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Human Embryogenesis: A Comparative Perspective

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Abstract

Understanding human embryology has historically relied on comparative approaches using mammalian model organisms. With the advent of low-input methods to investigate genetic and epigenetic mechanisms and efficient techniques to assess gene function, we can now study the human embryo directly. These advances have transformed the investigation of early embryogenesis in nonrodent species, thereby providing a broader understanding of conserved and divergent mechanisms. Here, we present an overview of the major events in human preimplantation development and place them in the context of mammalian evolution by comparing these events in other eutherian and metatherian species. We describe the advances of studies on postimplantation development and discuss stem cell models that mimic postimplantation embryos. A comparative perspective highlights the importance of analyzing different organisms with molecular characterization and functional studies to reveal the principles of early development. This growing field has a fundamental impact in regenerative medicine and raises important ethical considerations.

Contents

1. INTRODUCTION

Modern mammals share a common ancestor from approximately 166 million years ago (MYA), when the Prototheria lineage (e.g., platypus) diverged from the therian mammals (Bininda-Emonds et al. 2007, Luo et al. 2011). Metatherians are divided into American (e.g., opossum) and Australian (e.g., tammar wallaby) (Frankenberg 2018). Eutherians comprise various orders including primates (e.g., human and nonhuman primates), ungulates (e.g., cow and pig), rodents (e.g., mouse and rat), and lagomorphs (e.g., rabbit) (**Figure 1**) (Song et al. 2012). Despite the wide diversity of mammals, the early stages of their embryo development up to implantation are remarkably similar. Notable distinctions include placental diversification, regulation of the sex chromosomes, and possible differences in mechanisms that specify the few cells destined to form the embryo proper.

In this review, we discuss human embryogenesis from a comparative embryology perspective. We focus on preimplantation development, comparing human embryogenesis to that of other eutherians and metatherians. We also discuss in vitro stem cell–derived and postimplantation models of human embryogenesis that provide key insights into a poorly understood window of development and raise important ethical considerations.

2. PREIMPLANTATION DEVELOPMENT

After fertilization, the mammalian embryo travels from the oviduct to the uterus. The embryo subsequently implants, establishing a connection with maternal tissues. In this section, we discuss the main events required to achieve successful embryo formation and implantation.

2.1. Morphological Changes from Fertilization to Blastocyst Formation

In mammals, embryonic development begins with the fusion of a sperm with an oocyte to form a diploid zygote. The zygote undergoes a series of cleavage divisions, increasing the number of cells, known as blastomeres, without changing the overall volume of the embryo. After cleavage, the eutherian embryo undergoes compaction, whereby the blastomeres adhere to each other to form a tight ball of cells referred to as a morula. At this stage, the outer cells become polarized while the inner cells remain apolar. Subsequently, a morphological event called cavitation occurs, leading to the formation of a fluid-filled cavity, the blastocoel. The embryo at this stage is defined

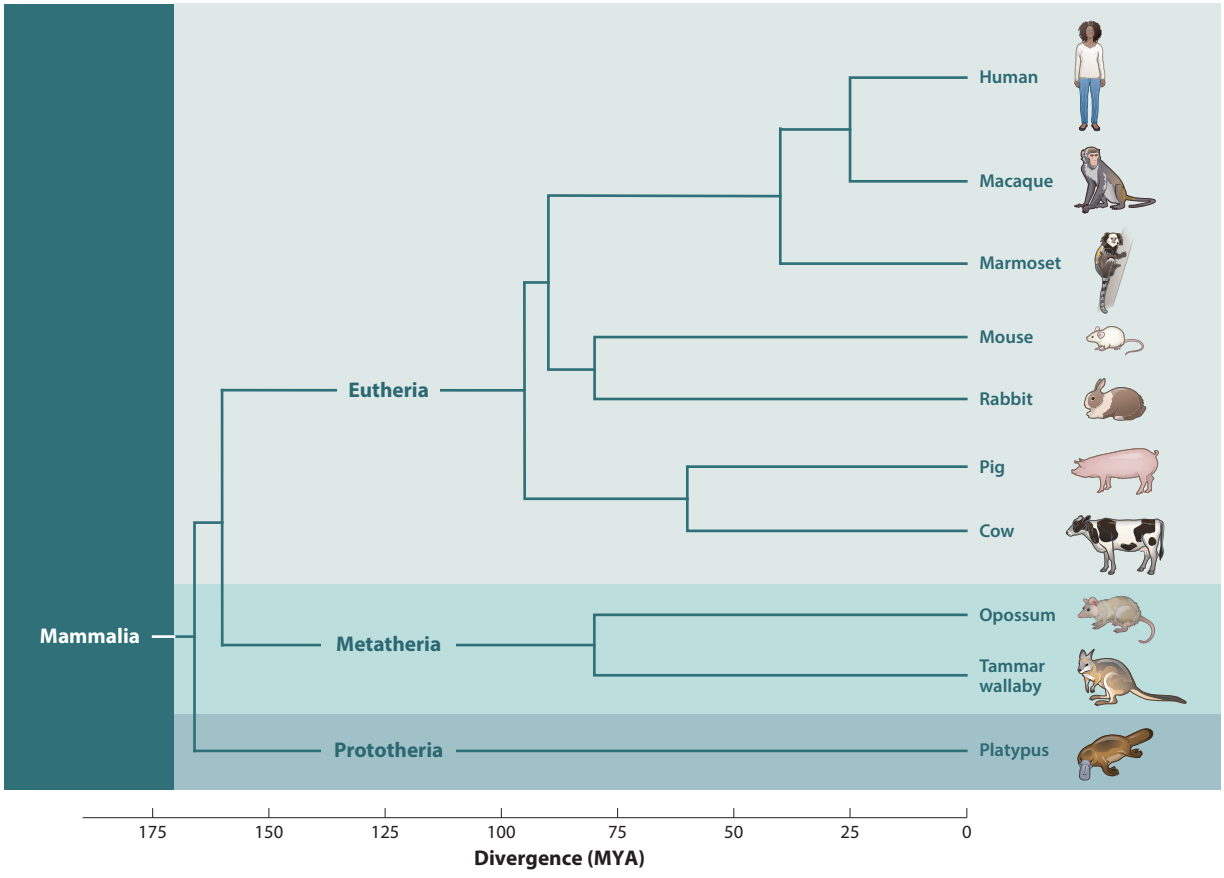

Figure 1

Phylogeny of the mammalian species discussed in this review. This cladogram shows the phylogenetic relationships among the main mammalian species that are referred to in this review following Bininda-Emonds et al. (2007) and Luo et al. (2011). For simplification, we report macaque as a general term for rhesus and cynomolgus monkeys. The scale indicates divergence in MYA (millions of years ago).

as a blastocyst. Metatherian embryos do not undergo compaction, polarization, or cavitation but still form a blastocyst (**Figure 2**).

2.1.1. Eutherians. This section covers changes from fertilization to blastocyst formation in rodents, humans, and other eutherians.

2.1.1.1. Rodents. Most of our mechanistic understanding of compaction comes from mouse studies. Compaction in the mouse occurs at the eight-cell stage, at 2.5 days postfertilization (dpf), and is characterized by blastomere flattening and accumulation at the cell–cell contact sites of E-cadherin and zonula occludens-1 (ZO-1), which are major components of adherens and tight junctions, respectively (Fleming et al. 1989). Live imaging has revealed E-cadherin-rich filopodia that extend from the apical border of cells and strongly adhere to neighboring cells (Fierro-González et al. 2013). In addition to E-cadherin, filopodia contain the myosin motor protein myosin-10, which is important for filopodia formation during compaction. The molecular manipulation of any of these components disrupts compaction. These findings indicate that filopodia contribute

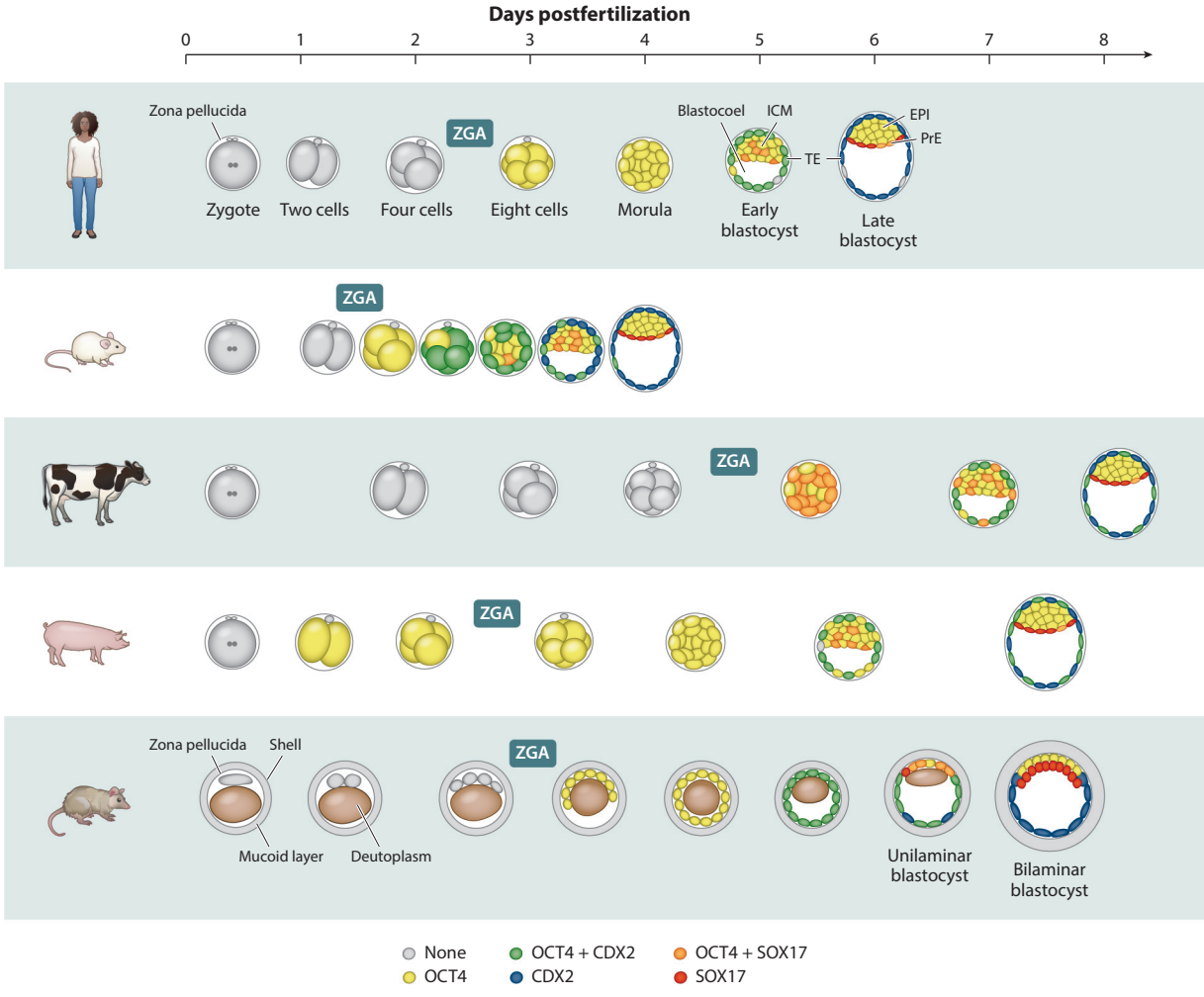

Figure 2

From fertilization to blastocyst formation. This schematic illustration shows fate specification events comparing human, mouse, cow, pig, and opossum embryos. Embryos are distributed along a temporal line starting with the zygote at 0.5 days postfertilization. Cells are colored according to the expression of key transcription factors that represent the three main lineages: OCT4 (epiblast marker), CDX2 (trophectoderm marker), and SOX17 (primitive endoderm marker). The timing of ZGA is indicated for each animal. Abbreviations: EPI, epiblast; ICM, inner cell mass; PrE, primitive endoderm; TE, trophectoderm; ZGA, zygotic genome activation.

to the tensile forces required to change cell shape during compaction (Maitre et al. 2015, 2016). However, how compaction is initiated remains unclear.

Eight-cell stage blastomeres establish a radial polarity that is characterized by the clustering of microvilli and the Par3-Par6-aPKC complex at the apical domain (Louvet et al. 1996). By contrast, E-cadherin, Par-1, and Na^+/K^+–ATPase become restricted to the basolateral domain (Vestweber et al. 1987, Vinot et al. 2005, Watson & Kidder 1988). Following compaction, microlumens are formed by the exocytosis of vesicles from the basal membrane of the outer cells (Aziz & Alexandre 1991). Sodium ions are actively transported across the outer cell layer through transmembrane channels (Watson & Barcroft 2001). This generates an osmotic gradient that

enables fluid to be pumped into the cavities (Barcroft et al. 2003), resulting in the coalescence of the microlumens into a single blastocoel cavity. To support expansion of the cavity, the embryo creates a paracellular permeability barrier that resists the increasing hydrostatic pressure and seals the outer cells by the maturation of tight junctions (Zenker et al. 2018). Increasing luminal pressure during cavitation induces tight junction maturation, thus influencing blastocoel size, cell shape, and division (Chan et al. 2019). Hydraulic fracturing of cell–cell contacts followed by fusion of the microlumens may define blastocoel positioning and therefore the first axis of the mouse embryo (Dumortier et al. 2019). It will be interesting to determine how mechanical forces are sensed by the cell and what impact they have on the expression of genes regulating cell fate.

To implant into the uterus, the mouse embryo hatches out of a glycoprotein shell called the zona pellucida (ZP) at 4 dpf (Cockburn & Rossant 2010). The emergence of the embryo from the ZP is a poorly understood process. It may be regulated by the mechanical pressure induced by the expanding blastocoel cavity, by dynamic cellular trophectodermal projections, by proteases, and by molecular signaling factors released from both the blastocyst and the maternal endometrium (Seshagiri et al. 2009).

2.1.1.2. Humans. In the human embryo, the earliest signs of polarization appear around the eight-cell stage. The basolateral localization of E-cadherin and the presence of gap junctions and apical microvilli have been observed in human eight-cell and morula-stage embryos (Nikas et al. 1996). Polarization and the establishment of cell–cell communication by tight and adherens junctions are highly conserved mechanisms among phyla. Therefore, PAR-complex activity, cytoskeleton interactions, and cell–cell communication are likely to be conserved in human embryos, but this has yet to be fully elucidated.

Human blastocysts express proteins associated with tight junction and desmosome formation, such as ZO-1 and occludins, as well as connexin gap junctions (Bloor et al. 2004, Ghassemifar et al. 2003). To implant, the expanded blastocyst hatches out of the ZP and attaches to the uterine wall at around 7–10 dpf (Wilcox et al. 1999). Failure to hatch may explain the low success rate of in vitro fertilization (IVF) embryos (Fong et al. 2001). Assisted hatching is therefore offered as a means of improving embryo implantation rates (Hammadeh et al. 2011). A better understanding of the molecular mechanisms underlying human blastocyst initiation, expansion, and hatching may improve IVF outcomes.

Time-lapse imaging of developing human embryos combined with one-step embryo culture have allowed the correlation of blastocyst development potential with morphokinetic parameters, such as blastomere size and length of cell division (Wong et al. 2010). Further insights can also be gained by live imaging. For example, are microlumens and hydraulic fractures involved in cavitation in human embryos? What are the mechanical forces involved in the compaction of these embryos, and is this process mediated by filopodia?

2.1.1.3. Other eutherians. Our understanding of nonhuman primate embryogenesis is limited to a few species: the rhesus monkey, the cynomolgus monkey, and the marmoset. These nonhuman primates diverged from the line that led to humans 25–40 MYA (**Figure 1**). Rhesus monkey embryos undergo compaction at 16–32 cells (4 dpf), followed by cavitation one day later, and implantation at 9–10 dpf (Boroviak & Nichols 2017). Similar timing has been observed in the marmoset embryo, with implantation at 11–12 dpf (Moore et al. 1985).

In other eutherians, the timing of compaction, blastomere polarization, and cavitation differs between species. For example, the bovine embryo reaches the morula stage at 5–6 dpf at 16–32 cells, while cavitation takes place at 7–8 dpf (Van Soom et al. 1997). Similar timing has been observed in pig embryos, in which the morula is formed around 4–5 dpf and the blastocyst around

6–7 dpf (Perry & Rowlands 1962). In the rabbit, a compacted morula composed of 32–64 cells is evident at 2 dpf, while cavitation takes places at 3 dpf (Ziomek et al. 1990). It is currently unclear whether the same proteins involved in tight and adherens junction formation and polarization are expressed in these species.

Interestingly, differences in trophectoderm (TE) proliferation and morphology before implantation reflect differences in the implantation and placentation processes in eutherian species (Bazer et al. 2009). Ungulate preimplantation development extends beyond blastocyst hatching. Embryo development in these species continues with the formation of a highly elongated blastocyst with a relatively small embryonic disc (Betteridge 1989, Stroband & Van der Lende 1990). In nonungulate species, such as the rabbit, the blastocyst continues to proliferate and grow in size before hatching (Denker 2000). At the time of implantation, the polar TE cells in contact with the epiblast (EPI) (Rauber's layer) disappear in species such as rabbit, cow, and pig. The EPI is therefore exposed, and only the mural TE is involved in implantation and subsequent placental development (Maddox-Hyttel et al. 2003, Sun et al. 2015, Williams & Biggers 1990). The mechanisms that underlie the disappearance of the polar TE and the subsequent exposure of the EPI are poorly understood, and it is also unclear if the EPI at this stage has undergone molecular changes as a consequence of differences in cell–cell contacts.

2.1.2. Metatherians. Early metatherian embryos show important differences in morphology when compared with eutherians. As in eutherians, the ZP surrounds the metatherian early embryo. However, two more enveloping layers coat the zygote: a mucoid layer and a shell, which may prevent polyspermy. Different regions of the oviduct and uterus secrete the glycoprotein constituents of the mucoid layer and shell as the zygote rolls down from the oviduct (Selwood 2000). Another evident feature in a metatherian zygote is the presence of a deutoplasm, a secondary cytoplasm that includes yolk bodies and vesicles (Frankenberg 2018). In many species, the position of the deutoplasm is polarized relative to the pronuclei of the zygote (Hill 1910, Selwood 1992). This organization defines an embryonic–abembryonic axis at the onset of development. During cleavage divisions, the deutoplasm is eliminated, along with small vesicles from blastomeres, into the extracellular space in a process called deutoplasmolysis (Frankenberg & Selwood 1998). Blastomeres are initially located in the embryonic pole and are attached to the ZP. The cells lining the ZP continue to divide without acquiring an inner position and therefore a compacted morula is not formed. The next steps vary depending on the species, but, in general, blastomeres divide symmetrically and spread toward the abembryonic pole until a unilaminar blastocyst is formed that is characterized by flattened cells along the ZP (Hill 1910, Selwood 1992). Following blastocyst expansion, around embryonic day (E) 7 in the opossum and E9 in the tammar wallaby, another cell layer is formed underneath the cells at the embryonic pole, which gives rise to a bilaminar blastocyst (Kress & Selwood 2006, Selwood 1992). As the blastocyst expands, the mucoid coat and the ZP are compressed and disappear. However, the external shell persists and breaks down only once somitogenesis has begun (Selwood 2000). Breakdown of the shell is presumably due to high proteinase activity at the interface between the trophoblast and the uterine epithelium (Denker & Tyndale-Biscoe 1986). Only then, around E11 in the opossum and E21 in the tammar wallaby, are the maternal endometrium and placenta in direct contact (Denker & Tyndale-Biscoe 1986, Mate et al. 1994).

2.2. Zygotic Genome Activation

The earliest stages of mammalian embryonic development progress in the absence of active transcription and rely on maternal messenger RNAs (mRNAs) and proteins deposited in the cytoplasm

of the oocyte. Transcriptional control is then passed to the embryo through a process known as the maternal-to-zygotic transition (MZT), during which the degradation of maternal products is coordinated with zygotic genome activation (ZGA), also referred to as embryonic genome activation. ZGA is critical for development beyond the early cleavage divisions. This process is conserved across animals, but the timing varies between species (Vastenhouw et al. 2019). Our understanding of the mechanisms underlying this crucial developmental milestone is still very limited in mammals, but such mechanisms have been extensively studied in other vertebrate organisms such as zebra fish (Giraldez et al. 2006).

2.2.1. Eutherians. This section discusses ZGA, or embryonic genome activation, in rodents, humans, and other eutherians.

2.2.1.1. Rodents. In the mouse, ZGA occurs between the early and late two-cell stages (Aoki et al. 1997). Similarly, in other rodents, such as the rat (Zernicka-Goetz 1994) and the hamster (Seshagiri et al. 1992), ZGA initiates at the two-cell stage. The double-homeobox (DUX) transcription factor family may be involved in ZGA (Hendrickson et al. 2017). DUX is restricted to the two-cell stage in mouse embryos and, when ectopically expressed in mouse embryonic stem cells (mESCs), induces an open chromatin state and the activation of retroviral elements as in two-cell-stage embryos (Hendrickson et al. 2017). The importance of the reactivation of transposable elements during this critical transcriptional transition in rodents and other species is currently unclear. Using the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 genome editing system (Jinek et al. 2012), recent studies (Chen & Zhang 2019, De Iaco et al. 2020) have shown that despite an impairment in the activation of a small set of ZGA-associated genes, *Dux* is dispensable for mouse development. These results highlight the differences between two-cell mouse embryos and two-cell-like ESCs and suggest that other genes contribute to this transition in vivo. Just how *Dux* expression is initiated in mouse embryos remains unclear, although work in mESCs suggests that the maternally contributed DNA-binding factors DPPA2 and DPPA4 may be important (De Iaco et al. 2019, Eckersley-Maslin et al. 2019). A high-throughput screening method combining CRISPR activation with single-cell transcriptomics in mESCs has identified DPPA2 and the chromatin-remodeling factor SMARCA5 as putative regulators of ZGA (Alda-Catalinas et al. 2020), which warrant further investigation.

2.2.1.2. Humans. In humans, ZGA occurs between the four- and eight-cell stages (Braude et al. 1988, Tesarik et al. 1987). Various genes and transposable elements are upregulated coincident with ZGA (Grow et al. 2015, Töhönen et al. 2015). How ZGA is initiated in humans is unclear but could involve DUX4, the human ortholog of *DUX* (Hendrickson et al. 2017). DUX4 mRNA and protein expression is restricted to the nucleus at the four-cell stage, and overexpression in human induced pluripotent stem cells (iPSCs) or human embryonic stem cells (hESCs) activates ZGA-associated genes. Interestingly, low-input chromatin accessibility and transcriptome sequencing (LiCAT-seq) assays have revealed activation from the four-cell stage of a large proportion of genes and endogenous retroviruses that possess *DUX4*-binding sites (Liu et al. 2019). Moreover, the same study has shown that a number of pluripotency-associated transcription factors, such as *POU5F1* (the gene encoding OCT4) and *SOX2*, are also expressed early during ZGA and may regulate aspects of this process. Consistent with this, DNase I hypersensitive site sequencing (DNase-seq) has shown enrichment of OCT4-binding sites coincident with ZGA in human embryos (Gao et al. 2018). Small interfering RNA (siRNA) knockdown of OCT4 leads to the downregulation of ZGA-expressed genes, suggesting a role for OCT4 in regulating human ZGA.

Chromatin profiling has demonstrated that in human zygotes, CpG-rich regulatory regions initially exhibit widespread open chromatin and enrichment in the permissive mark, trimethylated histone H3 lysine 4 (H3K4me3). Upon ZGA, regulatory regions controlling basic biological processes such as translation and blastocyst development resolve into an active state, while those controlling later development acquire the repressive mark H3K27me3 (Wu et al. 2018, Xia et al. 2019). Understanding the molecular mechanisms that regulate human ZGA may lead to a greater understanding of why the majority of human preimplantation embryos resulting after assisted reproductive technologies arrest between the four- and eight-cell stages (Wong et al. 2010).

2.2.1.3. Other eutherians. Transcriptome analysis suggests that ZGA occurs between the four- and eight-cell stages in marmosets and rhesus monkeys (Boroviak et al. 2018, Wang et al. 2017). In cows and sheep, ZGA occurs between the 8- and 16-cell stages (Crosby et al. 1988, Meirelles et al. 2004), while in pigs and rabbits it occurs at the 4-cell stage (Kanka 2003, Maddox-Hyttel et al. 2001). Similarly to mice and humans, chromatin accessibility in bovine embryos is established gradually throughout preimplantation development (Halstead et al. 2020). The analysis of binding motifs in accessible intergenic loci in the four- to eight-cell stages pointed to NF-κB family members and DUX factors as candidate bovine ZGA-regulating factors. Functional studies will be important to determine the role of putative regulators of ZGA in eutherians and to uncover conserved programs that drive the MZT.

2.2.2. Metatherians. In marsupials, little is known about the process of ZGA. Transcriptome analysis indicates that in the gray short-tailed opossum ZGA occurs around the eight-cell stage (Mahadevaiah et al. 2020). It will be interesting to investigate if transposable elements are activated, similar to what has been described in eutherians. In vitro culture conditions for metatherian embryos have not been efficiently developed, precluding the use of transcription-inhibition experiments to independently determine the time of ZGA in these mammals.

2.3. Cell Fate Specification

Mammalian preimplantation development results in the formation of a blastocyst with three distinct cell lineages. Significant progress has been made in our understanding of the mechanisms regulating the formation of these three lineages, principally from studies in the mouse. In the mouse, the first lineage segregation event forms the TE, which becomes distinct from the inner cell mass (ICM). Subsequently, ICM cells segregate into the pluripotent EPI, or primitive endoderm (PrE, also known as the hypoblast) (**Figure 2**). Only the EPI contributes to the embryo proper, while the two extraembryonic lineages, the TE and the PrE, contribute to the placenta and yolk sac, respectively. It is unclear whether this stepwise process of lineage segregation is conserved in other species, and which mechanisms underlie preimplantation cell fate specification.

2.3.1. Eutherians. This section covers cell fate specification in rodents, humans, and other eutherians.

2.3.1.1. Rodents. In the mouse, the establishment of inner and outer cell populations with different morphological asymmetries in terms of polarity and cell–cell contacts occurs at the morula stage and is required to trigger the first lineage-specific transcriptional programs (Korotkevich et al. 2017, Stephenson et al. 2010). Each cell relies on the balance between cell adhesion and polarity to regulate the activity of the Hippo signaling pathway and, consequently, the phosphorylation of yes-associated protein 1 (YAP1) (Sasaki 2017). In the outer cells of the embryo, unphosphorylated YAP1 translocates into the nucleus and interacts with the transcription factor

TEAD4 to promote the expression of TE-associated transcription factors, including *Cdx2* and *Gata3* (Nishioka et al. 2009, Ralston et al. 2010). YAP1 also represses the expression of *Sox2* as cells undergo TE initiation (Frum et al. 2018, Wicklow et al. 2014). In the inner cells, the activation of Hippo signaling leads to the phosphorylation of YAP1 by the kinase LATS1/2 (Frum et al. 2019). Phosphorylated YAP1 is excluded from the nucleus, preventing the induction of TE gene expression (Nishioka et al. 2009). Notch signaling, which acts in parallel to Hippo signaling, is activated heterogeneously between cells as early as the four-cell stage and triggers the onset of a TE program, including *Cdx2* expression in the early morula (Menchero et al. 2019, Rayon et al. 2014).

Until the early blastocyst stage, the mouse ICM is a heterogeneous population of cells coexpressing different levels of lineage-associated factors, including NANOG (EPI) and GATA6 (PrE) (Chazaud et al. 2006, Dietrich & Hiiragi 2007). FGF/FGF-receptor signaling, mediated via downstream MEK/ERK, underlies the subsequent divergence between EPI and PrE cells (Guo et al. 2010, Yamanaka et al. 2010). NANOG-expressing EPI precursors secrete FGF4, which binds to FGF receptors to reinforce a PrE program (Lanner & Rossant 2010). Heterogeneity in the expression of the FGF ligand and cognate receptor may be initiated by variations in SOX2/OCT4-dependent *Fgf4* transcription (Mistri et al. 2018). Different combinations of FGF receptor 1 and 2 in cells biased to differentiate into the EPI or PrE lead to distinct signaling transduction within the ICM (Kang et al. 2017, Molotkov et al. 2017). How a PrE program is initiated independently of FGF signaling, and how FGF-mediated feedforward and feedback loops lead to divergence of PrE and EPI cells, is unclear.

2.3.1.2. Humans. In the human embryo, it is unclear if differences in cell polarity drive the divergence of the first cell fate decision through the Hippo pathway. The nuclear expression of YAP1 has been detected in the early blastocyst in human embryos and is restricted to the TE in the late blastocyst (6 dpf) (Noli et al. 2015), but earlier expression has not yet been determined. Transcriptional differences are detectable once the blastocyst has formed at 5 dpf, suggesting that the mechanisms of divergence of early cell lineages may be distinct from the stepwise process in the mouse (Petropoulos et al. 2016). Interestingly, CDX2 is only detected in the human blastocyst after cavitation (Chen et al. 2009, Niakan & Eggan 2013), which may indicate an important distinction in the development of the human TE when compared with that of the mouse. GATA3 is also detected in the TE of the human blastocyst (Deglincerti et al. 2016). However, as for most factors, both a detailed time-course analysis of protein expression and functional studies have yet to be performed.

Among the few factors that have been characterized, NANOG is specifically expressed in the human EPI (Cauffman et al. 2009, Hyslop et al. 2005), suggesting that it may have a role in this cell type. OCT4 is initially expressed uniformly in all blastomeres from the eight-cell stage, similar to the mouse, and its expression persists in TE cells until the late blastocyst stage (Chen et al. 2009, Niakan & Eggan 2013). Moreover, OCT4 CRISPR-Cas9-targeted human embryos are compromised in blastocyst formation and, surprisingly, exhibit downregulation of genes associated with all three lineages (Fogarty et al. 2017). Many TE-associated genes and proteins are not expressed in OCT4-targeted embryos, suggesting that OCT4 either directly or indirectly positively regulates a TE program. By contrast, *OCT4*-null mouse embryos exhibit ectopic TE gene expression, suggesting that it is a negative regulator of the TE program in this species (Frum et al. 2013, Nichols et al. 1998). It will be important to further elucidate the extent to which molecular mechanisms driving embryogenesis differ between humans and other eutherians.

GATA6 is initially expressed early and broadly in early human blastocysts (Deglincerti et al. 2016, Roode et al. 2012), while SOX17 and GATA4 are expressed later and are more restricted to the PrE (Niakan & Eggan 2013, Roode et al. 2012), an expression pattern that is similar

Table 1 Conserved and species-specific markers of the first lineages specified in the mammalian embryo between mouse and human

Lineage	Gene/pathway	Expression		Reported function in mouse	References
		Mouse	Human		
TE	CDX2	✓	✓	Induces TE fate	Niakan & Eggan 2013, Strumpf et al. 2005
	GATA3	✓	✓	Induces TE fate	Deglincerti et al. 2016, Ralston et al. 2010
	YAP1	✓	✓	Activates CDX2 and GATA3. Induces TE fate	Nishioka et al. 2009, Noli et al. 2015
	NOTCH	✓	Unknown	Activates CDX2. Induces TE fate	Menchero et al. 2019, Rayon et al. 2014
	EOMES	✓	✗	TE maintenance and differentiation	Blakeley et al. 2015, Russ et al. 2000
	ELF5	✓	✗	TE maintenance	Blakeley et al. 2015, Ng et al. 2008
	PLAC8	✗	✓	Unknown	Blakeley et al. 2015
	CLDN10	✗	✓	Unknown	Blakeley et al. 2015
	ID2	✓	PrE-linked	Unknown	Blakeley et al. 2015, Guo et al. 2010
EPI	OCT4	✓	✓	Pluripotency maintenance	Niakan & Eggan 2013, Nichols et al. 1998
	SOX2	✓	✓	Pluripotency maintenance	Avilion et al. 2003, Cauffman et al. 2009
	NANOG	✓	✓	Pluripotency maintenance	Cauffman et al. 2009, Chambers et al. 2003, Mitsui et al. 2003
	KLF17	✗	✓	Unknown	Blakeley et al. 2015
	ESRRB	✓	✗	Pluripotency maintenance	Blakeley et al. 2015, Zhang et al. 2008
	KLF2	✓	✗	Pluripotency maintenance	Blakeley et al. 2015, Yeo et al. 2014
PrE	GATA6	✓	✓	Induces PrE fate	Chazaud et al. 2006, Roode et al. 2012
	GATA4	✓	✓	PrE maintenance	Plusa et al. 2008, Roode et al. 2012
	SOX17	✓	✓	PrE maintenance	Artus et al. 2011, Niakan & Eggan 2013, Roode et al. 2012
	FGF/ERK	✓	✓	Induces PrE fate	Petropoulos et al. 2016, Yamanaka et al. 2010

to that in the mouse. A detailed time-course analysis of NANOG, GATA6, and GATA4 has not been reported in humans. This would be informative to characterize whether there is a similar so-called salt-and-pepper expression of EPI- and PrE-associated proteins before lineage segregation. While some members of the FGF signaling pathway are transcribed in the human embryo (Petropoulos et al. 2016), FGF receptor or downstream MEK/ERK inhibition in human embryos does not appear to affect EPI and PrE segregation (Kuijk et al. 2012, Roode et al. 2012), unlike in the mouse embryo (Yamanaka et al. 2010). Thus, distinct and as-yet-uncharacterized mechanisms likely regulate the second cell fate–specification mechanism in human embryos. Beyond the conserved expression of these key lineage markers, some factors have been found to be enriched in certain lineages in a species-specific manner. Examples, including PLAC8 in the TE or KLF17 in the EPI of human but not mouse embryos (Blakeley et al. 2015), are detailed in **Table 1**.

2.3.1.3. Other eutherians. The specification of the TE program has not been completely elucidated in other eutherians, but the data point to more similarities with the human than with the mouse. In the cynomolgus monkey, pig, cow, and rabbit, CDX2 is detected in outer TE cells only once the blastocyst is formed (Bou et al. 2017, Chen et al. 2012, Kuijk et al. 2008, Nakamura et al. 2016). CDX2 may therefore be more important for TE maintenance than for initiation more broadly across eutherians. Other TE-associated factors, such as *GATA2* and *GATA3*, appear in the TE of the early pig (Ramos-Ibeas et al. 2019) and cynomolgus monkey blastocysts (Nakamura et al. 2016), but earlier expression has not yet been determined. This opens the question as to which mechanisms regulate TE initiation, and whether these mechanisms are conserved or divergent when compared with those in the mouse. YAP1 is expressed in the nucleus of outer cells from the morula stage in porcine and bovine embryos (Cao et al. 2014, 2019; Negrón-Pérez & Hansen 2018), while Notch activity has not been reported at these stages in any other mammal so far. Although direct regulation of *CDX2* and *GATA3* by the Hippo pathway has not been examined in these species, the nuclear expression of YAP1 suggests that it may have a conserved role. It is unclear why CDX2 expression is delayed until the blastocyst stage in most eutherians, and whether the earlier expression in the mouse reflects a distinct TE program.

In various eutherians, including cynomolgus monkey, pig, cow, and rabbit, OCT4 expression is present in all cells after ZGA and becomes restricted to EPI cells only in late blastocysts (Cao et al. 2014, Kobolak et al. 2009, Madeja et al. 2013, Nakamura et al. 2016). OCT4 is often shown to be coincidentally expressed with CDX2 in the TE of mid to late blastocysts compared with the earlier restriction in the mouse, and some evidence, particularly in the cow, suggests that the mutual inhibition shown in the mouse (Niwa et al. 2005) may not be conserved evolutionarily in other nonrodent eutherians. *OCT4*-null mutant cow embryos show impaired blastocyst formation and downregulation of TE protein expression (Daigneault et al. 2018), recapitulating the phenotype observed in humans (Fogarty et al. 2017). Moreover, detailed analysis of the *cis*-regulatory region of the cow *OCT4* locus reveals evolutionarily divergent mechanisms in gene regulation compared with those in the mouse (Berg et al. 2011). SOX2 follows similar dynamics to those of OCT4, where it is initially expressed in all cells in the cow morula and eventually becomes restricted to the EPI cells in the late blastocyst (Goissis & Cibelli 2014). Given the role of Hippo signaling in restricting the expression of SOX2 to the inner cells of the mouse morula (Frum et al. 2019), the broader expression observed in the cow may have implications for understanding the mechanisms regulating EPI establishment in other eutherians.

Similar to humans and mice, in cynomolgus monkey, marmoset, pig, and cow embryos NANOG and GATA6 are initially coexpressed and resolve into mutually exclusive EPI and PrE segregation in late blastocysts (Boroviak et al. 2015, Kuijk et al. 2008, Nakamura et al. 2016, Ramos-Ibeas et al. 2019). SOX17 expression is initiated at the morula stage in cow embryos and is eventually restricted to the PrE in late blastocysts (Canizo et al. 2019). While exogenous FGF4 treatment of cow embryos results in exclusive GATA6 expression in the ICM, the inhibition of MEK/ERK signaling does not abolish GATA6 expression (Kuijk et al. 2008), highlighting potentially important differences in the regulation of EPI and PrE divergence between species that require further investigation. In the presence of a MEK inhibitor, pig embryos exhibit downregulation of SOX17 in the PrE but do not ectopically express NANOG (Ramos-Ibeas et al. 2019). Unlike the situation in the mouse, it is therefore unclear which mechanisms regulate the second lineage decision in eutherians. This question is important for understanding embryogenesis, and its answer may inform pluripotency regulation with benefits for stem cell biology.

2.3.2. Metatherians. As in eutherians, metatherians establish three cell populations at the blastocyst stage. Given that all the cells forming the unilaminar blastocyst in marsupials are facing the ZP (with no inner cells) and have the same cell–cell contacts, a key question is how the cells sense their position to acquire a specific fate. One possibility is that maternal determinants are asymmetrically deposited in the oocyte or during early cleavage stages and serve as a positional signal. Blastomeres that spread toward the abembryonic pole may acquire a trophoblast fate, while blastomeres at the embryonic pole remain in an undifferentiated state called the pluriblast, which is equivalent to the eutherian ICM. The pluriblast gives rise to the EPI and PrE in the late blastocyst.

At the molecular level, several studies (Frankenberg et al. 2013, Morrison et al. 2013) have shown that key factors important for the specification of the first lineages in eutherians are conserved and expressed in the metatherian embryo despite some differences in expression patterns. In the tammar wallaby, OCT4, SOX2, CDX2, YAP1, and GATA6 are coexpressed in the nuclei of all cells in cleavage-stage embryos and in the unilaminar blastocyst (Frankenberg et al. 2013). The first differences in their domain of expression appear when the PrE cells emerge. OCT4 is more restricted to the embryonic pole than to the putative trophoblast, and GATA6 nuclear expression is observed in putative PrE precursors. At this stage, differences in Hippo signaling are also observed. YAP1 is localized in the nucleus of the EPI cells in the tammar wallaby, which is surprisingly distinct compared with the expression pattern in the mouse, while WWTR1, another cofactor of the pathway, is specifically localized in the nuclei of the TE (Frankenberg et al. 2013). Whether this distinction is functionally important and whether the expression patterns observed are conserved in other metatherian species are not yet known.

Single cell transcriptomic analysis of the gray short-tailed opossum indicates that transcriptional differences between lineages are not discernible until the late unilaminar blastocyst stage (Mahadevaiah et al. 2020). For example, GATA6 and POU5F3, an OCT4 paralog that is later specifically expressed in the EPI (Frankenberg et al. 2013), are coexpressed in all cells of the unilaminar blastocyst (E5.5), but their expression becomes restricted to different domains in the embryonic pole by E6.5. Similarly, TE-related genes such as TEAD4 are only detected from E6.5, and their expression does not overlap with GATA6-positive cells. The EPI, TE, and PrE lineages may be formed simultaneously in this species. Alternatively, an EPI–PrE pluriblast precursor may exist, albeit transiently. Altogether this suggests that the putative asymmetries between the embryonic and abembryonic poles are unlikely to trigger the differentiation of the first lineages, because all cells are transcriptionally equivalent until a relatively late stage (Mahadevaiah et al. 2020). Future studies addressing the emergence of polarity cues will be informative. Altogether, the comparison of key lineage markers and functional studies in different eutherian and metatherian species has provided a better idea of the conserved and divergent mechanisms driving cell fate specification events in the early mammalian embryo and warrants further investigation.

2.4. X-Chromosome Inactivation

In therians, sex chromosomes differ between the sexes, with males being heterogametic (XY) and females homogametic (XX). To avoid a disequilibrium in the dosage of X-chromosome genes, mammals developed a mechanism to silence one of the two X chromosomes in females (Lyon 1961). This mechanism, X-chromosome inactivation (XCI), takes place during early embryo development. Although the general goal is conserved, the strategy and molecular players involved vary between species.

2.4.1. Eutherians. This section discusses X-chromosome inactivation in rodents as well as humans and other eutherians.

2.4.1.1. Rodents. XCI takes place in all eutherians and is mediated by the long noncoding RNA (lncRNA) *Xist* (Brown et al. 1991, Marahrens et al. 1997, Penny et al. 1996). *Xist* acts in *cis* by coating and silencing the chromosome from which it is expressed (Clemson et al. 1996). XCI in adults is random, with either the paternal or the maternal X chromosome silenced.

Most studies on XCI have used the mouse model system. Unlike other eutherians, the mouse exhibits imprinted inactivation of the paternal X chromosome during preimplantation development, with gene silencing initiated at the four-cell stage (Okamoto et al. 2004). At the blastocyst stage, the paternal X chromosome is reactivated in the EPI, after which random XCI occurs (Mak et al. 2004, Okamoto et al. 2004). In contrast, imprinted XCI is maintained in the extraembryonic tissues (Takagi & Sasaki 1975). Imprinted XCI is achieved through the deposition of H3K27me3 on the mouse *Xist* locus during oogenesis. This repressive mark prevents maternal *Xist* expression in daughters, thus ensuring exclusive *Xist* expression from the paternal X chromosome (Inoue et al. 2017). During later development, imprinted XCI is maintained in extraembryonic tissues by *Tsix*, a noncoding RNA transcribed antisense to *Xist* (Lee et al. 1999, Sado et al. 2001).

2.4.1.2. Humans and other eutherians. XCI exhibits distinct properties between the mouse and other eutherians. Imprinted XCI does not occur in other eutherians studied to date (Okamoto et al. 2011), and *TSIX* is either truncated or absent in these species (Chang & Brown 2010, Migeon et al. 2001). Furthermore, relative to mice, XCI initiates later, from the blastocyst stage in rabbits (Okamoto et al. 2011) and pigs (Ramos-Ibeas et al. 2019) and from the blastocyst and early elongation stages in cows (Bermejo-Alvarez et al. 2011). In humans, XCI has been studied in detail (Briggs et al. 2015, Okamoto et al. 2011, Petropoulos et al. 2016), and *XIST* exhibits unusual expression dynamics. *XIST* is expressed from the four- to eight-cell stage, and unlike in the mouse, it is transcribed in both sexes. In females, *XIST* initially coats both X chromosomes, but this does not lead to immediate XCI. Instead, X-chromosome gene silencing occurs over a more protracted period. A minority of genes are inactivated from the eight-cell stage, while others are inactivated between the morula and blastocyst stages (de Mello et al. 2017). Even in the blastocyst, some X genes remain biallelically expressed despite the presence of *XIST* coating, an observation not seen in the mouse (Okamoto et al. 2011, Petropoulos et al. 2016). *XIST*-mediated silencing in human embryos may be antagonized by another lncRNA, *XACT*. *XACT* is observed only in hominoids, is coexpressed with *XIST* from both X chromosomes prior to XCI, and is subsequently downregulated (Casanova et al. 2019, Vallot et al. 2017).

Recently developed human implantation models (Deglincerti et al. 2016, Shahbazi et al. 2016) have enabled the ontogeny of XCI to be examined later in development (Zhou et al. 2019). This work has revealed that XCI is unsynchronized between the three main lineages. A higher proportion of TE-derived cells has undergone XCI as compared with EPI- and PrE-derived cells during the peri-implantation window (Zhou et al. 2019). In conclusion, the emerging picture is that XCI occurs relatively late in most eutherians, with the mouse being the exception to the rule.

2.4.2. Metatherians. In contrast to eutherians, XCI in metatherians is imprinted in all tissues (Sharman 1971). Furthermore, the *XIST* gene is absent. An alternative X-encoded lncRNA called *RSX* is thought to mediate XCI in these species (Grant et al. 2012, Sprague et al. 2019). *RSX* and *XIST* share little sequence homology and are located in different syntenic blocks, suggesting that they emerged independently following the eutherian–metatherian divergence.

In the opossum, *RSX* is expressed from the paternal X chromosome simultaneous with ZGA at the 8-cell stage, and imprinted XCI is completed by the 16-cell stage (Mahadevaiah et al. 2020). This scenario is reminiscent of that in the mouse, in which imprinted XCI is associated with *Xist* expression soon after ZGA. In humans, the initial biallelic expression of *XIST* is reversed in

the late blastocyst, where *XIST* coats only one X chromosome (Okamoto et al. 2011). It is not known if cells with biallelic XCI are counter-selected or if one X chromosome is reactivated in those cells. In the mouse and opossum, XCI occurs very early, and biallelic XCI at this point in development may severely compromise viability. Therefore, imprinted XCI may have evolved to avoid the detrimental effects of biallelic X-chromosome silencing in these species (Okamoto et al. 2011).

How imprinted XCI is regulated in metatherians is unknown. One candidate is *XSR*, an *RSX*-antisense lncRNA that is expressed only from the maternal X chromosome. *XSR* could antagonize *RSX* expression in a manner reminiscent of the *Tsix/Xist* paradigm (Mahadevaiah et al. 2020). The mechanisms regulating imprinted XCI in mice and marsupials may therefore have evolved convergently.

3. POSTIMPLANTATION DEVELOPMENT AND STEM CELL MODELS OF EMBRYOGENESIS

Ongoing development of the mammalian embryo is dependent on uterine implantation. Implantation has been defined as the so-called black box of human development because for practical and ethical reasons very little of this process is understood. In humans, there is limited access to fetal material at early stages, that is, within the first month of development, when key developmental processes occur. During this period, a complex program of cell fate decisions [e.g., three germ layers and primordial germ cell (PGC) specification] and critical morphogenetic changes in the embryo establish the formation of the embryo proper and extraembryonic tissues.

To further understand postimplantation development, efforts have been made primarily by two methods. The first is developing stem cell models to recapitulate postimplantation embryos, while the second is developing in vitro culture conditions to allow intact embryos to mimic implantation and progress in embryogenesis (**Figure 3**).

3.1. Eutherians

This section covers postimplantation development plus models of embryogenesis using stem cells in rodents, humans, and other eutherians.

3.1.1. Rodents. The mouse blastocyst implants at around 5 dpf (Wang & Dey 2006). After implantation, the EPI transforms into a cup-shaped epithelial tissue around an emerging lumen that is termed the proamniotic cavity (Coucouvanis & Martin 1995). The polar TE forms the extraembryonic ectoderm, which gives rise to the fetal portion of the placenta, forming structures such as the ectoplacental cone. The PrE forms two cell layers, the parietal and visceral endoderm, which surround the EPI and extraembryonic ectoderm (Hogan et al. 1980, Rossant 1995) and eventually give rise to the yolk sac. Different culture methods have been developed that show the intrinsic ability of mouse embryos to undergo limited postimplantation development in vitro (Hsu 1971, Jenkinson & Wilson 1970, Tam 1998). Coupling these methodologies with time-lapse imaging has revealed the morphogenetic events of the peri-implantation process: EPI polarization, rosette formation, and lumenogenesis (Bedzhov & Zernicka-Goetz 2014). The mural TE mediates implantation, and both the polar and mural TE give rise to trophoblast giant cells by endoreduplication. At the time of gastrulation, the amnion and yolk sac are formed. The amnion is a membrane surrounding the fetus that defines a fluid-filled cavity providing a protective niche, while the yolk sac is a blood supply source during development (Pereira et al. 2011). At E6.25, signals from both extraembryonic and embryonic tissues result in BMP/Wnt/Nodal/FGF

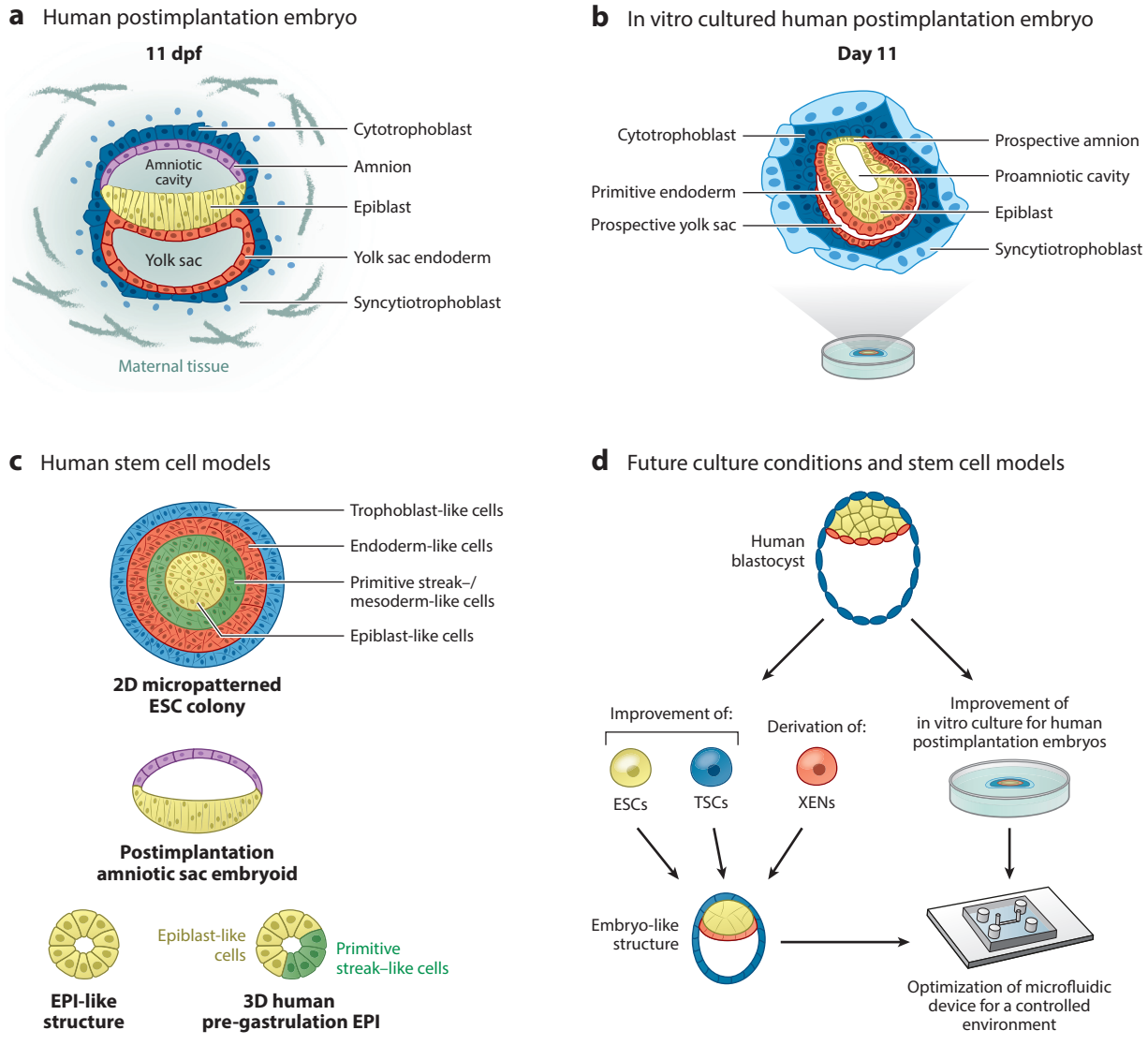

Figure 3

Postimplantation human embryos, current human stem cell models, and future perspectives. (*a*) Schematic representation of a human postimplantation embryo at 11 dpf based on the Carnegie and Boyd collection. (*b*) Representation of an in vitro cultured human postimplantation embryo at day 11. (*c*) Illustrations of stem cell models of human embryos. Panels *a–c* are redrawn with permission from Shahbazi et al. (2016) and Shahbazi & Zernicka-Goetz (2018). (*d*) Schematic representation of future postimplantation culture conditions and stem cell models of human embryos. Abbreviations: 2D, two dimensional; 3D, three dimensional; dpf, days postfertilization; EPI, epiblast; ESC, embryonic stem cell; TSC, trophoblast stem cell; XEN, extraembryonic endoderm cells.

signaling convergence, driving posterior EPI cells to undergo the epithelial-to-mesenchymal transition (EMT) to form the primitive streak (Arnold & Robertson 2009, Tam & Loebel 2007).

For decades, mESCs have been tested for their ability to recapitulate embryogenesis. mESCs can form cell culture–derived three-dimensional (3D) embryoid bodies (EBs) (Doetschman et al. 1985) and have been used to understand the mechanisms regulating key developmental processes

such as lumenogenesis (Coucouvanis & Martin 1995). EBs have also been used to derive organ-like structures called organoids (Clevers 2016, Eiraku et al. 2011) and embryo-like structures named embryoids (Fuchs et al. 2012, Marikawa et al. 2009, van den Brink et al. 2014). These models are characterized by spontaneous symmetry-breaking events that lead to the establishment of gene expression domains reminiscent of germ layer specification (Simunovic & Brivanlou 2017). Recent improvements have shown that culturing mESCs in 3D matrices can also recapitulate EPI cell polarization, rosette formation, and lumenogenesis (Bedzhov & Zernicka-Goetz 2014, Shahbazi et al. 2017).

As well as preimplantation ESCs, stem cells closely resembling the postimplantation EPI have been derived. These cells acquire a transitory 5.5-dpf-like pluripotent state and are defined as EPI stem cells (EpiSCs) (Brons et al. 2007, Tesar et al. 2007). Moreover, self-renewing stem cell lines have been derived from the extraembryonic tissues of embryos. Trophoblast stem cells (TSCs) derived from mouse blastocysts represent the extraembryonic ectoderm (Tanaka et al. 1998). Extraembryonic endoderm (XEN) cells have also been derived and represent the stem cell population of the PrE (Kunath et al. 2005). Coculturing of ESCs and TSCs in a 3D matrix leads to the generation of embryo-like structures, which recapitulate the early postimplantation embryo with proamniotic cavity formation, symmetry breaking, and specification of PGC-like cells and mesoderm (Harrison et al. 2017). Similarly, combining ESCs and TSCs promotes the formation of structures both morphologically and transcriptionally similar to mouse blastocysts named blastoids (Rivron et al. 2018). Embryo-like structures formed by ESCs, TSCs, and XENs initiate EMT and gastrulation (Peng et al. 2016, Sozen et al. 2018). How far these embryo-like structures can proceed in development is still to be defined. A recent study (Morgani et al. 2018) using a micropatterned culture platform showed that the induction of EpiSCs by FGF, BMP, Wnt, and NODAL signaling produces a variety of cells typical of gastrulation stages such as posterior EPI, primitive streak, mesoderm, and extraembryonic cells. These micropatterned systems offer a robust scalable method to generate regionalized cell types present in vivo. ESCs from the rat have also been derived (Buehr et al. 2008, Li et al. 2008), and rat trophoblast cell lines have been established from the placental labyrinth (Selesniemi et al. 2005). Increasing the repertoire of stem cell–derived models would be useful for molecular analysis and for understanding conserved mechanisms between closely related species.

3.1.2. Humans. Between 7 and 10 dpf, the human embryo implants in the uterus (Wilcox et al. 1999). Our understanding of human postimplantation development has been based on the archival collections at the Carnegie Institution for Science of Washington and the Boyd Collection at the University of Cambridge (Benirschke 1973; Hamilton & Mossman 1972; Hertig 1945, 1956; Shepard 1989). Studies of these collections have indicated that, after implantation, an amniotic cavity is formed within the EPI, which organizes into a polarized rosette-like structure. The human EPI forms a pseudostratified columnar epithelium, forming a bilaminar disc at the time of implantation (**Figure 3**) (Hertig 1945). Shortly after, a PrE-derived definitive yolk sac is observed (Luckett 1978). At this stage, the polar TE gives rise to the multinucleated syncytiotrophoblasts, which are specialized for nutrient and gas exchange, and the cytotrophoblasts, which differentiate into mononucleated extravillous cytotrophoblasts and mediate immunological acceptance of the conceptus (Moffett et al. 2017). Just how extravillous cytotrophoblast cells evade detection by the maternal uterine immune system is still unclear (Ander et al. 2019). Understanding the mechanism of this immune evasion could impact placental-related dysfunctions characterized by the failure of maternal tolerance such as preeclampsia and miscarriage (Sharkey et al. 2008). At approximately 14 dpf and shortly thereafter, gastrulation begins, the first sign of primitive streak formation occurs, and PGCs are thought to be specified (Witschi 1948).

Established hESCs (Thomson et al. 1998) have been used to recapitulate aspects of early postimplantation development (Itskovitz-Eldor et al. 2000). Human EBs are able to generate gastrula-organizer cells (Sharon et al. 2011), and in specific conditions can be directed to form organoids in vitro (Lancaster et al. 2013, Nakano et al. 2012). Culturing hESCs into geometrically controlled platforms in the presence of BMP4 is sufficient to induce ringlike organizations containing all three germ layers and extraembryonic cells (Warmflash et al. 2014). Treatment of these micropatterned colonies with WNT ligands also produces similar cell fate patterns but without extraembryonic differentiation (Martyn et al. 2019). In this model, BMP4 initiates dynamic waves of WNT and NODAL signaling (Chhabra et al. 2019). The precise mechanisms controlling the interdependence of these pathways, and whether they regulate human gastrulation in vivo, require further investigation. hESCs cultured in a 3D extracellular matrix can recapitulate amniotic cavity formation, albeit with low efficiency (Shahbazi et al. 2017, Taniguchi et al. 2015). A recently developed strategy has been used to generate hESC-derived gastruloids that undergo elongation along an anterior–posterior axis and patterning that reflects important elements of the body plan (Moris et al. 2020). Additionally, another study (Zheng et al. 2019) has developed a microfluidic device to culture hESCs that facilitates the modeling of human EPI and amnion development. While these in vitro models are tractable systems for molecular analysis and inform important aspects of human embryogenesis, they lack the 3D morphology and PrE- and TE-derived cell layers necessary for further development. They are therefore not equivalent in their potential to an intact human embryo in vivo and should not be treated as such.

Many different types of derived TSCs have been used to model human placental biology in vitro. However, a considerable lack of consensus exists about which cell line best models early placental biology (reviewed in Turco & Moffett 2019). Exogenous BMP treatment promotes the differentiation of hESCs into trophoblast cells (Amita et al. 2013, Xu et al. 2002) or extraembryonic mesoderm (Bernardo et al. 2011). A recent report (Okae et al. 2018) has generated TSCs from blastocysts and first trimester cytotrophoblasts. Moreover, 3D cultures of primary first trimester cytotrophoblasts, defined as trophoblast organoids, have been established to model the villous placenta (Haider et al. 2018, Turco et al. 2018). These culture systems will be useful to understand the genetic and epigenetic mechanisms involved in the specification of trophoblast lineages and may ultimately enable the study of placental diseases. Using naïve human pluripotent stem cells, self-renewing XEN-like cells resembling the human PrE can be established (Linneberg-Agerholm et al. 2019). However, XEN cells have yet to be derived directly from human embryos. In the future, the combination of human TSCs, ESCs, and XEN cells may be a useful tool to study aspects of early embryo development, such as signaling, cell–cell interactions, and morphogenesis, by generating embryo-like structures in vitro. Human embryo-like structures developed so far lack the potential to implant and develop into a fetus. The development of sophisticated methods that increasingly recapitulate important aspects of embryogenesis may test the current legal and regulatory frameworks originally intended for embryos arising from in vitro fertilization. Scientists are promoting an international discussion on this topic with the purpose of highlighting key aspects of this emerging area of research and providing recommendations for its ethical oversight (Hyun et al. 2020).

A number of studies have shown the successful coculturing of human blastocysts (Simón et al. 1999) or trophoblast spheroids with endometrial cells (Singh et al. 2010). This approach has been helpful to investigate both the chemokine interactions between the embryo and the epithelial endometrium (Dominguez et al. 2003) and the embryonic regulation of cell-surface molecules believed to be important for implantation (Aplin et al. 2001, Meseguer et al. 2001). It will be interesting to determine whether these conditions improve postimplantation morphogenesis. Experimental protocols have been developed to allow human embryos to self-organize in the

absence of maternal tissues up to the 14-day limit following fertilization (Deglincerti et al. 2016, Shahbazi et al. 2016). These developing embryos acquired important postimplantation features such as lineage segregation, amniotic cavitation, trophoblast differentiation, and yolk sac formation. Improved culture methods coupled with genetic studies could transform our understanding of this critical stage of embryogenesis.

3.1.3. Other eutherians. The pioneering work of Luckett (1975) and Enders and colleagues (1986), among others, has described the basic anatomy and major morphological transformations of postimplantation rhesus monkey embryos. A detailed study (Nakamura et al. 2016) in cynomolgus monkeys has provided molecular characterization of pre- and early postimplantation development. This work has revealed transcriptional changes in the EPI from pre- to postimplantation stages that have been informative when compared with in vitro–derived ESCs. Another study (Sasaki et al. 2016) has investigated the emergence of PGCs in cynomolgus monkeys and found that these cells appear in the dorsal amnion just prior to gastrulation. Similar studies of morphogenetic events in other nonhuman primates after implantation would provide a rich resource to better understand these species and to extrapolate aspects of human embryogenesis at a stage of development that is currently not possible to study.

Marmoset and rhesus monkey ESCs have been derived and have shown the ability to form postimplantation-like structures in vitro (Behr et al. 2005, Thomson et al. 1996). These nonhuman primate ESCs have been plated in a 3D Matrigel microenvironment, where they exhibit signs of implantation with trophoblast proliferation, invasion, and differentiation into cytotrophoblasts and syncytiotrophoblasts (Chang et al. 2018, Lopata et al. 1995). More recently, the establishment of improved in vitro culture systems has allowed the study of cynomolgus monkey embryos up to 20 dpf (Ma et al. 2019, Niu et al. 2019). Embryos in these conditions undergo in vivo postimplantation development events including embryonic lineage segregation, bilaminar disc formation, amniotic and yolk sac cavity formation, PGC emergence, and anterior–posterior axis establishment. The application of these methods to human embryos may allow for the study of critical events in embryogenesis at the time of implantation and shortly thereafter. While there have been recent calls for an extension (ASRM 2020), the culture of human embryos to study development beyond 14 days or the formation of the primitive streak would necessitate legal changes in some countries or approval from local, regional, or national ethics committees. For now, further detailed characterization of this critical stage of embryogenesis beyond 14 days in vitro is restricted to organisms that closely resemble the human.

The derivation of ESCs from other eutherian species has also been attempted in various domestic animals. While cow, pig, and sheep ESCs have been established, some of these ESCs have issues with consistency and stability, requiring further optimization of culture conditions (Blomberg & Telugu 2012, Ezashi et al. 2016). However, stable bovine ESCs similar to mouse and human ESCs in terms of transcriptome, karyotype, pluripotency marker gene expression, and epigenetic features have been recently developed (Bogliotti et al. 2018). These ESCs could be helpful in understanding basic biological processes and could be used to revolutionize breeding strategies in domestic animal production. Extensive effort is now focused on the derivation of PGCs and subsequently of haploid gametes from domestic animal ESCs. These could be used to facilitate the production of animals with high genetic value, thus having beneficial impacts on livestock health and economy (Goszczynski et al. 2019, Hou et al. 2018). Morphological descriptions of peri-implantation embryos of domestic animals such as pig or cow have also been reported (Bowen & Burghardt 2000). However, postimplantation in vitro models have not been developed, and our knowledge of the molecular and genetic mechanisms governing embryo elongation and implantation in domestic animals is limited.

3.2. Metatherians

Metatherians undergo a long period of preimplantation development, with organogenesis initiating prior to uterine attachment. The implantation period is very transient, only two days in the opossum, and the development of some structures, such as the hindlimbs, continues after birth (Chew et al. 2014, Selwood 1992). Thus, the morphogenetic changes that take place from the blastocyst to gastrulation in metatherians occur before implantation and are therefore more accessible than in eutherians. This has permitted study of the emergence of the primitive streak and characterization of the expression pattern of molecular markers during gastrulation in the gray short-tailed opossum (Mate et al. 1994, Yoshida et al. 2016). Despite this advantage, in vitro culture conditions for embryo development from fertilization, or during this later stage of development, have not yet been established.

A placenta is also formed in metatherians after the fusion of the chorion and the yolk sac. However, in most species the placenta is not invasive or is superficially invasive. Moreover, although an area of syncytium has been observed in some species, the placenta does not form a complex syncytial structure as in eutherians (Jones et al. 2014, Renfree 2010, Zeller & Freyer 2001). In the opossum, contact between the placenta and the maternal tissue at the time of implantation causes an inflammatory response in the mother (Griffith et al. 2017). The shift from an inflammatory reaction to a noninflammatory pregnancy may have been important to allow eutherians to develop an extended period of postimplantation development. Future molecular studies of the embryonic and extraembryonic compartments as well as the maternofetal interface could help to reveal the conserved principles and particularities of metatherian embryogenesis as they proceed through development before attachment to the maternal tissue.

4. CONCLUSIONS

While the mouse will continue to be an important model organism to study early embryogenesis, recent advances in molecular techniques, including single cell analyses and genome editing, now make it possible to study human embryogenesis directly. These techniques have revealed important differences and similarities between mouse and human development, further highlighting the importance of comparative embryology studies in multiple species. Interestingly, recent comparative studies have revealed intriguing similarities between human and nonhuman primates and other eutherian species, such as bovine and porcine embryos. Further comparison with other model organisms will increase our understanding of early development. If the zygote and early stages of embryo development prior to ZGA are very similar among species, what triggers the MZT earlier or later? Which mechanisms determine the early specification of the TE program in the mouse compared with the later specification in humans, pigs, or opossums? Are the upstream signaling pathways or transcription factors acting in these decisions different or conserved? What regulates the spatial domains of expression of some factors that switch from being ubiquitous to lineage specific?

Extending the duration of human embryo culture will provide a unique opportunity to study postimplantation development. The generation of more complex conditions in which to culture human embryos, for example with physically and chemically defined 3D matrices, endometrial cells, and microfluidics devices, would allow a more informed study of the interplay between the embryo and uterine environment. Moreover, a broader application of CRISPR-Cas9 methods, for example by generating genetic tags as reporters for gene expression, would further inform lineage-specification mechanisms in human embryos. Working with embryo-like structures has the advantages of easy manipulation, accessibility, scalability, and a finer control of several variables. However, key elements in this field still require further investigation and optimization; for

example, it will be important to further refine human embryonic and extraembryonic stem cells to improve the current human embryo-like structures and to develop tunable and finely controlled 3D matrices. Ultimately, the combination of studies in embryos from a variety of species, stem cells, and embryo-like structures will allow us to unravel the basic principles of human development.

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