Chapter 13

Molecular Mechanisms of Embryonic Stem Cell Pluripotency

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1. Introduction

Embryonic stem (ES) cells isolated from the inner cell mass (ICM) of blastocysts possess the defining pluroptency: unlimited self-renewal and giving rise to all cells of the organism[1, 2]. Thus, ES cells hold great promise for regenerative medicine to treat many diseases including heart failure, diabetes, Alzheimer's and Parkinson's disease by replacing the damaged cells with ES cell-derived healthy ones. The recent advent of induced pluripotent stem (iPS) cells reprogrammed from somatic cells has the potential to revolutionize the field of regenerative medicine since patient-derived iPS cells, in principle, circumvent the ethical problems and immune rejection associated with human ES cells[3]. Nevertheless, the future clinical translation of ES cells and iPS cells is facing numerous hurdles. Understanding the molecular mechanisms that impart ES cells with pluripotency may help address some of these challenges. The past few years have seen tremendous progress in understanding of mechanisms which govern ES cell pluripotency. In this chapter, we will review critical signaling and transcription factor networks that have been identified to maintain ES cell pluripotency.

2. Signaling pathways of ES cells

ES cells require extrinsic growth factors to maintain their pluripotency in culture. These extrinsic growth factors act on different signaling pathways to regulate intrinsic transcription factor networks to sustain ES cells in the undifferentiated state. The signaling pathways required to support pluripotency in mouse ES cell are distinct from those in human ES cells (Figure 1).

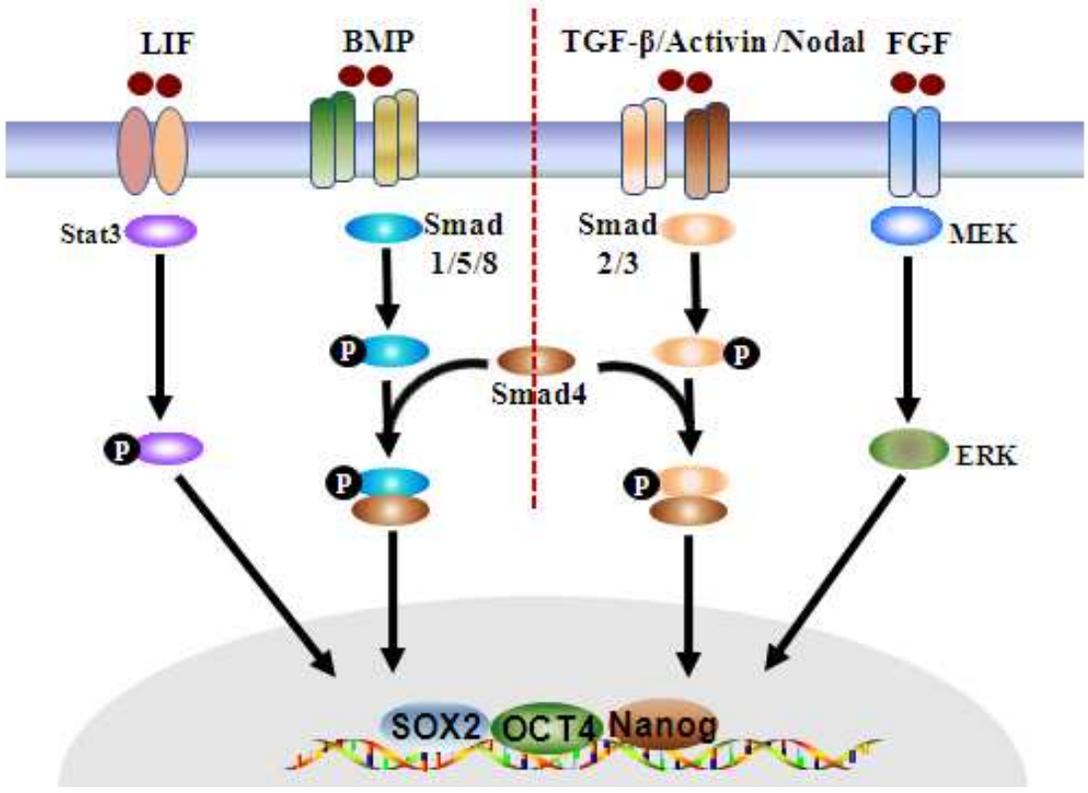

Figure 1. Exogenous growth factors signal through distinct signaling pathways to regulate transcription factors for ES cell pluripotency.

2.1. LIF/JAK/STAT3 pathway

Mouse ES cells were originally cultured on feeder layers derived from mouse embryonic fibroblasts (MEF). Later it was found that Leukaemia Inhibitory Factor (LIF), a member of the Interleukin-6 cytokines produced by MEFs, was the key factor to maintain pluripotency of mouse ES cells by inhibiting their differentiation[4]. Upon LIF binding, the LIF receptor recruits gp130 to form a heterodimer which subsequently activates Janus kinase (JAK) through transphosphorylation[5]. Activated JAK then phosphorylate gp130, creating a docking site to bind the SH2 domain of Signal Transducers and Activators of Transcription 3 (STAT3)[6-9]. Once STAT3 binds to the gp130 docking site, JAK then phosphorylates the recruited STAT3. Phosphorylated STAT3 forms a homodimer, which subsequently translocate into the nucleus, where it binds to gene enhancers to regulate target gene expression[10-12].

Although the LIF/JAK/STAT3 pathway has been well documented to maintain pluripotency of mouse ES cells in the presence of serum, the mechanisms by which activated STAT3 functions in this regard is poorly understood. Recently, studies in identification of STAT3 target genes have improved our understanding of activated STAT3 in maintaining pluripotency. Chen et al identified 718 STAT3-bound genomic sites that were co-occupied by pluripotency transcription markers (Oct4, Sox2 and Nanog) by using chromatin immunoprecipitation sequencing (ChIP-seq)[12]. In addition, Kidder and colleagues found that STAT3 target genes

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enriched in ES cells were downregulated in differentiated cells by mapping STAT3 binding targets in mouse ES cells and differentiated embryoid bodies (EBs)[13]. Along with these results, it has been demonstrated that knocking down STAT3-target genes induces activation of endodermal and mesodermal genes, supporting the conclusion that STAT3 prevents mESC differentiation by suppressing lineage-specific genes[14].

Interestingly, the LIF receptor and gp130 are also expressed in human ES cells and human LIF can induce STAT3 phosphorylation and nuclear translocation in human ES cells. However, human LIF is unable to maintain the pluripotent state of human ESs, suggesting that mouse and human ES cells require distinct signaling mechanisms to govern their pluripotency[15].

2.2. TGF-β signaling

TGF-β superfamily consists of more than 40 members, including TGF-β, Activin, Nodal, and bone morphogenetic proteins (BMPs). The TGF-β members transduce signals by binding to heteromeric complexes of serine/threonine kinase receptors, type I and type II receptors, which subsequently activate intracellular Smad proteins. Smads 2 and 3 are specifically activated by activin, nodal and TGF-β ligands, whereas Smads 1, 5 and 8 are activated by BMP ligands[16, 17] (Figure 1). The TGF-β-related signaling pathways play complex roles in regulating the pluripotency and cell fate of ES cells.

2.2.1. BMP signaling pathway

Bone Morphogenetic Protein (BMP) is a subset of the TGF-β superfamily[18]. When BMP ligands bind to type II BMP receptors (BMPRII), BMPRII then recruits and phosphorylates type I BMP receptors (BMPRI). Activated type I receptors subsequently phosphorylate BMP-responsive SMAD1/5/8 which then forms a complex with SMAD4 and translocates into nucleus to regulate target gene expression (Figure 1). In mouse ES cells, LIF can substitute MEF feeder layers in maintaining pluripotency in the presence of animal serum by activating the transcription factor STAT3. However, in serum-free cultures, LIF is insufficient to block neural differentiation and maintain pluripotency. Recently, Ying et al reported that BMP was able to replace serum to maintain pluripotency of mouse ES cells in the presence of LIF. BMP has been shown to phosphorylate SMAD1/5 and activate inhibitors of differentiation (*Id*) genes, which block neural differentiation by antagonizing neurogenic transcription factors[19]. In the absence of MEF and serum, exogenous LIF, in combination with BMP4 proteins, can sufficiently maintain the pluripotency of mouse ES cells derived from "permissive" mouse strains.

In contrast to a maintenance role in mouse ES cell pluripotency, BMP has been shown to promote human ES cells differentiation to trophoblasts, and inhibiting BMP signaling with the BMP antagonist, Noggin, sustains the undifferentiated state of human ES cells[20, 21]. In consistence, dorsomorphin and DMH1, small molecule BMP inhibitors previously identified in our lab, were shown to promote long-term self-renewal an pluripotency of human ES cells, presumably by inhibiting BMP induced extraembryonic lineage differentiation[22-25].

2.2.2. TGF-β/activin/nodal signaling pathway

Although MEFs feeder layers were initially used to co-culture both mouse and human ES cells, signal factors secreted from MEFs to maintain pluripotency of the two types of ES cells are fundamentally different. Sato et al first discoveried that TGF-β and Nodal genes were highly expressed in undifferentiated human ES cells[26]. Beattie et al later reported that Activin A, a member of the TGF-β superfamily, was secreted by MEFs, and medium enriched with activin A can replace MEF feeder-layers or MEF-conditioned media to maintain human ES cells in an undifferentiated state[27]. In consistence, James et al demonstrated that the TGF-β/Activin/Nodal pathway was activated through the transcription factors Smad2/3 in undifferentiated human ES cells[28]. The notion that TGF-β/Activin/Nodal signaling supports human ES self-renewal and pluripotency is further supported by the fact that recombinant Activin or Nodal stimulation induces higher levels of pluripotent protein expression (Oct4 and Nanog), while inhibition of TGF-β/Activin/Nodal signaling with Lefty or Follistatin decreases expression of these pluripotent proteins in human ES cells[29, 30].

Recent studies have focused on understanding the molecular mechanisms of TGF-β/Activin/Nodal signaling in retaining human ES cells pluripotency. Xu and colleagues showed that TGF-β/Activin/Nodal signaling activated Smad2/3 which subsequently binds to the Nanog promoter in undifferentiated human ES cells to induce expression of Nanog, a pluripotent transcription factor[31]. Additionally, mutating the putative Smad-binding sites reduced the response of Nanog to modulation of TGF-β signaling[31]. Nanog was also shown to coordinate with Smad2 in a negative-feedback loop to inhibit human ES cell differentiation[32]. In contrast to its important role in maintaining human ES cell pluripotency, the TGF-β/Activin/Nodal signaling is not essential for pluripotency of mouse ES cells. Although this pathway was shown to be active in undifferentiated mouse ES cells as assessed by phosphorylation of smad 2/3, inhibition of smad 2/3 phosphorylation by SB431542 had no effect on the undifferentiated state of mouse ES cells[28]. However, the TGF-β/Activin/Nodal signaling may play a role in mouse ES proliferation. A recent study showed that Inhibition of TGF-β/Activin/Nodal signaling by Smad7 or SB-431542 dramatically decreased mouse ES cell proliferation without effect on their pluripotency[33].

2.2.3. Growth and Differentiation factor 3 (GDF-3)

GDF-3 is another TGF-beta superfamily member that plays opposite roles in mouse and human ES cells. GDF-3, which acts as a BMP antagonist by direct binding to BMP-4, is specifically expressed in the pluripotent state of both mouse and human ES cells[34]. Ectopic expression of GDF-3 leads to the maintenance of pluripotency in human ES cells, whereas a similar effect is observed in mouse ES cells when GDF-3 levels are decreased. In the absence of LIF, GDF-3-deficient mouse ES cells can still sustain pluripotent markers[34]. These results are consistent with previously discussed BMP signals which can promote pluripotency of mouse ES cells, but cause differentiation of human ES cells. Thus lower concentrations of BMP antagonists, such as GDF-3, may enhance pluripotency in mouse ES cells, whereas higher levels of GDF-3 may favor pluripotency of human ES cells by abrogating BMP signaling.

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2.3. FGF/MEK signaling

The importance of Fibroblast growth factor (FGF) signaling for human ES cells pluripotency is highlighted by the facts that human ES cells are traditionally cultured in the presence of Fibroblast growth factors (FGFs) either on fibroblast feeder layers or in fibroblast-conditioned medium[35, 36]. Studies have demonstrated that all four FGF receptors (FGFR1, FGFR3 and FGFR4) and several components (SOS1, PTPN11 and RAF1) of their downstream activation cascade are significantly upregulated in undifferentiated human ES cells, in comparison to differentiated human ES cells[37-39]. In consistence, withdrawal of FGFs or inhibition of FGF signaling by a FGFR inhibitor, SU5402, rapidly induces human ES cell differentiation[40-42].

Although the pluirpotency maintenance role of exogenous FGFs in human ES cell has been known for a long time, the molecular mechanisms by which they function remain unclear. FGFs signal by binding to FGF receptors (FGFRs), and activate multiple signaling cascades, including Mitogen-Activated Protein Kinases (MAPKs), the Janus kinase/signal transducer and activator of transcription (Jak/Stat), phosphatidylinositol 3-kinase (PI3K) and phosphoinositide phospholipase C (PLCg) pathway[43]. Several studies have highlighted the FGF contribution to the maintenance of human ES cells mainly through the FGF/MEK pathway (Figure 1), [44, 45]. Studies have showed that FGF2 induces feeder layer cells to secret TGFβ1 and insulin-like growth factor 2 (IGF2), which can subsequently promote the undifferentiated state of human ES cells[46, 47]. Bendall et al further reported that the function of exogenous FGFs in promoting ES self-renewal could be replaced by addition of IGF2 alone, suggesting an indirect role of FGFs for human ES cell growth. However, this model was challenged in subsequent publications from Wang et al who reported that exogenous IGF2 alone was insufficient to maintain undifferentiated growth of human ES cells, and they proposed that FGFs may play a direct role in blocking caspase-activated apoptosis through anoikis in human ES cells[48]. Recently, Eiselleova and colleagues postulated a new model whereby endogenous FGF-2 signaling maintained the undifferentiated state and survival of human ESCs, while exogenous FGF-2 mainly suppress cell death and apoptosis genes, thus indirectly contributing to the maintenance of human ES cell pluripotency[49].

FGF signaling in mouse ES cells has also been extensively investigated. Mouse ES cells genetically deficient in Fgf4 and extracellular-signal regulated kinase 2 (Erk2) differentiate inefficiently. These results can be reproduced using inhibitors of FGF receptor and ERK, suggesting blockage of the FGF/MEK signaling pathway promotes mouse ES cell pluripotency[50-52]. Indeed, serum-free mouse ES cell medium supplemented with FGF/MEK inhibitors and LIF permits the derivation of mouse ES cells in the absence of feeders from strains normally considered non-permissive[53]. In addition, a recently identified compound, Pluripotin/SC1, has been shown to maintain mouse ES pluripotency by inhibiting ERK1 and activating the phophoinositide-3 kinase (PI3K) pathway through blocking RasGAP[54-56] [57, 58]. Although inhibition of FGF/MEK pathway can attenuate ES cell differentiation, it is insufficient to support mouse ES cell self-renewal. Combination of the MEK inhibitor PD0325901 with the Glycogen synthase kinase-3 (GSK-3) inhibitor CHIR99021 (known as 2i) can efficiently sustain the pluripotency of mouse ES cells in the absence of exogenous cytokines[59, 60]. Several groups dem-

onstrated that improvement of mouse ES cell pluripotency by inhibition of GSK-3 occurred via Wnt/β-catenin signaling, whereas many others argued that GSK3 was likely to exert β-catenin independent effects in ES cells[59, 61-67].

As demonstrated above, human and mouse ES cells are both derived from blastocyst-stage embryos, but they require different biological signals for maintaining pluripotency. In general, mouse ES cells maintain their pluripotency by activating LIF/STAT3 and BMP signaling, while human ES cells require TGF-β/Nodal and FGF/MEK pathways. Interestingly, several pathways, such as BMP and FGF/MEK, have completely oppositing effects on maintaining the pluriotency of mouse and human ES cells. Activation of BMP signaling and inhibition of the FGF/MEK pathway promote mouse ES self-renewal, whereas inhibition of BMP signaling and activation of FGF/MEK pathway sustain human ES cell pluripotency. These distinct signaling effects on pluripotency may reflect intrinsic differences between mouse and human ES cells. Recent studies have demonstrated that conventional human ES cells do not represent the "ground or naïve state" of stemness, but rather a more developmentally mature "primed state" resembling mouse epiblast stem cells (mEpiSCs) found in the post-implantation, pre-gastrulation stage of embryos [68-74]. Conventional human ES cells exhibit numerous similarities to the mouse EpiSCs over mouse ES cells (Table 1). For instance, conventional human ES cells and mouse EpiSCs display flattened cell colonies and epigenetic X-chromosome inactivation (XiXa), and require Activin and FGF for pluripotency maintanince. In contrast, mouse ES cells exhibit dome-shaped colony morphology and epigenetic activation of both X-chromosome (XaXa), and require LIF/STAT3 signaling to promote self-renewal. Subsequent studies have demonstrated that the medium containing "2i" (MEK inhibitor and GSK-3 inhibitor), when supplemented with other factors (such as forskolin), can efficiently convert conventional human ES cells into a ground or "naïve" state with display of hallmark features of mouse ES cells. This medium can also maintain human ES cell pluriptoency at the naïve state [69, 70, 72, 75-78].

property	mESCs	mEpiSCs	hESCs	hiPSCs
morphology	domed	flattened	flattened	flattened
clonogenicity	high (single cells)	low (clumps)	low (clumps)	low (clumps)
response to LIF/Stat3	self-renewal	none	none	none
response to Activin/bFGF	differentiation	self-renewal	self-renewal	self-renewal
response to BMP	self-renewal	differentiation	differentiation	differentiation
XX status	XaXa	XaXi	XaXi	XaXi
teratoma	yes	yes	yes	yes
chimaera	yes	no	ND	ND

Table 1. Comparison of the properties of mouse ES cells (mESCs), mouse epiblast stem cells (mEpiSCs), human ES cells (hESCs) and human iPS cells (hiPSCs).

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3. The regulatory network of pluripotency factors

ES cell pluripotency is conferred by a unique transcriptional network[79]. Early global transcriptional profiles and genetic studies have identified several critical transcription factors that are required for the pluripotency of ES cells, such as Oct4, Sox2, Nanog, Foxd3 and Id, etc [80-88]. Here we will mainly focus on Oct4, Sox2 and Nanog, three key transcription factors of the core pluripotency transcriptional network.

3.1. OCT4 and SOX2

OCT4 (also known as Oct3), a POU domain-containing transcription factor, was one of the first transcription factors identified as essential for both early embryo development and pluripotency maintenance in ES cells[84, 89]. The expression of Oct4 is activated at the 8-cell stage and is later restricted to the inner cell mass (ICM) and germ cells in early mouse embryogenesis *in vivo* [89-92]. Oct4 is highly expressed in both human and mouse ES cells, and its expression diminishes when these cells differentiate and lose pluripotency. Oct4 regulates a broad range of target genes including Fgf4, Utf1, Opn, Rex1/ Zfp42, Fbx15, Sox2 and Cdx2[93-95]. Repression of Oct4 activity in ES cells upregulates Cdx2 expression, leading to ES cell differentiation into trophectoderm[96]. Oct4 is also known to activate downstream genes by binding to enhancers carrying the octamer–sox motif (Oct–Sox enhancer), for synergistic activation with Sox2. In contrast with its target genes, little is known about Oct4 upstream regulators. The Oct4 promoter contains conserved distal and proximal enhancers that can either repress or activate its expression depending on the binding factors occupying these sites[97, 98]. The precise level of Oct4 is important for ES cell fate determination. Loss of Oct4 causes inappropriate differentiation of ES cells into trophectoderm, whereas overexpression of Oct4 results in differentiation into primitive endoderm and mesoderm[99, 100].

Sox2 is an HMG-box transcription factor that is detected in pluripotent cell lineages and the nervous system[101-103]. Inactivate Sox2 *in vivo* results in early embryonic lethality due to the failure of ICM maintenance[102]. Sox2 can form a complex with the Oct4 protein to occupy Oct–Sox enhancers to regulate target gene expression. Oct–Sox enhancers are found in the regulatory region of most of the genes that are specifically expressed in pluripotent stem cells, such as Oct4, Sox2, Nanog, Utf1, Lefty, Fgf4 and Fbx15[93, 94, 104-108].

3.2. Nanog

Nanog is another homeobox-containing transcription factor that is specifically expressed in pluripotent ES cells. The essential role of Nanog in maintaining the pluripotency of ES cells is highlighted by the facts that Nanog-deficient ES cells are prone to differentiation, whereas forced expression of Nanog partially renders ES cells self-renewal potential in the absence of LIF[85, 86, 109]. How Nanog regulates stem cell pluripotency remains entirely unknown. Studies have indicated that Nanog may maintain ES cell pluripotency by 1) downregulating downstream genes essential for cell differentiation such as Gata4 and Gata6 and 2) activating the expression of genes necessary for self-renewal such as Rex1 and Id[19, 85, 86]. Although it is widely accepted that Nanog, like Oct4 and Sox2, play a central role in

pluripotency maintenance, this dogma has been challenged by a subsequent report that Nanog protein levels are undetectable in a fraction of ES cells that express Oct4, and the pure populations of Nanog−/− ES cells can be propagated without losing expression of other pluripotency markers[110].

Little is known about the mechanism by which Nanog is regulated in ES cells. Recently, Suzuki et al showed that Nanog expression was upregulated by BrachyuryT and STAT3 in mouse ES cells[111]. In human ES cells and in mouse EpiSCs, Vallier et al reported that Activin/Nodal signaling stimulated expression of Nanog, which in turn prevents FGF-induced neuroectoderm differentiation [112]. In addition, several studies indicated that the Oct4/Sox2 complex was directly bound to the Nanog promoter to regulate target gene expression [106, 107, 113]. Genomic studies have revealed that Oct4, Sox2, and Nanog frequently bind the same regulatory regions in undifferentiated mouse and human ESCs, and that these binding sites are often in close proximity to one another[113-116]. These results indicate that Oct4, Sox2, and Nanog may physically interact with each other and coordinately regulate target genes in some cases. Additionally, Goke and colleagues reported that combinatorial binding sites of the Oct4/Sox2/Nanog were more conserved between mouse and human ES cells than individual binding sites were [113, 114, 117-119].

4. Summary

Understanding the molecular mechanism of pluripotency can greatly expand our knowledge of ES cell biology and facilitate future stem cell clinical applications. In the past few years, we have seen tremendous advances in understanding ES cell pluripotency. Although mouse ES cells and conventional human ES cells require distinct signaling pathways to maintain pluripotency, they display similar gene expression profiles, activities of transcription factors (such as Oct4, Nanog and Sox2) and transcription factor networks. Our understanding of pluripotency has been further expanded by the advent of iPS cells and the very recent discovery that conventional human ES cells are more equivalent to mouse EpiSCs, but rather "naïve state" of mouse ES cells. Nevertheless, our knowledge of the molecular mechanisms of ES cell pluripotency is still very limited. For instance, it remains unknown how growth factors establish and control transcriptional networks to regulate pluripoency and how ES cells respond so precisely to exogenous cues. Given the rapid advance in ES cell biology, we anticipate the molecular mechanisms underlying pluripotency of ES cells will soon be uncovered and pluripotent stem cells, such as ES cells and iPS cells, will be widely used for clinical applications in the near future.

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