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Leading Edge
Essay

Capturing Pluripotency

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In this Essay, we argue that pluripotent epiblast founder cells in the embryo and embryonic stem (ES) cells in culture represent the ground state for a mammalian cell, signified by freedom from developmental specification or epigenetic restriction and capacity for autonomous self-replication. We speculate that cell-to-cell variation may be integral to the ES cell condition, safe-guarding self-renewal while continually presenting opportunities for lineage specification.

Introduction

Mouse embryonic stem (ES) cells are permanent cell lines derived from the transient founder tissue in the preimplantation embryo, the epiblast (see Review by C.E. Murry and G. Keller and Essay by J. Rossant in this issue of *Cell*). ES cells have three defining properties: self-renewal, pluripotency, and primary chimera formation. After prolonged expansion in culture, they retain full responsiveness to developmental cues and show no intrinsic bias in the generation of different somatic lineages or germline cells upon reintroduction to the embryo. Similar self-renewing cells with broad developmental potency can also be generated by respecifying germ cells with extrinsic factors (Matsui et al., 1992) or reprogramming somatic cells using gene transfection (Takahashi and Yamanaka, 2006; see Review by R. Jaenisch and R. Young in this issue). Elucidating how cells with such capacities are established and propagated is a fascinating scientific challenge, charged with biomedical potential.

Considerable evidence indicates that a "trinity" of nuclear regulators, Oct4, Sox2, and Nanog, governs pluripotency in vivo and in vitro (Chambers and Smith, 2004; Niwa, 2007). Yet Oct4 and Sox2 also direct production of a destabilizing signal, fibroblast growth factor 4 (FGF4), which drives ES cells toward differentiation. Neutralizing this autoinductive pathway can preserve an uncommitted ground state, and we propose that this may be the key to capture of authentic ES cells from mammalian embryos or by reprogramming. However, recent evidence has also established that ES cells are not homogeneous. Individual ES cells exhibit variable expression of key factors such as Nanog, have distinct probabilities of self-renewal, and may have differing developmental potential. Moreover, ES cells appear subject to promiscuous and fluctuating transcription of lineage-affiliated genes. Delineating cellular transitions and hierarchies within the ES cell system should provide new insights into the fundamental nature of pluripotency and illuminate the incomplete capacities often observed in stem cells derived from human embryos and induced pluripotent stem (iPS) cells generated by reprogramming.

Transcription Factors Rule Pluripotency

Is the pluripotent condition a tabula rasa of unrestricted opportunity, or is differentiation prefigured by epigenetic encoding at specifier genes? Heritable modification of chromatin or DNA leading to fixation of gene expression programs is a key component in demarcating potential during development. The epigenetic signature of ES cells is characterized by coexistence of both activating and silencing chromatin modifications at multiple loci. It is posited that this bivalent status may be integral to pluripotency by providing a mechanism for repressing gene expression without permanent silencing. However, it is now apparent that bivalent domains are not exclusive to pluripotent cells (Mikkelsen et al., 2007). Moreover, ES cells remain undifferentiated in the complete absence of the repressive modification (trimethylation of histone 3 lysine 27) reported in bivalent domains, regardless of marginally increased expression of various lineage-affiliated genes (Schoeftner et al., 2006). Furthermore, ES cells can withstand deletion of DNA methyltransferases (Dnmts) and complete loss of DNA methylation without compromising self-renewal or genomic integrity, in marked contrast to the growth impairment and chromosomal abnormalities observed in other cell types (Tsumura et al., 2006). In general, absence of epigenetic components does not ablate the epiblast in the embryo or ES cell self-renewal in culture, indicating minimal dependency on such machinery for generation and maintenance of pluripotent populations (Niwa, 2007). In fact, early epiblast cells and ES cells are characterized by the erasure of a major epigenetic feature, the inactive X chromosome in female cells (Mak et al., 2004; Okamoto et al., 2004). Reactivation of the silent X coincides with the acquisition of full pluripotency in the nascent epiblast reflecting that this is a state created by removal rather than imposition of epigenetic restrictions.

However, perturbation of the chromatin-modifying machinery and DNA methylation often result in cell death during differentiation, indicating critical roles in the faithful prosecution of lineage commitment. It may be argued that cells that are blocked in differentiation have lost pluripotency. Consider, however, a determination gene such as *MyoD*. Mutation of *MyoD* will impair myogenic differentiation, but this does not imply any role for MyoD in pluripotency. For genes that act in the process of lineage commitment this distinction is often blurred. A critical test is whether repair of a gene defect is sufficient to restore differentiation capa-

bility. For example, ES cells in which the NuRD repressor complex is inactivated by deletion of the Mbd3 subunit are compromised in prosecuting differentiation, but this defect is eliminated upon re-expression of Mbd3, meaning that the pluripotent state has been preserved throughout (Kaji et al., 2006). A specific requirement in maintaining the pluripotent state should only be claimed when a change in developmental potential is irreversible, as for example when Oct4 or Sox2 is deleted (Niwa, 2007). Interestingly, Mbd3 appears necessary for derivation of ES cells, but this is because in its absence the epiblast does not form properly (Kaji et al., 2007) and not because Mbd3 is required by ES cells. In our view, current evidence suggests that although epigenetic mechanisms of gene marking and gene silencing might contribute in some degree to the overall stability of pluripotency, they are slaves not masters in this estate.

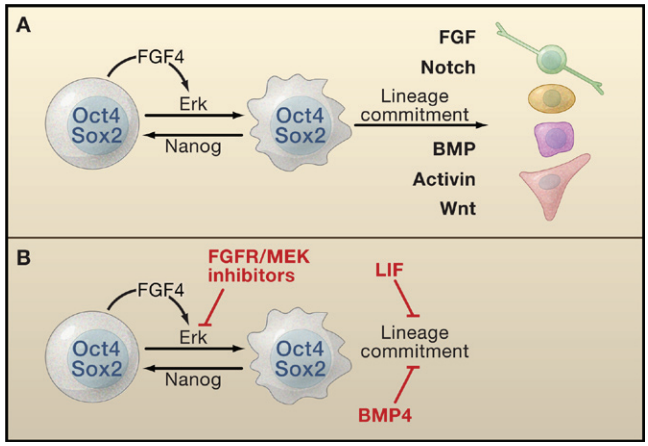

Figure 1. Maintaining Pluripotency

Autoinductive FGF4/Erk signaling poises embryonic stem (ES) cells for lineage entry and must be resisted to allow self-renewal.

(A) Oct4 and Sox2 direct expression of *fgf4* and poise ES cells for lineage commitment. Elevated Erk activity provides a signal that renders pluripotent cells susceptible to lineage inductive cues.

(B) Self-renewal of the pluripotent ES cell state requires overcoming the FGF4/Erk signal. The actions of FGF can be (1) blocked by selective pharmacological inhibitors of the FGF receptor (FGFR) and of Mek; (2) reversed by constitutive expression of Nanog; (3) counteracted by blockade of commitment effectors by the cytokine leukemia inhibitory factor (LIF) and the morphogen BMP4.

In contrast, much evidence indicates that naive pluripotency of early epiblast and authentic ES cells is critically dependent on the action of three transcriptional organizers, Oct4, Sox2, and Nanog (Chambers and Smith, 2004; Niwa, 2007). Coregulatory and autoregulatory mechanisms appear to link the three factors in a recursive self-reinforcing circuit. Each factor is essential for pluripotent epiblast cells in the blastocyst, whereas deletions from ES cells provoke unscheduled differentiation into trophoblast and hypoblast cells (reviewed in Niwa, 2007). Those fate choices are considered developmentally illegitimate because epiblast cells have passed beyond their segregation points prior to ES cell establishment (see Essay by J. Rossant). A key role of the trinity of transcription factors appears to be to suppress extraembryonic fate options through ongoing repression of trophoblast and hypoblast specifier genes, *Cdx2/Eomes* and *Gata-4/Gata-6*, respectively (Niwa, 2007). The pluripotent condition may thus be viewed as a transcription factor battlefield in which Oct4, Sox2, and Nanog are dominant and continuously suppress functional expression and activity of lineage specification factors (Niwa, 2007; Smith, 2005)

Autoinduced Erk Signaling Destabilizes Self-Renewal

Crucially, however, Oct4 and Sox2 also provide for developmental extinction of pluripotency by directing expression of FGF4. This acts as an autoinductive stimulus that propels ES cells toward lineage specification (Kunath et al., 2007) (Figure 1A). Importantly the FGF4/Erk signal does not specify lineage but renders ES cells responsive to further inductive signals. Consequently, ES cells or epiblast lacking FGF4 or deficient in downstream mitogen-activated protein kinase Erk1/2 signaling exhibit a general impairment of commitment.

Recently, a second class of embryo-derived multipotent stem cells has been described that actually depends on FGF signaling for propagation (see Essay by J. Rossant). ES cells are derived from mouse embryos prior to implantation into the uterus. After implantation, the epiblast undergoes dramatic expansion and morphogenesis, transforming from an unstructured cell mass into a columnar epithelium. The epithelialized epiblast (cylindrical in rodents, discoid in other mammals) is subject to potent lineage-specifying signals from adjacent extraembryonic tissues. Cultures of egg cylinder stage epiblast in the presence of FGF and activin give rise to continuous cell lines termed EpiSCs (Brons et al., 2007; Tesar et al., 2007). Unlike ES cells, EpiSCs show little or no capacity to colonize developing embryos when introduced into blastocysts. They do form multidifferentiated teratomas when injected into adult mice, however, demonstrating a degree of multilineage differentiation. It will be instructive to determine the relationship between EpiSCs and the FGF4-induced intermediate stage in ES cell lineage commitment. EpiSCs have culture requirements and properties distinct from mouse ES cells and similar to those stem cells derived to date from human embryos (Brons et al., 2007; Tesar et al., 2007). Notably their propagation in culture is stimulated by FGF. Human embryo cell lines vary in their efficiency of differentiation into particular lineages, perhaps indicating that they are partially specified. It could be informative to examine whether rodent EpiSCs exhibit lineage bias during in vitro differentiation and whether this may correlate with their original regionalization in the egg cylinder. If stem cells can be isolated and classified that are prespecified for individual germ layers this could be advantageous for therapeutic applications and drug screening, which require the generation of homogeneous cell lineages. This is because for ES cells it remains a major challenge to steer unidirectional commitment. Indeed, complete command of this primary fate choice may even be incompatible with the underlying nature of the pluripotent ground state (see below). A note of caution, however, comes from reports that both human embryo-derived stem cells and rodent EpiSCs differentiate into extraembryonic trophoblast without genetic manipulation (see Essay by

J. Rossant). This is difficult to reconcile with the argument that they are authentic representatives of postimplantation epiblast and may indicate that they are a corrupted state produced in vitro.

Perpetuating naive undifferentiated ES cells requires overcoming normal developmental progression engendered by the accumulating FGF4 signal downstream of Oct4 and Sox2. In fact, ES cells can be maintained by various in vitro artifices (Figure 1B). Conventional culture has relied on extrinsic stimulation of the Stat3 transcription factor by the cytokine leukemia inhibitory factor (LIF) and parallel induction of inhibitor of differentiation (ID) proteins by serum factors or bone morphogenetic proteins (BMPs) (Ying et al., 2003). These signals primarily act downstream of Erk signaling to block commitment instructions, although LIF/Stat3 also promotes ES cell growth and viability. Induction of IDs is entirely dispensable if FGF/Erk signaling is reduced. Furthermore, genetic disruption or pharmacological inhibition of the FGF4/Erk axis permits self-renewal without engagement of Stat3 (Q.L. Ying, J. Wray, J. Nichols, and A.S., unpublished data). Therefore, self-renewal appears to be the uninstructed or passive fate choice for ES cells. However, growth and viability are impaired under these conditions, either as a direct consequence of loss of Erk activity or possibly due to off-target effects of the chemical inhibitors. This collateral damage can be avoided by selective inhibition of glycogen synthase kinase-3 (GSK3). Stabilization of intracellular β-catenin seems to be a contributory factor downstream of GSK3 inhibition, either by simulating canonical Wnt signaling or by other interactions (Anton et al., 2007). Indeed, Wnt3a reinforces the suppression of neural commitment and may increase the probability of a self-renewal division. However, Wnt has only minor effects on ES cell growth and viability and does not fully reproduce the effects of GSK3 inhibition. We hypothesize that reduced GSK3 activity restores cellular growth and viability, principally via global derepression of biosynthetic capacity.

The biosynthetic capacity or "energy level" of the ES cell, counterregulated by GSK3 and Erk, might also directly influence the probability of self-renewal versus commitment. This possibility is suggested by quantitative analyses of MAPK regulation of the mating type switch in yeast. Modeling of these data reveals a feedback system whereby the threshold for a fate switch is reduced in relation to biosynthetic capacity of the cell (Colman-Lerner et al., 2005). An analogous inverse correlation in ES cells could create a situation whereby inhibition of Erk both directly suppresses effectors that mediate commitment but also indirectly lowers the level of effectors required. In this scenario, restoration of the biosynthetic capacity by GSK3 inhibition restores the threshold for commitment.

The Ground State

ES cells seem to exhibit a unique degree of autonomy for a mammalian cell. They are intrinsically self-sufficient, unprogrammed, and self-replicating, perhaps more akin to yeast than other metazoan cells. This likely underlies their propensity to give rise to multidifferentiated tumors known as teratocarcinomas (Chambers and Smith, 2004).

The question arises of whether ES cells are a tissue culture epiphenomenon, as has often been suggested, or whether early epiblast cells are in an equivalent state? The epiblast is generated from bifurcation of the primary inner cell mass (ICM) in the blastocyst. Inhibition of Erk signaling in early embryos suppresses emergence of hypoblast cells and results in development of the entire ICM into epiblast (Chazaud et al., 2006; J. Nichols, J.S., and A.G.S., unpublished data). Augmented by inhibition of GSK3 the nascent epiblast expands in situ under these conditions and if explanted gives rise to ES cells. Early epiblast cells are thus comparable to ES cells in their independence from but vulnerability to Erk signaling. Epiblast cells also give rise to teratocarcinomas if displaced from the powerful inductive environment of the early embryo (Stevens, 1983). ES cells may therefore be true counterparts of naive epiblast rather than culture creations. If epiblast and ES cell pluripotency are founded on a common ground state, this would be expected to be conserved in essential features between mammalian species. A testable prediction is that it should be possible to capture authentic ES cells from epiblasts of other species by targeting the Erk and GSK3 pathways.

Is the ground state hypothesis relevant to reprogramming? It is striking that in vitro the differentiated epigenome can be "collapsed" (that is, the restrictive epigenetic program of somatic cells can be deconstructed and erased) to recreate pluripotency. However, iPS cells produced by direct reprogramming (using a four-factor cocktail comprising Oct4, Sox2, cMyc, and Klf4) often exhibit compromised gene expression profiles, epigenetic features, and developmental potential compared with authentic ES cells (Takahashi and Yamanaka, 2006). This may be due to incomplete deprogramming creating an aberrant phenotype, or to precipitous transit through the ES cell state and conversion to a downstream cell such as an EpiSC. Intriguingly, the transcription factor Rex1 appears to be expressed at low or negligible levels in some iPS cells (Okita et al., 2007) and is also downregulated in EpiSCs. We suggest that creation of iPS cells is likely to entail a process of epigenetic deconstruction that reaches, and is stable in, the true ground state only if and when (1) Nanog is activated; (2) appropriate stoichiometry of the endogenous trinity factors is established: (3) extrinsic perturbations are minimized. A prediction is that inhibition of Mek and GSK3 should facilitate isolation of fully deprogrammed iPS cells by consolidating the ground state and eliminating EpiSCs and other phenotypes that cannot expand in these minimal conditions.

ES Cells: A Heterogeneous Coalition

Forced constitutive expression of Nanog is sufficient to prevent ES cell differentiation and render self-renewal constitutive even in the presence of active FGF/Erk signaling (Chambers et al., 2003; Ying et al., 2003). This implies that Nanog is normally limiting in ES cell cultures and that the Oct4/Sox2/Nanog trio of transcription factors is subject to a component of negative regulation. In fact, expression levels of Nanog are highly variable in ES cells (Figure 2A), contrasting with the apparent homogeneity of Oct4 and

Sox2. Furthermore, ES cells in which both alleles of Nanog have been deleted can remain undifferentiated and pluripotent (Chambers et al., 2007). Other pluripotency genes and many putative targets of Nanog identified by genome location analyses continue to be expressed in ES cells lacking Nanog. These findings challenge the dogma that Nanog acts in partnership with Oct4 and Sox2 in housekeeping transcriptional maintenance of pluripotency. However, cells lacking Nanog, whether by endogenous downregulation or gene deletion, do have a greatly increased tendency to differentiate (Chambers et al., 2007; Mitsui et al., 2003). This suggests that Nanog plays a pivotal role in buffering ES cells against the consequences of Erk signaling. In vivo deletion and cell fusion studies have demonstrated that Nanog functions in establishing de novo the unrestricted states of pluripotency (Mitsui et al., 2003; Silva et al., 2006) (J. Nichols, J.S., and A.S., unpublished data) and of germ cells (Chambers et al., 2007). In ES cell cultures, therefore, Nanog may reverse the effects of Erk signaling or of secondary inducers, resetting the ground state. Further insight should be obtained by examining whether absence of Nanog facilitates efficient conversion of ES cells into EpiSCs and whether EpiSCs lacking Nanog can be maintained or have altered developmental competence.

In the embryo, Nanog expression is also dynamic as it is downregulated at the time of implantation and re-emerges in the posterior egg cylinder. Creation of a green fluorescent protein (GFP) allele has established that mosaic expression in ES cells is determined primarily at the level of transcription and is fully reversible. The underlying mechanism is unknown. One possibility is an intrinsic oscillatory circuit mediated by autorepression (I. Chambers, personal communication). This may operate synchronously over the short time span of epiblast development but become asynchronous in expanded ES cell cultures. Alternatively, the fluctuations may be stochastic, as appears for initial Nanog expression in the embryo (Dietrich and Hiiragi, 2007). Whatever the mechanism, varying levels of Nanog impart functional heterogeneity in self-renewal probability upon individual ES cells (Chambers et al., 2007). Nanog is not the only pluripotency gene that exhibits nonuniform expression in ES cells. Rex1 (Zfp42) is also expressed differentially between interconvertible subpopulations with distinct growth and differentiation properties (Toyooka et al., 2008). Furthermore, ES cells exhibit transcriptional activity for many genes associated with lineage commitment and differentiation, similar to the phenomenon of multilineage priming (also known as multilineage preview) described in hematopoietic stem cells (Hu et al., 1997; Ye et al., 2003). This promiscuity has been widely exploited in promoterless gene trap screens (Skarnes et al., 1995). Some of these insertions show mosaic reporter profiles in undifferentiated ES cells (W. Skarnes, personal communication) indicating that lineage preview is subject to fluctuation. Therefore, the convention that ES cell cultures are homogeneous can no longer be maintained. Rather, they comprise a coalition of transitory transcriptional settings. Can this be reconciled with the ground state hypothesis?

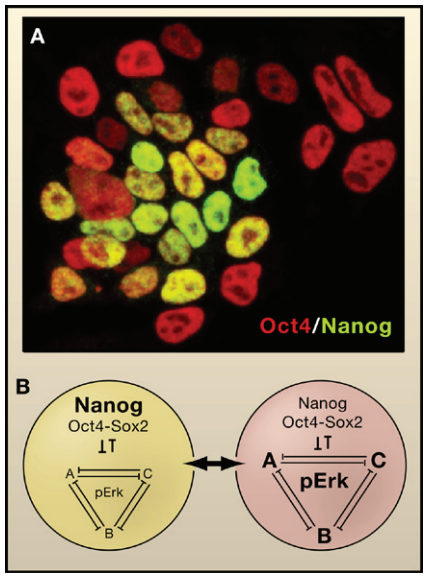

Figure 2. A Metastable Coalition

The transcription factor Nanog secures self-renewal of ES cells, and cell-to-cell variation creates the possibility for differentiation. (A) Embryonic stem (ES) cells are heterogeneous. Immunostaining shows highly variable levels of Nanog protein in Oct4-positive undifferentiated ES cells. (B) In our model, lineage-associated transcriptional circuits (A, B, and C) are maintained below threshold levels due to mutual antagonism and suppression by the three transcription factors Oct4, Sox2, and Nanog. A destabilized transitional state arises when downregulation of Nanog coincides with increased activation of Erk. Phosphorylated Erk (pErk) may activate inductive signaling pathways or directly promote lineage-affiliated transcriptional networks. The fluctuations in network activities generated by pErk confer an opportunity to establish a new stable cell state. However, if Nanog levels rise before commitment is effected, the actions of pErk are neutralized, the metastable ground state is restored, and the gate is closed. Photo courtesy of J. Silva and A. Smith.

A Platform for Multilineage Decisions

The foundation of pluripotency may be a fluid transcriptome. In open transcriptional space an uncommitted condition may be maintained if incipient gene regulatory networks are mutually antagonistic. This depends on crossinhibition restricting the probability of any single program attaining an activation threshold for dominance over the resident masters, Oct4/Sox2/Nanog. Neutralizing conflicts between multiple opposing transcription factor circuits could create a metastable state (Figure 2B). Analogous transcription factor duels have been proposed to underlie multilineage potential in the hematopoietic system (Huang et al., 2007). Promiscuous transcription may set the stage for lineage specification by parading the possibilities. For this competent state to be perpetuated, however, fluctuations in expression level should be constrained or their consequences reversed. Conversely, the Erk signal should destabilize the ground state to promote lineage specification. This might be achieved by increasing globally the amplitude of fluctuations in the system. Alternatively, Erk may selectively target particular circuits, for example to upregulate pathway responsiveness to secondary inducers. Whether chromatin modification is a direct target of Erk or of secondary inductive signals is currently unknown. In ES cell cultures, Stat3 and ID proteins may directly antagonize effectors downstream of Erk. Nanog acts in parallel by unknown mechanisms to restrict or undo consolidation of nascent transcriptional and epigenetic architecture of lineage specification. Optimal self-renewal requires both components, although either alone can be sufficient.

Cell-to-cell variation in Nanog levels creates graded differences in resistance to differentiation, ranging from highly refractory to highly susceptible. This heterogeneity likely contributes to two well-remarked features of ES cell

cultures: the incidence of background differentiation under conditions favoring self-renewal and the persistence of undifferentiated cells under conditions favoring differentiation (Smith, 2001). The regulation and dynamics of transitions in Nanog expression have yet to be fully characterized but do not appear related to cell cycle because downregulation of knockin GFP can be sustained for more than one round of cell division before re-expression (Chambers et al., 2007). Although it is clear that ES cells can move back and forth between high and low Nanog expression, it is also evident that many ES cells that downregulate Nanog then exit self-renewal. ES cells that lack Nanog may comprise a spectrum of phases between lineage specification and commitment.

There may also be significant heterogeneity within the Nanog-positive ES cell population. One possibility is that ES cells may continually spin-off derivatives that are in an EpiSC state. The reported mosaicism in Rex1 reporter gene expression might indicate that EpiSCs do coexist with ES cells, at least under some culture conditions. EpiSCs are otherwise indistinguishable from ES cells using the conventional pluripotency markers, Oct4, Sox2, and Nanog. They would be expected to persist at least transiently in coculture with ES cells due to paracrine provision of their supporting growth factors FGF4 and Nodal. Significantly, EpiSCs do not spontaneously convert back to ES cells. It is conceivable that ES cell cultures are structured hierarchically with only a portion of cells retaining full developmental potential. This would be consistent with data suggesting that many cells in ES cell cultures may not be competent to contribute to chimeras (Wang and Jaenisch, 2004). The heterogeneities described here (and those yet to be discovered) should be considered when interpreting transcriptomic and other global datasets generated from bulk ES cell populations. Adaptation of technologies developed in yeast (Colman-Lerner et al., 2005; McClean et al., 2007) for interrogating, measuring, and modeling cell-to-cell variation is likely to be crucial for further deconvolution of the ES cell condition. An interface between stem cell research and systems biology may now be key to understanding the decision pathways that face ES cells poised between pluripotency and lineage commitment.

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