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The Molecular Circuitry underlying pluripotency in embryonic stem cells

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Abstract

Cells in the pluripotent state have the ability to self-renew indefinitely and to differentiate to all the cells of the embryo. These cells provide an *in vitro* window into development, including human development, as well as holding extraordinary promise for cell-based therapies in regenerative medicine. The recent demonstration that somatic cells can be reprogrammed to the pluripotent state has raised the possibility of patient and disease specific induced pluripotent cells. Here we review the molecular underpinning of pluripotency. We focus on the transcriptional and signaling networks that underlie the state of pluripotency and control differentiation. In general, the action of each of the molecular components and pathways is dose and context dependent highlighting the need for a systems approach to understanding pluripotency.

The three germ layers of the mammalian embryo all derive from the cells of the epiblast which is itself a derivative of the inner cell mass (ICM) (Figure 1). Mouse embryonic stem cell (mESC) lines were initially derived by plating cells from the ICM on a layer of embryonic feeder cells[1, 2]. The cells cultured from the ICM meet the defining criteria for pluripotency in that they: 1) self-renewal indefinitely and 2) give rise to all the cell types which comprise the embryo. More recently, pluripotent cells meeting these same criteria have been isolated from the early human embryo (Movies 1 and 2) [3].

Functionally, pluripotency can be demonstrated by several experimental tests. These include differentiation to all three germ layers *in vitro* and *in vivo* (embroid body and teratoma formation, respectively), contribution to chimeric mice upon injection into blastocyst-stage embryos, and, most stringently, tetraploid complementation. In the latter technique, the pluripotent cells generate the entire mouse while the tetraploid cells contribute only to extraembryonic tissue[4, 5]. The pluripotency of mESCs has been demonstrating using all of the above techniques, while human embryonic stem cells (hESCs) have been used to generate embryoid bodies, teratomas and even mouse-human chimeric blastocyst-stage embryos[6].

The study of ESCs holds significant promise for problems of both fundamental and clinical significance. ESCs provided a technical means to manipulate the mouse germline.

Furthermore, while studies of mESCs *in vitro* can complement *in vivo* approaches, hESCs provide the only system for studying human development and its differences with other mammals. Finally, the ability to differentiate ESCs to specific cell types has the potential to lead to cell-based therapies for a wide range of disorders in regenerative medicine. The recent discovery that somatic cells can be reprogrammed into a pluripotent state[7] (known as induced pluripotent stem cells or iPSCs) has raised the possibility of generating patient- and disease-specific stem cells through reprogramming. In this article, we review the molecular basis of pluripotency focusing in particular on the signaling and transcriptional networks that ESCs use to maintain pluripotency and to differentiate.

Signaling pathways in pluripotency and differentiation

During embryogenesis, signaling pathways provide the cues to establish positional information within the embryo and to instruct cells to differentiate. Pathways typically begin at the cell surface with ligand binding to a receptor complex and terminate in the cell nucleus with the activation of transcription thus allowing a transfer of information from outside the cell to inside the nucleus. Proper signaling cues are essential both for self-renewal in the state of pluripotency and for instructing cells to differentiate to particular lineages. In this section, we review several emerging themes in signaling in pluripotent cells with a focus on the developmentally essential LIF, BMP, Activin/Nodal, FGF, and Wnt pathways (Table 1).

Proper signaling cues can maintain self-renewal by activating pluripotency and repressing differentiation-specific genes

Within the embryo, specific signals specify the ICM and allow its cells to remain pluripotent. Traditionally, *in vitro*, these signals have been replaced by culture on a feeder layer of mouse embryonic fibroblasts. These same cells are capable of maintaining the pluripotency of both mES and hESCs, however, elucidation of the signaling requirements to maintain each cell type without feeders has revealed large difference between mouse and human pluripotent cells.

Signaling pathways maintaining pluripotency in mESCs

Over twenty years ago, it was discovered that the feeder cells could be replaced by a combination of the signaling molecule LIF and serum[8]. LIF signals through the transcriptional activator STAT3 and activation of STAT3 alone is sufficient to replace the requirement for LIF in maintaining pluripotency[9]. The primary mechanism of action of LIF in maintaining pluripotency appears to be through STAT3 induction of Klf4, however, LIF also maintains Nanog expression by signaling through the PI3K pathway[10]. Thus, LIF appears to function mainly by directly inducing key pluripotency-associated genes.

More recently, it was discovered that under these culture conditions, the serum in the medium could be replaced with BMP ligands allowing the culture of mESCs in feeder-free, serum-free medium containing LIF and BMP[11]. In ESCs, BMP ligands signal through the Smad pathway to activate expression of Id genes that inhibit differentiation[11]. BMP also

functions by inhibiting the ERK and p38 MAPK pathways which promote differentiation in mES (see below)[12]. Thus, LIF and BMP function synergistically in mES by promoting pluripotency and suppressing differentiation, respectively.

It is natural to ask whether activation of these pathways is necessary or merely sufficient for the maintenance of pluripotency. In fact, mESCs deficient in either LIF or its receptor gp130 can be propagated and mice generated from these cells develop nearly normally[13]. Further, the pluripotent state of mESCs can be maintained solely by inhibition of the FGF differentiation pathway using both an FGF receptor inhibitor and a MEK inhibitor, however, growth under these conditions is improved when GSK3β is inhibited as well[14]. *Stat3*$^{-/-}$ cells which are incapable of transducing LIF signals can be maintained in this formulation demonstrating that this pathway is not strictly required for pluripotency. The maintenance of pluripotency by these three inhibitors has been termed the “ground state” of ES cell self-renewal[14].

Signaling pathways maintaining pluripotency in hESCs

Surprisingly, the LIF and BMP pathways do not play a role in self-renewal in hESCs (Table 2). Addition of LIF to hESC culture medium activates STAT3 but cannot substitute for the layer of feeder cells as is the case for mESC[15]. Additionally, BMP is a differentiation pathway in hESCs, and even relatively low doses cause differentiation to extraembryonic or mesodermal fates[16-18]. Instead, hESCs can be maintained in feeder-free, serum-free conditions through stimulation of the FGF[19-21] and Activin/Nodal pathways[22-24]. Thus the signaling requirements of mouse and human pluripotent cells are significantly different. It has been suggested that hESCs may represent a later stage of development than mESCs and indeed stem cell populations derived from the E5.5 epiblast share many features with hESCs (Figure 2)[25, 26]. It has also been shown that it is possible to revert hESCs to an earlier developmental state that resembles mESCs in its signaling requirements[27].

Similar to the roles LIF and BMP play in mESC, FGF and Actvin signaling both activate the expression of key pluripotency genes and suppress differentiation-related genes and pathways. Activin/Nodal signaling activates Nanog and the signal transducers Smad2/3 bind directly to the Nanog promoter[28]. This interaction has been suggested to occur *in vivo* in model organisms as well[29]. FGF signaling through the ERK pathway has been reported to sustain Nanog expression, however, this is likely an indirect effect acting through the Activin/Nodal pathway[17]. Independently, FGF activation of the PI3K pathway promotes pluripotency by directing Smad2/3 activity to pluripotency rather than differentiation genes (see below)[30, 31]. Further, both pathways play a role in suppressing the BMP differentiation pathway[28, 32], although the molecular mechanisms of these interactions remain unclear. In other contexts, Activin/Nodal signaling has been suggested to suppress BMP signaling through competition for common pathway elements such as Smad4[33], while FGF signaling through ERK has been shown to induce inhibitory phosphorylations in the linker regions of the BMP signal transducers Smad1/5/8[34].

Despite the differences between mouse and human, Wnt signaling has emerged as a signaling pathway that plays a role in maintaining pluripotency in both mouse and human. In mESCs, Wnt signaling can maintain pluripotency under conditions that would otherwise

promote differentiation[15, 35] and may function both by upregulating LIF/Stat3 signaling[36] and by suppressing the transition to the epiblast state[37]. Wnt signaling can also enhance the reprogramming of murine somatic cells to induced pluripotent cells[38, 39]. Wnt signaling appears to play a similar role in maintaining hESC pluripotency although the molecular mechanisms remain unclear[15]. As Wnt, FGF, and TGFβ all function as morphogens, it is very likely that particular concentrations are necessary for this activity as we now discuss.

The same signalling pathways recur in maintenance of pluripotency and induction of differentiation

Paradoxically, many of the pathways involved in maintaining pluripotency play a key role in differentiation as well. Decades of research in model organisms have delineated essential roles for the FGF, BMP, Activin/Nodal and Wnt signaling pathways in early developmental processes including mesoderm induction, dorsal-ventral patterning, and formation of Spemann's organizer[40-44] and these pathways directly activate key differentiation genes such as Brachyury, Gooscoid, and Sox17. Furthermore, under differentiation conditions, these pathways play similar roles in ESCs. Taken together, these observations raise a central question: how do signaling pathways maintain pluripotency under some conditions while directing differentiation under others.

In mESCs, the key to answering this question may lie in the fact that mESCs represent an early stage of development and are not primed for differentiation. Recent studies argue that mESCs transition to a primed epiblast stem cell (EpiSC) state before differentiating to any of the three germ layer lineages. This ES to epiblast transition is induced by upregulation of FGF signaling, consistent with the expression of FGF5 in the epiblast *in vivo*. Subsequently, embryonic lineages are specified by particular activities and/or combinations of ligands such as BMP, Activin/Nodal or retinoic acid (RA)[45, 46]. Importantly, while some signals are instructive, others may potentiate differentiation directed by other pathways. Further, RA signaling also appears to initiate the upregulation of FGF signaling that leads to the EpiSC state[45]. Thus, complex signaling relationships mediate pluripotency versus differentiation toward specific germ layers, and differences in cell state may determine whether a signaling pathway promotes self-renewal or differentiation.

In human cells, the issue is more problematic as hESCs already represent a later stage of development and are primed for differentiation. Upon stimulation with growth factors such as BMP or Activin/Nodal, hESCs show both morphological and molecular signs of differentiation within 24 hours[18, 47]. Thus, how it is that Activin/Nodal or Wnt signaling can both promote pluripotency and direct differentiation in hESCs remains an important question. Indeed recent studies showing that activation of Wnt signaling in hESCs leads to mesendoderm differentiation have been used to suggest that Wnt is primarily a differentiation, not self-renewal, pathway in hESCs[31, 48].

The answer to this issue may lie at least in part in the fact that nearly all of these pathways function as morphogens *in vitro* and *in vivo*, elucidating different outcomes depending on the concentration or duration of signaling[40, 42, 49]. In the case of Wnt signaling, recent evidence suggests that while low levels support pluripotency in hESCs, higher levels lead to

differentiation[50, 51]. This may also provide part of the explanation for the differing effects of Activin/Nodal signaling in hESCs as the concentrations used to maintain self-renewal are typically significantly lower than those used in differentiation[18, 47]. Thus, in this view, the state of pluripotency can be considered one of many possible fate outcomes induced by Activin/Nodal or Wnt morphogens and is induced by low but not high concentrations.

Signaling pathways form a network that dictates the balance of self-renewal and differentiation

Signaling pathways do not function in isolation but the status of signaling through one pathway can dictate the outcome when another is activated. Thus, another part of the explanation for how the same signaling pathways guide both self-renewal and differentiation likely lies in considering the status of a network of pathways rather than evaluating each pathway separately (Figure 3). Similar principles are needed to understand differentiation as the result of adding identical concentrations of a differentiating ligand can be altered depending on the status of other pathways.

An illustration of these ideas has recently been uncovered in the interactions between FGF and Activin/Nodal signaling governing the balance between pluripotency and differentiation in hESCs[31]. As discussed above, Activin/Nodal signaling mediates both self-renewal and differentiation to mesendodermal lineages. These effects depend on the status of the ERK and PI3K pathways which function downstream of FGF. In pluripotency conditions, the PI3K pathway is active and directs Activin/Nodal signaling to pluripotency-promoting genes such as Nanog. Under differentiation conditions, PI3K is suppressed, leading to upregulation of the ERK pathway as well as activation of Wnt signaling. These combined changes redirect Activin/Nodal from maintaining pluripotency to inducing differentiation. Thus considering the status of an integrated signaling pathway elucidates how Activin/Nodal signaling can play context-specific roles.

A similar phenomenon occurs during BMP-mediated differentiation. Results from model systems *in vivo* has identified a role for BMP in inducing mesodermal lineages[52]. In hESCs, however, treatment with BMP ligands leads to induction of genes and cell morphology associated with trophoectedermal lineages[16]. These differing results can be explained by the status of the FGF pathway. When the FGF pathway is active, it cooperates with BMP signaling to induce mesoderm, while in its absence, BMP induces trophectoderm[17]. A recent study has challenged whether BMP-differentiated hESCs represent a true trophectodermal population[18], however, whatever the outcome of that debate, it is clear that outcome of BMP-mediated differentiation depends on the status of the FGF pathway. These results highlight that the effects of signaling pathways on self-renewal and differentiation can only be unraveled by considering an integrated signaling network.

Epigenetic control of pluripotency and differentiation

The chromatin state of a cell provides the context in which the transcriptional changes that mediate self-renewal and differentiation must take place. Recently, modulators of chromatin have emerged as important players in the maintenance of pluripotency, in differentiation, and

in reprogramming. A thorough discussion of this topic is beyond the scope of this review and the reader is directed to recent reviews devoted to this subject[53, 54]. Here we review basic concepts and highlight recent studies relevant to signaling and transcriptional networks in pluripotency.

ESCs are generally characterized by an open chromatin configuration[54] and this is associated with a hyperactive transcriptome, including expression of low levels of many lineage specific genes[55]. This promiscuous transcription has been suggested to be associated with the plasticity of ESCs and differentiation is reflected in silencing genes from alternate lineages. *In vivo* studies have demonstrated a similar open chromatin configuration in cells from the E3.5 mouse blastocyst[56].

Generally, genes active in the pluripotent state are associated with the chromatin mark H3K4me3 while those associated with differentiation have a repressive mark such as H3K27me3[53]. Many differentiation genes have both active and repressive marks. This bivalent chromatin represents a state that is silent but poised for transcription[57]. These bivalent marks are recognized by the Polycomb complex that acts in a repressive role. Differentiation is accompanied by the loss of the repressive H3K27me3 mark and activation of transcription[58]. However, a simple role for Polycomb proteins in repressing differentiation-associated genes to maintain pluripotency is excluded by the observation that ESCs defective in a critical subunit of the Polycomb complex are pluripotent[59]. The maintenance of pluripotency in the absence of the Polycomb complex is likely due to the redundant role of several repressive complexes in silencing differentiation-related genes[60]. More recent studies have revealed a nuanced picture of Polycomb function with the composition and activity of the Polycomb complex changing during differentiation[53].

A recent study has highlighted the role of poised chromatin in triggering differentiation[61]. This study revealed that complexes of the Activin/Nodal signal transducers Smad2/3 with TIF1γ/TRIM33 recognized the poised chromatin mark H3K9me3 on key mesendodermal regulators specifically in response to activation by Activin/Nodal. This recognition was necessary for activation of these genes by the canonical Activin/Nodal active signaling complex containing Smad2 and Smad4. Thus, Activin/Nodal/nodal signaling uses the poised chromatin marks to switch these genes from poised to active states. These finding remain controversial, however, as in other contexts TRIM33 has been shown to be antagonistic to Activin/Nodal signals[62, 63] and the phenotype of the mouse knockout of TRIM33 is more consistent with overactive than repressed Activin/Nodal[64].

There are extensive associations between the network of transcription factors governing pluripotency and chromatin modifications. In particular, c-Myc has emerged as a key factor involved in the core circuitry of ESCs and linked to multiple activities involving chromatin modifications[65]. This study shows that loss of Myc leads to widespread changes in chromatin modifications in both mESCs and the early mouse embryo.

Transcriptional networks controlling pluripotency

Core Transcriptional Circuitry

Through microarray analysis, a global view of gene expression associated with the state of stemness has begun to emerge[66-69]. Prominent results in each of these studies included genes that had previously been shown to play critical roles in embryogenesis and formation of ESCs. These include genes that have been utilized for the reprogramming of mouse and human somatic cells, such as Klf4 and c-Myc. In addition, focused studies in mESCs and hESCs have confirmed requirements for other factors like FoxD3 and Id proteins in maintenance of self-renewal or pluripotency[70-72]. Relative to differences in signaling requirements and epigenetic status between mESCs, mEpiSCs and hESCs, transcriptional profiles appear to be more conserved between these cell types[25, 26]. Expression levels of some differentiation-associated genes are elevated in mEpiSCs and hESCs compared to mESCs at the RNA level, consistent with the notion of “primed” versus “naive” states of pluripotency[26, 27]. On the other hand, evidence that these differences exist at the protein level is limited. Further, pluripotency transcription factors Oct4, Sox2, Nanog, and Myc levels are consistent between cell types[26]. In this section, we will focus on Oct4, Sox2 and Nanog, three transcription factors that have been shown repeatedly to be at the heart of the transcriptional network that supports pluripotency.

Oct4, Sox2 or Nanog loss-of-function results in failure of epiblast formation and embryonic lethality by implantation stages. Consequently, mESCs cannot be established from Oct4$^{-/-}$, Sox2$^{-/-}$ or Nanog$^{-/-}$ blastocysts[73-75]. RNA interference-mediated knockdown of these genes in hESCs results in loss of pluripotency and self-renewal[76-78], consistent with results in mice. Gene expression and knockout studies established indispensible roles for Oct4, Sox2 and Nanog in pre- and peri-implantation murine development. Oct4 is expressed from the 8 cell stage, transiently in the extraembryonic endoderm and later becomes restricted to the epiblast[79, 80]. Similarly, Sox2 is expressed in all cells at morula stages and in the epiblast, as well as trophectoderm[81]. Nanog expression is initiated slightly later, in post-compaction morulae, and persists in the ICM and epiblast[74, 82]. Analysis of gene expression in human blastocysts indicates that Oct4, Sox2 and Nanog are expressed in the ICM[83, 84]. Recently, numerous genome-wide studies have been undertaken to determine how these key factors regulate the transcriptional repertoire of ESCs. Although the picture remains incomplete, several evolutionarily conserved trends have emerged.

Coordinators of the Pluripotency Network

Cooperative regulation—Genome-scale analyses of Oct4, Sox2 and Nanog binding sites reveal that they frequently bind the same regulatory regions in undifferentiated mouse and human ESCs, and that these binding sites are often in close proximity to one another[85-88]. Oct/Sox composite binding sites have been identified in individual promoters, directly adjacent or separated by less than five base pairs[89-91]. These data point not only to coordinated regulation of targets, but in some cases physical interactions between the transcription factors themselves[89, 90, 92]. Further, it appears that combinatorial binding sites may be significantly more conserved between mouse and human than individual binding

sites[93], suggesting that this may be a dominant mode of transcriptional control in the state of pluripotency.

In addition to one another, Oct4, Sox2 and Nanog activities are modulated by downstream effectors of signaling pathways, such as Tcf3 and Smad3, and by Polycomb Repressor Complexes (PRCs). Tcf3 and Smad3 binding sites are enriched in promoters that are also bound by the Oct4/Sox2/Nanog combination[94-96]. In the presence of Wnt signaling levels that support pluripotency in mESCs, Tcf3 is likely to be associated with repression of transcription, whereas elevated levels of Wnt signaling overcome the repressive effect of Tcf3 and lead to differentiation of mESCs[95, 97, 98]. In the reciprocal experiment, knockdown of Tcf3 can substitute for the Wnt requirement in maintenance of pluripotency[96]. A critical component of TGFβ signalling, Smad3, appears to be recruited by Oct4 to specific promoters on a genome-wide scale[94], providing another potential intersection between established signaling cues and the transcriptional network of ESCs. Finally, a subset of regulatory regions enriched for binding by each of these transcription factors is also enriched for binding by PRCs[58, 85]. Differential effects of Oct4, Nanog or Tcf3 knockdown on target gene expression (see below) may result from association of PRCs with some targets but not others[95]. Thus, a variety of inputs acting in concert, both cooperatively and antagonistically, are required to balance the transcriptional activity of the pluripotency network.

Targets—Oct4, Sox2 and Nanog have been shown to both activate and repress transcription in particular cases[89, 90, 99]. Indeed, global analyses have found Oct4, Sox2, and Nanog binding sites to be roughly equally distributed between genes that maintain pluripotency and those that promote differentiation[85, 87]. However, when Oct4 levels are experimentally manipulated, genes that are predicted to be activated by Oct4 are more likely to be affected than those predicted to be repressed[88, 94]. Suppression of Oct4 function in mESCs leads first to downregulation of targets that promote stemness, followed at later time points by upregulation of genes that promote differentiation[85, 88]. These observations suggest that Oct4 acts primarily through the activation of other pluripotency genes, which in turn repress genes associated with differentiation. In agreement with this hypothesis, overexpression of Oct4 coupled with cyclohexamide treatment to inhibit protein synthesis indicated that repressors of differentiation were far more likely to be direct targets than promoters of differentiation[100]. On the other hand, a slightly more even distribution between activated and repressed promoters was observed for Nanog binding sites[88], and Nanog and Oct4 have both been associated with repressive protein complexes in mESCs[101]. Thus, the precise activity of Oct4, Nanog and Sox2 is likely to be context- and promoter-specific.

Roughly equal numbers of Oct4 and Nanog binding sites are found in intragenic regions compared to promoter regions[86]. Although it remains unclear whether functional binding is equivalent between these two categories, these findings point to the need for careful attention to the genomic regions examined in published studies and further analysis.

Stoichiometry and combinatorial regulation of cell fate—In addition to regulating other genes, Oct4, Sox2 and Nanog have been shown to cross- and auto-regulate[75, 86, 100, 102-105]. Such feed-forward mechanisms support robustness of transcriptional programs, and also serve to maintain balanced levels of key regulatory

factors. Much like the case with signaling pathways, specific levels of transcription factor activity appear to be important for maintenance of pluripotency. Thus, even small perturbations of Oct4 or Sox2 levels lead to differentiation[78, 106-108]. Interestingly, upregulation of these transcription factors has different effects. For example, elevated levels of Oct4 expression lead to differentiation of ESCs to extraembryonic endoderm fates[108], while overexpression of Sox2 generates a variety of fates that specifically exclude endoderm[106]. Similarly, in a growth factor-mediated differentiation paradigm, slightly elevated levels of Oct4 or Sox2 correlated with distinct differentiation outcomes[109]. Further, this study found that in pluripotent cells Oct4 and Sox2 bound promoters together in agreement with previous reports, but that they became enriched at opposing loci during differentiation. For example, Sox2 becomes enriched over Oct4 at its own enhancer and in the Brachyury regulatory region during neurectodermal differentiation, signaling positive and negative regulation, respectively. Together with the high degree of cooperative binding observed in ChIP experiments, these data support the notion that correct stoichiometry of key transcription factors is critical. Thus, concentration- and activity-specific mechanisms function at multiple levels in regulation of pluripotency, including extracellular signaling and intracellular transcription factors.

Reprogramming to the pluripotent state

Induced pluripotent stem cells (iPSCs) present an opportunity to further analyze and test our knowledge of pluripotency. Further, the ability to generate disease- or patient-specific iPSC lines for study and eventually for therapeutics holds great promise for both basic biology and medicine. Still in its infancy, the field of iPSC research began with the reprogramming of mouse fibroblasts[7], followed closely by complementary experiments in human somatic cells[110, 111]. In these seminal studies, it was demonstrated that forced expression of a small number of transcription factors could alter the potency of highly derivative cells from embryos or adults, bringing them back to a status resembling embryonic stem cells. In the intervening five years, considerable progress has been made in our understanding of the reprogramming process. To thoroughly address this topic is the work of several reviews[112-114]. In this section, we focus on a few major developments in the field of reprogramming as they relate to our understanding of pluripotency.

The original study in mice found that four transcription factors, Oct4, Sox2, Klf4 and c-Myc (OSKM) were sufficient for reprogramming[7]. Although it was later postulated that these cells were only partially reprogrammed, the utility of OSKM has been upheld in numerous subsequent studies[115-117]. These requirements have further been dissected, revealing that different combinations of transcription factors are sufficient for the reprogramming of different somatic cell types[118-124]. In some cases, the function of individual transcription factors can be replaced by chemical or small molecule treatments[125-127]. Notably, the one requirement for which no substitute has been found is Oct4, underscoring the pivotal role that this transcription factor plays in pluripotency.

With very few exceptions, the efficiency of the reprogramming process continues to be low (< 1%)[114]. This suggests that numerous obstacles exist to the reacquisition of pluripotency by somatic cells[114, 128]. These include, but may not be limited to, deactivation of the somatic

cell gene program, activation of endogenous pluripotency genes (both the factors being used to reprogram and additional loci), epigenetic remodeling, and a number of cell divisions. It has further been postulated that only a small number of cells in any given population have the capacity to be reprogrammed[128]. It is likely that low reprogramming efficiency is the result of a combination of these factors – many barriers exist, and thus only a small number of cells manage to clear all the hurdles to be fully reprogrammed.

In order to be considered fully reprogrammed, iPSCs must pass all the tests of pluripotency, including the abilities to self-renew and to form derivatives of each of the three embryonic germ layers *in vitro* and *in vivo* (teratomas). The tetraploid complementation test, in which mouse iPSCs are challenged to form an entire embryo, was recently passed[129, 130] albeit with possible complications[131]. Global profiling methods for gene expression and DNA methylation have also been leveraged in characterization of iPSC lines[132-134]. However, our incomplete understanding of the state of pluripotency and of embryonic development in general leads to several key challenges in the effort to utilize iPSC technology[114, 135].

First, a more precise definition of the state of pluripotency is necessary. Focused and global studies of gene expression, promoter activation and repression, DNA methylation and other characteristics continue to refine this picture. Second, due to variability in the precise nature of iPSC lines derived using different methods, any study of disease-specific iPSC lines must have an isogenic wild-type iPSC line generated in the same manner, preferably in parallel. This increases confidence that defects in differentiation of the disease-specific iPSC line result from the disease defect, rather than an artifact of the reprogramming process. This leads to a third requirement, the ability to genetically modify iPSC lines. Although homologous recombination has been a standard technique in mESCs for decades, efficiencies in human cells have until recently been very low. However, use of zinc finger (ZF) and transcription activator-like effector (TALE) nucleases has significantly improved efficiency of this process[136, 137]. Finally, methods for directed differentiation of ESCs and iPSCs to specific cell types affected in a given disease must be established. This issue has and continues to be a major focus of the field.

The discovery of the possibility for somatic cells to reacquire pluripotency was a tremendous leap forward for both basic biology and medical applications. The field continues to move forward at an impressive pace, bringing new perspectives and tools to bear on questions of the nature of pluripotency.

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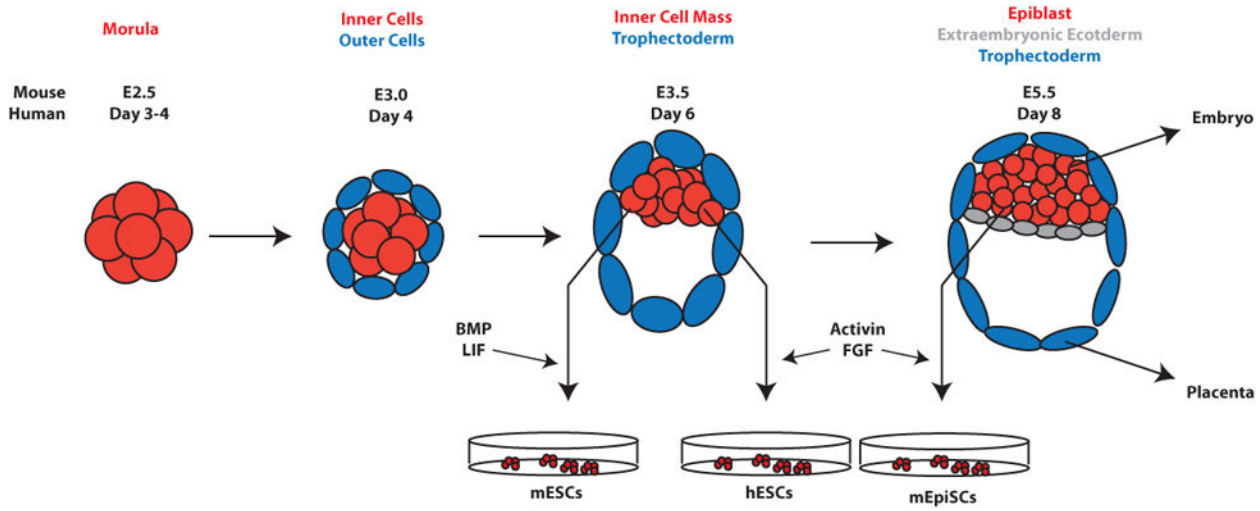

Figure 1. Early Mammalian Embryonic Development

After morula stages, the first cell fate decisions are made, in which cells sort to outer and inner populations. Outer cells give rise to the extraembryonic trophectoderm (TE), while inner cells form the inner cell mass (ICM). The ICM is located asymmetrically at one side of the blastocoel cavity within the TE. Subsequently, the ICM further differentiates to the extraembryonic endoderm (ExEn) and the epiblast, which gives rise to the embryonic ectoderm, mesoderm and endoderm. Mouse and human embryonic stem cells are derived in vitro by explanting the ICM.

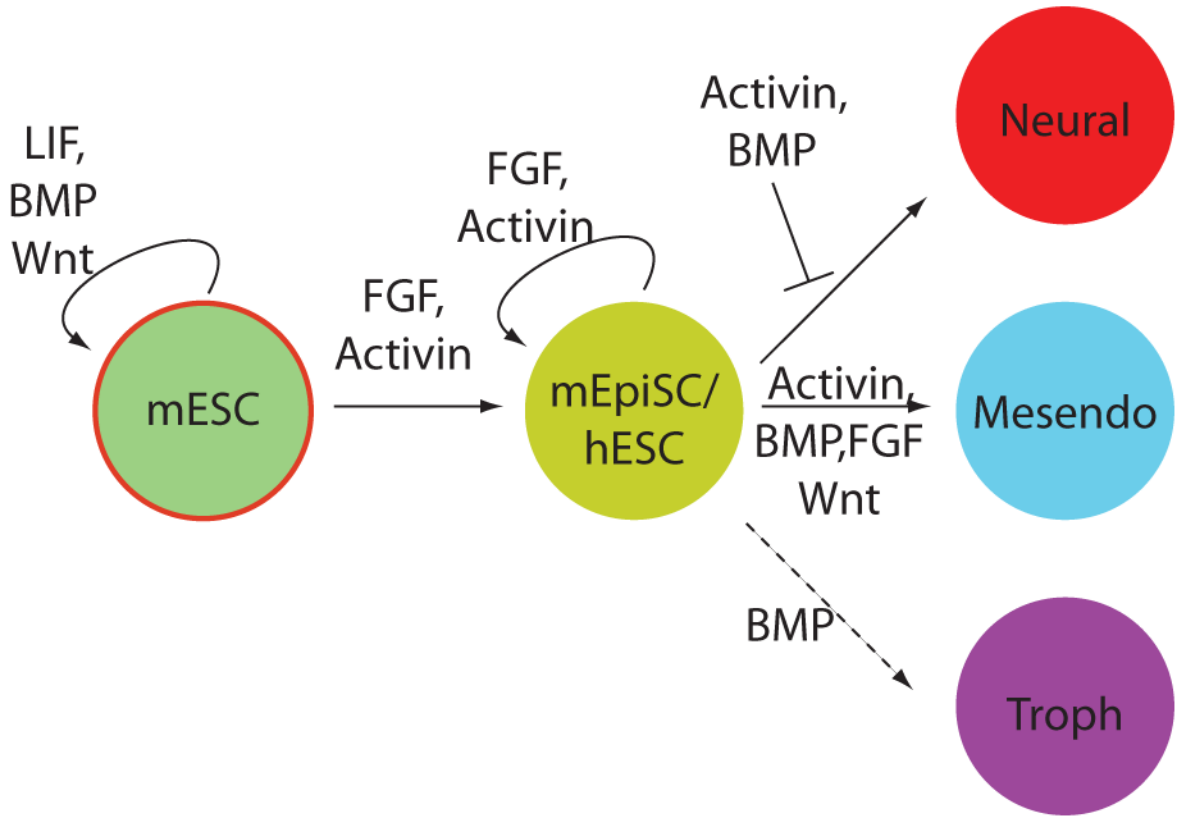

Figure 2. Relationships between signaling pathways, pluripotency, and differentiation

Schematic depicting the relationship between signaling pathways and the indicated cell states. The dashed line indicates a connection only present in human but not mouse ES cells while the red-circled state indicates a state only accessible to mouse but not human cells.

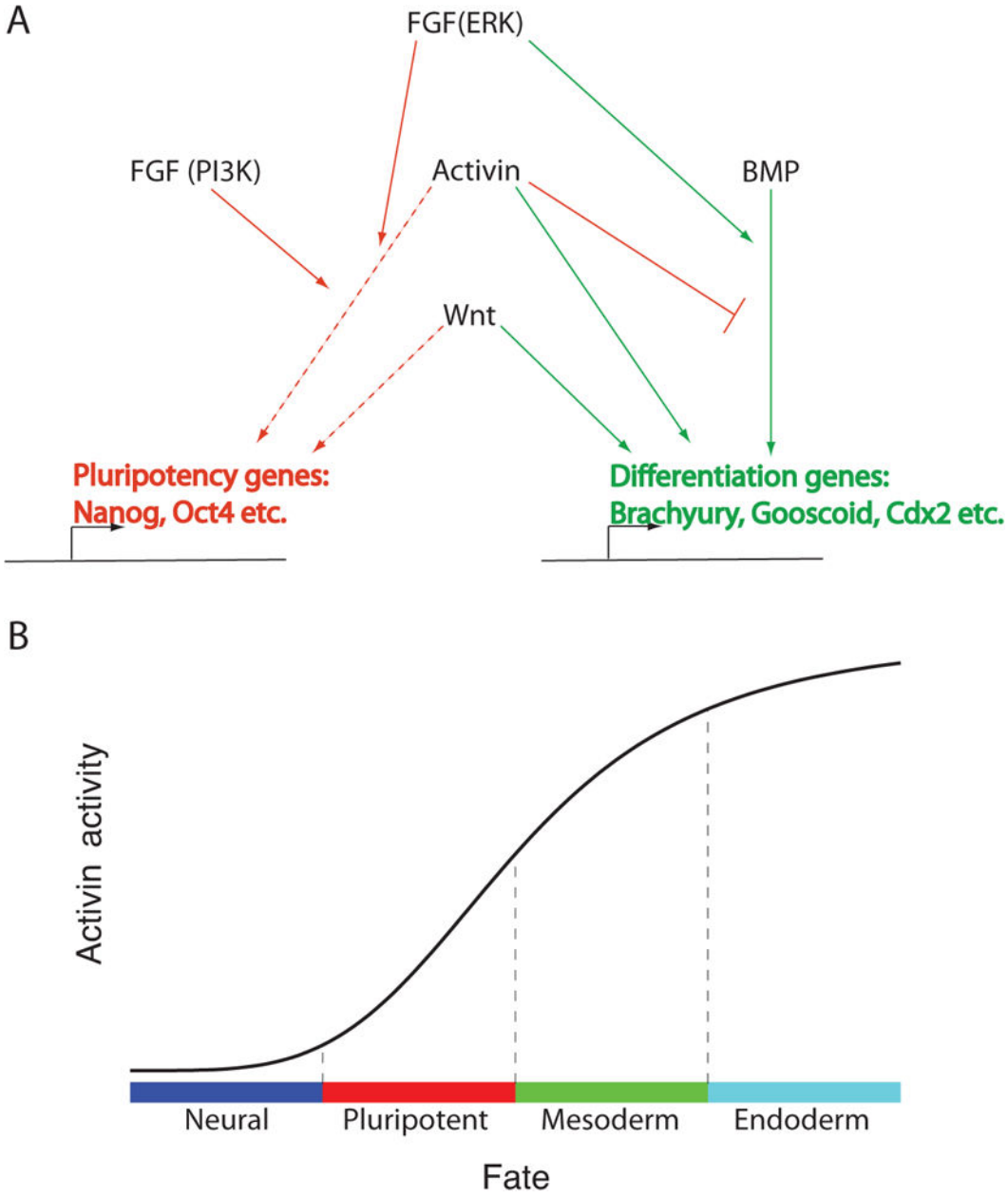

Figure 3. Network of signaling pathways governing pluripotency and differentiation

(A) Schematic depicting the relationships between signaling pathways and genes control cell fate. Red lines denote interactions promoting pluripotency and green lines denote interactions promoting differentiation. Dashed lines indicate interactions only operative at low to intermediate activity of the signaling pathway. (B) Pluripotency is one of a spectrum of possible fates that result from modulating the Activin/Nodal pathway. Lower or higher levels of pathway activity lead to neural or mesendodermal differentiation, respectively.

Table 1

Signaling pathways involved in the maintenance of pluripotency. Table summarizing properties of pathways that play a role in maintaining pluripotency either in mESCs or hESCs.

Pathway	LIF	BMP	Activin/Nodal	FGF	Wnt
Receptor	gp130	Alk2/3/6	Alk4/5/7	FGF-R	LRP5/6
Signal transducer	Stat3	Smad1/5/8	Smad2/3	MEK/ERK	β-catenin
mESCs?	+	+	-	-	+
hESCs?	-	-	+	+	+

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Table 2

Comparison of Mouse ESCs, mouse EpiSCs with Human ESCs. Many features of mESCs, mEpiSCs and hESCs have been evaluated singly and in parallel. A summary of key characteristics is provided here. For additional information, see refs [25, 26, 113, 114].

	mESCs	**hESCs**	**mEpiSCs**
Morphology	Rounded	Flattened	Flattened
Single cell survival	Good	Poor	Poor
Potency	All embryonic fates	All embryonic fates	All embryonic fates
Signaling inputs	BMP, LIF	Activin, FGF	Activin, FGF
Embryoid Body Formation	Yes	Yes	Yes
Teratoma Formation	Yes	Yes	Yes
Tetraploid Complementation	Yes	N/A	No
X inactivation	No	Yes	Yes