

Pluripotency in the Embryo and in Culture

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SUMMARY

Specific cells within the early mammalian embryo have the capacity to generate all somatic lineages plus the germline. This property of pluripotency is confined to the epiblast, a transient tissue that persists for only a few days. In vitro, however, pluripotency can be maintained indefinitely through derivation of stem cell lines. Pluripotent stem cells established from the newly formed epiblast are known as embryonic stem cells (ESCs), whereas those generated from later stages are called postimplantation epiblast stem cells (EpiSCs). These different classes of pluripotent stem cell have distinct culture requirements and gene expression programs, likely reflecting the dynamic development of the epiblast in the embryo. In this chapter we review current understanding of how the epiblast forms and relate this to the properties of derivative stem cells. We discuss whether ESCs and EpiSCs are true counterparts of different phases of epiblast development or are culture-generated phenomena. We also consider the proposition that early epiblast cells and ESCs may represent a naïve ground state without any prespecification of lineage choice, whereas later epiblasts and EpiSCs may be primed in favor of particular fates.

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1 INTRODUCTION

Embryogenesis is often considered as a progressive loss of developmental capacity from a “totipotent” zygote. In reality, however, the mammalian egg is a highly specialized and restricted cell. It is programmed to undergo a stereotyped process of cleavage divisions that accomplishes the oocyte-to-embryo transition (Selwood and Johnson 2006). Developmental potential is unlocked through formation of the epiblast cells in the inner cell mass (ICM) of the blastocyst. The notion that pluripotency is an emergent property that is realized in the epiblast is consistent with molecular analyses, notably expression patterns of critical transcription factors that are not present in the zygote. Newly formed epiblast cells are believed to have equivalent capacity to generate all somatic lineages of the embryo and the germline. They might therefore be considered “naïve.” In mice and rats, cells from the naïve epiblast can be propagated *in vitro*. These embryonic stem cells (ESCs) retain similar, if not identical, properties to the epiblast. They self-renew or differentiate into multiple tissue types, depending on the conditions of the *in vitro* environment. Most remarkably, they are able to participate in normal development when returned to the early embryo, even after extensive proliferation *in vitro*. Of particular note, ESCs can give rise to functional gametes and have therefore been used extensively to create genetically engineered lines of mice for developmental, genetic, and biomedical research (Capecci 2005). We review this evidence and the associated proposition that cells of the naïve epiblast and ESCs represent a developmental ground state for mammals. The epiblast is not a fixed state, however. Epiblast cells are subjected to specification signals from extraembryonic tissues preparatory to gastrulation (Beddington and Robertson 1999). Their naïve status may therefore be eroded even though they are not yet lineage committed. We contrast ESCs with pluripotent stem cells known as postimplantation epiblast stem cells (EpiSCs) that originate from this developmentally more advanced epiblast.

2 FORMATION OF THE BLASTOCYST

The mammalian embryo begins development packaged within the zona pellucida, a glycoprotein protective layer surrounding the oocyte. Cleavage divisions generate a cluster of cells known as blastomeres. In the mouse, differentiation initiates at the eight-cell stage with the onset of polarization of the outside cells. Microvilli appear on the apical surfaces and cytoplasmic microtubules and organelles become apicobasally distributed (Ducibella and Anderson 1975). The blastomeres then become closely apposed with one another in a cellular process called compaction.

Subsequent cell divisions result in the complete envelopment of some cells, which remain apolar, whereas the polar cells stay on the outside and develop into an epithelial layer, the trophoblast (Johnson and Ziomek 1981). Until the trophoblast and inner cells are fully segregated spatially, cells can be repositioned and will acquire the characteristics of cells in the new location (Tarkowski and Wroblewska 1967). This observation exemplifies both the regulative nature of the developing mammalian embryo and the progressive manner of lineage differentiation.

Specification of the trophoblast lineage appears to be mediated primarily by regionalized activity of the transcription factor *Tead4* in the cleavage-stage embryo (Nishioka et al. 2009). *Tead4* is active in the outside cells when the Hippo pathway is inactive but is inactivated in the inside cells (ICM) that perceive signaling through the Hippo pathway elicited by changes in cell contact and cell morphology (Wada et al. 2011). *Tead4* up-regulates the caudal-type homeobox transcription factor *Cdx2* and the T-box transcription factor *eomesodermin*, which drive commitment to the trophoblast lineage. A positive-feedback loop operates via the Ets-domain transcription factor *Elf5* (Ng et al. 2008). Methylation of the *Elf5* gene in ICM cells may provide an epigenetic barrier to subsequent trophoblast commitment.

Diversification of the trophoblast and ICM defines the progression of the embryo from the morula to the blastocyst stage of development. Coincident with specification of the trophoblast, the inside cells acquire a reciprocal expression profile of key transcription factors. They lose *Tead4*, *Cdx2*, and *eomesodermin*, but maintain the POU-domain transcription factor *Oct4* (*Pou5f1*), which is down-regulated in the trophoblast. *Oct4* is essential to establish the distinct identity of the ICM. In the absence of *Oct4*, inside cells form but are unable to develop ICM character and eventually differentiate along the trophoblast lineage (Nichols et al. 1998). Consequently, although mutant embryos can implant in the uterus, they do not develop any embryonic rudiment. *Oct4* often acts cooperatively with the SRY-box-containing transcription factor *Sox2* to regulate expression of multiple target genes, including fibroblast growth factor-4 (FGF4) (Basilico et al. 1997) and key transcription factors such as *Nanog* (Boyer et al. 2005). *Sox2*-null embryos do form normal blastocysts but fail development before gastrulation (Avilion et al. 2003). A requirement for embryo-derived *Sox2* in the ICM is believed to be masked by long-lived maternally encoded protein, although this has yet to be tested by ablation of the maternal gene product.

Trophoblast cells transfer fluid into the blastocyst to form a cavity, the blastocoel. After cavitation the ICM becomes partitioned into epiblast and hypoblast (also known as the “primitive endoderm”). In the mature blastocyst

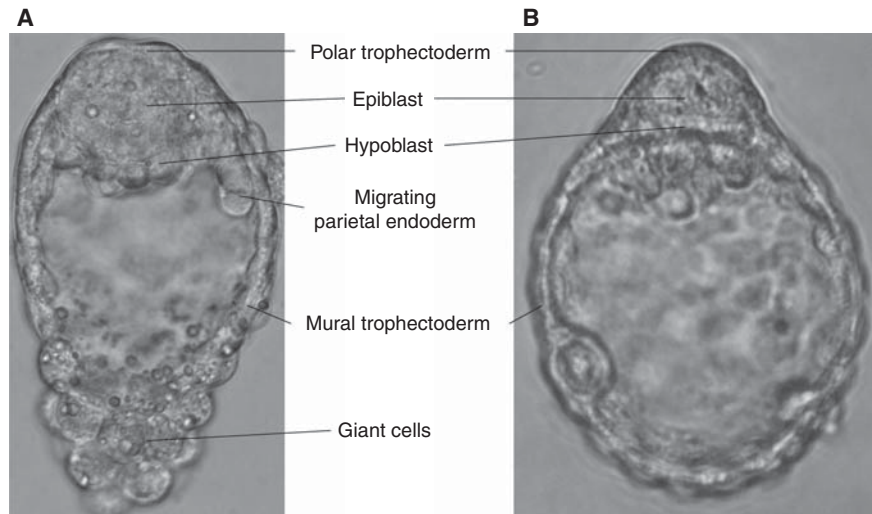


Figure 1. Bright field images of mature mouse blastocysts showing morphological segregation of epiblast and hypoblast before implantation in the uterus. (A) Peri-implantation at E4.5; (B) diapause. Embryos were freshly flushed from uteri and images captured on an Olympus IX50, with a magnification of 200 \times .

(Fig. 1A), the hypoblast is morphologically distinguishable as an epithelium overlying the blastocoelic surface of the ICM. The specification of epiblast versus hypoblast is still not fully understood. Genetic manipulations in ESCs suggested that the two transcription factors *Nanog* and *Gata6* may act in a mutually antagonistic manner to determine the two lineages. In the embryo, however, *Nanog* and *Gata6* both begin to be expressed in an apparently stochastic manner at around the eight-cell stage (Dietrich and Hiiragi 2007), and they can be colocalized in some cells of the ICM at least until the 32-cell stage (Plusa et al. 2008). Hypoblast markers, such as platelet-derived growth factor receptor α (PDGFR α) and *Sox17*, also initially show heterogeneous expression in the early blastocyst. Cells expressing these markers appear to sort out from *Nanog*-positive cells and if they end up located adjacent to the cavity will up-regulate additional transcriptional determinants *Gata4* and *Sox7* and become committed to hypoblast differentiation (Artus et al. 2011).

Formation of the epiblast is characterized by, and appears dependent on, expression of *Nanog*. *Nanog* protein is initially found at varying levels throughout the ICM (Dietrich and Hiiragi 2007; Plusa et al. 2008). By the late blastocyst stage, however, *Nanog* is precisely restricted to the new-formed epiblast, where it is present at similar levels in all cells. In female mouse embryos, reactivation of the silent paternal X chromosome occurs only in the *Nanog*-positive epiblast. *Nanog*-null embryos fail to establish epiblast identity because of degeneration of the ICM (Mitsui et al. 2003; Silva et al. 2009). X reactivation does not occur in these mutants, reflecting the absence of epiblast. Unexpectedly, the hypoblast is also lost even though *Nanog* is

not expressed in this lineage. *Nanog*-null hypoblast cells can be rescued by wild-type cells in chimeras, demonstrating a non-cell-autonomous requirement (Messerschmidt and Kemler 2010). Hypoblast development thus depends on paracrine signaling from the epiblast. Such interaction may provide a means for regulating the relative numbers of hypoblast versus epiblast cells. FGF4 is the principal candidate for a paracrine factor. Genetic deletion of *Grb2*, which couples the FGF receptor to the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) pathway, results in blastocysts that lack hypoblast (Cheng et al. 1998; Chazaud et al. 2006). Chemical inhibition of the FGF/Erk pathway in cultured embryos gives blastocysts in which all ICM cells become *Nanog*-positive epiblast (Nichols et al. 2009b; Yamanaka et al. 2010). This occurs by suppression of hypoblast specification such that all cells are converted to epiblast. Conversely, administering high concentrations of FGF4 to early embryos can drive the entire ICM to form hypoblast (Yamanaka et al. 2010). The segregation of the two lineages may be consolidated by reciprocal expression of FGF receptor in the hypoblast and FGF4 in the epiblast (Guo et al. 2010).

3 DERIVATION OF ESCs

ESCs were first derived by explanting blastocysts or ICMs on a layer of “feeder” cells (mitotically inactivated fibroblasts) in medium containing fetal calf serum (Evans and Kaufman 1981; Martin 1981). These supportive requirements had been determined empirically using pluripotent embryonal carcinoma (EC) cells (Martin and Evans 1975). They are sufficient to support unlimited expansion of

undifferentiated EC cells or ESCs. Like EC cells, ESCs form multidifferentiated teratocarcinomas when grafted to adult mice. Their full potency is revealed by blastocyst injection. This yields chimeric mice with extensive contribution from the injected ESC progeny to all tissues, including functional colonization of the germline (Fig. 2) (Bradley et al. 1984). Using embryo aggregation or tetraploid hosts, it is now possible to derive liveborn mice that are apparently entirely composed of ESC derivatives (Nagy et al. 1990, 1993). ESCs thus show an unprecedented developmental capacity after prolonged *in vitro* culture. Furthermore, ESC chimeras do not develop tumors. The continuous self-renewal that enables expansion in culture must be rapidly extinguished when ESCs are returned to the embryonic environment, but precisely how and when this occurs has not been defined.

Interestingly, Evans and Kaufman used embryos in diapause for their ESC derivations (Evans and Kaufman 1981). Diapause is a condition of embryo arrest at the peri-implantation blastocyst stage (see Fig. 1B). This occurs when implantation is prevented by estrogen deprivation consequent to persistent suckling, ovariectomy, or administration of an estrogen antagonist, such as tamoxifen. Blastocysts hatch from the zona pellucida, segregate hypoblast and epiblast, and then remain paused for up to several weeks because the uterus is unreceptive. When estrogen is restored, implantation can proceed and normal embryogenesis ensues. Diapause facilitates derivation of ESCs (Evans and Kaufman 1981; Brook and Gardner 1997), possibly because it synchronizes embryos at the naïve epiblast stage. Although development is suspended, the cell number

in the ICM increases slightly during diapause (Nichols et al. 2001), implying that the cells continue to cycle. This is a situation therefore when epiblast cells may undergo self-renewal *in vivo* (Smith 2001).

The traditional regime for ESC derivation is to explant intact blastocysts or immunosurgically isolated whole ICMs. ESC lines have also been established from single cells isolated following epiblast microdissection (Brook and Gardner 1997). That study provided definitive evidence that ESCs originate from the mouse epiblast at embryonic day 4.5 (E4.5). Furthermore, by removing the extraembryonic tissues completely, the investigators reported that the efficiency of ESC derivation was increased. This technique requires specialist expertise, however, and has not been widely adopted. Successful derivations have also been reported from morulae and even from isolated eight-cell-stage blastomeres, leading to the suggestion that ESCs might represent a very early stage of development (Eistetter 1992; Tesar 2005). In these studies, however, cultures are invariably left intact for several days. This may allow for developmental progression and cellular interactions that specify epiblast even if formation of an overt blastocyst is not apparent. Significantly, all mouse ESCs characterized to date show similar molecular and developmental phenotypes. There is currently no evidence that a distinct pre-epiblast cell state can be expanded.

4 EGG CYLINDER DEVELOPMENT AND EpiSCs

For implantation mouse embryos distribute along the length of the two uterine horns, associated with regionalized expression of epidermal growth factor (EGF) receptors in the uterus (Brown et al. 1989; Das et al. 1994). Physical contact initiates a localized decidual response in the uterus, and the embryo becomes directionally embedded in the deciduum. The giant cells of the mural trophoblast attach to a central crypt opposite the uterine mesometrium. The diploid polar trophoblast overlying the epiblast proliferates and forms the extraembryonic ectoderm. Meanwhile, the polar trophoblast derivatives overlying the extraembryonic ectoderm undergo giant cell transformation to form the ectoplacental cone. This contacts the mesometrial side of the uterus and initiates formation of the placenta. Hypoblast-derived parietal endoderm cells disperse along the inside surface of the mural trophoblast and secrete a thick basement layer known as Reichert's membrane. Hypoblast cells that maintain contact with the epiblast or extraembryonic ectoderm maintain an epithelial architecture and form the embryonic and extraembryonic visceral endoderm, respectively (Fig. 3). In addition to a nutritive function, the visceral endoderm is the source of regionalized signaling activity that is essential for patterning the embryo



Figure 2. Adult male mouse ESC chimera generated by injection of 129/Ola ESCs (sandy coat color) into a C57BL/6 blastocyst (black coat color), with pups produced by mating of the chimera to a wild-type albino MF1 female. The gray color of the pups indicates that the germ arose from colonization of the germline by the injected ESCs.

before and during gastrulation (Beddington and Robertson 1999).

After implantation the embryo is physically constrained and the continued growth of the epiblast is accommodated by its displacement into the blastocyst cavity. At this time the epiblast transforms within 12 hours from a compact cluster of cells (Fig. 3A) into a cup of columnar epithelium (Fig. 3B). The resulting structure is known as the egg cylinder. Epithelialization of the epiblast is believed to be regulated by both diffusible and cell-contact-dependent signals from the visceral endoderm (Coucouvanis and Martin 1995, 1999). It should be noted, however, that formation of the egg cylinder is a morphogenetic process unique to rodent embryos. In other mammals, including primates, the epiblast develops as a flat epithelial disk. This topological difference may influence the regulatory mechanisms that mediate progression from naïve pluripotency (Nichols and Smith 2009).

In addition to the clear morphological differences, pre- and postimplantation epiblast differ at the molecular level, for example, in the reciprocal expression of *Rex1* (*Zfp42*) and *FGF5* (Pelton et al. 2002). Furthermore, contribution of postimplantation epiblast cells to blastocyst chimeras has never been reported. It is not clear if this is because their cell-adhesion properties are incompatible with physical incorporation into the ICM or because they have lost

competence to respond to developmental cues in the blastocyst. Egg cylinder epiblast does have the capacity to give rise to teratocarcinomas and EC cells, however (Solter et al. 1970; Stevens 1970). Moreover, lineage tracing using vital dye labeling of single cells during early gastrulation *ex vivo* reveals progeny in all three germ layers (Lawson et al. 1991), and egg cylinder epiblasts can be induced to form primordial germ cells at high efficiency (Hayashi et al. 2011). Clearly, therefore, postimplantation epiblast cells are pluripotent.

Attempts to derive ESCs directly from postimplantation epiblasts met with repeated failure. However, cell lines that show pluripotent features have now been derived using different culture conditions (Brons et al. 2007; Tesar et al. 2007). These postimplantation epiblast-derived stem cells, or EpiSCs, can generate teratocarcinomas but do not contribute effectively to blastocyst chimeras. EpiSCs express *Oct4* but have reduced or no expression of *Rex1* and several other transcription factors expressed in ESCs. Compared with ESCs they show higher expression of factors present in the postimplantation epiblast, such as *FGF5* and *Brachyury*. EpiSCs share with ESCs a large nuclear-to-cytoplasmic ratio and prominent nucleoli, but their morphology is more two-dimensional and epithelial. They do not survive efficiently as isolated single cells and do not respond to ESC culture conditions, but instead are dependent on *FGF2* and *activin*. Strikingly, EpiSC lines differ

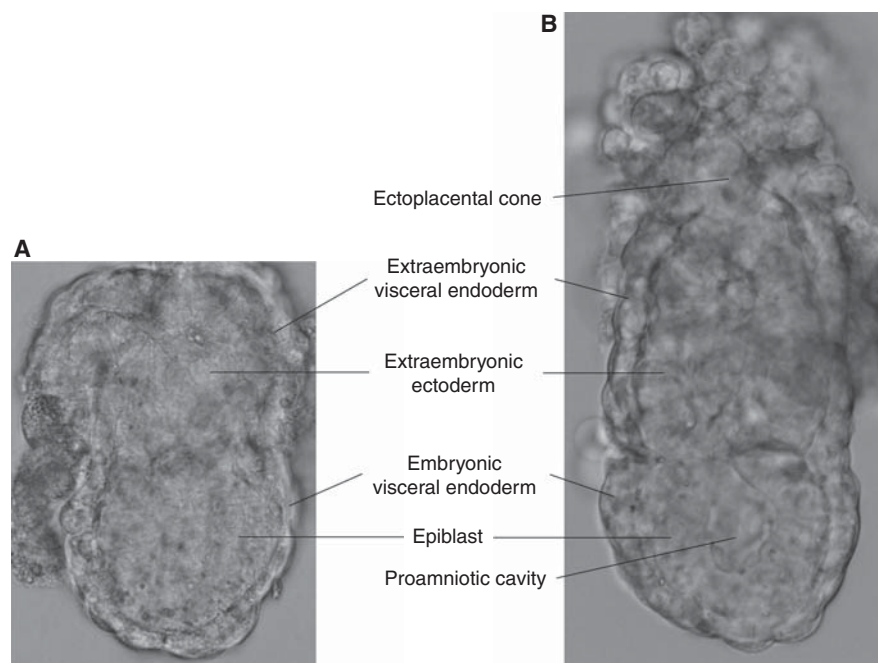


Figure 3. Bright field images of embryos just before and after epithelialization of the mouse epiblast at around E5.25. (A) The epiblast is still a compact ball. (B) The epiblast has epithelialized to form a cup-shaped structure and displays a prominent proamniotic cavity. Embryos were freshly dissected free from uteri, deciduum, and Reichert's membrane and images captured on an Olympus IX50, with a magnification of 200 \times .

markedly from one another, showing different differentiation biases (Bernemann et al. 2011). This variability could reflect heterogeneity within the egg cylinder. Alternatively, however, variability could arise from independent adaptations to the culture environment. Caution should therefore be exercised before considering EpiSCs as models of post-implantation epiblast cells. A key test will be to determine whether EpiSCs can engraft postimplantation epiblast in whole-embryo cultures.

Intriguingly, although most EpiSCs are clearly distinct from ESCs, some lines appear to harbor a subpopulation of cells that are more closely related (Han et al. 2010). If cultured to high density on feeder cells in the presence of leukemia inhibitory factor (LIF) and serum or transferred into 2i (see below) and LIF, such EpiSCs may spontaneously generate ESCs at low frequency (Hayashi and Surani 2009; Bernemann et al. 2011).

It has also been reported that ESC-like cell lines have been generated from dissociated postimplantation epiblasts following extended culture on feeder cells in medium supplemented with serum and LIF (Bao et al. 2009). Whether these cells arise directly from epiblast or via EpiSCs is uncertain. Another possibility is that they could develop via early germ cells. Primordial germ cells from early somite-stage embryos or isolated from genital ridges can convert in culture into pluripotent stem cells known as embryonic germ (EG) cells (Matsui et al. 1992; Resnick et al. 1992). Once established, EG cells are indistinguishable from ESCs apart from a variable degree of imprint erasure.

There is an intimate connection between the germline and pluripotency in mammals. Similar to primordial germ cells, it is possible to convert spermatogonial stem cells into pluripotent stem cells by exposing them to an appropriate regime of *in vitro* stimuli (Kanatsu-Shinohara et al. 2004; Ko et al. 2009). Indeed, it has even been suggested that all ESCs could be derived via germ cells (Zwaka and Thomson 2005). However, although germ cell specification might conceivably occur in ICM explants, this is not routinely observed. Furthermore, the high efficiency of establishing ESCs from early epiblast compared with a single report of derivation from postimplantation epiblast (Bao et al. 2009), which should more readily generate germ cell precursors, argues against this notion (Nichols and Smith 2011). Finally, derivation of ESCs from embryos lacking the essential germ cell determinant *Blimp1* undermines the case for an obligatory germ cell intermediate (Chu et al. 2011).

5 REFINED CONDITIONS FOR ESC PROPAGATION AND DEVELOPMENT OF “2i”

Culture of established ESCs on feeder layers is still favored by many researchers because this provides a robust en-

vironment for gene-targeting manipulations (Capecchi 2005). However, the derivation of mouse ESCs using feeders and serum is inefficient and variable, even from genetically identical inbred strains. Furthermore, the genetic background has a major influence on the capacity to give rise to ESCs. Until recently almost all the ESC lines in common use came from the 129 strain. It may be relevant that strain 129 males show an increased incidence of testicular teratomas. Unfortunately, however, these mice are generally poor breeders, perform inadequately in behavioral and cognitive studies, and are suboptimal for hematological and immunological research. Time-consuming back-crossing is often required to generate useful mouse models from the progeny of genetically modified 129 ESCs. Using the conventional culture regime, ESCs cannot reliably be derived from many important strains, such as FVB or nonobese diabetic (NOD) mice. Moreover, the derivation of ESCs from other species has not been successful, with the particular exception of certain primates (see below).

To exploit the full potential of ESCs and to clarify their relationship to cells in the mammalian embryo, it was necessary to refine the culture conditions and elucidate the molecular requirements for self-renewal. It was found that feeder cells could be replaced by a factor in conditioned medium (Smith and Hooper 1983, 1987; Koopman and Cotton 1984), later identified as the cytokine LIF (Smith et al. 1988; Williams et al. 1988). Germline-competent ESCs can be derived *de novo* using LIF instead of feeders (Nichols et al. 1990; Pease et al. 1990), but limited to the 129 strain. The subsequent replacement of serum with bone morphogenetic factor-4 (BMP4) (Ying et al. 2003) supported propagation of ESCs from several strains, although the *de novo* derivation of ESCs using just BMP and LIF appears limited to the 129 background (Ying et al. 2003).

LIF promotes ESC self-renewal by activation of the transcription factor *Stat3* (Niwa et al. 1998; Matsuda et al. 1999). However, LIF also stimulates the MAPK/Erk pathway, which acts to promote differentiation (Burdon et al. 1999). A more potent activator of Erk is FGF4, which is expressed by both epiblast and ESCs (Kunath et al. 2007). Genetic disruption or pharmacological inhibition of Erk signaling reduces differentiation in ESC cultures (Burdon et al. 1999; Kunath et al. 2007). Inhibition of this pathway increases persistence of Oct4-positive cells in primary explants during ESC derivation (Buehr and Smith 2003). In combination with LIF and BMP4, this enables ESC derivation from embryos of C57BL/6 and CBA strains in serum- and feeder-free culture conditions (Batlle-Morera et al. 2008).

Although FGF4/Erk stimulation is a major trigger for ESC differentiation (Kunath et al. 2007; Stavridis et al. 2007), Erk pathway inhibition alone is insufficient to support undifferentiated ESC expansion unless the cells are



maintained at high densities or provided with LIF (Ying et al. 2008). Parallel studies had indicated that inhibition of another enzyme, glycogen synthase kinase-3 (GSK3), could enhance ESC self-renewal (Sato et al. 2004; Cartwright et al. 2005; Ogawa et al. 2006). Addition of a selective inhibitor of GSK3, CH99021, “rescued” ESCs in which Erk signaling was blocked (Ying et al. 2008). Using a combination of three inhibitors, known as “3i,” comprising an FGF receptor inhibitor, an inhibitor of MAPK kinase (Mek) 1/2, and the GSK3 inhibitor, robust ESC self-renewal is obtained in the absence of serum or LIF. This system allows ESCs to be derived from embryos of various strains of mice with high efficiency (Ying et al. 2008; Nichols et al. 2009a; Kiyonari et al. 2010; Yamagata et al. 2010). Female cell lines, which are underrepresented among serum-derived ESCs for unknown reasons, are readily obtained using 3i (Nichols et al. 2009a; Kiyonari et al. 2010). The operational activity of the FGF receptor in driving ESC commitment is via the Erk pathway. Accordingly, use of a Mek inhibitor (PD0325901) with high potency and selectivity eliminates the need to block the FGF receptor. Hence, 3i evolved into 2i (Wray et al. 2010).

It is important to note that the optimal concentration of CHIR99021 for mouse ESCs, 3 μM , only partially inhibits GSK3 (Ying et al. 2008). GSK3 inhibition relieves negative regulation of multiple proteins. Pinpointing the critical target(s) in ESC self-renewal therefore presented a challenge. However, a major suspect was β -catenin. Recent

studies have confirmed that the effect of CHIR99021 is dependent on β -catenin and is largely reproduced by ablation of the β -catenin interactor Tcf3 (Wray et al. 2011; Yi et al. 2011). Tcf3 acts as a repressor of core pluripotency factors (Pereira et al. 2006; Cole et al. 2008), and deletion of Tcf3 suppresses ESC differentiation (Guo et al. 2011). Inhibition of GSK3 leads to stabilization of intracellular β -catenin, which interacts with Tcf3 to abrogate its repressor function (Wray et al. 2011). It is suggested that canonical Wnt signaling, which operates through β -catenin, may play a similar role in the blastocyst (Berge et al. 2011), but this remains uncertain in the absence of supporting genetic evidence (Merrill et al. 2004). Moreover, the canonical Wnt/ β -catenin pathway is a major inducer of axis formation and mesoderm differentiation in both the early embryo and ESC-derived embryoid bodies (ten Berge et al. 2008), which would imply a radical switch in function. The relative levels of different Tcfs may change in the late epiblast, but it also seems likely that Tcf3 may be regulated by pathways other than Wnt (Sokol 2011).

6 A TRANSCRIPTION FACTOR NETWORK THAT SUSTAINS NAÏVE PLURIPOTENCY

The ESC state is characterized by a unique network of transcription factors (Niwa 2007; Jaenisch and Young 2008). Table 1 lists key factors that have been functionally validated as playing pivotal roles, while Figure 4 presents a

Table 1. Phenotypes associated with core pluripotency transcription factors

Transcription factor	ESC overexpression	ESC loss of function	Embryo null	References
Oct4	Differentiation	Differentiation to trophoblast	ICM becomes trophectoderm	Nichols et al. 1998; Niwa et al. 2000
Sox2	May reduce differentiation	Differentiation to trophoblast; rescued by Oct4	Early postimplantation lethal	Avilion et al. 2003; Masui et al. 2007; Ura et al. 2011
Nanog	Autonomous self-renewal	Prone to differentiate, but can self-renew	Loss of epiblast and secondarily of hypoblast	Chambers et al. 2003, 2007; Mitsui et al. 2003; Silva et al. 2009
Stat3	Hormone-activatable fusion substitutes for LIF	No self-renewal in LIF, but maintained in 2i	Postgastrulation lethal	Takeda et al. 1997; Matsuda et al. 1999; Ying et al. 2008
Klf2	LIF-independent self-renewal	Not certain (none by RNAi)	Viable	Jiang et al. 2008; Hall et al. 2009
Klf4	LIF-independent self-renewal	Not certain (none by RNAi)	Viable	Jiang et al. 2008; Niwa et al. 2009
Esrrb	LIF-independent self-renewal	Partially impaired self-renewal (RNAi)	Placental failure; midgestation lethality	Luo et al. 1997; Ivanova et al. 2006; G Martello and AG Smith, unpubl.
Tbx3	LIF-independent self-renewal	Partially impaired self-renewal (RNAi)	Midgestation lethality	Davenport et al. 2003; Ivanova et al. 2006; Niwa et al. 2009
Tcf3	Differentiation	Reduced LIF dependence	Disrupted axial patterning; gastrulation lethality	Pereira et al. 2006; Guo et al. 2011; Wray et al. 2011
Mbd3 (NuRD)	Not described	Reduced LIF dependence	Early postimplantation lethal	Kaji et al. 2006, 2007

Abbreviations: ICM, inner cell mass; LIF, leukemia inhibitor factor; RNAi, RNA interference.

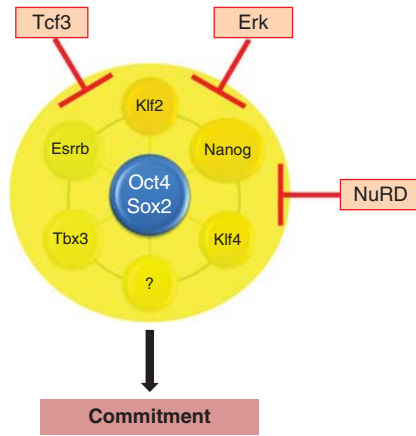
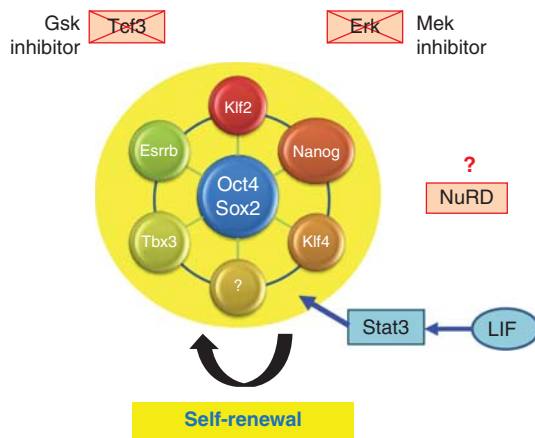
A Inner and outer core circuitry**B** Repressors and Erk target the outer circuit**C** 2i and LIF insulate and consolidate the outer circuit

Figure 4. Illustration of the interconnections between core pluripotency transcription factors and extrinsic stimuli. (A) Schematic of the functionally validated pluripotency factors depicting Oct4 and Sox2 as the essential hub linked to a circuit of factors that are individually more or less dispensable but collectively define and sustain the naïve state. These factors all cross-regulate one another. The question mark signifies a hypothetical additional pivotal self-renewal factor downstream from Stat3. (B) Collapse of the outer circuit of pluripotency factors leads to exit from ground-state pluripotency and ultimately lineage commitment and differentiation. (C). The 2i inhibitors prevent repression of the outer circuit, whereas LIF/Stat3 directly boosts expression of specific factors. How activity of NuRD is modulated in naïve ESCs is currently unclear.

diagrammatic summary. Paramount among the pluripotency factors is Oct4. Oct4 is distinguished by its relatively uniform and continuous expression throughout formation and maturation of the epiblast, and in both ESCs and EpiSCs in culture. Using conventional serial gene targeting *Oct4*-null ESCs cannot be recovered. A conditional strategy that induces acute elimination of Oct4 in ESCs triggers their conversion predominantly into trophoblast-like cells (Niwa et al. 2000). Overexpression of Oct4 does not stabilize pluripotency, however, but drives differentiation instead. This has been interpreted as reflecting participation of Oct4 in diverse transcription factor complexes that differentially regulate gene expression and may contribute either to maintenance of pluripotency or to lineage specification (Niwa et al. 2000). Protein interaction studies are consistent with the expectation that Oct4 has multiple partners (Pardo et al. 2010; van den Berg et al. 2010).

The best-characterized Oct4 partner is Sox2. Cooperativity between Oct4 and Sox2 was first described based on their functional binding to an FGF4 enhancer element (Ambrosetti et al. 1997). An Oct/Sox element has subsequently been identified at many pluripotency genes (Chambers and Tomlinson 2009). Conditional deletion of Sox2 in ESCs results in trophoblast differentiation, similar to deletion of Oct4 (Masui et al. 2007). Surprisingly, however, if Oct4 expression is maintained from a transgene, ESCs can be preserved in the absence of Sox2. The interpretation is that the only unique function of Sox2 may be in activation of Oct4 and that it may be substituted by alternative Sox proteins at other targets (Masui et al. 2007).

The variant homeodomain-containing protein, Nanog, was isolated in a functional cDNA screen in ESCs (Chambers et al. 2003). Overexpression of Nanog renders ESC self-renewal constitutive without requirement for LIF, BMP, or 2i (Chambers et al. 2003; Ying et al. 2003, 2008; Hall et al. 2009). Nanog is essential for formation of the epiblast in the embryo, as discussed above, yet it can be deleted from ESCs. *Nanog*-null ESCs are highly prone to differentiation, but nonetheless can sustain self-renewal (Chambers et al. 2007). Furthermore, they are pluripotent and can contribute extensively to chimeras (Chambers et al. 2007). They even make primordial germ cells, although these fail to mature in the genital ridge.

In an effort to identify additional components required for ESC self-renewal, Ivanova and colleagues tested >100 transcription factors in an RNA interference loss-of-function screen (Ivanova et al. 2006). An inducible rescue strategy was used to exclude off-target effects. From this functional assay, six genes were implicated in maintenance of self-renewal. These include *Oct4*, *Sox2*, and *Nanog*, as expected. Also identified were the nuclear receptor *Esrrb*, T-box transcription factor-3 (*Tbx3*), and T-cell leukemia

oncogene 1 (*Tcl1*). *Esrrb* has subsequently been found to interact with Oct4 and Nanog (van den Berg et al. 2008; Zhang et al. 2008) and to act as a reprogramming factor (Feng et al. 2009). However, during development *Esrrb* is required in the placenta, not in the embryo (Luo et al. 1997), suggesting that its function in pluripotent cells can be substituted by an alternative pathway. *Tbx3* overexpression has been shown to enhance ESC self-renewal, and it is proposed to operate by activating Nanog downstream from phosphatidylinositol-3-kinase and Akt (Niwa et al. 2009). *Tbx3* is also dispensable for early embryo development (Davenport et al. 2003). *Tcl1* is implicated in activation of Akt and appears to be a direct target of Oct4 (Matoba et al. 2006). It is abundant in oocytes and during early cleavage but is not required for blastocyst formation or postimplantation development (Narducci et al. 2002).

A search for transcription factors downstream from LIF first suggested a role for the Kruppel-like factor *Klf4* (Li et al. 2005). *Klf4* has been confirmed as a direct target of Stat3 (Bourillot et al. 2009; Hall et al. 2009) and when overexpressed can confer partial LIF independence to ESC propagation (Niwa et al. 2009). *Klf4* does not fully reproduce the effect of LIF, however, indicating that additional Stat3 targets contribute significantly. Interestingly, the related *Klf2* is not regulated by LIF or Stat3 but has similar, if not greater, capacity to support LIF-independent self-renewal (Hall et al. 2009). Embryos null for either *Klf4* or *Klf2* develop normally through gastrulation, and it is suggested that there may be substantial redundancy between the two factors (Jiang et al. 2008). The more distant relative *Klf5* is required for blastocyst development (Ema et al. 2008), but its expression is not restricted to the ICM. Both deletion and overexpression of *Klf5* produce only mild phenotypes in ESCs, suggesting that it does not serve a critical function in pluripotency.

Executing pluripotency requires exit from self-renewal and entry into lineage commitment guided by extrinsic cues (Smith 2009). Repressors that can suppress the pluripotency circuitry and drive ESCs out of the naïve state play a key role. One such factor is *Tcf3*, which, as previously discussed, functions by repressing key pluripotency factors, notably *Esrrb*, *Klf2*, and Nanog (Pereira et al. 2006; Cole et al. 2008; Guo et al. 2011; Wray et al. 2011). *Tcf3*-null ESCs are differentiation resistant (Pereira et al. 2006; Guo et al. 2011). *Tcf3*-null embryos develop normally until after implantation, when they show profound defects in axial patterning (Merrill et al. 2004). The nucleosome remodeling and deacetylase (NuRD) corepressor complex also plays a critical role in the exit from pluripotency. ESCs lacking NuRD owing to ablation of the scaffold protein *Mbd3* differentiate inefficiently and fail to silence pluripotency genes (Kaji et al. 2006). In the absence of *Mbd3*,

embryos become disorganized soon after implantation (Kaji et al. 2007).

The core gene regulatory network in ESCs is thus centered on Oct4 and Sox2, which are irreplaceable but are not capable of conferring self-renewal. A second group of transcription factors, which are individually dispensable, collectively sustain self-renewal (Fig. 4). These latter factors are expressed uniformly in 2i but are heterogeneous in serum (Chambers et al. 2007; Niwa et al. 2009). Unlike Oct4 they are down-regulated early during exit from the naïve state (T Kalkan and AG Smith, unpubl.) and are absent or expressed at low levels in EpiSCs. In contrast, Oct4 and Sox2 are present in all undifferentiated cells in serum and are maintained in EpiSCs. In serum, ESCs with low or absent expression of the outer factors may be susceptible to differentiation (Silva and Smith 2008; Smith 2009). Indeed, cells expressing low or undetectable levels of Nanog have a greater tendency to differentiate. However, some of these cells can reexpress Nanog and generate undifferentiated ESC colonies, so they are not yet committed (Chambers et al. 2007). It has been speculated that fluctuations in transcription factor expression may be an important feature of pluripotency that allows opportunity for lineage commitment (Graf and Stadtfeld 2008; Silva and Smith 2008). The lack of heterogeneity in 2i, however, indicates that metastability is not an intrinsic property of self-renewing ESCs (Smith 2010; Wray et al. 2010). Whether it is a prerequisite for differentiation remains to be determined.

7 EXPLOITING EMBRYONIC TRANSCRIPTION FACTORS TO CONVERT SOMATIC CELLS TO PLURIPOTENCY

Candidate ESC regulators have also been selected using digital differential display to identify pluripotency-associated expression. A group of such genes, known as Ecats, were functionally evaluated by gene knockout. Nanog was independently identified from this study (Mitsui et al. 2003), and ERas was discovered as a Ras family member that promotes ESC expansion (Takahashi et al. 2003). The inspired application of the Ecats, however, was to attempt to recreate pluripotency (Takahashi and Yamanaka 2006). Multiple Ecats were retrovirally transduced into fibroblasts that were then exposed to ESC culture conditions. Selection was applied for activation of the ESC-specific *Fbx15* promoter driving G418 resistance. Rare colonies were recovered, some of which morphologically resembled ESCs. By repeating this experiment and subtracting individual factors, a minimal group of four was found to be sufficient to reprogram fibroblasts to a pluripotent-like state. These are Oct4, Sox2, *Klf4*, and the oncogene *c-Myc* (Takahashi and Yamanaka 2006). The reprogrammed cells expressed

endogenous ESC markers such as Oct4, Nanog, and ERAs and were named induced pluripotent stem cells (iPSCs). Subsequently, by applying more rigorous selection for Nanog or Oct4 activation it was shown that a subset of iPSCs acquires authentic naïve pluripotency and can generate germline-competent chimeras (Okita et al. 2007; Wernig et al. 2007). Interestingly, the outer core pluripotency factors (Fig. 4), Nanog, Klf2, Esrrb, Tbx3, and Stat3, have each now been shown to contribute to reprogramming, although it is not clear to what extent these effects are redundant or additive.

8 HUMAN PLURIPOTENT STEM CELLS

The advent of iPSCs ignited ideas for potential medical applications. Disease-specific iPSCs may be used to dissect molecular mechanisms of disease progression and develop new pharmaceuticals, whereas patient-specific stem cells may one day provide a resource for autologous cell replacement therapies (Nishikawa et al. 2008; Yamanaka 2009). This requires human pluripotent stem cells, however.

The first primate pluripotent cell line was derived in 1995 from Rhesus macaque blastocysts (Thomson et al. 1995). In 1998, using donated supernumerary embryos from in vitro fertility treatment, human pluripotent cell lines were obtained (Thomson et al. 1998). They were designated as ESCs based on their origin from the blastocyst and capability to produce teratocarcinomas. However, closer investigation of these cells revealed a series of divergent properties compared with rodent ESCs (Chia et al. 2010). For example, the human cells do not respond functionally to LIF and self-renew in response to FGF and activin. These discrepancies were commonly attributed to species-specific differences. However, the derivation of EpiSCs from postimplantation mouse epiblasts (Brons et al. 2007; Tesar et al. 2007) supported the alternative explanation that the human cells represent a different developmental stage (Smith 2001). The process of derivation from primate embryos could involve progression in explant culture to the equivalent of a postimplantation state. As discussed above, primate epiblasts do not undergo egg cylinder morphogenesis, but expand in two dimensions as an embryonic disc. This pattern of development may proceed more readily in culture than egg cylinder morphogenesis. Moreover, mouse blastocysts cultured in FGF and activin produce EpiSCs rather than ESCs (Najm et al. 2011). It is now generally considered that current human pluripotent cells, whether embryo derived or generated by reprogramming, are more similar to EpiSCs than to ESCs. Like EpiSCs, they are heterogeneous between and within cell lines and can show marked biases in differentiation (Osafune et al. 2008). However, they show some molecular

distinctions from EpiSCs, so the precise relationship is uncertain (Chia et al. 2010).

Efforts have been made to reprogram human cells into a naïve state. If transgene expression is continuous, cells can be obtained that show many properties of mouse ESCs, including two active X chromosomes, LIF responsiveness, and the ability to self-renew in 2i (Hanna et al. 2010; Wang et al. 2011). However, these cells appear difficult to maintain without transgene expression, and their relationship to authentic ESCs remains questionable at this point.

Studying the molecular regulation of epiblast development in human embryos may provide insights to enable the derivation of ESCs (Roode et al. 2012). It must also be recognized, however, that the facility for extrinsic stabilization of naïve pluripotency may be a rodent-specific phenomenon. In this case genetic bioengineering may be the route to capture and propagate a human naïve stem cell.

9 CONCLUDING REMARKS

ESCs are sustained by a flexible signaling and transcription factor network that appears to be closely based on circuitry operative in the embryo. Elucidation of the fundamental requirements for self-renewal has removed the genetic strain barrier to the efficient derivation of ESCs from mouse embryos and allowed production of the first germline-competent rat pluripotent cell lines, from both preimplantation embryos and primordial germ cells (Buehr et al. 2008; Li et al. 2008; Leitch et al. 2010; Blair et al. 2012). To date, however, there are no reports of authentic ESC lines from other mammals, indicating that additional components to 2i and LIF may be required. The notion of naïve pluripotency implies that early epiblast cells and their counterpart ESC derivatives have equal unrestricted access to all somatic lineages and the germline, whereas later epiblast cells and EpiSCs may be biased for particular lineage choices through transcriptional priming and/or chromatin modification. It will be instructive to identify genome-wide molecular correlates of the naïve mouse ESC state at both transcriptome and epigenome levels. This may reveal expected constituents of a generic ground state.

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