# Mouse pluripotent stem cells at a glance

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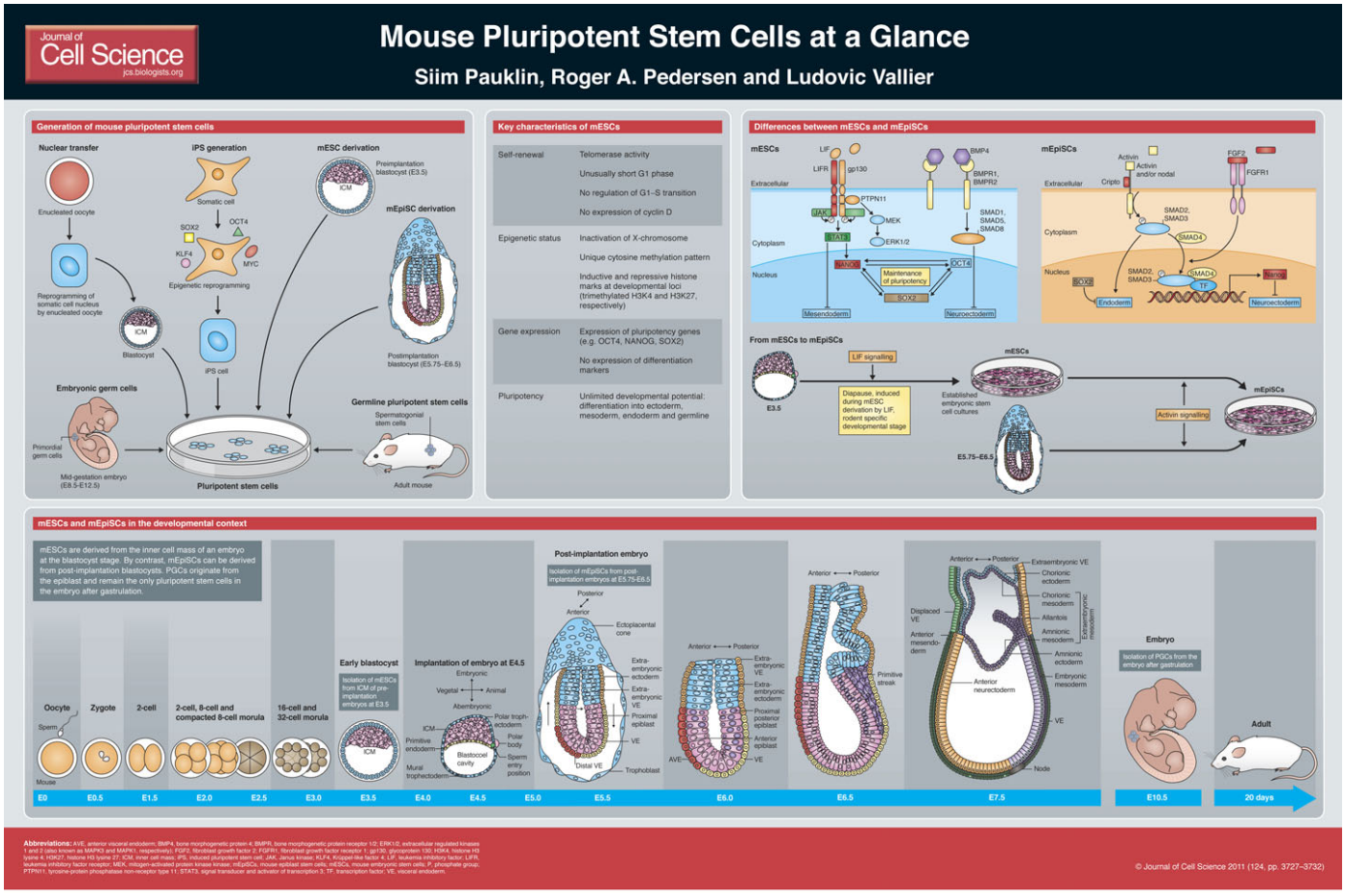
Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of an embryo at the blastocyst stage. They are characterised by the ability to proliferate indefinitely (or self-renew) in vitro while maintaining pluripotency, i. e. the capacity to differentiate into germ cells and a broad number of cell types that originate from the three primary germ layers endoderm, mesoderm and ectoderm. Defining the identity of embryonic pluripotent stem cells has been a main focus for the stem cell field since the derivation of the first mouse ESCs (mESCs) in 1981 (Evans and Kaufman, 1981; Martin, 1981). Indeed, mESCs could simply be the product of an in vitro culture system or be truly representative of pluripotent stem cells that are naturally present in the early embryo, and these two possibilities have immense consequences for the relevance of ESCs to model early mammalian development and for their use in clinical applications.

The technical difficulties involved in comparing a simplified in vitro system to a complex in vivo model have been a main challenge in answering the question of stem cell identity. Nevertheless, recent development, such as the derivation of ESCs from different species including the isolation of human ESCs (hESCs) (Thomson et al., 1998) and the possibility of isolating epiblast stem cells (EpiSCs) from the late epiblast of rodent post-implantation embryos (Brons et al., 2007; Tesar et al., 2007), have allowed new comparative studies and have helped to reveal the conserved mechanisms that control pluripotency across species. In this Cell Science at a Glance article, we will summarise our latest knowledge regarding the embryonic identity of pluripotent cells, as well as the implications this has for basic studies and future translational applications.

## Pluripotency during early embryonic development

Mouse development begins with the fertilised oocyte, which subsequently gives rise to the embryo and all extra-embryonic tissues that are necessary for supporting its development. This totipotent state is maintained from the zygote stage until the early blastocyst (or late cleavage) stage (Tarkowski et al., 2010). Thereafter, cells gradually lose their developmental potency and undergo functional specialisation (reviewed in Cockburn and Rossant, 2010; Surani et al., 2007). When the morula stage is reached, the cells form a compact sphere containing two different cell layers. The outermost layer becomes the trophectoderm (TE), which will form the fetal portion of the placenta following implantation, whereas cells within the embryo remain pluripotent. A fraction of these embryonic cells then differentiates into the primitive endoderm (PrE), which will give rise to extra-embryonic tissues, such as the yolk sac, allantois and amnion. By embryonic days 3–4 (E3–E4) in the mouse, the embryo comprises TE cells, PrE and the pluripotent stem cells of the inner cell mass (ICM). The cells of the ICM represent the

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population that is commonly isolated to obtain ESCs.

After implantation (at approximately E4.5 in mouse embryos), pluripotent ICM cells form an epithelial layer called the epiblast, and acquire a central cavity (reviewed in Hayashi et al., 2007). The pluripotent epiblast differentiates during gastrulation, when it forms the primitive streak at the posterior–proximal part of the embryo. Gastrulation gives rise to the three primary germ layers, namely endoderm, mesoderm and neuroectoderm, from which all adult tissues are derived (reviewed in Tam and Loebel, 2007). After gastrulation, primordial germ cells (PGCs) originating from the epiblast remain the only source of pluripotent stem cells in the embryo. This summary of early development illustrates that pluripotent cells exist during different stages of embryonic development, which suggests the existence of a diverse range of pluripotent states in vivo.

## Fundamental characteristics of mESCs

### Key characteristics of self-renewal in ESCs

In mESCs, the potential to self-renew is marked by two main characteristics: rapid proliferation and high telomerase activity. The mechanisms that maintain these characteristics are key to allowing ESCs to maintain their pluripotent status and grow indefinitely without resulting in the genetic anomalies usually observed in transformed cells. Importantly, those mechanisms are likely to rely on the transcription factors that are involved in regulating pluripotency. For example, the pluripotency transcription factor KLF4 has been shown to control telomerase reverse transcriptase (TERT) expression in mESCs (Wong et al., 2010), which prevents telomere shortening through the addition of TTAGGG repeats to chromosome ends. Interestingly, telomerase is strongly activated at the morula–blastocyst transition, which suggests that the mechanisms that maintain telomere length are conserved in vivo and in vitro (Bekaert et al., 2004).

The regulation of the cell cycle in mESCs also seems to be unique, because mESCs have an unusually short G1 phase and are devoid of regulation at the G1–S transition (reviewed in Burdon et al., 2002). This has been linked to the absence of cyclin D proteins (D1, D2 and D3), the presence of hyperphosphorylated retinoblastoma (RB1) protein and unresponsiveness to activity of cyclin-dependent kinase 4 (CDK4) (Savatier et al., 1996). In turn, induction of differentiation through the withdrawal of leukemia inhibitory factor (LIF) restores the expression of D-type cyclins and CDK4-associated activities, which provides additional evidence that such cell cycle regulation could be linked to the mechanisms that maintain pluripotency (Savatier et al., 1996). Interestingly, transcripts of cyclin D1, D2 and D3 cannot be detected in the epiblast of mouse embryo, whereas their expression is strongly activated in differentiating germ layers during gastrulation (Wianny et al., 1998). Thus, mechanisms that control proliferation of pluripotent stem cells in vivo could resemble those described in mESCs in vitro.

### Experimental characterisation of mESC pluripotency

Pluripotency represents the most important property that distinguishes ESCs from adult stem cells that are also capable of self-renewal but are unable to differentiate into different cell types. Therefore, demonstrating the capacity of mESCs to differentiate into a broad range of cell types remains the most significant step in their characterisation and this can be assessed in a variety of experimental settings with various stringencies. For example, the capacity of mESCs to differentiate into a broad number of tissues can be validated in vitro by assessing their ability to form embryoid bodies that contain differentiated derivatives of endoderm, mesoderm and ectoderm (reviewed by Jaenisch and Young, 2008). In vivo, mESCs injected into immune-compromised mice are able to form teratomas that contain tissues originating from all three germ layers. Germ line transmission through the generation of chimaeras by cell aggregation with eight-cell embryos or cell injection into blastocysts has further shown the potential of mESCs to contribute to all tissues of the adult organism including germ cells. This approach has been used for the past three decades to generate transgenic mice for functional genomic studies. The most stringent criterion for pluripotency to date is tetraploid complementation (Eggan et al., 2001; Nagy et al., 1990). In this approach, mESCs are aggregated with tetraploid host morulae, whose cells can only contribute to extraembryonic lineages but not embryonic lineages. Tetraploid complementation demonstrates that the donor cells are able to give rise to all cell types in the body and form a fully functional and fertile organism independent of any support from host cells.

### Epigenetic status of mESCs

The epigenetic status of the mESC genome is characterised by a unique DNA methylation pattern that includes hypomethylated imprinted genes and the presence of two activated X chromosomes (reviewed by Bernstein et al., 2007; Meissner, 2010). Furthermore, mESCs display a substantially higher level of hydroxymethylation of cytosines in DNA compared with differentiated cells (Ito et al., 2010), whereas developmental genes are marked by both transcriptionally repressive trimethylated H3K27 and active trimethylated H3K4 in mESCs (Bernstein et al., 2006). These 'bivalent domains' keep the developmental genes silenced in mESCs, but also keep the genes poised for rapid expression during cellular commitment to specific lineages.

These features collectively indicate a unique epigenetic status for mESCs that is characterised by a pattern of epigenetic marks that are distinct from somatic cells. Based on this characteristic, mESCs could be representative of a 'ground state' for pluripotency, meaning a basal proliferative state that is free of epigenetic restriction and has minimal requirements for extrinsic stimuli (reviewed in Nichols and Smith, 2009; Wray et al., 2010). Interestingly, some of these specific marks, including the bivalent domains, are present in the embryo at the blastocyst stage (Rugg-Gunn et al., 2010), which indicates some similarity between the epigenetic status of mESCs and the cells of the pluripotent epiblast (Ying et al., 2008). However, the lack of information concerning global methylation in vivo renders the overall picture incomplete. Thus it is possible that some aspects of the epigenetic profiles of mESCs, especially DNA methylation, are partially caused by culture conditions in vitro and do not reflect an accurate state of normal development.

## The pluripotency factors OCT4, NANOG and SOX2

The characteristic gene expression program of pluripotent ESCs is achieved through the interplay between signalling pathways, regulatory RNA molecules, chromatin-modifying enzymes and transcription factors (reviewed in Jaenisch and Young, 2008). An autoregulatory network of three transcription factors – OCT4 (officially known as POU5F1), NANOG and SOX2 – has a central function in the maintenance of pluripotency. These factors continuously suppress the expression of lineage-specific genes and maintain the expression of pluripotency genes (Boyer et al., 2005; Loh et al., 2006). Accordingly, genetic studies have shown that the absence of these factors in mESCs induces differentiation towards extra-embryonic lineages. NANOG is a notable exception, as mESCs with a mutation of the *Nanog* gene can maintain a pluripotent state (Chambers et al., 2007). Furthermore, several studies also suggest that OCT4, SOX2 and NANOG contribute to mechanisms that control early specification of the cell fate (Thomson et al., 2011). Importantly, the function of OCT4 and SOX2 is conserved in the pre-implantation

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embryo, because disruption of these genes results in differentiation of ICM into trophectoderm and extra-embryonic endoderm (Chambers et al., 2003; Nichols et al., 1998; Ying et al., 2003). The function of *Nanog* in vivo remains more elusive, as embryos with a mutation in this gene fail to develop after the blastocyst stage. These observations suggest that NANOG is essential for the maintenance of a pluripotent ICM (Chambers et al., 2007). Therefore, pluripotency mechanisms that are controlled by key transcription factors in vitro could be representative of a natural path of development but detailed studies in vivo with tissue-specific mutants could still reveal additional mechanisms for these processes. Moreover, microarray analyses have shown that the gene expression profile of mESCs and stem cells in the early epiblast vary to some extent, which suggests that some pluripotency factors are different in vivo and vitro.

Accordingly, a large number of additional transcription factors, including DAX1 (Niakan et al., 2006), SMAD1, SMAD5 and SMAD 8 (Ying et al., 2003), TBX3 (Niwa et al., 2009), KLF4 (Niwa et al., 2009), NR5A2 (Heng et al., 2010) and SALL4 (Zhang et al., 2006), have been shown to be involved in maintaining pluripotency in mESCs, and none of these factors is essential for the development of pre-implantation embryos. Such observations could be explained by functional compensation, since these genes are part of broad families of transcription factors that are often co-expressed. Alternatively, these factors could only be essential at the diapause stage (the delayed development of the blastocyst), similar to LIF signaling (see below). Thus, further experiments in vivo are necessary to fully demonstrate that the transcriptional network that maintains pluripotency in mESCs is representative of the normal mechanism that regulates pluripotency in vivo.

## Signaling pathways maintaining pluripotency in mESCs

Signalling through LIF represents the most important pathway for the maintenance of pluripotency in mESCs (Smith et al., 1988; Williams et al., 1988). Indeed, LIF is systematically required when mESCs are grown in combination with two small-molecule inhibitors (referred to as '2i') that block glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase kinases (MAP2Ks, also known as MEKs) or when grown in the presence of bone morphogenetic protein (BMP) (Ying et al., 2003; Ying et al., 2008). Importantly, although the role of the LIF signalling pathway is well established in pluripotent cells in vitro, genetic studies have shown that the absence of glycoprotein 130 (gp130) or signal transducer and activator of transcription 3 (STAT3) (Nichols et al., 2001; Takeda et al., 1997) – both of which are necessary for LIF signalling – has no consequence on early mouse development but are required for the diapause stage (reviewed in Lopes et al., 2004). These studies indicate that LIF-dependent mESCs are representative of pluripotent cells of the diapause embryo just before implantation. This last hypothesis can also explain the difficulty to derive 'ground state' pluripotent ES cells (i.e. cells that are permissive to germ-line transmission) in species other than rodents, because other mammalian species are not capable of halting development at the diapause stage. Overall, a number of questions concerning the correlation between the signalling pathways that control pluripotency in mESCs and the mechanisms that protect the pluripotent status of embryonic cells during pre-implantation stages remain unanswered.

## Differences between mESCs and mEpiSCs

For almost 25 years, LIF-dependent mESCs were the only pluripotent cell type derived form mouse embryos, despite the broad evidence that additional pluripotent state exist during early development. The discovery that EpiSCs (Table 1) can be derived from post-implantation blastocysts (E5.75–E6.5) (Brons et al., 2007; Tesar et al., 2007) provided a first indication that additional stages of pluripotency can be stabilised in vitro.

mESCs and mEpiSCs share the properties of self-renewal and pluripotency, but EpiSCs cannot colonise the ICM of an embryo at the blastocyst stage. Consequently, their capacity to participate to the germ line has not been demonstrated. Furthermore, EpiSCs express OCT4, SOX2 and NANOG but lack expression of other pluripotency factors that have been shown to be essential for mESCs, such as KLF4, Stella and DAX1. This suggests fundamental differences in the mechanisms that maintain pluripotency in the two cell types.

Importantly, mEpiSCs and mESCs also differ in phenotype and function (Table 1) (Brons et al., 2007; Tesar et al., 2007). mEpiSCs have a flattened morphology compared with the rounded domed colonies of mESCs, as well as a much lower efficiency of clonal propagation from single cells. Like mESCs, mEpiSCs express TERT and display telomerase activity, indicating that their capacity for self-renewal is intact. Whereas cell cycle regulation in mEpiSCs remains to be investigated in detail, the doubling time of mEpiSCs is 18 hours, compared with only 10–14 hours doubling time of mESCs, suggesting that a normal G1–S transition occurs in mEpiSCs (Brons et al., 2007; Tesar et al., 2007).

Two important differences between mESCs and mEpiSCs are that mEpiSCs display X-chromosome inactivation as well as stability of the genetic imprint (B. Sun, M. Ito, S. Mendjan, Y. Ito, A. Murrell, L.V., A. F. Ferguson-Smith and R.A.P., unpublished observations). This epigenetic status is shared with the late epiblast of the post-implantation embryo, which reinforces the similarity between cultured mEpiSCs and their in vivo counterpart. This similarity has been confirmed by gene expression profile experiments, which show that EpiSCs are closely related to the pluripotent stem cells located in the epiblast of the post-implantation embryo (Brons et al., 2007; Tesar et al., 2007).

Finally, mEpiSCs rely on activin–nodal and FGF2 signalling to sustain their pluripotent state, whereas LIF signalling is dispensable and BMP4 induces differentiation (instead of promoting self-renewal as it does in mESCs) (Brons et al., 2007; Tesar et al., 2007). Importantly, activin–nodal signalling has been shown to maintain the expression of *Nanog* through its downstream effectors SMAD2 and SMAD3, and simple overexpression of NANOG is sufficient to maintain the pluripotent status of mEpiSCs by blocking neuroectoderm differentiation that is induced by the absence of activin–nodal signalling (Vallier et al., 2009a). Thus, in mEpiSCs, pluripotency relies on inductive signals as opposed to the inhibition of GSK3 and MEK signalling pathways that take place in mESCs (Ying et al., 2003). Moreover, genetic studies in mouse have shown that absence of nodal–activin signalling blocks expression of pluripotency genes in the late epiblast, while resulting in ectopic expression of neuroectoderm markers. Thus, the function of activin–nodal signalling pathway in mEpiSCs could be representative of a mechanism that occurs in the pre-gastrulating embryo. mEpiSCs might, therefore, closely resemble their in vivo counterpart providing a unique system to study the mechanisms that control gastrulation.

## miPSCs – stem cells without an embryonic origin

While remaining the most commonly used mouse pluripotent stem cells, mESCs and mEpiSCs are now frequently associated with and compared to induced pluripotent stem cells (iPSCs). Indeed, this cell type provides for the first time a non-embryonic source for pluripotent stem cells, making them uniquely interesting for generating cell types from different genetic backgrounds and different species. Mouse iPSCs (miPSCs) are generated by direct reprogramming of somatic cells using the

**Table 1. Comparison of different types of pluripotent stem cells**

| | Early blastocyst (ICM) | mESC | Late epiblast | mEpiSC |
|---|---|---|---|---|
| Growth properties | | | | |
| Signals that maintain the cellular state | Unknown | LIF, STAT3, BMP4, WNT | Activin, nodal, TGFβ, FGF2 | Activin, nodal, TGFβ, FGF2 |
| Signals that control specification | Unknown | Activin, nodal TGFβ, FGF2, ERK1/2, GSK3β | BMP, nodal, WNT, FGF | BMP4, nodal, FGF, WNT |
| Morphology of colonies | n.a. | Domed | n.a. | Flattened |
| Clonal propagation from single cells | n.a | Efficient | n.a. | Inefficient |
| Doubling time | Unknown | 10–14 hours | Unknown | 18 hours |
| Telomerase activity | Yes | Yes | Yes | Yes |
| Cell cycle | Unknown | Absence of D-type cyclins | Unknown | Low expression of D-type cyclins |
| Gene expression profiles | | | | |
| Expression of pluripotency markers (NANOG, OCT3, OCT4, SOX2) | +++ | +++ | +++ | +++ |
| ICM transcripts (PECAM1, TBX3, GBX2) | +++ | +++ | –/+ | –/+ |
| Epiblast and early germ layer transcripts (OTX2, EOMES, FOXA2, brachyury, CER1, LEFTY, FGF5, NODAL) | –/+ | –/+ | +++ | +++ |
| Germ-cell-specific genes (stella, *PIWIL2*, *STRA8*, *DAZl*, BLIMP1) | +++ | +++ | –/+ | –/+ |
| Epigenetic status | | | | |
| Inductive and repressive histone modifications (H3K4me3 and H3K27me3, respectively) | Bivalent | Bivalent | Unknown | Unknown |
| X-chromosome inactivation | No | No | Yes | Yes |
| OCT4 enhancer activity | Distal | Distal | Proximal | Proximal |
| Developmental potential | | | | |
| Differentiation in vitro | | Yes | n.a. | Yes |
| Embryonic bodies | n.a. | Yes | n.a. | Yes |
| Teratoma formation | Yes | Yes | Yes | Yes |
| Chimaera production | Yes | Yes | No | No |
| Tetraploid complementation | Yes | Yes | No | No |

n.a., not available; +, upregulation; –, downregulation respectively.

overexpression of pluripotency factors such as OCT4, SOX2, KLF4 and MYC (Takahashi and Yamanaka, 2006). mESCs and mIPSCs share the same properties that are important for pluripotency (germline transmission and tetraploid complementation) and self-renewal (telomerase activity is present, but how cell cycle regulation of miPSCs is controlled remains unknown) (reviewed in Yamanaka, 2009; Hanna et al., 2010b). They also rely on the same signalling pathways to maintain their pluripotent status (LIF+2i), the same core transcription factors (OCT4, SOX2 and NANOG) and they share similar gene expression profiles with mESCs. The epigenetic status of miPSCs remains to be determined in detail but the cells display two active X chromosomes and bivalent domain marks (Fouse et al., 2008). Therefore, mIPSCs and mESCs are almost indistinguishable from one another, even at the molecular level. Interestingly, recent studies have shown that somatic cells can be directly reprogrammed into mEpiSCs by simply blocking LIF signalling and promoting activin–nodal signalling (Han et al., 2010). Consequently, the pluripotent state and, thus, the embryonic identity of miPSCs can be defined by the culture conditions used for their derivation. Nevertheless, these studies collectively suggest that mESCs and miPSCs share a common pluripotent state and – by extension – a common embryonic identity, whereas, divergence of the two cell types might simply represent defects in the reprogramming mechanisms.

## Perspectives

The broad similarities between mEpiSCs and pluripotent cells of the late epiblast suggest that both cell types are functionally equivalent. Surprisingly, the embryonic identity of mESCs that relies on LIF is more difficult to demarcate, because LIF signalling is only necessary for the diapause stage in rodents and because mESCs share limited similarities to early epiblast cells. Interestingly, recent reports have shown that mESCs can be derived in the absence of LIF by using three small-molecule inhibitors (referred to as '3i') (Ying et al., 2008). These cells, which are fully independent of LIF signalling, could represent a natural state of pluripotency that corresponds to cells of the early epiblast prior to implantation. However, further investigation will be necessary to define precisely the embryonic identity of these 3i-induced mESCs. Interestingly, a recent study has also shown that embryonic germ (EG) cells can be derived from primordial germ cells from post-implantation embryos using the 2i + LIF culture system (Leitch et al., 2010). This study suggests that EG cells share key characteristics with mESCs and, thus, reinforce the suggestion that mESCs have a germ cell origin (Zwaka and Thomson, 2005). Gene expression profiling on single cells might offer new opportunities in order to determine the relationship between mESCs and cells naturally present in the early embryo (Tang et al., 2010).

Several studies have recently shown that hESCs or human iPSCs (hiPSCs), which resemble LIF-dependent mESCs, can be generated by using specific transcription factors, such as KLF4, or specific combinations of chemical inhibitors (Hanna et al., 2010a). Using human pluripotent stem cells that correspond to an early embryonic stage (LIF-dependent hESCs) could be advantageous for large-scale amplification, as these cells should grow faster and should, therefore, be easier to expand. However, such cells might have problems regarding differentiation because their epigenetic status is likely to be unstable and they would need to pass through an epiblast-like stage in order to differentiate into the three germ layers. This additional step might prove difficult to fully reproduce in vitro and, thus, could

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introduce a main source of variability between experiments. Furthermore, optimal culturing conditions for the derivation of mESC-like hESCs might be difficult to establish, as it is not yet clear whether capturing a rodent diapause-like stage represents a normal pluripotent state in humans. Further studies are necessary to define signalling pathways that function in the pre-implantation embryo in humans and to develop new culture conditions that support human ICM or early epiblast stem cells.

Finally, the embryonic identity of pluripotent stem cells also defines its biological interest for developmental studies and for clinical applications. For example, EpiSCs are an advantageous model to study germ-layer specification, as these cells represent the last pluripotent state before gastrulation. In addition, mEpiSCs share several characteristics with hiPSCs and hESCs, including activin–nodal signalling for maintaining pluripotency (Vallier et al., 2005), stability of their epigenetic status and the capacity to differentiate in chemically defined conditions (Vallier et al., 2009b). This resemblance also suggests a similar embryonic identity and, thus, a shared pluripotent state. Recent functional studies in which both cell types were used in parallel have reinforced this hypothesis (Teo et al., 2011; Vallier et al., 2009a; Chng et al., 2010). Therefore, mEpiSCs could be used as a substitute for hESCs in large-scale studies and in vivo experiments that are difficult to perform using human cells. By contrast, mESCs represent an early stage of development that is relevant for studying the mechanisms that lead to extra-embryonic tissue specification; they also represent a unique model to study that epigenetic events that occur during early mammalian development. In conclusion, we now have a range of ESCs with divergent embryonic identities that represent different pluripotent states, and researchers should carefully consider the advantages and disadvantages of these specific pluripotent cell types for developmental studies or clinical applications.

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