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Conserved features of non-primate bilaminar disc embryos and the germline

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SUMMARY

Post-implantation embryo development commences with a bilaminar disc in most mammals, including humans. Whereas access to early human embryos is limited and subject to greater ethical scrutiny, studies on non-primate embryos developing as bilaminar discs offer exceptional opportunities for advances in gastrulation, the germline, and the basis for evolutionary divergence applicable to human development. Here, we discuss the advantages of investigations in the pig embryo as an exemplar of development of a bilaminar disc embryo with relevance to early human development. Besides, the pig has the potential for the creation of humanized organs for xenotransplantation. Precise genetic engineering approaches, imaging, and single-cell analysis are cost effective and efficient, enabling research into some outstanding questions on human development and for developing authentic models of early human development with stem cells.

INTRODUCTION

The foundation of the mammalian body plan is established during post-implantation development with the segregation of the primary germ layers at gastrulation. Whereas the events leading to this key developmental process are broadly similar in mammalian species, important species-specific differences exist among them, including the spatial organization of embryonic and extraembryonic regions, as well as in the molecular mechanisms regulating pluripotency, early somatic cell-fate decisions, and the specification of primordial germ cells (PGCs).

The mouse embryo has been widely used as a representative of mammalian development due to its easy husbandry, small size, short generation interval, large litters, and availability of genetic tools; thus, most of our understanding of the mechanisms of gene regulation and cellular differentiation come from fundamental studies of this organism. Pluripotent stem cells (PSCs) were also first derived from mouse embryos, which enabled genetic approaches to study mammalian development, including the first studies on gene knockout and transgenesis, complemented with methods such as chimeras. Many of the experimental approaches developed for studies on mice have been adopted and refined for studies in other mammals.

The mouse embryo undergoes a unique transformation between embryonic day 3.5 (E3.5) and E5.5, when it transitions from a round blastocyst to form the egg cylinder after implantation. During this transition, epithelialization of the inner cell mass (ICM) results in the formation of the cup-shaped epiblast that becomes surrounded by an outer layer of visceral endoderm (Figure 1). Proximally, the epiblast is juxtaposed by the extraembryonic ectoderm (ExE), a tissue derived from the polar trophoblast with critical functions in patterning the embryo (Guzman-Ayala et al., 2004; Lawson, 1999; Rodriguez et al., 2005). In this configuration, the pre-gastrula embryo initiates anterior-posterior axis specification, which is followed by the onset of gastrulation. Unique to mice, this process starts "inside-out," with the ectoderm as the internal germ layer, surrounded by mesoderm and endoderm. The embryo undergoes an inversion of the germ layers, followed by turning in the early somitic stages (Tam and Behringer, 1997). In contrast to the conspicuous geometry of the mouse embryo, the ICM of the blastocyst in other mammals develops into a single layered epithelium over several days (3 days in rabbit, 5 days in primates, and 6 days in ungulates) forming a "flat-disc" epiblast that becomes free of polar trophoblast. Notably, the planar morphology of the embryonic disc is also typical of monotremes, marsupials, and non-mammalian amniotes such as birds and reptiles (Hughes, 1993). Indeed, chick embryos have been fundamental models of vertebrate embryology (Stern, 1994).

Anterior-posterior patterning precedes the onset of gastrulation, although the precise mechanisms controlling these processes remain poorly understood (Blomberg et al., 2008; Yoshida et al., 2016). Importantly, many of the morphogenetic events characterizing early post-implantation human embryos, which are almost impossible to study *in vivo*, resemble those described in other animals, so information regarding fundamental mechanisms of early gastrulation can be extrapolated from studies in these species. Note, however, that development of the extraembryonic tissues varies considerably among mammalian species, reflecting distinct adaptive reproductive strategies. The anatomical diversity and evolution of extraembryonic membranes have been reviewed in detail previously (Carter

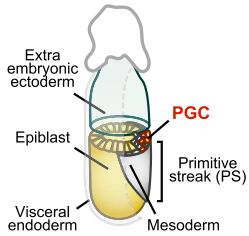
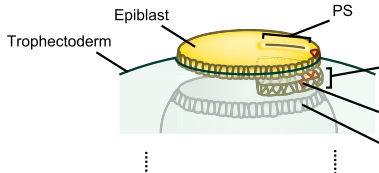
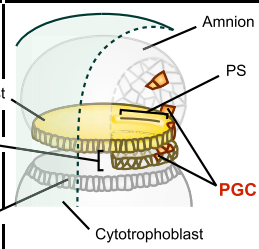
Species	Mouse	Rabbit	Pig	Cow	Human
Morphogy of gastrulating embryo					
Shape of epiblast	Egg cylinder	Flat disc	Flat disc	Flat disc	Flat disc
Cite of PGC specification	Posterior epiblast	Posterior epiblast (?)	Posterior epiblast	?	Nascent amnion and/or posterior epiblast
SOX2/SOX17 expression in PGC	SOX2	?	SOX17	?	SOX17
Accessibility of embryos	✓✓	✓	✓✓	✓	✕
Availability of pluripotent stem cells	✓✓	✓	✓	✓	✓✓

Figure 1. Comparative aspects of peri-gastrulation development in different species
Rabbit and pig embryos are easily accessible. Thus, investigations in these embryos can provide insights into the development of bilaminar disc embryos with relevance to humans. PGC, primordial germ cells; PS, primitive streak. The amnion in rabbit, cow, and pig forms later than in human.

and Enders, 2013; Roberts et al., 2016). Here, we discuss recent advances in our understanding of embryo development once it progresses past the blastocyst stage leading to the onset of gastrulation in flat bilaminar disc-forming embryos (Figure 1). We also discuss the advantages of using pig embryos as alternative *in vivo* model of flat-disc embryos, due to easy accessibility, large litter size, advanced assisted reproductive technologies, amenability to gene editing, and ethical acceptability. Besides, pigs are of significant medical importance; they are used to model human diseases and offer potential for xenotransplantation (Das et al., 2020; Telugu et al., 2017; Yao et al., 2016). We also briefly consider findings of the flat-disc rabbit and cow embryos, which have been used in many studies that help establish the principles of early gastrulation. Thus, investigations of bilaminar disc-forming embryos will be important for addressing questions relevant to human development.

PERI-IMPLANTATION DEVELOPMENT IN MAMMALIAN EMBRYOS WITH BILAMINAR DISCS

The formation of the blastocyst is accompanied by the first lineage segregation in eutherian mammals, consisting of the outer layer of trophectoderm (TE) and the ICM. Shortly thereafter, the second lineage segregation is marked by the emergence of hypoblast (or the primitive endoderm, as is known in the mouse) from the pluripotent epiblast. Studies on mouse embryos have shown that expression of *Cdx2*, a crucial gene for TE specification starts to be detectable at the compact morula stage, with the establishment of a mutually exclusive expression with *Oct-4* in the outer and inner cells, respectively (Niwa et al., 2005; Strumpf et al., 2005). Stimulation of *Cdx2* and *Gata3* expression occurs after the inactivation of the Hippo pathways in outer polarized cells (Hirate et al., 2013; Nishioka et al., 2009). *Cdx2*, which is activated by *Gata3* (Home et al., 2009), plays a key role in supporting the expression of downstream TE regulators *Eomes* (Russ et al., 2000) and *Tfap2c* (Kuckenberg et al., 2010), which may induce irreversible commitment of this lineage by the 16- to 32-cell stage (Posfai et al., 2017). Notable differences in other mammalian embryos, including differences in gene expression profiles and the tempo of lineage commitment, are known to suggest a protracted period of developmental plasticity in the TE (Berg et al., 2011; Sandra et al., 2017). *CDX2* is first detected in TE of blastocysts in human (Blakeley et al., 2015; Niakan and Eggan, 2013), 2013), pig (du Puy et al., 2011; Ramos-Ibeas et al., 2019) and cattle (Kuijk et al., 2008; Simmet

et al., 2018; Wei et al., 2017), whereas expression of *GATA3* is detected in morulae and persists to the blastocyst stage in all three species (Petropoulos et al., 2016; Ramos-Ibeas et al., 2019; Wei et al., 2018). *GATA3* expression is regulated by Hippo signaling, which is dependent on atypical protein kinase C (aPKC) sequestration of AMOT (Hirate et al., 2013; Korotkevich et al., 2017; Plusa et al., 2005). aPKC decorates the apical membranes of the outer cells of morulae, indicating that cell polarization triggers the transcriptional activation of the TE program in mouse, cow, and human (Gerri et al., 2020). Although the onset of the TE program may be common to multiple species, the irreversible TE lineage commitment in human and cow embryos is attained later than in the mouse (Berg et al., 2011; De Paepe et al., 2013) and is consistent with the later implantation in human (E7–E9) and attachment in cattle (~E21). This suggests that changes to the murine TE gene-regulatory network may have led to accelerated specification, ahead of implantation by day E4.5. There is further evidence for a longer period of developmental plasticity in non-murine species that emerges from investigations of the differentiation capacity of the ICM. In the mouse, the ICM is unable to differentiate into TE (Nichols and Gardner, 1984), whereas human ICM cells from E6 blastocysts readily differentiate into this lineage (Guo et al., 2020). In cattle, isolated ICMs can reconstitute a blastocyst (Kohri et al., 2019), demonstrating that a fully competent TE can emerge from ICMs. In experiments using mouse embryonic stem cells (ESCs), the *in vitro* equivalent of the pre-implantation naive epiblast shows that these cells cannot differentiate to TE in chimeras (Beddington and Robertson, 1989; Bradley et al., 1984). In contrast, human naive ESCs can give rise to TE through the upregulation of *GATA2/3*, *DAB*, and *TEAD* (Guo et al., 2020), suggesting that *CDX2* plays a less prominent role during TE specification in non-murine embryos.

Functional complementarity between human and other large mammalian embryos also find parallels in the signaling principles governing lineage decisions in the early embryo. During the first lineage decision segregating the TE and ICM, for example, inhibition of ERK signaling from the morula stage in pig and cattle promotes expansion of the TE in blastocysts (Canizo et al., 2019; Rodriguez et al., 2012). Although information on how human embryos respond to this treatment is lacking, experiments using naive human ESCs show that they readily differentiate into TE upon ERK inhibition alone (Guo et al., 2020). Later, during the second lineage segregation, ERK inhibition abrogates hypoblast formation in mouse and rabbit embryos (Nichols et al., 2009; Piliszek et al., 2017). In contrast, in human (Kuijk et al., 2012; Roode et al., 2012), pig (Rodriguez et al., 2012), and cattle (Canizo et al., 2019; Kuijk et al., 2012), ERK inhibition only partially blocks the hypoblast program, suggesting that additional signals regulate segregation of this lineage in non-murine embryos.

Other signaling pathways also show contrasting roles between mouse and other mammals. Of particular interest are those involved in the emergence of the pluripotent ICM. In the mouse, components of Jak/Stat3 and bone morphogenetic protein (BMP) signaling are highly expressed in the pre-implantation epiblast, while in the post-implantation epiblast transforming growth factor β (TGFβ)/Nodal signaling components become predominantly expressed (Boroviak et al., 2015). In contrast, in human, monkeys, pig, and cattle embryos, expression of multiple components of the TGFβ/Nodal signaling point to a key role from the early stages of epiblast development (Boroviak et al., 2015; Nakamura et al., 2016; Petropoulos et al., 2016; Ramos-Ibeas et al., 2019). Indeed, inhibition of TGFβ/Nodal signaling using small-molecule inhibitors from the morula stage does not affect the initial expression of NANOG in human, marmoset monkey, pig, and bovine ICM; however, inhibition after the blastocyst stage causes a significant reduction of NANOG-positive cells in the epiblast (Blakeley et al., 2015; Boroviak et al., 2015; Kuijk et al., 2012; Ramos-Ibeas et al., 2019). Equivalent treatment of mouse embryos does not affect pluripotency properties of the early epiblast (Blakeley et al., 2015; Granier et al., 2011). Thus, in non-murine embryos TGFβ/Nodal signaling plays a critical role soon after pluripotency is established in the ICM to support expansion of the epiblast during ~5 days (in primates) to 7 days (in pigs) that elapse until the onset of gastrulation (Blakeley et al., 2015; Nakamura et al., 2016; Ramos-Ibeas et al., 2019).

The distinct signaling environment of the pluripotent cells in the embryo has been instructive for designing culture conditions that capture equivalent cells *in vitro*. Naive mouse ESC cells, which approximately represent the nascent mouse epiblast, are dependent on LIF, MEK, and GSK3β inhibition (Boroviak et al., 2014; Ying et al., 2008). Under these conditions, these cells retain unique hallmarks of the early epiblast, including expression of naive specific transcription factors, reactivation of the inactive X in females, elevated expression of transposable elements, and DNA hypomethylation (Boroviak and Nichols, 2017; Nichols and Smith, 2009). The culture conditions used for mouse naive PSCs, however, are not sufficient to generate human naive PSCs, which require reduced GSK3β inhibition, additional inhibition of PKC, and the support of feeder cells (Guo et al., 2016; Takashima et al., 2014).

Human and mouse naive ESCs share expression of the core pluripotent transcription factors *SOX2*, *NANOG*, and *OCT-4*; however, expression of a different set of naive markers of the early primate epiblast *KLF17*, *TFCP2L1*, and *SOX15* occurs (Blakeley et al., 2015; Boroviak and

Nichols, 2017; Nakamura et al., 2016). Furthermore, *NODAL* is also expressed in human naive ESCs, consistent with the expression detected in the early epiblast (Blakeley et al., 2015; Petropoulos et al., 2016). Although Activin A supplementation is not required for growing these cells, Nodal inhibition affects their clonogenic potential and long-term proliferation, suggesting that autocrine signaling is required in human naive cells (Takashima et al., 2014). Interestingly, detailed analysis of pluripotency features of the pig embryo has also shown expression of *KLF17*, *TFCP2L1*, and *SOX15* as occurs in the human embryo (Ramos-Ibeas et al., 2019). Nascent pig epiblast cells from ~E6 embryos also show expression of *IL6* and Jak/Stat3 signaling components. Blocking this signaling pathway using a small-molecule inhibitor, AZD1480, reduces the number of Nanog-expressing cells. From E7–E8 until E11, the transcriptional signature of the pig epiblast changes to a more developmentally advanced stage, which in the mouse corresponds to the post-implantation epiblast of E5.5–E6.5 embryos, from which point epiblast stem cells can be established (Brons et al., 2007; Tesar et al., 2007). Conventional human ESCs share many features with these pig epiblast cells, such as inactive X chromosomes in females, increased DNA methylation, and dependence on fibroblast growth factor (FGF) and Nodal signaling for self-renewal. These cells are also known as primed ESCs, as they correspond to the cells in the embryo found just before the onset of gastrulation (Nichols and Smith, 2009). An important aspect for future interrogation is whether cells with the properties of formative pluripotency form during the development of the pig epiblast, which is the capacitation phase prior to lineage specification (Smith, 2017). If these cells do appear it will become possible to identify and capture them *in vitro*. Mouse formative stem cells can contribute to germline chimeras and can be captured from E5.5 embryos in the presence of low concentration of Activin and inhibition of Wnt and retinoic acid receptor (Kinoshita et al., 2020). These cells exhibit *OTX2* expression but lack primitive streak markers *T*, *GSC*, and low *FOXA2*. These molecular features are equivalent to those found in the pig epiblast from E8 to E11 (Ramos-Ibeas et al., 2019). Human formative stem cells established from pre-implantation embryos display molecular properties closely related to conventional human ESCs, suggesting they represent a developmental continuum with the post-implantation epiblast (Kinoshita et al., 2020). XPSCs are also intermediate PSCs derived from mouse, human, and horse embryos that are capable of contributing to chimeras and germline differentiation (Yu et al., 2020). In contrast to formative cells, XPSCs propagated in medium with Activin A and a WNT activator represent an earlier stage within the formative continuum. It will be interesting to find out whether such cells can be obtained from other livestock and whether they can also contribute to the germline *in vivo*.

Cross-species comparisons with *in vivo* human post-implantation stages are challenging. However, a time series of human embryos obtained after extended three-dimensional (3D) culture *in vitro* has provided valuable insights into the transcriptional changes during human epiblast maturation and embryo patterning. Initial signs of anterior-posterior polarity, depicted by *HESX1* and *TBXT* expression, are detected between days 12 and 14 (Xiang et al., 2020). In the pig, *HESX1* and *TBXT* expression is detectable in opposite ends of the embryo at E10–E11 (Hassoun et al., 2009; Kobayashi et al., 2017; Wolf et al., 2011; Yoshida et al., 2016). This pattern of *TBXT* expression is reminiscent of observations on rabbit, sheep, and cow embryos (Guillomot et al., 2004; Hue et al., 2001; Yoshida et al., 2016). Similarly, during this period *Nodal* expression becomes restricted to the posterior epiblast in rabbit and pig (Ploger and Viebahn, 2018; Yoshida et al., 2016), the region of the formation of the primitive streak, which has also been determined in 3D cultures of human embryos, and more recently in gastruloids (Moris et al., 2020b; Xiang et al., 2020). Although the temporal pattern may be skewed due to the *in vitro* culture of the human embryo, cross-species comparison of RNA-sequencing datasets show highest correlation of E10 and E12 pig epiblast cells with E8 and E10 human epiblasts, respectively (Liu et al., 2021). Conserved features of left-right patterning and axial elongation have also been studied in rabbit, pig, and cow embryos, which highlight some remarkable species-specific mechanisms (Ploger and Viebahn, 2018; Schroder et al., 2016). The information from these studies will be valuable for comparisons with *in vitro* human organoids and gastruloids.

Recent work from our laboratories has shown the developmental correspondence between human and pig germ cells during the initial period of gastrulation (Kobayashi et al., 2017; Zhu et al., 2021). We propose that building on these investigations into pig gastrulation using single-cell -omics and gene manipulation will offer new knowledge on the mechanisms of meso-endoderm and ectoderm specification with relevance to understanding human development.

ORIGIN OF THE GERMLINE IN MAMMALS

PGC specification occurs during the pre-gastrulation period, followed by the establishment of a cluster of founder population of PGCs at the caudal end, and before they commence migration to the gonads. Accordingly, studies on the origin and specification of PGCs are highly informative of early development as a whole. In this

context, the importance of embryo geometries and its relations with extraembryonic tissues in cell specification is also best demonstrated by analyzing PGC specification in mammals. In the mouse, the ExE is the primary source of the key germ cell specification molecule BMP4 (Lawson et al., 1999; Winnier et al., 1995), although BMP2 produced in the posterior visceral endoderm can also support mouse PGC (mPGC) induction (Chuva de Sousa Lopes et al., 2008). BMP induces *WNT3* expression in epiblast cells to trigger the mesoderm program, but critically also induces PGCs through the activation of *Blimp1* and *Prdm14* (Ohinata et al., 2009; Yamaji et al., 2008).

In bilaminar disc-forming embryos the source of BMP during peri-gastrulation has been studied in some detail. In the pig, BMP2 is first detected in the hypoblast of pre-gastrulation embryos, where it promotes the delamination of the extraembryonic mesoderm (ExM). BMP4 expression follows that of BMP2 and is highest in the ExM and in the posterior epiblast prior to the formation of the primitive streak (PS) (Valdez Magana et al., 2014). Expression of BMP4 coincides with expression *WNT3* and *TBXT*, both critical for the mesoderm program, in the posterior epiblast region (Kobayashi et al., 2017; Yoshida et al., 2016). WNT signaling is critical in conferring PGC competence to epiblast cells before BMP4 induction (Kobayashi et al., 2017).

Another example of disc-forming embryo is the rabbit, where some of these signaling effectors have been studied. In the rabbit embryo, BMP2 is also expressed in hypoblast cells of pre-PS streak embryos (Hopf et al., 2011). BMP4 becomes primarily restricted to the posterior epiblast during PS formation, which also coincides with *WNT3* and *BRACHYURY* expression in this area (Hopf et al., 2011; Yoshida et al., 2016). Furthermore, in pig and rabbit embryos the BMP inhibitor *DKK* and WNT inhibitor *CERBERUS* are expressed in the anterior PS region, probably restricting PGC induction to the posterior epiblast (Yoshida et al., 2016).

In *Cynomolgus* monkey, BMP2 is also expressed in the hypoblast of pre-gastrulation embryos and precedes the expression of BMP4, which is primarily expressed in the nascent amnion and in the posterior PS region (Sasaki et al., 2016). Thus, evidence from three bilaminar disc-forming species shows that the posterior epiblast is exposed to BMP during this critical period prior to the formation of the PS and emergence of the PGCs. An important difference between these species is the potential role of extraembryonic lineages in inducing the germline; the extraembryonic lineages show significantly diverse development among mammals. In *Cynomolgus* monkey the amnion expresses BMP4, and within this area a small number of cells with PGC-like identity (TFAP2C, BLIMP1, and SOX17, and no SOX2) has been reported. These cells apparently locate to the posterior epiblast region where their number increases (Sasaki et al., 2016), but lineage-tracing experiments are needed to confirm the findings. Notably, the cytotrophoblast and the amnion in the monkey express *WNT3A*, an essential germline competence factor, creating a suitable microenvironment for the induction of the PGC program upon BMP induction. Indeed, the nascent amnion epithelium derives from the post-implantation epiblast, retaining expression of pluripotency genes OCT-4, NANOG, and SOX2, suggesting that in conditions of equivalent signaling principles, a dual origin of the germ cells lineage in monkeys is possible (Kobayashi and Surani, 2018). However, evidence from other species suggesting the amnion as the site of the origin of PGC specification may not apply to all mammalian species with bilaminar disc embryos. In human, the amnion forms soon after implantation as a derivative of the epiblast and before the onset of gastrulation (Luckett, 1975). In the pig and the rabbit, the amnion derives from the ExM and forms by folding after the start of gastrulation (Hassan and Viebahn, 2017; Hassoun et al., 2010; Perry, 1981). Furthermore, in contrast to mouse embryos, the ExM in pig, sheep, cow, and horse emerges from delaminating posterior epiblast cells before the PS forms (Blomberg Le et al., 2006; Flechon et al., 2004; Guillomot et al., 2004; Hue et al., 2015).

Notably, pig PGCs are first found in the posterior epiblast expressing SOX17, BLIMP1, and TFAP2C, and the cluster expands to about 120 cells by induction of proliferating PGC-competent progenitors upon exposure to BMP. These newly formed pig PGCs (pPGCs) pause their proliferation briefly, only to resume cell-cycle progression after they start their migration (Kobayashi et al., 2017; Zhu et al., 2021). The PGCs originating in the amnion of *Cynomolgus* monkey appear to be in a proliferative phase soon after specification (Sasaki et al., 2016), but how PGCs migrate to posterior epiblast against the movement of amnion that expands toward the opposite direction merits further investigations. There is also a possibility that the cells found in the posterior epiblast derive, at least in part, directly from pre-gastrulation germ-cell-competent epiblast in response to BMP.

REGULATORY NETWORK OF PGCs

In mice, PGCs are specified from posterior proximal epiblast cells in response to BMP stimulation of WNT3, which induces *T* (Aramaki et al., 2013). *T* (or brachyury) is responsible for activating *Prdm14* and *Blimp1*, which together with *Tfap2c* establish the key tripartite gene expression network of mPGCs (Magnusdottir et al., 2013; Nakaki et al., 2013). This gene network inhibits the somatic differentiation program, promotes expression of

pluripotency genes, and triggers the onset of epigenetic reprogramming (Saitou and Yamaji, 2012). Reactivation of pluripotency genes *Sox2* and *Nanog* is a hallmark of mPGC development, and their expression is maintained until these cells reach the gonads. Mouse mutants for these genes lose PGCs by apoptosis and display reduced cell proliferation, indicating that that these genes act as survival factors and support the expansion of the initial pool of mPGCs (Campolo et al., 2013; Zhang et al., 2018).

In contrast, the regulatory network of PGC development in other mammals relies on other key genes (Irie et al., 2015; Kobayashi et al., 2017; Sasaki et al., 2015). First, SOX17 is a critical factor expressed ahead of BLIMP1 in human PGCs (hPGCs), monkey PGCs, and pPGCs (Irie et al., 2015; Kobayashi et al., 2017; Sasaki et al., 2016). SOX15 is also highly expressed during early PGC development, although it cannot replace SOX17 during hPGC specification (Pierson Smela et al., 2019). Second, SOX2 is repressed in human, monkey, pig, and cow, and PRDM14 is either absent in pre-gonadal PGCs (human and pig) or expressed at low levels in gonadal stages (human and monkey, pig and cow) (Kobayashi et al., 2017; Sasaki et al., 2016; Soto and Ross, 2021; Tyser et al., 2020; Zhu et al., 2021). In hPGC-like cells (hPGCLCs) PRDM14 plays a critical role but with a mechanism divergent from mPGCs (Sybirna et al., 2020). Third, PGC-competent cells require *EOMES*, but no *TBXT*, for hPGCLC induction (Chen et al., 2017; Kojima et al., 2017). This distinct gene expression profile among several species forming an embryonic disc suggests evolutionary conservation in the transcriptional network regulating PGC development. The basis for the evolutionary divergence in PGC specification mechanism merits further investigations. It will also be important to determine whether the mechanisms of other early cell-fate decisions have also diverged in different mammals.

EPIGENETIC RESETTING IN THE MAMMALIAN GERMLINE

DNA demethylation

Following specification of PGCs, they undergo comprehensive epigenetic resetting during migration to the gonads, which is a unique event not seen in somatic cells. The large-scale epigenetic resetting results in the erasure of parental epigenetic memory (Kurimoto and Saitou, 2018; Reik and Walter, 2001; Tang et al., 2016). Epigenome resetting occurs at several levels including genome-wide DNA demethylation, X chromosome reactivation in females, and chromatin remodeling. PGCs reach the lowest levels of DNA methylation at E12.5 in the mouse and by week 7 of development in human (Guo et al., 2015; Hill et al., 2018; Tang et al., 2015; Zhu et al., 2021). Similarly, a recent analysis of pPGCs also shows very low levels of CpG methylation by week 5 of embryo development (Zhu et al., 2021). Residual DNA methylation in all three species is primarily found at evolutionary young and potentially mobile TEs, such as IAPEZ in mice (Lane et al., 2003), AluY in humans (Tang et al., 2015), and SINE (PRE1 family) in pigs (Zhu et al., 2021), which might help to repress their retrotransposition.

Importantly, numerous repeat-poor loci are located at single-copy sequences that resist DNA demethylation in the germline (Guibert et al., 2012; Hackett et al., 2013; Seisenberger et al., 2012; Tang et al., 2015; Zhu et al., 2021). These sequences are found at gene-regulatory regions, such as promoters and enhancers, as well as at intergenic regions. Notably, we found that ~21% of these repeat-poor methylation-resistant loci have conserved synteny between human and pigs, whereas human and mouse share ~4% of sequences. Accordingly, human and pig have 265 common DNA methylation-resistant genes. Analysis of these genes revealed an association with metabolic and neurological syndromes in humans. The consequences of the transmission of reprogramming-resistant loci to subsequent generations merit investigation with pig as a model. The epigenetic inheritance of these loci and their contribution to some diseases have been indicated in genome-wide association study analysis.

The mechanisms of DNA demethylation have been studied in detail in the mouse, where a combination of passive and active demethylation has been proposed (Kagiwada et al., 2013; Seisenberger et al., 2012). Although Dnmt1 is expressed in mPGCs, the co-factor Uhrf1 is not, which will affect maintenance DNA methylation during proliferation. Furthermore, *de novo* methyltransferases Dnmt3A and Dnmt3B are expressed at low levels (Seki et al., 2005; Yabuta et al., 2006). Accordingly, these will lead to replication coupled dilution of DNA methylation. A role for active mechanisms mediated by the Ten-eleven translocation enzymes Tet1–Tet3 have been proposed in E9.5–E11.5 gonadal mPGCs, where the levels of these enzymes increase (Hackett et al., 2013; Kagiwada et al., 2013). Tet1 and Tet2 are required for imprinted gene demethylation in gonadal mouse PGCs (Vincent et al., 2013; Yamaguchi et al., 2013). Furthermore, Tet1 also acts as a transcriptional regulator of germline genes and contributes to the elimination of aberrant CpG methylation in gonadal PGCs (Hill et al., 2018). Little information is currently available on the epigenetic resetting in pre-gonadal human germ cells; however, the process is highly asynchronous, with "imprints" on some genes being erased before PGCs enter the gonadal ridges and others in later gonadal stages (Gkountela et al., 2013; Tang et al., 2015; Vertesy et al., 2018). Similarly, asynchronous demethylation takes place in pPGCs (Hyldig et al., 2011; Petkov et al., 2009). Thus, it will be

important to determine whether a Tet-mediated mechanism may participate in PGC demethylation in hPGCs and pPGCs. Analysis of pPGCs shows high levels of 5hmC in pre-migratory cells, which correlates with high expression of TET1 (Kobayashi et al., 2017; Zhu et al., 2021). Although early hPGCs have not been investigated, *in vitro*-produced hPGCLCs, which represent pre-migratory cells, also show the onset of an increase in 5hmC levels concomitantly with TET1 expression (Tang et al., 2015; Zhu et al., 2021), suggesting a role for additional mechanisms regulating DNA demethylation in humans and pigs.

Chromatin organization

Associated with the changes in DNA methylation, global changes in chromatin configuration also accompany PGC development (Hajkova et al., 2008; Seki et al., 2005). While H3K9me3 is retained in pericentric heterochromatin, H3K9me2 is almost completely depleted from PGCs soon after specification (Ancelin et al., 2006; Seki et al., 2007). This is probably a consequence of the reduction in histone methyltransferase G9a determined in pre-migratory mouse PGCs (Seki et al., 2007; Yabuta et al., 2006). Marked reduction of this histone mark is also typical of pre-gonadal pig and monkey PGCs as well as early gonadal hPGCs (Kobayashi et al., 2017; Sasaki et al., 2016; Tang et al., 2015). H3K27me3 levels increase in gonadal mPGCs and persist until late stages (de Sousa Lopes et al., 2007; Seki et al., 2007). In contrast, H3K37me3 levels are transiently increased in migratory hPGCs and pPGCs, but decrease sharply in gonadal stages (Gkountela et al., 2013; Hyldig et al., 2011; Tang et al., 2015; Zhu et al., 2021). It will be interesting to determine which regions of the genome retain this mark during the extensive DNA demethylation period in gonadal PGCs.

X chromosome reactivation

X chromosome reactivation (XCR) in females is another example of the extensive remodeling taking place during PGC development. Analysis of hPGCs shows that XCR is highly asynchronous and heterogeneous and may initiate in some cells prior to entering the gonadal ridges (Guo et al., 2015; Vertesy et al., 2018). Indeed, analysis of the H3K27me3 mark shows reduced number of such foci from week-4 hPGCs (Tang et al., 2015). The precise timing of when XCR starts in hPGCs is not known; however, analysis of pPGCs has shown that multiple hallmarks of XCR can also be determined in pre-migratory PGCs. We detected reduced expression of *XIST*, increased biallelic expression of X-linked genes, and high proportion of pPGCs without H3K27me3 foci (Zhu et al., 2021). These observations contrast with the findings in mPGCs, where a decrease in H3K27me3 foci is first detected in migratory cells from about E9.5 and biallelic expression of X-linked genes increases from E11.5 (Chuva de Sousa Lopes et al., 2008; Sugimoto and Abe, 2007).

The seemingly lengthy XCR process determined in pPGCs suggests alternative mechanisms regulating these events in this species. A notable difference with mice is the expression of the epigenetic regulator *Prdm14*, which has been linked with the reduction in X-associated H3K27me3 foci in migratory mPGCs (Mallol et al., 2019). Recent studies have shown very low or lack of *PRDM14* expression in early hPGCs and pPGCs (Tyser et al., 2020; Zhu et al., 2021). Functional analysis has also demonstrated a limited role in hPGC development (Sybirna et al., 2020). Thus, *PRDM14* might have a secondary role in XCR in pPGCs and hPGCs, in contrast to the suggested role in mPGCs (Mallol et al., 2019). Human *in vitro*-derived oogonia can partially recapitulate XCR, although the precise mechanism remains unknown (Yamashiro et al., 2018). Improvements in the *in vitro* gamete production methods might provide models for a deeper understanding of these epigenetic reprogramming steps. Thus, it will be important that future studies determine the sequence of events and the key molecules associated with the mechanism of XCR in pigs and humans.

IN VITRO MODELS FOR EARLY DEVELOPMENT AND THE GERMLINE

Advantages of late implantation model for building *in vitro* models

The parallels between the pig, cow, and rabbit embryonic disc morphology with the human embryo offer significant opportunities to establish the principles of gastrulation in these accessible species. Gastrulation in the pig, cow, and rabbit starts prior to implantation, which make these embryos readily accessible (Figure 2). Importantly, the pig conceptus has a superficial attachment within the uterine lining involving an epitheliochorial placenta. Thus, by the time placentation commences around day 14, the pig embryo has progressed to a stage with 6–8 somites, allowing investigations of this key phase of embryogenesis in a "free-floating" conceptus. How the embryo develops over this period might be relevant to early human development. These embryos could be used to address questions relating to the role of mechanical constraints and forces, as well as the impact of the geometry regulating cell-fate decisions during gastrulation *in vivo*. The importance of these factors has been subject of recent debates on the use of *in vitro* models derived from PSCs to understand cell-fate decisions during early mammalian development (Martinez Arias and Brickman, 2011).

With the development of novel approaches for growing embryo-like structures *in vitro*, new challenges have

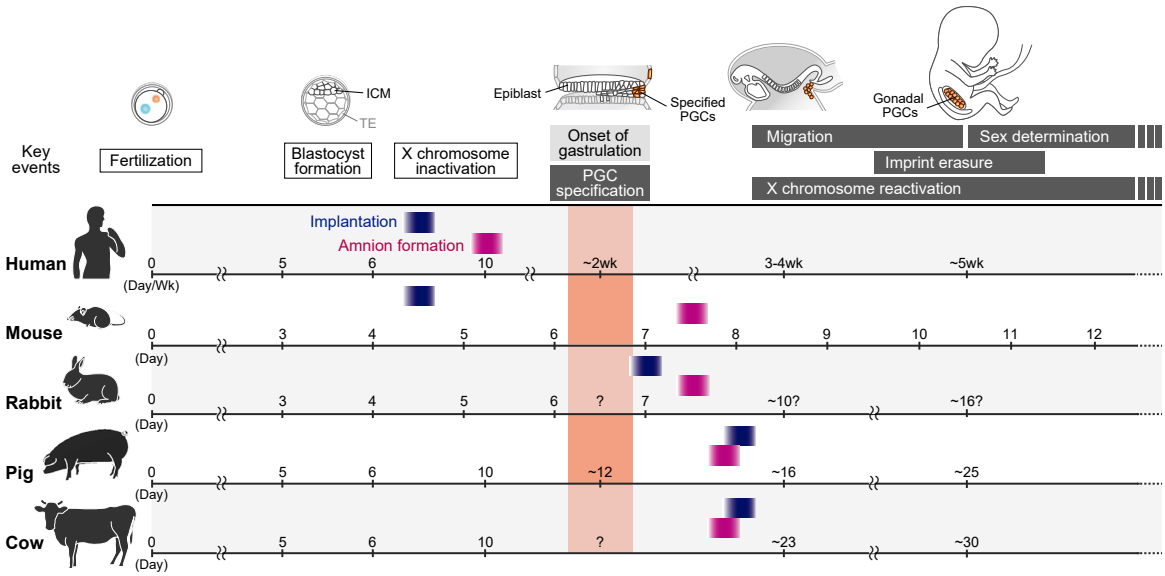


Figure 2. Diagrammatic representation of common developmental events during initial stages of embryogenesis

Primordial germ cells (PGCs) are specified during early gastrulation by different molecular mechanisms in mouse and human/pig (see text for details). The timing of PGC specification in the rabbit and cow are not yet known. Note that the onset of gastrulation and implantation/amnion development are not conserved across these species.

emerged for the attempts to recapitulate the culture conditions as close to those *in vivo* (Baillie-Benson et al., 2020; Xia and Izpisua Belmonte, 2019). Interpretation of the findings under these new *in vitro* methods requires careful consideration of the interplay of multiple variables, including geometry, mechanics, and signaling during cell-fate specification (Muncie et al., 2020). Importantly, it is imperative to compare development *in vitro* with the observations on embryos *in vivo* from species that follow similar events. The demonstration that 3D models of human epiblast development can be recapitulated to an extent *in vitro* without extraembryonic tissues (Simunovic et al., 2019; Zheng et al., 2019) and the recent report of gastruloids that recapitulate anterior-posterior elongation and the spatiotemporal organization of early human gastrula human embryos (Moris et al., 2020a) show that functional investigations into the roles of specific genes during germ layer induction using these systems may soon be possible. However, *in vivo* validation of the phenotypes will not be possible in the human. Thus, developing deeper understanding of the pig, cow, and rabbit gastrula may offer an alternative system for interrogating conserved mechanisms of cell-fate allocation during gastrulation.

In vitro models for advances in animal breeding

The derivation of PSCs from livestock species has thus far not achieved the same degree of success as in mouse and human. Attempts to establish PSCs have been based on the principles from mouse and human PSCs (hPSCs). Whereas the conditions used for naive mouse PSCs were not suitable, cells grown in media supplemented with Activin A and FGF, the key molecules used for hPSCs, supported the undifferentiated proliferation and pluripotent characteristics of pig PSCs (Alberio et al., 2010; Hou et al., 2016; Park et al., 2013). Recent refinements to the culture conditions have resulted in the derivation of region-specific bovine and sheep PSCs using FGF and an inhibitor of WNT signaling on cells grown on a feeder layer (Bogliotti et al., 2018; Vilarino et al., 2020). Other complex culture media including small-molecule inhibitors have also been reported to support the expanded potential of pig PSCs (Gao et al., 2019). These long-awaited cell lines from livestock species can be used to model embryo development in the same way as for human and mouse. Pig PSCs can also contribute to new approaches to animal breeding and genetic selection. For example, if the technology for the generation of *in vitro* gametes from pig PSCs becomes feasible, it will be possible to incorporate such advances for breeding, which will advance the pace of cumulative selection response and breed enhanced genotypes (Goszczynski et al., 2019). In future, the combination of such advances with precision gene-editing tools will facilitate the modification of alleles to generate enhanced phenotypes with the potential to transform agricultural production (Johnsson et al., 2019; Rexroad et al., 2019).

The complete cycle of the female mouse germline was reported following the aggregation of mouse PGCLCs with ovarian somatic cells (Hikabe et al., 2016). Using this

system, the oocytes produced were fertilized and gave rise to normal offspring, albeit at low frequency. More recently, advances in *in vitro* gametogenesis from hPSCs show that the aggregation of hPGCLCs with mouse gonadal somatic cells can support the development of oogonia/pro-spermatogonia after 120 days of culture, albeit at very low efficiency (Hwang et al., 2020; Yamashiro et al., 2018). These cells recapitulate many of the critical features of PGCs *in vitro* but fail to initiate meiosis. It will be important to determine whether failure to progress through meiosis is due to deficiencies in the supporting cells or to suboptimal culture conditions. Accomplishing the later phases of gametogenesis will unlock the potential of using *in vitro* gametogenesis as valuable tool for *in vitro* breeding in livestock.

Human organogenesis in pigs

The pig represents the most suitable xenogeneic source of human organs due to their size, breeding characteristics, and physiological similarities with humans (Sykes and Sachs, 2019). Pigs have a short gestation period (114 days), have large litters (6–12 offspring), and become sexually mature at 5 months. Moreover, many tissues and organs share anatomical and physiological properties with that of humans, including the pancreas, kidney, liver, lungs, eyes, and skin. Pigs have been genetically engineered to reduce their immunogenicity and increase compatibility with humans after xenotransplantation (Prather, 2013).

Recently, humanized pig hearts were shown to support long-term survival of baboons (up to 195 days) after orthotopic transplantation (Langin et al., 2018), demonstrating that immunological rejection can be prevented using genetic engineering. However, the recipient animals require continuous immunosuppression. The alternative approach to avoiding immune rejection would be to generate autologous human organs in pig chimeras (Wu et al., 2016).

Earlier reports showed limited contribution of hPSCs in interspecies chimeric fetuses (Gafni et al., 2013; James et al., 2006; Wu et al., 2017), which may be due to increased apoptosis or differences in cell-cycle progression of the injected cells, which might be mitigated by developmental stage matching with the host embryo (Mascetti and Pedersen, 2016). A species comparison between different types of hPSCs and pig embryonic cells showed broad equivalence (Ramos-Ibeas et al., 2019), indicating that strategies to closely match hPSCs with the recipient embryos may enhance overall chimeric integration. Overexpression of an anti-apoptosis factor or modulation of signal related to cell competition resulted in increased chimera efficiency when rodent- or human-primed PSCs are injected into mouse blastocysts (Masaki et al., 2016; Wang et al., 2018; Zheng et al., 2021). Preventing apoptosis using enhanced culture conditions can also improve interspecies chimerism between monkey and pig (Fu et al., 2020). Inhibition of apoptosis was also recently used to create pig chimeric fetuses containing a human endothelium. This was achieved through genetic ablation of *ETV2* gene in somatic cells used to generate embryos by nuclear transfer. These $ETV2^{-/-}$ blastocysts were complemented with hPSCs overexpressing *BCL2*, which resulted in all endothelial cells in these early embryos being of human origin (Das et al., 2020). Further follow-up studies may reveal whether such human-pig chimeras are viable at later stages of development. Nevertheless, this remarkable study provides proof of concept showing that combined incorporation of new tools such as gene editing, nuclear transfer, and stem cell technologies enable the robust and reproducible creation of engineered pigs with multiple applications in basic research and translational medicine.

AUTHOR CONTRIBUTIONS

R.A., T.K., and M.A.S. performed literature research and wrote the manuscript. All authors reviewed and approved the final version of the article.

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