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Editorial

The future of embryoids from a reproductive science perspective

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Background

In mammalian biology, is there a point at which a given generation begins? Historically, the answer was straightforward: it begins with the single-celled embryo, or zygote, which is the product of the successful union of an oocyte and a spermatozoon at fertilization. For the purposes of this Editorial, we call the product a 'sexual embryo', to which many also refer as 'normative development'. This sex-based definition was challenged by the first successful cloning of a mammal by somatic cell nuclear transfer (SCNT) into an 'enucleated' oocyte in 1996 (Campbell *et al.*, 1996). This way of cloning blurred the distinction between germline and soma, prompting us to revisit the historical definition of 'embryo' to include cases in which the genetic material of the embryo was not directly derived from both oocytes and spermatozoa. While spermatozoa seemed to be dispensable, the oocyte was still a minimum requirement. Indeed, following imprinted gene modification, non-fertilized oocytes can produce viable embryos and offspring via gynogenesis (Kono *et al.*, 2004) and parthenogenesis (Wei *et al.*, 2022).

The starting point of an embryo has recently been complicated by the development of embryo-like structures in mice, non-human primates, and humans. These structures are not directly derived from fertilized oocytes, but from aggregations of pluripotent stem (PS) cells. Since the first publication in this field in 2014 (Warmflash *et al.*, 2014), many more have followed (Rivron *et al.*, 2018; Sozen *et al.*, 2018; Li *et al.*, 2019; Liu *et al.*, 2021; Yu *et al.*, 2021). The series of improvements grows at a rapid pace (Li *et al.*, 2023; Oldak *et al.*, 2023; Pedroza *et al.*, 2023; Weatherbee *et al.*, 2023) and expands to include new species, with studies in a bovine model also recently published (Pinzon-Arteaga *et al.*, 2023), amidst a flurry of coverage in the popular press (Ball, 2023). In these remarkable advances, aggregates of diploid PS cells harbor the capacity to self-organize into three-dimensional structures with what appears to be a rudimentary body plan. These achievements are not trivial and lie beyond the reach of many laboratories. At present, it is a specialist endeavor, but as was the case with cloning by SCNT, it is likely to become more accessible.

This is not the first time that PS cells have been shown to organize themselves—they have, for example, been shown to do so by tetraploid embryo complementation (Nagy *et al.*, 1990)—but the degree of autonomy is new: in the tetraploid system, the stem cells were supported by a tetraploid embryo derived from fertilization. Let us take a look at how the aggregates come about and which forms, or embodiments, they take. The most common setting is one in which the aggregated cells (~100–200) all participate as founders, although cases of single stem cell founders have also been reported, in which, for instance, the cell was initially flanked by supporting cells that were then selectively removed (Li *et al.*, 2019; Zhang *et al.*, 2023). In those cases, the single cell is reminiscent of the degree of cellular potency ('totipotency') expressed by an early blastomere isolated from a sexual embryo at the 2–8 cell stage. However, the term 'totipotent' has been used in confusing ways in the scientific literature, sometimes even inappropriately (reviewed by Condic, 2014). In this Editorial, we use the term in the purest and strictest sense: an isolated cell (e.g. a blastomere) that is able to produce a complete adult individual—including the extraembryonic structures during development. Stem cell-derived embryo-like structures attain morphologies, gene expression, cellular compositions, and some outward basic functions that are similar to those of sexual embryos—the reason why we call them 'embryoids'. They do not, however, express cellular totipotency according to the definition we embrace in this Editorial, since they do not originate from a single cell entirely on its own. Having explained how the aggregates start out, let us take a look at their embodiments. There are two embodiments of embryoids: 'blastoids' and 'gastruloids', with the latter subdivided into 'peri-gastruloids'. The blastoid embryo model is a blastocyst-like structure (mouse: Harrison *et al.*, 2017; Rivron *et al.*, 2018; Sozen *et al.*, 2018; Li *et al.*, 2019; Sozen *et al.*, 2019; Zhang *et al.*, 2019; human: Yanagida *et al.*, 2021; Kagawa *et al.*, 2022; Karvas *et al.*, 2023; Oldak *et al.*, 2023; Yu *et al.*, 2023) that resembles the natural embryo in the preimplantation phase. To our knowledge, the term 'blastoid' was introduced by Geijsen and colleagues to define structures that resembled embryonic day 3.5 mouse blastocysts (Rivron *et al.*, 2018). In the other embodiment, the embryo model starts at a point in development that, in the case of sexual embryos, would be past the implantation stage and prior to gastrulation—hence the name 'peri-gastruloid' (Liu *et al.*, 2023). The term 'gastruloid' was first introduced by Martinez Arias and colleagues to describe small aggregates of mouse embryonic stem (ES) cells that appeared to recapitulate early embryonic events (van den Brink *et al.*, 2014). In both blastoids and gastruloids, the similarities to fertilization-derived, i.e. sexual counterparts, are remarkable considering the difference in their starting points: oocyte and spermatozoon (differentiated primary cells) vs PS cell lines (e.g. highly potent cell lines). The similarities are even more remarkable when one

considers that the stem cells used to build the aggregates do not exist during natural development but are experimentally derived from either the inner cell mass of blastocyst-stage embryos, in the case of ES cells, or from somatic cells, in the case of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). An important aspect to keep in mind is that some aspects of the ES and iPS cells are similar (for example they are both pluripotent) but, at the same time, there are also differences in terms of origin and epigenetic features. In particular, given that their somatic precursors are not directly meiotic products, iPS cell-derived embryo-like structures derived from homogeneous cell cultures could be viewed as clones of the somatic cell from which they originated.

Outward similarities to sexual embryos could make the stem cell-derived structures, or 'embryoids', highly attractive as models to investigate natural and medically assisted reproduction. At present, they are not intended as replacements of sexual embryos for reproductive purposes, but as we will discuss, if they keep improving, it is possible that the risk of dual-use (research vs reproduction) will at some point arise—at the latest when it has become clearer what exactly embryoids are (mere models or more than that). A model can be defined as, 'the generation of a physical, conceptual, or mathematical representation of a real phenomenon that is difficult to observe directly' (Encyclopedia Britannica). Modeling human embryos is much needed and overdue, considering the substantial proportion that are lost in the initial phases of natural pregnancy. It is difficult to calculate the magnitude of lost embryos because rigorous data are difficult to obtain ethically. While estimates of a 70% loss (Chard, 1991; Zinaman *et al.*, 1996; Macklon *et al.*, 2002) may seem excessive, a meta-analysis (Jarvis, 2016) indicated that 10–40% is a plausible range for preimplantation embryo loss, and overall pregnancy loss from fertilization to birth is ~40–60%. Examining why sexual human embryos fail to develop is, however, limited by experimental access being ethically and legally restricted in many countries (e.g. the '14-day rule'; Warnock, 1985), and also becomes anatomically intractable after implantation. Specifically, the '14-day rule' states that human embryos should not be cultured *in vitro* beyond 14 days post-fertilization or until the primitive streak forms (whichever comes first). This imposes limits on research, which embryoid research aspires to bypass and, thereby, illuminate the processes that govern early human early embryonic development and the causes of its failure (Shahbazi *et al.*, 2019; Shahbazi *et al.*, 2020), particularly in the Day-7 to Day-28 developmental window. This phase is often referred to as a 'black box', when, *in vivo*, human embryos become embedded in the endometrium. However, their very small size means they can barely, if at all, be detected using ultrasound imaging, and are thus 'difficult to observe directly'. Consequently, the possibilities offered by stem cell-derived embryoids generated directly *in vitro* are appealing.

We argue in this Editorial that embryoids, at their current research stage, are perhaps not so well-suited for studying the major problems of human reproduction, including oocyte- or sperm-related failures or other aspects of medically assisted reproduction, whereas they lend themselves well to studies in other areas, including reproductive toxicology and teratology. This limitation is a result of substantial, and we argue, significant, differences between embryoids and sexual embryos. Moreover, additional components appear to be necessary to complete the study of embryoids (e.g. models of the endometrium). We aim to stimulate a discussion of these differences, what embryoids are (or are not), and what they are (or are not) useful for studying. At the end of the discussion, we raise the possibility (a potential wild hypothesis) that embryoids represent a new route to obtaining full developmental potential—somatic embryogenesis—in addition to constituting a model.

Fundamental differences between embryoids and sexual embryos

Early losses during normative, i.e. sexual human development may arise from issues absent in embryoids but rooted in oocyte and spermatozoa. The potential of embryoids to reveal the reasons behind early human embryonic failure relies on the underlying assumption that they faithfully reproduce cellular processes and gene usage, despite having a distinct cellular provenance. After all, embryoids typically come into existence directly as diploid multicellular entities lacking a recent meiotic history, maternal-effect gene expression, sperm-associated attributes (other than the genome *per se*) that will potentially influence early embryo development (e.g. DNA methylation, chromatin modifications, RNA and proteins; Immler, 2018; Rutkowska *et al.*, 2020), sperm selection mechanisms, epigenome remodeling, early embryonic transcription and different mitochondrial characteristics. The list is probably even longer. Consequently, critics of embryoid research may wonder how embryoids can possibly rejoin the continuation of the developmental trajectory after omitting key early developmental processes and may ask if embryoids are bypassing 'quality controls'—only to fail later. Advocates of embryoids may counter that this is irrelevant if embryoids are only used as models of, for example, cell lineage segregation and differentiation during the time window of the 'black box'. However, it is reasonable to propose that some developmental failures of sexual embryos during the 'black box' period may be a manifestation of one or more issues previously suffered in the very processes that are omitted in embryoid formation.

Apart from gamete involvement, let us take a brief look at what sexual embryos do and what embryoids do not.

Prior to fertilization, prospective embryos:

- profit from a 'Darwinian' selection of the spermatozoa, mediated in part by the zona pellucida (the oocyte-specific extracellular coat) that acts as barrier to screen the less fit spermatozoa.

It follows that common paternal causes of infertility, such as defects in sperm motility or failure of the sperm-borne oocyte activating factor(s), are not represented in embryoids. To add, these differences are not unique to embryoids: some are also reflected in embryos obtained from ICSI. Gastruloids also bypass additional phases that pave the way to implantation.

Immediately after fertilization, nascent embryos:

- complete meiosis before embarking on mitosis (a transition accompanied by calcium ion oscillations);
- balance the two parental genomes—from sperm and oocyte—in terms of chromatin composition (e.g. sperm genome protamine exchange with maternal histones), DNA replication timing, transcription, and re-writing of epigenetic marks (with the exception of some imprinted loci)—summarized in the expression 'oocyte-to-embryo transition'; and

- give rise to mitotic blastomeres that progressively differentiate to form trophectoderm through a series of cell cycles of varying duration (embryonic cleavage phase), as a prelude to implantation *in utero*.

The non-gametic origin of embryoids clearly precludes the first three processes, and yet these have a significant bearing on the genomic stability of sexual embryos (Palmerola *et al.*, 2022). At fertilization, embryos inherit meiotic aneuploidy—when it occurs—mostly from the oocyte. After fertilization, the maternal and paternal genomes have different chromatin configurations that create an asymmetry in kinetochore attachment that can render zygotes prone to chromosome non-disjunction (van de Werken *et al.*, 2015). In addition, the duration of specific cell cycle stages is longer in early compared with later embryo divisions, while PS cells have a prolonged S-phase (Orford and Scadden, 2008), with implications for DNA repair mechanisms in embryos vs embryoids. In turn, the differences in cell cycle control checkpoints will impact the safeguarding of ploidy. This leads to a considerable proportion of aneuploid preimplantation embryos (Rubio *et al.*, 2003; Baart *et al.*, 2006; Vanneste *et al.*, 2009) that seem to be consistent with estimates of developmental failure. Not all aneuploidies are lethal, but most are incompatible with healthy development or are otherwise harmful. Therefore, it is not far-fetched to consider that the losses occurring immediately after implantation reflect the high proportion of aneuploid embryos, albeit with a note of caution from recent observations that suggest aneuploidy rates may be inflated i.e. they may be, in part, an artifact of their detection methods (Domingo-Muelas *et al.*, 2023). The main origin of *bona fide* aneuploidy is rooted in processes that occur during gametogenesis, particularly oogenesis, such as loading of meiotic cohesins onto chromosomes without replenishment after birth: this leads to a deterioration of cohesion and a susceptibility to chromosomal non-disjunction with advancing time—i.e. maternal age (Jessberger, 2010). This instability is compounded by DNA replication stress during the first embryonic mitosis (Palmerola *et al.*, 2022) and defective molecular pathways during subsequent mitoses (Tsuiko *et al.*, 2019). Embryonic aneuploidies are possibly exacerbated by ovarian stimulation and duration (Cascales *et al.*, 2021). In turn, the physiological response of women to ovarian stimulation may vary across human populations representing different gene pools (Altmäe *et al.*, 2011), diversity that is reduced in embryoids, perhaps more so in those derived from iPS cells. As a result of both faithful and aberrant embryonic mitoses, the first differentiated tissue product is the trophectoderm. We here consider it as an integral part of the embryo even though we acknowledge that, according to some lines of thought, the trophectoderm is more a 'life-support system required to support intrauterine existence before it can safely produce an embryo' (McLaren, 1987) rather than an integral part of the embryo proper. We leave it to the readers' common sense to draw their own conclusions as to whether the trophectoderm is or is not part of the embryo. In our opinion, a view that the trophectoderm is not part of the mammalian embryo would be akin to an eggshell not being part of a chicken egg (this is merely a simile, and we disregard here the different anatomical origins of trophectoderm and eggshell).

Early embryonic differentiation is guided by a genetic program preordained in oocytes, as underlined by many studies, including some that appeared while this Editorial was in preparation (Jentoft *et al.*, 2023). The maternal molecules responsible include maternal-effect gene products (Kim and Lee, 2014; Mitchell, 2022) such as the subcortical maternal complex (Li *et al.*, 2008) that are likely lacking in stem cells. A counterargument that maternal factors were also absent from stem cells in the tetraploid complementation system (Nagy *et al.*, 1990) overlooks the fact that they were provided by the tetraploid partner. Intriguingly, maternal products are traveling companions—if not outright integral components—of sexual embryos and are present during the late specification of the three primary cell lineages at the blastocyst stage, as revealed by immunofluorescence and proteomic analysis of mouse blastocysts (Li *et al.*, 2008; Gao *et al.*, 2017): although the maternal genes are transcriptionally silenced, the protein products persist as blastocysts continue to expand, with the recent discovery of a hitherto-overlooked intracellular deposit of zona pellucida (Israel *et al.*, 2023). In immature oocytes, the zona pellucida serves as a scaffold for the trans-zonal projections that mediate communication to granulosa cells and for the microvilli that mediate communication from the oocyte to the follicle (Zhang *et al.*, 2021). Thus, the oocyte incorporates functional molecules transported from granulosa cells—raising the question of whether maternal factors impact later stage development—whereas blastoids self-assemble from stem cells that may lack them, and the question is moot. To investigate if maternal factors are indeed missing in embryoids, we performed a search focusing on the class of maternal products known as maternal-effect genes (Mitchell, 2022). Although it would require writing a book to do justice to the number of maternal factors in embryos, we compared the relevant proteins of morula and blastocyst stages of sexual mouse embryos (Gao *et al.*, 2017) with those of Day-3 and Day-5 mouse blastoids (Min *et al.*, 2022) as detected using the same analytical method of TMT-based quantitative mass spectrometry in both studies. As shown in Fig. 1, the most famous maternal-effect proteins (e.g. Nlrp5 also known as 'Maternal antigen that embryos require': Mater) are present in sexual embryos but not in blastoids, although some others are present in both.

Other molecular traits of the embryo immediately following a sperm–oocyte union, but which may be absent from embryoids include the cytostatic factor, Emi2 (a.k.a. Fbxo43), which is unique to meiosis and persists in cleavage-stage development (Shoji *et al.*, 2006). It is beyond the scope of this Editorial to list the many other meiotic regulators whose traces are still present in the sexual embryo. Our point is that these traces are missing in embryoids, and while we know that they are not required for the establishment of a state relevant to embryonic character (otherwise embryoids could not form), we do not know whether they may also have roles in the maintenance of such a state. Meiotic regulation affects parental genome compartmentalization (pronucleus formation) in one-cell embryos, which facilitates segregation of epigenetic regulation (e.g. active DNA demethylation) and gene regulation. Transcription initiates in one-cell mouse and human embryos, but in both species, upregulated transcripts are removed well before pluripotency is established (Asami *et al.*, 2022; Asami *et al.*, 2023; Perry *et al.*, 2023): it is not known what the developmental significance of this is, if any, but in blastocysts and blastocyst-derived PS cells, there is no trace of the upregulated transcripts. In addition, any impact of non-genetic sperm contributions to the embryo—such as DNA methylation, RNA, modified nucleoproteins, and other proteins (Immler, 2018, for a comprehensive review)—on embryo development can only be meaningfully examined following fertilization. Consequently, embryoids derived from PS cells have different histories and associated processes.

The above applies mainly to blastoid-type embryoids, rather than gastruloids. Gastruloids, as *in vitro* models that reproduce key features of postimplantative embryonic events, obviously

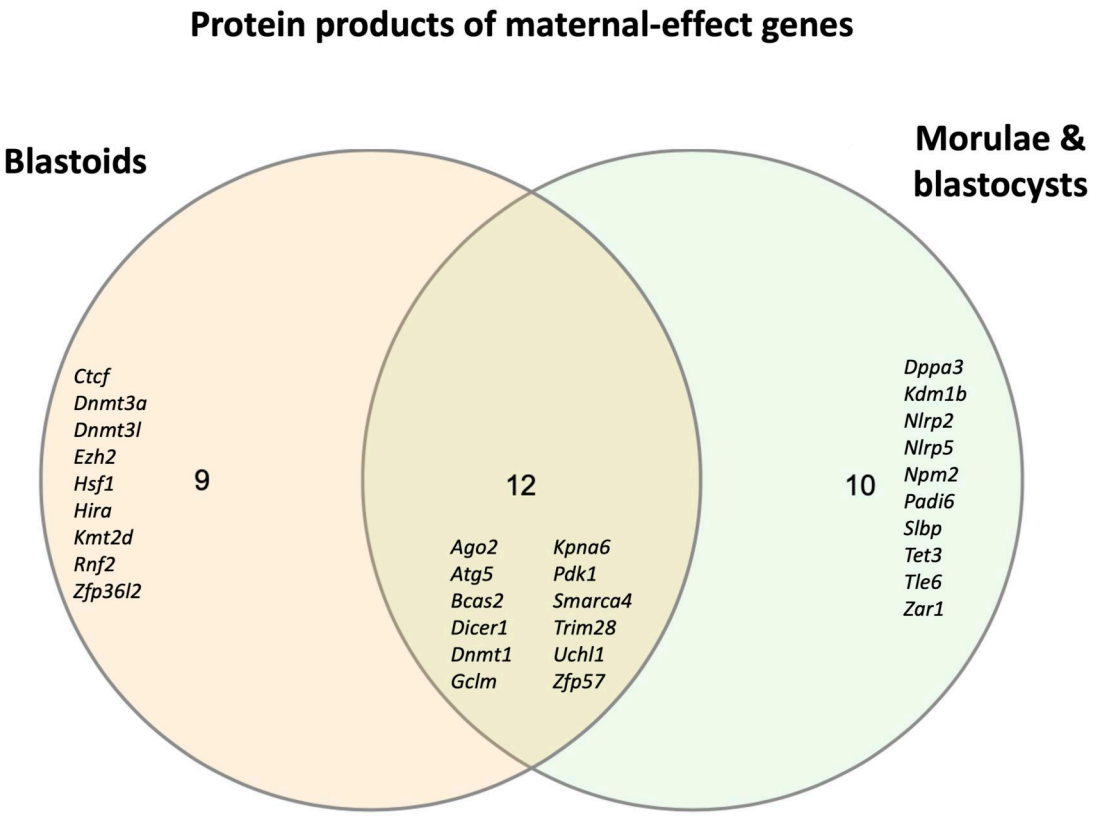

Figure 1. Venn diagram showing the proteins (gene names) of maternal effect genes (according to Table 2 of Mitchell, 2022) detected in Day-3 and Day-5 mouse blastoids vs morula- and blastocyst-stage mouse embryos. The mass spectrometry analyses were conducted by Min *et al.* (2022) (blastoids) and Gao *et al.* (2017) (morulae and blastocysts). The datasets were retrieved from the ProteomeXchange Consortium via the PRIDE partner repository (PXD031002, PXD003315).

lack crosstalk between mother and embryo *via* the placenta. Considering that flaws in this interaction can impact the development and health of SCNT embryos (Bauersachs *et al.*, 2009; Biase *et al.*, 2016), one wonders how representative gastruloids are, given that the crosstalk is not only defective but entirely lacking. Embryoids can be obtained from iPS cells in mice (Li *et al.*, 2019), cattle (Pinzon-Arteaga *et al.*, 2023), and humans (Liu *et al.*, 2021; Yu *et al.*, 2021; Oldak *et al.*, 2023), raising the possibility that embryoids have additional features that sexual embryos lack. These features are, for example, iPS cell mutations inherited from the precursor somatic cells (Zambelli *et al.*, 2018), including structural aberrations of mitochondria and mtDNA mutations related to the somatic origins, the significance of which may lie in the (mis)regulation of the epigenome by metabolism (Harvey *et al.*, 2018). These problems are not always observed and some iPS cells reacquire the peculiar mitochondrial traits of ES cells during derivation (Prigione *et al.*, 2010). Thus, the mitochondrial situation appears to be complex and may reflect different degrees of iPS cell reprogramming. Since these deviations occur during *in vitro* reprogramming, the question of whether *in vitro* culture has similar effects on embryoids is inevitable—certainly it does on the source material—ES and iPS cells (Davidson *et al.*, 2015)—that are used to make the embryoids. By contrast, in the case of sexual embryos, one tries to minimize the time spent in culture, due to stress and potential epigenetic perturbation that might reduce embryo viability (Ramos-Ibeas *et al.*, 2019). In essence, studies of sexual embryos strive to recapitulate physiological conditions, whereas studies of stem cells and embryoids rely on empirical tissue culture formulations. The resulting risks to the (epigenetic) health of embryoids have been flagged in a previous Editorial (Boiani and Duncan, 2022).

The differences between embryoids and embryos above do not necessarily represent fatal flaws for the new models. Cloned (SCNT) embryos, too, have epigenetic defects, yet >20 mammalian species have been cloned to date. Moreover, full-term mouse development can follow quite different early embryonic epigenetic trajectories (Suzuki *et al.*, 2016). Differences between embryoids and sexual embryos accordingly need not suggest a lack of inherent developmental capacity of the former. Instead, the differences may represent an opportunity to gain insights by looking at the matter from a different perspective, also considering the alluring possibility that the development of embryoids occurs *via* distinct pathways that do not recapitulate those active in sexual embryos.

The relative strengths of embryoids vs existing animal models

Despite our concerns about the use of embryoid models for investigating the causes of early embryo failure, they will help illuminate other developmental aspects. It is possible that studying the mechanisms of blastocyst cavitation will be feasible using blastoids. Animal models teach us that cavitation is a flexible process that also occurs under conditions quite different from natural conditions, exemplified by tetraploid embryo complementation (Nagy *et al.*, 1990). Blastoids—which model blastocysts whose prerogative is to implant in a receptive endometrium—could also help study the dialogue between embryoids and the

endometrium, under the assumption that the trophoblast of blastoids functions like that of blastocysts. Mouse and monkey embryoids can induce a decidual reaction in the endometrium after implantation (Rivron *et al.*, 2018; Li *et al.*, 2019, 2023), although no live births were achieved. This suggests a trophectoderm capable of at least partial function, although it might be noted that the decidual reaction is not a strong indicator of embryonic function, as it can also be induced by synthetic beads (McLaren, 1968). Regarding humans, the updated guidelines of the International Society for Stem Cell Research (Lovell-Badge *et al.*, 2021) prohibit the transfer of human embryo models into a human or non-human uterus (i.e. no embryo modeling activities aimed at reproductive use, reminiscent of the ban on reproductive cloning by SCNT). Implantation studies would therefore require an *in vitro* model of the endometrium: we would need a second model to study the first model. The human endometrium can be surrogated by means of mono- or multilayers of endometrial stromal cells (Weimar *et al.*, 2013; Schoen and Chen, 2018) and this has been explored: human blastoids have been shown to adhere to endometrial epithelial or stromal cells *in vitro* (Kagawa *et al.*, 2022; Yu *et al.*, 2023). This system could become enriched with endometrial gland organoids in future refinement (Rawlings *et al.*, 2021).

A dual model of this type would also lend itself to the study of, for example, drugs taken by pregnant women—on the assumption of comparable pharmacokinetics *in vitro* and *in vivo*. In some cases, drugs do not interfere with implantation *in utero* but act deleteriously afterwards. Thalidomide is a notorious example that was once offered to pregnant women in the late 1950s and early 1960s as a treatment for morning sickness but caused the birth of children with phocomelia. At that time, drug testing on pregnant animals was not mandated, leading to the unfounded conclusion that the models had failed to predict adverse clinical outcomes. Tests eventually conducted with thalidomide (e.g. during rat gestation) revealed teratogenicity (King and Kendrick, 1962). Thalidomide-inflicted damage affected all organs, whereupon most fetuses died before birth. Only fetuses with limb deformities survived, as limbs are not essential for life. The intake of thalidomide between the 20th and the 36th day after human fertilization was the most harmful (Vargesson, 2015), and gastruloids in the current state of the art are at an equivalent stage: Carnegie Stage 6–7 (Karvas *et al.*, 2023); Stage 7 embryos have an estimated age of 18–21 days. The effect of drugs *in vivo* is influenced by other organs, most notably the liver (but also potentially other hormone-secreting tissues), thus, here too—as in the case of the embryo–endometrium—there may be a need for a second *in vitro* model in order to complete and study the first model. Rather than replacing *in vivo* and other assays, embryoids could complement the assays and enhance our understanding. As well as in toxicology and teratology, opportunities most likely will not be limited to these areas.

Moreover, human embryoids can, in nominal terms, be mass-produced (Fig. 2), although this capacity is confined to a small number of laboratories at the present time. Despite the heterogeneity of the embryoids produced, mass production has the potential to reduce the number of human embryos required for research, thus contributing toward a human embodiment (Moris *et al.*, 2021) of the 3Rs (reduction, replacement, refinement) developed for animal research (Russell and Burch, 1959). This does not mean that animal models will be supplanted by embryoids. On the contrary, animal models are indispensable for determining the potential of embryoids, by revealing which biological processes they model and to what extent. Perhaps the apotheosis would be that of a model potentiated by another model, e.g. animal embryoids that attain full development in animal model. This would require transplantation of animal embryoids into *cis-uteri* (same species) or artificial uteri, and evaluation of term development. At present, the transfer of human embryoids into uteri, whether human or animal, or their combination with uterine explants, falls into the category of 'prohibited research' as defined by ISSCR guidelines (Lovell-Badge *et al.*, 2021). The situation is different in non-human species. Even though, up to this point, scientists have been unsuccessful in achieving live births after the implantation of embryoids in animals, the hypothetical scenario of embryoids developing to term impedes a sober scientific discussion around the possibilities and limitations of human embryoids.

Do embryoids create an ethical catch-22 situation?

A 'catch-22' can be defined as a tangled situation in which the solution to a problem is impossible because it is also the cause of the problem. In the case of embryoids, the more similar to sexual embryos they are, the more scientifically useful they become—but also more ethically problematic (Ahuja, 2023). The ethical debate surrounding embryoids cannot assume a lack of developmental potential like that of sexual embryos: first, full-term embryonic (and *ipso facto*, embryo-like) development can take different pathways (Suzuki *et al.*, 2016) and, second, while embryoids may currently have limited developmental potential, it is plausible that this could change as the technologies and conditions used in their generation evolve. The current prohibition of their *in utero* transfer seems to be practical solution that does not, however, settle the question of whether some, even a small percentage, of the embryoids have extensive developmental potential. It is simplistic to dismiss this possibility as scaremongering, or to say that embryoids have already gone too far in their developmental trajectory to implant and is analogous to arguing that sexual embryos cultured to 14 days can no longer implant. Given that recent studies have claimed improved production of extraembryonic tissues in blastoids (Liu *et al.*, 2023) and gastruloids (Oldak *et al.*, 2023), it seems all the more premature to exclude implantation potential. One can, for example, recall that reproductive cloning by SCNT began with extremely low success rates, but the technology improved. Although we have not yet arrived at a 100% efficient SCNT method despite almost 100 years of research since the initial idea of Hans Spemann (Spemann, 1938), we can say that this method has become more distributive and reproducible albeit in its limitations. An equivalent consideration is applicable to embryoids: even if they are not today regarded as developmentally competent, they could achieve this competence tomorrow (Rivron *et al.*, 2023). Indeed, the likelihood in this regard is potentially higher for embryoids than previously with SCNT, because: in contrast to the limited availability of oocytes and the high level of technical accomplishment in micromanipulation required to generate small numbers of cloned embryos, stem cell-derived embryoid generation is scalable (Fig. 2). It has been suggested that, at the current level of efficiency, >20 000 human blastoids could be generated in one experimental run (Yu *et al.*, 2023). It is difficult to eliminate the possibility that not even one among the 20 000 has extended developmental ability. One solution would be to genetically engineer the embryoids (or precursor stem cells) to preclude development past a certain stage, reminiscent of the proposal of 'altered nuclear transfer' originally put forward in the context of SCNT (Hurlbut, 2005).

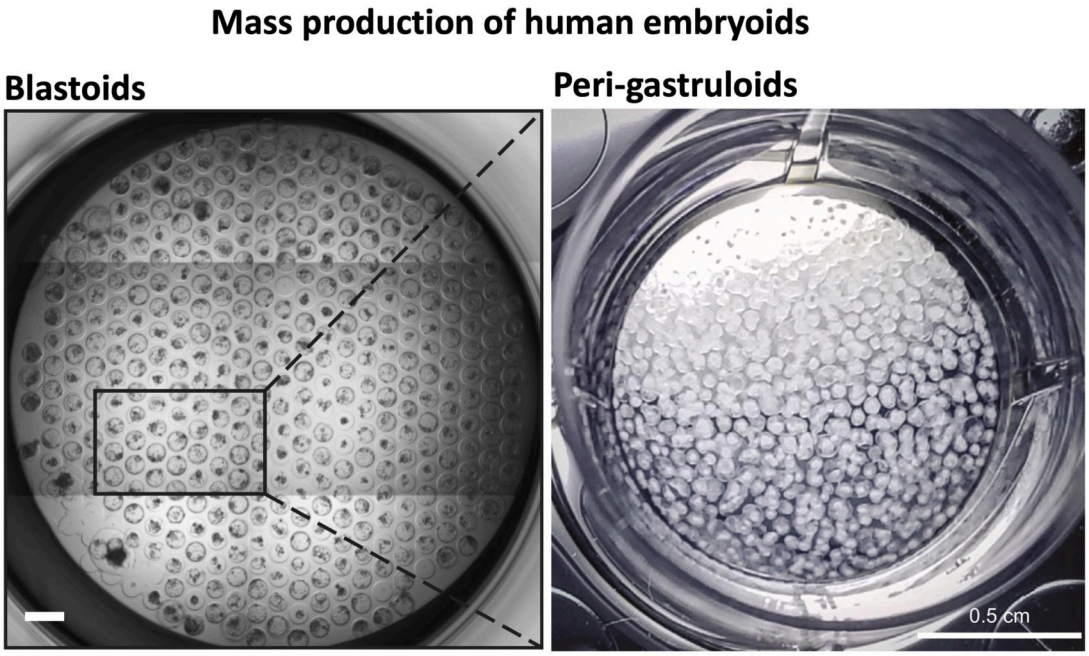

Figure 2. Images of human blastoids and peri-gastruloids exemplifying the high number of embryoids that can be produced in a single experiment. Left: image of blastoids reproduced without changes from Kagawa *et al.* (2022) (Figure 1C in the original publication) under the provisions of open access license CC BY 4.0 DEED (https://creativecommons.org/licenses/by/4.0/). Right: image of peri-gastruloids reproduced with permission and without changes from Liu *et al.* (2023) (Figure 1B in the original publication) (license number 5720190807395).

This topic might experience a revival in the context of pig–human chimeras and xenotransplantation, to allow human cells to make certain structures but not others (e.g. not the brain). Until they are rendered 'safe', the very properties that make embryoids attractive models—their recapitulation of sexual development—also raise ethical concerns regarding how they should be used. This is paradoxal: embryoids should be as similar as possible to human embryos, yet at the same time preserve cardinal distinctions. The question we should be asking (Rivron *et al.*, 2023), is at which point is the model so similar to the human embryo that it ceases to be a mere model and should legally be considered an embryo? Next to Rivron *et al.* (2023), we refer the reader to Pereira Daoud *et al.* (2020), Moris *et al.* (2021), and Denker (2023) for a more in-depth discussion of the ethical aspects and dilemmas of embryoids.

Wild hypothesis: might embryoids reflect a fundamentally distinct route of (somatic) embryogenesis in mammals?

'Nothing in biology makes sense except in the light of evolution' (Dobzhansky, 1973). Indeed, evolutionary considerations shed light on why embryoids may harbor developmental potential despite their anomalous beginnings. Apart from fertilization, mammals exhibit differences in their development prior to the formation of the three primary cell lineages (trophoblast, epiblast, and hypoblast). It has been argued that each mammalian group uses different morphogenetic strategies to reach this conserved phylotypic blastocyst stage (Sheng, 2015). This is reminiscent of the developmental hourglass model (Duboule, 1994), in which embryos are more divergent at the earliest and latest stages but more conserved in between. Given that some early processes exhibit heterogeneities (next to others that in turn are conserved), it is perhaps not surprising to find that stem cells, when they find themselves in a constellation (even though artefactual) are somewhat reminiscent of an embryo, including regulative mechanisms to 'sort things out'. This would imply that, whatever their developmental potential, embryoids achieve it differently from canonical embryogenesis (Aach *et al.*, 2017). Indeed, as has been noted, 'Further complications could also arise in assessing the ethical status of embryo-like structures because they do not necessarily follow "canonical embryogenesis" (Aach *et al.*, 2017)' (Moris *et al.*, 2021). Likewise, the *in vitro* behavior of embryoids seems to point to alternative routes to the same body plan: '[…] a deeper, evolutionarily conserved developmental mode which cells exhibit as they are released from their species-specific geometrical arrangements and mechanochemical signaling environments. […] Removing ES and ES-like cells from their native context allows cells to follow developmental trajectories guided by their inherent self-organizing capabilities' (Anlas and Trivedi, 2021).

Taking these reflections further, we propose that we should be open to the possibility that embryoids are not likely to take an equivalent route to that of sexual embryos, but a different, non-canonical route. A close natural precedent that comes to mind is somatic embryos of plants. Somatic embryogenesis is an artificial process in which asexual embryos can be induced from a single somatic cell or a clump of somatic cells and is a form of clonal propagation (Zimmerman, 1993; Winkelmann, 2016; Salaün *et al.*, 2021). Somatic embryogenesis is more common than many people think: banana trees, for example, are commercially propagated via somatic embryogenesis. It is clear that if we give importance to the origin, then embryoids are also asexual embryos, albeit with the same anticipated functional prerogative i.e. replication of the organism. Asexual embryogenesis would be something new to mammals. Albeit that headlines in the popular press have referred to parthenogenesis as asexual reproduction in vertebrates and mammals (Wahlquist, 2017), 'asexual' is not apposite in that, despite the absence of mating between a male and a female, a gamete was at the origin (Lampert, 2008; Moreira *et al.*, 2021). For the same reason, also twins obtained by splitting of 2-cell embryos are sexual—even though the embryo is engaged

in mitosis—and they belong to the same generation from the perspective of the nuclear DNA that underwent meiosis and ploidy restoration at fertilization. Thus, there are qualitative differences between embryoids and sexual embryos in nature (even in special cases of the latter, which are, yes, special, but not exceptions). Similar to embryoids, it may be argued that also iPS cells do not 'happen' in nature, yet they are used as tools with a profound impact on biomedical research. However, one difference is that stem cells are used in the context of pluripotency, while embryoids aspire to model an entity with full developmental potential. However, there is an overlap of the two fields in the case of embryoids derived from iPS cells, in which case, as already noted above, embryoids are a form of cloning of the individual from which the iPS cells were derived.

Conclusion

At present, the experimental production of embryoids is neither trivial nor within the reach of all laboratories, but this is likely to change, as has been the case with other technologies that were initially in the domain of few and then came into common use. If the improvement curve was equivalent to that of SCNT (which has been studied for almost 100 years, since Spemann (1938), and whose application in mammals is now 28 years old (Campbell *et al.*, 1996)), then between now and the end of this century we would have made a lot of progress but the method would still not be routine. Keeping this in mind, we proffer the following closing reflections on the assumption that embryoids are not a passing fad but are here to stay. To begin with, embryoids have undermined a dogma in embryology. Although many think in biology there is only the central dogma of molecular biology, whereby information flows from gene (DNA) to protein but not backwards (Crick, 1970), embryology also has its own central dogma. Dawkins (1982) proposed a 'central dogma of embryology', which states that while the genes code for the proteins and these lead to the bodily form of an organism, the code is irreversible in that bodily form may not be translated back into protein. Unfortunately, the 'central dogma of embryology' was not taken up by the scientific literature. From our perspective, if there were such a dogma, it would be that normative embryonic development of mammals goes from unicellular to multicellular; i.e. it did not start out as pluricellular. While sexual embryos progress from the one-cell stage through cleavage stages, this is not necessarily the case in embryoids—hence, the dogma is undermined. For these reasons, embryoids represent a revolution in the field of mammalian biology, second to SCNT cloning and the direct reprogramming of differentiated somatic cells to pluripotency (iPS cells). Embryoids also challenge the dogma that full mammalian development can only originate from gametes. As gametes are haploid cells, it will be interesting to see if an embryoid generation would be possible using haploid PS cells derived from unfertilized oocytes (Leeb and Wutz, 2011; Sagi *et al.*, 2016). Leaving the field of basic biology and thinking about applications, embryoids represent a golden opportunity in some areas (e.g. toxicology and teratology), but one must be clear about not creating false expectations in other areas. Producing an asexual embryo model (embryoid) that reflects some characteristics of normative (fertilization-derived: i.e. sexual) human embryos is quite different from having a tool to cure conditions of human sexual reproduction, which depend on many factors (considered above) represented differently (if at all) in embryoids. One should also be careful when implying that embryoids are useful and are an ethically neutral alternative to sexual embryos, since this position is based on the assumption that embryoids cannot develop, which would impose major restrictions on their utility. This can be summed up in the popular saying: 'you can't have your cake and eat it'. Perhaps the best way today to demonstrate beyond reasonable doubt that embryoids are an ethically neutral model is to take an experimentally tractable species such as the mouse and transfer high numbers (e.g. tens of thousands) of blastoids to the uteri of a large number (e.g. thousands) of recipients and get no offspring. It is impossible to prove that one thing does not happen, but let us be forgiving. In the absence of this experiment, or demonstration that, by contrast, embryoids *can* develop to term, we remain in the realm of wishful thinking and speculation as to what embryoids could or could not do *in vivo*. Our position as the *MHR*-ISSCR guidelines working group is that only a fertilized egg can reliably inform on the time window of developmental failure known as the 'black box', which is the crux of the debate regarding the '14-day rule' (Warnock, 1985) and petitions for its reform. Meanwhile, progress might be made *via* sensible alternatives *in vitro*. There are several potential applications of embryoids and we look forward with optimism to the progress of embryoids from a standpoint of basic science. However, today, it feels premature to couple their success with clinical applications.

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Appendix

The *MHR*-ISSCR guidelines working group who participated in the discussions preceding the drafting of the manuscript and its subsequent edits are (listed in alphabetical order): Bieke Bekaert, Michele Boiani, Julian Christians, Lynsey Cree, Alexandra Harvey, Francesca Gioia Klinger, Valentina Lodde, Bernard Roelen, Jan-Bernd Stukenborg, and Joachim Wistuba.

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