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All models are wrong, but some are useful: Establishing standards for stem cell-based embryo models

Eszter Posfai,[1,*] Fredrik Lanner,[2,3,4,*] Carla Mulas,[5] and Harry G. Leitch[6,7,8,*]
[1]Department of Molecular Biology, Princeton University, Princeton, NJ, USA
[2]Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden
[3]Division of Obstetrics and Gynecology, Karolinska Universitetssjukhuset, Stockholm, Sweden
[4]Ming Wai Lau Center for Reparative Medicine, Stockholm node, Karolinska Institutet, Stockholm, Sweden
[5]Wellcome - MRC Cambridge Stem Cell Institute, University of Cambridge, Cambridge, UK
[6]MRC London Institute of Medical Sciences, London, UK
[7]Institute of Clinical Sciences, Faculty of Medicine, Imperial College London, London W12 0NN, UK
[8]Centre for Paediatrics and Child Health, Faculty of Medicine, Imperial College London, London W2 1PG, UK
*Correspondence: eposfai@princeton.edu (E.P.), fredrik.lanner@ki.se (F.L.), h.leitch@lms.mrc.ac.uk (H.G.L.)
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Detailed studies of the embryo allow an increasingly mechanistic understanding of development, which has proved of profound relevance to human disease. The last decade has seen *in vitro* cultured stem cell-based models of embryo development flourish, which provide an alternative to the embryo for accessible experimentation. However, the usefulness of any stem cell-based embryo model will be determined by how accurately it reflects *in vivo* embryonic development, and/or the extent to which it facilitates new discoveries. Stringent benchmarking of embryo models is thus an important consideration for this growing field. Here we provide an overview of means to evaluate both the properties of stem cells, the building blocks of most embryo models, as well as the usefulness of current and future *in vitro* embryo models.

Introduction

Deciphering the mechanisms by which cells assemble into functional structures, such as tissues, and organs during embryonic development is fundamental to our understanding of how organisms develop and how errors in these processes result in developmental disorders and birth defects (Khokha et al., 2020).

How do we access these secrets of the embryo?

Descriptive embryological studies have cataloged early development across a range of species, revealing both the common principles and species-specific quirks in early embryogenesis. Insights gained from more basic model organisms were key to many early discoveries in mammals and continue to make an important contribution. The early experimental embryologists used cell transplantation assays to assess cell potential during embryogenesis (Beddington, 1982; Gardner and Rossant, 1979), and constructed cell fate maps based on laborious manual lineage tracing experiments (summarized in Lawson, 1999). The rise of genome editing techniques and the advances in molecular biology have allowed the generation of increasingly sophisticated animal models, including transgenic reporters, gene knockouts, and genetic approaches to lineage tracing. And finally, single-cell techniques, most notably RNA-sequencing, are enabling exhaustive cellular cataloging of embryos.

The insights gained from direct experimentation on embryos is indisputable. However, there are still major drawbacks to using embryos to advance our understanding of developmental mechanisms. Mammalian embryos, even those of the relatively prolific mouse, are scarce, contain few cells, and can be difficult to obtain because of ethical and cost considerations. Moreover, key events, such as gastrulation, often occur after the embryo implants and are not easily observable. Access to human embryos is naturally even more limited, due to practical, ethical, and legal limitations. Although advances have been made toward culturing human embryos past implantation, studies on donated embryos are constrained by the 14-day rule. Access to human embryos from termination of pregnancies is generally only available from the fifth week and later. For these reasons, methods to study mammalian development that do not rely on regular access to embryos would be advantageous. An early example of just such an approach was the study of transplantable teratocarcinomas, which had its origin in the pioneering work of Leroy Stevens (Stevens, 1967). These tumors are the source of embryonal carcinoma (EC) cells, which can be cultivated *in vitro*. When allowed to spontaneously differentiate, EC cells form 3-dimensional (3D) aggregates called embryoid bodies (EBs). Remarkably, EBs can form structures that are overtly similar to mouse embryos and that recapitulate many of the processes that occur in early postimplantation development (Martin and Evans, 1975; Martin et al., 1977). For this reason, EBs were seen as an important and promising tool by developmental biologists. In fact, the ability of EC cells to mimic development via EBs led to the notion that the early embryo itself might yield stem cell lines directly. This ultimately led to the derivation of mouse embryonic stem cells (mESCs) (Evans and Kaufman, 1981; Martin, 1981), a discovery that rendered EC cell research largely redundant. Unlike their karyotypically abnormal counterparts, mESCs were not only amenable to *in vitro* study but could efficiently reintegrate and take part in mouse embryogenesis; most critically allowing access to the germline.

Since the derivation of mESCs from the blastocyst-stage embryo, significant advances have been made in extending the repertoire of stem cell types in the mouse (reviewed in Rossant, 2008), as well as other mammalian species, notably humans (reviewed in Wu et al., 2016). There now exists a suite of early embryo-derived stem cell types which have opened major new avenues of research by creating an *in vitro*-grown and indefinitely self-renewing model of early embryonic cells (Table 1). Culturing and differentiating these stem cells *in vitro* has already advanced our understanding of the molecular mechanisms and cellular behaviors that underlie developmental processes in both healthy and pathological states, and as such have had a transformative effect on both basic and biomedical research. There are, however, drawbacks to experimentation with stem cells. First, most early embryo-derived stem cells are lineage restricted and cannot produce the cell type diversity that would support the entirety of embryonic development. Second, most protocols culture and differentiate stem cells in 2D, which cannot recapitulate the complex spatial interactions between cells and their environments that in many cases might be key to cellular function and tissue physiology.

These observations have led to a renewed interest in the development of more complex, often 3D, models of stem cell differentiation, which are designed to more accurately recapitulate the spatial interactions among cells observed in early embryonic development (Figure 1 and Table 2). Indeed, although EBs represented the first 3D differentiation system, it is only recently that EB-inspired approaches are once again being revisited (reviewed in Brickman and Serup, 2016). The aphorism of our title is oft-used and provokes a central question in this field: how useful are these models to the study of development and differentiation, and how can we improve and best utilize them going forward? Notably, despite renewed interest, no stem cell-based embryo model has come close to generating an embryo that is capable of giving rise to a viable animal or a functional tissue: the ultimate test of developmental fidelity. Here we first evaluate the stem cells commonly used to construct such systems, before discussing the various methods one might use to assess and validate stem cell-derived embryo models.

How good are the building blocks? Evaluation of current stem cells

An important aspect of building a stem cell-based embryo model is having the right starting cells that are able to differentiate and pattern into the desired structure. Since the first derivation of mESCs from the mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981), a number of different stem cell types representing distinct developmental stages have been derived from the epiblast (EPI) or the extraembryonic lineages of the early mouse or human embryo (Table 1). Alternatively, using either genetic or chemical reprogramming, different cellular states could also be converted to embryonic or extraembryonic stem cell states (reviewed in Nashun et al., 2015; Niwa, 2018). Different culture formulations capture different stages of developing lineages, with varying degrees of heterogeneity, chimeric potential, and differentiation ability (reviewed in De Los Angeles et al., 2015; Rossant, 2008). Therefore, the cellular identities and differentiation capacity of the starting stem cell state need to be evaluated, and considered prior to embarking on assessment of downstream model systems. So how good are currently available stem cell types? How can they be evaluated (Figure 2)?

Evaluation criteria

Morphology

Stem cells in a particular culture condition exhibit characteristic cell and colony morphologies. The degree of morphological heterogeneity within a cultured line can be an indication of their quality and a way to rapidly evaluate the degree of spontaneous differentiation (Nagasaka et al., 2017; Perestrelo et al., 2017). Recently, live-imaging coupled with deep learning of self-renewing and differentiating pluripotent stem has allowed identification of morphologies that appear to be unambiguously associated with differentiation (Waisman et al., 2019).

Genomic stability

Aneuploidies or large structural changes in chromosomes, arising due to genomic instability, are typically incompatible with normal embryonic development (reviewed in Garcia-Martinez et al., 2016); therefore, analysis of a stem cell line's karyotype to assess its quality is a standard technique. Certain chromosomal abnormalities occur more frequently, for example in mESC cultures, possibly due to conferring a growth advantage to cells (Codner et al., 2016; Zhang et al., 2016). X chromosome instability is also a well-described feature in mESC lines (Zvetkova et al., 2005), while human ESCs are prone to exhibit variability in X chromosome inactivation status (Silva et al., 2008). Cells with two active X chromosomes only exist transiently during normal development *in vivo*, and therefore are likely not an optimal state to maintain long-term *in vitro* (Silva et al., 2008). Loss of the Y chromosome is also a frequent occurrence (Eggan et al., 2002). Chromosomal abnormalities particularly impact germline transmission of the ESC genome, most likely due to a failure to navigate meiosis (Codner et al., 2016; Liu et al., 1997; Longo et al., 1997). However, other work has highlighted instances where certain chromosomal abnormalities have no apparent effect on somatic differentiation potential (Gaztelumendi and Nogués, 2014) and that certain tissue types tolerate chromosomal abnormalities better than

Table 1. Mouse, nonhuman primate, and human stem cell types representing early embryo development

	Derivation origin	Functional potential	Reference
MOUSE			
naive pluripotent stem cells	preimplantation EPI	EPI lineages, PGCs	Evans and Kaufman (1981) Martin (1981)
formative pluripotent stem cells	early postimplantation EPI, conversion from naive pluripotent stem cells	EPI lineages, PGCs	Kinoshita et al. (2020)
primed pluripotent stem cells	postimplantation EPI up until early head fold stage, conversion from naive and formative pluripotent stem cells	EPI lineages, not PGCs	Brons et al. (2007) Tesar et al. (2007) Osorno et al. (2012)
extended pluripotent or expanded potential stem cells	morula or blastocyst-stage embryos, conversion from naive pluripotent stem cells	EPI lineage, potentially PE lineage, no TE lineage	Yang et al. (2017a) Yang et al. (2017b)
Hex-positive embryonic stem cells	spontaneously occurring in naive embryonic stem cell cultures	EPI, PE, TE lineages claimed, but unconfirmed	Morgani et al. (2013)
2 cell-like cells	subpopulation of cells occurring in naive pluripotent cultures	EPI, PE, TE lineages claimed, but unconfirmed	Macfarlan et al. (2012)
Gata4/6-plused embryonic stem cells	transient overexpression of Gata4 or Gata6 in naive pluripotent cells	EPI and PE lineages	Schroeter et al. (2015)
trophoblast stem cells	blastocyst TE, postimplantation ExE	TE lineage (detailed analysis of differentiation into all cell types not available)	Tanaka et al. (1998)
extraembryonic endoderm stem cells	blastocyst (presumably PE lineage), early postimplantation embryo	parietal endoderm *in vivo*, can make visceral endoderm *in vitro*	Kunath et al. (2005) Lin et al. (2016)
naive and primitive extraembryonic endoderm stem cells	converted from naive pluripotent stem cells (naive), or derived from blastocyst embryos (primitive)	parietal and visceral endoderm *in vivo* (demonstrated for naive)	Anderson et al. (2017) Zhong et al. (2018)
NONHUMAN PRIMATE			
primed pluripotent stem cells	blastocyst ICM/EPI	cannot contribute to chimeras; EPI lineage, PGCs (*in vitro*), trophoblast potential debated	Thomson et al. (1995)
HUMAN			
naive pluripotent stem cells	blastocyst ICM/EPI	EPI lineages, extraembryonic lineages, PGCs (*in vitro*)	Gafni et al. (2013) Takashima et al. (2014) Theunissen et al. (2014) Ware et al. (2014) Guo et al. (2020) Io et al. (2020)

(*Continued on next page*)

Table 1. ***Continued***

	Derivation origin	Functional potential	Reference
formative pluripotent stem cells	blastocyst ICM/EPI, conversion from naive pluripotent stem cells	EPI lineages, PGCs (*in vitro*)	Kinoshita et al. (2020) Yu (2020)
primed pluripotent stem cells	blastocyst ICM/EPI, conversion from naive pluripotent stem cells	EPI lineages, extraembryonic lineages, PGCs (*in vitro*)	Thomson et al. (1998) Xu et al. (2002) Amita et al. (2013)
extended pluripotent or expanded potential stem cells	conversion from primed pluripotent stem cells	EPI lineages, extraembryonic lineages, PGCs (*in vitro*)	Gao et al. (2019) Yang et al. (2017b)
trophoblast stem cells	blastocyst TE, cytotrophoblast, conversion from naive pluripotent stem cells	TE lineage (*in vitro*)	Okae et al. (2018) Cinkornpumin et al. (2020) Dong et al. (2020)
extraembryonic endoderm stem cells	conversion from naive pluripotent stem cells	visceral endoderm (*in vitro*)	Linneberg-Agerholm et al. (2019)

EPI, epiblast; ExE, extraembryonic ectoderm; ICM, inner cell mass; PE, primitive endoderm; PGC, primordial germ cell; TE, trophectoderm.

others (reviewed in Garcia-Martinez et al., 2016). Smaller copy number variations also arise frequently during routine culture of stem cells (Liang et al., 2008), although whether the frequency with which they occur is higher than expected during normal cell divisions is less clear. Although the links between specific chromosomal abnormalities and stem cell potential remain an area of interest and investigation, we would recommend using cell lines with a normal chromosomal complement and the minimum of other genetic aberrations when attempting to model normal embryonic development. Regular screening of stem cell lines may well be needed to ensure their ongoing fidelity. Alternatively, the use of stem cell lines with known chromosomal abnormalities may provide a system to study how the resulting developmental defects subsequently emerge.

Gene expression

Comparing gene expression of stem cell types, whether by a handful of key marker genes or by genome-wide expression profiling, to representative embryo stages is a good first approximation of cell identity. However, there are inevitable deviations to be expected in the transcriptome due to adaptation to culture conditions (Tang et al., 2010), and therefore a transcriptomic comparison alone will never fully predict the functional performance of a particular stem cell type. It is important to note, however, that in species such as nonhuman primates (NHPs) and humans, where functional assays are limited, a transcriptional comparison remains a major pillar of stem cell quality control, given proper *in vivo* comparisons are available (discussed later).

Epigenetic characteristics

The epigenetic status of a stem cell line may impact gene expression, but is also an independent feature impacting differentiation potential (reviewed in Festuccia et al., 2017). For instance, mESCs in which DNA methyltransferase activity is disrupted are limited in their somatic differentiation potential but can undergo trophoblast differentiation (Ng et al., 2008; Sakaue et al., 2010), a clear indication that epigenetic characteristics of a given stem cell are a key consideration. Recently, there has been an interest in "epigenetic resetting" in reestablishing functionality in a range of developmental systems (for instance Lu et al., 2020). While DNA methylation is perhaps most studied, other epigenetic marks, processes, and characteristics are key in determining stem cell behavior (reviewed in Festuccia et al., 2017), and are again of particular interest in situations in which functional assays are not available.

In vitro *differentiation potential*

Evaluating the cell repertoire a stem cell is able to give rise to is a must in the process of determining potential. This can be done *in vitro* by guiding stem cells down defined differentiation trajectories. Differentiation protocols typically rely heavily on knowledge of embryonic development, which for some lineages is more established than for others (Keller, 2005). This therefore represents a limitation of *in vitro* differentiation: in many cases optimal differentiation protocols are not in place to yield desired cell types. In addition, analysis of differentiated cell types relies similarly on evaluating gene or protein expression in comparison with embryonic cells, which depending on the embryonic stage and species, may or

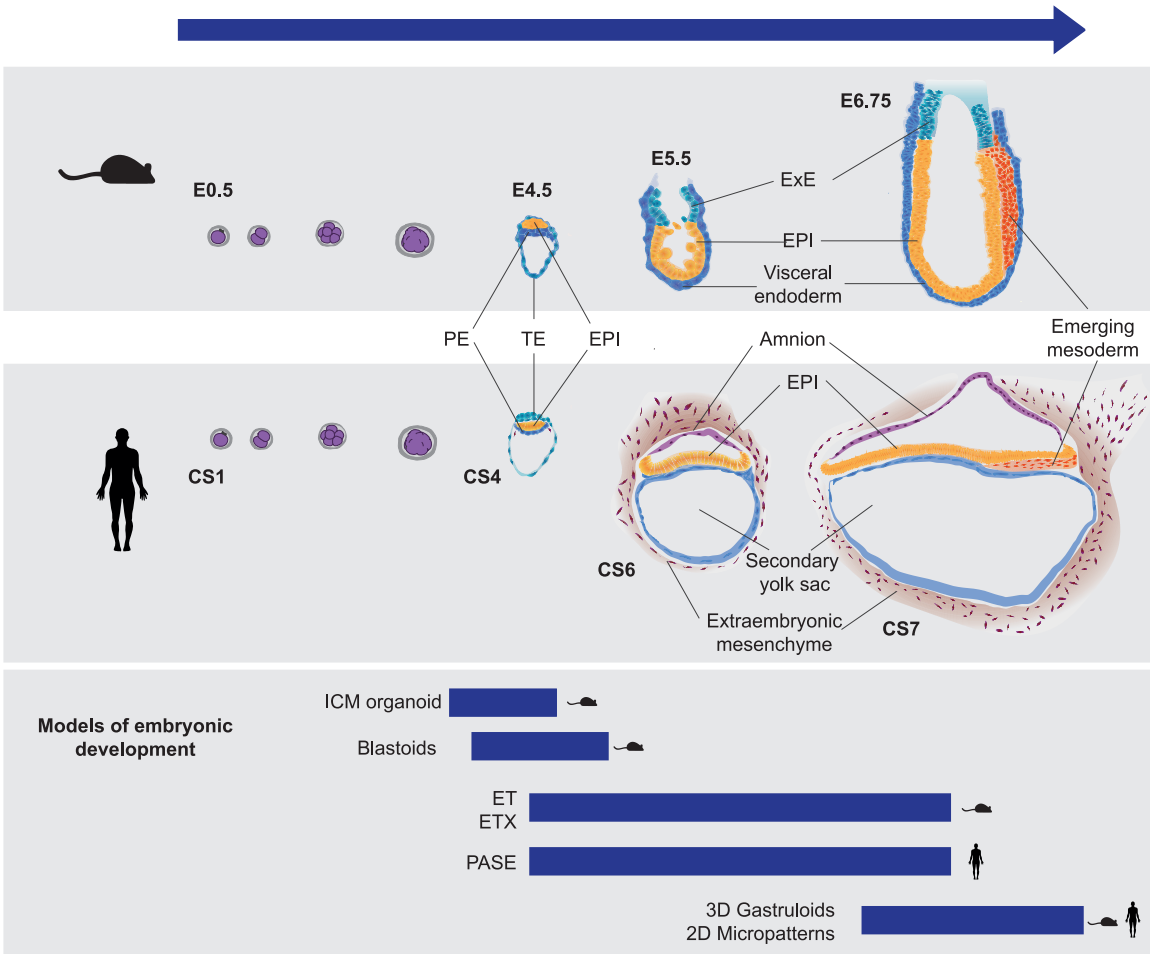

Figure 1. Timeline of embryonic development in mouse and human

Key cell types are shown across embryonic days (E) for the mouse, while for the corresponding Carnegie stage is shown for humans. Bottom panel shows a summary of the models of embryonic development (see Table 2), the corresponding embryonic stage they model, and species in which they have been developed so far. Abbreviations: PE, primitive endoderm (hypoblast); TE, trophectoderm; EPI, epiblast; ExE, extraembryonic ectoderm; CS, Carnegie stage.

may not be available. Nevertheless, when differentiation cues and adequate markers are known, *in vitro* differentiation provides an easily accessible option for testing cell potential.

In vivo *differentiation potential, ability of colonizing a host embryo*

A more stringent approach for testing differentiation potential is combining stem cells with host embryos to generate a chimera and analyzing which lineages the stem cell progeny contribute to at later developmental stages. Such *in vivo* differentiation assay has the benefits of allowing the embryonic environment to instruct differentiation. The disadvantages of this approach include low throughput capacity, the differential ability of stem cell types to colonize the host embryo, especially in situations where the stem cell is developmentally (Cohen et al., 2018) or phylogenetically (Masaki and Nakauchi, 2017) not matched to the host embryo, and the increased difficulty of characterizing the cellular identities of stem cell progeny within a complex tissue. Appropriate analysis and interpretation of chimera experiments is a major consideration (Posfai et al., 2021; Wu et al., 2016). The goal of such experiments is to assay for functional contribution to normal development, and as such, low contribution of cells that are not obviously functionally incorporated into tissues should be interpreted with caution. High contribution to adult tissues remains the best assessment of functionality. When embryos are assessed, expression of cellular markers of functional differentiation in donor cells (as assessed by immunofluorescence for instance), is a better indication than positional information alone.

Table 2. Mouse and human stem cell-based embryo models

Embryo model	Species	Constructed from:	Have been used to model aspects of:	Reference
ICM organoid	mouse	Gata6-plused mESC	EPI and PE sorting of inner cell mass	Mathew et al. (2019)
Blastoid	mouse	mESC + mTSC; mExtendedPSC + mTSC; mExtendedPSCor mExpandedPSC; 2C-like primed-to-naive conversion intermediate	formation of blastocyst morphology; crosstalk between EPI and TE compartments; initiation of decidualization	Rivron et al. (2018a) Kime et al. (2019) Li et al. (2019) Sozen et al. (2019)
ET embryoid	mouse	mESC + mTSC	AP axis formation; primitive streak and mesoderm formation; PGC specification; EPI and trophoblast compartment interaction	Harrison et al. (2017)
ETX embryoid	mouse	mESC + mTSC + mXEN; Gata6-pulsed mESC + mTSC	cavity formation; AP axis formation; primitive streak formation; EMT; mesoderm and endoderm formation; AVE migration; EPI, trophoblast and extraembryonic endoderm compartment interaction; PGC specification; initiation of decidualization	Sozen et al. (2018) Zhang et al. (2019) Amadei et al. (2021)
EB	mouse, human	mESC, hESC	germ layer formation	Martin and Evans (1975) Martin et al. (1977) Itskovitz-Eldor et al. (2000)
polarizing EB/3D gastruloid	mouse	mESC, mEC	primitive streak gene expression; AP, DV, and LR axis patterning; spatial gene expression of germ layers and patterning (e.g., *Hox* genes)	ten Berge et al. (2008) Marikawa et al. (2009) Boxman et al. (2016) van der Brink et al. (2014) Turner et al. (2017); Beccari et al. (2018) Moris et al. (2020) Simunovic et al. (2019)
3D gastruloid with ECM	mouse	mESC	same as 3D gastruloids; somite formation	van der Brink et al. (2020) Veenvliet et al. (2020)
2D micropatterned colony	mouse, human	mESC, hESC	germ layer formation (mesoderm, endoderm or ectoderm patterning); EMT	Warmflash et al. (2014) Deglincerti et al. (2016) Etoc et al. (2016) Britton et al. (2019) Morgani et al. (2018)
Gastrulation-like node	human	hESC	primitive streak formation; EMT	Muncie et al. (2020)
Postimplantation amniotic sac embryoid	human	hESC	amniotic sac formation; anterior and posterior epiblast fate specification	Shao et al. (2017) Zheng et al., (2019)

AVE, anterior visceral endoderm; EPI, epiblast; hESC, human embryonic stem cell; mESC, mouse embryonic stem cell; PE, primitive endoderm; PGC, primordial germ cell; PSC, potential stem cell; mTSC, mouse trophoblast stem cells; EMT, epithelial-to-mesenchimal transition; mXEN, mouse extraembyonic endoderm stem cells; mEC, mouse embryonic carcinoma; AP, anterior-posterior; DV, dorso-ventral; LR, left-right.

Mouse stem cells

Below we discuss different stem cell types that have been isolated from mouse embryos. There has been particular interest in recent times in distinguishing distinct pluripotent states. This is a rapidly evolving field. We have chosen to designate distinct stem cell types based on their competence for differentiation, as described below. In the future, further subdivisions or alternative categorizations may be necessary based on experimental data.

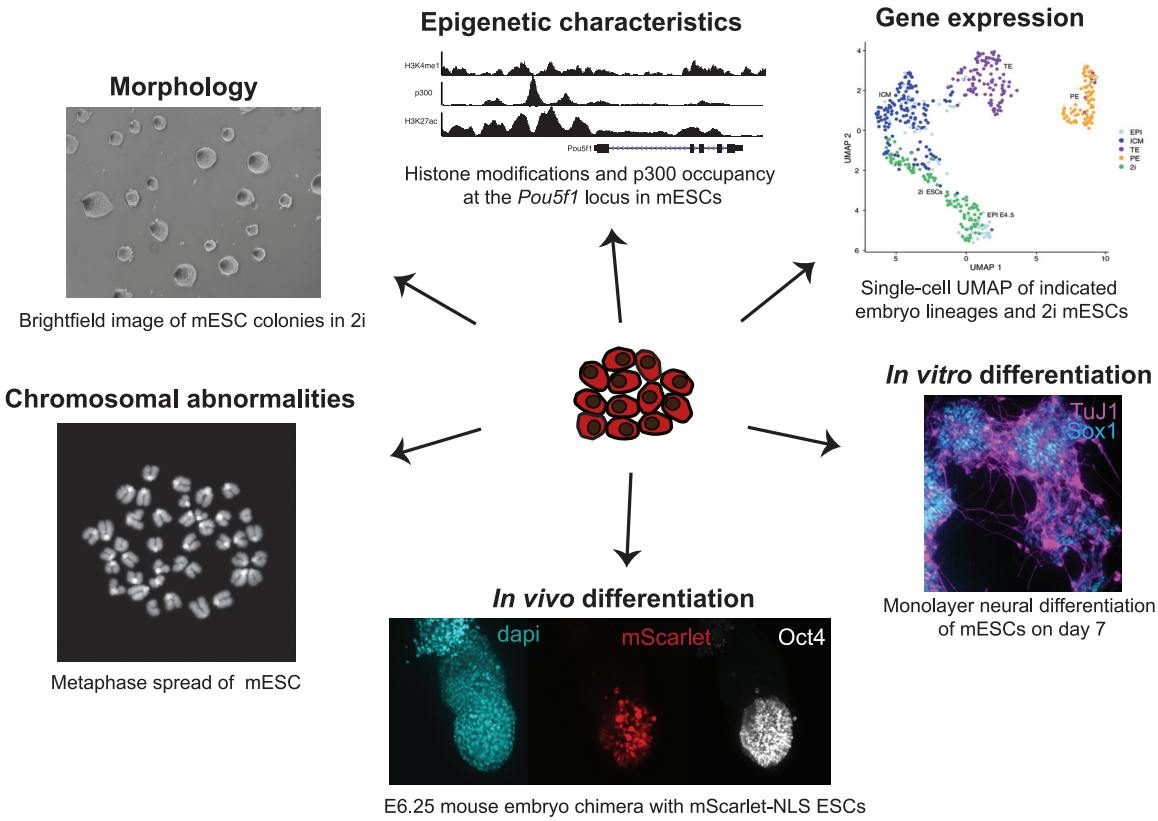

Figure 2. Ways to evaluate stem cells, illustrated by properties of mESCs
Single-cell UMAP based on data from (Posfai et al., 2021); epigenetic characteristics from (Buecker et al., 2014); remaining elements are unpublished data provided by the authors.

Naive pluripotent stem cells

The first pluripotent stem cell to be derived and the most extensively used, mESCs exhibit naive pluripotency, which is the ability of a single cell to make high contribution chimeras, including the germline (reviewed in De Los Angeles et al., 2015). Similarly, *in vitro*, naive pluripotent mESCs are able to differentiate into formative cells, which in turn can undergo germ layer differentiation and be induced to form PGC-like cells (Smith, 2017) (see next). Derived from the EPI of the blastocyst-stage embryo, their global gene expression profile most closely reflects that of E3.5-E4.5 EPI cells (Boroviak et al., 2014; Chen et al., 2016; Posfai et al., 2021). However, the culture conditions used to maintain the state can have profound effects on the morphology, expression profile, degree of heterogeneity, and epigenetic characteristics of cells (McEwen et al., 2013). The naive state can be captured in a relative homogeneity by 2i (Ying et al., 2008), 2i/LIF, or defined KOSR/LIF conditions (Gonzalez et al., 2016), while the traditional serum/LIF culture seems to sustain a highly heterogeneous cell population, composed of cells resembling various stages of preimplantation and early postimplantation development (reviewed in Morgani et al., 2017). Chimeric potential of serum/LIF ESCs is frequently low (especially from non-permissive genetic backgrounds), possibly due to cell heterogeneity, while 2i ESCs display higher chimeric contributions. Only mESCs to date have been shown to have the ability to rescue embryonic development in tetraploid embryos, an experimental technique that forces ESCs to take over the embryonic compartment from which tetraploid cells are gradually lost (Nagy et al., 1990). Culture with MEK and Wnt inhibitor (rather than agonist) captures cells in an intermediate rosette-like stage with features of the peri-implantation EPI, including cell polarization and epigenetic characteristics (Neagu et al., 2020). Cells can transit freely between conventional naive conditions and this rosette-like state, suggesting this represents an advanced naive state with additional features, or an intermediate cell type between naive and formative states. Future comparisons between the rosette-like state, formative stem cells, and the peri-implantation embryo will be of particular interest, and may necessitate a re-appraisal of the categories presented here.

Formative stem cells

In the embryo, the EPI exits the naive pluripotent state around the time of implantation and transitions to a formative state that is transiently competent for primordial germ cell (PGC) specification (Ohinata et al., 2009; Smith, 2017). This formative state can be induced from mESCs by differentiating them in Fgf/Activin to generate EPI-like cells (EpiLCs) (Hayashi et al., 2011), a transient intermediate required to induce PGC-like cells (PGCLCs) *in vitro*. Recent studies have reported capture of a formative state in self-renewing stem cell lines (Kinoshita et al., 2020). The formative stem (FS) cells reported by Kinoshita et al. (2020) are cultured in low Activin with inhibition of WNT and retinoic acid signaling. FS cells can be derived from but do not readily revert to the naïve pluripotent stem cells, indicative of a distinctive cellular state. They can directly give rise to PGCLCs and, following blastocyst injection, can colonize all three germ layers and the germline of the developing embryo, although germ line transmission has not yet been shown. The derivation of a self-renewing stem cell type that can directly give rise to PGCs is a major advance, and highlights the possibility that robust, direct formation of PGCs may emerge as an important functional test of peri-implantation pluripotent states. The chimera-forming efficiency of FS cells is lower than that of naïve ESCs, perhaps due to the heterochronicity between the E3.5 blastocyst and FS cells (which likely approximates to the E5.5-E6.5 EPI). Intriguingly, this observation led to a reevaluation of the capacity of the E5.5 EPI to contribute to chimeras following blastocyst injection, revealing a similar contribution pattern as reported for FS cells (Kinoshita et al., 2020). This highlights that observations *in vitro* can impact and extend our understanding of *in vivo* development, and demonstrates the benefits of iterating between *in vivo* and *in vitro* studies. Simultaneously Yu et al. (2020) reported the derivation of XPSCs. These cell lines maintain robust expression of naive pluripotency markers, as well as formative markers. They are derived from the preimplantation EPI (but not from postimplantation stages) and the extent to which they have irreversibly exited the naive state has not been reported. However, these cells can directly form PGCLCs *in vitro* and exhibit germline transmission following blastocyst injection. The culture conditions for XPSCs and FS cells are near opposite to each other, which in itself is an intriguing finding. Additional studies are needed to relate the significance of these signals to the native embryonic environment. Future work should also establish whether XPSCs are truly formative in nature or perhaps represent a distinctive naive or intermediate pluripotent state, such as that exhibited by rosette-like cells (to which their transcriptome best approximates).

Primed pluripotent stem cells

Primed epiblast stem cells (EpiSCs) can be derived from a range of developmental stages (Brons et al., 2007; Osorno et al., 2012; Tesar et al., 2007), but are most closely related to late-gastrulation anterior primitive streak (Kojima et al., 2014). They are typically cultured in fibroblast growth factor (FGF) and activin conditions, with FGF signaling likely the key driver from formative to primed pluripotency (Kinoshita et al., 2020). EpiSCs are heterogeneous in culture, although inhibition of Wnt signaling may lead to increased homogeneity (Kurek et al., 2015; Sugimoto et al., 2015). There is also significant line-to-line variability (Bernemann et al., 2011), which is at least partially due to variations in culture methodologies. EpiSCs display a distinctive gene expression profile, epigenetic status and differentiation capacity *in vitro,* perhaps most notably being recalcitrant to PGCLC induction (Hayashi et al., 2011). Without genetic or other manipulations that promote development reversion, they cannot readily contribute to live-born chimeras following blastocyst injection. However, in keeping with their cell type of origin and gene expression profile, they can reintegrate into the postimplantation embryo in *ex vivo* grafting experiments (Huang et al., 2012; Kojima et al., 2014).

Stem cells with extended or expanded potential

A number of studies have reported the identification of pluripotent stem cells that also harbor the ability to efficiently undertake extraembryonic lineage differentiation. Such stem cells, for example, were identified in 2i/LIF-cultured ESCs as a subpopulation that expressed the endoderm marker Hex (Morgani et al., 2013). However, the extent of extraembryonic differentiation and how generalizable such a feature is of 2i/LIF-cultured cells, remain open questions.

Expanded potential stem cells (Yang et al., 2017a) and extended pluripotent stem cells (Yang et al., 2017b) (hereafter both referred to as EPSCs) are some more recent arrivals on the embryo-derived stem cell stage. These stem cells can be derived from blastocyst or 8-cell stage embryos (Yang et al., 2017a), or converted from naive pluripotent mESCs using a cocktail of growth factors and inhibitors (Yang et al., 2017a, 2017b). Originally, they were found to express some genes characteristic of early-stage embryos. However, these were not significantly different from levels expressed by naive pluripotent mESCs and the overall expression profile of EPSCs most closely resembled that of E4.5 and E5.5 EPI (Posfai et al., 2021). In contrast to the original reports describing these stem cell types, later studies with more stringent criteria found no convincing evidence that these stem cell types exhibit potential to enter the trophoblast lineage (Posfai et al., 2021; Sozen et al., 2019). Interestingly, however, both of these stem cell types outperform 2i ESCs in their ability to chimerize

a host embryo (Li et al., 2019; Posfai et al., 2021) and there is indication these cells can indeed enter the primitive endoderm (PE) lineage in an *in vitro* blastoid setting (Sozen et al., 2019), properties that were not predicted from expression profiling. We also note that the capacity for low-level contribution to extraembryonic lineages has been reported since the earliest studies of chimeric contribution of mESCs (Beddington and Robertson, 1989) and so whether observations of enhanced potential represent a significant departure from the properties of standard naive pluripotent stem cells remains unclear. Considering that EPSCs display transcriptional signatures reaching toward E5.5 EPI, while still retaining high chimeric contribution potential, it would be interesting to contrast these with FS cells that share at least some similar properties.

Stem cells resembling the 2-cell embryo stage

In 2012, a small subpopulation (0.1%–0.4%) of stem cells were discovered in naive pluripotent mESC cultures which transiently expressed the endogenous retroviral transcript, MuERV-L, a marker of the 2-cell stage embryo (Macfarlan et al., 2012). This state, termed 2C-like, is an intriguing one: it occurs spontaneously and transiently in cultures. While in this state, cells express numerous other 2-cell-associated transcripts, such as *Zscan4* and Dux; pluripotency markers such as Oct4, Sox2, and Nanog are downregulated; histone mobility and chromatin accessibility are increased; and a distinct metabolic state is displayed (reviewed in Genet and Torres-Padilla, 2020). Since many of these features resemble characteristics of a 2-cell stage embryo, 2C-like cells have been dubbed the new *in vitro* model to study events taking place in the early embryo, most notably zygotic genome activation. Consequently, an increasing number of regulators, including transcription factors, microRNAs and epigenetic modifiers are being identified, which either promote or inhibit the 2C-like state in mESC cultures (reviewed in Iturbide and Torres-Padilla, 2020). There is abundant interest in understanding how accurately indeed this *in vitro* state models the 2-cell stage embryo. Despite subtle differences, the transcriptome of these cells remains more akin to unselected mESCs than to the 2-cell stage embryo (Kolodziejczyk et al., 2015). Furthermore, although *Dux* is necessary and sufficient for the 2C-like state *in vitro*, it may not be needed during embryonic development (Chen and Zhang, 2019; De Iaco et al., 2020). Critically, the *in vivo* developmental potential of 2C-like cells awaits clarification. Despite the abundant literature unpacking the regulators of this state, comprehensive assessment of the *in vivo* differentiation ability of 2C-like cells is lacking. Early studies suggested enhanced contribution to chimeras, including to the extraembryonic compartment (Choi et al., 2017; Macfarlan et al., 2012); however, stringent assessments were missing. Of note, Zscan4-expressing cells in mESC cultures, which are more abundant than MuERV-L-expressing 2C-like cells, but include the 2C-like population, reportedly have very poor chimeric potential (Amano et al., 2013). Therefore, from the *in vivo* experiments undertaken thus far it remains unclear if 2C-like cells exhibit any characteristics of 2-cell stage embryo cells or indeed have any distinctive functional properties.

Trophoblast stem cells

Trophoblast stem cells (TSCs) can be derived from the trophectoderm (TE) of the blastocyst embryo, as well as from the extraembryonic ectoderm (ExE) compartment of the postimplantation embryo all the way up to E8.5 (Tanaka et al., 1998; Uy et al., 2002). They were recently suggested to contain subpopulations that express markers of either preimplantation TE or postimplantation ExE (Frias-Aldeguer et al., 2020). Since the original derivation established the dependence of TSCs on FGF signaling (Tanaka et al., 1998), the development of defined culture conditions have since identified other signaling needs of TSCs, such as TGF-B signaling (Erlebacher et al., 2004; Kubaczka et al., 2014; Ohinata and Tsukiyama, 2014). In *in vitro* differentiation assays TSCs show a strong preference to differentiate into the giant cell lineage upon the withdrawal on self-sustaining signals, although when guided by additional signaling cues can be coaxed into differentiating into syncytial trophoblast types (reviewed in Latos and Hemberger, 2016). However, systematic analysis of whether TSCs can differentiate *in vitro* into all derivatives of the trophoblast lineage is lacking. Likewise, *in vivo* analysis of chimeras produced by TSCs is equally missing detailed analysis. A single report to date has shown that TSCs are able to rescue SOCS3−/− mutants, by rescuing the formation of the labyrinth layer in the placenta, which is otherwise absent in these mutants (Takahashi et al., 2006). However, it still remains an open question whether TSCs harbor the full developmental potential of the trophoblast lineage and whether they could generate the entire trophoblast compartment of the placenta. Overall TSCs display low chimeric potential (~9% reported in Ohinata and Tsukiyama, 2014). This, however, may very well be attributed to a limited ability to integrate into a preimplantation host embryo, as a large fraction of TSCs may resemble postimplantation stages (Frias-Aldeguer et al., 2020). Exciting efforts are on the way to attempt to capture TSCs at an earlier developmental stage, representing a homogeneous population of preimplantation TE, which could potentially enhance both their differentiation and chimerization abilities (Frias-Aldeguer et al., 2020; Lee et al., 2019; Nosi et al., 2017).

Extraembryonic endoderm stem cells

Extraembryonic endoderm (XEN) stem cells were originally derived from the PE layer of the blastocyst-stage embryo (Kunath et al., 2005), but were later also established

from early postimplantation stage embryos (Lin et al., 2016). The growth factor requirements of the first cultures are not well-defined, as XEN cells were demonstrated to thrive in a number of different conditions (Niakan et al., 2013). Under different culture conditions, ESCs have been shown to express PE marker genes and adopt XEN-like morphologies, highlighting that the barrier between naive pluripotency and a PE fate can be breached (Artus et al., 2010; Capo-Chichi et al., 2005; Soprano et al., 2007; Vrij et al., 2019). Both *in vitro* and *in vivo*, XEN cells demonstrated a strong bias to differentiate into postimplantation parietal endoderm and only rarely gave rise to visceral endoderm (Kunath et al., 2005; Lin et al., 2016). It was later demonstrated that the addition of Bmp4 *in vitro* is able to induce visceral endoderm differentiation of XEN cells, suggesting that the levels of Bmp4 supplied by the inner cell mass (ICM) and later the ExE in the embryo is likely too low to achieve such differentiation in chimeras (Artus et al., 2012; Paca et al., 2012). More recently XEN cell lines termed naive XEN (Anderson et al., 2017) and primitive XEN (Zhong et al., 2018) have been established using Wnt/ActivinA/Lif and low serum/Lif/Pdgf conditions, respectively. Both these new XEN lines share more markers with blastocyst-stage PE compared to conventional XEN cells. In addition, naive XEN cells were demonstrated to contribute to both visceral and parietal endoderm in chimeras (Anderson et al., 2017).

Intermediates and products of genetic reprogramming

It is not a requirement to start a stem cell-based embryo model from a stable cellular state. In fact, an increasing number of examples demonstrate that intermediates of genetic reprogramming may provide alternate starting states. For example, mESCs can be converted to XEN cells by overexpressing Gata6, a key marker of the PE (Fujikura et al., 2002; Shimosato et al., 2007). Overexpressing Gata6 for only 6 h in mESCs results in a state in which cells are double-positive for Gata6 and Nanog, resembling that of ICM cells of the blastocyst, prior to EPI/PE segregation (Schroeter et al., 2015). Indeed, these Gata6/Nanog double-positive cells were shown to be able to differentiate into an ESC or a XEN-like fate upon release from Gata6-overexpression, which was also exploited in a cell-based model of the ICM, termed ICM organoids (Mathew et al., 2019). Very recently, Gata6-pulsed mESCs were also used to build ETX embryoids, and were shown to be superior sources for EPI and PE-derived lineages, compared with using a mix of ESCs and conventional XEN cells as building blocks (Amadei et al., 2021).

Surprising properties of cells have been reported during the reprogramming of primed-to-naive pluripotency using defined culture conditions (Kime et al., 2019). Reprogramming intermediates expressing the 2-cell marker MuERV-L were reported, which subsequently formed cyst-like structures expressing some blastocyst lineage markers and embryo-like organization. Detailed investigation is still needed to determine the extent these structures resemble blastocyst embryos. In addition, overexpression of OSKM factors *in vivo*, in a mouse, led to the emergence of cells that were claimed to harbor expanded differentiation potential compared with standard pluripotent stem cells (Abad et al., 2013), although stringent evaluation of these cells was not performed.

In summary, in the case of the mouse, mESCs are the only cell type that have convincing potential to generate all downstream lineages. On the other hand, current extraembryonic stem cells may not be up to a similar task, which is not only concerning because they do not give rise to all the necessary cell types, but because this may impact the provision of appropriate signals to the embryonic compartment that pattern the developing EPI.

Human stem cells

Evaluating human stem cells poses additional challenges. As *in vivo* chimera assays cannot be performed, validating the true nature of human stem cells is problematic. Interspecies chimeras using human donor cells have been reported in a small number of studies (reviewed in Wu et al., 2016). Whether survival of human cells in this context is a meaningful assessment of cellular potential is at present unclear. Alternatively, surplus early human embryos could be used as hosts for chimera generation. Subsequent culture up to the 14-day limit, may allow some estimation of the donor's cells contribution to early embryonic development. Equally important, transcriptional reference material is generally lacking making transcriptional benchmarking difficult. These challenges have been illustrated with the first human embryonic stem cells (hESCs) derived from the preimplantation blastocyst-stage EPI (Thomson et al., 1998). In contrast to mESCs, classical hESCs do not represent the *in vivo* equivalent of preimplantation EPI but probably a postimplantation to gastrulation stage EPI correlating approximately to the mouse primed pluripotent state of EpiSCs (Messmer et al., 2019; Nakamura et al., 2016; Tyser et al., 2020; Xiang et al., 2020). In recent years, different strategies to generate mouse-like naïve cells have been established (Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Ware et al., 2014), each of which share at least some characteristics with the human preimplantation EPI. Naive and primed pluripotent stem cells can now be used to mimic pre- and postimplantation stages of human EPI, including aspects related to diverse morphology, signaling pathways, transcriptional networks, X chromosome inactivation, and epigenetics (Collier et al., 2017; Sahakyan et al., 2017; Shahbazi et al., 2019; Theunissen et al., 2014).

The capture of a formative state in human cells was also reported (Kinoshita et al., 2020; Yu et al., 2020), although it

is not clear how distinct human FS cells are to existing "primed" hESC lines that harbor at least some cells that are competent to make PGCs directly (Sasaki et al., 2015).

Establishment of human stem cells representing the extraembryonic lineages has been even more challenging. Early reports suggested that hESCs have the capacity to differentiate into the trophoblast lineage (Amita et al., 2013; Xu et al., 2002; Yang et al., 2015). The transcription factors GATA2, GATA3, TFAP2A, and TFAP2C have been shown to be part of the network activated in these protocols and that GATA3 overexpression in hESCs could mimic BMP4-driven differentiation (Krendl et al., 2017). Such trophoblast potential further highlights the difference with mESCs, that do not readily differentiate into trophectoderm lineages without overexpression of CDX2, or other significant genetic manipulations (reviewed in Latos and Hemberger, 2016). However, mouse EpiSCs seem to possess a similar capacity to differentiate into trophectodermal lineages (Brons et al., 2007). While it remains unclear why stem cell lines that likely best represent the postimplantation embryo retain (or reacquire) the capacity to give rise to trophoblast lineages, this *in vitro* peculiarity is apparently conserved in primed pluripotent stem cells from both mouse and human. Indeed, the trophoblast potential has proved controversial and several reports suggest the putative trophoblast cells are not true trophoblast but are mistaken mesoderm or amnion cells (Bernardo et al., 2011; Dong et al., 2020; Guo et al., 2020; Lee et al., 2016; Roberts et al., 2014). It is, however, intriguing how BMP4 induction can generate more mature cells with extravillous trophoblast-like character expressing HLA-G, multinucleated syncytiotrophoblast-like cells with hCG secretion (Amita et al., 2013; Krendl et al., 2017; Mischler et al., 2019; Xu et al., 2002; Yang et al., 2015).

More recently, a flurry of papers have been published on this topic. First, trophoblast stem cell lines have been established both from blastocysts and first trimester placentas (Okae et al., 2018). Subsequently, similar results were achieved by converting hESCs into either CDX2-positive trophectoderm stem cells or CDX2-negative TSCs (Mischler et al., 2019). Several reports have also now shown that adult cells can be reprogrammed directly into TSCs resembling the cytotrophoblast of the postimplantation embryo (Castel et al., 2020; Liu et al., 2020). Other researchers argue that only naive hESCs retain the trophectoderm lineage potential (Cinkornpumin et al., 2020; Dong et al., 2020; Guo et al., 2020; Io et al., 2020). In these conditions, conventional primed hESCs were not able to give rise to TSCs, but instead produced cells that resemble amnion-like cells. Whether the ability of human naivecells to generate extraembryonic lineages represents a species difference in cellular potential, or could be due to culture-induced epigenetic changes, such as DNA hypomethylation, in human naive cells has not been fully established. The picture is also not entirely clear *in vivo*, and work is ongoing to establish whether preimplantation lineage allocation in the early human embryo follows the same path as in mouse and other species. Transcriptional studies suggest that lineage specification may occur following blastocyst formation in the human which might explain hESC potential to make trophoblasts (Boroviak et al., 2018; Gerri et al., 2020; Meistermann et al., 2019; Petropoulos et al., 2016).

As discussed for the mouse, there have been reports of extended pluripotent human stem cells with capacity for extraembryonic differentiation (Gao et al., 2019; Yang et al., 2017b). Exactly how they relate to human naive and primed ESCs, both of which have been shown to exhibit extraembryonic differentiation, has not been definitively established. However, recent transcriptional analyses suggest extended pluripotent human stem cells most closely resemble primed human ESCs (Castel et al., 2020; Striparo et al., 2018).

Early efforts were made toward generating PE-like stem cells by adding retinoic acid to human EC cells which induced extraembryonic endodermal gene expression (Roach et al., 1994). In hESCs, overexpression of SOX7 or protein kinase C (PKC) stimulation has also been suggested to induce PE progenitors or differentiation (Feng et al., 2012; Séguin et al., 2008). More recently, naive hESCs with combined HHEX and FOXA2 reporters have been converted to stable naïve extraembryonic endoderm stem cells (nEnd) upon WNT, NODAL and LIF stimulation (Linneberg-Agerholm et al., 2019). Importantly, they compare the transcriptional profiles of the nEnd cells with human blastocyst expression data but also caution that a direct reference for the emerging definitive endoderm was not available. Recent transcriptional analysis of human postimplantation embryos could provide such reference (Tyser et al., 2020; Xiang et al., 2020).

Further comparative analysis of these different routes toward extraembryonic trophoblast and endodermal cells will be needed as well as further efforts to efficiently generate cells resembling the earlier trophectoderm. Following these developments, it would be expected that generation of human blastoids should be within reach.

A further consideration is that in humans and primates, in contrast to the mouse, both amnion and extraembryonic mesenchyme forms prior to gastrulation and there is high priority to establish stem cell models to study the formation and functional importance of these two additional extraembryonic lineages. For example, the origin of the extraembryonic mesenchyme needs to be clarified. Anatomical analysis of NHP embryos suggested that these mesenchymal cells developed from the parietal endoderm and not from the trophectoderm as had previously been

suggested (Enders and King, 1988). This notion is further supported by karyotype mosaicism of embryonic and extraembryonic tissues (Bianchi et al., 1993) and transcriptional analysis of NHP embryos (Nakamura et al., 2016). However, recent analysis of *in vitro* cultured NHP suggests that the origin may be from the epiblast prior to gastrulation (Yang et al., 2020).

Elegant studies culturing hESCs in soft 3D-gels and more recently in a controlled microfluidic system have generated structures called postimplantation amniotic sac embryoids (PASE) which consist of both an amnion and EPI compartment, supporting specification of primordial germ cells and primitive streak cells (Shao et al., 2017; Zheng et al., 2019). The amnion is suggested to have an important signaling role in inducing primitive streak cells (Yang et al., 2020; Zheng et al., 2019) though WNT and BMP4 signaling.

In future efforts in assembling postimplantation human stem cell-based embryo models it will likely be critical to add both amnion and extraembryonic mesenchyme components in addition to TE, EPI, and PE. Successful formation of these primate specific features would be important readouts of successful assembly of preimplantation blastoids.

Methods to evaluate whether an *in vitro* structure resembles *in vivo* development

Having considered the properties of the different stem cell types that are relevant to the embryo, and the methods used to evaluate these, the next question is how can we evaluate a model embryo made from these stem cells? We consider key features and discuss how one might consider whether any stem cell-derived structure represents a useful model of the embryo itself. An overview of different stem cell-based embryo models can be found in Figure 1 and Table 2.

As a general principle, comparison with an *in vivo* embryo of the same species and stage would be desirable. In humans, however, this is not always possible, and therefore cross-species comparisons are often necessary. Whereas the most closely related species, such as great apes, may theoretically provide the best comparators, their use carries greater ethical weight and is largely prohibited. Even the more commonly used nonhuman primate model organisms differ from humans in their embryology and so there is no perfect single model organism with which to compare human development. As such, benchmarking human stem cell-based models is uniquely challenging. This represents a significant hurdle and conundrum, as it is the opportunity to model human development with stem cells that has generated significant excitement in the field.

A valuable intermediate step is comparison with *ex vivo* cultured embryos. This has potential advantages in all species as it may allow identification of divergent features that simply reflect a model's *in vitro* environment, rather than meaningful biological difference with the *in vivo* embryo. Wherever possible, comparisons between *in vivo* embryos, *ex vivo* cultured embryos, and a given stem cell model may well prove most informative. Systems that support *in vitro* development of human embryos (Deglincerti et al., 2016; Shahbazi et al., 2016; Xiang et al., 2020; Zhou et al., 2019) could provide some insight into early postimplantation human development and serve as an important comparison. However, these are constrained by the 14-day rule, representing a further challenge to evaluating human model systems that progress to later stages (reviewed in Pera, 2017). Although we anticipate research into human stem cell-based embryo systems to continue at a pace, we would advocate for careful side-by-side experiments in diverse model systems in which meaningful comparisons, and functional experiments, can be performed. This will provide some indication that the model used might be a useful representation of the *in vivo* situation.

Evaluation criteria

Structure morphology

Overall structural resemblance to the embryo or to the tissues the model is purported to represent is a simple test and seems an obviously desirable feature. However, these are complex criteria with many considerations.

First, the extent to which spatial organization of cell types directs cell identity is worthy of further investigation. For instance, do structures with a greater degree of patterning generate cell types that more faithfully represent those of the embryo, compared with say a very basic EB? Structures with superficial resemblance to the early postimplantation mammalian embryo, appear to be able to produce cells of the anticipated identity and with some degree of appropriate spatial organization (e.g., 2D micropattern models, 3D gastruloids, and ETX embryoids). An interesting comparison can be made with a recent transcriptomic evaluation of teratomas formed from human cells, demonstrating many cell types with a transcriptomic signature similar to fetal tissue (McDonald et al., 2020), suggesting that morphologies incompatible with appropriate development can produce cell types of appropriate identity (see next section).

On the other hand, there are examples in which morphologies reminiscent of *in vivo* tissues or embryos can be formed with only a subset of relevant cells present. For example, the layered cortical structure present in cerebral organoids which (in their simplest form) lack non-neuronal cell types (Lancaster et al., 2013) or blastoids, which in terms of morphology look remarkably similar to blastocyst embryos, still contain a large fraction of developmentally non-relevant cell types (Li et al., 2019; Posfai et al., 2021). Ongoing

advances in spatial transcriptomics may be one route to combine morphological information with cell identification by RNA-sequencing (Moris et al., 2020).

Second, the relationship between cell-cell and cell-matrix contacts is also likely to direct fate. Signaling from integrins, epithelialization, luminogenesis, and transitions between epithelial and mesenchymal phenotypes are an intrinsic part of embryonic development, and defects in these processes compromise embryo development (Rivera-Pérez and Hadjantonakis, 2015). Intriguingly, this tissue ordering is recapitulated in some model systems (e.g., ETX) but not others (e.g., gastruloids) that nonetheless appear to generate many of the relevant cell types.

Finally, the physical characteristics of the model, such as the size, shape, and stiffness are also important considerations. Further investigation is needed to determine how they impact developmental processes (for example Orietti et al., 2020).

Cell identity

Assaying whether the correct numbers and types of cells are present in the embryo model through evaluating gene or protein expression is a highly informative method for benchmarking. Analysis of cell type-specific markers can yield valuable information on the composition and structure of the embryo model - however, these approaches have limitations. First, key marker genes are often repurposed during development and could lead to misinterpretation of cell type identity when used alone. For example, CDX2 is a marker of trophectoderm and ExE, but also mesoderm, amnion, and ectoderm. Using a combination of cell type markers is therefore required (Roost et al., 2015).

Second, evaluating key marker genes typically biases toward a set of characterized cell types. For charting the full spectrum of different cell types that compose an embryo model, single-cell RNA-sequencing is proving to be an extremely powerful tool (Minn et al., 2020; Posfai et al., 2021; Tyser et al., 2020; van den Brink et al., 2020). With this approach, global transcriptional similarities of *in vitro*-derived systems and *in vivo* embryo development can be compared, allowing for the identification of rare cell populations. However, caution should be taken when interpreting these correlations. First, the datasets of *in vitro* and *in vivo* samples are almost invariably produced during separate experiments, using different library preparation protocols and subject to different batch effects. Without a shared sample in both datasets, cross-sample comparisons inevitably rely on the assumption that samples *should* match. Current approaches do not allow easily distinguishing to what extent differences in correlation reflect differences in biology or batch effect. Second, there is a trade-off between number of samples analyzed and capture efficiency (i.e., how many genes can be detected). Mapping the transcriptional landscape of an embryo or embryo model requires high throughput approaches, typically achieved through microwell or droplet-based technologies. Large numbers of cells can be analyzed as part of a single batch, but additional barcoding is necessary to distinguish separate samples (Guo et al., 2019). Moreover, the capture efficiency of these methods is typically limited to 2,000 to 3,000 highly expressed genes. On the other hand, high capture efficiency methods (e.g., Smart-seq2) can be used to detect more genes but are typically more labor-intensive.

Finally, the selection of the reference dataset is crucial. The past decade has seen an accelerating expansion of single-cell datasets which capture different stages of embryonic development across multiple mammalian species (Table 3). Interspecies comparisons (Blakeley et al., 2015; Boroviak et al., 2018; Meistermann et al., 2019; Yang et al., 2020) can help fill in the gaps in data when *in vivo* samples are scarcer (e.g., during stages of human gastrulation). Assigning cell identity within a stem cell-based embryo model without appropriate reference datasets remains challenging (Zhou et al., 2019) and careful validation in species in which *in vivo* tissue is available, and the above-mentioned issues can be controlled for, may well be the most reliable route forward. The community would benefit greatly from integrated efforts to assemble all available high-quality datasets and build developmental road-maps to reference both stem cells and embryo models.

While transcriptional comparisons are a good start, cell identity is determined by a complex interplay of regulation at the transcriptional, chromatin, and protein activity level (see list of criteria for evaluating stem cells above). The development of single-cell multi-omics approaches (Argelaguet et al., 2019) allow mapping of cell identities across regulatory levels, although these still typically rely on data clustering and averaging features across cells (reviewed in detail elsewhere in Lee et al., 2020).

Transcriptional readouts can also be integrated with lineage information to establish cell identity as a combined feature of current state and past history. Traditional genetic-based lineage tracing is being supplemented by CRISPR-Cas9 barcode editing (summarized in Burgess, 2018) and combinatorial tagging (Kong et al., 2020).

Importantly, cellular phenotype (e.g., cell morphology) must also be considered. Can a cell sense a given signal and respond in the right way? Does the cell integrate appropriately in a specific tissue and/or display other relevant behaviors such as cell migration, invasion or elongation? Thus, a functional assessment of the constituent cells of the model, and not just the model itself (see below), may also indicate the usefulness of a given model system.

Table 3. Single-cell RNA-sequencing datasets on mouse, nonhuman primate, and human early embryo development

Mouse	*Oocyte*	*Zygote*	*2-cell*	*4-cell*	*8-cell*	*morula (E2.5)*	*early blastocyst (E3.0)*	*mid blastocyst (E3.5)*	*late blastocyst (E4.5)*	*E5.25*	*E5.5*	*E6.25*	*E6.5*	*E6.75*	*E7.0*	*E7.25*	*E7.5*	*E7.75*	*E8.0*	*E8.25*	*E8.5*	*E8.75*	
Tang et al., (2011)	x		x	x	x			x	x*														*epiblast
Xue et al., (2013)	x	x	x	x	x	x																	
Deng et al. (2014)	x	x	x	x	x	x	x	x	x														
Scialdone et al., (2016)													x**		x**		x**	x**					**epiblast and mesoderm
Mohammed et al., (2017)								x	x		x		x										
Posfai et al., (2017)						x	x	x															
Wen et al., (2017)											x		x										
Ibarra-Soria et al., (2018)																				x			
Pijuan-Sala et al., (2019)													x	x	x	x	x	x	x	x	x		
Cheng et al., (2019)										x	x	x	x										
Nowotschin et al., (2019)								x	x		x		x#				x#					x#	# endoderm
Chan et al. (2019)																			x		x		
Probst et al. (2020)														x			x						
Posfai et al. (2021)	x##	x##	x##	x##	x##	x, x##	x##	x##	x, x##		x##		x##		x##		x##		x##		x##		##integrated with Posfai et al., 2017, Mohammed et al., 2017, Deng et al., (2014), Pijuan-Sala et al., 2019
NONHUMAN PRIMATE (cynomolgus)	***E0***	***E2***	***E3***	***E5***	***E6***	***E7***	***E8***	***E9***	***E10***	***E11***	***E12***	***E13***	***E14***	***E15***	***E16***	***E17***	***E19***	***E20***					
Nakamura et al., (2016) (*in vivo*)					x	x	x	x				x	x		x	x							
Ma et al., (2019) (*in vitro*)										x	x	x	x		x	x							

(*Continued on next page*)

Table 3. ***Continued***

Mouse	*Oocyte*	*Zygote*	*2-cell*	*4-cell*	*8-cell*	*morula (E2.5)*	*early blastocyst (E3.0)*	*mid blastocyst (E3.5)*	*late blastocyst (E4.5)*	*E5.25*	*E5.5*	*E6.25*	*E6.5*	*E6.75*	*E7.0*	*E7.25*	*E7.5*	*E7.75*	*E8.0*	*E8.25*	*E8.5*	*E8.75*	
Niu et al., (2019) (*in vitro*)								x		x						x	x	x					
Yang et al., (2020) (*in vitro*)									x		x		x										E14 integrated with Tyser et al. (2020) and Nakamura et al., 2016
Nonhuman PRIMATE (marmoset)	***E0***	***E2***	***E3***	***E5***	***E6***	***E7***	***E8***	***E9***	***E10***	***E11***	***E12***	***E13***	***E14***	***E15***	***E16***	***E17***	***E19***	***E20***					
Boroviak et al., (2018)	x	x	x	x	x		x																integrated with Yan et al., 2013, Blakeley et al., 2015, Petropoulos et al., 2016, Deng et al. (2014) and Mohammed et al., 2017
HUMAN	***Oocyte***	***zygote***	***2-cell***	***4-cell***	***8-cell (E3)***	***morula (E4)***	***early blastocyst (E5)***	***mid blastocyst (E6)***	***late blastocyst (E7)***	***E8***	***E9***	***E10***	***E12***	***E14***	***~E16-19***								
Yan et al., (2013) (NSMB)	x	x	x	x	x	x		x															
Xue et al., (2013)	x	x	x	x	x	x																	
Blakeley et al., (2015)	x%	x%	x%	x%	x%	x%		x, x%															%integrated with Yan et al., 2013 and Deng et al. (2014)
Petropoulos et al., (2016)					x	x	x	x	x														
Meistermann et al. (2019) (*in vitro*)					x, x%	x, x%	x	x, x%	x														%integrated with Yan et al., 2013 and Petropoulos et al., 2016
Zhou et al., (2019) (*in vitro*)								x	x	x	x	x	x	x									
Xiang et al., (2020) (*in vitro*)								x	x	x	x	x	x	x									
Tyser et al. (2020) (*in vivo*)															x								

*epiblast; **epiblast and mesoderm; #endoderm; ##integrated with Posfai et al., 2017, Mohammed et al., 2017, Deng et al. (2014), Pijuan-Sala et al., 2019; %integrated with Yan et al., 2013. Boroviak et al., 2018, Yang et al., 2020, Blakeley et al., 2015, and Meistermann et al. (2019) also contain cross-species comparisons.

In conclusion, determining accurately the cell identity composition of an embryo model heavily relies on high-quality and detailed characterization of the *in vivo* counterpart. As new approaches are developed and improved, cell state characterization can move beyond transcriptional similarities and extend to other regulatory levels (e.g., proteins, chromatin), lineage hierarchies but more importantly function (see next).

Function

The ultimate functional test of a preimplantation stem cell based embryo model would be the demonstration of successful implantation and forward development, giving rise to a healthy fertile animal. Whether such an *in vitro* construct would continue to be identified as "a model" is an interesting philosophical question. While creating such a functional embryo is not the goal of many in the field, this undoubtedly would represent a major achievement. In the human context, any progress toward a similar model would be more ethically problematic and although this possibility seems some way off (see below), such ethical debates are already being rehearsed (Rivron et al., 2018b). Even if the ambition is not to construct a functional human embryo from stem cells, it seems within the realms of possibility that a future model could approximate a complete human embryo sufficiently that oversight and regulation might be needed.

In the meantime, more basic considerations and challenges remain, as even the early stages of implantation and postimplantation development of a stem cell-based preimplantation model embryo have not been demonstrated. Blastoids have been shown to interact with the maternal uterine wall and induce a decidual reaction (Rivron et al., 2018a). The latter stages of placentation, which is invasive in the mouse, have also not yet been demonstrated, nor have other key readouts of placental function such as hormonal secretion. Chorioallantoic fusion may well serve as a useful landmark. This represents the key point of contact between embryo-derived and extraembryonic tissues, and is a recurrent placental malformation phenotype (Perez-Garcia et al., 2018). The ability to derive a placental organoid could be one indication of developmental progression, but again whether this is a meaningful assay in the absence of embryo development is unclear. This raises the important issue of coordinated development, by which we mean the demonstration of functional interaction between the various components of any stem cell-based embryo model.

What are the major obstacles to making a fully functional mouse embryo (model) from stem cells? Currently, the major obstacle seems to be the availability of extraembryonic stem cells that can efficiently differentiate into all the necessary downstream cell types required to confer functionality (as discussed above). Thus, in terms of building a functional blastocyst from stem cells, improvements to the basic building blocks remain necessary. This highlights the even greater challenge in building a human blastoid, or blastocyst-like structure, in which none of the constituents' parts have been functionally validated.

If implantation can be achieved, there are numerous developmental milestones that could be assessed in the embryonic compartment (reviewed in Rossant and Tam, 2009). These tests are also relevant to stem cell models of the postimplantation embryo and would include processes such as lumen formation and proamniotic cavity formation. Thereafter, proximal-distal patterning and anterior-posterior axis formation are important next steps. In the mouse, the involvement of extraembryonic structures, or signaling molecules derived from them, is well described and are especially relevant here, with processes such as distal visceral endoderm (DVE) formation and migration to form the anterior visceral endoderm (AVE) being key milestones prior to primitive streak formation. Indeed, an intriguing aspect of gastruloid formation is the absence of extraembryonic tissue. Formation of anterior and posterior structures simultaneously has not hitherto been demonstrated, in either gastruloids or PASE. Recent evidence suggests regionalization of human gastruloids; however, this occurs without primitive streak formation. How this impacts later developmental milestones (see Figure 1) is unclear, and the extent to which pattern can continue to develop and emerge in these structures is of obvious interest. Combining morphological assessments with gene expression analysis has already been discussed as an important test. In addition, demonstrating cellular/tissue interactions and a coordinated developmental program would be useful indicators. While *in vitro* models of the gastrulation stage embryo have been heralded as a means to assess how to build an embryo, it is also necessary to demonstrate that what is made is in fact functional. Therefore, isolation and functional assessment of tissues or cells derived from such models will be helpful.

Functional assessment of early emerging cell types in embryo models should be undertaken where assays are available, with *in vitro*-derived cells benchmarked against stage-matched cells from the postimplantation embryo. Systems in which this is currently possible include testis repopulation with mouse PGCs (which is possible using *in vivo* PGCs as early as E8.5 [Chuma, 2004]) and neural crest transplantation (Cohen et al., 2018). Finally, *in vivo* the ability to readily give rise to naive pluripotent stem cells is lost shortly after implantation (Boroviak et al., 2014) and is reacquired only in PGCs from E7.5 onwards (Leitch et al., 2013a, 2013b). Therefore, loss of ability to form naive pluripotent stem cells serves as an important test to confirm appropriate exit from the naive pluripotent state in the context of embryo model systems.

Continued improvements and additions to the embryo model repertoire will likely lead to more sophisticated and complete model systems. This may allow recapitulation of early developmental disorders by using starting stem cell types that carry mutations in relevant genes. In the mouse, the benefits of this basic approach over preexisting *in vivo* strategies may not be immediately obvious, but this would allow rapid proof of principles experiments, as well as facilitating live-imaging and a range of other experimental manipulations. In addition, there are obvious benefits in assessing the impact of embryonic versus extraembryonic mutations, which is an arduous task using existing *in vivo* technologies (Perez-Garcia et al., 2018). Of course, it is the extension to human studies that carries greatest hope and expectation. In fact, engineering stem cells with expected embryo phenotypes may be one means of assessing the validity of any new stem cell-based model system.

Conclusions

The significant progress accomplished in recent years in the development of stem cell-based embryo models will likely continue at pace, opening opportunities to delve deeper into the mechanisms of embryonic development. Many of the standards we suggest have already begun to be adopted, but we hope this in-depth consideration of how to evaluate both the models themselves, as well as the stem cells used to construct them, will be of benefit to this growing community of researchers.

While embryo models may be able to substitute for animal studies in some contexts, ongoing work *in vivo* will be critical. Indeed, we would caution against a recent trend for arguing that *in vitro* constructs are likely to replace the mouse. Without regular benchmarking against *in vivo* development, and appropriate functional testing, we risk a precession of simulacra in which sight of the embryo is lost. Using those species in which cross-comparison is possible will allow identification of common pitfalls and the assessment of when and why stem cell based models deviate from normal development. This will be key information to feed into human model development, in which such comparisons will likely remain more challenging. Thus, it is evident that progress in human stem cell-based embryo systems will depend more than ever on work in other organisms.

Pluripotent stem cell types have historically received overwhelming attention in all species studied, and as a consequence, capture of multiple pluripotency states has been achieved, in the mouse at least. As this area of research continues to thrive, a more nuanced understanding of pluripotent states is emerging. The focus on this work, however, has left improvements on extraembryonic stem cell types trailing behind. Indeed, a major challenge remains the lack of validated extraembryonic stem cells that can make all the relevant downstream cell types, even in the mouse. In other species, including common model organisms, the outlook is even more challenging, as appropriately validated stem cells with which to start to build an embryo model are mostly lacking. While stem cell-based model systems would be a route to extend embryological studies into a broad range of species, without significant investment in the development of validated stem cell lines this will not be possible. We would therefore encourage investment in establishing these building blocks in other species to enable diversification of embryo models.

The models discussed extend across the early stages of development and include different starting cell types and entry points to development. Whether it is possible to stitch together these different systems in an attempt to achieve complete *in vitro* embryogenesis is an interesting question. Some would argue that this defeats the point of such models: rather the goal is not to perfectly recapitulate development but to have a suite of tools that allow specific mechanistic questions to be addressed. However, *in vitro* embryogenesis is a provocative goal in itself. This may be best attempted using embryos rather than stem cell-based embryo models in the first instance. There has been relatively limited advance in culturing techniques for later stage embryos, for instance after gastrulation and through somitogenesis. New methodologies would be of clear benefit if stem cell-based embryo models are to be matured to later developmental timepoints.

The field will continue to debate whether the future focus should be on making embryo models "less wrong" and whether this necessarily equates to "more useful." Ultimately, whichever approach is undertaken, success will be measured in the emergence of new discoveries that advance our understanding of developmental mechanisms and increase our knowledge of human development and disease. We hope that the standards suggested herein will help evaluate new, or update existing, model systems, but also anticipate these standards will require reevaluation based on future progress in the field and the direction of travel in the coming years.

AUTHOR CONTRIBUTIONS

E.P., F.L., C.M., and H.G.L. performed literature research and wrote the manuscript.

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