Pluripotency and Nuclear Reprogramming

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Abstract

Pluripotency is a "blank" cellular state characteristic of specific cells within the early embryo (e.g., epiblast cells) and of certain cells propagated in vitro (e.g., embryonic stem cells, ESCs). The terms pluripotent cell and stem cell are often used interchangeably to describe cells capable of differentiating into multiple cell types. In this review, we discuss the prevailing molecular and functional definitions of pluripotency and the working parameters employed to describe this state, both in the context of cells residing within the early embryo and cells propagated in vitro.

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Pluripotency: the unique ability of a cell to differentiate into all somatic and germ line cells of the developing embryo

1. INTRODUCTION

Mammalian development is characterized by an extremely well-orchestrated transition from an initial single cell, or zygote, to the entire spectrum of cells found in the body. This process is governed by an intrinsic regulatory program that employs transcription factors to decipher both sequence-specific and epigenetic information. Additional layers of control are provided by extrinsic information incorporating the spatial and temporal distributions of cells, cell shape, properties of the extracellular matrix, the actions of morphogens, the functions of other effectors intricately intertwined with those of transcription factors, and the activities of enzymes that modify epigenetic information.

Importantly, studies on pluripotent cells have identified intrinsic autonomous programs (defined by their ability to differentiate into precursor cells representing the founding populations of all three embryonic germ layers) that are of paramount importance in maintenance of the inherent pluripotency of these cells. Here, we review both the cellular and molecular design principles of pluripotency.

2. CELLULAR ORIGINS OF PLURIPOTENTIALITY

2.1. Totipotency and the Pre-Embryonic Stage

Totipotency is defined as the ability of a particular cell to give rise to all cell types of an organism, including the extraembryonic cell lineages that are required for appropriate development of the embryo (1). These extraembryonic lineages are crucial for proper implantation and maintenance of the embryo in the uterus and also provide inductive signals for pattern formation and axis development (2). In vivo totipotent cells exist only transiently in the early embryo and are generated via a natural reprogramming process initiated by fertilization of the oocyte. Although neither the oocyte nor the zygote is totipotent, the fertilized egg undergoes a transition from a specialized cell type with a restricted fate into equipotent blastomeres. At the two- and four-cell stages, murine blastomeres are totipotent, and each can develop into an entire animal (3).

A series of transplantation experiments demonstrated that maternal proteins and RNAs that had been deposited in the oocyte could reset the epigenetic state of somatic chromatin and transform the oocyte into totipotent blastomeres following fertilization (reviewed by Reference 4). Nevertheless, it has not been possible to establish totipotent cell lines in vitro. Indeed, this may be impossible because of the transient nature of the totipotent blastomeres in vivo; these blastomeres are not actually self-renewing or dividing but rather are generated via cleavage during the so-called pre-embryonic stage of embryonic development.

2.2. Primordial Pluripotency

As cleavage of the early embryo proceeds to the 16-cell stage, a gradual restriction in developmental potency is evident, and the cells are committed to development into two distinct lineages: the trophoblast lineage and the inner cell mass (ICM). Differentiation commences with flattening of the blastomeres and strengthening of cell-to-cell contact. This process has been termed compaction and is mediated by changes in cell-cell adhesion and expression of extracellular matrix proteins (5, 6). During compaction, cadherins, especially E-cadherin, play central roles (7). Cells located on the inside of the embryo at the compaction stage give rise to the ICM, whereas the outer cells form the trophectoderm, eventually leading to generation of the placenta. The ICM next gives rise to a second extraembryonic lineage, the hypoblast (or primitive endoderm), which will form the yolk sac. Simultaneously, the remaining cells transition into the epiblast (the primitive ectoderm), originating the embryo. In contrast to the totipotent precursors, cells of the epiblast (at this stage) are termed pluripotent because they can give rise to all somatic and germ line cells of the developing embryo but do not contribute to extraembryonic lineages. Hence, the epiblast cells exhibit "primordial" pluripotency, which has been termed the ground state of pluripotency (8); see **Figure 1** and **Table 1**.

2.3. Refined (Late) Pluripotency

Developmentally, upon implantation into the uterine wall, epiblast cells become rearranged into an epithelial structure that lines the central proamniotic cavity. During this early postimplantation period, termed the egg-cylinder stage, epiblast cells are alkaline phosphatase (AP) (9) and stage-specific embryonic antigen 1 (SSEA1) positive (10) and express high levels of Oct4 (for a detailed discussion of Oct4 see Section 3.1). At this stage, the epiblast cells exhibit "refined" pluripotency [also termed primed pluripotency (8)]. This definition refers to the restricted developmental potential of the late epiblast. Eventually, the primitive streak forms in a localized region of the epiblast adjacent to the embryonic/extraembryonic junction, and gastrulation commences at 6.5 days postcoitum (dpc).

In the interval from implantation to the onset of gastrulation, epiblast cells lose the ability to colonize the blastocyst, X chromosome inactivation takes place, the length of the cell cycle is reduced (10), and the extent of genome methylation increases. In the early embryo, the genome is demethylated, but the DNA next becomes progressively methylated, and by 6.5 dpc, the methylation pattern characteristic of adult tissues is established (11). During the subsequent steps of gastrulation, the embryo becomes transformed into a multilayered structure wherein all primordia are arranged according to the body plan, and the primitive streak marks the future posterior of the embryo.

2.4. Trapping Pluripotency

Pluripotent cell lines are of enormous interest especially in the field of regenerative medicine. Stem cells have been "trapped" in vitro by isolation from tissues at various stages of embryonic and postnatal development as well as from adult and tumor tissues. Such cells share certain features, but usually reflect aspects of their in vivo counterparts at the molecular level. Independent of origin, the gold standard used

Totipotency: the ability of the zygote and blastomeres to differentiate into all embryonic and extraembryonic lineages of the developing embryo

Epigenetic state: a summary of epigenetic modifications that collectively determine cellular identity in the lineage hierarchy

Trophoblast (trophectoderm): the outer layer of the blastocyst, generated during the first differentiation event when mammalian morula cells segregate into two lineages

Inner cell mass (ICM): this second lineage of the early embryo inside the blastocyst is surrounded by the trophectoderm and originates all embryonic tissues

Hypoblast (primitive endoderm): an epithelial layer derived from inner cell mass cells that are in contact with the blastocyst cavity forming the yolk sack

Epiblast (primitive/embryonic ectoderm): the second tissue derived from inner cell mass cells during the second differentiation event of embryonic development forming the embryo

Table 1 Properties of various pluripotent cell types in vitro[a]

Cell type	mESC	mEGC	mECC	miPSC	mEpiSC	hESC	hEG	hiPSC
Origin	Blastocyst	Embryonic gonad	Teratoma	Somatic cells	Late epiblast	Blastocyst	Embryonic gonad	Somatic cells
Blastocyst chimaera contribution	Somatic and germ line	Somatic and germ line	Somatic, low frequency of germ line	Somatic and germ line contribution	No	Not determined	Not determined	Not determined
Teratomas	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Spontaneous trophoblast differentiation	No	Not determined	Not determined	Not determined	Yes	Yes	Not determined	Not determined
Growth factor conditions	Lif, Bmp4	Derivation: Fgf2, Lif, SCF Maintenance: Lif and FBS	Lif, FBS	Lif	Fgf2, Activin	Fgf2, Activin, (BIO), MEF conditioned medium	Lif, Fgf2, Forksolin	Fgf2, Activin, MEF conditioned medium
Morphology in culture	Domed	Domed	Domed	Domed	Flat	Flat	Domed	Flat
XX status	XaXa	Not determined	XaXa	XaXa	XaXi	XaXi	Not determined	XaXi
Reference	17	26, 27	12	21, 56, 63	25, 24	38	28	58
Pluripotent state	Primordial				Refined	Not determined	Not determined	Not determined
Positive regulators	Lif/Stat3, Bmp4, Wnt, Igf				Tgf-β, Activin, Fgf2, Erk1 and -2, Wnt, Igf			
Negative regulators	Tgf-β, Activin, Fgf2, Erk1 and -2				Bmp4			
Pluripotency factors	Oct4, Nanog, Sox2, Klf2, Klf4				Oct4, Sox2, Nanog			
Response to Lif/Stat3	Self-renewal				None			
Response to Fgf/Erk	Differentiation				Self-renewal			
Response to 2i	Self-renewal				Differentiation/death			
Clonogenicity	High				Low			
XX status	XaXa				XaXi			

[a]Abbreviations: Fgf2, fibroblast growth factor 2; Bmp4, bone morphogenic protein 4; Erk, extracellular signal regulated kinase; FBS, fetal bovine serum; Fgf, fibroblast growth factor; hEGC, human embryonic germ cell; hiPSC, human induced pluripotent stem cell; hESC, human embryonic stem cell; Igf, insulin growth factor; Lif, leukemia inhibitory factor; mECC, mouse embryonic carcinoma cell; MEF, mouse embryonic fibroblast; mEGC, mouse embryonic germ cell; mEpiSC, mouse epiblast stem cell; miPSC, mouse induce pluripotent stem cell; mESC, mouse embryonic stem cell; SCF, stem cell factor; Stat3, signal transducer and activator of transcription 3; Wnt, wingless-type MMTV integration site family; Xa, acticated X chromosome; Xi, inactivated X chromosome; XX status, X chromosome status; 2i, two inhibitor cocktail targeting Erk and Gsk3.

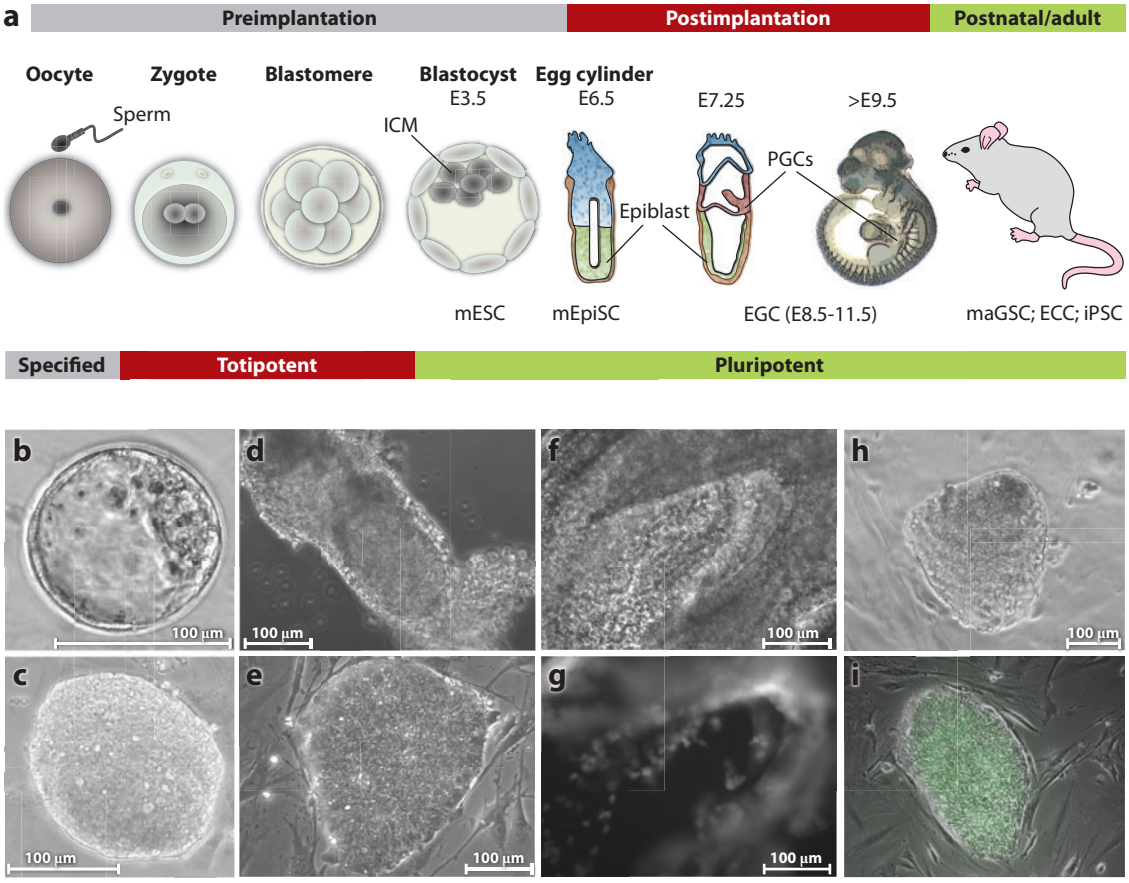

Figure 1

Origins of pluripotent stem cells and the morphology thereof in vitro. (*a*) Pluripotent stem cells can be trapped at various stages of embryonic development. (*b*) A phase-contrast micrograph of a blastocyst at embryonic day (E) 3.5 when the originating embryonic stem cells (ESCs) with domed morphology are evident (*c*). (*d*) A phase-contrast image of a mouse egg-cylinder stage embryo, which can give rise to epiblast stem cells (EpiSCs), at E6.5. (*e*) An EpiSC colony growing as a flat layer of cells. (*f*) A phase-contrast micrograph of a region from the hindgut of a mouse embryo at E8.5, wherein Blimp1-positive primordial germ cells (PGCs) may be noted. (*g*) Immunofluorescence from the region shown in (*f*) in which Blimp1-positive cells in a Blimp1-Cre reporter mouse are shown. (*h*) A phase-contrast image of a mouse induced pluripotent stem cell (iPSC) colony after transfection with DNAs encoding the transcription factors Oct4, Sox2, Klf4, and c-Myc. (*i*) Overlay of a phase-contrast image of a human embryonic stem cell (hESC) colony and an immunofluorescence image of the same colony after immunostaining using an anti-Oct4 antibody. Abbreviations: ECC, embryonic carcinoma cell; EGC, embryonic germ cell; ICM, inner cell mass; maGSC, multipotent adult germ line stem cell; mESC, mouse embryonic stem cell.

to test pluripotentiality is the ability to contribute to the formation of chimeric animals, including colonization of the germ line after injection into host blastocysts or via evaluation of the ability to form multi-lineage tumors, so-called teratomas, after injection into immunocompromised mice.

2.4.1. Embryonic carcinoma cells. Pluripotent stem cells were first derived from teratocarcinomas, germ line tumors that arise spontaneously from the adult testis or ovary of mice and humans (12). Studies in the early 1960s demonstrated the multilineage potential of single teratocarcinoma cells, and

ESC: embryonic stem cell

the first pluripotent embryonic carcinoma cell (ECC) lines were derived from such tumors about a decade later (12). Although ECCs can contribute to formation of chimeric animals, this contribution is often of low level, and germ line transmission occurs only sporadically (13). Additionally, many chimeric animals develop tumors derived from the injected cells, attributable to mutations and abnormal karyotypes that accumulated during the development of the teratocarcinoma from which the ECCs were initially derived (14).

2.4.2. Blastocyst-derived stem cells and mouse embryonic stem cells. At the time of implantation (~4.5 dpc), the blastocyst contains three distinct lineages of which the epiblast population is the smallest, with only 20–25 cells being embedded between the trophectoderm and the primitive endoderm. Under certain cell culture conditions, three stem cell types can be derived from this preimplantation stage, reflecting the three distinct cell lineages of the blastocyst. These are trophoblast stem (TS) cells from the trophectoderm, extraembryonic endoderm (XEN) cells from the primitive endoderm, and mouse embryonic stem cells (mESCs) from the epiblast (see **Figure 1**). TS cells require fibroblast growth factor (Fgf) and Activin/Nodal signaling to self-renew (see Section 3.3), and rely on the activity of TS-specific transcription factors, including Cdx2 and Eomes (15). In contrast, XEN cells are characterized by Gata6 and Sox7 expression, requiring Fgf signaling only during derivation (16).

mESCs were derived in 1981 from preimplantation blastocysts of the mouse strain 129 upon coculture on a feeder layer of irradiated mouse embryonic fibroblasts, in the presence of fetal bovine serum (17, 18). In this environment, the cytokine leukemia inhibitory factor (Lif) provides the principal self-renewal signal by activating the signal transducer and activator of transcription 3 (Stat3) pathway (19, 20). Lif acts together with bone morphogenic protein 4 (Bmp4) to support the pluripotent state of mESCs via induction of inhibitor of differentiation (Id) genes.

mESCs have been termed naive pluripotent cells, reflecting the primordial pluripotentiality of the early epiblast (blastocyst), but it remains possible that embryonic stem cells (ESCs) are in fact an artifact of tissue culture. mESCs resemble their in vivo counterparts in terms of expression of canonical pluripotency genes, such as *Oct4*, *Sox2*, and *Nanog*, and in expression of *AP* and *Ssea1*. Furthermore, ESC lines isolated from female embryos harbor two active X chromosomes. However, the cells are characterized by the ability to self-renew indefinitely and by possession of a hypermethylated genome (21).

It has been suggested, however, that mESCs are the counterparts of naive epiblasts, rather than being created in cell culture. This suggestion is based on the observation that inhibition of extracellular-regulated MAP kinase (Erk) signaling in early embryos suppresses hypoblast formation and causes the entire ICM to develop into an epiblast (22). Inhibition of glycogen synthase kinase-3 (Gsk3) leads to expansion of the nascent epiblast in situ and to generation of ESCs when such cells are explanted. Hence, the early epiblast may resemble ESCs in this regard (23).

2.4.3. Mouse epiblast stem cells. Epiblast stem cells (EpiSCs) have been derived from early postimplantation epiblasts between 5.5 and 7.5 dpc (**Figure 1**). Representing the egg-cylinder stage, they have already undergone X chromosome inactivation and exhibit a gene expression profile characteristic of that of postimplantation epiblast rather than the ICM. This profile is characterized by low-level expression of the pluripotency-related genes, *Nanog*, *Rex2*, and *Klf4*, but also by elevated levels of differentiation markers, such as *Fgf5* (24). Self-renewal of EpiSCs is maintained by Activin, Fgf2, Erk1 and -2, and transforming growth factor-β (Tgf-β) but not by Lif. Furthermore, growth is in fact inhibited by Bmp4 (24, 25). In contrast to the three-dimensional morphology of ESC colonies, EpiSC colonies are flat and do not expand well when diluted to single cells after

dissociation with trypsin. EpiSCs can form teratocarcinomas but contribute only minimally to chimeric animals. Therefore, EpiSCs may reflect the refined (late) pluripotency of the postimplantation epiblast.

2.4.4. Embryonic germ cells. The first ESC-like cells not derived from blastocysts were mouse and human embryonic germ cells (EGCs), originating from primordial germ cells (PGCs) derived from the developing gonad (26–28). During the specification process, such cells successively express *Fragilis*, *Blimp1*, and *Stella*. PGCs are large, round in shape, and these cells exhibit high-level expression of tissue-nonspecific alkaline phosphatase and *Oct4*. PGCs can be isolated in the presence of steel factor and Lif at 8.5 dpc or from genital ridges between 10.5 and 12.5 dpc. However, under such conditions, PGCs exhibit a finite proliferative capacity (26, 29). Addition of Fgf2 to the culture cocktail during the initial period leads to continuous division and establishment of EGC colonies. EGCs are similar to ESCs in terms of morphology, are able to form teratocarcinomas, and contribute to formation of chimeric animals, with apparent germ line transmission (26, 27). Interestingly, when EGCs are fused with somatic cells, they can induce demethylation of somatically imprinted genes, reflecting expression of an enzymatic activity that resets imprinting during development (30). Even though EGCs are derived from a later developmental stage than EpiSCs, they exhibit primordial rather than defined pluripotency. Study of the molecular events leading to PGC specification, and the molecular characterization of EGCs, has shown that EGCs are remarkably similar to ESCs. On the basis of this similarity, an alternative hypothesis on the true origin of ESCs has been advanced (31). Accordingly, the cells might transition through a PGC-like phase in vitro rather than arising directly from the epiblast. This route of ESC derivation is suggested by the presence of early PGC markers in ESCs, whereas late markers characteristic of mature germ cells are not expressed (32–34). In male mouse embryos, PGCs eventually develop into spermatogonial stem cells, which have been isolated from both newborn and adult male gonads and can give rise to embryonic stem (ES)-like cells in vitro (35–37).

2.4.5. Human embryonic stem cells. Almost two decades after the isolation of the first mESC lines from mouse blastocysts, the first human embryonic stem cells (hESCs) were derived from human blastomeres in the presence of Fgf2 (38). hESCs express high levels of telomerase and cell surface markers that are characteristic of undifferentiated nonhuman primate ES and human EC cells, including SSEA3, SSEA4, TRAl-60, TRA1- 81, and AP. hESCs maintain the potential to form derivatives of all three embryonic germ layers and to produce teratocarcinomas (38). Although both X chromosomes are active in mESCs, hESCs show a propensity toward X chromosome inactivation. Several pathways have been implicated in hESC self-renewal, including those involving Fgf2 (39), Tgf/Activin-A/Nodal (40), sphingosine-1-phosphate/platelet-derived growth factor (S1P/PDGF) (41), and insulin growth factor (IGF)/insulin (42), as reviewed in (43).

It is argued that hESCs are in fact the counterpart of mouse EpiSCs because of the similarities between the cell types. Both types of cells grow as flat colonies and do not grow well after dissociation into single cells. Both rely on Activin/Nodal signaling to maintain pluripotency, and in contrast to mESCs, neither cell type can be maintained in medium supplemented with Lif. Additionally, female cells of both cell types harbor one active and one inactive X chromosome (44). Nevertheless, crucial differences in gene expression profiles are evident. hESCs express SSEA3 and -4 on the surface and are AP positive, whereas mouse EpiSCs express Ssea1 and lack Ap. Additionally, hESCs express *DPPA3* (45) and *KLF4*, as do mESCs; these factors are not expressed by EpiSCs. Both Dppa3 and Klf4 play critical roles in conversion of EpiSCs into mESCs. Klf4 alone can in fact drive this conversion (46), and Dppa3 is activated during this process (47). Furthermore, hESCs express *REX1* (48, 49),

Induced pluripotent stem cells (iPSCs): cells generated by artificial induction of pluripotency in vitro

which is expressed in mESCs but not EpiSCs, and hESCs do not express the EpiSC marker *Fgf5* (50). Finally, hESCs absolutely require Fgf2, whereas mouse EpiSCs do not (51).

2.5. Artificial Acquisition of Pluripotency

2.5.1. Cell fusion. Cell fusion experiments generating pluripotent cells have been successfully performed using somatic cells and EGCs or ESCs (52–54). Tada et al. (52, 55) demonstrated that EGCs or mESCs could reprogram the nuclei of somatic cells after fusion with thymic lymphocytes. This procedure yields tetraploid cells resembling EGCs in which the originally inactive somatic X chromosome in female cells becomes reactivated and the differential DNA methylation of imprinted loci is erased. After introduction into diploid host blastocysts, those tetraploid cells are able to contribute to chimeric embryos. However, any such contribution is modest and is attributable to the tetraploid nature of the cells (52, 55). On the basis of these studies, it was shown that both bone marrow cells (54) and brain cells isolated from the central nervous system (53) could be fused with mESCs in culture, giving rise to cells that were stem cell–like in terms of morphology and other characteristics (53, 54).

2.5.2. Direct reprogramming of somatic cells into induced pluripotent stem cells. In an unprecedented study that appeared in 2006, Yamanaka's group (56) first described reprogramming of somatic cells into pluripotent cells via exogenous expression of only four transcription factors. Introduction of the reprogramming factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc* into mouse fibroblasts via retroviral infection, and subsequent selection in mESC medium, resulted in establishment of induced pluripotent stem cells (iPSCs). These cells exhibited all characteristic features of ESCs, including endogenous expression of pluripotency markers, reactivation of both X chromosomes in female cells, and (most importantly) the ability to generate chimeric animals, including contributions to the germ line (56).

On the basis of the initial protocol, different combinations of transcription factors, including *Nanog*, *Lin28*, or *Nr5a2*, and small molecules have been used in subsequent reprogramming strategies employing mouse and human cells (57, 58). Technically, the reprogramming process has been advanced via development of inducible (59) virus-free systems (60), whereby all transcription factors are encoded on a single DNA cassette (61) and integrated at a specific site in the genome. This cassette may even be excised after reprogramming, thus generating unmodified iPSCs (62, 63).

The simplicity of reprogramming is remarkable, but the molecular events involved in this process remain only poorly understood (for a review of current models, see Reference 64). Reprogramming of somatic cells is accompanied by extensive remodeling of epigenetic marks, including DNA demethylation of key pluripotency genes, such as *Oct4* and *Nanog*. A fundamental question is whether iPSCs are equivalent to ESCs. The major concern is that genetic or epigenetic aberrations will alter the differentiation and transplantation properties of iPSCs. Eventually, such abnormalities might lead to malignancy if such cells are used in regenerative medicine. Various studies have shown that iPSC clones appear to be indistinguishable from ESCs. Some studies demonstrated that small differences between iPSCs and ESCs are not attributable to distinct expression signatures but rather to experimental variation (65, 66). Furthermore, the notion of epigenetic memory of the donor cell has been raised (67), describing the reminiscence of functionally relevant epigenetic marks of the somatic donor cell after reprogramming. These may confer different properties to the reprogrammed cells under specific circumstances.

3. MOLECULAR CONTROL OF PLURIPOTENCY

In stem cells, transcription factors can be categorized into three core groups (see **Figure 2**): (*a*) those entrenching a position in the developmental hierarchy (factors of the

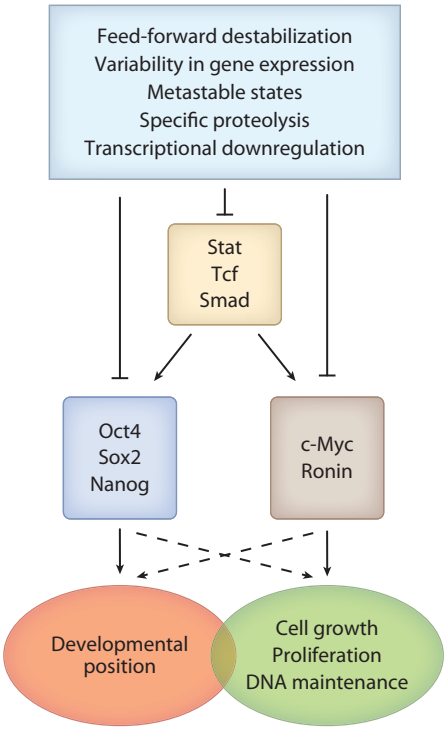

Figure 2

The transcription factor network in pluripotent stem cells. Abbreviations: c-Myc, myelocytomatosis oncogene; Nanog, Nanog homeobox; Oct4, POU domain class 5 transcription factor; Ronin, THAP domain containing 11; Smad, mad homology family; Sox2, SRY-box containing gene 2; Stat, signal transducer and activator of transcription; Tcf, transcription factor.

developmental module); (*b*) those controlling aspects of cell growth and homeostasis (factors of the cell growth module); and (*c*) factors functioning at the interface between complex cell context-specific signaling pathways of the former two modules (those of the cell signaling module). We consider the three genetic modules as separate entities. However, although these modules operate independently to a certain extent, in reality, significant interconnectivity is evident.

3.1. The Developmental Core Module

The best-understood pluripotency factor is Oct4. This protein belongs to the octamer class of transcription factors and is characterized by an ability to recognize the eight-base pair DNA sequence ATGCAAAT (68, 69). Oct4 contains a low-affinity DNA-binding domain (termed POU_S) and a homeotype domain with higher DNA affinity (termed POU_{HD}) (70). Although detailed structural studies on Oct4 have yet to be performed, analysis of Oct1 (Pou2f1) in a complex with target DNA has revealed that two principal configurations exist: Either POU_{HD} alone interacts with DNA, or both POU_S and POU_{HD} do so (71–74). Structural studies of Oct1 in conjunction with another core pluripotency factor of the developmental module, Sox2, have revealed that the latter protein reinforces binding of both forms of Oct1 to DNA (72). This synergy may well explain the peculiar stereotypic configuration found at Oct4 and Oct4/Sox2-recognition sites in the genome.

Oct4 is unique among pluripotency-associated factors in exhibiting an exceptionally confined expression pattern (exclusive to totipotent, pluripotent, and germ cells). Oct4 is also unique because the protein is absolutely required for pluripotency both in vivo (75) and in vitro (76) and for epigenetic reprogramming (64). Upon silencing of Oct4, ESCs spontaneously exit the self-renewal process and differentiate into trophoblast-like cells. This finding was totally unexpected because, in the developing embryo, lineage commitment to the trophoblast line precludes induction of pluripotency (see Section 2.1). It is perhaps even more remarkable that Oct4, in the appropriate molecular context, seems to promote differentiation of ESCs into cells of mesodermal and endodermal lineages (76–78).

Detailed studies have shown that Oct4 interacts with many supplementary transcription factors. Often, these cofactors interact directly with DNA. Specifically, Oct4 appears to interact with Sall4, Hdac2, Sp1, Nanog, Dax1, Nac1, Tcfp2l1, and Essrb (79–81). It is apparent that these various interaction partners do not form a single protein nexus but rather engage (with Oct4) in the formation of multiple complexes differing in composition. This

phenomenon may explain, at least in part, the existence of different pluripotent states (see Section 4.3).

The conspicuous interplay between Oct4 and Sox2 is of particular interest. Sox2 exhibits, to some extent, an expression pattern similar to that of Oct4 (82). However, genetic ablation studies indicate that silencing of *Sox2* affects a somewhat later stage of embryogenesis, possibly because of a stronger maternal contribution of Sox2 protein from the oocyte/zygote (82). Interestingly, although acute loss of *Sox2* mRNA results in unscheduled differentiation of ESCs into trophoblast-like cells, only a very small subset of Sox2 target genes appear to be affected by *Sox2* loss (83). Thus, the key feature of acute *Sox2* loss appears to be an inability to sustain appropriate *Oct4* levels. The observation that forced Oct4 expression can desensitize ESCs to effects to which such cells are normally responsive after Sox2 loss supports this suggestion (83).

The third principal member of the core developmental module is Nanog. This is a homeodomain-containing DNA-binding factor that is not markedly affiliated with any homeobox-containing protein family. Protein expression was originally found to be mandatory for maintenance of pluripotency and, indeed, *Nanog* expression alone is sufficient, under certain circumstances, to perpetuate pluripotency under adverse conditions both in vitro and in vivo (84, 85). Recently, however, functional studies on Nanog have shifted toward exploration of the role played by the protein in inauguration of pluripotency in vitro and in vivo rather than maintenance of the pluripotent state per se (86, 87). As is true of Oct4, Nanog interacts extensively with a number of protein partners including Smad1, Small3, Nr0b1, Nac1, Essrb, Zfp281, Hdac2, and Sp1 (79, 81, 88).

One of the most peculiar facets of the developmental module is that, whereas core pluripotency factors cooperate extensively at the protein level, they also collectively bind to a near-identical repertoire of target genes (**Figure 3**). This has given rise to the hypothesis that the factors form a regulatory circuit. Accordingly, expression of core transcription is self-modulated, and interconnected autoregulatory loops are formed. The core factors firmly control expression of an extensive set of genes required for maintenance of the pluripotent state (89–91). A direct correlation is evident between the number of pluripotency factors bound to each target gene and the level of gene expression. Furthermore, a hierarchical arrangement is apparent. For example, binding of Oct4 increases the likelihood that other factors will be recruited to the same site; such factors include Smad1, Stat3, Dax1, Essrb, and/or Xist (80, 90, 92). Although many genes to which core pluripotency factors bind are transcriptionally stimulated, a particular set of genes, associated with additional cofactors, is repressed by the core developmental module. Such genes are typically involved in lineage-specific regulation, and their expression is essential if a productive physiological differentiation process is to proceed (91, 93–97).

In summary, the core "engine" driving pluripotency consists of a set of highly specialized transcription factors that form a regulatory web. This web is based on extensive interactions and the formation of protein complexes that can vary in stoichiometry and size. Collectively, the core factors transcriptionally control an arsenal of developmental regulators that together

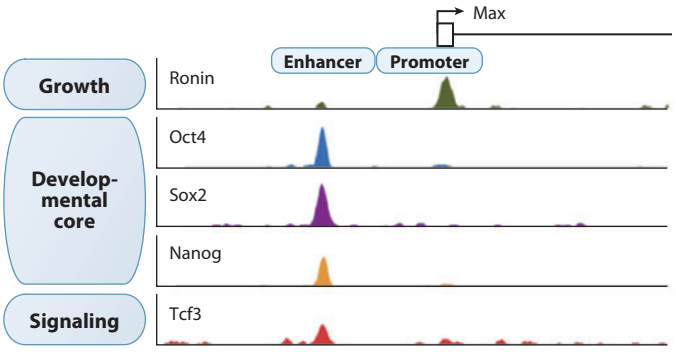

Figure 3

Simultaneous binding of multiple pluripotency-related transcription factors to target genes. Abbreviations: Max, myc-associated factor X; Nanog, Nanog homeobox; Oct4, octamer-binding protein 4; Ronin, THAP domain containing 11; Sox2, SRY-box containing gene 2; Tcf3, transcription factor 3.

regulate development in a lineage hierarchy equivalent to that of peri- or preimplantation epiblast.

3.2. Cell Growth Module

The transcription factor consortium discussed above firmly controls decisions relevant to developmental fate. However, a second independent gene network can be distinguished. This latter network enables pluripotent stem cells to grow and proliferate, see **Figure 2**.

An important member of this second regulatory module is the proto-oncogene c-Myc. It regulates ESC proliferation and is associated with activation of transcription and opening of chromatin. c-Myc controls the transcription of genes of various functional categories, including cellular metabolism, protein production, and cell cycle regulation (98). c-Myc expression enables ESCs to self-renew even under conditions that do not normally favor such activity (99). Genetic ablation of *c-Myc* causes premature exit from self-renewal (100). One role played by c-Myc is inhibition of expression of the prodifferentiation factor *Gata6* (101). Functional overlap with genes of the developmental module is evident (102, 103). At the protein level, c-Myc has been associated with both Tip60 (an acetyltransferase complex) and the chromatin-remodeling complex p400 (102). Although the Myc-controlled gene set supports ESC self-renewal, the genes also control specific aspects of cancer growth (102).

c-Myc expression is regulated by the transcription factor Ronin (Thap11) (104–106); this is an unorthodox zinc finger–like transcription factor (107, 108) that is a member of a small family of proteins carrying an N-terminal THAP (Thanatos-associated protein) domain. Genes encoding THAP domain-containing proteins evolved from the P element transposon, likely via a process termed molecular domestication (109). In contrast to Oct4 or Nanog, Ronin is exceptionally well conserved between mouse and human in terms of both amino acid sequence (over 95% when the polyQ tract is excluded) (108) and DNA target sequence (108, 110). Loss of Ronin expression is associated with cell death, whereas ectopic overexpression of the protein assists the undifferentiated growth of mESCs (108).

Ronin binds to the promoter regions of numerous metabolism-associated genes, and a significant target overlap with genes bound by c-Myc is evident (106). Genes that regulate development are conspicuously absent from the Ronin-binding set. As with c-Myc, *Ronin* expression is not strictly confined to pluripotent cells; an influence of Ronin activity is suspected in other cellular contexts, including cancer development (104, 105, 111–113). Ronin interacts extensively with other proteins, including the transcriptional coregulator Hcf-1. The antidifferentiation property of Ronin (after Lif removal) is entirely dependent on interaction with Hcf-1 (106); expression of the latter protein is regulated in a complex manner, and Hcf-1 can tether both histone deacetylase and histone lysine-4 methyltransferase at chromatin sites (114). This may explain how Ronin affects transcription of target genes. It appears relevant that Hcf-1 requires association with and proteolytic activation by O-linked *N*-acetylglucosamine transferase, which has been connected to cellular metabolic activity (115). Although core developmental factors interact extensively at the protein level, very few such interactions occur between proteins associated with c-Myc and Ronin. This suggests that, although the systems cooperate functionally, different protein classes are targeted.

3.3. Signaling and the Signaling Module

Several key signaling pathways, and the relevant target transcription factors, converge in the developmental core, permitting extrinsic modulation of pluripotency by developmental cues. These pathways directly reinforce self-renewal and/or maintain pluripotency by blocking differentiation cues. As outlined in **Figure 4**, important pathways in pluripotent cells include those involved in Lif/Stat, Bmp, Fgf2, Tgf-β, and Wnt signaling.

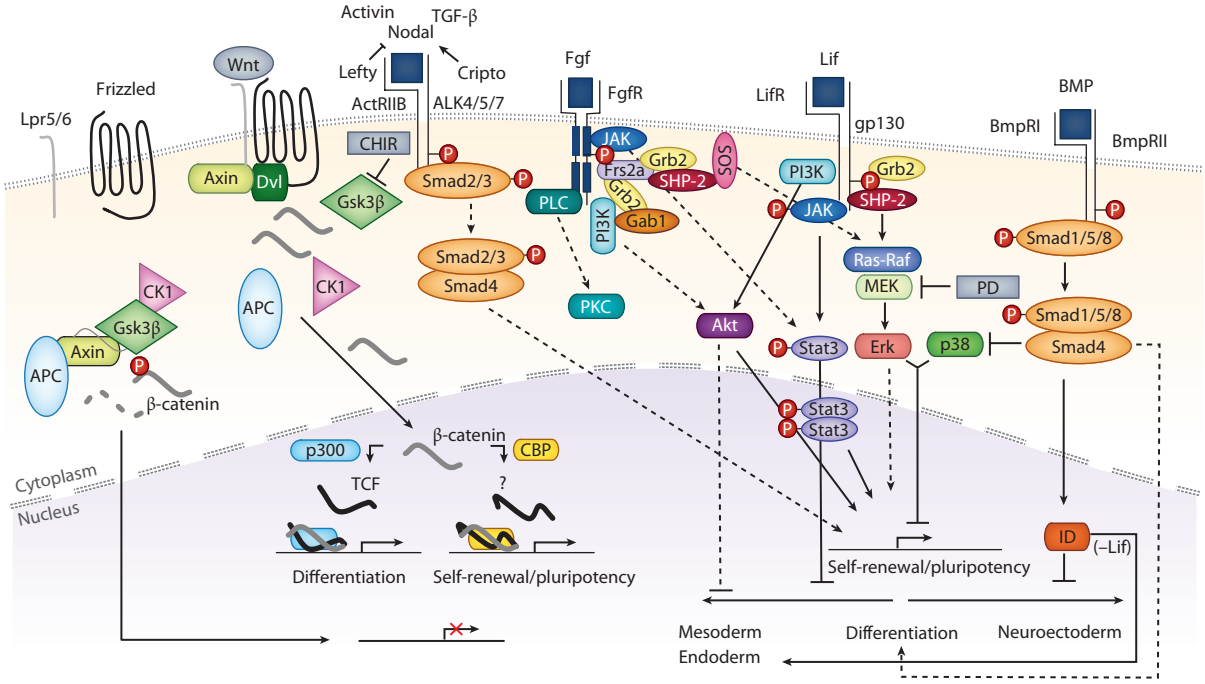

Figure 4

Signaling pathways involved in self-renewal and differentiation of pluripotent stem cells. Black lines and arrows indicate pathways common in primordial and refined pluripotent cells, whereas dashed lines and arrows depict events specific for human embryonic stem cells and cells with refined pluripotency. Abbreviations: ActRIIB, activin receptor type IIB; Akt, thymoma viral proto-oncogene; APC, adenomatosis polyposis coli; ALK4, ALK5, ALK7, activin receptor-like kinase 4, 5, and 7; β-catenin, cadherin-associated protein β1; BMP, bone morphogenic protein; BmpRI and -RII, bone morphogenic protein receptor I and -II; CBP, Creb-binding protein; CHIR, GSK3 inhibitor CHIR99021; CK1, casein kinase 1; Cripto, teratocarcinoma-derived growth factor 1; Dvl, Disheveled protein; Erk, extracellular regulated kinase; Fgf, fibroblast growth factor; Gab1, GRB2-associated binder 1; gp130, glycoprotein 130; Grb2, growth factor receptor-bound protein 2; Gsk3β, glycogen synthase kinase 3β; ID, inhibitor of differentiation; JAK, janus kinase; Lefty, left-right determination factor 1; Lpr5 and -6, low density lipoprotein receptor-related protein 5 and -6; Lif, leukemia inhibitory factor; LifR, leukemia inhibitory factor receptor; MEK, Mapk/Erk kinase; P, phosphorylated site; PD, Erk inhibitor PD184352; PI3K, phosphatidylinositol 3-OH kinase; PKC, protein kinase C; PLC, phosphoinositide phospholipase C; p38, protein kinase 38; p300, histone acetyltransferase p300; Ras-Raf, rat sarcoma-raf proto-oncogene; SHP-2, SH2 domain-containing protein tyrosine phosphatase-2; SOS, son of sevenless homolog; Smad1, -2, -3, -4, -5, and -8, Smad family members; Stat3, signal transducer and activator of transcription 3; TCF, transcription factor; TGF-β, transforming growth factor-β; Wnt, wingless-type MMTV integration site family.

3.3.1. Leukemia inhibitory factor/signal transducer and activator of transcription 3 signaling. Lif performs important functions in the early-stage mouse embryo (116) and can promote self-renewal of mESCs even in the absence of a feeder layer. Although Lif expression does not appear to increase the growth rate of mESCs, it enhances the probability that such cells will undergo self-renewal rather than differentiation (117). Lif is a member of the interleukin-6 family of cytokines, and the Lif-signaling effect is mediated via a heterodimeric complex composed of a specific low-affinitiy Lif receptor (LifR) chain and the gp130 protein. This complex has been shown to activate three pathways: (*a*) the Jak (Janus-associated tyrosine kinase)/Stat3 pathway (118, 119), (*b*) the phosphatidylinositol 3-OH kinase (PI3K)-Akt pathway, and (*c*) the mitogen-activated protein kinase (Mapk) pathway (120). Although the PI3K and Mapk pathways of ESCs are activated via multiple signals, the

Jak/Stat pathway is regulated exclusively by Lif. The Lif receptor complex activates Jak, which in turn phosphorylates specific tyrosine residues in the intracellular domain of gp130. These residues subsequently act as docking sites for proteins containing Src homology 2 (SH2) domains; Stat3 is one such protein. Phosphorylation of Stat3 promotes its dimerization and translocation into the nucleus, wherein the dimer acts as a transcriptional activator of pluripotency-related genes (119).

At the transcriptional level, Lif-mediated signaling via Stat3 and PI3K is integrated into the ESC developmental core module by means of enhanced expression of the transcription factors Klf4 and Tcf3; these proteins in turn activate transcription of *Sox2* and *Nanog*, whereas the Mapk/Erk pathway activity results in diminished amounts of nuclear of Tbx3 and therefore loss of its transcriptional activity (121).

3.3.2. Fibroblast growth factor signaling. FgfR1 is the most abundant receptor of ESCs (39). Importantly, stable interactions between Fgfs and their receptors require the presence of heparin or heparin sulfate. Fgf signaling is activated by ligand-receptor interactions at the cell surface, resulting in autophosphorylation of tyrosine residues in the intracellular region of an FgfR. These altered sites then recruit diverse proteins containing SH2 or phosphotyrosine-binding domains, facilitating assembly of signaling complexes, and in turn allocating signaling to any of four distinct pathways: (*a*) the Jak/Stat pathway, (*b*) the phosphoinositide phospholipase C-γ pathway, (*c*) the PI3K pathway, or (*d*) the Erk pathway (122).

Fgf2 was the first factor identified as being crucial for hESC maintenance, and it is widely accepted that a serum-free culture of hESCs on mouse feeder cells requires soluble Fgf2 if proliferation in an undifferentiated state is to continue (123). The precise mode by which Fgf2 influences cell fate decisions remains to be established.

3.3.3. Tgf-β superfamily. The Tgf-β superfamily contains structurally related signaling proteins, including Tgf-β, Activin, Nodal, growth differentiation factors (GDFs) and Bmps. All family members are important in maintaining pluripotency. Recent data suggest the existence of a subtle balance between the outcomes of Activin/Nodal- and Bmp-mediated signaling in hESCs; GDF3 may function at the intersection of these pathways, driving self-renewal and blocking differentiation (124, 125).

Canonical Bmp signaling is mediated by phosphorylation of Smad1, -5, and -8 by a BmpRI and -II receptor complex, which sequentially bind to Smad4 and mediate translocation of the complex into the nucleus, wherein specific promoter sequences are bound and expression of Bmp target genes results. Such target genes include those encoding the Id proteins (126), which are helix-loop-helix (HLH) proteins that lack the basic DNA-binding domain, resulting in the formation of inactive dimers with bHLH transcription factors. Such proteins have been shown to inhibit neural, but not mesodermal or endodermal, differentiation (127) and can substitute for Bmp to maintain the pluripotency of mESCs. Lif is still required under such circumstances. These findings were surprising beause it is well-established that, in addition to inhibition of neural cell fate via Id activity, Bmp can induce mesodermal differentiation of mESCs in the absence of Lif (128) and plays a conserved role in mesoderm and endoderm patterning during embryogenesis. This suggests that factors downstream of Lif and Bmp must interact to allow maintenance of pluripotency. It has been suggested that these interactions could involve binding of Lif to Smad1 or Stat3 (129), or to the core pluripotency factor Nanog (130). Such binding could negatively regulate expression of Bmp target genes, the transcription of which drives differentiation. Additionally, it has been shown, that Bmp4 can support self-renewal by inhibiting Mapk pathways (131). Interestingly, addition of Bmp to hESC medium can trigger differentiation toward a variety of cell types. Antagonization of Bmp signaling enhances ESC self-renewal and neural differentiation (132, 133).

Nodal and Activin are two distinct growth factors that share the same type I receptors (Alk4 and Alk7) and the same type II receptor (ActRIIB), whereas Tgf-β1 generally employs a different receptor (Alk5) to activate Smad2 and -3 signaling. Although the downstream effectors are common to both pathways, the receptors control distinct biological events and are therefore regulated by binding of numerous cofactors. For example, association with teratocarcinoma-derived growth factor 1 (Cripto) is necessary if Nodal is to activate Alk4/ActRIIb receptors, whereas the left-right determination factor 1 (Lefty) can block Nodal receptor binding (134). An additional layer of complexity is added by the involvement of a large number of Smad partners, including transcription factors (such as Runx1 and Gata3), transcriptional repressors (such as E2f4), a calcium-binding protein (calmodulin), and trafficking proteins (such as Importin) (reviewed in Reference 135). Smad signaling is important in control of pluripotency and influences the transcriptional regulation of key transcription factors of which Nanog appears to be the most responsive. In hESCs, Smad2 and -3 bind to the *Nanog* promoter and activate gene expression, whereas Smad1, -5, and -8 (activated by Bmps) bind to the promoter but rather inhibit *Nanog* expression. Additionally, Smad2 or -3 or Fgf2 signaling suppresses *Bmp4* expression, thus preventing spontaneous differentiation (51).

3.3.4. Wnt signaling. Wnt signaling plays a crucial role during embryonic development. Wnt signaling induces expression of mesodermal and endodermal markers, including *Brachyury*, *Flk1*, *Foxa2*, *Lxh1*, and *Afp* in mESCs; it has additionally been suggested that Wnt signaling probably maintains pluripotency via modulation of *Oct4*, *Sox2*, and *Nanog* expression (136).

The canonical Wnt pathway involves a series of reactions eventually resulting in translocation of β-catenin into the nucleus, to transiently activate target genes that are otherwise repressed by proteins of the Lef/Tcf protein family (137). Cytoplasmic β-catenin is normally phosphorylated at several N-terminal sites (Ser33, Ser37, Ser45, and Thr41) (138), resulting in ubiquitin-mediated proteolysis. Phosphorylation is catalyzed by the so-called destruction complex composed of Axin, Gsk3-β, casein kinase-1 (Ck1), and adenomatous polyposis coli (Apc). Binding of Wnt to the cognate Fz receptor and to the coreceptors low-density lipoprotein receptor-related proteins 5 and 6 (Lrp5 and -6) recruits the protein Dishevelled (Dvl) to the receptor complex. In turn, Dvl binds the scaffold protein Axin and phosphorylated Lrp, as well as Wnt-activated trimeric G proteins participating in degradation of the destruction complex (139). β-catenin remains unphosphorylated and can be translocated into the nucleus to activate its target genes. The protein cannot bind directly to DNA, interacting instead with a number of transcription factors, including Tcf/Lefs, Smads, and nuclear receptors, as well as transcriptional coactivators, such as p300 and Creb-binding protein (CBP) (140). Recent studies suggest that binding to p300 mediates differentiation, whereas formation of the β-catenin/CBP complex maintains the option of mESC self-renewal via interaction with binding partners, including Oct4 and Lrh1, thus activating expression of key pluripotency factors (141, 142). The upstream mechanisms controlling formation of one or the other complex remain under investigation (for a detailed review see Reference 143).

3.4. Transcriptional Control in Pluripotent Stem Cells

The transcriptional circuits considered above are distinguished by the presence of discrete target gene repertoires. In general, the core developmental regulators primarily recruit RNA polymerase II and associated factors and promote transcription initiation. Frequently, however, the RNA polymerase II transcription complex stops on the DNA template shortly after initiation (this is termed stalling or pausing) (144). At least one growth module member (c-Myc) appears, in some instances, to be

capable of overcoming this transcriptional impediment by enlisting specific antipausing factors, such as p-TEFb.

Accordingly, Oct4, Sox2, and Nanog typically bind to canonical enhancer regions of target genes. These DNA sequences are associated with nucleosomes bearing histone marks typically found at developmental enhancers, including H3K27me1 and H3K4me1, but are also associated with the acetyltransferase p300 and the mediator complex (90, 145–148).

The functional difference between Oct4 and analogous transcription factors, on the one hand, and Myc, on the other hand, is further emphasized by the presence of different histone marks and chromatin-associated proteins at the binding sites. In contrast to Oct4/Sox2 and Nanog, Myc gene targets are enriched in H3K4me3 and are almost completely devoid of H3K27me3 (90). Another feature characteristic of members of the developmental module is the presence of "bivalent" chromatin domains (see Section 3.5).

3.5. Pluripotency-Associated Chromatin Structure

Each cell type has a unique chromatin architecture, but several features render pluripotency-associated chromatin fundamentally different from that of any other cellular state (149). ESC's chromatin is thought to be more "open" and "dynamic." In pluripotent cells, heterochromatin-associated foci appear to be more diffusely distributed, and the associated histones are generally hyperacetylated (150).

In pluripotent stem cells, chromatin-modifying enzymes interact with the transcriptional modules, discussed above (those controlling development, growth, and cell signaling), by virtue of their control of genes encoding key enzymes capable of covalently modifying proteins. For example, Oct4 directly controls transcription of the H3K9 methyltransferase SetDB1. When histone marks characteristic of this enzyme are established, Polycomb group proteins are recruited, triggering subsequent events, including methylation of H3K27 residues, further enabling additional chromatin-associated modifications (151). Another example features the K9 demethylases of the Jumonji family. Transcription of *Jmjd1a* (*Kdm2a*) and *Jmjd2c* (*Kdm4b*) is directly controlled by Oct4. Loss-of-function studies have revealed prominent roles for these enzymes in terms of ESC self-renewal (152). Another instance of extensive cross connectivity between the developmental core and chromatin-modifying enzymes involves Jarid1b (Kdm5b), a K4 demethylase primarily targeting regions enriched in H3K36me3. This enzyme promotes cell self-renewal, at least in part, by inhibiting cryptic intragenetic transcription (153).

Finally, the Polycomb protein system plays an essential role in control of cell fate decisions in pluripotent stem cells (154). In general, gene regions occupied by these complexes, or by nucleosomes featuring the H3K27me3 modification, are transcriptionally silent. It has been suggested that many Polycomb group -targeted genes are locked in a "poised" state prior to productive expression (94, 95). In many instances, the K27me3 domain, which can span large regions (more than 100 kilobases) of the genome, is coaligned with smaller H3K4me-enriched domains. This configuration has been termed bivalent and has been proposed to provide provides the molecular basis for developmental gene priming (155). Recently, a Jmj protein (Jarid2) has been shown to be directly associated with the Polycomb complex, although the functional consequences of this linkage are not yet fully resolved (90, 156–158). Polycomb proteins are direct targets of several kinases that mediate cell signaling (159) and are thus intimately connected to cellular signaling processes (see Section 3.3).

The flip side of the "Polycomb coin" is canonical K4 methyltransferase activity. This activity is associated with proteins of the Trx group and therefore is linked to genes that are actively transcribed. Trx proteins have been firmly linked to the effective functioning of pluripotency-specific transcription factors and include Set1a and -b; Mll1-4; the histone mark

"reader" Wdr5; and the associated proteins Ash2, Rbp5, and Dpy-30 (160, 161).

Covalent histone modifications provide relatively "hard-wired" substrates affecting transcriptional control at the epigenetic level. A "softer" (thus more dynamic) higher-level nucleosome reconfiguration also plays a critical role in supporting the transcriptional framework necessary for pluripotency. Of particular relevance in this context is ATP-dependent nucleosome remodeling associated with ESC pluripotency and epigenetic reprogramming (93, 162–164). ESCs express a specialized form of the Swi/Snf-related protein complex, termed esBAF. The pluripotency-associated specificity is derived not only from its unique structure but also from the close associations that exist with the transcriptional networks described above. esBAF binds to many Oct4, Sox2, Nanog, Sall4, and c-Myc target genes (165). Functional studies have shown that overexpression of esBAF components enhances epigenetic reprogramming (164). Yet another critical remodeling factor linked to ESC pluripotency is Chd1, a member of the Snf helicase family. Chd1 can recognize H3K4me2 and H3K4me3 marks via an intrinsic chromodomain and is therefore associated with active genes. Chd1 knockdown causes differentiation, increases the presence of heterochromatin-associated features (including H3K9me3 and Hp1), and reduces the level of the exchange linker H1.

In addition to covalent and noncovalent mechanisms promoting the pluripotency-specific transcriptional program, other chromatin/epigenetic regulatory mechanisms have been characterized. Even though 60%–80% of all CpG sites in the ESC genome are methylated (166), ESC lines can be readily isolated and propagated from embryos deficient in DNA methyltransferase activity (167). However, such cells cannot properly differentiate (168); this has been partly attributed to the inability of the cells to appropriately silence pluripotency-associated factors, such as the genes encoding *Oct4* and *Nanog*, which are typically permanently modified in somatic tissues via DNA methylation. Hence, although loss of DNA methylation capacity can be compensated, or is irrelevant, in terms of ESC propagation, it is detrimental in terms of the ability of ESCs to appropriately differentiate.

Ultimately, one of the best-understood epigenetic phenomena (in the context of pluripotency) is X chromosome inactivation and reactivation. In fact, X chromosome status is frequently used as a marker of change in the cellular state of pluripotent cells because, in most somatic cells, one X chromosome is inactive (169). The activation state of the X chromosome is considered to be directly linked to the core developmental regulatory network because several pluripotency factors, including Oct4, Sox2, and Nanog, bind to a regulatory element in the first exon of *Xist* (92). In particular, reactivation of an inactive chromosome is associated with *Nanog* expression, and blastomeres lacking the *Nanog* gene fail to reactivate an inactive X chromosome (86).

3.6. Noncoding RNA and Pluripotency

An emerging major category of genes controlled by the core module is transcribed into noncoding RNAs. Among these, the best-understood class in terms of function is the family of microRNAs (miRNAs), short noncoding RNAs capable of destabilizing and repressing specific target RNAs. Generally, miRNAs are processed by the enzymes Dicer and Dcgr8, and genetic ablation of the genes encoding these enzymes affects the cell cycle and differentiation of ESCs (170–173). As noted above, when other pluripotency-controlling mechanisms were discussed, the key developmental regulatory core firmly controls expression of the miRNAs found in pluripotent cells. Oct4, Sox2, and Nanog bind directly to the miRNAs mir302 and mir290–295 (96). Conceptually, the dominant idea is that a major function of miRNAs in pluripotent stem cells involves control of the cell cycle.

Another very important member of the mRNA family is let7, which targets some pluripotency-associated genes (174, 175). This in turn provides the basis for establishment

of negative feedback loops in which let7 expression is negatively regulated by the RNA-binding protein Lin28. Upon differentiation of pluripotent cells, Lin28 is downregulated, resulting in stabilization of and an increase in the level of let7, which has differentiation-promoting activities itself (probably partly via downregulation of Myc) (176). In this context, it is relevant to note that mir290–295 enhances reprogramming in a Myc-dependent fashion (177, 178).

It has been reported that the mir302/367 cluster alone is capable of reprogramming somatic cells to the pluripotent state, possibly via targeting of specific epigenetic factors, such as Aof1, Aof2, Mecp1, and Mecp2 (179–181). This finding, if confirmed, indicates that the pluripotent state is fundamentally distinct from other cellular states in the sense that pluripotency is the common cellular form into which virtually all cell types convert. Recently, Oct4 has been shown to control and activate the expression of specific large intergenic noncoding RNAs (lincRNAs). Importantly, in some instances, knockdown of the expression of such RNAs caused growth defects and increased the level of apoptosis (182); some lincRNAs have been implicated in self-renewal and reprogramming (182, 183).

4. CONTROLLED SHUTDOWN OF PLURIPOTENCY DURING DIFFERENTIATION

Given that the pluripotent state is well-protected from the actions of differentiation stimuli via use of a series of autoregulatory feed-forward and feedback loops, it is logical to ask how robustly self-renewing pluripotent cells can eschew self-renewal.

4.1. Feed-Forward Generated Destabilization Signals

Although developmental regulators such as Oct4 and Sox2 firmly reinforce maintenance of the pluripotent state, by blocking expression of core developmental regulators of hypoblast and trophoblast differentiation, Oct4 and Sox2 also activate a set of genes that, paradoxically, promote differentiation and hence destabilize self-renewal. The first such factor characterized was the growth factor Fgf4. Oct4 and Sox2 transcriptionally activate Fgf4 (184). Fgf4 is a forceful activator of the Erk-signaling pathway, which promotes differentiation in cell types exhibiting primordial pluripotency (see Section 3.3). Hence, ESCs create, when self-renewing, an environment destructive of pluripotency via autoactivation of Erk signaling. However, elevation of Erk activity does not necessarily induce differentiation but renders cells more susceptible to the actions of additional differentiation-promoting signals (78). Indeed, the situation is even more complex. Oct4 activates not only Fgf4 signaling, but also triggers expression of a genetic program responsible for mesendoderm differentiation, whereas Sox2 inhibits the pathway (77). Accordingly, quantitative differences in stoichiometry, posttranslational modifications, and other means permit substates of pluripotency, some of which more prone to differentiation.

4.2. Variability in the Expression of Pluripotency Factors

Examination of a cross section of cells in ES culture at any given moment reveals a broad spectrum of Nanog expression among individual ESCs. This heterogeneity affects the propensity of ESCs to follow particular developmental pathways; the ESCs expressing *Nanog* at the highest level are relatively protected from the effects of differentiation stimuli and Erk signaling, but cells expressing low levels of or no Nanog exhibit a greatly increased tendency toward lineage commitment (86).

Thus, Nanog insulates ESCs from prodifferentiation signals, in particular those caused by Erk signaling. Nanog fluctuation provides an example in which heterogeneity in ESC culture defines subsets of cells that are more prone to differentiation stimuli. This could open a window of opportunity whereby such cells could exit the self-renewal mode. The mechanisms

underlying this fluctuation are currently unknown but may involve gene oscillation and/or stochastic (noisy) genetic expression (185).

4.3. Metastable States in Stem Cell Culture

4.3.1. Intrinsic factors that can stimulate transitions. Although Nanog expression is probably the best-characterized example of transcription factor heterogeneity in tissue culture, in line with phenotypic differences in stem cell behavior, other transcription factors exhibiting apparently similar behavior have been described (47, 186). For example, Stella expression can be used to divide the stem cell population into two subpopulations: Stella-positive cells that more closely resemble cells of the ICM, and Stella-negative cells that are more similar to epiblast cells. Conceptually, the underlying idea is that several separate transcriptional states exist. These individual states are frequently described as metastable states and would mathematically most closely resemble "attractor" states.

Although the idea that stable distinct states exist is attractive, it is equally possible that cell states vary over a much wider spectrum, with no cell permanently locked into any given state (187, 188). Finally, it is possible, although speculative, that the intrinsic heterogeneity of pluripotent stem cells, and the observed interconvertability among states, allow establishment of a lineage hierarchy within which only a small subset of cells is truly pluripotent. These cells spin off derivatives, all of which are to some extent compromised in terms of developmental and differentiation potential.

4.3.2. Extrinsic factors that stimulate transitions. If culture conditions are varied, or if the expression levels of transcription factors are changed, cellular properties diverge, leading to conversion of one pluripotent cell type into another. For example, ESCs can change into EpiSCs in the presence of Fgf2 and Activin. This process is accompanied by silencing of one X chromosome in female cells. The reverse conversion, including reactivation of one X chromosome, has been accomplished via artificial expression of Klf4 (46) and upon culture with Lif, Bmp4, and "2i" (a cocktail of small-molecule inhibitors targeting Erk and Gsk) in the presence of a feeder layer (189). Furthermore, EpiSCs can be converted into EGCs via PGC intermediates upon culture with Bmp4, followed by addition of Noggin, Chordin, Activin, and Fgf2. Once PGCs become established, addition of Lif, FCS, and Fgf2 triggers development of EGCs (47). Additionally, ESCs can give rise to TS cells upon conditional deletion of Oct4 or upon forced expression of *Cdx2* when cultured in MEF-conditioned medium in the presence of Fgf4 (190).

4.4. Rapid Proteolytic Removal of Pluripotency Factors

The pluripotency transcription factor network is a complex genetic system that includes network hubs, that is, proteins that interact with many other proteins. Oct4, Nanog, and Myc serve as such hubs. In general, cells are very sensitive to changes in the levels of these three proteins. A very effective approach toward a change in cellular state is obstruction of protein hub action. This can be achieved, in part, via site-specific proteolysis catalyzed by caspases. These enzymes are typically discussed in the context of programmed cell death (apoptosis) (191) but have recently been investigated in a different context, namely cell specialization (192, 193). In particular, caspase-3, a key "executor" caspase, is involved in cell fate decisions by specifically cleaving Nanog (194). Nanog depletion reduces self-renewal capacity and propels ESCs toward differentiation. In line with this notion, genetic ablation or blocking of caspase activity causes a significant delay in differentiation. The effect is tissue culture specific as caspase-3 knockout embryos survive beyond the interval during which pluripotent cells are present (195), likely owing to compensatory mechanisms (196). It is also likely that other critical transcription factors at hubs of

the pluripotency network are recognized and cleaved by caspases; these factors may include Ronin, a recently characterized member of the growth module (108). Finally, caspase activity seems not only to promote differentiation but also epigenetic reprogramming (197) by depleting the retinoblastoma (Rb) protein and thus promoting transition toward an ESC-like state.

4.5. Transcriptionally Mediated Downregulation of Pluripotency Factors

Direct downregulation of transcription factor synthesis is probably as important as the above-mentioned mechanisms in terms of deconstructing the pluripotent state. One critical factor destabilizing pluripotency is Gcnf (198), which binds directly to the Oct4 promoter and subsequently "locks" pluripotent stem cells into a differentiated state via recruitment of newly expressed DNA methyltransferases (199). Another enzyme that may silence Oct4 expression (either transiently after initiation of differentiation or more permanently later) is the histone H3K9 methyltransferase G9a (200). Similar chromatin-modifying activities are likely to act in other pluripotency-associated gene regions. Furthermore, direct binding of certain transcriptional repressors (including ARP-1/COUP-TFI and EAR3-COUP-TFII) to the Oct4 promoter has been reported (199). In general, it appears that some transcription factors, including Tcf3, antagonize the action of the core developmental module; Tcf3 and related proteins can rapidly shut down the pluripotency network (200).

5. CONCLUDING REMARKS

Although much information is available on the molecular regulation of pluripotency, a unifying view is lacking. At present we cannot with any degree of certainty predict whether a particular cell is totipotent, pluripotent, or multipotent, or whether it can contribute to the germ line. Similarly, we do not understand the major differences that certainly exist between pluripotent stem cells in vitro and in vivo. Only when this is possible will we be able to state that a major advance toward an understanding of pluripotency and nuclear reprogramming has been made.

SUMMARY POINTS

1. Pluripotency is acquired by a subset of cells during early embryonic development and is distinct from the totipotent state of the zygote and blastomeres. Totipotent cells can differentiate into all cell types of the embryo and all extraembryonic lineages, whereas pluripotent cells cannot contribute to the development of extraembryonic tissue.
2. Pluripotent stem cells are characterized by the ability to self-renew and to differentiate into any cell type of the developing embryo. They have been trapped in vitro, at various stages of embryonic development, and from postnatal and adult tissues.
3. Primordial and refined pluripotency are apparent during embryonic development, and in different forms of pluripotent stem cells in culture. Primordial pluripotency, equivalent to that exhibited by the preimplantation epiblast, is characteristic of cells that can differentiate into any embryonic cell lineage (including the germ line). Cells harboring a refined pluripotency, equivalent to that of the late epiblast, can differentiate into cells of all germ layers in teratoma formation assays but cannot contribute to chimeric animals, and therefore have restricted developmental potential.

4. Important pathways in pluripotent cells include the Lif/Stat, Bmp, Fgf2-, Tgfβ-, and Wnt-signaling cascades. It is not surprising that the activities of such pathways are also critical during early-stage embryogenesis.
5. The core factors transcriptionally control an arsenal of developmental regulators that together regulate development in a lineage hierarchy equivalent to that of a pre- or peri-implantation epiblast.
6. The cell growth network enables pluripotent stem cells to grow and proliferate. This function is highly adaptive and serves the very specific context-dependent needs of pluripotent stem cells.
7. Pluripotent stem cells have a unique chromatin architecture, differing fundamentally from that of any other cell type. ESC chromatin is thought to be more open and dynamic.
8. Key signaling pathways, and the relevant DNA-targeting transcription factors, converge in the developmental core, permitting extrinsic modulation by developmental cues.
9. Direct downregulation of transcription factor synthesis and protein degradation are probably the most important mechanisms operative in terms of deconstructing the pluripotent state.

FUTURE ISSUES

1. What is the true origin of mouse embryonic stem cells?
2. How can hESCs with primordial pluripotency be isolated?
3. What are the controls mediated by all transcriptional and epigenetic modules in pluripotent stem cells?
4. How is the pluripotent state deconstructed?

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