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How is pluripotency determined and maintained?

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Mouse embryonic stem (ES) cells are pluripotent, as they have the ability to differentiate into the various cell types of a vertebrate embryo. Pluripotency is a property of the inner cell mass (ICM), from which mouse ES cells are derived, and of the epiblast of the blastocyst. Recent extensive molecular studies of mouse ES cells have revealed the unique molecular mechanisms that govern pluripotency. These studies show that ES cells continue to self-renew because of a self-organizing network of transcription factors that prevents their differentiation and promotes their proliferation, and because of epigenetic processes that might be under the control of the pluripotent transcription factor network.

Introduction

Mouse embryonic stem (ES) cells, and the cells of the embryonic inner cell mass (ICM) from which mouse ES cells are derived, are pluripotent. According to recent consensus, pluripotency describes a cell's ability to give rise to all of the cells of an embryo and adult (Solter, 2006). Studies over the past few years have revealed the role that transcription factor networks and epigenetic processes play in the maintenance of ES cell pluripotency (Niwa et al., 2000; Mitsui et al., 2003; Chambers et al., 2003; Boyer et al., 2005; Niwa et al., 2005; Boyer et al., 2006). Among the findings to have emerged from these studies is that the functions of these transcription factors depend on the stage of development of a pluripotent cell, indicating that these factors function in combination with other processes (Sieweke and Graf, 1998). The activity of these transcription factors also depends on the accessibility of their target genes, which are made more or less accessible by the modification of their DNA, histones, or chromatin structure (Jaenisch and Bird, 2003). In this review, I discuss new insights into how transcription factor networks maintain mouse ES cell pluripotency and how these factors interface with epigenetic processes to control the pluripotency and differentiation of mouse ES cells.

An overview of mouse ES cell derivation, proliferation and differentiation

Pluripotent embryonic lineages and ES cell derivation

Mouse ES cells are derived mainly from the ICM of the mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981) (see Fig. 1). As the embryo develops, the ICM gives rise to two distinct cell lineages: the extraembryonic endoderm, which goes on to form the extraembryonic tissues; and the epiblast, which gives rise to the primitive ectoderm at the egg-cylinder stage of embryogenesis, from which the embryo proper arises. The primitive ectoderm is distinct from the ICM in several ways. It cannot give rise to the trophectoderm, nor to the primitive endoderm (see Fig. 1); it also has an epithelial morphology distinct from that of the ICM (Gardner and Rossant, 1979). Importantly, the primitive ectoderm is the only cell lineage in which pluripotency is maintained at this stage of development, enabling it to give rise to all three embryonic germ layers and to primordial germ cells (see Fig. 1). However, as it lacks the ability to differentiate into the extraembryonic, primitive endodermal and trophectodermal lineages, the primitive ectoderm is less pluripotent than the cells of the ICM and possesses 'restricted' pluripotency.

Traditionally, pluripotency has often been defined as the ability to generate all cell types of an embryo apart from the trophectoderm (the precursor to the bulk of the embryonic part of the placenta) (Bioani and Schöler, 2006). This is because an earlier analysis of chimeric mouse embryos, produced by the injection of ICM cells and ES cells into 8-cell embryos or blastocysts, had shown that ICM cells are excluded from the trophectoderm lineage (Beddington and Robertson, 1989). However, it has subsequently been found that the ICM does still possess the ability to differentiate into the trophectoderm lineage (Pierce et al., 1988), as do ES cells under particular culture conditions (Niwa et al., 2005). Therefore, in this review, I define pluripotency as the ability to generate all cell types, including the trophectoderm, without the self-organizing ability to generate a whole organism [see also Solter (Solter, 2006) for similar definitions of these terms].

ES cell proliferation

Pluripotency is maintained during ES cell self-renewal through the prevention of differentiation and the promotion of proliferation. In fact, ES cells can self-renew continuously for years if they are cultured under conditions that prevent their differentiation; for example, in the presence of leukemia inhibitory factor (Lif), a growth factor that is necessary for maintaining mouse ES cells in a proliferative, undifferentiated state (Suda et al., 1987). But how is pluripotency itself protected via self-renewal at the molecular level? This question is discussed in more detail below.

ES cell differentiation

Although ES cells are described as being pluripotent, they can only differentiate directly into three cell types: the primitive ectoderm, the primitive endoderm and trophectoderm cells, analogous to the differentiation ability of cells of the ICM.

The differentiation of mouse ES cells can be induced by the ectopic expression of certain transcription factors. For example, the expression of the transcription factor Gata6 in ES cells results in their differentiation into primitive endoderm (Fujikura et al., 2002). Likewise, the expression of the caudal-type homeobox transcription factor 2 (Cdx2) induces ES cells to differentiate into trophectoderm (Niwa et al., 2005). Therefore, both of these factors have to be tightly repressed for ES cells to self-renew, as discussed in more detail below.

Self-renewal by preventing differentiation

As mentioned above, ES cell pluripotency is maintained during self-renewal by the prevention of differentiation and the promotion of proliferation. For mouse ES cells, Lif is a key factor that prevents

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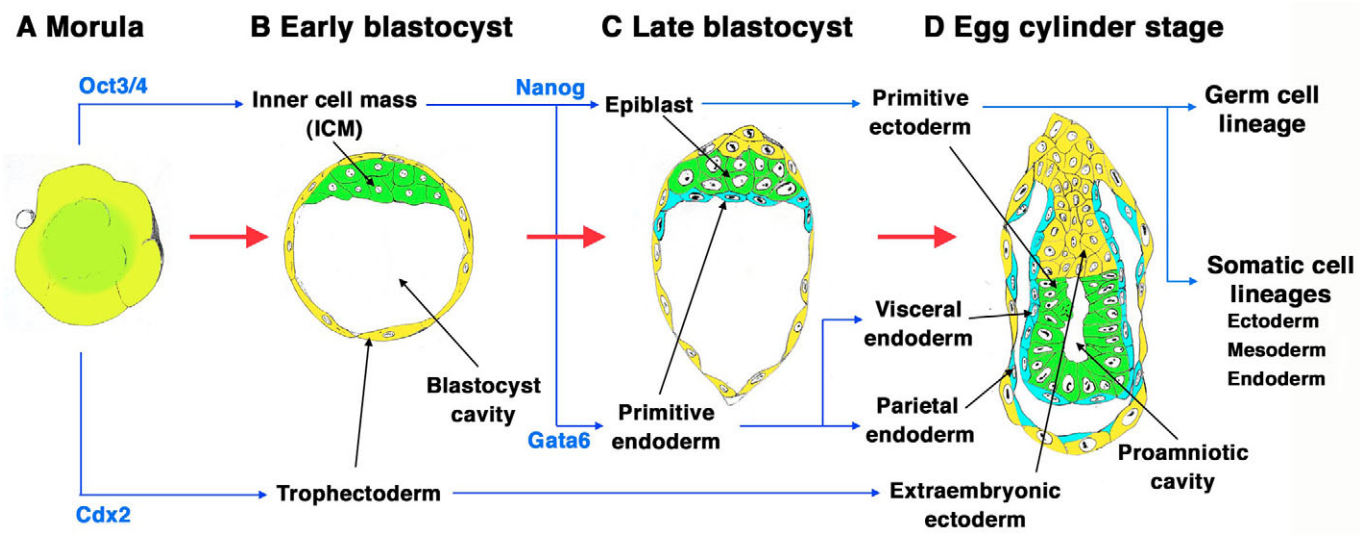

Fig. 1. Pluripotent lineages in the mouse embryo. A schematic view of mouse preimplantation development. (**A**) Pluripotent stem cells (green) are imaged in a morula as the inner cells, which (**B**) then form the inner cell mass (ICM) of the blastocyst. (**C**) After giving rise to the primitive endoderm on the surface of the ICM, pluripotent stem cells then form the epiblast and start to proliferate rapidly after implantation. (**D**) They then form the primitive ectoderm, a monolayer epithelium that has restricted pluripotency which goes on to give rise to the germ cell lineage and to the somatic lineages of the embryo. Certain key transcription factors (blue) are required for the differentiation of the various embryonic lineages.

differentiation. Lif belongs to the interleukin-6 cytokine family and binds to a heterodimeric receptor consisting of the Lif-receptor β and gp130 (Il6st – Mouse Genome Informatics). This binding results in the activation of the canonical Jak/Stat (Janus kinase signal transducer and activator of transcription) pathway. It has been reported that Stat3 activation is essential and sufficient to maintain the pluripotency of mouse ES cells (Niwa et al., 1998; Matsuda et al., 1999), and that c-Myc is a candidate target of Stat3 (Cartwright et al., 2005).

The POU family transcription factor Oct3/4, which is encoded by *Pou5f1*, is also a pivotal regulator of pluripotency (Nichols et al., 1998) that acts as a gatekeeper to prevent ES cell differentiation. Artificial repression of Oct3/4 in ES cells induces differentiation along the trophectodermal lineage; when overexpressed, ES cells differentiate mainly into primitive endoderm-like cells (see Fig. 2B) (Niwa et al., 2000).

Oct3/4 has been reported to directly prevent differentiation towards trophectoderm by interacting with Cdx2 (a trigger for trophectoderm differentiation; see Fig. 2D,E), to form a repressor complex. This complex interferes with the autoregulation of these two factors, giving rise to a reciprocal inhibition system that establishes their mutually exclusive expression (Niwa et al., 2005). As such, the downregulation of Oct3/4 results in an upregulation of Cdx2, and vice versa – a mechanism that might account for the two different pathways that lead to pluripotent stem cells and to trophectoderm cells.

Both the inhibition of Stat3 activity and the overexpression of Oct3/4 stimulate ES cells to differentiate into primitive endoderm-like cells (Fig. 2B) (Niwa et al., 1998; Niwa et al., 2000). The existence has been suggested of an unidentified co-factor of Oct3/4 that is activated by Stat3 (Niwa, 2001). The normal functions of this co-factor could be disrupted by an excess of Oct3/4, which might disrupt the functions of a ternary complex (consisting of Oct3/4, its co-factor and a general transcription unit, which activates target genes) via the saturation of protein interactions. This is supported by evidence that this 'overdose effect' of Oct3/4 on ES cell differentiation does not require Oct3/4 DNA-binding activity (Niwa et al., 2002). In such a model, the target gene(s) of this particular complex would normally prevent ES cells from differentiating into primitive endoderm by repressing the trigger factor, Gata6. Nanog is an NK-2 class homeobox transcription factor that is expressed throughout the pluripotent cells of the ICM. As overexpression of Nanog in mouse ES cells can maintain them in a pluripotent state in the absence of Lif, it is a good candidate for this hypothetical Gata6 repressor (Chambers et al., 2003; Mitsui et al., 2003). Indeed, *Nanog*-null ES cells differentiate into Gata6-positive parietal endoderm-like cells, which have a morphology that is similar to that of Gata6-induced cells (Fig. 2) (Mitsui et al., 2003). However, although it has been reported that *Nanog* expression is partly regulated by Oct3/4 and Sox2, a member of the Sox (SRY-related HMG box) family (Kuroda et al., 2005; Rodda et al., 2005), and although artificial *Nanog* expression can block the differentiation of ES cells into primitive endoderm cells [induced by either the withdrawal of Lif (Chambers et al., 2003) or the formation of embryoid bodies (EBs: ball-like structures that form when ES cells are kept in suspension culture and which mimic the egg-cylinder stage of embryogenesis] (Hamazaki et al., 2004), no direct evidence for the repression of *Gata6* by Nanog has yet been found.

The gatekeeper function of Nanog might not be restricted to preventing the differentiation of ES cells into primitive endoderm, as it has been reported that Nanog also blocks neuronal differentiation induced by the removal of Lif and bone morphogenetic protein (BMP) from serum-free culture (Ying et al., 2003). In addition, Nanog can also reverse mesoderm specification by repressing brachyury, which encodes the mesoderm-specific T-box transcription factor T. This factor directly activates *Nanog* expression, indicating that negative feedback is involved in the balance between self-renewal and mesodermal differentiation (Suzuki et al., 2006a). Thus, Nanog can block primitive endodermal differentiation, neuronal differentiation and mesodermal differentiation under different culture conditions

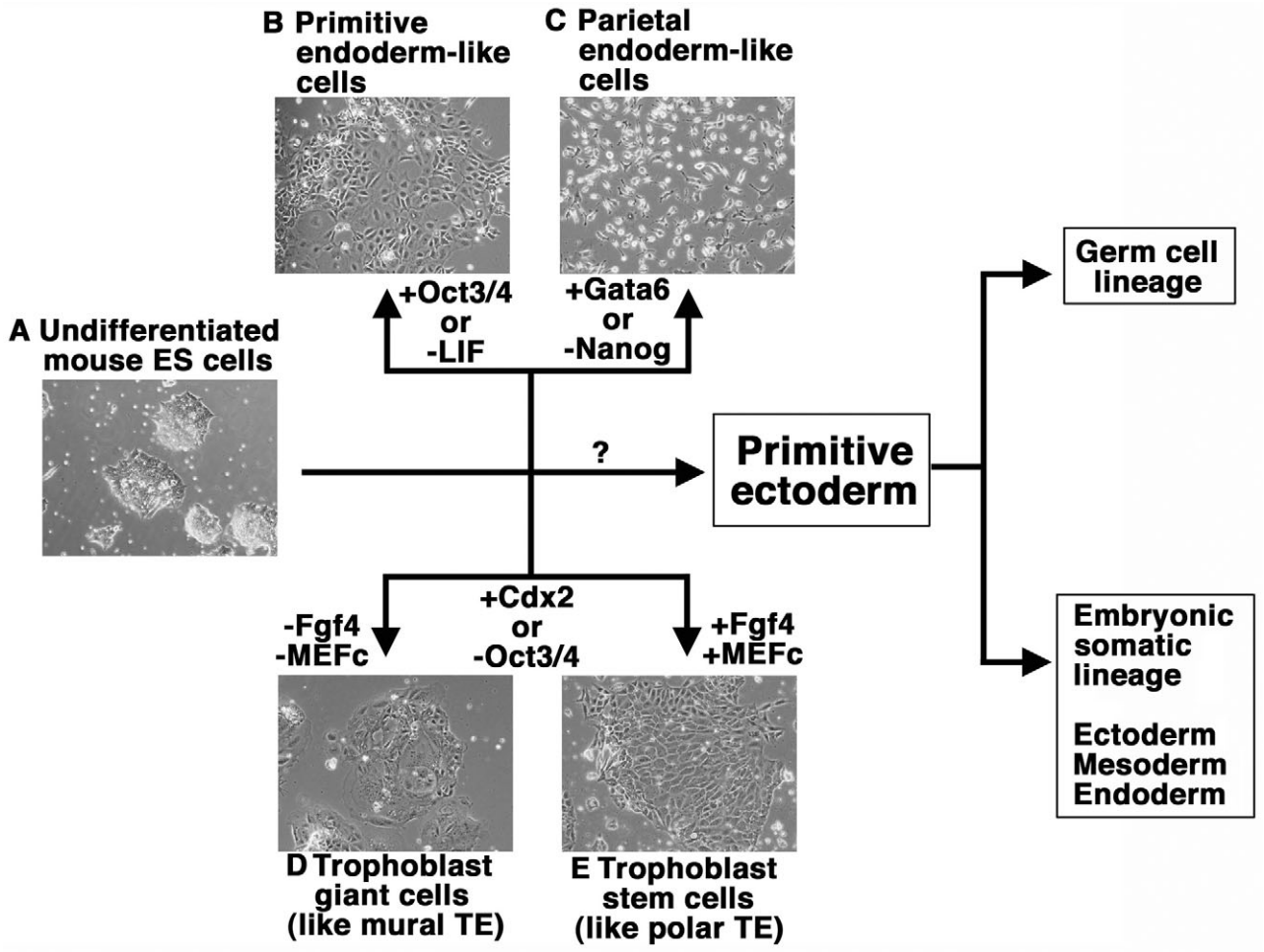

Fig. 2. Differentiation of mouse ES cells. (**A**) Mouse ES cells differentiate into three cell types – primitive endoderm, trophectoderm (TE) and primitive ectoderm – mimicking the differentiation potential of pluripotent stem cells in preimplantation embryos. (**B-E**) Different culture conditions can induce ES cells to differentiate into certain lineages. (B) In the absence of Lif and in the presence of an excess of Oct3/4, ES cells differentiate into primitive endoderm-like cells, whereas (C) in the absence of Nanog and in the presence of Gata6, they differentiate into parietal endoderm-like cells. (D,E) Removing Oct3/4 from, and adding Cdx2 to, ES cell culture induces TE-like differentiation. MEFc, mouse embryonic fibroblast conditioned medium.

Promoting self-renewal through proliferation

Under optimized culture conditions, in which Lif is essential (Smith et al., 1988), mouse ES cells divide symmetrically every 12 hours. During self-renewal, most ES cells are in the S phase of the cell cycle, with only a few in G1 (Burdon et al., 2002).

Recent findings suggest that the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway plays a pivotal role in promoting the proliferation, survival and/or differentiation of mouse ES cells (see Fig. 3). The deletion of *Pten*, which encodes a negative regulator of PI3K, in mouse ES cells has been reported to increase ES cell viability and proliferation (Sun et al., 1999), and it has recently been reported that the artificial activation of Akt is sufficient to maintain ES cell self-renewal in the absence of Lif (Watanabe et al., 2006).

Two modulators of the PI3K/Akt pathway are specifically expressed in ES cells, *Eras* and *Tcl1* (Fig. 3) (Takahashi et al., 2005). *Eras* encodes a constitutively active form of a Ras-family small GTPase that activates PI3K to stimulate ES cell proliferation and tumorigenicity after ectopic transplantation in vivo (Takahashi et al., 2003). The *Tcl1* gene product augments Akt activation by forming a stable heterodimeric complex with Akt (Teitell, 2005). Knockdown of *Tcl1* in mouse ES cells impairs self-renewal by inducing differentiation and/or repressing their proliferation (Ivanova et al., 2006; Matoba et al., 2006). However, the molecular mechanisms that direct the expression of *Eras* and *Tcl1* in ES cells have yet to be identified.

The transcription factor b-Myb has been reported to be an accelerator of cell-cycle progression in mouse ES cells. Overexpression of a dominant-negative form of b-Myb in these cells results in G1 arrest (Iwai et al., 2001), indicating that *b-Myb* is transcriptionally activated in G1 and promotes the transition to S phase by a complex mechanism (Joaquin and Watson, 2003). Moreover, *b-Myb*-null blastocysts show defective ICM outgrowth in vitro (Tanaka et al., 1999), suggesting that b-Myb might play an important role in promoting the cell cycle in ES cells. However, neither the transcriptional regulation of b-Myb nor its precise function in regulating the cell cycle in mouse ES cells have yet been analyzed.

The basic helix-loop-helix transcription factor Myc is a well-known accelerator of the cell cycle, acting via the transcriptional activation of cyclin E expression to promote G1-S transition (Hooker and Hurlin, 2006). Recently, Cartwright et al. (Cartwright et al., 2005) reported that *c-Myc* is a direct target of Stat3, and that overexpression of a dominant-active form of c-Myc that has a

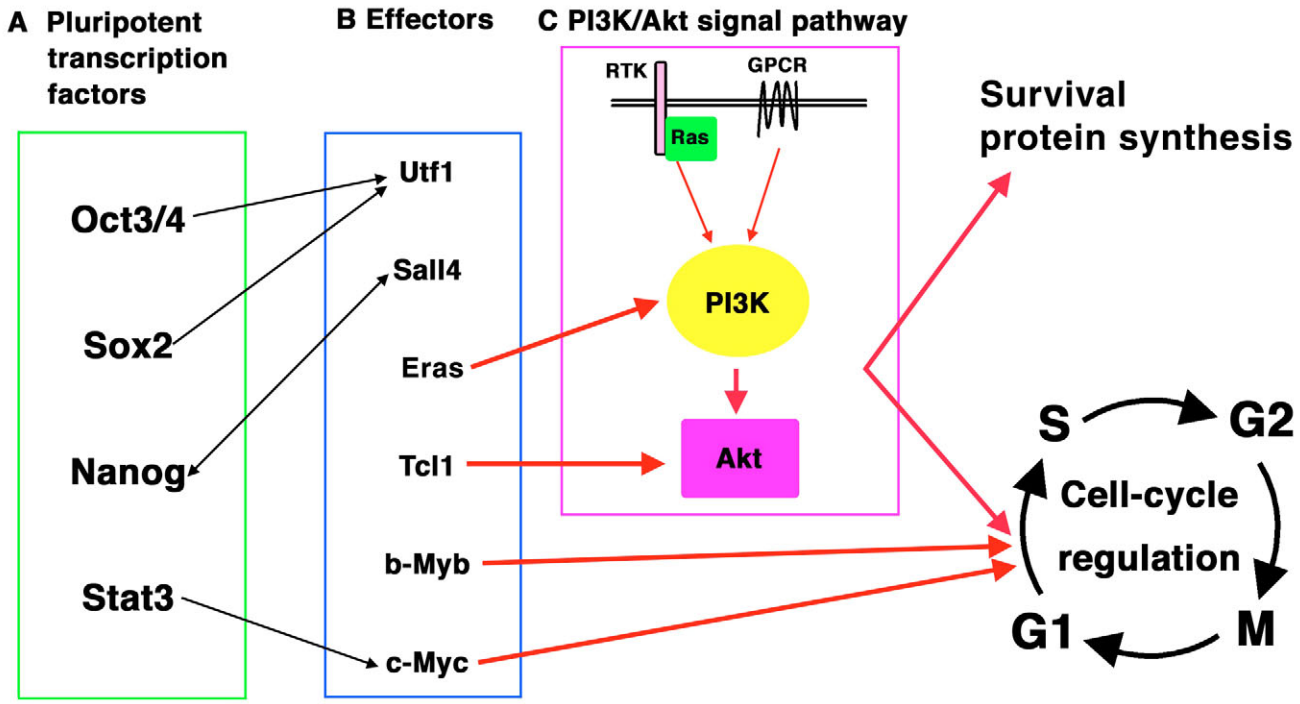

Fig. 3. Regulation of proliferation of mouse ES cells. (**A**) Pluripotent transcription factors activate the expression of (**B**) certain effectors that drive ES cell proliferation. Among these, Eras and Tcl1 stimulate the (**C**) phosphoinositide-3-kinase (PI3K)/Akt signaling pathway to promote the cell cycle, whereas b-Myb and c-Myc activate the progression of the cell cycle directly. How Utf1 and Sall4 affect ES cell proliferation remains unknown.

greater stability than the wild-type protein renders the self-renewal of mouse ES cells independent of Lif. By contrast, the overexpression of a dominant-negative form of c-Myc antagonizes mouse ES cell self-renewal and promotes differentiation. These findings suggest that the regulation of the G1-S transition may contribute to the maintenance of pluripotency, which is promoted by the Lif-Stat3 pathway in mouse ES cells (Burdon et al., 2002).

Undifferentiated embryonic cell transcription factor 1 (Utf1) was first identified as a transcriptional co-factor that is expressed in mouse ES cells in a stem-cell-specific manner (Okuda et al., 1998). Mouse ES cells with reduced expression of Utf1 show reduced proliferation in vitro and reduced tumorigenicity in vivo (Nishimoto et al., 2005). Utf1 possesses a stem-cell-specific enhancer that is activated by Oct3/4 and Sox2 (Nishimoto et al., 1999), so it can be regarded as a link between the pluripotent transcription factor network and the promotion of proliferation.

Mouse ES cells that lack *Sall4*, one of the mouse homologs of the *Drosophila* homeotic gene *spalt* that encodes a zinc-finger transcription factor, were recently reported to show reduced proliferation ability (Sakaki-Yumoto et al., 2006). Another study showed that Sall4 interacts with Nanog to activate *Sall4* and *Nanog* (Wu et al., 2006). However, *Sall4* expression is not restricted to mouse ES cells, and Nanog is still expressed in *Sall4*-null ES cells (Sakaki-Yumoto et al., 2006), so the physiological contribution of this positive-feedback loop to the maintenance of pluripotency remains to be confirmed.

Mechanisms to maintain self-renewal

In order to maintain the stable self-renewal of ES cells, the mechanisms that prevent their differentiation and promote their proliferation must be transmitted to their daughter cells. Thus, the expression levels of the genes that are involved in these mechanisms need to be stably maintained.

A transcription factor network that is stabilized by positive and negative regulation between its components is a good mechanism for maintaining the stable gene expression patterns that determine a particular cell phenotype (von Dassow et al., 2000). Moreover, the application of systems biological views, such as the Boolean network models, allows us to explain how small changes to a few components of a network can trigger the dynamic transition of a transcription factor network from one state to another (Kauffman, 2004). Random Boolean network models are a way of modeling networks that are composed of multiple factors which have multiple inputs in complex systems. They are based on Boolean logic, in which multiple logical operators, such as AND and OR, are united into expressions about the factor with binary values such as 1 and 0 (Kauffman, 2004).

Sox2 occupies an important position in the maintenance of the pluripotent transcription factor network (Fig. 4B). As discussed above, Sox2 is known to co-operate with Oct3/4 in activating Oct3/4 target genes (Yuan et al., 1995). To date, ES-specific enhancers that contain binding sites for Oct3/4 and Sox2 have been identified in several genes, including *Fgf4* (Yuan et al., 1995), osteopontin (*Spp1* – Mouse Genome Informatics) (Botquin et al., 1998), *Utf1* (Nishimoto et al., 1999), *Fbxo15* (Tokuzawa et al., 2003), *Nanog* (Kuroda et al., 2005; Rodda et al., 2005) and *Lefty1* (Nakatake et al., 2006). Interestingly, both *Oct3/4* and *Sox2* possess enhancers that are activated by the Oct3/4-Sox2 complex in a stem-cell-specific manner (Chew et al., 2005; Okumura-Nakanishi et al., 2005; Tomioka et al., 2002). *Sox2*-null embryos die immediately after implantation (Avilion et al., 2003), and knockdown of *Sox2* in mouse ES cells induces differentiation into multiple lineages, including trophectoderm, indicating its functional importance in the maintenance of pluripotency (Ivanova et al., 2006). The generation of *Sox2*-null ES cells would

help to elucidate the precise function of Sox2 and the identification of its target genes, as would also be the case for Oct3/4.

The identification of common target sites in the regulatory elements of *Oct3/4*, *Sox2* and *Nanog* by recent studies using chromatin immunoprecipitation (ChIP) together with genome-wide location techniques has suggested that *Oct3/4*, *Sox2* and *Nanog* might form a regulatory feedback circuit that maintains pluripotency in human and mouse ES cells; in this circuit, all three transcription factors regulate themselves, as well as each other (Boyer et al., 2005; Loh et al., 2006). Although this feedback model has not been confirmed in ES cells, a positive-feedback loop alone would be incapable of allowing the transcription factor network to maintain pluripotency because pluripotency is extremely sensitive to the expression levels of *Oct3/4* (Niwa et al., 2000).

Since even a slight overdose of Oct3/4 triggers differentiation, the network requires a negative-feedback loop in order to tightly regulate *Oct3/4* expression levels. An experimental model in prokaryotic cells has revealed that a simple negative-feedback loop can dramatically stabilize the expression level of a gene (Becskei and Serrano, 2000). Therefore, a direct or indirect negative-feedback loop could be sufficient to regulate the quantitative expression of *Oct3/4* within the range required to maintain pluripotency. To date, two regulatory elements, a distal and a proximal enhancer, have been identified as stem-cell-specific enhancers of *Oct3/4* (Yeom et al., 1996), to which many positive and negative regulators are recruited (Fig. 4A). Among them, members of the orphan nuclear receptor superfamily, which can bind to the proximal enhancer, are known to influence Oct3/4 expression. Liver receptor homolog 1 (Lrh1, also known as Nr5a2) is a putative positive regulator of *Oct3/4*, as *Oct3/4* expression is lost in the epiblast of *Lrh1*-null embryos and is quickly downregulated after the induction of differentiation in *Lrh1*-null ES cells (Gu et al., 2005a). By contrast, germ cell nuclear factor (Gcnf, or Nr6a1) is a potential *Oct3/4* negative regulator, as the expression domain of *Oct3/4* is enlarged and its expression prolonged in the neuroepithelium of *Gcnf*-null embryos (Fuhrmann et al., 2001). *Oct3/4* repression following the induction of differentiation is also delayed in *Gcnf*-null ES cells (Gu et al., 2005b). Chicken ovalbumin upstream promoter-transcription factors (Coup-tf) I and II, encoded by *Nr2f1* and *Nr2f2*, respectively, also function as negative regulators of *Oct3/4* expression (Ben-Shushan et al., 1995). The balance between these positive and negative regulators might determine the precise level of *Oct3/4* expression in response to extracellular stimuli (Fig. 4A).

A transcription factor network for self-renewal

The feedback regulatory circuit that maintains pluripotency interacts with the feedback loop shown in Fig. 4B, in which Oct3/4, Sox2 and Nanog function to maintain their expression to promote continuous ES cell self-renewal. This loop determines the differentiation fate of ES cells by influencing the expression of transcription factors, such as *Cdx2* (which promotes trophectodermal differentiation) and *Gata6* (which promotes primitive endoderm differentiation). Rapid transitions between the pluripotent state and one of these differentiation states have been theoretically confirmed to occur in a model in which two positive-feedback loops are connected by negative-feedback loops. In such a system, a small quantitative asymmetry in one loop can be converted into its exclusive expression (Becskei et al., 2001). Moreover, as *Gcnf*, *Nr2f1* and *Nr2f2* are upregulated after the induction of either trophectoderm or primitive endoderm differentiation (Fujikura et al., 2002; Niwa et al., 2005), these negative regulators might form the negative-feedback loop that shuts down *Oct3/4* in differentiated cells, and which could then be followed by epigenetic chromatin modifications that result in the repression of the *Oct3/4* promoter (Feldman et al., 2006).

The transition of the pluripotent transcription factor network to either the trophectodermal or extraembryonic-endodermal network is most likely to be regulated by the presence or absence of extracellular signals, such as the removal of Lif from mouse ES cells or the formation of EBs. However, the activation of *Cdx2* or the repression

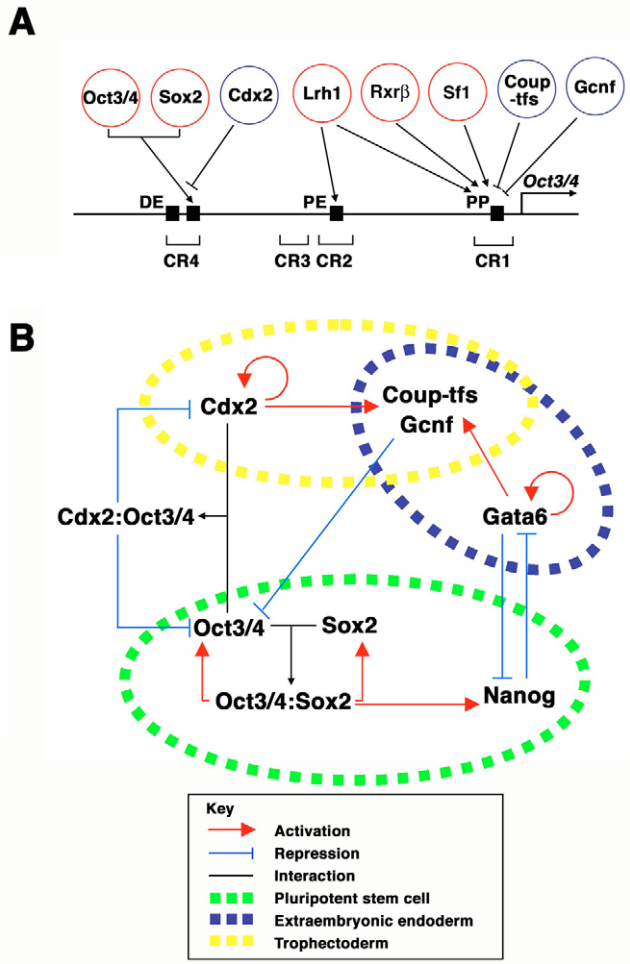

Fig. 4. A transcription factor network to control ES cell self-renewal and differentiation. (**A**) Transcriptional regulation of the mouse *Oct3/4* gene. There are four evolutionally conserved regions (CR1-4) that contain multiple transcription factor (TF) binding sites. The TFs that bind to these sites are shown above and either activate (red) or repress (blue) transcription. DE, distal enhancer; PE, proximal enhancer; PP, proximal promoter. (**B**) Transcription factor networks for pluripotent stem cells (green), trophectoderm (yellow) and primitive (extraembryonic) endoderm (blue). Positive-feedback loops between Oct3/4, Sox2 and Nanog maintain their expression to promote continuous ES cell self-renewal. Cdx2 is autoregulated and forms a reciprocal inhibitory loop with Oct3/4, which acts to establish their mutually exclusive expression patterns. A similar regulatory loop, not yet confirmed, might exist for Nanog and Gata6. A combination of positive-feedback loops and reciprocal inhibitory loops converts continuous input parameters into a bimodal probability distribution, resulting in a clear segregation of these cell lineages (see text for details). Coup-tfs and Gcnf act as a negative-feedback system to repress Oct3/4 completely.

of *Oct3/4* might occur in mouse ES cells through the infrequent spontaneous differentiation of these cells towards trophectoderm, which can occur under standard culture conditions (Beddington and Robertson, 1989). This tallies with evidence that Oct3/4 and Cdx2 compete with each other to be expressed during blastocyst formation, and with evidence that *Oct3/4* expression is dominant in the ICM (Niwa et al., 2005). Therefore, the gatekeeper function of Nanog, which is an Oct3/4 target and prevents extraembryonic endoderm differentiation, appears to be more important in mouse ES cells, as these cells are regulated by extracellular signals.

Indeed, Nanog could be at the hub of these multiple signal transduction pathways. As mentioned above, Nanog can block primitive endoderm differentiation (Chambers et al., 2003), neuronal differentiation (Ying et al., 2003) and mesoderm differentiation (Suzuki et al., 2006a) under different culture conditions. Recent studies have shown that Nanog interacts with Smad1 to inhibit the expression of brachyury (Suzuki et al., 2006b) and with Sall4 to form a positive regulatory loop for *Nanog* and *Sall4* (Wu et al., 2006); also, *Nanog* expression is activated by Foxd3 (Pan et al., 2006) and is repressed by Tp53 (Trp53 – Mouse Genome Informatics) (Lin et al., 2005), Gcnf (Nr6a1 – Mouse Genome Informatics) (Gu et al., 2005b), Tcf3 (Pereira et al., 2006) and the Grb2-Mek (Mdk – Mouse Genome Informatics) pathways (Hamazaki et al., 2006). However, during mouse development, *Nanog* transcription is downregulated in the epiblast and in early primitive ectoderm (Hart et al., 2004; Hatano et al., 2005), where Oct3/4 and Sox2 continue to be expressed (Avilion et al., 2003; Rosner et al., 1990). It is noteworthy that *Nanog* expression levels in P19 embryonal carcinoma (EC) cells is much lower than that in ES cells, although both EC and ES cells express similar levels of *Oct3/4* and *Sox2* (Chambers et al., 2003). This suggests that the positive-feedback circuitry in the pluripotent transcription factor network does not always require Nanog, and that the transcription factor network can establish a different stable circuit that maintains the levels of Oct3/4 and Sox2 expression required to maintain pluripotency with or without Nanog.

Two other factors have recently been reported to be necessary for the maintenance of ES cell self-renewal: estrogen-related receptor β (Esrrb) and T-box transcription factor Tbx3, both identified by functional screening mediated by RNA interference (Ivanova et al., 2006). Repression of *Esrrb* in mouse ES cells results in their differentiating into a mixture of extraembryonic and embryonic lineages, whereas knockdown of *Tbx3* triggers differentiation into mainly the embryonic lineages that are derived from the primitive ectoderm. Since the effect of repressing these genes can be cancelled out by the overexpression of *Nanog*, the maintenance of Nanog expression is one of their functions. The transcriptional regulation of their expression in ES cells has yet to be analyzed, but multiple binding sites for Oct3/4 and Nanog have been found in the mouse *Esrrb* gene (Loh et al., 2006). In addition, a recent protein interaction network analysis identified two transcription factors, the BTB-domain-containing protein Nac1 (Btbd14b – Mouse Genome Informatics) and the zinc-finger protein Zfp281, which interact with Nanog and are essential for maintaining the self-renewal of mouse ES cells (Wang et al., 2006). Further analyses will be required to integrate these genes into the current transcription factor network model described in this review.

An epigenetic mechanism for self-renewal

A series of recent studies have revealed that mouse and human ES cells possess certain novel epigenetic features. Polycomb-group (PcG) complex proteins mainly act to stabilize a repressive chromatin structure. Polycomb repressive complex 2 (PRC2), which consists of Ezh2, Eed and Suz12 in ES cells, functions as a histone methyltransferase on lysine 27 (K27) of histone H3, resulting in its tri-methylation (H3K27me3), a methylation mark that is associated with transcriptionally inactive genes (Cao and Zhang, 2004). In general, the distribution of this repressive chromatin mark is mutually exclusive to that of the tri-methylation mark H3K4me3, which is associated with transcriptionally active regions (Strahl and Allis, 2000; Lund and van Lohuizen, 2004). However, Bernstein et al. reported that in mouse ES cells, these histone marks co-localize in particular regions, which they named 'bivalent domains' (Bernstein et al., 2006). These domains, which are composed of short chromatin elements marked by H3K4me3 flanked by larger regions that contain H3K27me3, are associated with genes that are expressed at low levels (Fig. 5B) (Bernstein et al., 2006). Interestingly, the bivalent domains map to highly conserved non-coding elements (HCNEs) that have previously been identified as being conserved among the genomes of primates and rodents and which contain few retrotransposons (Bernstein et al., 2006). Moreover, half of these bivalent domains contain target sites that are common to Oct3/4, Sox2 and Nanog, as identified by genome-wide ChIP-on-Chip analysis (Boyer et al., 2005). Thus, these domains might signify the chromatin structure of genes that are in a differentiation-ready state, as proposed in the 'Localised Marking Model' by Szutoristz and Dillon (Szutoristz and Dillon, 2005). According to this model, most tissue-specific genes in ES cells would be targets for sequence-specific factors that can recruit histone-modifying enzymes, resulting in the formation of early transcription competence marks (ETCMs), which are enriched for histone H3 and H4 acetylation (H3Ac and H4Ac, respectively), and H3K4me3, all of which are histone marks associated with transcriptionally active regions. In both bivalent domains and ETCMs, H3K4me3 marks spread as genes near them become transcriptionally active, whereas H3K27me3 exclusively occupies those genes that are repressed during the differentiation of a particular cell type. Because the global level of H3K27me3 in ES cells is lower than that in differentiated cells, the mechanism by which this repressive mark targets such sites is of interest. Lee et al. (Lee et al., 2006) performed ChIP-on-Chip analysis for Suz12, Eed and H3K27me3, and revealed that Suz12- and Eed-binding sites significantly overlap with each other and with H3K27me3 marks on the highly evolutionarily-conserved regions of transcriptionally silent genes, including *Gata4* and *Cdx2*, in ES cells. The 1800 genes identified as targets of Suz12 included most of the targets repressed by Oct3/4, Sox2 and Nanog (Boyer et al., 2005). Boyer et al. (Boyer et al., 2006) also identified 512 common target genes of PRC2 and PRC1 by ChIP-on-Chip analysis and found that they were marked by H3K27me3, and that 87% were upregulated in the absence of PRC2 in *Eed*-null ES cells.

These findings suggest that the dynamic repression of developmental pathways in ES cells by epigenetic processes may be required for the maintenance of pluripotency; but this conclusion requires, in my view, further study. This is because observations made in ES cells that are deficient for members of the PRC2 and PRC1 complexes do not fit easily into this model. For example, *Eed*-null ES cells can still self-renew, maintain normal morphology and express Oct3/4, Sox2 and Nanog normally in the complete absence of PRC2 and despite a dramatic decrease in H3K27me3. These cells just show a high rate of spontaneous differentiation (Boyer et al., 2006; Azuara et al., 2006). Although the expression of *Gata4* and *Gata6*, as well as of several neural-specific genes, are upregulated in the absence of Eed, these ES cells can still produce all three germ

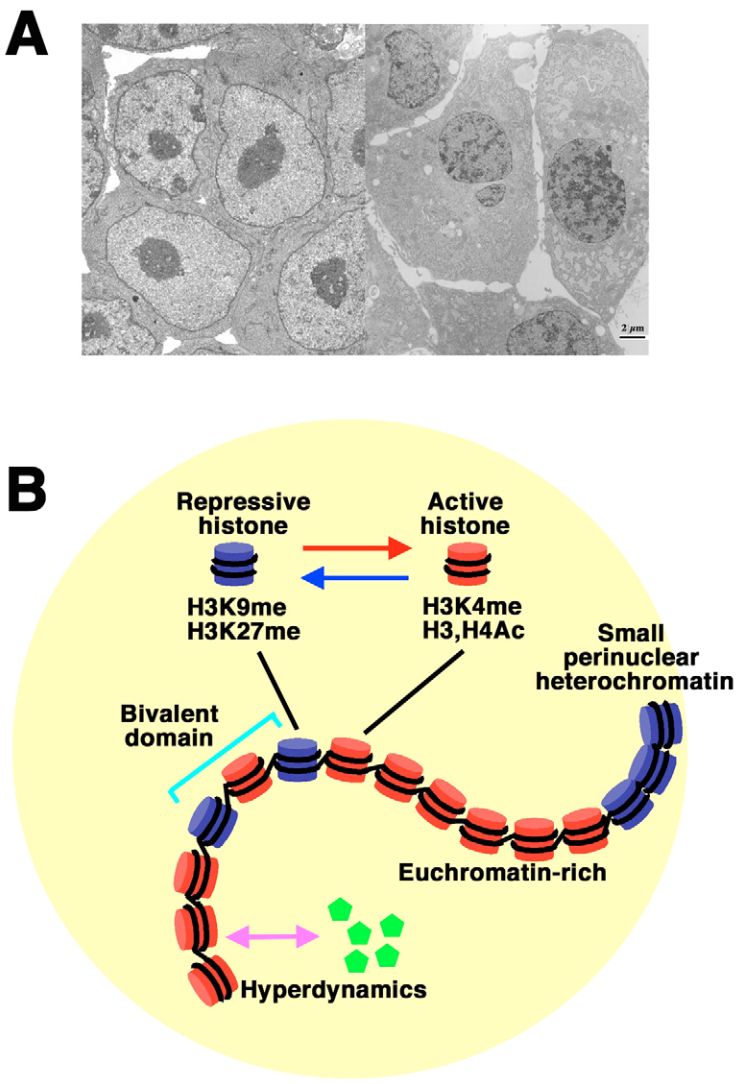

Fig. 5. Characteristics of the pluripotent epigenome. (**A**) Nuclei of undifferentiated (left) and differentiated (right) ES cells. The nucleus shrinks and the distribution of electron-dense areas, mainly heterochromatin, changes dramatically when ES cells are induced to differentiate into primitive endoderm by the ectopic expression of Gata6. (Electron micrographs courtesy of Naoko Ikue and Shigenobu Yonehara.) (**B**) Epigenetic features of the pluripotent cell nucleus. The volume of the nucleus is larger than that of a differentiated cell as a result of the relaxed chromatin structure. Small regions of perinuclear heterochromatin exist, but most of the chromatin exists as euchromatin, bearing histone marks associated with transcriptional activity. The hyperdynamics of chromatin proteins (green) might contribute to the maintenance of euchromatin. Bivalent domains are also a feature of the pluripotent epigenome, in which active histone marks (such as H3K4me) are flanked by transcriptionally repressive histone marks (such as H3K9me).

layers on injection into blastocysts (Montgomery et al., 2005; Azuara et al., 2006). *Suz12*-null ES cells also show features similar to those of *Eed*-null ES cells (Lee et al., 2006). The establishment of *Ezh2*-null ES cells has not been reported (O'Carroll et al., 2001), but it has been shown that Ezh2 protein becomes undetectable in *Eed*-null ES cells, and is restored by the introduction of an *Eed* transgene (Montgomery et al., 2005). ES cells lacking *Rnf2/Ring1β*, a component of PRC1, are also viable and show decreased amounts of histone H2A ubiquitination (Napoles et al., 2004). These findings indicate that the PcG proteins and the PRC1 and PRC2 complexes are not required for the maintenance of pluripotency.

Molecular mechanisms that determine pluripotency

If all genomic information is utilized at least once during the development of an organism, all genes should be ready to be expressed when they are required to execute pluripotency during development and, in general, the expression of a large number of genes is a common feature of stem cells (Zipori, 2004). Therefore, in pluripotent stem cells, many genes might be weakly expressed and, during differentiation, the expression levels of many might be reduced, whereas those of others are increased, determining the progeny's phenotype. Indeed, genome-wide gene expression profiling using microarrays has revealed that a variety of genes are expressed at low levels in ES cells (Carter et al., 2005). This might be a consequence of their chromatin structure being in an open configuration, allowing the leaky expression of genes by the general transcription machinery with neither positive nor negative regulation (Roeder, 2005) (Fig. 5B).

The leaky expression of a large number of genes characteristic of the ES cell pluripotent state is likely to be the result of both genetic and epigenetic mechanisms and processes. Through epigenetic processes, the pluripotent epigenome keeps the chromatin structure open to allow for rapid genetic regulation (Fig. 5B) (Zipori, 2004). The general abundance of transcriptionally active chromatin marks, such as H3K4me3 and H4Ac, in ES cells fits with this idea (Lee et al., 2004; Azuara et al., 2006). Hyperdynamic chromatin restructuring has been observed in mouse ES cells during self-renewal as rapid exchanges of histone H1 and HP1α (Meshorer et al., 2006), which might contribute to keeping the chromatin structure of ES cells open. The existence of such a globally relaxed chromatin structure is supported by the following evidence. Remarkable differences exist in the distribution and frequency of high electron density areas, which were originally designated as heterochromatin (Brown, 1966), between ES and parietal endoderm cells (Fig. 5B). DNaseI hypersensitive sites, which correlate with transcriptionally active

Table 1. Functions of epigenetic machineries in pluripotent stem cells

	KO embryos		KO ES cells				
Gene	Phenotype	ICM outgrowth	Proliferation	Marker expression	Differentiation	Restore by transgene	Reference
H3K9HMTases							
Suv39h1/h2	Viable	NT	Normal	Normal	Normal	NT	Peters et al., 2001; Lehnertz et al., 2003
G9a (*Ehmt2*)	Die at E9.5	NT	Normal	Normal	Defective	Restored	Tachibana et al., 2002
Glp (*Ehmt1*)	Die at E9.5	NT	Normal	Normal	Defective	Restored	Tachibana et al., 2005
Eset (Setdb1)	Die at E3.5-E5.5	Defective	NT	NT	NT	NT	Dodge et al., 2004
PRC2 (H3K27HMTase)							
Ezh2	Die at E3.5-E5.5	Defective	NT	NT	NT	NT	O'Carroll et al., 2001
Eed	Die at E8.5	Normal	Normal	Normal	Defective (mildly)	Restored	Faust et al., 1998; Montgomery et al., 2005
Suz12	Die at E8.5	Normal	Normal	Normal	NT	NT	Pasini et al., 2004; Lee et al., 2006
PRC1							
Rnf2 (*Ring1b*)	Die at E7.5	Normal	Normal	Normal	NT	NT	Voncken et al., 2003; Napoles et al., 2004
DNA methylation							
Dnmt1	Die at E9.5	NT	Normal	Normal	Defective	Restored	Lei et al., 1996; Gaudet et al., 1998
Dnmt3a/3b	Die at E11.5	NT	Normal	Normal	Defective	Restored	Okano et al., 1999; Chen et al., 2003
Dnmt1/3a/3b	NT	NT	Normal	Normal	Defective	Restored	Tsumura et al., 2006
Dnmt3l	Viable	Normal	Normal	Normal	Normal	NT	Hata et al., 2002
Cgbp (*Cxxc1*)	Die at E6.0	Normal	Normal	Normal	Defective	Restored	Carlone and Skalnik, 2001; Carlone et al., 2005
RNAi							
Dicer1	Die at E7.5	Defective	Retarded/ compensated	Normal	Defective	Restored	Bernstein et al., 2003; Kanellopoulou et al., 2005; Murchison et al., 2005
Chromatin remodeling/Histone exchange							
Snf2b (*Brg1*, *Smarca4*)	Die at E4.5-6.0	Defective	Not viable (F9 EC cells)	NT	NT	NT	Bultman et al., 2000; Sumi-Ichinose et al., 1997
Snf2h (*Smarca5*)	Die at E4.5-6.0	Defective	NT	NT	NT	NT	Stopka and Skoultchi, 2003
Snf5 (*Smarcb1*)	Die at E4.5-6.0	Defective	NT	NT	NT	NT	Klochendler-Yeivin et al., 2000
Srg3 (*Smarcc1*)	Die at E4.5-6.0	Defective	NT	NT	NT	NT	Kim et al., 2001
Mbd3	Die at E8.5	Defective	Retarded	Normal	Defective	Restored	Hendrich et al., 2001; Kaji et al., 2006
HirA	Die at E9.5	NT	Normal	Normal	Accelerated	NT	Roberts et al., 2002; Meshorer et al., 2006

NT, not tested.

chromatin (Weintraub and Groudine, 1976), are frequently detected in genes regardless of their expression levels in ES cells (Meshorer et al., 2006). Finally, nuclei in ES cells are about double the volume of those in differentiated cells (Faro-Trindade and Cook, 2006). As such, the guidance of cell fates could occur solely via the action of transcription factors, such as Gata6 and Cdx2, owing to the unprogrammed state of the pluripotent epigenome, which might allow transcription factors to freely access their target genes to control differentiation (Smith, 2005).

By contrast, as shown in Table 1, various epigenetic processes, including PcG/H3K27me3, DNA methylation, tri-methylation of lysine 9 of histone H3 (H3K9me3) and RNAi, are not essential for pluripotency. The requirement for H3K4me3 has not been assessed because a methyltransferase that allows H3K9me3 to be globally marked in ES cells has not yet been identified. The chromatin remodeling system, however, might be the exception because it has been reported that the inactivation of *Brg1/Snf2β*, a component of the SWI/SNF and ISWI complex family involved in ATP-dependent

chromatin remodeling, affects the viability of F9 EC cells (Sumi-Ichinose et al., 1997), although its specific involvement in the maintenance of pluripotency has not yet been confirmed. Conversely, we can conclude that epigenetic processes are required for proper ES cell differentiation. However, the inability of ES cells to differentiate in response to signals such as the withdrawal of Lif or the addition of retinoic acids, can be restored by the reactivation of the deleted epigenetic genes, indicating that pluripotency is maintained in the absence of these epigenetic mechanisms (Table 1). I propose, therefore, that epigenetic processes are likely to be responsible for the 'execution' of the pluripotent program, which is itself established by the transcription factor network, rather than for the 'maintenance' of pluripotency per se.

A comparison of ES and EC cells might shed light on the function of such epigenetic mechanisms in pluripotent stem cells. The ectopic expression of Gata4, a transcription factor related to Gata6, has different effects in ES and EC cells. During mouse development, *Gata4* is expressed in the primitive endoderm and its derivatives, and then in cardiac precursors (Kelley et al., 1993). When *Gata4* is ectopically expressed in ES cells, it directs differentiation into parietal endoderm, as does *Gata6* (Fujikura et al., 2002). By contrast, ectopic expression of *Gata4* in P19 EC cells enhances their differentiation into cardiomyocytes (Grepin et al., 1997). As mentioned above, P19 EC cells lack almost any expression of *Nanog* (Chambers et al., 2003) but nonetheless exhibit a poor capacity to differentiate into primitive endoderm (a differentiation pathway that is repressed by Nanog, as discussed above) (Mummery et al., 1990). This suggests that the genetic function of Gata factors in EC cells is different from that in ES cells because of the difference in pre-existing transcription factors in these cell types. However, both the prevention of differentiation into primitive endoderm and the change in response to the ectopic expression of Gata4 in P19 EC cells might reflect changes in their epigenetic state, perhaps owing to changes in the accessibility of their target genes. Since the phenotype of P19 EC cells is closer to that of primitive ectoderm than to ICM (Jones-Villeneuve et al., 1982), a restriction of pluripotency might be mimicked in P19 EC cells, in which the gatekeeper function of Nanog might be replaced by the epigenetic repression of its targets. Therefore, the function of Nanog might be limited to that of a gatekeeper, which blocks ES cells from following certain differentiation pathways but makes few other contributions to the state of pluripotency.

How does the transcription factor network determine the pluripotent state per se? As mentioned above, a combination of positive-feedback loops with reciprocal inhibitory loops allows continuous input parameters to be converted into a bimodal probability distribution (Becskei et al., 2001). This system was first applied to explain how the ICM and trophectoderm segregate into mutually exclusive *Oct3/4* and *Cdx2* expression domains and could possibly be applied to each differentiation event in development (Niwa et al., 2005). Epigenetic mechanisms might follow this process by locking one of the components that is transcriptionally inactivated by competition into a repressive state. If this is a general rule in the transition of the transcription factor networks, by which sequential differentiation events in development are mediated, what happens if all epigenetic repression is removed at once? During normal embryonic development, first ectoderm and mesoendoderm are segregated, and then the latter is separated into mesoderm and endoderm, in which ectodermal determination is repressed. The system consists of a combination of positive-feedback loops with reciprocal inhibitory loops, which work sequentially to choose one fate in these steps. If these systems start to work at once because of the epigenetic derepression of transcription, the positive- and negative-feedback loops could end up functioning chaotically and might result in a disordered state in which none of the transcription factor networks holds an exclusive position, resulting in there being no determination of cell phenotype. In addition, a feature of the random Boolean network is that small changes to a few components can mediate the transition of the stable condition of the network ('attractor') from one state to another, but this transition depends strongly on the initial state of the network. Only a particular change can trigger a transition, and other changes are cancelled out without any effect on the network, indicating that it might not be necessary to repress all tissue-specific transcription factor genes to prevent differentiation in the pluripotent state. This idea is supported by the fact that the ectopic expression of the tissue-specific transcription factors merely directs the differentiation of ES cells, and that the expression of many tissue-specific transcription factors, such as *Pax6* and *Pdx1*, are detected in ES cells (Lumelsky et al., 2001; Okada et al., 2004). Therefore, the function of the pluripotent transcription factor network might be limited to the activation of the epigenetic processes that generate the open chromatin structure required for rapid changes in the transcriptional status of tissue-specific genes during ES cell differentiation and development: for example, by activating the enzymes that result in transcriptionally repressive histone marks being exchanged for those of actively transcribed genes.

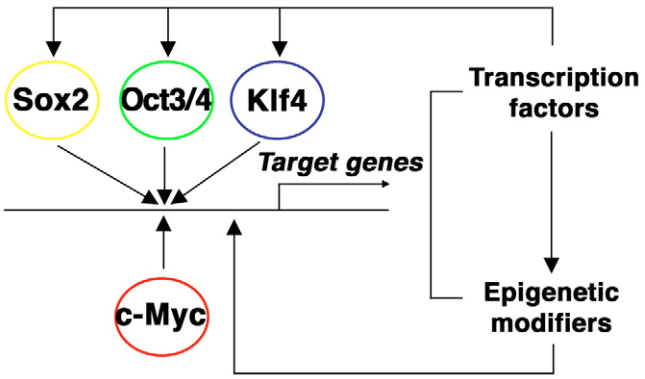

Fig. 6. Establishment of pluripotency in somatic cell nuclei. In a recent study (Takahashi and Yamanaka, 2006), four transcription factors, Oct3/4, Sox2, Klf4 and c-Myc, were found to be sufficient to establish pluripotency in the nuclei of fibroblasts. Oct3/4, Sox2 and Klf4 might function together to activate target genes to establish the stable pluripotent transcription factor network, as well as the pluripotent epigenome, whereas c-Myc might enhance the accessibility of target genes by stimulating DNA replication.

The establishment of pluripotency in vivo

During development, both genetic and epigenetic mechanisms could be involved in the establishment of the pluripotent state in the cells of the ICM through the reprogramming of nuclei in fertilized eggs. Such reprogramming activity is present in the cytoplasm of fertilized eggs, as proven by the generation of cloned embryos from somatic cell nuclear transfer (Wilmut et al., 1997). However, it is still unclear which mechanism contributes to this activity because the enzymes that modify the epigenetic state, as well as maternally transcribed and translated transcription factors, are present in fertilized eggs.

Recently, Takahashi and Yamanaka addressed this question. They reported that the co-introduction of four transgenes encoding the transcription factors Oct3/4, Sox2, c-Myc and Klf4 into somatic

cells, such as embryonic and adult tail-tip fibroblasts, resulted in the generation of induced pluripotent stem (iPS) cells, which gave rise to chimeric embryos following their injection into mouse blastocysts (Takahashi and Yamanaka, 2006). The functions of Oct3/4, Sox2 and c-Myc have been mentioned above. Klf4 is well known as an oncogene (Rowland and Peeper, 2006), but overexpression of *Klf4* in mouse ES cells reduces the differentiation ability of EBs (Li et al., 2005). Klf4 can also bind to the proximal promoters of Oct3/4 target genes, such as *Lefty1*, and helps to activate Oct3/4 and Sox2 (Nakatake et al., 2006). These four factors are thought to establish pluripotency in somatic cells as follows (Fig. 6). First, c-Myc promotes DNA replication, thereby relaxing chromatin structure, which allows Oct3/4 to access its target genes. Sox2 and Klf4 also co-operate with Oct3/4 to activate target genes that encode transcription factors which establish the pluripotent transcription factor network and which, together with Oct3/4, Sox2 and Klf4, result in the activation of the epigenetic processes that establish the pluripotent epigenome. The iPS cells have a similar global gene expression profile to that of mouse ES cells. Interestingly, *Nanog* is not required exogenously to establish pluripotency in iPS cells and its endogenous expression is not always activated in established pluripotent stem cells by these four factors, supporting the hypothesis that the function of Nanog in the maintenance of pluripotency is context dependent.

In iPS cells, the repressive histone marks in the promoter regions of Oct3/4 and Nanog are replaced by active marks, such as H3K4me and H4Ac, although DNA methylation is only partially erased. This suggests that Oct3/4, Sox2, c-Myc and Klf4 are indeed able to alter the epigenetic state of a cell and establish the pluripotent epigenome. This change should be mediated by enzymatic activities that erase the repressive histone marks (such as demethylases for H3K9 and H3K27) and generate active histone marks (such as H3K4 methyltransferase and histone acetyltransferase). Thus, to establish and maintain pluripotency, the genes encoding these enzymes would be activated by the pluripotent transcription factor network. Under such artificial conditions, the transcription factor network could orchestrate all the requirements for pluripotency.

Conclusion

Recent progress in understanding the establishment and maintenance of ES cell pluripotency has revealed the importance and functions of various key transcription factors. By contrast, although several features of the pluripotent epigenome have been discovered, their requirement for and involvement in the maintenance and establishment of pluripotency remain unclear. In the future, it will be necessary to confirm how genetic mechanisms determine the pluripotent epigenome and how the pluripotent epigenome functions to maintain the pluripotent transcription factor network.

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