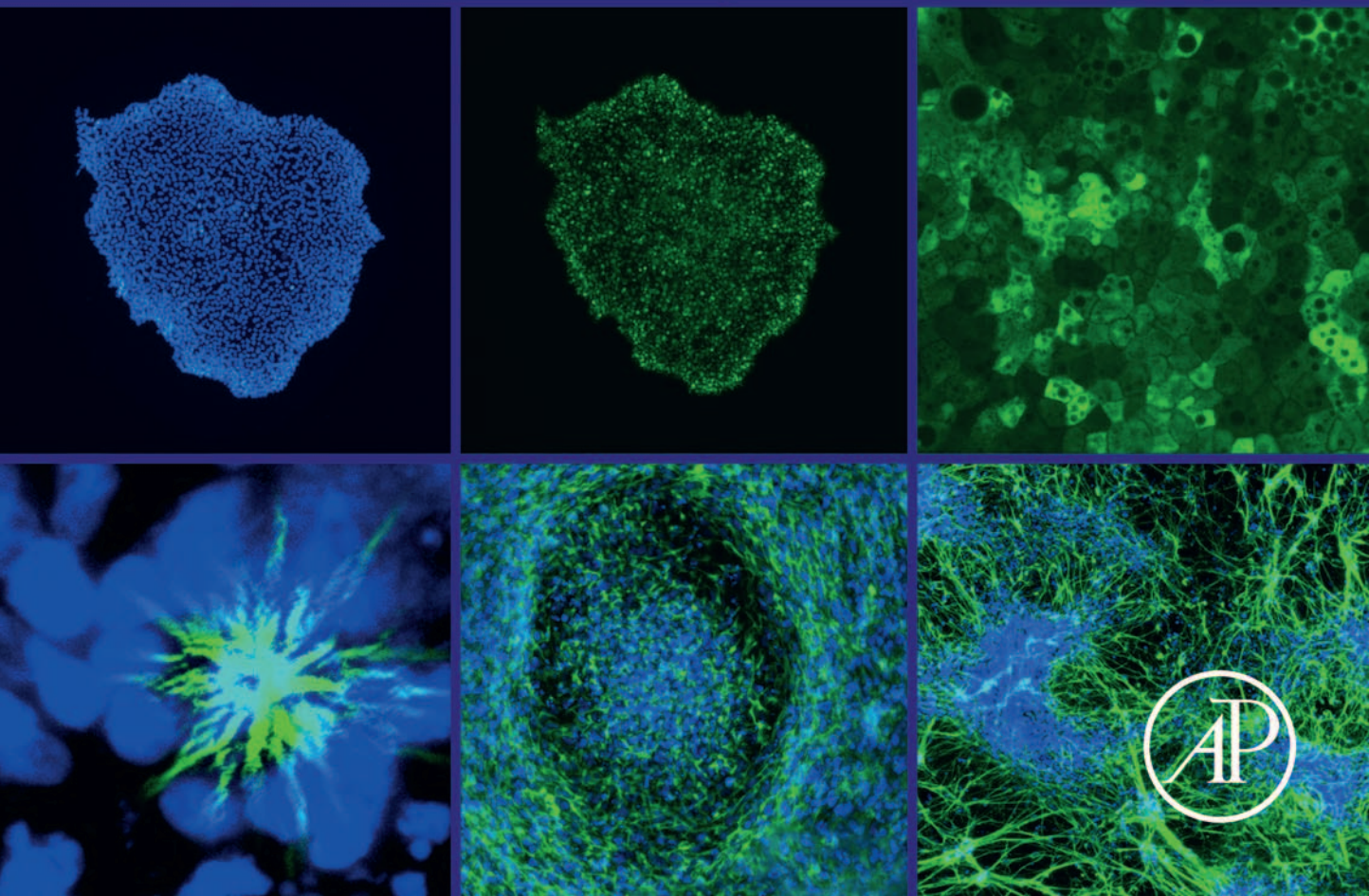
ADVANCES IN STEM CELL BIOLOGY

VOLUME 15

Novel Concepts in iPSC Disease Modeling

Edited by Alexander Birbrair



Novel Concepts in iPSC Disease Modeling, Volume 15

Advances in Stem Cell Biology

Series Editor
Alexander Birbrair

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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 beloved mom, Marina Sobolevsky, of blessed memory (July 28, 1959—June 3, 2020)

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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.*

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Preface

This book's initial title was "iPSCs: Recent Advances." Nevertheless, because of the ongoing strong interest in this theme, we were able 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 "Novel Concepts 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 iPSCs 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-the-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 iPSCs and their differentiation into several cell types, tissues, and organs using state-of-the-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of several disorders. Thus, this 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. Twelve chapters written by experts in the field summarize the present knowledge about *iPSC Disease Modeling*.

Amy L Ryan and colleagues from University of Southern California discuss iPSCs for modeling respiratory disease. N. Emmanuel Díaz-Martínez and colleagues from Center for Research and Assistance in Technology and Design of the State of Jalisco describe iPSCs for modeling metabolic and neurodegenerative disorders. Yiqin Du and colleagues from University of Pittsburgh compile our understanding of iPSCs for modeling Open-Angle Glaucoma. George J. Murphy and colleagues from Boston University School of Medicine update us with what we know about patient-specific iPSCs for modeling amyloid disease. Vasiliki Kalatzis and colleagues from University of Montpellier summarize current knowledge on modeling choroideremia with iPSCs. Kristen L. Boeshore and colleagues from National Institute of Health talk about the modeling of substance use disorders using iPSCs. Takeru Makiyama from Kyoto University Graduate School of Medicine addresses the importance of iPSCs for modeling Cardiac Sodium Channelopathies. Hung-fat Tse and colleagues from The University of Hong Kong present the modeling of Danon Disease using iPSCs. Paulina Ordonez from University of California San Diego gives an overview of iPSCs for modeling of Niemann-Pick Disease type C1. Dietmar Spengler and colleagues from Max-Planck Institute of Psychiatry introduce what we know so far about iPSCs for modeling of psychiatric

disorders. Michael G. Fehlings and colleagues from University of Toronto discuss iPSCs for Modeling of Spinal Cord Injury. Finally, Jessica Rosati and colleagues from Sapienza University of Rome focus on iPSCs for modeling functional outcomes of copy number variations in *chrna7* gene.

It is hoped that the chapters 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 Induced pluripotent stem cells: novel concepts for respiratory disease modeling

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Chapter outline

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Abstract

The past decade has witnessed the advent of induced pluripotency and methodology to direct these pluripotent stem cells to differentiate into many tissue-specific cell types, including those comprising the respiratory epithelium. Induced pluripotent stem cell-derived, (iPSC-derived), airway cells have expanded our capacity to replicate both respiratory development and disease. These relevant and reproducible models provide options to study disease-specific changes occurring in humans, which rodent models often do not readily recapitulate. Not only do these models use human cells, but they can also be patient-specific or engineered to reflect precise disease-causing mutations in controlled and isogenic cellular systems. Such advances represent a new era of precision medicine approaches for the evaluation and treatment of respiratory disease. This chapter presents a summary of recent advances in the generation of iPSC-derived cells, and their use in modeling human respiratory disease.

Keywords: Airways; Alveolar; Cystic fibrosis; Differentiation; Idiopathic pulmonary fibrosis; Induced pluripotent stem cells; Lung development; Organoids; Primary ciliary dyskinesia; Stem cells.

List of abbreviations

2-D	2-dimensional
3-D	3-dimensional
AAT	alpha-1-antitrypsin
AATD	alpha-1-antitrypsin deficiency
AAV6	adeno-associated virus 6
ABCA3	adenosine triphosphate binding cassette subfamily A member 3
ACE2	angiotensin-converting enzyme 2
AFE	anterior foregut endoderm
ALI	air–liquid interface
AM	alveolar macrophage
AQP5	aquaporin 5
ARDS	acute respiratory distress syndrome
AT1	alveolar type 1 cells
AT2	alveolar type 2 cells
ATP11A	ATPase phospholipid transporting 11A
BC	basal cells
BMP	bone morphogenic protein
C-KIT	tyrosine-protein kinase KIT (aka CD117, cluster of differentiation 117)
cAMP	cyclic adenosine monophosphate
Cas9	CRISPR associated protein 9
CC	club cells

CDX2	caudal type homeobox 2
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CHIR	CHRI99021, (a GSK-3 inhibitor)
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
COPD	chronic obstructive pulmonary disease
CPM	carboxypeptidase M
CRISPR	clustered regularly interspaced short palindromic repeats
CSF	colony stimulating factors
CXCR4	C-X-C motif chemokine receptor 4
DE	definitive endoderm
DMH-1	dorsomorphin homolog 1
DNAH5	dynein heavy chain 5
DPP9	dipeptidyl peptidase 9
DSP	desmoplakin
ECM	extracellular matrix
EGF	epidermal growth factor
EHT	endothelial-to-hematopoietic-transition
ERG	erythroblast transformation-specific (ETS)-related gene
ETV5	ATS variant transcription factor 5
FACS	flow activated cell sorting
FGF	fibroblast growth factor
FLT3	Fms like tyrosine kinase 3
FOXA2/M1/P2	forkhead box A2/M1/P2
GATA6	GATA binding factor 6
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GSK	glycogen synthase kinase
GWAS	genome-wide association studies
HAECs	human airway endothelial cells
HOPX	homeodomain-only protein homeobox
HOXA5/9/10	Homeobox A5/9/10
HPS1	Hermansky-pudlak syndrome 1 protein
HSC	hematopoietic stem cells
iAT2	induced AT2 cell
IBMX	3-isobutyl-1-methylxanthine
Id2	inhibitor of differentiation 2
IL	interleukin
ILD	interstitial lung diseases
IM	interstitial macrophage
IPF	idiopathic pulmonary fibrosis
iPSC	induced pluripotent stem cells
IRF7	interferon regulatory factor 7
JAK2	janus kinase 2
KGF	keratinocyte growth factor (or FGF7)

KLF4	krüppel like factor 4
KRT	cytokeratin
MCC	multiciliated cells
MIXL1	mix paired-like homeobox 1
MUC5B	mucin 5B
NANCI	NKX2.1 associated noncoding intergenic RNA
NDA	deoxyribonucleic acid
NGFR	nerve growth factor receptor
NKX2.1	NK2 homeobox 1
OBFC1	oligonucleotide-binding fold-containing protein 1
PAP	pulmonary alveolar proteinosis
PAX8	paired box 8
PC	phosphatidylcholine
PCD	primary ciliary dyskinesia
PDPN	podoplanin, aka T1alpha
PG	phosphatidylglycerol
PNEC	pulmonary neuroendocrine cells
POU5F1 (OCT4)	octamer-binding protein 4
PRR	pattern recognition receptor
PSC	pluripotent stem cells
RA	retinoic acid
RB	retinoblastoma protein
RNP	ribonucleoprotein
RUNX1	runt-related transcription factor 1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SCF	stem cell factor
SERPINA1	serpin family A member 1
SFTPC	surfactant protein C
Shh	sonic hedgehog
SMAD	similarity to drosophila gene mothers against decapentaplegic (MAD)
SOX2/17	SRY (sex determining region Y)-box 2/17
SPI1	transcription factor PU.1
STAT3	signal transducer and activator of transcription 3
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TGFβ	transforming growth factor beta
TMPSSR2	transmembrane protease serene 2
TOLLIP	toll interacting protein
TP63	tumor protein 63
TPO	thrombopoietin
vCC	variant club cells
VEGF	vascular endothelial growth factor
Wnt	wingless/int family
WT	wild-type

Introduction

While other diseases, such as cardiovascular disease, have seen a regression in morbidity and mortality rates due to an increased number of therapeutic approaches successfully reaching the clinic, respiratory disease rates continue to increase. Despite numerous promising therapeutics emerging from preclinical trials, respiratory disease has far fewer new drugs eventually being approved for patient use. One contributing factor is the limited number of disease and patient-specific model systems available to study human respiratory disease onset and progression. Murine models have provided some of the most informative data on lung development, reviewed in Rawlins and Perl (2012). For example, lineage tracing of SRY (sex-determining region Y)-box 2 (SOX2) helped to elucidate its role in lung budding and differentiation of tracheal mesenchyme and epithelium (Que et al., 2009). Unfortunately, for many respiratory diseases, differences in human and rodent lung structure and function reflect in models that do not fully capture disease phenotypes as they occur in humans. Models of cystic fibrosis (CF) in mice, for example, complete knockouts (O'Neal et al., 1993; Snouwaert et al., 1992), ΔF508 mutants (Colledge et al., 1995; van Doorninck et al., 1995), G551D mutants (Delaney et al., 1996; Semaniakou et al., 2018), variably represent the human manifestation of the disease (reviewed extensively in McCarron et al., 2018; Wilke et al., 2011). Similarly, idiopathic pulmonary fibrosis (IPF) models, often induced by intratracheal instillation of bleomycin in rodents (Antoniu and Kolb, 2009; Carrington et al., 2018; Williamson et al., 2015), share dissimilar pathogenesis, and vary in detectable alveolar cell death and inflammation. While characterized by the development of fibrotic foci, the distribution of fibrosis differs from that in humans (reviewed in Williamson et al., 2015). Such differences in the pathophysiology of IPF in rodent models are likely one of the reasons for a complete absence of therapeutics available to increase survival in patients with IPF (Raghu et al., 2011). While some lung diseases are better recapitulated in larger animal models, such as pig and ferret models of CF (Li et al., 2006; Meyerholz et al., 2018; Pezzulo et al., 2012; Rogers et al., 2008a; Rosen et al., 2018; Sun et al., 2010; Welsh et al., 2009; Xie et al., 2018), these are costly models to develop and are therefore not suitable for high throughput studies. While these models may increase our knowledge of disease onset and pathogenesis, the value of such models in translating therapeutics successfully into the clinic is still largely unknown. Establishing a reproducible human and patient-specific model that is sustainable and scalable has the potential to accelerate therapeutic development for lung disease, particularly for rare lung diseases, is likely a valuable addition to the tool-kit for studying human lung disease pathogenesis.

Primary patient cells fulfill some of the criteria for modeling human lung disease ex vivo. Unfortunately, access to patient cells is restricted to the availability of explant lungs from patients with end-stage disease or small samples which can be obtained by bronchoscopy, limiting the number of cells that can be acquired. In vitro expansion of primary lung basal cells (BCs) while retaining their multipotent

stem cell phenotype is limited, recent advances in the field are starting to elucidate methods for more robust long-term expansion of lung basal stem cells (Mou et al., 2016; Reynolds et al., 2016). Access to cells representing rare lung diseases for in vitro assays remains a significant limitation due to the low number of patients available for cell procurement. The discovery of human-induced pluripotency (induced pluripotent stem cells [iPSCs]) by the laboratories of Yamanaka and Thomson in 2007 provided a new technology that could be utilized for disease modeling in human cells, with the capacity to understand disease development and pathogenic mechanisms (Okita et al., 2007; Takahashi et al., 2007a,b; Yu et al., 2007). Directed differentiation of iPSCs can recapitulate key embryonic milestones in the development of the human lung to generate cells of the airway epithelium. Crucial studies of lung development in mice allowed us to understand some of the signaling pathways that direct lung organogenesis (Bellusci et al., 1997; Chung et al., 2018; Lu et al., 2001; Mucenski et al., 2003; Okubo and Hogan, 2004; Weaver et al., 1999, 2000; Zhang et al., 2007, 2008). Protocols involving the sequential and temporal addition of factors regulating activin/nodal, bone morphogenic protein (BMP), fibroblast growth factor (FGF), transforming growth factor bets (TGFβ), wingless (Wnt), and sonic hedgehog (Shh) signaling to pluripotent cells have evolved from early studies generating a relatively immature lung epithelium (Firth et al., 2014; Ghaedi et al., 2013; Green et al., 2011; Huang et al., 2014; Wong et al., 2012), to recent advances where functional cells can be generated and used for effective disease modeling (Ghaedi and Niklason, 2019; Happle et al., 2018; Hurley et al., 2020; Leibel et al., 2019; McCauley et al., 2017; Sahabian et al., 2019; Strikoudis et al., 2019). This chapter focuses on discussing some of the most recent advances in utilizing iPSC-derived cells to model human respiratory disease (Table 1.1).

Table 1.1 Key studies progressing toward obtaining autologous lung cells for cell therapy and precision medicine screening.

Key study features	Year	References
Generation of anterior foregut endoderm form iPSC	2011	Green et al. (2011)
First manuscript to describe differentiation of iPSC to CFTR expressing airway epithelium	2012	Wong et al. (2012)
Generation of proximal airway epithelium inclusive of abundant multiciliated cells	2014	Firth et al. (2014)
Gene correction of CFTR in iPSC-derived epithelium	2015	Crane et al. (2015), Firth et al. (2015)
Generation of airway progenitor cells from iPSC	2015	Huang et al. (2015)
Generation of lung bud organoids from iPSC	2015	Dye et al. (2015)
Multiciliated cell differentiation in iPSC-derived spheroids	2016	Konishi et al. (2016)
Dual inhibition of SMAD signaling enables long-term ex vivo expansion of epithelial basal cells	2016	Mou et al. (2016)

Table 1.1 Key studies progressing toward obtaining autologous lung cells for cell therapy and precision medicine screening.—*cont'd*

Key study features	Year	References
Isolation of NKX2.1 expressing lung progenitor cells from iPSC using CD47hi/CD26lo signature	2017	Hawkins et al. (2017)
Temporal regulation of Wnt signaling efficiently generated functional airway epithelium from iPSC	2017	McCauley et al. (2017)
Differentiation of iPSC to alveolar epithelial cells	2017	Jacob et al. (2017)
Single cell fate trajectories define lineage plasticity of iPSC-derived distal lung progenitors	2020	Hurley et al. (2020)
Derivation of basal stem cells from human pluripotent stem cells	2021	Hawkins et al. (2021)

CD26, *cluster of differentiation 26, also known as dipeptidyl peptidase-4 (DPP4) or adenosine deaminase complexing protein 2;* CD47, *cluster of differentiation 47, also known as integrin associated protein (IAP);* CFTR, *cystic fibrosis transmembrane regulator;* iPSC, *induced pluripotent stem cells;* NKX2.1, *NK2 homeobox 1, also known as thyroid transcription factor 1 (TTF-1);* SMAD, *similarity to drosophila gene Mothers Against Decapentaplegic;* Wnt, *wingless.*

Recapitulating lung development using induced pluripotent stem cells

Among the many organs that arise from the endoderm, the lung has received considerable attention over the past few years, driven by a need to develop better disease models specific to the human respiratory system. Although in depth, lineage-tracing studies have elegantly demonstrated stages of fetal lung development in rodents, human lung development is less well-understood and rodent signaling pathways guiding lung development are not necessarily conserved humans (Bellusci et al., 1997; Hogan et al., 1997; Lu et al., 2001; Morrisey, 2018; Mucenski et al., 2003; Rawlins et al., 2008; Stabler and Morrisey, 2017; Swarr and Morrisey, 2015; Wang et al., 2013; Weaver et al., 2000; Zhang et al., 2007). Consequently, there is an incomplete understanding of the underlying mechanisms involved in early human fetal lung development. In humans, the respiratory epithelium of the lung differentiates from the anterior foregut endoderm (AFE) and the splanchnic mesoderm gives rise to the pulmonary mesenchyme. Lung development begins during weeks 4–7 of embryonic development when Wnt2/Wnt2b expressing cells in the ventral anterior mesoderm are thought to interact with the cells of the AFE (Goss et al., 2009). Additionally, BMP4 expressed in the surrounding mesenchyme, inhibits SOX2 expression in these early progenitor cells which concomitantly increases NK2 homeobox 1 (NKX2.1) expression, a key transcription factor that specifies thyroid and lung development (Domyan et al., 2011). The embryonic stage is followed by the fetal period consisting of the pseudoglandular stage (weeks 5–17), the canalicular stage (weeks 16–26), and finally the saccular stage (weeks 24 to birth). Lung alveolarization occurs during the postpartum period and continues throughout childhood (weeks 36 up to 8 years Narayanan et al., 2012). The lung is partitioned

into two core regions, the airways and parenchyma. The airways form the conduits between the outside world and the primary gas exchanging unit, the alveoli. The conducting airways comprise of the trachea which divides into two primary bronchi that enter the lung at each hilus. The primary bronchi then branch repeatedly into smaller bronchi and bronchioles eventually enter a terminal bronchiole. Distal airways comprise of those with a diameter of less than 2 mm leading to the terminal bronchioles and the alveolar ducts (Jain and Sznajder, 2007).

In 2007, Takahashi and Yamanaka discovered that a combination of four transcriptions factors (POU5F1 (OCT4), krüppel like factor 4 (KLF4), c-Myc and SOX2) could be used to reprogram somatic, differentiated cells into pluripotent cells akin to embryonic stem cells (Takahashi et al., 2007a,b). These cells are known as iPSCs and can be generated from any accessible and proliferating cell source including fibroblasts, keratinocytes, and peripheral blood mononuclear cells (Aasen et al., 2008; Loh et al., 2009; Takahashi et al., 2007b). iPSCs have the same unique properties as embryonic stem cells including a seemingly unlimited self-renewal capacity and differentiation into all three germ layers. The differentiated progeny is attractive for the creation of disease-specific models enabling analysis of complex cellular and molecular interactions occurring during human lung development and disease manifestation and progression, in addition to being a promising source of cells for tissue regeneration. iPSCs can be patient-specific and, therefore, provide an autologous cell source that is genetically identical to the patient, allowing for the potential to engraft cells or tissue generated from these cells while avoiding the need for immune suppression. Specific differentiation of iPSCs toward cells comprising the adult lung follows a stepwise strategy striving to mimic the above-mentioned stages of lung development and maturation in vitro. This results in compression of the timeline from months to days in culture. This challenge is not trivial and over the past 10 years methodology has been established and protocols refined to generate cells and structures that resemble the human adult conducting airways and more distal alveolar cells (Dye et al., 2015; Firth et al., 2014; Hawkins et al., 2017; Huang et al., 2015; Jacob et al., 2017; Konishi et al., 2016; McCauley et al., 2018; Wong et al., 2012). These methodologies will be discussed in more detail below, and lung development is summarized in Fig. 1.1.

Early lung specification: definitive endoderm and anterior foregut endoderm

Definitive endoderm (DE) consists of the first cells in the developing foregut that can give rise to tissues of the lung, liver, pancreas, and thyroid (Cardoso and Kotton, 2008). Induction of Nodal signaling through addition of Activin A in pluripotent stem cells (PSCs) is able to robustly induce expression of DE-associated genes, including primitive streak marker, MIXL1 (mix paired-like homeobox 1), transcription factors, SRY-box 17 (SOX17) and forkhead box A2 (FOXA2) and cell surface markers, cellular homolog of the feline sarcoma viral oncogene v-kit (c-KIT), and C-X-C motif chemokine receptor 4 (CXCR4) (Green et al., 2011). This step

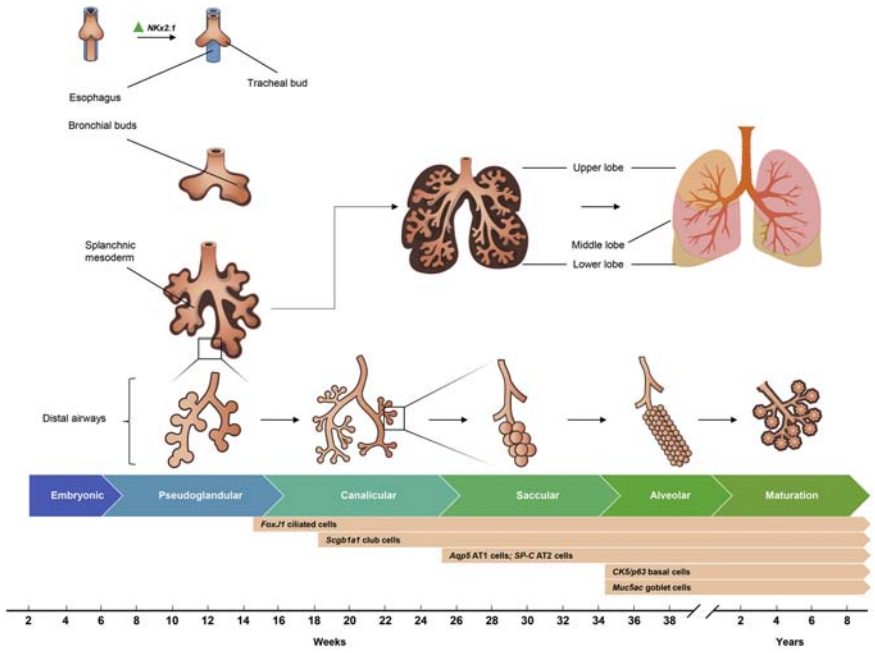

FIGURE 1.1 Timeline of lung development showing the developmental stages and key events.

During the embryonic stage, cells on the ventral side of the AFE begin to express NKX2.1, a key marker that specifies lung and by the end of this stage, the trachea has completely separated from the esophagus forming two tracheal buds. During the pseudoglandular stage, the tracheal buds begin to form thousands of terminal bronchioles in a highly ordered process called branching morphogenesis. Finally, during the canalicular and saccular stages, these terminal branches narrow further forming epithelial sacs that will ultimately develop into alveoli and mature during the alveolarization stage. Highlighted above the timeline are key genes that identify cells at the specific stages.

efficiently generates >95% DE cells; however, cell surface markers can be used to purify DE further via fluorescence-activated cell sorting (FACS) (Diekmann et al., 2017). Subsequent generation of AFE, the most rostral part of the endoderm has proven more challenging. AFE is critical to generating trachea and lung tissue which rise from the caudal region of the AFE (Que et al., 2006). Through examination of the morphogens that were able to augment anterior endoderm, associated with reemergence of pluripotency factor SOX2 and suppression of posterior endoderm markers such as caudal type homeobox 2 (CDX2), Green and colleagues were able to define a cocktail comprising Noggin, an inhibitor of BMP signaling, and SB-431542, an inhibitor of Activin/Nodal and TGFβ signaling to support $SOX2^{+}$, $CDX2^{-}$, and $FOXA2^{+}$ AFE (Green et al., 2011). PSCs can also be advanced through AFE using a three-dimensional (3-D) organoid differentiation protocol where similarly inhibition of BMP/TGFβ signaling pathways, with the simultaneous

stimulation of Wnt and FGF signaling, is able to favor specification of SOX2 expressing AFE over an intestinal fate (Dye et al., 2015). While these protocols are reproducible, improvements are still needed to refine the efficiency of AFE generation through tight regulation of the concentration and temporal application of exogenous cytokines and growth factors.

Generation of the NKX2.1 expressing primordial lung progenitor cells

As mentioned above, the adult lung comprises of a complex branched network of proximally located conducting airways and the parenchyma comprising of more distally located smaller airways and the alveolar air spaces. During branching morphogenesis in the human fetal lung, bud tip progenitor cells are rapidly proliferating cells found at the branch tips that are able to give rise to both proximal airway cells during the earlier branching stages and to alveolar cells at later branching stages (Rawlins et al., 2009). All cells must first progress through a primordial progenitor stage defined by the upregulation of the ventral marker NKX2.1 (homeodomain-containing transcription factor). NKX2.1 is the earliest known transcription factor associated with commitment to thyroid and lung lineages, occurring around 28 days gestation in human development (Goss et al., 2009; Herriges et al., 2014; Yuan et al., 2000). The mesenchyme plays a critical role in lung development providing key morphogens, such as FGFs and Wnts, demonstrated in mouse models of lung development where cell lineages are traced in transgenic mice (Goss et al., 2009; Harris-Johnson et al., 2009). FGF signaling followed by induction of sonic hedgehog signaling (Shh) has been shown to correlate with induction of NKX2.1 in lung progenitors emerging from the foregut endoderm (Hebrok et al., 1998; Morrisey and Hogan, 2010; Rankin and Zorn, 2014). Similarly, in foregut spheroids generated from developing human embryonic bud tips, or from human iPSCs, addition of FGF2 is able to induce NKX2.1, in addition to both Shh and thyroid lineage marker, paired box-8 (PAX8) (Miller et al., 2018). Through systematic evaluation, FGF and hedgehog signaling pathways Shh was shown to be downstream of FGF2 signaling and capable of selectively activating NKX2.1 without concomitant specification of $PAX8^{+}$ thyroid cells (Miller et al., 2018). Similar induction of lung progenitors was also achieved in 2-D cultures by a number of research groups (Firth et al., 2014; Huang et al., 2014; Wong et al., 2015). These initial protocols used several combinations of FGFs (Abo et al., 2020; Antony et al., 2013; Bartoszewska et al., 2017), BMP4, and CHIR (a glycogen synthase kinase (GSK) 3 inhibitor which induces Wnt signaling) to stimulate proximal lung progenitor cell induction from AFE, all succeeded with variable efficiencies across multiple iPSC lines. To date, the efficient specification of lung NKX2.1 expressing progenitor cells remains highly variable, a problem that can be circumnavigated by purification of the lung progenitor cells. Through a prospective analysis of green fluorescent protein (GFP)-tagged NKX2.1 expressing cells, a cell surface signature of $CD47^{hi}$ and $CD26^{lo}$ was found to be capable of selecting NKX2.1 expressing lung progenitor

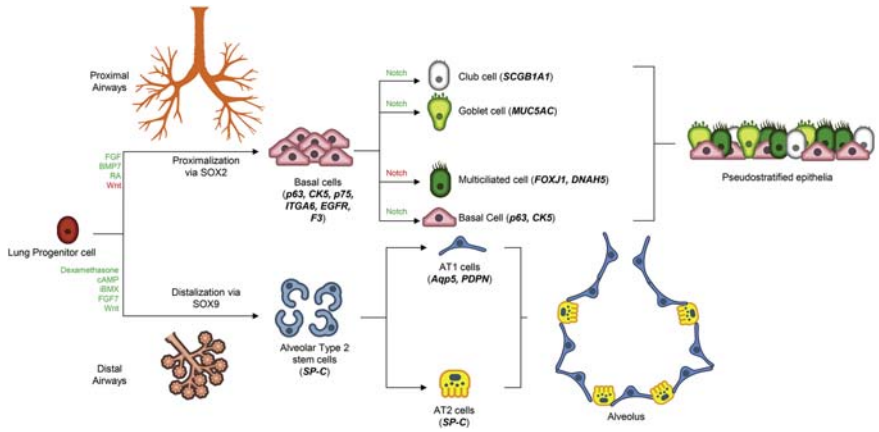

FIGURE 1.2 Differentiation of iPSC-derived lung progenitor cells to mature airway cells.

Differentiation pathways strive to follow that of airway development. iPSCs can be induced into proximal or distal fates through the activation (green) or inhibition (red) of the identified signaling pathways. Basal cells, Club cells, Goblet cells, multiciliated cells, AT1, and AT2 cells are all shown in the differentiation process toward a pseudostratified proximal airway epithelium or an alveolar epithelium. Key genes specific to each cell type are indicated in parentheses after the cell name.

cells with over 90% efficiency (Hawkins et al., 2017). In addition, membrane-bound enzyme Carboxypeptidase M (CPM) is expressed on NKX2.1$^+$ AFE cells, and also able to purify lung progenitor cells from directed lung differentiation of iPSCs. CPM is also capable of identifying the same cells during human and murine embryonic development (Gotoh et al., 2014). These sorted cells are able to differentiate to multiciliated proximal airway epithelium and distal alveolar epithelium, in both 3-D organoid structures and cells at the air–liquid interface (ALI) (Gotoh et al., 2014; Konishi et al., 2016). Interestingly, at the pseudo-glandular stage of fetal human lung development, epithelial progenitors at the tips have been shown to coexpress SOX2 and SOX9 (Danopoulos et al., 2018), differing from those originally identified as being *Sox9*$^+$ and *Id2*$^+$ in mice (Nikolic et al., 2017). Using 3-D in vitro models, these cells have been shown to be critical for proximal-distal patterning and lung branching in the human lung (Nikolic et al., 2017). Divergence of proximal and distal lineage fates of iPSC-derived lung progenitor cells will be discussed in more detail below and summarized in Fig. 1.2.

Specification of proximal airway basal stem cells

BCs are self-renewing populations of epithelial progenitor cells capable of differentiating into all the cells comprising of the surface airway epithelium. As such, they are regarded as key stem cells of pulmonary epithelium and are a desirable cell to generate from iPSC-differentiation methods. BCs can be expanded, although to a

limited capacity, in vitro and are capable of both self-renewal and differentiation. Several studies have demonstrated the generation of functional airway epithelium derived from PSCs (Dye et al., 2015; Firth et al., 2014; Konishi et al., 2016; McCauley et al., 2017; Wong et al., 2012); however, currently none have purified a self-sustaining culture of BCs akin to primary human or murine counterparts. Methods are robustly established for the isolation and expansion of primary airway BCs in vitro (Fulcher and Randell, 2013; Mou et al., 2016), and these primary cells are used routinely in human lung biology research. BCs are chiefly characterized through expression of tumor protein 63 (TP63) and cytokeratin 5 (KRT5) (Warner et al., 2013); however, a number of recent studies have indicated a potential heterogeneity in BCs with subpopulations existing within the proximal airways (Carraro et al., 2020a; Deprez et al., 2020; Duclos et al., 2019) with the putative multipotent BC expressing nerve growth factor receptor (NGFR) and KRT15. The precise identity and function of the other subpopulations is currently being investigated and may provide valuable insight into airway regeneration in response to disease and aging (Carraro et al., 2020b). In situ BCs are located typically beneath the pseudostratified epithelium of upper airway structures lining the basolateral membrane. In lineage traced mouse models, TP63 activity is present early during fetal development and key in generating NKX2.1-specific lung progenitor cells. However, these cells lack the genetic profile of BCs in adult tissues and are regarded as basal progenitors. Isolating BCs is challenging as the molecular pathways that control their specification and maintenance of multipotency are not well established. Studies have shown that FGF10 may help to maintain BCs by inhibiting differentiation to mature epithelium (Volckaert et al., 2013), though it is important to note that these studies are predominantly based on information in rodent models whose signaling pathways and cellular interactions can be different to that of human tissues (Danopoulos et al., 2019). Specification of iPSC-derived BCs from lung progenitors likely involves temporal regulation of Wnt signaling found to be high in human lung progenitors (McCauley et al., 2017). Additionally, TGFβ/BMP/similarity to drosophila gene mothers against decapentaplegic (SMAD) pathway signaling is suppressed in multipotent TP63 expressing BCs compared to much higher activity in both luminal and suprabasal cells in the trachea: dual inhibition of SMAD with DMH-1 (dorsomorphin, a selective inhibitor of BMP signaling) and A-8301 (TGFβ kinase/activin receptor-like kinase inhibitor) was recently shown to be important in the sustained expansion of a multipotent BC in vitro (Mou et al., 2016). Although several studies have demonstrated generation of basal cells (Dye et al., 2015; Firth et al., 2014; Konishi et al., 2016; McCauley et al., 2017; Wong et al., 2012), it was only recently that the first study was published in which BCs were able to be purified and expanded (Hawkins et al., 2021). In this study, Hawkins and colleagues expanded their original differentiation protocol pushing BC-specific differentiation of purified lung progenitors in 3-D spheroid cultures. Using a TP63-TdTomato reporter iPSC line, the NKx2.1-expressing lung progenitor cells can be pushed to a proximal fate in the presence of FGF2, FGF10, cAMP, dexamethasone, and IBMX, BCs are then specified and matured in the presence of

commercially available pneumacult Ex+ media supplemented with DMH1 and A8301 (dual SMAD inhibition) and rho-A kinase inhibition (ROCKi) (Hawkins et al., 2021). Over a period of 40+ days, the spheroids upregulated nerve growth factor expression (NGFR) while maintaining TP63 expression and these cells could be dissociated and successfully differentiated at the ALI (Hawkins et al., 2021). Differentiation resembled that of primary cells and generated functional multiciliated cells. As a final proof-of-principle, the authors also demonstrated differentiation upon engraftment into a tracheal xenograft. It is important to note that this protocol expands the BCs in a 3-D culture, when the BCs are placed in a more traditional 2-D culture system they tend to undergo a phenotypic switch and adopt a more squamous epithelial phenotype, more akin to high passaged primary BCs (unpublished data, Ryan laboratory). To understand the differences between primary and iPSC-derived BCs, a complete characterization of iBCs and their primary human counterparts is still ongoing and will be critical in determining their utility in both modeling disease and their potential for regenerative cellular therapy. There is still a need to address fundamental knowledge-gaps in lung epithelial stem/progenitor cell biology to develop successful gene-editing and cell-based therapies for lung disease. Such knowledge gaps include precise identification and functional understanding of endogenous stem/progenitor cell populations of the airway epithelium, including the regional-specific cues that control their undifferentiated state. Furthermore, there are no studies that have yet considered the impact of epigenetic regulation on BC stemness and fate decisions, this may be particularly important when comparing iPSC-derived to primary airway-isolated BC which have very different environmental exposures likely regulating cellular function.

Specification of distal airway alveolar type 2 stem cells

The alveolar epithelium comprises of two specialized cell types: alveolar type 1 (AT1) cells, elongated cells covering over 95% of the alveolar surface to facilitate gas exchange into the capillaries, and alveolar type 2 (AT2) cells, smaller cuboidal cells that produce and secrete pulmonary surfactant to reduce surface tension. These two cell types form a protective epithelial barrier that allows for the passive leakage of fluid between the interstitium and vasculature, and the alveolar space. During development, a bipotent progenitor cell expressing both At1 and AT2 cell markers is able to give rise to both mature cell types (Desai et al., 2014; Treutlein et al., 2014) AT1 cells are generally considered terminally differentiated although, under specific conditions such as pneumonectomy, some plasticity had been observed in which a dedifferentiation of AT1 cells to AT2 cells may occur (Jain et al., 2015). AT2 cells are typically considered the stem cell of the distal airways, with the capacity to differentiate and replenish damaged AT1 cells (Whitsett and Weaver, 2002). In embryogenesis, AT2 cell differentiation begins at gestational week 24 in humans, derived from the endoderm. NKX2.1 remains a critical transcription factor in alveolar morphogenesis by regulating key functional properties of differentiated alveolar cells, for example, the synthesis of surfactant proteins (DeFelice et al., 2003).

Additionally, NKX2.1-associated noncoding intergenic RNA (NANCI), which was recently identified as a long noncoding RNA required for AT2 differentiation, is also regulated by NKX2.1 (Herriges et al., 2017). In vitro studies of AT2 cells have been limited due to our capacity to isolate and expand them in culture. AT2s poorly proliferate ex vivo and differentiate into podoplanin (PDPN/T1α), aquaporin 5 (AQP5), and homeodomain-only protein homeobox (HOPX) AT1-like cells within 4−7 days of isolation from human lung tissues (Bove et al., 2014; Liebler et al., 2016; Marconett et al., 2014; Sucre et al., 2018; Wang et al., 2018a). Adapted protocols for more successful culture have included evaluating the addition of dexamethasone, cyclic adenosine monophosphate (cAMP), growth factors (such as keratinocyte growth factor, KGF/FGF7), and 3-D culture; none of these, however, provides an ideal system for ex vivo sustainment of AT2 cells (Borok et al., 1998; Isakson et al., 2001; Qiao et al., 2008). Most recently new serum and feeder-free protocols for the 3-D expansion of AT2 cells have been developed, capitalizing on data showing that inhibition of BMP signaling can prevent AT2 cell differentiation (Chung et al., 2018; Katsura et al., 2020; Salahudeen et al., 2020). While enhancing the capacity to study primary AT2 cells ex vivo, the techniques are still challenging and limited to available donor lungs. Directed differentiation of iPSCs to generate AT2s de novo would provide increased opportunity to study normal human AT2 development and to understand the pathogenesis of alveolar diseases (Korogi et al., 2019; Yamamoto et al., 2017).

Recently, scientists have reported methodology to generate AT2 cells from iPSC-derived lung primordial lung progenitor cells (Ghaedi et al., 2013, 2014; Gotoh et al., 2014; Jacob et al., 2017; McCauley et al., 2017). After induction of the NKX2.1, primordial progenitor cells are cultured as 3-D spheroids in Matrigel and distal/alveolar differentiation was performed in "CK+DCI" medium, consisting of a custom serum-free differentiation media base (cSFDM) supplemented with CHIR99021, KGF, dexamethasone, cAMP, and 3-Isobutyl-1-methylxanthine (IBMX). Complex and temporal regulation of cellular signaling, specifically Wnt signaling (McCauley et al., 2017) and gene transcription networks, is involved in the fate decisions of lung progenitor cells with NKX2.1, FOXA family members, KLF5, GATA6, STAT3, ETV5, FOXM1, and FOXP2 genes all interact in gene networks active during the differentiation of AT2 cells (Whitsett et al., 2019). Development of robust protocols has been greatly enhanced through the generation of a dual NKX2.1-GFP and surfactant protein C (SFTPC)-TdTomato reporter iPSC line (Jacob et al., 2017) enabling purification of putative AT2 cells through sorting. Such purification would be otherwise challenging due to a current lack of specific cell surface markers for AT2 cells. Using this reporter, putative AT2 cells have been isolated and successfully expanded long term in 3-D spheroid cultures (Jacob et al., 2019). Other protocols for 3-D expansion of iPSC-derived spheroids have also been developed with varying levels of AT2 cell maturity and differentiation capacity observed (Gotoh et al., 2014; Yamamoto et al., 2017, 2020). The restricted differentiation capacity of iPSC-derived AT2 cells to AT1 cells may, in part, be due to the lack of an air interface within the 3-D cultures. Through use of a rotating bioreactor,

differentiation of iPSC-derived AT2 cells to AT1 cells was stimulated: this system creates an ALI providing a cellular microenvironment more akin to the human lung (Ghaedi et al., 2014). Other studies have generated AT2 cells through development of more complex organoids, generating branching-like structures (Chen et al., 2017); analysis of these organoids alongside human lung fetal tissues suggested that the cells present resembled the second trimester of development more closely than a mature alveolar cell. A greater understanding of the differentiation trajectories of NKX2.1 expressing primordial lung progenitors to AT2 cells has highlighted a cellular plasticity that can be regulated during the earlier fate decisions to produce a more stable AT2 phenotype (Hurley et al., 2020). AT2 dysfunction underlies the pathogenesis of several lung diseases, and we will discuss surfactant disorders resulting in neonatal respiratory distress and early onset interstitial lung disease and IPF later in this chapter (Gong et al., 2004; Hong et al., 2017; Katzen et al., 2019; Kinting et al., 2018; Kroner et al., 2017; Peca et al., 2011; Rindler et al., 2017; Schindlbeck et al., 2018).

Specification of pulmonary neuroendocrine cells

Differentiation to some of the rarer cell types in the lung, including pulmonary neuroendocrine cells (PNECs) and ionocytes, has not been a primary focus of many of the pioneering studies using iPSC to study lung disease and development. The rarity of these cells in the human lung and a lack of specific cell surface markers for their isolation had made them virtually impossible to study ex vivo. As these cells become of increased interest in lung developmental biology and disease, studies are starting to emerge, demonstrating the successful differentiation to these infrequent cells. iPSC-derived PNECs have been shown sporadically in several of the original airway differentiation protocols; however, it was not until the last year that iPSC-derived PNECs were generated. The first study focused on the generation of PNEC as the putative precursor cell for small cell lung cancers and generated PNECs with retinoblastoma protein (RB) and TP53 mutations induced (Chen et al., 2019). A subsequent study focused on the specification of PNECs through inhibition of Notch signaling and culture at the ALI (Hor et al., 2020). These cells expressed PNEC specific markers and were transcriptomically similar to primary fetal PNEC's (Hor et al., 2020). iPSC generation of cell types such as PNECs allows for the potential of an indefinite cell pool of these otherwise incredibly rare primary cell populations. Purification, expansion, and direct functional comparison of induced PNECs to primary airway PNECs remain a challenge to the field.

Cellular plasticity in iPSC-derived airway progenitors

Despite specification of cells which are transcriptomically and functionally similar to their primary counterparts, there is still notable plasticity in the differentiated iPSC progeny. Hurley et al. (2020) used single-cell RNAseq analysis to determine cell fate trajectories identifying plasticity in iPSC differentiation that leads to lung

and nonlung endodermal cell fates. At day 17 of differentiation toward AT2 cells, the iPSC-progenitors showed 4 distinct cell clusters based on expression of identity marker genes for the distal lung epithelium, nonlung endoderm, and 2 smaller clusters of pulmonary neuroendocrine cells and gut (Hurley et al., 2020). These data suggest that maintaining a lung-fate throughout differentiation is perhaps more complex and challenging than initially presumed. As mentioned above, NKX2-1 is a core transcription factor regulating lung specification, its expression is unstable in iPSC-derived lung progenitor cells and can be lost in a subset of cells in which Wnt target genes LEF1, NKD1, and AXIN2 become upregulated deviating cells away from the lung lineage (Hurley et al., 2020). As such, inhibition of the Wnt pathway has been pursued as a means to reduce the percentage of cells deviating from lung fate. Downregulation at the NKX2.1^{+} primordial progenitor stage (iPSC day 15) induces proximal airway patterning, while downregulation in the later stages of differentiation is associated with distal lung maturation. Withdrawal of Wnt activator, CHIR, beginning on differentiation day 17 of differentiation is able to specify a distal lung epithelial fate, maintaining NKX2-1, and acquiring SFTPC expression. Temporal regulation of NKX2.1 expression is, therefore, a critical factor in determining both lung and nonlung fate. When sorting by NKX2-1 at day 30 of differentiation, for example, a mixed population of lung and nonlung cells results. All of which have the capacity to eventually form epithelial spheres with alveolar specification. This suggests a time-dependent loss of plasticity and stabilization or restriction of the cell lineage in such iPSC models (Hurley et al., 2020).

In vivo, cell fate decisions are largely regulated by the immediate cellular environment in addition to responding to morphogens secreted by neighboring cells. To date, the direct role of the cellular microenvironment, or niche, in specifying iPSC fate decisions and sustaining stem cell function has been largely overlooked in current protocols and, from our understanding of lung development and disease pathogenesis, is a critical component in defining cellular function. Cell: cell and cell: matrix interactions are important considerations when considering the stability of airway stem cells and their potential for functional airway restoration to establish effective mucociliary clearance (reviewed extensively in Burgstaller et al., 2017; Meiners et al., 2018). With specific reference to the performance of iPSC-derived lung stem cells, Ghaedi et al. (2014) have shown that the culture matrix used for ALI differentiation has a substantial impact on the expression of key AT2 cell markers in iPSC-derived AT2 cells. They compared several extracellular matrices (ECMs) including collagen IV, collagen I, fibronectin, Matrigel, and native human lung ECM (Aasen et al., 2008). iPSC-derived AT2 cells expressed higher levels of pro-surfactant protein C (SFTPC), surfactant protein A (SPC), and surfactant protein B (SPB) when cultured on collagen IV, a prevalent matrix protein in the alveolar basement membrane (Ghaedi et al., 2014). In addition, the authors found that when the cultures were continuously and alternately exposed to media and air, iPSC-derived AT2 cells are able to differentiate to become AT1-like as indicated by expression of aquaporin 5 (AQP5), podoplanin (PDPN), and caveolin-1 (Ghaedi et al., 2014). Several groups have evaluated cellular engraftment through repopulation of

decellularized lung scaffolds with cells representing a variety of iPSC-derived lung progenitor cells (Ghaedi et al., 2018; Gilpin et al., 2014). In these models, the cells have variable differentiation capacity and phenotype compared to traditional culture techniques; however, overall epithelial gene expression in the scaffold cultures is generally much lower than that of native lung samples (Ghaedi et al., 2018; Gilpin et al., 2014). iPSC-derived AT2 and airway epithelium progenitors are able to attach and differentiate on acellular human and rat lung scaffolds, diffusing into both the alveolar structures and conducting airways (Ghaedi et al., 2018; Gilpin et al., 2014). On human scaffolds, in the airways, differentiation to secretory and ciliated cells was confirmed by expression of club cell secretory protein (CCSP) and FOXJ1, and cells in the alveolar compartments expressed SFTPC (Ghaedi et al., 2018), polarization of the cells, and functional differentiation was not fully confirmed in these studies. Furthermore, regional-specific localization of the cells was not discrete with airway cells differentiating in the alveolar region and vice versa (Ghaedi et al., 2018). While these studies report some engraftment, none performed an extensive analysis of mucociliary function or validated the impact of the cellular microenvironment on cell adhesion and differentiation. It should be noted that differentiation protocols for iPSC-derived lung cells have been modified and improved since these initial recellularization studies were completed; however, functional engraftment of primary airway cells still remains a challenge to the field. Further studies are necessary to understand cell: cell and cell: matrix interactions to improve upon generation a repopulates lung tissues, such as the trachea, capable of long-term functional mucociliary clearance in a clinical setting. More recently, a tracheal xenograft model has proven successful in generating a pseudostratified epithelium from iPSC-derived BCs (iBCs) (Hawkins et al., 2021). Decellularized rat tracheas were seeded with iBCs and implanted subcutaneously into the flanks of immune-compromised mice. Airflow was maintained through tracheas using open-ended tubing. After 3 weeks, a pseudostratified epithelial layer of cells had formed with BCs ($KRT5^+$) occupying their expected position along the basement membrane, and secretory cells ($SCGB1A1^+$, $MUC5AC^+$, and $MUC5B^+$) and multiciliated cells ($ATUB^+$) oriented toward the air-filled tracheal lumen (Hawkins et al., 2021). These results show that iBCs can form an airway epithelium that resembles the structure and function of native airways. However, the functionality of such repopulated tracheas has not been demonstrated. Taken together, these results indicate that while the region-specific lung ECM helps to further direct iPSC-derived lung progenitors toward the regional phenotype (i.e., airway progenitors to airway epithelial cells in the airways), it does not prevent the attachment or change the fate of cells intended for another region (i.e., alveolar cells in the airways). Importantly, when compared to cells grown on traditional culture plates, cells seeded onto the lung scaffolds generally exhibited higher expression of lung epithelial markers including SFTPC, PDPN, KRT5, MUC5AC, FOXJ1, and TP63 (Ghaedi et al., 2018). NKx2.1 expression also increased in the scaffold-cultured cells proportionally to the length of time in culture (Gilpin et al., 2014), and the proliferative capacity, as indicated by Ki67, was also maintained (Gilpin et al., 2014).

While progress has been made in terms of successfully generating airway progenitor cells from iPSCs and directing their differentiation toward specific cell types, evaluation of their function in context of their cellular microenvironment will be critical before clinical applications can be explored. Indeed, the iPSC field is largely dependent on advances made in this area through the understanding of endogenous airway stem and progenitor cells in the adult human lung. Fundamental details of cell: matrix and cell: cell, such as inflammatory cell, interactions remain to be elucidated. Understanding the endogenous human airway stem cell regulation will allow for better maintenance of differentiated iPSCs leading to regeneration of functional lung tissues.

Modeling lung disease in the conducting airways

iPSCs have been used to model the proximal airways with studies focusing on disease modeling, therapeutic development, and cellular therapeutics. Application of iPSC-derived lung models has been more prominent in genetic and rare lung diseases such as CF and primary ciliary dyskinesia (PCD), in addition to inflammatory airway disease. Each of these will be discussed in more detail below.

Cystic fibrosis

CF is an autosomal recessive disorder affecting approximately 30,000 Americans. Disease pathogenesis is driven by mutations in the cystic fibrosis transmembrane regulator (CFTR) leading to impaired chloride and bicarbonate conductance, dehydration of the airway mucus, impaired mucociliary clearance and bacterial colonization, eventually leading to chronic lung infection and fibrosis. While recent developments in small molecule therapeutics have improved quality of life for many CF patients, there remain at least 10% who have no therapeutic options and no existing cure (Egan, 2020; McGarry, 2020). These patients typically fall into the class 1 CFTR mutations, which result in no CFTR protein production, due to the presence of nonsense mutations (Cooney et al., 2018; Sharma et al., 2018). iPSCs have already been generated from CF patients (Firth et al., 2015; Fleischer et al., 2018; Merkert et al., 2017) allowing for a seemingly infinite pool of CF mutant and patient-specific cells for research. Refining of the iPSC differentiation protocols, discussed above, should enable iPSC-derived models to be applied to understanding CF pathogenesis and developing patient and mutation-specific models for targeted screening and efficacy studies. Studying rare mutations, present in only a small number of individuals in the USA, significantly limits access to primary cellular material for targeted studies. Fortunately, the advances in gene-editing technologies have made possible the site-specific correction of mutant genes or the targeted insertion of specific disease-causing mutations into iPSC (Firth et al., 2015; Fleischer et al., 2020; Merkert et al., 2019; Suzuki et al., 2016). While the original studies to successfully correct CFTR in iPSCs used zinc finger nucleases (ZFNs)

(Crane et al., 2015) or Tal effector nucleases (TALENs) (Firth et al., 2015) it has been the invention of CRISPR-Ca9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein) that has made gene-editing accessible to all research laboratories (Cong et al., 2013; Jinek et al., 2012; Ran et al., 2013). This technology had evolved rapidly in its wide range of gene-editing applications (reviewed in Hille and Charpentier, 2016; Nidhi et al., 2021). CRISPR-based editing technology has been applied to correct CFTR in a number of human cellular models including patient-BCs, using different gene-editing strategies (Geurts et al., 2020; Vaidyanathan et al., 2020). One study used Cas9 ribonucleo proteins (RNP) in combination with adeno-associated virus 6 (AAV6) to deliver a donor vector to airway BCs with ∼28% efficiency. The second study adapted Cas9 fusion proteins, known as base editors, which work through a fusion of a cytidine deaminase or a TadA heterodimer to partially inactive the nickase Cas9 protein; this enables efficient C-G to T-A base editing on single-stranded DNA, using this they demonstrate functional rescue of CFTR activity but potentially traded efficiency for specificity achieving a 1%−9% correction rate (Geurts et al., 2020; Ryan, 2020; Vaidyanathan et al., 2020). However, there are limitations in the use of corrected primary airway stem cells in autologous cellular therapies. Primary cells from CF patients are difficult to obtain and are usually entangled with bacterial infections that are challenging for establishing cell culture (Peters-Hall et al., 2018). For this reason, researchers have developed strategies to gene correct patient-derived iPSCs (Cardoso and Kotton, 2008; Cindric et al., 2020). Successfully, CFTR restoration is evaluated through its mature glycosylation and the measurement of chloride conductance. Wild type (WT) and successfully edited CFTR proteins undergo N-glycosylation, which causes conformational changes, and is needed for the protein to translocate to the cell membrane (Firth et al., 2015). The specific sites of glycosylation can be detected through western blots, where they produce bands of 150kDA (core glycosylation) and 170kDA (complex glycosylation) (Crane et al., 2015; Firth et al., 2015). CFTR chloride conductance can be measured through methods such as patch-clamp assays, Ussing chamber analysis, and iodine efflux assays (Crane et al., 2015; Firth et al., 2015; Simsek et al., 2016; Suzuki et al., 2016). The availability of autologous pairs of mutant and edited iPSC provides human and patient/mutation specific models for a precision medicine approach to drug screening and additionally provides hope for the future development of a cellular therapy to restore function in the CF lung.

Studies employing animal models are being developed and applied to evaluate practical methods of deploying cellular therapy to CF patients. In 2018, the first *in vivo* iPSC-derived lung epithelial cell engraftment into a mouse lung injury was reported (Miller et al., 2018). In this study, iPSC-derived lung-bud tip cells were engrafted intratracheal into immunocompromised mice after a naphthalene-induced airway injury. The engrafted cells persisted for 6 weeks and endured a secretory cell fate with a small number of ciliated cells. Although informative of the practicality of cell engraftment, the systemic differences between mouse and human fetal lung is significant, illuminating rodents as a poor model for lung

disease therapeutics. However, due to their similarities in airway epithelium structure, physiology, and pathophysiology, ferret and pig have proven to be exemplary animal models. CF models of both organisms have been established, along with protocols for deriving iPSCs, indicating a clear step toward developing an autologous cell therapy animal model for CF (Gao et al., 2020; Godehardt et al., 2018; Rogers et al., 2008b; Sun et al., 2014).

Aside from cellular therapies, iPSCs have the potential to provide a therapeutic benefit for CF patients through patient-specific theratype modeling. The existence of many CFTR mutant variants producing multiple molecular phenotypes creates difficulty for the development of uniform treatments. Moreover, less common variants are not as thoroughly investigated, characterized, or pursued for CF therapeutics. Patient-specific iPSCs provide a unique opportunity to analyze CFTR variants and test their responsiveness to modulators through methods such as high-throughput screening (Clancy et al., 2019; Merkert et al., 2019). In a recent study performed by Merkert et al. (2019) CF disease-specific iPSC-based high-throughput assays were performed to identify functional modulators of CFTR ΔF508 mutants. The authors identified multiple modulators that partially rescued CFTR function, providing evidence for the success of iPSC-based screening methods. Further validation of mutant theratypes could help to predict in vivo clinical response of variants and provide patients with rare mutations, access to modulators that cater to their specific needs. To fully develop functional treatments for CF, more research is needed to address causality for differing levels of severity among CF patients; including the role of microRNA, epigenetic changes and consideration of other organs affected by CFTR mutations. Patient-specific iPSC-derived models have been a practical starting point to evaluate these questions as shown in a recent study that developed iPSC-derived airway organoids from CF and gene-corrected cell lines. Here microRNAs known to regulate CFTR were indicated to have differing levels of expression, indicating a possible role of microRNAs in CF development and severity (Bartoszewska et al., 2017; Mitash et al., 2019). It is hoped that iPSC-derived cells representative of mutations in CF will provide opportunities to learn more about the regulation of the airway in CF and increase effective strategies for drug discovery, toxicology, and therapeutic development.

Primary ciliary dyskinesia

As a genetically recessive and heterogeneous lung disease, PCD has been recognized as a potential target for patient-specific iPSC disease modeling and iPSC-based gene and cellular therapies. Like CF, PCD patients have impaired motile cilia and mucociliary clearance, resulting in situ inversus, a loss of host defense in the lung, chronic infection, and bronchiectasis (Knowles et al., 2013a, 2016). Characterization and diagnosis of PCD is challenging because of the broad range of similar symptoms that are found in other respiratory diseases, coupled with the lack of universally accepted screening tests available. Screening is now available for 36 genetic mutations; however, this will fail to capture defects in patients with

unidentified underlying mutations, where over one-third of patients fall into this category. Most of these known causative mutations are in genes that encode proteins involved in cytoplasmic preassembly of axonemal components, axonemal structure, or cilia motility and can lead to structural defects and dysfunctional phenotypes of cilia motility (Antony et al., 2013; Boon et al., 2014; Chivukula et al., 2020; Cindric et al., 2020; Daniels et al., 2013; Dougherty et al., 2016; Hjeij et al., 2013; Horani et al., 2018; Knowles et al., 2012, 2013b,c, 2014; Loges et al., 2018; Paff et al., 2017; Zariwala et al., 2013). The current gold standards for diagnosis are nasal nitric oxide measurements, cilia ultrastructure, and beat frequency, all of which require significant expertise to successfully perform. With limited cells for analysis and the high rate of inconclusive test results, having an unlimited supply of iPSC-derived multiciliated cells from patients can provide the capacity for extensive analysis of cilia function and could enable precise genotype/phenotype correlations to be fully evaluated. Moreover, an increasingly important strategy for the diagnosis of PCD is likely to be personalized medicine based on high throughput sequencing, especially as the cost of deep sequencing continues to decline dramatically. Several models have been used to study PCD, including unicellular, multicellular, mammal, human bronchial epithelial cells (HBECs), and, most recently. iPSC-derived epithelium. Cellular organisms such as *Chlamydomonas reinhardtii* and *Tetrahymena thermophila* are easy to culture and expand and have well-characterized phenotypes; however, studies have shown major differences in their cilia and flagella structure, protein composition, and function compared to human airway epithelial cells (O'Toole et al., 2012; Pennarun et al., 1999). This causes major discrepancies between model and patient PCD phenotypes, making the evaluation of potential therapies challenging. Mammalian models are more similar in cilia structure and function; however, differences in lung physiology and pathophysiology result in an inability to recapitulate PCD pleiotropic phenotypes. Murine models have been found to show ciliary dyskinesia, and substandard mucociliary clearance, but do not develop bronchiectasis (Fricker et al., 2014; Mall, 2008). Furthermore, many murine PCD models develop hydrocephalus and cardiac defects, which are not seen in human PCD patients (Fricker et al., 2014).

Cell culture models such as primary HBECs and iPSC-derived airway epithelial cells allow *for* in vitro modeling of PCD. Primary HBECs differentiated at the ALI and 3-D organoid models closely resembling the in vivo airway epithelium and provide patient-specific models to evaluate the variances in disease phenotypes (Marthin et al., 2017). Multiple studies have demonstrated the ability of human iPSCs to undergo directed differentiation to functional airway epithelium and multiciliated cells (Firth et al., 2014; Konishi et al., 2016; McCauley et al., 2017). Differentiation of these cells resulted in functional beating cilia, that can be characterized based on the correct $9 + 2$ microtubule doublet arrangement (Konishi et al., 2016). Moreover, similar results were reported from a recent study investigating the generation and differentiation of iPSCs from a PCD patient homozygous for a common dynein heavy chain mutation in the DNAH5 gene (Hawkins et al., 2021). Multiciliated cells derived from these iPSCs were found to recapitulate

the lower ciliary beat, hypokinetic cilia, and outer dynein arm structural defects observed in the donor primary nasal epithelium (Hawkins et al., 2021). These results provide evidence for an iPSC model that can represent multiple PCD-related genetic variants. This model could open doors for providing significant new insights into mechanisms underlying inherited and acquired disease characterized by ciliary dysfunction.

Inflammatory airway disease

Most respiratory disorders have facets of immune-inflammation including chronic obstructive pulmonary disease (COPD, comprising of bronchitis and emphysema) (Eapen et al., 2017; Wang et al., 2018b), asthma (Gillissen and Paparoupa, 2015; Mishra et al., 2018), CF (Cantin et al., 2015; Nichols and Chmiel, 2015) and lung cancer (Gomes et al., 2014), as well as being a more obvious characteristic of pneumonia and bronchiectasis (Fuschillo et al., 2008; Kang et al., 2009; Martinez-Garcia et al., 2015). Inflammation is a typical homeostatic response of the host immune system toward pathogenic infection or mechanical injury. Upon infection or injury, the epithelium responds by secreting an array of cytokines and chemokines to orchestrate an immune response. As a result, innervated blood vessels become dilated, allowing fluid and leukocytes to migrate to the site of injury, causing swelling, heat, and pain. This is typically known as the acute-phase inflammatory response. Under pathological conditions, such as those in respiratory disease, inflammatory responses persist and, if left unresolved, chronic inflammation ensues exacerbating disease. Chronic inflammation is a primary component of respiratory disease, suggestive of an underlying pathological dysregulation of tissue-resident immune cells.

As inflammation is a cellular response to a pathogenic challenge, be it infection or mechanical, it is somewhat ambiguous to model inflammation with iPSCs. Instead, the challenge is focused on generating leukocytes responsible for specific lung inflammatory diseases from iPSCs. All leukocytes are generated from hematopoietic stem cells (HSCs) *In situ,* HSCs are first derived from endothelial precursor cells in a bio-process known as endothelial-to-hematopoietic-transition (EHT) (Ottersbach, 2019) and are subsequently maintained in the bone marrow, a vascular rich microenvironment. Generating HSCs from iPSCs de novo is challenging and, although possible, most methods do not maintain the multipotent level of HSC and instead push to a more differentiated and stable population of mature leukocytes over attempting to maintain HSCs in vitro (Cherry and Daley, 2013; Choi et al., 2011; Lim et al., 2013; Sugimura et al., 2017). Generation of HSC from iPSC was recently demonstrated using seven transcription factors (ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, and SPI1) (Sugimura et al., 2017) and while this seemingly represents the current gold standard for generating HSC from iPSC, there is still much to be desired in the increased complexity and lack of efficiency using this method. Instead, the propagating stem cell cultures are typically maintained at the more specific progenitor cells, depending on the ultimate cell

of interest. In simple terms, HSCs diverge into two separate lineages. The lymphoid lineage, which results in B-cells, T-cells, and Natural Killer (NK) cells, and the Myeloid lineage which produces innate leukocytes, such as neutrophils, monocytes and their derivative cells, macrophages and dendritic cells, eosinophils, basophils and mast cells. Each arm has its own common progenitor, the common lymphoid progenitor and common myeloid progenitor (CMP), respectively, and it is from here that cultures are typically propagated and maintained.

It is challenging to justify the use of iPSC-derived leukocytes to model respiratory inflammatory diseases. The majority of these leukocytes can be isolated relatively easily from donated human blood draws and yield much purer populations of cells that are more physiologically relevant than their iPSC counterparts. Furthermore, isolating these cells from blood is much quicker, utilizing FACS or magnetic-activated cell sorting (MACS) technology, yielding cells of interest in a matter of hours as opposed to weeks using iPSC-based techniques. Indeed, the focus of generating iPSC-derived leukocytes is better applied in the field of cell-based therapies. There is, however, an exception to this. In the lungs, the primary tissue-resident cells are macrophages, of which there are two major subtypes. The tissue resident, often labeled as "alveolar macrophages" (AMs), which reside within the respiratory airspaces, and the interstitial macrophages (IMs), which reside within the tissue parenchyma. Interestingly, these macrophage subtypes have two distinct developmental stem origins, during embryonic development and only the IMs are derived from their common precursor, circulating monocytes (Tan and Krasnow, 2016). Instead, the AMs are derived from fetal liver monocytes and migrate to the tissue and subsequent airspaces *before birth.* These AMs then reside within the airspaces and propagate throughout life, with a slow turnover that is a-typical in other macrophage populations, which are primarily postmitotic. Conversely, the interstitial macrophages are derived from bone marrow-derived blood monocytes and migrate into the tissue upon stimulation (Guilliams et al., 2013; van de Laar et al., 2016). It is regarded that the primary function of the AM is to maintain homeostasis in the airways, rather than respond to pathogens. Rationally, the IM's response to pathogens or mechanical stress would require significant damage to the epithelial barrier. Due to its developmental origin, utilizing these lung-specific tissue-resident AMs for inflammatory modeling and research can be quite challenging due to the fact that this specific macrophage population is not derived from circulating monocytes, and instead would require explanted or postmortem lung tissue in order to isolate, which can be quite a rare commodity. As a result, a strong case could be made for generating alveolar-like macrophages from iPSC and understanding the AM responses to respiratory diseases would benefit from an AM-like iPSC model.

Macrophages have been a primary focus in respiratory inflammatory research, including research centered around iPSCs. As myeloid cells are the primary source of inflammation in respiratory disease, research has focused on generating myeloid lineage cells from iPSCs. Cells that typically resemble macrophages are generally derived from circulating monocytes. In turn, monocytes are derived from the common myeloid progenitor cell which is the first step required for generating

mature myeloid effector cells (Karlsson et al., 2008). BMP4 and FGF2 are used to commit iPSC to mesendoderm (Yu et al., 2011), followed by vascular endothelial growth factor (VEGF) to support EHT and maintain the HSCs (Leung et al., 2013). Wnt activation and Activin A stimulation of nodal signaling has also been shown to be required for EHT and HSC maintenance (Pandya et al., 2017; Takata et al., 2017). Maturation of HSC to myeloid progenitors is promoted by the addition of cytokines, including interleukins 6, 3 (IL-6, IL-3) and stem cell factor *(SCF)* (Metcalf, 2008). These CMPs can be purified by sorting for CD38+, CD123+, CD10-, CD45RA-, with other potential markers available (Will and Steidl, 2010). After EHT, propagation can vary depending on the end-stage macrophage type required (Lee et al., 2018). Most commonly, cultures will be stimulated with colony stimulating factors (CSF) 1 and/or 3, IL-3 and IL-6 (van Wilgenburg et al., 2013). Other factors for progenitor cell propagation, including IL-34, SCF, fms-like tyrosine kinase 3 (FLT3), VEGF, and thrombopoietin (TPO), may be important. The cultures yield single cells that are released in suspension and can be harvested, and these cells are now synonymous to cells at the granulocyte-monocyte progenitor, transitioning to monocytes and require terminal differentiation. CSFs are typically used at this stage with other factors added to skew the polarization of the resulting iPSC-derived macrophage cultures, including IL-3, IL-34, and TGFβ (Amos et al., 2017).

Pragmatically speaking, the protocols outlined above likely resemble the IMs of the lung and not AMs. Generation of AMs could present a potential avenue for cell-based therapy in respiratory disease and as mentioned above, a viable option for inflammatory lung disease research. In 2018, Happle et.al demonstrated that iPSC-derived human macrophages were able to reverse the pulmonary alveolar proteinosis (PAP) phenotype in a humanized mouse model (Happle et al., 2018; Mucci et al., 2018). This study demonstrates how the iPSC-derived macrophages alter their phenotype based on localized signals over prolonged time periods, demonstrating the need for correct localized signaling to correctly develop the phenotype of macrophages. Notably, this model requires the physiological, in vivo, microenvironment to fully differentiate the macrophage to a mature phenotype. However, it is important to note that whilst some of these studies have made efforts to demonstrate their findings using human material (Guilliams et al., 2013; Litvack et al., 2016), they have not been completely validated in a human system. There are naturally practical reasons restricting validating the development of lung macrophages in human embryogenesis. Therefore, we must consider the possibility that the human development does not mimic that which we have seen in limited animal models. Furthermore, animal models seldom have robust construct validity when studying human diseases in vitro. It is therefore important to develop in vitro models of human iPSC recapitulating lung macrophage development during embryogenesis and disease.

Efforts are focusing on the generation of an AM phenotype in vitro. For example, the development of a Myb-alveolar-like macrophage phenotype from mouse embryonic stem cells has been demonstrated (Litvack et al., 2016). These cells are expandable, a unique feature of tissue-resident macrophages, such as AMs,

and not synonymous with monocyte-derived macrophages. Further, Myb-alveolar-like macrophages are capable of bacterial clearance and rescuing a rat model from fecal sepsis (Jerkic et al., 2021). It is important to note, however, that while this work has shown some translatability to a human stem cell model (Litvack et al., 2016), there have always been challenges of translating promising results from animal models to human, in terms of efficiencies and complexity. Furthermore, the models that have so far generated the Myb-alveolar like macrophages have come from embryonic stem cell lines and not iPSC, including that of the human model. Translation from embryonic to iPSC has not been without its challenges also. However, the generation of an alveolar-like macrophage phenotype, that is self-replicating, without the need of monocyte-derived replenishment is a valuable in vitro asset for lung inflammatory research.

Modeling lung disease in the distal airspace

After specification of the primordial lung progenitor cell from iPSCs, differentiation can be directed to specify distally fated lung cells comprising the alveoli, an induced AT2 cell or iAT2. These cells are increasingly being used to model distal airway disease and evaluate novel therapeutics. Most recently, they have been applied to evaluate infectivity, virulence, and lung damage caused by the novel coronavirus SARS-CoV-2. Below we will discuss genetically driven surfactant deficiencies, alpha-1-anti-trypsin deficiency (AATD), IPF, and Lymphangioleiomyomatosis (LAM).

Surfactant protein dysfunction

Secretion and concentration of surfactant within the lung airspaces can be a primary hallmark of respiratory disease, summarized in Fig. 1.3. Pulmonary surfactant dysfunction is an underlying cause of respiratory disease in both neonates and pediatric populations with significant contributions to propagating morbidity in these patient populations. Such deficiencies in natural homeostatic control of surfactant proteins can lead to Acute Respiratory Distress Syndrome (ARDS) and interstitial lung diseases (ILD) (Agrawal et al., 2012; Bullard et al., 2005; Crossno et al., 2010; Hamvas et al., 2007; Magnani and Donn, 2020; Somaschini et al., 2012). In humans, SPB and SPC are synthesized and stored in lamella bodies and secreted into the alveolar airspaces by AT2 cells. Once secreted, these proteins function by controlling surface tension, important for gaseous exchange and maintaining natural lung homeostasis (Klay et al., 2018; Whitsett and Weaver, 2002). There are also associated proteins involved such as adenosine triphosphate binding cassette subfamily A member 3 (ABCA3), which shuttles phospholipids such as phosphatidylcholine (PC) and phosphatidylglycerol (PG) toward the lamellar body structures (Ban et al., 2007; Cheong et al., 2007; Fitzgerald et al., 2007; Rindler et al., 2017).

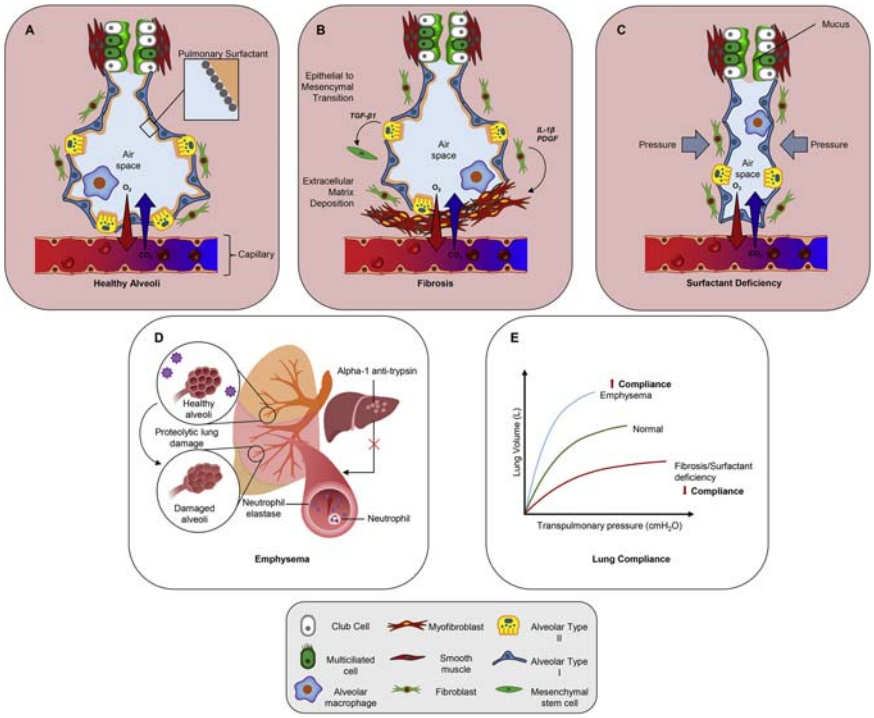

FIGURE 1.3 Effects of disease on alveolar structure and overall lung compliance.

(A) Healthy lung alveoli showing the structural organization of AT2 and AT1 cells, pulmonary surfactant lining the apical surface, alveolar macrophages in the alveolar space, and oxygen diffusion gradients between the alveoli airways and the pulmonary microvasculature. (B) In pulmonary fibrosis, damaged lung alveoli lead to aberrant wound healing and tissue remodeling involving extensive extracellular matrix deposition. Epithelial to mesenchymal transition is enhanced by TGF-β1 and IL-1β signaling. (C) In diseases associated with pulmonary surfactant deficiency the alveolar space collapses. (D) AATD leads to recruitment of neutrophils and neutrophil elastase-induced alveolar damage and eventually emphysema. (E) Hypothetical graph showing changes in lung compliance expected in the above-mentioned diseases. The lower panel includes a key to the cell types included in the diagram.

Mutations within these genes are rare, however, can lead to deficiencies and potentially life-threatening illnesses.

iPSC models have successfully been generated to model surfactant deficiencies to better understand the disorder providing a promising tool for screening into novel therapies (Korogi et al., 2019; Lachmann et al., 2014; Leibel et al., 2019). Cells from a patient harboring mutations in the SFTPB gene have been isolated and reprogrammed to iPSC (Baekvad-Hansen et al., 2010; Leibel et al., 2019; Tredano et al., 1999). Organoids were successfully generated from these iPSCs and expressed

a mix of DE and vimentin expressing mesenchymal cells. Following similar protocols established for AT2 differentiation cells within the organoid were induced to produce surfactants SPB and SPC, encoded by genes SFTPB and SFTPC, respectively (Leibel et al., 2019). In comparison with WT controls, both SFTPB- and SFTPC-positive cells were significantly decreased, as well as ABCA3 expressing intracellular lamellar bodies. The phenotype could be rescued by overexpressing SFTPB in these cells. While delivery of ex vivo gene-edited cells would be an ideal therapeutic strategy for these patients, like with the other diseases discussed efficient delivery, long-term engraftment, and retention of functional AT2 cells in the human lung will require significant methodological advances to become a reality.

Accumulation of proteins and phospholipids in the alveolar space is the primary characteristic of PAP, another rare and life-threatening lung disease (Lachmann et al., 2014). In adults, PAP is primarily caused by autoimmunity toward granulocyte macrophage-colony stimulating factor (GM-CSF). However, early onset PAP or PAP like syndromes can have early onset genetic causes involving mutations in SFTPB, SFTPC (Tredano et al., 2004), ABCA3 (Young et al., 2008), NKX2.1 (Guillot et al., 2010), and GM-CSF receptor α or β subunit (Suzuki et al., 2010), among others (Borie et al., 2011). GM-CSF is a cytokine, whose function is to regulate and mature localized myeloid cells including, monocytes, neutrophils, and eosinophils. Lack of bioavailability of GM-CSF because of autoimmunity or involved mutations impairs the maturation of these myeloid cells in lung tissue, resulting in the accumulation of surfactant proteins within the lung, in particular the alveolar spaces. By utilizing patient-specific mutation, researchers have developed iPSC-derived models of PAP in vitro (Suzuki et al., 2014), which when gene-corrected reverses the PAP phenotype (Lachmann et al., 2014). iPSC technology has also demonstrated potential therapies toward ameliorating PAP. As GM-CSF primarily influences macrophage phenotype and function and is clearly impaired in PAP (Trapnell et al., 2009), studies have investigated if iPSC-derived macrophages can provide a therapeutic benefit in animal models. As described above, iPSC-derived macrophages (Happle et al., 2017; Mucci et al., 2018) can successfully reverse the PAP pathology.

Alpha-1-anti-trypsin

AATD is an inherited, autosomal codominant genetic disease that manifests as pulmonary emphysema in adults (Blank and Brantly, 1994). Like many rare diseases, it is likely underdiagnosed with an estimated prevalence of around 1 in 2,500 people in the USA today. Alpha-1-anti-trypsin (AAT) is a circulating antiprotease, coded by the gene SERPINA1, that is predominantly synthesized in the liver and circulates around the body. In the lung, it inactivates neutrophil elastase after diffusing into the alveolar lining, where it functions to modulate immune responses and protect the alveolus from protease-mediated damage, summarized in Fig. 1.3 (Fregonese et al., 2008). Excess neutrophil elastase destroys alveoli leading to ineffective gaseous exchange and increased susceptibility to further damage

from cigarette smoke and airborne particulates. In the absence of a cure, current therapeutic approaches involve weekly delivery of recombinant AAT, augmenting alveolar damage. AATD is a monogenic disorder and is therefore, an ideal target for gene and/or cell therapy. In terms of basic research to better understand the disease, AATD patient-specific iPSCs have been generated, including a relatively large repository of available AATD-specific iPSC lines available for investigative research (Kaserman et al., 2020; Kaserman and Wilson, 2018; Segeritz et al., 2018; Tafaleng et al., 2015; Wilson et al., 2015). These cells have successfully been utilized to investigate specific facets of the liver pathologies involved in AATD; however, studies using lung models have not been robustly established. Currently, studies have started to generate lung epithelium from iPSC cell lines with specific ATT deficiencies (Kaserman et al., 2020) using the dual NKX2.1/TP63 reporter described above. This study successfully generated fully characterized iPSC lines from ATTD patients with known genotypes PiZZ and PiSZ associated with AATD, as well as generating lines from rarer genotypes. They went on to perform transcriptomic profiles of derived lung progenitors that demonstrated expression of known lineage-specific markers, including NKX2-1 and SOX9. Subsequently, they demonstrated that gene correction of SERPINA1 in iPSCs was able to restore typical AAT function in derived hepatocytes. This gene correction approach demonstrates the potential for future therapeutics, and other studies have demonstrated that specific point mutations, such as the JAK2-V617F mutation seen in AATD, can be targeted, and corrected using CRISPR/Cas9 technology (Smith et al., 2015). As for CF, the potential for in vivo gene correction or ex vivo generation of gene-corrected iPSC-derived cells for cellular therapy exists and remains the hope of many for a cure.

While the underlying pathology for AATD is centered around a deficiency in AAT secretions from hepatocytes, the primary disease phenotype is a destruction of lung parenchyma. iPSC studies have typically aimed at modeling the hepatic aspect of AATD while the respiratory disease phenotype is left to be desired. This is most likely bottlenecked through lack of robust methods and models to derive accurate lung cultures from iPSCs. Direct and modeling AATD with iPSCs would also likely require incredibly complex coculture systems, including mesenchyme and likely hepatic cultures along with epithelial structures. From a potentially therapeutic standpoint of iPSC-based cell therapy would therefore need to target the liver. Protocols generating hepatocytes from SERPINA1 mutant iPSCs have been developed (Tafaleng et al., 2015), resulting in functional hepatocytes with the pathological accumulation of the misfolded AAT associated with AATD. Furthermore, studies have successfully been able to gene correct SERPINA1 mutant iPSC which had restored AAT function in the subsequently derived hepatocytes (Yusa et al., 2011). While this provides promise in the potential for autologous cell therapies in AATD, it is bottlenecked by the limitations of common issues currently affecting this field of research, namely, successful transplantation/engraftment of the host's iPSC-derived cells.

Idiopathic pulmonary fibrosis

IPF is a chronic and progressive fibrotic lung disease in which the alveoli are destroyed, aberrant epithelial−fibroblast interactions lead to the induction of matrix-producing myofibroblasts, extracellular matrix (ECM) accumulates lead to scarring, lung remodeling, decreased lung compliance, disrupted gas exchange, and ultimately respiratory failure, summarized in Fig. 1.3A, B. IPF affects around 100,000 people in the United States, most commonly men and between the ages of 50 and 70. After diagnosis, IPF individuals are likely to only survive for an additional 3−5 years. While the exact cause of IPF is unknown, it likely involves a combination of genetic factors increasing risk and environmental exposures, for example, cigarette smoking, viral infections, and air particulates. Genome-wide association studies (GWAS) have identified common genetic variants; however, causality has yet to be thoroughly established. Potentially relevant genes identified are involved in host defense (MUC5B, ATP11A, TOLLIP), telomere maintenance (TERT, TERC, OBFC1), and barrier function (DSP, DPP9) (Allen et al., 2017; Hobbs et al., 2019; Kropski et al., 2015; Lee and Lee, 2015; Lorenzo-Salazar et al., 2019; Noth et al., 2013). A notable single-nucleotide polymorphism (SNP) located upstream of the MUC5B promoter (rs35705950) was identified as a significant risk factor associated with IPF susceptibility (Seibold et al., 2011). This variant was subsequently detected in about a third of patients with IPF (Lee and Lee, 2015). Surprisingly, despite the strong association, this MUC5B variant is also a prognostic indicator and is correlated with improved survival compared to noncarriers, although the mechanisms by which this variant increases survival remains unknown (Biondini et al., 2021; Gally et al., 2021; Hancock et al., 2018; Helling et al., 2017).

Although there are several genome-wide and linkage studies available that have identified variants that contribute to IPF susceptibility and have provided invaluable data to IPF researchers, these alone have proven insufficient in understanding the pathogenesis of IPF which has long been an obstacle in the development of effective pharmaceutical therapies (Behr et al., 2021a,b; King et al., 2014). Attempts at identifying therapeutic targets using animal models have been somewhat successful. Preclinical safety studies using animal subjects paved the way for clinical trials in humans and have resulted in FDA approval of two drugs, pirfenidone and nintedanib, which slow the progression of the disease (Behr et al., 2021a,b; Justet et al., 2021; King et al., 2014; Richeldi et al., 2014; Yoon et al., 2018). However, attempts at clarifying disease pathogenesis through traditional animal models have remained elusive as no animal model fully recapitulates the progressive nature of IPF seen in humans. Although the murine model of pulmonary fibrosis is well established and is the choice for identifying and testing new compounds (Moeller et al., 2008; Tashiro et al., 2017), it not without any disadvantages. For example, researchers investigating the effects of rare variants on telomere maintenance by challenging *TERC* and *TERT*-null mice with low-dose bleomycin, a chemotherapeutic, pro-fibrotic drug used to treat Hodgkin's lymphoma, produced mixed results, and highlights the difficulties in recapitulating IPF pathogenesis and performing

mechanistic studies in animal models (Degryse et al., 2012; Liu et al., 2007; Povedano et al., 2018). Furthermore, accurate modeling of a disease with no specific identifiable etiology has always remained challenging and, given the number of common and rare variants associated with IPF susceptibility, it is clear a newer approach needs to be added, one that accounts for the subtle genetic differences between IPF patients.

Fortunately, the recent advances in stem cell technology have provided another avenue for researchers to study IPF. 3-D lung organoids have shown potential to recapitulate several features of human lung disease and lung development. 3-D cell structures have several advantages over 2-D cultures. Perhaps chief among which allows for the investigation of multitissue and complex structures that are more physiological. Indeed, a recent study was able to induce a specific HPS1 mutation, known to cause pulmonary fibrotic disease in humans which leads to the accumulation of ECM and increased volume of mesenchymal cells, key hallmarks of fibrotic lung disease (Chen et al., 2017; Mulugeta et al., 2015). Another study was able to recapitulate the progressive nature of the disease by using TGF-β1 on fetal lung fibroblast organoids or iPSC-derived mesenchymal organoids and then demonstrated that the progressive scarring phenotype resembles that of IPF (Wilkinson et al., 2017). Using a more traditional ALI model iPSC-derived AT2-like cells, application of a cocktail of IPF relevant cytokines that mimic the milieu known to be present in IPF lungs during differentiation was able to increase secretion of known IPF biomarkers demonstrating overlap with data from IPF patients. The cocktail was able to impair AT2 differentiation and increased an airway epithelial signature, including upregulation of SOX2, FOXJ1, SCGB1A1, KRT5, and MUC5B, supporting recent data identifying a considerable number of more proximal-associated cells in the distal airspaces, a phenomenon becoming known as IPF-associated bronchiolization.

Lymphangioleiomyomatosis

LAM is a rare progressive multisystemic and cystic disease of the lung characterized by an abnormal, tumorous smooth-muscle proliferation alongside blood vessels, lymphatics, and bronchioles occurring almost exclusively in young premenopausal women. LAM is classified by the WHO as a PEComa (perivascular epithelioid cell tumor), a perivascular mesenchymal tumor coexpressing myogenic and melanocytic markers (Johnson and Tattersfield, 2000; Travis et al., 2015). This excessive smooth muscle cell proliferation is driven by a mutation in the tuberous sclerosis complex 1 and 2 (TSC1/TSC2) genes causing severe, mostly obstructive lung disease. LAM can present as part of tuberous sclerosis complex, an autosomal dominant genetic disease with a germline mutation of TSC1/TSC2 leading to benign tumors at various sides of the body with a variety of symptoms and severity levels or can occur sporadically due to a somatic mutation in these genes causing a genetic mosaicism in those patients (Carsillo et al., 2000; Sato et al., 2002). There has been little therapeutic development for the treatment of these patients, due to lack of availability of tissues and cells for research due to a rare LAM population. Recently Julian et al. successfully

differentiated LAM patient-derived iPSCs and iPSCs with TSC2 knocked out to smooth muscle cells (SMCs) (Julian et al., 2017). These SMCs recapitulated several of the molecular and functional characteristics of pulmonary LAM cells including hyperactive mTORC activation, reduced autophagy, and metabolic reprogramming. A lack of SMC integrity in the diseased cells also leads to a destabilization of the vascular network. This study was the first to report iPSC-derived LAM cells, and it will be interesting to follow work on iPSC-derived cells for studying LAM and see how closely they are able to mimic the in vivo disease state, including the multiple cellular interactions with the lymphatic endothelium and alveolar cells.

These advances overcome the missing compatibility of animal models with the actual human disease as well as the lack of biological samples needed for studying. Not only provide insight into the status quo of the disease but rather give us the possibility to decipher developmental issues from the pluripotent stem cell to the very end LAM cell and to determine the cell of origin. Delaney et al. have suggested the neural crest cell lineage as a possible cell of origin due to a high EMT ability and as an explanation for the variety of tumors in different parts of the body otherwise developing from different germ layers (Delaney et al., 2014). Further investigation at different stages of embryogenesis as well as coculture models with cell types characterizing the disease, such as airway epithelial cells and lymphatic endothelial cells, are needed to enhance our understanding of the pathogenesis of LAM.

Modeling viral infection of the lung

According to the World Health Organization, respiratory infections are the leading cause of death worldwide among communicable diseases. Viruses are typically transmitted through contact and inhalation of airborne particles and can lead to respiratory disease with a broad spectrum of disease severity. Infections of the respiratory system can generally be divided into 2 main categories, upper and lower respiratory tract infections. Upper respiratory tract infections typically include influenza and common cold viruses, such as rhino virus. The lower respiratory tract infections which tend to affect children at higher rates include the respiratory syncytial virus (RSV), adenoviruses, influenza viruses, parainfluenza viruses, and corona viruses, including the novel SARS-CoV-2. The recent coronavirus disease 2019 (COVID-19) pandemic to sweep the world significantly accelerated the development of research models to facilitate research focusing on respiratory infections, and iPSC-based models have been proven to be a valuable addition. The multipotent potential of iPSCs has proven valuable in investigating COVID-19 as well as other viral infections, in multiple cellular and tissue types from singular genetic backgrounds. Further, iPSCs can be used to investigate genetic susceptibilities to viral infection, by either deriving iPSC from specific genetic backgrounds or by inducing mutants in WT iPSC lines

iPSCs have successfully been used to investigate several inflammatory diseases caused by viruses. These include encephalitis caused by the human cytomegalovirus (Belzile et al., 2014; D'Aiuto et al., 2012) and/or by the vesicular stomatitis virus

(Lee et al., 2012), hepatitis caused by hepatitis B and C (Liu et al., 2011; Shlomai et al., 2014; Wu et al., 2012), and myocarditis caused by the coxsackievirus (Sharma et al., 2014). iPSC models have also been used to investigate antiviral therapies, with a particular emphasis on antiretrovirals for HIV infections, using iPSC derived T-cells, monocytes, and macrophages (Jerebtsova et al., 2012; Kambal et al., 2011; Kang et al., 2015; Ni et al., 2011). A number of studies have also employed similar approaches to investigate respiratory infections. Like most iPSC modeling that studies viral infections, the respiratory models will either focus on a system of directly modeling the disease caused by the virus, or by modeling susceptibility to certain viral infections (Trevisan et al., 2015), such as genetic mutations. Parainfluenza virus infection, which causes lower respiratory tract infections in infants, was utilized in an iPSC-derived lung organoid system. This model demonstrated that parainfluenza particles successfully infected SFTPC+ AT2 cells within the iPSC-derived organoid (Fig. 1.4). Further, the study was able to demonstrate

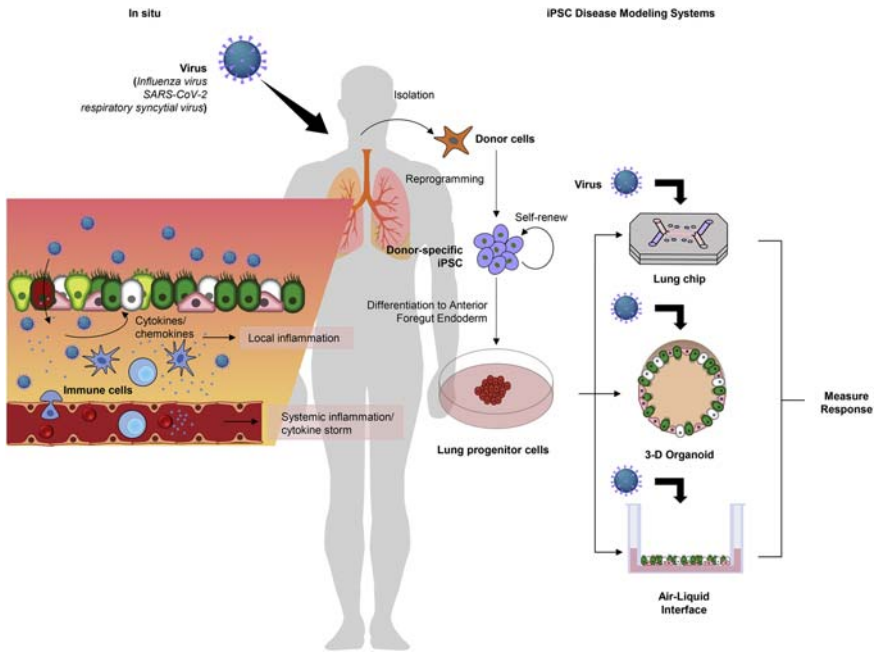

FIGURE 1.4 Models for evaluating viral infection of the human lung.

Studies of human primary tissue in situ include the analysis of fixed pathological lung tissue and analysis of peripheral blood samples or saliva for makers of inflammation as shown on the left of the diagram. Human iPSC can be used and differentiated into lung-specific models of lung tissues. Models include the commonly used air—liquid interface differentiation to a pseudostratified proximal airway epithelium, 3-D organoids which spontaneously develop mimicking lung development and branching, 3-D spheroids (tracheospheres or alveolarspheres) or more recent technologies such as the lung-lung-on chips.

viral shedding into the lumen of the organoids, which is challenging to recapitulate using primary cell counterparts (Porotto et al., 2019). This model also demonstrated how the measles virus can induce formation of syncytium in the same organoids. Susceptibility to infections has also been investigated using iPSC-derived human lung models. Patients deficient for Interferon Regulatory Factor 7 (IRF7) are known to have a severe susceptibility to infections (Zhang and Pagano, 1997), in particular to H1N1 influenza (Ciancanelli et al., 2015). IRF7 is part of the IRF superfamily of transcriptions factors important in regulating type 1 interferons (Ning et al., 2011), involved in innate and humoral responses to infections. iPSC cells derived from a patient with a heterozygous compound mutation if the IRF7 gene, resulting in a loss of function, were utilized to develop pulmonary epithelial cells (Huang et al., 2014). This model then went on to demonstrate an increase in H1N1 viral replication, compared to WT controls (Ciancanelli et al., 2015). The comparatives here were made between pulmonary epithelial cells generated from 3 iPSC clones derived from the same patient to pulmonary epithelial cells generated from an embryonic stem cell line—RUES2. Though this study does provide an excellent clinical example of the impart IRF7 has on influenza infections, it is unclear what impact donor variability may have. Generating other patient-specific IRF7 mutations, however, will be challenging due to its rarity, and that most patients are a-symptomatic in nature. Potentially, inducing IRF7-specific mutations in WT lines, including the control ESC line used here (RUES2), could provide further insights and represent better pair-matched controls.

SARS-CoV-2, the virus responsible for the COVID-19 global pandemic is of continued research interest. Much like original SARS-CoV viruses, it targets the angiotensin-converting enzyme 2 (ACE2) protein (Li et al., 2003, 2005), with subsequent data suggesting SARS-CoV-2 has a higher affinity to ACE2 resulting in the increased viral propensity for COVID-19 compared to previous SARS outbreaks (Wan et al., 2020). ACE2 is expressed on a number of different tissue types, most notably in vascular and cardiac tissue (Hamming et al., 2004). ACE2 is also expressed in the lung with high expression values noted in AT2 cells (Zhao et al., 2020), as well as expression in ciliated cells of upper airway epithelium (Lee et al., 2020). In terms of iPSC modeling, a large focus has been looking into infectious susceptibility of different tissues and a number of different iPSC-derived cellular models have now been applied to advance our understanding of SARS-CoV2 infection. These iPSC models include iPSC-derived cardiomyocytes (Bojkova et al., 2020; Perez-Bermejo et al., 2021; Sharma et al., 2020), hepatocytes (Yang et al., 2020), intestinal epithelium (Lamers et al., 2020), pancreatic (Yang et al., 2020), and indeed lung organoids (Huang et al., 2020b; Leibel et al., 2020; Surendran et al., 2020; Tiwari et al., 2021). iPSC-derived AT2 (iAT2) cells as well as putative progenitors express both ACE2 and transmembrane protease serine 2 (TMPSSR2) important in SARS-CoV-2 infection (2, however, do so at a reduced capacity of their primary cell counterparts). Further studies using this model demonstrated infection with SARS-CoV-2 within the iAT2 that activates interferon-related inflammatory cascades (Huang et al., 2020a), although conflicting studies

that included SARS-CoV-2 infection in iAT2 cells suggest that IFN activation is minimal (Li et al., 2021). These models are limited in their incapability of generating other pneumocytes, including AT1 cells, which have also been demonstrated to be important in SARS-CoV-2 infections. However, these lung models have provided an excellent platform for investigating potential therapeutics by providing a drug screening tool (Esmail and Danter, 2021; Han et al., 2020). This type of work has demonstrated the significant advantages that iPSC modeling has to offer. The organoid modeling that PSCs allow for can provide the necessary complexity of tissue structures in host physiology (Fig. 1.4). They also allow for the generation of multiple cell types derived from the same genetic background which is invaluable in determining viral infectivity of different tissues and the related pathology that this can cause.

Concluding remarks

While the journey to a cure for many lung diseases is still far from a reality, iPSCs and their differentiated progeny still have substantial capacity to impact lung health through patient-specific screening in a precision medicine approach to their potential as a cellular therapy. In this chapter, we have described some of the latest developments in iPSC-based modeling of the human lung and lung disease. Although new therapeutics have been developed using cell lines and animal models, translation rates into effective therapeutic approaches for human lung disease are poor with many potential therapeutics failing to make it into the clinic. iPSC-derived cells provide an opportunity for patient specific and precision medicine approaches for therapeutic development and the hope remains for a cellular therapy for a cure for all lung diseases.

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CHAPTER

2 iPSC for modeling of metabolic and neurodegenerative disorders

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Chapter outline

Abstract

Metabolic and neurodegenerative disorders, such as cardiovascular dysfunction, diabetes, and Parkinson's disease, are fundamental health concerns all around the

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world. The development of novel treatments and new techniques to address these disorders is being actively studied by researchers and medical personnel. In the present chapter, we will discuss the utilization of induced pluripotent stem cells (iPSCs) for cell-therapy replacement and disease modeling in chronic conditions of high incidence. The aim of iPSCs is to restore the functionality of the damaged tissue by replacing the death cells with competitive ones. To achieve this objective, iPSCs can be properly differentiated in virtually any cell fate and can be strongly translated into human health via in vitro and in vivo disease modeling.

Keywords: Alzheimer; Cardiovascular; Cardiovascular dysfuction; Cell diferentiation; Cell reprogramation; Cell therapy; Diabetes; Disease modeling; iPSC therapy; iPSCs; Metabolism; Neurodegenerative; Parkinson; Pluripotency; Stem cells.

Introduction

Throughout history, stem cells (SCs) have been widely employed, even before knowing their pluripotency mechanisms. The first successful therapeutic application of SCs was the bone marrow transplantation in 1968 (Bach et al., 1968), from that date to the present the development technology has preside over multiple advances on identification, understanding, and SCs applications; the best example is the breakthrough development of induced pluripotent stem cells (iPSCs) from somatic cells, establishing a watershed in science progress (Doss et al., 2019; Shi et al., 2017; Takahashi et al., 2006). Cell reprogramming mediated by the Yamanaka factors expanded the view around SCs, which already showed a very promising picture.

Since the isolation of human embryonic stem cells (hESC), SCs were proposed as a platform of study of human developmental biology, disease modeling, drug screening, and cell therapy (Gearhart, 1998; Thomson, 1998). Recent trends in regenerative and personalized medicine aim to develop specific metabolic disease models, where iPSCs cells can offer an additional advantage to achieve a more representative approach (Ittner et al., 2015; Chao et al., 2018; Nugraha et al., 2019; Schüssler-Fiorenza et al., 2019; Stoddard-Bennett et al., 2019). In this chapter, we will recapitulate the origins, obtention methods, novel research, applications, and future perspectives of iPSC for the modeling of neurological, cardiovascular, and diabetic metabolic disorders, since these pathologies are of major concern due to their incidence worldwide.

What are iPSCs?

iPSCs are a cell lineage with similar characteristics to embryonic SCs, they both express the same pluripotency factors, surface markers, and possess the capacity to differentiate into the three germ layers (Yu et al., 2007). The advantages of iPSCs include their human origin, accessibility, quick expansion, ability to self-renew, and pluripotent state providing the ability to form practically any cell type, in addition to the avoidance of ethical concerns associated with the obtention and management of hESCs (Shi et al., 2017).

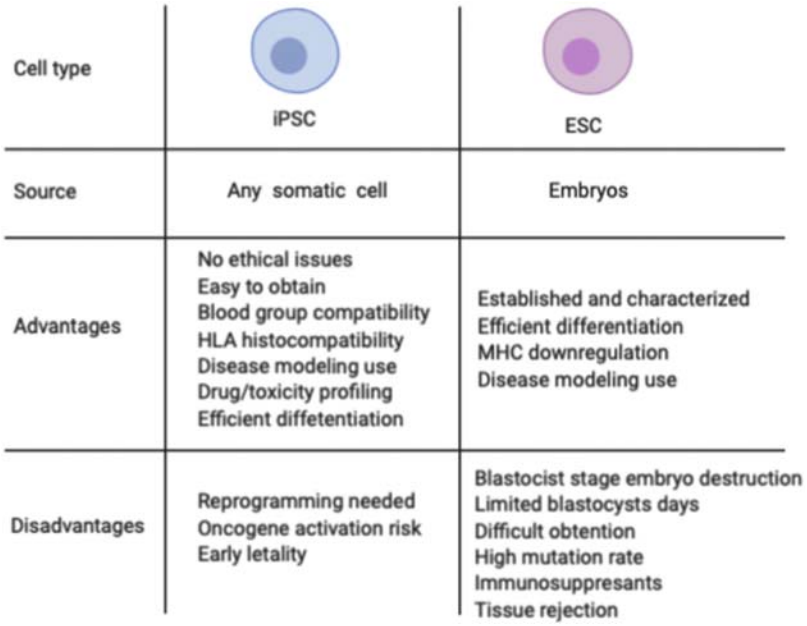

FIGURE 2.1

Induced pluripotent stem cells versus embryonic stem cells. Table indicating the differences between iPSCs and ESCs, including sources, advantages, and disadvantages.

In the early 2000s, a great discovery was achieved by Yamanaka, S. and Takahashi, K., in which they successfully generated new kinds of SCs similar to ESCs (Fig. 2.1) from mouse embryonic and adult fibroblasts cultures, these were called iPSCs. They were able to reprogram the gene expression of the fibroblasts by adding four transcription factors to the culture media: OCT4, SOX2, KLF4, and C-MYC, factors known as the Yamanaka factors, which help to induce and maintain the pluripotent state in the iPSCs generated (Shi et al., 2017; Takahashi et al., 2006). iPSCs have been widely used for disease modeling, drug screening, and cell therapy. Furthermore, the combination of iPSCs with organoids and gene editing, such as CRISPR-Cas9, turns these cells into a highly versatile tool for regenerative medicine and iPSCs-based drug screening to evaluate compounds with the potential to treat several diseases, resulting in clinical candidates being identified and some approved for their application (Shi et al., 2017; Mullard, 2015).

How are iPSCs obtained?

The generation of iPSCs was first conducted by reprogramming somatic cells, such as fibroblasts, introducing the Yamanaka factors: Oct4, Klf4, c-Myc, and Sox2, via

integrating viral vectors, in particular, retroviral and lentiviral transduction. Nevertheless, these methods might generate insertional mutagenesis in the host cells, turning these cells inadequate for their clinical application. To overcome this issue and reduce risks associated with vectors, nonintegrating methods have been developed in the later years, including episomal DNAs, synthetic mRNAs, Sendai virus, among others (Shi et al., 2017). Genome editing technologies provide the possibility to introduce genetic changes into iPSCs in a site-specific manner, generating isogenic iPSCs lines, distinctly important in sporadic and polygenic diseases (Hockemeyer et al., 2016).

iPSCs can be generated, technically, from any tissue of the body; nevertheless, the most employed sources are fibroblasts and peripheral blood mononuclear cells due to their easy access. The process of generation of iPSCs initiate by recollection of sample cells from the patient or animal model with the correct technique, then the somatic cells are transduced with the virus containing the reprogramming factors, either lentivirus, retrovirus, and sendai virus might be used. Once the reprogramming is achieved, morphological changes can be appreciated in the cultured cells forming ESCs-like colonies, which may be collected and clonally expanded. These clonal cells are cultured for several passages to ensure they conserve their characteristic morphology (Fig. 2.2; Zhang et al., 2020).

Subsequently, the obtained cells are tested for pluripotency gene expression, including Oct4, Sox2, Klf-4, c-Myc, and absence of virus derived transgenes that might compromise the iPSCs quality and functionality. Every iPSCs cell should express ESCs antigens, such as NANOG, SSEA-4, and TRA-1-80 to ensure their similarity, in addition to analyzing the alkaline phosphatase and telomerase activity to verify their cell cycle functionality. The previous steps may be achieved with the polymerase chain reaction technique. Additional steps that might be performed include the chromosomal analysis for a normal karyotype or identify possible translocations (Petrenko et al., 2020).

To verify the iPSCs pluripotency potential, it is necessary to test their capacity to form specialized tissue from the three germ layers. When cultured in suspension, the ESCs-like reprogrammed cells start to transform into embryoid bodies (EBs) and after approximately 2 weeks the EBs must have differentiated, in a spontaneous manner, to the three germ layers. This differentiation is analyzed by immunostaining of the corresponding markers of each germ layer, the most commonly used antibodies are alpha-fetoprotein for the endoderm layer, beta−III−tubulin for the ectodermal layer, and smooth-muscle antibody for the mesodermal layer (Holmqvist, 2016).

Current iPSCs models overview

Complex iPSC-based differentiation systems are currently being developed to conscientiously recapitulate human tissue-level and organ-level dysfunction, incorporated into disease modeling, drug screening and the study of host−pathogen

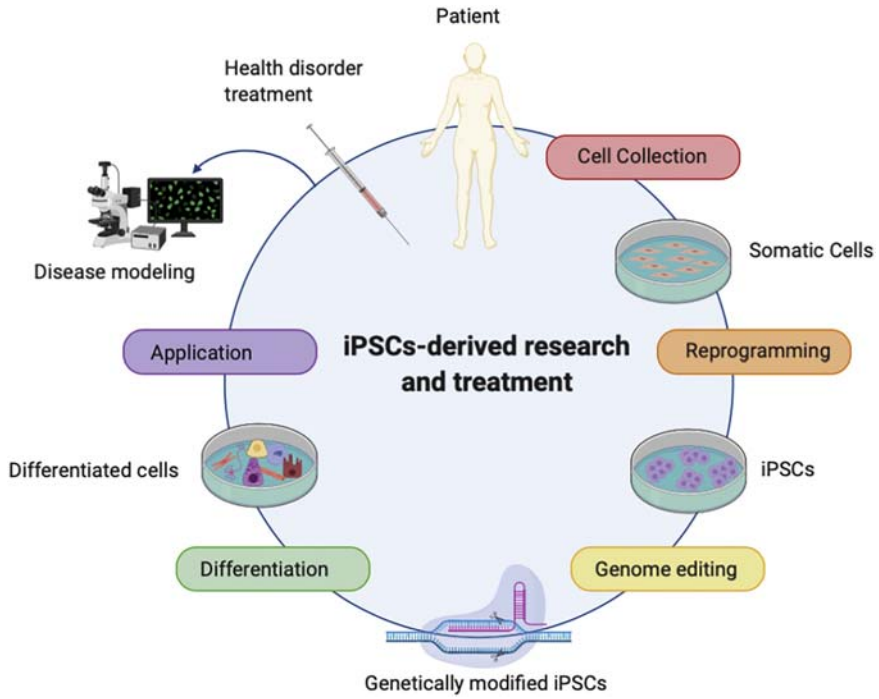

FIGURE 2.2

A graphic of human iPSCs-based therapy. Somatic cells are collected from affected patients and cultured, then the somatic cells are reprogrammed into iPSCs. Additionally, with genome editing technology or a viral transduction method it is possible to genetically correct the patient-derived iPSCs. The corrected iPSCs are differentiated into desired cell types to serve as genetically matched healthy donor cells. Finally, the genetically matched healthy cells are available for their application for research and disease modeling or clinical treatments by transplanting into patients for cell therapy.

Graphic adapted from Shi, Y., Inoue, H., Wu, J. C., Yamanaka, S., 2017. Induced pluripotent stem cell technology: a decade of progress. Nat. Rev. Drug Discov., 16 (2), 115–130. https://doi.org/10.1038/nrd.2016.245.

interactions (Rowe et al., 2019). Currently, the available models are in vitro culture and animal models. Animal models have provided several tools for modeling human diseases, contributing to the identification of pathological molecular mechanisms in an in vivo setting. However, species differences obstruct the possibility to capture the complete vision of the human disease, making urgent the need to establish human disease modeling platforms for biomedical research (Onos et al., 2016; Shi et al., 2017). Disease modeling applying iPSCs requires cells harboring the disease-causing mutation or damage, to differentiate them into the predisposed cell type needed to analyze the disease and identify the pathological mechanisms involved in its etiology.

The phenotype of cells differentiated from iPSCs provides a good model for diseases with an early onset rather than late-onset due to their fetal-like properties (Studer et al., 2015). If necessary, aging in cells could be induced by treating them with cellular stressors, which target mitochondrial function or protein degradation pathways (Nguyen et al., 2011).

To improve disease modeling, it has been developed a coculture method of more than one cell type, in order to study their interaction (Shi et al., 2017). Yet, the interaction between different cell types could be better employing iPSCs-derived 3D organoids, due to their resemblance to endogenous cell organization and organ structure, enabling a cellular context that mimics physiology and development, with the potential to model drug response in a spatiotemporal context (Lancaster et al., 2014). 3D cultures still need to be improved with a more standardized culture medium and extracellular matrix, in order to correctly mimic their physiological environment, with greater vascularization, growth, maturation, and nutrient supply.

Metabolic disorders

Metabolic disorders are a heterogeneous group of disorders that may be inherited or may occur as the result of spontaneous mutation, involving failure of the metabolic pathways or intermediary metabolism leading to multiple-system manifestations, affecting millions of people worldwide (Stenton et al., 2020). They can be classified according to Dasgupta and Wahed in amino acid metabolism disorders, carbohydrate metabolism disorder, urea cycle disorder, organic acid disorders, fatty acid oxidation disorder, mitochondrial disorders, peroxisomal disorders, lysosomal storage disorders, purine or pyrimidine metabolic disorders, and disorders of porphyrin metabolism (Dasgupta et al., 2014). These disorders are also associated with lifestyle and inherited epigenetics, both altering gene and protein expression (Martínez et al., 2014).

This kind of disorder is characterized by the incapacity of usage and storage of energy derived from alimentation (Huynh et al., 2016). There is some controversial information regarding the role of microRNAs, a small class of noncoding RNAs (ncRNAs) that regulate the expression of most protein-coding genes, in the pathogenesis of metabolic disorders, since they may represent potential biomarkers to diagnose a disease, besides possible molecular targets for their treatment (Assmann et al., 2019).

Human iPSC disease modeling provides an authentic genetic signature of human tissue and unlimited resources for identifying the mechanisms underlying the pathologies development (Park et al., 2008). In addition to the maturation setting, we can test novel pathophysiological approaches for generating patient-specific medicine. Studies involving international collaboration and increased numbers of patient samples and iPSCs quality controls would greatly facilitate the development of novel therapies (Kim, 2015). There is strong evidence of the use of iPSCs to successfully model metabolic disorders associated mainly with heart diseases (Chanana et al., 2016), neurodegenerative diseases (McKinney, 2017), and diabetes (Stepniewski et al., 2015).

Cardiovascular disorders

Cardiovascular diseases (CVD) are one of the leading causes of death worldwide with an increasing prevalence, according to the Global Burden of Disease, there were 422.7 estimated million cases of CVD and 17.92 million deaths in 2015. Some of the most significant CVD are heart failure, ischemic heart disease (IHD), myocardial infarction, stroke, hypertensive heart disease, cardiomyopathy, aortic aneurysm, atrial fibrillation, rheumatic heart disease, endocarditis, and peripheral arterial disease (Roth et al., 2017).

The cardiovascular system is complex with a broad interdependence with blood circulation, blood vessels, and blood constituents that act as the human body's transport system (Lafta Mossa, 2008; Savoji et al., 2019). The heart, considered as one of the main components of this system, is highly specialized, organized, and complex itself, it is made up of different cell types, ventricular or atrial cardiomyocytes (CMs), cardiac fibroblasts, endothelial cells (ECs), and perivascular cells (Zhou and Pu, 2016).

Since CMs are the most abundant constituent of the heart, approximately 30%−40% of mammalian heart cells are CMs, occupy around 70%−85% of the total volume and their role for generating contractile force and rhythmic beating in the heart they are considered as an important tool and approach to study the etiology of CVDs (Nakano et al., 2012; Pingzhu et al., 2016).

However, technical and ethical restrictions, as well as the limited possibility of isolation of human primary CMs hinder the establishment of models based on human surgical material (Brodehl et al., 2019), due to this, iPSC-based CMs are being studied to overcome the above difficulties.

In this section of the chapter, we will discuss the application of CMs derived from iPSC-CMs as a resource to model CVD. As well as in other metabolic diseases, motivation for developing accurate and predictive CVD models has three approaches: increasing knowledge of CVD pathophysiology; studying, testing, and discovering new treatments for CVDs; screening for adverse heart drug reactions, considered as a primary risk in drug development.

Cardiomyocytes

CMs are enigmatic and incredible highly organized cells that fulfill countless functions as they are engaged in the generation of contractile force and regulation of the heart rhythmic beating (Woodcock et al., 2005). Almost half of the total volume of a single CM is made up by myofibrils and a quarter is occupied by mitochondria's (Nakano et al., 2012). CMs are characterized by specialized structures such as sarcolemma and sarcoplasmic reticulum, whose functions remain uncertain. Along with the sarcoplasmic reticulum, sarcolemma forms the T- tubular system, forming a branched and interconnected network that is critical in calcium signaling and excitation-contraction necessary for beat-to-beat heart contraction. Sarcoplasmic reticulum regulates Ca^{2+} cytosolic concentration, and it is responsible for the Ca^{2+} source in excitation−contraction coupling (Walker et al., 1999).

Cardiomyocyte differentiation

CMs derived from embryonic stem cells (ESCs) or iPSCs can be achieved by three strategies: spontaneous differentiation, monolayer cultures, and inductive coculture (Leitolis et al., 2019).

Spontaneous differentiation based on suspension culture in vitro produces 3D aggregates named as EBs, which not only develop CM but also cells from other germ layers, according to Zhang et al. (2009), EBs can be cultured in attachment plates and treated with differentiation medium for further development of beating areas. As for inductive coculture, this method was reported for the first time by Christine et al. (2003), it basically consists of coculturing SCs with visceral endoderm (VE)-like cells from the mouse. Similarly, monolayer-based protocols require the addition of growth factors to increase differentiation efficiency (Fig. 2.6). Among these protocols, it is important to consider the critical factors and signaling pathways involved such as BMP4, Wnt, and FGF (Leitolis et al., 2019).

CVDs modeled by iPSC-CMs

IHD also known as coronary heart disease (CHD) is common among developed countries and according to the American Heart Association approximately 15.4 million people older than 20 years have IHD in the United States (Ferreira-González, 2013). The term ischemia refers to the lack of oxygen and blood flow, thus CHD is due to narrowed heart arteries responsible for blood supply to the heart (Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria, 2010). Recently, Wei et al. (2019) developed an IHD model using CMs differentiated from human induced pluripotent cells (hiPSC-CMs) and optimizing ischemic conditions. Results were encouraging due to the observed consistencies within in vivo conditions in the heart, as the mimicry of the in vivo IL8 increased.

In addition, genome editing techniques as CRISPR/Cas9 or TALEN might be used to induce genetic mutations in iPSC-CMs development to investigate the pathological changes caused by gene mutations. Hypertrophic cardiomyopathy (HCM), a genetic disorder commonly related to left ventricular diastolic function is caused by mutations in genes associated with sarcomere proteins (Marian and Braunwald, 2017). In order to study the role of PRKAG2 mutation in the pathophysiology of this disease, patient's iPSC-CMs were generated and then the PRKAG2 mutation was corrected by CRISPR/Cas9, this emendation notably improved the electrophysiological activity in comparison with PRKAG2-mutated iPSC-CMs (Ben Jehuda et al., 2018). Diastolic dysfunction (DD) has also been modeled based on iPSC-CMs in order to unveil its mechanisms. iPSC-CMs were obtained from healthy and HCM patients with DD, Ca^{2+} in HCM iPSC-CMs was analyzed as well as proteins involved in its handling. Results revealed novel information about DD in HCM physiology that is useful to develop new therapeutic targets (Wu et al., 2019).

Moreover, iPSC technology has also been used to mimic rare genetic cardiac disorders that cannot be equally reproduced using animal models and remain having

nonunderstood gaps such as LEOPARD syndrome, an autosomal dominant disorder caused by missense mutations in PTPN11. Research groups have developed iPSC from LEOPARD patients that could work as a platform for drug and treatment development (Carvajal-Vergara et al., 2010).

Therefore, it is crucial to consider novel disease modeling techniques as iPSC application, where derived CMs offer a new opportunity and novel approach in the study of cardiovascular pathologies and rare cardiac-related genetic disorders.

Diabetes

Diabetes mellitus (DM) is a chronic disease comprising a group of metabolic disorders involving carbohydrate, protein, and fat-causing elevated blood glucose concentrations (hyperglycemia) due to insulin deficiency or resistance. Thus, it has an elevated incidence worldwide, there are approximately almost 425 million cases reported and the rate keeps going up. DM is mainly classified into two types, type 1 and type 2 (T1DM and T2DM, respectively). T1DM is generated by an autoimmune response that destroys the insulin-secreting β-cells of the pancreas while T2DM manifests through poor insulin secretion and insulin resistance. As a result of hyperglycemia, comorbidities as CVDs, nephropathy, and tissue or organ damage might develop (Kruszynska, 2004; Dadheech et al., 2019; Harreiter et al., 2019; Hossain et al., 2016).

Since DM is a high incidence disease, efforts to study and look for new therapeutic options have been made for several years; however, there is a lack of diabetes cellular models because of the complexity of functional human pancreatic β-cells obtention. Up to date, the most representative model of human β-cells is the EndoC-βH1 cell line, generated from the human fetal pancreas by lentiviral transduction (Scharfmann et al., 2014; Tsonkova et al., 2018). However, the high costs and limited access to human pancreatic β-cells and the necessity of functional cells for DM treatment have promoted the iPSC disease modeling approach.

The obtention of insulin-producing cells derived from iPSC (INS-iPSC) is based on mimicking embryo developmental processes via multiple differentiation stages and it is important to consider the definitive endoderm (DE) differentiation and the spheroid formation for the differentiation functional pancreatic β-cells (Yabe et al., 2017).

iPSC-based pancreatic β-cells as a model of disease

Successful differentiation of iPSC to pancreatic β-cells is the first and key step for modeling DM disorders and its schematic representation can be found in Fig. 2.7. Footprint free β-cells can be obtained from healthy patients while cells carrying monogenic mutations can be obtained whether from Diabetic patients iPSCs (DiPSC) or by genome editing techniques, additionally, DiPSC genetic abnormalities can also be repaired or modified by novel genome editing techniques to explore

the viability of gene therapy (Kawser Hossain et al., 2016). In 2013, iPSC-derived β-cells that modeled diabetes due to glucokinase deficiency were successfully generated and it demonstrated that SC-derived β-cells maintained the physiological phenotype of diabetic subjects (Hua et al., 2013).

A novel example of iPSC implementation to model and study DM is the representation of neonatal or congenital diabetes which is caused by mutations in the insulin gene (INS) that can lead to β-cell apoptosis (Lemelman et al., 2018). In order to delve into the cellular mechanisms of this disorder, Balboa et al. (2018) generated β-cells derived from iPSCs of patients carrying INS mutations, differentiation to pancreatic β-cell was carried out after previous correction of the mutation by CRISPR/Cas9 system. These experiments showed that INS mutations related to neonatal diabetes promote the disease development by leading to defective β-cell mass expansion.

Neurodegenerative disorders

Neurodegenerative disorders a prominent class of neurological diseases without cure, however, rather treated by pharmacological or stimulus therapy. They are characterized by an inexorable loss of a specific type of neuron according to the disease (Fu et al., 2018). Complex morphological characteristics prone those to defects, such as long-range neural projections and broad synaptic connections, making them selectively vulnerable due to increased metabolic demands to maintain the structural complexity of the cells involved. These disorders share molecular and cellular characteristics, such as protein aggregation, mitochondrial dysfunction, glutamate toxicity, calcium load, proteolytic stress, oxidative stress, neuroinflammation, and aging. All of the anterior contribute to neuronal death and disease development (Fig. 2.3; Gan et al., 2018; Pacelli et al., 2015).

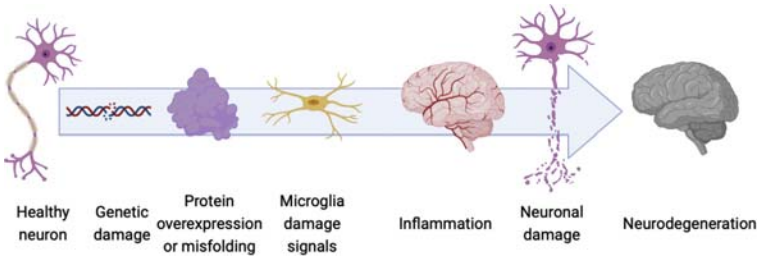

FIGURE 2.3

Cellular neurodegenerative process. The neurodegeneration process is initiated by metabolic disruption events in the neurons, such as protein overexpression or misfolding. These events activate microglial cells and the BBB, sending signals (cytokines) which induce inflammation in the brain. Inflammation leads to neuronal damage and finally neurodegeneration.

Aberrant production of misfolded proteins and their aggregation in the brain may cause complex and distinctive pathophysiological profiles in several neurodegenerative proteinopathies. Some features attributed to these diseases include β-amyloid (Aβ) plaques, phosphorylated Tau-containing tangles, in Alzheimer's disease (AD) (Masters et al., 2015), and Lewy bodies associated with α-synuclein protein in Parkinson's disease (PD) (Dettmer et al., 2015). However, the majority of AD and PD cases are idiopathic, which causes studying these disorders mechanisms very difficult without access to damaged tissue in the patient's nervous system. Postmortem brain tissues have provided essential pathological information in order to understand neurodegenerative mechanisms, nonetheless, it is not suitable for identifying the biological changes during initial stages of the disease.

Furthermore, transgenic animals provide valuable models, but the microenvironment and the species differences may be major reasons why transgenic animals have been largely unable to sufficiently recapitulate disease phenotypes (Wu et al., 2019). Recent advances in reprogramming patient somatic cells into iPSCs have provided a novel means to generate disease-relevant cells for in vitro disease modeling reports (Hotta et al., 2015; Keller et al., 2000; Shi et al., 2017).

Parkinson's disease

The core feature of PD involves selective loss of A9-type dopaminergic neurons that project from the *substantia nigra pars compacta* (SNPc) in the midbrain to the dorsal striatum (Alexander, 2004). Dopamine dyshomeostasis is highly related to the metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), resulting in the alteration of functional protein residues, protein aggregation, oxidative stress, and finally cell death. These fatal results are due to the DOPAL triggering of α-synuclein oligomerization leading to synapse impairment, including dopamine leakage from synaptic vesicles (Masato et al., 2019). Therefore, α-synuclein metabolism plays an important role in the development of PD, some factors that promote this protein aggregation are mitochondrial dysfunction, oxidative stress, neurotoxins, and oxidation, trafficking or mutation of α-synuclein (Hashimoto et al., 2004).

There is some clinical convergence between PD and metabolic disorders such as lysosomal storage diseases leading to systemic manifestations in the liver, spleen, and bone marrow to progressive neurodegenerative diseases, for example, cholesterol deposition in neurons (Baris et al., 2014); disorders in metal metabolism like copper or iron generating oxidative damage in basal ganglia, increased oxidative stress, toxic oligomers formation, membrane breakage, enhanced α-synuclein fibrillation, and neuronal damage (Carboni et al., 2015); aminoacid metabolism disorders influence in PD pathophysiology via chronic reduction of neurotransmitters levels, free radicals generation, suppression of brain energy metabolism through the inhibition of the respiratory chain (Sgaravatti et al., 2003; Limphaibool et al., 2018).

PD can be categorized into two types: genetic or familial and sporadic or idiopathic, the genetic form only represents the 10% of the total population diagnosed with this disease, while the other 90% represents a sporadic form. A great discovery

achieved with iPSCs-PD models is the neuronal death induced by mitochondrial dysfunction due to oxidative stress, playing an important role in the sporadic PD research (Ambasudhan et al., 2013; Ludtmann et al., 2018).

The first gene that was linked to PD was the SNCA gene, which encodes the protein α-synuclein, although the function is not well understood. PD-iPSCs models have shown that SNCA is associated with α-synuclein aggregation and Lewy body-like deposition in dopaminergic neurons. One gene that has been correlated with both genetic and sporadic forms of PD is the LRRK2 gene, and it is associated with upregulation of the α-synuclein protein, elevated expression of key oxidative stress-response genes, and mitochondrial dysfunction in dopaminergic neurons that were derived from PD-iPSCs (Cooper et al., 2012). Another related gene is PARK2 or Parkin, this is an E3 ubiquitin ligase that targets mitochondria, mutations in PARK2 are associated with an autosomal recessive early onset familial PD and are correlated to loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons (Shaltouki et al., 2015). One event that results in the loss of Parkin expression is exon deletion, leading to increased oxidative stress, reduced dopamine (DA) uptake and increased spontaneous DA release in dopaminergic neurons derived from PD-iPSCs. These observations suggest that Parkin is involved in controlling DA neurotransmission and suppressing DA oxidation in human midbrain dopaminergic neurons (Jiang et al., 2012). In the case of sporadic PD, GBA1 encodes a lysosomal enzyme, β-glucocerebrosidase, involved in glycolipid metabolism, mutations in GBA1 were correlated to lowered β-glucocerebrosidase activity and α-synuclein accumulation in dopaminergic neurons derived from PD-iPSCs (Woodard et al., 2014).

One alternative to study PD is through the recently developed method to generate brain organoids: these may help to create complex 3D models of midbrain tissue from iPSCs. 3D brain or midbrain organoids will allow the exploration of multiple factors that contribute to PD and broaden the potential targets for drug development to neuron-adjacent cells (Wu et al., 2019) due to the inclusion of well-characterized neurons, astroglia, and oligodendrocytes in a coculture manner (Monzel et al., 2017). A large number of studies show that human iPSCs can be used as a reliable source for the generation of disease-relevant cell types to investigate the development of the disorder. Through continuous propagation and efficient differentiation of patient-derived iPSCs into specific neuronal subtypes and 3D organoids, we are now able to better recapitulate the cellular progression toward neurodegeneration in vitro allowing researchers to discover new methods to manipulate iPSC-derived neurons, uncovering novel disease phenotypes and eventually identifying novel successful clinical interventions (Fig. 2.4; Wu et al., 2019).

Models of PD in the actuality focus on the two principal characteristics of the disease: loss of functionality of dopaminergic neurons and α-synuclein protein aggregates (Falkenburger et al., 2016). One of the most used models is the SH-SY5Y neuroblastoma cell line and the PC12 cell line, because they are able to produce catecholamines and develop neuron-like properties, but differentiation of these lines is difficult, they are used for pharmacological interventions (Jang et al., 2016).

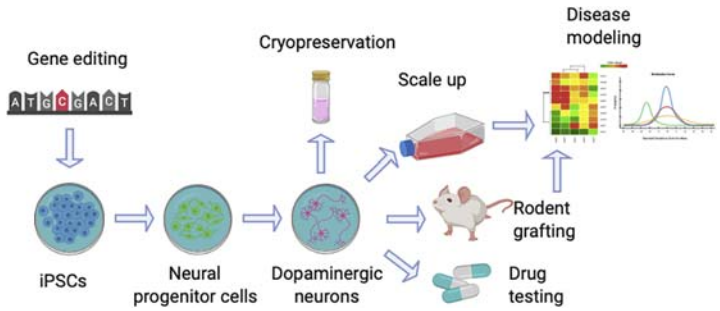

FIGURE 2.4

Parkinson's disease model. In a model of PD, iPSCs might be genetically corrected or produced when the disorder is associated with a genetic aberration in order to generate neural progenitor cells and dopaminergic neurons by a differentiation protocol. These cells can be used for different purposes such as cryopreservation for further use, drug testing, scaling up and rodent/animal grafting for disease modeling and research.

A second line used for modeling PD is the lund human mesencephalic cell line (LUHMES), providing a more stable dopaminergic phenotype, since they show neuronal markers, long processes, and electrical properties similar to dopaminergic neurons (Lotharius et al., 2005).

Another approach is the use of iPSCs from patients to generate more accurate models of the disease, providing a better tool for understanding pathogenic mechanisms of neurological disorders, the capacity to develop personalized therapeutic strategies, and the possibility to study early pathogenic mechanisms. It has been developed a model for a comparative analysis of multipotent mesenchymal stromal cells, derived from adult bone marrow, adipose tissue, and wharton's jelly, to determine if they could be a future therapeutic tool for regenerative medicine, with a focus on neuroregenerative potential (Petrenko et al., 2020). They found that the secretome of these cells had neurotrophic potential to stimulate the neurite outgrowth and reduce death of neural SCs after oxidative stress. Also, a model generated from iPSCs of young-onset PD from patients with the disorder confirmed the implication of lysosomal degradation pathways, mitochondrial dysfunction, and impaired mitophagy in PD pathogenesis. In addition, they tested two neuroprotective compounds, phorbol esters, in their iPSCs-PD models, resulting in reduced α-synuclein aggregation, increased TH-positive neurons in mesencephalic dopaminergic neuron cultures (Laperle et al., 2020). It is important to mention that the creation of libraries of iPSCs from patients is becoming an important source of preclinical models of PD for future developments (Smith et al., 2020).

Alzheimer's disease

AD is characterized by the brain volume reduction and hippocampal degeneration. The pathological hallmarks are extracellular Aβ plaques and aggregation of

hyperphosphorylated tau protein in neurofibrillary tangles, which tend to aggregate, causing neuronal dysfunction and cell death (Wray et al., 2009). Researchers found that iPSC-derived neurons from different patients showed different pathophysiology of AD, especially in the accumulation of Aβ oligomers (Kondo et al., 2013). Other common signs of AD include gliosis, inflammation, abnormalities of the Blood-Brain Barrier (BBB), altered pathways of cellular and endocytic degradation, and elevated DNA damage (De Strooper et al., 2016).

Glucose is the principal energy source of neurons and astrocytes in the brain requiring glucose transporters and insulin for optimal utilization, therefore type 2 diabetes is a great risk factor to AD. Oxidative stress also contributes to pathogenesis aggravating glucose dyshomeostasis, raising intracellular calcium levels, which damage DNA, interfering with gene transcription and promoter functionality, and RNA, impairing protein translation, all leading to AD development (Butterfield et al., 2019).

There are some investigations relating impaired mitochondrial energy metabolism as the defining characteristic of several cases of AD, due to a compensatory mechanism of energy production to maintain challenged neuronal cells (Demetrius et al., 2013).An increase in molecular disorder in mitochondrial metabolism in neurons and energy production in astrocytes generate higher thermodynamic entropy, thus inducing detrimental effects in molecular fidelity, such as conformational changes and covalent modifications in structure, resulting in protein misfolding and amyloid formation (Cornwell and Westermark, 1980; Demetrius et al., 2004). Regularly, mature neurons do not enter the cell cycle, nor complete mitosis, however, if there is DNA damage due to oxidative stress. Thus, leading to cell cycle entry, activation of DNA repair mechanisms, and if repair is not successful, apoptotic pathways will be triggered and cause neuronal loss (Zhu et al., 2007).

There are studies carried out for AD that use brain cells derived from iPSC, to generate neurons with a mutation that demonstrate high expression in Aβ, p-tau, and GABAergic neuron degeneration in APOE4 neurons, apolipoprotein E4 (APOE4) is a strong genetic risk factor for AD (Israel et al., 2012; Wang et al., 2018). The generation of neural precursors from iPSC has also been extensively studied, in the production of astrocytes, the mutation in PSEN1 increased Aβ production and oxidative stress, altered cytokine release and Ca^{2+} homeostasis, thus reducing neuronal support function in PSEN1 astrocytes (Oksanen et al., 2017). Another studied mutation has been APOE3 and APOE4 which manifests impaired Aβ clearance and increased cholesterol content of APOE4 astrocytes. For microglia, the isogenic APOE3 and APOE4 mutations have demonstrated a reduced Aβ uptake from media and organoids (Lin et al., 2018). Kondo et al. and Israel et al. report the discovery that Aβ-oligomers accumulating neurons from individual iPSC cell damage are associated with increased reactive-oxygen-species (ROS). For that, AD patient iPSC-derived neurons might be a competent therapeutic model and testing ground to find the patient's optimal pharmacological regimen and with the use of 3D in vitro cultures, explore the pathophysiology of AD (Fig. 2.5).

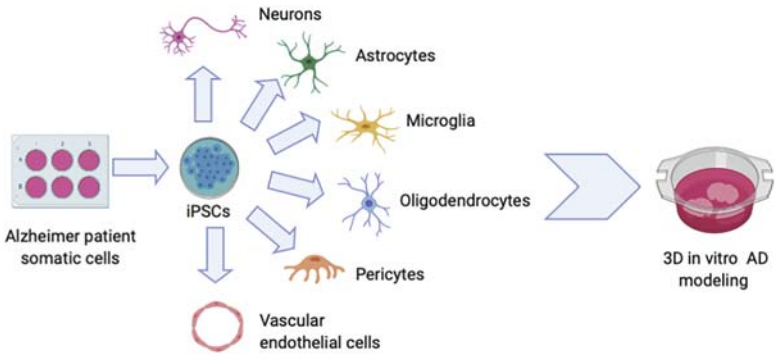

FIGURE 2.5

Alzheimer's disease model. Differentiation of somatic cells from patients can be reprogrammed to iPSCs and then differentiated into several brain cell types for 3D in vitro AD modeling, to examine interactions between the different cell types.

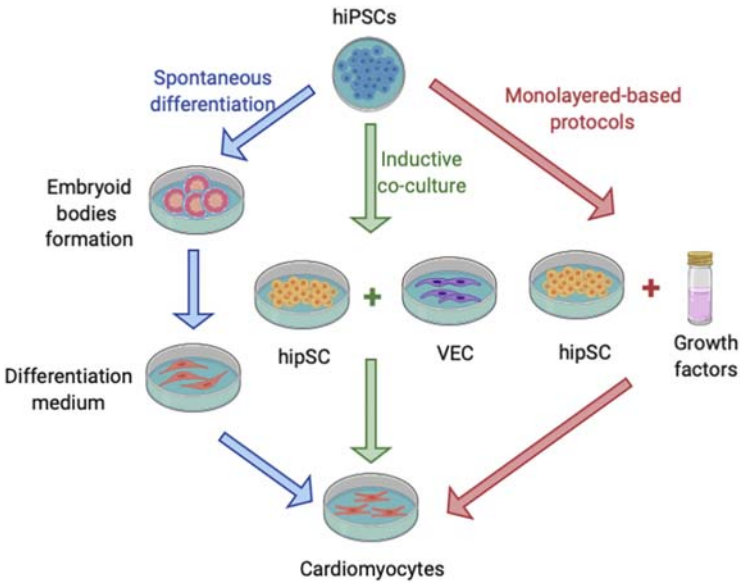

FIGURE 2.6

Cardiovascular disease models. Spontaneous differentiation based on EB formation (*blue arrows*), Inductive co-culture (green) and monolayer-based protocols employing the addition of growth factors (red).

Future applications

Massive progress has been made investigating disease mechanisms and potential treatments through patient-derived iPSCs in combination with gene-editing technologies, such as CRISPR-Cas9, making available new tools for understanding biological mechanisms underlying iPSC pluripotency, maintenance, and differentiation.

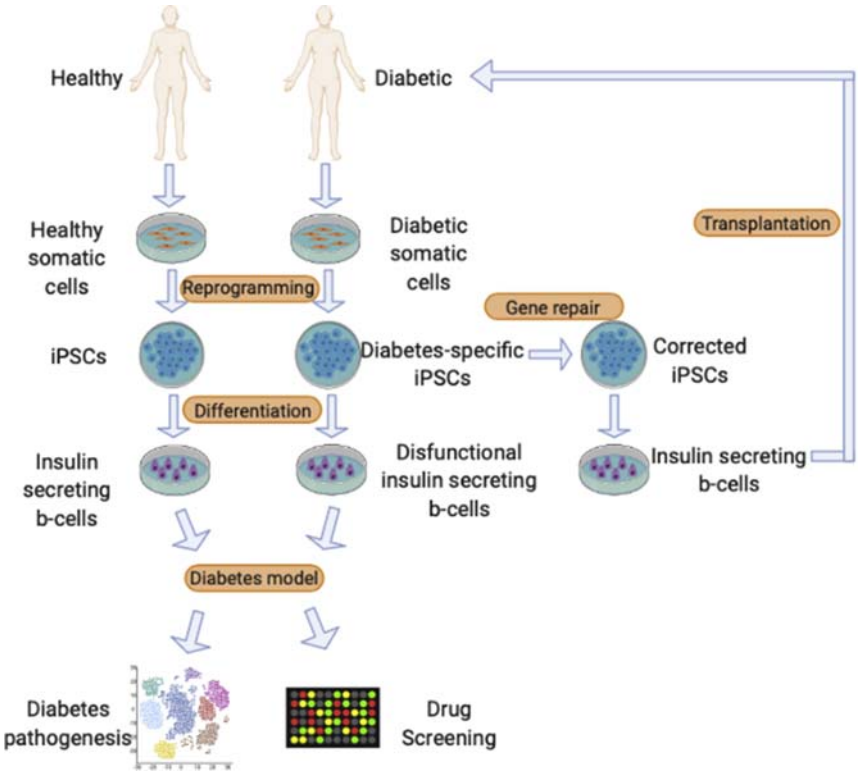

FIGURE 2.7

Schematic representation of iPSC-derived β-cells from healthy and diabetic patients used for modeling and studying DM pathogenesis.

Autologous iPSCs might avoid the serious side effects associated with lifelong immunosuppression required for allogeneic cell transplantation, representing a major advantage in the use of these cells for clinical purposes in the future (Pearl et al., 2012). The clinical utility of iPSCs-derived products relies on the state of the art of directed differentiation, cell state conversion, and tissue engineering (Doss et al., 2019).

In addition to pathological mechanisms of diseases via in vitro 2D modeling and future 3D models representing a better overview to pathophysiological conditions, enabling the spatiotemporal cellular interactions analysis, although there are several limitations in the present status of this technology that must be overcome in order to exploit the potential of the novel tools and techniques (Yin et al., 2016).

Limitations

Even though iPSCs are a well-established technology, there are still some obstacles associated with iPSC-based therapy before enabling it for clinical applications. One

issue is the risk of tumorigenicity due to their pluripotency state; besides, they can accumulate karyotypic abnormalities if maintained in culture for long periods of time. If these barriers are overcome, and cells have been correctly delivered, patients should be monitored for potential tumors and the immune system reactivity (Lund et al., 2012). Because of these concerns, iPSCs-derived products need to be completely characterized and screened for the lack of potentially genetic alterations and ensure their quality and innocuousness. There are still challenges to overcome for iPSCs; regarding the mechanisms of pluripotency, reprogramming and the derivation of some functional mature cells; nevertheless, several groups are looking behind these fundamental processes and it will be a hotspot for SC research in the upcoming years.

Conclusions

The iPSCs models can be induced from somatic cells and can be differentiated into specific cell types to mimic many worldwide known diseases. Novel studies generate new opportunities for medical treatments, but they also face many challenges such as the improvement of differentiation protocols of hiPSCs, in addition to prioritizing the safety and effectiveness of transplanted cells. We must keep in mind that SCs may provide an important tool for SC therapy, but it also results in some ethical concerns, the great advantage is that derivation of human-iPSCs from somatic cells avoids the destruction of human embryos and their manipulation for research, thus bypassing ethical problems arising from ESCs obtention and manipulation. iPSCs models of metabolic and neurodegenerative diseases, such as PD AD, and Diabetes, are just the beginning of a new era of medical advancements and technology that will be available for clinical treatments, disease prevention, finding novel cures for diseases, and more in a not so far future.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER

Induced pluripotent stem cells for modeling open-angle glaucoma

3

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Chapter outline

Abstract

Glaucoma is the leading cause of irreversible blindness worldwide affecting millions of people. None of the current treatments is ideal, and investigations on new treatments are needed. In this chapter, we will review the current treatment options for glaucoma and discuss the possibility and feasibility of using induced pluripotent stem cells (iPSCs) to model glaucoma. The trabecular meshwork cells and retinal ganglion cells are the main cell types affected by glaucoma. iPSCs from normal subjects and from glaucoma patients have the potential to differentiate into

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these cell types which can be used to mimic the cellular glaucomatous changes in vitro. These cells with glaucomatous characteristics can be used to investigate glaucoma pathophysiology and novel treatment options (Graphic Abstract shown as Fig. 3.1).

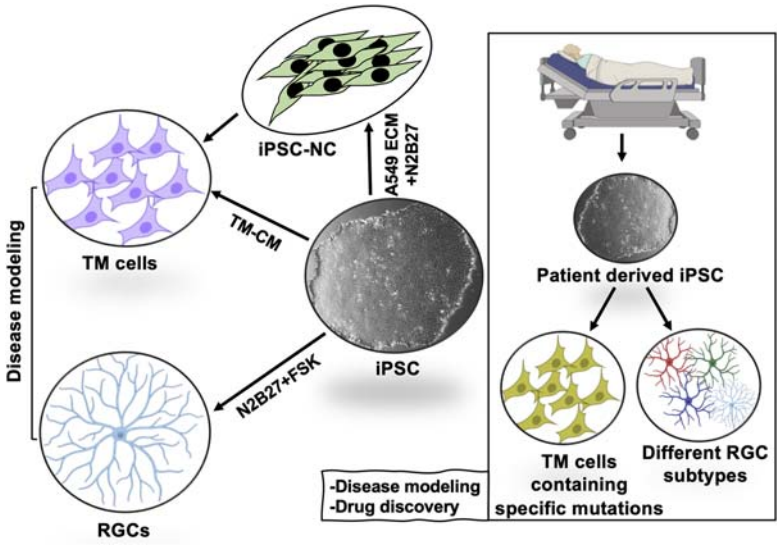

FIGURE 3.1 Graphic abstract.

iPSC can be induced to differentiate into TM cells and RGCs using different approaches to model glaucoma. Patient-derived iPSCs can differentiate into various RGC subtypes and can be used for disease modeling and drug discovery. *CM*, conditioned medium; *ECM*, extracellular matrix; *FSK*, Forskolin; *iPSCs*, induced pluripotent stem cells; *NC*, neural crest; *RGCs*, retinal ganglion cells; *TM*, trabecular meshwork.

The graphic was created partially using Biorender.com.

Keywords: Cellular model; Dexamethasone; Extracellular matrix; Glaucoma; Induced pluripotent stem cells; Neural crest; Retinal ganglion cells; Trabecular meshwork; Treatment.

List of abbreviations

CHI3L1	chitinase 3-like 1
CLANs	cross-linked actin networks
CM	conditioned media
Dex	dexamethasone
ER	endoplasmic reticulum
ESCs	embryonic stem cells
FSK	forskolin

IOP	intraocular pressure
iPSC-NC	iPSC derived neural crest cells
iPSC-NC-TM	iPSC-NC derived trabecular meshwork cells
iPSCs	Induced Pluripotent Stem Cells
MGP	matrix gla protein
MMP	matrix metalloproteinase
MYOC	myocilin
NC	neural crest
NOS	nitric oxide synthase
OPTN	optineurin
PACG	primary angle-closure glaucoma
POAG	primary open-angle glaucoma
RGCs	retinal ganglion cells
ROCK	Rho-associated kinase
TIMP3	tissue inhibitor of metalloproteinase 3
TM	trabecular meshwork

Introduction

Vision is one of the most important senses that humans possess. Glaucoma is the leading cause of irreversible vision loss, affecting more than 60 million people worldwide (Khan et al., 2016; Quigley and Broman, 2006). The estimated number of people afflicted with glaucoma would be increased to 76 million in 2020 (Teotia et al., 2017). Additionally, primary open angle glaucoma (POAG) is the leading cause for vision loss in African Americans (Teotia et al., 2017). POAG causes irreversible blindness gradually and a lot of patients do not know they are suffering from POAG since it is normally asymptomatic with gradual visual field loss. Glaucoma is primarily characterized by increased intraocular pressure (IOP) in the eye which pushes against the optic nerve and damages the retinal ganglion cells (RGCs) in the retina (Chamling et al., 2016). The axons of RGCs gather in the optic nerve that is crucial for the transmission of visual information from the eyes to the brain (Chamling et al., 2016). Glaucoma is categorized as primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), secondary, and developmental/congenital glaucoma (Foundation, 2019). Among these, POAG is the most common type (Weinreb et al., 2016) (https://www.nei.nih.gov/learn-about-eye-health/eye-conditions-and-diseases/glaucoma/types-glaucoma). In this chapter, we mainly focus on studies involving POAG and using stem cells to model glaucoma for exploring glaucoma pathophysiology and discovering new treatments.

Pathophysiology of POAG

POAG is a disease of two parts of the eye tissues: the trabecular meshwork (TM) and Schlemm's canal conventional outflow pathway which is responsible for IOP regulation, and the RGCs and optic nerve complex which is responsible for visual

function (Epstein, 2012). Up to 90% of the aqueous humor in the eye is drained through the conventional outflow pathway including the TM and Schlemm's canal (Wang et al., 2019; Stamer et al., 2015). The abnormality of cells and extracellular matrix (ECM) of the TM and Schlemm's canal reduces the outflow facility and increases IOP. Contrastingly, the RGC and optic nerve complex in the posterior part of the eye is the target of glaucoma damaging the vision. In POAG patients, the anterior chamber angle remains open, but the resistance of the TM and Schlemm's canal pathway is increased. So the aqueous humor cannot be drained efficiently while aqueous humor is continuously produced, leading to accumulation in the anterior chamber which elevates IOP. There is currently a knowledge gap in the exact pathogenesis of glaucoma. Researchers have not completely characterized how glaucoma is manifested, but have been working on investigating why this outflow resistance increases. The discovery will direct further effective treatment options for glaucoma. The primary hypothesis is that damage occurs to the TM tissue which compromises the ability of the tissue to regulate outflow of the aqueous humor; when the resistance increases, aqueous outflow decreases and IOP elevates. It has been discovered that, IOP elevation is associated with the TM cellularity reduction (Alvarado et al., 1984; Xiong et al., 2021), the abnormality of TM cell phagocytic function (Buller et al., 1990), and the differential deposition of extracellular matrix in the TM tissue (Gong, 2016; Keller et al., 2009). IOP elevation leads to RGC apoptosis and necrosis which interferes with the RGC's function to send visual information to the brain (Kaur et al., 2008). Another cause of RGC death in glaucoma is the occurrence of hypoxia. Hypoxia promotes the synthesis of nitric oxide synthase (NOS) which is toxic to RGCs and increases cell death (Kaur et al., 2008).

Genetics of POAG

Some POAG-associated genes have been identified such as myocilin (MYOC), optineurin (OPTN), and WDR36 (Park et al., 2006; Youngblood et al., 2019; Resch and Fautsch, 2009). Mutant MYOC can cause protein misfolding and the disfigured proteins cause stress on the endoplasmic reticulum (ER) in TM cells. TM cells with chronic stress have a higher rate of cell death which impairs the TM function, hence causing glaucoma (Jain et al., 2017). OPTN associates with the Golgi apparatus but can sometimes fragment or destroy it when OPTN undergoes a mutation called E50K (Park et al., 2006). The fragmentation of the Golgi apparatus would then interfere with the cell's ability to transport necessary proteins and promote cell death in TM cells, leading to POAG. WDR36 has been debated about its role in glaucoma. Some researchers believe that mutations in this gene cause damage to the TM tissues on the periphery of the iris (Chi et al., 2010). Other studies point to WDR36 as not being a factor that contributes to POAG because there was no significant correlation between WDR36 mutations and POAG (Liu et al., 2017). Further research is needed to explore the link of these genes with the development of glaucoma.

Risk factors of POAG

Aging is a common risk factor in the occurrence of glaucoma. Overall, most people diagnosed with POAG are older than 40 years old at first diagnosis (Quigley and Broman, 2006; Youngblood et al., 2019). People who have relatives with POAG are also at a high risk of developing the disease from around 4% to 16% if in direct family members (Youngblood et al., 2019).

PACG

Contrastingly, PACG is caused by the iris of the eye physically blocking the conventional outflow pathway, the TM and Schlemm's canal, and increasing the IOP (Foundation, 2019). Individuals of Asian or Indian descent have a higher risk of developing this condition (Chelerkar et al., 2018). In 2015, there were 15.7 million cases of PACG worldwide with projected numbers to reach 21 million in 2020. The highest risk factor that has been reported is the actual dimensions of the eye. Certain measurements that may contribute to increased likelihood of PACG are short axial length, small corneal diameter, shallow anterior chamber, steep curvature, and a lens that is closer to the anterior of the eye (Ahram et al., 2015). Researchers have identified five genes that are involved in the development of PACG which are *HGF*, heat shock protein 70 (*HSP70*), membrane-type Frizzled-relation protein (*MFRP*), *MMP9*, and *NOS3* (Liu et al., 2020). Although identified, these genes have not been confirmed under genome-wide association studies so they cannot be exactly attributed for causing PACG (Liu et al., 2020). While PACG is less prevalent than POAG, it remains a common neuropathy that affects many people worldwide.

Current treatment options for POAG

Several treatment options have been used clinically for POAG including, but not limited to, medicine, laser treatment, and surgeries. All medications and procedures focus on reducing the aqueous humor production or increasing the aqueous humor outflow to lower the IOP of the eye, hence protecting the RGCs from damage and preserving visual acuity.

Medicine

Medicines that are commonly used are latanoprost, travoprost, unoprostone, and bimatoprost. All of these are classified as prostanglandin analogs and prostamides which act on the uveoscleral pathway to increase aqueous humor outflow (Bucolo et al., 2018). Another category of medicines that are used in treatment of POAG is $\alpha 2$ adrenergic agonists like brimonidine and apraclonidine that decrease aqueous humor secretion and increase outflow. A newer category of medicines for treating

POAG is Rho-associated kinase (ROCK) inhibitors, such as Netarsudil and Ripasudil. These are targeting the conventional outflow pathway to relax TM cells and affect the ECM with antifibrotic activity of the TM tissue (Inoue and Tanihara, 2013; Tanna and Johnson, 2018; Castro and Du, 2019; Li et al., 2021).

Laser

Laser treatment is another method to treat POAG. The most common method is laser trabeculoplasty which involves exposing TM cells to laser light (Young and Caprioli, 2019). This decreases their resistance to outflow of the aqueous humor. Alternatively, patients can undergo a laser diode cyclophotocoagulation. Contrasting laser trabeculoplasty, this laser is focused on the white sclera of the eye instead of the cornea and damages the pigmented ciliary processes, reducing the amount of aqueous humor secreted (Ndulue et al., 2018). These treatments are usually only temporarily effective and lose their effect after a few months, making them only a temporary solution for a long-term problem.

Surgeries

One surgical treatment option is trabeculectomy. Trabeculectomy is the most commonly used surgical procedure to promote increased drainage of the aqueous humor. It involves removing small portions of the TM and local tissue which increases the area that the aqueous humor can drain (Weinreb and Khaw, 2004; Binibrahim and Bergstrom, 2017). It has been developed minimally invasive glaucoma surgeries aiming to recover the function of outflow pathway for treating glaucoma (Xin et al., 2020). Although there are a variety of treatment methods, POAG is still difficult to treat and the exact pathophysiology of its manifestation is still under research.

Induced pluripotent stem cells as cellular models of diseases

Induced pluripotent stem cells (iPSCs) are commonly employed in different areas of research, ranging from disease modeling to treatment (Kumar et al., 2018; Rowe and Daley, 2019). These cells are especially attractive because they dodge the moral quandary of using embryonic stem cells. iPSCs have undergone many tests to compare them to embryonic stem cells including immunofluorescent staining, methylation assays, teratoma formation assays, karyotyping, and bisulfite genomic sequencing. Studies have provided convincing evidence that iPSCs share characteristics similar to embryonic stem cells (Raab et al., 2014; Halevy and Urbach, 2014; Marei et al., 2017). Another important aspect of iPSCs is that they can be collected from individuals who suffer from different diseases, thus recapitulating all the features of the disease and can be used as a suitable disease model. These cells

exhibit similar genetic makeup as that of the donors, making them less likely to be rejected and also mimicking the disease in the dish serving as cellular models (Raab et al., 2014). iPSCs can be generated by transfecting any somatic cells with transcription factors *Oct4, Sox2, c-Myc,* and *Klf4* (Wuli et al., 2020; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Lin et al., 2012). Fibroblasts are usually used to culture iPSC populations because they can generate large populations easily and were the first cells used to reprogram iPSCs. Some cautions for using them are that they have a relatively low reprogramming efficiency and can cause discomfort to the donors when sample collection occurs. They are collected via skin biopsies or neonatal foreskin biopsies which cause intense pain that must be anesthetized during the procedure. These cuts can also increase risk of infection, allergic reaction, and the formation of scar tissue (Raab et al., 2014). It has been reported that iPSCs can be reprogrammed from peripheral blood mononuclear cells (Ye et al., 2013; Zhou et al., 2015) or from exfoliated renal epithelial cells present in the urine (Zhou et al., 2011, 2012; Zhang et al., 2015; Afzal and Strande, 2015). Regardless of the collection methods, iPSCs are coveted in research because of their pluripotency.

iPSCs can differentiate into a variety of somatic cell types such as neurons, adipocytes, and osteocytes (Sheyn et al., 2016; Taura et al., 2009; D'Aiuto et al., 2014). This ability allows them to be prime candidates in the modeling of diseases because they can emulate cell types and tissues that are the specific targets of diseases. By creating a microcosm of the tissue, the pathophysiology and treatment options of diseases can be studied without in vivo animal experiments. Their potential for the generation of medical and scientific knowledge is truly extraordinary. Using various methodologies, scientists can characterize diseases more coherently when they have sample tissues.

As mentioned previously, one big appeal of iPSCs is that they can generate cells that have characteristics of the donor. This then allows researchers to test treatments on these cells. Since the cells have a similar genetic makeup, the effects on the iPSC-derived organoids in vitro can provide evidence on possible effects when the donor is treated with the same procedure. One such case is the generation of iPSCs from a diagnosed glaucoma patient with OPTN E50K mutation. Synthetic mRNAs with a microRNA booster kit were transfected to the fibroblasts from the OPTN E50K mutation patient. These cells were cultured for weeks, and eventually iPSC colonies were identified and cultured independently (Ohlemacher et al., 2016). These iPSCs had the same genetic characteristics as the donor and can be differentiated into cells relevant to glaucoma studies.

In the case of POAG, iPSCs can be induced to become RGCs and TM cells which are cells primarily affected by glaucoma. These cells can be utilized for disease modeling and for drug screening to identify targets for the treatment of glaucoma. TM cells are responsive to stimulation of steroid dexamethasone (Dex) (Keller et al., 2018) which can induce glaucoma-like conditions. Dex increases the outflow resistance in TM tissue and decreases the tissue's ability to regulate outflow which are the characteristics of POAG (Tian et al., 2020). The responsive

TM cells can be exposed to substances which can be tested for regenerative capability or protein expression which are related to glaucoma pathology or treatment mechanisms. The ability of iPSCs to differentiate into RGCs and TM cells make them as prime models for exploring possible treatment options for POAG. Also, since many retinal diseases are monogenic which makes it easy to model them using iPSCs due to their low allele frequencies and high genetic variation (Lidgerwood et al., 2019). Diseases involving multiple genes, namely, as heterogeneous diseases, are difficult to be modeled using iPSCs. Due to this, there are currently efforts going on worldwide that are attempting to build banks of iPSCs that are generated to model those diseases (Lidgerwood et al., 2019).

iPSC-TM cells as a model of glaucoma

We have successfully isolated TM stem cells by side population sorting or by clonal culture (Du et al., 2012). After long-term cryopreservation, TM stem cells retain their stemness with expression of stem cell markers (CD90, CD166, CD105, CD73, OCT4, SSEA4, Notch1, KLF4, ABCG2, Nestin, HNK1), the capability to form spheroids and colonies, multipotency, and the ability to differentiate into TM cells responsive to Dex treatment (Kumar et al., 2020a,b). These cells have been approved to have the ability to home to the TM tissue and regenerate the TM tissue to reduce IOP for glaucoma treatment in mouse models (Xiong et al., 2020, 2021; Du et al., 2013; Yun et al., 2018). But there are still limitations for using TM stem cells from glaucoma patients to make in vitro disease models. Because TM stem cells are located at the insert region between the TM and the corneal endothelium (Castro and Du, 2019; Braunger et al., 2014) where biopsy needs to go inside the eye, and in vitro disease models require huge cell numbers, and it is not feasible to gather large amounts of TM stem cells by biopsy in glaucoma patients for making disease models.

Other stem cell types, such as mesenchymal stem cells (Manuguerra-Gagne et al., 2013; Roubeix et al., 2015) and adipose-derived stem cells (Zhou et al., 2020), have been discovered with the ability to regenerate the TM and reduce IOP in animal models.

iPSCs have their unique property of pluripotency with unlimited lifespan and ability to differentiate into almost all kinds of somatic cells. iPSCs have been tested for regenerative capabilities on the TM tissue to reduce IOP (Zhu et al., 2016). These iPSCs can be induced to differentiate to TM cells using various approaches, providing an autologous source of TM cells for disease modeling or transplantation for treatment. Mouse iPSCs derived from dermal fibroblasts were induced to differentiate into TM cells using a paracrine growth factor milieu from immortalized primary TM cells which were layered on the top of a cell culture insert and mouse iPSCs were seeded beneath the inserts in cell culture plates (Ding et al., 2014). The differentiation was achieved by coculturing cells for 21 days which resulted in the generation of iPSC-TM cells that expressed TM cell markers matrix gla protein, tissue inhibitor of metalloproteinase 3 (TIMP3), Col4a5, and lost the expression of pluripotency markers OCT4, Nanog, and Sox2. These cells were able to

phagocytose pHrodo green-conjugated *E. coli* particles. These induced iPSC-TM like cells responded to Dex treatment with elevated expression of MYOC and matrix metalloproteinase-3 (MMP-3). This finding indicates that the iPSC-TM cells can be a great cell source as cell-based therapy to restore the TM tissue in glaucoma eyes.

We recently reported that human iPSCs can be induced into TM cells by two steps with an intermediate stage of neural crest (NC) cells. Since TM cells are derived from NC embryonically, this two-step protocol can generate a stable TM progenitor pool. The induced NC cells can then differentiate into TM-like cells, responsive to Dex treatment (Kumar et al., 2020a,b). The undifferentiated iPSCs were positive to pluripotent stem cell markers OCT4, alkaline phosphatase, SSEA4, and SSEA1 (Fig. 3.2).

This protocol employed ECM from A549 cells and N2/B27 medium to induce a NC stage from iPSCs grown in monolayer culture conditions. After induction on the A549 cell-derived ECM and in the culture medium containing N2/B27, the iPSCs differentiated into NC cells expressing NC markers HNK1 and p75 (Fig. 3.3). A549 cell ECM was used in this protocol for NC induction because A549 cells express α5 chain of laminin-10/11 and secrete it into the conditioned medium, which is important for mediating adhesion, proliferation, and differentiation of neuronal cells after its interaction with integrin α3β1 (Kikkawa et al., 1998). Laminin 511 has been isolated from the conditioned medium of A549 cells (Kikkawa et al., 1998; Gu et al., 2001). Laminin 511 can maintain self-renewal and proliferation of ESCs in feeder-free conditions by increasing cell-cell contact by interaction with integrins α1, α6β1, and α5β1 of ES cells, making laminin 511 an ideal surface

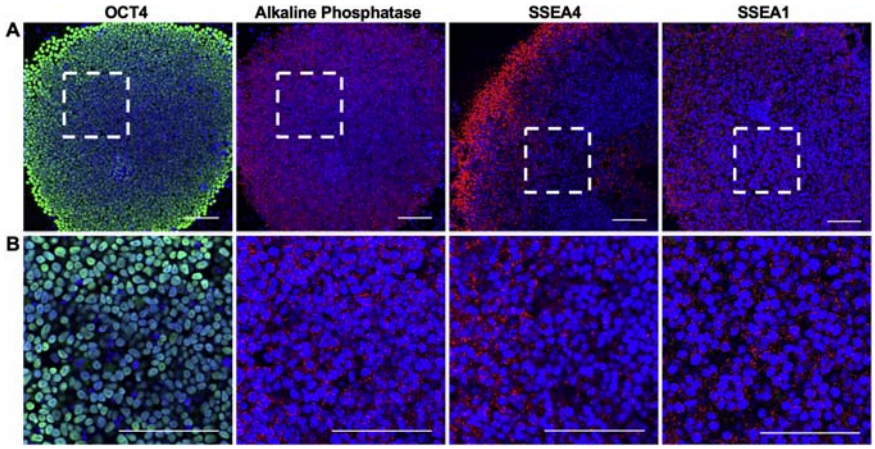

FIGURE 3.2 iPSC colonies express pluripotent cell markers.

Immunofluorescent staining shows expression of pluripotency markers OCT4, alkaline phosphatase, SSEA4, and SSEA1 in iPSCs grown as colonies. (A) are the magnified pictures of in framed region. (B) DAPI stains nuclei as blue. Scale bars, 100 μm.

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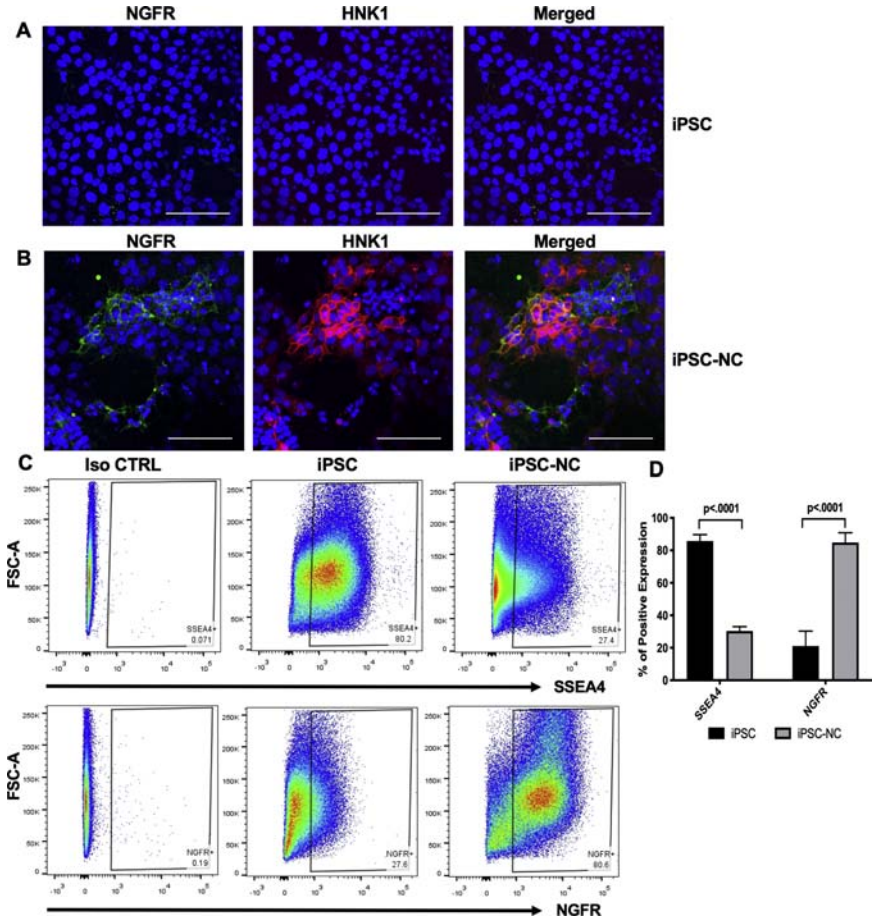

FIGURE 3.3 iPSCs can differentiate into neural crest cells (iPSC-NC).

Immunofluorescent staining shows iPSC were negative to neural crest markers NGFR and HNK1, (A) while iPSC-NC cells expressed NGFR and HNK1. (B) DAPI stains nuclei as blue. Scale bars, 100 μm. (C) Representative figures of flow cytometry analysis showing most of iPSCs were positive to SSEA4 and negative to NGFR. After induction, iPSC-NC reduced expression of SSEA4 and increased expression of NGFR. (D) Percentage of iPSCs and iPSC-NC cells labeling positive by SSEA4 and NGFR ($n = 6$, mean ± SD, mean ± SD). One-way ANOVA followed by Tukey's multiple comparisons test.

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coating system for feeder-free maintenance of ESCs and iPSCs (Domogatskaya et al., 2008). Laminin 511 and other isoforms have been reported to induce directed differentiation of human iPSCs into cells of neural crest, neural, and retinal origin by interaction with various integrin receptors (Shibata et al., 2018), making A549 ECM including secreted laminin 511 an idea substrate for iPSC-NC induction.

The induced NC cells were grown in a conditioned medium (CM) derived from primary human TM cells, mixed with DMEM/HAM's F12 (1:1). The successfully differentiated TM cells (iPSC-NC-TM) expressed TM cell marker CHI3L1 (Fig. 3.4A and D). The iPSC-NC-TM cells were responsive to Dex treatment with increased expression of MYOC and ANGPTL7, a characteristic of primary TM cells, and reduced expression of CHI3L1 (Fig. 3.4B and D). The differentiated iPSC-NC-TM cells also formed cross-linked actin networks (CLANS) after Dex treatment (Fig. 3.4C) which is an important feature of Dex-induced primary TM cells (Keller et al., 2018).

The formation of promigratory NC cells has been reported after culturing of human pluripotent stem cells in N2B27 medium and withdrawing TGF-β inhibition for 10 days in culture (Noisa et al., 2014). These cells maintained their differentiation capacity even after 20 passages, suggesting the generation of stable NC cells. The induced cells showed expression of NC cell markers HNK1 and p75 and loss of hESC markers (Menendez et al., 2013). Our two-step protocol can generate a stable neural crest cell pool to allow the same TM cell source for further disease modeling, TM cell transplantation for glaucoma, and for drug discovering.

iPSC-RGCs as a model of glaucoma

RGCs are the other cell type that is affected by glaucoma. They are one of the important components of the retina in the posterior part of the eye. The axons of the RGCs gather together to form the optic nerve that transmits information to the brain. iPSCs have been successfully induced to differentiate into RGCs (Chamling et al., 2016). An important factor in differentiating cells from stem cells is to make them similar to the specific cells they are supposed to be imitating. It is important that the iPSC-derived cells have similar morphology, electrophysiology, and protein expression to their analogs found in humans (Lidgerwood et al., 2019).

Various steps need to be taken to differentiate the iPSCs to RGCs. As mentioned previously, iPSCs are reprogrammed from somatic cells and turned into embryoid bodies in culture. The embryoid bodies can commonly differentiate into different types of cells found in all three germ layers, including neural cells during embryonic development. iPSCs can differentiate into RGCs with a variety of methods such as utilizing culture media containing various supplements, genetic modification, and three-dimensional (3D) organization (Gill et al., 2014).

The creation of retinal cells, retinogenesis, is modulated by a variety of growth factors and proteins. Some molecules that can be added to the culture with iPSCs to induce RGC differentiation are fibroblast growth factor 2 (FGF2), insulin-like growth factor 1 (IFG1), epidermal growth factor (EGF), bone morphogenetic protein antagonist noggin, nodal antagonist left-right determination factor 2 (LEFTY2), Wnt signaling pathway inhibitor dickkopf 1 (DKK1), forskolin, and vitamin supplements N2 and B27. These growth factors and proteins can induce iPSCs to differentiate into RGCs (Gill et al., 2014).

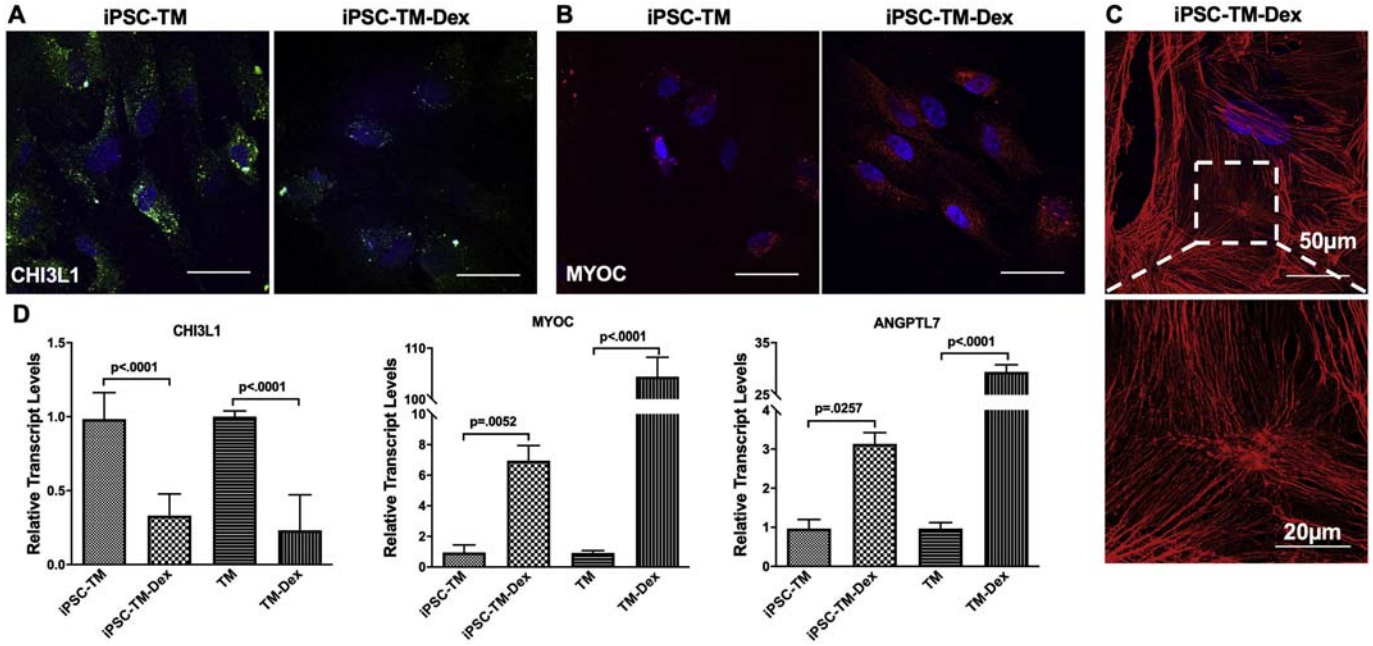

FIGURE 3.4 iPSC-NC cells can differentiate into TM-like cells.

(A) Immunofluorescent staining shows iPSC-NC differentiated TM-like cells (iPSC-TM) expressed TM cell marker CHI3L1 and after treatment with Dex, the cells (iPSC-TM-Dex) reduced CHI3L1 expression. (B) iPSC-TM-Dex cells had increased expression of MYOC. DAPI stains nuclei as blue. Scale bars, 50 μm. (C) Phalloidin staining shows F-actin in the iPSC-TM-Dex cells formed cross-linked actin networks (CLANs) and the enlarged figure. (D) qPCR shows mRNA expression of CHI3L1, MYOC, ANGPTL7 comparing iPSC-TM and iPSC-TM-Dex, TM cells and TM-Dex treated cells ($n = 6$, mean ± SD). One-way ANOVA followed by Tukey's multiple comparisons test.

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Another method that can be used for RGC differentiation is genetic modification. This method relies on the overexpression of transcription factors that shift the iPSC cell fate toward RGCs (Gill et al., 2014). Transcription factor, Pax6, is an example of these. ESCs transfected with Pax6 were shown to express markers of retinal neuron progenitor cells. These markers were Tubb3, Nefm, Nestin, Brn3, Thy1, Isl1, and Opn4 (Kayama et al., 2010). These markers provide evidence that after transfection with Pax6, stem cells can be induced to differentiate into RGCs. In another study, Pax6 was also concluded to induce iPSC differentiation into RGCs. The differentiated cells expressed nestin, Tubb3, Nefm, Brn3, and cone-rod homebox (Suzuki et al., 2012). Other transcription factors that have been tested for RGC reduction are Atoh7 and Math5 (Chen et al., 2011). Cells induced with these transcription factors expressed common RGC markers (Chen et al., 2011), hence reflecting the genetic modification of iPSCs to RGCs.

iPSCs can also be induced to differentiate to RGCs using 3D organization. When the eye is forming the retina, there is a process organizing the cells in neat structure systems in a 3D space (Chamling et al., 2016). This process allows stem cells to aggregate into a structure called optic cup which then can differentiate into the multilayered structure found in a fully developed eye. This multilayered structure includes cells such as RGCs and photoreceptors (Chamling et al., 2016). This method involves placing iPSCs on Matrigel plates and culturing them. The arrangement of the cells will promote the formation of the optic cup and develop the complex 3D structure (Maekawa et al., 2016). But RGCs are produced in low populations as compared to other cell populations in this 3D structure (Chamling et al., 2016). The limited cell number is not ideal for testing pathologies of diseases or generating simulation tissue. This method is still being studied to determine if this technique has potential to generate large amounts of RGCs.

Once RGCs have been properly differentiated and their populations are high enough, these cells can be used to study retinal diseases like POAG. Although increased IOP is commonly associated with glaucoma, it is possible that those afflicted with POAG may not have high IOP but still have the effects of POAG. This is known as normal-tension glaucoma. Two genes, OPTN and TBK1, have been identified as factors that cause normal-tension glaucoma and RGC death. TBK1 is involved in the activation of a pathway that regulates autophagy; the increased levels of TBK1 induce this pathway into hyperactivity which causes an increased rate of autophagy and cell death (Giacalone et al., 2016). iPSC-derived RGCs can be used to study various genes, proteins, and transcription factors like TBK1 and characterize their effects or contributions toward retinal diseases like glaucoma.

Patient-derived iPSCs as a model of glaucoma

iPSCs can be isolated from many sources around the body, such as skin fibroblasts, peripheral blood mononuclear cells, or exfoliated cells in the urine. Although using these iPSCs for modeling diseases is useful, it would be ideal if the iPSCs being used

come from the person afflicted with the disease, in this case, glaucoma. Cells collected from these patients could shed light on pertinent information relevant to finding treatment options for these specific patients, meaning personalized medicine. Some characteristics of these cells to consider scrutinizing are the specific mutations and pathophysiologic mechanisms, the effects of novel gene augmentation, gene silencing, small molecule therapies, and the restoration of function posttransplantation of iPSC-derived cells and tissues (Giacalone et al., 2016). This allows researchers and doctors to get a better understanding of the specific genetic landscape of the individual they are trying to treat. Since all the cells produced from these patients will contain the same genetic information, this would also reduce the possibility of tissue rejection and destruction because the donor's body would recognize these patient-derived cells as their own. These patient-derived cells also have the same genetic markers as the donor, so these cells can be created to form tissues that simulate those found in the donor. This can be used to run tests to glean more information about the pathophysiology of POAG. This may involve inducing mutations, exposure to drugs and stressors, or simulation of glaucoma-like conditions. Patient-derived iPSCs are an emerging type of cells that is becoming increasingly attractive for research into the characterization and modeling of diseases.

Drug discovery using iPSCs as a glaucoma model

Patient-derived iPSCs are ideal sources for drug discovery because they can provide information about donors that cannot be collected normally. As discussed in the previous section, it is hard or impossible to collect glaucoma-associated TM cells or RGCs from living patients due to the damage extraction would cause to the tissues, especially the RGCs. Patient-derived iPSCs are very useful in learning new information and exploring personalized medicine. It has been discovered that RGCs exist in different subtypes. These subtypes may have slightly different structures and function so single cell RNA sequencing (scRNA-seq) was used to identify specific gene variations between these RGC subtypes (Langer et al., 2018). Based on this finding, patient-derived iPSCs could be controlled to differentiate into more specific RGC subtypes (Langer et al., 2018). Characterization of these RGC subtypes can offer more information in discovering new possible drug targets. Drugs could eventually be designed to target specific transcription factors or genes found only in those RGC subtypes that POAG affects.

The drug development process is a long, expensive undertaking that involves much testing on both human and animal models. In the existing system, iPSC-derived stem cells can modify the second and third clinical trial phases by allowing the drugs to be tested on tissues from humans (Ko and Gelb, 2014). In the second phase of clinical trials, iPSCs can be used to test for drug efficacy. During the third phase of clinical trials, iPSCs can be used to determine the drug efficacy on populations of individuals from a multitude of genetic backgrounds (Ko and Gelb, 2014). These cells populations could be cultivated from a variety of people both

with and without diseases to establish a vast bank of cells from different genetic profiles. Glaucoma studies can also utilize this method and test drug efficacy for multiple genetic backgrounds and their effects on healthy and glaucoma-afflicted individuals.

Some factors may disrupt this process and make it difficult to use patient-derived iPSCs. One challenge is that iPSC reprogramming may be insufficient for differentiation into desirable cell types (Ko and Gelb, 2014). This study drew increased emphasis in understanding the cellular processes and machinery necessary for iPSC differentiation. Currently, we only understand the differentiation procedure for a few cell types but not all of them (Ko and Gelb, 2014). If more is understood about these functions, more types of patient-derived iPSCs can be used for novel drug development. Drug discovery is a crucial step in the exploration of treatment of diseases and patient-derived iPSCs look like an attractive avenue for modeling of disease targets.

Conclusion

iPSCs can be reprogrammed from normal subjects or from patients with a specific disease and can be induced to differentiate into specific functional cell types, such an TM cells and RGCs in glaucoma, for modeling diseases, exploring disease pathophysiology, discovering novel drug treatments, and cell replacement for regeneration to treat glaucoma (Fig. 3.1 Graphical abstract).

Acknowledgments

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CHAPTER

4

Patient-specific induced pluripotent stem cells for understanding and assessing novel therapeutics for multisystem transthyretin amyloid disease

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Chapter outline

Abstract

The systemic amyloidoses represent a devastating class of multisystem protein folding disorders whose etiologies involve complex interactions between multiple

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tissue types (e.g., aberrant protein producing effector and downstream damaged target organs). Problematically, this hallmark multiorgan involvement presents unique challenges for developing robust *in vitro* and *in vivo* preclinical models of these diseases, ultimately slowing the discovery of novel therapeutics. To these ends, induced pluripotent stem cells (iPSCs), capable of differentiating into any cell type of the body while at the same time maintaining the entire genetic context of the individual from which the line was derived, have the potential to allow for novel insights into systemic amyloid diseases such as transthyretin amyloidosis (ATTR amyloidosis). In this chapter, we will discuss the contribution of iPSCs to the study of this class of complex, devastating disorders, with a special emphasis on ATTR amyloidosis. In the process, we will highlight how patient-specific iPSC-based models of amyloid disease can lend novel insight into disease progression, revolutionize the drug discovery pipeline, and improve clinical management of disease through development of novel diagnostic biomarkers.

Keywords: Amyloid; Directed differentiation; Disease modeling; Drug discovery; Gene editing; Hereditary ATTR amyloidosis; Induced pluripotent stem cells; Light chain amyloidosis; Precision medicine; Protein folding disorders; Proteostasis; Systemic amyloid disease; Transthyretin (ATTR) amyloidosis; Unfolded protein response; Wild-type ATTR amyloidosis.

Introduction

Systemic amyloid disease refers to a class of complex, devastating protein folding disorders impacting over one million individuals worldwide (Blancas-Mejía and Ramirez-Alvarado, 2013; Buxbaum, 2004; Merlini and Westermark, 2004; Wechalekar et al., 2016; Falk et al., 1997). In these diseases, effector cells secrete proteins which misfold and travel throughout circulation, eventually depositing at downstream target tissues resulting in cell death and organ dysfunction (Fig. 4.1) (Blancas-Mejía and Ramirez-Alvarado, 2013; Buxbaum, 2004; Merlini and Westermark, 2004; Wechalekar et al., 2016; Falk et al., 1997). These disorders are often heritable, with destabilizing mutations increasing the propensity of proteins to misfold and eventually aggregate into proteotoxic species. At the same time, the quantity and functionality of many protein homeostasis (proteostasis) mechanisms decrease throughout aging, increasing the abundance of misfolded protein species in circulation, and resulting in pathologies which mimic many of the systemic amyloid diseases (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015; Hipp et al., 2019; Kaushik and Cuervo, 2015; Labbadia and Morimoto, 2015). In turn, insights gained through studying these complex disorders can have wide reaching implications—ranging from the development of broad-acting, novel therapeutics for many disorders to the understanding of cellular and molecular mechanisms of multisystem aging-associated biological decline.

One of the most common systemic amyloid diseases, transthyretin amyloidosis (ATTR amyloidosis), can be caused by over 100 described destabilizing mutations in the transthyretin (TTR) gene or the sporadic misfolding of the wild-type protein in an aging-related fashion (termed hereditary or hATTR amyloidosis, and wild-type or

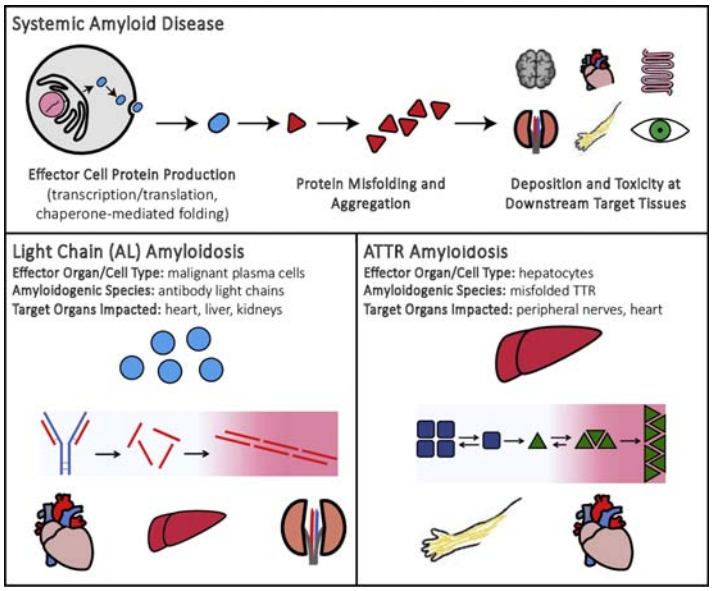

FIGURE 4.1 Systemic amyloid disease pathogenesis.

(Top) Representation of the overall steps comprising systemic amyloid disease pathogenesis, from protein production and initial folding, to secretion and misfolding, followed by deposition at diverse tissue types. (Bottom) Descriptions of light chain-associated AL and ATTR amyloidosis including effector organs, overall formation of amyloid species, and target organs impacted.

wtATTR amyloidosis, respectively) (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015; Giadone et al., 2018). In both forms of the disease, the liver produces misfolded TTR which subsequently travels throughout circulation and deposits extracellularly at downstream target organs (e.g., peripheral nerves and/or cardiac tissue), most often resulting in cardiomyopathy and/or peripheral neuropathy (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015; Giadone et al., 2018). Although historically, both wild-type and hereditary ATTR amyloidosis have been considered rare diseases, recent advances in clinical treatment, diagnostic technologies, and awareness for the disease will likely lead to an accelerated rise in prevalence (Schmidt et al., 2018; Grogan et al., 2017).

ATTR amyloidosis (both hATTR and wtATTR) is a complex disorder involving interaction of multiple target organs (i.e., TTR-producing effector tissues such as the liver, and target organs such as the heart and peripheral nerves). Moreover, patients with hATTR amyloidosis exhibit further complexity as seen in diverse pathologies (e.g., target organs impacted) and disease progression, due in part to the sex and genetic background of the patient (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015). Due to the multitissue etiology

of the disease and the strong association with the age and genetic background of the affected individual (hurdles common to many other systemic amyloid diseases), preclinical models of ATTR amyloidosis (both *in vivo* and *in vitro*) have remained extremely limited. In turn, cellular and molecular understandings of the fundamental processes involving ATTR amyloidosis disease ontogeny (e.g., proteotoxic TTR secretion and deposition) remain elusive. In an effort to combat this, researchers now employ induced pluripotent stem cell (iPSC)-based models in a *precision medicine* approach to study and develop novel therapeutics for TTR-mediated and other systemic amyloid diseases (Giadone et al., 2018, 2020; Leung et al., 2013; Leung and Murphy, 2016).

Here, we will describe current therapeutic and investigative approaches for ATTR amyloidosis, including present limitations and considerations for developing novel models of the disease. We will also discuss the potential for iPSCs to model systemic amyloid diseases and improve the efficiency of drug discovery in the field. In doing so, we will survey recent achievements in iPSC platforms for modeling protein folding disorders as well as discuss future applications for the technology in the modeling of ATTR amyloidosis and other systemic amyloid diseases.

ATTR amyloidosis pathogenesis

ATTR amyloidosis pathogenesis involves the concentration-dependent aggregation of misfolded TTR at downstream target organs (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015; Giadone et al., 2018, 2020). TTR is produced chiefly by the liver (with some synthesis in the pancreas and choroid plexus), where it comes together to form a tetramer and serves its normal physiological role as a minimal transporter of thyroxine and retinol-binding protein throughout the body (Power et al., 2000). In both wtATTR and hATTR amyloidosis, tetrameric TTR is destabilized, resulting in monomer formation. Dissociated monomers can subsequently misfold, aggregate into low molecular weight oligomers, travel throughout circulation, and deposit extracellularly at downstream target organs such as the heart and peripheral nerves as large, insoluble Congophilic amyloid fibrils (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015).

Hereditary ATTR amyloidosis (resulting from destabilizing mutations) is understood to be a rare disease, with an estimated prevalence of 50,000 cases worldwide (Gertz, 2017). Aging-associated wtATTR amyloidosis, involving the deposition of wild-type TTR fibrils, is thought to be much more common, with postmortem studies identifying cardiac TTR amyloid fibrils (associated with late-stage disease progression) in 25% of individuals over 80 years of age (Ruberg and Berk, 2012; Tanskanen et al., 2008). Clinically, hATTR amyloidosis symptoms typically manifest in the fifth or sixth decade of life (though dependent on the mutation), while wild-type disease manifests in the 8th decade (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015). Presently, TTR

amyloid disease is diagnosed by histologically staining fat aspirate biopsies with Congo Red—a dye which preferentially stains amyloid fibrils. Mass spectrometry (MS) is then performed to identify Congophilic protein species. Complicating our understanding, treatment, and accurate assessment of the prevalence and penetrance of the disease, a lack of approved diagnostic biomarkers coupled with a subtle and systemic onset of symptomatology makes the disease notoriously difficult to diagnose (Reixach et al., 2004; Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015; Giadone et al., 2018, 2020).

Current standards of care for patients with ATTR amyloidosis

Historically, standards of care for patients with ATTR amyloidosis have included liver transplantation in an effort to decrease the concentration of misfolded, amyloidogenic TTR in circulation (Fig. 4.2). Despite this, however, not all patients are candidates for surgery due to age or disease progression, while at the same time donor organ deficits exist. Alternatively, small molecule kinetic stabilizers such as diflunisal (a repurposed NSAID) and tafamidis (a synthetic compound, Vyndaqel) work by binding and stabilizing tetrameric TTR, limiting monomerization and subsequent misfolding from occurring (Berk et al., 2013; Ando et al., 2016; Maurer et al., 2017, 2018; Buxbaum, 2019; Coelho et al., 2012). Tafamidis recently received FDA approval for treatment of peripheral neuropathy, while diflunisal is approved for both cardiomyopathy and peripheral neuropathy (Berk et al., 2013; Coelho et al., 2012). Despite success in clinical trials for both compounds, patients exhibit large variations in responsiveness, likely due to their inherited TTR mutation or other disease modifying factors (Berk et al., 2013; Ando et al., 2016; Maurer et al., 2017, 2018; Buxbaum, 2019; Coelho et al., 2012). In addition, first-in-class RNA interference (RNAi)-based therapeutics, Inotersen and Patisiran, have been

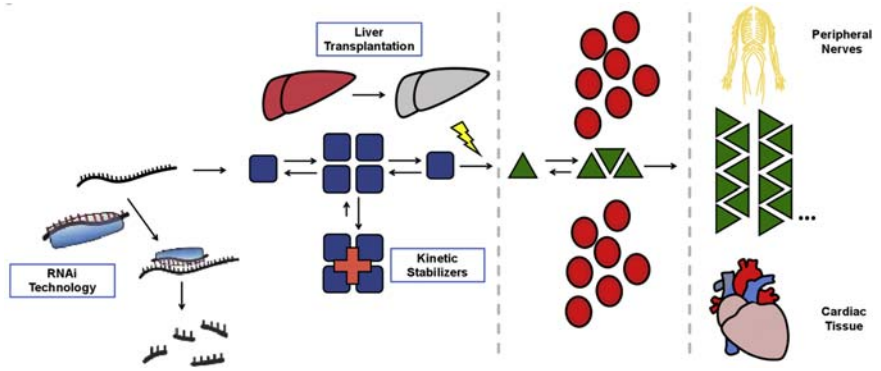

FIGURE 4.2 ATTR amyloidosis pathogenesis and novel therapeutic strategies.

Mechanisms of ATTR amyloidosis pathogenesis including novel therapeutic strategies for treating the disorder.

developed for hATTR amyloidosis. Inotersen (TEGSEDI) and Patisiran (ONPATTRO) work via slightly different mechanisms (antisense oligonucleotide vs. siRNA, respectively), but are both delivered via GalNac-coated lipid nanoparticles which selectively target the liver (Adams et al., 2018; Butler et al., 2016; Benson et al., 2018; Buxbaum, 2018). Both have recently received FDA approval for use in patients with ATTR amyloidosis-associated polyneuropathy. Despite the recent advent of therapeutics for the disease, the most effective treatment regimen (including multi-drug combination and dosage) is likely dependent on the individual.

Complicating the development of broadly efficacious therapeutics is that the genetics of ATTR amyloidosis are highly complex, with wide-ranging prevalence and penetrance depending on the inherited mutation, sex, and/or ethnicity. Worldwide, the most prevalent TTR mutation is the peripheral-neuropathy associated TTR^{V30M} variant. In the United States, however, it is estimated that approximately 4% of African Americans are carriers for the TTR^{V122I} mutation, primarily associated with cardiomyopathy (i.e., TTR amyloid fibrils deposited in the cardiac tissue) (Buxbaum and Ruberg, 2017). Interestingly, the penetrance of certain mutations changes drastically depending on geography. Penetrance of the peripheral neuropathy-associated TTR^{V30M} mutation, for example, ranges from 18% of carriers developing the disease by age of 50 in France to 80% by the age of 50 in Portugal (Parman et al., 2016; Hellman et al., 2008). At the same time, there appears to be correlations between inheritance pattern (i.e., paternal vs. maternal origin) and age-of-onset as well as sex of the patient (e.g., a majority of wtATTR amyloidosis being male) (Lemos et al., 2014; Connors et al., 2009).

In addition to variation in disease progression dependent upon geographic location, a substantial amount of variability in clinical manifestation and responsiveness to treatments depends on the inherited TTR mutation. This is largely due to the relative stabilities of the natively folded monomer and/or tetramer conferred by the mutation. Highly destabilizing mutations such as TTR^{A25T} and TTR^{L55P}, for example, have a large propensity to misfold, and as a result, are less responsive to kinetic stabilizers which rely on physical interactions with the natively folded tetramer (Sekijima et al., 2003; McCutchen et al., 1993). This is contrasted to the relatively successful stabilization of homo- and heterotetramers made up of TTR^{WT} and/or TTR^{V30M} (Waddington Cruz and Benson, 2015). In order to develop broadly effective therapeutics for ATTR amyloidosis, preclinical models and the drug discovery pipeline must take into account the diverse genetic backgrounds and driver mutations of the affected patient population — a prospect enabled by iPSC technology.

Limitations of current pre-clinical disease models

Hindering the development of effective treatments for diseases such as ATTR amyloidosis, the current drug discovery and clinical trial paradigm is both costly and time-consuming (Brodniewicz and Grynkiewicz, 2010; Wong et al., 2019; Avorn, 2015; Roses, 2008). Potential candidate compounds are first identified for

follow-up study and subsequently evaluated for efficacy and toxicity in preclinical models, consisting of both *in vitro* immortalized cell culture-based experiments and *in vivo* animal models, prior to entering multiphase clinical trials (Giadone et al., 2018; Brodniewicz and Grynkiewicz, 2010; Roses, 2008).

Traditionally, *in vitro* cell culture-based studies rely on the use of immortalized cell lines often derived from human primary tissue (Giadone et al., 2018; Brodniewicz and Grynkiewicz, 2010). Though insightful, cell lines represent the genetic background of a single person and as a result, make it difficult to extrapolate findings made in one individual cell line to other lines, animals, and eventually humans. This limitation is likely a contributor to the large failure rates seen in drug discovery. At the same time, the process of generating immortalized cell lines often leads to large, karyotypic abnormalities — an additional source of factors confounding investigations into the cells or tissues from which these lines are derived (Toouli et al., 2002).

Alternatively, *in vivo* animal models have long been valued as a gold standard for understanding disease and determining the efficacy of preclinical pharmaceutical agents (Ericsson et al., 2013; Rosenthal and Brown, 2007). For most animal models of human disease to be effective, the model should recapitulate some aspect of the human disease-associated phenotype and that this phenotype should be driven by etiological mechanisms shared by both the human and model organism (McGonigle and Ruggeri, 2014). Problematically, however, this limits the robustness of animal models for diseases whose causal mechanisms are unclear. Similarly, animal models of complex diseases, whose causes are either unknown or involve combinatorial interactions of multiple driver mutations and/or noncoding variants (e.g., Alzheimer's disease, Parkinson's, multiple sclerosis, etc.) have had limited success in predicting efficacy of novel therapeutic compounds (McGonigle and Ruggeri, 2014). Lastly, as with immortalized cell lines, animal models that require knocking-in disease-associated mutations are developed from overexpression of a mutation in a single genetic background — severely limiting the understanding of how diverse genetic backgrounds affect therapeutic potential.

ATTR amyloidosis has proven difficult to study both *in vitro* and *in vivo* due to the multitissue etiology and highly aging-related component of the disease (Giadone et al., 2018, 2020; Leung et al., 2013; Leung and Murphy, 2016; Buxbaum, 2009). To this point, several mouse models of the disease have been developed; however, none currently recapitulates key aspects of ATTR amyloidosis pathogenesis (Buxbaum, 2009). The earliest models, involving knocking-in human TTR^{V30M} cDNA or genomic DNA (including upstream regulatory sequences), had no amyloid fibrils formed in the peripheral nerves (Buxbaum, 2009). Later mouse models, involving introducing a large number of copies of genomic wild-type and TTR^{L55P}, achieve fibril deposition with approximately 100 copies of transgene (Buxbaum, 2009). Curiously, upon transferring TTR^{V30M} mice to a sterile facility, the mice no longer formed amyloid fibrils — implying an importance of the immune system in the formation of fibrils and disease (Buxbaum, 2009; Inoue et al., 2008).

In addition to the limitations of ATTR amyloidosis mouse models for recapitulating disease phenotype, present animal models are also unable to consider the genetic background of the individual, which has strong effects on the progression

of the disease. Developing more robust, patient-specific preclinical models, better equipped to determine efficacy and toxicity of a novel compound in multiple genetic backgrounds and cell types, represent a potential avenue for reducing the failure rate, time, and cost of drug discovery for ATTR amyloidosis and other systemic amyloid diseases.

Developing IPSC-based models of hereditary ATTR amyloidosis

In order to address the limitations of current mouse models, while also taking into account the importance of genetic backgrounds of patients, several groups now employ iPSCs to model various aspects of ATTR amyloidosis. In 2013, Leung et al. developed the first iPSC-based platform for studying hereditary ATTR amyloidosis (Fig. 4.3) (Leung et al., 2013). In the model, peripheral blood

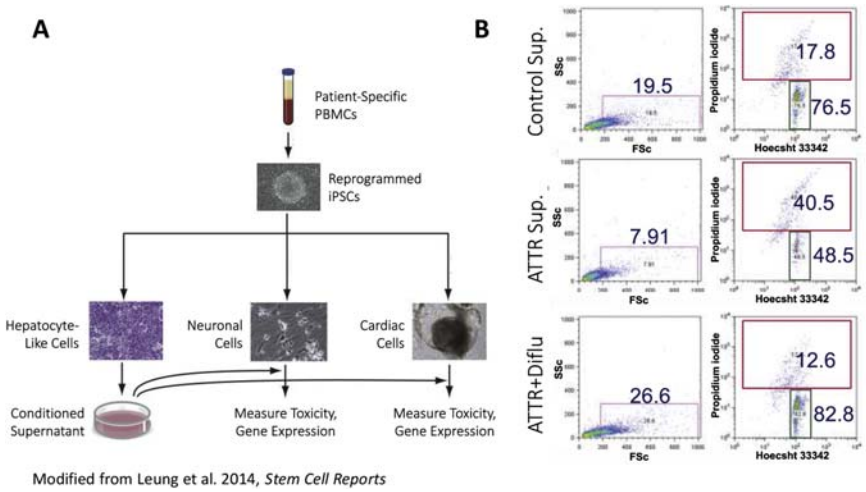

FIGURE 4.3 Multisystem iPSC-based modeling of hereditary ATTR amyloidosis recapitulates key aspects of human disease pathogenesis.

(A) Schematic representation of model consisting of the reprogramming of patient-specific iPSCs and differentiation into hepatocyte-like, neuronal, and cardiac cells to measure responsiveness to TTR-containing hepatic supernatant. (B) Flow cytometry-based, PI viability assay representing an increase in toxicity upon exposing iPSC-derived motor neurons to ATTR amyloidosis hepatic supernatant compared to cells dosed with wild-type control hepatic supernatant (40.5% dead/dying vs. 17.8%). Upon exposing motor neurons to ATTR amyloidosis hepatic supernatant with diflunisal, toxicity returned to levels comparable to wild-type control (12.6%).

Modified from Leung, A., Nah, S.K., Reid, W., et al., 2013. Induced pluripotent stem cell modeling of multisystemic, hereditary transthyretin amyloidosis. Stem Cell Rep. 1 (5), 451–463. (Published 2013 Oct 31). https://doi.org/10.1016/j.stemcr.2013.10.003, the Murphy laboratory's iPSC-based platform for modeling ATTR amyloidosis.

mononuclear cells (PBMCs) or fibroblasts are obtained from diagnosed patients and subsequently reprogrammed into iPSCs. Patient-specific iPSCs are then directed to differentiate into effector hepatocyte-like cells (HLCs) via a two-dimensional, chemically defined feeder-free directed differentiation protocol. Importantly, these HLCs produce and secrete destabilized, amyloidogenic mutant TTR variants into their supernatants, which are detected through proteomics methods like MS (Giadone et al., 2018, 2020; Leung et al., 2013). Concurrently, patient-matched iPSCs are directed to differentiate into the target cells involved in the disease, including neuronal and cardiac lineage cells. The TTR-containing supernatant from iPSC-derived HLCs is dosed onto iPSC-derived target cells and toxicity can be measured via flow cytometry-based cell death assays or expression of stress marker genes via qRT-PCR. Models such as these can be used to assess the efficacy of novel therapeutics in decreasing toxicity seen at downstream target cell types. To this end, upon combining TTR^{L55P} iPSC-derived hepatic supernatant with kinetic stabilizer diflunisal, and subsequently dosing patient-matched iPSC-derived neuronal cells, toxicity in exposed neuronal cells decreases in the presence of the stabilizer compared to cells dosed with mutant supernatant without stabilizer (Fig. 4.3) (Leung et al., 2013).

For genetically complex disorders such as hereditary ATTR amyloidosis, with disease progression varying with ethnicity and driver mutation, it is important to perform experiments in a number of different iPSC lines. Due to both time (e.g., length of differentiation protocols) and cost restrictions, however, this can be unrealistic. It is therefore worthwhile to carefully choose iPSC lines that are most appropriate to answer the biological question at hand (e.g., a cardiomyopathy-associated mutation when studying cardiac system dysfunction). In order to facilitate this, a library of genetically diverse iPSC lines derived from ATTR amyloidosis patients with a variety of driver mutations, ethnicities, and pathologies has been recently developed (Giadone et al., 2018).

Hepatic proteostasis remodeling in IPSC-based models of ATTR amyloidosis

Utilization of the above described patient-specific iPSC-based model of ATTR amyloidosis has furthered investigations into the contribution of proteostasis and hepatic disease modifying factors to the peripheral organ toxicity observed in patients with the disease.

Recent work utilizing the previously described iPSC-based platform demonstrates the potential for UPR-associated signaling pathways in modulating ATTR amyloidosis pathogenesis (Giadone et al., 2020). To this end, TALEN-mediated gene editing was utilized to generate isogenic, corrected, and uncorrected syngeneic iPSCs derived from a patient with the TTR^{L55P} mutation. Uncorrected and corrected iPSCs were directed to differentiate into HLCs, and single-cell RNA sequencing (scRNAseq) was performed in order to identify a transcriptional signature resulting

from expression of disease-causing TTR^{L55P}. HLCs expressing mutant TTR were found to upregulate expression of chaperone genes and genes associated with the adaptive (ATF6 and IRE1) arms of the unfolded protein response (UPR). It was further shown that ATF6 branch-specific activation of the UPR in ATTR amyloidosis patient-specific iPSC-derived HLCs selectively reduced secretion of the toxic TTR^{L55P} (Giadone et al., 2020). After being transcribed and translated, nascent polypeptides are shuttled into the ER to undergo a number of complex chaperone-mediated folding events, eventually forming a functional protein product. Proper folding of newly translated polypeptides and degradation of misfolded protein products is regulated by complex signaling pathways collectively termed proteostasis networks (Hipp et al., 2019; Giadone et al., 2020; Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Hetz, 2012; Walter and Ron, 2011; Glembotski et al., 2019; Romine and Wiseman, 2020; Christis et al., 2010). These networks, including the UPR, play important roles in maintaining the fidelity of the proteome in instances of homeostasis, stress, and disease. The UPR is regulated by three transmembrane receptors within the lumen of the ER: IRE1, ATF6, and PERK (Fig. 4.4) (Hipp et al., 2019; Giadone et al., 2020; Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Hetz, 2012; Walter and Ron, 2011; Glembotski et al., 2019; Romine and Wiseman, 2020; Christis et al., 2010). When unfolded or misfolded proteins accumulate within the cell (perhaps due to destabilizing mutations), molecular chaperones such as HSPA5 (BiP) interact with these proteins and can in turn activate the three arms of the UPR. Activation of these pathways leads to translocation of cognate transcription factors to the nucleus, upregulation of distinct, yet overlapping transcriptional signatures, and various functional consequences. The adaptive arms

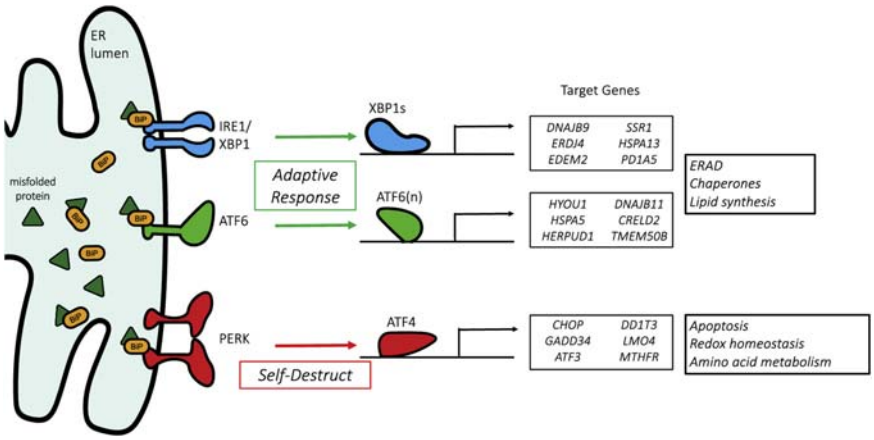

FIGURE 4.4 Activation of UPR-associated signaling pathways upon detection of misfolded proteins.

The detection of misfolded proteins within the lumen of the ER by molecular chaperones such as *HSPA5* (*BiP*) results in activation of IRE1, ATF6, or PERK signaling. Activation of each arm results in transcriptional activation of specific target genes as well as activation of functional pathways to cope with the production of misfolded protein.

of the UPR, mediated by IRE1 and ATF6 signaling, lead to upregulation of components of the ubiquitin proteasome system and ER-associated degradation (ERAD) pathway (Hipp et al., 2019; Giadone et al., 2020; Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Hetz, 2012; Walter and Ron, 2011; Glembotski et al., 2019; Romine and Wiseman, 2020; Christis et al., 2010). Functionally, these pathways work to selectively target and degrade misfolded, potentially toxic, protein species, thereby limiting their secretion. At the same time, adaptive UPR activation leads to upregulation of genes associated with chaperone function as well as an increase in the overall size of the ER to facilitate an increase in folding capacity (Hetz, 2012; Walter and Ron, 2011; Christis et al., 2010). Alternatively, chronic UPR activation, triggered by excessively large quantities of misfolded proteins, activates the PERK signaling pathway, whose functional downstream consequence includes synthesis and activation of pro-apoptotic machinery (Hetz, 2012; Walter and Ron, 2011).

Employing a novel iPSC-based model for ATTR amyloidosis has demonstrated that expression of amyloidogenic TTR results in transcriptional and functional changes in patient-specific hepatic cells. This result is surprising to many, as the field currently holds that the livers of patients with ATTR amyloidosis are the same as healthy livers (a fact underscored by the use of domino liver transplantations using ATTR amyloidosis donor livers) (Ruberg and Berk, 2012; Benson, 2012; Ando et al., 2005; Gertz et al., 2015; Adams, 2013; Monteiro et al., 2004). Additionally, these experiments suggest that the liver employs protective mechanisms via adaptive UPR-associated signaling pathways in order to cope with the production of misfolding-prone TTRs. Furthermore, this work provides evidence that modulation of UPR-associated ATF6 signaling results in a selective decrease in the secretion of misfolded proteins in patient-specific HLCs, and therefore, potentially represents a broadly applicable therapeutic strategy for ATTR amyloidosis and other systemic amyloid disorders. To this end, much work is currently underway to develop small molecules which selectively target each arm of the UPR in a stress-independent manner (Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Glembotski et al., 2019; Romine and Wiseman, 2020). The systemic amyloid diseases, including both forms of ATTR amyloidosis, can result from the misfolding of over 20 structurally distinct proteins. This variation in protein structure limits the broad utility of current therapeutics (e.g., kinetic stabilizers targeting natively folded proteins, antiamyloid breaking antibodies, etc.) for these disorders, thereby slowing the drug discovery pipeline. In spite of this large diversity, however, the common pathway shared by all is the production and secretion of destabilized proteins via effector organs. As a result, studies seeking to understand proteostasis machinery within systemic amyloid disease effector cells (e.g., HLCs in ATTR amyloidosis) aim to identify common endogenous signaling pathways which may represent druggable, broadly efficacious therapeutic strategies capable of decreasing distal aggregation of toxic protein species (Giadone et al., 2018, 2020; Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Glembotski et al., 2019; Romine and Wiseman, 2020).

The clinical trial in a test tube: revolutionizing the drug discovery pathway for systemic amyloid disease

In previous sections, we discussed how data using iPSC-based models of ATTR amyloidosis suggest the utility of UPR modulating therapeutics in potentially treating systemic amyloid disease. With the advent of iPSC technology, it is now possible to evaluate the efficacy and toxicity of a new chemical entity such as small molecule activators of the UPR in a number of diverse genetic backgrounds and cell types (Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Glembotski et al., 2019; Romine and Wiseman, 2020). For example, putative small molecules can be added to proteotoxic TTR-secreting patient-specific iPSC-derived HLCs from diverse backgrounds. Supernatant from these cells could then be interrogated for the presence and quantities of secreted TTR species in order to determine the ability of the novel compound to limit their secretion into circulation. Effectiveness can be further interrogated by dosing conditioned hepatic supernatant onto patient-matched iPSC-derived target (e.g., neuronal or cardiac) cells, and assessing toxicity.

The utility of iPSCs in preclinical evaluations of novel compounds can be further understood by evaluating their toxicity in a number of different cell types. Regarding hepatic cells, patient-derived HLCs could be generated, drug could be administered, and metabolomics could be performed to assess potentially deleterious metabolic side effects of the drug. Alternatively, if a novel compound has the potential to cross the blood–brain barrier, patient-specific iPSC-derived neuronal cells or three-dimensional organoids could be exposed to the compound in an effort to assess off-target effects and toxicity.

Implementing iPSC technology in preclinical evaluations of novel therapeutics has the potential for revolutionizing the drug discovery pipeline by identifying problematic toxicity and efficacy issues prior to entering the investigational new drug and clinical trial phases. Precision medicine-based approaches for preclinical models of pharmacologic efficacy and toxicity can decrease the overall time and cost of drug development.

Future directions

Developing novel biomarkers for ATTR amyloidosis

In addition to assessing therapeutic strategies, iPSC-based platforms can also be utilized to aid in the development of novel biomarkers for ATTR amyloidosis. To this end, recent work has proposed the use of serum levels of TTR as a potential marker for disease progression in patients with cardiomyopathy-associated ATTR amyloidosis (Hanson et al., 2018). Healthy individuals have circulating levels of TTR of approximately 0.2–0.4 mg/mL, while decreased concentrations are often associated with worse disease outcome (Hanson et al., 2018). The mechanism of this inverse correlation of serum TTR levels and disease progression, however, remains unclear.

An alternative approach being developed utilizes TTR tetramer-specific fluorescently labeled small molecules to assess the percentage of natively folded tetrameric TTR relative to total serum levels (Chen et al., 2016). With this method, the ratio of circulating tetramer to total TTR could assist in detecting early stages of TTR misfolding and disease progression (Chen et al., 2016). Future studies could involve measuring in vitro equivalents of the abovementioned putative biomarkers in iPSC-derived cell types (e.g., HLCs) derived from patients with varying levels of disease severity in order to assess diagnostic efficacy.

Evaluating small molecule activators of the UPR

As discussed, achieving stress-independent activation of the UPR-associated ATF6 signaling pathway through small molecule activators is capable of selectively decreasing the secretion of toxic TTRs (Chen et al., 2014; Plate et al., 2016; Shoulders et al., 2013; Glembotski et al., 2019; Romine and Wiseman, 2020). Toward a *clinical trial in a test tube* approach, future studies should employ libraries of diverse patient-specific iPSCs to determine the efficacy of this approach in decreasing hepatic secretion of mutant TTR across a number of genetic backgrounds (Giadone et al., 2018; Park et al., 2017). In the case of chronic stress, UPR activation can be linked to employment of pro-apoptotic machinery via PERK signaling (Hetz, 2012; Walter and Ron, 2011). Going forward, it will be critical to evaluate the potential for activators of the adaptive UPR to trigger cell death pathways.

Building an iPSC-based model for light chain amyloidosis

Like ATTR amyloidosis, the most prevalent systemic amyloid disease, light chain (AL) amyloidosis, suffers from a lack of robust *in vitro* and *in vivo* preclinical models (Fig. 4.1). In the disease, cancerous plasma (B) cells rapidly proliferate and secrete quantities of antibody light chains (Merlini et al., 2018; Sanchorawala, 2006). Free light chains subsequently misfold and aggregate, depositing in distal target tissues (e.g., the heart, kidney, and liver) (Merlini et al., 2018; Sanchorawala, 2006). Current treatment options for patients with the disease are both highly limited and invasive — involving administration of chemotherapeutic agents to limit malignant plasma cell growth, coupled with autologous bone marrow transplantation (Merlini et al., 2018; Sanchorawala, 2006). In an iPSC-based model for AL amyloidosis, PBMCs could be isolated from patients and subsequently reprogrammed. Reprogrammed clones would subsequently need to be screened for the presence of the pathology-driving mutation to ensure that the model contains the genetic background of the malignant plasma cell. As with models of ATTR amyloidosis, AL amyloidosis iPSCs could be differentiated into the respective effector cell (i.e., plasma/B cells), and secretion of light chain species and requisite downstream cell type toxicity could be evaluated via MS and flow cytometry-based cell death assays, respectively.

Profiling misfolded protein-mediated signatures of cellular stress and toxicity

Investigating the secretion and deposition of TTR aggregates in ATTR amyloidosis using iPSC-derived cell types has the potential to lend insight into other multisystem protein folding disorders. To this end, the development of gene signatures resulting from the interaction of mutant, misfolded TTR aggregates with multiple cell types can potentially aid in the development of robust *in vitro* models of systemic amyloid diseases (also involving the interaction of diverse cell types with misfolded proteins). Future work should aim to understand similarities in transcriptional signatures resulting from exposure to diverse proteotoxic aggregates — from ATTR to LCs. Once accomplished, these profiles can be employed as outputs for tracking damage and toxicity in future cell-based models for other protein folding disorders.

Conclusions

The systemic amyloid diseases are a class of highly complex disorders involving the production of proteins which misfold, travel throughout circulation, and deposit as proteotoxic aggregates at distal target organs — mechanisms broadly applicable to many aspects of human biology. Investigations into multisystem protein folding disorders such as ATTR amyloidosis, through iPSC-based platforms, have the potential to lend novel insight into many aspects of general biological dysfunction at the cell and molecular levels, while at the same time lay the foundation for novel therapeutics such as proteostasis network remodeling agents.

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CHAPTER

iPSCs for modeling choroideremia

5

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Chapter outline

Abstract

This chapter will cover the ways that induced pluripotent stem cells (iPSCs) have been used to study the inherited retinal dystrophy choroideremia (CHM). Following a general introduction into the eye and its diseases, the chapter will more specifically focus on the clinical phenotype, genetics, and pathophysiology of CHM. Previous efforts to study the disease using diverse animal and cellular models, will then be covered. Then, following a brief introduction into iPSC generation and characteristics, studies using iPSCs specifically in the context of CHM will be outlined. Furthermore, and particularly pertinent for the disease, is the description of iPSC-derived retinal models for CHM, namely, the retinal pigment epithelium. The chapter will cover the characteristics of this tissue and the ways it has been used, to date, for modeling CHM and testing the efficacy of novel

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therapies (gene supplementation and translational read-through). Future directions for iPSC-based modeling of CHM will be discussed.

Keywords: AAV; CHM; Choroid; Choroideremia; Inherited retinal dystrophies; iPSCs; Phagocytosis; Photoreceptors; Prenylation; PTC124; Rab27a; REP1; Retina; Retinal pigment epithelium; Trafficking.

Eye structure and function

The human eye is a liquid-filled sphere (Fig. 5.1A) made up of an anterior segment (the cornea, iris, and lens) and a posterior segment (the vitreous, retina, choroid, and sclera) (Hejtmancik and Nickerson, 2015). The **sclera** constitutes the outermost layer of the posterior eye. It is a white fibrous tissue, which maintains eye shape. It becomes the transparent cornea on the anterior side. The **cornea** acts as a protective barrier to physical and pathogenic injury and also constitutes the major surface

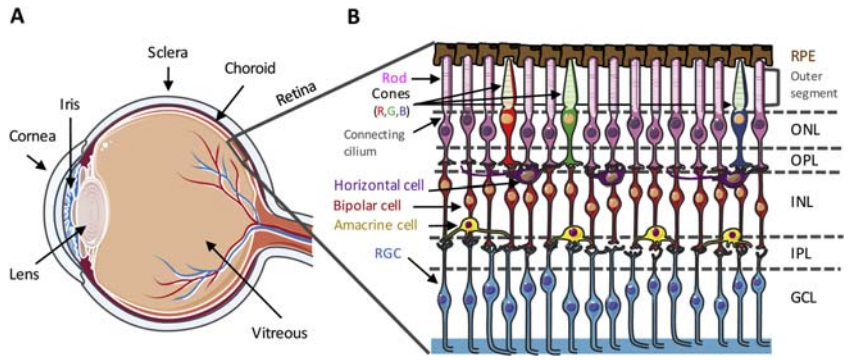

FIGURE 5.1 Schematic representation of the human eye.

(A) The human eye comprises the cornea, iris and lens, on the anterior side and the vitreous, retina, choroid, and sclera, on the posterior side. (B) The retina lines the back of the eye. It consists of the retinal pigment epithelium (RPE) and the neuroretina containing the photoreceptors, namely the rods, and the *red* (R), *green* (G) and *blue* (B) cones. From posterior to anterior, the multilayered neuroretina comprises: the outer segments of the photoreceptors; the outer nuclear layer (ONL) with the nuclei-containing cell bodies and inner segments of the photoreceptors, which are connected to the outer segments by the connecting cilium; the outer plexiform layer (OPL) containing the synapses between the axons of the photoreceptors and the neuronal cells; the inner nuclear layer (INL) containing the bipolar, amacrine, and horizontal cells; the inner plexiform layer (IPL) containing the synapses between the neuronal cells and the retinal ganglion cells (RGCs), and the ganglion cell layer (GCL) containing the RGCs. The axons of the RGCs converge to form the optic nerve.

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for light entry and refraction (Eghrari et al., 2015). The **iris** is the colored ring of tissue of the eye that surrounds the pupil, which dilates or constricts to regulate the amount of light entering the eye. The light then traverses the **lens**, situated just behind the pupil, which changes its shape to allow correct focus onto the retina (Hejtmancik and Shiels, 2015). The **retina** is the tissue that lines the posterior segment. It is composed on the posterior side by the retinal pigment epithelium (RPE) and on the anterior side, by the multilayered neuroretina (Grossniklaus et al., 2015). Lastly, between the retina and sclera is situated the **choroid**, a highly vascularized and pigmented tissue, which is important for the health of the retina (Zhang and Wildsoet, 2015).

The **neuroretina** is composed of three layers of specialized neurons that are interconnected by two synaptic layers (Fig. 5.1B). On the poster side and adjacent to the RPE are the light-sensing **photoreceptors**, which constitute the outer nuclear layer. There are two types of photoreceptors, rods and cones, which are used predominantly under dim or bright light, respectively. Furthermore, there is only one type of rod that allows us to see in black and white, whereas there are three types of cones (red, green, and blue) that allow us to see in color. The photoreceptors have a characteristic morphology comprising an axon, a cell body containing the nucleus, an inner segment rich in mitochondria and which is attached, via a connecting cilium, to an outer segment filled with lipid discs. It is within these outer segment discs that the phototransduction process to convert the light signal into an electrophysiological signal takes place (Grossniklaus et al., 2015). On the anterior side, the photoreceptors synapse with the **bipolar cells**, which are situated in the inner nuclear layer (INL). This synaptic layer is known as the outer plexiform layer. The bipolar cells then transmit the electrophysiological signal to the **retinal ganglion cells** (RGCs) via a second set of synapses in the inner plexiform layer (IPL). Lateral connections also exist within the INL via **horizontal** and **amacrine** cells, thus further modulating the signaling pathway from photoreceptors to RGCs. The RGCs constitute the most anterior layer of the neuroretina and, as their axons merge together to form the optic nerve, the electrophysiological signal is hence transmitted from the eye to the brain for image interpretation.

The outermost layer of the retina is the **RPE**, a polarized cuboidal epithelium. The RPE lies upon the highly collagenous Bruch's membrane, which separates it from the underlying choroid (Fig. 5.2). On the apical side, the RPE has numerous microvilli that are in are in close contact with the photoreceptor outer segments (POS). The apical pole of each RPE cell contains a high level of melanosomes that contribute to its pigmentation, and the basal pole contains a nucleus and predominant mitochondria. The RPE is essential for the support and survival of the neuroretina (Sparrow et al., 2010). Via its pigmentation, the RPE provides protection by absorbing excess light to prevent light-induced toxicity (Fig. 5.2). The apical microvilli are involved in the phagocytosis of the most-aged tips of the POS that are shed daily to prevent the build-up of photo-oxidative radicals. Furthermore, the RPE forms a close partnership with the photoreceptors for regeneration of the chromophore 11-*cis*-retinal following light interaction by a process known as the

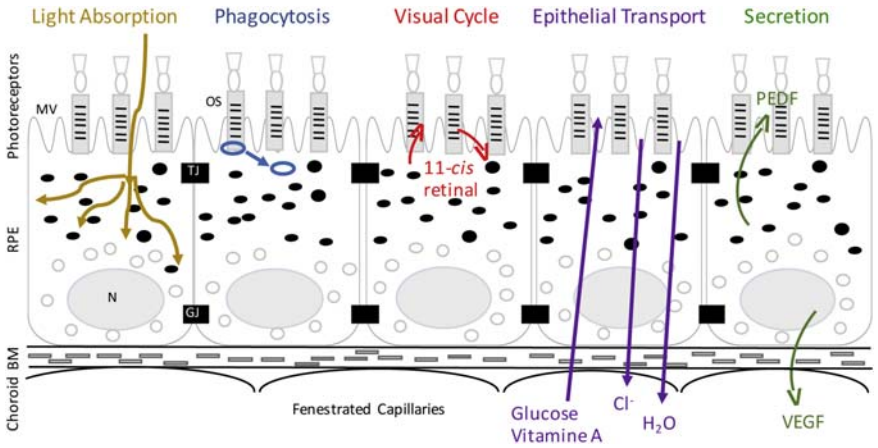

FIGURE 5.2 Graphical representation of RPE structure and functions.

The RPE is in close contact with the photoreceptors on the apical side and is separated from the underlying choroid on the basal side by Bruch's membrane (BM). Each RPE cell has apical microvilli (MV) in close contact with the POS, apical melanosomes (filled black ovals), a basal nucleus (N) and basal mitochondria (open circles). Apical tight junctions (TJs) and basal gap junctions (GJs) exist between cells. The RPE is responsible for the: absorption of excess light (indicated by brown arrows) that cannot be processed by the photoreceptors; phagocytosis of the shed tips (blue ovals) of the POS; regeneration of the chromophore 11-*cis* retinal via the visual cycle; epithelial transport of nutrients toward the photoreceptors and evacuation of water toward the choroid, and the polarized secretion of growth factors. PEDF, pigment epithelial-derived factor. VEGF, vascular endothelial growth factor.

visual cycle (Strauss, 2005). Each RPE cell is in close contact with its neighbor via a series of gap junctions on the basolateral side and tight junctions on the apical side. Consequently, the RPE is capable of polarized epithelial transport, which is highly regulated by a series of ion channels (Reichhart and Strauss, 2014). Via this polarized transport, the RPE removes water and by-products of retinal metabolism from the photoreceptors and provides them with glucose and other nutrients from the choriocapillaris (Fig. 5.2). Similarly, by polarized growth factor secretion, the RPE maintains the structure and integrity of the retina and underlying choroid.

Lastly, the **choroid** is a highly vascularized conjunctive tissue. It contains collagen fibers for maintaining its structure and pigmented melanocytes to help prevent light-mediated toxicity. Its vascular network, the choriocapillaris, is made up of fenestrated capillaries, which feed the outer retina. However, a distinct barrier sequesters the eye from the systemic circulation (Fields et al., 2019). This is referred to as the **blood–retinal barrier** (BRB), and it is composed of the tight junctions of the RPE, the highly collagenous underlying Bruch's membrane, and the nonfenestrated retinal vessels. The BRB thus prevents unhindered travel of any molecule to and from the eye and renders this organ relatively immune-privileged.

Inherited retinal dystrophies

An interruption in the conversion or transmission of the light signal within the retina is a primary cause of visual impairment worldwide. This can be due to trauma, combinatorial risk factors including age (two common examples are age-related macular degeneration and glaucoma) or a secondary effect of another pathology (e.g., diabetes). Another important cause is the inheritance of a genetic defect that affects the development, structure, or function of the retina, either exclusively (nonsyndromic) or conjunctly (syndromic) with other tissues (Berger et al., 2010). Such diseases are generally referred to as inherited retinal dystrophies (IRDs) and, although some stationary forms do exist, IRDs are most often characterized by a progressive loss of vision due to photoreceptor death. Although the RGCs are part of the retina, their degeneration generally gives rise to an atrophy of the optic nerve with a normal retinal aspect, thus these disorders are not classified as IRDs but as hereditary optic neuropathies (Hamel, 2014).

IRDs are a large group of clinically and genetically heterogeneous disorders (Sullivan and Daiger, 1996). Collectively, they have a prevalence of approximately 1/2000 live births, but individually they fall under the category of rare diseases. IRDs have been linked to mutations in over 270 genes and their mode of transmission can be autosomal dominant, recessive, or X-linked (https://sph.uth.edu/retnet/). Intriguingly, mutations in the same gene can be responsible for either dominant or recessive forms, as well as for distinct clinical phenotypes. In some cases, this can be either due to the type of mutation, with deleterious mutations leading to a more severe phenotype, whereas missense mutations lead to a milder phenotype, or the localization of the mutation with regard to domains important for the function of the encoded protein (Dias et al., 2018).

The majority of causative IRD genes have been described to affect the structure of the photoreceptor, most notably the connecting cilium (Hamel, 2014). These pathologies are also referred to as ciliopathies and are often syndromic, as extraretinal symptoms in other ciliated tissues, such as the ear or kidney, can also be associated. Another prevalent group of causative IRD genes comprises those that affect the phototransduction process. The third group comprises genes affecting photoreceptor development or gene and protein regulation. In addition, IRD causative genes can also directly affect the function of the RPE, notably phagocytosis and retinoid metabolism. Lastly, some causative IRD genes are ubiquitously expressed but with a restricted retinal phenotype, which is thought to be due to the intense metabolic activity of this tissue.

Clinically, IRDs are categorized into subgroups based on the type of photoreceptor that degenerates first. Rod-cone dystrophies are initially characterized by night blindness and peripheral vision loss due to the degeneration of rods, which are more highly concentrated in the peripheral retina. The most common example is retinitis pigmentosa (Verbakel et al., 2018). By contrast, cone-rod dystrophies are initially characterized by a loss of fine vision due to the degeneration of cones, which are more highly concentrated towards the center of the retina (Hamel, 2007).

A specific subclass of cone dystrophies purely affects the central cone-rich macula and is hence referred to as maculopathies. The most common example is Stargardt disease (Tanna et al., 2017). Furthermore, one particular subgroup of IRDs is characterized by an early and severe onset of peripheral and central vision loss and is referred to as Leber congenital amaurosis (Kondkar and Abu-Amero, 2019). Lastly, in some IRDs, the retinal degeneration can be also associated with a degeneration of the choroid (Berger et al., 2010). These disorders are referred to as chorioretinopathies, and the most common example is choroideremia.

Choroideremia

Choroideremia (CHM), meaning the total absence of choroidal vessels in the fundus, was first described in 1872. In 1952, Sorby et al. established that CHM was a progressive disease, distinct from retinitis pigmentosa, and inherited as an X-linked recessive trait, primarily affecting males (Sorsby et al., 1952). The disease prevalence is estimated to be 1/50,000 (Kalatzis et al., 2013). Affected boys have difficulty seeing at night in the first decade of life and then progressively lose peripheral vision by their teens and in their twenties. By their fourth decade of life, CHM males have a significantly constricted peripheral visual field and many are considered legally blind. In the fourth to fifth decade of life, the majority of patients continue to have preserved central vision. Impairment of visual acuity occurs at a late age and, beyond the sixth decade, only a few patients retain any central vision (Roberts et al., 2002).

Accompanying this disease course, areas of RPE hypopigmentation and thinning throughout the fundus are early manifestations of the disease, already seen in boys younger than 10 years of age (Kalatzis et al., 2013). Optical coherence tomographic imaging in later phases shows shortening of the inner and outer segments of the photoreceptors, reduced thickness of the outer nuclear layer, and depigmentation of the RPE. Areas of chorioretinal atrophy with loss of the RPE and of the choriocapillaris appear over time. These expose large choroidal vessels and, due to the disappearance of RPE and choroidal melanocytes, give the characteristic whitish appearance of the fundus of CHM patients (Fig. 5.3A).

Most heterozygous female carriers are asymptomatic in childhood and, even later in life, present a milder phenotype without serious visual impairment and good preservation of the visual field (Preising et al., 2009). They can, however, be identified clinically by the presence of patchy areas of RPE atrophy (Fig. 5.3B), which progress mildly with age. Rarely, female carriers with more severe phenotypes similar to affected males have been described (Potter et al., 2004). The clinical findings in affected females could be explained by the Lyon hypothesis of unbalanced X chromosome inactivation (Vajaranant et al., 2008). Alternatively, it has been proposed that the causative gene can escape X-linked inactivation and maintain detectable but reduced expression in different cell lines from the inactivated X (Carrel and Willard, 1999). Both hypotheses could explain why the phenotype is so heterogeneous and, generally, milder.

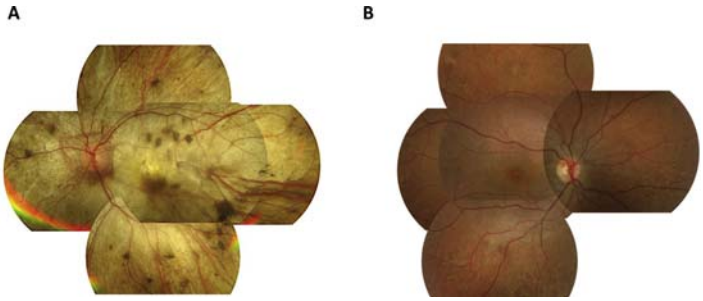

FIGURE 5.3 Fundus images of choroideremia patients.

(A) Typical whitish appearance of the fundus of a male choroideremia patient showing large atrophic areas of the retina and choroid. (B) Fundus of an asymptomatic female carrier showing pigment mottling in the peripheral retina.

Images courtesy of Pr. C.P. Hamel.

Genetics of choroideremia

Like most IRDs, CHM is a monogenic disease. The causative gene, *CHM*, is located at Xq21.2 and spans a 186-kb region (Cremers et al., 1990). *CHM* consists of 15 exons encoding a 653-aa polypeptide, the Rab Escort Protein 1, REP1 (van Bokhoven et al., 1994). The majority of reported *CHM* mutations lead to truncated or absent REP1 and are thus considered as loss-of-function mutations. Currently, 430 unique pathogenic *CHM* variants, and 731 total variants, have been reported (https://databases.lovd.nl/shared/genes/CHM). These variants most commonly comprise substitutions and deletions, followed by duplications, indels and insertions. Atypical variants have also been reported: deep intronic mutations resulting in pseudoexon insertion and exon skipping (van den Hurk, 2003); L1 retrotransposon (van den Hurk, 2003) and SINE (Vache et al., 2019) insertions that alter splicing; large X chromosome translocations (Garcia-Hoyos et al., 2005; Lorda-Sanchez et al., 2000; Mukkamala et al., 2010; Siu et al., 1990) and contiguous gene deletions (Merry et al., 1989; Yntema et al., 1999) that result in extraretinal symptoms; exon duplications (Chi et al., 2013; Edwards et al., 2018), and mutations in the *CHM* promoter (Vache et al., 2019; Radziwon et al., 2017). Furthermore, somewhat strikingly, missense mutations are rarely associated with CHM, as only three such mutations, p.Leu550Pro (Sergeev et al., 2009), p.His507Arg (Esposito et al., 2011), and p.Leu457Pro (Torriano et al., 2017) have been validated as pathogenic to date. Thus, as most *CHM* mutations are loss-of-function, there is no real evidence for a genotype–phenotype correlation (Freund et al., 2016; Sanchez-Alcudia et al., 2016; Simunovic et al., 2016). The only exception is a recent study showing that a patient with a milder phenotype carried a splice site mutation with residual full-length transcript production (Fry et al., 2019).

Interestingly, a second gene that encodes a protein highly homologous to REP1 has also been identified in mammals. Considering its similarity, this gene was called

CHM-like (*CHML*). Human *CHML* is located on chromosome one and encodes a protein named REP2. This 656-aa polypeptide shares ~75% homology with REP1 and has the same biochemical role (Cremers et al., 1992). *CHML* is a gene that lacks introns, and it is thought to arise from a retrogene insertion of the *CHM* mRNA transcript during vertebrate evolution. Both REP1 and REP2 are ubiquitously expressed in human tissues, including the retina.

Pathophysiology of choroideremia

REP1 is responsible for the posttranslational prenylation of the Rab GTPase protein superfamily and, hence, plays a major role as a regulator of intracellular vesicular trafficking (Alexandrov et al., 1994; Andres et al., 1993; Seabra et al., 1993, 1992). Currently, more than 60 Rab proteins have been identified (Hutagalung and Novick, 2011). Rabs are characteristic of certain cellular compartments (Fig. 5.4) and are known to be involved in the transport, secretion, and fusion of vesicles (Seabra et al., 2002). To accomplish this role, Rabs need to be activated by both GTP binding and the addition of a hydrophobic prenyl group (Pereira-Leal et al., 2001). REP1 recognizes unprenylated cytosolic Rabs and acts as a chaperon presenting them to the enzyme Rab geranyl-geranyl transferase II (RGGTaseII). RGGTaseII recognizes the REP1-Rab complex, and promotes its prenylation by adding one or two 20-carbon geranylgeranyl groups via a stable thioether linkage. This binding occurs in two cysteines situated in the Rab carboxyl terminal domain. The recognition is mediated by the α subunit of the RGGTaseII, while the enzymatic activity resides in the β subunit. The prenyl donor is a molecule of geranylgeranyl pyrophosphate (GGPP), which, once added, acts as a negative regulator of the prenylation cycle, as the REP1-Rab complex then dissociates from the enzyme. REP1 can then chaperon the prenylated Rab to its target membrane and, once delivered, the prenylation cycle is complete (Fig. 5.5), and REP1 is free to associate with another unprenylated Rab (Alexandrov et al., 1994; Seabra et al., 2002; Pereira-Leal et al., 2001; Alory and Balch, 2001).

Both REP1 and REP2 share the same chaperon function in Rab prenylation and, as their substrate specificity can overlap, they seem to be able to substitute for each other in certain contexts. When REP1 is absent, as in CHM, REP2 activity appears to compensate REP1 deficiency in almost all the tissues of the body, with the exception of the retina (Cremers et al., 1994); this would explain the associated retina-specific phenotype. Although Rab proteins can be prenylated in vitro by both REPs, in the context of CHM, all Rabs are in competition for binding and prenylation by REP2. Therefore, Rabs that present a lower affinity for REP2 may be underprenylated.

Seabra et al. showed that Rab27a was the underprenylated Rab protein associated with CHM in patient lymphoblasts (Seabra et al., 1995). Furthermore, they consistently showed that Rab27a was highly expressed in the RPE and the choroid of the rat retina. It was subsequently demonstrated that, although Rab27a binds REP1 and

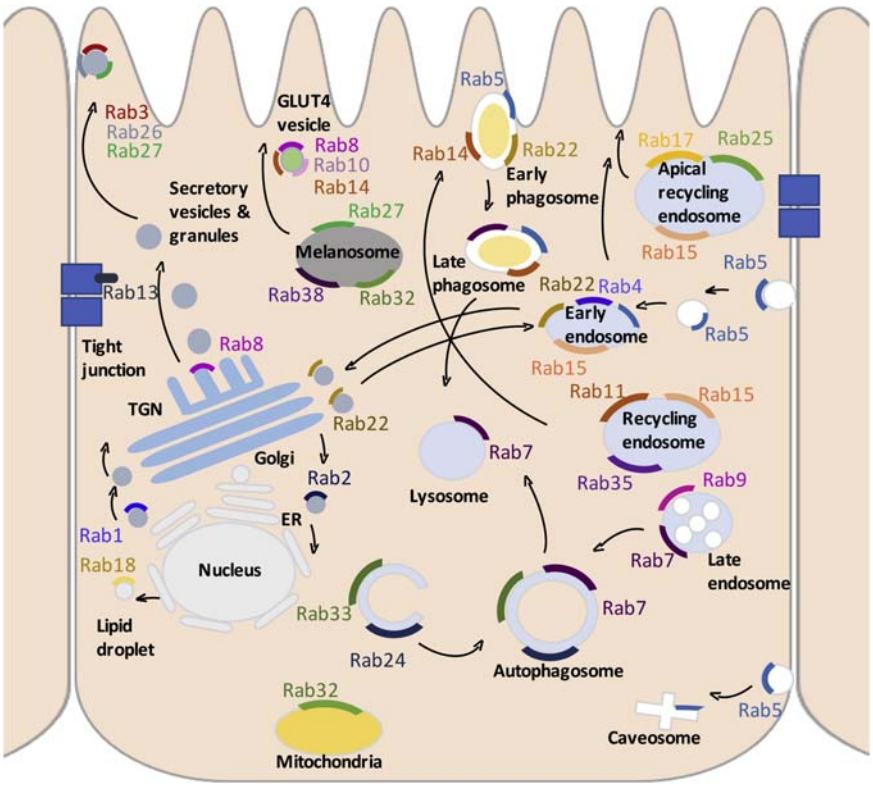

FIGURE 5.4 Localization and function of Rab GTPases in RPE cells.

Non exhaustive representation of Rab proteins involved in the different pathways of trafficking, fusion and secretion of vesicles in the RPE. Each Rab protein is localized to a specific compartment within the cell. Particularly relevant to CHM, Rab27, as well as Rab38 and Rab32, are involved in melanosome biogenesis and trafficking. Rab27 is also involved in secretion and exocytosis. Rab5, Rab14 and Rab22 are involved in phagocytosis and endocytosis. Rab7 mediates late endosome and phagosome maturation and lysosomal fusion. TGN, *trans*-Golgi network. ER, endoplasmic reticulum.

REP2 with equal affinity, the REP2-Rab27a complex has a lower affinity for RGGTaseII than the REP1-Rab27a complex (Larijani et al., 2003). Moreover, the REP2-Rab27a reaction can be effectively inhibited by other Rab proteins (Rak et al., 2004). However, despite the undisputed connection between REP1 and Rab27a, it is not clear how underprenylation of Rab27a gives rise to the clinical phenotype of CHM.

One of the major roles of Rab27a is to regulate melanosome distribution to the apical pole of RPE cells. It acts as part of a molecular complex with MYRIP and myosin VIIA, to likely bridge retinal melanosomes to the actin cytoskeleton, thus mediating their local trafficking (Klomp et al., 2007; Lopes et al., 2007). In addition,

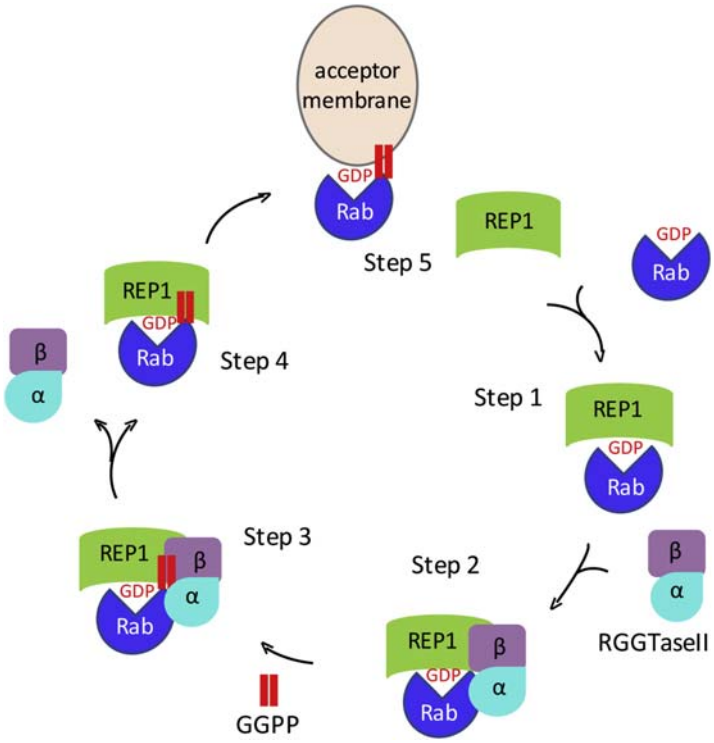

FIGURE 5.5 Schematic representation of the REP1 prenylation cycle.

Step 1, the prenylation cycle is initiated by REP1 binding an unprenylated Rab. Step 2, the REP1-Rab complex is recognized by the enzyme RGGTaseII, made up of its α and β subunit. Step 3, RGGTaseII adds the GGPP donor to the Rab. Step 4, RGGTaseII dissociates from the complex. Step 5, REP1 escorts the prenylated Rab to the acceptor membrane and is then free to associate with another unprenylated Rab.

Rab27a is used by secretory vesicles and granules to exocytose their cargo (Ejlerskov et al., 2012; Munafo et al., 2007; Yamaoka et al., 2016; Yi et al., 2002). This is consistent with a recent report showing that Rab27a modulates the L-type calcium channel, $Ca_v1.3$, which underlies secretory processes in various cell types (Catterall, 2011). $Ca_v1.3$ and Rab27a were shown to colocalize and physically interact at the basolateral membrane of the RPE. Furthermore, overexpression of Rab27a was found to inhibit channel function and reduce secretion of vascular endothelial growth factor (VEGF) (Reichhart et al., 2015).

A subsequent study showed that, in addition to Rab27a, Rab27b, Rab38, and Rab42 (also known as Rab7b) have much slower prenylation rates in vitro than the majority of other Rab proteins studied (Kohnke et al., 2013). Rab27b and Rab38, like Rab27a, are also localized to melanosomes (Hutagalung and Novick, 2011). More recently, Rab42 was shown in keratinocytes to be localized to

compartments containing melanosomes, where it is involved in protein degradation (Marubashi and Fukuda, 2020). Therefore, it is possible that the underprenylation of other Rabs, in addition to Rab27a, may also be involved in the pathophysiology of CHM.

To conclude, to date, although the underlying gene and biochemical defect of CHM is well characterized, it is still unknown how the underprenylated Rab pool leads to the associated chorioretinal degeneration.

Animal models of choroideremia

Many spontaneous or generated mouse models carrying mutations in IRD genes exist that have helped advance research (Baehr and Frederick, 2009; Dalke and Graw, 2005). However, numerous mouse models that have been generated do not mimic the patient phenotype nor undergo photoreceptor degeneration. This may be due to the short lifespan of the mouse or to the difference in retinal structure between the two species. An excellent example of the latter are the murine models generated for the family of IRD genes responsible for Usher syndrome (USH). In humans, mutations in these genes associate with vision and hearing loss. However, the mouse models do not present with a retinal phenotype (Williams, 2008). This is because the USH family proteins are localized to calyceal processes, microvillus-like structures that form a collar around the base of the outer segment at the level of the connecting cilium in the primate retina. By contrast, murine photoreceptors lack these processes (Sahly et al., 2012).

Furthermore, the murine retina contains only two (green and blue) types of cones compared to the three types in humans, and the distribution pattern of cones and rods varies between the two species with the absence of a cone-rich macula in mice. The dog eye has proven more suitable for studying IRDs as it resembles the human eye in both size and structure, and many IRDs in man have canine counterparts (Tsai et al., 2007). However, for certain diseases, the identification of a corresponding canine model has proven elusive. In recent years, zebrafish have become more popular for modeling IRDs. This is because the zebrafish retina contains all the major cell and tissue layers found in the human retina, including the calyceal processes and the three cone types (Slijkerman et al., 2015). Furthermore, zebrafish have a short reproductive cycle and their offspring develop *ex utero*, thus eye development can be monitored easily.

The first animal model for CHM was identified from a mutagenic screen in zebrafish (Starr et al., 2004). This model, referred to as chm^{ru848}, carries a nucleotide change in the second exon of the *chm* gene, which gives rise to a premature UAA stop codon at position 33 (p.Gln33*) of the encoded rep1. The phenotype is severe and systemic, resulting in lethality at 5- to 6-days postfertilization (dpf). The cause of this extreme phenotype is explained by the fact that zebrafish lack a homolog of the human *CHML* gene (Moosajee et al., 2009). Up until six dpf,

chm^{ru848} larvae can survive because the mutant zebrafish still benefit from *chm* expression provided by the yolk sac and present in different tissues, including the eye. Survival thus continues until the maternal transcript is degraded (Starr et al., 2004). Mutant zebrafish larvae show degeneration in both RPE and photoreceptors, with ineffective phagocytosis of POS observed in the mutant RPE (Krock et al., 2007). Furthermore, it was reported that photoreceptor degeneration was nonautonomous and required contact with the mutant RPE, suggesting that photoreceptor death was a secondary consequence.

The first *Chm* knock-out mouse model was generated in 1997 (van den Hurk et al., 1997). However, *Chm* inactivation was discovered to be lethal at the embryonic stages in hemizygous male mice due to a defective trophoblast development and vascularization in extraembryonic tissues (Shi et al., 2004). The viable heterozygous females, which were equivalent to human female carriers, showed a mild loss of photoreceptors (van den Hurk et al., 1997). A subsequent conditional knockout mouse model, whereby *Chm* was inactivated in photoreceptors and/or the RPE, was then generated in 2006 to overcome the embryonic lethality (Tolmachova et al., 2006). Heterozygous *Chm*$^{null/WT}$ females showed progressive degeneration of the photoreceptors accompanied by reduced eletroretinogram responses, patchy depigmentation of the RPE and Rab prenylation defects, similar to patients. Furthermore, when *Chm* was knocked out specifically in the RPE, this tissue degenerated without major effects observed on the photoreceptors. Similarly, when *Chm* was knocked out specifically in the photoreceptors, these cells degenerated without significant effects on the RPE. These results suggested that a *Chm* defect can give rise to cell autonomous degeneration of both the RPE and photoreceptors. However, when a *Chm* defect is present in the photoreceptors and RPE concurrently, the diseased RPE accelerated photoreceptor degeneration (Tolmachova et al., 2010).

Interestingly, although the majority of pathogenic *CHM* variants in humans are loss-of-function mutations that result in a retinal-specific phenotype, the invalidation of *CHM* homologs in zebrafish and mice is lethal. Similarly, the identification of a canine model for CHM also proved elusive, suggesting that *CHM* invalidation may also be lethal in this species (Robert et al., 2010). Thus, in the absence of an appropriate animal model for CHM, human cell culture is an essential complement for research (Seymour and Fisher, 2009).

Cellular models of choroideremia

In an effort to further unravel the pathophysiology of CHM, initial studies were carried out on primary cell lines generated from patients. It was shown that intracellular transport was affected in fibroblasts and monocytes of CHM patients (Strunnikova et al., 2009). These studies identified a defect in the proteolytic degradation of FluoroSphere beads, which was associated with an increased lysosomal pH. A decrease in the secretion of cytokines and growth factors, such as monocyte chemoattractant

protein (MCP)-1, interleukin (IL)-8, tumor necrosis factor alpha, basic fibroblast growth factor (bFGF), and pigment epithelial-derived factor (PEDF), was also identified, whereas VEGF secretion was unchanged.

These results were confirmed later by studies on human fetal RPE (hfRPE) cells. Following REP1 depletion in hfRPE, mediated by siRNA knockdown, a delay in the clearance of POS due to reduced phagosomal acidification was observed following phagocytosis (Gordiyenko et al., 2010). Furthermore, there was a decrease in the association of the phagosomes with the late endosomal markers, Rab7 and LAMP-1, suggesting defective phagosome-lysosome fusion. In addition, an increase in the production of MCP-1 and IL-8 was observed, suggesting an onset of inflammation, which could be correlated with the infiltration of inflammatory cells in the choroicapillaris of CHM patients (MacDonald et al., 2009).

Although these studies did provide insights into CHM pathophysiology, the results were somewhat contradictory. This is likely due to the fact that the model systems were either cells that were not truly affected in the disease or nondiseased tissue. The subsequent advent of induced pluripotent stem cell (iPSC) technology held the promise of generating retinal tissue from CHM patients, which represents a more pertinent model for studying the disease.

Human induced pluripotent stem cells

As CHM is characterized by a retinal-specific phenotype, the information that can be gleamed from nonretinal cells is limiting. This coupled to the lack of a pertinent animal model that reproduces the human condition in affected males has pushed for the development of an alternative human model. Along this line, pluripotent stem cells (PSCs), in particular those induced from somatic tissues (iPSCs), have revolutionized the field of biomedical research. The fact that iPSCs can be generated from somatic cells of patients with inherited disorders, and that these cells can be differentiated theoretically into any tissues, has made these iPSC-derived tissues or even organs an invaluable tool for disease modeling and therapeutic studies.

The ground-breaking work of Yamanaka showed that a somatic cell, whether murine (Takahashi and Yamanaka, 2006) or human (Takahashi et al., 2007), could be reprogrammed into iPSCs with a cocktail of four transcription factors: OCT3/4, SOX2, KLF4, and c-MYC. The first step of reprogramming is heterochromatin reorganization to allow for a more open conformation, which is characterized by higher plasticity. c-MYC plays a major role in this process because it can recruit the histone acetyltransferase to promote transcription of target genes. KLF4 is a zinc finger transcription factor for maintenance of self-renewal. It binds the *NANOG* promoter and, with SOX2 and OCT3/4, mediates *NANOG* expression. SOX2 and OCT3/4 heterodimerize together and they are associated with self-renewal, pluripotency, and the maintenance of an undifferentiated phenotype.

The initial vectors used to vehicle the transcription factor cocktail into somatic cells were retro- and lentiviral vectors, which were characterized by an efficiency of <0.1% and represented downstream safety risks, as well as potential interference with read-outs, due to the random integration of exogenous genes into the host genome (Gonzalez et al., 2011). Nonviral delivery of reprogramming factors (such as plasmids, mRNAs, proteins) was subsequently investigated, and although safer, they showed a lower efficiency when compared to virus-based vectors. The most popular method today, which represents an interesting compromise between safety and high efficiency, is the use of Sendai virus (SeV)-based vectors (Fusaki et al., 2009). SeV vectors are nonintegrative; thus, the vectors and exogenous genes are progressively lost during cell division and result in a reprogramming efficiency of up to 1%.

Once reprogrammed, iPSCs represent an indefinite supply of self-renewing cells that can undergo multilineage differentiation (ectoderm, mesoderm, and endoderm). Despite the significant advantages associated with their renewal capacities, iPSCs do harbor some limitations. The renewal potential of PSCs in the blastocyst is only a transient step of embryogenesis (Weissbein et al., 2014). By contrast, with iPSCs, this renewal characteristic is exploited indefinitely and, hence, is associated with the risk of the occurrence of genetic instability. PSCs have shorter cell cycles than differentiated cells due to a shortened G1 phase (Becker et al., 2006). Thus, when iPSCs are reprogrammed from somatic cells, they begin to proliferate rapidly and acquire short cell cycles. This, in turn, incurs a high risk of acquiring genetic aberrations. The DNA machinery is challenged due to the increase in successive rounds of DNA replication, and, consequently, replication defects and defective chromosomal segregation slip through weakened checkpoints (Weissbein et al., 2014). The highly documented genetic instability associated with iPSCs, ~20% of human iPSC lines generated exhibit at least one large chromosomal aberration, can have unforeseen consequences on pathophysiological or therapeutic read-outs. Therefore, it is essential to check the genetic stability of iPSCs once they have been generated and regularly thereafter during culture.

Human iPSCs for choroideremia

To date, we have generated a bank of genetically stable iPSC lines from over five patients with CHM (Torriano et al., 2017; Cereso et al., 2014; Erkilic et al., 2019; Torriano et al., 2018) and unpublished data). In our experience, preservation of the genetic integrity of these lines in recent years was helped by technological advances that allowed us to reprogram under feeder-free conditions and to subculture cells rapidly using chemical dissociation protocols. The first of the CHM lines that we generated was reprogrammed using an inefficient lentiviral method (Cereso et al., 2014), but the remainder have been generated using the highly efficient SeV method. When we first switched to the SeV method, we were unprepared for the contrast in efficiency, and, consequently, we were confronted with a large

number of clones simultaneously emerging and requiring subculture. As a result, many of the newly generated iPSC lines showed evidence of genetic instability and would normally have been discarded.

However, an intriguingly high level of complex genetic instability was detected in the iPSCs generated from two brothers of a CHM family carrying a large deletion of the entire *CHM* gene (Erkilic et al., 2019). The karyotype anomalies were complex and involved combinations of the chromosomes 12, 20, and/or 5. A core translocation, t(12; 20) (q24.3; q11.2), was found to be common among all the iPSC clones. We thus performed complementary karyotype analyses on blood cells of all family members and determined that this novel reciprocal translocation was segregating in the family. Therefore, its presence in the original somatic cells bred further instability following reprogramming into iPSCs. This study teaches us that unstable iPSCs should not be automatically discarded, as they can uncover chromosomal aberrations segregating in unsuspecting families. This in turn has important consequences for genetic counseling, due to the high rate of association of such rearrangements with reproductive failure or birth defects.

Generally, iPSC lines for CHM have been generated from patient peripheral blood mononuclear cells (PBMC) (Duong et al., 2018; Vasireddy et al., 2013) or fibroblasts isolated from punch biopsies (Torriano et al., 2017; Cereso et al., 2014; Erkilic et al., 2019; Torriano et al., 2018). As *CHM* is ubiquitously expressed, patient fibroblasts have also proven useful as an intermediate step to assay the effect of unusual mutations on *CHM* transcript and REP1 protein levels (Vache et al., 2019; Torriano et al., 2017). Furthermore, fibroblasts can also be used to study the underprenylation defect characteristic of CHM using an in vitro prenylation assay (Fig. 5.6A). Prenylation allows Rabs to attach to vesicular membranes to accomplish their roles as regulators of intracellular transport, whereas unprenylated Rabs remain free, and nonfunctional, in the cytosol. In the in vitro prenylation assay, the cytosolic fraction of the cell lysate is incubated with recombinant REP1, RGGTaseII, and a biotinylated prenyl donor. The free, cytosolic Rab pool can thus be prenylated in vitro and the biotinylated Rabs subsequently detected by western blot analysis. The intensity of the signal provides a semiquantitative analysis of the original unprenylated Rab pool; the stronger the signal, the more cytosolic Rabs present.

We previously showed that regardless of the type of mutation, CHM patient fibroblasts contain 4-10-fold higher unprenylated Rab levels than control cells (Torriano et al., 2017; Sanchez-Alcudia et al., 2016). Similarly, iPSCs (Fig. 5.6B) generated from CHM patient fibroblasts also show higher unprenylated Rab levels, as compared to controls (Fig. 5.6C and D). Furthermore, using immunofluorescence studies, Vasireddy et al. suggested a trafficking defect of Rab27a in iPSCs generated from PBMC of a CHM patient. In the REP1-deficient iPSCs, Rab27a showed a perinuclear localization, whereas in the presence of REP1, Rab27a appeared localized to the membrane (Vasireddy et al., 2013).

In conclusion, although not a true model of the disease, CHM fibroblasts and even iPSCs have helped to further CHM research by addressing questions that did not require a retinal context to be answered.

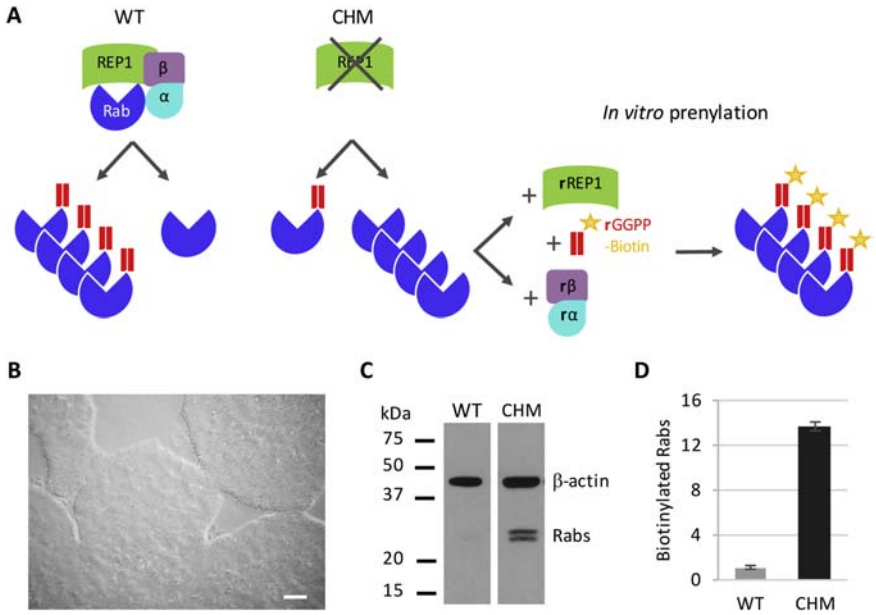

FIGURE 5.6

In vitro prenylation of choroideremia iPSCs.

(A) Simplified schematic representation of the prenylation status in wild type (WT, left panel) and CHM (middle panel) cells. REP1 presents Rab proteins to the enzyme RGGTaseII for addition of a prenyl donor (in *red*). As this is a dynamic process, the WT cell will contain a majority of prenylated Rabs (in *blue* and *red*) and a minority of Rabs destined for prenylation (in *blue*). By contrast, the CHM cell, which is deficient in REP1, will contain an excess of unprenylated Rabs (in *blue*); a proportion of prenylated Rabs (in *blue* and *red*) will be present due to prenylation via REP2. In the in vitro prenylation test (right panel), the cytosol containing the unprenylated Rab pool is incubated with recombinant REP1 (rREP1), RGGTaseII (rα and rβ) and a biotinylated prenyl donor (rGGPP; *yellow* star). In this way, the unprenylated Rabs (in *blue*) undergo in vitro prenylation (in *blue*, *red* and *yellow*), and the addition of the biotinylated prenyl group allows their subsequent detection via western blot analysis. (B) iPSCs of a CHM patient generated using SeV vectors and cultured under feeder-free conditions. Scale bar, 200 μm. (C) Western blot analysis following in vitro prenylation of iPSCs from the same patient showing a readily detectable biotinylated Rab population, as compared to WT iPSCs. (D) Graph showing the semi-quantification of the biotinylated Rab levels, normalized to β-actin levels and expressed as relative units, in the WT (*gray* bar) and CHM (*black* bar) iPSCs shown in (C); data are expressed as mean ± SEM ($n = 2$).

Human iPSC-derived retinal pigment epithelium

As mentioned above, it is not yet definitively accepted as to whether the retinal defects associated with CHM are due to autonomous degeneration of the photoreceptors or RPE, but it does seem that the diseased RPE exacerbates photoreceptor degeneration. Moreover, it is generally accepted that the choroid is a secondary consequence of RPE degeneration. Therefore, a highly pertinent model to study CHM is iPSC-derived RPE. RPE was initially generated from iPSCs as early as 2009 (Buchholz et al., 2009; Hirami et al., 2009; Meyer et al., 2009), only 2 years after the initial reports of human iPSC generation. The particular advantage of RPE is that it can be spontaneously differentiated from iPSCs (Buchholz et al., 2009). Following removal of bFGF from iPSC culture media, pigmented foci spontaneously appear in culture plates (Fig. 5.7A). These can be manually dissected using a light microscope and further subcultured to obtain a homogenous tissue.

iPSC-derived RPE is comprised of a monolayer of cells with a pigmentation visible to the naked eye (Fig. 5.7B). The monolayer is composed of tightly packed cells with a characteristic cobblestone morphology (Fig. 5.7C). iPSC-derived RPE cells have numerous microvilli on their apical surface and a basally located nucleus (Fig. 5.7D). Furthermore, the pigmentation is due to the presence of numerous apically located melanosomes. iPSC-derived RPE cells also express characteristic RPE markers in a polarized fashion: on the apical side, the tight junction marker ZO1 and the microvilli marker MERTK; in the cytosol, the visual cycle proteins LRAT, CRALBP, and RPE65; on the basolateral side, the integral membrane protein Bestrophin-1 (Torriano et al., 2017; Schwarz et al., 2015). Moreover, iPSC-derived

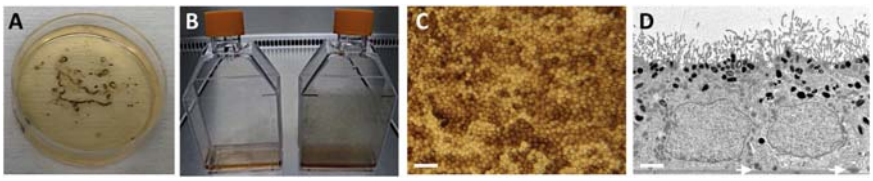

FIGURE 5.7 Generation and morphology of choroideremia iPSC-derived RPE.

(A) Pigmented foci spontaneously appearing in a 35-mm dish of confluent CHM iPSCs during the differentiation protocol. (B) Visibly pigmented iPSC-derived RPE cultured in a 75 cm^2 culture flask (on the right) compared to non-pigmented cultured fibroblasts (on the left). (C) Pigmented cobblestone appearance of a CHM iPSC-derived RPE monolayer. Scale bar, 40 μm. (D) Transmission electron micrograph of a CHM iPSC-derived monolayer showing two adjacent cells with, on the apical side, microvilli, tight junctions (asterisks) and melanosomes, and, on the basal side, nuclei and underlying secreted deposits (arrows). Scale bar, 2 μm.

Images courtesy of N. Erkilic.

RPE reproduces the functions of the RPE in vivo. It is capable of active apico-basal fluid transport due to the presence of apical tight junctions (Cereso et al., 2014), consistent with ZO1 labeling. Similarly, iPSC-derived RPE is capable of polarized growth factor secretion, such as PEDF apically and VEGF basally (Simonin et al., 2019). Furthermore, this tissue can perform phagocytosis of POS via its apical microvilli (Singh et al., 2013) and can actively participate in the visual cycle following the addition of recombinant all-*trans* retinol to the culture media (Maeda et al., 2013; Muniz et al., 2014).

Taken together, a morphologically characteristic and functional iPSC-derived RPE model would potentially hold much interest for CHM, both in terms of pathophysiology studies, as well as for the testing of novel therapeutic molecules.

Human iPSC-derived RPE for modeling choroideremia

The first example of iPSC-derived RPE generated for CHM was published in 2014 (Cereso et al., 2014). We generated iPSCs from a patient carrying a complex duplication-deletion event, which resulted in the skipping of exon eight and premature REP1 termination. The CHM iPSC-derived RPE showed a normal morphology, expressed characteristic markers, and was capable of fluid transport and internalization of fluorescently labeled microspheres. Moreover, we showed that the patient iPSC-derived RPE mimicked the biochemical defect of CHM. Firstly, using the in vitro biotinylated prenylation assay detailed above, only a faint biotinylated Rab signal could be detected in control iPSC-derived RPE. By contrast, a fourfold stronger signal could be detected in the CHM iPSC-derived RPE, indicative of a larger unprenylated Rab pool in these cells. Secondly, by differential centrifugation and western blot analysis, we showed that the CHM iPSC-derived RPE contained a significantly higher content of cytosolic Rab27a, and a lower content of membrane-bound Rab27a, as compared to control RPE. Thus, taken together, iPSC-derived RPE from CHM patients mimics the underprenylation defect associated with the disease.

In a complementary study in 2018, Duong et al. suggested that CHM iPSC-derived RPE from four patients carrying loss-of-function mutations showed decreased phagolysosomal activation (Duong et al., 2018), similar to what had been previously reported in patient lymphoblasts (Strunnikova et al., 2009). After internalization of pHrodo Biospheres, the fluorescence of which increases within the lysosome, the fluorescence of CHM iPSC-derived RPE did decrease over time as in control RPE, but the fluorescence levels were lower at all time points (Duong et al., 2018). Furthermore, the authors reported a differential distribution pattern of Rab27a in iPSC-derived RPE by immunofluorescence studies, as was previously reported in iPSCs (Vasireddy et al., 2013). In control RPE, Rab27a showed a diffuse labeling in the majority of cells, whereas in a minority (20%), Rab27a labeling was found to be perinuclear. This was similar in patient iPSC-derived RPE, except that a

slightly but significantly higher proportion of cells (30%) showed a perinuclear Rab27a localization (Duong et al., 2018), thus suggestive of defective trafficking of Rab27a.

In conclusion, the differential phenotypes observed between control and patient iPSC-derived RPE provide an insight into CHM pathophysiology, as well as pertinent readouts to assess the efficacy of novel therapies.

Human iPSC-derived RPE for proof-of-concept studies of gene supplementation for choroideremia

Currently, clinical trials for gene supplementation in CHM are ongoing using an AAV2/2 vector expressing *CHM* (MacLaren et al., 2014). However, as mentioned above, an appropriate animal model reproducing the disease in affected males does not exist. Thus, preclinical proof-of-concept studies prior to these clinical trials were performed initially on patient fibroblasts and on heterozygous $Chm^{wt/null}$ females (Tolmachova et al., 2013). Although the use of these models showed that the therapeutic vector could reduce unprenylated Rab levels in patient cells and that it was safe in wild-type mice, the studies were not highly informative in terms of functional rescue of electroretinogram responses in $Chm^{wt/null}$ females at 4 weeks of age.

A similar study was performed on this animal model using an AAV2/8 vector expressing *CHM*. The authors showed the appearance of functional anomalies using pupilometry tests from 32 weeks of age, and histological anomalies at 50 weeks of age, that were corrected by the therapeutic vector. Thus, the late appearance of anomalies in this heterozygous animal model renders it challenging for proof-of-concept studies. As an intermediate step between fibroblasts and iPSC-derived retinal cells, Vasireddy et al. showed that patient iPSCs could be used to assess the efficacy of therapeutic vectors. By transducing CHM iPSCs (as well as fibroblasts) with an AAV2/2 vector expressing *CHM*, the authors showed that they could induce Rab27a out of the perinuclear region to the membrane of the cells 48-h posttreatment (Vasireddy et al., 2013).

In 2014, Cereso et al. were the first to show the pertinence of human iPSC-derived RPE for testing the efficiency of AAV vectors (Cereso et al., 2014), the most currently used vectors for gene supplementation in IRDs (Trapani et al., 2014). By studying the transduction efficiency of a panel of AAV serotypes in control iPSC-derived RPE, we showed that transduction efficiency increased over time and that AAV2/5 was the most efficient serotype (Cereso et al., 2014). By four-weeks posttransduction, AAV2/5 showed the highest percentage of transduced cells, followed by AAV2/2, then 2/4, and lastly 2/8 and 2/9. These results were particularly thought-provoking due to the fact that *CHM* gene supplementation trials had already begun using an AAV2/2 vector. We then developed an AAV2/5 vector expressing the *CHM* gene and, following transduction of the aforementioned patient iPSC-derived

RPE, the unprenylated Rab pool was reduced to control levels. Similarly, following transduction, the membrane-associated Rab27a pool in CHM iPSC-derived RPE was increased significantly compared to untreated cells.

Furthermore, in 2017, Torriano et al. generated iPSC-derived RPE carrying the pathogenic missense mutation p.Leu457Pro and performed proof-of-concept studies using the aforementioned AAV2/5 vector expressing *CHM* (Torriano et al., 2017). We showed that transduction of patient iPSC-derived RPE significantly reduced unprenylated Rab levels, as compared to nontreated RPE, despite the presence of a mutant protein (expressed at ~14% of control levels). Thus, the p.Leu457Pro mutation did not hinder the function of the exogenous REP1 protein. This topical observation confirmed that CHM patients carrying missense mutations could be included in ongoing clinical trials. In addition, this study showed the predictive value of iPSC-derived RPE for therapy screening prior to inclusion.

In 2018, Duong et al. performed a short-term comparative study between AAV2/5 and a novel serotype, AAV2/7m8, in iPSC-derived RPE and showed a higher transduction efficiency of AAV7m8 at 96-h posttransduction (Duong et al., 2018). Furthermore, they suggested an improvement in phagolysosomal degradation and Rab27a trafficking following transduction of CHM iPSC-derived RPE. Interestingly, the authors report that there is a delayed onset of transgene expression from AAV2/7m8 at 24-h posttransduction in CHM iPSC-derived RPE as compared to control. They suggest that this could be due to a defective endocytosis, of which Rab27a is considered to be a mediator (Yamaoka et al., 2016). This observation could have implications for the potential efficiency of AAV-mediated gene therapy in the case of CHM compared to other IRDs.

Taken together, even in the absence of an appropriate animal model, relevant proof-of-concept studies of *CHM* gene supplementation were made possible by pertinent iPSC-derived RPE models. Moreover, these studies raised observations that could be of relevance for current and future clinical trials.

Human iPSC-derived RPE for proof-of-concept studies of translational read-through for choroideremia

Strikingly, 30% of the mutations associated with CHM are nonsense mutations (Moosajee et al., 2014). CHM thus lends itself to treatment by a particular class of therapeutic compounds known as translational read-through inducing drugs or TRIDs. TRIDs promote ribosomal misreading of premature stop codons and enhance incorporation of a near-cognate amino acid (Davies et al., 1965). This can result in the production of a full-length protein, which in some cases could be functional. As the zebrafish *chm*^{ru848} model carries a nonsense UAA mutation, p.Gln33*, in exon two of *chm*, it has been used to test the efficiency of TRID therapy with promising results (Moosajee et al., 2008). Treatment with one particular TRID,

PTC124, resulted in increased survival, preservation of retinal structure, and an increase in rep1 expression of ~20% over the residual maternal rep1 deriving from the yolk sac (Moosajee et al., 2016).

As a clinically pertinent complement to this study, we generated iPSC-derived RPE from a patient carrying a UAA nonsense mutation, p.Lys258*, in exon six of *CHM* (Torriano et al., 2018). The p.Lys258* variant was associated with low mutant RNA levels, no detectable REP1 protein and an increase in the unprenylated Rab pool, similar to that observed with other loss-of-function mutations (Sanchez-Alcudia et al., 2016; Cereso et al., 2014; Erkilic et al., 2019). Although treatment with PTC124 showed a tendency to decrease the unprenylated Rab pool in the iPSC-derived RPE, there was no significant difference from untreated cells (Torriano et al., 2018). Furthermore, treatment did not result in the production of a detectable REP1. It has been shown that during translational read-through, not all mino acids are introduced into the target site at the same frequency (Keeling et al., 2012). In the case of a UAA nonsense mutation, either a tyrosine (60%) or a glutamine (40%) are most frequently introduced. However, in the case of REP1, in silico predictions showed that both of these substitutions would be damaging, as the lysine at position 258 is highly conserved (Torriano et al., 2018). By contrast, in the case of the zebrafish p.Gln33* mutation, the wild-type glutamine would be incorporated relatively frequently. Furthermore, this amino acid is not evolutionarily conserved, suggesting that even incorporation of a tyrosine would be tolerated. These factors, coupled to the presence of residual maternal rep1 derived from the persistent yolk sac, could account for the partial rescue observed posttreatment in the zebrafish chm^{ru848} model, and explain why these results were not translatable to a human context.

To conclude, to render TRID therapy more effective, a personalized approach may need to be considered, whereby patient iPSC-derived RPE is screened for drug efficiency prior to inclusion in a potential clinical trial. Only by such a model can effectiveness in the context of the patient's genetic mutation be truly evaluated.

Future directions for modeling choroideremia

Taken together, CHM is an ideal candidate for an iPSC-based approach due to the absence of a relevant animal model. A further advantage is that the primary defect of the disorder affects the RPE, which is readily differentiated from iPSCs. To date, work on iPSC-derived RPE from CHM patients has been highly informative for proof-of-concept studies and has shown its predictive value for personalized screening of drug efficacy. Although insights into CHM pathophysiology have been obtained, there are still many exciting avenues that can be studied using iPSC-derived retinal models.

The functionality of CHM iPSC-derived RPE has not yet been fully explored and could hold potential clues to disease pathophysiology. There still remains work to be done concerning phagolysosomal degradation following internalization of POS,

which would be biologically more relevant than the work performed to date using biospheres. Due to the capacity of iPSC-derived RPE to be cultured long term without loss of morphology or functionality, one could study the degradation kinetics of the ingested POS over multiple days or even weeks and thus evaluate the consequential effects of oxidative stress linked to defective degradation (Singh et al., 2013).

Furthermore, if Rab27a trafficking is truly affected in CHM, then this should have downstream functional or morphological consequences. For example, there have been rare cases of neovascularization observed in CHM (Endo et al., 2000; Robinson and Tiedeman, 1987; Sawa et al., 2006), which could be explained by secretory dysregulation of pro-angiogenic cytokines that is consistent with the role of Rab27a in secretory processes. Polarized cytokine and growth factor secretion can be finely studied in iPSC-derived RPE by assaying apical and basal secretion separately following compartmentalized culture (Simonin et al., 2019). The results obtained could also be correlated to the activity of specific ion channels known to regulate RPE functions (Reichhart and Strauss, 2014). For example, intracellular calcium imaging could be used to assay the activity of calcium channels and whole-cell patch-clamp electrophysiology studies to assay a wider spectrum, such as potassium or chloride channels (Mamaeva et al., 2021).

In addition, the eye fundus of CHM patients is initially characterized by pigment mottling, which then gives way to RPE atrophy (Hoyng et al., 2004). It is tempting to speculate that the pigment mottling could be associated with dysregulation of melanosomes. This would be consistent with the observation that decreased Rab27a expression is linked with melanosome aggregation (Kawasaki et al., 2008). Alternatively, the pigment mottling could be due to subretinal deposits, as have been observed in the conditional knockout *Chm* mouse model (Wavre-Shapton et al., 2013). To address these possibilities, ultrastructural analyses can be performed on iPSC-derived RPE, in which melanosome biogenesis (Simonin et al., 2019), as well as basal deposits (Galloway et al., 2017), can be quantitatively and/or qualitatively assayed in comparison with controls.

Moreover, a major question surrounding CHM is if photoreceptors autonomously degenerate or if degeneration is due to the diseased RPE. Emerging techniques will now pave the way to generating other iPSC-derived retinal models, which may help answering this question. It is already possible to recapitulate eye development in vitro with the generation of an optic cup and formation of a stratified retina containing all retinal cell types (Eiraku et al., 2011; Nakano et al., 2012). Technological advances over the years have allowed the generation of so-called "retinal organoids" containing a mature outer nuclear layer comprising photoreceptors with an inner segment, connecting cilium, and nascent outer segment (Gonzalez-Cordero et al., 2017; Parfitt et al., 2016). Furthermore, these organoids mature in the absence of an RPE layer. Thus, retinal organoids derived from CHM patients would be an ideal model to determine whether REP1 is essential for photoreceptor integrity and/or function independently of the RPE.

In addition, RPE and endothelial coculture models used to mimic RPE-choroid interactions have been investigated with encouraging results (Skottman et al., 2017). Along this line, a recent study reported an iPSC-derived RPE-choroid model generated by bioprinting iPSC-derived endothelial cells, pericytes, and fibroblasts on the one side of a biodegradable scaffold, and seeding iPSC-derived RPE on the other side (Song et al., 2017). The endothelial cells reportedly organize into a vascularized tissue that resembles the native choroid. The biodegradable scaffold provides the framework for the RPE and endothelial cells to secrete extracellular matrices and to communicate. Such a coculture model would be highly pertinent for CHM to address the effect of the diseased RPE on the health of the choroid and proliferation of the choriocapillaris. Moreover, the coculture of varying combinations of control and CHM RPE or choroid may help determine whether the diseased choroid also plays a role in RPE degeneration.

These novel models further expand the possibilities for better modeling CHM in the future. It is vital to understand the link between the underlying molecular/biochemical defect and the clinical phenotype, as this in turn may lead to complementary, albeit noncurative, therapeutic possibilities, aimed at either slowing or halting retinal degeneration. The ultimate way to fully recapitulate CHM in vitro would be an iPSC-derived model comprising an intact neuroretina, RPE, and choroidal layer. To our knowledge, such a model has not yet been developed, but the advances in bioprinting on biodegradable scaffolds hold much promise for the future and it may only be a matter of time.

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CHAPTER

6 Applications of human induced pluripotent stem cell and human embryonic stem cell models for substance use disorders: addiction and neurodevelopmental toxicity

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Chapter outline

Abstract

Drug abuse is a major public health concern throughout the United States and the rest of the world, both due to the adverse effects of addiction in drug-taking adults and because of neurodevelopmental toxicity in children exposed in utero. Model systems derived from either human embryonic stem cells or induced pluripotent stem cells (hiPSC) can be used to examine the mechanisms underlying both addiction in adults and drug-induced neurodevelopmental deficits. Here we discuss

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the use of hiPSC to examine genetic factors that influence the susceptibility of an individual to addiction. In the past several years, the approach of comparing induced pluripotent stem cells (iPSC) derived from individual drug-addicted patients to those of nonaddicted individuals after in vitro differentiation to specific neuronal subtypes, such as dopaminergic neurons, has been developed. This approach can be used to examine the effects of specific suspected allelic variations of genes involved in neurotransmitter systems common to the mesolimbic reward pathway. Initial studies of this type have been used to verify the correlation of specific genetic polymorphisms with addiction to a drug of abuse. Additionally, iPSC-derived neurons have been used to test drugs for potential efficacy in treatment of addiction. In terms of studying the neurodevelopmental effects of drugs of abuse, the use of stem cell technology is especially promising. For example, we have generated three-dimensional (3D) neocortical organoids from human pluripotent stem cells to examine the effects of cocaine on neocortical development, demonstrating an important and selective role of the cytochrome P450 enzyme CYP3A5 in adverse neurodevelopmental effects of cocaine. The use of in vitro neuronal differentiation models and 3D brain organoids generated from hiPSC allows for a direct study of genetic risk factors and cellular mechanisms that underlie substance-induced developmental abnormalities of the brain.

Keywords: 3D brain organoid; Addiction; Alcohol; Cocaine; Copy number variations (CNVs); CYP3A5; Dopaminergic neurons; Human induced pluripotent stem cells (hiPSC); Neocortical development; Single-nucleotide polymorphisms (SNPs).

Introduction

In this chapter, recent studies utilizing neuronal cell lines derived from both human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) as models for drug addiction and toxicity will be reviewed. hiPSC lines derived from addicts and nonaddicts can potentially be used to examine the effects of putative or known addiction-related genetic variations such as single-nucleotide polymorphisms (SNPs) or copy number variations (CNVs) on responsiveness to certain pharmacological treatments, such as anticraving drugs. The use of hiPSC from individual users could also potentially be informative for developing therapeutic regimens specifically tailored for that individual, taking into account the individual's unique genetic background.

A more immediate application of both hiPSC and hESC is that these cells can be employed to examine mechanisms involved in the neurodevelopmental toxicity of drugs of abuse. Many drugs of abuse, especially nicotine, alcohol, and cocaine have adverse effects on development of the brain. Neurodevelopmental effects may involve entirely different mechanisms, separate from those that mediate drug abuse and addiction in adults. Although such effects can be detected in humans, it is difficult or impossible to determine the relevant neurobiological and molecular mechanisms using animal material or human surveys. Especially for development of the cerebral cortex, developmental mechanisms that are present in human brain

are not adequately modeled in rodents. Thus, hiPSC- and hESC-based models can provide a method for understanding toxicity of drugs on development of the human brain that allows for examination of molecular and biological processes.

The epidemic of drug abuse and addiction

Drug abuse and addiction continues to be one of the largest public health challenges in the United States. According to the National Vital Statistics System-Mortality (NVSS-M), a total of 70,237 individuals died of drug overdose in 2017, with 39% of deaths involving the potent synthetic opioid fentanyl, 23% involving heroin, 21% involving cocaine, and 13% involving methamphetamine (Hedegaard et al., 2019; Kariisa et al., 2019). The overall number of drug overdose deaths increased 70% from 2011 to 2017 (Hedegaard et al., 2018, 2019), and throughout that 6-year period the majority of overdose deaths were due to opioids, including fentanyl, heroin, oxycodone, and methadone. The death toll, however, only tells part of the story. It can be estimated that approximately 22.7 million people in the United States are substance dependent (National Survey on Drug Use and Health; https://www.drugabuse.gov/publications/drugfacts/nationwide-trends), resulting in an estimated annual economic burden of $740B (National Institute of Drug Abuse; https://www.drugabuse.gov/related-topics/trends-statistics#supplemental-references-for-economic-costs). This burden includes not only health care costs, but also loss of productivity in the workplace and costs related to crime.

Identification of genetic targets for treatment of substance use disorders

Drugs of abuse affect the mesolimbic pathway, otherwise known as the reward pathway, in the brain, mediated by the neurotransmitter dopamine (Berridge and Kringelbach, 2015; Dackis and O'Brien, 2001; Koob and Volkow, 2016; Wise, 1987, 1998). The process of "reward" has recently become understood to involve two main components, usually designated as "liking" and "wanting." The mesolimbic pathway is believed to mediate the "wanting" component of reward (Berridge and Kringelbach, 2015). Long periods of drug abuse lead to alterations in brain circuitry and chronic dysregulation of neurotransmitter systems even after withdrawal (e.g., Grimm et al., 2001; Mendez et al., 2009). Lasting alterations in brain chemistry result in patients being highly susceptible to relapse, for example, when confronted with environmental cues associated with previous drug use (Volkow et al., 2006). Opioids, cocaine, amphetamines, nicotine, and alcohol all seem to elicit their rewarding effects or desire to repeatedly use the drug, at least in part, by increasing the amount of available dopamine at synapses between the ventral tegmental area (VTA) and the nucleus accumbens in the ventral striatum

(Dackis and O'Brien, 2001; Koob and Volkow, 2016; Wise, 1987, 1998); thus, there have been many attempts to develop anticraving drugs that modulate the increased availability of dopamine (Douaihy et al., 2013; Lin, 2013; O'Brien, 2005).

The knowledge that many different drugs of abuse co-opt overlapping elements of neurotransmitter pathways in the midbrain has led to the search for genetic markers that may make individuals particularly vulnerable to development of addiction, and many research groups have focused on dopaminergic pathway genes as candidates for therapy. In addition to this traditional candidate gene approach, genome-wide association studies (GWAS) utilizing high-throughput methodologies to interrogate thousands of sequences simultaneously can identify SNPs that correlate with the progression of specific substance use disorders. Most of these GWAS studies have focused on alcohol or nicotine dependence (Hancock et al., 2019; Hart and Kranzler, 2015), but studies have also been carried out for opioids (Jensen, 2016; Smith et al., 2017), cocaine and other stimulants (Gelernter et al., 2014; Jensen, 2016; Sun et al., 2020), and cannabis (Agrawal et al., 2018). These studies have identified new candidate genes and SNPs to examine further in the context of substance use disorders (reviewed by Palmer et al., 2015; Prom-Worley et al., 2017). However, genetic variation between individuals occurs in several forms. In addition to SNPs, CNV accounts for a major proportion of genetic variation between individuals (Iafrate et al., 2004; Hastings et al., 2009). Additional potential variations include variable tandem DNA repeats and epigenetic effects such as imprinting (Chuang et al., 2017; Haggarty, 2015; Talseth-Palmer and Scott, 2011).

All of the aforementioned types of genetic variations are likely contributors to drug addiction susceptibility. Although some genetic factors may contribute substantially, it is probable that there are also many that contribute only a minimal or negligible effect, making drug addiction a polygenic disorder. Most other common human disorders, such as heart disease, high blood pressure, bipolar depression, and schizophrenia, are also believed to be polygenic. Like these disorders, drug abuse should not be thought of as being caused directly by genetic factors; rather, genetic factors may increase an individual's susceptibility. For any individual drug-abusing subject, the number of contributing genetic factors is likely to be large, perhaps with hundreds of contributing genetic factors and a similar number of contributing environmental factors. The smaller the contribution of any individual genetic variant, the more difficult it is to identify.

Provided the potential genetic risk factors are known, identification of combinations of contributing genetic factors can be used to determine a polygenic risk score (PRS) for addiction. Similar scores have been developed for other complex health conditions like schizophrenia and bipolar disorder (Dudbridge, 2013; Purcell, 2009). However, since the number of genetic variants that are important for drug abuse susceptibility is likely to be large compared to other human disorders, each individual variant may make less of a contribution to overall drug abuse. Importantly, there are also other nongenetic factors that contribute to drug abuse, including environmental factors, prenatal drug exposure (see below), and epigenetic factors

that alter gene expression. The relative contribution of genetic versus nongenetic factors to overall drug abuse is currently unknown and may vary between individuals. Contributions of these other factors would be missed in a measure like a PRS. Because of the complexity of the genetic and nongenetic contributions to drug abuse, it is unlikely that genetic factors could be identified solely by comparing hiPSC lines from drug abusers to those of nondrug abusers. Nonetheless, hiPSC models may provide an important avenue of research for genetic studies of drug abuse in that genetic factors that are identified by other means may be modeled by genetically modifying hiPSC lines or by comparing preexisting hESC lines. These may be differentiated to appropriate neural lines and used to examine mechanisms involved in the functioning of neurotransmitter systems or potential therapeutic agents. Furthermore, genetic factors that are only suspected to contribute to drug abuse, but that cannot be verified statistically, can be tested for their effects on development of neurons or even brain systems.

Finally, one might imagine that a large number of different and unrelated genetic aberrations could influence one final common pathway; for example, there might be many genetic mechanisms that could all influence one neurotransmitter system, or even expression of a single protein. The dopamine transporter (DAT) gene, to use one example, could be altered by mutations in the coding or noncoding regions, CNVs, differential methylation of promoter sequences, changes in expression of transcriptional regulators, or changes in pathways that regulate differentiation or plasticity of dopaminergic neurons expressing DAT. The number of genetic variations involved in DAT expression could be very large. In this manner, there could be genetic or epigenetic effects on drug abuse that are mediated by a single protein in a single neuronal subtype that are, therefore, essentially undetectable by GWAS or association studies. One might employ hiPSCs to examine differences in, for example, dopaminergic neuron function or dopamine transport using hiPSC lines from drug abuse subjects as compared to controls. The studies by Sheng et al. (2016a, 2016b) were an initial step directed at this approach.

It is now possible to differentiate almost any type of neuron, glial cell, or brain cell assembly, from either hESC or hiPSC. If a genetic factor is known, or suspected, to be involved in drug abuse, these differentiated neurons or brain cell structures can be employed to study how that genetic factor affects the functioning of hiPSC or hESC-derived neurons in vitro, or even after transplantation in rodent models.

Generation of human induced pluripotent stem cell-derived neuronal cell lines as models for drug addiction

hiPSC are derived by genetically reprogramming adult somatic cells (e.g., fibroblasts) to a pluripotent state (Takahashi and Yamanaka, 2006). hiPSC can subsequently be differentiated to specific adult cell types of choice in vitro by adding differentiating factors in the appropriate sequence and manipulating other culture

conditions. Virtually any brain cell type can be differentiated from human pluripotent stem cells (hPSC), both hESC or hiPSC (Liu and Zhang, 2011), including midbrain dopaminergic neurons (Arenas et al., 2015; Cai et al., 2010; Hartfield et al., 2014; Nolbrant et al., 2017; Sundberg et al., 2013; Vazin et al., 2009; Lee et al., 2015b; Kriks et al., 2011; Yan et al., 2005) forebrain glutamatergic and GABAergic neurons (Lieberman et al., 2012; Shi et al., 2012), cultures of interconnecting neurons of different types (Lee et al., 2015a), and even complete two-dimensional (2D) or three-dimensional (3D) structures such as layered cortical organoids (Kindberg et al., 2014; Lancaster et al., 2013; Lee et al., 2017; Velasco et al., 2019). In most models, either hESC or hiPSC lines can be employed with similar results, although in some cases hiPSC lines can differ markedly in differentiation capacity (e.g., Lee et al., 2015b). Generation of iPSC is intended to revert cells back to pluripotency, including a restoration of embryonic DNA methylation patterns. However, several studies have shown low-passage iPSC has a residual epigenetic memory in which some adult methylation patterns of the donor tissue are preserved (Chin et al., 2009; Ji et al., 2015; Kim et al., 2010; Lister et al., 2011). This epigenetic memory may influence the differentiative ability of iPSCs derived from different somatic tissues. A further complication is the tendency of hiPSC lines to undergo chromosomal aberrations during reprogramming. iPSC have been shown to have an increased incidence of chromosomal duplications (Mayshar et al., 2010), an increased incidence of CNVs (Hussein et al., 2011), and an increased incidence of mutation to coding regions (Ji et al., 2012).

In 2012, Lieberman and colleagues were the first to produce neuronal cell lines from iPSC of addicted and nonaddicted subjects. They aimed to model the effects of alcohol, looking specifically at alcohol's effects on the NMDA receptor (Lieberman et al., 2012). The NMDA receptor mediates the excitatory effects of the neurotransmitter glutamate within the brain and is inhibited by ethanol (reviewed by Chandrasekar, 2013). Previous research carried out in rodents has shown that acute ethanol exposure leads to decreased responsiveness of NMDA receptors, and thus an inhibition of glutamatergic excitatory transmission (Nie et al., 1994; Puglia and Valenzuela, 2010) while chronic exposure leads to increased NMDA receptor expression and enhanced functioning (reviewed by Nagy, 2008). This increase in expression and function of NMDA receptors leads to ethanol tolerance. Lieberman and colleagues (2012) were interested to see if they could show the same chronic effects of ethanol on NMDA receptor responsiveness and expression in iPSC-derived neuronal lines from alcohol-addicted human subjects. In their study, hiPSC were derived from skin punch biopsies of four alcohol-addicted subjects (according to established DSM-IV criteria) and three nonaddicted social drinkers. Differentiation to neural cultures containing both neurons and glial cells was verified by immunostaining for neuronal, glial cell, and synaptic markers. Electrical activity characteristic of glutamate signaling via NMDA and AMPA receptors and GABA signaling via $GABA_A$ receptors was observed, and the ability of the neurons to produce action potentials was verified. Chronic 7-day exposure to 50 mM ethanol significantly increased the expression

level of several NMDA receptor subunits (NR1, NR2A, NR2D) in cultures derived from addicted individuals but not in cultures derived from social drinkers. The results support the hypothesis that increased expression of NMDA receptors in chronic drinkers may lead to tolerance and addiction. Furthermore, the fact that significant changes in NMDA receptor expression after chronic exposure were only observed in cells derived from alcohol-addicted subjects and not from nonaddicted social drinkers may reflect an underlying heritable difference in the addicted individuals that predisposes them to addiction.

Further studies by Lieberman et al. (2015) used neural cultures derived from iPSC of 9 social drinkers and 12 heavy drinkers to analyze expression levels of $GABA_A$ receptor subunits. In adults, GABA (γ-amino butyric acid) is a major inhibitory neurotransmitter in the brain, and its effects are mediated via the ionotropic $GABA_A$ receptor, which, when bound to GABA, allows Cl- to enter neurons and inhibit their firing (Olsen and Sieghart, 2009). However, during early brain development, the chloride gradient is reversed, and $GABA_A$ therefore mediates excitatory effects by allowing Cl- to exit and depolarize the neuron (Lee et al., 2005; Wang and Kriegstein, 2009). Genes encoding the $GABA_A$ receptor subunits are found in clusters on four different human chromosomes, allowing for coordinated regulation of the clustered genes (Steiger and Russek, 2004). The GABRA2 gene, located in a cluster on chromosome 4p12, encodes the $\alpha 2$ subunit of $GABA_A$ and exists as two distinct alleles which arise from a T-to-C SNP within exon 5. The C polymorphism (rs279858*C) had been previously indicated through GWAS as a heritable factor associated with not only addiction to alcohol (Olfson and Bierut, 2012), but also addiction to other drugs of abuse (Agrawal et al., 2006), indicating a common mechanism for addiction to several drugs.

Lieberman and coworkers (2015) produced a total of 36 iPSC lines from skin punch biopsies of 12 heavy drinkers and 9 social drinkers and differentiated them to neuronal lines as described previously (Lieberman et al., 2012). Quantitative RT-PCR analysis was used to determine the genotypic expression of GABRA2 in neuronal lines, which were then categorized as either T/T, T/C, or C/C. Expression of GABRA2 receptor expression was shown to be significantly decreased in both the T/C heterozygotes and the C/C homozygotes compared to T/T homozygotes. Furthermore, qRT-PCR and RNA sequencing revealed a positive correlation between frequency of the C allele and low expression of all $GABA_A$ subunit genes in the chromosome 4p12 cluster. This correlation was not seen when the expression of $GABA_A$ receptor genes on the other three chromosomal clusters was examined. This suggests that the rs279858*C polymorphism within GABRA2 negatively effects the expression of the entire cluster of $GABA_A$ receptor genes on chromosome 4. Interestingly, when Lieberman and colleagues analyzed a dataset of postmortem adult brain tissues, no correlation between rs279858*C, GABRA2, and the other 4p12 GABAA genes was found. Since gene expression profiles of the neuronal lines derived from iPSC more closely resemble embryonic gene expression profiles, this suggests that T-to-C polymorphism has its effect on increasing susceptibility to alcoholism via a developmental mechanism. Taken together, the initial and subsequent

studies of Lieberman et al. (2012, 2015) using iPSC-derived neural cell lines have suggested that allelic differences at several loci, including loci for NMDA and $GABA_A$ receptors, could predispose an individual to alcohol addiction.

A recent study has also used neural cell lines derived from iPSC to examine the role of rs1799971, an A118G SNP in the OPRM1 gene that encodes the μ-opioid receptor (MOR) (Halikere et al., 2019). The MOR mediates the highly addictive effects of the opioids. Expressed on GABAergic neurons, an agonist-bound MOR will inhibit the firing of these inhibitory interneurons, resulting in an indirect disinhibition of dopaminergic neurons (Fields and Margolis, 2015). The A118 allele of OPRM1 encodes a MOR variant with an asparagine at position 40 (the N40 variant) while the G118 allele encodes a variant with an aspartate at position 40 (the D40 variant). The D40 variant leads to greater inhibition of GABAergic neurons (Popova et al., 2019) and has therefore been linked to greater susceptibility to addiction (Mague et al., 2010; LaForge et al., 2000). Halikere and coworkers (2019) obtained lymphocyte samples from individuals of similar backgrounds and representing both sexes. These individuals were not identified as either opioid-addicted or nonaddicted, and their opioid use status was not known. The cells were reprogrammed to form hiPSC and then differentiated to GABAergic neurons. Differentiation was verified via qRT-PCR to look for expression of general neuronal markers, inhibitory neuronal markers, synaptic markers, and MOR. Cell lines were identified as either N40 or D40 expressers and were then tested with exposure to the MOR agonist DAMGO. While DAMGO inhibited GABAergic neurons in both the N40 and D40 lines, the inhibitory effect was significantly greater in the D40 line, which would be expected to result in a greater disinhibitory effect downstream. This suggests that individuals expressing the D40 MOR variant would exhibit greater responsiveness to opioids and therefore be more susceptible to addiction.

Halikere and coworkers took their analysis a step further by using CRISPR/Cas9 editing to convert a human ES cell line homozygous for the A118 allele to an isogenic line that is homozygous for the G118 allele (Halikere et al., 2019). They also used one of the lines produced in their study from a homozygous G118 donor and used CRISPR/Cas9 editing to convert it to an isogenic line that is homozygous for the A118 allele. Subsequent differentiation of the parental and edited lines to GABAergic neural cells was performed and verified as before, and the neural lines were then subjected to DAMGO exposure. Within both pairs of isogenic cell lines, the DAMGO resulted in a greater inhibition of GABAergic neurons expressing the D40 MOR variant than in those expressing the N40 MOR variant. The use of CRISPR/Cas9 editing to generate isogenic lines that differed only in the expression of the MOR allele is a robust way to control for other potentially confounding genetic influences.

Another recent example of iPSC-derived neurons as models for addiction comes from Sheng and coworkers (2016a, 2016b), who modeled opioid addiction in iPSC-derived dopaminergic neurons. The human DAT1, which clears dopamine from a synapse by transporting it back into presynaptic terminals, is encoded by a gene that contains polymorphisms in the 3′VNTR (variable number tandem repeat)

region that results in 3−13 repeats, with most individuals containing either the 9- or 10-repeat alleles (Kang et al., 1999). Overexpression studies of 9- or 10-repeat alleles have led to conflicting results on the potentially modulatory effect of the number of repeats on DAT1 expression (Fuke et al., 2001; Mill et al., 2005; Miller and Madras, 2002; VanNess et al., 2005). Sheng and colleagues (2016a, 2016b) aimed to overcome obstacles inherent to overexpression studies by producing neuronal lines from iPSC of four individuals: one nonaddicted control subject homozygous for the 9-repeat allele, one opioid-addicted subject homozygous for the 9-repeat allele, one nonaddicted control subject homozygous for the 10-repeat allele, and one opioid-addicted subject homozygous for the 10-repeat allele. Differentiation of the iPSC lines into dopaminergic neurons was carried out following the methods of Kriks and colleagues (2011) and was verified by both immunohistochemistry and qRT-PCR for expression of general neuronal markers and dopaminergic neuronal markers (Sheng et al., 2016a, 2016b). Dopamine release from the cells was also verified by HPLC and was shown to be greater for opioid-dependent lines than for control lines (Sheng et al., 2016a, 2016b). When examining expression of neuronal marker TUJ-1 and dopaminergic marker TH, no significant differences were observed between the 4 cell lines (Sheng et al., 2016a, 2016b).

Lines derived from the control and addicted individuals homozygous for the 9-repeat 3′VNTR allele of DAT exhibited greater expression of DAT than lines from individuals homozygous for the 10-repeat allele, indicating that the number of repeats affects DAT density (Sheng et al., 2016b). There were no observed differences in DAT expression between the control and addicted lines from individuals homozygous for the 9-repeat allele or between control and addicted lines from individuals homozygous for the 10-repeat allele, indicating no effect of drug addiction on DAT expression levels in individuals with the same number of repeats (Sheng et al., 2016b). An effect of drug usage was seen for expression of dopamine receptor D2 (Drd2). Specifically, Drd2 expression was significantly decreased in the opioid-dependent lines compared to the control lines, regardless of repeat number (Sheng et al., 2016a). This finding is in agreement with previously reported decreases in Drd2 in opioid-addicted patients detected by neuroimaging studies (Volkow et al., 2004).

Having established the dopaminergic cell lines, Sheng and coworkers then used them to test the effects of valproic acid (VPA) on the expression of proteins of the dopaminergic pathway (Sheng et al., 2016b). VPA treatment increased the expression of DAT, TH, and Drd2 in all 4 cell lines, while it decreased the expression of Nurr1 in all cell lines. Interestingly, VPA had a significantly greater effect on Drd2 expression in the opioid-dependent lines, regardless of DAT 3′VNTR repeat number. Finally, VPA significantly decreased DA release in all 4 cell lines. Taken together, these results have demonstrated the effect of 3′VNTR repeat number on the expression of DAT, suggesting that individuals with nine repeats have a greater expression of DAT and therefore an increased intrinsic ability to clear DA from synapses than individuals with 10 repeats. They have also demonstrated an effect of

VPA in increasing DAT levels and decreasing dopamine release in all lines of dopaminergic neurons, regardless of repeat number. This suggests the use of VPA as a potential therapy for opioid-addicted patients, as it could decrease amounts of dopamine in the synapses via mechanisms affecting release and re-uptake. The study also serves as a proof of principle that neuronal cultures derived from addicted patients could be used as model system to test for mechanism and efficacy of treatments.

Limitations of human induced pluripotent stem cell models of drug abuse

As noted above, drug addiction is polygenic, and there are many heritable changes that predispose an individual to addiction, with each one by itself having a minimal effect. Identification of all genetic risk variants in an individual and elucidating those that are critical for the subject's disease will be difficult or perhaps impossible. GWAS studies may provide information regarding combinations of individual heritable changes that predispose an individual to addiction. Such combinations may differ from one individual to another. However, performing GWAS studies on drug addicts proves challenging because of (1) the likelihood that the addict uses multiple different drugs of abuse and (2) the importance of nongenetic contributing factors. Identifying a significantly large GWAS cohort of cocaine abusers, for example, who do not also abuse other substances or are not significantly confounded by other nonheritable factors, such as elements of socioeconomic status, is difficult to say the least.

Use of human embryonic stem cells and human induced pluripotent stem cells as models for neurodevelopmental effects

Various drugs of abuse, especially alcohol, nicotine, and cocaine, are believed to have deleterious effects on development of the human brain. Unraveling the nature of these effects and the pathophysiological mechanisms has been fraught with difficulty for several reasons. First, animal studies and especially rodent studies do not adequately reflect development of the human cerebral cortex. Surveys in humans are complicated by difficulties in obtaining adequate information on prior drug abuse during pregnancy. Usually, neurodevelopmental effects are separate from those that mediate drug abuse.

In the case of cocaine, one of the most thoroughly studied drugs, there is a large and conflicting literature on neurodevelopmental effects. Some laboratory studies suggest that cocaine has minimal effects on progenitor cell proliferation (McCarthy and Bhide, 2012) while others suggest that cell proliferation is a principal mechanism of neurodevelopmental defects (Kosofsky et al., 1994; Lidow et al., 2001;

Lidow and Song, 2001a, 2001b; Rando et al., 2013). Since the studies by Lidow et al. (2001) and Lidow and Song (2001a; 2001b) involved subhuman primates rather than rats, their conclusions may be suspected to be more relevant to humans. Grewen et al. (2014) identified substantial effects of prenatal cocaine on brain structure in early infancy. Bateman and Chiriboga (2000) found that cocaine significantly decreased overall head circumference, strongly suggesting a deleterious effect of cocaine on overall development of brain size.

Large-scale surveys of prenatal cocaine exposure in humans have detected adverse effects of cocaine. Some of the effects of prenatal cocaine have been reported to be modest (Richardson and Day, 1994), but others were relatively severe (Bandstra et al., 2004, 2011; Chiriboga et al., 2014; Richardson et al., 2015; Singer et al., 2000). Although post hoc determination of dose, time of exposure, and the role of other contributing factors are difficult, most studies have carefully attempted to control for other contributing factors. It is, of course, impossible to determine biological mechanisms in human surveys; thus, if there are contributing genetic factors these would have to be identified by other means prior to examining their contribution to variation in cocaine's developmental toxicity in developing human subjects.

It is worth noting that when children in these studies were followed up for long periods, into teenage years, some of the adverse effects of prenatal cocaine were found to be serious or debilitating. Substantial deficits in executive function, for example, decision making, were noted in several studies (Conradt et al., 2014; Min et al., 2014; Minnes et al., 2016; Noland et al., 2003; Richardson et al., 2015). Numerous studies have consistently found substantial increases in the frequency of drug abuse in adolescents exposed prenatally to cocaine (Delaney-Black et al., 2011; Frank et al., 2011; Min et al., 2014; Minnes et al., 2014; Richardson et al., 2013, 2019). Although there has been some dispute concerning whether these increases in drug abuse liability were due to other factors, such as the presence of drugs in the household (Warner et al., 2011), most studies have attempted to control for these factors and have nonetheless detected substantial effects of prenatal cocaine. We might note that the increases in drug abuse liability caused by prenatal cocaine exposure were in some studies, and for certain drugs, more than twofold, thus exceeding the contribution of any known genetic risk factor.

To address the uncertainty regarding mechanisms mediating the effects of cocaine on brain development, we initially employed a nondopaminergic neural progenitor cell line derived from the rat mesencephalon, AF5, to examine effects of cocaine on neural progenitor cell proliferation (Lee et al., 2008; Truckenmiller et al., 2002). Cocaine inhibited proliferation of this neural progenitor cell line by causing cell cycle arrest in G1 without causing apoptosis. This effect was associated with decreased expression of cyclin A2. The findings in AF5 cells were confirmed in primary human fetal CNS cells (Lee et al., 2008). Cocaine decreased cyclin A2 expression in primary human neural progenitor cells and A2B5 progenitor cells only, with no effect in primary human microglia or neurons. Primary human astrocytes, in contrast, exhibited increased cyclin A2 in response to cocaine, associated with activation of the JNK pathway and increased rates of cell proliferation

(Lee et al., 2008, 2016). The pathway involved in the inhibition of cell proliferation by cocaine in neural progenitor cells was found to involve increased ROS production due to CYP450-mediated N-oxidative metabolism of cocaine, ER stress, and activation of PERK, causing increased ATF4 and thereby decreasing cyclin A2 and cell proliferation. Furthermore, cocaine-induced down-regulation of cyclin A2 and interruption of progenitor cell-cycle progression were confirmed in fetal rat brains (Lee et al., 2008).

Due to the species' differences in brain development and CYP450-dependent drug metabolism, it has been difficult to translate findings from rat models directly to humans (Rakic, 2009; Hill and Walsh, 2005; Martignoni et al., 2006). We therefore used hPSC to investigate the relationship of CYP450 and the effects of cocaine on human neocorticogenesis. We established a 2D in vitro model of human neocortical development using hPSC, which generated radial glia scaffolding that supported cortical glutamatergic and GABAergic neuronal migration (Fig. 6.1) (Kindberg et al., 2014). Notably, the sequential development of the glutamatergic projection neurons, with deep-layer $CTIP2^+$ neurons appearing first, followed by generation of upper-layer $SATB2^+$ neurons, mimicked in vivo human neocortical development (Rakic, 2009; Hill and Walsh, 2005). Cocaine was found to induce oxidative stress, interrupt neural progenitor proliferation, and induce premature neuronal differentiation of various cortical neuronal subtypes in this 2D model (Kindberg et al., 2014). This 2D cell culture is unable to recapitulate *in vivo*-like cytoarchitectural organization and connectivity of the brain, limiting its possibilities for determining mechanisms of cocaine-induced brain developmental pathogenesis.

We recently adapted this 2D model system to recapitulate the 3D cytoarchitecture of the developing human neocortex. We established a human PSC-based 3D

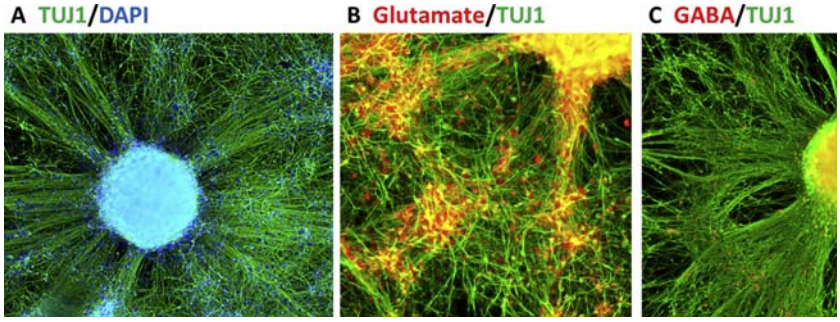

FIGURE 6.1 A 2D model of neocortical development using hPSCs.

(A–C) Expression of TUJ1, glutamate, and GABA by immunocytochemistry.

Data from Kindberg, A.A., Bendriem, R.M., Spivak, C.E., Chen, J., Handreck, A., Lupica, C.R., Liu, J., Freed, W.J., Lee, C.-T., 2014. An in vitro model of human neocortical development using pluripotent stem cells: cocaine-induced cytoarchitectural alterations. Dis. Model. Mech. 7, 1397–1405.

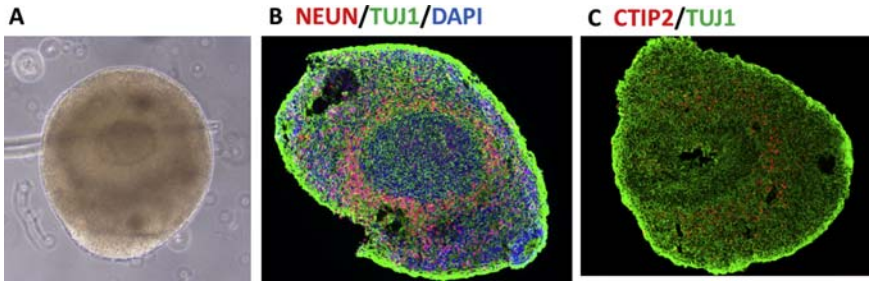

FIGURE 6.2 Generation of 3D neocortical organoids with single ventricle-like cavities from hPSCs.

(A) Phase-contrast image of a floating neocortical organoid derived from hPSCs. (B, C) Cryosections of neocortical structures immunostained for various cortical markers (B) NEUN/TUJ1 and (C) CTIP2/TUJ1.

Data from Lee, C.-T., Chen, J., Kindberg, A.A., Bendriem, R.M., Spivak, C.E., Williams, M.P., Richie, C.T., Handreck, A., Mallon, B.S., Lupica, C.R., Lin, D.T., Harvey, B.K., Mash, D.C., Freed, W.J., 2017. CYP3A5 mediates effects of cocaine on human neocorticogenesis: studies using an in vitro 3D self-organized hPSC model with a single cortex-like unit. Neuropsychopharmacology 42, 774–784.

neocortical organoid model that comprises a single rosette-like structure (Fig. 6.2) (Lee et al., 2017). Using a single rosette, rather than aggregates, allows for the development of measurable cortical layers. This 3D neocortical organoid model retains essential features of human neocortical development, including proliferative neuroepithelium during the earlier differentiation stage, neurogenesis, neuronal migration, and later expansion of the neocortical region. The model was similarly effective using either hESC or hiPSC lines (Lee et al., 2017). Significantly, expression of markers of cortical layer formation, including reelin expression in superficial layers, was retained and organized appropriately in this model. In that respect, this model is an advance over other 3D brain organoid methods in which multiple aggregates are combined (Lancaster et al., 2013; Pasca et al., 2015; Kadoshima et al., 2013). In addition, other models use embedding in a Matrigel scaffold (Lancaster et al., 2013; Pasca et al., 2015; Qian et al., 2016), which would interfere with drug diffusion and thus complicate administration of drugs. Using this 3D hPSC-based brain organoid model, we identified a single, specific isoform of the cytochrome P450 3A (CYP3A) family, CYP3A5, as being responsible for the adverse effects of cocaine on cerebral cortical development (Lee et al., 2017).

It is worth noting that the cytochrome P450 inhibitor cimetidine was effective in blocking the adverse effects of cocaine in each of the models that were tested, including AF5 cells (Lee et al., 2008), the developing rat brain (Lee et al., 2008), and the 2D neocortical development model (Kindberg et al., 2014). Cimetidine has previously been used to inhibit toxicity of other drugs mediated by cytochrome P450 (e.g., Dreyfuss et al., 1996; Lewis et al., 1992; Sztajnkrycer et al., 2003). Cimetidine, marketed under the name Tagamet, is used primarily to inhibit

production of stomach acid and is not toxic even in very high doses (Freston, 1982). Cimetidine is a general cytochrome P450 inhibitor, not selective for CYP3A5. Also, the dosages that would be required to prevent cocaine neurodevelopmental toxicity in humans are unknown, and determination of its potential efficacy would require studies in subhuman primates. On the other hand, the use of cimetidine to prevent cocaine toxicity during pregnancy could have adverse consequences, such as potentiation of cocaine's primary pharmacological effects or increasing the severity of cocaine toxicities that occur via CYP3A5-independent mechanisms. Another approach could be the development of selective CYP3A5 inhibitors for use during pregnancy to prevent cocaine toxicity. It would also be difficult to predict cocaine abuse during pregnancy, although at-risk individuals could be selected for prophylactic treatment. If such an approach was found to be effective there is, however, a possibility of alleviating not only the toxicity of cocaine for brain development, but also the increased risk of later drug abuse that is consistently seen in the offspring of cocaine-abusing mothers.

Interestingly, the CYP3A5 gene shows wide variations in expression in different human populations. There are common mutations which cause a loss of expression in groups of European heritage, whereas ethnic groups with African heritage generally lack these mutations. For example, the original study by Kuehl et al. (2001) found that 60% of African-Americans, but only 33% of Caucasians, express normal CYP3A5. Other studies in specific or localized ethnic groups within Africa and Europe have found even larger differences, while other groups, such as Asians, tend to show frequencies between these extremes (Lamba et al., 2012; Lee et al., 2003; Kurose et al., 2012; Thompson et al., 2004). Recognizing the influence of CYP3A5 polymorphisms on cocaine-induced adverse developmental effects may explain individual differences in cocaine-induced developmental disorders and help identify cocaine-exposed infants at risk for adverse neuropsychological outcomes (Fig. 6.3). It may be possible to develop compounds with selective effect of CYP3A5 which can serve to ameliorate adverse developmental effects of cocaine in at-risk individuals.

These studies also provide a potential target for hiPSC-based studies of developmental mechanisms related to cocaine. The principal function of CPY3A5 in the brain is, of course, not metabolism of cocaine. hiPSC with genetic CYP3A5 variants could therefore be used to examine the function of CYP3A5 in brain development, and the potential role in effects of cocaine.

Notably, although we proposed that this genetic factor might account for cocaine's adverse effects on neocortical development, we do not maintain that this is the only mechanism whereby cocaine might alter brain development. In our in vitro microarray studies of cocaine on gene expression in various human CNS cell types, changes in cyclin A were only one of many changes that were observed (Lee et al., 2009). For example, there have been a number of studies linking WNTs to cocaine-induced abnormalities in brain cytoarchitecture (Cuesta et al., 2017a, 2017b; Dias et al., 2015; Novikova et al., 2005). We recently reported that hPSC lines with a duplication of 17q21.31−17q21.32, including WNT3 and WNT9B,

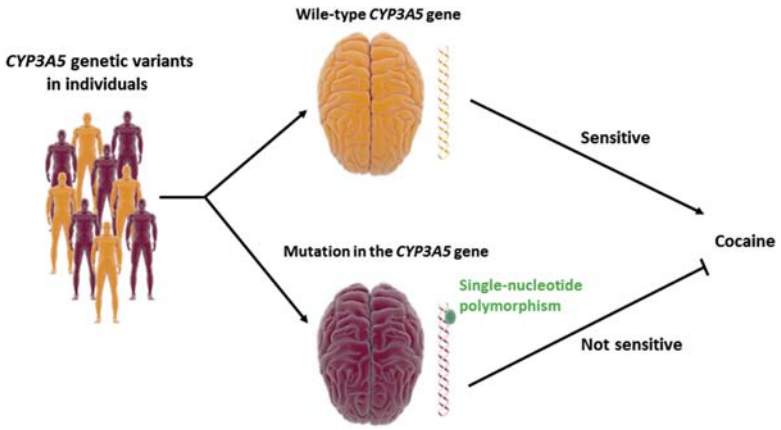

FIGURE 6.3 A hypothesis regarding the role of CYP3A5 polymorphisms in cocaine-induced neocortical developmental impairment.

Several single-nucleotide polymorphisms (SNPs) are known to result in variant CYP3A enzyme expression and activity. Using hPSC-derived 3D brain organoids, we previously found that CYP3A5 might play a dominant role in the N-oxidative metabolism of cocaine and associated adverse effects in the developing human neocortex. Therefore, individuals expressing wildtype CYP3A5 might be more sensitive to cocaine-induced brain development disorder as compared to individuals with the mutant allele of CYP3A5.

exhibit enhanced neuronal differentiation (Lee et al., 2015a, 2015b). Therefore, changes in WNT signaling could be another biological driver of pathogenesis of cocaine-induced brain developmental disorder.

In terms of changes in the developing brain, there appear to be several distinct effects of cocaine, which are probably at least to some degree mediated by different mechanisms. The first is an alteration in dendritic development and organization, which seems to be related to the presence of dopamine D1 receptors, although the transduction mechanism still is unclear (Jones et al., 1996, 2000). A second effect, reported by Crandall et al. (2004), is a decrease in the migration of GABAergic neurons from the medial ganglionic eminence to the cerebral cortex, and this has been associated with dopaminergic mechanisms as well (Crandall et al., 2007). Cocaine also increases parvalbumin-positive interneurons and causes changes in pyramidal neuron morphology (Thompson et al., 2010). Finally, there is a decrease in neural progenitor proliferation reported by our group which is entirely independent of dopaminergic mechanisms, depending instead on cocaine metabolism by CYP3A5 (Lee et al., 2015b).

Prenatal nicotine exposure has also been found to have adverse neurodevelopmental effects (Lewis et al., 2007; Noland et al., 2003). Recently, a pluripotent human stem cell model has been employed to examine the neurodevelopmental effects of nicotine (Wang et al., 2018). Nicotine caused a decrease in neural progenitor proliferation and premature neuronal differentiation similar to that caused by

cocaine (Lee et al., 2008, 2011). Whether the molecular mechanisms through which nicotine produces these effects are entirely separate from, or similar to, those of cocaine have not yet been reported.

Conclusions

For certain disorders, the genetic factors that underlie disease are best examined by the direct study of human patients. In schizophrenia, for example, there is no clear-cut animal model, and certainly no cellular model that fully recapitulates the disorder. In contrast, animals can readily be used to mimic substance abuse; however, the study of genetic predisposition is complicated by the highly polygenic nature of the disorder and by the myriad epigenetic and environmental factors that contribute to susceptibility. The use of hiPSC from addicted subjects is one way of modeling substance abuse to examine the contribution of specific genetic variants or combinations of variants in vulnerability to addiction. As shown in the recent work by Sheng and colleagues (2016a, 2016b), these models can also be used to test the effects of potential new drug treatments.

For substance-induced neurodevelopmental impairment in the developing fetus, pathophysiological mechanisms and genetic contributions to the disorder cannot be understood solely through direct study of human subjects. Furthermore, it is difficult or impossible to precisely determine the amount of exposure of any individual subject who is suspected of having been exposed to substances prenatally. Also, it is difficult to determine the baseline level of intellectual function for that individual. Thus, although prenatal exposure to some substances is known to have adverse effects on brain development and intellectual function, little is known about the mechanisms and genetic factors that are involved. The use of in vitro 3D brain organoid models based on hiPSC affords an uncomplicated study of genetic risk factors and cellular mechanisms that underlie substance-induced developmental abnormalities of the brain. Moreover, the use of the 3D organoid methodology can readily be adapted for use in a wide range of neurodevelopmental disorders with toxic or genetic etiologies (Amin and Pasca, 2018).

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CHAPTER

Induced pluripotent stem cells for modeling cardiac sodium channelopathies

7

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Chapter outline

Abstract

Cardiac sodium channelopathies are caused by mutations in the *SCN5A* gene, coding α subunit of voltage-gated cardiac sodium channels, and has been reported to be associated with a variety of phenotypes, including long QT syndrome type 3, Brugada syndrome, cardiac conduction disease, and dilated cardiomyopathy, as well as overlap between these phenotypes. The disease-causing mechanisms have been analyzed in cultured cells, using a heterologous expression system, which posed limitations in analysis as it is a non-myocardial model and is unable to completely emulate the myocardial electrical activity including action potential. However, the recently developed induced pluripotent stem cell technology offers the possibility to produce the relevant cell types and enables us to analyze the human-derived in vitro models of genetic diseases including cardiac ion channel diseases. In this chapter, we review the iPSC model of cardiac sodium channelopathies.

Keywords: Arrhythmia; Brugada syndrome; Cardiac sodium channel; Induced pluripotent stem cell; Long QT syndrome.

Introduction

The cardiac voltage-gated sodium channel, Nav1.5, is responsible for the generation and subsequent propagation of cardiac action potential (AP) through the heart

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(Balser, 1999; Benson et al., 2003). The α subunit of Nav1.5 is encoded by the *SCN5A* gene. *SCN5A* is located on chromosome 3p21 which contains 28 exons and is associated with two, non-pore forming, β subunits. Mutations in *SCN5A* reportedly cause a variety of cardiac arrhythmia disorders; "cardiac sodium channelopathies," including long QT syndrome (LQT) type 3^3 (Bennett et al., 1995), Brugada syndrome (BrS) (Brugada and Brugada, 1992), atrial fibrillation (Darbar, 2008; Ellinor et al., 2008; Makiyama et al., 2008), sinus node dysfunction (SND) (Schott et al., 1999), cardiac conduction disease (CCD) (Benson et al., 2003), and, in rare cases, dilated cardiomyopathy (DCM) (Bezzina et al., 2003; Frigo et al., 2007). In addition, single *SCN5A* mutations often present with multiple clinical manifestations as a consequence of the various biophysical defects associated with the mutations, known as overlap syndromes (Remme et al., 2006).

Human-induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007) have a pluripotency almost equal to that of embryonic stem cells. There is a great deal of ongoing research in regenerative and transplantation therapy, drug discovery, and elucidation of the pathogenesis of diseases using iPSC techniques.

iereditary arrhythmia disease studies have traditionally involved mutant channel current recordings using the patch-clamp method in a heterologous expression system in cultured cells. However, the interaction with myocardial-specific proteins and APs could not be measured because there is a discrepancy between noncardiomyocyte (CM) model and real human CMs. iPSC technology enables us to analyze the disease-causing mechanisms of inherited arrhythmia disorder using self-beating CMs with the same genetic background as the patient. A number of studies of disease-specific iPSC-based disease modeling have been reported (LQT1 (Moretti et al., 2010), LQT2 (Itzhaki et al., 2011), Timothy syndrome (Yazawa et al., 2011), catecholaminergic polymorphic ventricular tachycardia (Itzhaki et al., 2012, etc.). In this chapter, the current state of research on cardiac sodium channelopathies using iPSCs is described.

Induced pluripotent stem cell model of sodium channelopathies (Table 7.1)

LQT type 3

Congenital LQT is an inherited arrhythmogenic disease associated with lethal arrhythmic events and sudden cardiac death. Patients with LQT are currently classified into over 15 genetic subtypes, LQT1-3 accounts for approximately 90% of the genotyped patients, specifically: 40−55%, 30−45%, and 5−10% are LQT1, LQT2, and LQT3, respectively (Schwartz et al., 2012; Mizusawa et al., 2014). LQT1 and LQT2 are caused by loss-of-function mutations in *KCNQ1* and *KCNH2*, which encode cardiac slowly (I_{Ks}) and rapidly (I_{Kr}) activating delayed rectifier potassium

Table 7.1 Induced pluripotent stem cell model of cardiac sodium channelopathies.

Phenotype	Gene	Mutation (amino acid)	Function (compared to control cells)	Drug testing	Comments	Refs.
LQT3	*SCN5A*	1795insD, mouse 1798insD	Increased I_{NaL} reduced I_{Na}, Prolonged APD, reduced V_{max}	GS-458967 (GS967, I_{NaL} inhibitor) (Portero et al., 2017)	Overlapping phenotypes	(Davis et al., 2012), (Portero et al., 2017)
	SCN5A	F1473C	Increased I_{NaL}, right-shifted steady-state inactivation, Faster recovery from inactivation, Prolonged APD	Mexiletine		Terrenoire et al. (2013)
	SCN5A	V1763M	Increased I_{NaL}, right-shifted steady-state inactivation, Faster recovery from inactivation, prolonged APD	Mexiletine		Ma et al. (2013)
	SCN5A	V240M, R535Q	I_{Na}: Longer time to 90% inactivation tendency for prolonged APD in R535Q,			Fatima et al. (2013)
	SCN5A	N406K	Prolonged APD, Ca^{2+} transient, FPD (Yoshinaga et al., 2019)	Tetrodotoxin (Spencer et al., 2014), mexiletine (Yoshinaga et al., 2019)	E_m was hyperpolarized by LiCl via NCX blockade, Cyclopiazonic acid prolonged plateau phase of cytosolic Ca^{2+} via SERCA blockade	(Spencer et al., 2014), (Yoshinaga et al., 2019)
	SCN5A	R1644H	Faster recovery from inactivation, Prolonged APD and FPD, A high incidence of EADs	Mexiletine, ranolazine, phenytoin		Malan et al. (2016)
	SCN5A	N1774D	Prolonged APD, hyperpolarized shift of steady-state activation, increased late sodium current	Propranolol	I_{NaL} inhibition by propranolol was unrelated to β-adrenergic receptor signaling pathway.	Hirose et al. (2020)

Continued

Table 7.1 Induced pluripotent stem cell model of cardiac sodium channelopathies.—*cont'd*

Phenotype	Gene	Mutation (amino acid)	Function (compared to control cells)	Drug testing	Comments	Refs.
BrS	*SCN5A*	R620H/ R811H, K1397fs	Reduced I_{Na}, reduced V_{max}, increased triggered activity, abnormal Ca^{2+} transients, Peak-to-peak interval variability.			Liang et al. (2016)
	SCN5A	A226V/ R1629X, T1620M	Reduced I_{Na}, Reduced V_{max} and APA, An increased phase-1 repolarization pro-arrhythmic AP morphology		Dynamic clamp was employed to electronically express I_{K1}	Ma et al. (2018)
	SCN5A	R367H	Reduced I_{Na}, Right-shifted activation and left-shifted inactivation, Faster recovery from inactivation		I_{Na} kinetics changes were not observed in tsA201 cells.	Selga et al. (2018)
	SCN1B	L210P/ P213T	Reduced I_{Na}, APA and V_{max}, Right-shifted activation and left-shifted inactivation, Slow recovery from inactivation		Ajmaline showed stronger I_{Na} suppression effect, Carbacol increased beating rates and arrhythmia-like events	El-Battrawy et al. (2019)
CCD	*SCN5A*	D1275N	Reduced I_{Na}, beating frequency, APA and V_{max}, Right-shifted activation Reduced the ratio of membrane/ cytosolic $Na_V1.5$ Levels	MG132, a proteasome inhibitor	Abnormal I_{Na} properties were not obvious in a heterologous expression system.	Hayano et al. (2017)
	SCN5A	I230T (homo, hetero; located in exon 6)	Reduced I_{Na}, V_{max}, and right-shifted activation in long culture		Long culture increased the fraction of adult *SCN5A* isoform (exon 6/6a), dynamic clamp was employed	Veerman et al. (2017)
DCM	*SCN5A*	R219H	Gating pore current at hyperpolarized potentials Prolonged APD and depolarized RMP More acidic in intracellular pH Higher diastolic Ca^{2+} levels Abnormal sarcomere organization Impaired contractility by AFM			Moreau et al. (2018)

AFM, *atomic force microscopy;* APD, *action potential duration;* BrS, *Brugada syndrome;* CCD, *cardiac conduction disease;* DCM, *dilated cardiomyopathy; EAD, early after depolarization;* Em, *membrane potential;* FPD, *field potential duration;* I_{Na}, *sodium current;* I_{NaL}, *late sodium current;* LQT, *long QT syndrome;* RMP, *resting membrane potential;* V_{max}, *maximum upstroke velocity.*

channels, respectively (Moss et al., 2007; Shimizu et al., 2009). On the other hand, LQT3 is caused by gain-of-function mutations in *SCN5A*, which encodes cardiac voltage-gated sodium channels (Bennett et al., 1995).

In 2012, Davis et al. firstly reported an SCN5A-related human iPSC (hiPSC)-model derived from a patient with SCN5A-1795insD (Davis et al., 2012). The SCN5A-1795insD mutation was originally identified in a Dutch family with overlapping features of LQT3, BrS, and CCD (Bezzina et al., 1999; van den Berg et al., 2001). Transgenic mice carrying the mouse equivalent (1798insD) of the human SCN5A-1795insD mutation, displayed SND accompanied with conduction defect and QTc prolongation (Remme et al., 2006, 2009). Davis et al. (Davis et al., 2012) generated iPSCs from Scn5a-heterozygous mice and the differentiated CMs demonstrated a significant decrease in sodium current (I_{Na}) density, a larger late sodium current (I_{NaL}), a prolonged AP duration (APD), and a decreased AP upstroke velocity (V_{max}) compared to those in wild-type (WT) mice which recapitulated findings from primary CMs isolated directly from adult Scn5a-het mice. Finally, hiPSCs were generated from a patient with the equivalent SCN5A-1795insD mutation, the patient-derived hiPSC-CMs exhibited similar electrophysiological changes to the mouse iPSC-CMs.

Thus far, several LQT3-related hiPSCs have been reported to recapitulate the electrophysiological abnormal features of the patients (V240M, R533Q (Fatima et al., 2013), F1473C (Terrenoire et al., 2013), V1763M (Ma et al., 2013), N406K (Spencer et al., 2014), R1644H (Malan et al., 2016)). The hiPSC model is a useful platform not only for studying the pathophysiological mechanisms, but also for evaluating the efficacy of candidate drugs and developing novel diagnostic and therapeutic strategies for LQT3.

Recently, we investigated the cellular mechanism by which β-blockers affected I_{NaL} currents in human LQT3 CMs (Hirose et al., 2020). We established a hiPSCs from a young male carrying the SCN5A-N1774D mutaion and whose repetitive torsades de pointes were effectively treated with propranolol. In N1774D-hiPSC-derived CMs, prolonged APDs and increased I_{NaL} at baseline were attenuated after propranolol administration (Fig. 7.1). In addition, using guanosine diphosphate βs (a G protein inhibitor), we determined that propranolol blocked sodium channels by means of a unique mechanism unrelated to β-adrenergic signaling pathway, by preferentially inhibiting late rather than peak I_{Na}. Terrenoire et al. established LQT3-related hiPSCs carrying SCN5A-F1473C and which exhibited increased I_{NaL} resulting in prolonged depolarization (Terrenoire et al., 2013). Using the hiPSC model, they demonstrated that a sodium channel blocker, mexiletine, reduced I_{NaL}. Mexiletine was also effective in reducing I_{NaL} in SCN5A-V1763M hiPSC-CMs (Ma et al., 2013). Similarly, mexiletine, phenytoin, and ranolazine attenuated prolonged repolarization and the high incidence of early after depolarizations in SCN5A-R1644H hiPSC-CMs (Malan et al., 2016). In addition, administration of a specific I_{NaL} inhibitor, GS-458967 (GS967), did not affect the amplitude of peak I_{Na}, but attenuated I_{NaL} and prolonged APDs in SCN5A-1795insD hiPSC-CMs (Portero et al., 2017). As for its application as a diagnostic

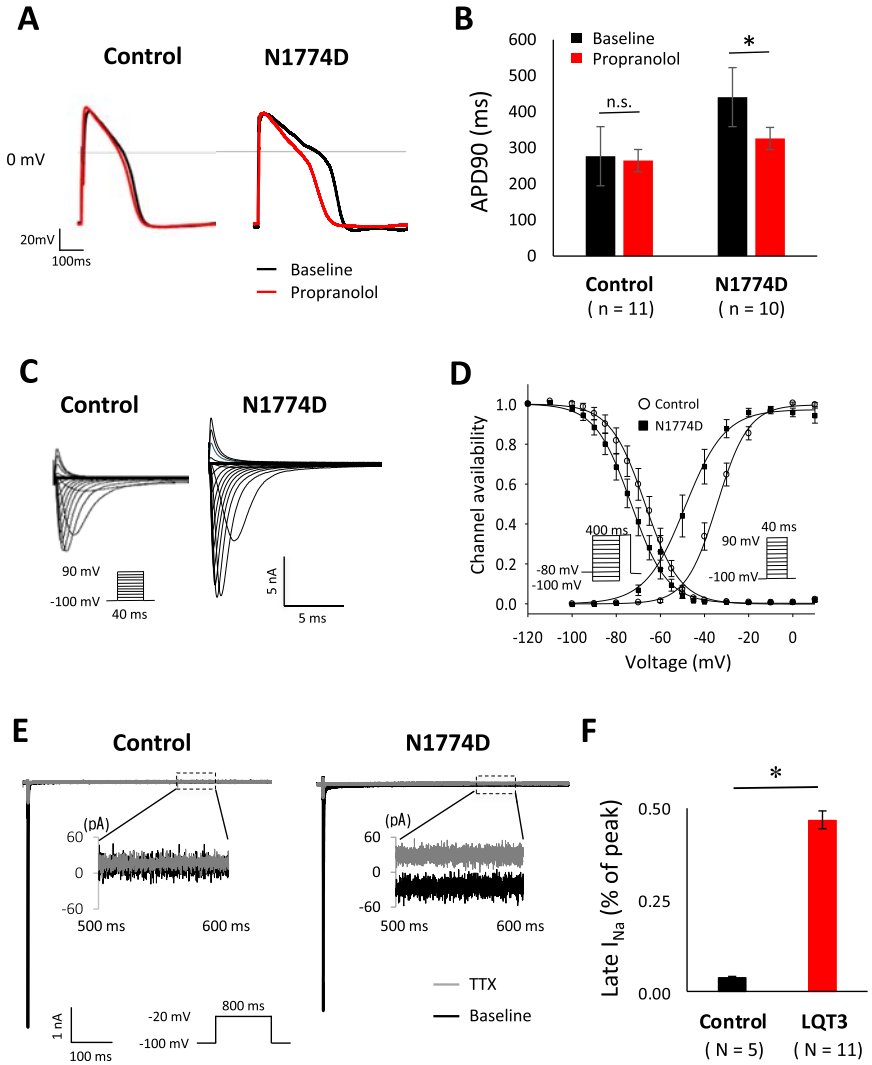

FIGURE 7.1 Effect of propranolol in control and N1774D-hiPSC-CMs (Modified from Hirose et al., 2020)

(A) Representative traces of paced ventricular-type action potential (AP) at 1 Hz pacing at baseline (black line) and after the administration 5 μM propranolol (red line) in control (201B7 and 253G1, left) and N1774D-hiPSC-CMs (right). (B) Summarized data in effects of propranolol on AP duration of control and N1774D-hiPSC-CMs. The data pooled from different lines or clones among the control and N1774D group were analyzed. $*p < 0.001$, vs. control. APD_{90} was measured at 90% repolarization (APD_{90}). APD90 values in N1774D-hiPSC-CMs were significantly prolonged compared with those in control. Propranolol significantly shortened the values of APD_{90} in N1774D-hiPSC-CMs. $*p < 0.001$, vs. baseline. (C) Representative traces of sodium currents in control and N1774D-hiPSC-CMs. The pulse protocol is shown in the inset.

tool to recognize pathogenic variants and distinguish multiple subtypes of LQT, we proposed a phenotypic cell-based diagnostic assay using hiPSC-CMs by specific ion-channel blockade (e.g., mexiletine for LQT3) (Yoshinaga et al., 2019).

Brugada syndrome

Brugada syndrome is an inherited disorder, characterized by sudden death from ventricular tachyarrhythmias (especially during sleep), ST-segment elevation in the right precordial leads, and conduction slowing in the absence of structural heart diseases (Brugada and Brugada, 1992, 1997). In approximately 15% of Brugada patients, mutations of *SCN5A* have been identified, functional analyses of some of these mutations revealed a loss-of-function type modulation (Chen et al., 1998; Priori et al., 2000; Antzelevitch, 2001; Makiyama et al., 2005).

In 2016, Liang et al. firstly reported a BrS hiPSC model carrying R620H/R811H (a compound heterozygous mutation) and K1397fs (Liang et al., 2016). BrS hiPSC-CMs showed reductions in inward I_{Na} density and reduced V_{max} of AP compared with healthy control hiPSC-CMs. Furthermore, BrS hiPSC-CMs demonstrated an increased burden of triggered activity, abnormal Ca^{2+} transients, and peak-to-peak interval variability. Thus, the patient-specific hiPSC-CMs were able to recapitulate single-cell phenotype features of BrS.

The second study reporting a BrS hiPSC model was performed by Ma et al. (Ma et al., 2018). BrS hiPSCs were generated from patients with A226V/R1629X (a compound heterozygous mutation) and T1620M. Dynamic was employed to "electronically express" I_{K1} to restore normal resting membrane potentials and allow normal recovery of the inactivating currents: I_{Na}, I_{Ca}, and I_{to}. BrS hiPSC-CMs exhibited a loss of peak sodium current density and a loss-of-INa basal AP morphology manifested by a reduced maximum upstroke velocity and AP amplitude, as well as an increased phase-1 repolarization pro-arrhythmic AP morphology. Moreover, Ito densities of BrS and control hiPSC-CMs were similar. This study indicates that a repolarization deficit could be a mechanism underlying BrS. Selga et al. (Selga et al., 2018) studied the biophysical properties of I_{Na} in BrS

(D) Voltage dependence of steady-state inactivation and activation in control and N1774D-hiPSC-CMs. Curves were fit using the Boltzmann equation. The activation curve negatively shifted by 13 mV. (E) Representative traces of sodium currents in the absence (black line) and presence (gray line) of 20 μM tetrodotoxin (TTX). The used protocol is shown in the lower panel. Inset shows late sodium current between 500 and 600 ms. Tetrodotoxin-sensitive current was calculated by subtraction. (F) Mean late sodium current of control and N1774D channels. Late sodium current is presented as the percentage of late sodium current to peak sodium current. The data pooled from different lines or clones among the control and N1774D group were analyzed. The late sodium current was significantly increased. $^{*}p < 0.001$, vs. control.

SCN5A-R367H hiPSCs and demonstrated a reduction in I_{Na} density, a shift in both activation and inactivation voltage-dependence curves, and faster recovery from inactivation. Interestingly, the changes in the kinetics of I_{Na} were not observed in a conventional heterologous expression system using tsA201 cells. Their work highlighted the need for assessment of the pathophysiological mechanisms of sodium channel mutations in a cardiac- and patient-specific model.

In rare cases, variants in *SCN1B*, encoding the β subunit of sodium channels, have been reported to be implicated in BrS (Watanabe et al., 2008). El-Battrawy et al. (El-Battrawy et al., 2019) investigated the cellular phenotype of BrS with *SCN1B* variants using hiPSC-CMs. They generated hiPSCs from a patient with recurrent syncope harboring two heterozygous variants, L210P and P213T. BrS SCN1B hiPSCs showed significantly reduced peak I_{Na} and I_{NaL} level, a positive shift of the activation curve, a negative shift of the inactivation curve, and decelerated recovery from inactivation. In AP recordings, AP amplitude and V_{max} were reduced, and ajmaline (a sodium channel blocker) showed a stronger suppressive effect on I_{Na} in BrS cells as compared to cells from healthy donors. Interestingly, carbachol, a parasympathetic stimulator, increased the beating frequency and arrhythmia-like events in BrS hiPSC-CMs. Thus, hiPSCs with *SCN1B* variants successfully recapitulated some key features of BrS.

Other phenotypes of cardiac sodium channelopathies (e.g., CCD, DCM)

The *SCN5A* missense mutation, D1275N, has been associated with several unusual phenotypes involved in reduced sodium channel function, including DCM, SND, CCD, and atrial and ventricular tachyarrhythmias (McNair et al., 2004; Olson et al., 2005; Laitinen-Forsblom et al., 2006). The reported electrophysiological properties of SCN5A-D1275N channels vary with experimental system; studies using heterologous expression systems showed no major differences between the mutant and WT channels, whereas peak I_{Na} densities were lower in SCN5A-D1275N knock-in mice than in WT ones (Watanabe et al., 2011). To investigate the biophysiological properties of D1275N channels in human in vitro model, we generated hiPSCs from a patient with familial CCD and demonstrated that D1275N hiPSC-CMs exhibit reduced $Na_V1.5$ protein expression and reduced the maximum sodium conductance, which was consistent with the SCN5A phenotypes associated with reduced sodium channel function observed in the patient (Hayano et al., 2017). Furthermore, treatment with a proteasome inhibitor, MG132, rescued the membrane $Na_V1.5$ protein levels, suggesting that ubiquitin-dependent proteolysis might be the major underlying mechanism resulting in $Na_V1.5$ loss-of-function in D1275N channels.

In fetal and infant heart, alternatively spliced $Na_V1.5$ which contains exon 6a is dominantly expressed instead of the adult Nav1.5 isoform with exon 6 (Murphy et al. 2012). Veerman et al. (Veerman et al., 2017) generated hiPSCs from one heterozygous and one homozygous SCN5A-I230T (located in exon 6) individual, both from a

family with recessive CCD. In patient-derived hiPSC-CMs after short-term culture (20 days), the alternatively spliced exon 6a was dominant instead of the exon 6. Heterozygous I230T hiPSC-CMs displayed no reduction in sodium current. Following prolonged culture for 66 days in I230T hiPSC-CMs, the fraction of the adult *SCN5A* isoform increased while I_{Na} was decreased, especially the changes were marked in homozygous cells. The results suggest that the electrophysiological immaturity associated with low-level expression of the adult sodium channel isoform should be taken into account in modeling *SCN5A* variants in exon 6.

DCM is a structural heart disease that causes dilatation of cardiac chambers and impairs cardiac contractility. *SCN5A* mutations have been identified in patients with arrhythmic disorders associated with DCM (Hershberger and Siegfried, 2011). Moreau et al. (Moreau et al., 2018) studied the hiPSC-CMs generated from a patient with complex cardiac arrhythmias and DCM carrying SCN5A-R219H located in the voltage sensor domain. The biophysical properties of I_{Na} recorded from R219H CMs were not altered by the presence of the Nav1.5 R219H mutation. Interestingly, R219H hiPSC-CMs exhibited a gating pore current at hyperpolarized potentials which was absent in WT hiPSC-CMs, this was consistent with the previously reported results regarding heterologous expression systems (Gosselin-Badaroudine et al., 2012). In AP recordings, APD was prolonged, resting membrane potential was depolarized in R219H hiPSC-CMs, and in intracellular pH recordings, R219H cells were more acidic than WT cells. In addition, R219H cells showed poorly organized sarcomere striation and impaired contractility by atomic force microscopy. In this study, they described electrical, structural, and contractile disturbances which could explain the cardiac arrhythmias associated with DCM.

Summary and future perspectives

Cardiac sodium channelopathy presents with a variety of phenotypes ranging from arrhythmias to cardiomyopathy, with overlapping syndromes. Electrophysiological findings have also been reported for a variety of mechanisms, including gain, loss, and mixed function modulation by electrophysiological changes in I_{Na}, I_{NaL}, and channel availability, trafficking defect, ubiquitination abnormality, etc. Indeed, the detailed mechanisms of disease causative mechanisms, including why they present with such a variety of clinical phenotypes, are not well understood, future analysis in a more patient-like model of hiPSC-CMs is highly necessary. The iPSC model has the advantage of being an in vitro human-derived CMs, but attention must be paid to the limitations; purity, immaturity, interclonal variation, as well as mixed types of differentiated CMs. These challenges have been gradually addressed with improved methods of cardiac differentiation and maturation (Parikh et al., 2017; Ronaldson-Bouchard et al., 2018), as well as methods of differentiation into specific types of CMs such as atrial cells and sino-atrial cells (Devalla et al., 2015; Lee et al., 2017; Protze et al., 2017). As more mature and specific types of CM differentiation

methods are characterized, more accurate recapitulations of cardiac sodium channelopathies in hiPSC-CMs will be possible, accelerating their application as models in pathophysiological analysis and drug discovery.

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CHAPTER

iPSCs for modeling Danon disease

8

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Chapter outline

Abstract

Danon disease is a dominant X-linked disorder caused by a mutation of the *LAMP2* gene that is located on the X-chromosome and encodes the lysosome-associated membrane protein-2 (LAMP2). LAMP2 plays important roles in mediating autophagy. Deficiency in LAMP2 leads to the accumulation of autophagic waste in affected cells. Clinically, affected male patients have early onset of symptoms during their teenage years and present with proximal myopathy, hypertrophic cardiomyopathy, retinopathy, and varying degrees of mental retardation. Almost all patients develop progressive cardiomyopathy and fatal ventricular arrhythmia and are unlikely to survive beyond 30 years of age without cardiac transplantation. Female patients with Danon disease usually have later onset of the disease due to random inactivation of the X-chromosome in somatic cells and thus only a proportion of the somatic cells, including cardiomyocytes, will manifest the phenotypes of Danon disease. Nonetheless the majority of affected female patients will develop progressive cardiomyopathy, fatal arrhythmia, and death. Unfortunately, due to the lack of a human cardiomyocyte-based model of Danon disease, there is no clear understanding of its pathophysiology and thus no effective therapy.

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Adult somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by transient overexpression of four specific factors. These iPSC-lines can be further differentiated into various cell types, including cardiomyocytes, and so provide a new and important source of human cardiomyocytes for disease modeling and drug development.

In this chapter, we will summarize the latest development in the application of iPSC-based technologies for modeling Danon disease. It should be noted that like other X-linked diseases, the somatic cells of a female heterozygous carrier contain a population of cells with a mosaic pattern of X-chromosome inactivation. Making use of this property, we will be able to produce an isogenic iPSC-line for better modeling of disease phenotype. The technical aspects of this method will also be described in this chapter.

Keywords: Autophagy dysfunction; Calcium handling dysfunction; Danon disease; Dilated and hypertrophic cardiomyopathies; Glycogen accumulation; Intracellular vacuole; Isogenic iPSCs; LAMP2; LAMP2protein deficiency; LC3function; Lysosomal membrane instability; Random X-chromosome inactivation; Reactivation of the silenced X-chromosome; Transgenic mouse model; X-linked inheritance.

Introduction

Danon disease is a dominant X-linked disorder caused by a primary deficiency of the lysosome-associated membrane protein-2 (LAMP2) due to loss-of-function *LAMP2* mutations(Danon et al., 1981; Nishino et al., 2000). LAMP2 mediates the transport of materials across the lysosomal membrane and autophagy (Saftig et al., 2008; Ruivo et al., 2009). Although the exact pathophysiological mechanisms of Danon disease may vary with different *LAMP2* mutations, studies from our group and others have shown that autophagic failure and associated lysosomal instability are two important pathways that contribute to the cell injury or death associated with LAMP2 deficiency (Nishino et al., 2000; Ng et al., 2016; Law et al., 2016; Tanaka et al., 2000).

Clinically, Danon disease is characterized by proximal muscle weakness, severe cardiomyopathy, and varying degrees of mental retardation (Danon et al., 1981). Although Danon disease appears to be a rare condition, it is widely reported around the world (Cheng and Fang, 2012), including Hong Kong and China (Cheng et al., 2012; Tse et al., 1996) and associated with high morbidity and early mortality (Boucek et al., 2011). In male patients, cardiac symptoms such as hypertrophic cardiomyopathy typically appear during the teenage years (Cheng and Fang, 2012; Boucek et al., 2011). They rapidly progress to end-stage cardiac failure and fatal ventricular arrhythmias. *Affected individuals seldom survive beyond 30 years of age without cardiac transplantation* (Maron et al., 2009).

Owing to the random X-chromosome inactivation, heterozygous female patients usually experience later disease onset (Boucek et al., 2011). Nonetheless progressive dilated cardiomyopathy and fatal arrhythmias will eventually develop at around age 50 years.

The application of iPSC-based modeling of Danon disease

Limitations of current animal models of Danon disease

Animal and cell-based models are important for revealing disease mechanisms and drug discovery. In 2000, Tanaka et al. described the first animal model for studying Danon disease (Tanaka et al., 2000). They created a transgenic mouse deficient in LAMP2 protein to mimic the genetic condition of Danon disease. Using this model, they provided evidence that the cardiac hypertrophy and the accumulation of autophagic vacuoles were directly associated with a lack of LAMP2 protein. Nonetheless although transgenic mice are widely used for disease modeling, some diseases may not develop in the same way in a mouse as they do in humans because of the large physiologic and genetic differences. As confirmed by Ueda et al., animal models of conditions such as paroxysmal nocturnal hemoglobinuria have not been able to completely replicate the disease phenotype of humans (Ueda et al., 2016). The difference between mice and humans also means a particular drug that works in mice may be ineffective in humans. In addition, many genetic disorders such as Danon disease may be caused by a multitude of mutations. Although some treatments may work for patients with specific mutations, they may not work for other patients with different mutations. To test a specific treatment for an individual patient, a unique model would need to be created for each patient. This would be highly labor and capital intensive, and impractical for most laboratories. Finally, specifically for many heart diseases, it is more difficult to study arrhythmia in mouse models. This is because the heart rate of a mouse, around 580 bpm as measured by Uechi et al. (1998), is much faster than the normal 60−100 bpm of humans. This makes it difficult to observe and isolate abnormalities on a mouse ECG.

A brief introduction of iPSCs

Induced pluripotent stem cells (iPSC) are a type of pluripotent stem cell that can be reprogrammed directly from a terminally differentiated somatic cell. The technology was first introduced in 2006 by Shinya Yamanaka's lab in Japan, and the first human iPSCs were produced by overexpressing the four factors: Oct4, Sox2, Klf4, and cMyc, and these factors were referred to as the Yamanaka factors. Pluripotent stem cells are used mostly for research in regenerative medicine since they possess the ability to propagate indefinitely and are able to differentiate into every cell type in the body (neurons, hepatocytes, cardiomyocytes, etc.).

Prior to the introduction of iPSCs, embryonic stem cells (ESCs), another type of pluripotent stem cell derived from embryos, were used for research in regenerative medicine. There were many limitations to their use as preimplantation stage embryos were required for their production. As a result, there was much controversy as well as ethical issues surrounding the production and use of ESCs. In addition, it was neither feasible nor practical to create patient-specific ESC lines, as they could only be differentiated from embryos.

The introduction of iPSCs eliminated these ethical issues since embryos were no longer required to create pluripotent stem cells for research. The creation of patient-specific cell lines was made possible, as they could be derived directly from the patient's own cells. This made it possible to test and create treatments specific to the patient.

The advantages of an iPSC-based model

Although iPSC-based disease models are unable to simulate multiorgan disease progression in the body, they have many advantages over mouse models. First, as mentioned above, it is easier to create a patient-specific model. By correcting the mutation in the patient's cell line and using the corrected cell line as a control for experiments, irregularities and inconsistencies observed in the results caused by individual genetic variations can be eliminated, producing more accurate results and conclusions. Second, it is much cheaper to create and maintain iPSC models than mouse models. To create a mouse model, materials for gene editing and many mice embryos are needed, whereas only Sendai virus and patient cells are needed to create an iPSC model. The cost of maintaining mice (food, housing, breeding, etc.) is also higher than that of the Sendai virus (USD ~3500 for seven to eight cell lines) and of maintaining the cell lines (cell culture medium, plasticware, etc.). Third, it is easier to test drugs in iPSC models. The drug to be tested can be dissolved in an appropriate solvent (water, PBS, DMSO) and added to the cells. In contrast, in a mouse model, a variety of methods may be required that may range from simply feeding the drug to the animal to injecting the drug capsulated in a vehicle into the target region of interest. Finally, as mentioned above, the many differences between mice and humans, both genetic and physiological, may cause drugs to react differently. The working concentration, effectiveness, and efficacy of drugs can be tested more accurately if a human patient cell-based iPSC model is used.

The procedures for producing iPSCs

Most iPSC lines in current use are produced by introducing the four Yamanaka factors into donor cells using Sendai virus as the vector. Common donor cell types include fibroblasts, renal epithelial cells, and peripheral blood mononuclear cells (PBMCs) such as lymphocytes and monocytes. Obtaining donor fibroblasts involves a painful skin biopsy, so most iPSCs produced in recent years have been generated from renal epithelial cells or PBMCs. The procedures for producing iPSCs are shown and illustrated in Fig. 8.1.

It should be noted that as soon as a sufficient number of cells is available, genotyping of every picked colony should be carried out to check if the desired mutation is present in the iPSCs of that colony. This is especially important for X-linked diseases such as Danon disease. If the patient is a male, the cell lines will usually contain the mutation since males have only one X-chromosome. Nonetheless if

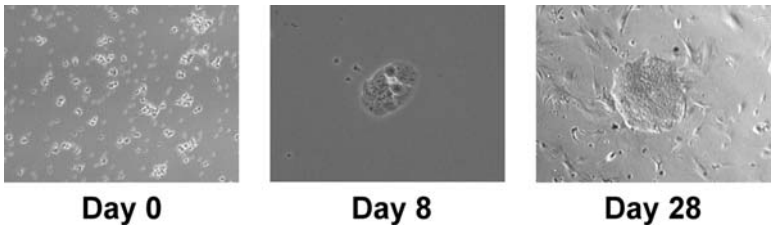

FIGURE 8.1

The generation of iPSC-colonies from PBMCs. At Day 0: PBMCs are isolated from the patient's blood sample and cultured on a 15 cm round dish. At Day 3: Sendai virus is added to the cells and seeded on matrigel and feeder cells (MEFs) in PBMC medium. At Day 8: Colonies start to form from transduced cells and medium used is changed to iPSC medium. At Day 28 (a): Large cell colonies are formed. These colonies will then be passaged, expanded and maintained.

the patient is a heterozygous female, the generation of iPSCs with specific active allele may be less predictable due to the mosaic pattern of X-chromosome inactivation. Based on our observation that the X-chromosome inactivation status remains unchanged during reprogramming, one should consider separating cells (skin fibroblasts or PBMCs) with different X-chromosome inactivation status before proceeding to reprogramming. This can be achieved by diluting the cells into single cells and then checking the expression of wild-type or mutant LAMP2 by immunostaining or mRNA-based methods (Fig. 8.2). Using this approach, we were able to create an isogenic pair of iPSCs that were genetically identical but differed only in the expression of the specific *LAMP2* allele and use it to model Danon disease (Ng et al., 2016).

Differentiation of established iPSC lines to cardiomyocytes

For our studies of Danon disease, we are interested in producing iPSC-derived cardiomyocytes for drug testing. There are many ways to do this, with most of them involving regulation of the WNT signaling pathways. One protocol similar to that developed by Lian et al. (2013) is briefly described below:

1. iPSCs are initially cultured on plates coated with matrigel in iPSC medium until they reach 80% confluence
2. iPSC medium is removed and RPMI/B27 medium without insulin and containing a Gsk3 inhibitor (e.g., CHIR99021) is added and cells cultured for 24 h
3. The Canonical Wnt pathway is then inhibited by adding Wnt signaling inhibitors such as IWP2 and IWP4
4. Functional contracting cardiomyocytes are then developed when cultured in RPMI/B27 medium

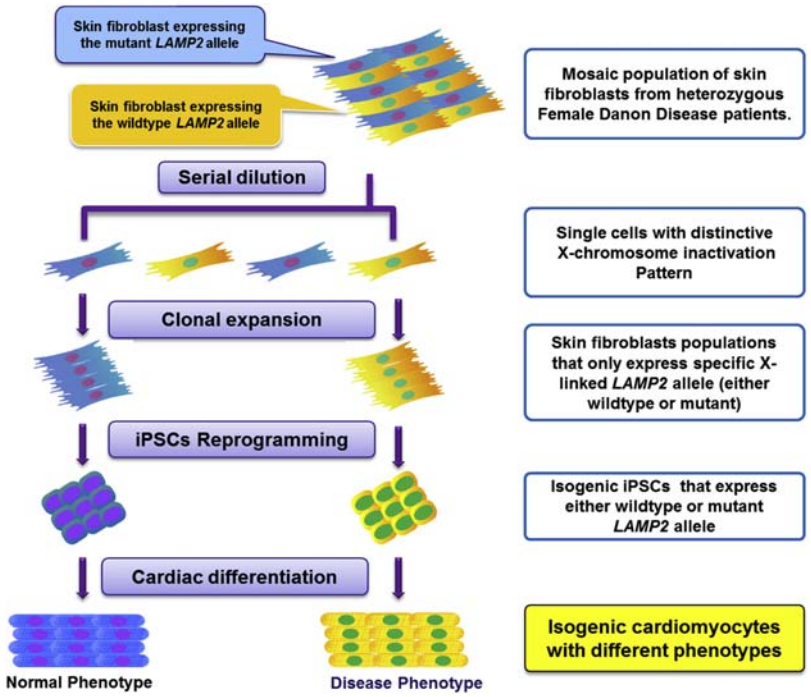

FIGURE 8.2

The generation of isogenic iPSCs with different X-chromosome inactivation status.

Although other similar protocols exist, commercial differentiation kits have been developed recently and made cardiac differentiation much easier with less time required to prepare the medium.

Immunostaining of cardiac markers such as cardiac Troponin-T (cTnT) could be used to verify that the cells have differentiated into cardiomyocytes (Fig. 8.3).

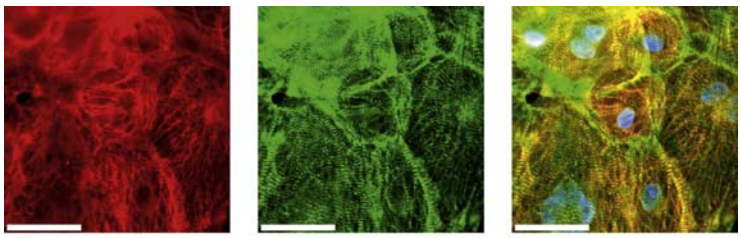

FIGURE 8.3

Immunostaining of the iPSCs-derived cardiomyocytes. Scale bar: 50 μm.

Application of iPSC-cardiomyocytes to evaluate autophagic dysfunction

Although Danon disease is a severe disorder, it is also very rare. Fewer than five studies have been published on the application of patient-specific iPSCs to model the cardiac dysfunction associated with *LAMP2* mutations (See Table 8.1).

In 2015, Hashem et al. provided the first report on the generation of iPSC-lines from Danon disease patients (Hashem et al., 2015). In their study, iPSC lines were generated from two patients with different *LAMP2* mutations (129−130 insAT and IVS-1 c.64 + 1 G > A). Although the two mutations were located at different regions (the former in exon 2 and the later in intron 1), both abolished the production of LAMP2 protein. Both iPSC lines were differentiated into cardiomyocytes and used for functional evaluation. Compared with the wild type, the cardiomyocytes with *LAMP2* mutations exhibited various pathological hallmarks of Danon disease, such as increased cell size, accumulation of vacuoles, and autophagy impairment. More interestingly, the authors also demonstrated the mitochondrial dysfunctions in the iPSC-derived cardiomyocytes with *LAMP2* mutations, which likely contributes to the increased oxidative stress and apoptosis. Nonetheless it should be noted that since physiological parameters may vary among individuals, some differences observed between the wild type and the LAMP2-deficient cardiomyocytes may have been due to the different genetic backgrounds of the samples. This is especially important when one looks at parameters such as internal calcium transient, where the observed difference was less than 10%.

Addressing this issue, our laboratory has used the isogenic iPSC-lines generated from a heterozygous Danon disease female as a unique platform to model the cardiomyopathy associated with Danon disease. In brief, due to the mosaic pattern of X-chromosome inactivation in somatic cells, skin fibroblasts obtained from our heterozygous female Danon disease patient consisted of two populations of cells that expressed the genes from two X-chromosomes. Although the two populations of cells are genetically identical, the one with the wild-type *LAMP2* allele on the active

Table 8.1 iPSCs-based Danon disease models.

Target phenotype	Mutation	Reference	Year
Cardiomyocytes apoptosis, Increased cell size, Impaired calcium transient	Exon 2129−130 insAT, IVS-1 c.64 + 1 G > A	PMID: 25826782	2015
Isogenic iPSCs	c.520C > T	PMID: 27678261	2016
Mitochondrial dysfunction	Exon 2129−130 insAT, IVS-1 c.64 + 1 G > a	PMID: 28526246	2017
Impaired autophagy, no cardiac functions provided	IVS6+1_4delGTGA	PMID: 29175505	2018

X-chromosome will produce LAMP2 protein while the one with mutant *LAMP2* allele will be deficient in LAMP2 protein. By reprogramming these cells individually, we were able to obtain a pair of isogenic iPSC lines that differed only in the production of LAMP2. Since the iPSCs lines are genetically identical, this allows us to evaluate the functional differences in the absence of any other factors that may arise with different genetic backgrounds.

Using this isogenic iPSC-based platform and some other LAMP2-deficient lines derived from the same family, we were able to show that the cardiomyocytes derived from the LAMP2-deficient iPSCs recapitulated the physiological hallmarks of Danon disease. For example, using electron microscopy, we were able to observe the abnormal accumulation of glycogen and autophagic vacuoles inside the LAMP2-deficient cardiomyocytes (Fig. 8.4).

Probably due to the accumulation of this autophagic waste, the LAMP2-deficient cardiomyocytes were significantly larger in size and more likely to have disorganized myofibrils (Fig. 8.5). Functionally, although we observed no significant difference in the action potential profile, the LAMP2-deficient cardiomyocytes exhibited significantly impaired calcium handling ability, notably the reuptake of calcium, in accordance with the observation reported by another research group (Hashem et al., 2015). Using special-line scan analysis, the delayed calcium reuptake is probably due to the accumulation of autophagic vacuole that physically hinders the local transfer of calcium (Ng et al., 2016) (Fig. 8.6).

One of the key questions about Danon disease is how does a deficiency of LAMP2 protein cause autophagic dysfunction. It is now clear that the three isoforms of LAMP2 protein may participate in different processes associated with autophagy, although it should be noted that in most patients, the *LAMP2* mutation occurs in exons one to eight, which leads to the disruption of all forms of LAMP2 isoform. As such, it is not surprising that most LAMP2 mutations cause severe autophagy

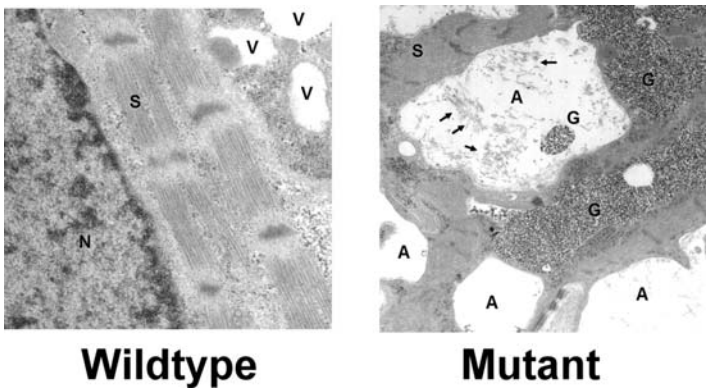

FIGURE 8.4

Electron microscopy analysis of the iPSCs-derived cardiomyocytes. *A*, Autophagosome; *G*, glycogen granules; *S*, sarcomere; *V*, vacuoles.

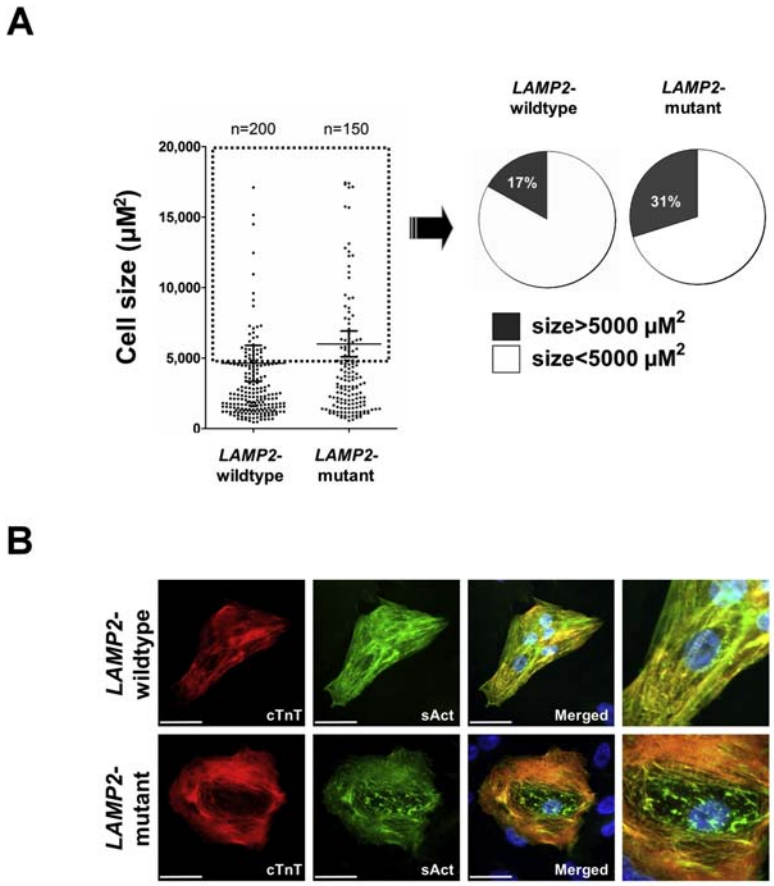

FIGURE 8.5

Morphological features of LAMP2-deficient iPSCs-derived cardiomyocytes. (A) Size distribution of the iPSC-derived cardiomyocytes expressing wildtype- or mutant- *LAMP2* alleles. (B) Immunostaining analysis. cTnt: cardiac troponin T, sAcT: sarcomeric α-actinin. Scale bar: 50 μM.

dysfunction. In the iPSC-based system, Western blot analysis is one of the most frequent methods used to access autophagic dysfunction and evaluate the LC3-II/LC3-I ratio. Theoretically, the formation of autophagosomes involves cleavage of LC3-I to LC3-II, where LC3-II is the membrane component of the autophagosome (Glick et al., 2010). As such, the accumulation of autophagosome is reflected by the decrease in LC3-I level and increase in level of LC3-II. Nonetheless since autophagy is a very dynamic process, the increase in LC3-II may also be due to activation of autophagy rather than failure in the clearance of autophagosome. Therefore, to evaluate autophagic flux, it may be necessary to examine the effects of autophagy

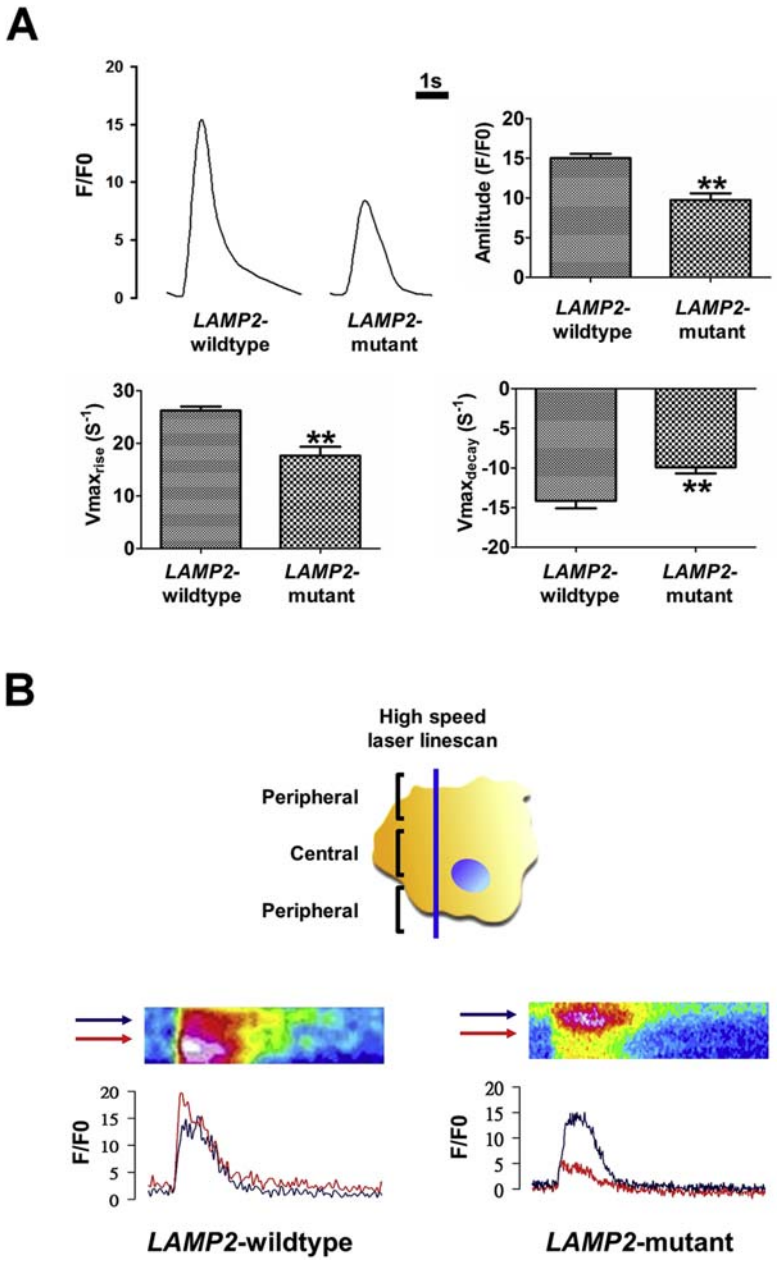

FIGURE 8.6 Calcium handling analysis.

(A) recordings of intracellular calcium transient. (B) Linescan analysis of the calcium transients across the cells.

activation and blockade of autophagosome clearance. To achieve this, cells can be treated with rapamycin and bafilomycin before determining LC3-II levels. Using this approach, we have demonstrated that even in the absence of bafilomycin, LC3-II accumulated in the LAMP2-deficient cardiomyocytes in the presence of rapamycin, indicating that the LAMP2-deficient cardiomyocytes failed to clear the autophagosome. This was supported by the observation that P62 (autophagosome cargo protein) accumulated in the LAMP2-deficient cells in the absence of bafilomycin (Ng et al., 2016).

Although the role of LAMP2 in mediating autophagy is well documented, its involvement in the degradation of autophagosomes remains unclear. We hope that in the near future, the iPSC-based model may facilitate exploration of the mechanism involved.

The application of an iPSC-based model for drug testing

In addition to disease modeling, cardiomyocytes derived from LAMP2-deficient iPSCs also provide a platform for drug testing and development of personalized medicine. For example, using the iPSC-based model of Danon disease, Hashem et al. demonstrated the involvement of increased oxidative stress in cardiomyocytes derived from Danon disease-specific iPSCs. Based on this observation, they tested the effects of antioxidants in reducing the cardiac dysfunction associated with Danon disease. They reported that NAC, a clinically approved agent, can be used to prevent the apoptosis of LAMP2-deficient cardiomyocytes (Hashem et al., 2015). In addition, using the isogenic iPSC pairs generated from a heterozygous female Danon disease patient, our group demonstrated that reactivation of the silenced wild-type *LAMP2* allele by global demethylating treatment may restore LAMP2 protein production and attenuate the cardiac dysfunction (Ng et al., 2016). It is possible that cardiomyocytes derived from this patient may also help in the evaluation of therapeutic and side effects of particular drugs prior to their use in patients. Nonetheless it should be noted that cardiomyocytes derived in vitro are usually less mature than adult cardiomyocytes so their use when testing antiarrhythmic agents should be cautious since they may not reflect the actual condition.

Future perspectives

Danon disease is a fatal disorder with no cure. As cardiac manifestations are the major cause of death, treatment strategies principally aim to attenuate the cardiac dysfunction. This mainly involves the use of antiarrhythmic drugs as well as the insertion of artificial pacemakers. As the disease progresses, cardiac transplantation is the only and final means of expanding the life of an affected individual. Currently, AAV-based gene therapy to restore LAMP2B expression (RP-A501) is undergoing

PHASE 1 clinical trials, ClinicalTrials.gov Identifier: NCT03882437. From the perspective of an iPSC-based model, and with the development of the CAS9/CRISPR-based gene editing technique, any mutations can be corrected in vitro. Such an approach may enable correction of the pathogenic LAMP2 mutations in iPSCs generated from the Danon disease patient. These gene-corrected iPSCs could then be differentiated into a desired cell type for autologous transplantation. These represent important tools in regenerative medicine for treating Danon disease as well as other genetic disorders.

Acknowledgment

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CHAPTER

9 Human-induced pluripotent stem cells for modeling of Niemann-Pick disease type C1

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Chapter outline

Abstract

Advances in the generation, differentiation and genetic manipulation of induced pluripotent stem cells (IPSCs) have expanded the approaches available to model disease in a dish. Neural cells derived from IPSC offer the unprecedented opportunity to analyze and target early pathologic events leading to neurodegeneration in disease-relevant cells. Niemann Pick type C1 (NPC1) is a rare but devastating neurodegenerative disease that shares many similarities with Alzheimer's disease (AD). Unlike AD, onset of NPC1 typically occurs in childhood and progression can be rapid, leading to significant disability early in life. Most IPSC-based models of NPC1 have been generated from reprograming patient-derived fibroblasts. However, ablation of NPC1 expression by knockdown of *NPC1* or gene-editing is a feasible strategy, as NPC1 is a loss of function disorder. Other than satisfying the urgent need to identify new therapeutic targets, IPSC-based models of NPC1 can help us understand the role of lysosomal failure in the disruption of neuronal homeostasis that leads to neurodegenration and death in these patients. Differentiation protocols to generate specific populations of neurons and glial cells from IPSCs are readily available. Furthermore, novel technologies to generate brain organoids from

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IPSCs add to the potential of using IPSC technology to model NPC1 and related neurodegenerative disorders.

Keywords: Autophagy; Disease modeling; Drug discovery; Human-induced pluripotent stem cell; Neurodegeneration; Niemann-Pick type C1 disease.

Introduction

Niemann-Pick type C1 disease (NPC1D) is a rare, progressive, and incurable pediatric neurodegenerative disease leading to significant morbidity and death. NPC1D is a lysosomal storage disease, as one of the hallmark abnormalities include accumulation of unesterified cholesterol in lysosomes (Sturley et al., 2004; Vanier, 2010); however, the mechanism underlying neurodegeneration in NPC1D is likely to reach beyond the accumulation of lipids in the lysosome. Multiple downstream effects of abnormal cholesterol distribution within the cell have been described including abnormalities of neurotransmission, autophagy, and mitochondrial failure, among others (Ordonez et al., 2012; Maetzel et al., 2014; Davis et al., 2021). NPC1D affects about 1:100,000 live births and is caused by mutations in the *NPC1* gene (Imrie et al., 2015; Sturley et al., 2004) encoding for the cholesterol transporter NPC1 which resides within the lysosomal membrane. There are currently over 300 described *NPC1* mutations associated with human disease (McKay Bounford and Gissen, 2014). These highly heterogeneous mutations cause total or partial loss of function of NPC1 with resulting entrapment of cholesterol in lysosomes and ultimately neuronal death (Sturley et al., 2004). However, the exact mechanism or mechanisms underlying neurodegeneration in NPC1D have not been fully elucidated and are a subject of active research.

The spectrum of clinical presentation of NPC1D resembles its genetic heterogeneity, as more than one organ system can be affected, and manifestations vary significantly among individuals (Walkley and Suzuki, 2004). Common clinical features include but are not limited to: ataxia, lack of coordination, dysphagia, dystonia, hepatosplenomegaly, developmental delay, and cognitive decline (Imrie et al., 2015; Vanier, 2010). To further illustrate the wide clinical spectrum of NPC1D, clinical onset of disease can range from the first few months of life to late childhood (Imrie et al., 2015; Vanier, 2010), and patients with mild mutations can survive into adulthood, albeit with significant disability.

There are currently no FDA-approved treatments for NPC1D. The most promising therapy, 2-hydroxypropyl-β-cyclodextrin (2HPBCD), is currently in Phase 2b/3 clinical trials (https://clinicaltrials.gov/ct2/show/NCT04958642). Earlier clinical trials of 2HPBCD showed beneficial results in managing NPC1D, however, the drug does not cross the blood−brain barrier making it necessary to use an intrathecal route of administration (Ory et al., 2017). Another potential therapy that has undergone clinical studies and is approved for treatment of NPC1D outside of the US is miglustat. The main role of miglustat is to stabilize disease and slow symptom progression, underscoring the need for early diagnosis and initiation of therapy (Wraith et al., 2010). Other potential therapies are in the pipeline, including the compelling possibility of gene therapy (Chandler et al., 2017). The complex

pathophysiology of neuronal failure in NPC1D suggests that patients will benefit from more than one therapeutic approach, and there is still room for the development of new therapies.

Traditional models of NPC1 and rationale for human stem cell-derived models

The NPC1 gene is highly conserved among species, creating opportunities to establish a variety of cell-based and animal models to study NPC1D (Fog & Kirkegaard, 2019). A large repository of NPC1D patient-derived fibroblasts is available to a highly collaborative community of NPC1 researchers (https://catalog.coriell.org/1/NIGMS), and these have been widely used to dissect the pathogenesis of cholesterol sequestration and for drug screening assays. Many mouse models have been established, including the standard $Npc1^{nih}$ mouse model described by Pentchev et al. in 1980, and the more recent $Npc1^{I1061T}$ mouse engineered to harbor the I1061T mutation of NPC1 that is responsible for most cases of human disease (Praggastis et al., 2015). Colonies of cats with spontaneous mutations of NPC1 have been established and are well suited for drug studies due to their larger size and well-defined natural history (Vite et al., 2008). NPC1 cats have been used to delineate the effects, dosing strategy, and toxicity of candidate therapeutic compounds. Both mouse and cat models of NPC1D have been central to the efforts for therapeutic development in NPC1D, with special mention to the success in advancing 2HPBCD to human trials.

Despite the remarkable progress made to date in characterizing the molecular and cellular phenotypes of NPC1D, the field still lacks sufficient understanding of the exact pathogenesis of neuronal failure in NPC1D. Perhaps more urgently, the number of clinically available therapies is fairly limited, stressing the need to identify therapeutic targets that are direct effectors of neuronal failure in response to abnormal cholesterol trafficking. For these reasons, there is an urgent need for further research in models that are relevant to disease pathogenesis and clinical outcome, such as human neurons. Human-induced pluripotent stem cell (hiPSC) technology allows for the unique ability to observe and learn from human cells in healthy and diseased states. Despite its neuronal selectivity, NPC1D can affect different organ systems and types of cells in the body (Imrie et al., 2007; Wraith et al., 2009); therefore, hiPSCs are a powerful disease-modeling platform as they can differentiate into a variety of cell types. Even though utilizing hiPSC-derived platforms to study NPC1D is relatively new, a variety of hiPSC-derived cell types have been described in the literature to study NPC1D. Different groups have demonstrated the ability to successfully culture NPC1D hiPSCs, neural progenitor cells (NPCs), neurons, hepatocytes, and glial cells (Efthymiou et al., 2015; Maetzel et al., 2014; Peter et al., 2017; Soga et al., 2015; Trilck et al., 2013; Yu et al., 2014, Lee et al., 2020). The variety of hiPSC-derived cellular platforms developed by research groups in the field is being used to investigate the pathogenesis of NPC1D and aid in the development of potential new therapies.

Human-induced pluripotent stem cell-derived models of NPC1

Patient-specific hiPSC-derived neural cells and glia

Because mutations of NPC1 disrupt primarily neuronal health and cause neurodegeneration, most patient-specific hiPSC models created to study the disease have focused on the generation of neural cells. Neural progenitor cells (NPCs) are neural precursor cells that can be generated from hiPSCs (Mertens et al., 2018). Their ability to undergo cell division and proliferate makes them a robust modeling platform; however, it is possible that certain phenotypes of disease are not evident in actively proliferating cells that have not completed neuronal differentiation. The highly polarized and postmitotic nature of neurons makes them especially vulnerable to phenotypes of disease that have been postulated in NPC1D, such as those involving vesicular trafficking and cellular disposal pathways such as autophagy. Using lineage-specific expression markers, multiple groups have demonstrated the ability to successfully generate NPC1D NPCs (Efthymiou et al., 2015; Soga et al., 2015; Trilck et al., 2013; Yu et al., 2014; Sung et al., 2017). Gene expression analysis by microarray did not find significant differences between NPC1D NPC and control lines (Efthymiou et al., 2015). Most of the described NPC1D NPC models recapitulate the hallmark phenotype of NPC1D; cholesterol accumulation (Soga et al., 2015; Trilck et al., 2013; Yu et al., 2014), which provides basic validation of these NPCs as viable models for the study of NPC1D. In addition to observing abnormal cholesterol phenotypes, autophagy has been investigated as a driver of disease in NPC1D (Soga et al., 2015). By probing the indicators of autophagic activity and progression LC3-II and p62 (Klionsky et al., 2016; Soga et al., 2015), it has been shown that autophagy is impaired in NPC1D NPCs. This autophagy dysfunction mimics the same abnormalities seen in other cell type models of NPC1D such as hiPSC-derived neurons and hepatocytes (Maetzel et al., 2014; Soga et al., 2015) that are discussed below. Saito et al. (2021) showed accumulation of cholesterol and glycolipids in both undifferentiated hiPSCs and derived NPCs, however abnormalities of lysosomal morphology and autophagic activity were only observed in differentiated NPC1 neurons. Lipid droplet accumulation and enlarged lysosomes were more prominent in neuronal cells rather than NSCs. Similarly, it was only in NPC1 neurons that phenotypes indicative of autophagy dysfunction were obvious, such as the accumulation of membranous cytoplasmic bodies and disruption of the p62/SQSTM1-KEAP1-NRF2 axis.

Human iPSC-derived neurons constitute the most common and relevant hiPSC-derived model to study NPC1D. As previously noted, mutations of NPC1 preferentially affect neurons and neurodegeneration is the main source of morbidity and mortality in NPC1D (Sturley et al., 2004). Multiple groups have demonstrated the ability to successfully generate NPC1D neuronal cell lines from hiPSC. Basic characterization of neuronal identity includes documentation of typical morphology and expression of neuron-specific markers. Some groups have also provided

functional characterization indicative of incipient electrophysiological activity of NPC1D neurons such as presence of voltage-dependent Na^+ and K^+ channels and spontaneous action potentials after prolonged differentiation (Trilck et al., 2013). NPC1D neurons have been reported to have decreased viability (Efthymiou et al., 2015; Lee et al., 2014; Maetzel et al., 2014) which is a cellular phenotype that aligns with the neurodegeneration observed in NPC1D patients (Sturley et al., 2004). Of note, Efthymiou et al. reported decreased viability in NPC1D neurons but not in glia derived from the same hiPSC lines, consistent with our own observations of decreased survival in NPC1D hiPSC neurons but not in parental fibroblast lines of similar genetic background (unpublished data). Similar to NPC1D NPC models, most neuronal models described in the literature demonstrate the hallmark characteristics of cholesterol accumulation and increased numbers of enlarged lysosomes (Lee et al., 2014; Maetzel et al., 2014; Trilck et al., 2017).

Replicating the established phenotypes of NPC1D in human neurons is necessary and can provide readouts for drug screening experiments in disease-relevant cells. An even more compelling application of a neuronal modeling platform is to identify the mechanism(s) that underlie the preferential neuronal failure caused by mutations of *NPC1*. A common abnormal phenotype that has recently gained attention in the field is autophagy dysfunction (Davis et al., 2021; Ordonez et al., 2012; Lee et al., 2014; Maetzel et al., 2014). Various groups have reported impaired autophagy flow in NPC1D neurons as measured by abnormal levels of LC3-II and p62, two proteins that are turned over by autophagy (Klionsky et al., 2016; Lee et al., 2014; Maetzel et al., 2014). Basal autophagy is restored in NPC1D neurons and hepatocytes after genetic correction of the *NPC1* mutation (Maetzel et al., 2014), providing further evidence that autophagy disruption is directly linked to loss of NPC1 function. Recently, an elegant study by Davis et al. used non-neuronal cells and NPC1 knockout (KO) hiPSC-derived neurons to characterize the role of the master autophagy regulator mTORC1 in NPC1 disease pathogenesis as an effector downstream of cholesterol sequestration (Davis et al., 2021). Lysosomes from NPC1 null non-neuronal cells display widespread proteolytic defects and increased lysosomal membrane fragility as evidenced by increased susceptibility to membrane damage. Data generated in this study suggest that loss of NPC1 function and the subsequent failure to export cholesterol out of the lysosome underlie dysregulated mTORC1 signaling and lysosomal defects in enzyme-mediated degradation and membrane integrity. Because mitochondrial turnover is specifically dependent on efficient autophagy progression involving the lysosome, mitochondrial function was probed in NPC1 knockout hiPSC-derived neurons. NPC1 neurons showed mitochondrial fragmentation and loss of mitochondrial membrane potential, suggesting that loss of NPC1 function impairs mitochondrial function by interfering with mitochondrial turnover by mitophagy. Supportive of these findings, we have observed accumulation of cholesterol, decreased neuronal viability, and autophagy dysfunction in NPC1D patient-specific neurons (unpublished data). Using NPC1D patient-derived hiPSC neurons, we have observed phenotypes consistent with autophagy dysregulation including upregulation of Beclin-1, impaired turnover of

autolysosomes and autophagosomes, and mitochondrial dysfunction. Our findings suggest NPC1D patient-specific neurons have dysregulation of autophagy that is likely to impact mitochondrial turnover and neuronal health. We have documented loss of mitochondrial membrane potential and increased oxidative stress in NPC1 neurons. Consistent with this, Jurs et al. (2020) found increased reactive oxygen species (ROS) in NPC1 patient-derived neurons coupled with decreased antioxidative activity mediated by catalase. Furthermore, other irregularities seen in NPC1D neuronal models include but are not limited to abnormal expression of genes involved in calcium and WNT signaling (Efthymiou et al., 2015; Rabenstein et al., 2017), as well as aberrant VEGF characteristics, dysfunctional sphingolipid processing (Lee et al., 2014), and abnormal ganglioside accumulation (Trilck et al., 2017). A brain organoid model of NPC1 was developed and characterized by Lee et al. (2020). NPC1 organoids were smaller and had reduced proliferative capacity. They exhibited accumulation of cholesterol, impaired autophagic flux and lysosomal dysfunction, showing that they can recapitulate the main phenotypes of NPC1 patients. Interestingly, NPC1 organoids also showed deficient neuronal differentiation, a phenotype that is not obvious in 2D NPC1 neuronal cultures. Pathological phenotypes were reversed by treatment with valproic acid and HPBCD, both of which are postulated as treatments for NPC1 patients. Brain organoids are interesting models that may better reflect the interaction between different cell types in a disease state, and improve the accuracy of drug screening studies.

To our knowledge, only one well-characterized NPC1D hiPSC-derived glial model has been documented in the literature (Peter et al., 2017). Similar to all other NPC1D models previously described, the glial model demonstrates the hallmark phenotype of cholesterol sequestration, thus validating its ability to reflect NPC1D pathology. This group reported increased number of proliferative glial cells and increased number of $GFAP^+$ and $vimentin^+$ cells, suggestive of gliosis in NPC1D hiPSC-derived glial cells, as well as irregular phosphorylation and bundling of astrocyte-related intermediate filament proteins, GFAP, and vimentin. Of note, pharmacologic activation of protein kinase C rescued gliosis and restored the pattern of cholesterol distribution in NPC1D glial cells. These data suggest that despite been preferentially affected, neurons may not be the only cell type in the brain affected by NPC1D.

Patient-specific hiPSC-derived hepatocytes

While NPC1D is a neurodegenerative disorder, it also affects other organ systems and is known to cause liver dysfunction (Imrie et al., 2007; Wraith et al., 2009). Therefore, studying the cellular and molecular mechanisms of NPC1D in hepatocytes is relevant, both to dissect mechanisms of cholesterol trafficking and to identify pathways that can be targeted to treat the fraction of NPC1D patients that develop debilitating liver disease. NPC1D hepatocytes with typical morphology and expression of cell-specific markers have been successfully generated from hiPSC (Maetzel et al., 2014; Soga et al., 2015). Maetzel et al. showed that

sequestration of cholesterol in the lysosomal compartment of NPC1D hepatocytes impairs cholesterol esterification as measured by decreased incorporation of radiolabeled oleate into cholesteryl esters. The cholesterol sensing machinery was also found to be impaired in these cells as shown by lack of SREBP2 cleavage suppression upon serum treatment. These defects were rescued by genetic correction or by treatment with HPBCD. Similar to what has been described in neurons, NPC1D hepatocytes have abnormal autophagy flow as shown by accumulation of LC3-II and p62 that was not explained by increased synthesis but rather to insufficient clearance. Maetzel et al. also observed decreased hepatocyte viability, suggesting that despite of the striking neurodegeneration that drives progression of disease in NPC1D patients, impaired cell viability may not be neuron-specific but rather a phenotype seen in other cell types that are more sensitive to NPC1 mutations such as hepatocytes. Analysis of NPC1 hiPSC-derived hepatocytes by Soga et al. also reported abnormalities of the autophagy pathway as shown by higher expression levels of LC3-II and p62 proteins and excessive p62 aggregation in NPC1D hepatocytes, suggestive of upregulation of autophagy and impaired autophagy flow.

Gene-edited hiPSC lines for the study of NPC1D

Gene-editing using TALENs or CRISPR technology has been used to generate isogenic hiPSC lines to study NPC1D. Gene-editing allows for induction or correction of disease-causing point mutations for modeling or therapeutic applications. Maetzel et al. (2014) used TALENs to repair the most common disease-causing mutations of *NPC1* in humans ($NPC1^{I1061T}$ mutation) in two heterozygous hiPSC lines with one affected allele. Corrected hiPSC lines did not lose pluripotency and were capable of differentiation into neurons and hepatocytes. The authors confirmed restoration of NPC1 protein levels, normal cholesterol distribution, and recovery of cholesterol sensing as shown by serum-induced suppression of SREBP2 cleavage in edited NPC1 hiPSC lines. Furthermore, basal autophagy was examined in NPC1 hiPSC-derived neurons and hepatocytes, and their isogenic controls. Genetic correction of the $NPC1^{I1061T}$ mutation was sufficient to restore autophagic flux in NPC1 neurons and hepatocytes, suggesting that impaired autophagy is directly caused by loss of NPC1 function. Our lab has taken an inverse approach by using CRISPR- Cas9 technology to generate isogenic lines with complete knockout of *NPC1*. This strategy allows us to study the effects of ablation of *NPC1* without the genetic bias that can be associated with patient-derived lines. We used CRISPR/Cas9 gene-editing to generate an *NPC1* knock-out (KO) hiPSC line by inserting a frame-shift mutation in Exon 4 that engineered a premature stop codon leading to complete ablation of *NPC1* (Davis et al., 2021). CRISPR-engineered *NPC1* KO neurons show established phenotypes of NPC1D such as sequestration of cholesterol in lysosomes and decreased neuronal viability, as well as novel phenotypes of NPC1D such as autophagy and mitochondrial dysfunction.

Drug discovery applications

In addition to expanding our understanding of the molecular and cellular abnormalities involved in NPC1D pathogenesis, hiPSC technology has contributed to efforts in developing new therapies for NPC1D. As a relatively new method to model NPC1D, hiPSC-derived models have been used to further investigate already identified potential therapies for NPC1D such as 2HPBCD and Miglustat (Soga et al., 2015; Yu et al., 2014). Additionally, new potential therapies and treatment strategies have been identified by targeting pathological phenotypes that have been identified using hiPSC models (Efthymiou et al., 2015; Lee et al., 2014; Maetzel et al., 2014; Peter et al., 2017). As an example, Maetzel et al. (2014) characterized abnormal autophagy phenotypes in their NPC1D hiPSC-derived neurons and hepatocytes. These observations drove them to investigate the ability of autophagy enhancers and regulators to rescue NPC1D models, alone or in conjunction with agents that mobilize lysosomal cholesterol such as 2HPBCD. Similarly, our group has engaged in hypothesis-driven investigation into potential NPC1D therapies with a focus on regulation of mitochondrial turnover by autophagy (Davis et al., 2021, Ordonez et al., 2012, and unpublished data). Using rescue of autophagy and mitochondrial dysfunction as primary outcomes, we have systematically screened hundreds of compounds and identified various candidates that rescue viability of NPC1D hiPSC-derived neurons by providing mitochondrial protection, including some that are FDA-approved for other applications and can be repurposed for the treatment of NPC1D (unpublished data). In addition, strategies that modulate mTORC1 activity while avoiding off-target effects resulting from complete mTORC1 inhibition are attractive therapeutic options that bypass the effects of cholesterol sequestration while preserving normal autophagy and other important metabolic queues arising from the lysosome. Other strategies that have been studied in NPC1D neurons include compounds that regulate the WNT pathway, calcium flux, and calcium signaling. Taken together, these studies highlight the potential of using hiPSC-derived cell lines, in conjunction with established animal models, as platforms for drug screening and validation.

Conclusions and future directions

Reprogramming and gene-editing technologies are powerful tools that have dramatically expanded the development of cell-based human models of disease. These strategies have been exploited to generate modeling platforms for multiple monogenic and multifactorial diseases. The case for hiPSC-based models for the study of NPC1D is especially compelling. The deleterious effects of *NPC1* mutations have strong neuronal selectivity, despite the fact that the NPC1 transporter is ubiquitously distributed in all cell types where it has a universal role in cholesterol homeostasis. Despite of the enormous contribution of traditional models to our knowledge of NPC1D, it is evident that undertaking mechanistic studies and drug testing in a disease-relevant system such as human neurons can increase the odds to successfully

identify much needed therapeutic targets. Challenges remain, such as imperfect differentiation protocols, the potential for off-target effects from genetic manipulation inherent to reprogramming, and limitations inherent to disease-modeling in a dish. Progress has been made in the generation of specific subpopulations of neurons that are relevant to the study of NPC1D such as cortical (Shi et al., 2012) and cerebellar neurons (Wang et al., 2015); however, variability of differentiation can be observed between individual hiPSC lines and may be affected by genetic background, reprogramming strategy, or technical issues. Variability of differentiation and lack of maturity is especially problematic in hepatocyte differentiation, another disease-relevant cell type for the study of NPC1D. Multiple studies have documented off-target genetic or epigenetic changes induced by reprogramming of somatic cells to hIPSCs, or propagated by selection of preexisting mutations in parental fibroblasts. It is therefore indispensable to perform rigorous quality control of patient-specific hiPSC lines and their products. Generation of isogenic hiPSC lines by gene-editing is a complementary strategy that can help avoid off-target effects by more precisely modifying a chosen locus with minimal impact on the remaining genome (Howden et al., 2018). Lastly, we strongly believe that a mutually complementary approach that includes hIPSC-derived, other cell-based, and animal models of NPC1D will offer the best chance for success in our quest to develop new treatments for this devastating disease.

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CHAPTER

10 iPSC-based modeling in psychiatric disorders: opportunities and challenges

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Chapter outline

Abstract

The high heritability of major psychiatric disorders suggests that disease risk is significantly encoded in the human genome. Patient-specific iPSCs capture a donor's genotype and enable to investigate genetic risk in disease-relevant cell types at varying stages of neural development in vitro. A body of iPSC studies from recent years has provided a wealth of information on molecular and cellular phenotypes that are thought to recapitulate alterations from patients' brains including deficits in neural proliferation, migration, differentiation, and maturation among others. Despite this remarkable progress, cardinal features of major psychiatric disorders

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comprising impairments in local and distant connectivity, complex cell—cell interactions beyond a purely neurocentric view, and above all, the uncharted territory of polygenicity, are still understudied. Here, we discuss recent progress to expand our knowledge in this direction, as well as potential caveats in the interpretation of current findings.

Keywords: (epi-) genetic editing ($n = 7$); Cerebral organoid; iPSC; Microcircuit; Neurodevelopment; Psychiatric disorders; Synapse; Transplantation.

List of abbreviations

2D, 3D	two dimensional, three dimensional
ASD	autism spectrum disorder
ATAC	assay for transposase-accessible chromatin
BD	bipolar disorder
CA3	cornu ammonis
cIN	cortical interneurons
CLCN3	chloride voltage-gated channel 3
CNTN4	contactin 4
CNV	copy number variation
COS	childhood onset of schizophrenia
CRISPR/CAS9	clustered regulatory interspaced short palindromic repeats/CRISPR associated protein 9
CRISPRa	CRISPR mediated gene activation
dCAS9	dead CAS9 (endonuclease deficient)
DG	dentate gyrus
E/I	excitation/inhibition balance
eQTL	expression quantitative trait locus
FURIN	paired basic amino acid cleaving enzyme
GABA	γ-aminobutyric
GPC	glia precursor cell
GWAS	genome-wide association study
iN	induced neuron
iPSC	induced pluripotent stem cell
MD	major depressive disorder
NPC	neural progenitor cells
OCR	open chromatin region
SCZ	schizophrenia
SNAP91	synaptosomal-associated protein 91-Kd
SNP	single nucleotide polymorphism
TF	transcription factor
TSNARE1	t-SNARE domain containing 1

Introduction

All of us are touched, directly or indirectly, by major psychiatric disorders. In 2015, ~300 m people were affected by major depression (MD), ~60 m by bipolar disorder (BD), and ~21 m by schizophrenia (SCZ). For comparison, less than ~35 m people were affected by cancer (WHO). First episodes of thought disorders like SCZ and mood disorders like MD and BD occur typically in adolescence to early adulthood. Thereafter, episodes tend to recur, disease-free intervals to shorten, and successive episodes to get worse. All of these factors contribute to insidious hospitalization over time. Although mainstay psychopharmacological drugs and adjuvant psychotherapy can ameliorate symptoms in most patients, remission remains often incomplete, and up to one-third of patients with SCZ do not respond at all. With no cure in sight, major psychiatric disorders place an enormous burden on family and society and are among the leading cause of disability worldwide (James et al., 2018; Mental Health). This situation is expected to worsen as a result from demographic aging and will increase the need for advances on diagnosis, therapy, and prevention.

In the last decades, our knowledge of psychiatric disorders has grown stepwise (Charney et al., 2018; Principles of neural science, 2013; Sadock, 2017) despite the intricacy of the human brain, its inaccessibility in life, and a shortage of histopathological clues to disease mechanisms. For instance, the introduction of structural magnetic resonance imaging has been evidenced in patients with SCZ thinning of prefrontal and temporal cerebral cortices (Cannon et al., 2002). Yet, contrary to other common diseases (e.g., hypertension, diabetes), causal mechanisms remain still elusive with barely any insight into primary molecular and cellular risk factors in psychiatric disorders. This deficit reflects in major part the fact that the substrates of our thoughts, emotions, perceptions, actions, and memories are far more difficult to localize, quantify, and model than other somatic functions. Diagnosis of psychiatric disorders is usually based on patients' self-report and physicians' observation of cognitive and behavioral symptoms rather than on direct measurement of any etiological factor. This descriptive approach has led to numerous discontinuous categories for psychiatric disorders (Diagnostic and statistical, 2013) that are better conceptualized as complex syndromes cutting across historically grown diagnostic boundaries (Stephan et al., 2016).

Until recently, our lack of insight into primary risk factors resulted also from a paucity of genetic data and of methods for the investigation of living brain cells from diseased and healthy individuals. In the last century, there have been persistent efforts to understand the genetics of psychiatric disorders (Sullivan et al., 2012); however, it took decades to pass till advances in the field of molecular genetics and bioinformatics enabled for querying the entire human genome in a hypothesis-free manner for the identification of genetic variations associated with disease. These genome-wide association studies (GWASs) have detected an increasing number of common single nucleotide polymorphisms (SNPs) that interact with rare gene-coding mutations and structural variants (i.e., copy number

variations, CNVs) in the mediation of genetic risk (McIntosh et al., 2019; Sullivan and Geschwind, 2019). Individually, disease-associated SNPs have only small effect size; however, in sum they account for a significant part of heritability. Interestingly, disease-associated SNPs are also shared significantly among SCZ, BD, and MD showing that these disorders are genetically closely related (Cross-Disorder, 2019). This outcome strengthens the recent conceptualization (Stephan et al., 2016) that major psychiatric disorders represent quantitative deviations from health rather than distinct nosologies.

In contrast to recent insights into genetics, causal mechanisms of psychiatric disorders still linger in the dark. This reflects the circumstance that the vast majority of disease-associated SNPs maps to noncoding regions and thus fulfills possibly a gene-regulatory role (Freedman et al., 2011). Further studies are needed to narrow down candidate causal genes regulated by disease-associated SNPs and the cell-type(s) in which this regulation takes place. The complex correlation structure of numerous variants within the implicated GWAS loci poses a major challenge for fine-mapping of potential candidates yet. More promisingly, bioinformatic tools have allowed to infer cell types and biological pathways through which disease-associated SNPs may operate. These studies suggest that common risk SNPs tag genomic loci that are enriched for genes with a role in neuronal tissues and cell types from early development to adulthood (Bryois et al., 2019; Coleman et al., 2019; Collado-Torres et al., 2019; Nagel et al., 2018; Polioudakis et al., 2019; Schork et al., 2019; Skene et al., 2018). Integrative network and pathway level analyses further support that these genes converge on mechanisms relevant to ion channel activity, synaptic plasticity, nervous system development, and neuronal differentiation (Girdhar et al., 2018; O'Dushlaine, 2015). Latter predictions match well previous epidemiological and clinical evidence that early neurodevelopmental deviations could contribute to the formation of a vulnerable brain that interacts with adolescent maturation processes in the onset of disease (Owen and O'Donovan, 2017).

Despite this significant gain of information on pathways, cell types, and developmental periods associated with polygenic risk, we would like to caution that most, if not all, of these findings are merely correlative in nature. Accordingly, they cannot answer the question which molecular and cellular mechanisms are causally affected by polygenic risk, how they converge on altered neurocircuitries, and how these translate into perturbed higher brain function in patients. Until recently, this shortcoming reflected also a dearth of suitable human model systems to dissect the functional consequences of polygenic risk in disease-relevant cell types.

In this chapter, we will discuss how the advance of patient-specific induced pluripotent stem cells (iPSCs) has provided a powerful tool to start to address these questions. Firstly, we will highlight the rational of iPSC-based modeling of psychiatric disorders. Following this, major findings from case/control studies on SCZ and BD, and more recently, on MD, will be briefly surveyed. Against this backdrop, we will explore the question how current in vitro systems can be improved to

approximate higher level brain functions. Progress on cell culture techniques and transplantation studies in mice may offer new opportunities to address this topic. Looking forward causality, we discuss how the use of (epi-) genetic editing techniques can help to uncover causal variants and mechanisms in iPSC-based modeling. Finally, potential caveats of these emergent developments are considered.

The rationale of iPSC-based disease modeling

The advent of patient-specific iPSCs has opened the opportunity to break up barriers that prevented studies on cellular and molecular abnormalities in living neurons from patients with psychiatric disorders. As a matter of fact, brain tissues from live patients are inaccessible, while postmortem tissues are confounded by secondary disease processes, psychopharmacological drugs, and life history. Nowadays, iPSCs can be generated from various sources of somatic cells, preferentially, from nuclear cells contained in blood samples routinely drawn from in-patients (Induced pluripotent, 2016). Importantly, iPSCs capture the donor's genotype and allow for investigation of the corresponding polygenic disease architecture in a wide range of disease-relevant neuronal and glial cells. Comparative gene expression profiling of differentiating iPSCs and postmortem brains supports that iPSC-derived cells closely track progression from early embryogenic to perinatal stages in vivo and produce neuronal and glial cells of varying maturity (Brennand et al., 2015; Burke et al., 2018; Mariani et al., 2012; Nicholas et al., 2013; Paşca et al., 2015; Stein et al., 2014). These data also suggest that iPSC-derived cells match early neural stages in vitro rather than stages of the mature brain. Hence, iPSCs are an opportunity to model the role of GWAS risk genes in early neural development in vitro, and by extrapolation, in increasing the risk for later disease (Brennand and Gage, 2011).

Moreover, patient-specific iPSCs can serve as versatile platform to chart the largely unknown territory of polygenicity in major psychiatric disorders. At present, single, or at best a small number, of GWAS variants can be introduced into human embryonic stem cells (ESCs) or iPSCs from healthy donors to study their effect on cellular and molecular phenotypes. This approach is of particular interest to rare high-penetrance CNVs associated with psychiatric disorders (Hoffmann et al., 2018a, 2020). By contrast, genetic engineering can barely embrace the huge amount of common risk variants identified in current GWAS. This reservation applies alike to the generation of transgenic animal models carrying human risk variants. Beyond the question of sheer numbers, GWAS risk variants also localize typically to noncoding regions, which are less conserved among species than their coding regions, and thus less likely to reproduce faithfully regulatory features from humans.

Collectively, iPSCs offer the possibility to identify and dissect genetically encoded molecular and cellular phenotypes during early neural stages in vitro, and by inference, to elucidate potential disease processes in the human brain.

Major results from iPSC-based modeling of psychiatric disorders

In a seminal study, Brennand et al. (2011) firstly introduced iPSC-based modeling in the realm of psychiatric disorders. The researchers measured reduced neuronal connectivity, neurite numbers, synaptic maturation, and glutamate receptor expression in iPSC-derived forebrain neurons from patients with familial SCZ relative to neurons from healthy donors. In support of clinical relevance, this phenotype was partly ameliorated by treatment in vitro with the antipsychotic loxapine. These findings have sparked off a number of subsequent studies reviewed comprehensively elsewhere (Hoffmann et al., 2018a; Ahmad et al., 2018). Briefly, neuronal progenitors (NPCs) from patients with SCZ were found to show aberrant migration and cellular polarity (Brennand et al., 2015; Yoon et al., 2014), perturbed wingless signaling (Srikanth et al., 2015; Topol et al., 2015), increased oxidative stress (Brennand et al., 2015; Ni et al., 2020; Paulsen et al., 2012; Robicsek et al., 2013), and altered responses to environmental stressors (Hashimoto-Torii et al., 2014). Beyond NPCs, iPSC-derived cortical, hippocampal or dopaminergic neurons from patients with SCZ exhibited reduced synaptic maturation and neuronal activity (Brennand et al., 2011; Robicsek et al., 2013; Wen et al., 2014; Yu et al., 2014), as well as attenuated neuronal activity-dependent transcription when compared to samples from healthy donors (Roussos et al., 2016). Additional reports have identified abnormal miRNA profiles (Narla et al., 2017; Siegert et al., 2015; Topol et al., 2016; Zhao et al., 2015) and increased L1 retrotransposition (Bundo et al., 2014) in patient-derived neuronal cells.

A similar picture has emerged for iPSC-based modeling of BD (for review (Hoffmann et al., 2018b) with alterations in developmental patterning (Chen et al., 2014), cell adhesion (Wang et al., 2014), NPC proliferation, wingless signaling (Madison et al., 2015), neuronal excitability, mitochondrial function (Mertens et al., 2015), miRNA profiles (Bavamian et al., 2015), and immune-regulatory pathways (Vizlin-Hodzic et al., 2017). Interestingly, many of these alterations were ameliorated by treatment in vitro with lithium (Chen et al., 2014; Wang et al., 2014; Mertens et al., 2015; Stern et al., 2018; Tobe et al., 2017), a medication used only in patients with BD.

In contrast to SCZ and BD, patient-specific iPSC-based modeling of MD has been only recently explored by Vadodaria et al. (2019a,b), who focused on responders and nonresponders to serotonin-uptake inhibitors. More specifically, iPSC-derived serotonergic neurons from responders formed more refined circuitries compared to nonresponders and were less active following application of serotonin.

Overall, these studies have provided important insights into molecular and cellular alterations in iPSC-derived cells from patients with major psychiatric disorders at various stages of differentiation (neural stem cells, NPCs, and neurons) and of various identities (cortical, hippocampal, dopaminergic, and serotonergic neurons). Broadly speaking, they support the hypothesis that deficits in early neural

development and differentiation contribute to major psychiatric disorders. Moreover, these cellular and molecular deficits coupled in part to signaling pathways and subcellular structures thought to be potentially affected in psychiatric disorders.

Advances in cell culture systems for iPSC-based modeling of psychiatric disorders

It is important to stay aware that above findings from iPSC-based modeling of psychiatric disorders can only serve as a distant proxy to impaired higher system-level functions in patients. In the living brain, each GWAS variant confers only tiny effects at the molecular and cellular level, mostly in a tissue-specific manner (Collado-Torres et al., 2019; Skene et al., 2018). These effects will converge on the formation of altered microcircuit and macrolevel connectivity during early brain development and beyond. Neurocircuitries are the substrate from which higher system-level functions like cognition, mood, and behavior among others, emerge (Principles of neural science, 2013). Conversely, changes in the activity of neurocircuitries are hypothesized to contribute to impairments in higher level functions, singly or globally, in patients with psychiatric disorders (Schmitt et al., 2011). Therefore, it will become necessary to use iPSC-based techniques additionally for the modeling of neurocircuitries to approximate differences in higher level functions between patients and healthy individuals, and to gain new insights into actionable therapeutic targets.

Toward microcircuitries and complex cell–cell interactions

Conventional iPSC-derived culture systems reflect only poorly the cellular complexity of the living brain, and even less, do they recapitulate local and distant connectivity. Almost all studies from above have sought to establish neuronal monolayer cultures (hereafter referred to as 2-dimensional (2D) cultures) containing fairly homogenous populations of a defined subtype (e.g., excitatory cortical neurons), yet lacking the complex in vivo network architecture. For example, accumulating evidence from neuroimaging studies suggests that neuronal connectivity in the prefrontal cortex is impaired in SCZ and could underpin reduced hippocampus-dependent cognitive functions (Wadehra et al., 2013). The hippocampus consists of distinct cell types, whose gene expression profiles and connectivity are well-characterized. More specifically, presynaptic dentate gyrus (DG) axons connect via the mossy fiber pathway to postsynaptic cornu ammonis (CA3) neurons. This local circuitry is reshaped by developmental and environmental cues in a highly plastic manner that impacts hippocampal information processing and memory formation. Recent progress on compartmentalized microfluidic devices allows for modeling human brain circuitry in vitro by segregating cell bodies of different neural subtypes while axonal connections can still develop between compartments (Rothbauer et al., 2018). In this regard, Sarkar et al. (2018) introduced recently a microfluid chip

platform to investigate the DG-CA3 circuit of iPSC-derived hippocampal neurons from patients with SCZ in comparison with healthy donors. Presynaptic DG neurons were seeded in one compartment and postsynaptic CA3 neurons in the opposing compartment of a microfluid device with both compartments connected by narrow channels (Fig. 10.1). This allowed for the formation of synaptic connections as shown by rabies virus tracing experiments. Importantly, multielectrode array (MEA) recordings and whole-cell patch clamping techniques further evidenced that CA3 neurons derived from patients with SCZ exhibited reduced spontaneous and electrophysiological activities following circuit formation in CA3/DG cocultures. Collectively, this study firstly introduced a reductionist microfluid chip platform for studying the polygenetic effects of psychiatric disorders on microcircuit formation in vitro (Fig. 10.1).

As an alternative to microfluid chip devices, multiple cell lineages can be combined in 3D aggregates (so-called brain spheroids) (Paşca, 2019) to investigate complex cell−cell interactions in vitro. This is of interest to excitatory and inhibitory

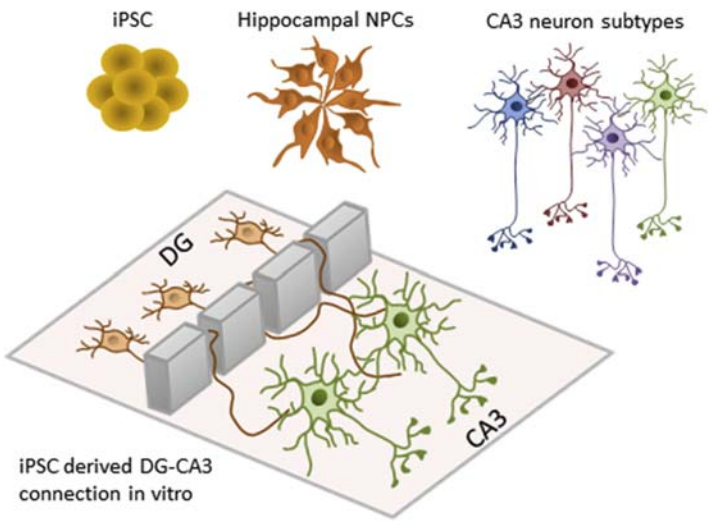

FIGURE 10.1

Microfluid device for investigation of CA3/DG cocultures. The microfluid device consists of two separate chambers connected by narrow channels. iPSC-derived DG neurons are seeded in one compartment and postsynaptic CA3 neurons in the opposing compartment. DG axons connect to CA3 neurons via narrow channels. Thus, this device enables investigation of CA3/DG circuit formation in vitro and of potential differences between neurons from patients with SCZ and healthy controls.

Adapted from Sarkar, A., Mei, A., Paquola, A.C.M., Stern, S., Bardy, C., Klug, J.R., Kim, S., Neshat, N., Kim, H.J., Ku, M., et al., 2018. Efficient generation of CA3 neurons from human pluripotent stem cells enables modeling of hippocampal connectivity in vitro. Cell Stem Cell 22, 684–697.e9; attributable license 4780771131092.

neurons of the cerebral cortex that are derived from the germinal zones of the dorsal and ventral telencephalon, respectively. These neurons populate cortical layers at defined ratios in vivo that cannot be recapitulated by conventional 2D-culture or 3D-brain organoids (see below). Altered neuronal connectivity and changes in the excitation/inhibition (E/I) balance of cortical microcircuits have long been suspected to play a key role in SCZ etiology (Anticevic and Lisman, 2017; Foss-Feig et al., 2017). Brain spheroids could offer the possibility to study differences in the E/I balance of iPSC-derived neurons from patients with SCZ relative to healthy donors.

In another approach, brain organoids are derived from neural precursors that are differentiated in the presence of external patterning cues into specific brain regions. They contain a broad array of cell types that resemble in vivo counterparts (Quadrato et al., 2017) and many of these seem to track developmental trajectories from the human brain with similar variability (Velasco et al., 2019). As of yet, only one study (Stachowiak et al., 2017) has sought to explore this new technique for the study of psychiatric disorders by investigating potential differences in forebrain organoid formation between patients with familial SCZ and healthy controls. The researchers observed that in cases proliferative NPCs were retained in the subcortical intermediate zone and developed into atypically placed deep subcortical neurons. Moreover, T-box brain one positive pioneer neurons were delayed in entering upper cortical layers, while calretinin-positive interneurons exhibited perturbed intercortical connectivity. Taken together, this study takes previous findings on impaired migration and differentiation of iPSC-derived neurons from patients with SCZ (Major results from iPSC-based modeling of psychiatric disorders) an important step further by linking them to aberrant subcortical neurogenesis in a reductionist model of human brain development.

The work of Stachowiak et al. (2017) raises further the question of whether patient-specific brain organoids could also enable insight into disease-associated alterations in neuronal connectivity. Extracellular recording of brain organoids has evidenced spontaneous action potentials and coordinated bursting activity in support of functional neuronal networks (Quadrato et al., 2017). Moreover, from 6 months onward in culture, cortical organoids exhibit nested oscillatory network activity raising the prospect to model the development of neural networks (Trujillo et al., 2019). As encouraging these findings are, we would like to caution that the hurdle for case/control studies in psychiatric disorders remains still high at the level of organoids. In addition to high costs for extended organoid cell culture, organoid-to-organoid variability is high and increases over time. A possible explanation for this variability is that brain organoids do not recapitulate faithfully distinct cellular subtype identities and appropriate progenitor maturation from cortical development in vivo (Bhaduri et al., 2020). Moreover, molecular signatures of cortical areas emerging in organoid neurons are less spatially segregated than in primary samples. Hence, future studies are necessary to clarify to what degree brain organoids can be used to model microcircuit formation in health and disease.

Toward in vivo systems for iPSC-based modeling of psychiatric disorders

While the study by Bhaduri et al. (2020) revived the debate on the fidelity of brain organoids for modeling early brain development in vitro, the same researchers also showed that abnormal development in vitro was rescued following transplantation of dissected brain organoids into the cortex of newborn mice brain. This result is of particular relevance to two recent iPSC-based case/control studies on SCZ that have applied transplantation of human precursor cells into mice (Shao et al., 2019; Windrem et al., 2017). These studies were originally based on the rationale that the microenvironment in mice cortices might be more permissive to unmask rather subtle neurodevelopmental phenotypes expected from psychiatric disorders. Additionally, Windrem et al. (2017) chose children with early onset of SCZ (COS), who show greater neurodevelopmental deviance early in life, while the clinical and neurobiological course of the disease is continuous with adult onset SCZ (Gogtay, 2008). Human glial precursor cells (hGPCs) derived from iPSCs of patients with COS or age-matched healthy individuals were engrafted into neonatal immunedeficient shiverer mice (Fig. 10.2). Owing to the lack of myelin basic protein (MBP), this strain of mice develops congenital hypomyelination. Control transplanted hGPCs gave rise to both astrocytes and myelinogenic oligodendrocytes that efficiently populated the host forebrain. By contrast, hGPCs from COS entered the host cortex prematurely, produced less donor cells, and developed into less differentiated oligodendrocytes and astrocytes (Fig. 10.2).

Interestingly, myelin wild-type mice engrafted with hGPCs from COS showed a significantly reduced auditory prepulse inhibition (a proxy to sensorimotor gating deficits in some patients with SCZ) when compared to counterparts engrafted with hGPCs from healthy donors. Furthermore, glial chimerization with hGPCs from COS was associated with increased anxiety and fear in recipient mice and concomitant deficits in socialization, cognition, and sleep patterning, relative to mice counterparts engrafted with hGPCs from healthy individuals (Fig. 10.2). All of these behavioral symptoms are frequently found in patients with SCZ and suggest altered circuit and synapse formation in mice engrafted with hGPCs from patients with COS.

In another transplantation study in mice, Shao et al. (2019) sought to address the E/I balance of cortical microcircuits in SCZ. For this purpose, patients with SCZ were selected based on prior clozapine treatment, a second line medication for severe SCZ. Firstly, the researchers showed that iPSC-derived GABA-ergic cortical interneurons (cINs) from both controls and cases undistinguishably developed into authentic and fully functional interneurons in engrafted mice. In a complementary approach, RNA-sequencing of in vitro differentiated cINs from controls and cases uncovered downregulation of the protocadherin alpha family in cases, which was specific to GABA-ergic fate. Well-fitting, in vitro differentiated cINs from cases showed reductions in somatic neurite numbers, total branch numbers, and total neurite length when compared to healthy controls. Consistent with these in vitro results,

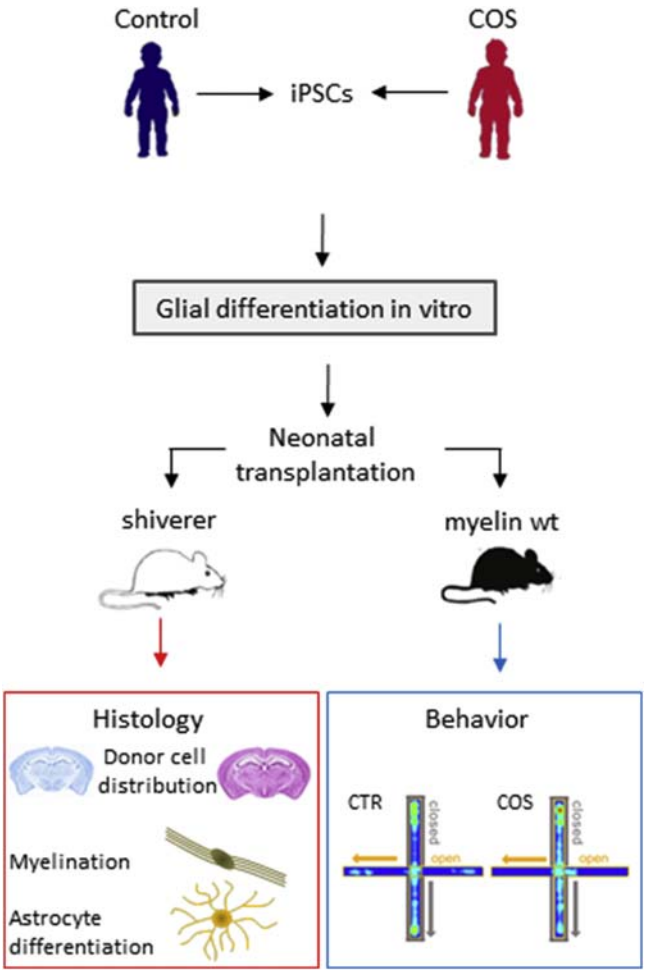

FIGURE 10.2

Flowchart for the investigation of iPSCs-derived glial precursor cells from age-matched healthy children and children with COS. In vitro differentiated glial precursor cells were engrafted in myelin-deficient shiverer or myelin wild-type neonate mice. The distribution of donor cells in the recipient mice brains was assessed by histological analysis. In addition, the extent of myelination and of differentiation into astrocytes was investigated (*left*). In a complementary set of experiments, control or case glial precursor cells were engrafted in myelin wild-type neonate mice that underwent a battery of behavioral tests thought to mimic symptoms from patients with SCZ.

Adapted from Windrem, M.S., Osipovitch, M., Liu, Z., Bates, J., Chandler-Militello, D., Zou, L., Munir, J., Schanz, S., McCoy, K., Miller, R.H., et al., 2017. Human iPSC glial mouse chimeras reveal glial contributions to schizophrenia. Cell Stem Cell 21, 195–208.e6; attributable license 4780780133937.

cINs from case-engrafted mice showed a significant reduction in inhibitory synapse formation in vivo relative to control-engrafted mice. Overall, Shao et al. (2019) established an integrated iPSC-based approach for the investigation of the E/I balance of cortical microcircuits in SCZ. Remarkably, iPSC-derived cINs from patients with SCZ formed less inhibitory synapses in transplanted mice and in vitro culture. This phenotype developed in the absence of other potential abnormalities in cortical circuitry and thus pointed to a cell autonomous defect of iPSC-derived cINs from patients with SCZ.

While both iPSC-transplantation studies (Shao et al., 2019; Windrem et al., 2017) support a role of disrupted neural circuits in SCZ, it is important to realize that neurons, and the networks they form, do not operated outside the world of other cell types populating the living brain. In fact, Windrem et al. (2017) argued that impaired oligodendrocytic and/or astrocytic differentiation may be a primary cause of impaired neuronal network function in transplanted mice. Oligodendrocytes perform myelination of axons in white mater to boost neuron conduction, while astrocytic networks enhance synaptic efficacy and plasticity and underpin the differential competence of hominid glia (Han et al., 2013; Oberheim et al., 2009). Taking these considerations into account, it will become important to study microcircuit function under more naturalistic conditions than afforded by current 2D/3D systems. An ideal in vitro system should contain neural, glial, immune, and vascular cells in appropriate proportions to recapitulate the intricate balance of interactions between these cell types in vivo. This would allow to assess iPSC-derived cells from patients and cases with greater fidelity for differences in neuronal circuits, and potentially affected high level systems functions. While we are aware that rebuilding multicellular systems ab initio is a daunting task (Park et al., 2018), refinements in transplantation studies appear more feasible on intermediate terms. In fact, two recent reports (Mansour et al., 2018; Real et al., 2018) on transplantation of human stem cell-derived cortical progenitors/neurons or brain organoids into adult mice brain lend further support to this suggestion. Engrafted brain organoids developed corticothalamic, subcerebral, and intracortical projections neurons, though these neurons did not faithfully trace the laminar organization of the host cortex. Additionally, engrafted human organoids gave rise to astrocytes and oligodendrocytes, were vascularized within 2 weeks by the host tissue, and were invaded by host microglia. Cortical and subcortical long-range connections from transplanted neurons underwent with maturation structural refinements and integrated functionally into existing host circuits (Mansour et al., 2018). Lastly, engrafted brain organoids acquired progressively synchronous network activity and oscillatory patterns, and responded to sensory stimuli indicating circuit-level maturation (Real et al., 2018). This finding may open the perspective to investigate iPSC-derived case/control cells not only under resting conditions, but also in response to environmental exposures of recipient mice. If yes, circuit-level integration of human iPSC-derived cells may also offer a tool to dissect genes environment interactions in a living model system.

Uncovering causality in iPSC-based modeling of psychiatric disorders

In contrast to our rapidly expanding knowledge on genetic risk variants associated with major psychiatric disorders, our insight into how these variants contribute causally to disease is still in its infancy. This is due to the fact that risk SNPs typically localize to noncoding regions and capture all the genetic variation localized in an SNP-associated haplotype block (Sullivan and Geschwind, 2019). Hence, risk-associated SNPs do not encode necessarily the causal regulatory variation in disease, but may merely flag a critical genomic region. The advance of genetic and epigenetic editing techniques has provided powerful tools to narrow down and dissect the causal role of single, or multiple SNPs, on molecular and cellular phenotypes in disease-relevant living cells from diseased and healthy donors. While editing techniques have been readily embraced for the investigation of monogenic diseases (Soldner and Jaenisch, 2018), their potential for the exploration of polygenic psychiatric diseases has been recognized only recently.

Genetic editing of GWAS risk SNPs in iPSC-based modeling of psychiatric disorders

Mature micro-RNAs (miRNAs) are noncoding RNAs of ~22 nucleotides in size (Bartel, 2009) that associate with the cytoplasmatic RISC complex (RNA induced silencing complex). Subsequently, miRNAs target through a 6- to 8-base pair complementary seed region single or multiple mRNAs and downregulate their expression. Although miRNAs have been originally recognized for their roles in cell lineage and cell fate decisions during (neuro-) development, more recent evidence supports additional roles in neuronal differentiation and maturation (Rajman and Schratt, 2017; Sun et al., 2013), and possibly, in higher brain functions (O'Connor et al., 2016). Compatible with this view, postmortem brains from patients with SCZ, MD, or ASD (autism spectrum disorder) showed altered miRNAs expression profiles. Among these, miR-137 maps to a locus previously implicated in SCZ (Lee et al., 2013; Ripke et al., 2013). In further support of a potentially causal role in SCZ, miR-137 regulates the expression of additional GWAS risk genes like *TCF4* (transcription factor 4) and *CACNA1C* (calcium channel voltage-dependent, l-type, α-1C subunit) (Kwon et al., 2013; Lett et al., 2013).

To gain insight into the role of *MIR137* risk SNPs for gene expression, Siegert et al. (2015) converted directly fibroblasts containing either the major or minor allele (each carrying all four noncoding risk SNPs) into early neuron-like cells. In the presence of the minor allele, miR-137 expression was significantly increased when compared to the major allele and associated with a stronger downregulation of neuronal activity-dependent synaptic vesicle release and of presynaptic plasticity genes. Since these experiments left unanswered, which *MIR137* allele was actually involved in disease, as well as which SNPs contributed causally to gene expression,

Forrest et al. (2017) sought to prioritize GWAS risk SNPs prior to functional analyzes in iPSC-derived neuronal cells. For this purpose, the researchers used chromatin analysis (ATAC-sequencing) of neurally differentiated iPSCs to map open chromatin regions (OCRs). Differentiation into excitatory cortical neurons associated with dynamic changes in OCRs and both neuronal OCRs and transcription factor (TF) binding sites revealed a significant enrichment for GWAS risk variants from SCZ (Fig. 10.3). More relevant to this section, OCR mapping allowed assigning to a rare large effect SNP (rs1198588) at *MIR137* a potential regulatory role. To explore the hypothesized mechanistic link between the OCR at this risk SNP and *MIR137* expression, CRISPR/CAS9 editing was used to convert this SNP into a nonrisk allele in an iPSC line derived from a patient with SCZ. When compared to iPSCs containing the risk allele, isogenic iPSCs harboring the nonrisk allele exhibited altered chromatic dynamics at the *MIR137* locus and increased gene expression in cortical induced neurons (iNs). In turn, elevated levels of miR-137 associated with signs of reduced cortical maturation.

Taken together, this study (Forrest et al., 2017) firstly defined a causal role for a rare GWAS risk SNP in SCZ by integrating chromatin mapping, SNP editing, and comparative differentiation of isogenic iPSCs. Notably, presence of the risk allele associated with improved cortical maturation when compared to the nonrisk allele. While this result appears at first sight counterintuitive, gene-editing in a patient-specific iPSC-line support its relevance. Yet, the present study did not examine additional *MIR137* risk SNPs with a potential regulatory role in the same or opposite direction in cortical neurons. In fact, given the extraordinary genetic complexity of psychiatric disorders, we should not feel surprised when (epi-) genetic editing of single risk SNPs or genes (see below) produces molecular or cellular phenotypes opposite to "common sense".

Epigenetic editing of GWAS risk variants in iPSC-based modeling of psychiatric disorders

Polygenicity of psychiatric disorders implicates that many common variants of small effect size act together in disease manifestation. The nature of these interactions and the mechanisms involved are largely unknown at present. In a recent study, Schrode et al. (2019) sough to throw new light on this question by perturbing singly or jointly the expression of candidate genes nearby SCZ risk variants in isogenic iPSC-derived neuronal cells. For this purpose, variants and associated genes (Pardiñas et al., 2018; Ripke et al., 2014) were prioritized by integrating GWAS data and postmortem gene expression profiles. This strategy enabled for identifying variants predicted to impact risk for SCZ owing to their effect on gene expression (i.e., so-called expression quantitative trait loci; eQTLs) (Fig. 10.4). Among the prioritized candidate loci, five contained a single protein-coding gene: *FURIN*, *TSNARE1*, *CNTN4*, *CLCN3,* and *SNAP91*. In a nutshell, the proprotein convertase FURIN activates various regulatory proteins in the constitutive exocytic and endocytic pathway; TSNARE1 (t-SNARE domain containing 1) regulates intracellular protein transport and

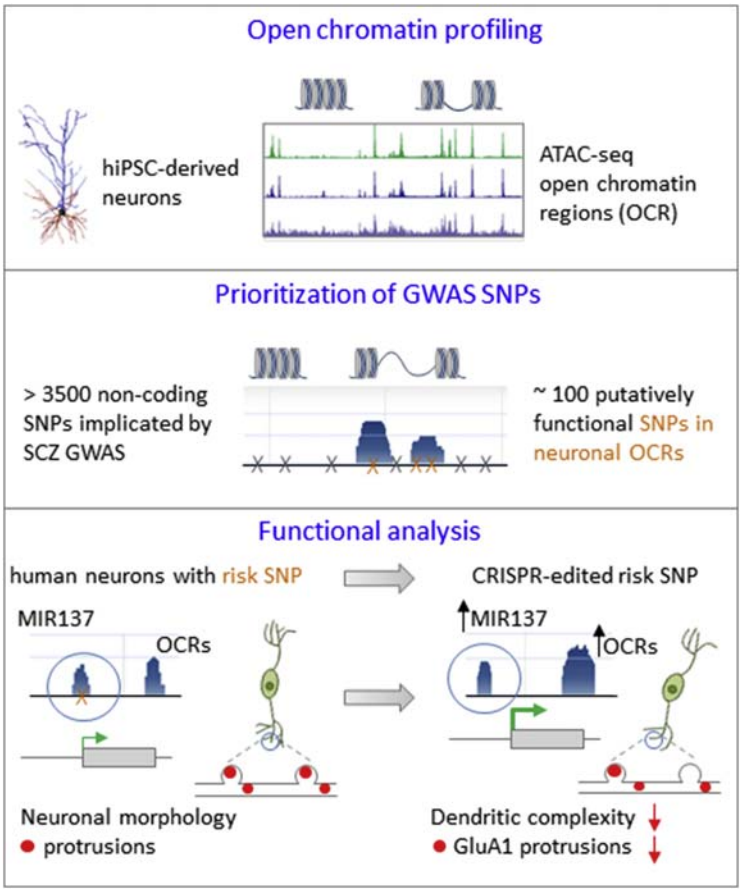

FIGURE 10.3

A common GWAS risk SNP at *MIR137* regulates dendritic arborization in vitro. (A) Open chromatin regions (OCRs) are defined by ATAC-sequencing during differentiation of human iPSCs toward excitatory cortical neurons. (B) GWAS SNPs from SCZ are enriched in OCRs and are prioritized accordingly. (C) CRIPSR/CAS9 editing was used to generate an isogenic iPSC line from a patient with SCZ differing solely at the predicted functional GWAS risk SNP in *MIR137*. When compared to the risk allele (left), conversion to a nonrisk allele enhanced chromatin dynamics at the MIR137 locus and associated with increased miR-137 expression in excitatory iNs (right). Presence of the risk allele also associated with more mature dendritic arbors and neuronal protrusion of excitatory iNs relative to isogenic cells carrying the nonrisk allele.

Adapted from Forrest, M.P., Zhang, H., Moy, W., McGowan, H., Leites, C., Dionisio, L.E., Xu, Z., Shi, J., Sanders, A.R., Greenleaf, W.J., et al., 2017. Open chromatin profiling in hiPSC-derived neurons prioritizes functional noncoding psychiatric risk variants and highlights neurodevelopmental loci. Cell Stem Cell 21, 305–318.e8; attributable license 4780780427601.

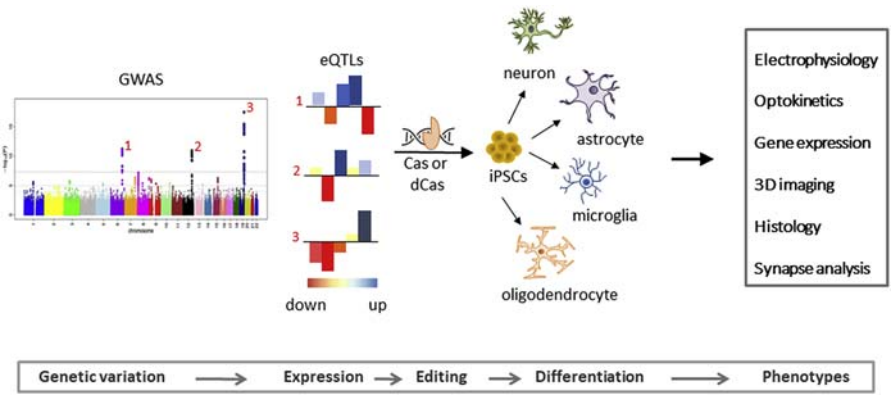

FIGURE 10.4

Functional genomics of psychiatric disorders. GWAS enable identification of common genetic variants, typically SNPs, associated with disease. These risk variants can be prioritized by eQTL analysis (or any other method) to narrow down potentially regulatory SNPs affecting gene expression. Such credible SNPs are gene-edited by means of CRISPR/CAS to generate nonrisk alleles in human iPSCs. Then isogenic iPSCs are differentiated into distinct disease-relevant neuronal and glial cell types for further analysis including electrophysiology, gene expression, and histology, among other methods. In case prioritization of candidate SNPs is inapplicable, potential candidate genes can be regulated, singly or jointly, by CRISPR/dCAS mediated activation or inhibition. Taken together, this strategy allows the identification and functional characterization of causal regulatory risk variants in disease-relevant cells in vitro.

synaptic exocytosis; CNTN4 (contactin 4) is an axon-associated cell adhesion molecule with a role in neuronal network formation; CLCN3 is a chloride voltage-gated channel; and SNAP91 (synaptosomal-associated protein, 91-KD) regulates synaptogenesis and presynaptic maturation.

In order to assess the role of the prioritized cis-eQTL in the 3′ untranslated region of *FURIN*, CRISPR/CAS9 editing was used to produce homozygous nonrisk alleles in iPSCs from healthy donors. Thereafter, isogenic iPSCs were differentiated into glutamatergic or GABAergic iNs, or induced astrocytes. Among terminal differentiated cells, conversion to the nonrisk allele associated with *FURIN* downregulation concomitant to reduced neurite length and neuronal activity solely in excitatory neurons. By contrast, the nonrisk alleles associated with *FURIN* upregulation concomitant to increased cell migration at the NPC stage.

Since no single cis-eQTL could be defined for *TSNARE1*, *CLCN3,* and *SNAP91*, disease-relevant CRISPR/dCAS9-mediated activation (CRISPRa) of each candidate gene singly or jointly (including FURIN downregulation via RNAi) was used to assess their role for iPSC-derived neural cells from healthy donors. Remarkably, single perturbations resulted in transcriptional effects opposite to disease-associated

changes in iPSC-derived neurons and postmortem brain tissues from patients with SCZ. By contrast, joint regulation in the direction of disease, converged on transcriptional changes concordant with those from disease. Under this condition, a subgroup of upregulated (7%) and downregulated (11%) genes were more strongly regulated than predicted by the additive effects of singly regulated risk genes. These subgroups were enriched in genes relevant to synaptic functions, genes harboring rare CNVs or nonsynonymous de novo mutations in SCZ, and genes identified in SCZ GWAS.

Collectively, Schrode et al. (2019) tied up three complementary lines of experiments to gain insight into the nature of polygenicity in SCZ: Prioritization of risk SNPs based on their effect on gene expression in postmortem brains, comparative differentiation of risk SNP-edited isogenic iPSCs, and joint CRISPRa-mediated regulation of GWAS risk genes in the direction of disease in differentiated iPSCs. Beyond current findings, this proof of concept study provides a blueprint for future investigations in the field of functional genomics of psychiatric disorders (Fig. 10.4).

Conclusion and outlook

Patient-specific iPSC modeling of major psychiatric disorders has provided a wealth of information on molecular and cellular phenotypes that are thought to recapitulate to varying degrees alterations from diseased brains (for recent reviews, see (Hoffmann et al., 2018a,b; Ahmad et al., 2018). Progress on differentiation protocols, high throughput measurements (e.g., high content imaging, multielectrode systems), and patient stratification is expected to increase reproducibility within and across iPSC studies further and thus to strengthen statistical power.

Irrespective of these upcoming advancements, we argued here that cardinal features of major psychiatric disorders are neglected by so far owing to technical challenges deemed unsurmountable in iPSC-base modeling until recently. These include impairments in local and distant connectivity, complex cell−cell interactions that extend beyond a purely neurocentric view, and above all, the uncharted territory of polygenicity. We discussed key studies that open up the horizon to expand our knowledge in this direction, but that also raise new questions in terms of feasibility and interpretation. Undoubtedly, progress on microfluid systems, 3D-culture, transplantation, and (epi-) genetic editing involves cost-intensive techniques that require a high degree of technical skills and analytical training unaffordable to many laboratories. Even then, when these limitations do not apply, we need to accept that whatsoever sophisticated experimental in vitro and in vivo systems remain models that cannot recapitulate the complexity of the living brain in its entirety. In fact, these models can yield results difficult to reconcile with those from independent research lines. Examples discussed here include unexpected "beneficial" effects from risk alleles when compared to nonrisk alleles (e.g., *MIR137* and *FURIN* Forrest et al., 2017; Schrode et al., 2019) or unexpected synergistic effects of SCZ GWAS risk variants at the transcriptional level (Schrode et al., 2019). As we poorly know how single cellular effects cascade up- and downstream on higher level systems function in

living brains or how the transcriptional logic of cellular in vitro systems couples to polygenicity in psychiatric disorder, we have to abstain from premature conclusions—exciting as these may appear. As the human brain functions as a whole, not as the sum of its parts, no single iPSC-based experimental approach is likely to deliver final answers to all the challenges outlaid here. Nonetheless, the combination of different levels of iPSC-based modeling, and their integration with clinical and genetic findings, bears a high potential to accelerate progress on psychiatric disorders and therapy on intermediate terms.

Author contributions

All authors contributed to the conceptualization, literature search, draft preparation, and review and editing. A.H. performed visualization.

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Fig. 10.1 is adapted after (Sarkar et al., 2018), attributable license 4780771131092; Fig. 10.2 is adapted after (Windrem et al., 2017), attributable license 4780780133937; Fig. 10.3 is adapted after (Forrest et al., 2017), attributable license 4780780427601; and Fig. 10.4 is partly adapted after (Ikram et al., 2010), attributable open access.

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CHAPTER

11 Research applications of induced pluripotent stem cells for treatment and modeling of spinal cord injury

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Abstract

Spinal cord injury introduces complex pathophysiological consequences that contribute to devastating behavioral outcomes. In recent years, induced pluripotent stem cell (iPSC)-based technologies have emerged as tools that can advance cell-based therapeutics and disease modeling for SCI. iPSCs can be used as the starting source for a number of potential cell therapies, including oligodendrocyte precursor cells, neural cells, Schwann cells, mesenchymal stem cells, pericytes, and inflammatory cells, all of which aim to ameliorate the injury milieu. Three-dimensional tissues and organoids can also be developed using iPSCs in an effort to promote bridge formation across the lesion. Moreover, iPSC-derived organoids introduce novel strategies for injury modeling, which can ultimately help guide research efforts. As such, iPSC-based cell therapies and disease models can help to advance SCI therapeutics.

Keywords: Induced pluripotent stem cells; Neural cells; Oligodendrocyte precursor cells; Organoids; Schwann cells; Spinal cord injury.

Introduction

Molecular SCI pathophysiology

Spinal cord injury (SCI) pathology is biphasic in nature, comprising an initial primary mechanical trauma and a secondary phase, which constitutes the induction of detrimental injury cascades. Overall, the postinjury spinal cord milieu represents a harsh, pro-inflammatory microenvironment that inhibits central nervous system (CNS) regeneration and impairs functional recovery. Acute and subacute pathophysiology is characterized by apoptosis, demyelination, excitotoxicity, hemorrhaging, ionic imbalance, ischemia, necrosis, neuroinflammation, neuronal loss, and oxidative stress (Alizadeh et al., 2019, Oyinbo, 2011). One of the earliest sequalae of the primary injury is the disruption of spinal cord vasculature (Popa et al., 2010), which results in ischemia, hemorrhaging, and subsequent cellular death due to ATP deprivation, oxygen loss, and oxidative stress (Alizadeh et al., 2019). Neuronal and glial necrosis occurs as a result of direct plasma membrane disruption (Alizadeh et al., 2019), as well as ionic imbalance. The cellular disruption causes an increase in the extracellular glutamate concentration. Glutamatergic excitotoxicity in neurons and oligodendrocytes results in caspase-3 mediated apoptosis and an associated reduction in the perilesional cellular density by day seven (Xu et al., 2008). Moreover, the resultant ionic imbalance also damages axons by impeding mitochondrial ATP production via cytotoxic increases in $[Ca^{2+}]i$. This permits an increase in [Na+]i via the impairment of Na^+/K^+ ATPases, which in turn activates voltage-gated Na^+ channels (Li and Stys, 2001). Na^+ influx facilitates the entry of chloride and water down their electrochemical gradients, resulting in cellular swelling and

membrane permeation (Alizadeh et al., 2019). Immediately after injury (0−2 day postinjury (dpi)), damage-associated molecular pattern signaling activates astrocytes and microglia, which act to contain the lesion epicenter (Alizadeh et al., 2019). Signaling the secondary stage of inflammation (≈3 dpi), astrocytes upregulate the production of molecules that promote the extravasation and polarization of bloodborne monocytes (David and Kroner, 2011). Invading monocytes form macrophages which, depending on signals within their microenvironment, adopt either a neuroprotective M2-like, or pro-inflammatory M1-like, phenotype. The extracellular environment postinjury, mediated by TNF-α, tends to favor M1-like adoption (Kroner et al., 2014). Depending on their polarization, macrophages accordingly implement either remedial or inflammatory processes. M1-like macrophage contributions include axonal repulsion, pro-inflammatory cytokine production (e.g., IL-1β, IL-6, TNF-α) and inducible nitric oxide synthase expression, which is linked with exacerbated glial and neuronal apoptosis. M2 macrophages, however, contribute to debris scavenging, lesion containment, and antiinflammatory cytokine production (e.g., IL-4, CD206). Microglia adopt similar markers and roles to monocyte-derived macrophages but are to be treated as distinct. SCI triggers the establishment of a glial scar that separates the penumbra from the epicenter (Kroner and Almanza, 2019). The glial scar is complex in nature and includes hypertrophied reactive astrocytes, pericytes, and the deposition of extracellular matrix molecules, which form an inhibitory chemophysical mesh barrier of intersecting filaments that acutely reduce the lesion and inflammatory expansion but chronically prevent axonal regeneration (Kroner and Almanza, 2019).

Current induced pluripotent stem cell treatment of spinal cord injury

Cell therapies in traumatic SCI have faced a number of difficulties. There is a lack of readily accessible CNS cells, and the expansion of remedial, multipotent, and pluripotent cell types is arduous. Embryonic stem cells, while rich in proliferative capacity, introduce issues of immunogenicity as well as those of an ethical nature, limiting their clinical viability (Jin et al., 2019, Khazaei et al., 2017). Due to their limitless supply, proliferative capacity, and ability to circumvent the aforementioned obstacles facing ESC-based methods, induced pluripotent stem cells (iPSCs) represent the current front runner of stem cell therapy in SCI treatment. Human iPSC-derived (hiPSC) cells can be derived from somatic cells following the addition of four Yamanaka factors (c-Myc, Klf4, Sox2, and Oct4) (Takahashi et al., 2007). Importantly, these cells have demonstrated a robust therapeutic capacity in preclinical models of subacute SCI, as compared to chronic SCI, which appears less targetable. While the specific cell types used differ between studies, the reported benefits of hiPSC-derived transplantation include axonal outgrowth, axonal sparing, angiogenesis, modulation of the extracellular environment, relay formation, sensorimotor recovery, and trophic support (Fujimoto et al., 2012, Lu et al., 2014). Fujimoto et al. generated hiPSC-derived neural progenitor cells (NPCs) that reconstructed corticospinal circuitry to ameliorate hindlimb motor

functionality following a contusive, subacute, thoracic injury (Fujimoto et al., 2012). Romanyuk et al. showed how hiPSC-derived NPCs in rodent models provide rapid improvements in motor functionality (<14 days of postinjury, dpi) as well as long term, widespread white matter sparing (Romanyuk et al., 2014). Tuszynski's group further showed that caudalized hiPSC-derived NPCs with spinal cord properties also exhibit a strong capacity for corticospinal tract reconstruction and motor recovery (Kumamaru et al., 2018, Kadoya et al., 2016). These studies report on the purported prospect of hiPSCs to generate donor neural cells in the treatment of SCI. iPSCs have also shown utility in other avenues of SCI research.

Appropriate disease modeling of SCI pathophysiology is instrumental for the development of novel therapeutics. However, in vivo modeling of SCI is expensive, time-consuming, and impractical due to the high maintenance and mortality rates incurred via current injury models. Moreover, the ability to reconstruct complex, 3D, cytoarchitectural structures, comprising diverse cellular populations, represents a key advantage of iPSCs in SCI disease modeling. It therefore stands to reason that hiPSC-derived spinal organoids represent an alternative, more phylogenetically relevant, method of modeling isolated pathophysiological processes in vitro. Briefly, in vitro human models of SCI pathology may possess superior anatomical and synaptic relevance as compared to current animal models, as well as the ability to study individual pathological events. Moreover, iPSC technology permits the study of developmental cellular interactions in the healthy spinal cord; knowledge that is pivotal to the mechanistic understanding underpinning the reported benefits of iPSC therapy. Ultimately, iPSC applications may facilitate the continued advancement of SCI research, which is currently encumbered with inefficient methods of studying SCI pathology (Fig. 11.1).

Application of iPSCs for SCI cell-based therapies

Depending on several factors, including level of injury, force of impact, and type of external force, SCI patients may experience a myriad of functional deficits. Moreover, the overwhelming pathophysiological response is further complicated by the diverse cellular niche of the healthy spinal cord. As such, the convoluted pathology of SCI demands an equally complex solution. Biasing or differentiating iPSCs toward different terminal cell fates can help address the modalities of SCI, including the reduction of cellular death, restoring appropriate neural circuitry and myelination patterns, promoting a time-pertinent response to inflammation, as well as supporting angiogenesis.

iPSC-derived oligodendrocyte precursor cells

Oligodendrocytes are highly sensitive to unfavorable changes in the spinal microenvironment, and thus are highly susceptible to cellular death postinjury. As a result, a prominent feature of SCI is the disruption of white matter tissue, in which injured

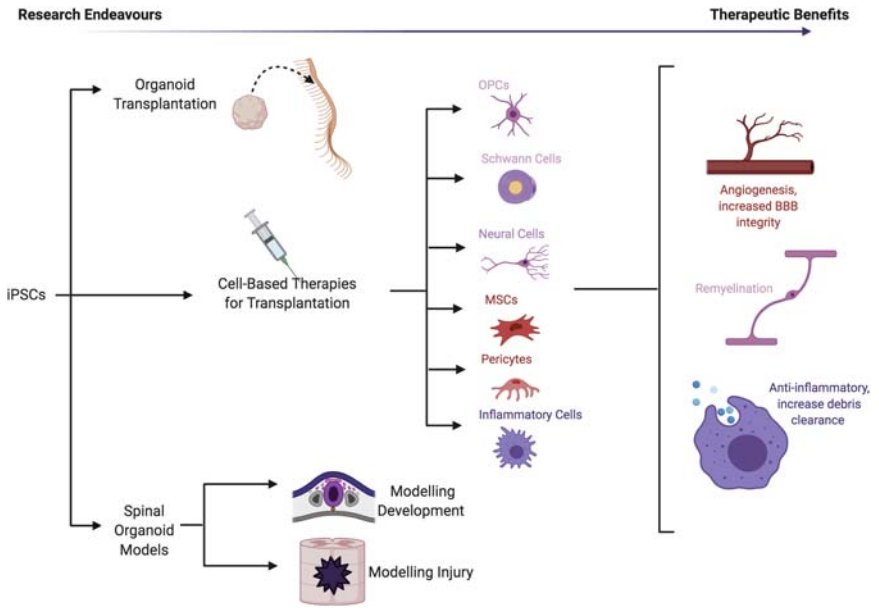

FIGURE 11.1 The role of iPSC Technologies in Spinal Cord Injury (SCI) Research.

iPSCs have a number of potential applications in SCI. They can be differentiated into mature cells that can be transplanted into the site of injury, or used for the derivation of organoids. Organoids can be transplanted into the site of injury or they can be used as models of development or injury in vitro.

axons become progressively demyelinated (Pukos et al., 2019). Surviving postmitotic oligodendrocytes are unable to remyelinate injured axons, and thus the majority of endogenous remyelination that occurs is through the recruitment of NG2+ oligodendrocyte precursor cells (OPCs). Native OPCs in the spinal cord are robustly recruited to the lesion border from progenitor cell niches, which initiates shortly after injury (Zai and Wrathall, 2005). Despite the correlation of OPC recruitment to remyelination postinjury, endogenous remyelination is insufficient in itself to restore functional recovery (Hassannejad et al., 2019). Regardless, insufficient endogenous remyelination may require supplemental OPC transplantation.

Although the majority of studies utilize primary embryonic tissue, few studies have derived OPCs from iPSCs. OPCs can be produced from iPSCs following the induction of embryoid bodies (EBs) and subsequent induction of neural-like supplements (DMEM/F12, N2, basic fibroblast growth factor, and heparin), followed by caudalization with retinoic acid (RA). Once colonies of neural stem/progenitor cells have formed from neural rosettes (PAX6+/SOX1+), isolating colonies are introduced to purmorphamine (sonic hedgehog agonist) and vitamin B27 supplements for 27 days. Isolated colonies of OPCs (OLIG2+ or NKX2.2+) can then be used to induce a neuroglial phenotype (Wang et al., 2013). Similar to embryonically

derived tissue, iPSC-OPCs can functionally improve Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale scores, mechanical allodynia/pain sensation, reduce cavity size, and promote a greater extent of remyelinated axons in traumatically injured animals (Yang et al., 2018, All et al., 2015, Kim et al., 2017a). Apart from traumatic injury, iPSC-derived OPCs can also rescue hypomyelinated states (Wang et al., 2013, Fan et al., 2017), which suggests they can be applied to similar demyelinated states such as SCI. While prepared in culture, differentiated human iPSC-OPCs express mature oligodendrocyte markers (Livesey et al., 2016, Magnani et al., 2019) where differentiation can be improved by supplementing culturing protocols with neurotrophic support. This can promote oligodendrocyte survival, differentiation, and remyelination posttransplantation but does not consistently improve locomotor recovery (Cao et al., 2010).

An important caveat of iPSC-OPC generation is encountered during the culturing process, as the cells are time-consuming to grow in culture, thus translating to challenges from a logistical and financial perspective. Longer OPC generation protocols also present a higher risk of contamination, as well as a greater extent of variation in cell identity with higher passage numbers (Myers et al., 2016). However, relatively new methods that select OPCs using PDGF/insulin-like growth factor and sonic hedgehog signaling can reduce the time needed for these culturing methods by half (Kim et al., 2017a). The harsh microenvironment also presents as an issue for clinical translation, in which myelin promotes a prolonged activation of pro-inflammatory macrophages. This is particularly troubling, as the extent to which myelin can stifle or facilitate repair through macrophage activation is currently unknown. In isolated culturing paradigms, myelin debris can promote a pro-inflammatory macrophagic reaction, where pro-regenerative activation can be observed over time. However, in an already pro-inflammatory state, myelin activation potentiates the pro-inflammatory polarization (Gaudet and Fonken, 2018, Kopper and Gensel, 2018). Ultimately, this suggests that recruited OPCs, alongside exogenous OPC transplantation, may worsen macrophage polarization, which is highly dependent on specific injury microenvironments. Most notably, OPC transplantation does not restore the diverse cellular niche of neurons that are present before injury, as OPCs only differentiate into mature neuroglia. Thus, OPCs may be beneficial in injured patients who have primarily damaged axons with limited neuronal death. Future studies are warranted to directly compare the effects of iPSC-derived OPCs and NPCs to determine if restoring neuronal loss, or promoting remyelination in injured axons, could present an optimal improvement to functional recovery.

iPSC-derived neural cells

NPCs are the developmental precursors that give rise to the three neural cells found in the CNS: neurons, oligodendrocytes, and astrocytes. As such, NPCs are particularly suitable for neural repair and regeneration following SCI. NPCs can be easily derived for use in preclinical studies due to their existence in the subventricular and hippocampal regions of the adult nervous system (Nagoshi and Okano, 2018).

Such preclinical studies have shown that NPCs not only survive, but migrate, myelinate, promote signal conduction, reduce astrogliosis, and ultimately promote functional recovery (Wilcox et al., 2014). Nonetheless, adult CNS-derived NPCs do not represent a practical option for clinical application, as CNS tissue is not safely accessible in adult humans. Similarly, embryonic tissue is not a suitable NPC source for transplantation into humans due to logistical and ethical concerns. As such, iPSCs represent an ideal alternative source of NPCs. Indeed, various protocols have been generated for the differentiation of human iPSCs into NPCs (hiPSC-NPCs) by imitating developmental signaling cascades via dual small mothers against decapentaplegic (SMAD) inhibition (Chambers et al., 2009) or neural rosette differentiation from EBs (Zeng and et al., 2010). Moreover, these hiPSC-NPCs can be further caudalized toward a spinal identity using RA in order to more closely recapitulate the lost spinal cells (Nutt et al., 2013). Notably, hiPSC-NPC transplantation has contributed to trophic support, myelination, synaptogenesis, axonal outgrowth, and functional recovery at varying degrees, depending on the injury severity and animal model (Qin et al., 2018). While autologous hiPSC-NPCs represent a promising emerging therapy, there are important limitations that need to be optimally addressed in order to promote clinical translation. To begin, the capacity of NPCs to contribute to repair and regeneration is often inhibited by the intense SCI microenvironment, which both alters the ultimate NPC differentiation and physically restricts repair across the lesion site. Indeed, NPC differentiation following transplantation remains inconsistent, and it often favors the maladaptive astrocytic lineage. Therefore, recent advances have been aimed at further restricting NPCs in vitro along an intended oligodendrogenic (Nagoshi et al., 2018, Biswas et al., 2019) or neuronal lineage (Chu et al., 2015). Furthermore, iPSC derived neurons can be specified into either motor neuron progenitors (Du et al., 2015) or interneurons (Butts et al., 2019) in order to optimally address particular functional deficits; however, further investigation is needed to clarify how these particular neuronal subtypes would be able to integrate with the appropriate endogenous circuitry. In addition, the accumulation of chondroitin sulfate glycosaminoglycans forms a glial scar that physically restricts the integration of the transplanted cells and their bridging of neural circuits. Nonetheless, NPCs can be transduced with chondroitinase ABC—an enzyme that digests the scar via cleavage (Jin et al., 2011). This process promotes the bridging of circuits across the scar cavity by eliminating the scar tissue that inhibits regeneration. As such, NPCs can be manipulated in order to overcome the intense SCI microenvironment and to optimally promote repair and regeneration through the replacement of particular cell types and the digestion of physical barriers. These advances will inevitably inspire future clinical trials.

iPSC-derived Schwann cells

Schwann cells represent a therapeutically relevant cell therapy for SCI. As these cells develop from the neural crest, they inhabit the peripheral nervous system where they myelinate neurons. However, following SCI, the cells display plasticity such

that they are able to contribute to remyelination of the CNS (Kim et al., 2017b). Importantly, transplantation of Schwann cells in SCI models has revealed that these cells protect and remyelinate neurons, restore signal conduction and, ultimately, promote locomotor behavior (Honmou et al. 1996, Mousavi et al., 2019). As such, clinical trials have been launched and completed in order to assess the safety of Schwann cells in both subacute (Anderson et al., 2017) and chronic SCI patients (ClinicalTrials.gov Identifiers: NCT01739023, NCT02354625). In these studies, Schwann cells have been isolated from autologous peripheral nerves, thus allowing for patient-specific treatments. While these studies have confirmed the safety, autologous peripheral Schwann cells must be expanded in culture prior to transplantation. Such expansion of autologous peripheral Schwann cells is often limited by inefficient division as well as fibroblast contamination in culture (Kim et al., 2017b). As such, iPSCs introduce an alternative method by which autologous Schwann cells can be generated for transplantation into SCI models. The proliferative capacity of iPSCs makes them an excellent candidate for derivation of Schwann cells for SCI (Ma et al., 2015). Lineage specification of iPSCs has been reported in two-step protocols, which first differentiate iPSCs into neural crest cells followed by further Schwann cell specification (Lee et al., 2010, Kim et al., 2017b). Although such iPSC-derived Schwann cells have not been tested in the context of SCI, they are able to secrete neurotrophic factors, promote myelination, and promote peripheral nerve regeneration in vivo (Kim et al., 2017b). As such, iPSC-derived Schwann cells represent a plausible treatment strategy that should be tested for SCI.

iPSC-derived mesenchymal stem cells

Mesenchymal stem cells (MSCs) are defined as multipotent cells that have a mesodermal identity as well as self-renewal capacity (Uccelli et al., 2008). They are an attractive cell candidate for cell replacement therapy as they can be isolated from a number of tissues including adipose tissue, bone marrow, umbilical cord blood, and dental pulp (Lv et al., 2014), which give rise to osteoblasts, chondrocytes, and adipocytes (Lv et al., 2014, Dominici et al., 2006). Moreover, MSCs can modulate the nonpermissive SCI environment and promote neural regeneration and survival by secreting antiinflammatory and neurotrophic factors such as BDNF, GDNF, and NTF (Cofano et al., 2019). Additionally, MSCs play an important role in immunomodulation following SCI. MSCs have been shown to mitigate circulating and splenic cytokine levels as well as immune cell responses, ultimately reducing signs of inflammation (Badner et al., 2018, Vawda et al., 2019, DePaul et al., 2015, Nakajima et al., 2012). Such changes are associated with locomotor benefits (Vawda et al., 2019, DePaul et al., 2015, Nakajima et al., 2012) and reduced pain (Watanabe et al., 2015), thus contributing to a plethora of MSC clinical trials on ClinicalTrials.gov. Of note, variations in MSC derivation introduce limitations that may complicate their application in a clinical setting. In an effort to promote personalized treatments, MSCs can be derived from patients' bone marrow or adipose tissue (Vawda and Fehlings, 2013). However, these tissues are not easily

accessible and require invasive procedures that may not be suitable for immunocompromised SCI patients. An easier alternative involves deriving MSCs from cord blood which has been banked by the patient, yet this introduces limitations for individuals that have not banked their MSCs (Cofano et al., 2019, Vawda and Fehlings, 2013). Even with the successful isolation of autologous MSCs from one of these sources, the cells are often difficult to expand in culture (Wang et al., 2018). Considering cell therapies require a large number of cells for transplantation, a source of MSCs that is not only easily accessible, but also easily expandable, is required. In addition to this, MSC derivation heterogeneity contributes to variations in differentiation, and MSCs also display considerable differences in gene expression across individuals (Frobel et al., 2014). Together, these factors may potentially give rise to variable therapeutic outcomes posttransplantation. For this reason, standardization of MSCs can be achieved through the development of iPSC-derived MSCs (Frobel et al., 2014). Derivation of MSCs from iPSCs has been described by many groups using several unique differentiation protocols (Chen et al., 2012, Giuliani et al., 2011, Hynes et al., 2013, Lian et al., 2010, Liu et al., 2013, Moslem et al., 2013, Sun et al., 2012, Tang et al., 2014, TheinHan et al., Dec. 2013). One of these protocols involves culturing iPSCs in nonadherent conditions to make EBs, followed by treatment with Activin and BMP4 (Bloor et al., 2020). Although such iPSC-derived MSCs have yet to be used in the context of SCI, they have previously been shown to have a similar gene expression profile to MSCs and have proven their capacity for immunomodulation in other contexts, thus making them applicable for future testing in SCI (Wang et al., 2018, Frobel et al., 2014, Ng et al., 2016).

iPSC-derived pericytes

Pericytes are a perivascular cell type that regulate angiogenic functions throughout the CNS, which involves the integrity of microvasculature and the blood-brain/spinal cord barrier (BBSB) through the lining of blood capillaries (Armulik et al., 2010, Bergers and Song, 2005). Pericytes can also promote neuronal regeneration in the context of injury, where they recruit immune cells, contribute to the formation of the fibrotic glial scar, and establish microvasculature growth as late as 21 dpi (Hesp et al., 2018). Improving microvascular integrity can potentially restore blood flow surrounding the traumatic lesion, which can reduce hypoxia and nutrient deprivation to the spinal cord.

Considering the roles of pericytes in the healthy and damaged spinal cord, iPSC-derived pericytes could potentially address the angiogenic deficits of SCI. Thus, it is theoretically practical to develop iPSC-derived pericytes to investigate their potential therapeutic benefit in SCI. Subsequent purification in culture can be obtained through cell separation via flow cytometry of PDGFRβ+ NG2+ cells (Jamieson et al., 2019), with further neuronal pericyte characterization using either FOXF2+, VTN+, and FOXC1+ cell types (Faal et al., 2019). Thus far, iPSC-pericytes have contributed to in vitro cell aggregation models of neurovascular units, which contain essential features of the CNS including neurons, neuroglia, and endothelial cells to

model development. In these models, the therapeutic potential of iPSC-pericytes is outlined through their ability to increase barrier properties by regulating isogenic pressure through sodium-potassium fluxes (Stebbins et al., 2019). Maintenance of ionic pressure is important because it can restore hemodynamic and cellular metabolic functions impaired in SCI. Apart from human-iPSC literature, peripherally injected murine pericyte-like stromal cells can mitigate BBSB disruption by providing structural support to CNS-vasculature, thus reducing BBSB permeability (Badner et al., 2016). Altogether, the aforementioned studies suggest that pericyte-like cells can be therapeutically useful to maintain endothelial vasculature integrity, which can reduce the angiogenic deficits of SCI through reduced immune cell infiltration and improved angiogenesis functionality. Despite not restoring neuronal circuitry, iPSC-derived pericytes could potentially restore microvasculature permeability, thereby promoting a healthier microenvironment for regeneration of neuronal tissue to occur.

iPSC-derived inflammatory mediators

The endogenous tissue repair that occurs in the CNS following trauma is at most, insufficient. At a molecular level, tissue repair and wound healing involves phases of macrophage subtype activation. The coordination between a pro-inflammatory cascade of M1 macrophages, followed by pro-regenerative M2 macrophage activation, can aid in wound healing through initial debris clearance and subsequent inflammatory homeostasis, respectively (Gensel and Zhang, 2015). However, the disruption of the BBB in neuronal injury can lead to an overwhelming inflammatory response of resident (microglia) and infiltrating macrophages. This leads to excessive and prolonged M1 activation, which inhibits the initiation of tissue remodeling through limited M2 activation. As a result, an imbalance of phagocytosis, reactive oxygen species (ROS) accumulation, and pro-inflammatory cytokines contributes to the subpar regenerative microenvironment of the CNS following trauma (Zhang et al., 2016). Thus, restoring the imbalance in macrophage phenotypes present after neuronal injury presents a promising therapeutic strategy to combat excessive inflammation in SCI.

Transplantation of macrophages may be beneficial when considering the antagonizing effects excessive M1 has on M2 activation. Experimentally increasing M2 activation following SCI through pharmacological treatment correlates with improved functional locomotor recovery and reduced pro-inflammatory signaling (Pomeshchik et al., 2015, Francos-Quijorna et al., 2017). Thus, transplantation of terminally differentiated human M2 identifying macrophages presents a unique approach to address the phenotype imbalance in neuronal injury. Deriving iPSC-macrophages (iMacs) can yield an undefined, embryonic-like macrophage phenotype (M0). This macrophagic state can be derived through a stepwise induction of pluripotent cell gastrulation, the development of hemogenic endothelial cells, and expansion of erythromyeloid progenitors (Lee et al., 2018). Theoretically, iMacs can be further differentiated into terminal, mature tissue repairing macrophages

through introducing M2-stimulating ligands (IL-4, IL-10, glucocorticoids, among others) into the culturing protocol (Martinez and Gordon, 2014, p. 2). Considering the sensitivity of macrophage phenotypes to environmental cues, transplantation of terminally differentiated M2-iMacs provides a unique dual benefit of using more translational human tissue, while transplanting a more rigid macrophage phenotype. Despite the lack of applications using iMacs in the context of SCI, information regarding the potential therapeutic benefit of macrophage transplantation and culturing purification can be cumulated to create a potential therapeutic avenue for SCI therapy. Future studies investigating how iMacs behave in the transplantation environment can elucidate if they retain their pro-repair macrophage phenotype-identity, which is required to restore the homeostatic balance of macrophage activation following SCI.

Application of iPSCs to generate 3D tissue for grafting into SCI

Bridging large gaps in the injured spinal cord using 3D-printed tissue

The idea of bridging the physical tissue gap in SCI using a support for successful nerve regeneration via three-dimensional (3D) tissue-like structure has produced moderate success (Kim et al., 2019, Zhu et al., 2015). During these extraneous efforts, various parameters, including the shape of the tissue-like structure, inner channel configuration, and pore size of the scaffold tissue have been tested. Neuro-Spinal Scaffolds have even entered Phase I clinical trials (Kim et al., 2019). However, one of the largest criticisms for using this approach is that it requires removal of a piece of host tissue in order to make enough space for the insertion of the scaffold. This may damage some of the spared tissue surrounding the scar and lead to more functional deficits. Despite this, the cavity space created from SCI trauma may be a potential space that can be filled by the 3D bio-printed tissue, if the 3D printed tissue can be carefully inserted in the cavity area without damaging the surrounding tissue.

Recent advances in tomography imaging and 3D bio-printing methods can increase the opportunity to carefully print 3D scaffolds and fabricate scaffolds with cells based on the exact dimensions and cavity shape in each patient. With this technology, a biological structure that highly mimics natural tissue properties can be fabricated using "bio-inks." By combining bio-printed scaffolds with NPCs or neural cells, it is now possible to make cellular 3D tissues that can be inserted into the cavity in order to fill the gap (Chen et al., 2017). Exemplifying their potential efficacy, the Tuszynski lab has recently transplanted a biomimetic 3D printed scaffold combined with NPCs (Koffler et al., 2019). They found that injured host axons can regenerate into 3D biomimetic scaffolds and synapse onto transplanted NPCs. In turn, transplanted NPCs extend axons out of the scaffold and into the host spinal

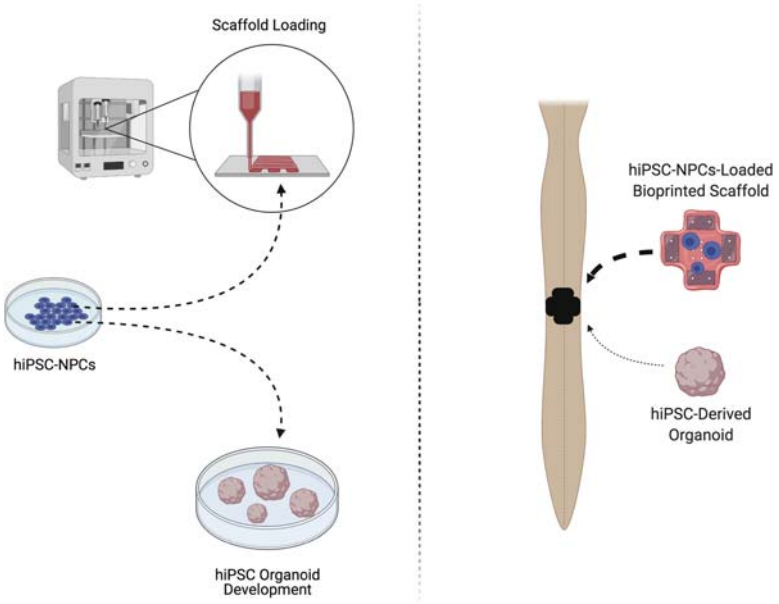

FIGURE 11.2 Generating 3D Tissues for Spinal Cord Injury (SCI).

3D tissues can be generated by loading 3D scaffolds with iPSCs or using iPSCs to create 3D organoids to physically, and literally, bridge the gap of neural tissue that follows SCI.

cord to restore synaptic transmission and significantly improve functional outcomes (Koffler et al., 2019). In a recent effort, Joung et al. attempted to combine a 3D bio-printed scaffold with hiPSC-NPCs, biased toward a spinal identity, as well as hiPSC-OPCs to generate a tissue with a similar structure to the spinal cord (Joung et al., 2018). Although promising, the challenge of safely inserting this bio-printed tissue in the cavity is yet to be optimized (Fig. 11.2).

Engraftment and transplantation of human organoids for SCI

Neural organoids have recently gained a lot of popularity in regenerative medicine. Neural organoids are 3D mini organ mimetics that can be derived from hiPSCs in vitro. Neural organoids are excellent physiological models for human neurological diseases, which cannot be recapitulated using animal models. The most feasible applications for organoid technologies at the moment are drug testing, disease modeling (see next section for SCI modeling), and studying human development. Recent success with engineering human organoids has created great enthusiasm and expectations, especially for their potential as tissue substitutes. Several studies have shown that transplantation of cells in the form of tissue and 3D matrices has a higher rate of cell survival compared to transplantation of single cells (Sekine et al., 2011, Daviaud et al., 2018). Brain organoids have also been tested in several studies

for the treatment of rat brain injury, which demonstrated the feasibility and efficacy of organoid transplantation for traumatic neuronal injury (Wang et al., 2020). Although generation of region-specific brain organoids is well established and several optimized protocols have been developed, generation of spinal organoids is still in the beginning of its field. Besides their identity, the spinal cord organoids should have distinct morphology and tissue architecture that suit the damaged tissue it is meant to replace. Recently, Hor et al. established a protocol to generate spinal organoids (Hor et al., 2018) using RA-treated and caudalized EBs within Matrigel droplets. These spinal organoids resembled the ventral spinal cord containing different spinal cell types including limb-innervating motor neurons, excitatory V2a interneurons, inhibitory Renshaw interneurons, and spinal-identifying astrocytes (Hor et al., 2018). This is a promising step, yet further refinement is required to derive spinal organoids that are best suited for transplantation for SCI. Once protocols have been optimized, organoids will provide an opportunistic method by which the cavity area in the injured spinal cord can be filled (Fig. 11.2).

Application of iPSC-derived organoids for spinal cord modeling and research

Using iPSCs-organoids to model spinal cord injury *in vitro*

Traumatic SCI leads to a diverse cellular response, involving an imbalance of extracellular components and excitotoxicity of neural cells, programmed cell death signaling, and immune cell infiltration. Thus, the premise surrounding SCI research suggests that this complex and step-wise pathophysiology can only be modeled through in vivo paradigms. Notably, in vivo modeling cannot isolate the direct pathological consequences that result from the mechanical trauma. Appropriately, in vitro injury paradigms have been developed to compartmentalize the pathophysiological consequences of SCI, such as the primary injury response to trauma, secondary injury inflammatory cascade, and the chronic glial scar formation. Current in vitro modeling of traumatic CNS-injuries has been aptly characterized through ex vivo organotypic slice cultures. In addition to being a comparable SCI model to in vivo exemplars, in vitro modeling of SCI presents several advantages over animal modeling of SCI. Primarily, animal models rely on spinal tissue for the coordination of muscle movement, respiratory and diaphragmic activity, sensory processing, and bladder control. When introduced to experimental injury, animals correspondingly exhibit functional deficits that require high maintenance of pain mitigation and physical rehabilitation of motor, sensory, respiratory, and urinary functions. Due to invasive modeling and extensive postoperative care, a large subset of these animals often do not survive. Thus, in vitro modeling provides a flexible and less time-consuming method to investigate SCI pathology.

Despite these illustrated benefits, limitations to ex vivo slice culture may impact their use and translation to human physiology. To date, in vitro modeling of SCI is

conducted solely in murine CNS tissue. Species diversity presents a confounding factor in translating in vitro modeling to the clinic, as murine models are commonly used to study neuronal injury, despite drastic anatomical and synaptic differences between quadrupeds and bipeds (Filipp et al., 2019). Additionally, modeling injury in quadruped animals may underestimate the extent of innate neuroplasticity and recovery in humans (Filipp et al., 2019). To circumvent this translational concern, human iPSC-derived organoids provide a unique alternative. Human iPSC-derived organoids can reflect diverse cell populations that accurately capture the in vivo environment following experimental injury, all the while ensuring the aforementioned benefits including high reproducibility, low maintenance, and high-throughput modeling.

Injury modeling to organoid culture

Pluripotent stem cells exhibit a remarkable ability to self-organize and assemble into miniature organ-like structures. Specifically, cervical Homeobox (HOX) B4+ and thoracic HOXC8+ identifying cells can be developed in spinal organoids that represent the morphogen gradient of cellular rostral-caudal positioning (Fig. 11.3) (Hor et al., 2018). Considering their proper cellular positioning,

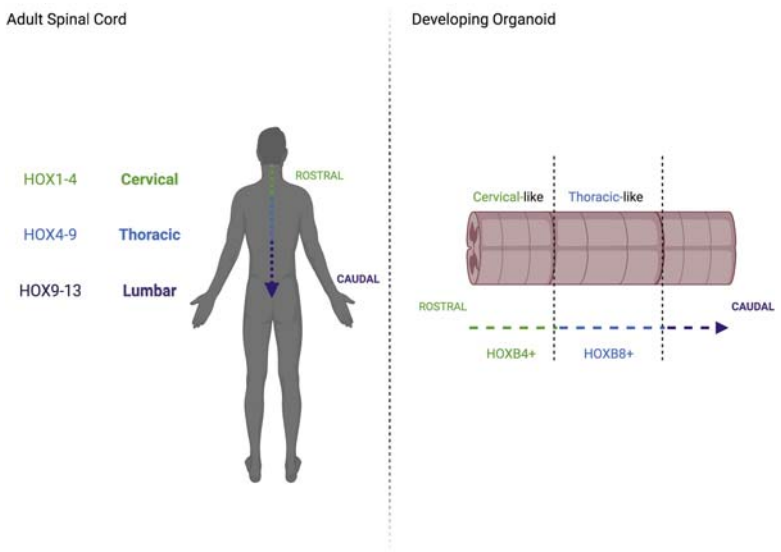

FIGURE 11.3 Cultured Spinal Organoids Recapitulate a Rostral-Caudal Axis.

During development of the mammalian central nervous system (CNS), segmentation of all cell types is dictated by Homeobox (HOX) genes. In the spinal cord, these factors regionalize the spinal cord into three main segments: cervical, thoracic, and lumbar regions. Remarkably, spinal organoids in culture can mimic this same developmental process, suggesting that these organoids can develop structural and functional components similar to the mature, adult spinal cord.

modeling of a variety of traumatic lesions can be sufficiently applied to these spinal-like structures. Similar to in vivo archetypes, primary injury mechanisms have been modeled through compression and transection forces using ex vivo cultures. Theoretically, mechanical injury models can be developed using organoids. Transection and axotomy can also be modeled with a scalpel blade to the organoid's surface, which models aspects of mechanical injury, with modest reproducibility of experimental results (Weightman et al., 2014, Slovinska et al., 2016). Considering there is limited description of how spinal organoids respond to mechanical injury, the potential efficacy of this injury design can be supported by comparisons with hypoxia-induced neural injury on cerebral organoids. Cortical organoids exposed to trauma can effectively model astrogliosis, as well as an increase in Tau pathology, which is correlated with a majority of neurodegenerative diseases (Lai et al., 2020). Specifically, the response to hypoxia in cerebral-region organoids can be mimicked in primary human tissue (Paşca et al., 2019), which illustrates how environmental factors can impact CNS-derived organoids similar to an in vivo injury environment.

Organoid model optimization: microfluidic platforms

Microfluidics presents a robust tool to introduce into a culturing system that can optimize the current modest injury reproducibility (Ao et al., 2020). Briefly, this may involve embedded organoid cultures in a 3D gel equipped with media channels that can regulate the biochemical environment, as well as outlet channels that can isolate axons to measure distance outgrowth and axonal guidance. Platforms can be incorporated into a single-dish protocol that involves EB formation, cellular embedment, and subsequent proliferation of organoids, which are equipped with an air–liquid interface to reduce hypoxia of medial organoid tissues (Ao et al., 2020, Srinivasan et al., 2014). Complex mechanisms involving synaptogenesis require axon terminals to identify appropriate synaptic targets. Axonal guidance, as well as axonal fascicle formation, is a crucially underdeveloped concept that is complicated by the complexity of in vivo cellular populations releasing extracellular factors, many of which include cellular adhesion proteins, growth factors, and morphogen gradients (Mukherjee et al., 2020, Kawada et al., 2017). In addition to chemical cues, microfluidics can also mimic the flow of cerebrospinal fluid in vivo. This is especially important, as fluid flow can shape organogenesis through axonal migration and turning angles (Gu et al., 2014, Sawamoto, 2006). Theoretically, these platforms can be used to model injury mechanisms, as well as ligand-based drug screening, to treat inflammatory and excitotoxicity consequences of neuronal injury.

Organoid model optimization: neurovascular units

Organoids exhibit neuronal maturation through diverse neurotransmitter and synaptic protein expression, but they do not typically contain a diverse population of neuroglia nor vasculature. As a result, it is difficult to represent immune or angiogenic dysregulation following injury. This presents another area of interest in spinal

organoid modeling to coordinate an accurate and translational response to mechanical injury. An assembloid, or a combination of cerebrospinal organoids with non-neuronal cell types such as microglia, endothelial cells, or pericytes, can appropriately recapitulate the complex cell environment of the CNS. Recently, an assembloid comprised of a human brain organoid and iPSC-derived microglial cells helped determine that iPSC-microglia respond similarly to mechanical injury through iPSC-derived organoid modeling, where they secreted cytokines in response to inflammatory cues, as well as contributed to synaptic pruning in organoid development (Abud et al., 2017). The fusion of organoids can promote a more translational model of human disease, which may involve the interaction of organ systems such as BBB disruption (Nzou et al., 2018), or domain-specific modeling within the human cortex (Xiang et al., 2017).

Using iPSC organoids to model development

The spinal cord is a very intricate system in which bundles of neural circuits are organized into distinct tracts that govern unique functions. These circuits involve coordinated interactions between sensory organs, sensory neurons, interneurons, motor neurons, and muscle (Chédotal, 2019). When the relay of these circuits is disrupted in SCI, function is lost accordingly. As previously mentioned, advances using iPSC technology have aimed to restore these relays through the generation and replacement of particular neuronal cell types, such as motor neurons (Du et al., 2015) or V2A interneurons (Butts et al., 2019). Although preliminary evidence suggests that transplanted cells seem to be able to integrate within appropriate circuits while avoiding the pathways that are irrelevant, the mechanisms underlying such integration require further research (Kadoya et al., 2016). The absence of such instructive cues would have important implications in the context of regeneration, and it may imply that transplanted cells need to be instructed by external artificial cues. Nonetheless, the investigation of this integration process is complicated by the fact that there is an inadequate understanding of how these cellular interactions are programmed during the development of the healthy cord (Chédotal, 2019). To optimize repair following injury, pluripotent cells, including ESCs and iPSCs, can be beneficial due to their ability to self-organize into 3D organoid structures, which can allow researchers to study interactions between multiple cells. Since their discovery in 2013, cerebral organoids (first described using ESCs) have provided an important platform by which axon guidance, synaptogenesis, and circuit formation can be studied (Giandomenico et al., 2019). For example, cerebral organoids have been shown to display various guidance molecules that help instruct and manipulate neuronal connectivity, such as WNT5A, Netrin, EphrinB1, L1CAM, and NRN1(Giandomenico et al., 2019). Similar studies using spinal cord organoids would help elucidate the mechanisms that govern interactions between different subtypes of neurons in order to achieve spinal circuit formation (Hor et al., 2018). Indeed, self-organizing motor nerve organoids have been generated. Importantly, the resulting nerve organoids mimic the unidirectional motor pathways seen

in vivo (Kawada et al., 2017). Moreover, iPSC-derived neuromuscular organoids can be used to further elucidate motor system formation by modeling the neuromuscular junction (Faustino Martins et al., 2020). This may allow us to gain an understanding about how motor neuron axons exit the CNS and interact with muscle. Most importantly, such circuit-mimicking organoids could then be screened and analyzed in order to identify the particular cues that may instruct circuit formation. This knowledge—generated with iPSC-derived organoids—would help optimize iPSC therapies by advising researchers whether or not transplanted cells need to be extrinsically guided toward the intended tracts.

Conclusion

Although SCI presents a complex and multifaceted disease etiology, iPSC technology aims to advance the therapeutic field by enhancing cell-based therapies and developing new injury models. Notably, iPSCs can be used for the derivation of optimal mature cells or 3D organoids that can be transplanted into the site of injury in order to promote repair. In addition, iPSC-derived organoids may present unique models that can aid in the development of SCI research. As such, iPSC technologies hold the potential to significantly advance SCI prognosis in the future.

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CHAPTER

12

Functional outcomes of copy number variations of Chrna7 gene: current knowledge and new insight from induced pluripotent stem cells studies

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Chapter outline

Novel Concepts in iPSC Disease Modeling, Volume 15. https://doi.org/10.1016/B978-0-12-823882-0.00012-6

Abstract

The discovery of genomic rearrangements, known as copy number variations (CNVs), is relatively new; their contribution to genetic heterogeneity and their impact on human diseases is still largely unknown. CNVs within the human 15q13.3 region have been associated with neuropsychiatric and neurodevelopmental disorders such as schizophrenia, autism spectrum disorders (ASD), intellectual disability, and epilepsy. Several recent studies suggest that, within this chromosomic region, the gene *CHRNA7,* encoding for the human alfa7 subunit of the nicotinic acetylcholine receptor, plays a major role in the observed pathological phenotypes. Consistently, patients carrying deletions or duplications of CHRNA7 present symptoms comparable to patients with a larger 1.5 Mb deletion of the 15q13.3 region. The penetrance of CHRNA7 CNVs is variable, and the exact pathogenic mechanisms are currently being elucidated.

In this chapter, we have critically reviewed all up-to-date studies regarding the functional outcomes of CNVs involving the alpha7-nicontinic receptor (α7nAChR), highlighting the advantages and disadvantages of the methodologies and models utilized. We have described the structure, functionality, and physiological role of the α7nAChR, analyzing the mechanisms that determine the occurrence of CNVs and the clinical features of patients carrying *CNRNA7* CNVs. We have examined and compared the mice models used to study the role of these CNVs and the new human model of induced pluripotent stem cells, which is proving very useful in clarifying the clinical and phenotypic features pertaining specifically to humans.

Keywords: alpha7 nicotinic acetylcholine receptor; CHRNA7; CNV; Copynumber variation; Epilepsy; Induced pluripotent stem cell; iPSC; Microdeletion; Microduplication; nAChR; NAHR; Neural stem cells; Neurodevelopmental disease; Neuropsychiatric disease; Schizophrenia.

Introduction

Genetic variations in the human genome are responsible for the great variability in the inheritance of human traits. In 2007, during the first sequencing of the human genome, it first became evident that genetic content varied within the chromosomal regions (Levy et al., 2007). These structural variations are now known as Copy Number Variations (CNVs). In the following years, the ability to detect CNVs improved progressively, and the recent development of higher resolution genomic technologies, in particular in the field of clinical diagnostics, has led to the discovery of new CNV-dependent disorders (Pang et al., 2010; Conrad et al., 2010). It has thus become clear that CNVs are not only an important source of genetic diversity within the general population but are also significantly associated with many human diseases, the so-called genomic disorders. Although the role of certain CNVs in the human genome appears to be benign, numerous CNVs have been implicated in the etiology of a wide variety of human diseases, among which neurodevelopmental disorders, multiple congenital anomaly syndromes, complex neurodegenerative and neuropsychiatric disorders, cancer and immune deficiency

(Stankiewicz and Lupski, 2010; Girirajan et al., 2012; Karaca et al., 2015; Larson et al., 2018; Pizzo et al., 2019). When present, these genomic variants can modify specific phenotypic traits, which would explain why individuals presenting the same CNV(s) might not display a pathological phenotype (incomplete penetrance) or why individuals carrying the same variant might show different phenotypes (variable expressivity). This model could be also applied to single-gene disorders, in as much as further hits caused by CNV(s) could potentially explain why there may be discordant clinical features reported among affected carriers of the same molecular alteration. To further complicate the landscape, it is emerging that the same disorder can be caused by different CNVs (genetic heterogeneity) and that a specific CNV can be associated with different disorders (pleiotropy).

This chapter on genetic disorders arising from CNVs hitting the CHRNA7 genomic region of chromosome 15 will be divided into three parts:

In the first part, we will focus on the mechanisms that determine the occurrence of these CNVs, observing the chromosome structure that mediates their formation, and we will explore the genetic conditions that might lead to disease development by influencing gene/protein expression.

Secondly, we will be taking a closer look at nicotinic receptors with particular attention to α7. We will examine the protein structure of nicotinic receptors, the mechanism at the base of the action of α7, its pharmacological characteristics, its role during normal brain development, and the clinical features of patients carrying CNVs on the alpha7-nicotinic acetylcholine receptor (α7nAChR).

Subsequently, we will consider the mice models used to study the role of these CNVs in the brain and then describe how the new human model of induced pluripotent stem cells has helped to clarify the clinical and phenotypic features pertaining specifically to humans. The iPSC lines, obtained directly from patients, provide an excellent human model for studying the role of this receptor during neuron differentiation and for developing pharmacological molecules capable of influencing human pathological behavior dependent on these CNVs.

In the conclusion, we will discuss a new approach for considering CNV-dependent diseases, thanks to the studies developed on iPSC lines.

CHRNA7 locus and CNVs (copy number variations)

Taking a more detailed look at these variations, we will take up the following questions regarding the exact definition of these CNVs, the consequences of their presence, and what causes them to form inside the human genome.

Copy number variations (CNVs) are defined as changes in large DNA regions, differing among individuals, which consist of duplications and/or deletions larger than 1 Kb of one or both alleles (Feuk et al., 2006; Chance et al., 1994; Stankiewicz and Lupski, 2002). CNVs play an important role in modulating the phenotypic spectrum, both in monogenic and polygenic diseases, and are most likely responsible for about one-fifth of the known neurodevelopmental disorders

(Stankiewicz and Lupski, 2002), including autism, schizophrenia, intellectual disability, attention-deficit hyperactivity disorder, developmental delay, and epilepsy (Battaglia et al., 2013). CNVs are located both in chromosomal regions containing genes and in regions without genes. Rather than being randomly distributed across the genome, their presence correlates with specific structural features of the chromosomes (e.g., inverted repeats, segmental duplications or low copy repeats (LCRs), highly homologous small regions, etc.) (Chance et al., 1994; Cooper et al., 2007; Perry et al., 2006; Sharp et al., 2005; Shaw et al., 2002).

LCRs, or segmental duplications, are duplicated genomic sequences of a size ranging from 1 to 400 Kb, with a sequence homology >95% (Sharp et al., 2005), mainly located in peri-centromeric and subtelomeric regions (Zarrei et al., 2015). These sequences, which, from an evolutionary point of view, are themselves CNVs, cause several genomic regions to be particularly prone to genomic rearrangements. These sequences, in fact, are *in cis* substrates that mediate the nonallelic homologous recombination mechanism (NAHR). NAHR is a recombination event in which, during meiosis or mitosis in these regions, nonallelic copies of LCR may align themselves irregularly. This misalignment can take place between different loci, either on the same chromosome or on different chromosomes. The result of these ectopic recombination events is the main mechanism of further CNVs (Stankiewicz and Lupski, 2002).

In this chapter, we will focus on the CNVs affecting the 15q13.3 chromosomal region encompassing the CHRNA7 gene (CHRNA7-CNVs). *CHRNA7* encodes for the α7 subunit of nicotinic acetylcholine receptors (nAChRs), which represents a family of ligand-gated ion channels (Schaaf, 2014). This gene includes ten exons: exon 1 encodes the signal peptide sequence, which allows the α7 protein to enter into the endoplasmic reticulum (ER), where it undergoes a maturation process; exons 2 through 6 contribute to the formation of the extracellular ligand-binding domain, which contains the glycosylation sites, which are necessary for the correct functioning of the α7 homomeric receptors (Chen et al., 1998). Exons 7-8 encode for the membrane spanning region, while the last two exons encode for the intracellular portion. These exons produce a transcript that is translated into a protein of approximately 57 kDa (OMIM number * 118,511). *CHRNA7* gene maps into the proximal region of the long arm of chromosome 15, particularly enriched by a series of Low Copy Repeats (LCRs), whose members are called BP1−BP5. In particular, BP4 and BP5 members share a large amount of homology, with three regions (in inverted orientation from one another) of 95 kb, 140 kb, and 218 kb, containing sequences which are 99.6% identical. Moreover, a further LCR element distal to *CHRNA7* has been mapped between exons 1 and 2 of *OTUD7A* (D-CHRNA7-LCR). The region involved in the rearrangement can vary in size, depending on which members of these LCRs pair during meiosis. NAHR between the BP4 and BP5 members leads to the formation of the most frequent reciprocal 1.6 Mb 15q13.3 microdeletion/microduplication, encompassing six genes, *MTMR15, MTMR10, TRPM1, KLF13, OTUD7A*, and *CHRNA7* and one microRNA: hsa-miR-211 (van Bon et al., 2009). Moreover, the smallest recurrent CNVs

encompassing only the *CHRNA7* gene have been described, mediated by BP4 and D-CHRNA7-LCR, as measuring approximately 680 Kb (Shinawi et al., 2009; Szafranski et al., 2010) (Fig. 12.1).

This genomic region is complicated by the presence of the chimeric *CHRFAM7A* gene, composed of exons 5−10 of the *CHRNA7* gene fused with four exons of *FAM7A* gene, located 1.6 Mb upstream of the CHRNA7 gene (Gault et al., 1998; Sinkus et al., 2009; Sinkus et al., 2015). The four exons of FAM7 gene encode 27 amino acids while exons belonging to CHRNA7, encode for a protein which has lost the sequence motif for endoplasmic translocation (Goder and Spiess, 2001; Kozak, 1999). The formation of *CHRFAM7A* is human specific and relatively recent in human evolution, as it is not found in either rodents or in primates. It is expressed in the human brain at low levels (approximately one order of magnitude less than *CHRNA7* in the hippocampus), but more abundantly in peripheral lymphocytic cells and in numerous other tissues (Gault et al., 1998; Sinkus et al., 2009; Riley et al., 2002; Flomen et al., 2006). The *CHRFAM7A* copy number differs among individuals, ranging from zero to three copies, and encodes for a product lacking part of the *CHRNA7* ligand binding site (Sinkus et al., 2009).

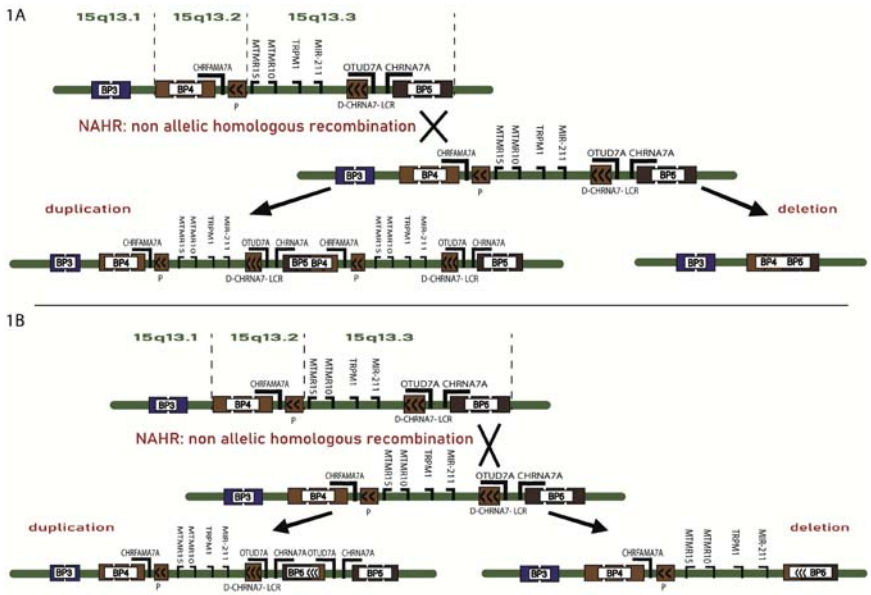

FIGURE 12.1

Schematic overview of the regions 15q13.2 and 15q13.3: (A) Nonallelic homologous recombination between BP4 and BP5 members leads to the formation of the reciprocal 1.6 Mb 15q13.3 microdeletion/microduplication; (B) Nonallelic homologous recombination mediated by BP4 and D-CHRNA7-LCR.

The ancestral allele lacking the fusion gene is rare in the population today (1% circa), while nearly the entire human population harbors the fusion gene, which might imply a selective advantage. The CHRFAM7A null allele is probably part of a chromosome structure that precedes the emergence of CHRFAM7A: two possible haplotypes are illustrated in Fig. 12.2A.

Regarding the CHRFAM7A genotype, two differing hypotheses were proposed (Riley et al., 2002; Flomen et al., 2008) to explain its emergence: the researchers modeled two possible routes through which successive rounds of deletion, duplication, and inversion might have produced the chromosome that we have today. Interestingly, when present, *CHRFAM7A* exists in two orientations with reference to *CHRNA7* (Flomen et al., 2008) (Fig. 12.2B). When the orientation of CHRFAM7A is the same with respect to CHRNA7, it is generally accompanied by a 2-bp deletion functional polymorphism, within exon 6, which has been associated with schizophrenia and bipolar disorder (Fig. 12.2C).

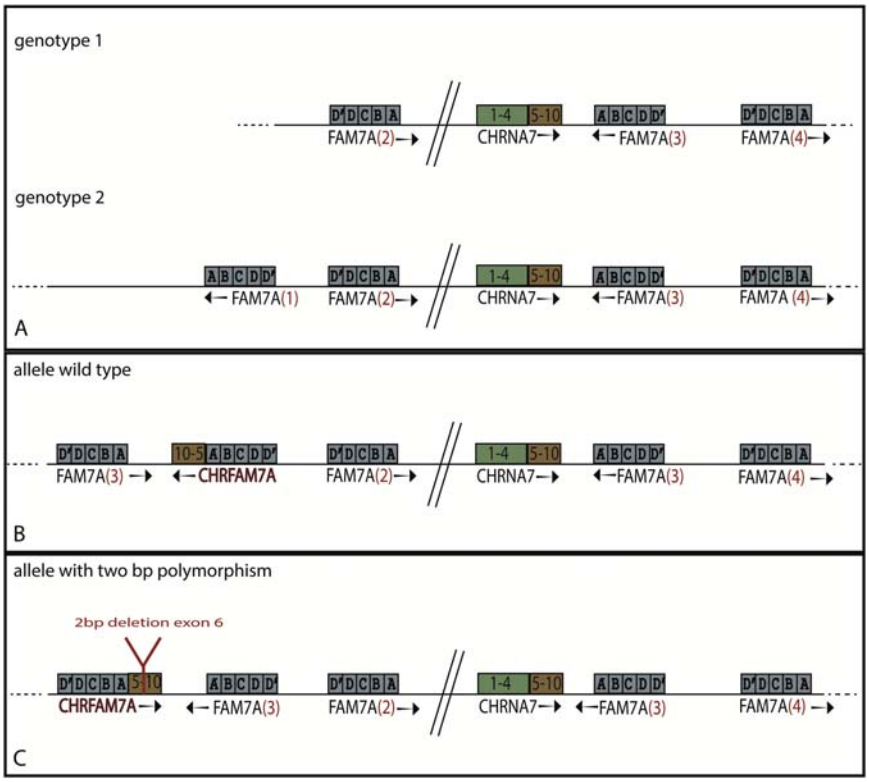

FIGURE 12.2

Genomic map of CHRNA7 and its partial duplication, CHRFAM7A: (A) two possible haplotypes of CHRFAM7A null allele; (B) wild-type CHRFAM7A allele; (C) 2 bp polymorphism and inversion of CHRFAM7A.

The mechanisms of action in CNVs can be multiple. It is possible that CNVs, altering gene expression, can create, on the one hand, a neutral phenotype if the genes affected are not dosage sensitive. On the other hand, the alteration in gene expression can create a pathological phenotype in the case of dosage-sensitive genes mapping within the CNV (Lupski and Stankiewicz, 2005). A dosage sensitivity model implies that a 50% increase or decrease in gene copy number is deleterious. Various mechanisms can bring this about: (1) a change in stoichiometric balance between CNV-dependent gene products and other proteins. When the gene is duplicated, the protein product aggregates pathologically because it is present in too high concentrations. When the gene is deleted, protein function may be compromised because it cannot reach the minimum required concentration; (2) the unmasking of a mutated recessive-allele, which can be understood as follows: when a CNV creates a deletion on an allele, and there is a pathological mutation on the allele situated on the homologous chromosome, this sole remaining recessive (mutated) allele is unmasked, becoming functionally dominant, and causing the disease; (3) the perturbation of chromosome functional domains interfering with right gene expression regulation, through the modification of the relative distance between the gene and its regulatory region, in the case of a duplication, or the removal of regulatory elements, such as enhancers, in the case of a deletion (Rice and McLysaght, 2017) (Fig. 12.3).

Nicotinic receptors

In the next paragraphs, we will focus on the structure of nicotinic receptors, with a closer look at α7nAChR, at its structure, at the pharmaceutical molecules that have been developed on this protein, and finally on its role in brain development and maintenance. The nAChRs belong to the family called "cholinergic receptors," thanks to their ability to bind to the neurotransmitter acetylcholine (ACh). These ionotropic receptors are channels that conduct Na^+, K^+, and Ca^{2+} ions across the plasma membrane, subsequent to the ligand binding. Nicotinic receptors were first identified in muscle; the first four muscle-type nAChR subunits were called α1, β1, γ, and δ. The other subunits, correspondently called α2−α10 and β2−β4, are largely expressed in the nervous system, both in neurons and glial cells (Changeux and Edelstein, 2005). The α and non-α subunits are encoded by separate genes and display around 30%−40% of amino acid sequence homology. In the membrane, these subunits are organized as heteropentamers or homopentamers, forming the receptor channel (Fig. 12.4A). The functional and structural diversity of nicotinic receptors depends on the possible combinations of these subunits. Each subunit is made up of different domains: the N-terminal extracellular domain, which carries the agonist-binding site, four transmembrane domains (TM1, TM2, TM3, TM4) with the TM2 domain forming the walls of the ion pore, and a variable intracellular cytoplasmic loop that contains sites for receptor modulation and channel conductance determinants (Morales-Perez et al., 2016; Lendvai et al., 2013; Corradi and Bouzat, 2016). Interfacing between the extracellular and

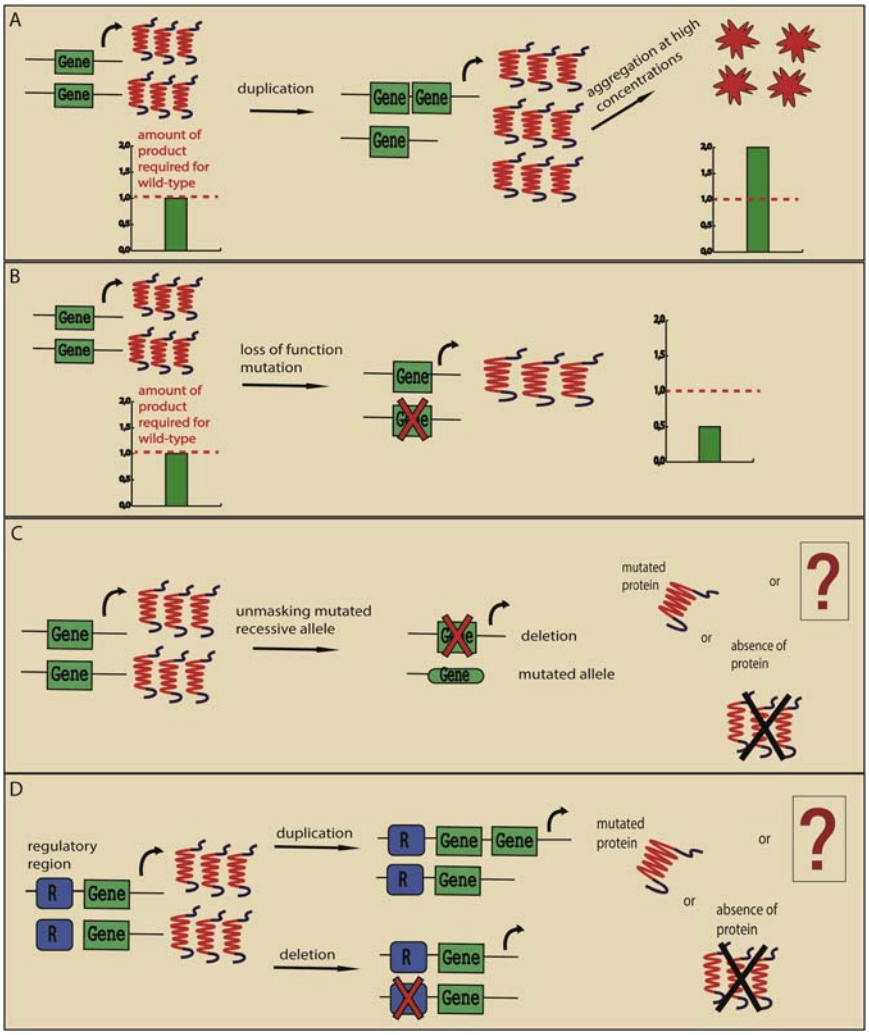

FIGURE 12.3

Various mechanisms of dosage sensitivity: (A) When the gene is duplicated, the protein product aggregates pathologically; (B) when the gene is deleted, protein function may be compromised; (C) a CNV creates a deletion in an allele, and there is a pathological mutation on the allele situated on the homologous chromosome; the remaining recessive (mutated) allele is unmasked; (D) in the case of a duplication or a deletion, the relative distance between the gene and its regulatory region is modified.

transmembrane domains, there is a disulfide-linked loop which makes an essential contact with the loop connecting the TM2 and TM3 sequences, forming a network that relays structural changes from the binding site toward the pore. This region, named the coupling region, contributes to the fundamental mechanism of receptor

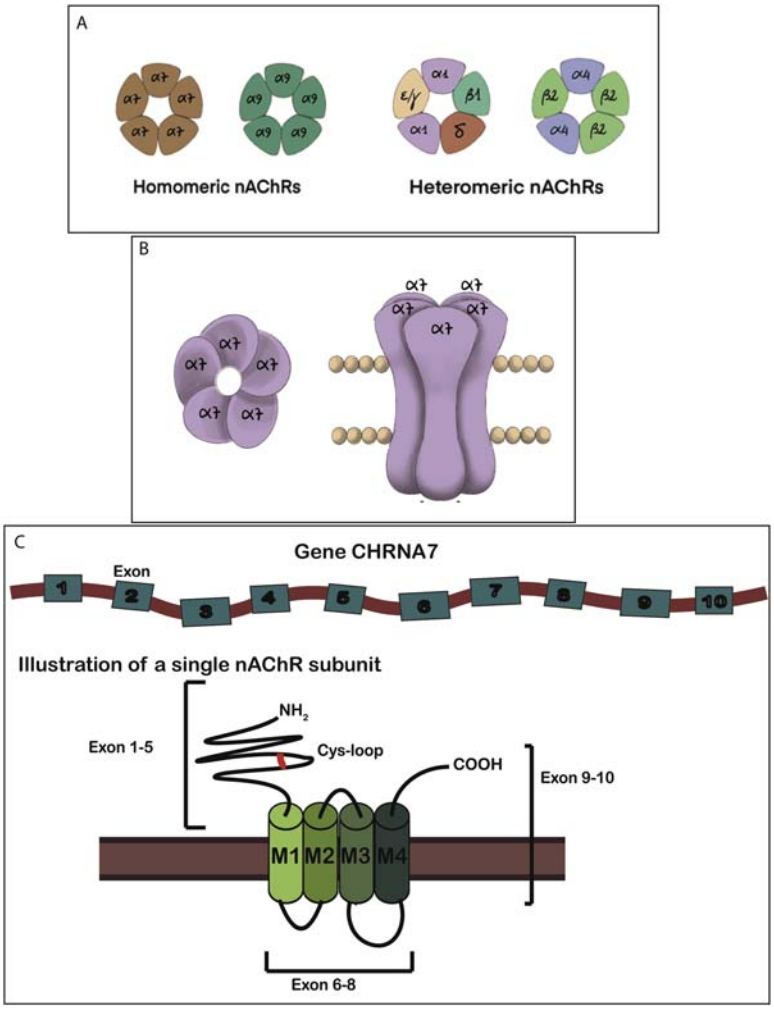

FIGURE 12.4

(A) Schematic representation of some of homo- and heteromeric nicotinic receptors types; (B) The organization of α-7 nicotinic receptor subtype; (C) Coding sequence of α-7 nicotinic receptor and relative organization of NH_2-, COOH- and transmembrane domains.

activation (Bouzat et al., 2004; Lee and Sine, 2005; Bartos et al., 2009). In particular, each binding site is formed at the interfaces between pairs of subunits, while each coupling region is confined within each subunit. In particular, the homomeric α7 receptor has five identical ACh-binding sites; however, ACh occupancy of only one site is sufficient for activation (Andersen et al., 2013). In heteromeric receptors, the non-α subunits (β, γ, ε, γ) do not participate in the ACh binding but

may be relevant for the binding of allosteric ligands (De Jaco et al., 2017). The composition of the subunits confers the pharmacological and functional properties of each receptor subtype (Fig. 12.4).

α-7 nicotinic receptor subtype: functional characteristics and pharmacology

α7nAChR is one of the main receptor subtypes expressed in the brain, where it has a central role in correct fetal brain development and also, in correct adult brain functioning, such as learning and synaptic plasticity. Numerous papers have been published in the last 40 years on this protein and on the development of molecules designed to influence its functions. The first approach taken was to mutagenize single residues or to delete part of the protein and use these recombinant proteins in order to measure calcium flux associated with the binding of acetylcholine (Ach); these experiments gave the researchers the capacity to define important regions, such as ligand-binding sites, antagonist-binding sites, allosteric sites modifying the protein structure, and intracellular sites implicated in protein−protein interaction. Later, numerous molecules (agonists, antagonists, positive allosteric modulators [PAMs]) were analyzed for their capacity to influence channel function.

Compared to other nicotinic receptors, α7 exhibits unique functional characteristics which include:

- a high permeability to calcium. Generally, the binding of the acetylcholine with α7nAChRs triggers a flow of Ca^{2+} and Na^{+}, which enter the cells through the opened channel. Normally this gradient also involves the increased release of Ca^{2+} from intracellular storage, for example, from the ER.
- A rapid activation and desensitization phase (in the order of milliseconds). The calcium current has a short duration and rapidly brings the channel receptor to its desensitized state, in which, even if the ligand is present at the binding site, it is not able to conduct the ionic flow. In neurons, the release of intracellular Ca^{2+} mediated by α7 contributes to the mechanisms of synaptic plasticity (Shen and Yakel, 2009).
- a selective inhibition given by α-bungarotoxin and methylcyconitin (MLA) and a low affinity for nicotine.

Recently, many papers have shown that α7nAChR-mediated calcium signaling occurs via ionotropic calcium influx, due to the opening of the α7 channel and, also, via metabotropic activity, through G protein coupling (King et al., 2015; King and Kabbani, 2016; King et al., 2018) (Fig. 12.5). In neurons, both ionotropic and metabotropic α7nAChR functions are observed. Activated ionotropic α7nAChRs in synapses can involve a wide range of Ca^{2+} sensitive targets, including enzymes such as cyclic-dependent AMP protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase. These kinases are sensitive to Ca^{2+} levels and can regulate various synaptic ion channels as well as cytoskeletal and trafficking proteins, which control vesicle release and mobility (Shen and Yakel, 2009). Furthermore, cell calcium signaling

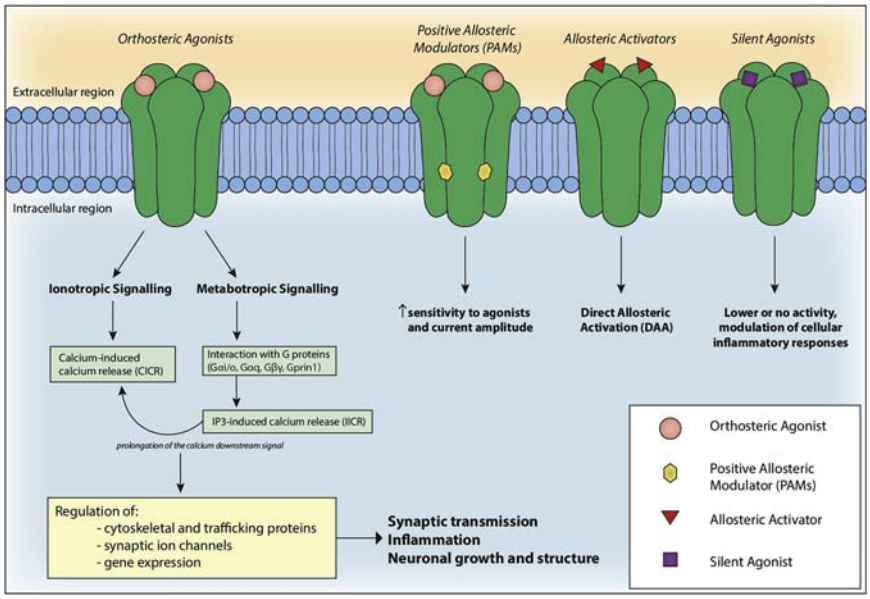

FIGURE 12.5

α-7 nicotinic receptor subtype: calcium signaling and pharmacology. On the left, orthosteric agonists and α7nAChR-mediated calcium signaling via ionotropic and metabotropic activities. On the right, classes of ligands that selectively modulate the α7 nicotinic receptor activity: positive allosteric modulators (PAMs), allosteric activators, and silent agonists.

mediated by nAChR regulates gene expression in neurons, controlling the activation of transcription factors such as CREB, which play an important role in memory and learning (Chang and Berg, 2001).

It has been suggested that ligand-driven structural transition of the α7nAChR to the open channel state can also enable a metabotropic signaling state during channel desensitization. In this regard, metabotropic signaling by the α7 receptor can provide greater spatial and temporal receptor influence in the cell (Kabbani and Nichols, 2018). King and collaborators defined a G protein-binding cluster (GPBC) within the TM3−TM4 loop of the α7nAChR, which mediates interactions with G protein. The lack of the G protein-binding cluster generates a dominant negative α7nAChR mutant (a7345−348A), impaired in downstream G protein signaling (King et al., 2015). An interesting feature of this receptor is that it exhibits ligand-mediated metabotropic-like signal transduction behavior, which appears to be independent of detectable ion-currents (Kabbani and Nichols, 2018). This can be pharmacologically significant, as explained below.

A large number of ligands that selectively modulate the α7 nicotinic receptor activity were initially developed and tested for their actions on the functionality of this receptor. Subsequently, their therapeutic properties were tested on those

mechanisms in which this receptor is implicated, regarding inflammation, memory, and behavioral disorders. Today, several drugs targeting the nAChRs are currently in the clinical trial stage. Several α7 full agonists have been characterized: the natural neurotransmitter acetylcholine, SEN 12333/WAY-317538; PNU- 282907; AR-R17779 (Levin et al., 1999), EVP6124 and TC5619; their functions are to bind the orthosteric ligand site and to drive conformational transitions through open-channel states, then into desensitized states (Hibbs et al., 2009). Agonist activation of nAChRs may also occur via an allosteric site (Direct Allosteric Activation, DAA). Moreover, several studies also focused on partial agonists for nAChRs, ligands that activate the ion channel with lower efficacy than the endogenous agonists (i.e., nicotine, GST-21 (Meyer et al., 1997). Among these ligands, the S24795 has been studied for AD. Recently, attention has been drawn to a new class of drugs, termed silent agonists (Horenstein and Papke, 2017), which produce very little channel activation (having $< 10\%$ efficacy compared to Ach) but are profoundly desensitizing. One example is NS6740 (Papke et al., 2018), which produces very stable and long-lived desensitization (Godin, 2019). PAMs are another kind of potential drug candidate. PAMs can selectively modulate the activity of Ach at α7nAChRs, preserving the activation and deactivation kinetics of the receptor, without leading to desensitization after prolonged binding to the receptor (Eskildsen et al., 2014; Unal et al., 2020) (Fig. 12.5).

A few words should be said regarding the protein product of the CHRFAM7 gene found inside the CNV, which is associated with α7. In recent years, its role has been allotted more and more importance as its presence/absence/duplication provokes pharmacological responses quite different from those resulting in mouse models.

In humans only, the *CHRFAM7A* gene encodes a specific deleted isoform of the α7nAChR called dupα7 subunit. Several investigators have demonstrated that the dupα7 subunit alters the assembly and function of the α7nAchR complex by forming a heteropentamer of α7/dupα7 subunits, which alters ligand binding to the receptor (Sinkus et al., 2015; Costantini et al., 2015). Lasala and collaborators (Lasala et al., 2018) demonstrated in recipient cells, following a quantitative dupα7 overexpression, that: (1) dupα7 alone does not form functional ion channels; (2) the dupα7 subunit can assemble with α7, forming a variety of heteromeric α7/dupα7 receptors, whose functional capacity depends on stoichiometric subunit binding. For functional heteromeric α7/dupα7 receptors, in fact, at least two α7 subunits are required and the activation of heteromeric receptors requires a α7/α7 interfacial-binding site. Heteromers containing four dupα7 subunits are nonfunctional and those with three or less dupα7 subunits, despite being functional, have reduced Ach sensitivity, due to the reduced number of active Ach-binding sites (Andersen et al., 2013; Andersen et al., 2011). An additional negative modulatory action of dupα7 might be associated with a decrease in the number of surface α7 receptors. The authors focused their attention on ionotropic responses; no data are as yet available regarding the dual ionotropic/metabotropic receptor function. It has further been reported that CHRFAM7A expression likewise alters binding to the α7nAChR in neuronal cells as well (Chan et al., 2019) (Fig. 12.6).

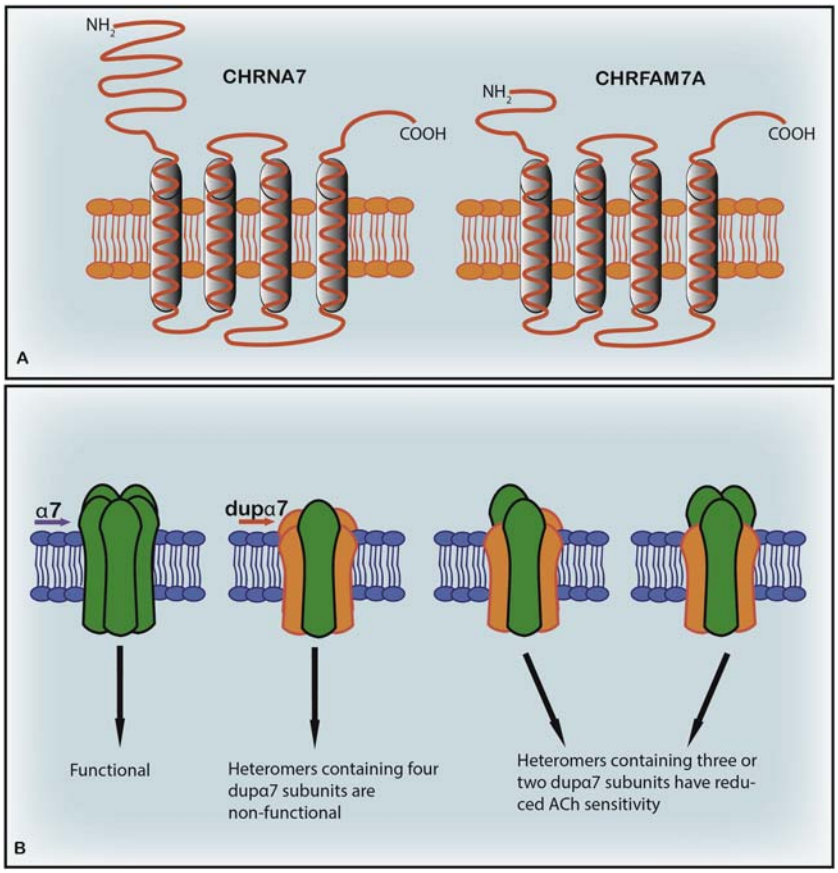

FIGURE 12.6

(A) Structure of CHRNA7 and CHRFAM7A gene products; (B) Pentameric structure of the normal α7nAChR and three type of pentamer containing the peptide of the duplicated subunit (dupα7).

Distribution and functions of α7 nicotinic receptors in the nervous system

Three approaches have been developed to study a7nAChR in mice models: (1) studies on the expression and function of this receptor during prenatal development and, following birth, during adult-onset neurogenesis (Otto and Yakel, 2019; Broide et al., 2019); (2) behavioral and functional studies performed on transgenic mice through the deletion and mutagenesis of CHRNA7; (3) studies using molecules targeting α7nAChR, directly infused into normal mice and mice carrying several pathological conditions, such as neurodevelopmental/neuropsychiatric/neurodegenerative diseases, in which nicotinic receptor involvement was demonstrated by the therapeutic

effects (De Jaco et al., 2017; Lykhmus et al., 2020; Wang, 2017; Neves and Grace, 2019). The analysis of α7nAChR mRNA expression throughout normal mouse whole-body during embryonic and neonatal development, published in 2019, highlighted information on the brain, previously established in other articles on chickens (Erkman et al., 2000; Keiger et al., 2003), rodents (Broide et al., 1995; Messi et al., 1997; Adams et al., 2002), and humans (Hellström-Lindahl et al., 1998; Falk et al., 2002), regarding its abundant distribution in the central (CNS), peripheral (PNS), and enteric nervous systems. During brain development in mice, in fact, it was found that there was an overall increase in α7 mRNA expression, in particular in the autonomic and parasympathetic ganglia, as well as in cortical structures, comprising the hippocampal anlage expression, which became more committed to specific brain structures and nuclei. Low α7 mRNA expression was also observed in various non-neural tissues throughout the body, including adrenal medulla, kidney, tongue, tooth, nasal epithelium, prostate, testis, and muscle, while expression levels were higher in the adrenal medulla. This widespread expression of α7nAChRs throughout the developing nervous system and the adrenal medulla suggests that these receptors have important functions during development. Great attention was given to α7nAChRs distribution in the hippocampus during development. In this region, the α7nAChR, located pre- and postsynaptically, contributes to cognition, attention, learning, and memory (Hasselmo, 2006; Levin, 2012). Moreover, this receptor, mediating the stimulus of Ach, is implicated in hippocampal adult-neurogenesis, a life-long process whereby neural stem cells (NSCs), located in both the subventricular zone (SVZ) and the subgranular zone (SGZ) of the mammalian dentate gyrus (DG) divide, differentiate, mature, and integrate into the local circuitry (Aimone et al., 2014; Altman and Das, 1965; Bonaguidi et al., 2012; Eriksson et al., 1998; Gonçalves et al., 2016; Spalding et al., 2013). α7nAChRs, present in the SVZ, are also important for cell proliferation, differentiation, and survival (Inestrosa and Arenas, 2010; Kaneko et al., 2006). Two interesting articles published in 2019 and 2020 (Otto and Yakel, 2019; Baradaran, 2020), respectively, have taken into consideration the influence of α7nAChRs in hippocampal lateralization. Two characteristics are typical of the hippocampus: asymmetry and sexual dimorphism in structure and function. It is believed that hippocampal asymmetry developed for the purpose of simplifying functions such as memory and navigation (Eichenbaum and Cohen, 2014). In humans, a high level of cognitive capacity is associated with increased hippocampal lateralization (Gotts et al., 2013), while defects in left-right asymmetry have been observed in autism and schizophrenia ((Schumann et al., 2004; Hanlon et al., 2005). Sexual dymorphism may contribute to cognition, influencing hippocampal adult neurogenesis. Concerning mice models, there was an association between levels of adult neurogenesis and performance in tasks measuring pattern separation, spatial discrimination, or cognitive flexibility (Clelland et al., 2009; Cushman et al., 2012; Hollands et al., 2017; Pan et al., 2012; Sahay et al., 2011). Interestingly, these papers demonstrated that: (1) the expression of the α7nAChR subunits in the left hippocampus was significantly higher than those on the right side; (2) sexual dimorphism disappeared when α7nAChR was removed. Male mice showed both loss of neural stem cells and

decreased performance in the task subsequent to the loss of α7nAChR, while this was not seen in females. These results highlighted that the presence of a7 receptors is important for correct hippocampal homeostasis.

Another important aspect in brain structure that has been reported is that functional nAChR responses can be found not only in neurons but also in nonexcitable cells, including microglia, astrocytes, and myelinating glial cells (De Simone et al., 2005; Fields et al., 2017). The expression of α7nAChRs in the immune system and in microglia is of particular interest, owing to its crucial role in the regulation of the cholinergic antiinflammatory pathway, both in peripheral areas and in the brain, which has been largely demonstrated (Fields et al., 2017; Reale et al., 2015). Moreover, α7nAchR appears to be one of the major regulators in the initiation, progression, and metastasis of various forms of cancer.

Clinical characteristics of CHRNA7 CNV in human patients: heterozygous deletion and duplication, homozygous deletion

The clinical features of individuals carrying CHRNA7-CNVs are highly variable. Our description is based on papers selected from the literature in which the observation of the phenotypic characteristics is accomplished via genetic analyses. We will examine the clinical phenotypes of the entire spectrum of CHRNA7-CNVs, including heterozygous deletions, homozygous deletions, heterozygous duplications, and triplications.

Several published articles have reported that the deletion of the one copy of *CHRNA7* gene (Masurel-Paulet et al., 2010) or of the region comprehending the *CHRNA7* gene (van Bon et al., 2009; Ben-Shachar et al., 2009; Miller et al., 2009; Sharp et al., 2008; Stefansson et al., 2008) produces a clear pathological phenotype which becomes much more evident when the deletion is present in homozygosis (Hoppman-Chaney et al., 2013). There are no evident clinical differences among CNVs of the single CHRNA7 gene or of the largest, and most common CNVs comprehending several genes including *CHRNA7* (Lowther et al., 2015). Thus, the size of the deletion does not consistently correlate with the severity of symptoms, corroborating the hypothesis that *CHRNA7* is the most significant gene in all of these genomic alterations. The heterozygous BP4-BP5 microdeletion, 1.6 Mb in size, represents the most frequent CNV affecting *CHRNA7*. Described for the first time in 2008 by Sharp et al. (2008), it is associated with a wide range of neurodevelopmental disorders, such as intellectual disability (ID), developmental delay (DD) with language or speech impairments and idiopathic generalized epilepsies, whereas congenital abnormalities are less common (<5%) and do not present with distinctive dysmorphic features. Neuropsychiatric phenotypes are very common, affecting more than 80% of individuals, and include ASD, attention deficit hyperactivity disorder (ADHD) or attention difficulties, mood disorders, such as

anxiety and bipolar disorder, abnormal behaviors, including aggression and hyperphagia and schizophrenia. This deletion is inherited in approximately 85% of the cases, with significantly more cases with maternal segregation (Lowther et al., 2015; Dibbens et al., 2009; Helbig et al., 2009). Such microdeletion has also been found in apparently normal individuals, consistent with incomplete penetrance. To conclude, the high clinical variability also present among close family members, exposed to similar environmental factors, yet differing significantly in their clinical manifestations (Hassfurther et al., 2016), demonstrates that the clinical symptomatology derives from a multifactorial origin, part of which will be explained further on (in the section discussing iPS and mice). The few patients carrying homozygous 1.5 Mb microdeletion had similar clinical features consisting of epilepsy, profound intellectual disability, muscular hypotonia, and congenital retinal dysfunction (Hoppman-Chaney et al., 2013; Le Pichon et al., 2013; Liao et al., 2011; Spielmann et al., 2011; Endris et al., 2010; Lepichon et al., 2010; Prasun et al., 2014).

While loss of *CHRNA7* has been found to have a variable phenotype but highly pathological penetrance, gains of *CHRNA7* have been more enigmatic. Their clinical significance and pathogenicity is less well understood, mainly due to the fact that BP4−BP5 reciprocal microduplications, as well as nested duplications only encompassing *CHRNA7* have been found in control individuals without clinical impairment (van Bon et al., 2009; Miller et al., 2009; Zhou et al., 2016; Turco et al., 2018), with a frequency not significantly different with respect to affected subjects. Affected individuals present cognitive deficits; language or speech impairment was reported. As in the case of CHRNA7 microdeletions, neuropsychiatric phenotypes included schizophrenia, ASD, ADHD, and mood disorders, abnormal behaviors, including disruptive behavior disorder, pica, and alcohol abuse seizures. Epilepsy was also recorded. Finally, some patients showed dysmorphic features, although not specific.

There is only one case report of CHRNA7 triplication (Soler-Alfonso et al., 2014), regarding four affected individuals spanning three generations. The family members were affected by cognitive disabilities in the borderline intellectual functioning range, and various degrees of neuropsychiatric pathologies. Both the proband and his twin brother were affected with a pervasive developmental disorder not otherwise specified (PDD-NOS), dysthymia, and anxiety disorder. Their mother had been diagnosed in her twenties with bipolar disorder, anxiety disorder, attention deficit disorder, learning disability, and seizures. The maternal grandfather, who carried the triplication, was impacted by alcoholism and drug addiction, and characterized by abusive behavior toward others, including his own family members. Despite very high variability in expressivity within the family, the penetrance of behavioral phenotypes was complete.

In conclusion, patients with either *CHRNA7* gains or losses have been found to manifest a similar range of neuropsychiatric phenotypes, suggesting that the human brain is sensitive to *CHRNA7* dosage. This idea is supported by the fact that the low number of patients showing Childhood-Onset-Schizophrenia are carriers of deletions as well as duplications, which occur at overlapping sites containing the last two introns plus one exon of the *CHRNA7* gene (Zhou et al., 2016; Ahn et al., 2014).

Two independent CNVs genome-wide association studies (GWAS) reported an association between *CHRFAM7A* dosage and Alzheimer's Disease; lower copy number and, as a consequence, lower levels of the fusion gene expression have been found associated with AD (Lew et al., 2018; Szigeti et al., 2014; Swaminathan et al., 2012). In contrast, upregulation of *CHRFAM7A* was observed in the brain of individuals affected by schizophrenia and bipolar disorder (Kunii et al., 2015) and association studies suggest a correlation with inverted orientation (2 bp deletion).

Functional aspects of CHRNA7 mutation in animal models: modeling CNS features

The role of CHRNA7 CNVs in neurodevelopmental and neuropsychiatric diseases has been modeled in animals by testing three genotype modifications (Fig. 12.7):

- Single deletion of the *CHRNA7* gene (*CHRNA7*-ko).
- Deletion of the 15q13.3 orthologous region in the murine genome, to mimic patients with the 15q13.3 microdeletion syndrome (1.5-MB deletion).
- Insertion in the mouse genome of the human gene *CHRFAM7A* (*CHRFAM7A* transgenic mice).

We will focus on: (1) the reliability and accuracy of behavioral phenotypes observed in mice models with respect to patients' symptomatology and (2) the assessment of CNV-induced pathogenic pathways.

α7-nAChR knock-out mice

Human studies have led to the hypothesis that *CHRNA7* is the key element causative for the 15q13.3 microdeletion syndrome. The first *CHRNA7-ko* model was created on mice back in 1997, by deleting exons 8−10, which encode for its transmembrane domain (Orr-Urtreger et al., 1997).

Behavioral impairments

This same mouse model has not been changed since 1997, so investigations have continued to be carried out on the behavioral, physiological, and electrophysiological phenotypes based on this model. Regarding behavior, most of the studies on mice models, showed that *CHRNA7*-ko mice were by and large normal, with no overt deficits in sensorimotor reflexes, anxiety levels, motor function, learning, and memory auditory sensory gating (Paylor et al., 1998; Yin et al., 2017; Azzopardi et al., 2013), thus suggesting that loss of function in *CHRNA7* is not sufficient to cause statistically significant social behavioral or neuropsychiatric-like alterations in mice. Only a few studies showed subtle impairments in working memory, attention, and/or visual acuity accompanied by depression-like behavior (Fernandes et al., 2006; Hoyle et al., 2006; Young et al., 2007; Young et al., 2011; Origlia et al., 2012; Zhang et al., 2016; Lewis et al., 2018; Young et al., 2004). These variable results (Table 12.1) may be due to the use of different behavioral tests/mouse

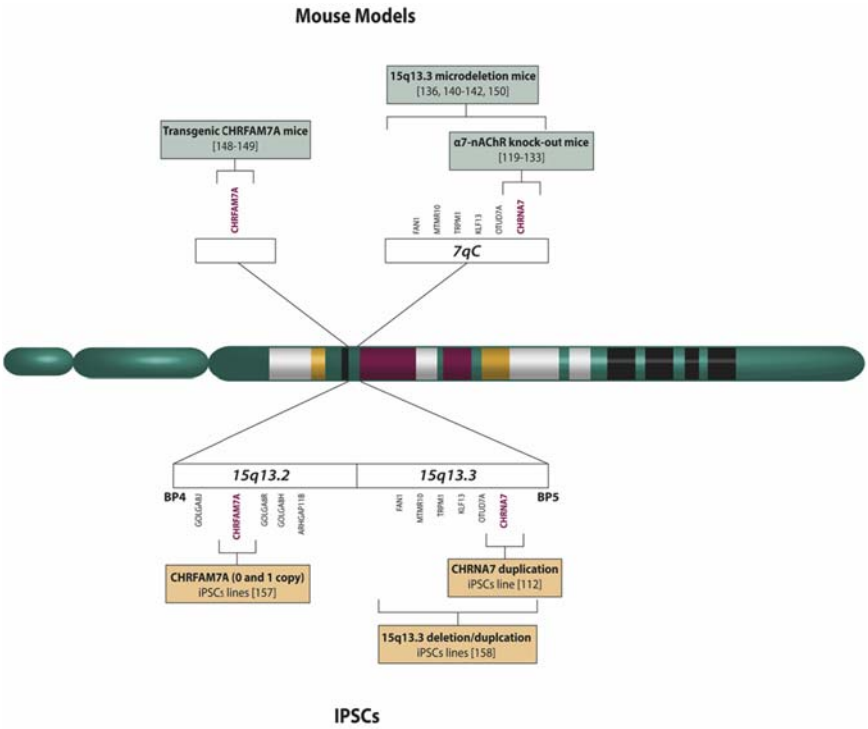

FIGURE 12.7

Mouse models and IPSC lines carrying CNVs in the chromosome 15q13.2-q13.3. *Area above chromosome band: mouse models syntenic to human chromosome 15q13.2-q13.3; area below chromosome band: IPSC lines obtained from patients carrying 15q13.2-q13.3 CNVs.*

strains used to assess pathogenic phenotypes. For instance, Lewis and colleagues (Lewis et al., 2018) demonstrated that CHRNA7-ko mice present with an enhanced propensity for aggressive behavior, a phenotypic tract that was previously overlooked in the same model (Yin et al., 2017). The study by Lewis showed that aggressivity can be recapitulated in animals by transferring the mutation on an intrinsically more aggressive mouse strain - from C57BL/6 (Orr-Urtreger et al., 1997) to Balb/c mice (Lewis et al., 2018) and provoking mice aggressivity with a more stressful test (resident intruder test after a social-isolation period).

Brain circuitry and neuronal electrophysiology

Beyond behavioral evaluation, *CHRNA7*-ko mice did not show electrophysiological phenotypic differences with respect to wild-type animals (Yin et al., 2017). Other studies (Zhang et al., 2016; Lewis et al., 2018; Campbell et al., 2010; Adams et al., 2012; Lin et al., 2014) focused on the individuation of possible entangled

Table 12.1 The table resumes and compares the main behavioral phenotypes linked to CHRNA7 ko mice (Orr-Urtreger et al., 1997; Paylor et al., 1998; Yin et al., 2017; Azzopardi et al., 2013; Fernandes et al., 2006; Hoyle et al., 2006; Young et al., 2007; Origlia et al., 2012; Zhang et al., 2016; Lewis et al., 2018; Young et al., 2004) and 15q13.3 microdeletion mice models (Kogan et al., 2015; Forsingdal et al., 2016; Nilsson et al., 2016; Fejgin et al., 2014).

Behavioral phenotypes patient-related	CHRNA7-KO	15Q13.3 microdeletion models
Basic physiological features		
Basic behavior: Locomotion, exploration, rearing, sniffing, body weight, rotarod	No significant differences respect to wt were detected. In (Paylor et al., 1998), KO mice were slightly less active.	HET Df(h15q13)/+ (Fejgin et al., 2014) and D/+ (Kogan et al., 2015) mice were overall normal with mildly reduced locomotion, the hypoactive phenotype was more pronounced in Df(h15q13)/- mice (Forsingdal et al., 2016). Df(h15q13)/+ (Fejgin et al., 2014), but not, D/+ (Kogan et al., 2015) mice showed slightly increased body weight.
Anxiety		
Reduced propensity to explore "open" and/or uncomfortable (elevated/dark) spaces: Locomotion in open field, light/dark transition, zero-maze task	No significant differences respect to wt were detected in light/dark exploration (Paylor et al., 1998), elevated plus maze (Yin et al., 2017) or open field. In (Paylor et al., 1998), KO showed a trend toward walking in the center in the open field test (low anxiety); an opposite indication was shown in (Fernandes et al., 2006).	No significant differences respect to wt were detected in open field. D/+ (Kogan et al., 2015) mice spend significantly more time in open arms of the zero-maze (low anxiety).
Schizophrenia related features		
Deficits in auditory-sensory processing: Startle-response to auditory stimulus and prepulse inhibition	No significant differences respect to wt were detected in tactile- (Paylor et al., 1998) or auditory-evoked (Paylor et al., 1998; Yin et al., 2017), mild impairments were observed when comparing only males animals (Azzopardi et al., 2013).	Deficits were detected in Df(h15q13)/+ (Fejgin et al., 2014) mice. Df(h15q13)/- (Forsingdal et al., 2016) showed a more severe phenotype.

Continued

Table 12.1 The table resumes and compares the main behavioral phenotypes linked to CHRNA7 ko mice (Orr-Urtreger et al., 1997; Paylor et al., 1998; Yin et al., 2017; Azzopardi et al., 2013; Fernandes et al., 2006; Hoyle et al., 2006; Young et al., 2007; Origlia et al., 2012; Zhang et al., 2016; Lewis et al., 2018; Young et al., 2004) and 15q13.3 microdeletion mice models (Kogan et al., 2015; Forsingdal et al., 2016; Nilsson et al., 2016; Fejgin et al., 2014).—*cont'd*

Behavioral phenotypes patient-related	CHRNA7-KO	15Q13.3 microdeletion models
Attention deficits: 5 choice serial reaction time	Mild impairments in visual attention were more evident in KO mice respect to HET (Hoyle et al., 2006; Young et al., 2007; Young et al., 2011; Young et al., 2004), and in tasks with an elevated attentional load (Young et al., 2007).	Impairments in visual attention were detected in Df(h15q13)/+ (Nilsson et al., 2016) mice.
Nest building		Df(h15q13)/+, Df(h15q13)/- mice showed a (Forsingdal et al., 2016) reduced nest building activity. The phenotype was more severe for Df(h15q13)/- mice.
Cognitive impairments		
Deficit in memory and learning: spatial memory and learning (water maze test, Y-maze tests); *olfactory memory and learning* (odor span test); *Fear conditioning*	No significant deficits were detected in basic test (Paylor et al., 1998; Azzopardi et al., 2013), deficits were detected only in more complex tasks (Fernandes et al., 2006; Young et al., 2011), olfactory working memory (Young et al., 2007) and procedural learning (Young et al., 2011).	No or mild deficits detected for Df(h15q13)/+ (Fejgin et al., 2014) or D/+ (Kogan et al., 2015), significant impairments were detected for Df(h15q13)/- (Forsingdal et al., 2016).
Visual acuity		
Behavior-based measure of visual acuity: Visual water task	Significant reduction of visual acuity detected in KO mice (Origlia et al., 2012).	

Table 12.1 The table resumes and compares the main behavioral phenotypes linked to CHRNA7 ko mice (Orr-Urtreger et al., 1997; Paylor et al., 1998; Yin et al., 2017; Azzopardi et al., 2013; Fernandes et al., 2006; Hoyle et al., 2006; Young et al., 2007; Origlia et al., 2012; Zhang et al., 2016; Lewis et al., 2018; Young et al., 2004) and 15q13.3 microdeletion mice models (Kogan et al., 2015; Forsingdal et al., 2016; Nilsson et al., 2016; Fejgin et al., 2014).—*cont'd*

Behavioral phenotypes patient-related	CHRNA7-KO	15Q13.3 microdeletion models
Depression		
Propensity to behavioral despair: tail suspension test, forced swimming test	Significant propensity to behavioral despair detected in KO mice (Zhang et al., 2016). In (Yin et al., 2017) only KO female mice showed depressed behavior in one task.	
Impairment in reward mechanisms (anhedonia): Sucrose preference test	Anhedonia detected in KO mice (Zhang et al., 2016).	
Aggressive behavior		
Tube test or resident induced test	No significant deficits were shown (Yin et al., 2017). Significantly more aggressive behavior detected in more stressing test, when mice are backcrossed for BalbC mice (Lewis et al., 2018).	Df(h15q13)/+ were more aggressive when returned to the cages (Fejgin et al., 2014).
Autism spectrum disorders		
Repetitive behavior: Self-grooming, holeboard exploration, marble burying test, jumping	No significant differences respect to wt were detected in HET or KO mice. Impairments were limited to KO males in some task (Yin et al., 2017).	D/+ (Kogan et al., 2015) and Df(h15q13)/− (Forsingdal et al., 2016) mice showed repetitive behavior.
Impairments in social interactions: Preference of social/nonsocial object, familiar/new partners, nonsocial/social behavior	No significant differences respect to wt were shown in HET or KO mice (Yin et al., 2017).	No significant impairments in social interaction were shown for Df(h15q13)/+ (Forsingdal et al., 2016), while phenotype was significantly detected for D/+ (Kogan et al., 2015) and Df(h15q13)/− (Forsingdal et al., 2016).

Continued

Table 12.1 The table resumes and compares the main behavioral phenotypes linked to CHRNA7 ko mice (Orr-Urtreger et al., 1997; Paylor et al., 1998; Yin et al., 2017; Azzopardi et al., 2013; Fernandes et al., 2006; Hoyle et al., 2006; Young et al., 2007; Origlia et al., 2012; Zhang et al., 2016; Lewis et al., 2018; Young et al., 2004) and 15q13.3 microdeletion mice models (Kogan et al., 2015; Forsingdal et al., 2016; Nilsson et al., 2016; Fejgin et al., 2014).—*cont'd*

Behavioral phenotypes patient-related	CHRNA7-KO	15Q13.3 microdeletion models
Impairments in communication skills: Ultrasonic vocalization in pups		Not significant impairments detected in Df(h15q13)/+, significant deficits detected in Df(h15q13)/ − (Forsingdal et al., 2016) and in D/+ mice (Kogan et al., 2015).
Hepilepsy		
Convulsant-induced seizure: three stages of epilepsy (early stage seizure, myoclonic jerks, clonic seizure)		Df(h15q13)/+ (Fejgin et al., 2014) mice showed mostly early stage seizure and myoclonic jerks, resistant to clonic seizure. Analogous phenotype for D/+ (Kogan et al., 2015). The phenotype was more strongly indicated among Df(h15q13)/− (Forsingdal et al., 2016): With no myoclonic jerks or clonic seizure. Epilepsy phenotype more likely to be absence seizure as in microdeletion patients.

In the first column, the behavioral tests most commonly used to assess the specific patient-related feature are listed as examples, in each of the studies different versions/tests may have been used to assess the same impairment. CHRFAM7A transgenic mice (Costantini et al., 2019; Jiang et al., 2019) did not show behavioral impairments, therefore are not listed in the table. Het, *heterozygous;* KO, *homozygous;* Wt, *wild type.*

pathogenic cellular and molecular pathways in *CHRNA7* loss in mice, evidencing alterations in specific brain circuitry (hippocampus, nucleus accumbens, cortical inhibitory pathways) compatible with the pathological phenotypes encountered in patients.

Campbell and collaborators evaluated the impact of CHRNA7 loss on hippocampal neuronogenesis in the mature nervous system (Campbell et al., 2010). The generation of new neurons in adult mice and their integration into the hippocampal circuitry is a crucial mechanism for sustaining hippocampal-dependent learning and memory tasks and may also play a role in diminishing addictive

behavior (Noonan et al., 2010). In *CHRNA7*-ko mice, adult-born neurons presented with short and less complex dendritic arbours, retained an immature electrophysiological state longer than wild-type animals and were more prone to die during the critical period, when adult-born neurons are normally integrated into behaviorally relevant networks. A prolonged condition in adulthood of a reduced availability of new neurons could determine a progressive failure of hippocampal functionality that might in turn be responsible for the cognitive and behavioral symptoms of CHRNA7 patients. Consistently, findings from wild-type and *CHRNA7*-ko mice suggest that also aggressive behavior, a recurrent feature in patients, could be caused by the imbalance of another hippocampal network modulated by cholinergic signaling through alfa7 receptors (Lewis et al., 2018, 2015). Cholinergic stimulation of GABAergic interneurons reduces the activation of a specific subset of dentate gyrus neurons involved in aggressive behavior. Lewis et al. (2018) showed that hostile behavior is enhanced in *CHRNA7*-ko mice and cannot be corrected by partial agonists or nicotine (as happens in wild-type animals). These results suggest that genetic-induced imbalance of the cholinergic modulation of hippocampus might strongly contribute to the pathogenic phenotype in patients.

Finally, Zhang et al. (2016) showed a possible association between *CHRNA7* loss and molecular pathogenic mechanisms involved in depression. Considering that inflammation and deregulation of brain-derived neurotrophic factor (BDNF) pathway through the TrkB receptor, in specific brain areas (nucleus accumbens), have been observed in depressed patients, these factors may be implicated in the development of this psychiatric disorder. Research was thus carried out on CHRNA7-ko mice whose behavior suggested a possible depressed phenotype. Interestingly, these mice showed an elevated level of tumor necrosis factor-α and interlukin-1β in the serum (two cytochines that are altered in patients) and an increased activation of BDNF/TrkB signaling. Anatomically, an increase in synaptogenesis and spine density in the nucleus accumbens was observed. These findings suggest that the loss of CHRNA7 might induce a depression-like phenotype by increasing BDNF-TrkB signaling and, consequently, synaptogenesis in the nucleus accumbens.

15q13.3 microdeletion models

Additional studies have addressed the possibility of modeling the 15q13.3 microdeletion syndrome in mice, by removing the genome segment syntenic to the deleted 1.5 MB-region in patients. The corresponding orthologous region is present in the mouse genome on chromosome 7 (7qC) and is partially conserved among humans and mice. Recently, two mouse models of the 15q13.3 microdeletion have been described: the Df(h15q13)/+ (Gass et al., 2016; Lewis et al., 2005; Daskalakis et al., 2007; Javitt et al., 2008) and the D/+ (Kogan et al., 2015) models. The first model (Df(h15q13)/+) was obtained by Cre/Lox-mediated excision of a 1.28 megabase region on Chromosome 7, spanning from a breakpoint between exon 9 and 10 of the *Chrna7* gene to a breakpoint between exon 3 and 4 of the *Fan1* gene, while the

second model (D/+) was generated by a slightly different approach: by FLP/FRT-mediated deletion of a 1.2 megabase region between exon 10 of *Chrna7* and exon 1 of *Fan1*. Both methodologies caused an overall deletion of the genes *Fan1, Mtmr10, Trpm1, Klf13, OTUD7a*, and *Chrna7*.

Behavioral phenotype

The deletion did not affect basic behavior or physiological functions, such as locomotion, exploration, rearing, or sniffing. However, when animals were tested for specific behavioral tasks to unmask autism (stereotypic repetitive behavior, alteration of social interaction), schizophrenia (irregular auditory sensory processing), cognitive impairments (long-term spatial memory), or epilepsy (absence seizure, i.e., freezing), they showed altered functionality comparable to the symptomatic spectrum of human patients (Table 12.1), although with slight differences between the two models (Df(h15q13)/+ and D/+), which might reflect the phenotypic diversity in human subjects bearing the 15q13.3 microdeletion. In addition, the phenotype was more accentuated in the homozygous animals with respect to heterozygous mice (Forsingdal et al., 2016), suggesting a dose-dependent effect of the deletion, thus reproducing the human pathology (Table 12.1).

Brain circuitry and neuronal electrophysiology

More recently (Nilsson et al., 2016), the physiology of brain circuitry functioning was examined in more depth on Df(h15q13)/+, focusing on schizophrenia-related imbalances within prefrontal cortical circuitry, usually observed as reduction in markers for cortical inhibitory signaling (Lewis et al., 2005; Daskalakis et al., 2007; Javitt et al., 2008). This 15q13.3 feature is particularly interesting in the context of CHRNA7, because of its involvement in cortical inhibitory transmission (Adams et al., 2012; Lin et al., 2014). Similar to symptoms in human patients, Df(h15q13)/+ animals showed marked, medial prefrontal cortical dysfunctions, as measured by multiple parallel tests of inhibitory transmission and attentional functioning. These dysfunctions were observed as decreased baseline activity of putative interneurons and reduced interneuronal and pyramidal neuronal sensitivity to auditory stimulation.

Transgenic CHRFAM7A models

Results obtained from clinical and in vitro studies (Lasala et al., 2018; Chan et al., 2019; Kunii et al., 2015; Lasala et al., 2019; de Lucas-Cerrillo et al., 2011; Maldifassi et al., 2018; Wang et al., 2014) point to a substantial contribution of the *CHRFAM7A* gene to the modulation of α7nAchR-mediated physiological and pathophysiological pathways. The lack of the *CHRFAM7A* in the mouse genome might account for the incomplete phenotype observed in *CHRNA7*-ko mice and for the unreliable clinical translatability of pharmacological treatments developed in animal models and impinging on α7nAchR (Lewis et al., 2017). In order to unravel these issues, a humanized transgenic model was developed to study the role of CHRNA7 and CHRFAM7A more comprehensively in human

health and disease (Costantini et al., 2019; Jiang et al., 2019). Transgenic animals did not show any pathogenic/peculiar feature regarding general health and behavior. Initial studies (Costantini et al., 2019) focused on hematopoiesis and showed that the presence of CHRFAM7A increases the stem cell compartment in the bone marrow and protects mice from depletion of the hematopoietic stem cell reservoir caused by the systemic inflammatory response syndrome. Concerning the neurological role of CHRFAM7A, Jiang et al. (2019) showed that expression of CHRFAM7A into the brain did not alter the CHRNA7A expression level, but decreased the percentage of α−bungarotoxin-binding brain cells, thus suggesting that in the central nervous system as well, CHRFAM7A expression modulates the functionality of α7nAchR. Proteomic profiling of whole brain tissue showed that almost 100 proteins were differentially expressed in transgenic versus wild-type animals. In agreement with the potential role of CHRFAM7A in the pathogenesis of neuropsychiatric and/or neurodegenerative diseases, in the group of the most up/down-regulated proteins, mitochondria-associated enzymes/proteins were present, whose dysfunction likely plays a critical role in the occurrence and progression of these diseases.

Novel concepts on the functions of the α7nAChR obtained through the use of iPSC models

Studies on neurodevelopmental and neuropsychiatric diseases, which up to roughly a decade ago could only be carried out on animal models and on postmortem sections of the human brain, have made a quantum leap in recent times, thanks to the techniques provided by the production of induced pluripotent stem cells (iPSCs). In particular, the use of human-induced pluripotent stem cells to generate neural cells has, in fact, revolutionized our ability to access and study human neurons and glial cells directly from patients affected by neuropsychiatric diseases, where rare but penetrant genetic abnormalities are likely to play a role (Dolmetsch and Geschwind, 2011; Grskovic et al., 2011; Marchetto et al., 2010; Park et al., 2008; Paşca et al., 2011).

To date, only six papers have been published regarding iPSCs and the role of CHRNA7/CHRFAM7 during development (Turco et al., 2018; Larsen et al., 2019; Ihnatovych et al., 2019; Gillentine et al., 2017; Chatzidaki et al., 2015; Gill et al., 2013) Four of them defined the role of this receptor in healthy donors and two focused on its deregulation caused by a Copy Number Variation which influences CHRNA7 and CHRFAM7 dosages (Fig. 12.7). The results obtained with iPSCs lead us to believe that the iPSC model will be the best approach for comprehending the mechanistic bases of neuropsychiatric disorders. In Larsen's study (Larsen et al., 2019), through the differentiation of three healthy donor-derived iPSCs into neural stem cells (NSC), researchers linked the α7nAChR expression in hiPSC-derived neurons to an intracellular signaling influencing synaptic

transmission. The research was carried out in four stages: (1) characterization of the native expression of the α7nAChR in hiPSC-derived neurons, (2) evaluation as to whether α7 stimulation resulted in the activation of relevant cellular downstream processes, (3) characterization of the hiPSC-derived neurons as a model for synaptic transmission, and finally (4) investigation of the involvement of α7nAChR in synaptic transmission. Prior to this publication, other groups had characterized voltage-dependent channels in neuron-derived iPSCs (Chatzidaki et al., 2015; Dage et al., 2014). Their results were interesting because they demonstrated the effective presence and function of different channels during neuronal differentiation but, as the authors stated, the development of other differentiation protocols was essential in order to obtain terminally differentiated neurons. In Larsen's paper, this problem was overcome: neuronal differentiations of the NSCs were highly reproducible, and according to the authors, the scalability, reproducibility, and cellular identity of their model qualified it for pharmaceutical research. After 21 days of differentiation, on the basis of pan-neuronal mRNA and protein marker expression, the authors investigated the effects of neurotransmitters on intracellular calcium levels mediated by CHRNA7-derived protein. In particular, they demonstrated that intracellular calcium increased in response to neurotransmitters such as GABA, acetylcholine, and glutamate, whereas no effects were observed with the addition of dopamine and serotonin. Subsequently, the changes in gene expression of nAChR subunits during neural maturation were examined, showing differing increases of the subunits α4- α7- β2 and interestingly, a strong upregulation of the human-specific hybrid α7 gene (CHRFAM7A), a dominant negative modulator of the α7 receptor function. Through the use of various molecules, such as agonists, antagonists, and PAM, the presence of functional α7nAChR was demonstrated. Nicotinic receptors were functionally active, since they were capable of activating intracellular signaling mediated by ERK1/2 phosphorylation and c-Fos overexpression. α7 was capable of increasing the levels of intracellular calcium through two mechanisms: calcium influx via voltage-gated calcium channels and release from intracellular stores, as demonstrated on other cell lines (Guerra-Álvarez et al., 2015; Dajas-Bailador et al., 2002). To study the synaptic transmission in these neurons, the differentiation was extended to 5 weeks. At this time-point, the authors, after demonstrating the presence of chemical synapses, confirmed the involvement of the α7nAChR in synaptic transmission. The use of agonists and antagonists showed that acetylcholine signaling through the α7nAChR had no effects on basal synaptic transmission, but rather played a central role in increasing synaptic activity. These results, obtained directly on human neuronal cells, have broken new ground for potential use of this differentiation protocol in α7 drug discovery, considering that a translation gap had previously emerged, when preclinical studies on mice, demonstrating the beneficial cognitive effects of agonists and PAMs, had not been confirmed in human trials (Lewis et al., 2017; Sadigh-Eteghad et al., 2015).

The production of the first iPSC lines directly derived from human patients allowed researchers to produce the first cell lines from patients affected by

neurodevelopmental/neuropsychiatric diseases caused by CNVs, which have been utilized to study the role of nicotinic acetylcholine receptors in both pathological (Gillentine et al., 2017) and nonpathological settings (Turco et al., 2018).

Gillentine et al. (2017) developed human iPSC lines from both individuals with 15q13.3 deletions and 15q13.3 duplications. These iPSC lines, along with the induced neuronal cell lines that were derived, offer researchers a useful model for studying the molecular consequences of a CHRNA7 CNV and may represent a tool for developing therapies for affected individuals. By using this kind of human model, genetic characteristics and clinical features can be associated to investigate cellular/functional behavior. In the abovementioned experiments, gene expression, α7nAChR-dependent calcium flux, and consequences of altered calcium signaling in iPSCs and NPCs derived from 15q13.3 CNV probands were explored. As was expected, it was found that α7nAChR-dependent calcium flux was decreased in 15q13.3 deletion, while, surprisingly, in the neural precursor cells (NPCs) carrying the duplication, despite having a double dosage of CHRNA7 mRNA confirmed by the genomic and expression data, the same decrease in calcium flux was observed. The decreased calcium flux in both groups mirrored the clinical observations: patients with deletions often have a similar but more severe phenotype than duplications. The authors focused their attention on the necessary process of folding, assembly, and trafficking of α7nAChRs, which allows them to reach the plasmatic membrane. Although the findings showed an increase in the two chaperones implicated in nicotinic receptor processing found in the 15q13.3 duplication NPCs, the α7 subunits were retained inside the ER, instead of reaching the plasmatic membrane, creating ER stress, as a result of inefficient or diminished processing. To support this hypothesis, it was verified that both CNV groups JAK2 (MIM: 147796), directly activated by a7nAChR-dependent calcium flux, had significantly decreased expression compared to controls, with its downstream target, PI3K, having decreased mRNA expression in both groups, but only significantly for deletions. Moreover, having observed this mechanism, which is affected by changes in calcium flux in the cells of probands with 15q13.3 CNVs, it can be hypothesized that there may be other processes stemming from the same calcium flux variations which could contribute to explaining the variable expressivity of phenotypes observed. The findings in this paper have significant implications because they suggest possible itineraries for further research. In particular, it is not unlikely that drugs capable of mitigating ER stress could benefit individuals with a variety of neuropsychiatric disorders associated with 15q13.3 duplications, a plausible hypothesis which has yet to be explored.

One of the unique features of α7nAChR in the human context is the presence of the fusion gene, CHRFAM7A (Gault et al., 1998; Sinkus et al., 2015). Considering the high frequency of CHRFAM7A CNV in the human population, the use of iPSC lines can contribute to the comprehension of the functional impact of CNVs on α7nAChR in human cells. The findings will be valuable for clinical trials in the near future. To study the functional consequences of the presence of the CHRFAM7A gene product on α7nAChR, Ihnatovych and collaborators

(Ihnatovych et al., 2019) produced two iPSC lines from skin biopsies of subjects affected by Alzheimer's disease. The CHRFAM7A gene was lacking in UB068 (0 copies), while UB052 had only one copy of CHRFAM7A (Fig. 12.2A). This interesting article highlighted that the presence of one copy of CHRFAM7, as opposed to zero copies, altered α7nAChRs electrophysiological properties, suggesting that, based on their genotypes, individuals may respond differentially to PAMs, agonists, and antagonists. Thus, it may prove useful to take these pharmacogenetic correlations into account during drug development when targeting α7nAChR. The authors also demonstrated a regulatory effect of CHRFAM7A on Aβ1−42 uptake, which was concentration-dependent. Linear uptake of Aβ1−42 was observed in the 0 line, while the presence of CHRFAM7A mitigated the dose response of Aβ1−42 uptake at higher concentrations, suggesting a protective effect not correlated to the physiological concentrations. Their results suggested a negative modulatory effect of CHRFAM7A on synaptic transmission (relevant in schizophrenia, where increased CHRFAM7A indicates risk) and a modulatory effect on Aβ1−42 uptake (relevant in AD, where loss of CHRFAM7A indicates risk). The described data obtained from iPSCs derived from healthy and affected donors highlighted two important findings: (1) that there is a strong functional difference between nicotinic receptors from mice models and human models, and (2) that pharmacological drugs which have positive effects in mice do not always have positive effects in humans. This is due to the fact that the mechanisms in the two model systems are different and, above all, that the genetic background found in humans (as demonstrated by the findings on CHRFAM7) is not reproduced in mice models.

Conclusions

This chapter has been conceived to allow the reader to understand that symptomatically complex diseases like neurodevelopmental/neuropsychiatric disorders may have a genetic cause, such as the CNV of the α7nAChR, or that this same CNV may represent a risk/susceptibility factor for these diseases. We have also focused on the various approaches taken in these years for studying the functions of this receptor and on the current pharmacological treatments that may be capable of modulating this receptor, influencing its many functions. We have illustrated the utility of studying mouse model systems and moved on to examine the new frontiers represented by iPSCs. The initial idea of comparing the complex neurodevelopmental and neuropsychiatric symptoms developed by patients to mice models has been found to be inconclusive. Behavioral tests for uncovering deficits related to autism, schizophrenia, and depression in mice are very difficult to design and execute, which may have contributed to the variability of the described results. Of note, patients with the single *CHRNA7*-CNV gene and those with the larger 1.5 MB deletion do not show differing phenotypes; on the contrary, in animals, the spectrum of neurological and neurobehavioral symptoms of the 15q13.3 syndrome can be reproduced only by the complete deletion of the mouse genome tract

corresponding to the entire 1.5 MB sequence; a deletion of CHRNA7 is not sufficient by itself to evoke phenotypes in mice. Another relevant difference between the two species in this context is the role of the human-specific gene CHRFAM7A. Recent findings (Chan et al., 2019; Costantini et al., 2019; Jiang et al., 2019; Araud et al., 2011), both in vitro and in vivo, show that CHRFAM7A acts as an inhibitor/modulator of CHRNA7; thus it may play an important role in regulating the CHRNA7 signaling involved in cognition and behavior, which is not reproduced in mice. The genetic environment (strain-related effects) could be an additional factor, for example, certain genetic modifiers necessary for phenotypic expression of *CHRNA*7 deletion might not be present in C57BL/6J mice. Indeed, the genetic background of C57BL/6 masks the CHRNA7ko-induced aggressivity, which is instead apparent in Balb/c CHRNA7 ko mice. It is evident that the genetic background has a powerful effect on the function and regulation of the receptor; in fact, clinical trials performed with pharmacological molecules bound to the receptor, whose efficacy in mice models had previously been demonstrated, did not give the expected results. All of these discrepancies between human and mouse models can be avoided by using patient-derived iPSCs as a disease model (Casamassa et al., 2020). Owing to the fact that the iPSCs are directly derived from the patient's cells, with a genetic background that is identical to that in which the symptoms developed, they can highlight the links between signal transduction, cellular pathological phenotype, and clinical symptoms, which is not possible using mice. It is obvious that working with iPSCs has changed both the perspectives and the approach to the problem. With iPSCs, we begin by observing the biological function that normally corresponds to a molecular mechanism, such as calcium flux activation, analyzing the causes and consequences of this flux. Using this kind of approach, associated with the opportunity of having large quantities of iPSCs from patients with differing genetic characteristics (and in the cases we are considering, having opposite CNVs, that is both a duplication and a deletion) can yield a wealth of information which could not have been obtained from studies on mice. These two opposite CNVs have a common result: reduced calcium flux. This similar result is caused by two different mechanisms, one directly caused by the deletion of the gene, and the other indirectly caused by gene duplication creating endoplasmatic reticulum stress. The same information is reflected in the clinical characteristics of the patients, who display the same symptomatology, with more marked features with the deletion, and milder effects with the duplication. The information obtained with iPSCs allows us to develop different kinds of pharmaceuticals directly on human cells, capable of regulating the mechanisms impaired by the CNVs. Regarding cells carrying the deletion, the molecule will have to be capable of maintaining the channels open, whereas for cells carrying the duplication, the therapy will function by acting on plasmatic reticulum stress. The creation of a personalized drug modulating the α7nAChR, thus restoring healthy mechanisms, will be made possible, thanks to the use of iPSCs.

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ADVANCES IN STEM CELL BIOLOGY VOLUME 15

Novel Concepts in iPSC Disease Modeling

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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.

Novel Concepts in iPSC Disease Modeling, Volume 15 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: open-angle glaucoma, amyloid disease, choroideremia, substance abuse disorders, Danon disease, Niemann-Pick disease type C1, spinal cord injury, 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.

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