CHAPTER 7

Mechanisms of Stem Cell Self-Renewal

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7.1 SELF-RENEWAL OF PLURIPOTENT STEM CELLS

The capacities for self-renewal and differentiation are the two most prominent characteristics of stem cells. Self-renewal can be defined as making a complete phenocopy of stem cells through mitosis, which means at least one daughter cell generated by mitosis possesses the same capacity of self-renewal and differentiation. In stem cell self-renewal, symmetric cell division generates two stem cells; asymmetric cell division results in one stem cell and either one differentiated progeny or a stem cell with a restricted capacity for differentiation. Self-renewal by symmetric cell division is often observed in transient stem cells appearing in early embryonic development to increase body size. In contrast, self-renewal by asymmetric cell division can be found in permanent stem cells in embryos in later developmental stages, and also in adults to maintain the homeostasis of the established body plan.

Stem cells are categorized by their abilities for differentiation. Pluripotency is defined as the ability of a cell to differentiate into any of three germ layers. Pluripotent stem cells can be found in both pre- and postimplantation embryos. Mouse ES cells are authentic pluripotent stem cells established from the inner cell mass (ICM) of blastocyst-stage embryos. Mouse ES cells continue self-renewal in a leukemia inhibitory factor (LIF)-dependent manner, and retain the ability to contribute to embryonic development after injection into blastocysts, which is the characteristic now designated as naïve pluripotency. In contrast, mouse epiblast stem cells (EpiSCs) are derived from epiblasts or primitive ectoderm of postimplantation embryos. Mouse EpiSCs maintain self-renewal in the presence of fibroblast growth factor 2 (Fgf2) and Activin and they rarely contribute to chimeric embryos after blastocyst injection, which is the characteristic now designated as primed pluripotency.

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X chromosome inactivation occurs in female mouse embryos immediately after implantation, and it was reported that mouse ES cells retain two active X chromosomes whereas EpiSCs have one inactive X chromosome, indicating a difference in their developmental stages. Human ES cells are derived from ICM but demonstrate characteristics of primed pluripotent stem cells. It was also demonstrated that mouse ICMs are capable of giving rise to EpiSCs in the presence of Fgf2 and Activin, suggesting that pluripotency is determined by culture conditions rather than the origin of the stem cells.

7.1.1 Molecular Mechanisms to Retain ES Cell Self-Renewal

The ability for continuous self-renewal *in vitro* is one of the characteristic phenotypes of ES cells. As found in other cellular phenotypes, continuous self-renewal should be regulated via transcription in the nucleus, which in turn is triggered by extracellular signals. In this section, the minimal molecular mechanism to maintain naïve pluripotency in mouse ES cells will be described, based on functional analysis data (see Figure 7.1).

7.1.1.1 Extracellular Signals for ES Self-Renewal

7.1.1.1a Leukemia Inhibitory Factor

LIF is a soluble factor that is sufficient for maintaining the pluripotency of mouse ES cells. LIF belongs to the interleukin (IL)-6 cytokine family, whose members share the transmembrane glycoprotein Gp130 as a common component for signal transduction of their receptors. The high-affinity LIF receptor consists of a heterodimer of Gp130 and LIF receptor β (Lifrβ). One major signal transduction pathway mediated by Gp130 is JAK–STAT, and it has been shown that the activation of Stat3 is necessary and sufficient to maintain pluripotency. The phosphatidylinositol-3-OH kinase (PI3K)–Akt pathway is also activated by LIF under Gp130, and artificial activation of Akt was able to replace the requirement for LIF. These two intracellular pathways integrate the LIF signal into the nuclei in parallel, which activates the transcription factors responsible for maintaining pluripotency. In contrast, the Grb2-mitogen-activated protein kinase (MAPK) pathway is also activated by LIF, but acts as a negative regulator of pluripotency. Among these intracellular signal pathways, JAK–STAT is exclusively activated by LIF in ES cells, whereas the PI3K–Akt and the Grb2–MAPK pathways are regulated by LIF as well as other extracellular signals such as insulin/insulin-like growth factor (IGF) and Fgf4, respectively.

Although LIF sustains self-renewal of mouse ES cells in media containing fetal calf serum, or in serum-free N2B27 media in combination with bone morphogenetic protein (BMP) 4, its physiological action during development appears to be restricted. Elimination of the function of *Lif*, *Gp130*, *Lifrβ*, or *Stat3* by gene targeting did not interfere with self-renewal of pluripotent

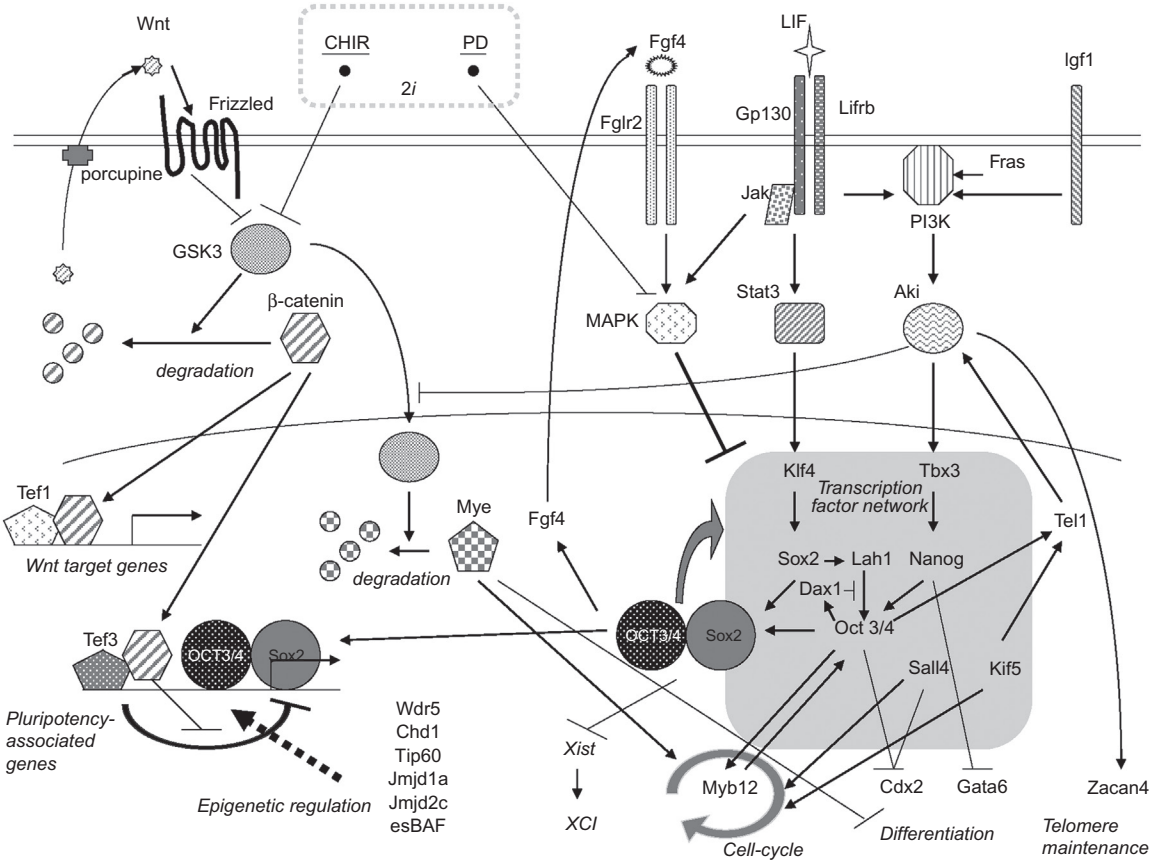

FIGURE 7.1 **A bird's-eye view of the molecular mechanism working in mouse ES cells to maintain pluripotency.**
See text for the abbreviations in the figure.

stem cells during early embryogenesis. The role of Gp130 in the pluripotent cell phenotype was only evident when the delayed blastocysts were carefully analyzed. The ICM of delayed blastocysts normally maintains pluripotency, but *Gp130*-null blastocysts could not maintain pluripotency during the delayed period. Since the maintenance of blastocysts in the uterus without implantation is a characteristic feature in rodents, the responsiveness of ES cells to Gp130 signaling has its origin in this adaptive physiological function. Moreover, it may be the reason why LIF does not show obvious effects on ES cells of other species, especially primates. However, the function of the Gp130–Stat3 pathway in germ cell development is evolutionally conserved, since it can be found in invertebrates, which may suggest that the role of this system in rodent ES cells is a small evolutionary cooption derived from the maintenance of germ cells.

7.1.1.1b Fibroblast Growth Factor 4

Fgf4 is an autocrine factor that promotes the differentiation of mouse ES cells. Either elimination of Fgf4 secretion by gene targeting or blockage of Fgf receptor kinase activity by a specific inhibitor promotes self-renewal and inhibits spontaneous differentiation in the medium containing LIF and serum. The negative signal for pluripotency from Fgf4–Fgfr2 is transduced by the Grb2–MAPK pathway, and the targeted disruption of Grb2 or blockage of the MAPK activity by a specific inhibitor also promotes self-renewal. Since *Fgf4* expression is regulated by the pluripotency-associated transcription factors Oct3/4 and Sox2, destabilization of pluripotency also seems to be one of the important contradictive effector pathways of pluripotency-coupled transcription factor network. Nuclear localization of Tbx3 is negatively regulated by MAPK, but there might be other targets to mediate this negative effect.

7.1.1.1c Wnt

Wnt is also an autocrine factor that stabilizes naïve pluripotency. Inhibiting the secretion of Wnt family ligands by inhibiting the activity of porcupine causes mouse ES cells to change to EpiSC-like cells. In contrast, inhibiting glycogen synthase kinase (GSK) 3 activity, which is negatively regulated in the canonical Wnt signaling pathway, stabilizes self-renewal when combined with inhibition of MAPK in serum and LIF-free culture media (also called *2i* culture conditions). However, neither addition of recombinant Wnt ligand nor inhibition of GSK3 is sufficient to support self-renewal of mouse ES cells without inhibition of MAPK activity or LIF. Since LIF shows a synergistic effect with inhibition of GSK3 and MAPK, they may have different integration points in the pluripotency-associated transcription factor network. Inhibition of GSK3 causes stabilization of β-catenin and promotes its nuclear localization. Nuclear-localized β-catenin interacts with Tcf1 to activate its transcriptional activity as well as with Tcf3 to inhibit its repressor function. GSK3 might not be regulated exclusively by Wnt and it was suggested that the PI3K–Akt pathway negatively regulates its nuclear localization.

7.1.1.2 Transcriptional Regulation for ES Cell Self-Renewal

7.1.1.2a Oct3/4

Oct3/4 encoded by *Pou5f1* was initially identified as Oct3 or Oct4 for undifferentiated, state-specific expression in embryonal carcinoma cells. Oct3/4 is absolutely essential for establishing and maintaining pluripotency in embryonic development, since *Oct3/4*-null embryos die immediately after implantation and ICM isolated from *Oct3/4*-null blastocysts undergoes differentiation toward trophectoderm. Inducible loss of *Oct3/4* in ES cells also causes their differentiation toward trophectoderm, indicating that Oct3/4 is essential for self-renewal of mouse ES cells. Oct3/4 forms a heterodimer with Sox2, and regulates multiple pluripotency-associated genes including

both *Oct3/4* and *Sox2*, indicating that a positive feedback mechanism may be involved in the maintenance of ES cell self-renewal. Oct3/4 acts as a transcriptional repressor for the genes involved in the induction of differentiation toward trophectoderm such as *Cdx2*, which explains the reason why loss of Oct3/4 function leads exclusively to trophectoderm differentiation. Overexpression of Oct3/4 induces differentiation to a mixture of differentiated cells other than trophectoderm, which might be due to the retention of the repressor function that blocks trophectoderm differentiation.

7.1.1.2b Sox2

Sox2 is a member of the Sry-related HMG-box transcription factor family. It acts as a partner of Oct3/4 to direct transcriptional activation, and is essential for maintaining a pluripotent cell population in embryos, since *Sox2*-null embryos die after implantation. Inducible loss of *Sox2* in ES cells causes their differentiation toward trophectoderm, indicating that Sox2 is essential to maintain ES cell self-renewal. However, artificial maintenance of *Oct3/4* expression from a transgene at physiological expression level restores self-renewal of *Sox2*-null ES cells with keeping the expression of the known target genes of Oct3/4–Sox2 complex. Therefore, the primary role of Sox2 is deduced to the indirect transcriptional activation of Oct3/4 and the activator function with Oct3/4 can be compensated by other Sox family members.

7.1.1.2c Nanog

Nanog encodes an NK2-family homeobox transcription factor. *Nanog* comes from *Tir na nÓg*, the name of the land of the ever-young in Celtic mythology, because its forced expression in mouse ES cells allows self-renewal in the absence of LIF. This is a quite unique feature of *Nanog*, because artificial maintenance of either *Oct3/4* or *Sox2* cannot replace the requirement of LIF in ES cell self-renewal. Nanog should be required for normal preimplantation development since *Nanog*-null embryos die after implantation. However, inducible deletion of *Nanog* in ES cells gives rise to *Nanog*-null ES cells that are still able to continue self-renewal, although they show higher incidence of spontaneous differentiation, indicating that Nanog is not essential for ES cell self-renewal. Since Nanog co-localizes to the target sites in the genome with Oct3/4 and Sox2, it may contribute to stabilizing their binding or enhancing their function. It was reported that *Nanog* expression is regulated by LIF via the PI3K–Akt pathway.

7.1.1.2d Klf4

Klf4 is a Krüppel-type zinc-finger transcription factor. Forced expression of *Klf4* maintains ES cell self-renewal in the absence of LIF as found in the case of *Nanog*. However, *Klf4*-null embryos pass through early developmental stages without abnormality and die immediately after birth due to the defect

of the epithelial seal in skin and colon. Although the establishment of *Klf4*-null ES cells has not yet been reported, siRNA-mediated knockdown suggests that Klf4 shares its function with other Klf family members Klf2 and Klf5 to maintain ES cell self-renewal. No single gene knockout showed any defect, whereas triple knockdown of all three Klf factors caused prevention of ES cell self-renewal. However, since *Klf5*-null ES cells showed defective proliferation, each Klf factor has unique functions, as well as functions in common with other Klf factors. It was reported that *Klf4* is regulated by LIF via the JAK–STAT pathway whereas *Klf2* is mainly regulated by Oct3/4, suggesting different regulatory functions in ES cell self-renewal. Klf4 cooperates with Oct3/4 and Sox2 to activate the transcription of a particular set of target genes including *Lefty1*.

7.1.1.2e Tbx3

Tbx3 belongs to the T-box transcription factor family. Knockdown of *Tbx3* in mouse ES cells induces differentiation, whereas its forced expression maintains LIF-independent self-renewal. However, *Tbx3*-null embryos die at E13.5 due to a defect in the yolk sac, indicating that its function is not required for the maintenance of pluripotent cell population *in vivo*. *Tbx3* expression is positively regulated by LIF via the PI3K–Akt pathway, while the nuclear localization of Tbx3 protein is negatively regulated by MAPK.

7.1.1.2f Tcf3

The T-cell factor (Tcf) family is regarded as a target of the canonical Wnt pathway. In mouse ES cells, Tcf1 acts as a conventional target to mediate transcriptional activation of nuclear-localized β-catenin. In contrast, Tcf3 works with a unique mode of action in mouse ES cells. Tcf3 co-localizes to the target sites of Oct3/4, Sox2, and Nanog and negatively regulates their transcriptional activation, which is competed by nuclear-localized β-catenin. This model is supported by the evidence that *Tcf3*-null ES cells show stabilized self-renewing ability and that *Tcf3* overexpression induces differentiation.

7.1.1.2g Myc

Myc is known as a common regulator of cellular proliferation and metabolism in various stem cells. Forced expression of *Myc*, especially its mutant form *MycT58A*, supports ES cell self-renewal without LIF. It was reported that nuclear-localized GSK3 phosphorylates Myc at T58 leading to its degradation, and nuclear localization of GSK3 is negatively regulated by PI3K–Akt under the LIF signal. Although *Myc*-null ES cells continue self-renewal, double knockout of *Myc* and *Mycn* induces differentiation to primitive endoderm, suggesting a shared function of Myc and Mycn to prevent differentiation in mouse ES cells. However, their function is context-dependent, because ES cells lacking *Max*, which is a common dimerization partner of the Myc family, can be maintained in *2i* culture although they die in fetal bovine serum-containing culture. Therefore, the requirement for Myc in mouse ES cells seems to prevent

the differentiation to primitive endoderm which is enforced by MAPK activation, and its function in cellular proliferation is dispensable.

7.1.1.3 Nuclear Receptors

Nuclear receptors are suggested as being involved in the pluripotency-associated transcription factor network, especially in the regulation of *Oct3/4*. SF1/Nr5a1 is known to bind to the *Oct3/4* promoter with RAR to activate its transcription. Lrh1/Nr5a2 is reported to bind to the SF1 binding sites in mouse ES cells, and *Lrh1*-null ES cells showed rapid downregulation of *Oct3/4* in primitive ectoderm, suggesting Lrh1 has a major function in the primed pluripotent state. Tr2/Nr2c1 and Tr4/Nr2c2 are also suggested to be involved in the transcriptional activation of *Oct3/4* in ES cells. The repressive nuclear receptor family members such as Gcnf/Nr6a1 and Couptfs (Nr2f1–3) occupy SF1 binding sites in somatic cells, which may contribute to the blocking of aberrant activation of *Oct3/4* in differentiated cells. Dax1/Nr0b1 encodes a negative regulator of Nr5a1 and Nr5a2 and is specifically expressed in naïve pluripotent stem cells. *Dax1* expression is positively regulated by Oct3/4 and Stat3, and Dax1 inhibits the activity of Nr5a1 and Nr5a2 as well as Oct3/4 to repress *Oct3/4* expression, suggesting a role in fine-tuning *Oct3/4* gene expression within an appropriate range. Indeed, forced expression of *Dax1* in ES cells causes induction of differentiation toward trophectoderm along with repression of *Oct3/4*.

7.1.1.3a Stat3

Stat3 is a pivotal mediator of the LIF signal. Artificial activation of Stat3 using the Stat3–ER fusion is sufficient to substitute the requirement of LIF for ES cell self-renewal. However, it is not absolutely required for maintaining pluripotency because *Stat3*-null ES cells can be propagated in *2i* culture as in the case of *Myc:Mycn*-null ES cells. Therefore, the function of Stat3 is limited to the transduction of the LIF signal into the core transcription factor network.

7.1.1.3b Rex1

Rex1 encodes a C2H2 zinc-finger transcription factor that is also known as *Zfp42*. Although it is specifically expressed in pluripotent stem cells both *in vitro* and *in vivo*, its function is absolutely dispensable for maintaining pluripotency. Indeed, *Rex1*-null ES cells maintain self-renewal and an ability to contribute to chimeric embryos. A similar unexpected observation was reported for Esg1 and Rest.

7.1.1.4 Mediator Complex

The transcriptional activation by tissue-specific transcription factors is transmitted to the recruitment of RNA polymerase II to the promoter site via mediator complex. This general mechanism is shared in mouse ES cells for transcriptional activation by pluripotency-associated transcription factors. Mediator complex

also contributes to chromatin architecture modulation by the interaction with cohesin, which is also a ubiquitous mechanism.

7.1.1.5 Epigenetic Regulation of ES Cell Self-Renewal

A unique epigenetic feature of mouse ES cells provides the basis of the pluripotency-associated transcription factor network. In general, all epigenetic regulation in mouse ES cells aims to keep genome-wide open chromatin conformation.

7.1.1.5a Histone Marks

Polycomb group complex (PRC) 2 consists of Suz12, Eed, and Ezh2 and mediates tri-methylation at Lys27 of histone H3, which contributes to gene silencing by establishing bivalent domains at the regulatory elements of the developmentally regulated genes. On the other hand, it was also shown that PRC2 is dispensable in maintaining ES cell self-renewal, since *Suz12*-null, *Eed*-null, and *Ezh2*-null ES cell lines all continue to self-renew. PRC1, which consists of Ring1a and Ring1b, mediates histone ubiquitination. ES cell self-renewal is abolished by disruption of both *Ring1a* and *Ring1b* loci. In general, PRC2 recruits PRC1 to the target sites but they may have shared function since only *Eed:Ring1b*-null ES cells, but not ES cells lacking either PRC1 or PRC2 function, lose the ability to differentiate properly. It is known that the trithorax group (trxG) mediates methylation of Lys4 of H3 to counteract the silencing effect by PRC. Recently, Wdr5 has been identified as a member of trxG, and is involved in the maintenance of ES cell self-renewal.

Methylation of Lys9 of histone H3 is involved in heterochromation formation by the recruitment of heterochromatin protein (HP)-1. Although H3K9 di- and tri-methyl transferases G9a and Glp are dispensable for ES cell self-renewal, knockout of H3K9 mono-methyl transferase *Eset* in ES cells causes de-repression of endogenous retroviruses. A similar phenotype was observed in *Kap1/Trim28*-null ES cells probably because Kap1 is required for the recruitment of Eset to the target sites.

Jumonji-family proteins mediate demethylation of methylated histones. Jumonji/Jarid2 is not required for ES cell self-renewal, but it is involved in fine-tuning H3K27 methylation by interacting with PRC2. Knockdown of *Jmjd1a* or *Jmjd2c* leads to the differentiation of ES cells, suggesting their involvement in the positive regulation of pluripotency-associated genes via demethylation of H3K9.

7.1.1.5b DNA Methylation

Methylation of cytosine residues in DNA, which generates 5-methylcytosine, is a major heritable epigenetic mark. Dnmt3a and Dnmt3b act as a *de*

novo methyltransferase, while Dnmt1 works for the maintenance of methyltransferase during DNA replication. Although mouse ES cells express high levels of *Dnmt3a* and *Dnmt3b*, double knockout ES cells of both *de novo* methyltransferases continue self-renewal. However, these ES cells lose DNA methylation during long-term culture and then show a higher incidence of spontaneous differentiation toward trophectoderm than wild-type ES cells. Triple knockout ES cells lacking all three DNA methyltransferases are still viable, indicating that DNA methylation is not required for ES cell self-renewal.

5-Hydroxymethylcytosine is a demethylation-product of 5-methylcytosine by tet-family DNA demethylase. *Tet1* is strongly expressed in ES cells, resulting in abundant distribution of 5-hydroxymethylcytosine in mouse ES cell genome. However, it was demonstrated that *Tet1*-null ES cells, although showing a partial reduction of 5-hydroxymethylcytosine, maintained pluripotency.

7.1.1.5c Chromatin Remodeling

Chromatin remodeling is involved in the regulation of proper transcriptional activation at the promoter region by altering the histone-DNA contact. Chromatin remodeling proteins can be divided into four families: SWI/SNF (switch/sucrose nonfermentable), CHD (chromodomain helicase DNA binding), ISWI (imitation switch), and INO80 (inositol-requiring 80). It was reported that mouse ES cells express a specific type of SWI/SNF complex designated as esBAF. Knockdown of its major component *Brg1* causes defects in proliferation and differentiation, and knockout of *BAF250a*/*Arid1a* or *Baf250b*/*Arid1b* affect proliferation and differentiation of ES cells, indicating its functional significance. Recently, it was reported that esBAF co-localizes with Stat3 in the genome to exclude PRC2 from its target sites. In contrast, disruption of *Mbd3*, that is a component of NuRD complex of CHD family, results in stable self-renewal in the absence of LIF, suggesting its negative role in ES cell self-renewal. Tip60 of the INO80 family mediates acetylation of H3K4 and was suggested to be involved in ES cell self-renewal, since its knockdown causes prevention of self-renewal, and Chd1 of the CHD family was suggested to be required for the maintenance of proper pluripotency by knockdown experiment. Both of these hypotheses are awaiting confirmation by gene targeting.

7.1.1.6 miRNA in ES Cell Self-Renewal

miRNA regulates a set of gene expression by binding to its target sequence. Processing of miRNA is mediated by Drosha/Dgcr8, and *Dgcr8*-null ES cells show slow proliferation ratios with a lack of all miRNA, indicating that the role of miRNA in ES cells is reduced to the positive regulation of their proliferation.

7.2 PREVENTION OF DIFFERENTIATION

To maintain ES cell self-renewal by keeping pluripotency, pluripotency-associated transcription factors must counteract the induction of differentiation. Mouse ES cells directly differentiate into *only* three cell lineages; primitive ectoderm, primitive endoderm, and trophectoderm. Mouse ES cells rarely differentiate to trophectoderm after withdrawal of LIF or by formation of embryoid body. However, when *Oct3/4* expression is artificially reduced, ES cells are homogeneously converted into trophectoderm, indicating that Oct3/4 blocks the differentiation program toward trophectoderm. *Cdx2* and *Eomes* are upregulated immediately after repression of *Oct3/4*, and their forced expression is sufficient to induce trophectoderm differentiation, suggesting that these trophectoderm-associated transcription factors are critical repressive targets of Oct3/4 in ES cells. It was revealed that Oct3/4 and Cdx2 form a repressor complex that inhibits their own expression, suggesting the reciprocal inhibitory loop between pluripotency- and trophectoderm-associated transcription factors involves a sharp segregation of these cell lineages.

In the case of primitive endoderm, the Gata-family transcription factors Gata4 and Gata6 have a pivotal role to determine this cell fate. Primitive endoderm differentiation is induced in embryoid bodies as well as in culture without LIF. Induction of *Gata4* and *Gata6* is coupled with these differentiation events, and forced expression of either *Gata4* or *Gata6* in mouse ES cells triggers differentiation toward primitive endoderm which resembles the character of eXtraembryonic ENdoderm stem (XEN) cells derived from blastocysts. Since *Gata4* and *Gata6* link via cross auto-regulation, their activities should be repressed in ES cells. It was suggested that Nanog works as a repressor of *Gata6* expression, because the Nanog expression pattern is reciprocal to Gata6 in preimplantation embryos, and also because *Gata6* is directly repressed by Nanog *in vitro*. In addition, the original report of *Nanog*-null ES cells showed that they acquire an XEN cell-like character, although later reports showed no such phenotype in *Nanog*-null ES cells. To establish a reciprocal expression pattern, *Nanog* and *Gata6* may form a reciprocal inhibitory loop as in the case of *Oct3/4* and *Cdx2*, but so far there is no direct evidence of repression of *Nanog* by Gata6. The repression of *Nanog* might be indirect via activation of the repressive nuclear receptors such as Gcnf and Couptfs.

Conversion of mouse ES cells to primitive ectoderm occurs naturally in embryoid body formation. However, no transcription factor that acts as a trigger of this conversion, as Cdx2 in trophectoderm and Gata6 in primitive endoderm differentiation, has been identified. When the expression of pluripotency-associated transcription factors is compared in naïve and primed pluripotent stem cells, many transcription factors such as *Klf4*, *Tbx3*, *Dax1*, and *Rex1* are expressed at higher levels in the naïve state than in the primed

state. These naïve-specific genes may have functional significance to trigger conversion to the primed state by their downregulation, but there is no direct evidence to support this idea to date.

7.3 MAINTENANCE OF STEM CELL PROLIFERATION

Mouse ES cells proliferate continuously, with a doubling time of 12–14 hours in the conventional culture with LIF. Such rapid proliferation is based on the characteristic cell-cycle regulation, which allows unlimited transition from G1 to S phase. It was shown that Rb, which acts as a major regulator of G1-S checkpoint by inhibition of E2F transcriptional activity, is constitutively inactivated by phosphorylation, and mouse ES cells lacking all three *Rb*-related genes proliferate normally, although the precise mechanism mediating hyperphosphorylation of Rb is unknown.

Mybl2 is a pleiotropic regulator of cell-cycle promotion, and one of its major functions is the promotion of G2-M transition. It was demonstrated that Mybl2 (also known as B-Myb) is required for preimplantation development and normal ES cell proliferation. Interestingly, *Mybl2* is a putative target of Oct3/4, and Mybl2 activates the transcription of *Oct3/4*, suggesting a positive regulatory loop between cell-cycle promotion and pluripotency-associated transcription factor network.

The PI3K–Akt pathway has a dual role in mouse ES cells. One is the transduction of the LIF signal to activate the pluripotency-associated transcription factors such as *Tbx3* and *Nanog*. Another is the promotion of proliferation as found in many other cell types. Eras is a member of the Ras family small-GTP binding protein and is specifically expressed in ES cells. *Eras*-null ES cells show reduced proliferation ratio whereas its forced expression promotes proliferation, indicating its positive function in ES cell proliferation. Eras constitutively activates PI3K but not the Raf–MAPK pathway, suggesting that its function is mediated by the PI3K–Akt pathway. Tcl1 encodes an adaptor protein that promotes dimerization of Akt and their phosphorylation. *Tcl1* is a direct target of Oct3/4 and knockdown of *Tcl1* causes reduction of proliferation of ES cells, suggesting the positive regulation of the PI3K–Akt pathway by the pluripotency-associated transcription factors to promote proliferation.

Sall4- and *Klf5*-null ES cells show slower proliferation than wild-type ES cells, suggesting their positive role on ES cell proliferation. Sall4 belongs to the spalt-like zinc-finger transcription factor family. *Sall4*-null embryos die after implantation, and ICM isolated from *Sall4*-null blastocysts shows defective growth *in vitro*, indicating its function in proliferation of pluripotent cell

population *in vivo*. *Sall4*-null ES cells still possess pluripotency, as shown by their contribution to three germ layers after blastocyst injection. However, they show higher incidence of differentiation toward trophectoderm, indicating its function in repressing trophectoderm-associated transcription factors, which could be mediated by interaction with Oct3/4. Klf5 encodes a Krüppple-type, zinc-finger transcription factor, and *Klf5*-null ES cells show a fairly similar phenotype to that of *Sall4*-null ES cells. It is speculated that the function of Klf5 in promoting proliferation is partly mediated by transcriptional activation of *Tcl1*.

7.4 MAINTENANCE OF TELOMERE LENGTH

Mouse ES cells are supposed to have the ability to proliferate indefinitely. It was demonstrated that mouse ES cells can be maintained up to about 250 cumulative doublings with no indication of crisis or transformation. They indeed have high telomerase activity, and telomerase RNA component (*Terc*)-null ES cells show gradual loss of telomere length as well as reduction of proliferation rate after 300 divisions followed by virtual termination of cell growth at 450 divisions.

An additional mechanism for maintaining the telomere length of mouse ES cells was reported. Zscan4 (zinc-finger and SCAN domain containing 4) was first noticed for its 2-cell-stage-specific expression in embryos, and its heterogeneous expression in ES cells. When *Zscan4* was knocked down, telomeres were shortened and karyotype abnormalities accumulated, resulting in crisis by passage eight (~50 divisions), although telomerase activity was maintained. Zscan4 forms a complex with meiosis-specific homologous recombination proteins such as Dmc1 and Spo11, and mediates the maintenance of telomere length by homologous recombination between telomeres when *Zscan4* expression is transiently activated. Interestingly, *Zscan4* is listed as a putative target of the PI3K–Akt pathway as are Tbx3 and Nanog, suggesting a function as a mediator of the effect of this pathway on cell proliferation.

7.5 X CHROMOSOME INACTIVATION

In female somatic cells, one of the X chromosomes is randomly inactivated for dosage compensation. This event, termed random X chromosome inactivation (XCI), occurs in the epiblast of postimplantation embryos at around E5.0. As mentioned earlier, female naïve pluripotent stem cells, such as female mouse ES cells, possess two active X chromosomes, whereas female primed pluripotent stem cells such as EpiSCs carry one inactive X chromosome, indicating that the maintenance of active X chromosomes by inhibiting random XCI is a unique feature of mouse ES cells. XCI is initiated by

the expression of a non-coding RNA on the X chromosome, *Xist*. *Xist* mRNA coats the X chromosome carrying transcriptionally active *Xist* and mediates the recruitment of epigenetic machinery such as PRC2 to silence the entire chromosome. It was reported that *Xist* expression is negatively regulated by Oct3/4, Sox2, and Nanog, and that Rnf12, the positive regulator of *Xist*, is also negatively regulated by these three transcription factors. In contrast, *Tsix*, a negative regulator of *Xist*, is positively regulated by Rex1, Klf4 and Myc. Moreover, the involvement of Oct3/4 was demonstrated in X chromosome pairing and counting in cooperation with Ctcf. This evidence indicates that the pluripotency-associated transcription factor network is tightly coupled with XCI regulation to inhibit its activity in the undifferentiated state and promote its activity immediately after differentiation.

Imprinted XCI is a characteristic event found in extra-embryonic cell lineages. In female embryos, the paternal X chromosome is reactivated after fertilization, but selectively inactivated in 4- to 8-cell stages. This state is kept in the trophectoderm and primitive endoderm, whereas reactivation occurs in pluripotent stem cells in the ICM. Imprinted XCI is mediated by the epigenetic marks on both paternal and maternal X chromosomes. Although imprinted XCI is tightly coupled with lineage restriction toward extra-embryonic cell lineages, female ES cells undergo random X inactivation when they are directed to differentiate into primitive endoderm and trophectoderm by forced expression of *Gata6* and *Cdx2*, respectively, suggesting the complete erasure of the epigenetic marks for imprinted XCI in female ES cells.

7.6 SUMMARY

Mouse ES cells are the best analyzed among stem cells. Pluripotency is a unique stem cell state that allows the generation of all cell types which appear in development. The artificial activation of particular transcription factors triggers the induction of homogeneous differentiation, providing a good model system for studying how self-renewal switches to differentiation. Extensive analyses of ES cell self-renewal and differentiation at the molecular level will provide a basic concept of how stem cell self-renewal is regulated in general.

FOR FURTHER STUDY

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