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In vitro modeling of early mammalian embryogenesis

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Abstract

Synthetic embryology endeavors to use stem cells to recapitulate the first steps of mammalian development that define the body axes and first stages of fate assignment. Well-engineered synthetic systems provide an unparalleled assay to disentangle and quantify the contributions of individual tissues as well as the molecular components driving embryogenesis. Experiments using a mixture of mouse embryonic and extra-embryonic stem cell lines show a surprising degree of self-organization akin to certain milestones in the development of intact mouse embryos. To further advance the field and extend the mouse results to human, it is crucial to develop a better control of the assembly process as well as to establish a deeper understanding of the developmental state and potency of cells used in experiments at each step of the process. We review recent advances in the derivation of embryonic and extraembryonic stem cells, and we highlight recent efforts in reconstructing the structural and signaling aspects of embryogenesis in three-dimensional tissue cultures.

Introduction

During the first two weeks after fertilization, the human embryo goes through a series of concerted cell movements, cell lineage specifications, and large-scale morphogenetic events that transform a uniform cluster of cells into highly organized layers of tissues [1]. This process is accompanied by key developmental milestones, including implantation of the embryo into the uterus, the separation of embryonic and extraembryonic tissues, and gastrulation, which breaks the embryonic symmetry to establish the body axes. While the first steps in mammalian development from fertilized egg to the pre-implantation delineation of embryonic and extra-embryonic tissues are stereotyped, the morphology of subsequent

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events is quite variable among species. For instance, the implantation of the mouse and human embryos takes place shortly after the first lineage segregation event, however their 3D shapes after implantation are different (Figure 1). The shape of the human embryo after the first lineage segregation is similar to a cow embryo, however the cow embryo implants much later in development, after embryonic symmetry has been broken [2,3].

Although mammalian development is accompanied by considerable variation in strategies around the time of gastrulation, many of the key genes involved are manifestly shared among all vertebrates. Since biologists have characterized the building blocks of embryos, there is need for the engineering community to assist in their proper assembly, not just to imitate nature, but to understand how cells and tissues interact to promote a robust outcome. The field of organoid biology has shown the surprising capacity of stem cells to self-organize out of context, given suitable media and physical environments [4,5].

In this context, recapitulating early development (henceforth ‘synthetic embryology’) has several advantages over later stages (which are more complex in terms of tissue geometry and constituent cell types) as an assay to uncover the cell’s potential for self-organization. The stem cells one uses are closest to the natural starting point, tissue interactions are best understood at this stage, and for the non-human system the emergence of a viable fetus is the ultimate test of function. Success in this area requires full exploitation of the variety of cellular building blocks, their physical manipulation, and an appreciation for the in-vivo context, topics we review here.

Blastocyst

During early preimplantation development of a mammalian embryo, cells arrange into a structure called the blastocyst, comprising three molecularly, morphologically, and spatially distinct lineages (Figure 1) [6]. The three lineages are: the epiblast, which will predominantly give rise to the embryo-proper, and two extraembryonic lineages, the primitive endoderm (PrE), which will give rise to the visceral endoderm (VE), also known as the hypoblast, and trophectoderm (TE), which will give rise to the fetal portion of the placenta. The epiblast is a compact mass of cells, while the trophectoderm and primitive endoderm display apicobasal polarity and are held together by adherent and tight junctions. These two epithelia envelop the epiblast in a niche bound by basement membranes composed of laminin, collagen, and fibronectin. Soon after the blastocyst forms, the epiblast tissue will become polarized and form a single-layered epithelium surrounding a pro-amniotic cavity (Figure 1). In humans and mice, epiblast polarization and formation of the amniotic cavity takes place shortly after uterine implantation.

Embryonic stem cells for synthetic embryology

Stem cell lines can be derived by culturing embryo outgrowths in vitro under appropriate medium conditions [7]. They can be indefinitely propagated in culture (referred to as self-renewal), and they represent the morphology, marker expression and developmental potential of their parent embryonic lineages. Stem cells representing the epiblast, extraembryonic endoderm, and TE, have been derived from mouse blastocysts [8] (Figure

2a). Most attention was focused on pluripotent embryonic stem cells (ESC) which give rise to all tissues of the body and have tremendous potential for regenerative medicine. Initially the derivation of mouse ESC (mESC) was inefficient and limited to so-called permissive backgrounds. The advent of a dual inhibition (2i) medium that represses FGF/RTK signaling and activates WNT has greatly expanded the mouse strains that allow stem cell derivation and diminished variability within cohorts of cells [9].

Mouse ESCs are considered to be in the naïve state of pluripotency and are derived from pre-implantation blastocyst [10]. However, when derived from the post-implantation epiblast these stem cells are considered to represent the primed state of pluripotency and are referred to as epiblast stem cells (EpiSCs). Naïve state pluripotent cells can contribute to all the cells of the embryo-proper (being excluded from extra-embryonic tissues) in preimplantation embryo chimaeras, whereas the primed state pluripotent cells have the same potential and only integrate into the post-implantation epiblast (Figure 2c) [11,12]. Human and monkey ESCs, despite being derived from the pre-implantation embryo, at the transcriptomics level resemble the pluripotent epiblast cells of an early gastrulating monkey embryo and are much closer to mouse EpiSCs [13], with similar developmental potential. However, there is evidence that hESCs can be shifted to extraembryonic fates by exposure to high levels of the growth factor (a secreted signal) BMP4 (Figure 2c).

Recently, efforts have been devoted to formulating defined culture conditions that would maintain human pluripotent ESCs cells in a state more similar to the mouse naïve state, and these naïve hESC were derived either directly from early blastocysts or by converting hESCs in vitro (reviewed in [14]). Common to these protocols is promoting the activity of the leukemia inhibitory factor (LIF) pathway and repressing the mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3) pathways. However, given the diversity in the presence of inhibitors and agonist of many other pathways in these media, precisely which state of embryonic development these cells represent and how far they are on the spectrum of stem cell naïvete is not very well understood.

Very recently, by modulating a number signaling pathways, mESCs could be derived from individual eight-cell blastomeres displaying developmental potential of both embryonic and extraembryonic lineages in mouse chimaera experiments. These expanded potential stem cells (EPSCs) can also be derived by culturing mESCs and primed hESCs in the appropriate medium. The single cell transcriptome of mEPSCs and hEPSCs revealed these cells as distinct compared to ESC, EpiSCs, and early embryo cells [15–17]. Although the precise correspondence of EPSCs to cells in embryo still needs to be investigated, given their demonstrated developmental potential [16], they are extremely promising in modeling early human embryogenesis.

Even though naïve and primed mouse pluripotent stem cells only contribute to embryonic tissues in chimaera assays, it is possible to differentiate hESCs into extraembryonic trophectoderm and, when starting from naïve hESCs, into extraembryonic endoderm tissues by directed induction *in vitro* (see next section).

We have detailed this menagerie of stem cells and their extraembryonic counterparts in this and the next section to emphasize to an engineering audience, that the choice of starting materials is crucial for the creation of synthetic embryo-like structures. Since we are not starting from a fertilized egg, success depends on juxtaposing the correct type of cells, in the appropriate numbers, physical environment, and with appropriate timing, all aspects of bioengineering. The 'correct' conditions are implicit in the embryo, but as yet have been only crudely defined. Precisely engineered synthetic systems can aid in discovering those conditions and also define the limits to the self-organizing capacity of cells. Synthetic embryology seems the best context in which to quantify and engineer the cell state starting points, secreted signals, forces, and physical environments necessary to build organs.

Extraembryonic stem cells:

The complexity of the extra-embryonic lineages, particularly the endoderm, is best understood for mouse and defines expectations for the analogous stem cell lines [18]. At the blastocyst stage, the polar TE lies adjacent to the epiblast, and is fated to form the extraembryonic ectoderm and ectoplacental cone, which will subsequently form the fetal portion of the placenta. While the mural TE initially encloses the blastocyst cavity and eventually forms the outer layer of the parietal yolk sac. The PrE differentiates into two cell types, the parietal endoderm (ParE) and the visceral endoderm (VE) [19]. ParE cells are distributed over mural TE cells, eventually forming the endodermal component of the parietal yolk sac. The VE possesses the machinery for polarized absorption and transcytosis and functions as the primary site of gas, nutrient, and waste exchange before the establishment of the maternal–embryonic circulation. The visceral endoderm is further distinguished (subclassified) by whether it encapsulates the extra-embryonic ectoderm (exVE) or the embryonic epiblast (emVE).

An outstanding question is whether this diversity of components has to be created before assembly or will emerge by self-organization from simpler precursors. The limitations of current synthetic embryo systems plausibly hinge on this level of detail, and the engineering community ignores it at their peril.

XEN cells are the *in-vitro* counterpart of the PrE lineage of the mouse embryo since they contribute to the VE and the ParE in chimeras [20,21]. Culture conditions critically influence XEN cell identity; in the presence of BMP4 they differentiate into exVE [22,23], but revert upon BMP4 withdrawal. Treatment of XEN cells with Nodal or Cripto causes differentiation toward emVE, as well as the activation of anterior visceral endoderm (AVE)-specific genes [21].

While, XEN cells have not yet been isolated from human embryos, recently, self-renewing human and mouse PrE cells were derived by applying standard Activin + WNT endoderm agonists to naïve ESCs, but in the presence of LIF and low insulin [24,25].

Self-renewing mouse trophoblast stem cells (mTSCs) were isolated from preimplantation mouse embryos by culturing blastocysts or extraembryonic ectoderm in the presence of FGF4 [26]. The same selection does not work for human due to differences in signaling, and

so for years the human TSCs (hTSCs) were not available. Recently, a screen of conditions revealed that it is possible to isolate and maintain stem cells that resemble human trophoblast from 6–9 week placentas and from blastocysts, but by a more comprehensive set of agonists and inhibitors than for mouse [27]. These cells are plausibly hTSCs, their transcriptomics profile is most similar to the villous cytotrophoblast and under specific conditions they can differentiate into post-implantation trophectoderm tissue, namely the extravillous cytotrophoblast and syncytiotrophoblast (Figure 2c). Interestingly, as mentioned previously, hESCs can potentially be directly differentiated into cells that resemble the trophoblast cells *in vitro* with a high dose of BMP4, enhanced by NODAL and FGF2 inhibitors [28], although under these conditions they are transient and therefore not stem cells. The identity of these cells is controversial because it was challenging to explain how ESC, that are expected not to contribute to extraembryonic lineages in chimaeras, can give rise to trophectoderm cells [29]. Recent transcriptomic analyses suggested ESC induced by BMP4 for 8 days are most similar to invasive syncytiotrophoblast [30,31]. Applying the same differentiation protocol followed by maintenance in published hTSC medium, EPSCs can be differentiated into self-renewing TSCs [16]. Although TS and BMP4-induced trophoblast have been compared with transcriptomics data of first trimester placenta [27,30,31], it will be interesting to compare these cells with recently published single cell transcriptomics data of post-implantation E14 human [32] and E21 monkey embryos [33,34].

Finally, following implantation, the quasi-spherical epiblast breaks proximal-distal symmetry by differentiating one side of the epiblast cells into amnion cells. To date, this process has only been inferred based on morphological changes of the cells from columnar epiblast to squamous amniotic cells. Recent long-term cultures of human embryos up to 12 days has not captured this transition [35,36], however in monkey embryos *in vitro*, it is seen on E13–14 based on immunofluorescence staining [33,34]. These works have not identified a distinct transcriptomic profile of these cells, either due to technical reasons or because the amnion and trophectoderm cells of the pre-gastrulating embryo do not have a sufficiently distinct transcriptomic profile.

Self-organization in two and three dimensions.

The capacity of human and mouse pluripotent stem cells to recapitulate gastrulation when confined to two dimensional micropatterns has recently been reviewed elsewhere [37]. This system remains the best quantitative assay for signaling dynamics in apical-basal polarized epithelia, enhanced through live endogenous reporters for TGFβ and WNT signaling pathways [38–40]. Nevertheless, the presence of cell movements, the progressive induction of activators (BMP to WNT to NODAL), and their associated secreted inhibitors, in addition to the complex cell biology within the epithelia, make it impossible to fully connect the signaling history of a cell to its fate. Events in 3D with multiple cell types will be more complex.

Embryonic development, beginning with the blastocyst, requires contact mediated by the ECM between embryonic and extraembryonic lineages [41–43]. The ability to derive and maintain ES, TS and XEN stem cell lines representing all three blastocyst lineages in the

mouse, and their developmental potential assessed in vivo and in vitro, has led investigators to combine these stem cells with the goal of self-assembling cohorts of cells in the hope of generating synthetic embryo-like structures.

Mouse blastoids

Rivron and colleagues [44] combined mESCs and mTSCs *in vitro* to generate structures that they propose resemble early mouse blastocyst which they termed blastoids. They first aggregated ESCs in non-adherent microwell arrays, for 24hr, and then added TSCs which would coat the ESC aggregate, thereby encapsulating it in a configuration reminiscent of the blastocyst. A WNT signaling activator and cAMP analogue were added to the culture medium that led to cavitation with a lozenge of ESC on one side, akin to the inner cell mass. The diameter of blastoids was generally comparable to that of E3.5 blastocysts recovered from pregnant females. As with many foreign bodies, for example BioRad Affigel blue beads, when transferred to the uterus of pseudopregnant females, blastoids would implant promoting a decidual tissue reaction [45,46]. This is not a demonstration per se of the ability of the TS-cell derived trophectoderm-like layer to initiate the physiological implantation response. Blastoids exhibited more extensive cell proliferation when compared to trophoblast vesicles [47]. It argues for cross-talk between the epiblast and trophoblast components in mediating proliferation and self-renewal in both compartments. In their original incarnation, blastoids do not contain a primitive endoderm layer and thus are unable to develop further. By screening media conditions, however, ref. [48] derives a PrE population that envelopes a cavitated epiblast epithelia, and by this route introduces the third lineage into the blastoids.

Subsequent studies using mEPSCs generated the full repertoire of three blastocyst lineages in the correct configuration [49,50]. EPS-derived blastoids resembled blastocysts in morphology and cell lineage allocation and recapitulated key morphogenetic events taking place during preimplantation development *in vitro*. Upon transfer, some EPS-derived blastoids underwent implantation into the uterus and induced decidualization (as mentioned, this observation is to be expected for any foreign body introduced into the uterus of a hormonally primed female), and generated disorganized tissues *in utero*. Single-cell and bulk RNA sequencing revealed that EPS-derived blastoids contain all three cell lineages of the mouse blastocyst and share transcriptional similarity with blastocysts recovered from pregnant females. Even so, the characterization of the cells representing the PrE/hypoblast lineage has not been comprehensively analyzed in any of the blastoid studies thus far. Moreover, it has not been shown whether PrE cells polarize and come to form an epithelium on the surface of the inner cell mass, nor whether the basement membranes, one separating the polar TE from the epiblast and the other which lies between PrE and epiblast, are present in blastoids. These basement membranes are essential for normal embryo development, as mouse mutants lacking components of these basement membranes are embryonic lethal at these stages. The membranes separate the adjacent tissue layers, but also likely provide signaling and structural platforms that facilitate normal developmental progression. More intensive analysis of the peri-implantation mouse embryo should reveal shortcomings of synthetic systems [51].

Models of gastrulating mouse embryos

The most dramatic studies to date to recreate the post-implantation mouse embryo [52,53] simply mix the three stem cell lines, ES, TS, and XEN, to generate mouse embryo-like structures. In a prior study, instead of XEN cells, Matrigel was used to replace the polarity signal [54]. These structures correctly assemble at low frequencies, arguing for improvements through titrating the exact numbers of each or the three stem cells, as well as the sequential addition of each component to the assemblage. Even so, the authors propose those structures that do develop, go on to execute an epithelial-to-mesenchymal (EMT) transition within the ES-derived epiblast layer, comparable to EMT in mouse gastrulation [55]. They also suggest that the EMT event leads to mesoderm and endoderm specification, as is the case of real embryos. They observe that the XEN-derived visceral endoderm that encapsulates the embryo-like structure, is patterned into a distal squamous morphology overlying the ES-derived epiblast, representing the emVE, and a proximal cuboidal morphology overlying the TS-derived extra-embryonic ectoderm, representing the exVE [18]. While the patterning of the VE adjacent to the ES and TS descendants is suggested by the morphology of cells, though it is not yet entirely confirmed with markers.

An alternative approach begins by creating an aggregate of mESCs with a defined size then supplying a pulse of uniform WNT activation. Over the course of ~6 days, these aggregates express markers of anterior-posterior patterning and elongation, as defined by Hox genes and other axial mesendodermal markers [56]. There is even segregation of some dorsal-ventral markers. Curiously, despite expressing markers beyond gastrulation, morphogenetic features such as coherent cell polarity and lumen formation are lacking, and there is considerable variation in shape.

Models of human embryogenesis

Results obtained with hESC in 3D to date have been more modest compared to the mouse, in part for lack of clear analogues for the extraembryonic lineages, as well as a human gastrulation benchmark. A key feature of primate pre-gastrulation embryos is the discoid morphology shared with rabbit and chick embryos [57,58]. The first hint of a spontaneous morphological symmetry-breaking, in the absence of external signals, was in a system that induced cyst formation at the interface between a soft gel and liquid [59]. A much more robust protocol developed subsequently uses microfluidics to impose a consistent hESC cyst assembly and application of a high BMP4 concentration on one side. BMP4 induced an asymmetric morphological change that resemble the proximal-distal symmetry breaking of the primate embryo, with columnar pluripotent cells overlaid by squamous cells resembling the amnion [60]. As argued earlier, no transcriptomics data distinguish primate amnion cells from trophectoderm at this stage, including recent single cell sequencing of human E14 and monkey E21 embryos, where the putative amnion cells in the transcriptomics data have been proposed yet not distinguished from trophectoderm [33]. However, in this synthetic system, certain cells showed markers of primordial germ cells in the putative posterior amnion, possibly recapitulating the *in vivo* primate data [13], however they were also seen in the epiblast. Following the asymmetric morphology change, epiblast cells undergo EMT, activate gastrulation markers, and disperse into the gel.

A second instance of symmetry-breaking in a 3D human stem cell system uses a uniformly applied low dose of BMP4 on pluripotent spherical hESC epithelia. After two days, in a fraction of colonies (dependent on BMP4 concentration), markers of the primitive streak, as well as an EMT, appear on one side of the colony [61]. This anterior-posterior symmetry breaking requires WNT signaling and its inhibitor DKK1, produced in response to WNT, consistent with a Turing model. Live-cell imaging shows that the EMT happens concurrently on one side of the hESC cysts and not by the coalescence of scattered cells, thus indicating a WNT gradient. Again, the mesenchymal cells are not confined and disperse into the supporting gel.

For models that begin as epithelia, gastrulation as defined by molecular makers, occurs concurrently over a sector of the epiblast and the cyst then soon disintegrates. *In vivo*, the situation is different. This process is best studied in the chick, in which the apical connections remain and cells flow into the streak where, they delaminate, undergo EMT, and flow away confined by the VE/hypoblast [62].

Mechanics plausibly plays a role in promoting flow of the chick epiblast [63], but the same effects have not been studied in stem cell systems or even mouse embryos. Engineering a support system that enabled morphogenesis resembling natural gastrulation in a discoid embryo would be very informative.

Conclusions:

The development of synthetic embryology is just beginning and in human provides the only way to study the crucial period between implantation and 4–5 weeks post-fertilization when the embryo has taken on a recognizable fetal morphology and the anlages of the major organs have formed. (Beyond this time fetal samples are available.) Mammalian embryos cultured ex vivo have so far only been implanted onto a plastic surface without any particular coating on the surface [35,36]. Synthetic embryo systems will benefit from developing better implantation models, such as using a cellular layer (mimicking the maternal endometrium) that permits invasion by the trophoblast and ultimately signals back to the embryo in ways that remain to be defined. To our knowledge, mouse and human embryos differ considerably in how they implant, making the development of synthetic human implantation models crucial [1].

We possess a plenitude of single cell transcriptomics and epigenomics data on the early mouse embryo and we are beginning to acquire important data on the same period in non-human primate embryos [33,34,64–67]. Genome-scale assays of synthetic embryo-like systems are needed, but require an embryo for comparison. Are all genes of equal relevance when comparing synthetic to real, and what is the relative weight of assays showing functional equivalence? Synthetic systems have the potential to separate sufficiency from necessity. How do the properties of stem cell lines change when in contact with other lineages? Is morphologically normal gastrulation possible with VE and no TE in human? What is the self-organization potential of the extra-embryonic lineages?

Synthetic systems are powerful because they are highly amenable to genome editing technologies, bioengineering, or optogenetic tools. Mice (and embryos) can be made to order from mESCs by tetraploid complementation or 8-cell injection circumventing the need to breed animals [68]. Thus, synthetic and natural embryos can be compared for the same cells. The Allen Institute is fluorescently tagging interesting genes in human and mouse pluripotent stem cells, for public distribution. The synthetic embryologist needs to be conversant with all molecular and cellular tools.

The 720 page reference anthology on gastrulation devotes two pages to human [58] and we expect synthetic systems will help fill these lacunae in our knowledge.

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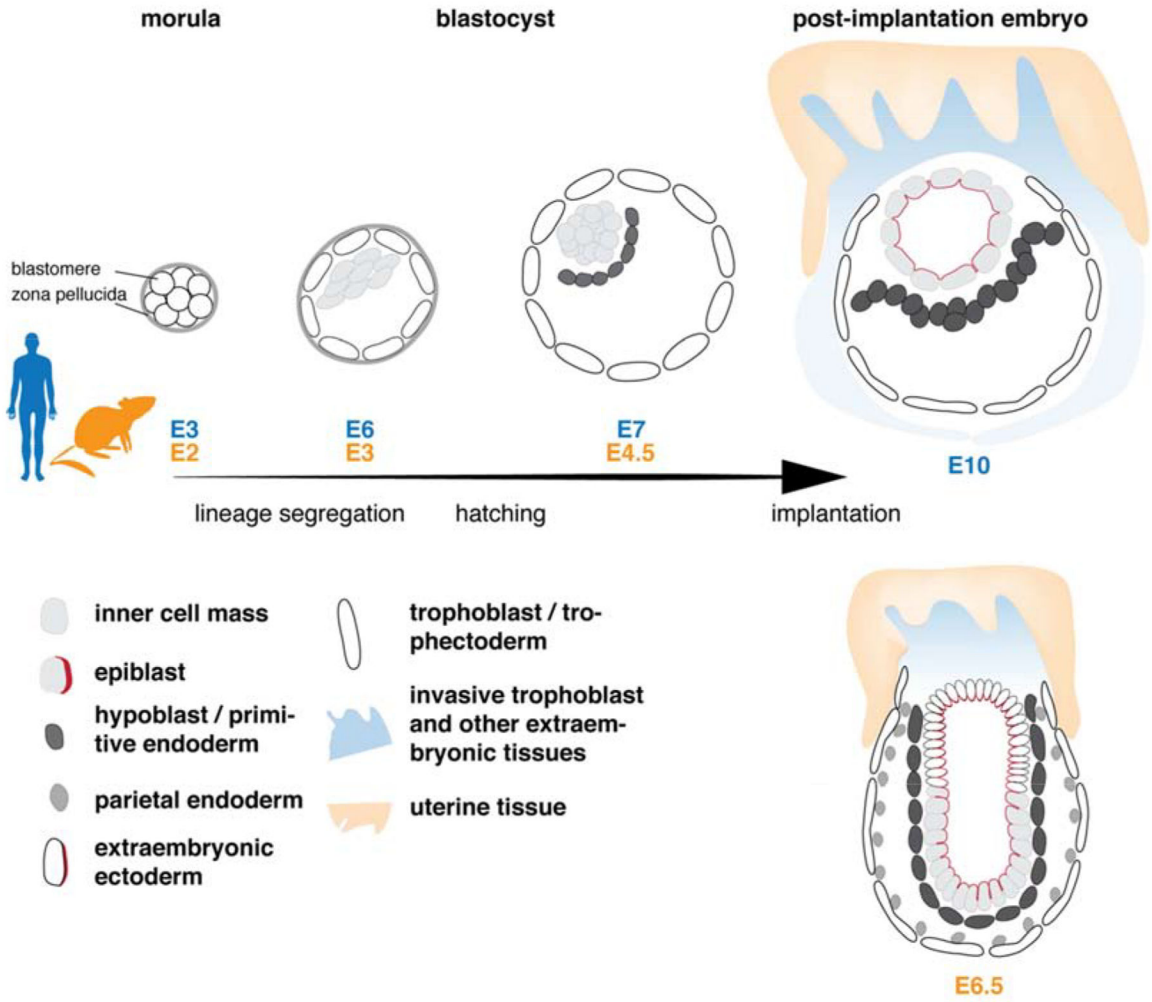

Figure 1:
Schematics of early mouse and human embryo development. In the human embryo, the hypoblast derivatives are not drawn given that their identity and morphology at E10 has not yet been elucidated.

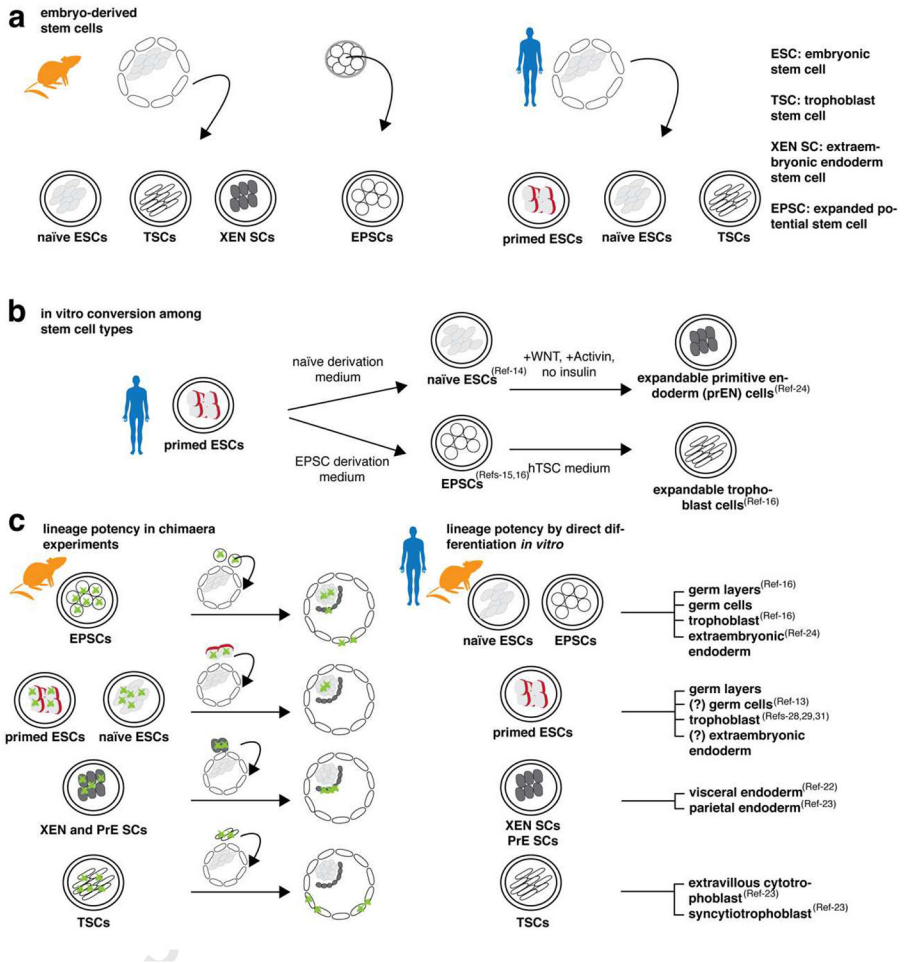

Figure 2: An overview of embryonic and extraembryonic stem cells for synthetic embryology.

(a) Summary of major embryonic and extraembryonic stem cell types derived from mouse and human embryos. (b) Schematics of conversion between stem cell types reported in the literature. (c) Illustration of multipotency of various stem cell types, based on their developmental potential in mouse chimaeras (left) and based on directed differentiation of human and mouse pluripotent cells *in vitro* with various protocols (right). Question mark in the protocol indicate that there is possibility that primed cells could give rise to germ cells in vitro, although it has not been reported yet. The conversion of primed ESCs to germ layers has been shown on numerous occasions so references were omitted. The conversion from naïve to germ cells has been demonstrated, however its discussion goes beyond the scope of this review.

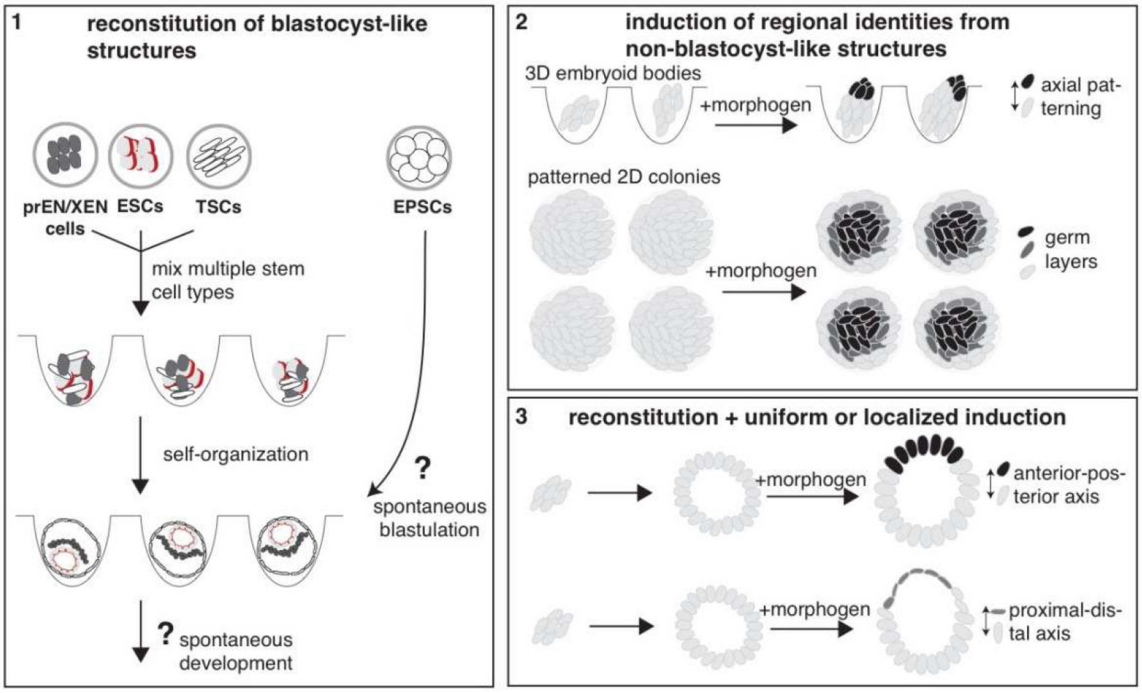

Figure 3: Current strategies in modeling early mammalian embryogenesis.

Three approaches can be distinguished: (1) Controlled assembly of blastocyst-looking structures (blastoids) by mixing the three stem cell types derived from the three blastocyst lineages. The aim of this approach is to reconstitute a structure that resembles the conceptus which could potentially recapitulate some of the subsequent elements of embryogenesis. It was reported that a cluster of mouse EPSCs is sufficient to spontaneously form a blastocyst [49], although another study purports that multiple cell types are still required [50]. Further examples: [44], [52], [53]. (2) Morphogen-induced embryonic events in a cluster of stem cells without creating blastocyst-looking structures. Examples include pulsing embryoid bodies with a small-molecule WNT agonist to generate 3D gastruloids [56] and inducing radially-symmetric germ layers on patterned 2D stem cell colonies, reviewed in [37]. (3) A combination of both approaches, where certain morphological features of the blastocyst are reconstituted followed by induction with morphogens. Examples include creating the peri-implantation pluripotent and polarized epiblast cyst which is uniformly stimulated with BMP4 to produce the breaking of the anterior-posterior symmetry [61] and asymmetric stimulation of ESC-generated cysts with BMP4 to break the proximal-distal axis [60].