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Review

Regulatory Principles of Pluripotency: From the Ground State Up

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Pluripotency is the remarkable capacity of a single cell to engender all the specialized cell types of an adult organism. This property can be captured indefinitely through derivation of self-renewing embryonic stem cells (ESCs), which represent an invaluable platform to investigate cell fate decisions and disease. Recent advances have revealed that manipulation of distinct signaling cues can render ESCs in a uniform "ground state" of pluripotency, which more closely recapitulates the pluripotent naive epiblast. Here we discuss the extrinsic and intrinsic regulatory principles that underpin the nature of pluripotency and consider the emerging spectrum of pluripotent states.

Introduction

The pluripotent state is the fundamental building block at the root of embryonic development. Cells that acquire pluripotency harbor the functional capacity to give rise to all the somatic lineages of the embryo and to the germline. In vivo, the pluripotent state emerges during development of a totipotent zygote toward a blastocyst. This process delineates two lineages: the inner cell mass (ICM), which is the pluripotent founder population, and the trophectoderm (TE), which forms an extraembryonic epithelial layer that envelopes and supports the ICM. At the late blastocyst stage (embryonic day 4.0 [E4.0]) the ICM consolidates to establish the pluripotent *Nanog*-expressing epiblast lineage and an overlying extraembryonic layer of *Gata6*-expressing primitive endoderm (also known as hypoblast) (Silva et al., 2009). At this point the partitioned epiblast cells enter the developmental "ground state," the origin of all future embryonic lineages (Figure 1).

Acquisition of the ground state in the epiblast is characterized by uniform expression of key pluripotency factors and reactivation of the paternal X chromosome in female embryos. This is paralleled by the establishment of a derestricted epigenome, including global DNA hypomethylation. The ground state can therefore be considered a cellular condition that is liberated from epigenetic and developmental constraints, a so-called blank slate (Silva and Smith, 2008). Functionally, ground state epiblast cells are described as being in a "naive" state of pluripotency, which reflects their unbiased developmental potential. As postimplantation development proceeds however (from E5.0), powerful inductive stimuli trigger naive epiblast cells to transit to a "primed" phase of pluripotency that is poised to initiate lineage-specification programs and is epigenetically restricted (Nichols and Smith, 2009). Naive pluripotency in the embryo is, consequently, an inherently transient condition. Nevertheless, the ephemeral nature of naive pluripotency can be captured indefinitely in vitro, through derivation of embryonic stem cells (ESCs) from the ICM or through experimental reprogramming strategies (Evans and Kaufman, 1981; Martin, 1981; Yamanaka and Blau, 2010). Under permissive culture conditions, ESCs retain naive pluripotent capacity while undergoing self-renewal—cell division without loss of cellular identity—and thus represent a surrogate model of the naive epiblast that is effectively kept in stasis.

Remarkably, when reintroduced back into the early embryo, ESCs that have undergone extensive in vitro expansion are induced to exit self-renewal and can efficiently contribute to all aspects of embryonic development (Bradley et al., 1984). If introduced into a tetraploid donor blastocyst, which cannot complete fetal development, healthy adult mice can be derived that are exclusively composed of hitherto in vitro cultured ESCs, stringently underscoring their unrestricted potency (Nagy et al., 1993). Importantly, this occurs without tumorigenesis, demonstrating that in the appropriate context, ESCs retain full control over the developmental program. Because ESCs can efficiently colonize the germline, they represent a highly tractable system for constructing genetically engineered lines of mice such as gene knockouts. Additionally, the unique properties of ESCs make them an invaluable tool for modeling developmental processes and disease. More recently, a model of the primed pluripotent state termed epiblast stem cells (EpiSCs) have also been derived, which provide a complementary system to investigate pluripotency and cell fate decisions (Brons et al., 2007; Tesar et al., 2007).

In this Review, we discuss the fundamental principles that underpin the self-renewing pluripotent state(s). We consider the emerging spectrum of pluripotency that ranges from the naive "ground state" to the developmentally "primed" state. In particular, we focus on the accumulating knowledge of the functional, epigenetic, and signaling properties that contribute to propagating ground state pluripotency in murine ESCs. The progress in this field has paved the way for defining and establishing naive human ESCs (hESCs) and we consider these recent advances.

Propagating Naive Pluripotency

Classically, mouse ESCs are derived through explanting an intact blastocyst or an isolated ICM onto a layer of mitotically

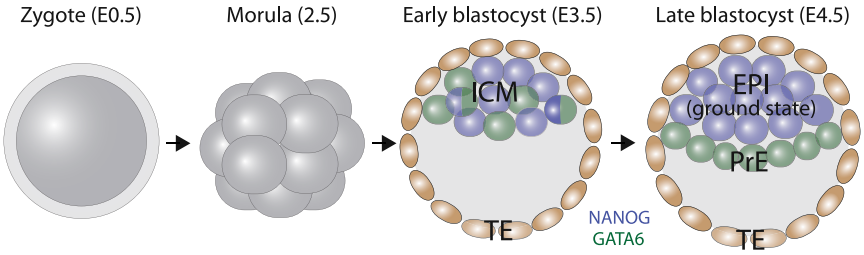

Figure 1. Establishment of the Pluripotent Ground State In Vivo

Development of the zygote proceeds through cleavage divisions to form a morula, which elaborates the extraembryonic trophectoderm (TE) lineage and the pluripotent inner cell mass (ICM) of the blastocyst at E3.5. The ICM expresses both *Gata6* (green) and *Nanog* (blue) but subsequently segregates into the extraembryonic primitive endoderm (PrE) and pluripotent naive epiblast (EPI), which exhibit mutually exclusive expression of these genes, respectively. The EPI is the source of all embryonic lineages, including the germline, and is therefore the developmental "ground state."

inactivated fibroblasts. These fibroblasts, known as "feeders," endow trophic factors that together with fetal calf serum engender a complex culture medium that supports self-renewal. ESC culture media is typically supplemented with the signaling molecule leukemia inhibitory factor (LIF), which can substitute for feeders in the presence of an appropriate extracellular matrix such as gelatin (Smith et al., 1988; Williams et al., 1988). Under serum/LIF conditions (+/− feeders) ESCs are denoted as "conventional" or "serum" ESCs. These cells have a high nuclear/cytoplasmic ratio, form domed colonies of tightly packed but heterogeneous cells, and are competent to be passaged as colony forming single cells, a property referred to as clonogenicity. A hallmark of such ESCs is the expression of *Oct4*, *Sox2*, and to some extent *Nanog*, which together with a suite of interconnected regulators, underpin pluripotency (Young, 2011; Dunn et al., 2014). Historically it has only been possible to efficiently derive ESCs from the 129 strain of mice using serum/LIF conditions, implying that competence for self-renewal is significantly influenced by genetic background (Gardner and Brook, 1997).

The derivation of ESCs from the ICM in serum is linked with ongoing transcriptional changes and it has been suggested that such derivation may occur via a primordial germ cell (PGC)-like program (Chu et al., 2011; Tang et al., 2010). However, loss of the obligatory PGC factor *Blimp1* does not impede ESC derivation, confirming that ESCs represent direct capture of the naive epiblast state (Bao et al., 2012). Nevertheless, one consequence of the derivation and maintenance of ESCs in serum is that undefined and often conflicting signaling pathways are activated. While such conditions sustain naive pluripotency at the level of the cellular population, they also promote a significant degree of morphological, transcriptional, and ultimately functional heterogeneity among cells (Chambers et al., 2007; Hayashi et al., 2008; Toyooka et al., 2008). As a result conventional ESCs exist in at least two distinct populations that broadly correspond to a naive state, which is functionally comparable to the preimplantation epiblast, or the more developmentally advanced "primed" state linked with expression of lineage-associated genes and poor performance in pluripotency assays. These subpopulations do however interchange their identity in culture (Abranches et al., 2013), suggesting that the transcriptional and functional differences between states are in dynamic equilibrium, at least to some extent, and thus that ESCs in serum can be considered "metastable," cycling in and out of naive status. Further subpopulations may also persist in serum that, for example, are primed toward extraembryonic primitive endoderm fate or have irretrievably exited a pluripotent state, highlighting overall population heterogeneity (Canham et al., 2010).

The accumulated knowledge of the molecular circuitry that underpins pluripotency has recently led to the development of novel conditions that preferentially stabilize the naive state in culture. Principally, the use of two small-molecule kinase inhibitors, termed "2i," harnesses ESCs in a distinct transcriptional and epigenetic state that includes uniform expression of key pluripotency factors, such as *Nanog* and *Prdm14*, and global DNA hypomethylation (Leitch et al., 2013; Yamaji et al., 2013; Ying et al., 2008). This is paralleled by a consistent relatively spherical colony morphology, with defined borders that lack differentiating cells, and enhanced clonogenicity. The 2i components comprise a specific inhibitor of the FGF/ERK signal transduction pathway, known as PD03 (PD0325901) and a specific inhibitor of GSK3, referred to as CHIRON (CHIR99021), that collectively shield ESCs from inductive differentiation stimuli (discussed below) and select against differentiating cells (Ying et al., 2008). Importantly, ESCs maintained in 2i are consistently competent to form high-contribution mouse chimeras with germline transmission—often with notably greater efficiency than ESCs from serum conditions—and thus robustly satisfy the defining test of naive pluripotency.

Culture using the 2i system is typically carried out in both a feeder- and serum-free growth medium, such as N2B27, with optional addition of LIF. Standard 2i conditions are therefore highly chemically defined and thus theoretically more reproducible than serum-based culture. The use of the 2i culture system also bears the significant advantage that it facilitates derivation of pluripotent ESCs from all tested strains of mice, including biologically important but previously recalcitrant strains, such as nonobese diabetic (NOD) and FVB (Kanda et al., 2012; Nichols et al., 2009; ten Berge et al., 2011). Indeed, pluripotent stem cells can also be established from other developmental sources using 2i, such as rat preimplantation epiblast or PGCs, that adopt almost identical properties to mouse ESCs, suggesting that 2i promotes a generic naive state, at least in rodents (Buehr et al., 2008; Leitch et al., 2010). It remains to be determined however whether ESCs maintained in 2i over an extended culture period are karyotypically and epigenetically stable (at genomic imprints for example), an important feature of ESCs propagated in serum/LIF.

Defining the Ground State In Vitro

The "ground state" is considered here as the unrestricted naive pluripotent state established in vivo in the epiblast cells of the mature blastocyst (Figure 1). This differs subtly from "naive pluripotency" per se, which is strictly a functional property attributed to any cell that exhibits the unbiased capacity to give rise

to all embryonic lineages following blastocyst injection, irrespective of how closely or not it mirrors the developmental ground state established in vivo. Both serum/LIF and 2i/LIF culture conditions are conducive for maintenance of naive pluripotency, as judged by chimera contribution. However, most ESCs in serum/LIF exhibit an altered transcriptional and epigenetic profile relative to preimplantation epiblast cells and thus, at the population level, are considered to be functionally naive but not ground state.

In contrast, the molecular and functional properties of ESCs in 2i/LIF are consistent with most of these cells being in an optimized state of naive pluripotency that is closely comparable to the developmental ground state in vivo. For example, ESCs in 2i exhibit significantly reduced/absent expression of lineage-associated genes, a permissive epigenetic landscape, and cluster closely with E4.5 epiblast cells at the single-cell transcriptome level (Boroviak et al., 2014; Marks et al., 2012). For this reason ESCs in 2i (+/−LIF) are referred to as being "ground state," since they are both functionally naive and a close molecular approximation of the epiblast cells of the blastocyst. It is important to consider however that while the 2i system represents the best available approach to model the developmental ground state, it remains an inherently imperfect recreation, at least because self-renewal is not part of the in vivo program. Thus, in the context of pluripotent cells in vitro, the ground state can be considered to be the most pristine or optimized state of naive pluripotency on a spectrum of multiple naive states.

Extrinsic Signaling Pathways for Naive Pluripotency

The capacity for ESCs to indefinitely retain naive pluripotency in culture requires a continuous input from extrinsic signals. Such exogenous cues are requisite owing to ESC-derived autocrine factors that promote exit from the self-renewal program and in particular fibroblast growth factor (FGF). Secretion of FGF4 in culture feeds back through the MEK/ERK signaling cascade to sensitize ESCs to instructive differentiation signals, which in turn direct commitment to specific lineages (Kunath et al., 2007; Stavridis et al., 2007). Because *Fgf4* expression in ESCs is activated by OCT4 and SOX2, its prodifferentiation influence is directly wired into the core pluripotency circuitry (Yuan et al., 1995). Other influences intrinsic to ESCs, such as the NuRD corepressor complex, also antagonize maintenance of naive status (Reynolds et al., 2012). Thus, without culture conditions that counterbalance or inhibit inductive differentiation signals emanating from ESCs themselves, self-renewal is inherently destabilized. As such, modulation of key extrinsic pathways such as JAK/STAT3 activation, WNT signaling, BMP4 activity, or FGF is important to maintain ESC identity and is considered here in the context of the principal culture conditions serum/LIF and 2i.

Serum/LIF Culture

The conventional regime of serum-supplemented culture influences multiple signaling pathways. However, an essential factor is LIF, which acts via binding the gp130/LIF-R cell-surface receptor complex (Yoshida et al., 1994). Downstream, JAK kinases phosphorylate and activate the transcription factor STAT3, which is the critical effector of LIF. Consistently, the absence of *Stat3* is incompatible with ESC self-renewal in serum/LIF, whereas its overexpression is sufficient to drive LIF-independent self-renewal (Niwa, 2007). The key role of STAT3 is further revealed by its requirement to sustain the pluripotent ICM in vivo (Do et al., 2013).

Mechanistically, phosphorylated STAT3 translocates to the nucleus in ESCs, where it regulates several pluripotency-promoting targets including *Klf4*, *Gbx2*, and possibly *c-Myc* (Cartwright et al., 2005; Hall et al., 2009; Niwa et al., 2009; Tai and Ying, 2013). Because forced expression of these targets does not fully recapitulate STAT3 activity however, LIF is expected to modulate further genes. Indeed, elegant recent studies have demonstrated that the transcription factor *Tfcp2l1* is likely the primary target promoting pluripotency downstream of LIF/STAT3 (Martello et al., 2013; Ye et al., 2013). As such, *Tfcp2l1* is necessary for ESC responsiveness to LIF in conventional conditions and, conversely, forced *Tfcp2l1* expression is sufficient to support self-renewal in the absence of LIF. However, LIF does additionally activate the PI3K/AKT and MEK/ERK signal cascades, as well as bona fide STAT3 target *Klf4*, implying that while *Tfcp2l1* is the critical mediator, the full activity of LIF may be realized through integrating multiple direct targets (Figure 2) (Niwa et al., 2009). Paradoxically, this includes weak stimulation of the differentiation-inducing MEK/ERK pathway.

In conjunction with LIF, fetal calf serum sustains self-renewal in conventional conditions. The crucial component of serum is BMP4, which acts via downstream SMAD signaling pathways to activate *Inhibitor of Differentiation* (*Id*) genes. Forced expression of *Id* genes or addition of exogenous BMP4 to culture substitutes for serum to maintain self-renewal. This regulation may function in part by promoting expression of *E-Cadherin*, which restrains cell fate commitment (Malaguti et al., 2013). In the absence of BMP4, ESCs progressively differentiate toward neuroectoderm derivatives, whereas absence of LIF from culture results in extensive nonneural differentiation (Ying et al., 2003). The prevalence of reciprocal differentiation pathways when either component is removed has fostered the assumption that LIF and BMP4 together support self-renewal by each suppressing differentiation toward specific fates, collectively restricting access to all lineages.

2i Culture

Culture in serum/LIF sustains self-renewal by overriding or counteracting differentiation stimuli downstream of their effects and, thus, ESCs in conventional conditions exist in a battleground of competing signals that precipitates in metastability. It is desirable therefore to identify conditions that insulate from, rather than counteract, differentiation-inducing signals, thereby stabilizing the upstream naive state. The key candidate for modulation is the FGF-ERK pathway. *Fgf4*$^{-/-}$ ESCs are severely compromised in differentiation toward both neural and mesendoderm lineages, implying FGF4 is upstream of cell fate commitment. A comparable phenotype is observed in *Erk2*$^{-/-}$ ESCs, highlighting ERK1/2 as the downstream effector of FGF4 (Kunath et al., 2007; Stavridis et al., 2007). The FGF/ERK pathway thus appears to drive transition out of naive status to a primed state that is susceptible to further lineage-specifying cues. Mechanistically, activated ERK2 phosphorylates the pluripotency protein KLF2 leading to its proteosomal degradation and, consequently, acts to destabilize the naive network (Yeo et al., 2014). In parallel, ERK1/2 directly promotes competence for the primed state by

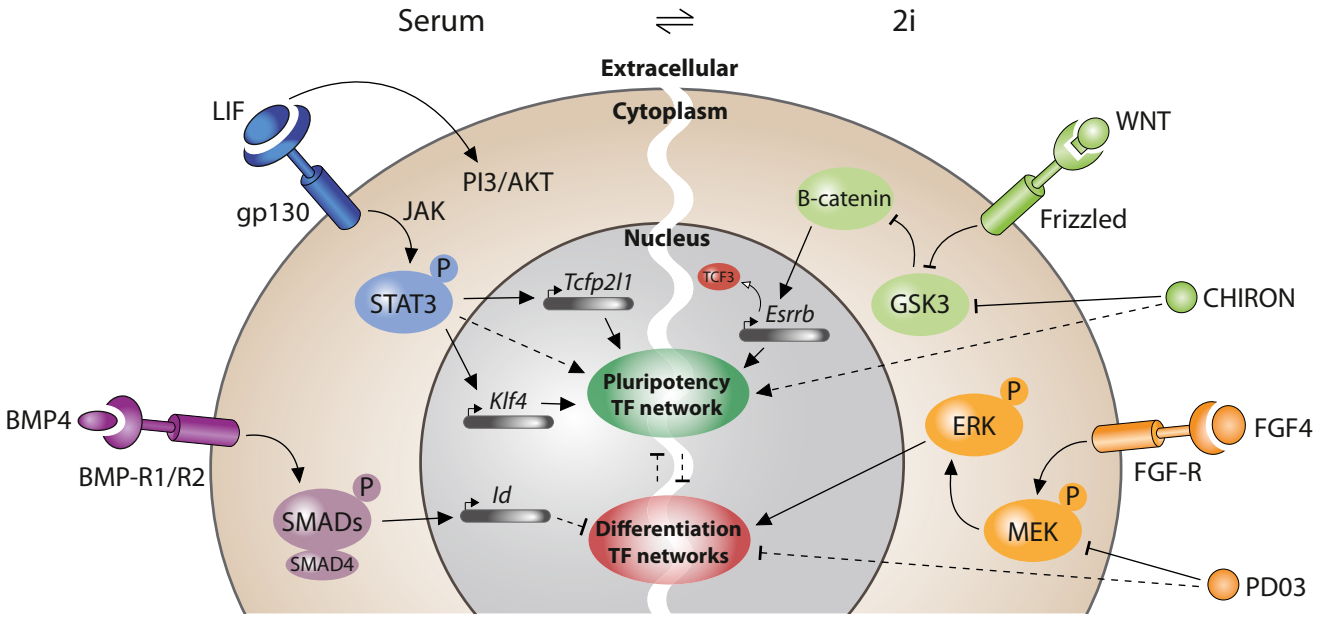

Figure 2. Extrinsic Signaling Pathways that Feed into Reinforcing or Antagonizing Naive Pluripotency

Simplified schematic of various signaling cascades that affect self-renewal. Filled arrows indicate activation, whereas bars show inhibition or blockade of target activity. A solid line implies a direct or known downstream target and a dashed line indicates an indirect or inferred effect. Clockwise: BMP4 is present in serum and functions via SMADs to activate *Id* genes. LIF signaling affects many pathways but primarily acts via JAK-mediated phosphorylation of STAT3, which activates *Tcfp2l1* and *Klf4*. Canonical WNT signaling blocks GSK3 activity leading to stabilization of β-catenin, which in turn abrogates TCF3-mediated repression of pluripotency genes including *Esrrb*. CHIRON closely mimics WNT signaling by inhibiting GSK3. FGF signaling activates the MAPK pathway leading to phosphorylation of MEK, which in turn phosphorylates and activates ERK. Activated ERK promotes transition to "a primed" state, which is therefore blocked by the MEK inhibitor PD03.

targeting poised chromatin to developmental genes (Tee et al., 2014).

It was therefore hypothesized that blockade of ERK activity would robustly capture the upstream naive state (Silva and Smith, 2008). Indeed, the potent MEK inhibitor PD03, which blocks phosphorylation and hence activation of ERK1/2, promotes long-term self-renewal and colony formation in defined media (Figure 2) (Ying et al., 2008). However, while inhibition of ERK suppresses differentiation, PD03 is insufficient to support ESC viability in the absence of LIF. Based on knowledge that inhibition of another kinase, GSK3, enhanced ESC self-renewal (Ogawa et al., 2006; Sato et al., 2004), it was found that addition of the GSK3 inhibitor CHIRON to PD03 culture stabilizes ESC propagation, even without LIF or BMP4/serum. This rescue of ESCs is closely phenocopied in *Gsk3a/Gsk3b*-null ESCs, demonstrating that GSK3 is the specific target of CHIRON (Ying et al., 2008). Collectively PD03 and CHIRON comprise the "2i" culture system.

The effects of GSK3 inhibition on self-renewal are principally mediated through stabilizing β-catenin. Indeed, ESCs lacking *B-catenin* do not respond to CHIRON, confirming a direct effect (Lyashenko et al., 2011; Wray et al., 2011). Such stabilization of β-catenin effectively mimics stimulation of canonical WNT signaling, which is thought to restrict progression from naive to primed status (ten Berge et al., 2011; Yi et al., 2011). Mechanistically, stabilized β-catenin translocates to the nucleus, where it may enhance expression of pluripotency factors through interactions with OCT4 or via weakly expressed TCF1 (Kelly et al., 2011; Yi et al., 2011). However, it appears that the primary role of nuclear β-catenin in self-renewal arises through interactions with its definitive partner and transcriptional repressor TCF3. In ESCs, TCF3 is colocalized with OCT4 and SOX2 at core pluripotency genes, where the repressor activity of TCF3 competes to antagonize OCT4/SOX2 activity. However, the interaction between TCF3 and β-catenin disrupts TCF3-mediated repression of these targets, thus stabilizing the naive self-renewal program (Faunes et al., 2013; Wray et al., 2011). Inhibition of GSK3 with CHIRON therefore relieves suppression of pluripotency genes by TCF3 and, consistently, genetic studies reveal *Tcf3* null ESCs exhibit enhanced self-renewal (Guo et al., 2011; Yi et al., 2011).

The critical target antagonized by TCF3 is *Esrrb*, since forced expression of *Esrrb* substitutes for GSK3 inhibition to restore viability, whereas ablation of *Esrrb* largely eliminates the self-renewal response to 2i conditions (Martello et al., 2012). GSK3 inhibition with CHIRON therefore effectively represents an extrinsic stimulus that operates through WNT signaling to reinforce the pluripotency gene network (Loh and Lim, 2011). This suggests that the ground state is not an inherently stable condition that can only be perturbed by differentiation cues but instead requires continuous extrinsic input. Thus, 2i conditions elicit the in vitro ground state through both shielding ESCs from differentiation stimuli and also via engaging with the core pluripotency circuit to reinforce its expression. The 2i system additionally appears to be inherently selective against cells that have exited naive status (most cell types, including primed cells, differentiate or die in 2i), and indeed this property can be exploited to select for transition from partially reprogrammed pre-iPS to naive induced pluripotent stem cells (iPSCs) (Silva et al., 2008).

Parallel Pathways Support Naive Pluripotency

An emerging theme is that naive pluripotency can be maintained by modulating several independent extrinsic signaling pathways (Figure 2). Moreover, because these various pathways signal through parallel routes, they act additively to reinforce pluripotency. Indeed, the optimal culture condition for robust self-renewal of ground state ESCs comprises the three additive 2i/LIF supplements (CHIRON, PD03, LIF), which primarily affect canonical WNT, FGF/ERK, and JAK/STAT signals. Notably, however, any combination of two of these tripartite supplements is sufficient to maintain naive self-renewal, at least in tested genetic backgrounds (Wray et al., 2010; Dunn et al., 2014). The extrinsic pathways impinged on by PD03, CHIRON, and LIF are therefore to some extent redundant and can compensate for the absence of one another to engender a naive state. Indeed the combination of FGF/ERK inhibition and BMP4 pathway augmentation also elicits an apparent self-renewing ground state (Hassani et al., 2014). While each extrinsic regulator has unique critical targets, there is also a degree of crosstalk and overlap as CHIRON, LIF, and even PD03 all converge on activation of *Tfcp2l1* (Ye et al., 2013).

The redundancy between extrinsic inputs for self-renewal is evidenced by deletion of the critical LIF effector *Stat3* or its key target *Tfcp2l1*. This loss renders ESCs nonviable in serum/LIF conditions, yet is inconsequential for self-renewal in 2i because essential LIF/STAT3 functions are compensated for by activation of canonical WNT signaling and blockade of FGF/ERK (Martello et al., 2013; Ying et al., 2008). Conversely, the CHIRON effector β-*catenin* and downstream target *Esrrb* are absolutely required for efficient self-renewal in 2i but are dispensable in serum conditions, where LIF/STAT3 and BMP4 activity can bypass their function to sustain ESC identity (Martello et al., 2012; Wray et al., 2011). The same is true for *Klf2*, which appears to be an essential component of the pluripotency circuit in 2i but not serum (Yeo et al., 2014). Notably, however, the tripartite 2i/LIF condition robustly maintains a naive state when any of these genes is absent, underscoring the redundancy generated by multiple pro-self-renewal pathways.

Thus, naive pluripotency, and indeed an unrestricted "ground state," can apparently be elicited through independent inputs that operate through both unique and convergent targets, with ESCs able to well-tolerate removal of one (or more) input as long as others are in play. Above a threshold level of extrinsic influences that either block differentiation or activate naive genes, pluripotency is robustly stabilized. Notably, the theoretical threshold level to stabilize naive status may vary in different genetic backgrounds, where the sensitivity to specific pathways is altered. For example, non-129 ESCs may exhibit enhanced susceptibility to prodifferentiation FGF/ERK signaling, as may XY as opposed to XX ESCs, implying a heightened dependence on blockade of this stimulus to maintain naive status (Hanna et al., 2009; Schulz et al., 2014). Moreover, other influences intrinsic to ESCs can encourage exit from self-renewal, such as the translational regulator PUM1, the zinc-finger protein ZFP706, the subcellular localization of the transcription factor TFE1, and the NURD corepressor complex, and may need to be overcome to differing extents in different genetic contexts (Betschinger et al., 2013; Leeb et al., 2014; Reynolds et al., 2012). It will be of interest to elucidate the context-dependent molecular signatures generated by combinations of culture supplements to dissect precisely how various extrinsic pathways impinge on the "state" of pluripotency.

Intrinsic Networks for Naive Pluripotency

Downstream of extrinsic signals, regulatory networks of transcription factors (TFs) and cofactors propagate the gene expression programs that underpin naive pluripotency (Young, 2011). Such intrinsic genetic networks have been extensively interrogated and two TFs, *Oct4* and *Sox2*, emerge as the fundamental lynchpins. These factors are required for both the acquisition and maintenance of pluripotency, and *Oct4* and *Sox2* are therefore defined here as "core" pluripotency factors, albeit the critical role of *Sox2* may be to activate *Oct4* (Avilion et al., 2003; Masui et al., 2007; Nichols et al., 1998). Both *Oct4* and *Sox2* are expressed continually and relatively uniformly in ESCs under all standard conditions. In the acute absence of *Oct4* or *Sox2*, ESCs progressively differentiate toward trophectoderm derivatives, while overexpression also elicits lineage specification, implying that precisely regulated OCT4 and SOX2 levels are crucial for the balance between self-renewal and differentiation (Masui et al., 2007; Niwa et al., 2000; Thomson et al., 2011). Indeed, limiting the range of *Oct4* expression to only intermediate levels by using heterozygous ESCs actually stabilizes a uniform naive state (Karwacki-Neisius et al., 2013).

In addition to *Oct4* and *Sox2*, *Nanog* is historically included as a key TF as it is crucial for the acquisition, but not maintenance, of naive pluripotency (Chambers et al., 2007; Silva et al., 2009). The requirement for *Nanog* to acquire naive pluripotency in vitro can however be bypassed, implying that *Nanog* plays a distinct functional role from *Oct4* and *Sox2* (Carter et al., 2014; Schwarz et al., 2014). Nonetheless, the *Oct4*/*Sox2*/*Nanog* (OSN) triumvirate acts cooperatively in ESCs to bind overlapping genomic targets, including their own promoters, and thus forms a robust autoregulatory network that self-maintains pluripotency. Prominent among OSN targets are *cis*-acting regulatory elements, where OSN acts both directly and as a platform to recruit diverse coactivators and TFs that collectively execute the pluripotency expression program. Importantly, OSN occupancy correlates with cobinding by STAT3, β-catenin, and SMAD1, which are the effectors of the propluripotency signaling cascades activated by LIF, WNT, and BMP4, respectively, thus directly coupling these signaling pathways to the genes regulated by the core pluripotency circuitry (Chen et al., 2008; Loh et al., 2006; Young, 2011). The repressor TCF3 also engages with OSN to antagonize activation of its targets (Cole et al., 2008), and thus CHIRON-mediated abrogation of TCF3 activity via stabilized β-catenin enhances self-renewal. Notably, when signaling cues are switched to primed FGF/Activin conditions, OCT4 redistributes away from naive genes to occupy enhancers associated with early development through interactions with OTX2, underscoring how core TFs can integrate extrinsic cues in a context-dependent manner (Buecker et al., 2014; Factor et al., 2014).

ESCs also express a repertoire of "ancillary" pluripotency regulators such as *Klf2*, *Esrrb*, *Klf4*, *Prdm14*, *Sall4*, *Tfcp2l1*, and *Tbx3* that reinforce and buffer the pluripotency network against prodifferentiation influences but are typically individually dispensable for pluripotent identity. Indeed, the expendable role of *Nanog* for pluripotency means it is also included as an ancillary

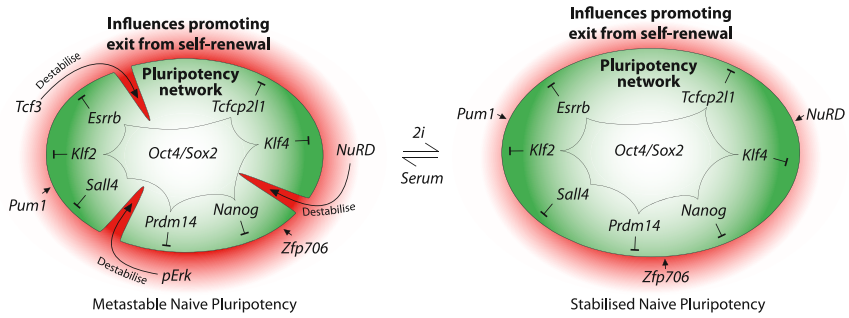

Figure 3. Genetic Networks for Maintenance of Naive Pluripotency

The pluripotency network includes essential core components (*Oct4/Sox2*) and multiple ancillary factors (shown in green) that collectively form a self-organizing circuitry. Ancillary factors generally act to buffer the network against fluctuations and perturbations that promote exit from self-renewal (shown in red). In serum (left), ancillary factors are heterogeneous among the population and thus render individual ESCs in varying states of susceptibility to inherent influences that promote exit from self-renewal such as the FGF/ERK pathway or NuRD corepressor complex activity (shown as canals in the ancillary bubble). When the pluripotency network is stabilized through blocking key differentiation influences and/or by directly reinforcing expression of ancillary factors (e.g., 2i/LIF, right), the influences that drive exit from self-renewal are mitigated and robust naive pluripotency emerges.

factor. In general, ancillary factors stabilize each other's expression through feedback loops, at least indirectly, and thus form a redundant self-organizing circuit (Dunn et al., 2014). In conventional conditions, many ancillary factors exhibit heterogeneous expression, fluctuating between on and off states at the single-cell level and thus render individual ESCs in varying degrees of susceptibility to distinct lineage-specifying cues (Figure 3) (Chambers et al., 2007; Martello et al., 2012; Toyooka et al., 2008; Yamaji et al., 2013). In 2i however these factors appear to be homogenously expressed among the population, which may both reflect and contribute to stabilized naive pluripotency (Wray et al., 2010).

When active, ancillary factors can act through both overlapping and distinct mechanisms to affect pluripotency. For example, SALL4 associates with OCT4 to promote repression of trophectoderm genes and stabilizes expression of *Oct4* itself (Zhang et al., 2006). ESSRB on the other hand directly integrates into the pluripotency TF circuitry by fine-tuning the expression level of key targets cooperatively with NANOG, while PRDM14 represses FGF signaling and DNA methylation (Festuccia et al., 2012; Grabole et al., 2013; Yamaji et al., 2013). In serum conditions, other groups of collaborative TFs or "modules" are also considered important. The "*Myc*" module, for example, promotes rapid transition through G1 cell cycle and suppresses differentiation, in part by activating *Dusp2* and *Dusp7* phosphatases that repress FGF/ERK (Cartwright et al., 2005; Chappell et al., 2013). These modules and the extended genetic networks that underpin pluripotency in serum have been extensively reviewed (Chambers and Tomlinson, 2009; Jaenisch and Young, 2008; Loh et al., 2011; Ng and Surani, 2011; Young, 2011).

The current knowledge of the molecular determinants of pluripotency is largely inferred from conventional ESC conditions. This is an important consideration because such serum conditions sustain self-renewal through distinct pathways, and hence through at least subtly altered networks, to ground state ESCs. Thus, while the genetic networks in serum conditions represent one route to engender functional pluripotency, they by no means represent the only or defining molecular signature of self-renewing naive pluripotency per se. For example, the *Myc* module, which is critical in serum, is almost entirely silenced in 2i. Indeed, approximately 25% of active ESC genes are differentially expressed between 2i/LIF and serum/LIF conditions (~3,500 genes, >2-fold) (Marks et al., 2012). Functional pluripotency is therefore apparently maintained across a surprisingly wide bandwidth of transcriptional (and epigenetic) variation. Consequently, while important work has elucidated a persuasive model to describe pluripotency in the context of conventional ESCs, the extent to which existing paradigms are absolute requirements or context-dependent nodes for eliciting naive pluripotency is unclear.

This ambiguity is exemplified by *Prdm14*, which has been shown to be a critical component of the genetic framework that supports ESC self-renewal in serum, thus implying an essential function in pluripotent identity in general. However, *Prdm14* is dispensable for both the self-renewal of ESCs in 2i and for executing all differentiation pathways, highlighting a strictly context-dependent role (Grabole et al., 2013; Yamaji et al., 2013). Such ambiguities are also probably relevant, at least to some extent, for the precise roles of other TFs, small RNAs, epigenetic mechanisms, and *cis*-regulatory elements that input into pluripotency. Understanding how various regulatory components integrate into pluripotency in distinct contexts and their interplay will be an important aspect of future studies.

Insights into the transcriptional foundations of conventional and ground state ESCs have however recently been established, with several conclusions deducible (Dunn et al., 2014; Marks et al., 2012). For example, cell-cycle genes are downregulated in 2i/LIF, whereas metabolic genes are highly upregulated, which may be related to parallels between ground state ESCs and early development where shifts in metabolic pathways occur (Zhou et al., 2012). Alternatively, the high metabolic activity in 2i conditions could be indicative of increased cellular stress. We consider here significant distinctions and overlaps between conventional serum/LIF ESCs and ground state ESCs in 2i/LIF.

Pluripotency Gene Networks

First, *Oct4* and *Sox2* are expressed at comparable levels between serum and 2i/LIF, suggesting that at the population level, there is no inherent difference in the core circuitry between states. Most ancillary pluripotency genes, including *Tbx3*, *Esrrb*, *Nanog*, *Klf4*, and *Klf2*, are however expressed moderately higher in 2i/LIF conditions (typically <2-fold up). This primarily reflects a switch from mosaic expression in serum to expression by all cells in 2i/LIF, since differences are mitigated by comparing the naive *Rex1*-positive population from serum to ground state ESCs (Marks et al., 2012). Some pluripotency factors/markers, including *Prdm14*, *Stella*, *Tcl1*, and *Tfcp2l1*, are nevertheless directly upregulated in 2i/LIF (typically 2- to 5-fold up), which may be a consequence of stabilizing the naive circuitry or

alternatively indicate that these genes are themselves important for reinforcing the ground state downstream of 2i signaling. Consistent with the latter, forced expression of *Prdm14* can drive a homogenous *Rex1*-positive state, even in conventional conditions, while *Tcfp2l1* overexpression efficiently reprograms primed EpiSCs to naive pluripotency (Grabole et al., 2013; Martello et al., 2013).

Despite the general trend of higher expression of ancillary factors in 2i/LIF, several previously pluripotency-associated genes, such as *Utf1*, *Lin28b*, and *Id* genes, are downregulated in ground state ESCs. This highlights both that these targets are invoked through distinct pathways and also that their high expression is not an absolute requirement for pluripotency. A further question is the degree to which allelic regulation impinges on the stability and state of pluripotency. Indeed, it was observed that *Nanog* may be predominantly monoalleically expressed specifically in serum-cultured ESCs, thereby predisposing them to differentiation cues (Miyanari and Torres-Padilla, 2012). Nevertheless, recent reports have suggested *Nanog* transcription is biallelic in both 2i/LIF and serum, implying this mode of regulation is unlikely to underlie significant differences between pluripotent states/conditions (Faddah et al., 2013; Filipczyk et al., 2013).

Lineage-Associated Genes

A prominent distinction between ground state ESCs and conventional ESCs is the significant expression of lineage-associated transcripts in the latter. In particular, genes related to mesoderm and ectoderm are active in serum conditions but are near undetectable in 2i/LIF (Marks et al., 2012). To some extent, this is related to heterogeneity in serum. Nevertheless the *Rex1*-positive naive population in serum still exhibits considerable expression of lineage-associated genes. Indeed, the global transcriptomes between *Rex1*-positive ESCs in serum and ESCs in 2i/LIF are clearly distinct (Marks et al., 2012). Thus, as of yet there is no subpopulation in serum that is transcriptionally equivalent to ground state ESCs. This collectively suggests that serum ESCs may represent capture of a more developmentally advanced state linked with onset of some early differentiation programs, relative to ESCs in 2i/LIF where developmental gene expression is diminished.

Nonetheless, transcription of certain lineage-associated genes is detectable in 2i/LIF conditions, particularly germline and endoderm transcripts. Enhanced germline gene expression is primarily a consequence of global DNA hypomethylation. Expression of endoderm genes on the other hand may be related to the capacity of some ESCs in 2i/LIF to generate extraembryonic primitive endoderm and/or trophectoderm, in addition to maintaining naive potential (Morgani et al., 2013). This is consistent with 2i/LIF conditions supporting capture of at least some cells in an earlier developmental stage, perhaps comparable to nascent ICM cells (E3.5) or earlier, rather than the naive epiblast (E4.5). Significantly, however, expression of a reporter for the primitive endoderm gene *Hex* is heterogeneous across the population and delineates the subpopulation most primed toward extraembryonic fate. Thus, while 2i/LIF conditions establish a relatively homogenous population with respect to naive pluripotency, there may still be significant functional and transcriptional heterogeneity related to extraembryonic potential.

In contrast, heterogeneity in serum involves dynamic cycling in and out of various embryonic "lineage-primed" states, and it has been posited that such metastability is an essential component of pluripotent identity per se. It is also suggested that the concomitant expression of multiple lineage specifiers in serum establishes a balance whereby distinct differentiation pathways mutually counteract the dominance of each other, with the upshot being all fates remain accessible and thus an unrestricted pluripotent state is engendered (Loh and Lim, 2011). Indeed, forced expression of lineage specifiers can reprogram somatic cells to pluripotency, supporting the concept that pluripotency can be established through the equilibrium of counteracting differentiation forces (Montserrat et al., 2013; Shu et al., 2013). Nevertheless, the absence of multilineage gene expression in 2i/LIF coupled with an apparently uniform naive capacity, argues that metastability is not an inherent property of naive pluripotency but rather may represent a culture epiphenomenon due to the conflicting stimuli in serum (Smith, 2013). Importantly, however, the onset of mosaic expression profiles and heterogeneity may be crucial aspects that underlie competence for unbiased cell fate commitment during exit from pluripotency.

Epigenetic Landscapes in Pluripotent States

DNA Modification

DNA methylation at CpG dinucleotides is a repressive epigenetic modification typically associated with transcriptional silencing but also has diverse roles in regulating transposable elements, splicing, and genome integrity (Smith and Meissner, 2013). Once established, DNA methylation (5mC) is faithfully propagated through cell divisions and stabilizes restriction of cellular identity (Bröske et al., 2009; Hemberger et al., 2009; Ng et al., 2008; Oda et al., 2013). However, during early development, 5mC is dynamically remodeled, leading to a globally hypomethylated state in the ICM (Smallwood et al., 2011; Smith et al., 2012). The global erasure of 5mC is considered important to remove epigenetic barriers against the acquisition of pluripotency (Hackett and Surani, 2013). Nevertheless, despite being derived from the hypomethylated ICM, ESCs in serum accumulate high levels of global DNA methylation usually associated with primed or lineage-restricted cells (Meissner et al., 2008).

In contrast, recent studies have shown that ground state ESCs exhibit a globally hypomethylated DNA methylome, with 5mC ~3-fold lower in 2i/LIF-derived ESCs relative to ESCs in serum (Ficz et al., 2013; Habibi et al., 2013; Leitch et al., 2013). The levels and distribution of 5mC in 2i/LIF ESCs thus appear comparable with the derestricted ICM or naive epiblast cells (E3.5–E4.5), whereas 5mC in serum ESCs is closer to the hypermethylated state of primed postimplantation epiblast (E6.5). Moreover, the apparently naive subpopulations in serum (*Rex1*/*Nanog*-positive) still retain elevated global 5mC, underscoring that they are not directly equivalent to ground state ESCs (Ficz et al., 2013; Habibi et al., 2013), albeit the quintile of ESCs expressing the highest level of *Rex1* do appear relatively hypomethylated (Singer et al., 2014). This may imply that ESCs in serum dynamically transition into a hypomethylated state at low frequency. Such dynamic DNA methylation variation, particularly at *cis*-regulatory enhancers, may play a key role in modulating the functional heterogeneity of serum ESCs, since ablation of

DNA methyltransferases promotes uniform self-renewal (Jasnos et al., 2013; Lee et al., 2014). Conversely, stable hypomethylation in 2i/LIF is probably directly linked with competence for uniform ground state pluripotency by minimizing epigenetic restrictions and intercellular variation.

The epigenetic memory imposed by 5mC is surprisingly plastic in ESCs as switch from serum to 2i/LIF induces comparatively rapid hypomethylation, while the reciprocal switch results in appropriate acquisition of DNA methylation (Leitch et al., 2013; Shipony et al., 2014). Some loci are resistant to demethylation, however, such as genomic imprints and IAP elements, which is consistent with their escape from reprogramming during preimplantation development. Mechanistically, resistant loci are enriched with H3K9me3, which may act as a platform that blocks demethylation or that preferentially attracts residual DNA methylation activity, perhaps via UHRF1, which directly binds H3K9me3 (Bostick et al., 2007; Habibi et al., 2013; Rothbart et al., 2012). On the other hand, the mechanism that mediates global 5mC erasure in 2i/LIF may include oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) by TET enzymes since 5hmC is transiently, but weakly, enriched at some genomic loci during ESC transition to ground state pluripotency. Indeed, in the absence of *Tet1* and *Tet2*, both the rate and extent of 5mC erasure is compromised (Ficz et al., 2013; Hackett et al., 2013). Nevertheless, *Tet1/Tet2*$^{-/-}$ ESCs can still undergo significant demethylation, whereas, strikingly, *Prdm14*$^{-/-}$ ESCs fail to become hypomethylated in 2i/LIF. This seems to reflect a key role for PRDM14 in repressing the de novo methylases *Dnmt3a* and *Dnmt3b*, which are required for maintenance of 5mC in ESCs (Chen et al., 2003; Grabole et al., 2013; Leitch et al., 2013; Yamaji et al., 2013). Furthermore, PRDM14 also directly targets TET activity (Okashita et al., 2014), highlighting *Prdm14* as a key hub that integrates multiple synergistic DNA demethylation mechanisms. Thus, DNA hypomethylation in ground state ESCs appears to be mediated via decommissioning the de novo DNA methylation machinery and, in parallel, through targeted 5hmC conversion.

Interestingly, in contrast to male XY ESCs, which are predominantly studied owing to their higher derivation efficiency, female XX ESCs in serum do exhibit global DNA hypomethylation. This is a direct consequence of two active X chromosomes promoting repression of de novo methyltransferases and pERK activity (Ooi et al., 2010; Schulz et al., 2014; Zvetkova et al., 2005). The magnitude of hypomethylation in XX ESCs relative to XY counterparts can vary from 10% to 3-fold depending on the genetic background and precise culture conditions, such as the presence of feeders (Hackett et al., 2013; Schulz et al., 2014). Nevertheless, the observation that XX ESCs exhibit significantly depleted 5mC coupled with blockade of pERK activity suggests that female ESCs in serum conditions may occupy a pluripotent state closer to the in vitro ground state. Indeed, DNA hypomethylation per se may serve as a useful marker for benchmarking pluripotent states and, in particular, for denoting ESCs that have entered ground state pluripotency.

Histone Modification

The copresence of the activating modification H3K4me3 and repressive polycomb mark H3K27me3 on chromatin is termed bivalent. Bivalency is observed in multiple cell types but is a particularly prominent feature of developmental promoters in conventional ESCs. The bivalent signature is thought to dampen transcription but maintain a flexible poised state that can be rapidly reactivated in response to lineage-specifying cues, albeit evidence is accumulating that bivalency may be functionally dispensable (Denissov et al., 2014; Voigt et al., 2013). Significantly, epigenetic profiling revealed that H3K27me3 is depleted in ground state ESCs, and consequently the number of bivalent domains is dramatically reduced. The reduction of H3K27me3 in 2i/LIF may be a direct effect of ERK inhibition, since ERK is necessary for EED activity at target promoters (Tee et al., 2014). Nevertheless, the developmental genes that lose H3K27me3 in 2i/LIF are generally not derepressed, implying that alternative mechanisms constrain their expression (Marks et al., 2012). Notably, polycomb is dispensable for ESC self-renewal, as is DNA methylation (Leeb et al., 2010; Tsumura et al., 2006), but their absence abrogates differentiation, indicating that these repressive epigenetic systems primarily function in the initiation and maintenance of cell fate restriction rather than pluripotent identity per se.

We await detailed reports on the relative abundance and distributions of other chromatin modifications between various pluripotent states. One observation however is that pluripotent cells in 2i/LIF may exhibit a global reduction of the repressive marks H3K9me2 and H3K9me3 (Leitch et al., 2013). There is also a reported concomitant increase in H3K4me3, while PADI4, which citrulinates linker histone H1 to decompact chromatin, is also upregulated in ground state conditions (Christophorou et al., 2014). Thus, multiple repressive modifications (5mC, H3K27me3, H3K9me2, H3K9me3) are apparently depleted, or at least redistributed, in 2i/LIF conditions, while several epigenetic mechanisms linked with decondensed chromatin are active. This observation is consistent with ground state cells acquiring a derestricted epigenetic state that is conducive to the onset of all developmental programs, upon appropriate cues.

Transcriptional Pausing

One potential consequence of a general reduction of repressive epigenetic modifications is precocious transcription. Nevertheless despite previous suggestions (Efroni et al., 2008), neither ESCs cultured in 2i/LIF nor in serum appear to exhibit global transcriptional hyperactivity, suggesting that alternative mechanisms may be in place to regulate transcription (Marks et al., 2012). One possibility is that RNA polymerase II (Pol II) pausing at promoter proximal sites has a prominent role. Such a regulatory mechanism may be important at lineage-specific genes in early embryonic cells and, indeed, promoter proximal Pol II pausing is prevalent in ESCs in serum. This may be mediated in part by ERK1/2 activity, which directly phosphorylates the CTD at developmental genes thereby promoting Pol II pausing (Tee et al., 2014). Strikingly, however, ESCs grown in 2i/LIF exhibit a significantly greater extent of promoter proximal pausing relative to ESCs in serum, and this effect is particularly evident at developmental genes, among others (Marks et al., 2012). Pol II pausing may therefore be a crucial regulatory mechanism associated with ground state pluripotency. However, as ERK activity is blocked in 2i/LIF conditions, it is unclear what mechanisms direct such elevated transcriptional pausing.

The Extended Spectrum of Pluripotent States

The emerging theme from multiple studies is that pluripotency, as a functional property, is not restricted to a specific underlying

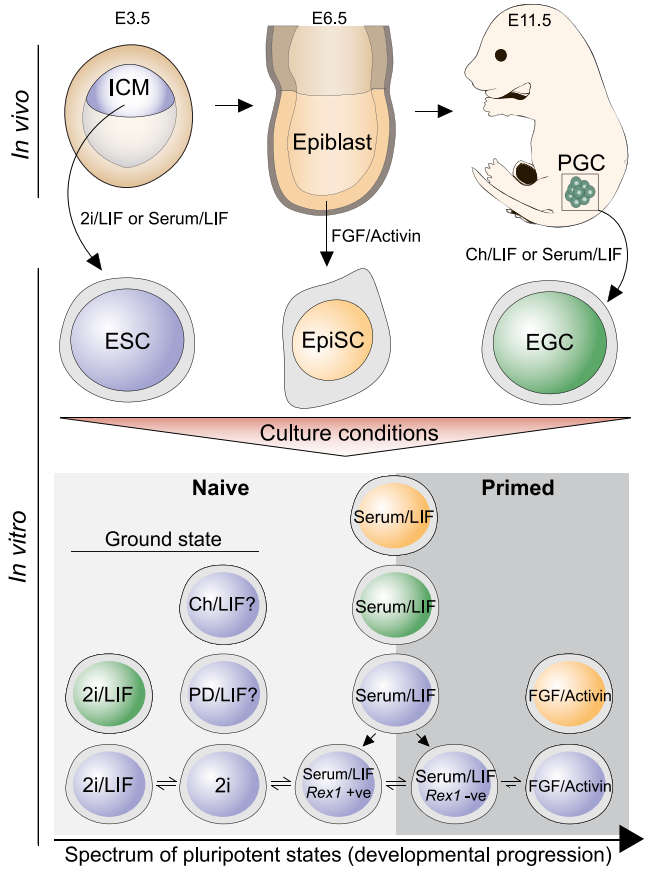

Figure 4. Embryonic Origin and Spectrum of Pluripotent Stem Cell States

The pluripotent cells of a blastocyst between E3.5 and E4.5 can give rise to functionally naive ESCs (blue). Between E5.5 and E8.0 postimplantation epiblast can establish EpiSCs (orange), which occupy a primed pluripotent state. Additionally, primordial germ cells (PGCs), which are the founders of the germline lineage, can give rise to naive EGCs (green), which are highly comparable to ESCs. Depending on the culture/derivation conditions these pluripotent stem cells occupy discrete molecular states that can be broadly classed as naive or primed. The most optimized state of naive pluripotency, which closely recapitulates the naive epiblast cells of the blastocyst, is termed ground state. An interchangeable spectrum of pluripotent states may arise that ranges from ground state to primed pluripotency. The state of pluripotency adopted in vitro is primarily dictated by the combination extrinsic signals in the culture environment rather than the developmental source of the pluripotent cells. CH, Chiron; PD, PD03.

molecular signature or specific culture parameters. Indeed, there appears to be a surprisingly broad bandwidth of molecular states that can confer pluripotent properties. This plasticity/flexibility effectively results in a spectrum of pluripotency, upon which pluripotent states are progressively more restricted, or at least altered, but nonetheless still exhibit some defining features of pluripotency (Figure 4). At one end of the continuum is the ground state, which perhaps represents the state of broadest and most unrestricted developmental potential. Indeed, a fraction of ground state ESCs in 2i/LIF may be functionally totipotent, capable of contribution to embryonic and extraembryonic lineages (Morgani et al., 2013). At the other end of the spectrum is the so-called "primed" pluripotent state, as exemplified by EpiSCs.

EpiSCs are established from mouse postimplantation epiblast and beyond (E5.5–E8.0) and require bFGF and Activin A signaling to maintain self-renewal (Brons et al., 2007; Tesar et al., 2007). EpiSCs exhibit pluripotent features such as the capacity to undergo multilineage differentiation in vitro and efficiently form teratomas in vivo, while they also express the core pluripotency factors *Oct4* and *Sox2*. However, *Oct4* expression in EpiSCs is primarily driven by its proximal enhancer, as opposed to the distal enhancer that is active in naive ESCs, and this distinction is often used as a proxy to distinguish between primed and naive pluripotent states. Transcriptionally, EpiSCs exhibit reduced/absent expression of many ancillary pluripotency factors, including *Klf4*, *Klf5*, *Prdm14*, *Rex1*, and *Esrrb*, which is in part due to attenuated *Nanog* expression (Festuccia et al., 2012). Moreover, EpiSCs accumulate epigenetic barriers incompatible with a naive state such as female X chromosome inactivation and promoter DNA methylation at pluripotency genes. Consequently, primed EpiSCs contribute poorly or not at all to blastocyst chimeras and are thus functionally distinct from naive ESCs. Instead, EpiSCs appear to functionally resemble the anterior primitive streak (Kojima et al., 2014), albeit there is considerable heterogeneity at the population level and forced expression of *E-Cadherin* can restore naive potential (Ohtsuka et al., 2012). Notably, an alternative model of the primed state known as epiblast-like cells (EpiLCs) has recently been developed, and although EpiLCs more closely parallel the postimplantation epiblast (E5.5–E6.5) than do EpiSCs, they do not self-renew (Hayashi et al., 2011). Nonetheless, the transient EpiLC population is a highly useful system to model pluripotent state transitions.

Between the states characterized by ground state ESCs and lineage-primed EpiSCs, there may be a continuum of self-renewing pluripotent states with distinct functional and transcriptional signatures and biases. These appear to be primarily a consequence of extrinsic influences modulated by culture conditions, with serum conditions, for example, perhaps being intermediate on the spectrum. Like 2i/LIF culture, such conventional serum conditions promote cells with naive potential (germline competent), which are at least *Rex1* positive. However, as judged by several criteria, the *Rex1*-positive ESCs in serum appear more developmentally advanced than ground state ESCs in 2i/LIF and thus occupy a discrete molecular state of naive pluripotency. Serum conditions additionally support cells with apparent "primed" properties (*Oct4* positive/*Rex1* negative). This subpopulation is however functionally distinct from primed EpiSCs as inferred from the observation that EpiSCs, but not ESCs, can readily contribute to all germ layers when engrafted onto cultured postimplantation embryos (Huang et al., 2012). Collectively, this suggests that the pluripotent state(s) that arise in serum conditions occupy positions along the spectrum between ground state and primed cells. Another so-called "2C" state is also reported to exist in serum, which apparently closely parallels two-cell embryos at the transcriptional level (Macfarlan et al., 2012). Further pluripotent "states" may be generated according to precise culture conditions that intercalate between ground state, serum, and primed states. For example, various combinations and/or concentrations of PD03, CHIRON, BMP4, and LIF could engender distinct pluripotent cells, with different properties to bona fide ground state ESCs in 2i/LIF (or 2i) (Figure 4) (Chen

et al., 2013). More subtle alterations to conditions may also have profound effects. The addition of vitamin C or knockout serum replacement (KSR) to conventional culture conditions, for example, promotes TET catalytic activity, leading to global DNA hypomethylation and an altered transcriptional profile (Blaschke et al., 2013). The presence or absence of feeders also impinges on the precise molecular signature of ESCs.

Thus, a range of pluripotent states exist that are, in general, a direct response to culture conditions. It remains to be determined precisely what the functional differences are between such states and whether they might correspond to successive pluripotent phases during ontogeny. Notably, however, most nodes along the pluripotent spectrum in vitro are reversible just by interchanging culture parameters, albeit EpiSCs are somewhat restricted (Bao et al., 2009). Indeed, pluripotent stem cells from distinct developmental origins also assume the state dictated by conditions rather than the embryonic source. Embryonic germ cells (EGCs) derived from PGCs, for example, are almost indistinguishable from ICM-derived ESCs, when both are in the same media (2i/LIF or serum) (Leitch et al., 2013). Moreover, the ICM forms self-renewing EpiSCs, rather than ESCs, when derived in appropriate media, while postimplantation epiblast can form ESCs rather than EpiSCs in serum/LIF (Bao et al., 2009; Najm et al., 2011). This collectively implies that up to a threshold level of progressive restriction, pluripotency is plastic and can revert through multiple molecular states through altering culture parameters, which are therefore the dominant influence over which pluripotent state emerges (McEwen et al., 2013). It is therefore crucially important to consider the precise culture environment when inferring absolute conclusions related to pluripotent identity.

Toward Naive Human ESCs

The matter of pluripotent "state" is particularly relevant when considering human ESCs (hESCs), which have been considered to occupy a phase of pluripotency with more similarity to the murine primed rather than naive state and thus a relatively advanced position on the spectrum (De Los Angeles et al., 2012). This classification of hESCs as "primed" is based on several lines of evidence including expression of *Oct4* driven by its proximal enhancer, relatively high primed/lineage-associated gene expression (such as *Fgf5* and *Lefty1*), global DNA hypermethylation, morphological similarity to EpiSCs, and reliance on FGF/Activin for self-renewal. Moreover, hESCs are not LIF responsive, while conventional 2i conditions elicit neural differentiation, implying that unlike mouse ESCs, 2i does not select against the expansion of differentiated cell types in hESC cultures (Hirano et al., 2012; Theunissen et al., 2014). As such, it has become a question of great interest whether hESCs, and by extension human iPSCs, can be coaxed into the naive state. This may enhance their developmental potential, facilitate genetic/experimental manipulation, and potentially enable purer populations of differentiated cells to be generated, all of which would have implications for development of disease models and regenerative therapies.

Several studies have reported that after transgenic interventions, hESCs/iPSCs with naive properties can be derived and maintained (Buecker et al., 2010; Hanna et al., 2010; Wang et al., 2011). However, the requirement for continued genetic manipulation limits their clinical utility and it is desirable to identify culture conditions that directly support human naive pluripotency independently of transgenes. A precedent for this possibility is that primed EpiSCs revert to a naive state at low frequency when switched to mouse naive culture conditions (Bao et al., 2009; Gillich et al., 2012), albeit primed hESCs do not respond equivalently.

To address this, human-specific conditions for putative naive states have recently been developed. For example, hESCs cultured in basal media containing FGF and TGF supplemented with 2i, LIF, and Dorsomorphin (AMP kinase and BMP inhibitor) upregulate multiple naive markers including *NANOG*, *KLF4*, and *TBX3* (Chan et al., 2013). Alternative culture parameters include 2i, FGF, and KSR either supplemented with LIF and ROCK inhibitor (Valamehr et al., 2014) or without (Ware et al., 2014). Indeed, monkey iPSCs are also reported to acquire several naive traits using variations of the 2i/LIF/FGF condition (Fang et al., 2014). Another study using a panel of six inhibitors including 2i/LIF reported hESCs with many apparent naive features and contribution to interspecies chimeras, albeit it is unclear whether this can be used as a robust test for naive pluripotency (Gafni et al., 2013). Finally, using a targeted reporter for *OCT4* expression from its naive-specific distal enhancer, a comprehensive screen identified an alternative combination of six inhibitors (2i, ROCKi, BRAFi, SRCi, and JNKi) supplemented with LIF and Activin. Under these conditions, hESCs appear to exhibit a more compelling upregulation of naive markers, as judged by equivalence with mouse ESCs, but lack other naive features such as two active X chromosomes in female cells (Theunissen et al., 2014).

A significant issue toward establishment of naive hESCs is that, at present, there is no universal defining test for naive pluripotency in a human system, unlike murine ESCs where chimera contribution to blastocysts is the benchmark. Assigning naive status to hESCs is therefore generally based on a molecular rather than a functional basis. One key parameter to consider is similarity to the global gene expression profile of human preimplanation epiblast cells (Yan et al., 2013), which represent the human ground state. Additionally, global DNA hypomethylation (<40%) appears to be a fundamental feature of naive pluripotency both in vitro and during preimplantation development in human and mouse (Guo et al., 2014; Smith et al., 2014; Wang et al., 2014) and should be used as a critical benchmark for bona fide ground state status in hESCs. Given this, the recent report that hESCs exhibit significant global DNA hypomethylation and cluster more closely with in vivo epiblast at the transcriptional level is of great interest (Takashima et al., 2014). Here, transient expression of NANOG and KLF2 followed by switch to 2i/LIF (with titrated CHIRON) in conjunction of PKC inhibitor enabled expansion of "reset" hESCs that show all tested molecular features of ground state pluripotency, including two active X chromosomes and diminished lineage-associated expression. Functionally, these cells rely on *TCFP2l1* analogous to mouse ESCs in 2i and can contribute to the ICM in early mouse embryos, unlike conventional hESCs. While there is an initial requirement for forced transgene expression, this can also be achieved without genetic intervention implying that human pluripotent stem cells have the intrinsic capacity to occupy a state closely comparable to mouse ground state ESCs.

The conditions reported to date considerably narrow the gap between hESCs and ground state epiblast cells in vivo. As such, important steps have been taken toward generating bona fide naive human stem cells and have led to multiple novel pluripotent "states" being established. Similarly, to the pluripotent spectrum in mice, these distinct states of human pluripotency probably have discrete features and functional benefits for future research and may potentially reflect successive pluripotent phases in vivo.

Perspective

The capture of naive pluripotency has enabled unparalleled investigations into development and disease. Studies are also uncovering the extrinsic and intrinsic regulators of pluripotency per se, revealing a broad bandwidth of influences that feed into the balance between maintenance or exit from self-renewal. One consequence of this is that a spectrum of self-renewing pluripotent "states" emerges depending on extrinsic cues in the culture environment. Ongoing refinements to such parameters will help to shed more light on the underlying mechanisms of pluripotency, and importantly, how this property can be further exploited for research and biomedical purposes. The insights gained from murine models are now being applied to human ESCs and iPSCs, which hold great promise for therapeutic applications.

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