Deconstructing and reconstructing the mouse and human early embryo

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Abstract

The emergence of form and function during mammalian embryogenesis is a complex process that involves multiple regulatory levels. The foundations of the body plan are laid throughout the first days of post-implantation development as embryonic stem cells undergo symmetry breaking and initiate lineage specification in a process that coincides with a global morphological reorganization of the embryo. Here we review experimental models and how they have shaped our current understanding of the post-implantation mammalian embryo.

Main

The development of an organism entails cellular differentiation, tissue morphogenesis and growth. Deciphering how these processes are regulated and coordinated to generate form and function is therefore complex. Historically, the first insights were inspired by descriptive observations of embryos developing in vitro. Wilhelm Roux realized the need to establish causality or, in other words, determine the effects of experimental manipulations on normal development1, a notion that pioneered the field of experimental embryology. From initial cell separation2 and transplantation experiments to the generation of chimeras3,4 and the creation of genetically modified organisms5-7, the use of experimental embryology techniques to study mammalian embryos has led to the establishment of a number of key developmental principles8. This method can be thought of as a ‘top-down’ approach, with the starting point being the embryo, which is deconstructed to its constituent pieces through experimental manipulation (Figure 1).

The alternative way to verify a basic developmental principle that applies to a complex organism would be to construct a simple system based on that principle, an approach inspired by the field of synthetic biology8. The core concept of this reductionist ‘bottom-up’ approach is the use of individual blocks (for example, stem cells) to build simplified in vitro models of real embryos, so-called embryoids or synthetic embryos. Although this idea was already introduced at the beginning of the 20th century, it has been revived over the past years and the number of embryo stem cell models is growing. These simplified
models are highly relevant for the study of mammalian development, in particular for the early phases of post-implantation development, when embryos become inaccessible to view and experimentation. Moreover, ethical guidelines limit the developmental timeframe of human embryos that is amenable to scientific study in the laboratory. In this Review, we will discuss top-down strategies and innovations used to elucidate the cellular and molecular mechanisms that shape mouse and human post-implantation embryos, as well as bottom-up approaches devised to reconstruct embryos using stem cells.

**Elucidating early development in the mouse**

Experiments in the 1950s have pioneered the establishment of *in vitro* culture methods for mouse pre-implantation embryos. These methods were optimized and simplified, leading to the generation of the standard medium used nowadays, which allows embryo development up to the blastocyst stage (embryonic day E4.5). With these culture techniques in place, methods were developed to manipulate gene expression during embryo development, resulting in the discovery of specific developmental gene functions (Figure 1). In combination with long-term time-lapse imaging, which allows the tracking of individual cell dynamics in living embryos, these improved methodologies led to an understanding of how the blastocyst is formed and its lineages are specified. These lineages are the embryonic epiblast, precursor of the future fetus, and two extra-embryonic epithelial tissues, the primitive endoderm and the trophectoderm, precursors of the yolk sac and placenta, respectively. Together, these techniques have shaped our current understanding of the first days of mammalian development, the so-called pre-implantation stage.

The mouse blastocyst implants into the uterus at E4.75-5.0. Implantation marks the initiation of a series of morphogenetic reorganizations and changes in stem cell identity that set the stage for the establishment of the body plan. However, given the small size of the implanting embryo and its inaccessibility in the uterus, the sequence of events during this period has remained largely unexplored, and hence this stage was coined the black box of development. The analysis of mouse embryos recovered at successive developmental stages in combination with the generation of knockout models provided an opportunity to study the signaling interactions and cellular mechanisms involved in post-implantation morphogenesis. Upon implantation, the apolar epiblast transforms into a cup-shaped epithelial tissue that flanks an emergent luminal space, the pro-amniotic cavity. The polar (embryonic) trophectoderm forms the extra-embryonic ectoderm, which is adjacent to the epiblast and contains trophoblast stem cells (TSCs). These cells will go on to form the mature placenta, and during the first days of post-implantation development they will generate structures such as the ectoplacental cone. The mural (abembryonic) trophectoderm gives rise to terminally differentiated trophoblast giant cells, which are required during the implantation of the embryo. The primitive endoderm forms the differentiated parietal endoderm cells and the visceral endoderm, which develops to envelop both the epiblast and the extra-embryonic ectoderm (Figure 2 and 3). The extra-embryonic tissues play a key role in
patternning during post-implantation development, and the crosstalk established between embryonic and extra-embryonic cells breaks the initial symmetry of the epiblast, leading to the establishment of anterior and posterior domains and formation of the primitive streak. We will summarize this process in the paragraph below, and for additional details refer the reader to excellent Reviews covering these topics elsewhere.\textsuperscript{15, 21, 25-27.}

As the embryo implants and starts to grow, the extra-embryonic ectoderm secretes proteases Furin and Pace that promote the maturation of Nodal protein, which is secreted from the epiblast.\textsuperscript{28} In turn, Nodal leads to BMP4 expression in the extra-embryonic ectoderm, which activates Wnt3 in the epiblast and further amplifies Nodal signaling.\textsuperscript{29} Concomitantly, a discrete population of visceral endoderm cells is specified in the distal part of the embryo (distal visceral endoderm, DVE), which subsequently migrates towards the extra-embryonic ectoderm, marking the future anterior domain.\textsuperscript{30, 31} These anterior visceral endoderm (AVE) cells secrete Wnt and Nodal antagonists, therefore creating an anterior-posterior gradient of Wnt and Nodal activity that leads to the specification of the primitive streak in the posterior epiblast and the onset of gastrulation by E6.5.\textsuperscript{32} Posterior epiblast cells initiate the expression of mesoderm markers, undergo epithelial-to-mesenchymal (EMT) transition and ingress through the primitive streak to form mesoderm and definitive endoderm.\textsuperscript{15} In addition, extra-embryonic ectoderm-derived BMP signals lead to the induction of 4 to 8 primordial germ cells (PGCs) in pre-streak posterior epiblast cells.\textsuperscript{33, 34}

Despite this overall basic understanding of the signaling cross-talk between the embryonic and extra-embryonic tissues, many fundamental questions have remained unanswered. What are the morphogenetic events that take place in the implanting embryo? How are they regulated at the cellular and molecular level? What are the transcriptional and epigenetic changes that drive these morphogenetic transformations? What is the influence of the uterine environment or, alternatively, are these morphogenetic events embryo autonomous? Thanks to the development of methods to culture mammalian embryos in vitro beyond implantation we are just beginning to address these questions.

Several studies in the 1970s indicated that mouse blastocysts have the intrinsic ability to undergo post-implantation morphogenesis in vitro.\textsuperscript{35-39} These culture methods varied in complexity, composition and substrate used, and were not commonly adopted in the field. The first systems relied on collagen gels and lens explants to promote attachment, but later successful development was also reported on plastic dishes, or in co-culture with uterine cells. Advancing these methodologies and coupling them with high-resolution time-lapse imaging has led to the discovery of the morphogenetic events that shape the epiblast, leading to its polarization and the formation of the pro-amniotic cavity at the transition from pre- to post-implantation. Epiblast cells polarize in response to $\beta_1$-integrin signaling, which is initiated by the interactions with the extra-cellular matrix, secreted by the extra-embryonic tissues. This polarization transforms the epiblast into a transitory rosette-like structure that undergoes lumenogenesis to form the pro-amniotic cavity. This process does not require cell death, contrary to a hypothesis proposing
apoptosis as the main mechanism driving pro-amniotic cavitation\textsuperscript{47}. Whereas the transformation of the epiblast now has been largely uncovered, the cellular and molecular mechanisms that reshape the extra-embryonic tissues still await discovery.

Another breakthrough has been the development of single-cell technologies, which allow developmental biologists to study cell fate decisions at the single-cell level in an embryonic context\textsuperscript{48}. This approach has been particularly useful to identify events leading to lineage specification\textsuperscript{49} and differential allelic gene expression\textsuperscript{50, 51}. Single-cell sequencing studies also led to the discovery that molecular differences are already present between individual cells of 2-cell stage and 4-cell stage embryos, and that lineage specification is affected by this cellular heterogeneity\textsuperscript{18, 19, 52-54}. In addition, the route from pluripotency establishment at the blastocyst stage (E4.5) to lineage commitment at gastrulation (E7.5) can now be efficiently followed\textsuperscript{55-57} (Figure 3). During these three days, epiblast cells are pluripotent and able to form any cell type of the fetus. However, epiblast cells present marked transcriptional, epigenetic and metabolic differences that define distinct pluripotent states, from the naïve epiblast of unrestricted potential at E4.5 to the lineage-biased primed pluripotent state characteristic of the E6.5/E7.5 epiblast\textsuperscript{58-60}. These pluripotent state transitions are drivers of epiblast morphogenesis and the formation of the pro-amniotic cavity\textsuperscript{61}. Mouse blastocysts may be locked in the naïve pluripotent state in a state of dormancy, diapause, which can be mimicked \textit{in vitro} by inhibiting proliferation or downregulating the metabolic activity of the blastocyst\textsuperscript{62, 63}.

We are just beginning to open the black box of implantation development. Our understanding of the complex interplay between tissue morphogenesis and cell fate specification and the intercellular communication between distinct tissue types is still far from complete. A more comprehensive knowledge of these processes may be achieved using \textit{in vitro} models of the embryo, as discussed in the following section.

\textbf{Stem cell models of the mouse embryo}

Soon after the derivation of mouse embryonic stem cells (ESCs) in 1981\textsuperscript{64, 65}, their potential to mimic embryogenesis was tested through formation of 3D aggregates called embryoid bodies (EBs)\textsuperscript{66}. This work was based on experiments investigating the developmental potential and differentiation capabilities of embryonic carcinoma cells\textsuperscript{67, 68}. EBs became a widely used tool to explore the mechanisms of cell fate specification and differentiation, and to direct ESCs into specific developmental lineages\textsuperscript{69}. At that point it was unclear whether these lineage specification events in EBs occurred in an organized manner. The pioneering work of ten Berge et al. led to the conclusion that self-organizing morphogen gradients can be established in EBs, leading to polarized Wnt signaling and mesoderm specification\textsuperscript{70}. More recently EBs have been used to generate organ-like structures, termed organoids\textsuperscript{71, 72}, and embryo-like structures, or embryoids\textsuperscript{73-76}. These models share the intrinsic symmetry breaking events that likely arise as a consequence of fluctuations in gene expression, which are amplified under appropriate experimental conditions\textsuperscript{77}. These spontaneous symmetry breaking events\textsuperscript{70} can be generated more robustly
by providing a short pulse of the GSK3 inhibitor (Wnt activator) Chiron\textsuperscript{73, 76} during a precise time window. Under these conditions EBs can develop to establish distinctive gene expression domains suggestive of germ layer specification\textsuperscript{73} (Figure 4). EBs-derived embryoids are very valuable tools to study self-organization and lineage specification, but they do not recapitulate the initial morphogenetic steps of the epiblast, such as polarization into a 3D rosette structure that undergoes lumenogenesis\textsuperscript{45}. Inspired by the fundamental discoveries of Mina Bissell and Keith Mostov, among others\textsuperscript{78-81}, we recently showed that individual ESCs cultured in a 3D matrix recapitulate the processes of cell polarization, rosette formation and lumenogenesis via vesicular exocytosis, following naive pluripotency exit\textsuperscript{45, 61}. When the chemical, physical and mechanical properties of the 3D matrix are controlled, a self-organized circular arrangement of gene expression is generated as a consequence of differential cell adhesion and cortical tension\textsuperscript{82}. Globally, these studies highlight an intrinsic ability of ESCs to self-organize and generate patterns\textsuperscript{83}.

The derivation of stem cells more akin to the post-implantation epiblast represents another interesting avenue that might help to explore the molecular mechanisms of cell lineage allocation and pluripotent state transitions. Culture of post-implantation epiblasts in the presence of Fgf2 and Activin-A generates epiblast stem cells (Epi SCs), which capture a lineage-biased primed pluripotent state similar to the E6.5/E7.5 epiblast\textsuperscript{84-86} (Figure 3). When mouse ESCs are cultured in this medium they acquire a transitory E5.5-like formative pluripotent state\textsuperscript{87, 88}. The resulting epiblast-like stem cells (EpiLCs) do not express lineage factors and are competent for PGC specification\textsuperscript{87}. When EpiLCs are grown in micropatterns of a defined size they generate a circular arrangement of cellular fates that can be modulated by changing the external growth factor environment\textsuperscript{89}.

Self-renewing stem cell lines representative of the extra-embryonic tissues of the mouse embryo have also been established (Figure 3). Extra-embryonic endoderm (XEN) cells represent the stem cell population of the primitive endoderm, but they are molecularly and functionally more similar to the parietal endoderm than the visceral endoderm\textsuperscript{90}. TSCs derived from mouse blastocysts represent the stem cell population of the extra-embryonic ectoderm\textsuperscript{91}. Recently it has been shown that fostering the self-assembly of TSCs and ESCs in a 3D matrix leads to the generation of embryo-like structures, which recapitulate the morphogenesis of early post-implantation embryos including pro-amniotic cavity formation, and undergo symmetry breaking and specification of mesoderm and PGC-like cells\textsuperscript{92} (Figure 4). Mixing ESCs and TSCs has also led to development of structures morphologically and transcriptionally similar to mouse blastocysts\textsuperscript{93}. These blastoids can also be generated by reprogramming primed cells into the naïve state\textsuperscript{84}[bioRxiv], but in both cases they do not robustly develop to post-implantation stages, at least at present. Most recently, complete embryo-like structures composed of ESCs, TSCs and XEN cells have been generated\textsuperscript{95} that establish all three tissues: ESC-derived epiblast, TSC-derived extra-embryonic ectoderm and XEN-derived visceral endoderm. These embryo-like structures initiate EMT and gastrulation, which leads to the specification of mesoderm and definitive endoderm-like cells, and globally to the
acquisition of a gene expression signature similar to E7.0 mid-gastrula stage embryos\textsuperscript{95, 96}.

An important conclusion that can be drawn from these studies is that ESCs have the potential to undergo patterning events \textit{in vitro}. A comparison of the different \textit{in vitro} models of embryogenesis generated so far may help to reveal the role of the extra-embryonic tissues in directing the self-organizing capabilities of ESCs. In addition, how far these embryoids and embryo-like structures can develop remains to be determined.

**Elucidating early development in the human**

The first month of human development has remained elusive for decades. A major breakthrough in our understanding of human embryogenesis came from the work of John Rock, Miriam Menkin and Landrum Shettles in the US, and Robert Edwards, Barry Bavister and Patrick Steptoe in the UK, who reported the \textit{in vitro} fertilization (IVF) of a human egg\textsuperscript{97, 98} (Figure 1). Refined culture conditions endowed the resulting zygotes with the ability to cleave, blastulate and hatch\textsuperscript{99, 100}. As a result, the first seven days of human embryogenesis became accessible to observation and experimentation. The use of surplus donated IVF human embryos in combination with single-cell profiling has permitted researchers to describe major events in human pre-implantation development, including embryonic genome activation and cell lineage allocation\textsuperscript{101-106}. The recent application of genome editing technology further revealed the function of early developmental genes, such as the pluripotency transcription factor OCT4\textsuperscript{107}. On day 7, the human blastocyst, composed of the embryonic epiblast and two extra-embryonic epithelial tissues, primitive endoderm (hypoblast) and trophectoderm, is ready to implant in the maternal uterus\textsuperscript{108}. Although failure to implant represents one of the main causes of early pregnancy loss and an important limitation of assisted reproductive techniques\textsuperscript{109}, our understanding of human implantation morphogenesis is scant given the technical difficulties to study human embryos beyond day 7. In this context, a number of studies have reported successful co-culture of human blastocysts with endometrial cells beyond day 7\textsuperscript{110, 111}. This approach represents a valuable tool to determine the signaling crosstalk between the endometrium and the embryo at the time of implantation. However, whether human embryos undergo proper post-implantation morphogenesis in these settings remains unexplored.

Due to these difficulties, the knowledge of early human post-implantation morphogenesis has been based on the contributions of the Carnegie Institution of Washington, which has collected and described samples of \textit{in vivo} developing human embryos since 1914\textsuperscript{112-114}. These observations have established that upon implantation (day 7-8), an amniotic cavity is formed within the epiblast, which organizes into a polarized rosette-like structure, resembling the process of pro-amniotic cavity formation in the mouse\textsuperscript{45}. However, although both mouse and human epiblasts develop similarly and form a pseudostratified columnar epithelium, the mouse epiblast acquires a cylinder-like morphology and the human epiblast forms a disc. More importantly, in human embryos epiblast cells adjacent to the trophoblast are specified to form a squamous epithelium known as the amnion\textsuperscript{112} (Figure 2 and 3). The trophoderm at the embryonic pole,
which mediates the implantation of the embryo into the uterus, transforms into the villous cytotrophoblast. This tissue harbors bipotent stem cells that give rise to the differentiated extravillous cytotrophoblast, which infiltrates the maternal decidua, and the multinucleated syncytiotrophoblast, which forms lacunar spaces to allow the supply of maternal blood (day 11-12)\textsuperscript{115,116}. At this stage a definitive yolk sac, derived from the hypoblast, can be observed\textsuperscript{117}. This is in contrast to the mouse, where the mural trophectoderm mediates the implantation of the embryo into the uterus, the polar trophectoderm gives rise to the extra-embryonic ectoderm, and both amnion and yolk sac are formed at the time of gastrulation\textsuperscript{118}. By day 14 of human embryogenesis, the primitive streak emerges, gastrulation is initiated and PGCs are specified\textsuperscript{119}.

These major morphogenetic transformations have also been observed in Rhesus monkey embryos\textsuperscript{120}, highlighting a high degree of similarity in the early post-implantation embryogenesis of higher primates\textsuperscript{121}. The work of Patrick Luckett and Allen Enders, among others, established the basic anatomy of early post-implantation Rhesus monkey embryos, including yolk sac development, extra-embryonic mesenchyme formation, and amnion specification and cavitation\textsuperscript{117,120,122,123}. In cynomolgus monkeys PGCs were shown to originate from the amnion prior to gastrulation\textsuperscript{124}, whereas in porcine embryos PGCs are specified at the posterior epiblast\textsuperscript{125}. Despite this divergence, the mechanisms of PGC specification described in both species and in PGC-like cells\textsuperscript{125-127} seem to be conserved\textsuperscript{124,125}. Analyses of Carnegie stage human embryos revealed the presence of PGCs in the yolk sac at E24\textsuperscript{119}. However, where exactly human PGCs are specified remains unknown.

The establishment of a culture system allowing human embryo development beyond implantation would open the doors to explore the basic mechanisms of human post-implantation morphogenesis. Embryos cultured in these settings would undergo the major morphogenetic events of early post-implantation morphogenesis, namely: lineage segregation, amniotic cavitation, trophoblast differentiation, yolk sac formation and amnion specification (Figure 2). Based on the self-organizing capabilities of mouse embryos\textsuperscript{43}, it was plausible to hypothesize that early human post-implantation morphogenesis could happen in the absence of maternal tissues, and thus in an embryo-autonomous fashion. Using an experimental protocol and culture media previously developed for mouse embryos\textsuperscript{43}, we and the Brivanlou laboratory showed that human embryos can attach, survive and develop under these conditions\textsuperscript{128,129}. Moreover, approximately 30% of the developing embryos displayed the major hallmarks of post-implantation morphogenesis up to day 12-13\textsuperscript{128,129}. This \textit{in vitro} method provides a powerful tool to understand the mechanisms of early human embryogenesis, causes of early pregnancy loss, and the biology of human ESCs in the physiological context of the embryo. But it also raises fundamental questions about the limits of self-organising capabilities of human embryos. Would the presence of endometrial cells and/or a 3D scaffold affect the morphogenesis of the embryo? What are the transcriptional and epigenetic regulators in different cell types as the embryo undergoes morphogenesis? A study in cynomolgus monkeys has identified global transcriptional changes in the epiblast as the embryo implants in the uterus\textsuperscript{130}. These changes are suggestive of a pluripotent state transition in the implanting epiblast\textsuperscript{130}, similar to what has been described
in mouse embryos\textsuperscript{56, 57, 88}, although timing and identity of the involved genes differ. In favor of such a pluripotent state transition, evidence suggests that if human embryos are blocked in the naïve pluripotent state, amniotic cavitation is impaired\textsuperscript{61}. However, the cellular and molecular mechanisms that trigger exit from pluripotency and cell lineage specification in human embryos are unknown.

Advances in culture\textsuperscript{128, 129} and genome editing of human embryos\textsuperscript{107, 131-133} provide a unique opportunity to investigate gene function at the time of implantation, when the embryo needs to undergo a dramatic reorganization and many pregnancies fail. However, it has to be noted that these studies are limited to the first 7 days of post-implantation development. The 14-day rule establishes a legal limit for the \textit{in vitro} culture of human embryos in many countries, including the UK. This rule mandates that embryos can be cultured to day 14 of development or the appearance of the first signs of primitive streak formation, whichever event takes place first\textsuperscript{69}. Thus, at present, the mechanisms of cell fate specification at gastrulation cannot be studied in the context of \textit{in vitro} human embryo culture. However, alternative models to study post-implantation human embryogenesis are being explored. For example, human ESCs have been shown to be able to colonize mouse embryos as well as embryos of other non-rodent species, differentiating into the three germ layers, and hence generating interspecies chimeras\textsuperscript{134-137}. This approach allows the investigation of human cell lineage allocation \textit{in vivo}. Other strategies are based on the generation of \textit{in vitro} stem cell-derived models that recapitulate certain aspects of human embryogenesis. In the next section we will discuss the efforts to mimic human embryogenesis using stem cells.

\textbf{Stem cell models of the human embryo}

Since the initial derivation of human ESCs in 1998\textsuperscript{138}, extensive efforts have been made to devise robust differentiation protocols to generate homogenous cell populations for regeneration\textsuperscript{69}. As in the mouse, the first approximation to investigate whether human ESCs are capable of self-organising was the generation of EBs\textsuperscript{139}, which contain representative cell types of the three germ layers. Subsequent work showed that EBs can develop to specify gastrula organizer cells\textsuperscript{140}, and given the appropriate signals, they can be directed to form complex organoids \textit{in vitro}\textsuperscript{141, 142}. However, in doing so they do not reproduce the spatial patterning of the post-implantation human embryo\textsuperscript{77}. Hence, the question remained of how to exploit the self-organizing capabilities of human ESCs to mimic embryogenesis. A hint that this could be possible came from studies of non-human primate ESCs, which showed an intrinsic capability to form post-implantation-like structures \textit{in vitro}\textsuperscript{143, 144}. The use of circular micropatterns has shown that self-organizing patterns of human ESCs can be induced by confinement\textsuperscript{145} (Figure 3). A geometrically controlled culture and a chemical cue (BMP4) are sufficient to generate ring-like arrangements of different cellular fates, similar to those observed at gastrulation\textsuperscript{145, 146}. This self-organizing pattern emerges as a consequence of the interplay between receptor localization (and thus accessibility to the ligand) and production of the BMP inhibitor Noggin\textsuperscript{147}. Exposure of such micropatterned colonies to ACTIVIN-A and WNT3A leads to the formation of a structure equivalent to the human organizer\textsuperscript{148}. This system
represents an in vitro model ideally suited to decipher the complex interplay between signaling, fate and shape and to explore symmetry breaking events and the self-organizing properties of pluripotent stem cells. It lacks, however, some of the key morphological features of the early post-implantation human embryo, such as the formation of the amnion and the amniotic cavity. Recent reports have shown that amniotic cavity formation can be recapitulated in vitro using human ESCs by addition of a 3D extracellular matrix. Moreover, these structures undergo spontaneous symmetry breaking forming a squamous amniotic epithelium that is contiguous to an epiblast columnar epithelium, as observed in early post-implantation human embryos. They also initiate gastrulation-like events such as mesoderm specification and upregulation of EMT transcription factors. Interestingly, these events appear to be controlled by a gradient of BMP4, in agreement with observations in monkey embryos. However, in contrast to the micropattern technology the formation of these embryoids is less efficient and robust, at least at present.

These models are just the beginning of a surge of interest in synthetic embryo research. There are still a number of unresolved questions, technical difficulties and ethical concerns that await future investigation. First, the initial culture parameters may have a profound impact in the final outcome. It is becoming increasingly apparent that ESCs can be maintained in different pluripotent states in vitro by modifying culture conditions, and these states may endow cells with different functionalities. Secondly, the contribution of extra-embryonic tissues to the development of the human epiblast remains to be determined. The derivation of human TSCs and the generation of human ESCs with embryonic and extra-embryonic potential may be useful tools to generate organized structures comprising both embryonic and extra-embryonic lineages. The third aspect to take into consideration is reproducibility. Devising robust protocols will help to obtain quantitative measurements and to assess gene function. The fourth issue relates to the lack of an appropriate in vivo control. Given that human embryos can only be cultured in vitro until day 14 and prior to the appearance of the primitive streak, our knowledge of human gastrulation is limited to a few electron microscopy images of embryos developing in vivo. This limitation could be partially overcome by the use of non-human primate embryos, which display a similar developmental program to humans, both in terms of morphogenesis and gene expression. Finally, the generation of synthetic human embryo-like structures raises a number of fundamental ethical questions that need to be carefully evaluated.

Given the numerous potential scientific and clinical benefits of this research, such as improving ESC differentiation protocols or deciphering the causes of early pregnancy loss, it is important that updated guidelines and limits should be established to enable scientific progress within an ethical and legal framework.

Future perspectives

The study of embryo development has been shaped by the concepts of experimental embryology. This top-down approach contrasts with the recent generation of stem cell models of the embryo. In this exciting time for developmental biologists we envisage that only by combining studies on natural
and synthetic embryo-like structures we will be able to unravel the basic principles of human development. There are still a number of key elements that need further investigation in the coming years. First, the use of physically and chemically defined and tunable 3D matrices as an alternative to the sarcoma-derived Matrigel. Second, the establishment of human embryonic and extra-embryonic stem cell lines, which may be used to produce improved forms of human synthetic embryo-like structures. Third, the generation of more complex in vitro culture systems for human embryos that can mimic the 3D uterine environment. Fourth, the use of non-human primates as in vivo reference for the characterization of synthetic human embryos. Fifth, a broad application of CRISPR/Cas9 technology to study gene function during human embryogenesis and, finally, further development of long-term high resolution 4D imaging techniques to analyze cellular behaviors as embryos start to grow.

Working with synthetic embryo-like structures has the main advantages of scalability, ease of genetic manipulation, accessibility, and the possibility to finely control a limited number of variables or elements. For these reasons, we envisage that they will become a fundamental model to decipher the cellular and molecular principles of mammalian development. However, it is important to keep in mind that the mimicking of embryogenesis in vitro using stem cells generates simplified models of the real embryo, which do not recapitulate the whole complexity of developing organisms. Hence, to comprehend the development of form and function constructing synthetic embryos will complement deconstructing natural ones.
Figure legends:

**Figure 1. Timeline of technical breakthroughs in early mouse and human embryo research.** Major milestones are divided into descriptive and top-down or bottom-up approaches. The first category includes strategies and innovations based on observation of normal development and/or experimental manipulation. The second category contains findings that enabled the application of synthetic biology concepts to the developmental biology field, and the different attempts at recreating embryogenesis using stem cells. This timeline does not include fundamental discoveries in the fields of organoid research, stem cell differentiation, reprogramming and reproduction, or conceptual advances in mammalian embryo research. EB: embryoid body; EpiLCs: epiblast-like cells; EpiSCs: epiblast stem cells; ESCs: embryonic stem cells; IVF: *in vitro* fertilization; KO: knock-out; TSCs: trophoblast stem cells; XEN: extra-embryonic endoderm.

**Figure 2. Overview of mouse and human post-implantation development.** By E4.5 in mice and E7 in humans, the blastocyst is ready to implant into the uterus. At this stage it is composed of one embryonic tissue, the naïve pluripotent epiblast, and two extra-embryonic tissues, trophectoderm and primitive endoderm (mouse) or hypoblast (human). Implantation is initiated through the abembryonic trophectoderm (mural) in the mouse or the embryonic trophectoderm (polar) in the human embryo. Upon exit from naïve pluripotency and in response to the extracellular matrix secreted by the extra-embryonic tissues, the epiblast undergoes a process of polarization, rosette formation, and subsequent lumenogenesis. In the mouse embryo, by E5.5, the polar trophectoderm has formed the extra-embryonic ectoderm, which is contiguous to the epiblast, and undergoes lumen formation. The fusion of the extra-embryonic ectoderm and epiblast cavities generates the pro-amniotic cavity. Both tissues are surrounded by the visceral endoderm derived from the primitive endoderm. The primitive endoderm also forms the parietal endoderm, which is adjacent to trophoblast giant cells derived from the mural trophectoderm. A subset of visceral endoderm cells is specified as DVE and AVE, which migrate towards the epiblast-extra-embryonic ectoderm boundary to determine the future anterior side of the embryo. By E6.5 in the posterior epiblast the primitive streak is formed, gastrulation is initiated and PGCs become specified at the boundary between the posterior epiblast and the extra-embryonic ectoderm. By contrast, during early post-implantation development of human embryos a subset of epiblast cells becomes specified as extra-embryonic amnion and the pluripotent epiblast acquires a disc shape. By E11 the hypoblast has formed a prospective yolk sac and the trophectoderm has differentiated into cytotrophoblast and syncytiotrophoblast. By E14 gastrulation is initiated in the posterior epiblast. In monkey embryos PGCs are specified in a population of amniotic cells, but whether this is also the case in human embryos remains to be determined. AVE: anterior visceral endoderm; DVE: distal visceral endoderm; PGCs: primordial germ cells; TE: trophectoderm.
Figure 3: Cell types in early post-implantation mammalian embryos. A summary of the major cell types present in early post-implantation mammalian embryos, formed from the three tissues of the blastocyst. Cellular features and molecular markers are specified. Stem cell lines are highlighted in blue, next to their in vivo cellular counterparts. AVE: anterior visceral endoderm; DVE: distal visceral endoderm.

Figure 4. Stem cell models of the mouse and human embryo. Blastoids: a suspension culture of mouse ESCs and TSCs leads to the generation of structures transcriptionally and morphologically similar to the blastocyst. Reprogramming of primed pluripotent cells into naive conditions can also generate blastocyst-like structures with embryonic and trophoblast lineages. Mouse epiblast-like structures: ESCs cultured in 3D Matrigel recapitulate the formation of rosettes that evolve to form a luminal cavity. Polarized EBs: aggregates of ESCs, known as EBs, undergo a process of symmetry breaking, specify a region of mesoderm, and form a primitive streak-like domain. EB-derived gastruloids: under appropriate experimental conditions EBs are able to elongate and specify ectoderm-like, mesoderm-like and endoderm-like domains. The resulting structures are thus called gastruloids. Organized germ layers: ESC rosettes evolve to generate organized germ layers when cultured in a scaffold of defined physical properties. Patterned EpiLC colonies: EpiLCs cultured in micropatterns of a defined size generate a radial organization of cellular fates. Different combinations of growth factors and inhibitors lead to distinct cell fate patterning. The diagram depicts cellular identities observed in a medium containing Activin-A, Wnt3a, Fgf2 and Bmp4. Polarized embryo-like structures: co-culture of ESCs and TSCs in 3D Matrigel generates composite structures that specify mesoderm and PGC-like cells at the extra-embryonic/embryonic boundary. Gastrulating embryo-like structures: a suspension culture of ESCs, TSCS and XEN cells leads to the generation of structures that resemble E7.5 mouse embryos morphologically and transcriptionally. Human epiblast-like structures: human ESCs cultured in 3D Matrigel recapitulate the formation of a luminal cavity. Post-implantation amniotic sac embryoids: ESC embryoids break symmetry and generate amnion-like cells and an epiblast-like region that undergoes EMT. Patterned ESC colonies: ESC confinement in the presence of BMP4 generates a circular arrangement of cellular fates, mimicking the generation of embryonic germ layers. In this model, treatment with WNT3A and ACTIVIN-A leads to the formation of a gastrula organizer. AVE: anterior visceral endoderm; EB: embryoid body; EpiLC: epiblast-like cells; ESC: embryonic stem cell; PGC: primordial germ cells; TSC: trophoblast stem cells; XEN: extra-embryonic endoderm.

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References


<table>
<thead>
<tr>
<th>No.</th>
<th>Author(s)</th>
<th>Title</th>
<th>Journal</th>
<th>Volume</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>Liang, P. et al.</td>
<td>CRISPR/Cas9-mediated gene editing in human triploid zygotes.</td>
<td>Protein Cell</td>
<td>6</td>
<td>363-372</td>
</tr>
<tr>
<td>137</td>
<td>Thomson, J.A. et al.</td>
<td>Embryonic stem cell lines derived from human blastocysts.</td>
<td>Science</td>
<td>282</td>
<td>1145-1147</td>
</tr>
<tr>
<td>138</td>
<td>Itskovitz-Eldor, J. et al.</td>
<td>Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers.</td>
<td>Mol Med</td>
<td>6</td>
<td>88-95</td>
</tr>
<tr>
<td>139</td>
<td>Sharon, N., Mor, I., Golan-lev, T., Fainsod, A. &amp; Benvenisty, N.</td>
<td>Molecular and functional characterizations of gastrula organizer cells derived from human embryonic stem cells.</td>
<td>Stem Cells</td>
<td>29</td>
<td>600-608</td>
</tr>
<tr>
<td>142</td>
<td>Thomson, J.A. et al.</td>
<td>Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts.</td>
<td>Biol Reprod</td>
<td>55</td>
<td>254-259</td>
</tr>
<tr>
<td>143</td>
<td>Behr, R., Heneweer, C., Viebahn, C., Denker, H.W. &amp; Thie, M.</td>
<td>Epithelial-mesenchymal transition in colonies of rhesus monkey embryonic stem cells: a model for processes involved in gastrulation.</td>
<td>Stem Cells</td>
<td>23</td>
<td>805-816</td>
</tr>
<tr>
<td>148</td>
<td>Taniguchi, K. et al.</td>
<td>Lumen Formation Is an Intrinsic Property of Isolated Human Pluripotent Stem Cells.</td>
<td>Stem Cell Reports</td>
<td>5</td>
<td>954-962</td>
</tr>
</tbody>
</table>
Implantation (mural TE) → Epiblast polarization and cavity formation → Epiblast rosette → Extra-embryonic cavity formation

E5.5

Implantation (polar TE) → Epiblast polarization and lumenogenesis → Epiblast disc → Amnion specification and amniotic cavity formation

E7

Extra-embryonic ectoderm → DVE specification → DVE/AVE migration → Pro-amniotic cavity formation

E9

Symmetry breaking?

E11

Naive pluripotency exit → Epiblast rosette → Epiblast disc → Amnion

E14

Gastrula stage

PGCs

Pro-amniotic cavity

Nascent mesoderm

Primitive streak

Syncytiotrophoblast

PGCs?
MOUSE

Trophectoderm

Epiblast
Mouse embryonic stem cells

Epi-like stem cells
Columnar epithelium
Formative pluripotent
Oct4+ Nanog- Rex1-

Primordial germ cell
Unipotent
Oct4+ Blimp1+ Prdm14+

Nascent mesoderm
Mesenchymal
Multipotent
Oct4+ Nanog+ Otx2+

Villous cytotrophoblast
Trophoblast stem cells
Epithelial, bipotent
CDX2+ ELF5+ ITGA6+

Extravillous trophoblast
Syncytiotrophoblast
Invasive
Differentiated
HLAG+ ITGA5+

Visceral/Yolk sac endoderm
Extra-embryonic mesenchyme
Polarized epithelium
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PRIMITIVE ENDODERM

HUMAN

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MOUSE

Blastoid
Epiblast-like structure
Polarized embryo-like structure
EB-derived gastruloid
Organized germ layers
Patterned EpiLC colony
Polarized embryo-like structure
Gastrulating embryo-like structure

HUMAN

Epiblast-like structure
Post-implantation amniotic sac embryoid (PASE)
Patterned ESC colony

Legend:
- Yellow: Epiblast-like cells (Oct4+)
- Dark Blue: Ectoderm-like cells (Sox2+ Sox1+)
- Green: Trophoblast-like cells and TSCs (Cdx2+)
- Grey: PGC-like cells (Stella+ AP2γ+)
- Red: Endoderm-like cells (Sox17+)
- Orange: Amnion-like cells (Gata3+ T+ AP2β+)
- Pink: AVE-like cells (Lefty1+)
- Brown: Extra-embryonic mesoderm-like cells (Cdx2+ Sox17+ T+)
- Brown: Primitive streak-like and mesoderm-like cells (T+)