becoming transcriptional repressors and sequestering the other proteins into the cytoplasm (Carmona-Mora and Walz, 2010). Moreover, RAI1 point mutations may result in the production of proteins that localize to the nucleus, where they may affect protein−protein interactions and target gene regulation. Further analysis of disease mechanisms may ultimately lead to the development of a therapy for this incurable disease. Because RAI1 dysfunction is implicated in schizophrenia and autism spectrum disorder, it is possible that any advancement toward a therapy for SMS may have an important impact in the field of neurodevelopmental disorders.

We are grateful to the families of SMS patients in Italy and all over the world for supporting and for their interest in our research. We apologize with those authors whose work was not cited for space limitations.

Acknowledgment

This work was supported by Lejeune Foundation, Cycle 2018a—Project #1758 to JR, Italian Ministry of Health, Ricerca Corrente 2020/2021 to JR, Fondazione Just Italia to MP and JR, Program Rare Diseases CNCCS-Scarl-Pomezia to MP, Prin-Miur Italy (2017F2AC5 to MP, Telethon-Italy (GGP19128 to MP).

References

Aasland, R., 1995. The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem. Sci. 20, 56−59. https://doi.org/10.1016/S0968-0004(00)88957-4.

Abad, C., Cook, M., Cao, L., et al., 2018. A rare de novo RAI1 gene mutation affecting BDNF-enhancer-driven transcription activity associated with autism and atypical Smith-Magenis syndrome presentation. Biology 7, 31. https://doi.org/10.3390/biology7020031.

Acquaviva, F., Sana, M.E., Della Monica, M., et al., 2017. First evidence of Smith-Magenis syndrome in mother and daughter due to a novel RAI mutation. Am. J. Med. Genet. 173, 231−238. https://doi.org/10.1002/ajmg.a.37989.

Alaimo, J.T., Hahn, N.H., Mullegama, S.V., Elsea, S.H., 2014. Dietary regimens modify early onset of obesity in mice haploinsufficient for RAI1. PLoS One 9, e105077. https://doi.org/10.1371/journal.pone.0105077.

Alaimo, J.T., Barton, L.V., Mullegama, S.V., et al., 2015. Individuals with Smith-Magenis syndrome display profound neurodevelopmental behavioral deficiencies and exhibit food-related behaviors equivalent to Prader-Willi syndrome. Res. Dev. Disabil. 47, 27−38. https://doi.org/10.1016/j.ridd.2015.08.011.

Allanson, J.E., Greenberg, F., Smith, A.C., 1999. The face of Smith-Magenis syndrome: a subjective and objective study. J. Med. Genet. 36, 394−397.

Altieri, F., Turco, E.M., Vinci, E., et al., 2018. Production and characterization of CSSI003 (2961) human induced pluripotent stem cells (iPSCs) carrying a novel puntiform mutation in RAI1 gene, Causative of Smith−Magenis syndrome. Stem Cell Res. 28, 153−156. https://doi.org/10.1016/j.scr.2018.02.016.

Atanesyan, L., Günther, V., Dichtl, B., et al., 2012. Polyglutamine tracts as modulators of transcriptional activation from yeast to mammals. Biol. Chem. 393, 63−70. https://doi.org/10.1515/BC-2011-252.

Barboni, M.T.S., Bueno, C., Nagy, B.V., et al., 2018. Melanopsin system dysfunction in Smith-Magenis syndrome patients. Invest. Opthalmol. Vis. Sci. 59, 362. https://doi.org/10.1167/iovs.17-22612.

Bi, W., Park, S.-S., Shaw, C.J., et al., 2003. Reciprocal crossovers and a positional preference for strand exchange in recombination events resulting in deletion or duplication of chromosome 17p11.2. Am. J. Hum. Genet. 73, 1302−1315. https://doi.org/10.1086/379979.

Bi, W., Saifi, G.M., Shaw, C.J., et al., 2004. Mutations of RAI1, a PHD-containing protein, in nondeletion patients with Smith-Magenis syndrome. Hum. Genet. 115, 515−524. https://doi.org/10.1007/s00439-004-1187-6.

Bi, W., Ohyama, T., Nakamura, H., et al., 2005. Inactivation of Rai1 in mice recapitulates phenotypes observed in chromosome engineered mouse models for Smith−Magenis syndrome. Hum. Mol. Genet. 14, 983−995. https://doi.org/10.1093/hmg/ddi085.

Bi, W., Saifi, G.M., Girirajan, S., et al., 2006. RAI1 point mutations, CAG repeat variation, and SNP analysis in non-deletion Smith−Magenis syndrome. Am. J. Med. Genet. 140A, 2454−2463. https://doi.org/10.1002/ajmg.a.31510.

Brendal, M.A., King, K.A., Zalewski, C.K., et al., 2017. Auditory phenotype of Smith−Magenis syndrome. J. Speech Lang. Hear. Res. 60, 1076−1087. https://doi.org/10.1044/2016_JSLHR-H-16-0024.

Burns, B., Schmidt, K., Williams, S.R., et al., 2010. Rai1 haploinsufficiency causes reduced Bdnf expression resulting in hyperphagia, obesity and altered fat distribution in mice and humans with no evidence of metabolic syndrome. Hum. Mol. Genet. 19, 4026−4042. https://doi.org/10.1093/hmg/ddq317.

Capra, V., Biancheri, R., Morana, G., et al., 2014. Periventricular nodular heterotopia in Smith-Magenis syndrome. Am. J. Med. Genet. 164, 3142−3147. https://doi.org/10.1002/ajmg.a.36742.

Carmona-Mora, P., Walz, K., 2010. Retinoic acid induced 1, RAI1: a dosage sensitive gene related to neurobehavioral alterations including autistic behavior. Curr. Genom. 11, 607−617. https://doi.org/10.2174/138920210793360952.

Carmona-Mora, P., Encina, C.A., Canales, C.P., et al., 2010. Functional and cellular characterization of human retinoic acid induced 1 (RAI1) mutations associated with Smith-Magenis syndrome. BMC Mol. Biol. 11, 63. https://doi.org/10.1186/1471-2199-11-63.

Carpizo, R., Martínez, Á., Mediavilla, D., et al., 2006. Smith-Magenis syndrome: a case report of improved sleep after treatment with β1-adrenergic antagonists and melatonin. J. Pediatr. 149, 409−411. https://doi.org/10.1016/j.jpeds.2006.04.055.

Chen, K.-S., Manian, P., Koeuth, T., et al., 1997. Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. Nat. Genet. 17, 154−163. https://doi.org/10.1038/ng1097-154.

Chen, L., Mullegama, S.V., Alaimo, J.T., Elsea, S.H., 2015. Smith-Magenis syndrome and its circadian influence on development, behavior, and obesity − own experience. Dev. Period Med. 19, 149−156.

Cheng, J., Maquat, L.E., 1993. Nonsense codons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half-life of cytoplasmic mRNA. Mol. Cell Biol. 13, 1892−1902. https://doi.org/10.1128/MCB.13.3.1892.

Chik, C.L., Rollag, M.D., Duncan, W.C., Smith, A.C.M., 2010. Diagnostic utility of daytime salivary melatonin levels in Smith-Magenis syndrome. Am. J. Med. Genet. 152A, 96−101. https://doi.org/10.1002/ajmg.a.33158.

Dardour, L., Verleyen, P., Lesage, K., et al., 2016. Bilateral renal tumors in an adult man with Smith-Magenis syndrome: the role of the FLCN gene. Eur. J. Med. Genet. 59, 499−501. https://doi.org/10.1016/j.ejmg.2016.09.005.

Darvekar, S., Johnsen, S.S., Eriksen, A.B., et al., 2012. Identification of two independent nucleosome-binding domains in the transcriptional co-activator SPBP. Biochem. J. 442, 65−75. https://doi.org/10.1042/BJ20111230.

Darvekar, S., Rekdal, C., Johansen, T., Sjøttem, E., 2013. A phylogenetic study of SPBP and RAI1: evolutionary conservation of chromatin binding modules. PLoS One 8, e78907. https://doi.org/10.1371/journal.pone.0078907.

Di Cicco, M., Padoan, R., Felisati, G., et al., 2001. Otorhinolaringologic manifestation of Smith−Magenis syndrome. Int. J. Pediatr. Otorhinolaryngol. 59, 147−150. https://doi.org/10.1016/S0165-5876(01)00475-X.

Dittwald, P., Gambin, T., Gonzaga-Jauregui, C., et al., 2013. Inverted low-copy repeats and genome instability − a genome-wide analysis. Hum. Mutat. 34, 210−220. https://doi.org/10.1002/humu.22217.

Dosier, L., Vaughn, B., Fan, Z., 2017. Sleep disorders in childhood neurogenetic disorders. Children 4, 82. https://doi.org/10.3390/children4090082.

Dubourg, C., Bonnet-Brilhault, F., Toutain, A., et al., 2014. Identification of nine new RAI1-truncating mutations in Smith-Magenis syndrome patients without 17p11.2 deletions. Mol. Syndromol. https://doi.org/10.1159/000357359.

Edelman, E., Girirajan, S., Finucane, B., et al., 2007. Gender, genotype, and phenotype differences in Smith-Magenis syndrome: a meta-analysis of 105 cases. Clin. Genet. 71, 540−550. https://doi.org/10.1111/j.1399-0004.2007.00815.x.

Elsea, S.H., Girirajan, S., 2008. Smith−magenis syndrome. Eur. J. Hum. Genet. 16, 412−421. https://doi.org/10.1038/sj.ejhg.5202009.

Falco, M., Amabile, S., Acquaviva, F., 2017. RAI1 gene mutations: mechanisms of Smith−Magenis syndrome. Appl. Clin. Genet. 10, 85−94. https://doi.org/10.2147/TACG.S128455.

Fragoso, Y.D., Stoney, P.N., Shearer, K.D., et al., 2015. Expression in the human brain of retinoic acid induced 1, a protein associated with neurobehavioural disorders. Brain Struct. Funct. 220, 1195−1203. https://doi.org/10.1007/s00429-014-0712-1.

Gamba, B.F., Vieira, G.H., Souza, D.H., et al., 2011. Smith-Magenis syndrome: clinical evaluation in seven Brazilian patients. Genet. Mol. Res. 10, 2664−2670. https://doi.org/10.4238/2011.October.31.17.

Gerber, H., Seipel, K., Georgiev, O., et al., 1994. Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science 263, 808−811. https://doi.org/10.1126/science.8303297.

Girirajan, S., 2005. RAI1 variations in Smith-Magenis syndrome patients without 17p11.2 deletions. J. Med. Genet. 42, 820–828. https://doi.org/10.1136/jmg.2005.031211.

Girirajan, S., Vlangos, C.N., Szomju, B.B., et al., 2006. Genotype–phenotype correlation in Smith-Magenis syndrome: evidence that multiple genes in 17p11.2 contribute to the clinical spectrum. Genet. Med. 8, 417–427. https://doi.org/10.1097/01.gim.0000228215.32110.89.

Gnanavel, S., 2014. Smith-Magneis syndrome: Behavioural phenotype mimics ADHD. Case Rep. https://doi.org/10.1136/bcr-2013-201766.

Guérin-Moreau, M., Colin, E., Nguyen, S., et al., 2015. Dermatologic features of Smith-Magenis syndrome. Pediatr. Dermatol. 32, 337–341. https://doi.org/10.1111/pde.12517.

Han, J.C., 2016. Rare syndromes and common variants of the brain-derived neurotrophic factor gene in human obesity. Prog. Mol. Biol. Transl. Sci. 75–95.

Hayes, S., 2000. CAG repeat length in RAI1 is associated with age at onset variability in spinocerebellar ataxia type 2 (SCA2). Hum. Mol. Genet. 9, 1753–1758. https://doi.org/10.1093/hmg/9.12.1753.

Heery, D.M., Kalkhoven, E., Hoare, S., Parker, M.G., 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387, 733–736. https://doi.org/10.1038/42750.

Hildenbrand, H.L., Smith, A.C.M., 2012. Analysis of the sensory profile in children with Smith–Magenis syndrome. Phys. Occup. Ther. Pediatr. 32, 48–65. https://doi.org/10.3109/01942638.2011.572152.

Huang, W.-H., Guenthner, C.J., Xu, J., et al., 2016. Molecular and neural functions of RAI1, the causal gene for Smith-Magenis syndrome. Neuron 92, 392–406. https://doi.org/10.1016/j.neuron.2016.09.019.

Huang, W.-H., Wang, D.C., Allen, W.E., et al., 2018. Early adolescent RAI1 reactivation reverses transcriptional and social interaction deficits in a mouse model of Smith–Magenis syndrome. Proc. Natl. Acad. Sci. USA 115, 10744–10749. https://doi.org/10.1073/pnas.1806796115.

Huisman, S., Mulder, P., Kuijk, J., et al., 2018. Self-injurious behavior. Neurosci. Biobehav. Rev. 84, 483–491. https://doi.org/10.1016/j.neubiorev.2017.02.027.

Imai, Y., Suzuki, Y., Tohyama, M., et al., 1994. Cloning and expression of a neural differentiation-associated gene, p205, in the embryonal carcinoma cell line P19 and in the developing mouse. Mol. Brain Res. 24, 313–319. https://doi.org/10.1016/0169-328X(94)90144-9.

Imai, Y., Suzuki, Y., Matsui, T., et al., 1995. Cloning of a retinoic acid-induced gene, GT1, in the embryonal carcinoma cell line P19: neuron-specific expression in the mouse brain. Mol. Brain Res. 31, 1–9. https://doi.org/10.1016/0169-328X(95)00020-S.

Joober, R., Benkelfat, C., Toulouse, A., et al., 1999. Analysis of 14 CAG repeat-containing genes in schizophrenia. Am. J. Med. Genet. 88, 694–699. https://doi.org/10.1002/(SICI)1096-8628(19991215)88:6<694::AID-AJMG20>3.0.CO;2-I.

Kieliszewski, M.J., Lamport, D.T.A., 1994. Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. Plant J. 5, 157–172. https://doi.org/10.1046/j.1365-313X.1994.05020157.x.

Klionsky, D.J., Abdelmohsen, K., Abe, A., et al., 2016. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd ed.). Autophagy 12, 1–222. https://doi.org/10.1080/15548627.2015.1100356.

Kolb, S.J., 2011. Spinal muscular atrophy. Arch. Neurol. 68, 979. https://doi.org/10.1001/archneurol.2011.74.

Laje, G., Bernert, R., Morse, R., et al., 2010. Pharmacological treatment of disruptive behavior in Smith-Magenis syndrome. Am. J. Med. Genet. C Semin. Med. Genet. 154C, 463−468. https://doi.org/10.1002/ajmg.c.30282.

Li, Z., Shen, J., Liang, J., Sheng, L., 2015. Congenital Scoliosis in Smith−Magenis syndrome. Medicine 94, e705. https://doi.org/10.1097/MD.0000000000000705.

Liburd, N., Ghosh, M., Riazuddin, S., et al., 2001. Novel mutations of MYO15A associated with profound deafness in consanguineous families and moderately severe hearing loss in a patient with Smith-Magenis syndrome. Hum. Genet. 109, 535−541. https://doi.org/10.1007/s004390100604.

Martin, S.C., Wolters, P.L., Smith, A.C.M., 2006. Adaptive and maladaptive behavior in children with Smith-Magenis syndrome. J. Autism Dev. Disord. 36, 541−552. https://doi.org/10.1007/s10803-006-0093-2.

Maya, I., Vinkler, C., Konen, O., et al., 2014. Abnormal brain magnetic resonance imaging in two patients with Smith-Magenis syndrome. Am. J. Med. Genet. 164, 1940−1946. https://doi.org/10.1002/ajmg.a.36583.

Miller, D.T., Adam, M.P., Aradhya, S., et al., 2010. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. Am. J. Hum. Genet. 86, 749−764. https://doi.org/10.1016/j.ajhg.2010.04.006.

Myers, S.M., Challman, T.D., 2004. Congenital heart defects associated with Smith-Magenis syndrome: two cases of total anomalous pulmonary venous return. Am. J. Med. Genet. 131A, 99−100. https://doi.org/10.1002/ajmg.a.30290.

Nag, H.E., Nærland, T., 2020. Age-related changes in behavioural and emotional problems in Smith−Magenis syndrome measured with the Developmental Behavior Checklist. J. Intellect. Disabil. https://doi.org/10.1177/1744629519901056.

Osório, A., Cruz, R., Sampaio, A., et al., 2012. Cognitive functioning in children and adults with Smith-Magenis syndrome. Eur. J. Med. Genet. 55, 394−399. https://doi.org/10.1016/j.ejmg.2012.04.001.

Pennuto, M., Sambataro, F., 2010. Pathogenesis of polyglutamine diseases. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, UK.

Poisson, A., Nicolas, A., Cochat, P., et al., 2015. Behavioral disturbance and treatment strategies in Smith-Magenis syndrome. Orphanet J. Rare Dis. 10, 111. https://doi.org/10.1186/s13023-015-0330-x.

Potocki, L., Bi, W., Treadwell-Deering, D., et al., 2007. Characterization of Potocki-Lupski syndrome (dup(17)(p11.2p11.2)) and delineation of a dosage-sensitive critical interval that can convey an autism phenotype. Am. J. Hum. Genet. 80, 633−649. https://doi.org/10.1086/512864.

Rubenstein, B., Ahlqwist, M., Bengtsson, C., 1996. Hyperacusis, headache, temporomandibular disorders and amalgam fillings: an epidemiological study. In: Proceedings of the Fifth International Tinnitus Seminar, pp. 657−658.

Schoumans, J., Staaf, J., Jönsson, G., et al., 2005. Detection and delineation of an unusual 17p11.2 deletion by array-CGH and refinement of the Smith−Magenis syndrome minimum deletion to ∼650 kb. Eur. J. Med. Genet. 48, 290−300. https://doi.org/10.1016/j.ejmg.2005.05.004.

Seranski, P., Hoff, C., Radelof, U., et al., 2001. RAI1 is a novel polyglutamine encoding gene that is deleted in Smith−Magenis syndrome patients. Gene 270, 69−76. https://doi.org/10.1016/S0378-1119(01)00415-2.

Shaw, C.J., Lupski, J.R., 2005. Non-recurrent 17p11.2 deletions are generated by homologous and non-homologous mechanisms. Hum. Genet. 116, 1−7. https://doi.org/10.1007/s00439-004-1204-9.

Shayota, B.J., Elsea, S.H., 2019. Behavior and sleep disturbance in Smith−Magenis syndrome. Curr. Opin. Psychiatr. 32, 73−78. https://doi.org/10.1097/YCO.0000000000000474.

Slager, R.E., Newton, T.L., Vlangos, C.N., et al., 2003. Mutations in RAI1 associated with Smith−Magenis syndrome. Nat. Genet. 33, 466−468. https://doi.org/10.1038/ng1126.

Smith, A.C.M., McGavran, L., Robinson, J., et al., 1986. Interstitial deletion of (17)(p11.2p11.2) in nine patients. Am. J. Med. Genet. 24, 393−414. https://doi.org/10.1002/ajmg.1320240303.

Smith, A.C., Dykens, E., Greenberg, F., 1998. Sleep disturbance in Smith-Magenis syndrome (del 17 p11.2). Am. J. Med. Genet. 81, 186−191.

Smith, A.C.M., Magenis, R.E., Elsea, S.H., 2005. Overview of Smith-Magenis syndrome. J. Assoc. Genet. Technol. 31, 163−167.

Spilsbury, J., Mohanty, K., 2003. The orthopaedic manifestations of Smith-Magenis syndrome. J. Pediatr. Orthop. B 12, 22−26. https://doi.org/10.1097/01.bpb.0000043565.17315.36.

Stankiewicz, P., Lupski, J.R., 2002. Genome architecture, rearrangements and genomic disorders. Trends Genet. 18, 74−82. https://doi.org/10.1016/S0168-9525(02)02592-1.

Tarpey, P.S., Smith, R., Pleasance, E., et al., 2009. A systematic, large-scale resequencing screen of X-chromosome coding exons in mental retardation. Nat. Genet. 41, 535−543. https://doi.org/10.1038/ng.367.

Tomona, N., Smith, A.C.M., Guadagnini, J.P., Hart, T.C., 2006. Craniofacial and dental phenotype of Smith−Magenis syndrome. Am. J. Med. Genet. 140A, 2556−2561. https://doi.org/10.1002/ajmg.a.31371.

Toulouse, A., Rochefort, D., Roussel, J., et al., 2003. Molecular cloning and characterization of human RAI1, a gene associated with schizophrenia. Genomics 82, 162−171. https://doi.org/10.1016/S0888-7543(03)00101-0.

Truong, H.T., Dudding, T., Blanchard, C.L., Elsea, S.H., 2010. Frameshift mutation hotspot identified in Smith-Magenis syndrome: case report and review of literature. BMC Med. Genet. 11, 142. https://doi.org/10.1186/1471-2350-11-142.

van der Zwaag, B., Franke, L., Poot, M., et al., 2009. Gene-network analysis identifies susceptibility genes related to glycobiology in autism. PLoS One 4, e5324. https://doi.org/10.1371/journal.pone.0005324.

Van Thillo, A., Devriendt, K., Willekens, D., 2010. Slaapproblemen bij Smith-Magenissyndroom: Behandeling met melatonine en bètablokkers. Tijdschr Psychiatr. 52, 719−723.

Vilboux, T., Ciccone, C., Blancato, J.K., et al., 2011. Molecular analysis of the retinoic acid induced 1 gene (RAI1) in patients with suspected Smith-Magenis syndrome without the 17p11.2 deletion. PLoS One 6, e22861. https://doi.org/10.1371/journal.pone.0022861.

Vlangos, C.N., Wilson, M., Blancato, J., et al., 2005. Diagnostic FISH probes for del(17)(p11.2p11.2) associated with Smith-Magenis syndrome should contain the RAI1 gene. Am. J. Med. Genet. 132A, 278−282. https://doi.org/10.1002/ajmg.a.30461.

Walz, K., Caratini-Rivera, S., Bi, W., et al., 2003. Modeling del(17)(p11.2p11.2) and dup(17)(p11.2p11.2) contiguous gene syndromes by chromosome engineering in mice: phenotypic consequences of gene dosage imbalance. Mol. Cell Biol. 23, 3646−3655. https://doi.org/10.1128/MCB.23.10.3646-3655.2003.

Wilde, L., Oliver, C., 2017. Brief report: contrasting profiles of everyday executive functioning in Smith−Magenis syndrome and down syndrome. J. Autism Dev. Disord. 47, 2602−2609. https://doi.org/10.1007/s10803-017-3140-2.

Wilde, L., Silva, D., Oliver, C., 2013. The nature of social preference and interactions in Smith−Magenis syndrome. Res. Dev. Disabil. 34, 4355−4365. https://doi.org/10.1016/j.ridd.2013.09.014.

Williams, S.R., Zies, D., Mullegama, S.V., et al., 2012. Smith-magenis syndrome results in disruption of CLOCK gene transcription and reveals an integral role for RAI1 in the maintenance of circadian rhythmicity. Am. J. Hum. Genet. 90, 941−949. https://doi.org/10.1016/j.ajhg.2012.04.013.

Wolters, P.L., Gropman, A.L., Martin, S.C., et al., 2009. Neurodevelopment of children under 3 Years of age with Smith-Magenis syndrome. Pediatr. Neurol. 41, 250−258. https://doi.org/10.1016/j.pediatrneurol.2009.04.015.

Zhang, J., Guan, J., Wang, H., et al., 2019. Genotype-phenotype correlation analysis of MYO15A variants in autosomal recessive non-syndromic hearing loss. BMC Med. Genet. 20, 60. https://doi.org/10.1186/s12881-019-0790-2.

Zieger, E., Schubert, M., 2017. New insights into the roles of retinoic acid signaling in nervous system development and the establishment of neurotransmitter systems. Int. Rev. Cell Mol. Biol. 1−84.

CHAPTER

Induced pluripotent stem cells for modeling of cardiac arrhythmias

10

Zeina R. Al Sayed[1], **Marzia Corli**[1], **Nihar Masurkar**[1], **Jean-Sébastien Hulot**[1,2]

[1]*Université de Paris, INSERM, PARCC, Paris, France;* [2]*CIC1418 and DMU CARTE, AP-HP, Hôpital Européen Georges-Pompidou, Paris, France*

Chapter outline

Abstract

Arrhythmias are a critical health burden, responsible for a significant proportion of sudden cardiac death. They arise from inherited or acquired abnormalities in cardiac ion channels or their associated regulatory proteins functioning within cardiomyocytes. In opposite with the classical in vitro models, human induced pluripotent stem cells—derived cardiomyocytes (hiPSC-CMs) offer a patient-specific model that recapitulates disease phenotype and mirrors the reaction to pharmacotherapy. Thus far, the hiPSC-CMs model has genuinely improved our knowledge concerning both, the pathogenicity of genetic variants in inherited arrhythmias and the toxicity of some factors implicated in acquired forms of arrhythmia. Besides, as these cells preserve patient genetic background, they enable the customization of therapies and open

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00002-3

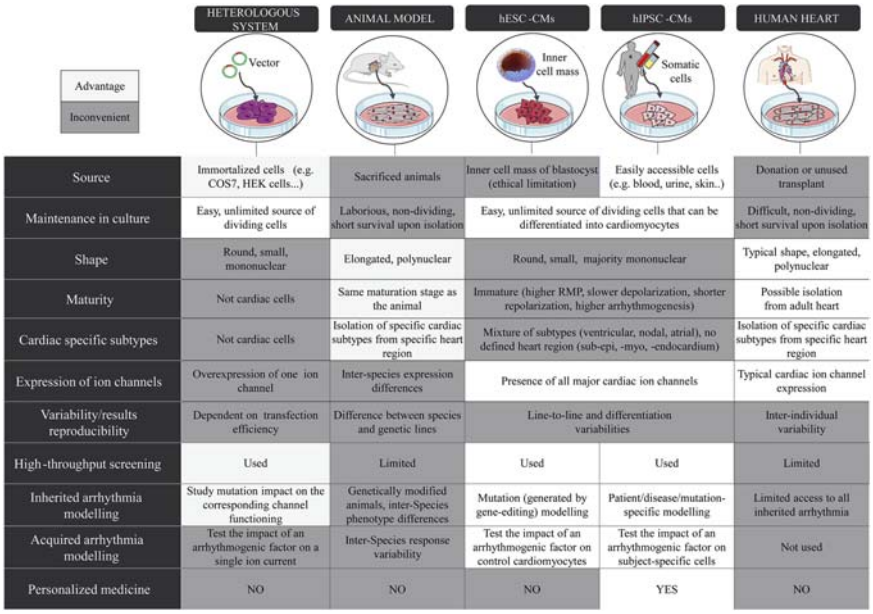
thereby the door for personalized medicine. In this chapter, we will outline studies that leveraged the hiPSC-CMs model to explore the mechanism underlying inherited or acquired arrhythmic phenotypes and will point out its sustained limits.

Keywords: Acquired arrhythmias; Action potential; Atrial fibrillation; Brugada syndrome; Catecholaminergic polymorphic ventricular tachycardia (CPVT); ECG; hiPSC-CMs; Inherited arrhythmias; Ion channel; Ion current; Long QT; Maturation; Short QT; Sudden cardiac death; Torsade de pointe.

Introduction

The coordination between different functions of the heart is intimately linked to the proper regulation of its dynamic electrical activity mediated by hundreds of ion channels. Changes in the expression or function of these ion channels can engender arrhythmias potentially leading to sudden cardiac death (SCD). According to the World Health Organization, cardiovascular disease is the most common cause of death (17.9 million deaths [31%]) worldwide and SCD, mainly triggered by arrhythmias, is considered as a substantial contributor to this number. This has fueled considerable interest among researchers to elucidate the pathomechanisms underlying cardiac arrhythmias and propose pharmacological strategies for either prevention or treatment. Several models intending to recapitulate the human disease phenotype have been proposed. A summarized comparison between currently available in vitro models is presented in Fig. 10.1.

Over the past years, small animals such as genetically modified mice have been employed to investigate human arrhythmic diseases. These models have provided a testbed for pharmacology and become an inexorable step for preclinical drug approval. Nevertheless, considerable species differences reside between human and animal cardiomyocytes, including differences in beating rates, expression of key ion channels, and Ca^{++} cycling (Clauss et al., 2019). Therefore, human arrhythmias cannot be completely recapitulated with the use of transgenic animal models. On the other hand, in vitro cellular models have helped us understand the genotype-phenotype correlation. Indeed, the overexpression of mutant ion channel genes in heterologous cell expression systems has facilitated the characterization of mutation repercussions on individual ion channel functions (Makielski Jonathan et al., 2003; Song and Shou, 2012). While immortalized cells exhibit human origin, they lack the actual cardiac cellular context. Occasionally, primary cardiomyocytes isolated from the human heart have been used to investigate pathophysiological mechanisms underlying some human arrhythmias (Nguyen et al., 2017; Jost et al., 2005). Despite their physiological relevance, the use of isolated cardiomyocytes is restricted owing to ethical barriers and technical challenges concerning their limited availability and limited maintenance in cell culture. A landmark discovery of 1998 was the isolation of human embryonic stem cells (hESCs) from the blastocyst inner cell mass that are donned with self-renewal and pluripotency properties. Following the first derivation of cardiomyocytes



	HETEROLOGOUS SYSTEM	ANIMAL MODEL	hESC-CMs	hIPSC -CMs	HUMAN HEART
Source	Immortalized cells (e.g. COS7, HEK cells...)	Sacrificed animals	Inner cell mass of blastocyst (ethical limitation)	Easily accessible cells (e.g. blood, urine, skin..)	Donation or unused transplant
Maintenance in culture	Easy, unlimited source of dividing cells	Laborious, non-dividing, short survival upon isolation	Easy, unlimited source of dividing cells that can be differentiated into cardiomyocytes		Difficult, non-dividing, short survival upon isolation
Shape	Round, small, mononuclear	Elongated, polynuclear	Round, small, majority mononuclear		Typical shape, elongated, polynuclear
Maturity	Not cardiac cells	Same maturation stage as the animal	Immature (higher RMP, slower depolarization, shorter repolarization, higher arrhythmogenesis)		Possible isolation from adult heart
Cardiac specific subtypes	Not cardiac cells	Isolation of specific cardiac subtypes from specific heart region	Mixture of subtypes (ventricular, nodal, atrial), no defined heart region (sub-epi, -myo, -endocardium)		Isolation of specific cardiac subtypes from specific heart region
Expression of ion channels	Overexpression of one ion channel	Inter-species expression differences	Presence of all major cardiac ion channels		Typical cardiac ion channel expression
Variability/results reproducibility	Dependent on transfection efficiency	Difference between species and genetic lines	Line-to-line and differentiation variabilities		Inter-individual variability
High-throughput screening	Used	Limited	Used	Used	Limited
Inherited arrhythmia modelling	Study mutation impact on the corresponding channel functioning	Genetically modified animals, inter-Species phenotype differences	Mutation (generated by gene-editing) modelling	Patient/disease/mutation-specific modelling	Limited access to all inherited arrhythmia
Acquired arrhythmia modelling	Test the impact of an arrhythmogenic factor on a single ion current	Inter-Species response variability	Test the impact of an arrhythmogenic factor on control cardiomyocytes	Test the impact of an arrhythmogenic factor on subject-specific cells	Not used
Personalized medicine	NO	NO	NO	YES	NO

FIGURE 10.1 Comparison of in vitro model used to study arrhythmia.

Major advantages and inconvenient of the five commonly used cells to study arrhythmia in a dish are outlined. The table shows that human induced pluripotent stem cells—derived cardiomyocytes (hiPSC-CMs) are more advantageous than the other in vitro models for investigating arrhythmia in humans. *RMP*, resting membrane potential.

from hESCs (Kehat et al., 2001), hESC- cardiomyocytes (CMs) have been subjected to several cell-level analyses to investigate their electrophysiological, molecular, pharmacological, and contractile properties. While these cells rise above interspecies differences, the ethical limitations have impeded their applications. Following hESC-CMs, another breakthrough was the reprogramming of somatic cells, obtained using minimally invasive protocols into an embryonic-like state called induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007). In addition to the beneficial properties of hESC-CMs, human induced pluripotent stem cells—derived cardiomyocytes (hiPSC-CMs) offer the advantage of being patient-specific. HiPSC-CMs have genuinely metamorphosed our knowledge concerning human arrhythmias. Since their first derivation in 2009 (Zhang et al., 2009), there has been a remarkable increase in the use of hiPSC-CM models to study human arrhythmia (Fig. 10.2). In this chapter, we will highlight the major findings from studies that have modeled inherited and acquired arrhythmias using hiPSC-CMs. We will describe the inherent limitations, overview accomplishments directed to circumvent these limits and discuss future expectations toward precision medicine.

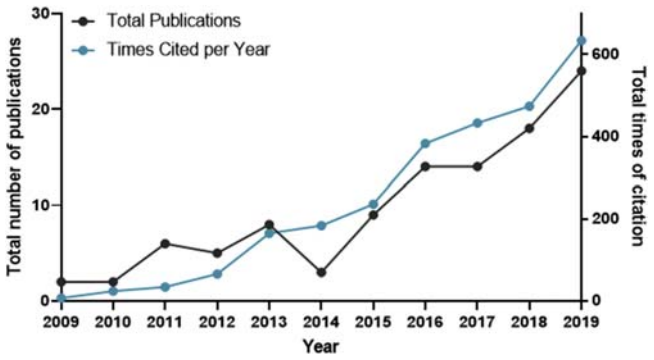


FIGURE 10.2 Number of published and cited articles where the human induced pluripotent stem cells–derived cardiomyocytes (hiPSC-CMs) model was used to study cardiac arrhythmias since their first derivation in 2009 and over the last 10 years.

Data were extracted from web of science with search terms: iPSC, cardiomyocytes, and arrhythmia. Data from the year 2020 were excluded from the search.

The use of human induced pluripotent stem cells–derived cardiomyocytes to investigate inherited arrhythmias

Patients with inherited arrhythmias may present with different clinical profiles ranging from developing no or minor symptoms to major symptoms such as loss of consciousness and SCD. Despite the plethora of knowledge and the major advances in genetic screening to define causal mutations associated with inherited arrhythmias, yet the translation of their subsequent biological outcomes into effective clinical approaches along with the definition of the genotype-phenotype causal relationship remains limited. The list of candidate genetic variants is continuously expanding, posing more challenges such as overlap syndromes (same variant causing multiple syndromes) and variant with uncertain significance (VUS) (Vermersch et al., 2020). Hence, patient-specific hiPSC-CMs coupled with genome editing strategies such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 have offered a reliable platform to model inherited diseases, evaluate the pathogenicity of genetic variants, develop drugs, and personalize medicine (Vermersch et al., 2020). A concise overview of the main inherited channelopathies modeled using hiPSC-CMs is depicted below, listed in Table 10.1, and displayed in Fig. 10.3.

Long QT syndrome

The long QT syndrome (LQTS) is a cardiac repolarization disorder characterized by the prolongation of the QT interval that may degenerate into ventricular tachyarrhythmias, notably torsade de Pointes (TdP), eventually leading to SCD. At the cellular level, this defective ventricular repolarization disorder arises from the prolongation of the action

Table 10.1 List of major studies of inherited arrhythmic diseases accomplished using the human induced pluripotent stem cells–derived cardiomyocytes model.

Inherited disease	Gene	Mutation	Hallmark common findings	References
LQT1	KCNQ1	R190Q (heterozygous)	Prolongation of the repolarization phase, Decreased I_{Ks}	Moretti et al. (2010)
		P631 fs/33I		Egashira et al. (2012)
		G589D, splicing mutation in intron 7 (IVS7-28A > G)		Kiviaho et al. (2015)
		Exon 7 deletion		Ma et al. (2015)
		M437V		Sogo et al. (2016)
		G589D		Kuusela et al. (2016)
		G589D, splicing mutation in intron 7 (IVS7-2A > G)		Kuusela et al. (2017)
		A341V		Takaki et al. (2019)
		A344Aspl		Yoshinaga et al. (2019)
LQT2	*KCNH2*	A561T	Prolongation of the repolarization phase, Decreased I_{Kr}	Matsa et al. (2011)
		A614V		Itzhaki et al. (2011)
		R176W		Lahti et al. (2012)
		N996I		Bellin et al. (2013)
		A422T		Spencer et al. (2014)
		A561V		Mehta et al. (2014)
		N996I		Sala et al. (2019)
		Splicing mutation in intron 9 (IVS9-28A/G)		Mura et al. (2017)
		S428X, R366X, A561V, splicing mutation in intron 9 (IVS9-28A/G)		Metha et al. (2018)
		R752W		Chai et al. (2018)
		T983I		Garg et al. (2018)
		A422T, G601S		Yoshinaga et al. (2019)
	TBX20	R311C		Caballero et al. (2017)

Continued

Table 10.1 List of major studies of inherited arrhythmic diseases accomplished using the human induced pluripotent stem cells –derived cardiomyocytes model.—*cont'd*

Inherited disease	Gene	Mutation	Hallmark common findings	References
LQT3	*SCN5A*	V1763M V240M, R535Q N406K R6144H R1193Q	Prolongation of the repolarization phase Increased late I_{Na}	Ma et al. (2013) Fatima et al. (2013) Spencer et al. (2014) Malan et al. (2016) Kroncke et al. (2019)
LQT7	*KCNJ2*	R67W, R218W, R218Q	Repolarization phase not prolonged Ca^{2+} impairment delayed after depolarization (DAD)	Kuroda et al. (2017)
LQT8	*CACNA1C*	G406R N639T	Prolongation of the repolarization phase Ca^{2+} impairment	Yazawa et al. (2011) Chavali et al. (2019)
LQT14	*CALM1*	F142L	Prolongation of the repolarization phase Ca^{2+} impairment	Rocchetti et al. (2017)
LQT15	*CALM2*	D132H, D132V D130G N98S	Prolongation of the repolarization phase, Ca^{2+} impairment	Pipilas et al. (2016) Limpitikul et al. (2017) Yamamoto et al. (2017)
SQTS	*KCNH2*	N588K T618I	Shortening of the repolarization phase, Increased I_{Kr}	El-Battrawy et al. (2018a) Shinnawi et al. (2019) Zhao et al. (2019) Guo et al. (2019)
AF	*UNKNOWN*		Ectopic beating Alterations of I_f and I_{CaL} DADs	Benzoni et al. (2019)
CPVT	*RyR2*	F243I S406L P2328S M4109R E2311N	Ca^{2+} impairment	Fatima et al. (2011) Jung et al. (2011) Kujala et al. (2012) Itzhaki et al. (2012) Di Pasquale et al. (2013)

		Exon 3 deletion, P2328S, T2538R, L4115F, Q4201R, V4653F		Penttinen et al. (2015)
		R420Q		Novak et al. (2015)
		L3741P		Preininger et al. (2016)
		F2483I		Wei et al. (2018)
		D3638A	Increased DADs with isoproterenol	Acimovic et al. (2018)
		Exon 3 deletion, L4115F		Pölönen et al. (2020)
		I4587V		Sasaki et al. (2016)
		P2328S		Paavola et al. (2016)
		R4651I		Park et al. (2019)
	CASQ2	D307H		Novak et al. (2012)
				Novak et al. (2015)
				Maizels et al. (2017)
		Premature stop codon		Lodola et al. (2016)
CPVT/ LQTS	*TECRL*	R196Q, splicing mutation (c.331+1G > A)	Prolongation of the repolarization phase Ca^{2+} impairment DADs	Devalla et al. (2016)
BrS	Unknown	Unknown	No change	Veerman et al. (2016)
				Miller et al. (2017)
	SCN5A	A735V	Decreased I_{Na}	de la Roche et al. (2019)
		R620H, R811H	Increased arrhythmogenesis, decreased I_{Na}	Liang et al. (2016)
		R367H		Selga et al. (2018)
	SCN1B	L210P, P213T		El-Battrawy et al. (2019b)
	SCN10A	R1268Q, R1250Q		El-Battrawy et al. (2019a)
	RRAD	R211H	Prolongation of the repolarization phase, decreased I_{Na} early after depolarization	Belbachir et al. (2019)
BrS/LQT3	*SCN5A*	1795insD	Prolongation of the repolarization phase, Increased late $I_{Na,}$ decreased peak I_{Na}	Davis et al. (2012)
		E1784K		Okata et al. (2016)

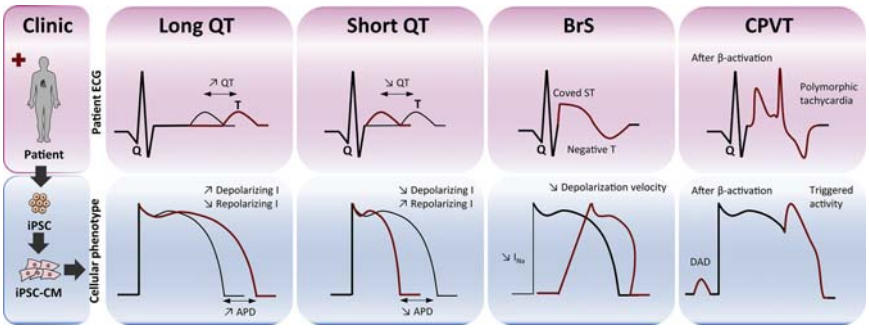


FIGURE 10.3 The reflection of inherited arrhythmia clinical phenotype (ECG) in human induced pluripotent stem cells—derived cardiomyocytes (hiPSC-CMs) electrical activity.

Long QT syndrome characterized by a prolongation of the QT interval in ECG is recapitulated in corresponding hiPSC-CMs which present a prolongation of the APD due to reduced repolarizing currents or increased depolarizing currents. In contrast, hiPSC-CMs derived from patients harboring an LOF mutation in depolarizing currents or gain of function (GOF) mutation in repolarizing currents exhibits a decrease in APD. This recapitulates the short QT interval in corresponding patient ECG. As for BrS, to date, the major cellular phenotype reported in hiPSC-CMs from the diagnosed patients is a decrease in I_{Na}. This is in favor with the repolarization theory of the BrS mechanism, which suggests a late activation of the RVOT as a cause for the elevation of the ST segment and the negative T wave in patients ECG. In hiPSC-CMs from CPVT patients presenting polymorphic tachycardia after the administration of β-agonist, abnormal transient calcium is detected and causes DADs and triggered activity following β-activation. *APD*, action potential duration; *BrS*, Brugada syndrome; *CPVT*, catecholaminergic polymorphic ventricular tachycardia; *DADs*, Delayed after depolarization; I_{Na}, sodium current.

potential duration (APD) in ventricular cardiomyocytes. It is also associated with the high propensity of arrhythmias, typically early after depolarization, which are believed to be the origin of the life-threatening arrhythmias, TdP (Gan-Xin et al., 2001). To date, mutations in 16 genes have been linked to congenital LQTS; however, 20% of instances are still genetically unresolved (Neira et al., 2019). Mutations in these genes implicate either loss of function (LOF) in repolarizing currents or gain of function (GOF) in depolarizing currents (Barsheshet et al., 2013). Three major LQTS subtypes (LQT1, LQT2, and LQT3) account for about 90% of mutation-positive patients. LOF mutations in *KCNQ1* that generate I_{Ks} in LQT1 are the most common form of inherited LQTS and account for 35% of cases. The second most common LQTS accounting for 30% cases is LQT2 that arises from LOF in *KCNH2*, generating I_{Kr}. Increased late sodium current ($I_{Na,L}$) generated by *SCN5A* is linked to LQT3, which accounts for 10% of all cases. The rest of the genes related to inherited LQTS encode either ion channel subunits (*KCNE1*, *KCNJ5*, *KCNE2*, and *SCN4B*) or proteins that regulate ion channel functions (*SNT1*, *ANKB*, *AKAP9*, *CAV3*, *CALM1*, and *CALM2*) (Bezzina Connie et al., 2015). The recent advances in genetic tools have unveiled different variants of these genes. However, it is difficult to establish the causal relationship between each variant

and LQTS owing to interindividual genome heterogeneity and variable penetrance, resulting in a rise in the number of VUS without any evidence of pathogenicity (Wu Joseph et al., 2019). Since the first iPSC-CM model of LQTS in 2010 (Moretti et al., 010), multiple studies have demonstrated the reliability of hiPSC-CMs in recapitulating the cardinal cellular features of the disease, i.e., prolonged repolarization phase and high propensity of spontaneous and triggered arrhythmias (Table 10.1). Interestingly, hiPSC-CMs coupled with the CRISPR technique can be used to identify pathogenic VUS. This rationale was adopted in a study on a novel VUS (T983I) in the *KCNH2* gene in LQTS patients. Selective correction of this VUS in hiPSC-CMs of LQTS patient resulted in the normalization of the abnormal electrical profile, whereas the introduction of the same variant in control cells using the CRISPR technique induced the LQTS phenotype (Garg et al., 2018). Thus, the causative role of VUS in LQT induction can be corroborated with such strategies regardless of the genetic background noise (Garg et al., 2018; Chavali et al., 2019). Moreover, high throughput techniques can accelerate the classification of VUS into potentially pathogenic variants (Yoshinaga et al., 2019). Mutation-specific drugs can similarly be examined, offering an unprecedented opportunity for the development of more targeted therapy, e.g., the use of lumacaftor for specifically rescuing the pathological phenotype of LQT2 with a mutation affecting hERG channel trafficking (Mehta et al., 2018). Finally, the comparison of hiPSC-CMs derived from LQTS-afflicted patients presenting different expressivity of the same mutation (different severity of the disease) may shed light on the modulatory role of genetic modifiers (Matsa et al., 2011).

Short QT syndrome

The short QT syndrome (SQTS), first described in 2000, is a rare autosomal dominant arrhythmic disease (Gussak et al., 2000) that predisposes patients to atrial and ventricular tachyarrhythmia and increases the propensity of sudden cardiac arrest. In contrary to the LQTS, the SQTS is characterized by an abnormal shortening of the QT interval (<330 ms) in the ECG, reflective of accelerated ventricular repolarization (Campuzano et al., 2019; Patel et al., 2010). The GOF mutations in repolarizing currents (*KCNH2*, *KCNQ1*, and *KCNJ2*) and LOF mutations in depolarizing currents (*CACNA1C*, *CACNB2*, *CACNA2D*, *SLC4A3*, and *SCN5A*) have been attributed to this disorder (Campuzano et al., 2018). The first report elucidating the ability of hiPSC-CMs to recapitulate the clinical phenotype of SQTS was provided by El-Battrawy et al. who observed increased I_{Kr} along with shortened APD in hiPSC-CMs derived from SQTS patients harboring the *KCNH2*−N588K mutation (Ibrahim et al., 2018). Consistently, hiPSC-CMs from patients carrying a different GOF mutation of *KCNH2* (T618I) showed a similar cellular phenotype that was rescued using quinidine (Guo et al., 2019), the drug of choice against SQTS (El-Battrawy et al., 2018a). Other drugs such as ivabradine, mexiletine, ajmaline (Zhao et al., 2019), BmKKx2 (Guo et al., 2019), and disopyramide (Shinnawi et al., 2019) tested on SQTS-hiPSC-CMs could also suppress the abnormal electric-phenotype in vitro, serving as alternative candidates for the treatment and prevention of lethal tachyarrhythmias in SQTS-afflicted patients.

Brugada syndrome

In 1992, Pedro and Josep Brugada described a complex disease as "right bundle branch block, persistent ST-segment elevation, and SCD," which was later termed as "Brugada syndrome" (BrS) following its characterization (Miyazaki et al., 1996). The diagnosis of BrS is carried out based on clinical history and ECG features, typically involving an ST elevation in the right-precordial leads V1−V3 that may occur spontaneously or be induced by sodium blockers. BrS is frequently a silent disease reported in a structurally normal heart. The occurrence of SCD, notably during rest or at night, is the first manifestation of the disease in young patients in the fourth decade of their life (Bezzina Connie et al., 2015; Romero et al., 2019). To date, pathogenic mutations in 23 different genes have been linked to BrS and the list continues to grow (Wu et al., 2017). These mutations (over 300) predominantly implicate LOF in *SCN5A* gene (∼30% of instances) (Sieira and Brugada, 2016; Aiba, 2019). However, BrS susceptibility genes have been observed only in 30%−35% of clinically diagnosed BrS, leaving ∼65% of patients without a known genetic cause (Brugada et al., 2016; Sarquella-Brugada et al., 2016). Recent evidence suggests a polygenic mode of inheritance of BrS, involving multiple genetic variants acting through different pathways (Ingles et al., 2019; Bezzina et al., 2013). Aside from its genetic heterogeneity and complexity, the pathophysiological mechanism (depolarization or repolarization imbalance) underlying ST elevation in the ECG of BrS patients is still unclear (Brugada et al., 2018). A major limit hampering our understanding of BrS is the paucity of an appropriate model to recapitulate the disease phenotype. So far, the best genetic models of BrS are *Scn5a*-KO mouse and pig carriers of the E558X nonsense mutation. However, interspecies differences and interindividual genetic variations confound the ability to translate these findings from animal models to humans (Sendfeld et al., 2019). In contrast, patient-specific hiPSC-CMs carrying mutations in *SCN5A* (de la Roche et al., 2019; Pérez-Hernández et al., 2018; Ma et al., 2018; Selga et al., 2018; Liang et al., 2016; Okata et al., 2016; Davis et al., 2012) or genes affecting its expression or functions (El-Battrawy et al., 2019a,b; Belbachir et al., 2019) presented reduction in peak I_{Na}, slower depolarization velocity, and exaggerated ajmaline sensitivity, recapitulating the plausible proarrhythmic mechanism associated with BrS. Aside from the use of cell lines modulated with *SCN5A* mutations, differences in sodium channel gating properties were unveiled for the first time in hiPSC-CMs derived from a patient harboring the same mutation (de la Roche et al., 2019; Selga et al., 2018). This observation is thought to be related to the presence of Nav1.5 regulatory proteins in hiPSC-CMs but not in heterologous systems. Moreover, hiPSC-CMs derived from patients carrying *SCN5A*-1795insD that led to an overlap of Brugada/LQT3 syndrome could recapitulate both the reduction in BrS-associated peak I_{Na} and the increase in LQT3-linked persistent I_{Na} (Davis et al., 2012). Overall, these findings underscore the use of hiPSC-CMs to model diseases associated with dysfunctional cardiac sodium channels. On the other hand, hiPSC-CMs derived from patients lacking *SCN5A* or other BrS-related gene variants showed no remarkable changes in I_{Na}, $I_{Ca,L}$, I_{to}, or AP characteristics (Veerman et al., 2016) and exhibited

no modifications in the field potential following ajmaline application (Miller et al., 2017). BrS is viewed as a problem that originates at the tissue level, in particular at the right ventricular outflow tract (RVOT), which is the BrS favorite arrhythmogenic site (Kofune et al., 2011). This has restricted BrS modeling at the cellular level. In this direction, to gain insights into the molecular mechanism underlying BrS and hunt potential therapeutic targets, more efforts should be concerted in tissue-modeling of BrS.

Catecholaminergic polymorphic ventricular tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a malignant, inherited arrhythmia syndrome characterized with bidirectional ventricular tachycardia induced by physical or emotional stress in young patients with a structurally normal heart. This CPVT-specific ECG signature is rarely presented, and its diagnosis is challenging. While β-blockers are the first-line treatment for CPVT, they may be sometimes ineffective in preventing life-threatening arrhythmias (Hayashi et al., 2009; Pflaumer et al., 2019). The most common mutations responsible for CPVT are located in *RyR2* (∼60% of CPVT patients) and less frequently in *CASQ2*. As both genes encode key factors of intracellular calcium handling, their mutations may result in the abnormal release of calcium from the sarcoplasmic reticulum (SR) during diastole. The consequences include delayed after depolarization (DAD), leading to triggered activity and ventricular tachyarrhythmia (Bezzina Connie et al., 2015; Napolitano et al., 2020). To date, several studies have used hiPSC-CMs to model CPVT in a dish. Irregular transient calcium and increased incidence of DADs, which are exacerbated by β-agonist stimulation, are the typical features highlighted in hiPSC-CMs from CPVT patients carrying *RyR2* (Jung et al., 2012; Novak et al., 2012, 2015; Paavola et al., 2016; Preininger et al., 2016; Sasaki et al., 2016; Acimovic et al., 2018; Kujala et al., 2012; Itzhaki et al., 2012) or *CASQ2* mutation (Novak et al., 2015; Lodola et al., 2016; Haron-Khun et al., 2017). Hence, the presence of a cellular phenotype associated with CPVT allowed the use of hiPSC-CMs for drug screening (Jung et al., 2012; Preininger et al., 2016; Itzhaki et al., 2012; Haron-Khun et al., 2017; Penttinen et al., 2015; Pölönen et al., 2018, 2020). Interestingly, clinical observations validated the findings on hiPSC-CMs where flecainide and dantrolene were both capable of curing arrhythmia related to CPVT (Preininger et al., 2016; Penttinen et al., 2015). Furthermore, in an overlap syndrome of CPVT and LQT, hiPSC-CMs carrier of the *TECRL* homozygous mutation captured salient arrhythmic features of both diseases which were suppressed by Flecainide (Devalla et al., 2016). In addition to drug testing, CPVT hiPSC-derived cardiomyocytes were exploited to investigate the effectiveness of gene therapy in abolishing arrhythmia. Viral vector-mediated transfer of *CASQ2* into hiPSC-CMs from a patient carrying a *CASQ2* LOF mutation rescued transient calcium abnormalities and decreased the percentage of DADs (Lodola et al., 2016). Moreover, overexpression of a potent Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitory peptide in CPVT-hiPSC-CMs could reverse the arrhythmia phenotype, confirming the findings from a CPVT mouse model in a human model (Bezzerides Vassilios, 2019).

Atrial fibrillation

Atrial fibrillation (AF) represents the most common sustained arrhythmia in clinics (1% of worldwide population) especially in elderly people. The risk of AF is high with comorbidities such as hypertension, diabetes, obesity, heart failure, cardiac myopathies, and channelopathies (Enriquez et al., 2016; Kalstø et al., 2019; Zulkifly et al., 2018). Treatment for AF mainly comprises surgical ablation or nonselective antiarrhythmic drugs, which are often associated with serious complications and may be efficient only in a select number of patients. The presence of a heritable component underlying AF, notably lone AF, has been recently reported in a rare subset of familial instance; however, incomplete penetrance and the lack of adequate AF model complicate the definition of the causal relationship (Kalstø et al., 2019). In this regard, besides exploring a potential genetic implication, the advent of subtype-specific patient-hiPSC-CMs differentiation strategies presents an unprecedented opportunity to dig through AF physiopathology and screen for surrogate treatments. Benzoni et al. were the first to use this approach to investigate the cellular mechanism of a familial case of untreatable persistent AF. These authors reported higher beating rates, enhanced I_f and $I_{Ca,L}$ currents, and prolonged APD. Further, patient-derived hiPSC-CMs were more prone to arrhythmia (i.e., DADs and ectopic beats) under stressful conditions as compared to control cells. In conclusion, these findings suggest the implication of I_f-mediated automaticity and prolonged APD that may trigger dispersion in repolarization, in the initiation of AF (Benzoni et al., 2019). Henceforward, future studies employing hiPSC should be devoted to explore the genetic basis and the mechanism behind AF and the development of efficient therapies.

Application of human induced pluripotent stem cells—derived cardiomyocytes to investigate acquired arrhythmias

Rhythm disturbances, aberrant conduction, and abnormal repolarization are most commonly acquired rather than being inherited (Keating and Sanguinetti, 2001) and can develop in patients with a predisposing genetic background. In the absence of any variant of ion channel genes, arrhythmias appear as a consequence to structural heart diseases, hormone stimulation, infections, and most frequently the use of medications. The development of arrhythmias following exposure to these elements is unpredictable and depends on unspecified genetic, epigenetic, and environmental factors. This renders therapeutic strategies even more complicated and stresses the need for sophisticated risk stratification tools and personalized patient care (Isbister and Semsarian, 2019). Since their development, hiPSC-CMs have successfully served to recapitulate induced arrhythmia in vitro and acted as a powerful test-tool to evaluate cardiotoxic reactions. In the following paragraphs, we describe the major findings established using hiPSC-CMs with respect to cardiac arrhythmia induced by infection, hormones, and drugs. Fig. 10.4 schematically explains the importance of hiPSC-CMs in modeling acquired arrhythmia.

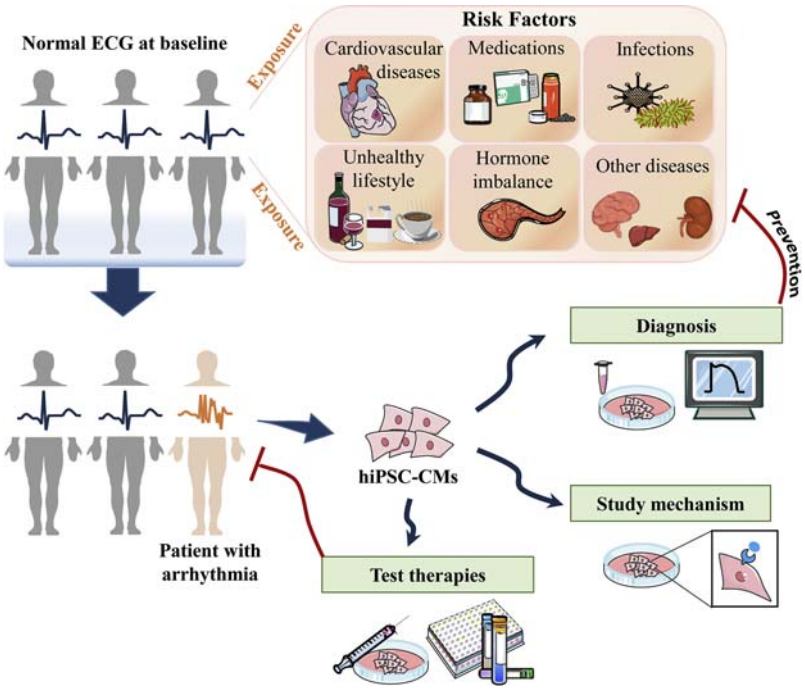


FIGURE 10.4 Human induced pluripotent stem cells—derived cardiomyocytes (hiPSC-CMs) offer an unprecedented opportunity to unveil, study, and treat acquired arrhythmia.

Some individuals are more prone to develop arrhythmia upon their exposure to risk factors such as cardiovascular and noncardiovascular diseases, unhealthy lifestyle, drugs, hormones disequilibrium, and infections. Deriving hiPSC-CMs from these individuals allow the identification of the risk factor and support thereby preventive care. It confers also a platform to study the mechanism underlying the induction of arrhythmia. Finally, it helps to assess and innovate personalized therapies that fit each individual needs.

Infection-induced arrhythmia

Myocarditis is an inflammation of the heart muscle that can lead to heart failure and SCD, especially in young individuals (Gaaloul et al., 2016). Viral infection is considered as a major contributor to myocarditis. Viruses exploit the host translational machinery to enhance their replication. Viral factors or the host immune response can prompt cardiac electrophysiological and structural remodeling, and increase the propensity of life-threatening arrhythmias (Tse et al., 2016). HiPSC-CMs confer a key tool to model these arrhythmias and examine potential effective treatments. A good example of this statement is the study of coxsackievirus-induced myocarditis using hiPSC-CMs (Sharma et al., 2014). Coxsackievirus uses a receptor expressed by cardiomyocytes for invasion. Once inside the host cell, the viral protease targets an intracellular structural protein called dystrophin and disrupts the

sarcolemma integrity, resulting in abnormal calcium transients, irregular beating, and ultimately cessation of beating. Gene expression profiling of infected hiPSC-CMs led to the development of an antiviral treatment regimen using interferon β1 (Sharma et al., 2014). In line with these outcomes regarding hiPSC-CMs efficiency to investigate viral-induced arrhythmias, Es-Salah-Lamoureux et al unraveled the impact of a viral transactivator, Tat, generated by human immunodeficiency virus (HIV) on repolarizing currents, I_{Kr} and $I_{Ks,}$ in control hiPSC-CMs. AP was lengthened in hiPSC-CMs exposed to Tat (Es-Salah-Lamoureux et al., 2016), consistent with the prolongation of QT interval (Moreno et al., 2013) and a high risk of TdP in HIV patients (Kocheril et al., 1997). This observation also explains the relatively high mortality rate (about 10%) observed with SCD (Tseng et al., 2012). In the end, it is worth noting that the heart's reaction to a virus is patient-specific. Genetic predisposition is thought to be one of the factors rendering some patients more susceptible to develop cardiac complications such as arrhythmias following viral infection (Tse et al., 2016). This emphasizes the use of patient hiPSC-CMs to decipher the specific mechanisms and tailor appropriate treatment.

Hormone-induced arrhythmia

Among healthy individuals, corrected QT interval is shorter in postpubertal men than in women (Vicente et al., 2014). In addition to gender-related variations in the expression of some ion channel genes (Gaborit et al., 2010), sex hormones are thought to influence cardiac repolarization. A higher risk of LQT-related TdP and SCD was reported in men with hypogonadism and in those on androgen blockers (Salem et al., 2016). For instance, enzalutamide, an androgen receptor antagonist used to treat men with prostate cancer, is associated with adverse effects of LQT and SCD. Salem et al. explored the impact of acute and chronic exposure to enzalutamide on hiPSC-CMs and reported an increase in APD and cellular arrhythmias after enzalutamide application regardless of the exposure period. Furthermore, this drug was shown to reduce I_{Kr} and increase $I_{Na,L}$ (Salem et al., 2019). In women, repolarization is likely regulated by both estradiol and progesterone. While studies have speculated a protective role of progesterone against LQT, consistent with that of testosterone (Odening et al., 2012), conflicting results concerning the role of estradiol in repolarization regulation have been noted (Salem et al., 2016; Odening et al., 2012). A recent study on hiPSC-CMs cultured in conditions mimicking catecholamine stress showed that estradiol prevented APD prolongation induced by excessive isoprenaline (El-Battrawy et al., 2018b). In contrast, several studies conducted in animal cardiomyocytes have revealed APD lengthening following estradiol addition (Cheng et al., 2012; Tanabe et al., 1999). Thus, the understanding of the mechanism underpinning the effects of sex hormones on cardiac repolarization and arrhythmia may reveal gender-related risks and pave a way for hormone-based antiarrhythmic therapies. HiPSC-CMs serve as a suitable investigation platform for this purpose.

Drug-induced arrhythmia

Over the years, the pharmaceutical sector has tremendously suffered from the failure of several drugs that could not surpass the preclinical tests outlined by the FDA owing to safety concerns. Cardiovascular complications (8.5% rate) are considered as the main causes of drug exclusion (Arrowsmith and Miller, 2013). Some drugs with undetermined cardiotoxic effects have managed to escape the FDA evaluation. Between 1953 and 2013, 462 drugs prescribed for cardiovascular or noncardiovascular diseases were withdrawn from the market for toxicity reasons. Of these, 15% of drugs were associated with cardiotoxic adverse effects (Onakpoya et al., 2016). QT prolongation, a common side-effect on the heart, is a predominant form of fatal acquired arrhythmia induced by many drugs (Antoniou et al., 2017). This raises awareness about the reliability of models used in preclinical studies to identify proarrhythmic risks. To face this dilemma, the Cardiac Safety Research Consortium launched a "Comprehensive in vitro Proarrhythmia Assay" in 2014 as a new paradigm to evaluate the risk of drug cardiotoxicity. The program integrated hiPSC-CMs to verify and consolidate the results obtained from heterologous systems and in silico models. The list of drugs included antiarrhythmic, antidepressants, antibiotics, and, intriguingly, anticancer medications (Colatsky et al., 2016). Cardio-oncology, combining the first and the second most leading causes of death worldwide, has emerged as a new field that intrigues many researchers (Kostakou et al., 2019). Several studies have relied on hiPSC-CMs to (1) unmask potential proarrhythmic effects of anticancer drugs and (2) investigate the mechanism underlying the influence of these treatments on heart electrophysiology (Gary et al., 2019; Hanf et al., 2019). However, a looming challenge concerning anticancer drugs is the unavoidable lifetime treatment. HiPSC-CMs can help overcome this limit during drug development by allowing investigation of long-term exposure (Sakamoto et al., 2019) and presumably finding alternatives to deal with potentially adverse effects.

Another common challenge confronted while prescribing medications is that patients respond differently in terms of effects and side-effects. Genetic factors are suspected to modulate an individual's response to drugs. On this concern, besides offering patient-specific prediction of drug cardiotoxicity in vitro, hiPSC-CMs enable effective query of transcriptomic predisposition to develop adverse effects and/or variations upon drug application (Knowles et al., 2018; Zhao and Zhang, 2017; Stillitano et al., 2017). We have previously addressed the variable susceptibility to sotalol-induced LQT using a panel of subject-specific hiPSC-CMs. Our library of hiPSC-CMs faithfully recapitulated the drug sensitivity observed in the clinic. Moreover, transcriptomic profiling uncovered five genetic modulators with potential implications in predisposing the high sensitive group to arrhythmia following drug administration (Stillitano et al., 2017). Such studies could solve the puzzling reason behind the undesired side-effects of drugs in some groups of patients and may be promising for preventing these effects by adjusting the expression of genetic modulators.

Taken together, the systemic inclusion of hiPSC-CMs into drug safety screening protocol appears to be crucial to produce more effective drugs, save patients from unwanted side-effects, ameliorate their life quality, and offer opportunities to tailor personalized drug prescriptions.

Limitations

Despite the tremendous effort to improve differentiation protocols, hiPSC-CMs are reported to lack the structural, electrophysiological, and metabolic maturity of adult cardiomyocytes. In fact, current differentiation strategies produce round small cells that are similar to prenatal cardiomyocytes. In-depth comparative transcriptomic analyses showed that derived hiPSC-CMs are arrested at embryonic developmental stages (E8-E11). Although long-term culture improves their maturation, hiPSC-CMs fail to gain at least the neonatal-like transcript levels (Zhou et al., 2017; Uosaki et al., 2015). Furthermore, in terms of ion channel gene expression, hiPSC-CMs express low levels of *KCNJ2* (Kodama et al., 2019) and the lack of I_{K1} results in the switch in their resting membrane potential (RMP) to around −60 mV versus −85 mV reported for adult ventricular cells (Scuderi and Butcher, 2017). In addition to the higher RMP, high expression of *HCN4*, engendering $I_{f,}$ enables hiPSC-CMs to beat spontaneously, which is in contrast, the sole feature of pacemaker cells in adulthood (Scuderi and Butcher, 2017). Further, despite the normal expression of *SCN5A* (Kodama et al., 2019), in more depolarized membranes, $I_{Ca,L}$ takes over I_{Na} during the depolarization phase that is substantially slower in immature cardiomyocytes (Scuderi and Butcher, 2017). To circumvent this obstacle that hinders the modeling of arrhythmic diseases, some studies opted in silico addition of I_{K1} via dynamic patch clamp or *KCNJ2* overexpression. These methods engender more hyperpolarized RMP with values closer to those in adult cardiomyocytes and enhance sodium channel participation in AP depolarization (Ma et al., 2018; Vaidyanathan et al., 2016; Goversen et al., 2018; Li et al., 2017). Electrophysiological differences between adult cardiomyocytes and hiPSC-CMs are schematized in Fig. 10.5.

The structural machinery of hiPSC-CMs is also underdeveloped. In contrast to their adult counterparts, these cells lack the regular ultrastructure and T-tubule network, which affect RyR2/SERCA (responsible for Ca^{++} release/recapture) distribution. As a consequence, Ca^{2+} handling is slower and shows spatial inhomogeneous distribution in hiPSC-CMs as compared to that in adult cardiomyocytes with SR stores (Hwang et al., 2015; Koivumäki et al., 2018). Further, the contribution of sarcolemmal Ca^{2+} ($I_{Ca,L}$ and I_{NCX}) is prominent in hiPSC-CMs, influences excitation-contraction coupling, and renders these cells more susceptible to arrhythmic events such as DADs, leading to misinterpretations while studying diseases with impaired Ca^{2+} transients such as CPVT (Koivumäki et al., 2018). To address structural weaknesses and improve contractile performance, three-dimensional (3D) cardiac culture protocols are increasingly employed. The aggregation of hiPSC-CMs into a tissue-like structure is proven to promote cardiomyocyte maturation, enhance their sarcomeric organization, and ameliorate their electrical performance (Correia et al., 2018; Zhang et al., 2013; Lemoine et al., 2017). Studying cardiac arrhythmia in 3D structures provides an insight into the electrophysiological characteristics of a multicellular tissue model. For instance, conduction defects and reentry, the hallmark arrhythmogenic features of SQTS and CPVT, were effectively recapitulated in a 3D model of patient-derived hiPSC-CMs (Shinnawi et al., 2019; Park et al., 2019).

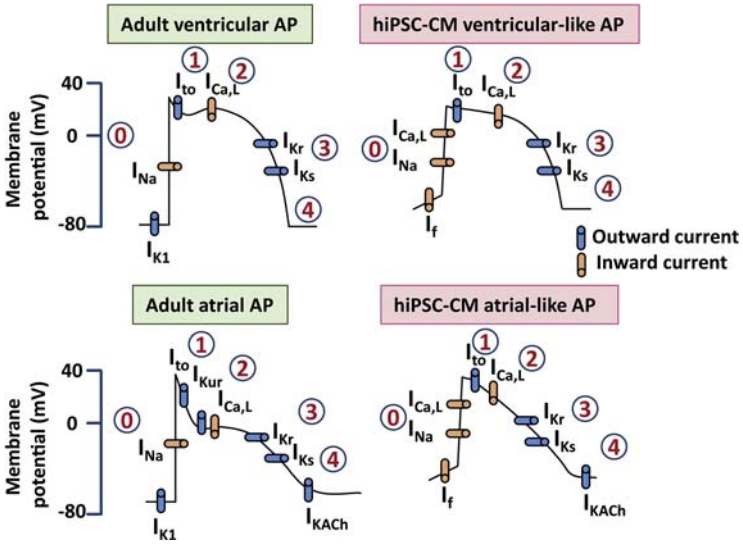


FIGURE 10.5 Comparison of action potentials of ventricular-like and atrial-like human induced pluripotent stem cells–derived cardiomyocytes (hiPSC-CMs) versus action potentials of adult cardiomyocytes.

Phases 0–4 are the fast depolarization, early repolarization, plateau phase, late repolarization, and diastole, respectively. The major difference between adult and hiPSC-derived cardiomyocytes resides in the presence of a prominent pacemaker current (I_f) and the absence of inward rectifier potassium currents (I_{K1}). The presence of I_f is responsible for the spontaneous AP in hiPSC-CMs, while the absence of I_{K1} results in more depolarized membrane potential in hiPSC-CMs. At higher resting membrane potentials as compared to adult cardiomyocytes, less I_{Na} is activated during the depolarization phase of hiPSC-CMs. In consequence, phase 0 is slower in both atrial-like and ventricular-like hiPSC-CMs as compared to the adult counterparts. Besides, phase 1 is less prominent in hiPSC-CM notably in atrial-like hiPSC-CMs where the notch phase is significantly abridged, due to reduced activation of I_{to}.

Moreover, in addition to the single-cell characterization of LQT with hiPSC-CMs, lethal TdP were also reproduced in 3D tissue models (Kawatou et al., 2017), highlighting the potential nodes for efficient pharmaceutical assays and therapeutic interventions.

Another limitation related to the current hiPSC-CM technology is that the resulting cardiomyocytes display a mix of cardiac subtypes with electrophysiological consequences. Based on AP morphology, cardiomyocyte mixture can be categorized into nodal-like, atrial-like, and ventricular-like cells (Kane and Terracciano, 2017). An obvious disadvantage of this heterogeneous combination is realized while studying the phenotypes related to specific types of myocytes. So far, most classical differentiation protocols mainly (up to 70%) produce a ventricular-like cellular population (Burridge et al., 2015). Approaches that aim to enrich a specific cell subtype

have been developed. For example, atrial-subtype enrichment necessitates the addition of retinoic acid during differentiation (Cyganek et al., 2018; Lee et al., 2017; Zhang et al., 2011). Other methods took into account lineage-specific transcription factors that act at different stages of fetal cardiac development as well as overexpression or selection of specific transcription factor-positive cardiomyocytes, e.g., COUP-TFII as a reporter for atrial lineage (Schwach et al., 2017), SHOX2 specific to nodal cells (Ionta et al., 2015), and IRX4 as a marker of ventricular progenitors (Ban et al., 2015).

Researchers around the world have been grappling to minimize the above-asserted limitations. Over the past decade, we have witnessed a plethora of methods particularly focusing on improving hiPSC-CM maturity which remains so far the major given criticism. Such studies are of great interest. Besides endorsing the reliability of hiPSC-CMs in disease modeling and drug screening, they broaden our knowledge concerning the process of human cardiomyocyte development and functioning.

Conclusion and perspective

Despite inherent limitations and considering the to date research outcomes, hiPSC-CM model remains more relevant to native human cardiomyocyte electrophysiology and provides a more reliable representation of both acquired and inherited arrhythmias than other in vitro models. The robust argument in favor of hiPSC-CMs is being patient-specific. This lends solid support to the concept of personalized medicine. Future projects can envisage incorporating an experimental assay using patient iPSC-CMs in parallel to translational studies. This would facilitate the diagnosis of the pathogenicity of different genetic variants in inherited arrhythmias or to calculate the risk of developing acquired arrhythmia in the presence of the overriding modulating player, which is the unique genetic background of each individual. Moreover, assessing the effects of drugs on patient-specific hiPSC-CMs allows customization of accurate prescriptions to fit patient's response. Finally, concerted efforts are now devoted to push the field of regenerative medicine by combining hiPSC-CMs and genome editing approaches with an eye toward designing novel therapeutic approaches, notably gene correction. To sum up, hiPSC-CMs have been revolutionizing our knowledge regarding arrhythmia and hold great promises for the invention of novel therapies as well as personalized and preventive medicine.

Funding

This work was supported by Foundation Leducq: grant 18CVD05.

References

Acimovic, I., Refaat, M.M., Moreau, A., et al., 2018. Post-translational modifications and diastolic calcium leak associated to the novel RyR2-D3638A mutation lead to CPVT in patient-specific hiPSC-derived cardiomyocytes. J. Clin. Med. 7, e423.

Aiba, T., 2019. Recent understanding of clinical sequencing and gene-based risk stratification in inherited primary arrhythmia syndrome. J. Cardiol. 73, 335−342.

Antoniou, C.-K., Dilaveris, P., Manolakou, P., et al., 2017. QT prolongation and malignant arrhythmia: How serious a problem? Eur. Cardiol. 12, 112−120.

Arrowsmith, J., Miller, P., 2013. Phase II and Phase III attrition rates 2011−2012. Nat. Rev. Drug Discov. 12, 569.

Ban, K., Wile, B., Cho, K.-W., et al., 2015. Non-genetic purification of ventricular cardiomyocytes from differentiating embryonic stem cells through molecular beacons targeting IRX-4. Stem Cell Rep. 5, 1239−1249.

Barsheshet, A., Dotsenko, O., Goldenberg, I., 2013. Genotype-specific risk stratification and management of patients with long QT syndrome. Ann. Noninvasive Electrocardiol. 18, 499−509.

Belbachir, N., Portero, V., Al Sayed, Z.R., et al., 2019. RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome. Eur. Heart J. 40, 3081−3094.

Bellin, M., Casini, S., Davis, R., et al., 2013. Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. EMBO 32 (24), 3161−3175.

Benzoni, P., Campostrini, G., Landi, S., et al., 2019. Human iPSC modelling of a familial form of atrial fibrillation reveals a gain of function of if and ICaL in patient-derived cardiomyocytes. Cardiovasc. Res. cvz217.

Bezzerides Vassilios, J., Caballero, A., Wang, S., et al., 2019. Gene Therapy for Catecholaminergic polymorphic ventricular tachycardia by inhibition of Ca^{2+}/calmodulin-dependent kinase II. Circulation 140, 405−419.

Bezzina Connie, R., Najim, L., Priori Silvia, G., 2015. Genetics of sudden cardiac death. Circ. Res. 116, 1919−1936.

Bezzina, C.R., Barc, J., Mizusawa, Y., et al., 2013. Common variants at SCN5A-SCN10A and HEY2 are associated with Brugada syndrome, a rare disease with high risk of sudden cardiac death. Nat. Genet. 45, 1044.

Brugada, J., Campuzano, O., Arbelo, E., et al., 2018. Present status of Brugada syndrome: JACC state-of-the-art review. J. Am. Coll. Cardiol. 72, 1046−1059.

Brugada, R., Campuzano, O., Sarquella-Brugada, G., et al., 2016. Brugada syndrome. GeneReviews® [Internet]. Seattle (WA). University of Washington, Seattle, pp. 1993−2015.

Burridge, P.W., Sharma, A., Wu, J.C., 2015. Genetic and epigenetic regulation of human cardiac reprogramming and differentiation in regenerative medicine. Annu. Rev. Genet. 49, 461−484.

Caballero, R., Utrilla, R.G., Amorós, I., et al., 2017. Tbx20 controls the expression of the KCNH2 gene and of hERG channels. PNAS 114 (3), E416−E425.

Campuzano, O., Sarquella-Brugada, G., Cesar, S., et al., 2018. Recent advances in short QT syndrome. Front. Cardiovasc. Med. 5, 149.

Campuzano, O., Fernandez-Falgueras, A., Lemus, X., et al., 2019. Short QT syndrome: a comprehensive genetic interpretation and clinical translation of rare variants. J. Clin. Med. 8, e1035.

Chai, S., Wan, X., Ramirez-Navarro, A., et al., 2018. Physiological genomics identifies genetic modifiers of long QT syndrome type 2 severity. J. Clin. Invest. 128 (3), 1043−1056.

Chavali, N.V., Kryshtal, D.O., Parikh, S.S., et al., 2019. Patient-independent human induced pluripotent stem cell model: a new tool for rapid determination of genetic variant pathogenicity in long QT syndrome. Heart Rhythm 16, 1686−1695.

Cheng, J., Ma, X., Zhang, J., et al., 2012. Diverse modulating effects of estradiol and progesterone on the monophasic action potential duration in Langendorff-perfused female rabbit hearts. Fundam. Clin. Pharmacol. 26, 219−226.

Clauss, S., Bleyer, C., Schüttler, D., et al., 2019. Animal models of arrhythmia: classic electrophysiology to genetically modified large animals. Nat. Rev. Cardiol. 16, 457−475.

Colatsky, T., Fermini, B., Gintant, G., et al., 2016. The comprehensive in vitro proarrhythmia assay (CiPA) initiative — update on progress. J. Pharmacol. Toxicol. Methods 81, 15−20.

Correia, C., Koshkin, A., Duarte, P., et al., 2018. 3D aggregate culture improves metabolic maturation of human pluripotent stem cell derived cardiomyocytes. Biotechnol. Bioeng. 115, 630−644.

Cyganek, L., Tiburcy, M., Sekeres, K., et al., 2018. Deep phenotyping of human induced pluripotent stem cell−derived atrial and ventricular cardiomyocytes. JCI Insight 3, 99941.

Davis, R.P., Casini, S., van den Berg, C.W., et al., 2012. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. Circulation 125, 3079−3091.

de la Roche, J., Angsutararux, P., Kempf, H., et al., 2019. Comparing human iPSC-cardiomyocytes versus HEK293T cells unveils disease-causing effects of Brugada mutation A735V of Na V 1.5 sodium channels. Sci. Rep. 9, 1−14.

Devalla, H.D., Gélinas, R., Aburawi, E.H., et al., 2016. TECRL, a new life-threatening inherited arrhythmia gene associated with overlapping clinical features of both LQTS and CPVT. EMBO Mol. Med. 8, 1390−1408.

Di Pasquale, E., Lodola, F., Miragoli, M., et al., 2013. CaMKII inhibition rectifies arrhythmic phenotype in a patient-specific model of catecholaminergic polymorphic ventricular tachycardia. Cell Death Dis. 4, e843.

El-Battrawy, I., Besler, J., Liebe, V., et al., 2018a. Long-term follow-up of patients with short QT syndrome: clinical profile and outcome. J Am Heart Assoc 7, e010073.

El-Battrawy, I., Zhao, Z., Lan, H., et al., 2018b. Estradiol protection against toxic effects of catecholamine on electrical properties in human-induced pluripotent stem cell derived cardiomyocytes. Int. J. Cardiol. 254, 195−202.

Egashira, T., Yuasa, S., Suzuki, T., et al., 2012. Disease characterization using LQTS-specific induced pluripotent stem cells. Cardiovasc. Res. 95, 419−429.

El-Battrawy, I., Albers, S., Cyganek, L., et al., 2019a. A cellular model of Brugada syndrome with SCN10A variants using human-induced pluripotent stem cell-derived cardiomyocytes. Europace 21, 1410−1421.

El-Battrawy, I., Müller, J., Zhao, Z., et al., 2019b. Studying Brugada syndrome with an SCN1B variants in human-induced pluripotent stem cell-derived cardiomyocytes. Front. Cell Dev. Biol. 7, 261.

Enriquez, A., Antzelevitch, C., Bismah, V., et al., 2016. Atrial fibrillation in inherited cardiac channelopathies: from mechanisms to management. Heart Rhythm 13, 1878−1884.

Es-Salah-Lamoureux, Z., Jouni, M., Malak, O.A., et al., 2016. HIV-Tat induces a decrease in I_{Kr} and I_{Ks} via reduction in phosphatidylinositol-(4,5)-bisphosphate availability. J. Mol. Cell. Cardiol. 99, 1−13.

Fatima, A., Xu, G., Shao, K., et al., 2011. In vitro Modeling of Ryanodine Receptor 2 Dysfunction Using Human Induced Pluripotent Stem Cells. Cell Physiol. Biochem. 28 (4), 579−592.

Fatima, A., Kaifeng, S., Dittmann, S., et al., 2013. The Disease-Specific Phenotype in Cardiomyocytes Derived from Induced Pluripotent Stem Cells of Two Long QT Syndrome Type 3 Patients. PLOS One 8 (12), e83005.

Gaaloul, I., Riabi, S., Evans, M., et al., 2016. Postmortem diagnosis of infectious heart diseases: a mystifying cause of Sudden Infant Death. Forensic Sci. Int. 262, 166−172.

Gaborit, N., Varro, A., Le Bouter, S., et al., 2010. Gender-related differences in ion-channel and transporter subunit expression in non-diseased human hearts. J. Mol. Cell. Cardiol. 49, 639−646.

Gan-Xin, Y., Wu, Y., Liu, T., et al., 2001. Phase 2 early afterdepolarization as a trigger of polymorphic ventricular tachycardia in acquired long-QT syndrome. Circulation 103, 2851−2856.

Garg, P., Oikonomopoulos, A., Chen, H., et al., 2018. Genome editing and induced pluripotent stem cells in cardiac channelopathy. J. Am. Coll. Cardiol. 72, 62−75.

Gary, G., Paul, B., Lior, G., et al., 2019. Use of human induced pluripotent stem cell−derived cardiomyocytes in preclinical cancer drug cardiotoxicity testing: a scientific statement from the American heart association. Circ. Res. 25, 125:e75-92.

Goversen, B., van der Heyden, M.A.G., van Veen, T.A.B., et al., 2018. The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: special focus on I_{k1}. Pharmacol. Ther. 183, 127−136.

Guo, F., Sun, Y., Wang, X., et al., 2019. Patient-specific and gene-corrected induced pluripotent stem cell-derived cardiomyocytes elucidate single-cell phenotype of short QT syndrome. Circ. Res. 124, 66−78.

Gussak, I., Brugada, P., Brugada, J., et al., 2000. Idiopathic short QT interval: a new clinical syndrome? Cardiology 94, 99−102.

Hanf, A., Oelze, M., Manea, A., et al., 2019. The anti-cancer drug doxorubicin induces substantial epigenetic changes in cultured cardiomyocytes. Chem. Biol. Interact. 313, 108834.

Haron-Khun, S., Weisbrod, D., Bueno, H., et al., 2017. SK4 K+ channels are therapeutic targets for the treatment of cardiac arrhythmias. EMBO Mol. Med. 9, 415−429.

Hayashi, M., Isabelle, D., Fabrice, E., et al., 2009. Incidence and risk factors of arrhythmic events in catecholaminergic polymorphic ventricular tachycardia. Circulation 119, 2426−2434.

Hwang, H.S., Kryshtal, D.O., Feaster, T.K., et al., 2015. Comparable calcium handling of human iPSC-derived cardiomyocytes generated by multiple laboratories. J. Mol. Cell. Cardiol. 85, 79−88.

Ibrahim, E.-B., Lan, H., Lukas, C., et al., 2018. Modeling short QT syndrome using human-induced pluripotent stem cell−derived cardiomyocytes. J. Am. Heart Assoc. 7, e007394.

Ingles, J., Macciocca, I., Morales, A., et al., 2019. Genetic testing in inherited heart diseases. Heart Lung Circ. 29, 505−511.

Ionta, V., Liang, W., Kim, E.H., et al., 2015. SHOX2 overexpression favors differentiation of embryonic stem cells into cardiac pacemaker cells, improving biological pacing ability. Stem Cell Rep. 4, 129−142.

Isbister, J., Semsarian, C., 2019. Sudden cardiac death: an update. Intern. Med. J. 49, 826−833.

Itzhaki, I., Maizels, L., Huber, I., et al., 2011. Modelling the long QT syndrome with induced pluripotent stem cells. Nature 471 (7337), 225−229.

Itzhaki, I., Maizels, L., Huber, I., et al., 2012. Modeling of catecholaminergic polymorphic ventricular tachycardia with patient-specific human-induced pluripotent stem cells. J. Am. Coll. Cardiol. 60, 990-1000.

Jost, N., Virág, L., Bitay, M., et al., 2005. Restricting excessive cardiac action potential and QT prolongation: a vital role for IKs in human ventricular muscle. Circulation 112, 1392−1399.

Jung, C.B., Moretti, A., Mederos y Schnitzler, M., et al., 2011. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. EMBO 4 (3), 180−191.

Jung, C.B., Moretti, A., Mederos, y, Schnitzler, M., et al., 2012. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. EMBO Mol. Med. 4, 180−191.

Kalstø, S.M., Siland, J.E., Rienstra, M., et al., 2019. Atrial fibrillation genetics update: toward clinical implementation. Front. Cardiovasc. Med. 6, 127.

Kane, C., Terracciano, C.M.N., 2017. Concise review: criteria for chamber-specific categorization of human cardiac myocytes derived from pluripotent stem cells. Stem Cells 35, 1881−1897.

Kawatou, M., Masumoto, H., Fukushima, H., et al., 2017. Modelling Torsade de Pointes arrhythmias in vitro in 3D human iPS cell-engineered heart tissue. Nat. Commun. 8, 1−11.

Keating, M.T., Sanguinetti, M.C., 2001. Molecular and cellular mechanisms of cardiac arrhythmias. Cell 104, 569−580.

Kehat, I., Kenyagin-Karsenti, D., Snir, M., et al., 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J. Clin. Invest. 108, 407−414.

Kiviaho, A.L., Ahola, A., Larsson, K., et al., 2015. Distinct electrophysiological and mechanical beating phenotypes of long QT syndrome type 1-specific cardiomyocytes carrying different mutations. Int. J. Cardiol. Heart Vasc. 8, 19−31.

Knowles, D.A., Burrows, C.K., Blischak, J.D., et al., 2018. Determining the genetic basis of anthracycline-cardiotoxicity by molecular response QTL mapping in induced cardiomyocytes. eLife 7, e33480.

Kocheril, A.G., Bokhari, S.A., Batsford, W.P., et al., 1997. Long QTc and torsades de pointes in human immunodeficiency virus disease. Pacing Clin. Electrophysiol. 20, 2810−2816.

Kodama, M., Furutani, K., Kimura, R., et al., 2019. Systematic expression analysis of genes related to generation of action potentials in human iPS cell-derived cardiomyocytes. J. Pharmacol. Sci. 140, 325−330.

Kofune, M., Watanabe, I., Ohkubo, K., et al., 2011. Clarifying the arrhythmogenic substrate for Brugada syndrome. Int. Heart J. 52, 290−294.

Koivumäki, J.T., Naumenko, N., Tuomainen, T., et al., 2018. Structural immaturity of human iPSC-derived cardiomyocytes: in silico investigation of effects on function and disease modeling. Front. Physiol. 9, 80.

Kostakou, P.M., Kouris, N.T., Kostopoulos, V.S., et al., 2019. Cardio-oncology: a new and developing sector of research and therapy in the field of cardiology. Heart Fail. Rev. 24, 91−100.

Kroncke, B., Yang, T., Roden, D.M., et al., 2019. Multiple mechanisms underlie increased cardiac late sodium current. Heart Rhythm 16 (7), 1091−1097.

Kujala, K., Paavola, J., Lahti, A., et al., 2012. Cell model of catecholaminergic polymorphic ventricular tachycardia reveals early and delayed afterdepolarizations. PloS One 7, e44660.

Kuroda, Y., Yuasa, S., Watanabe, Y., et al., 2017. Flecainide ameliorates arrhythmogenicity through NCX flux in Andersen-Tawil syndrome-iPS cell-derived cardiomyocytes. Bioch. Biophys. Rep. 9, 245−256.

Kuusela, J., Kujala, V.J., Kujala, A., et al., 2016. Effects of cardioactive drugs on human induced pluripotent stem cell derived long QT syndrome cardiomyocytes. Springerplus 5 (1), 234.

Kuusela, J., Larsson, K., Shah, D., et al., 2017. Low extracellular potassium prolongs repolarization and evokes early afterdepolarization in human induced pluripotent stem cell-derived cardiomyocytes. Biol. Open. 6 (6), 777−784.

Lahti, A., Kujala, V.J., Chapman, H., et al., 2012. Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. Dis. Model Mech. 5 (2), 220−230.

Lee, J.H., Protze, S.I., Laksman, Z., et al., 2017. Human pluripotent stem cell-derived atrial and ventricular cardiomyocytes develop from distinct mesoderm populations. Cell Stem Cell 21, 179−194.e4.

Lemoine, M.D., Mannhardt, I., Breckwoldt, K., et al., 2017. Human iPSC-derived cardiomyocytes cultured in 3D engineered heart tissue show physiological upstroke velocity and sodium current density. Sci. Rep. 7, 1−11.

Li, M., Kanda, Y., Ashihara, T., et al., 2017. Overexpression of KCNJ2 in induced pluripotent stem cell-derived cardiomyocytes for the assessment of QT-prolonging drugs. J. Pharmacol. Sci. 134 (2), 75−85.

Liang, P., Sallam, K., Wu, H., et al., 2016. Patient-specific and genome-edited induced pluripotent stem cell-derived cardiomyocytes elucidate single cell phenotype of Brugada Syndrome. J. Am. Coll. Cardiol. 68, 2086−2096.

Limpitikul, W., Dick, I.E., Tester, D.J., et al., 2017. A Precision Medicine Approach to the Rescue of Function on Malignant Calmodulinopathic Long-QT Syndrome. Circ. Res. 120 (1), 39−48.

Lodola, F., Morone, D., Denegri, M., et al., 2016. Adeno-associated virus-mediated CASQ2 delivery rescues phenotypic alterations in a patient-specific model of recessive catecholaminergic polymorphic ventricular tachycardia. Cell Death Dis. 7, e2393.

Ma, D., Wei, H., Zhao, Y., et al., 2013. Modeling type 3 long QT syndrome with cardiomyocytes derived from patient-specific induced pluripotent stem cells. Int. J. Cardiol. 168 (6), 5277−5286.

Ma, D., Wei, H., Lu, J., et al., 2015. Characterization of a novel KCNQ1 mutation for type 1 long QT syndrome and assessment of the therapeutic potential of a novel IKs activator using patient-specific induced pluripotent stem cell-derived cardiomyocytes. Stem Cell Res. Ther. 6, 39.

Ma, D., Liu, Z., Loh, L.J., et al., 2018. Identification of an I_{Na} -dependent and I_{to} -mediated proarrhythmic mechanism in cardiomyocytes derived from pluripotent stem cells of a Brugada syndrome patient. Sci. Rep. 8, 11246.

Maizels, L., Huber, I., Arbel, G., et al., 2017. Patient-Specific Drug Screening Using a Human Induced Pluripotent Stem Cell Model of Catecholaminergic Polymorphic Ventricular Tachycardia Type 2. Circ. Arrhythm. Electrophysiol. 10 (6), e004725.

Makielski Jonathan, C., Ye, B., Valdivia Carmen, R., et al., 2003. A ubiquitous splice variant and a common polymorphism affect heterologous expression of recombinant human SCN5A heart sodium channels. Circ. Res. 93, 821−828.

Malan, D., Zhang, M., Stallmeyer, B., et al., 2016. Human iPS cell model of type 3 long QT syndrome recapitulates drug-based phenotype correction. Basic Res. Cardiol. 111 (2), 14.

Matsa, E., Rajamohan, D., Dick, E., et al., 2011. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. Eur. Heart J. 32, 952–962.

Mehta, A., Sequiera, G.L., Ramachandra, C., et al., 2014. Re-trafficking of hERG reverses long QT syndrome 2 phenotype in human iPS-derived cardiomyocytes. Cardiovasc. Res. 102 (3), 497–506.

Mehta, A., Ramachandra, C.J.A., Singh, P., et al., 2018. Identification of a targeted and testable antiarrhythmic therapy for long-QT syndrome type 2 using a patient-specific cellular model. Eur. Heart J. 39, 1446–1455.

Miller, D.C., Harmer, S.C., Poliandri, A., et al., 2017. Ajmaline blocks INa and IKr without eliciting differences between Brugada syndrome patient and control human pluripotent stem cell-derived cardiac clusters. Stem Cell Res. 25, 233–244.

Miyazaki, T., Mitamura, H., Miyoshi, S., et al., 1996. Autonomic and antiarrhythmic drug modulation of ST segment elevation in patients with Brugada syndrome. J. Am. Coll. Cardiol. 27, 1061–1070.

Moreno, T., Pérez, I., Isasti, G., et al., 2013. Prevalence and factors associated with a prolonged QTc interval in a cohort of asymptomatic HIV-infected patients. AIDS Res. Hum. Retrovir. 29, 1195–1198.

Moretti, A., Bellin, M., Welling, A., et al., 2010. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N. Engl. J. Med. 363, 1397–1409.

Mura, M., Mehta, A., Ramachandra, C.J., et al., 2017. The KCNH2-IVS9-28A/G mutation causes aberrant isoform expression and hERG trafficking defect in cardiomyocytes derived from patients affected by Long QT Syndrome type 2. Int. J. Cardiol. 240, 367–371.

Napolitano, C., Priori, S.G., Bloise, R., 2020. Catecholaminergic polymorphic ventricular tachycardia. GeneReviews.

Neira, V., Enriquez, A., Simpson, C., et al., 2019. Update on long QT syndrome. J. Cardiovasc. Electrophysiol. 30, 3068–3078.

Nguyen, N., Nguyen, W., Nguyenton, B., et al., 2017. Adult human primary cardiomyocyte-based model for the simultaneous prediction of drug-induced inotropic and pro-arrhythmia risk. Front. Physiol. 8, 1073.

Novak, A., Barad, L., Zeevi-Levin, N., et al., 2012. Cardiomyocytes generated from $CPVT^{D307H}$ patients are arrhythmogenic in response to β-adrenergic stimulation. J. Cell Mol. Med. 16, 468–482.

Novak, A., Barad, L., Lorber, A., et al., 2015. Functional abnormalities in iPSC-derived cardiomyocytes generated from CPVT1 and CPVT2 patients carrying ryanodine or calsequestrin mutations. J. Cell Mol. Med. 19, 2006–2018.

Odening, K.E., Choi, B.-R., Liu, G.X., et al., 2012. Estradiol promotes sudden cardiac death in transgenic long QT type 2 rabbits while progesterone is protective. Heart Rhythm 9, 823–832.

Okata, S., Yuasa, S., Suzuki, T., et al., 2016. Embryonic type Na^+ channel β-subunit, *SCN3B* masks the disease phenotype of Brugada syndrome. Sci. Rep. 6, 34198.

Onakpoya, I.J., Heneghan, C.J., Aronson, J.K., 2016. Post-marketing withdrawal of 462 medicinal products because of adverse drug reactions: a systematic review of the world literature. BMC Med. 14, 10.

Paavola, J., Väänänen, H., Larsson, K., et al., 2016. Slowed depolarization and irregular repolarization in catecholaminergic polymorphic ventricular tachycardia: a study from cellular Ca^{2+} transients and action potentials to clinical monophasic action potentials and electrocardiography. Europace 18, 1599–1607.

Park, S.-J., Zhang, D., Qi, Y., et al., 2019. Insights into the pathogenesis of catecholaminergic polymorphic ventricular tachycardia from engineered human heart tissue. Circulation 140, 390−404.

Patel, C., Yan, G.-X., Antzelevitch, C., 2010. Short QT syndrome: from bench to bedside. Circ. Arrhythm. Electrophysiol. 3, 401−408.

Penttinen, K., Swan, H., Vanninen, S., et al., 2015. Antiarrhythmic effects of dantrolene in patients with catecholaminergic polymorphic ventricular tachycardia and replication of the responses using iPSC models. PloS One 10, e0125366.

Pérez-Hernández, M., Matamoros, M., Alfayate, S., et al., 2018. Brugada syndrome trafficking-defective Nav1.5 channels can trap cardiac Kir2.1/2.2 channels. JCI Insight 3, e96291.

Pflaumer, A., Wilde, A.A.M., Charafeddine, F., et al., 2019. 50 years of catecholaminergic polymorphic ventricular tachycardia (CPVT) − time to explore the dark side of the moon. Heart Lung Circ. 29, 520−528.

Pipilas, D.C., Johnson, C.N., Webster, G., et al., 2016. Novel calmodulin mutations associated with congenital long QT syndrome affect calcium current in human cardiomyocytes. Heart Rhythm 13 (10), 2012−2019.

Pölönen, R.P., Penttinen, K., Swan, H., et al., 2018. Antiarrhythmic effects of carvedilol and flecainide in cardiomyocytes derived from catecholaminergic polymorphic ventricular tachycardia patients. Stem Cell. Int. 2018, 9109503.

Pölönen, R.P., Swan, H., Aalto-Setälä, K., 2020. Mutation-specific differences in arrhythmias and drug responses in CPVT patients: simultaneous patch clamp and video imaging of iPSC derived cardiomyocytes. Mol. Biol. Rep. 47, 1067−1077.

Preininger, M.K., Jha, R., Maxwell, J.T., et al., 2016. A human pluripotent stem cell model of catecholaminergic polymorphic ventricular tachycardia recapitulates patient-specific drug responses. Dis. Model Mech. 9, 927−939.

Rocchetti, M., Sala, L., Dreizehnter, L., et al., 2017. Elucidating arrhythmogenic mechanisms of long-QT syndrome CALM1-F142L mutation in patient-specific induced pluripotent stem cell-derived cardiomyocytes. Cardiovasc. Res. 113 (5), 531−541.

Romero, D., Behar, N., Mabo, P., et al., 2019. Differences in Brugada syndrome patients through ventricular repolarization analysis during sleep. Conf. Proc. IEEE Eng. Med. Biol. Soc. 5638−5641.

Sakamoto, K., Sakatoku, K., Sugimoto, S., et al., 2019. Continued exposure of anti-cancer drugs to human iPS cell-derived cardiomyocytes can unmask their cardiotoxic effects. J. Pharmacol. Sci. 140 (4), 345−349.

Sala, L., Yu, Z., Oostwaard, D.W., et al., 2019. A new hERG allosteric modulator rescues genetic and drug-induced long-QT syndrome phenotypes in cardiomyocytes from isogenic pairs of patient induced pluripotent stem cells. EMBO 8 (9), 1065−1081.

Salem, J.-E., Alexandre, J., Bachelot, A., et al., 2016. Influence of steroid hormones on ventricular repolarization. Pharmacol. Ther. 167, 38−47.

Salem, J.-E., Yang, T., Moslehi, J.J., et al., 2019. Androgenic effects on ventricular repolarization: a translational study from the international pharmacovigilance database to iPSC-cardiomyocytes. Circulation 140, 1070−1080.

Sarquella-Brugada, G., Campuzano, O., Arbelo, E., et al., 2016. Brugada syndrome: clinical and genetic findings. Genet. Med. 18, 3−12.

Sasaki, K., Makiyama, T., Yoshida, Y., et al., 2016. Patient-specific human induced pluripotent stem cell model assessed with electrical pacing validates S107 as a potential therapeutic agent for catecholaminergic polymorphic ventricular tachycardia. PloS One 11, e0164795.

Schwach, V., Verkerk, A.O., Mol, M., et al., 2017. A COUP-tfii human embryonic stem cell reporter line to identify and select atrial cardiomyocytes. Stem Cell Rep. 9, 1765−1779.

Scuderi, G.J., Butcher, J., 2017. Naturally engineered maturation of cardiomyocytes. Front. Cell Dev. Biol. 5, 50.

Selga, E., Sendfeld, F., Martinez-Moreno, R., et al., 2018. Sodium channel current loss of function in induced pluripotent stem cell-derived cardiomyocytes from a Brugada syndrome patient. J. Mol. Cell. Cardiol. 114, 10−19.

Sendfeld, F., Selga, E., Scornik, F.S., et al., 2019. Experimental models of Brugada syndrome. Int. J. Mol. Sci. 20, 2123.

Sharma, A., Caleb, M., Ryoko, H., et al., 2014. Human induced pluripotent stem cell−derived cardiomyocytes as an in vitro model for coxsackievirus B3−induced myocarditis and antiviral drug screening platform. Circ. Res. 115, 556−566.

Shinnawi, R., Shaheen, N., Huber, I., et al., 2019. Modeling reentry in the short QT syndrome with human-induced pluripotent stem cell−derived cardiac cell sheets. J. Am. Coll. Cardiol. 73, 2310−2324.

Sieira, J., Brugada, P., 2016. Management of Brugada syndrome 2016: should all high risk patients receive an ICD? All high-risk patients should receive an implantable cardiac defibrillator. Circ. Arrhythm. Electrophysiol. 9, e004195.

Sogo, T., Morikawa, K., Kurata, Y., et al., 2016. Regen. Ther. 4, 9−17.

Song, W., Shou, W., 2012. Cardiac sodium channel Nav1.5 mutations and cardiac arrhythmia. Pediatr. Cardiol. 33, 943−949.

Spencer, C.I., Baba, S., Nakamura, K., et al., 2014. Calcium transients closely reflect prolonged action potentials in iPSC models of inherited cardiac arrhythmia. Stem Cell Rep. 3 (2), 269−281.

Stillitano, F., Hansen, J., Kong, C.-W., et al., 2017. Modeling susceptibility to drug-induced long QT with a panel of subject-specific induced pluripotent stem cells. eLife 6, e19406.

Takahashi, K., Tanabe, K., Ohnuki, M., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861−872.

Takaki, T., Inagaki, A., Chonabayashi, K., et al., 2019. Erratum to "Optical Recording of Action Potentials in Human Induced Pluripotent Stem Cell-Derived Cardiac Single Cells and Monolayers Generated from Long QT Syndrome Type 1 Patients". Stem Cell Int. 2020, 8765895.

Tanabe, S., Hata, T., Hiraoka, M., 1999. Effects of estrogen on action potential and membrane currents in Guinea pig ventricular myocytes. Am. J. Physiol. 277, H826−H833.

Tse, G., Yeo, J.M., Chan, Y.W., et al., 2016. What is the arrhythmic substrate in viral myocarditis? Insights from clinical and animal studies. Front. Physiol. 7, 308.

Tseng, Z.H., Secemsky, E.A., Dowdy, D., et al., 2012. Sudden cardiac death in patients with human immunodeficiency virus infection. J. Am. Coll. Cardiol. 59, 1891−1896.

Uosaki, H., Cahan, P., Lee, D.I., et al., 2015. Transcriptional landscape of cardiomyocyte maturation. Cell Rep. 13, 1705−1716.

Vaidyanathan, R., Markandeya, Y.S., Kamp, T.J., et al., 2016. I_{k1}-enhanced human-induced pluripotent stem cell-derived cardiomyocytes: an improved cardiomyocyte model to investigate inherited arrhythmia syndromes. Am. J. Physiol. Heart Circ. Physiol. 310, H1611−H1621.

Veerman, C.C., Mengarelli, I., Guan, K., et al., 2016. hiPSC-derived cardiomyocytes from Brugada Syndrome patients without identified mutations do not exhibit clear cellular electrophysiological abnormalities. Sci. Rep. 6, 30967.

Vermersch, E., Jouve, C., Hulot, J.-S., 2020. CRISPR/CAS9 gene-editing strategies in cardiovascular cells. Cardiovasc. Res. 116, 894−907.

Vicente, J., Johannesen, L., Galeotti, L., et al., 2014. Mechanisms of sex and age differences in ventricular repolarization in humans. Am. Heart J. 168, 749−756.e3.

Wei, H., Zhang, X., Clift, C., et al., 2018. CRISPR/Cas9 Gene editing of RyR2 in human stem cell-derived cardiomyocytes provides a novel approach in investigating dysfunctional Ca2+ signaling. Cell Calcium 73, 104−111.

Wu, Y., Ai, M., Bardeesi, A.S.A., et al., 2017. Brugada syndrome: a fatal disease with complex genetic etiologies − still a long way to go. Forensic Sci. Res. 2, 115−125.

Wu Joseph, C., Garg, P., Yoshida, Y., et al., 2019. Towards precision medicine with human iPSCs for cardiac channelopathies. Circ. Res. 125, 653−658.

Yamamoto, Y., Makiyama, T., Harita, T., et al., 2017. Allele-specific ablation rescues electrophysiological abnormalities in a human iPS cell model of long-QT syndrome with a CALM2 mutation. Hum. Mol. Genet. 26 (9), 1670−1677.

Yazawa, M., Hsueh, B., Jia, X., et al., 2011. Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. Nature 471 (7337), 230−234.

Yoshinaga, D., Baba, S., Makiyama, T., et al., 2019. Phenotype-based high-throughput classification of long QT syndrome subtypes using human induced pluripotent stem cells. Stem Cell Rep. 13, 394−404.

Zhang, J., Wilson, G.F., Soerens, A.G., et al., 2009. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ. Res. 104, e30−41.

Zhang, Q., Jiang, J., Han, P., et al., 2011. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. Cell Res. 21, 579−587.

Zhang, D., Shadrin, I., Lam, J., et al., 2013. Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. Biomaterials 34, 5813−5820.

Zhao, L., Zhang, B., 2017. Doxorubicin induces cardiotoxicity through upregulation of death receptors mediated apoptosis in cardiomyocytes. Sci. Rep. 7 (1), 44735.

Zhao, Z., Li, X., El-Battrawy, I., Lan, H., Zhong, R., et al., 2019. Drug testing in human-induced pluripotent stem cell−derived cardiomyocytes from a patient with short QT syndrome type 1. Clin. Pharmacol. Ther. 106, 642−651.

Zhou, Y., Wang, L., Liu, Z., et al., 2017. Comparative gene expression analyses reveal distinct molecular signature between differentially reprogrammed cardiomyocytes. Cell Rep. 20, 3014−3024.

Zulkifly, H., Lip, G.Y.H., Lane, D.A., 2018. Epidemiology of atrial fibrillation. Int. J. Clin. Pract. 72, e13070.

This page intentionally left blank

CHAPTER

11 Modeling heritable kidney disease using human kidney iPSC-derived organoids

Thomas A. Forbes[1,2,3], **Melissa H. Little**[1,2,4]

[1]*Murdoch Children's Research Institute, Parkville, VIC, Australia;* [2]*Department of Paediatrics, The University of Melbourne, Parkville, VIC, Australia;* [3]*Department of Nephrology, Royal Children's Hospital, Parkville, VIC, Australia;* [4]*Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, VIC, Australia*

Chapter outline

Abstract

Progressive chronic kidney disease in both children and adults can result from genetic disorders, often requiring dialysis and/or kidney transplantation for the patient. For most of these diseases, no effective, targeted therapies are in clinical use in part due to an incomplete molecular understanding of disease pathobiology. Next generation, high-fidelity genomic sequencing has increased the detection of variants of unknown significance in known and novel genes for patients with genetic kidney disease. These variants require validation, which has traditionally involved studying gene function in animal models and/or cell culture. Kidney organoids represent in vitro models of human kidney tissue generated from pluripotent stem cells, including induced pluripotent stem cells derived directly from patients. Unlike any previous approach, this enables the three-dimensional modeling of the affected organ including the effects of patient's own genetic

Current Progress in iPSC Disease Modeling, Volume 14. https://doi.org/10.1016/B978-0-323-85765-9.00003-5

background. It also has the potential to personalize therapeutic screening providing increased translational relevance to clinical care. This chapter appraises kidney organoids as models of genetic kidney disease. We review existing applications of kidney organoids to disease modeling and illustrate the capabilities and limitations of this novel technology. We also highlight what is still required to develop clinically relevant outcomes for kidney disease using kidney organoids.

Keywords: Cell culture; Ciliopathies; Differentiation; Disease modeling; Functional genomics; Genetics; Induced pluripotent stem cells; Kidney disease; Kidney organoid; Polycystic kidney disease; Stem cells; Steroid resistant nephrotic syndrome.

Introduction

Chronic kidney disease (CKD) refers to the gradual deterioration in kidney function due to a variety of inherited or acquired disease processes. Genetic kidney diseases contribute significantly to CKD burden, especially in children (Fletcher et al., 2013; Mallett et al., 2014). In fulminant (Class 5) CKD, dialysis and transplantation may extend patient survival but with high healthcare expenditure, poor health outcomes, and quality of life (AIHW. Projections, 2014; Cass et al., 2010). For genetic renal disease in particular, an incomplete understanding of disease pathobiology has led to a paucity of disease-modifying treatments. Clinical translation of research into genetic renal disease has been hampered by lack of in vitro, human, renal models. Kidney organoids, differentiated from induced pluripotent stem cells (iPSCs), are small, self-organizing, in vitro, miniature kidney tissues derived from stem cells, which resemble the cell types and structure of the in vivo kidney (Little and Combes, 2019; Takasato et al., 2015). While great excitement surrounds the future potential for organoid technology to culture organs for renal replacement therapy, disease modeling for novel gene and drug discovery is more immediately realizable. In this approach, primary cells from an individual patient can be reprogrammed into iPSC, differentiated to kidney and tested against multiple candidate treatments in an effort to determine the most appropriate individualized therapy. Kidney organoids overcome the limitations posed by existing cellular and animal disease modeling platforms, offering an in vitro, three-dimensional (3D), human kidney tissue which can be regenerated from any patient's primary cells. However, as immature, avascular and somewhat variable microtissues, the study of kidney organoids brings with it a new set of challenges.

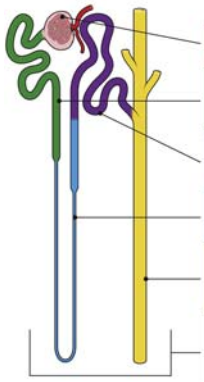
Kidney organoids are accurate models of the developing kidney

The human kidney is a remarkably complex organ containing over 25 different cell types arranged into between 200,000 and 1.8 million functional units called nephrons (Hoy et al., 2003; Hughson et al., 2003). Each nephron filters the blood in

the glomerulus and processes this filtrate within the renal tubule which is subdivided into multiple segments with specified functions determined by transporter expression (Fig. 11.1). The appropriate development, anatomical distribution, and biological function of cells within these nephrons is essential for the meticulous regulation of serum biochemistry, acid-base balance, total body water content, excretion of nitrogenous waste, and numerous hormonal responses.

Organoids are small, stem-cell derived, self-organizing, in vitro microtissues composed of similar cell types in a tissue architecture reminiscent of the in vivo organ (Lancaster and Knoblich, 2014). Protocols for the differentiation of many human organoids from stem cells have been characterized including brain (10), heart (11), liver (12), stomach and intestine (13, 14). While the first differentiation protocols used embryonic stem cells, reprogrammed iPSCs (Takahashi et al., 2007) are more readily available and make possible the regeneration of organoids from patients with inherited diseases.

The common principle of these protocols has been to accurately reproduce the signaling cues that would be experienced by a cell during the embryological development of the in vivo organ, much of which has been learned from the study of animals. The mammalian kidney is formed from the interaction of two distinct embryological structures, the metanephric mesenchyme (MM) and the ureteric bud (UB). Both derive from the intermediate mesoderm which arises from the



Segment	Disease Phenotype	Examples of Segment Specific Diseases	Published Organoid Models
Glomerulus	Proteinuria Vairiable reduction in GFR	SRNS (*NPHS1, NPHS2*) Alport Syndrome (*COL4A5*)	(Freedman et al., 2015) (Hale et al., 2018) (Tanigawa et al., 2018)
Proximal Convoluted Tubule	Specific or global loss of electrolytes, glucose, amino acids in the urine	Specific: Renal glycosuria (*SLC5A2*) Renal Fanconi Syndrome: Nephropathic cystoinuria (*CTNS*)	(Przepiorski et al., 2018) (Hollywood et al., 2019)
Distal Convoluted Tubule	Specific retention or urinary wasting of specific electolytes ADTKD – interstitial fibrosis and tubular atrophy (IFTA)	Gitelman Syndrome (*SLC12A3*) ADTKD (*MUC1, UMOD, REN*)	(Dvela-Levitt et al., 2019)
Loop of Henle	Hyponatraemia, hypokalaemia, hypochloraemia, hypomagnesaemia, nephrocalcinosis	Bartter Syndrome (*SLC12A1*)	n/a
Collecting Duct	Specific retention or urinary wasting of specific electrolytes	Nephrogenic Diabetes Insipidus (*AVP2R, AQP2*) Liddle Syndrome (*SCN1B*) Distal renal tubular acidosis (*ATPVV0A4*)	n/a
Pantubular	Tubulointerstital fibrosis PKD – overwhelming polycystic change NPHP-RC – variable cyst development Progressive reduction in GFR	ADPKD (*PKD1, PKD2*) ARPKD (*PKHD1*) NPHP-RC (*NPHS1, NPHS2*)	(Forbes et al., 2018) (Cruz et al., 2017) (Czerniecki et al., 2018) (Low et al., 2019)

FIGURE 11.1

Schematic of a human nephron depicting glomerulus, proximal tubule, loop of Henle, distal tubule, and collecting duct. The accompanying table describes the variety of disease phenotypes associated with each segment, examples of segment-specific diseases with their associated genes and published examples of functional genomic research featuring organoids. (*AD/ARPKD*, autosomal dominant/recessive polycystic kidney disease; *ADTKD*, autosomal dominant tubulointerstitial kidney disease; *GFR*, glomerular filtration rate; *NPHP-RC*, nephronophthisis and related ciliopathies; *SRNS*, teroid resistant nephrotic syndrome).

posterior primitive streak following gastrulation (Little and McMahon, 2012). A number of published protocols detail the directed differentiation of kidney organoids, all following the principle of recapitulating the developmental signaling cues seen during in vivo kidney development to arrive at very similar renal end points (Takasato et al., 2014, 2015; Morizane et al., 2015; Taguchi et al., 2014; Freedman et al., 2015). These protocols are discussed in detail in previous reviews (Little and Combes, 2019; Little et al., 2017). As an example, our own protocol is presented in Fig. 11.2A. In this protocol, Wnt agonist CHIR99021 mimics in vivo conditions to pattern to $MIXL1^{+}/T^{+}$ posterior primitive streak. At this point, FGF9 is substituted for CHIR99021 to pattern to a balanced intermediate mesoderm ($LHX1^{+}/PAX2^{+}$). Cells are dissociated and centrifuged to aggregates which are transferred to a transwell air-liquid interface. Cultured over FGF9 for a further five days before withdrawal of all growth factors, nephron structures spontaneously self-organize within these aggregates (Fig. 11.2A). Immunofluorescence and single cell RNA sequencing of organoids has established the presence of glomerular podocytes ($NPHS1^{+}$, $NPHS2^{+}$), proximal ($LTL^{+}/HNF4A^{+}$) and distal ($ECAD^{+}/GATA3^{+}$) tubules, vascular ($SOX17^{+}/CD31^{+}$) and stromal ($MEIS1^{+}$) components (Fig. 11.2B–G) alongside a variety of off-target cell types (Wu et al., 2018; Combes et al., 2019b; Subramanian et al., 2019). Importantly, a detailed transcriptional analysis of the robustness of the protocol has demonstrated a high reproducibility between differentiation experiments (Subramanian et al., 2019; Phipson et al., 2019).

Because of the cellular and architectural complexity of the kidney, genetic renal diseases encompass a varied spectrum of disease phenotypes depending on the cell type and nephron segment affected, as summarized in Fig. 11.1. In the below discussion, we bisect inherited kidney diseases into those affecting the glomerulus and those affecting the renal tubule.

Genetic diseases of the glomerulus

The most proximal segment of the nephron is the glomerulus, a collection of fenestrated capillaries wrapped in specialized visceral epithelial cells called *podocytes*. A trilayer glomerular filtration barrier (GFB) consists of the podocytes, glomerular endothelium, and a collagen-based glomerular basement membrane (GBM) formed between them. In the healthy state, the GFB permits the filtration of water, electrolytes, and small molecules (for example, glucose, urea, bicarbonate) but prevents the filtration of larger molecules (for example, proteins) by exerting both size and charge selectivity.

The most common inherited glomerular disease phenotype is proteinuria which in severe cases is called steroid resistant nephrotic syndrome (SRNS). SRNS is the second most common cause of progressive CKD in children (Fletcher et al., 2013; NAPRTCS, 2014). In their healthy state podocytes form intercellular foot processes, bridged by a network of proteins called the slit diaphragm (Noone et al., 2018;

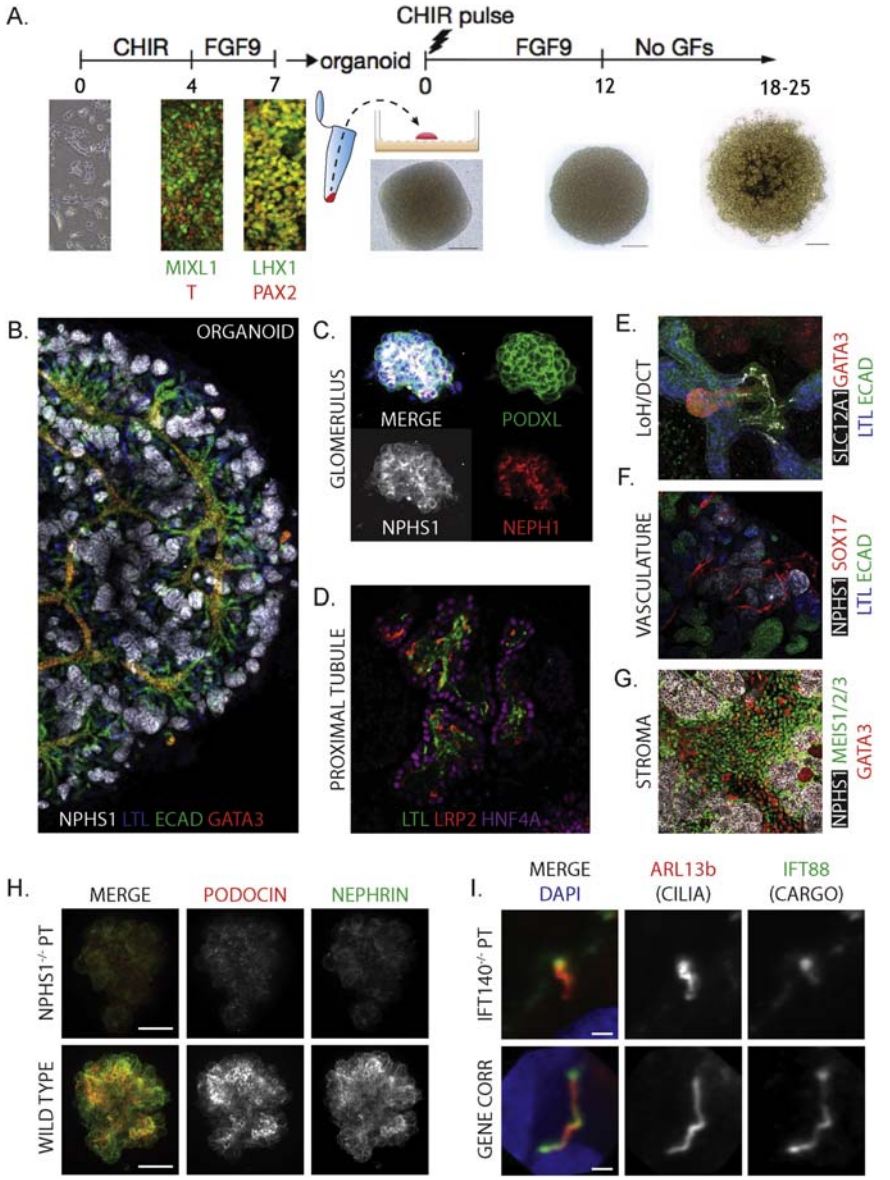


FIGURE 11.2

(A) Example of kidney organoid differentiation protocol. Induced pluripotent stem cells (iPSCs) are sequentially exposed to Wnt agonist CHIR99021 and FGF9 to pattern posterior primitive streak and intermediate mesoderm, respectively. Each individual iSPC clone requires optimization of the duration of each treatment. Cells are dissociated and reaggregated by centrifugation. Cell pellets are on cultured on transwells over FGF9 for a

Fissell and Miner, 2018). In SRNS, this structure is lost along with the size and charge selectivity of the GFB, allowing negatively charged, high molecular weight proteins to be filtered into the urine. The majority of gene mutations associated with SRNS has been linked to the regulation of the slit diaphragm and regulation of cytoskeletal dynamics within podocytes (Lovric et al., 2016).

Kidney organoids for the study of glomerular disease

A number of reports have examined the role of organoids in modeling inherited diseases of the glomerulus. The first examined organoids differentiated from *podocalyxin* (*PODXL*) knockout hPSC (Freedman, 2015). Glomeruli in *PODXL* knockout organoids demonstrated a diffuse and punctate immunofluorescent expression of SYNPO and ZO-1 compared to a more polarized, linear expression in control organoids (Freedman et al., 2015). This finding was restricted to the glomerular segment, consistent the association of *PODXL* variants with SRNS (Kang et al., 2017; Barua et al., 2014).

Two groups, including our own, have examined glomeruli within organoids differentiated from the iPSC of patients with congenital nephrotic syndrome due to variants in *NPHS1,* which encodes the critical slit diaphragm protein NEPHRIN

further five days before withdrawal of all growth factors. Structures are observed to self-organize within three–five days of aggregate culture. (Scale 500 μm) (B) Immunofluorescent image of organoid demonstrating contiguous expression of all four major nephron segments including glomeruli ($NPHS1^+$), proximal tubule (LTL^+), distal tubule ($ECAD^+/GATA3^-$), and putative collecting duct ($ECAD^+/GATA3^+$). (C) Glomeruli sieved from dissociated organoids express a variety of glomerular specific markers including NPHS1, NEPH1, and PODXL. (D) Proximal tubules within organoids express HNF4A, LRP2 (MEGALIN), and bind LTL. (E) Loop of Henle (LoH) and distal convoluted tubule (DCT) expresses SLC12A1 and ECAD. (F) SOX17 reported demonstrating vascular progenitors wrapping around $NPHS1^+$ glomeruli. (G) $MEIS1/2/3^+$ stroma between $NPHS1^+$ glomeruli. (H) Sieved glomeruli from organoids derived from iPSC of a patient with congenital nephrotic syndrome due to variants in *NPHS1* demonstrate reduced immunofluorescent expression of NPHS1/NEPHRIN and NPHS2/PODOCIN compared to control glomeruli. (I) Distal tubular cilia within tubules of organoids derived from iPSC of a patient with compound heterozygote *IFT140* demonstrate short, club-shaped cilia (ARL13B) with accumulation of IFT cargo (IFT88) at the tip. This phenotype is rescued to wild type by CRISPR-mediated correction of one of the variants.

(A–H), Adapted from Hale, L.J., Howden, S.E., Phipson, B., Lonsdale, A., Er, P.X., Ghobrial, I., et al., 2018. 3D organoid-derived human glomeruli for personalised podocyte disease modelling and drug screening. Nat. Commun. 9 (1), 5167. Scale 20 μm. Adapted from Forbes, T.A., Howden, S.E., Lawlor, K., Phipson, B., Maksimovic, J., Hale, L., et al., 2018. Patient-iPSC-derived kidney organoids show functional validation of a ciliopathic renal phenotype and reveal underlying pathogenetic mechanisms. Am. J. Hum. Genet. 102 (5),816–831. Scale 1 μm. Image credits: Jessica Vanslambrouck, Sean Wilson, Siebe Spijker, Lorna Hale, Shahnaz Khan.

(Hale et al., 2018; Tanigawa et al., 2018). Both studies demonstrated loss of NEPHRIN expression by immunofluorescence of patient glomeruli (Hale et al., 2018; Tanigawa et al., 2018). Our report used nonisogenic iPSC organoids as a control and also documented reduced immunofluorescent expression of PODOCIN and CD2AP in isolated glomeruli (Fig. 11.2I) (Hale et al., 2018). Tanigawa et al. (2018) utilized an isogenic, CRISPR gene-corrected control and demonstrated restoration of NEPHRIN expression and the widening of gaps (implying restoration of a slit diaphragm) between foot processes on transmission electron microscopy (EM) of organoids transplanted into mice.

Together, these reports substantiate a role for kidney organoids in modeling genetic glomerular disease. In this regard, a clear benefit of organoids as glomerular disease models lies in the ability to culture podocytes in three dimensions. In other cell types, 3D culture has increased cell survival and permitted physiological cell−cell and cell−ECM interactions more faithful to the in vivo circumstance (Pampaloni et al., 2007; Ghosh et al., 2005; Duval et al., 2017). Our own transcriptional analysis of glomeruli isolated from kidney organoids demonstrated increased expression of key podocyte genes in a 3D culture compared to conditionally immortalized podocytes or two-dimensional (2D) podocytes extruding from 3D organoid glomeruli (Hale et al., 2018). This work also demonstrated improved maturation of ECM within 3D glomeruli, with evidence of collagen and laminin switching (Hale et al., 2018).

The absence of a bona fide glomerular vasculature, however, remains a limitation of the glomerular disease model. While a vascular progenitor population is present in organoids (Fig. 11.2F) (Wu et al., 2018; Combes et al., 2019b; Subramanian et al., 2019), these cells rarely penetrate glomeruli sufficiently to allow the development of a mature GBM which requires a matrix contribution from both endothelial cells and podocytes in the in vivo context (Chew and Lennon, 2018). This currently impedes the modeling of Alport syndrome, which arises due to pathological variants in Type IV collagens critical to the GBM.

One well-demonstrated strategy of increasing the interaction between endothelium and podocytes within organoids is murine subrenal capsular transplantation (Tanigawa et al., 2018; Sharmin et al., 2016; van den Berg et al., 2018). Invading host vasculature matured organoid vessels which then penetrate podocyte clusters and establish a more representative GFB with improved foot process morphology (Tanigawa et al., 2018; van den Berg et al., 2018). Evidence of tubular flow also suggested active glomerular filtration which in turn improved tubular epithelial morphology (van den Berg et al., 2018). These improvements in morphology and maturity, however, currently come at the expense of key distinct advantages of in vitro organoid culture: throughput speed and scale. As a result, significant efforts to vascularize organoids in vitro are underway. In a specialized microfluidic culture device, kidney organoids exposed to fluid flow sheer stress increased the vasculature and the transcriptional maturity of the organoid (Homan et al., 2019). The addition of vascular endothelial growth factor (VEGF) to cultures increased the amount of vasculature but reduced their proximity to the epithelial structures, concluding

that relying on the endogenous VEGF production from organoid glomeruli was more ideal (Homan et al., 2019). Indeed this endogenous organoid VEGF production has appeared sufficient to induce convincing glomerular vascularization in the context of murine transplantation (van den Berg et al., 2018). While glomerular vasculature was observed by EM in the sheer stress model, the development of a GFB remained primitive and no convincing evidence of perfusability was demonstrated (Homan et al., 2019). An array of complex bioengineering vascularization techniques, including sacrificial inks and microfluidic chips, are approaching this challenge (Grebenyuk and Ranga, 2019). Engineering an endothelium-podocyte interaction will remain a critical hurdle for the field to overcome in the modeling of inherited glomerular disease.

Inherited diseases of the renal tubule

Inherited tubular disease is phenotypically far more heterogenous than glomerular disease. Pathogenic variants in segment-specific ion and water transporters within the tubule give rise to a group of disorders called tubulopathies which manifest inappropriate wasting or retention of salts and/or free water. In the proximal convoluted tubule (PCT), a dense apical brush border performs an extensive reabsorption of glucose, phosphate, bicarbonate, low molecular weight proteins, and other electrolytes (Curthoys and Moe, 2014). Inherited PCT disease can produce a specific defect in reabsorption, such as renal glycosuria [OMIM 233100] due to dominant variants in glucose transporter gene *SLC5A2*. Alternatively, global PTC failure (also known as renal Fanconi syndrome) can result from metabolic or storage disorders of the proximal tubule, such as nephropathic cystinosis [OMIM 219800] (recessive variants in *CTNS*) where cystine crystals accumulate in the proximal tubule, affecting a global reabsorptive failure.

The filtrate then passes through the ascending and descending loop of Henle which together establishs a medullary concentration gradient while also contributing to water and salt reabsorption. Finally the distal convoluted tubule (DCT) performs the fine tuning of salt, water, and acid-base balance. The DCT communicates the concentration of the urinary filtrate (a surrogate for total body hydration) to the juxtaglomerular apparatus which, via the renin-angiotensin-aldosterone hormonal axis, controls salt and water reabsorption in the DCT and collecting duct (CD). Beyond the proximal tubule, inherited disease phenotypes manifest with the gain or loss of function of the gene product affected, often a membrane ion or water transporter. For example, pathogenic variants in *AQP2* or the vasopressin receptor *AVPR2*, which control water reabsorption in the CD, lead to excessive urine production and life-threatening dehydration without free access to drinking water, called nephrogenic diabetes insipidus [OMIM 125800; 304800].

Ciliopathies represent a broad group of disorders characterized by variable cystic and fibrotic change within the renal tubulointerstitium causing an insidious progression of CKD. As the name suggests gene products implicated in ciliopathies are

localized to the primary cilium and centrosome (Braun and Hildebrandt, 2017). This group includes autosomal dominant polycystic kidney disease (ADPKD; *PKD1*, *PKD2,* and *GANAB*) and autosomal recessive polycystic kidney disease (ARPKD; *PKHD1*), which present with overwhelming cystic dilation of renal tubules obliterating normal functioning kidney disease. In contrast, the ciliopathies also include nephronophthisis and related ciliopathies (NPHP-RC) which has over 60 described autosomal recessive genotypes and presents with tubular atrophy, tubular basement membrane abnormalities, and variables renal cysts (Braun and Hildebrandt, 2017). Autosomal dominant tubulointerstitital kidney disease (ADTKD), previously called medullary cystic kidney disease, is caused by variants in *UMOD*, *REN,* and *MUC1* and also presents with primary, progressive interstitial fibrosis, tubular atrophy, and variable medullary cyst development (Eckardt et al., 2015).

Kidney organoids and the study of tubular disease

ADPKD is the most common genetic cause of CKD and is predominantly caused by heterozygous mutations in *PKD1* or *PKD2* (Mallett et al., 2014; NAPRTCS, 2014). Multifocal renal cysts are believed to arise from a homozygous epithelial lineage following a "second hit" somatic mutation in the wild-type allele (Tan et al., 2018). Using CRISPR-Cas9 gene-editing, Freedman et al. derived homozygous knockout $PKD1^{-/-}$ knockout iPSC lines and demonstrated in vitro cystogenesis in kidney organoids (Freedman et al., 2015). Large, LTL^{+}/$ECAD^{+}$ epithelial, ballooning cystic structures arose from 6% of $PKD1^{-/-}$ organoid tubules, compared to 0% of control cultures (Freedman et al., 2015). Transferring these organoids to suspension culture increased the efficiency of cystogenesis from both $PKD1^{-/-}$ and $PKD2^{-/-}$ knockout lines (Cruz et al., 2017). Cysts were noted to be hyperproliferative by immunofluorescence for phosphohistone H3 compared to controls. Microarray analysis of cystic compared to noncystic tissue suggesting increased MYC and mTOR activity in the cystic epithelium (Cruz et al., 2017). Most recently this group screened eight factors thought to modulate the "cell to microenvironment" interaction in 96 well plates, in an impressive demonstration of the potential for compound screening using kidney organoid tissues (Czerniecki et al., 2018). In this work, treatment with a nonmuscle myosin II B (NMIIB) inhibitor, blebbistatin, increased cyst swelling establishing a hypothesis of NMIIB activators as potential novel therapy for ADPKD-*PKD1* (Czerniecki et al., 2018; Helms et al., 2019). The use of CRISPR/Cas9 engineered $PKD1^{-/-}$ and $PKD2^{-/-}$ homozygous iPSC, however, may not specifically illustrate the constitutional genotype of ADPKD patients and these findings are yet to be validated using patient iPSC.

A recent report modeled ARPKD with patient iPSC-derived kidney organoids, demonstrating striking differential whole-organoid swelling compared to both isogenic and unrelated controls following extended exposure to forskolin, which was rescued by pretreating with thapsigargin (sarcoendoplasmic reticulum

Ca^2-ATPase inhibitor) or CFTR inhibitor 172 (Low et al., 2019). While this study advocates feasibility for disease modeling of ARPKD using kidney organoids, the presence of a bona fide CD segment is not demonstrated in this model and cyst swelling in this report commenced in the proximal tubule (Low et al., 2019). While transient, self-limited proximal tubule dilation has been reported in knockout mouse models(51) and one human fetal kidney report (prior to the discovery of PKHD1 as the associated gene) (Nakanishi et al., 2000), in vivo ARPKD cystogenesis has been reproducibly isolated to the CDs (Fischer et al., 2009; Faraggiana et al., 1985; Verani et al., 1989; Holthofer et al., 1990). Some controversy arises as to whether a true CD is represented within the nephrons of kidney organoids. *SIX2* lineage tracing suggests that $GATA3^+$ epithelial cells do not derive from a $SIX2^+$ MM precursor (Howden et al., 2019), and both principal and intercalated cells are identified within organoids on EM following murine transplantation (van den Berg et al., 2018). However, the organoid morphology of these $ECAD^+/GATA3^+$ segments is *not* reminiscent of dichotomous UB branching seen in vivo and single cell RNA sequencing of mouse kidney suggests that many markers traditionally used to identify ureteric epithelium (such as *Apq2*, *Krt8*, *Krt18,* and even *Hoxb7*) are expressed in the distal tubule and connecting segment and hence derived from the MM and not the UB (Combes et al., 2019a). Some elaborate protocols for the culture of isolated ureteric epithelium have been published (Mae et al., 2018; Taguchi and Nishinakamura, 2017), which may prove more relevant to the modeling of ARPKD.

Our group have modeled NPHP-RC, using iPSC-derived organoids from a patient with Mainzer Saldino syndrome due to compound heterozygous variants in *IFT140*, which produces a gene product essential for maintenance of the primary cilium (Forbes et al., 2018). Gene-corrected and matched, isogenic iPSC were derived from patient fibroblasts using combined reprogramming and CRISPR/Cas9 techniques (Forbes et al., 2018; Howden et al., 2015). Tubules from patient organoids demonstrated shortened, club-shaped primary cilia (Fig. 11.2I) (Forbes et al., 2018). This morphology was consistent with prior animal and cellular models and was rescued to a long, straight, wild-type morphology following gene-correction (Forbes et al., 2018). Differential gene expression analysis of sorted epithelial cell fractions from patient and gene-corrected organoids identified differences in cell polarity, cell junction, and axonemal dynein motor assembly (Forbes et al., 2018). Single epithelial cells sorted from patient organoids were less able to form polarized cysts in matrigel culture compared to gene-corrected control (Forbes et al., 2018). No gross morphological differences between patient and control organoids were evident, and differential gene expression analysis required adjustment to remove genes recognized to be highly variable between differentiation experiments, usually due to differences in nephron segmentation (Phipson et al., 2019).

An impressive, recent demonstration of the strength of the patient-derived organoid platform in novel drug discovery involved the modeling of ADTKD-*MUC1* (Dvela-Levitt et al., 2019). This disease is exclusively caused by frameshift variants in the *mucin 1* (*MUC1*) gene, and the mutant protein accumulates in the endoplasmic

reticulum (ER) of the DCTs, activating the unfolded protein response (Dvela-Levitt et al., 2019; Kirby et al., 2013). Using an antibody specific for the mutant protein, this group validated the immunofluorescent clearance of the protein from the ER following treatment with a novel candidate compound on organoids derived from the iPSC of multiple patients (Dvela-Levitt et al., 2019). This novel therapeutic discovery was reported with much greater impact as a result of a demonstrated utility in a regenerated patient-derived human kidney tubule.

The pathogenic mechanism underlying the renal fibrosis commonly seen in epithelial dysfunction in vivo is poorly understood, and the ability for kidney organoids to adequately model this is unclear. Furthermore, single cell RNA sequencing analysis of kidney organoids has established that they contain a variable component of off-target stromal cell types (Wu et al., 2018; Combes et al., 2019b; Subramanian et al., 2019). Characterization, optimization, and reproducibility of the stromal component of primary kidney and organoids will be critical before any research can be performed examining "fibrosis" of the organoids as a readout of disease.

Interestingly, ADPKD and ADTKD are adult onset diseases, challenging initial presumptions that organoids may be limited to the modeling of pediatric disease. This hypothesis was built on the observation that whole-organoid transcriptional profiling most closely correlates to trimester one to two human fetal kidney (6) and maturation arrests with the withdrawal of growth factors (Phipson et al., 2019). Rather, it would appear that the prerequisite for modeling a genetic disease in organoids is the adequate expression of the gene of interest within the organoid. This poses a challenge for modeling diseases caused by mutations in specific transporter genes, most of which demonstrate low expression both in developing kidneys and current organoid protocols.

In the primary report of a scaled up organoid bioreactor protocol that utilized knockout serum replacement in place of FGF9 for intermediate mesoderm patterning, Przepiorski et al. (2018) engineered *HNF1B*$^{-/-}$ knockout iPSC and compared their differentiation to the parental isogenic control iPSC clone in an attempt to model renal cysts and diabetes syndrome. They document downregulation of key *HNF1B* transcriptional target genes as well as failure of the proximal and distal tubular segments to develop within the organoids, consistent with human phenotype and mouse models of the disease (Przepiorski et al., 2018). A separate article from the same group establishes an additive repurposed role for the mTOR inhibitor Everolimus to the existing standard of care in cysteamine in the treatment of lysosomal storage disease nephropathic cystinosis (Hollywood et al., 2020). Organoids from both CRISPR/Cas9-engineered CTNS knockout iPSC and patient-derived iPSC demonstrated recognized pathologies of (i) enlarged lysosomes accumulating cystine and cysteine and (ii) reduced cellular basal macroautophagy (Hollywood et al., 2020). While cysteamine effectively resolved the former, Everolimus was demonstrated to rescue the latter (Hollywood et al., 2020).

Strengths and limitations of kidney organoids as models of inherited kidney disease compared to existing animal and two-dimensional cell culture platforms

While organoids offer solutions to many of the limitations of traditional functional genomic models such as animal models and 2D cell culture, they have their own fallibilities and perform an adjunctive role in functional genomic research. These differences are summarized in Table 11.1 and discussed in detail below.

Skin fibroblasts represent an easily obtainable and expandable surrogate cell type for other less obtainable primary tissues for functional genomic interrogation. However, increasing knowledge of differential tissue specific isoform splicing and protein function between cell types challenges this practice. For example, there has been a long held assumption that primary cilia (an almost ubiquitous cellular organelle) maintained the same structure and function in all cell types (Wheatley, 1995). Challenging this dogma, a mutant *Tctn1* (a ciliary transition zone protein) mouse model demonstrated abnormal cilia number and morphology at the embryonic node and neural tube but undisturbed ciliogenesis in the limb bud and perineural mesenchyme (Garcia-Gonzalo et al., 2011). In another example, *CEP290* patients with an isolated ophthalmic phenotype demonstrated ciliogenesis defects in iPSC-differentiated optic cups but not in fibroblasts, while in patients with *CEP290* Joubert syndrome, both tissues were affected (Shimada et al., 2017). In a further example, RNA analysis of an *NPHP3* variant of unknown significance identified splicing abnormalities in human urine derived renal epithelial cells that were not evident in RNA derived from a peripheral blood sample (Molinari et al., 2018). In all these examples, examination of the tissue of interest proved advantageous to the more easily accessible surrogate cell type. This advocates that renal disease should be studied in a renal cell type and demonstrating an advantage of kidney organoid culture.

However, 3D, in vitro tissue culture is not without its disadvantages. 2D cell culture is straightforward, cheap, fast, and highly reproducible. In contrast, the isolation of a purified single cell type from a multicellular organoid for molecular interrogation requires extended dissociation and technically complex protocols. This limits the number of conditions that can be simultaneously researched and introduces technical artifact and variation arising during dissociation and purification.

Mouse models have been a pillar of functional genomic research for decades and generally provided phenotypes reflecting those of human disease for a given orthologous genotype (Hofmeister et al., 2017). However numerous differences exist between the two species. Human kidneys are multipapillate and develop over 30 weeks with nephrogenesis ceasing at approximately 36 weeks, whereas mouse kidneys are unipapillate, develop over one week and nephrogenesis ceases during the first postpartum week of life (Little, 2015; Hartman et al., 2007; Hinchliffe et al., 1991). The observation of human cortical nephrons inserting into other nephrons away from the ureteric tree, a process called arcading, has

Table 11.1 Characteristics of kidney organoids compared to other functional genomic models.

	Kidney organoids	Animal models	Immortalized renal cells	Patient primary fibroblasts
Dimensionality	Three-dimensional (3D) multicellular tissue; Improved cell—cell and cell—matrix interaction		Two-dimensional unicellular culture or simple 3D unicellular structures (epithelial cysts)	
Experimentation/ Analysis	Complex		Straightforward	
Phenotype	Patient-specific, renal tissue	Renal and extrarenal	Renal epithelial cellular	Nonrenal, nonepithelial, patient-specific
Maturity	Immature	Mature	–	–
Species	Human	Animal	Generally animal	Human
Throughput	Moderate—high	Low	High	High
Environment	In vitro Avascular No urinary drainage	In vivo Physiological developmental environment	In vitro	

not been reported in murine renal development (Zhai et al., 2006). Transcriptional differences in kidney development highlight examples where translation between the species may also be limited (Lindstrom et al., 2018a,b,c). In the developing mouse kidney, *Six2* expression is critical to maintain the cap mesenchyme while *Six1* is downregulated whereas in human, the two genes appear to demonstrate a functional redundancy and dominant variants in both *SIX1* and *SIX2* are associated with congenital abnormalities of the kidney and urinary tract (CAKUT) (O'Brien et al., 2016; Combes et al., 2018; Weber et al., 2008; Faguer et al., 2012; Hwang et al., 2014). Many attempts to model ARPKD in mice have failed to recapitulate an early onset polycystic kidney disease, rather an early onset liver disease and variably penetrant polycystic kidney disease manifesting around three−six months (Woollard et al., 2007; Bakeberg et al., 2011; Moser et al., 2005; Williams et al., 2008). In another example of phenotype discrepancy, dominant variants in *HNF1B* are a common cause of a CAKUT in humans (87−89), but mice only develop disease in the context of homozygote deletions (Desgrange et al., 2017; Massa et al., 2013). These observed discrepancies in kidney development, anatomy, transcriptional profiling, and genetic disease expression impede the translation of findings in mouse models to human disease and illustrate the capacity for human cellular models to contribute.

In spite of the difficulties extrapolating findings between species, animal models offer many other benefits that organoids cannot fulfill. Firstly, murine disease modeling allows interrogation of nonrenal phenotype in syndromic monogenic disease. Furthermore, mice are capable of modeling nonrenal diseases that extrinsically damage the kidneys, such as thrombotic microangiopathies or primary hyperoxaluria. Because the genes responsible for these diseases are not expressed by the kidney, they cannot be modeled within kidney organoids. Organoids exhibit subtle technical variation in differentiation end-point including degree of off-target cell populations and nephron patterning, between individual experiments (25), while a naturally developed animal kidney will reliably arrive at the same end-point within a given developmental timeframe. Kidney organoids lack the fidelity to portray some inherited phenotypes, for example, CAKUT which describes a broad array of dysplastic anomalies with a poor genetic understanding. Finally, animal models achieve a greater degree of maturity than current organoid protocols can provide allowing for a broader repertoire of candidate genes.

Functional genomic inquiry within iPSC-derived tissues can use one of two approaches: reprogramming of patient iPSC or gene-editing of desired genetic variants into wild-type iPSC. In both cases, an isogenic control iPSC creates an experimental comparison whereby the patient variant is an isolated variable (Fig. 11.3) (Tanigawa et al., 2018; Low et al., 2019; Forbes et al., 2018; Howden et al., 2015; Dvela-Levitt et al., 2019). Reprogramming patient iPSC theoretically allows for the investigation of genetic modifiers that may alter the phenotypic expression of an individual's genotype. However, this requires a gene-edited control iPSC clone to be engineered and differentiated for each patient studied. In the situation where a studied candidate gene variant is proven not to be

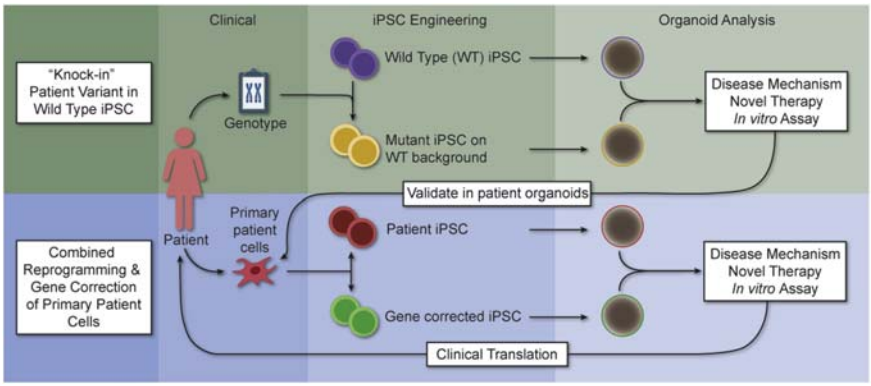


FIGURE 11.3

Illustration of the two approaches to induced pluripotent stem cell (iPSC)-based modeling of patient-derived genomic variants. Depicted in green, the "knock-in" approach involves editing a wild-type iPSC line [purple (gray in print version)] to carry a genomic variant [yellow (light gray in print version)] derived from a patient with a specific phenotype. The wild-type iPSC clone acts as an isogenic control for the "knock-in" clone. This approach can be performed without patient material and more easily facilitates the study of multiple variants. However, this knock-in approach account for the potential contribution of gene polymorphisms extraneous to the variant of interest which may alter the expression of disease or response to treatment. Therefore any relevant findings need to be validated using true patient iPSC clones [red (dark gray in print version)] and isogenic gene-corrected controls, both of which can be clonally derived using combined gene-editing and reprogramming protocols (Forbes et al., 2018; Howden et al., 2015, 2018). The demonstration of a differential phenotype or treatment response within organoids differentiated from this iPSC clones can be more confidently translated back to patient care or clinical trials.

disease-associated, the opportunity to retrospectively identify the true pathogenic variant remains with this approach, as expression of the correct gene is retained in the model. In situations where multiple patient variants are to be examined, editing those variants into a single wild-type iPSC clone allows more focused comparison between variants and obviates the need to optimize protocols for multiple control iPSC clones. In this circumstance, a representative iPSC clones must be generated for each variant of interest. In summary, as a recapitulation of a 3D human tissue, kidney organoids overcome many deficiencies of existing functional genomic models but equally rely on those models to overcome their own limitations. Thus, while organoids are likely to become an essential component of function inquiry into human genetic diseases, they are likely to compliment the role of mouse and 2D-cellular models rather than replace them.

Conclusions

Stem cell–derived kidney organoids represent a novel functional genomic model, clearly capable of expanding our knowledge of inherited glomerular and tubular kidney diseases. Short term outcomes of kidney organoids research include novel gene discoveries, patient-specific disease modeling, and clinical translation of novel therapies. Current protocols, however, do not allow the modeling of all genetic kidney diseases and thus organoids stand to complement existing functional genomic models, rather than replace them. The diseases currently best suited to modeling with kidney organoids are those where expression of the gene of interest is strong within the organoid and a discrete immunofluorescent and/or morphological readout of protein localization, structural tissue development, or cell function can be obtained. As new differentiation and analysis techniques and bioengineering technologies are superimposed on existing kidney regeneration techniques, the breadth of diseases able to be modeled and thus the clinical translation of discoveries is likely to increase.

Acknowledgments

Thomas A Forbes has been supported by an NHMRC Postgraduate Scholarship (GNT1114409) and a Royal Australasian College of Physicians Jacquot Award and is a Clinician Scientist Fellow and the Murdoch Children's Research Institute. Melissa H Little is an NHMRC Senior Principal Research Fellow (GNT1136085). We acknowledge Bill Reid, Creative Studios, The Royal Children's Hospital, Melbourne for assistance in Figures.

References

AIHW. Projections, 2014. Of the Prevalence of Treated End-Stage Kidney Diseasein Australia 2012–2020. Cat. AIHW, Canberra no. PHE 176. Release Date 6 June 2014.

Bakeberg, J.L., Tammachote, R., Woollard, J.R., Hogan, M.C., Tuan, H.F., Li, M., et al., 2011. Epitope-tagged Pkhd1 tracks the processing, secretion, and localization of fibrocystin. J. Am. Soc. Nephrol. 22 (12), 2266–2277.

Barua, M., Shieh, E., Schlondorff, J., Genovese, G., Kaplan, B.S., Pollak, M.R., 2014. Exome sequencing and in vitro studies identified podocalyxin as a candidate gene for focal and segmental glomerulosclerosis. Kidney Int. 85 (1), 124–133.

Braun, D.A., Hildebrandt, F., 2017. Ciliopathies. Cold Spring Harb. Perspect. Biol. 9 (3), a028191.

Cass, A.C.S., Gallagher, M., Howard, K., Jones, A., McDonald, S., Snelling, P., White, S., 2010. The Economic Impact of End-Stage Kidney Disease in Australia. Kidney Health Australia, Melbourne.

Chew, C., Lennon, R., 2018. Basement membrane defects in genetic kidney diseases. Front. Pediatr. 6, 11.

Combes, A.N., Wilson, S., Phipson, B., Binnie, B.B., Ju, A., Lawlor, K.T., et al., 2018. Haploin sufficiency for the Six2 gene increases nephron progenitor proliferation promoting branching and nephron number. Kidney Int. 93 (3), 589−598.

Combes, A.N., Phipson, B., Lawlor, K.T., Dorison, A., Patrick, R., Zappia, L., et al., 2019a. Single cell analysis of the developing mouse kidney provides deeper insight into marker gene expression and ligand-receptor crosstalk. Development 146 (12).

Combes, A.N., Zappia, L., Er, P.X., Oshlack, A., Little, M.H., 2019b. Single-cell analysis reveals congruence between kidney organoids and human fetal kidney. Genome Med. 11 (1), 3.

Cruz, N.M., Song, X., Czerniecki, S.M., Gulieva, R.E., Churchill, A.J., Kim, Y.K., et al., 2017. Organoid cystogenesis reveals a critical role of microenvironment in human polycystic kidney disease. Nat. Mater. 16 (11), 1112−1119.

Curthoys, N.P., Moe, O.W., 2014. Proximal tubule function and response to acidosis. Clin. J. Am. Soc. Nephrol. 9 (9), 1627−1638.

Czerniecki, S.M., Cruz, N.M., Harder, J.L., Menon, R., Annis, J., Otto, E.A., et al., 2018. High-throughput screening enhances kidney organoid differentiation from human pluripotent stem cells and enables automated multidimensional phenotyping. Cell Stem Cell 22 (6), 929−940.

Desgrange, A., Heliot, C., Skovorodkin, I., Akram, S.U., Heikkila, J., Ronkainen, V.P., et al., 2017. HNF1B controls epithelial organization and cell polarity during ureteric bud branching and collecting duct morphogenesis. Development 144 (24), 4704−4719.

Duval, K., Grover, H., Han, L.H., Mou, Y., Pegoraro, A.F., Fredberg, J., et al., 2017. Modeling physiological events in 2D vs. 3D cell culture. Physiology 32 (4), 266−277.

Dvela-Levitt, M., Kost-Alimova, M., Emani, M., Kohnert, E., Thompson, R., Sidhom, E.H., et al., 2019. Small molecule targets TMED9 and promotes lysosomal degradation to reverse proteinopathy. Cell 178 (3), 521−535 e23.

Eckardt, K.U., Alper, S.L., Antignac, C., Bleyer, A.J., Chauveau, D., Dahan, K., et al., 2015. Autosomal dominant tubulointerstitial kidney disease: diagnosis, classification, and management–A KDIGO consensus report. Kidney Int. 88 (4), 676−683.

Faguer, S., Chassaing, N., Bandin, F., Prouheze, C., Chauveau, D., Decramer, S., 2012. Should SIX2 be routinely tested in patients with isolated congenital abnormalities of kidneys and/or urinary tract (CAKUT)? Eur. J. Med. Genet. 55 (12), 688−689.

Faraggiana, T., Bernstein, J., Strauss, L., Churg, J., 1985. Use of lectins in the study of histogenesis of renal cysts. Lab. Invest. 53 (5), 575−579.

Fischer, D.C., Jacoby, U., Pape, L., Ward, C.J., Kuwertz-Broeking, E., Renken, C., et al., 2009. Activation of the AKT/mTOR pathway in autosomal recessive polycystic kidney disease (ARPKD). Nephrol. Dial. Transplant. 24 (6), 1819−1827.

Fissell, W.H., Miner, J.H., 2018. What is the glomerular ultrafiltration barrier? J. Am. Soc. Nephrol. 29 (9), 2262−2264.

Fletcher, J., McDonald, S., Alexander, S.I., 2013. Australian, New Zealand pediatric nephrology A. Prevalence of genetic renal disease in children. Pediatr. Nephrol. 28 (2), 251−256.

Forbes, T.A., Howden, S.E., Lawlor, K., Phipson, B., Maksimovic, J., Hale, L., et al., 2018. Patient-iPSC-Derived kidney organoids show functional validation of a ciliopathic renal phenotype and reveal underlying pathogenetic mechanisms. Am. J. Hum. Genet. 102 (5), 816−831.

Freedman, B.S., Brooks, C.R., Lam, A.Q., Fu, H., Morizane, R., Agrawal, V., et al., 2015. Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. Nat. Commun. 6, 8715.

Freedman, B.S., 2015. Modeling kidney disease with iPS cells. Biomark. Insights 10 (Suppl. 1), 153−169.

Garcia-Gonzalo, F.R., Corbit, K.C., Sirerol-Piquer, M.S., Ramaswami, G., Otto, E.A., Noriega, T.R., et al., 2011. A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. Nat. Genet. 43 (8), 776−784.

Ghosh, S., Spagnoli, G.C., Martin, I., Ploegert, S., Demougin, P., Heberer, M., et al., 2005. Three-dimensional culture of melanoma cells profoundly affects gene expression profile: a high density oligonucleotide array study. J. Cell. Physiol. 204 (2), 522−531.

Grebenyuk, S., Ranga, A., 2019. Engineering organoid vascularization. Front. Bioeng. Biotechnol. 7 (39).

Hale, L.J., Howden, S.E., Phipson, B., Lonsdale, A., Er, P.X., Ghobrial, I., et al., 2018. 3D organoid-derived human glomeruli for personalised podocyte disease modelling and drug screening. Nat. Commun. 9 (1), 5167.

Hartman, H.A., Lai, H.L., Patterson, L.T., 2007. Cessation of renal morphogenesis in mice. Dev. Biol. 310 (2), 379−387.

Helms, L., Gomez, I., Cruz, N.M., Freedman, B.S., 2019. Myosin Activation as a Novel Therapeutic Strategy to Treat PKD in Human Kidney Organoids and Mice. American Society of Nephrology; 9th November 2019, Kidney Week 2019. Washington D.C.

Hinchliffe, S.A., Sargent, P.H., Howard, C.V., Chan, Y.F., van Velzen, D., 1991. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. Lab. Invest. 64 (6), 777−784.

Hofmeister, A.F., Komhoff, M., Weber, S., Grgic, I., 2017. Disease modeling in genetic kidney diseases: mice. Cell Tissue Res. 369 (1), 159−170.

Hollywood, J.A., Przepiorski, A., D'Souza, R.F., Sreebhavan, S., Wolvetang, E.J., Davidson, A.J., et al., 2020. Use of human induced pluripotent stem cells and kidney organoids to develop a cysteamine/mTOR inhibition combination therapy for cystinosis. J. Am. Assoc. Nephrol. 31 (5), 962−982.

Holthofer, H., Kumpulainen, T., Rapola, J., 1990. Polycystic disease of the kidney. Evaluation and classification based on nephron segment and cell-type specific markers. Lab. Invest. 62 (3), 363−369.

Homan, K.A., Gupta, N., Kroll, K.T., Kolesky, D.B., Skylar-Scott, M., Miyoshi, T., et al., 2019. Flow-enhanced vascularization and maturation of kidney organoids in vitro. Nat. Method 16 (3), 255−262.

Howden, S.E., Maufort, J.P., Duffin, B.M., Elefanty, A.G., Stanley, E.G., Thomson, J.A., 2015. Simultaneous reprogramming and gene correction of patient fibroblasts. Stem Cell Rep. 5 (6), 1109−1118.

Howden, S.E., Thomson, J.A., Little, M.H., 2018. Simultaneous reprogramming and gene editing of human fibroblasts. Nat. Protoc. 13 (5), 875−898.

Howden, S.E., Vanslambrouck, J.M., Wilson, S.B., Tan, K.S., Little, M.H., 2019. Reporter-based fate mapping in human kidney organoids confirms nephron lineage relationships and reveals synchronous nephron formation. EMBO Rep. 20 (4), e47483.

Hoy, W.E., Douglas-Denton, R.N., Hughson, M.D., Cass, A., Johnson, K., Bertram, J.F., 2003. A stereological study of glomerular number and volume: preliminary findings in a multiracial study of kidneys at autopsy. Kidney Int. Suppl. (83), S31−S37.

Hughson, M., Farris 3rd, A.B., Douglas-Denton, R., Hoy, W.E., Bertram, J.F., 2003. Glomerular number and size in autopsy kidneys: the relationship to birth weight. Kidney Int. 63 (6), 2113−2122.

Hwang, D.Y., Dworschak, G.C., Kohl, S., Saisawat, P., Vivante, A., Hilger, A.C., et al., 2014. Mutations in 12 known dominant disease-causing genes clarify many congenital anomalies of the kidney and urinary tract. Kidney Int. 85 (6), 1429−1433.

Kang, H.G., Lee, M., Lee, K.B., Hughes, M., Kwon, B.S., Lee, S., et al., 2017. Loss of podocalyxin causes a novel syndromic type of congenital nephrotic syndrome. Exp. Mol. Med. 49 (12), e414.

Kirby, A., Gnirke, A., Jaffe, D.B., Baresova, V., Pochet, N., Blumenstiel, B., et al., 2013. Mutations causing medullary cystic kidney disease type 1 lie in a large VNTR in MUC1 missed by massively parallel sequencing. Nat. Genet. 45 (3), 299−303.

Lancaster, M.A., Knoblich, J.A., 2014. Organogenesis in a dish: modeling development and disease using organoid technologies. Science 345 (6194), 1247125.

Lindstrom, N.O., Guo, J., Kim, A.D., Tran, T., Guo, Q., De Sena Brandine, G., et al., 2018a. Conserved and divergent features of mesenchymal progenitor cell types within the cortical nephrogenic niche of the human and mouse kidney. J. Am. Soc. Nephrol. 29 (3), 806−824.

Lindstrom, N.O., McMahon, J.A., Guo, J., Tran, T., Guo, Q., Rutledge, E., et al., 2018b. Conserved and divergent features of human and mouse kidney organogenesis. J. Am. Soc. Nephrol. 29 (3), 785−805.

Lindstrom, N.O., Tran, T., Guo, J., Rutledge, E., Parvez, R.K., Thornton, M.E., et al., 2018c. Conserved and divergent molecular and anatomic features of human and mouse nephron patterning. J. Am. Soc. Nephrol. 29 (3), 825−840.

Little, M.H., Combes, A.N., 2019. Kidney organoids: accurate models or fortunate accidents. Genes Dev. 33 (19−20), 1319−1345.

Little, M.H., McMahon, A.P., 2012. Mammalian kidney development: principles, progress, and projections. Cold Spring Harb. Perspect. Biol. 4 (5), a008300.

Little, M.H., Takasato, M., Soo, J.Y.-C., Forbes, T.A., 2017. Recapitulating development to generate kidney organoid cultures. In: Tsuji, T. (Ed.), Organ Regeneration Based on Developmental Biology. Springer Singapore, Singapore, pp. 193−222.

Little, M.H., 2015. Improving our resolution of kidney morphogenesis across time and space. Curr. Opin. Genet. Dev. 32, 135−143.

Lovric, S., Ashraf, S., Tan, W., Hildebrandt, F., 2016. Genetic testing in steroid-resistant nephrotic syndrome: when and how? Nephrol. Dial. Transplant. 31 (11), 1802−1813.

Low, J.H., Li, P., Chew, E.G.Y., Zhou, B., Suzuki, K., Zhang, T., et al., 2019. Generation of human PSC-derived kidney organoids with patterned nephron segments and a de novo vascular network. Cell Stem Cell 25 (3), 373−387.

Mae, S.I., Ryosaka, M., Toyoda, T., Matsuse, K., Oshima, Y., Tsujimoto, H., et al., 2018. Generation of branching ureteric bud tissues from human pluripotent stem cells. Biochem. Biophys. Res. Commun. 495 (1), 954−961.

Mallett, A., Patel, C., Salisbury, A., Wang, Z., Healy, H., Hoy, W., 2014. The prevalence and epidemiology of genetic renal disease amongst adults with chronic kidney disease in Australia. Orphanet J. Rare Dis. 9, 98.

Massa, F., Garbay, S., Bouvier, R., Sugitani, Y., Noda, T., Gubler, M.C., et al., 2013. Hepatocyte nuclear factor 1beta controls nephron tubular development. Development 140 (4), 886−896.

Molinari, E., Decker, E., Mabillard, H., Tellez, J., Srivastava, S., Raman, S., et al., 2018. Human urine-derived renal epithelial cells provide insights into kidney-specific alternate splicing variants. Eur. J. Hum. Genet. 26 (12), 1791−1796.

Morizane, R., Lam, A.Q., Freedman, B.S., Kishi, S., Valerius, M.T., Bonventre, J.V., 2015. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nat. Biotechnol. 33 (11), 1193−1200.

Moser, M., Matthiesen, S., Kirfel, J., Schorle, H., Bergmann, C., Senderek, J., et al., 2005. A mouse model for cystic biliary dysgenesis in autosomal recessive polycystic kidney disease (ARPKD). Hepatology 41 (5), 1113−1121.

Nakanishi, K., Sweeney Jr., W.E., Zerres, K., Guay-Woodford, L.M., Avner, E.D., 2000. Proximal tubular cysts in fetal human autosomal recessive polycystic kidney disease. J. Am. Soc. Nephrol. 11 (4), 760−763.

NAPRTCS, 2014. Annual Report 2014. Available from: https://web.emmes.com/study/ped/annlrept/annualrept2014.pdf.

Noone, D.G., Iijima, K., Parekh, R., 2018. Idiopathic nephrotic syndrome in children. Lancet 392 (10141), 61−74.

O'Brien, L.L., Guo, Q., Lee, Y., Tran, T., Benazet, J.-D., Whitney, P.H., et al., 2016. Differential regulation of mouse and human nephron progenitors by the Six family of transcriptional regulators. Development 143 (4), 595−608.

Pampaloni, F., Reynaud, E.G., Stelzer, E.H., 2007. The third dimension bridges the gap between cell culture and live tissue. Nat. Rev. Mol. Cell Biol. 8 (10), 839−845.

Phipson, B., Er, P.X., Combes, A.N., Forbes, T.A., Howden, S.E., Zappia, L., et al., 2019. Evaluation of variability in human kidney organoids. Nat. Method 16 (1), 79−87.

Przepiorski, A., Sander, V., Tran, T., Hollywood, J.A., Sorrenson, B., Shih, J.H., et al., 2018. A simple bioreactor-based method to generate kidney organoids from pluripotent stem cells. Stem Cell Rep. 11 (2), 470−484.

Sharmin, S., Taguchi, A., Kaku, Y., Yoshimura, Y., Ohmori, T., Sakuma, T., et al., 2016. Human induced pluripotent stem cell-derived podocytes mature into vascularized glomeruli upon experimental transplantation. J. Am. Soc. Nephrol. 27 (6), 1778−1791.

Shimada, H., Lu, Q., Insinna-Kettenhofen, C., Nagashima, K., English, M.A., Semler, E.M., et al., 2017. Vitro modeling using ciliopathy-patient-derived cells reveals distinct cilia dysfunctions caused by CEP290 mutations. Cell Rep. 20 (2), 384−396.

Subramanian, A., Sidhom, E.H., Emani, M., Vernon, K., Sahakian, N., Zhou, Y., et al., 2019. Single cell census of human kidney organoids shows reproducibility and diminished off-target cells after transplantation. Nat. Commun. 10 (1), 5462.

Taguchi, A., Nishinakamura, R., 2017. Higher-order kidney organogenesis from pluripotent stem cells. Cell Stem Cell 21 (6), 730−746 e6.

Taguchi, A., Kaku, Y., Ohmori, T., Sharmin, S., Ogawa, M., Sasaki, H., et al., 2014. Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells. Cell Stem Cell 14 (1), 53−67.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861−872.

Takasato, M., Er, P.X., Becroft, M., Vanslambrouck, J.M., Stanley, E.G., Elefanty, A.G., et al., 2014. Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. Nat. Cell Biol. 16 (1), 118−126.

Takasato, M., Er, P.X., Chiu, H.S., Maier, B., Baillie, G.J., Ferguson, C., et al., 2015. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature 526 (7574), 564−568.

Tan, A.Y., Zhang, T., Michaeel, A., Blumenfeld, J., Liu, G., Zhang, W., et al., 2018. Somatic mutations in renal cyst epithelium in autosomal dominant polycystic kidney disease. J. Am. Soc. Nephrol. 29 (8), 2139−2156.

Tanigawa, S., Islam, M., Sharmin, S., Naganuma, H., Yoshimura, Y., Haque, F., et al., 2018. Organoids from nephrotic disease-derived iPSCs identifyimpaired NEPHRIN localization and slit diaphragm formation in kidney podocytes. Stem Cell Rep. 11 (3), 727−740.

van den Berg, C.W., Ritsma, L., Avramut, M.C., Wiersma, L.E., van den Berg, B.M., Leuning, D.G., et al., 2018. Renal subcapsular transplantation of PSC-derived kidney organoids induces neo-vasculogenesis and significant glomerular and tubular maturation in vivo. Stem Cell Rep. 10 (3), 751−765.

Verani, R., Walker, P., Silva, F.G., 1989. Renal cystic disease of infancy: results of histochemical studies. A report of the Southwest Pediatric Nephrology Study Group. Pediatr. Nephrol. 3 (1), 37−42.

Weber, S., Taylor, J.C., Winyard, P., Baker, K.F., Sullivan-Brown, J., Schild, R., et al., 2008. SIX2 and BMP4 mutations associate with anomalous kidney development. J. Am. Soc. Nephrol. 19 (5), 891−903.

Wheatley, D.N., 1995. Primary cilia in normal and pathological tissues. Pathobiology 63 (4), 222−238.

Williams, S.S., Cobo-Stark, P., James, L.R., Somlo, S., Igarashi, P., 2008. Kidney cysts, pancreatic cysts, and biliary disease in a mouse model of autosomal recessive polycystic kidney disease. Pediatr. Nephrol. 23 (5), 733−741.

Woollard, J.R., Punyashtiti, R., Richardson, S., Masyuk, T.V., Whelan, S., Huang, B.Q., et al., 2007. A mouse model of autosomal recessive polycystic kidney disease with biliary duct and proximal tubule dilatation. Kidney Int. 72 (3), 328−336.

Wu, H., Uchimura, K., Donnelly, E.L., Kirita, Y., Morris, S.A., Humphreys, B.D., 2018. Comparative analysis and refinement of human PSC-derived kidney organoid differentiation with single-cell transcriptomics. Cell Stem Cell 23 (6), 869−881 e8.

Zhai, X.-Y., Thomsen, J.S., Birn, H., Kristoffersen, I.B., Andreasen, A., Christensen, E.I., 2006. Three-dimensional reconstruction of the mouse nephron. J. Am. Soc. Nephrol. 17 (1), 77−88.

Further reading

Clissold, R.L., Hamilton, A.J., Hattersley, A.T., Ellard, S., Bingham, C., 2015. HNF1B-associated renal and extra-renal disease-an expanding clinical spectrum. Nat. Rev. Nephrol. 11 (2), 102−112.

Decramer, S., Parant, O., Beaufils, S., Clauin, S., Guillou, C., Kessler, S., et al., 2007. Anomalies of the TCF2 gene are the main cause of fetal bilateral hyperechogenic kidneys. J. Am. Soc. Nephrol. 18 (3), 923−933.

Giacomelli, E., Bellin, M., Sala, L., van Meer, B.J., Tertoolen, L.G.J., Orlova, V.V., et al., 2017. Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. Development 144 (6), 1008−1017.

Heidet, L., Decramer, S., Pawtowski, A., Moriniere, V., Bandin, F., Knebelmann, B., et al., 2010. Spectrum of HNF1B mutations in a large cohort of patients who harbor renal diseases. Clin. J. Am. Soc. Nephrol. 5 (6), 1079−1090.

Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., et al., 2013. Cerebral organoids model human brain development and microcephaly. Nature 501 (7467), 373−379.

McCracken, K.W., Catá, E.M., Crawford, C.M., Sinagoga, K.L., Schumacher, M., Rockich, B.E., et al., 2014. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. Nature 516 (7531), 400−404.

Spence, J.R., Mayhew, C.N., Rankin, S.A., Kuhar, M.F., Vallance, J.E., Tolle, K., et al., 2011. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470 (7332), 105−109.

Wu, F., Wu, D., Ren, Y., Huang, Y., Feng, B., Zhao, N., et al., 2019. Generation of hepatobiliary organoids from human induced pluripotent stem cells. J. Hepatol. 70 (6), 1145−1158.

Index

Note: 'Page numbers followed by "*f*" indicate figures and "*t*" indicate tables.'

D

E

F

G

H

I

S

T

This page intentionally left blank

ADVANCES IN STEM CELL BIOLOGY VOLUME 14

Current Progress in iPSC Disease Modeling

Edited by
Alexander Birbrair
Department of Pathology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
Department of Radiology, Columbia University Medical Center, Medical Center, USA

The series *Advances in Stem Cell Biology* is a timely and expansive collection of comprehensive information and new discoveries in the field of stem cell biology.

Current Progress in iPSC Disease Modeling, Volume 14 addresses how induced pluripotent stem cells can be used to model various diseases. Somatic cells are reprogrammed into induced pluripotent stem cells by the expression of specific transcription factors. These cells are transforming biomedical research in the last 15 years. This volume teaches readers about current advances in the field. This book describes the use of induced pluripotent stem cells to model several diseases in vitro, enabling us to study the cellular and molecular mechanisms involved in different pathologies. Further insights into these mechanisms will have important implications for our understanding of disease appearance, development, and progression. In recent years, remarkable progress has been made in the obtention of induced pluripotent stem cells and their differentiation into several cell types, tissues, and organs using state-of-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of several disorders.

The volume is written for researchers and scientists in stem cell therapy, cellular and molecular biology, and regenerative medicine and is contributed by world-renowned authors in the field.

Key Features

- Provides overview of the fast-moving field of induced pluripotent stem cell technology, regenerative medicine, and therapeutics.
- Covers the following diseases: Noonan, Costello, MELAS/LEIGH, Smith–Magenis, and polycystic ovary syndromes, neurofibromatosis type 1, multiple sclerosis, chronic mountain sickness, inherited retinal dystrophies, hereditary spastic paraplegias, cardiac arrhythmias, heritable kidney disease, and more.
- Contains description of cutting-edge research on the development of disease-specific human pluripotent stem cells. These cells allow us to study cellular and molecular processes involved in several human diseases.

ELSEVIER
ACADEMIC PRESS
An imprint of Elsevier
elsevier.com/books-and-journals

ISBN 978-0-323-85765-9
9 780323 857659