Cell Stem Cell
Perspective

Different Flavors of Pluripotency, Molecular Mechanisms, and Practical Implications

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Pluripotent stem cells (PSCs) have been classified into two distinct states: a primitive, naive LIF-dependent state represented by murine ESCs, and a primed bFGF-dependent state observed in murine and rat epiblast stem cells (EpiSCs). The vast similarities between EpiSCs and human ESCs suggest that, despite their blastocyst origin, human ESCs exist in a primed pluripotent state. Recent findings demonstrate that the naive and primed pluripotent states are interconvertible, even in human cells, and hint that growth factor-mediated Nanog expression may be an important factor regulating the balance between them.

Pluripotent Stem Cells

Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass (ICM) of the preimplantation blastocyst, which have the ability to generate all three germ layers, ectoderm, mesoderm, and endoderm (Yu and Thomson, 2008). ESCs have been derived from both murine and human blastocysts, but curiously, ESC lines from these two species display profound differences in colony morphology, proliferation rate, growth factor requirements, and epigenetic status (Table 1). Murine ESCs grow in three-dimensional, tightly packed colonies with a population doubling time of approximately 16 hr and require LIF and BMP4 growth factor signaling for their continued self-renewal. In contrast, human ESCs form flattened two-dimensional colonies and are maintained in a bFGF and ActivinA/TGF-β signaling-dependent manner. Human ESCs proliferate slowly, with a population doubling time averaging 36 hr. Epigenetically, human and murine ESCs display a different pattern of X chromosome inactivation and promoter occupancy by pluripotency transcription factors (Hanna et al., 2009). In addition, cultures of human ESCs are split by mechanical- or collagenase-mediated passaging as small clusters of cells, and unlike murine ESCs, most human ESC lines cannot be passaged as single cells by trypsin digest (Yu and Thomson, 2008). Similar differences are observed between murine and human induced pluripotent stem cells (iPSCs), which are derived through the ectopic expression of pluripotency transcription factors (Oct4, Sox2, Klf4, and c-Myc) in somatic cells (Takahashi and Yamanaka, 2006; Okita et al., 2007; Takahashi et al., 2007).

Distinct Pluripotent States

Until recently the differences between mESCs and hESCs were thought to be variations of an otherwise comparable pluripotent population. This view was challenged by the derivation of so-called epiblast stem cells (EpiSCs) from explanted murine or rat postimplantation epiblasts. EpiSCs demonstrate striking similarities to hESCs with regard to their molecular properties, growth factor requirements, colony morphology, X-inactivation status, and culture dynamics (Tesar et al., 2007; Brons et al., 2007; Guo et al., 2009). These results demonstrate that in the mouse, two functionally distinct pluripotent states exist: a "naive" LIF-dependent pluripotent stem cell (PSC) state that is compatible with the preimplantation ICM and a "primed" bFGF-dependent PSC state that is reminiscent of the postimplantation epiblast (Nichols and Smith, 2009). The naive pluripotent state represents a more primitive stem cell population than the primed PSCs, in that they are uniquely capable of integrating with the blastocyst ICM and contribute to chimera formation. In addition, naive PSCs exhibit a more open chromatin structure, as demonstrated by the lack of X-inactivation (Hanna et al., 2010). In accordance with this notion, EpiSCs fail to integrate into the preimplantation blastocyst and rarely contribute to chimera formation (Tesar et al., 2007). The strong observed overlap of the key characteristics of EpiSCs and human ESCs suggest that despite their blastocyst origin, human ESCs exist in a primed pluripotent state. It has been argued that the blastocyst origin of human ESCs is evidenced by their expression of Rex1 (Zfp42), known to be a specific marker of the ICM in murine embryos. However, the ICM specificity of this marker has not been established for the human embryo. Molecular examination of blastocysts across different species, including humans, demonstrates that a wide variation exists in the developmental timing of the expression of early regulators of embryonic development such as Oct4 and Cdx2 (Kuijk et al., 2008; Pant and Keefer, 2009; Chen et al., 2009). It is possible that in human development, Rex1 expression is similarly extended into the early postimplantation epiblast, explaining its expression in human ESCs and iPSCs.

The naive murine PSC state is maintained by an intricate balance between LIF and BMP4 growth factor signals. LIF activates three distinct intracellular signaling pathways: the Jak/Stat3 and PI(3)K/Akt pathways that stimulate self renewal and the MEK/ERK pathway that drives mESC differentiation (Figure 1; Niwa et al., 2009; Hamazaki et al., 2006). BMP4 serves to inhibit this differentiation-inducing effect of the MEK/ERK pathway through upregulation of Inhibitor of Differentiation (Id) proteins (Ying et al., 2003). Thus, it appears that in naive murine

Table 1. Characteristics of Pluripotent Stem Cell States

Species	Culture Conditions	Passed Pluripotency Test	X-Chromosome (Female)	Cell Surface Marker	Alkaline Phosphatase	Reference
Naive Pluripotent						
Mouse (permissive)	LIF/Bmp4 or 3i[a]	teratoma, germline transmission, tetraploid complementation	active	SSEA1	positive	Ying et al., 2008
Mouse (nonpermissive)	2i[b]+LIF	teratoma, germline transmission	N/D	SSEA1	positive	Hanna et al., 2009
Rat	2i[b]+LIF or 3i[a]	teratoma, germline transmission	active	SSEA1	positive	Buehr et al., 2008
Rat	LIF/Bmp4, 4 factors[c]	teratoma	N/D	SSEA1	positive	Liao et al., 2009
Human	2i[b]+LIF+Forskolin, 2 factors[d]	teratoma	active	SSEA3/SSEA4/ Tra1-60/Tra1-81	positive	Hanna et al., 2010
Human	LIF, 5 factors[e]	not pluripotent in LIF conditions, teratoma after conversion to primed iPSC state	N/D	SSEA1	negative	Buecker et al., 2010
Human	2i[b]+LIF, 4 factors[f]	teratoma	N/D	SSEA4/Tra1-60/ Tra1-81	positive	Li et al., 2009
Primed Pluripotent						
Mouse (permissive)	FGF/ActivinA	teratoma	inactive	SSEA1	negative	Tesar et al., 2007
Mouse (nonpermissive)	FGF/ActivinA	teratoma	N/D	SSEA1	negative	Brons et al., 2007
Rat	FGF/ActivinA	teratoma	N/D	SSEA1	negative	Brons et al., 2007
Human	FGF/ActivinA	teratoma	inactive	SSEA4/Tra1-60/ Tra1-81	positive	Thomson et al., 1998

N/D: not done.
[a] FGFR, GSK3β, and MEK/ERK inhibition.
[b] GSK3β and MEK/ERK inhibition.
[c] Constitutive expression of Oct4, Sox2, Klf4, and cMyc.
[d] Transient expression of Oct4+Klf4 or Klf2+Klf4.
[e] Constitutive expression of Oct4, Sox2, Klf4, cMyc, and Nanog.
[f] Lentiviral expression of Oct4, Sox2, Nanog, and Lin28.

PSCs, pluripotency is maintained by cross-inhibition of differentiation signals downstream of the LIF and BMP4 receptors. In line with this idea, Ying and colleagues demonstrated that mESCs can be maintained in the absence of ectopic signals when intracellular differentiation inducing signals are eliminated. Simultaneous inhibition of the FGF receptor tyrosine kinase, MEK/ERK signaling, and GSK3β, the so-called 3i culture regime, are able to sustain LIF-independent growth and self-renewal of mESCs (Ying et al., 2008), demonstrating that in the absence of differentiation cues, mESC self-renewal prevails over differentiation.

The primed pluripotent stem cell state is maintained by a combination of ActivinA/TGF-β and bFGF signaling. The ActivinA/TGF-β pathway leads to activation of SMAD2/3, which is a direct upstream inducer of NANOG expression (Figure 1; Xu et al., 2008; Vallier et al., 2009). The function of the bFGF signaling pathway is less clear and may be divergent between hESCs and murine EpiSCs. In human ESCs, bFGF inhibits neural development and cooperates with SMAD2/3 to maintain NANOG expression, whereas in murine EpiSCs, bFGF was not observed to have a stimulatory effect on Nanog expression (Greber et al., 2010).

Importantly, and in support of the model that naive and primed states represent different "flavors" of pluripotency, naive and primed cells are readily interconvertible. For example, naive mESCs adopt a primed EpiSC state upon a simple growth factor switch to bFGF/ActivinA (Guo et al., 2009). The conversion of primed PSCs to the naive pluripotent state is less efficient but can be facilitated by the overexpression of Klf4, Klf2, Nanog, or Nr5a (Guo et al., 2009; Guo and Smith, 2010; Silva et al., 2009). In addition, Bao and colleagues demonstrated that EpiSCs can also revert to ESC-like cells (rESCs) upon prolonged passaging of the stem cells in the presence of LIF and selection for cells that express the germ cell-specific marker Stella (Dppa3) (Bao et al., 2009). However, this growth factor-mediated conversion of EpiSCs to rESCs may be indirect. With the same Stella-GFP reporter cell line, Hayashi and colleagues recently demonstrated that EpiSCs can form primordial germ cells (PGCs) in vitro (Hayashi and Surani, 2009). PGCs can give rise to embryonic germ cells (EGCs) that are nearly indistinguishable from ESCs, so it is possible that the rESCs reported by Bao and colleagues are in fact EGCs derived from intermediate PGCs (Bao et al., 2009; Guo and Smith, 2010). Whether indirect or

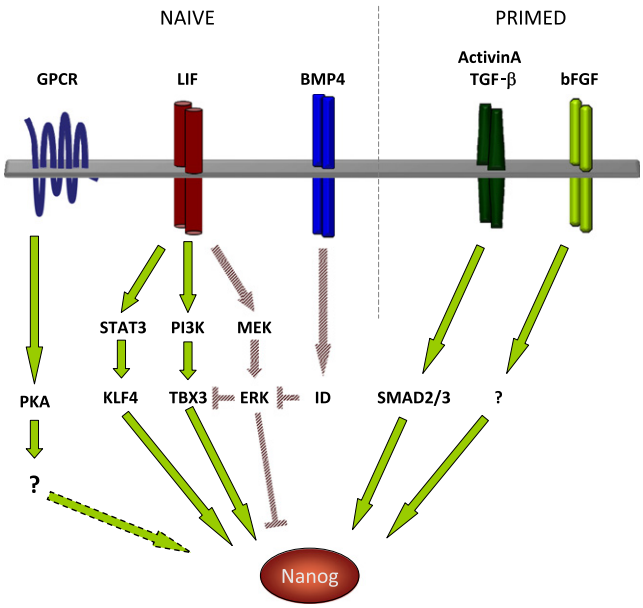

Figure 1. Exogenous Growth Factor Signals Regulate Nanog Expression Levels

In naive pluripotent stem cells, LIF stimulation results in activation of the PI(3)K/Akt, Stat3, and MEK/ERK signaling pathways. Stat3 and PI(3)K/Akt positively regulate self-renewal (as indicated by solid arrows): PI(3)K/Akt acts via T-Box3 and Stat3 acts via stimulating Klf4 expression. In contrast, activation of MEK/ERK signaling inhibits Nanog expression (dashed arrow), but this pathway is inhibited by BMP4 signals through upregulation of Id proteins. Activation of PKA signaling by G protein-coupled receptors can stabilize the naive pluripotent state by maintaining Nanog expression levels through an unknown signaling mechanism. In primed pluripotent stem cells, ActivinA/TGF-β growth factor signaling induces Nanog expression via the SMAD2/3 binding site found in the Nanog promotor. Additional cooperative activation via bFGF was shown in human ESCs, but may not play a role in Nanog expression in murine EpiSCs.

not, it is clear that LIF signaling plays an active role in the EpiSC to rESC conversion. Whereas the 3i regime can support the maintenance of naive murine PSCs, the conversion of murine EpiSCs into rESCs is impaired in the absence of LIF signaling (Yang et al., 2010). Ectopic expression of constitutively active Stat3 restored the EpiSC to rESC conversion, demonstrating that this pathway plays a critical role in establishing the naive pluripotent state (Yang et al., 2010).

Metastable Pluripotent Stem Cells

Curiously, the naive PSC state is only maintained in lines derived from a limited set of "permissive" inbred mouse strains. Until recently, naive PSC lines could not be derived from nonpermissive mouse strains such as the widely used nonobese diabetic (NOD) mice. This limitation severely hampered the development of novel genetic models in these backgrounds. Hanna and colleagues demonstrated that in nonpermissive genetic backgrounds, the naive PSC state depends on the continued presence of exogenous factors, either the constitutive expression of ectopic reprogramming factors (Klf4 or cMyc) or the presence of small molecule inhibitors of the GSK3β and MEK/ERK signaling pathways (Hanna et al., 2009), so-called 2i+LIF conditions. Upon withdrawal of these exogenous factors, the nonpermissive naive PSCs rapidly differentiate. Similar strategies allowed the derivation of naive PSCs from the rat either by ectopic expression of all four reprogramming factors, Oct4, Sox2, Klf4, and cMyc (Liao et al., 2009), or by using a chemical inhibitor cocktail in the form of 3i or 2i+LIF (Buehr et al., 2008). Importantly, naive rat PSCs were shown to be amenable to homologous recombination and allowed the generation of knockout rats (Tong et al., 2010), demonstrating that the naive PSC state offers functional advantages over primed PSCs.

The results discussed above demonstrate that the naive pluripotent state is not restricted to murine PSCs but requires the stabilization by exogenous factors in order to be maintained in other species. Three recent reports suggest that alternative states exist for human PSCs as well. Li and colleagues demonstrated that human iPSCs could be derived in the presence of 2i+LIF (Li et al., 2009). In these conditions, the human iPSCs adopt murine ESC-like colony morphology and are propagated as single cells by trypsin digest. However, the molecular and functional properties of the cells were not reported and their cell-surface marker profile suggests that the cells retain a degree of similarity to "traditional" primed human iPSCs. More recently, our lab demonstrated that reprogramming of somatic cells via Oct4, Sox2, Klf4, c-Myc, and Nanog under naive growth factor conditions results in cells termed hLR5 (human, LIF, and 5 reprogramming factors) that resemble murine ESCs with regard to their colony morphology, culture dynamics, and certain molecular properties (Buecker et al., 2010). Maintenance of the hLR5 state depends on the continued expression of ectopic reprogramming factors. Furthermore, hLR5 cells did not form differentiated derivatives and are therefore strictly speaking not pluripotent. However, hLR5 cells convert to a stable primed pluripotent state when switched to "standard" bFGF culture conditions. Importantly, hLR5 cells allow efficient homologous recombination-mediated gene targeting via standard protocols that are routinely used for murine ESCs and can thus serve as a powerful intermediate for the genetic manipulation of human PSCs. With a similar approach, Hanna and colleagues derived naive human PSCs by combining ectopic expression of Oct4 and Klf4 or Klf4 and Klf2 with small molecule inhibitors of GSK3β and MEK/ERK (Hanna et al., 2010). Importantly, the authors demonstrated that forskolin, an agonist of the Protein Kinase A pathway, can transiently replace the need for ectopic reprogramming factors. Interestingly, forskolin is also used for the derivation of embryonic germ cell lines from embryonic gonads, suggesting a close relationship between naive pluripotency and the germline. Indeed, the expression of germ cell-specific genes appears to be a hallmark property of naive PSCs (Chou et al., 2008; Tesar et al., 2007; Hanna et al., 2010). In the absence of ectopic reprogramming factors, the maintenance of human naive LIF-dependent PSCs is limited to approximately 15 passages. Taken together, these combined results suggest that a naive LIF-dependent pluripotent state can exist in multiple species, including humans. The specific growth factor conditions that maintain this state in the absence of ectopic reprogramming factors or small molecule inhibitors remain poorly understood.

Nanog as a Mediator of the Pluripotent State

The transcription factors Oct4, Sox2, and Nanog are at the heart of the stem cell transcriptional pluripotency network and together maintain pluripotency (reviewed in Barrero et al., 2010

[this issue of *Cell Stem Cell*]; Cole and Young, 2008; Chambers and Tomlinson, 2009). So how can two distinct sets of growth factors (LIF and Bmp4 versus bFGF and ActivinA) signal to define and maintain two discrete pluripotent states via the same transcriptional regulators of pluripotency, Oct4, Sox2, and Nanog? Of the three core factors, only Nanog has been shown to be a downstream target of both sets of growth factor pathways and thus Nanog is an attractive candidate as a key regulator of the pluripotent stem cell state.

Nanog was first described by two independent groups in a screen for genes that can bestow LIF-independent growth on mESCs (Chambers et al., 2003; Mitsui et al., 2003). Nanog knockout studies demonstrated that although Nanog is not required for maintaining pluripotency, *Nanog*$^{-/-}$ ESCs are more prone to differentiation (Chambers et al., 2007). In addition, in the absence of Nanog, the derivation of naive PSCs is impaired, demonstrating that Nanog plays an important role in establishing a pluripotent state (Silva et al., 2009). Several signaling pathways emanating from the LIF and ActivinA/TGF-β receptors were recently shown to regulate Nanog expression. Niwa et al. demonstrated that in mESCs, LIF-induced activation of PI(3)K/AKT induces T-Box 3 (Tbx3) expression, which in turn activates Nanog transcription (Figure 1; Niwa et al., 2009). In addition, parallel activation of the Jak/Stat3 pathway induces Klf4 expression, which binds to the Nanog promotor and further simulates Nanog expression (Niwa et al., 2009; Zhang et al., 2010). Xu and colleagues discovered SMAD2/3 binding sites in the human Nanog promotor, suggesting a direct effect of ActivinA/TGF-β signaling on Nanog expression in human ESCs. Indeed, ActivinA stimulation of human ESCs leads to enhanced activity of the Nanog promoter, whereas mutations of the SMAD2/3 binding sites abrogate this responsiveness (Figure 1; Xu et al., 2008).

Interestingly, Nanog expression levels are heterogeneous within a mESC colony and heterogeneity is reestablished when Nanog-GFP mESCs are sorted into Nanog^{high} and Nanog^{low} subpopulations (Chambers et al., 2007; Kalmar et al., 2009). The percentage of Nanog^{high} cells is greatly increased under 3i culture conditions, demonstrating that high Nanog expression levels are closely linked to the inhibitor-enforced naive ESC state (Wray et al., 2010). In contrast, Nanog^{low} cells within the ESC culture are more prone to differentiation, suggesting that low Nanog levels lead to a more primed pluripotent state (Chambers et al., 2007). Indeed, overexpression of Nanog facilitates the LIF-mediated conversion of EpiSCs into mESCs (Silva et al., 2009). These combined data suggest that the different sets of exogenous growth factors that support naive and primed PSCs promote a particular pluripotent state by maintaining different levels of Nanog expression. The recent generation of Nanog-GFP knockin human ESCs demonstrates that Nanog exhibits heterogeneous expression in human ESCs as well, but it remains to be analyzed whether similar functional differences exist between NANOG^{low} and NANOG^{high} human ESCs (Fischer et al., 2010).

Nanog and Naive Pluripotent Stem Cells

The above data suggest that in nonpermissive species, the naive pluripotent state is stabilized by the induction of Nanog expression, either by ectopic reprogramming factors or by small molecule inhibitors of differentiation. Indeed, Klf4 and/or Klf2 are upstream regulators of Nanog and are required to induce the transition of primed human PSCs to a naive pluripotent state (Hanna et al., 2010). In human PSCs, the combined inhibition of GSK3β and MEK/ERK is still insufficient to maintain the naive PSC state in the absence of ectopic reprogramming factors. However, the addition of forskolin, a PKA activator, allows the maintenance of naive iPSCs for up to 15 passages. PKA is commonly activated as a result of G protein coupled receptor (GPCR) activation and concomitant elevation of intracellular cAMP levels. The role of GPCR signaling in PSC maintenance is not well understood, but two reports suggest that elevation of intracellular cAMP levels stabilizes Oct4 and Nanog expression and can delay mESC differentiation upon LIF withdrawal (Faherty et al., 2007; Layden et al., 2010).

Considering how growth factor signals, reprogramming factor activity, and/or small molecule inhibitors seem to converge on Nanog, it seems reasonable to propose that additional growth factor signals that enhance Nanog expression levels may stabilize the naive human PSC state. Further exploration of the PKA signaling pathway, the identification of the relevant GPCR receptor and ligand, and the link between PKA signaling and Nanog expression may identify additional growth factor signals that sustain the stable, long-term propagation of naive human iPSCs and perhaps allow the derivation of naive human ESCs from blastocyst embryos.

New Applications of Naive Human PSCs

The naive human pluripotent state offers several advantages over existing human ESC and iPSC lines. Homologous recombination-mediated gene targeting is much enhanced in naive pluripotent stem cells, perhaps because of the more open chromatin structure of these cells (Hanna et al., 2009; Buecker et al., 2010; Tong et al., 2010). As such, naive PSCs can enable the generation of mutant or transgenic human cell lines to model disease and development. In addition, the targeted correction of mutations in patient-derived naive iPSCs would be an elegant way to restore normal organ function in future cell therapies. Moreover, maintaining human PSCs in the naive pluripotent state may help eliminate the large variation in developmental propensity observed among primed human PSC lines and even within a single, heterogeneous cell line (Osafune et al., 2008; Newman and Cooper, 2010; Guenther et al., 2010; Chin et al., 2010), increasing consistency in the results generated by different labs. Finally, naive pluripotent stem cells are uniquely able to integrate into the blastocyst ICM and functionally contribute to the developing embryo. Although no responsible group would propose to apply blastocyst complementation to developing human embryos, there are both basic and applied experimental settings that could eventually benefit from the unique functional properties of naive human PSCs. For example, in a series of elegant experiments, Kobayashi and colleagues recently demonstrated that naive rat pluripotent stem cells can integrate across species boundaries into mouse blastocyst embryos and form viable chimeras (Kobayashi et al., 2010). Although the overall percentage of rat cells contributing to the hybrid embryo was low, the rat donor cells were able to form an entire pancreas in recipient mouse embryos in which the pancreas was genetically ablated. Human ESCs have been injected into murine blastocysts in the past but did not

significantly contribute to the developing embryo (James et al., 2006). The above experiments with naive rat PSCs suggest that in the naive state, human PSCs may be developmentally compatible with blastocysts of other species. Such experiments will undoubtedly raise justifiable concerns and debate over the moral implications of cross-species chimeras (discussed in Solter, 2010). Nonetheless, in the right genetic background and perhaps in a larger animal host such as the pig, cross-species chimeras may serve as a feasible approach for the generation of autologous human tissues for organ transplantation.

In basic research, naive human PSCs will provide a critical tool to model the earliest steps in human embryonic development, as they are expected to be more closely related to the blastocyst ICM than current human ESC lines. Their unique primitive state will allow the study of critical epigenetic processes during early differentiation, such as, for example, X-inactivation. However, although initial attempts at deriving naive human iPSCs are promising, the culture (growth factor) conditions that support the long-term maintenance of these cells remain elusive. Ultimately, naive human ESCs will need to be generated from blastocyst embryos as well. Such experiments will raise ethical challenges of their own that should be subject to strict oversight. Nonetheless, it is clear that comparison studies regarding the functional behavior of embryo- and reprogramming-derived pluripotent cells from different species has been, and will continue to be, a valuable area of experimental inquiry.

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