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# Regulation of Cell Fate Decisions in Early Mammalian Embryos

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## Keywords

mammals, embryo, gene regulatory network, RNAseq, cell fate

## Abstract

Early embryogenesis is characterized by the segregation of cell lineages that fulfill critical roles in the establishment of pregnancy and development of the fetus. The formation of the blastocyst marks the emergence of extraembryonic precursors, needed for implantation, and of pluripotent cells, which differentiate toward the major lineages of the adult organism. The coordinated emergence of these cell types shows that these processes are broadly conserved in mammals. However, developmental heterochrony and changes in gene regulatory networks highlight unique evolutionary adaptations that may explain the diversity in placentation and in the mechanisms controlling pluripotency in mammals. The incorporation of new technologies, including single-cell omics, imaging, and gene editing, is instrumental for comparative embryology. Broadening the knowledge of mammalian embryology will provide new insights into the mechanisms driving evolution and development. This knowledge can be readily translated into biomedical and biotechnological applications in humans and livestock, respectively.

## INTRODUCTION

**Lineage specification:** cells initiating differentiation; can be induced to change their fate until they become lineage restricted

Mammals belong to a large and diverse class of animals (~5,400 species) (1) that share a common body plan and key anatomical features, such as a dorsal nervous system, segmented trunk muscles, vertebrae located along the anterior–posterior axis, and complex heads with multisensory organs (2). The foundations of mammalian development have been historically studied in rodents as a model system; however, recent improvements in assisted reproductive technologies and availability of sequenced genomes from human and domestic animals have enabled the emergence of the field of comparative mammalian embryology. These new studies broaden our understanding of the basic cellular and molecular mechanisms controlling mammalian development, thus providing insights into reproductive adaptations, speciation, and evolution.

The formation of the zygote marks the beginning of mammalian embryogenesis. For the first few days, the embryo develops independently of the maternal environment and can self-organize into a blastocyst composed of three foundational lineages. Messenger RNAs and proteins encoding for cell cycle and nucleic acid synthesis regulators stored in the oocyte sustain this early period of embryo development, characterized by a series of cleavage divisions. After embryonic genome activation (EGA), which takes place between the 2- (mice), 4–8- (rabbits, pigs, and humans), or 8–16-cell (cattle) stages, the embryo proper directs development autonomously until the formation of the blastocyst (**Table 1**). The notably early EGA in mice appears to be a rodent adaptation of a basal process of gradual and progressive genome activation characteristic of other mammals (3). The mouse's conspicuously rapid and synchronous embryo development, as well as its small size, make it a very robust experimental system that has been widely adopted for studying mammalian biology. Yet large mammals (e.g., primates and domestic animals) follow rules consistent with slower developmental progression. Therefore, this begs the question of whether adaptation to rapid development was enabled in part through changes in the gene regulatory network (GRN) controlling lineage specification and lineage allocation in rodents, and whether these changes conferred an evolutionary advantage. Could these changes help explain why 40% of mammals are rodents? This review discusses the understanding of processes controlling early embryogenesis and offers an interpretation of the potential consequences of these adaptations for the reproductive physiology of different mammals.

**Table 1 Comparative features of early mammalian embryo development**

| Feature | Embryonic stage | | | | |
|---|---|---|---|---|---|
| | Mouse | Rabbit | Human | Pig | Cattle |
| EGA | 2-cell | 4–8-cell | 4–8-cell | 4–8-cell | 8–16-cell |
| Cell polarization | 8-cell | 32-cell | 8–16-cell | 16-cell | Partial 16-cell stage |
| Embryo compaction | 8-cell | 32-cell | 16–20-cell | 32-cell | 32-cell |
| Lineage segregation | 1st TE-ICM<br>2nd Epi-Hypo | ? | 1st TE-ICM<br>2nd Epi-Hypo | 1st TE-ICM<br>2nd Epi-Hypo | 1st TE-ICM<br>2nd Epi-Hypo |
| Lineage restriction | TE: 16–32 cells<br>ICM: 32–64 cells | ND | TE: Blastocyst (?)<br>ICM: ? | ND | TE: Blastocyst (?)<br>ICM: ? |
| Implantation/MRP | E4.5 | E6–7 | ~E7–9 | E11–13 (MRP) | E12 (MRP) |

Abbreviations: E, embryonic day; EGA, embryonic genome activation; ICM, inner cell mass; MRP, maternal recognition of pregnancy; ND, not determined; TE, trophectoderm.

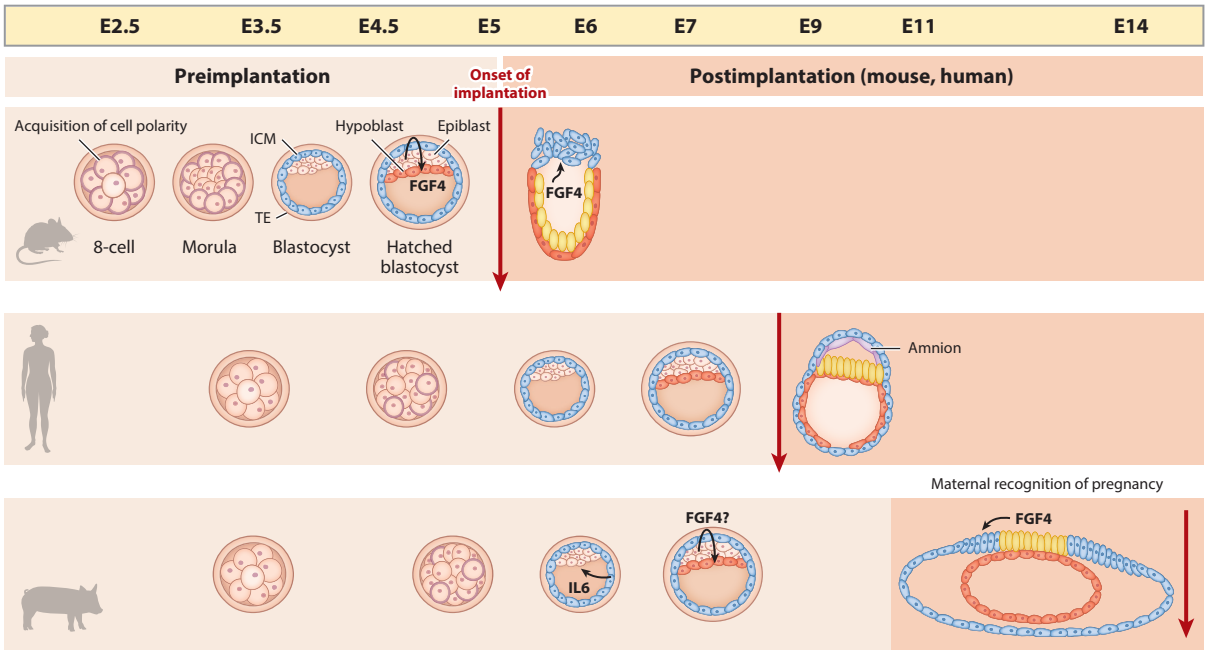


**Figure 1**

Chronology of early embryo development in different mammals. Blastomeres establish cell polarity from the 8-cell stage in the mouse, whereas in human and pig this starts in some cells from the 16-cell stage. Time of implantation varies between species. The polar trophoblast of the mouse embryo undergoes extensive proliferation and differentiation during implantation. The mouse conceptus forms an egg cylinder that contains a conical epiblast. In other mammals, the polar trophoblast either plays a less significant role (human) or is absent after the blastocyst stage. In these species, the epiblast expands to form a flat embryonic disc. FGF4 and IL6 play important roles in the communication between cell compartments within the conceptus. Abbreviations: E, embryonic day; ICM, inner cell mass; TE, trophectoderm.

## Establishing the Feto-Maternal Interphase

After a short period of autonomous development characterized by cleavage divisions and extensive epigenetic reprogramming of the maternal and paternal genomes (4, 5), the 8-cell mouse embryo begins to self-organize through a process of cell polarization and compaction (**Figure 1**). Cell polarization, established in the contact-free area of the blastomeres, consists of the formation of an apical domain enriched for microvilli, F-actin, and aPKC (6, 7). Polarization leads to changes in mechanical sensing important for defining the fate of inner and outer cells, whereby apolar cells are biased toward inner cell mass (ICM) cells and outer cells toward trophectoderm (TE) (8). Embryo compaction, in contrast, is an E-cadherin-mediated process that reinforces tensile forces of polarized blastomeres, contributing to cell localization to either the outside or the inside (9). These two events occur almost simultaneously in the mouse embryo; however, there are some variations in other mammals. Microvilli are found first at the 16-cell stage in human, pig, and cattle embryos and even later in rabbits (32-cell stage) (10–12). Compaction is reported to start in the 8-cell stage and continue up to the morula stage in human embryos (12–14), whereas it is first detected in 32-cell-stage pig and cattle embryos (10, 15).

In mouse embryos, mechanical changes are associated with the activation of the transcription factor (TF) network responsible for TE specification and development. For instance, YAP protein levels, which activate *Tead4*, increase in polarized outside cells. The activation of *Tead4* leads to

**Lineage restriction:** a cellular state refractory to change in response to local stimuli

inactivation of the pluripotency factor *Sox2* and upregulation of *Cdx2* and *Gata3* (7, 16–18). The activation of *Cdx2* causes downregulation of *Oct4*, and this event establishes one of the earliest known antagonistic relationships between two key factors identified in mammalian embryos responsible for the segregation of TE and ICM (19, 20). Although *Cdx2* is activated between the 8- and 16-cell stages in the mouse, experimental manipulations have shown that the emergent TE becomes lineage restricted between the 16- and 32-cell stages (21–23). After this stage, *Cdx2* reinforces the GRN controlling TE differentiation by targeting other TE markers, such as *Gata3*, *Eomes*, *Tfap2c*, *Ets2*, and *Elf5* (24–27). *Elf5*, which is activated by *Eomes*, acts as a gatekeeper of this lineage by preventing precocious differentiation of TE precursors (27, 28). Through positive feedback loops, gradual progressive lineage delineation determines the development of the TE. This process is a classic example of how developmental programs are executed during animal development, whereby transcriptional noise in early precursors and stochasticity in lineage specification gradually resolve into the lineages of the embryo (29). In other animals, differences in the temporal expression profile, as well as the type of genes involved in the TE GRN, have been reported (30). These observations highlight physiological differences, such as timing of implantation or signaling to the mother of the presence of the conceptus in the uterus. For example, in the mouse, implantation begins at embryonic day 4.5 (E4.5), whereas in humans it starts by E7–9 (31). In ungulates, attachment to the uterine lining occurs between E13 and E15 in pigs (32) and from E21 in cattle (33) (**Table 1**). In the latter species, a functionally developed TE plays a critical role prior to implantation by eliciting maternal recognition signals between E11 and E13 in pigs (34) and from E12 in cattle (35–37). This suggests that TE lineage restriction in species with late implantation can, and may, occur later than in mice. Notably, experimental evidence shows that TE cells from the blastocyst can contribute to the hypoblast lineage in cattle embryos (38). Similarly, blastocyst-derived TE cells can contribute to the ICM in humans (39). This indicates that TE from cattle and human blastocysts maintains remarkable developmental plasticity, in contrast to in mouse embryos, in which it becomes restricted in early blastocysts (21) (**Figure 1**). It is worth noting that the developmental plasticity of TE cells from more advanced stages has not been experimentally tested in other species.

The transcriptional signature of the emerging TE across other species studied is characterized by the expression of a common set of genes, including *TEAD4*, *GATA3*, *GATA2*, and *DAB2* (40–42). *CDX2* is expressed in the TE of mid–late blastocysts in human (40, 43), pig (42, 44), and cattle (45). Similarly, in pig and cattle, CDX2 protein is detected in all TE cells from blastocysts, whereas it is found only in scattered cells in earlier stages (38, 46, 47). This expression profile suggests a different role for this gene in these species compared with mice. Functional experiments show that *CDX2* knockdown in the pig disrupts cell polarity rather than lineage specification (46). In cattle, *CDX2*-knockdown embryos can form a TE and express other lineage markers, such as *KRT8* and *IFN-T* (48). In addition, *CDX2* target genes *EOMES* and *ELF5* are not expressed in human, pig, and cattle TE at the blastocyst stage (40, 42, 45, 49, 50), but they are activated in more advanced stages when *CDX2* is also expressed (49). Although in mice *Cdx2* represses *Oct4* in the TE lineage (19, 51), in human, pig, and cattle embryos both *CDX2* and *OCT4* are expressed in the TE of blastocysts (38, 52, 53). It is not clear what the function of maternal *OCT4* is in the early TE lineage; however, studies in human and pig embryos suggest that it may play a role in the activation of *CDX2* (54, 55). Zygotic *OCT4*, in contrast, seems critical for maintaining expression of *NANOG* in the epiblast of cattle blastocysts, rather than supporting TE lineage development (55, 56). This evidence suggests that *Cdx2* in rodents has evolved to acquire a novel role during TE segregation, perhaps to accommodate for the earlier lineage restriction and fate commitment ahead of embryo implantation by E4.5. In other mammals, in which implantation occurs at a later stage, the TE lineage is specified over a prolonged period and additional cell divisions.

In addition to evidence from experimental manipulation of cells, studies using single-cell RNAseq (scRNAseq) technology have provided detailed molecular understanding of the events leading to the segregation of different lineages in preimplantation embryos. scRNAseq of preimplantation embryos shows that a distinct population of TE and ICM can be identified in mouse blastocysts (57). Similarly, pig and cattle blastocysts show clear demarcation of TE and ICM cells (42, 45, 58). In contrast to these findings, a report analyzing hundreds of cells from human embryos showed that TE segregates at the same time as the epiblast and hypoblast in E7 blastocysts (41), which placed primates at odds with other animals. This controversy led researchers to reassess the data of this study, and they determined that embryo staging differences could have accounted in part for the reported differences in lineage segregation between human and other species (59). In agreement with this analysis, a recent study using a combination of live imaging and single-cell transcriptomics showed that human embryos are highly asynchronous (60). Thus, using day after fertilization as a staging parameter for human embryos does not reflect precise developmental staging, in contrast to mouse embryos. Importantly, this study showed that an initial segregation of TE and ICM can be defined by the transcriptional profile, prior to epiblast and hypoblast in the human embryo (60). This study also showed that emerging TE and ICM cells establish a close relationship, as evidenced by the expression of many ligand receptors, including *IL6/IL6R*, *FGF/FGFR*, *TGFB/TGFBR*, and *BMP/BMPR*, that may play important roles in the coordinated development of the TE in preparation for implantation. A cross talk between these two compartments, previously demonstrated in the mouse, showed that FGF4 provided by the epiblast supported expansion of the trophoblast stem cell niche (61) (**Figure 2**). Notably, recent studies from mouse in vitro–created blastoid embryos reached similar conclusions (62). A similar cross talk between these compartments is also operating in the pig embryo, where FGF4 has a trophic effect during TE segregation (63) and elongation (49, 64), resulting in the formation of a one-meter-long

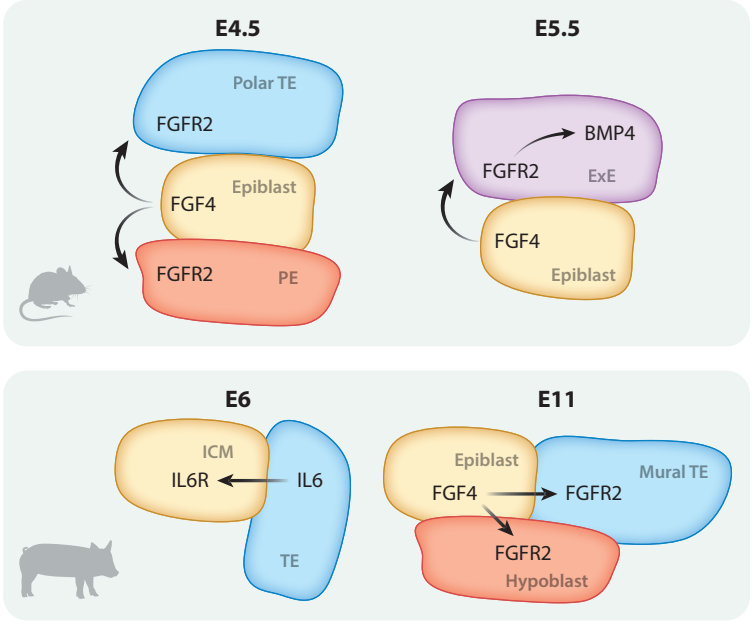


**Figure 2**

FGF4 and IL6 ligands induce responses in neighboring cellular compartments during blastocyst formation. Abbreviations: E, embryonic day; ICM, inner cell mass; TE, trophectoderm.

trophoblast within a few days (65, 66). Thus, the evidence from different species suggests that the segregation of the TE lineage is a primal event in mammalian embryos. TE development is closely coordinated by the developing epiblast to establish an embryonic–extraembryonic–maternal communication highway critical for ensuring timely recognition of pregnancy and successful implantation.

## Pluripotency in the Preimplantation Embryo and In Vitro Stem Cell Lines

The complementary lineage emerging after TE segregation is the ICM. Mouse ICM cells exist transiently from E3.5 (32-cell stage) until E4.5 (64-cell stage), when they begin to segregate into epiblast and primitive endoderm (or hypoblast, as it is known in other species) (21, 67, 68). During the early period (32-cell embryo) of ICM emergence, these cells have the potential to reconstitute a full blastocyst after experimental manipulations. However, by the 64-cell stage, these cells are unable to generate TE cells (21, 22). The changes in developmental potential reflect the establishment of a new GRN consistent with a transition from totipotency to pluripotency that gradually emerges in the ICM (57, 67). Functionally, the emergent pluripotent stage captured in vitro from isolated E4–4.5 epiblasts can give rise to embryonic stem cells (ESC) (69). These cells are pluripotent and can give rise to all the lineages of a fetus. Their molecular characterization reveals a unique genetic and epigenetic signature that is also known as naïve or ground-state pluripotency (70). This state differs from more developmentally advanced pluripotent cells derived from the E5.5 embryos, known as epiblast stem cells (EpiSC) (71, 72). EpiSC have different molecular features compared with ESC and also represent a primed state of pluripotency (70). They can differentiate into all somatic lineages of a fetus but cannot give rise to germ cells in chimeras (71, 72). The classification of different states of pluripotency is useful when comparing features of ESC lines from different species. Human ESC, which were first derived from blastocysts, have molecular features corresponding with primed pluripotency. When this was achieved, after decades of failed attempts at establishing human ESC by using the mouse culture conditions, it became clear that the requirements of human pluripotent cells were different. It was also evident that the type of cells growing in vitro represented a different embryonic cell type, as demonstrated by molecular, physiological, and morphological differences. Since then, appreciation of the differences in embryonic development between mammals has grown, and it has informed novel strategies for the derivation of ESC in domestic species. Initial approaches, primarily based on conditions used for mouse ESC derivation, were unsuitable for the establishment of bona fide farm animal ESC (73–76). Pig ESC (pESC) were produced under culture conditions that included LIF (77, 78), bFGF (79–82), or combinations thereof (83, 84). Although the evidence that LIF receptors are expressed in pig embryos is controversial (82, 85), scRNAseq data show that they are expressed in subsets of ICM cells of early blastocysts and then become confined to the hypoblast lineage in full blastocysts (42). Similarly, IL6R/IL6ST, which use the Jak/Stat pathway for signal transduction, are highly enriched in the early pig ICM, are retained in the hypoblast of late blastocysts, and decrease in the epiblast. These data suggest that Jak/Stat signaling triggered by LIF/IL6 may have two different roles: in the establishment of the pluripotent ICM in early blastocysts and in the development of the hypoblast lineage in later embryos. It was also suggested that LIF is important for pESC self-renewal via stimulation of the PI3K/AKT pathway (83) and Stat3 (86). In attempts to capture naïve cells, GSK3β and MEK inhibitors (2i medium) supplemented with LIF have also been used (87). However, all pESC lines have shown limited self-renewal ability over many passages. Only one study reported long-term survival and generation of chimeric fetuses, but fetal contribution was low, and many cells integrated in the trophoblast (84). Cattle ESC show limited proliferation and cannot generate teratomas in immunodeficient mice when grown in culture conditions combining LIF and bFGF (88, 89). Cells derived under 2i conditions show features of naïve

pluripotency, such as dome-shaped colonies and *LIFR* and *KLF4* expression, and can contribute to chimeric fetuses at low efficiency (90). Ovine ESC-like cells derived in media containing GSK3β inhibitor and bFGF can form teratomas but fail to contribute to chimeric animals (91). Goat ESC-like cells derived in media supplemented with LIF show long-term proliferation (>120 passages) and can form teratomas, but germline competence has not been determined (92). Canine ESC grown in media supplemented with LIF and bFGF grow for more than 30 passages and can form teratomas (93). All these studies show that capturing naïve cells in vitro for the establishment of ESC lines requires further refinements to the culture conditions and selection of embryonic stages suitable for isolation of these cells. Some progress in this direction was reported in humans recently, in which naïve ESC could be derived under stringent culture conditions. However, these cells become unstable in culture and have a propensity for DNA demethylation at imprinted loci (94).

Other strategies have concentrated on adapting conditions used for the derivation of primed pluripotent stem cells, such as mouse EpiSC or human ESC. The first report of pig EpiSC (pEpiSC) derived from late epiblasts showed dependence on Activin A and bFGF for self-renewal. Upon differentiation, these cells can give rise to all somatic layers, TE lineage, and germ cell precursors in vitro (95). Later reports showed similar results using pig induced pluripotent stem cells (79, 81). However, the culture conditions were not fully optimized, requiring the use of feeder cells and serum replacement. Despite the need for technical improvements, it seems that capture of primed cells is consistent with a prevalent pluripotent state during pig embryo development. Cells expressing markers of primed pluripotency exist in the epiblast between E6 and E11, a period when the epiblast grows from 25 to ~180–200 cells. Expansion of the epiblast during this period requires Nodal/TGFβ/Activin A signaling, as shown by the sharp reduction in the number of Nanog cells in the epiblast when this pathway is inhibited in mid–late blastocysts (42). Consistent with these principles, rabbit ESC, which depend on bFGF and Activin A/Nodal/TGFβ signaling, have been derived by several groups (96–98). One report described derivation of rabbit ESC from single cells in bFGF-free medium; however, the chimeric contribution in vivo was not tested (99).

Similarly, in cattle, stem cells with primed pluripotency characteristics derived using FGF2 and an inhibitor of WNT signaling on a feeder layer can be established at high efficiency. These cells have long-term self-renewing capacity and can differentiate to derivatives of the three germ layers in teratomas (100). Primed horse ESC-like cells have also been derived with bFGF supplementation; however, teratoma formation was not efficient (101, 102). Therefore, the studies described above suggest that conditions that capture primed pluripotency offer the most promising culture system for the derivation of ESC in domestic species. Considering that primed pluripotent cells align with the mature epiblast of a developing embryo (42), and that in large mammals the germline precursors develop from early mesodermal precursors (103), it would be important to establish the potential of these cells for germline contribution in chimeras.

Recent refinements in culture conditions have led to the successful derivation of novel stem cell lines from mouse and pig blastocysts (104, 105). These cells, named expanded potential stem cells (EPSC), grow on feeder layers in media containing Activin A, WNTi, LIF, and GSK3βi, plus the addition of SRC kinase inhibitors and vitamin C. Pig EPSC contribute to chimeras; can differentiate into germ cells when SOX17 is transiently overexpressed (~12%), albeit at low efficiency (~1.5%); and contribute to TE in vivo. However, the fact that these pig EPSC have limited differentiation capacity toward germ cell progenitors raises questions about their state of pluripotency. Is it possible that they fail to exit pluripotency in a timely fashion upon differentiation because their pluripotent features resemble a semi-naïve/primed state? In contrast, mouse EPSC contribute to germline chimeras and to TE with high efficiency. Do these

differences reflect specific requirements by non-rodent ESC? Further refinements, based on new understanding of the mechanisms of pluripotency in the early embryo, should serve to improve the derivation of highly competent cells for differentiation into all lineages of the chimeric fetuses.

## Gene Regulatory Network of Pluripotency

Prior to the first lineage segregation, the three core pluripotency genes, *Oct4*, *Sox2*, and *Nanog*, are expressed from the 8-cell stage and become confined to the ICM of the mouse blastocyst (67). This triad of transcription factors (TF) sits atop a core pluripotency network that associates with multiple other key genes with functions in transcriptional and epigenetic remodeling, metabolism, and cell division (106). As naïve pluripotency emerges, a specific set of genes, including *Klf2*, *Nr0b1*, *Grb2*, and *Esrrb*, identifies this stage in vivo and in vitro. Core pluripotency genes are also expressed in human and pig embryos; however, there are differences in expression of specific naïve pluripotency markers. *KLF2*, *NR0B1*, and *GBX2* are not expressed in human, marmoset, macaque, and pig embryos, but instead *KLF4*, *KLF5*, *KLF17*, and *TCFP2L1* are detected (40, 42, 107, 108). Whether the differences in gene expression of naïve pluripotency genes between mice and other animals are of functional significance is still unclear. However, based on the finding that cells from diapausing mouse embryos express all the naïve markers (107), this GRN may have evolved in rodents that acquired the novel mechanism of embryonic arrest.

Cells with naïve properties exist for a very transient period in mouse development. A clear transition to primed pluripotent gene expression is evident in E5.5 epiblasts (109). This transition is characterized by the activation of other genes, including *Nodal*, *Fgf5*, *Dnmt5*, *Otx2*, and *Lef1*, whose biological functions point to preparation for the next stage of embryo development, the onset of gastrulation. Investigations of human embryos beyond the blastocyst stage are not possible, and thus determining the progress of pluripotency in vivo is intractable. Notably, human ESC share remarkable molecular and physiological similarities with mouse EpiSC, and studies have demonstrated that these cells are arrested in a primed state of pluripotency (110). As an alternative to investigating the human embryo, a developmental time series of *Cynomolgus* monkey embryos analyzed by scRNAseq showed expression of naïve markers in early-preimplantation embryos, followed by primed pluripotency genes in late-preimplantation and postimplantation epiblasts (108). A similar time series in pig embryos sampled between the morula (E5) and the mature epiblast (E11) stage showed a clear transition toward a primed state of pluripotency, characterized by the expression of *PRDM14*, *NODAL*, and *DNMT3B* and downregulation of naïve pluripotency markers (42). Remarkably, in *Cynomolgus* monkeys and in pigs, this period is estimated to last at least 5–6 days, whereas it is extinguished in 2 days in mice. This suggests that in mice the epiblast has fewer cells than in other species at the start of gastrulation. Interestingly, a previous study estimated that the number of epiblast cells required to initiate mouse gastrulation is ~500–600, whereas for baboons and pigs it is ~2,500 (111). Because the length of the cell cycle does not accelerate before the onset of gastrulation, it seems that larger mammals compensate the need for more cells by extending the period of epiblast growth. Cell number and tissue size (volume of the epiblast) have been proposed to play key roles in mechano-sensing and in triggering signal gradients critical in determining future morphogenetic events (112). Therefore, reaching the correct epiblast size may be an important step before the exit of pluripotency.

## Signaling Pathways Driving Progression of Pluripotency

The establishment of pluripotency is closely linked to cell–cell interactions mediated by cytokines through ligand–receptor interactions. During the emergence of naïve pluripotency, expression of

*Lifr* and *Il6st*, together with downstream effectors of the Jak/Stat signaling pathway, such as *Stat3*, *Klf4*, and *Tfcp2l1*, is detected in the mouse ICM (107). Similarly, expression of WNT (*Wnt6*, *Wnt7b*, and *Axin2*) and BMP (*BMP*, *Id1*, and *Id3*) signaling-related genes is also high at this stage. Stimulation of these pathways in culture is used for the efficient derivation of naïve cells from early mouse embryos (69) and is, together with the inhibition of ERK signaling, the basis for maintaining naïve stem cells in culture (113). By E4.5, a gradual transition to a primed pluripotent state is evident, and the signaling pathways operating in this scenario differ considerably. Components of the TGFβ pathway (*Acvr1b*, *Acvr2a*, and *Tdgf1*) and ligands involved in signaling (*Nodal*, *Gdf3*, and *Lefty*) are upregulated at this stage, whereas naïve markers are downregulated (40, 69). Notably, inhibition of TGFβ signaling does not affect expression of Oct4 and Nanog in the emerging ICM (40) but is critical by E4.5 (114). Consistent with expression of naïve marker genes in the early pig embryo, components of the Jak/Stat signaling pathways, such as *IL6R* and *IL6ST*, are also detected (42). *IL6* is also expressed in the neighboring TE, suggesting a paracrine signal between the extraembryonic and embryonic domain at this stage of development. Knocking out the *IL6* gene affects the proliferation of TE cells, but with no effect on the proportion of ICM cells. This indicates that IL6 does not play a critical role in naïve pluripotency in the pig embryo. However, both *IL6R* and *IL6ST* are expressed in hypoblast cells of more advanced blastocysts, which would point to a role for this pathway during hypoblast segregation and development. This possibility was tested using small-molecule inhibitors of the Jak/Stat pathway during different stages of pig development. A reduced number of Nanog cells was detected in the early embryo, supporting an active role of the Jak/Stat pathway in the emergence of the founder population of the ICM. The trigger responsible for this Jak/Stat signal remains unknown, because in contrast to in mice, *LIF* is not expressed, and *LIFR* is found in only a few cells of the developing pig embryo (42, 44). Alternative signaling pathways, such as PI3K-Akt and MAPK, could be involved (115), as these are also found in the pig ICM (42). In humans and marmosets, *LIFR* is not expressed in the epiblast, which instead expresses *IL6ST* (40, 107). However, there is no functional evidence of its role in the embryo.

In human, marmoset, *Cynomolgus*, pig, and cattle, there is compelling evidence for an active TGFβ signaling from the early stages of epiblast development. Concurrent with the transition from naïve to primed pluripotency from early to mid–late blastocyst, expression of signaling components of the TGFβ pathway increases gradually in these cells (41, 42, 107, 108). Functionally, inhibition of TGFβ/Nodal/Activin A signaling from the premorula to the mid-blastocyst stage does not affect development of the pig epiblast, but it affects later stages (42). Similar findings were reported in human embryos (40), although differences in the response to inhibition of TGFβ have been reported (116). Thus, although features of pluripotency are shared significantly between species, the period during which cells with naïve or primed characteristics can be found in an embryo varies considerably. This has probably been a major confounding problem for experiments aimed to establish ESC from different species.

## Segregation of the Hypoblast

After the emergence of the pluripotent ICM, the second lineage segregation takes place during the formation of the epiblast and hypoblast. The early ICM (E3.5) is made of cells expressing the key pluripotency genes *Oct4*, *Nanog*, and *Sox2* and markers of the hypoblast lineage, including *Gata6*, *Pdgfrα*, and *Sox17*, which gradually resolve into distinct cellular domains of mutually exclusive expression by E4.5 (117–119). These E3.5 bipotential cells respond to FGF4, whose expression is controlled by Oct4/Sox2 in ICM cells, to initiate cell differentiation toward PE (120, 121). Mutations of the *FGF4* gene (122) or its cognate receptors (*Fgfr1/2*) (123), or chemical inhibition of

FGF/MEK signaling, results in inhibition of PE differentiation in the mouse embryo (124, 125). Conversely, supplementation of early embryos with FGF4 leads to the complete differentiation toward PE at the expense of pluripotent epiblast cells (124). The seemingly critical role played by FGF4 in promoting PE segregation in the mouse appears to be determined by local sensing of FGF4 produced by internalized cells expressing *Fgfr2* (126). More recent evidence shows that FGF4 can promote epiblast fate in cells expressing *Fgfr1* (123, 127), suggesting that the balance between epiblast expansion and PE segregation triggered in response to FGF4 stimulation may be determined by the expression of specific receptors within the ICM. Segregation of the hypoblast in other mammals is less well understood; however, there is evidence of common features in the GRN of other species. For instance, GATA6 is ubiquitous in all lineages of the blastocyst in human, monkey, pig, and cattle and becomes gradually restricted to the hypoblast in advanced stages, therefore making it a poor lineage-specific marker (40–42, 45, 108, 128). *PDGFRα* is first expressed in bipotent precursor ICM cells in primate, pig, and cattle embryos and gradually becomes restricted to hypoblast cells in late blastocysts (42, 45, 59, 107). SOX17 is first detected in hypoblast-fated cells that co-express NANOG but becomes mutually exclusive by the mid-blastocyst stage in human, marmoset, pig, and cattle embryos (42, 59, 107). Therefore, this gene can be used as a reliable marker of specified hypoblast. By using small-molecule chemical inhibitors, it was shown that blocking of FGF receptors, as well as the ERK signaling pathway, does not completely eliminate the formation of the hypoblast in human, monkey, pig, and cattle embryos (63, 107, 128–130). Even at high concentrations of these inhibitors, there are still cells that differentiate into hypoblast (42, 130, 131), suggesting that other pathways may be operating during hypoblast segregation in other mammals. As indicated above, a potential role for IL6 signaling was uncovered in the pig by using scRNAseq (42). Another potential candidate is the WNT signaling pathway. Although dispensable for lineage segregation in the mouse embryo (132), inhibition of WNT signaling together with MEK inhibition reduces the number of cells contributing hypoblast in marmosets (107). In cattle embryos, however, inhibition of WNT signaling promotes hypoblast differentiation (133). No effect of WNT inhibition on hypoblast segregation was observed in the pig embryo (42). The apparently different signaling requirements for the segregation of the hypoblast lineage in mammals suggests a divergent developmental program, which may have evolved because it might be under fewer developmental constraints than other cell types, such as the epiblast. The hypoblast is indeed a remnant of the ancestors to mammals, like amphibians and fish, in which it plays a key role in providing energy-rich yolk to sustain the development of the embryo as a free-living organism. The physiological role of the hypoblast in mammals has certainly changed, as is evidenced from its reduction in size and brief existence. The mouse primitive endoderm may have evolved into a structural role, in, for example, positioning the primitive streak, separating embryonic domains, and ensuring vascular connectivity (134). Thus, from an evolutionary perspective, the role of the hypoblast/primitive endoderm in mammals appears to have changed significantly. These changes therefore may be reflected by subtle changes in the GRN that resulted in different signaling requirements for segregation and differentiation of this lineage.

## CONCLUSIONS AND PERSPECTIVES

This review presented evidence showing the value of comparative mammalian embryology in providing a detailed picture of the conserved mechanisms governing the initial steps of development in this group of animals. Until recently, the mouse served as a prime model for mammalian development; however, evidence from studies in other species has demonstrated key differences in the control of early development. If we are to understand how mammals evolved, we must study

the molecular features of embryos from more species. Genome sequence information and reproductive biotechnologies are available for humans and most domestic animal species, thus enabling detailed investigations of embryos from other mammals. A good example of how comparative embryology has been critical for understanding early development relates to the questions of lineage segregation. This key event during the early period of embryogenesis was first described in detail in the mouse, showing a sequential segregation of the TE and the hypoblast in blastocysts (135). The universality of these findings has, however, been questioned by recent studies with human embryos, which were shown to simultaneously segregate TE and hypoblast in blastocysts (41). The observed differences were later attributed to differences in embryo staging (59), which are due to the asynchronous development of the human embryo (60), resembling that of other large mammals. Furthermore, new detailed studies in nonhuman primates and domestic animal embryos provide further detailed evidence demonstrating that sequential lineage segregation occurs in all the mammalian species studied so far, although there are differences in the timing of the events. Integration of the molecular features observed in different mammalian embryos with their respective reproductive strategies helps us to place potential adaptive mechanisms in a physiological context. For instance, restriction of the TE lineage occurs earlier in the mouse than it does in human or ungulate embryos, suggesting that in the mouse the GRN responsible for TE specification has changed. Indeed, the role and expression profile of *Cdx2* in the mouse and its relationship with *Oct4* have not found equivalents in the TE of other mammals. The changes in the early TE GRN are attributed to the co-option of existing genes, together with gene duplications and acquisition of novel enhancers via the integration of retroviral sequences (136), which enabled rapid growth of the placenta (137). These evolutionary trajectories have influenced placental rate of development, which may indirectly affect fetal development.

Another notable finding from comparative molecular embryology is the lengthy period of epiblast expansion in non-rodent mammals of approximately 6–7 days, which culminates with the formation of the embryonic disc (ED). The ED, made of columnar epithelial cells, is conserved in mammals, including basal rodents such as the plains vizcacha (138). It seems that in mice and rats, changes in polar trophoblast have led to reconfiguration of this basal structure to form the egg cylinder, containing a conical epiblast. The consequences of this remarkable anatomical change in the geometries of peri-gastrulation embryos are just beginning to be appreciated (139). For instance, the mouse polar trophoblast is the main source of BMP4, which induces germ cell specification in the mouse epiblast via a GRN consisting of *Prdm1*, *Prdm14*, and *Tfap2c* (140, 141). In other mammals that do not have a polar trophoblast covering the ED (pigs), or that develop a precocious amnion (*Cynomolgus* monkeys), the sources of BMP4 are the extraembryonic mesoderm and the syncytial trophoblast, respectively (103, 142). In these mammals, the germ cells are induced in the posterior primitive streak (and possibly in the amnion in *Cynomolgus*) via activation of a different GRN initiated by *SOX17*, *PRDM1*, and *TFAP2C*. Whether changes in the GRN governing embryo development are prerequisites that enable novel adaptations in extraembryonic lineages to evolve without affecting conceptus viability is still an open question. Evidence from basal amphibians suggests that developmental constraints imposed by the mode of germline development may be critical factors driving evolution and speciation in vertebrates (143). In conclusion, additional molecular comparative studies will elucidate whether similar constraints exist in mammals, as well as the impact they had in the GRN controlling cell fate specification.

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