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Pluripotency in the light of the developmental hourglass

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ABSTRACT

The hourglass model of development postulates divergence in early and late embryo development bridged by a period of developmental constraint at mid-embryogenesis. Recently, molecular support for the hourglass model of development has accumulated, with the emphasis on studies using zebrafish and *Drosophila* species. Across mammals, the hourglass model and specifically divergence in early development has thus far received little attention. Divergence in mammalian pre-implantation development is particularly interesting because of its potential impact on derivation of pluripotent embryonic stem cells. Here, we review recent findings that support the hourglass model of development. We provide striking examples of variation in key events in mammalian peri-implantation development and their potential consequences for pluripotency of embryonic stem cell lines, including mechanisms of cell signalling and differentiation, gene regulatory networks, X-chromosome inactivation, and epigenetic regulation. The variation in these processes indicates divergence in early mammalian development as was postulated by the hourglass model of development. We discuss the naive and primed states of pluripotency in light of this developmental divergence and their implications for human pluripotent stem cell states.

Key words: hourglass, phylotypic period, phylotypic stage, development, pluripotency, stem cells, embryonic stem cells, mammals, divergence.

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I. INTRODUCTION

(1) The developmental hourglass

According to the classical or early conservation model, first proposed by by Karl Ernst von Baer, young embryos of different species initially resemble each other and increasingly diverge as development progresses (Brauckmann, 2012). This idea that the earliest stages of embryogenesis are more conserved has resulted in the funnel-like model of development (also known as the 'developmental lock'). According to this model, diversity increases additively and progressively as development progresses. The funnel-like model of development is based on the intuition that changes in early development affect vital processes in later development, whereas changes in late development have no consequences for earlier stages. Accordingly, early development is more resistant to evolutionary change (Rasmussen, 1987; Irie & Kuratani, 2011). Technical advances in microscopy and more recently in transcriptomics have challenged this view and have demonstrated that despite similarities in appearance, at the cellular and molecular level, early embryonic stages are highly divergent among species (Hazkani-Covo, Wool & Graur, 2005; Domazet-Lošo & Tautz, 2010; Kalinka *et al.*, 2010; Irie & Kuratani, 2011; Kalinka & Tomancak, 2012). These observations lend support to the so-called hourglass model of development, which was first proposed in the 1990s (Duboule, 1994). It postulates that both early and late stages of embryogenesis are highly divergent among different species within a phylum and these diverging developmental stages are bridged by a period of developmental constraint. The stage of development at which an animal most closely resembles other species has been designated the phylotypic period. In vertebrates, this represents the stages that involve organogenesis (Slack, Holland & Graham, 1993; Richardson, 2012) (Fig. 1). The hypothesis of a morphological phylotypic period has been controversial (Hall, 1997; Richardson *et al.*, 1997). Indeed, the idea of divergence of early development to result in convergence at mid-embryogenesis is rather counterintuitive. During the earliest stages of development there is a transient population of pluripotent cells that give rise to all differentiated cell types. It is hard to understand how evolutionary adaptations can modify early development without interfering with the essential functions of the pluripotent cell population.

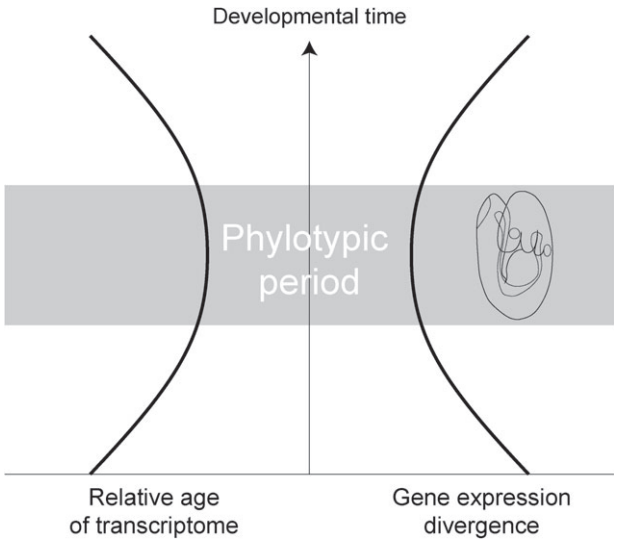

Fig. 1. The hourglass model of development postulates divergence in early and late development and evolutionary constraint at the phylotypic period. From this model it follows that expression of evolutionarily young genes is highest at early and late stages of development, whereas expression of evolutionarily old genes is enriched during mid-embryogenesis. Depicted is a Theiler stage 14 mouse embryo in the vertebrate phylotypic period.

Evolutionary constraint can be expected to be high in young embryos if it is assumed that errors during early embryonic development will impact on all subsequent developmental stages. Nevertheless, large-scale gene expression studies have provided molecular support for mid-embryonic transcriptional convergence and transcriptional divergence at early and late stages of development, which will be discussed further below.

(2) Transcriptional support for the hourglass model of development

In one of the earlier studies to address the hourglass model of development at the molecular level, the expression of nearly 1600 protein coding genes in mouse (*Mus musculus*) development was examined, using publicly available data from the gene expression database (Hazkani-Covo *et al.*, 2005). Protein divergence with their human (*Homo sapiens*) orthologs was calculated to measure the degree of conservation for each gene and the protein distance was subsequently plotted against the developmental stages in which the

genes were expressed. From this analysis, it could be concluded that at Theiler stages 12–15 of mouse embryonic development (a period spanning from the formation of the first somites until the formation of the forelimb bud), gene expression is more conserved between mouse and human than at earlier or later stages of development (Hazkani-Covo *et al.*, 2005). In a more recent study, comparison of the transcriptomes of a large number of consecutive developmental stages from the well-studied vertebrate model organisms mouse, chicken (*Gallus gallus*), African clawed frog (*Xenopus laevis*), and zebrafish (*Danio rerio*), revealed that pharyngular-stage embryos (i.e. the stage at which a series of paired branchial grooves on the inside of the throat have formed) have the highest transcriptome similarity in terms of orthologous gene expression (Irie & Kuratani, 2011). Notably, there were less transcriptional similarities from the cleavage stage up to the blastula stage among the different species and also later stages in development showed less similarity in gene expression (Irie & Kuratani, 2011). Additionally, a recent comparison of transcription factor orthologs in zebrafish and *X. laevis* revealed that their expression patterns were most similar in mid-embryonic development, peaking at the mid/late segmentation stage of zebrafish development and at the late tailbud stage for *X. laevis* embryos, which is slightly earlier than the aforementioned pharyngular stage (Irie & Kuratani, 2011; Schep & Adryan, 2013).

In another study, molecular support for the hourglass model was found by using a method called 'phylostratigraphy' (Domazet-Lošo & Tautz, 2010). In this method, the evolutionary age of genes is estimated by tracing the origin of each gene through sequence comparison across the genomes throughout the whole tree of life. Using a combination of phylostratigraphy and stage-specific gene expression data the authors were able to estimate the transcriptome age at given ontogenic stages of zebrafish development. Expression of evolutionarily conserved genes was observed at the phylotypic period, whereas expression of evolutionarily younger genes was enriched at earlier and later stages of zebrafish development. Using the same approach on series of transcriptome data at different stages of development of *Drosophila melanogaster*, the authors found that the evolutionarily oldest transcriptome is expressed at the arthropod phylotypic phase (Domazet-Lošo & Tautz, 2010). Further substantiation for the hourglass model comes from developmental stage-specific gene expression analysis in six *Drosophila* species, separated by up to 40 million years. At the arthropod phylotypic stage, conservation of gene expression patterns was highest, while gene expression patterns diverged in early and late stages of embryo development. Moreover, selective constraint was highest during the phylotypic period for genes with similar evolutionarily optimal expression levels across species (Kalinka *et al.*, 2010). In yet another study, a pairwise comparison of orthologous transcription factors in *Drosophila melanogaster* and *Anopheles gambiae* revealed that their expression patterns are more conserved at 16–18 h of *D. melanogaster* development and 40 h of *A. gambiae* development (Schep & Adryan, 2013). This time point is later in *D. melanogaster* development than the arthropod phylotypic stage at 8–10 h as described previously (Kalinka *et al.*, 2010), which could be explained by the different approaches used. However, when the authors compared genome-wide transcriptome expression instead of focusing on the expression dynamics of just the transcription factors, they did observe the highest similarity at the earlier identified arthropod phylotypic stage of *D. melanogaster* development (Schep & Adryan, 2013). In general, the expression pattern of transcription factors followed the hourglass model of development; similar expression patterns at mid-embryogenesis while diverging at early and late development (Schep & Adryan, 2013).

In the phylum Nematoda an hourglass model of expression divergence has also been observed. Comparison of expression profiles at 10 developmental stages (ranging from the four-cell stage to the first juvenile stage) in five *Caenorhabditis* species separated by at least 30 million years of evolution revealed expression conservation at mid-embryogenesis (stage 7), which has been designated the nematode phylotypic stage (Levin *et al.*, 2012). This period coincides with upregulation of homeobox transcription factors and other developmental regulators, as has been described for the phylotypic stage in other phyla (Slack *et al.*, 1993). Interestingly, genes expressed at the nematode phylotypic stage were more likely to result in a severe knockdown phenotype after RNA interference, indicating stronger evolutionary constraint at this stage than at earlier and later stages of development (Levin *et al.*, 2012).

In addition to the above findings from the animal kingdom, a recent study also found evidence for an hourglass model of development in plants, indicating convergent evolution of this developmental profile across kingdoms and a conserved logic of embryogenesis (Quint *et al.*, 2012).

(3) Proximate and ultimate explanations for the transcriptional hourglass

Precise temporal and spatial control of gene expression relies on the activity of *cis*-acting regulatory DNA regions, such as transcriptional enhancers. Enhancers are located at variable distances from their target genes and generally reside in non-coding parts of the genome (de Laat & Duboule, 2013). Through looping and binding to the promoters of genes and through their ability to recruit transcription factors and transcriptional activators, such as p300 and CREB binding protein, enhancers can modulate gene activity (Heintzman *et al.*, 2007; Xi *et al.*, 2007; Kim *et al.*, 2010). The binding of

transcription factors depends on sequence motifs in the enhancer region.

Because regulatory DNA sequences are occupied by sequence-specific transcription factors they are free of nucleosomes and as a result these sites are sensitive to DNase I treatment (Xi *et al.*, 2007). In a recent study, genome-wide mapping of DNase I hypersensitive sites (DHSs) was performed in 49 different human cell types, ranging from human embryonic stem (ES) cells to terminally differentiated fates (Stergachis *et al.*, 2013). Surprisingly, cluster analysis resulted in a dendrogram that reflected the developmental origin of the various cell types investigated. This means that mesoderm, ectoderm and endoderm derivatives clustered within their respective lineages, because they share common regulatory regions. From this dendrogram, the authors could infer which regulatory regions arose at seven distinct developmental branch points (i.e. the epiblast, mesoderm, hemangioblast, paraxial mesoderm, endothelia, hematopoietic, and lymphoid lineages). Evolutionary conservation of DHSs was less constrained at early and late developmental branch points and more constrained at mid-embryonic branch points (Stergachis *et al.*, 2013).

Enhancer elements can also be identified by the presence of histone 3 lysine 27 acetylation (H3K27ac) and histone 3 lysine 4 monomethylation (H3K27me1) (Creyghton *et al.*, 2010). In line with the above findings, enhancers that are active at mid-embryogenesis of zebrafish development (as determined by the presence of H3K27ac) are more conserved than enhancers that are active at early or late embryogenesis (Bogdanovic *et al.*, 2012). Together, these results provide a proximate explanation for the hourglass-shaped transcriptomes in development.

Different hypotheses have been put forward to explain the resistance to evolutionary change at mid-embryogenesis. A possible explanation could be that the mid-embryonic stages are isolated from the selective forces of the environment and are therefore less subject to evolutionary change (Domazet-Lošo & Tautz, 2010). It has also been suggested that the mid-embryonic period is constrained to support the spatial and temporal regulation that is required for complex multicellular life (Duboule, 1994; Irie & Kuratani, 2011; Quint *et al.*, 2012). The *Hox* gene cluster is a good example, because it regulates the body plan of the embryo along the anterior–posterior axis and any interference with *Hox*-regulated networks could be catastrophic to development (Duboule, 1994; Irie & Kuratani, 2011). Alternatively, interdependencies between the developing organ systems might impose morphological constraints on this developmental period (Irie & Kuratani, 2011). As an evolutionary explanation for the divergence in early embryogenesis it has been proposed that species-specific adaptation to ecological niches results in different reproductive strategies that impose selective pressures on embryo development (Kalinka & Tomancak, 2012).

In summary, the various examples discussed above demonstrate the accumulating evidence supporting the notion of a phylotypic period. Developmental stages that precede or follow the phylotypic period are more susceptible to evolutionary change. The hourglass model of development has not yet been extensively studied and verified in mammals. Nevertheless, various studies indicate that seemingly fundamental molecular mechanisms in the earliest stages of embryo development are not conserved among mammalian species. These studies will be discussed below.

II. FUNDAMENTAL DIFFERENCES AMONG MAMMALIAN PRE-IMPLANTATION EMBRYOS

(1) Blastocyst development

After fertilization, the mammalian zygote initially goes through a number of cleavage divisions, resulting in an increase in cell number with simultaneous reduction of cell volume (Fig. 2). The initial phase of development, before zygotic genome activation (ZGA), depends on maternally stored RNAs. The developmental stage at which ZGA occurs differs among species (Table 1). The mouse zygotic genome becomes activated at the two-cell stage, whereas in human and pig (*Sus scrofa*) ZGA occurs at the 4–8-cell stage and in cattle (*Bos taurus*), sheep (*Ovis aries*) and rabbit (*Lepus curpaeums*), at the 8–16-cell stage (Li, Lu & Dean, 2013; Xue *et al.*, 2013). At the morula stage, the mouse embryo first compacts and subsequently cavitates, resulting in a spherical epithelial layer of cells called the trophectoderm (TE), which encloses a cluster of cells called the inner cell mass (ICM; Fig. 2). Shortly after cavitation, TE cells become committed to the TE lineage, after which it gives rise to the placenta and the chorion (Suwinska *et al.*, 2008). After blastocyst formation, a second lineage segregation in the ICM results in the formation of the epiblast and the hypoblast (in rodents: primitive endoderm) lineages (Fig. 2). The primitive endoderm (PE) gives rise to parietal endoderm and the visceral endoderm of the yolk sac after implantation, whereas the epiblast gives rise to the embryo proper, the umbilical cord and the amnion. Notably, all three cell types of mouse blastocyst-stage embryos can give rise to stem cell cultures. The epiblast precursors can give rise to ES cells, which have the capacity to self-renew while preserving pluripotency; i.e. the potential to give rise to all differentiated cell types including germ cells (Evans & Kaufman, 1981; Martin, 1981). The PE cells can give rise to self-renewing PE stem cells, also known as XEN-cells (Kunath *et al.*, 2005), and the TE can give rise to trophectoderm stem (TS) cells (Tanaka *et al.*, 1998). Similar to ES cells, XEN and TS cells can differentiate within the lineage they were derived from.

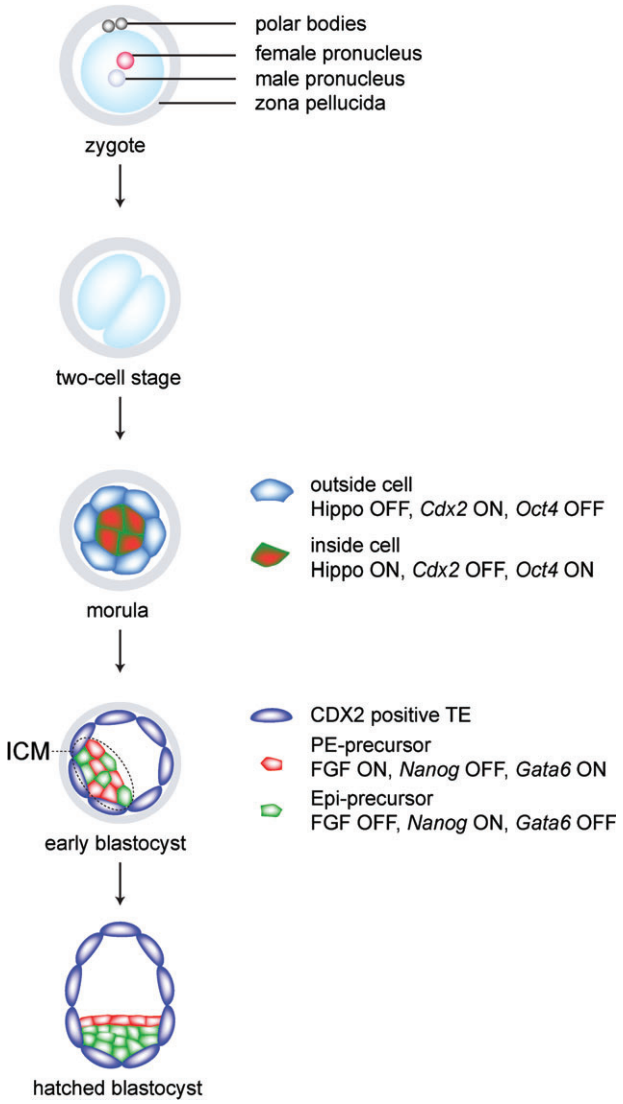

Fig. 2. Lineage segregation in mouse pre-implantation development. In mouse preimplantation development, the embryo undergoes two consecutive lineage segregation events, resulting in a late-blastocyst-stage embryo with three distinct cell lineages: trophectoderm (TE), epiblast (Epi), and primitive endoderm (PE). FGF, fibroblast growth factor; ICM, inner cell mass.

(2) Morphological divergence in early development

There are several remarkable variations on early mammalian embryo development. We will briefly discuss the most striking examples. Unlike the majority of eutherian mammals, marsupial blastocysts consist of a single layer of cells without ICMs. At one pole of these unilaminar blastocysts, a disc of cells is formed called the pluriblast. Subsequently, the pluriblast gives rise to hypoblast cells and epiblast cells, with the latter overlying the first (Selwood *et al.*, 1997; Eakin & Behringer, 2004). A unilaminar blastocyst stage has also been described for some eutherian mammals, such as the lemur (*Eulemur* spp.), the tenrec (*Hemicentetes* spp.) and the elephant shrew (*Elephantulus* spp.) (Edwards & Beard, 1997; Eakin & Behringer, 2004). Another striking morphological difference is the cup-shaped structure of E5.5 mouse egg cylinder-stage embryos. In most other mammals the epiblast develops into an embryonic disc with planar morphology. In non-rodent mammals the mesoderm lines the exterior of the yolk sac, but as a consequence of the egg cylinder morphology the mesoderm lines the interior of the yolk sac in mouse embryos, which is also known as 'inversion of the germ layers' (Eakin & Behringer, 2004). Furthermore, it has been known for a long time that armadillos (*Dasypus* spp.) exhibit obligate polyembryony, giving rise to a litter of four genetically identical progeny (Newman & Patterson, 1910). This remarkable reproductive strategy is unique to armadillos and it is the result of a division of the embryonic disc after implantation (Enders, 2002; Eakin & Behringer, 2004).

The above examples highlight variations in the formation and development of the epiblast. There are also prominent differences in implantation and the development of the extraembryonic lineages. For example, in human post-implantation embryos the entire trophoblast surrounding the embryo begins to differentiate, whereas in egg cylinder-stage mouse embryos cytotrophoblast (i.e. the precursor cell of all other trophoblasts) differentiation is mainly evident at the mesometrial pole. As opposed to the invasive implantation strategies of mouse and man, implantation in ungulates such as the pig is non-invasive. Unlike the mouse, porcine blastocysts do not immediately implant after hatching from the zona pellucida, which occurs at day 7–8 (Oestrup *et al.*, 2009). Before implantation, the porcine TE becomes dramatically elongated, changing the morphology from a blastocyst to a tubular structure that can reach 1 m in length (Oestrup *et al.*, 2009; Mummery *et al.*, 2011). Because of this elongation, implantation of porcine embryos is relatively late and occurs at day 13–14 of gestation, after the formation of the primitive streak (Oestrup *et al.*, 2009). Horse embryos implant at an even later stage in development. After hatching from the zona pellucida, horse embryos are enclosed in a glycoprotein structure called the capsule, which encloses the embryo until day 30–35 (Allen & Wilsher, 2009). At day 35 of equine development, just prior to implantation, essential organogenesis has already completed and fore and hindlimb buds have formed (Allen & Wilsher, 2009).

(3) Divergence in lineage segregation

(a) *Segregation of the ICM and the TE*

In the developing mouse embryo, segregation of the TE from the ICM depends on the Hippo signalling pathway (Cockburn *et al.*, 2013; Manzanares & Rodriguez,

Table 1. Important differences in early mammalian development

	ZGA	H3K27me3 patterns	DNA methylation patterns	Imprinted X-chromosome inactivation
Mouse	Two-cell stage	ICM > TE	ICM > TE	Yes
Rat	Late two-cell stage	ICM = TE	Unknown	Yes
Human	4–8-cell stage	ICM = TE	ICM < TE	No
Cow	8–16-cell stage	ICM = TE	ICM > TE	Inconclusive
Pig	4–8-cell stage	ICM < TE	ICM ≥ TE	No
Rabbit	8–16-cell stage	Unknown	Unknown	No
Sheep	8–16-cell stage	Unknown	ICM > TE	Unknown
References	Li *et al.* (2013), Xue *et al.* (2013) and Zernicka-Goetz (1994)	Erhardt *et al.* (2003), Gao *et al.* (2010), Okamoto *et al.* (2011), Ross *et al.* (2008), Saha *et al.* (2013) and Teklenburg *et al.* (2012)	Fulka *et al.* (2004) and Ma *et al.* (2012)	Dupont & Gribnau (2013)

H3K27me3, histone 3 lysine 27 trimethylation; ICM, inner cell mass; TE, trophectoderm; ZGA, zygotic genome activation.

2013). Trophectoderm development is initiated at the eight-cell stage from when embryonic cells progressively acquire an inner or outer character (Fig. 2). In outside cells, cell polarity sequesters the junction associated protein angiomotin (AMOT) to the apical protein complex, which results in suppression of Hippo signalling (Hirate *et al.*, 2013). As a consequence, YAP is not phosphorylated allowing its transportation into the nucleus, where it operates as a co-factor for the Hippo transcription factor TEAD4 to drive the expression of the TE transcription factor *Cdx2* (Nishioka *et al.*, 2009). CDX2 inhibits the expression of the pluripotency factor *Oct4*, resulting in an upregulation of TE genes and a downregulation of pluripotency genes (Niwa *et al.*, 2005). On the contrary, in inside cells the Hippo pathway is activated resulting in exclusion of YAP from the nucleus. In the absence of *Cdx2* upregulation the expression of *Oct4* and other pluripotency genes is sustained in the inside cells (Niwa *et al.*, 2005; Nishioka *et al.*, 2009; Cockburn *et al.*, 2013; Hirate *et al.*, 2013). Little is known about the role of Hippo signalling in the first lineage segregation in mammals other than the mouse. However, in rabbit, bovine, and human embryos, OCT4 expression is retained in the TE despite the clearly detectable presence of CDX2 protein. The simultaneous expression in the TE of these species suggests that OCT4 and CDX2 do not mutually repress each other as they do in the mouse (Kirchhof *et al.*, 2000; Niwa *et al.*, 2005; Kuijk *et al.*, 2008; Cauffman *et al.*, 2009; Chen *et al.*, 2009; Kobolak *et al.*, 2009; Berg *et al.*, 2011).

The expression of OCT4 in the CDX2-positive TE of expanded bovine and human blastocysts, but not in that of the mouse, might reflect differences in the timing of cellular commitment of this lineage (Kirchhof *et al.*, 2000; Niwa *et al.*, 2005; Kuijk *et al.*, 2008; Cauffman *et al.*, 2009; Chen *et al.*, 2009; Berg *et al.*, 2011). From approximately the 64-cell blastocyst stage onwards, mouse ICM cells become committed to their lineage and can no longer contribute to the TE (Handyside, 1978; Rossant & Vijh, 1980; Balakier & Pedersen, 1982). By contrast with mouse embryos, elegant embryo-manipulation experiments have demonstrated that bovine and human TE cells of full blastocysts are not restricted to the TE lineage, but can still give rise to ICM derivatives and should formally be considered pluripotent (Berg *et al.*, 2011; De Paepe *et al.*, 2013). It might be expected that this variation in timing of lineage commitment will also affect the differentiation potential of ES cell lines. Indeed, it appears that human ES differentiate more easily into TE cells than do mouse ES cells (Beddington & Robertson, 1989; Xu *et al.*, 2002).

(*b*) *Segregation of the epiblast and the PE*

The segregation of the PE and the epiblast depends on differential fibroblast growth factor (FGF) signalling between ICM cells (Feldman *et al.*, 1995; Arman *et al.*, 1998; Cheng *et al.*, 1998; Chazaud *et al.*, 2006). FGF4 activates the mitogen-activated protein (MAP) kinase signal transduction pathway, which results in activation of the PE-specific transcription factor GATA6 leading to the mutually exclusive expression of the pluripotency factor NANOG in the epiblast precursor cells and GATA6 in the PE precursor cells (Fig. 2). Mouse embryos that lack crucial components of this pathway (e.g. *Fgf4*, *Fgfr2* or *Grb2*) will develop ICMs that lack PE precursors and that are entirely composed of epiblast-like cells (Feldman *et al.*, 1995; Arman *et al.*, 1998; Cheng *et al.*, 1998; Chazaud *et al.*, 2006). When mouse embryos are cultured in the presence of small chemical compounds that interfere with FGF signalling they fail to develop GATA6-positive PE precursors and have ICMs composed entirely of NANOG-positive cells (Fig. 3) (Nichols *et al.*, 2009*b*; Yamanaka, Lanner &

Rossant, 2010). Surprisingly, chemical interference with FGF signalling failed to block hypoblast formation, and inhibition of the downstream component MEK only partially blocked hypoblast formation in developing bovine embryos (Fig. 3) (Kuijk *et al.*, 2012). In developing human embryos hypoblast formation is not blocked by FGF-receptor inhibition or MEK inhibition (Roode *et al.*, 2011; Kuijk *et al.*, 2012), although good-quality embryos contained more NANOG-positive ICM cells upon MEK-inhibition (Van der Jeught *et al.*, 2013). Apparently, the mechanism behind one of the earliest differentiation events in mammalian development is not conserved among mouse, bovine and human embryos.

From E4.5 onwards, the epiblast of the mouse embryo can no longer contribute to the PE lineage (Grabarek *et al.*, 2012). In line with these findings, mouse explanted post-implantation epiblasts fail to generate parietal endoderm. In marked contrast, explanted rat (*Rattus norvegicus*) epiblasts of gastrulating post-implantation E7.5 embryos still give rise to this PE derivative (Buehr *et al.*, 2008). As with human ES cells, this divergence in commitment could affect the differentiation potential of rat ES cell lines and it has indeed been described that rat ES cells give rise to differentiating colonies under conditions that completely block mouse ES cells differentiation (Shen *et al.*, 2011).

(4) Divergence in gene regulatory networks

Cellular identity depends on the activity of gene regulatory networks (GRNs) that determine global transcription profiles. In mouse and human ES cells, the transcription factors OCT4, SOX2 and NANOG have central roles in the GRN that maintains the pluripotent phenotype. Cooperatively, these factors bind to the promoter regions of a large number of genes to regulate their transcriptional activity. Thereby, they activate genes that promote pluripotency and self-renewal, and inhibit genes that are associated with lineage commitment and differentiation. Through auto-regulatory feedback loops OCT4, SOX2, and NANOG reinforce the pluripotent state (Boyer *et al.*, 2005; Loh *et al.*, 2006; Chen *et al.*, 2008; Sharov *et al.*, 2008).

A cross-species comparison of transcriptome dynamics revealed substantial differences in GRN structures between different stages of rat and mouse pre-implantation development (Casanova, Okoniewski & Cinelli, 2012). In mouse, bovine, and human pre-implantation development more than 40% of orthologous genes were differentially regulated (Xie *et al.*, 2010). Transcripts that were differentially regulated in development showed species-specific changes in transcription factor binding sites for the shared GRN master regulator OCT4. Several factors, such as the transcription factor OCT4, are shared between the GRNs of mouse, bovine, and human blastocysts, but by contrast

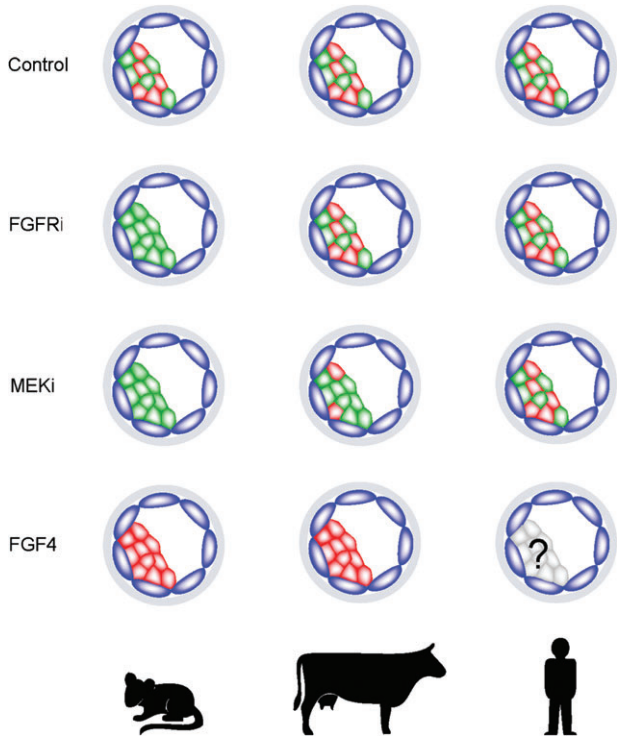

Fig. 3. The effects of modulation of the fibroblast growth factor (FGF) and mitogen-activated protein (MAP) kinase signalling pathway on developing mouse, bovine and human embryos. Without modulation of the FGF and MAP kinase signalling pathways, mouse, bovine, and human blastocysts have inner cell masses (ICMs) that heterogeneously express NANOG and GATA6. Upon FGF receptor inhibition (FGFRi), mouse embryos have ICMs composed entirely of NANOG-positive cells. FGFRi has no effect on ICM composition of bovine and human blastocysts. In mouse embryos, MEK inhibition (MEKi) results in all-NANOG positive ICMs. In bovine embryos, MEKi only partially alters the balance between NANOG- and GATA6-positive cells. In human embryos, the ratio between NANOG-positive cells and GATA6-positive cells is unaltered by MEKi. Stimulation of developing mouse and bovine embryos with FGF4 results in all GATA6-positive, NANOG-negative ICMs. The effect of FGF4 stimulation on developing human embryos is as yet unknown. Green cells, NANOG-positive epiblast precursors; red cells, GATA6-positive primitive endoderm precursors; blue cells, CDX2-positive trophectoderm cells. Images for cow and man were adapted from isotypes derived from the Gerd Arntz archive (http://www.gerdarntz.org/content/gerd-arntz#isotype).

with human and mouse, the pluripotency factor SOX2 is not included in the bovine GRN and the role of SOX2 appears to have been replaced by high mobility group box 1 (HMGB1) (Xie *et al.*, 2010). It should be noted that for this specific study entire embryos were used. The observed differences might therefore be the result of transcriptional variation in specific embryonic cell types such as the TE or precursors of the hypoblast

and may not apply to the epiblast precursors, since all of these cell types can be recognized at the blastocyst stage of mouse, human, and bovine development (Niwa *et al.*, 2005; Chazaud *et al.*, 2006; Kuijk *et al.*, 2008, 2012; Chen *et al.*, 2009). It would therefore be particularly interesting to determine the cell lineage that expresses HMGB1 and to examine the effects of interference with the expression of this gene on the formation of the bovine epiblast.

(5) Single-cell analysis

Single-cell analyses could overcome potentially confounding effects of heterogeneity in cell populations. In an important recent study, single-cell RNA sequencing was performed on maturated oocytes, zygotes, and blastomeres of two-, four-, and eight-cell-stage embryos, and on entire morula-stage embryos to uncover gene networks in mouse and human pre-implantation development (Xue *et al.*, 2013). By weighted gene co-expression network analysis on the RNA-seq datasets 25 modules of correlated transcripts were detected in human pre-implantation development, 9 of which were restricted to a single developmental stage. Function annotation revealed that embryos sequentially activated modules involved in gene transcription (four-cell stage), post-transcriptional RNA processing (eight-cell stage), and protein translation and cell energetics (morula stage). Interestingly, a comparison with transcriptome data of mouse pre-implantation embryos revealed that seven out of nine modules specific to human pre-implantation development were at least moderately preserved. The modules have in common that they are involved in general cellular processes such as cell cycle control, transcriptional and translational regulation, RNA processing, and cell metabolism. Mouse and human sometimes differed in the developmental stage at which certain modules were activated. For example, mouse oocyte and single-cell-stage modules were found to contain genes that were also expressed in human oocytes and zygotes, but in human embryos the expression was retained until the four-cell stage, possibly reflecting the later onset of ZGA in the human. In short, these findings demonstrate that, until the morula stage, human and mouse pre-implantation embryos share core-transcriptional programs involved in fundamental cellular processes (Xue *et al.*, 2013). It is worth mentioning that module preservation was restricted to the minority (7) of the initially 25 identified modules, in concordance with divergence in early embryo development. In order to understand better the mechanisms of the formation and the differentiation of the pluripotent stem cell population, it would be highly informative to complement the findings of this valuable study (Xue *et al.*, 2013) with a similar analysis on individual cells at later stages of development, after the first lineage decisions have been made.

(6) Divergence in binding of pluripotency factors

The pluripotency GRN can be imposed on mouse and human somatic cells through the ectopic overexpression of the transcription factors OCT4, SOX2, KLF4, and C-MYC, which results in the formation of so-called induced pluripotent stem (iPS) cells. iPS cells share many features with ES cells including the ability to differentiate into all three germ layers and into germ cells (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007). The capacity of the same set of factors to reinstate pluripotency in both mouse and human somatic cells is suggestive of conservation of the pluripotency GRN. However, there is little conservation of the genomic regions that are bound by the pluripotency factors OCT4 and NANOG in mouse ES cells. Just over half of the regions are conserved between mouse and rat and no more than one-third is conserved in other mammalian species (Fernandez-Tresguerres *et al.*, 2010). *Vice versa*, there is little conservation between the regions bound by human NANOG and OCT4 in human ES cells and orthologous regions in the mouse. The binding profiles of OCT4 and NANOG in the mouse are indeed markedly different from the binding profiles of these factors in human, with just ∼5% of the homologous target sites occupied in both species (Kunarso *et al.*, 2010). A large fraction of the OCT4 and NANOG binding regions is composed of transposable elements and there is little overlap in the contribution of the different families of repeats between the mouse and the human factors. Thus, in addition to divergence of the target sites, the rewiring of the pluripotency GRN could have occurred through transposition events of these elements, redistributing the binding regions for one or multiple transcription factors through the genome, resulting in pronounced differences in binding of key players of the GRN to their target genes (Kunarso *et al.*, 2010; Xie *et al.*, 2010). It is worth mentioning that, in addition to the lack of conservation of the orthologous regions of OCT4 and NANOG binding sites, the observed differences between mouse and human in binding profiles of OCT4 and NANOG might be caused by the different states of pluripotency of these cell types (for details, see Section III). However, such rewiring of the DNA binding sites is not unusual, as exemplified by the species-specific binding preferences of functionally conserved transcription factors in the livers of five different species, four of which are mammals (Schmidt *et al.*, 2010).

By contrast with other members of the pluripotency network, the sequence of the transcription factor NANOG shows relatively low conservation (Theunissen *et al.*, 2011). For example, the human ortholog has an identity score of 54% and the zebrafish ortholog has an identity score of just 13% relative to mouse NANOG. Although *Nanog* is dispensible for reprogramming (Carter *et al.*, 2014; Schwarz *et al.*, 2014), reprogramming of mouse *Nanog* null embryonic

fibroblasts is approximately 100-fold less efficient than reprogramming of Nanog$^{+/+}$ fibroblasts (Carter *et al.*, 2014). NANOG facilitates the transition to a stable self-sustaining pluripotency network at the late stage of the reprogramming process (Schwarz *et al.*, 2014). In spite of the high divergence in *NANOG* sequence, *NANOG* orthologs, including zebrafish *Nanog* are also capable of rescuing the decreased generation of iPS cells from mouse *Nanog* null cells (Theunissen *et al.*, 2011). Successful rescue depends on the homeodomain (i.e. the DNA-binding part of NANOG), which in itself is sufficient to induce pluripotency in reprogrammed *Nanog* null cells (Theunissen *et al.*, 2011). It is conceivable that Nanog orthologs are differentially regulated or interact with different protein partners as a result of the low degree of conservation outside the homeodomain, which may hint at divergence in the regulation of the pluripotency network.

In conclusion, species-specific variation in GRN-structures in stem cells and development could have considerable influence on the identity and behaviour of the pluripotent stem cell population, specifically if key factors with a node function are not conserved, as appears to be the case for cattle (Xie *et al.*, 2010). It is important to realize that when pluripotency GRNs are constructed on expression data derived from whole-blastocyst-stage embryos, the hypoblast and TE lineages could confound the discovery of factors with apparent node functions. It is therefore important to confirm that pluripotency GRN factors are indeed localized to the epiblast lineage, for example by immunofluorescence or *in situ* hybridization. Alternatively, single-cell analyses could overcome the issues associated with heterogeneous cell populations.

(7) Divergence in telomere lengthening

The ends of mammalian chromosomes are protected by telomeres, highly repetitive sequences of hexameric TTAGGG repeats. Due to incomplete replication, telomeres shorten with each mitotic division until they reach a lower threshold after which somatic cells enter senescence. In stem cells, telomeres are enzymatically lengthened by a reverse transcriptase called telomerase, the expression of which is regulated by the WNT/ß-catenin pathway that also drives stem cell self-renewal in a variety of stem cell systems including ES cells (Hoffmeyer *et al.*, 2012). Independent of its catalytic role in adding telomeric repeats, telomerase also induces the expression of WNT pathway components in ES cells (Park *et al.*, 2009). *In vivo*, telomere lengthening in early embryonic development and in the germ lineage safeguards genome integrity for future generations. In mouse cleavage-stage embryos, telomeres are maintained by a recombination-based mechanism and at around the early blastocyst stage, telomeres are lengthened enzymatically by telomerase. This process appears to be biased to the ICM, because these cells have longer telomeres than the TE cells. On the contrary, bovine embryos appear to have higher telomerase activity and concomitant telomere lengths in the TE and shorter telomeres in the ICM (Iqbal *et al.*, 2011). These differences indicate lower telomerase activity in the ICMs of bovine embryos compared to mouse embryos. Considering the molecular coupling of the WNT/ß-catenin and telomerase pathways, reduced telomerase activity in bovine ICMs may suggest there is also decreased WNT activity in these cells, which may affect their stem cell properties including the growth factor requirements of derivative cell lines. Furthermore, since high telomerase activity in mouse ES cells is a prerequisite for their infinite self-renewal capacities, decreased telomerase activity in bovine embryonic cells may lessen their self-renewal potential in culture, which could explain the lack of success in establishing long-term self-renewing bovine ES cell lines (Gandolfi *et al.*, 2012; Nowak-Imialek & Niemann, 2013).

(8) Divergence in histone modifications

Epigenetic modifications are heritable non-genetic alterations that influence the transcriptional activity of genes. A well-studied epigenetic modification is trimethylation of histone 3 at lysine residue 27 (H3K27me3), which is associated with transcriptional repression. In mouse ES cells, peaks of H3K27me3 can be detected at the promoters of developmentally important genes. In addition, the promoters of the same lineage-specific genes are often acetylated at lysine residue 9 of histone 3 and methylated at lysine 4 of histone 3, which are both epigenetic marks of open chromatin (Azuara *et al.*, 2006; Bernstein *et al.*, 2006; Pan *et al.*, 2007; Zhao *et al.*, 2007). These opposing epigenetic modifications are called bivalent domains and it has been hypothesized they may enable pluripotency of ES cells by silencing genes encoding developmentally important transcription factors while keeping them poised for activation (Azuara *et al.*, 2006; Bernstein *et al.*, 2006). The importance of epigenetic modifications on cellular phenotypes is further illustrated by the finding that interspecies differences in epigenetic marks are predictive of interspecies differences in gene expression and transcription factor binding (Xiao *et al.*, 2012). In mouse development, H3K27me3 can be detected in all blastomeres of four-cell-stage embryos. At the blastocyst stage, levels are very high in the ICM and relatively low in the TE (Table 1) (Erhardt *et al.*, 2003). This pattern is retained in embryo-derived stem cell lines; mouse TS cells (derived from the TE) and extraembryonic endoderm stem cells (derived from the PE) have lower global H3K27me3 levels than mouse ES cells (Rugg-Gunn *et al.*, 2010). Compared to the mouse, a reversed pattern was observed in *in vivo*-derived porcine blastocysts with higher H3K27me3 levels in the TE than in the ICM, which is surprising, given its alleged role in bivalent domains of mouse and human

pluripotent stem cell lines (Azuara *et al.*, 2006; Bernstein *et al.*, 2006; Gao, Hyttel & Hall, 2010). Differences in H3K27me3 levels between ICM and TE have not been described in bovine or human peri-implantation-stage embryos (Ross *et al.*, 2008; Okamoto *et al.*, 2011; Teklenburg *et al.*, 2012; Saha *et al.*, 2013). It is unresolved whether these species-specific patterns of H3K27me3 reflect global differences in transcriptional activity, which genomic regions are enriched for H3K27me3, and what the effects of different H3k27me3 patterns are on cellular phenotypes. Furthermore, little is known on how other chromatin marks (e.g. acetylation of H3K9 and methylation of H3K4) behave in early mammalian development. Finally, future studies that address these questions preferably take into account the heterogeneity of the ICM and distinguish epiblast from PE precursors.

(9) Divergence in X-chromosome inactivation

In female mammalian embryos, one of the X-chromosomes is inactivated to compensate for differences in gene dosage between males (XY) and females (XX). H3K27me3 plays an important role in this process. X-chromosome inactivation (XCI) is initiated by the binding of the non-coding RNA *Xist* to the X-chromosome, which leads to the establishment of further repressive epigenetic changes including the accumulation of H3K27me3. XCI in the mouse is a dynamically orchestrated process. Shortly after zygotic genome activation, monoallelic *Xist* transcription from the paternal X-chromosome results in its inactivation in all blastomeres (imprinted XCI). Imprinted XCI is inherited by the tissues derived from the PE and the TE. However, at the blastocyst stage, the inactivated paternal X-chromosome is reactivated exclusively in the epiblast precursors shortly resulting in the presence of two active X-chromosomes (XaXa). Subsequently, one of the two X-chromosomes is randomly inactivated in all the epiblast cells after which the inactivation pattern is epigenetically inherited by all their somatic daughter cells resulting in populations with either maternal or paternal inactive X-chromosomes. In mammals other than the mouse, imprinted XCI has thus far only been observed in rat and bovine extra-embryonic tissues. In the placentae of equids and primates, including humans, allele frequencies of X-linked gene expression are supportive of random X-inactivation in this tissue (Table 1) (Dupont & Gribnau, 2013). Bi-allelic expression of *XIST* in early pig, bovine, rabbit, rhesus monkey, and human embryos and bi-allelic expression of X-linked genes in the TE of rabbit, monkey, and human blastocysts also indicates the absence of imprinted XCI in these species (Okamoto *et al.*, 2011; Tachibana *et al.*, 2012). Apparently, the orchestrations of the epigenetic events that lead to XCI are not evolutionary conserved.

(10) Divergence in DNA methylation

In addition to histone modifications, epigenetic modifications can also be established at the DNA level. DNA methylation is well characterized and occurs most frequently on cytosines that are next to guanine nucleotides (CpG dinucleotides). DNA methylation patterns are dynamically regulated in early mouse development. Shortly after fertilization, the paternal genome is actively demethylated, a phenomenon that has also been observed in human and bovine development (Ma *et al.*, 2012). In the course of the cleavage divisions, the maternal genome is passively demethylated, resulting in a hypomethylated genome when the embryo reaches the blastocyst stage. At this stage of mouse development, active remethylation occurs exclusively in the ICM, while the TE remains hypomethylated. In sheep and rabbits, the paternal pronucleus is not actively demethylated as it is in mouse, human, and bovine zygotes. There appears to be much less decline in global DNA methylation when sheep embryos proceed through the cleavage divisions, resulting in a relatively hypermethylated morula-stage embryo. Through active demethylation exclusively in the TE cells, the ICM is relatively hypermethylated at the blastocyst stage of sheep development (Ma *et al.*, 2012). Remarkably, contrasting with mouse and sheep, the ICMs of human blastocysts and rhesus macaques are hypomethylated compared to the TE (Table 1) (Fulka *et al.*, 2004; Yang *et al.*, 2007). It is as yet unclear how these remarkable differences impact on the pluripotent stem cell population. Triple knockout for the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b has demonstrated that at least in mouse ES cells DNA methylation is not essential (Tsumura *et al.*, 2006). It is still an open question if this also applies to pluripotent stem cells of other species than the mouse.

III. DIFFERENT TYPES OF PLURIPOTENCY

(1) Naïve *versus* primed states of pluripotency

As we have discussed above, the hourglass model of development postulates evolutionary divergence in early and late stages of development and a period of evolutionary constraint in mid-embryogenesis. Divergence in early mammalian development has thus far received little attention, but as is evident from the examples above, there appears to be considerable variation in early embryonic processes among mammals, as may be expected if the hourglass model of development holds true for mammals. The highlighted processes are also directly or indirectly important to the proper development of the pluripotent epiblast and this may underpin the difficulties in identifying generic conditions that allow derivation of ES cells from mammals. Indeed, notable differences exist between mouse and human pluripotent stem cell lines. Mouse ES cells grow

in dome-shaped colonies, have the ability to contribute to chimaeras, and in the case of female cells both copies of the X-chromosome are active. Active LIF/STAT3 signalling and BMP4 signalling cooperatively maintain mouse ES cells in their pluripotent state (Table 2). Human ES cells grow as an epithelial monolayer reminiscent of the post-implantation epiblast and female human ES cells often have inactivated one of their X-chromosomes (Buecker & Geijsen, 2010; Kuijk *et al.*, 2011). Moreover, human ES cell self-renewal depends on the TGFß and FGF/MAP kinase signalling pathways (Table 2) (Vallier, Alexander & Pedersen, 2005).

From the mouse and the rat, post-implantation epiblast stem cell (EpiSC) lines can be derived that have several features in common with human ES cells such as colony morphology, growth factor requirements, and X-chromosome inactivation in female lines (Brons *et al.*, 2007; Tesar *et al.*, 2007). Unlike mouse ES cells, EpiSCs hardly contribute to chimaerism after injection into blastocyst-stage embryos. Because the post-implantation epiblast is primed for lineage specification and commitment, EpiSCs are considered to be in a primed pluripotent state. The profound similarities between EpiSCs and human ES cells suggest that human ES cells are also in a primed pluripotent state reflecting the human post-implantation epiblast. Thus, pluripotency appears to come in two different forms; a naïve pluripotent state represented by murine ESCs and a primed pluripotent state, resembling the post-implantation epiblast, represented by EpiSCs and human ES cells (Nichols & Smith, 2009).

(2) The pluripotent ground state of mouse embryonic stem cells

Mouse ES cells are generally cultured on a feeder layer of mitotically inactivated fibroblasts in the presence of fetal calf serum and LIF. Under these conditions, there are various subpopulations as determined by the heterogeneous expression of the pluripotency factors NANOG, REX1, and STELLA and the differentiation factors BRACHYURY, GATA6, HEX and SOX17 (Chambers *et al.*, 2007; Singh *et al.*, 2007; Hayashi *et al.*, 2008; Toyooka *et al.*, 2008; Canham *et al.*, 2010; Niakan *et al.*, 2010). This has led to the idea that under regular mouse ES cell conditions some cells are more primed towards lineage differentiation and other cells are in a more naïve state of pluripotency. Simultaneous inhibition of the FGF/Erk pathway and of glycogen synthase kinase 3ß (GSK3ß) is sufficient to maintain mouse ES cells in the absence of feeders, serum, and LIF (Ying *et al.*, 2008). These conditions, commonly known as 2i, are considered to maintain mouse ES cells in the ground state of ES cell self-renewal (Table 2). FGF/Erk inhibition prevents differentiation towards the PE lineage and blocks the progression from a naïve to a primed pluripotent state. GSK3ß is a negative regulator of WNT signalling, and its inhibition mimics activation of canonical WNT signalling. In the pluripotent ground state, mouse ES cells reinforce the pluripotency GRN and homogeneously express the pluripotency genes REX1, KLF4, and NANOG. Moreover, mouse ES cells cultured in 2i have significantly less H3K27me3 at promoter regions of silenced genes, resulting in considerably fewer bivalent domains. In regular mouse ES cell culture conditions, approximately 3000 genes are classified as bivalent, while under 2i conditions less than 1000 genes are classified as bivalent (Marks *et al.*, 2012). 2i conditions have also allowed the derivation of ES cells from previously refractory strains of mice and from rats (Buehr *et al.*, 2008; Nichols *et al.*, 2009*a*).

(3) Human ground-state naïve pluripotent stem cells

2i conditions fail to block hypoblast formation in human embryos and, unsurprisingly, 2i conditions fail to capture human ground-state pluripotency (Roode *et al.*, 2011; Kuijk *et al.*, 2012). However, in a recent landmark paper, Gafni *et al.* (2013) reported the culture conditions that allow stable maintenance of human pluripotent stem cells in a naïve state. When cultured in this naive human stem cell (NHSC) medium,

Table 2. Signal transduction pathways involved in the different types of mouse and human pluripotent stem cells

	Ground state	Naïve state without small chemical compounds	Primed
Mouse	LIF GSK3ß inhibition MEK inhibition	LIF Feeders/BMP4	FGF2 Nodal/activin
Human	LIF TGFß1 FGF2 MEK inhibition GSK3ß inhibition JNK inhibition p38 inhibition	N/A	FGF2 TGFß1/nodal/activin

N/A, not applicable.

human pluripotent stem cells share defining hallmarks of naïve pluripotency, including DNA hypomethylation, the ability to contribute to mouse development, and relatively high homologous recombination efficiency in gene targeting experiments (Gafni *et al.*, 2013). Importantly, gene expression analysis of nearly 10000 mouse–human orthologous genes revealed that mouse and human naïve pluripotent stem cells cluster together and apart from mouse EpiSCs and human ES cells.

The human naïve pluripotent ground state depends on a combination of growth factors and small chemical compounds that interfere with multiple cell signal transduction cascades (Table 2). It consists of a combination of essential growth factors that otherwise sustain primed human pluripotent stem cells (i.e. TGFß1 and FGF2), combined with the 2i conditions that support the murine pluripotent ground state (LIF and inhibitors of MEK and GSK3ß), and of chemical compounds that interfere with the c Jun N-terminal kinase (JNK) and p38/MAP kinase signal transduction pathways. It is not yet entirely clear how this cocktail of growth factors and chemical compounds imposes naïve pluripotency on human pluripotent stem cells. The simultaneous stimulation of FGF2 signalling and inhibition of MEK implies that the beneficial effect of FGF2 is not exerted through MEK. In addition to the MAP kinase pathway, FGF can also activate the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K)/AKT pathway. In primed human ES cells, the beneficial effects of FGF2 are exerted through this pathway, because inhibition of the PI3-K/AKT pathway has adverse effects on pluripotency of human ES cells (Armstrong *et al.*, 2006; Ding *et al.*, 2010). Moreover, FGF2 can be substituted by heregulin and insulin-like growth factor (Igf-I), potent activators of the PI3-K/AKT pathway, or a constitutively active AKT to maintain primed human ES cells undifferentiated (Singh *et al.*, 2012). Future experiments should determine whether the beneficial effects of FGF2 on the human ground state of pluripotency also depend on activated PI3-K/AKT-signalling, as is the case for human ES cells that are in the primed state of pluripotency.

The positive effects of interference with the JNK pathway on naïve human pluripotency probably relate to the activation of this pathway upon differentiation of mouse and human ES cells and to the negative role of this pathway in reprogramming to pluripotency (Van Hoof *et al.*, 2009; Yao *et al.*, 2013). The requirement for inhibition of the p38/MAP kinase pathway on naïve pluripotency is more surprising, since inhibition of this pathway promotes cardiomyocyte formation from human ES cells (Graichen *et al.*, 2008). Under serum-free culture conditions, but without chemical inhibitors, mouse ES cells require active BMP4 signalling. In addition to the induction of expression of *Id* genes (Ying *et al.*, 2003), BMP4 exerts its positive effects on mouse ES cell self-renewal through inhibition of the p38/MAP kinase pathway. The importance of this effect is illustrated by the observation that inhibition of the p38/MAP kinase pathway allows the establishment of mouse ES cells from embryos that have compromised BMP4 signalling (Qi *et al.*, 2004). In contrast to mouse ES cells, addition of BMP4 to human ES cells results in the differentiation towards trophoblast (Xu *et al.*, 2002). It would be interesting to learn if the chemical compound that blocks p38/MAPK signalling can be substituted with BMP4 in the context of NHSC medium to support naïve human ES cell pluripotency. Another important question to address is what the effects of the growth factors and chemical compounds that support human naïve pluripotency are on developing human embryos. Furthermore, does NHSC medium support the generation of ES cells from species that hitherto have been refractory to ES cell derivation?

The important study of Gafni *et al.* (2013) builds on the findings of recent decades, in which there has been a dissection of the mouse and human pluripotency GRNs and the cell signalling pathways that converge onto these networks, and demonstrates that this knowledge enables better control over the pluripotent state. Another important lesson from this study is that despite huge differences in early development, the pluripotent stem cell populations of mouse and human embryos have many features in common (Gafni *et al.*, 2013).

Nevertheless, the fact that human naïve pluripotency requires interference with other signal transduction pathways than mouse naïve pluripotency underscores the early embryonic differences that can exist between mammalian species, as we have discussed herein and which might be expected based on an hourglass model of development. Future studies can focus on these essential differences between mouse and human naïve pluripotent stem cells.

IV. CONCLUSIONS

(1) There has recently been a rapid increase in the molecular evidence that supports the hourglass model of development.

(2) Events that are seemingly fundamental from a mouse's perspective appear to be regulated differently in early development of other mammalian species indicating that the hourglass model of development, which postulates divergence between early embryos, also applies to mammals.

(3) Studies aimed at elucidating epigenetic patterns and activity of gene regulatory networks in early development should preferably try to discriminate between epiblast-, hypoblast-, and trophectoderm-specific patterns to avoid confounding the results from one cell type with another.

(4) A thorough comparison at the transcriptional and epigenetic levels is necessary to understand fully the scale of divergence in early embryo development, but also to distill the common denominators.

(5) In the near future we can expect to get a much more complete picture of the variation among species in early developmental processes, which will be instrumental to control pluripotency and differentiation fully in each species.

(6) The recent establishment of naïve human pluripotent stem cells provides a powerful new tool to study the degree of divergence with the mouse in one specific cell type.

(7) The difference in culture conditions between mouse and human naïve pluripotent stem cells emphasizes the existence of developmental differences between these species.

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