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Chapter 8

Molecular Mechanisms Underlying Pluripotency

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Additional information is available at the end of the chapter

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1. Introduction

Pluripotency and self renewal are the two primary characteristics of pluripotent stem cells (PSCs) [1]-[4]. **Pluripotency** refers to the capacity of a single cell to give rise to any cell type of an embryo or an adult animal [5],[6]. A mammalian organism is developed from a single fertilized egg, the zygote, in an extremely ordered and error-proof fashion [7]. The zygote and the subsequent 2 to 4-cell stage blastomere are considered to be **totipotent** since they can give rise to the entire fetus, including the embryo and the extra embryonic tissue such as the placenta and the umbilical cord (Fig.1) [7]. As embryo development proceeds to 8-cell stage and beyond depending on the species, the cells in the blastomere gradually lose their totipotency. At about embryonic day 3.5 (E3.5) in mouse (about E5 in human) the blastomere compacts into a blastocyst in which two distinct cell populations reside. Cells in the outer layer of the blastocyst form the trophectoderm (TE) which eventually give rise to the extra embryonic tissue, trophoblast of the placenta, whereas cells in the inside of the blastocyst form the inner cell mass (ICM). The ICM then gives rise to additional two lineages of cells, the primitive endoderm (PrEn or hypoblast) and the primitive ectoderm (PrEc or epiblast) (Fig.1). The PrEn produces the secondary extra embryonic tissues, such as yolk sac, allantois and amnion, while the PrEc gives rise to all three germ layers of the embryo, namely the ectoderm, the mesoderm and the endoderm (Fig.1). Although the extra embryonic tissues are indispensible for mammalian embryonic development, it is the ICM derived PrEc (or epiblast) cells that form all the cells of an embryo and adult animal, thus these cells are defined as **pluripotent** [5],[8].

As the embryo implants into the uterus and development further commences to E5-E6.5 days in mouse, some of the post-implantation epiblast cells are found to maintain the capability of producing all derivatives of the three embryonic germ layers [1],[5]. The difference between cells derived from ICM of the pre-implantation blastocyst and those from post-plantation epiblast is that the ICM derived cells express stage-specific embryonic antigen 1 (SSEA1), give rise to all three embryonic germ layers, and most importantly, contribute to chimeric mouse

and germ line transmission, while those from post-implantation epiblast do not express SSEA1 and do not contribute to chimeric mouse and germ line [1],[5],[9]-[11]. Thus the ICM derived cells are defined to be in a "naive (or ground, primordial)" state of pluripotency, and those from post-implantation epiblast are defined to be in a "primed (or refined)" state of pluripotency [1],[5],[12],[13]. Other *in vivo* sources of pluripotent cells include the germline cells extracted either from embryonic or adult male reproduction organs [5],[8] (Fig. 1).

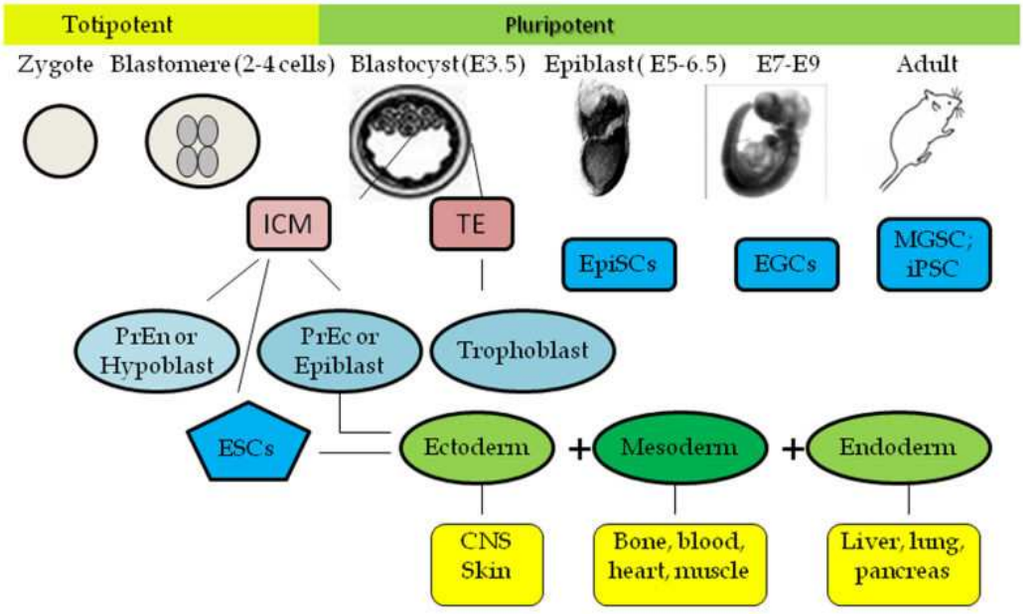

Figure 1. Early embryonic development and sources of PSCs. ICM, inner cell mass; TE, trophectoderm or Trophoblast; EpiSCs, epiblast stem cells; EGC, embryonic germ cells; iPSCs, induced pluripotent stem cells; MGSCs, male germ stem cells; PrEn, primitive endoderm; PrEc, primitive ectoderm; ESCs, embryonic stem cells.

Both the innate totipotency and pluripotency are transient developmental stages in the beginning of embryogenesis [5],[7]. Because of their finite number and transient nature, these cells are very challenging to study, although scientists have showed immense interest to understand them since they hold key answers to many aspects of biology and life.

Intriguingly, pluripotency can be captured or induced in cell cultures with defined growing conditions [14]-[16]. Mouse ESCs (mESCs) are one of the first and best-established ICM-derived cells (Fig.1 and Table 1). Well defined culture conditions allow mESCs to self renew infinitely while maintaining a pluripotent state *in vitro*, providing an invaluable source of cells for molecular studies and differentiation into a variety of desired cell types (Table 1) [17]-[21].

Despite the genetic similarities between mouse and human, it was until two decades later that the first human ESCs (hESCs) were established in cell culture [9]. These cells give rise to all lineages of the primary germ layers and form teratomas (Table 1). Although they were also derived from ICM of pre-implantation embryos, the hESCs demonstrate many striking differences from mESCs (discussed in detail below). Since germ line transmission studies cannot be performed with these cells, it is not known at what exact pluripotency state the hESCs

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are. However, hESCs resemble more closely mouse epiblast stem cells (mEpiSCs), which were derived from post-implantation epiblast [16],[22], indicating that the hESCs are probably in a primed state of pluripotency (Table 1).

Decoding the molecular basis of pluripotency and self renewal is fundamental to the understanding of stem cell biology, embryonic development, and clinical application of regenerative medicine. The *in vitro* culture of these PSCs, especially those from induced pluripotent stem cells (iPSCs), has provided an unprecedented tool to investigate deeper into the molecular mechanism governing pluripotency. Gradually we have uncovered that pluripotency is regulated by a complex network of factors, including transcription factors and epigenetic regulators, which trigger multiple signaling transduction pathways, such as the TGF-β pathway and Wnt pathway. Since the concise molecular mechanism controlling pluripotency varies among the different kinds of PSCs, we will first give a brief introduction of their properties.

	mECCs	**mESCs**	**miPSCs**	**mEpiSCs**	**hESCs**	**hiPSCs**
Origin	Teratoma	ICM of Blastocyst	Somatic cells	Late epiblast	ICM of Blastocyst	Somatic cells
Teratoma formation	Yes	Yes	Yes	Yes	Yes	Yes
Chimera and germ line contribution	Yes	Yes	Yes	No	Not determined	Not determined
Culture conditions	LIF, FBS	LIF, BMP4	LIF	Fgf2, Activin	Fgf2, Activin, MEF CM	Fgf2, Activin, MEF CM
Morphology	Domed shape	Domed shape	Domed shape	Flat	Flat	Flat
X chromosome	XaXa	XaXa	XaXa	XaXi	XaXi	XaXi
Pluripotency status	Naive state			Primed state	Not determined, possibly primed state	
Pluripotency factors	Oct4, Nanog, Sox2, Stat3, Klf2, Klf4,				Oct4, Nanog, Sox2	
Response to LIF	Self renewal and pluripotency				None	
Response to Fgf2	Differentiation				Self renewal and pluripotency	
Response to 2i	Self renewal and pluripotency				Differentiation and cell death	
References	7, 17-19	10-14, 22-28	32-35	3,9,16	4,15	51, 53, 54

Table 1. Properties of some PSCs

2. Properties of PSCs

Mouse ECCs: Mouse embryonic carcinoma cells (mECCs), the first PSCs established in cell culture, were derived in 1964 from teratomas from an inbred mouse line, which produces spontaneous testicular teratomas (Table 1) [14],[23]. These cells show many aspects of naive PSCs, such as the expression of antigen SSEA1, differentiation into all three germ layers when stimulated, and generation of chimeric mice when injected into blastocysts [14],[23]-[25]. However, since the ECCs carry many mutations, the chimeric mice derived from these cells develop spontaneous tumors [26].

Mouse ESCs: Based on the findings made from mECCs, derivation of mESCs directly from ICM of normal developing embryos became possible and faster (Fig.1). Two groups, Kaufman's and Martin's, isolated such cells in culture from the ICM of pre-implantation blastocysts using different protocols in 1981 [15],[27]. And it was Martin who coined the term ESCs [27].

mESCs satisfy all the characteristics of naive pluripotency with a normal karyotype, resembling their *in vivo* counterparts in terms of expressing the pluripotency factors Oct4, Sox2, and Nanog, and SSEA1 and alkaline phosphatase (AP) (Table 1). They can be differentiated into all derivatives of the three germ layers; grow in a dome-shaped morphology; display a high nuclei/cytoplasm ratio; and most importantly, form teratomas and give rise to germ line transmission when injected into blastocysts (Table 1) [17]-[21].

The cell cycle control in mESCs also seems to be unique. They have an unusually short G1 phase and no regulation at the G1–S transition, the presence of hyperphosphorylated retinoblastoma (RB) protein, and unresponsiveness to activity of cyclin-dependent kinase 4 (CDK4) [11],[28]. Epigenetically, mESCs possess a hypermethylated genome and both X-chromosomes are activated if isolated from female embryos [29]-[31].

To grow mESCs, a feeder cell layer of mouse embryonic fibroblasts is typically used, with medium containing ES qualified fetal bovine serum or knockout serum replacement, leukemia inhibitory factor (LIF), non-essential amino acid, and β-mercaptoethanol [20],[21],[32],[33]. LIF serves as the extrinsic factor for pluripotency and self renewal by activating the signal transducer and activator of transcription 3 (Stat3) pathway [21],[34],[35]. Later it was discovered that LIF and Bone morphogenic protein 4 (BMP4) can support mESCs pluripotency without serum (Table 1) [36],[37]. More recently, it has been demonstrated that mESCs can be derived and maintained using medium containing small molecule inhibitors of glycogen synthase kinase (GSK3) (which functions to activate the Wnt signaling pathway) and the mitogen-activated protein kinase (MAPK) signaling pathways, completely devoid of serum and extrinsic growth factors [5],[38]. This culture media with defined components is termed the 2i (2 inhibitors) system [5],[38].

Mouse EpiSCs: Mouse Epiblast Stem cells (mEpiSCs) have been derived from post-implantation blastocysts (E5–E6.5) (Fig.1) [16],[22]. These cells demonstrate the properties of self-renewal and pluripotency, but they cannot colonize the ICM of a blastocyst and produce germ line transmission [13],[16],[22]. Thus mEpiSCs are in the "primed" state of pluripotency. Furthermore, mEpiSCs express high levels of Oct4, Sox2, and Nanog but relatively low levels

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of other pluripotency factors that have been shown to be essential for mESCs, such as Klf4 and Stella [5],[13],[16]. This suggests fundamental differences in the mechanisms that maintain pluripotency in mESCs and mEpiSCs [8],[16].

Epigenetically, mEpiSCs display X chromosome inactivation (XCI) as well as stability of the genetic imprint [3],[5],[8]. This epigenetic status is shared with the late epiblast of the post-implantation embryo, which reinforces the similarity between cultured mEpiSCs and their *in vivo* counterpart. This similarity has been confirmed by gene expression profile experiments, which show that EpiSCs are closely related to the pluripotent cells located in the epiblast of a post-implantation embryo [16],[22]

Mouse EpiSCs also differ with mESCs regarding growing conditions, phenotypes and function. To keep them in a self renewing state, activin, fibroblast growth factor 2 (Fgf2), and transforming growth factor β (TGF-β) are needed, whereas LIF is dispensable and BMP4 leads to differentiation (Table 1) [16],[22]. Instead of growing in a dome-shaped morphology as mESCs, mEpiSCs exhibit a flattened shape and do not propagate well as single cells. Whereas cell cycle regulation in mEpiSCs remains to be investigated in detail, the doubling time of mEpiSCs is 18 hours, compared with only 10–14 hours doubling time of mESCs, suggesting that a normal G1–S transition occurs in mEpiSCs [16],[22].

Human ESCs: Like mESCs, human ESCs (hESCs) were isolated from the ICM of the pre-implantation blastocyst almost two decades after the isolation of mESCs [9]. hESCs possess the potential to differentiae into all three primary germ layers and to produce teratomas when injected into blastocysts [1],[9]. They express high levels of pluripotency factors Oct4, Nanog, and Sox2, and are positive for SSEA3/4 and AP. However, hESCs share multiple defining features with mouse EpiSCs rather than mESCs. These characteristics include flat morphology, dependence on FGF2/Activin signaling to self renew, inclination for XCI, and reduced tolerance to single-cell dissociation by trypsinization (Table 1). These molecular and biological similarities with mEpiSCs suggest that hESCs correspond, at least partially, to the primed pluripotency state rather than to the naive state.

iPSCs: In 2006, Shinya Yamanaka's research group at Kyoto University made a milestone achievement by converting adult mouse cells back to a ground pluripotent stem cell-like state through exogenous expression of only four transcription factors, Oct4, Sox2, Klf4, and c-Myc [39]. These miPSCs exhibit all characteristics of mESCs, including expression of pluripotency marker protein, activation of both X chromosomes, and most importantly, the ability to generate chimeric animals and contributing to germ line transmission (Table 1). Later on, similar cells were also induced from human somatic cells. hiPSCs resemble more hESCs and mEpiSCs than mESCs (Table 1)[40]-[42]. As their production efficiency rapidly improved, iPSCs have soon been able to compete with traditional embryonic and adult stem cells [39]-[50]. The primary advantages of iPSCs compared to other stem cells are: a) iPSCs can be created from the tissue of the same patient that will receive the transplantation, thus avoiding immune rejection, and b) the lack of ethical implications because cells are harvested from a consent individual. These patient-specific cells can be used to study diseases *in vitro*, to test drugs on a human model without ethical concerns, and to hopefully be used as a source of tissue replacement for diseased and damaged cells.

3. Embryonic Germ stem Cells (mEGC) and Male Germ Stem Cell (MGSCs)

PSCs have also been derived from reproductive system cells. When cultivated in adequate growth conditions, reproductive system cells generate ES-like stem cells (it is termed embryonic germ cells (EGCs) if isolated from mouse embryonic day 8.5 embryos [51], or male germ stem cells (MGSCs) if derived from postnatal male gonads [52]) (Fig.1). The EGCs and MGSCs are both naive stem cells, capable of generating all three embryonic germ layer cells, teratomas and chimeras. EGCs and MGSCs have also been derived from human sources, but their characteristics are not as well defined [53]-[55].

Next we will focus on the mESCs, which are in the naive state, and hESCs, which are probably in the primed state, to discuss the molecular mechanism of pluripotency maintenance.

4. Transcription factors regulatating pluripotency

An interplay of transcription factors and epigenetic factors participates in the maintenance of pluripotency of stem cells [34],[35],[56]-[62]. Among them Oct4 (or POU5F1), Nanog, and Sox2 are generally accepted as the core pluripotency factors, since they are vital to maintian the pluripotency of both the hESCs and mESCs, which are in a different pluripotency state [1],[8],[63]. These three factors also collectively bind to an array of genes that are essential for pluripotency and differentiation [1],[8],[63].

4.1. Core pluripotency factors and their transcription cotrol

The POU transcription factor Oct4 is a central player for stem cell pluripotency (Fig.2). Its expression is strictly confined to the totipotent, pluripotent, and germ cells during early development. *In vitro*, the cellular level of Oct4 must be tightly controlled to maintain the pluripotency status, up- or down-regulation by 50% leads to ESC differentiation [64],[65]. *In vivo*, Oct4 deletion in mice leads to ICM failure [57].

The homeoprotein Nanog is another central factor for pluripotency (Fig.2) [66]. The ICM in Nanog-deficient mice fails to generate epiblast and only produces endoderm-like cells [66]. Furthermore, ESCs derived from Nanog-deficient mice cannot maintain pluripotency and instead differentiate into extraembryonic endoderm lineages [66]. Mechanistically, Nanog functions by inhibiting NFκB and cooperating with Stat3 to inhibit cell differentiation in mESCs [66]-[68].

The third central factor is Sox2 (Fig.2) [3],[5],[63],[69]. Sox2 exhibits an expression pattern similar to that of Oct4 during development [70]. Genetic ablation studies indicate that silencing of *Sox2* affects a somewhat later stage of embryogenesis, possibly because of a stronger maternal contribution of Sox2 protein. Key feature of acute *Sox2* loss appears to be an inability to sustain appropriate Oct4 levels [70].

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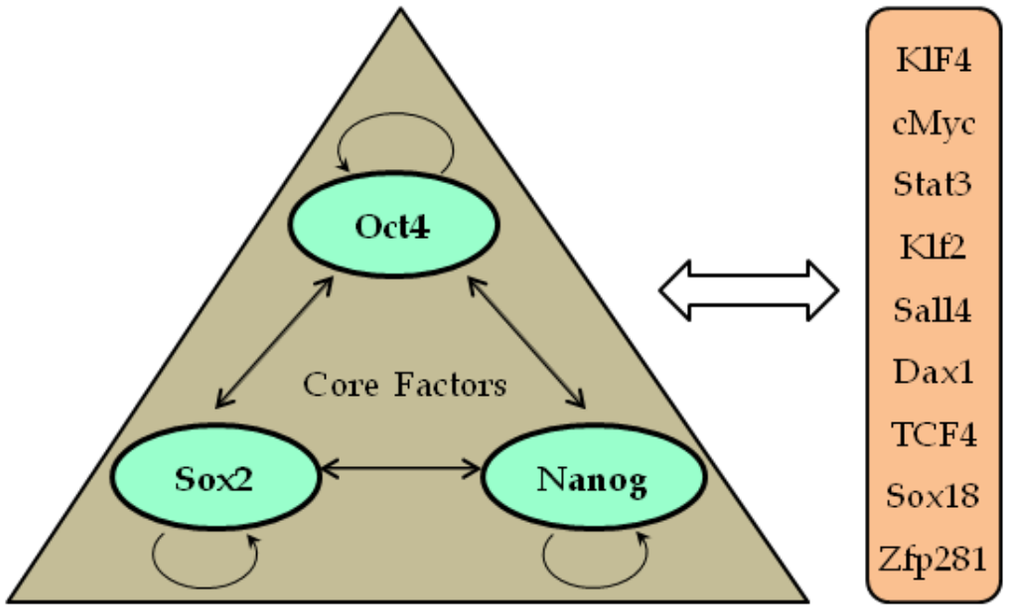

Figure 2. Schematic representation of the transcriptional regulation of core pluripotency factors and the extended factors

These three core factors do not function by themselves. Instead they are involved in a multiple-gene complex to regulate stem cell pluripotency (Fig.2). Their interacting partners have been extensively studied by coimmunoprecipitation (Co-IP) or chromatin immunoprecipitation (ChIP) assays in both mESCs and hESCs. Oct4 has been found to associate with Sox2, Nanog, Smad1, Stat3, TCF3, Rest, Hsp90, etc. [4],[21],[71]-[73]. Nanog seems to interact with Oct4, Smad1, Nac1, Zfp281, and Hsp90 [21],[72]-[74]. And Sox2 associates with Oct4, Nanog, Klf4, Rpa1, Sall4, and Npm1 [75],[76].

One unique aspect of the regulation of these core factors is that they act together to regulate their own promoters, forming an interconnected auto-regulatory feedback loop (Fig.2) [5], [51], [71]. Another unique aspect is that they co-occupy and active/enhance expression of other genes necessary to maintain ESC status, while contributing to repression of genes encoding differentiation signals (Fig.2) [5],[51],[71],[77]-[80]. For example, binding of Oct4 to a promoter region of a gene increases the likelihood of Nanog, Sox2, and other regulatory factors to bind to the same promoter [5],[51],[71],[77]-[80].

Apart from transcriptional control of these core factors, post-translational modifications also play an essential role. Oct4 has been reported to be ubiquitinated in differentiating mouse embryonic carcinoma cells but not in mESCs [81],[82]. Phosphorylation of Nanog promotes its interaction with the prolyl isomerase Pin1, leading to increased Nanog stability by suppressing its ubiquitination[83]. In addition, a recent report demonstrates that Hsp90, a molecular chaperone, associates with Oct4 and Nanog and maintains their cellular level, possibly through protecting them against degradation by the ubiquitin protesome pathway [21]. These studies demonstrate that post-translational modifications and protein stability of the pluripotency factors is also vital for stem cell pluripotency maintenance.

4.2. Extended network of regulatory factors

Besides the core pluripotency factors, many other transcription factors participate in the regulation of stem cell pluripotency, including cMyc, Klf2, Klf4, Stat3, Rex1, Sall4, Zfp281, and the proteins associated with the three core factors [1],[3],[5]. These transcription factors participate in the pluripotency regulation in a state or species-specific fashion. For example, Stat3 plays an important role in mESC pluripotency since target deletion of Stat3 resulted in early embryonic lethality, and ectopic expression of a dominant-negative Stat3 in ESCs leads to loss of pluripotency [34],[35]. However, Stat3 is not suficient to maintain the pluripotency of hESCs and mEpiSCs [21],[84].

5. Signal transduction pathways in pluripotency maintenance

Innate signal transduction pathways are crucially important for understanding the regulation of the stem cell pluripotency. Extensive efforts, including high throughput genetic and chemical screening, have been invested into identifying genes and pathways that affect the core pluripotency factors Oct4, Nanog, and Sox2, or their associated genes. We have now gradually obtained a glimpse of the intrinsic signalling pathways that are involved in the regulation of stem cell pluripotency and differentiation. These signallng pathways include the Wnt pathway, TGF-β pathway, LIF/Stat3 pathway, Fgf pathway, insulin geowth factor (IGF) pathway, Notch pathway, Hedgehog pathway, etc [3]-[5],[71],[74],[85],[86] (Fig.3). Among them, the Wnt and TGF-β pathways are most heavily studied and best understood in terms of their roles in stem cell pluripotency maintenance. Here, we will focus on discussing the Wnt pathway, TGF-β signaling, LIF/Stat3 pathway, and the Fgf pathway (Figs. 3 and 4).

5.1. WNT signaling pathway

The Wnt pathway plays an important role in tissue development by regulating a wide range of cellular processes such as proliferation, adhesion, morphology, and migration [87]-[91]. It consists of over 30 extracellular ligands that bind to Frizzled (FZD) and low-density lipoprotein receptor related protein (LRP) receptors at the cell surface (Fig.3) [91]. The Wnt ligands are able to activate both the canonical pathway and the non-canonical pathway [88],[92]. The activation of Wnt pathway in the canonical pathway results in the preservation of β-catenin and its subsequent nuclear translocation, which enables downstream gene activation by the TCF/LEF family transcription factors [87]-[95]. The non-canonical pathway is independent of β-catenin and involves the activation of several other signaling pathways, such as the JNK pathway [3]-[5],[88],[91],[92].

The Wnt signaling pathway is directly linked to the core transcriptional network of pluripotency and is demonstrated to be essential for self renewal and pluripotency of both naïve and primed PSCs, when LIF is absent. Evidences for this notion include: 1), Wnt signaling is activated in both mESCs and hESCs, and is down-regulated during differentiation [90]; 2), activation of the canonical Wnt pathway is required to maintain the expression level of core pluripotency factors Oct4 and Nanog, through which the self-renewal and pluripotency are

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sustained (Figs. 3 and 4); and 3), ectopic expressing of an constitutively active form of β-catenin maintains the expression levels of Oct4 and Nanog and thus self renewal and pluripotency in ESCs [96].

Moreover, Wnt signaling inhibits the differentiation of ESCs, especially to neural differentiation [90],[97],[98]. Mutation of Apc, an important mediator in the Wnt pathway, leads to impaired differentiation both *in vitro* and in teratomas [99]. Furthermore, ESCs with highly elevated β-catenin levels also have a compromised ability to differentiate [10].

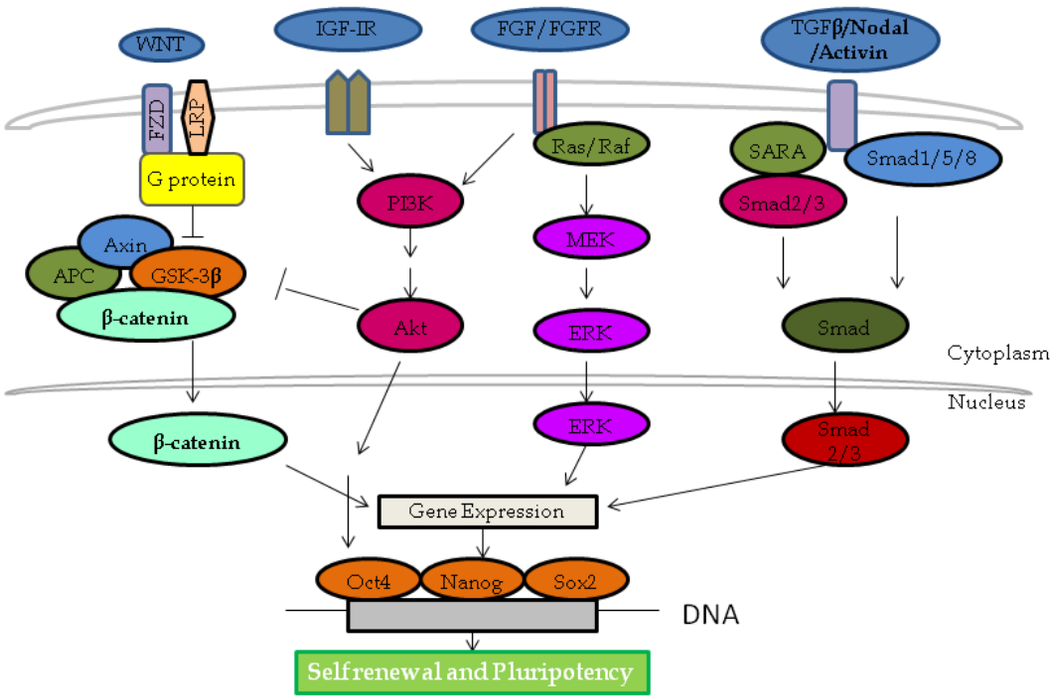

Figure 3. Signaling pathways regulating pluripotency of the primed stem cells, such as mEpiSCs and possibly hESCs

The role of the Wnt signaling in stem cell pluripotency is further confirmed by its down-stream effectors, the TCF/LEF family of transcription factors [72],[88],[92],[100],[101]. In ESCs, TCF3 is the most abundantly expressed member of this transcription factor family. TCF3-null ESCs have an increased resistance to differentiation and up-regulation of various Oct4 and Nanog-regulated genes [74],[102],[103]. Activation of Wnt converts TCF3 into an activator, elevating the expression of these same targets and suppressing differentiation [103]. TCF3 may also suppress the expression of Oct4 and Nanog, although its ability to activate these targets is unclear [104],[105].

However, Wnt signaling alone is not sufficient to support the ground state pluripotency [38]. It has been shown that inhibition of GSK3 in mESCs enhances growth capacity and suppresses neural differentiation, but it also promotes non-neural differentiation [37], [38]. To block differentiation of mESCs, the combination of a GSK3 inhibitor and an FGF-Erk inhibitor (the 2i system) [5],[38] is necessary.

5.2. TGF-β signaling pathway

The TGF-β signaling pathway plays a vital role in both the developmental and adult life of a mammalian organism by regulating many processes including apoptosis, proliferation, senescence, inflammation, cell fate, and tissue repair [94],[106],[107]. The TGF-β super family contains more than 30 growth factors including TGF-βs, BMPs, growth and differentiation factors (GDFs), Activin, and Nodal [2],[106],[108]. The canonical signaling cascade of TGF-β pathway involves the ligands of the TGF-β super family binding to cell surface receptors that activate the Smad proteins in the cytoplasm, which leads to their nuclear translocation and transcriptional activation of target genes [108]. The noncanonical TGF-β signaling includes intracellular signaling pathways activated by TGF-β family members that do not activate Smad proteins [108]. The TGF-β pathway can also be regulated by other key signaling pathways such as Wnt signaling pathways.

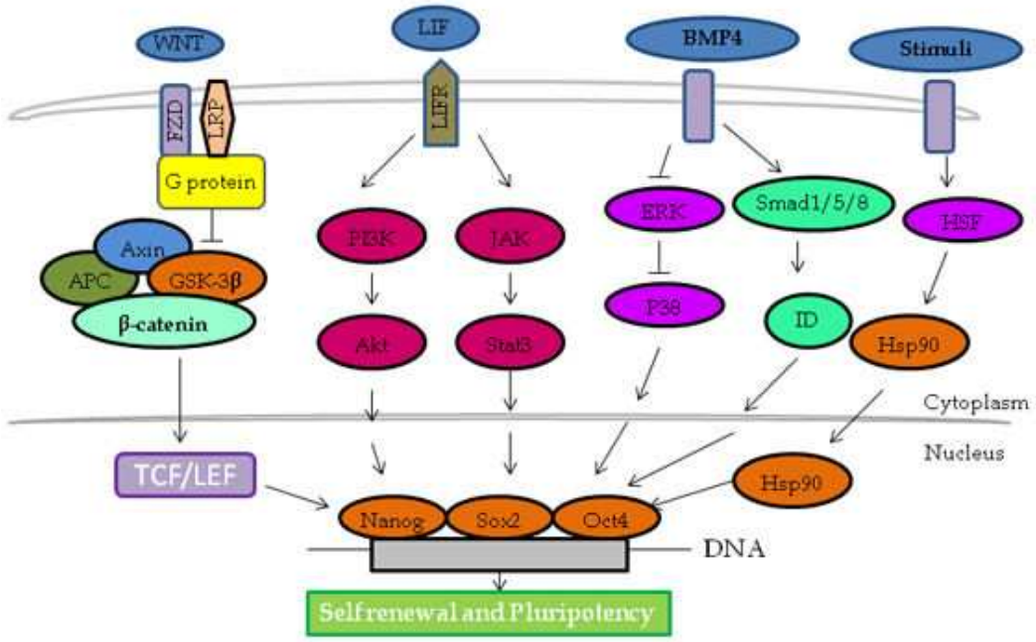

Figure 4. Signaling pathways regulating pluripotency of the naive stem cells, such as mESCs and miPSCs

All members of this family are important for stem cell pluripotency and self-renewal of both mESCs and hESCs, although the role of these signaling molecules appears to differ between the two types of cells [109]. In mESCs, BMP4 maintains self-renewal through inhibition of the MAPK/ERK pathway and the expression of Id protein [36],[37], and promotes mESC proliferation via an increase in Wnt expression (Fig.4) [86]. In contrast, BMP4 promotes hESC differentiation through down-regulation of Nanog and Oct4 [110]. Long-term maintenance of hESC pluripotency therefore requires down-regulation of BMP activity by Noggin and Fgf2 [85]. In hESCs, on the other hand, it is other members of the TGF-β super family that maintain their pluripotency. Phosphorylation and nuclear localization of Smad2 induced by TGF-β, Activin, or Nodal signaling was observed in undifferentiated hESCs and is decreased upon

early differentiation (Fig.3) [111]. Activin A is demonstrated to be able to support long-term feeder-free culture and maintenance of pluripotency in hESCs by inducing the expression of Oct4 and Nanog, and suppressing BMP (Fig.3) [87]. Nodal expression also plays a role in the maintenance of human ES cell pluripotency through the inhibition of neuroectodermal differentiation, a default differentiation pathway of ESCs (Fig.3) [112]. Furthermore, inhibition of the TGF-β/Activin/Nodal pathways initiated differentiation and resulted in the decreased expression of stem cell marker proteins [111],[113].

In addition, Activin and Nodal signaling has been shown to promote mESC self-renewal in serum-free conditions [114]. It is therefore clear that TGF-β signaling plays an important role in the maintenance of self-renewal and pluripotency, although the exact mechanism of action for this family of growth factors appears to differ between family members, pluripotency state, and species (Figs. 3 and 4).

BMPs are also potent inhibitors of differentiation in mouse embryos. Knockdown of their down-stream mediator, Smad1 and Smad4, in mESCs leads to a change of the expression pattern of germ layer markers during differentiation [37],[115],[116]. TGF-β signaling also participates in the cell fate decision making of mESCs. Multiple cell lineages, including neural, hematopoietic, cardiomyogenic, and hepatic, have been found to be affected by the TGF-β family [115],[117]. For example, BMP4 regulates mesodermal cell commitment to the hematopoietic lineage and specifies blood lineages at the later stages of differentiation [118]-[120]. Another study found that BMP4 and Activin induce mesoderm differentiation into cardiac lineage [121]. In addition, BMP2-induced mesodermal and cardiac specification results in full cardiogenic differentiation, leading to an enrichment of cardiomyocytes within embryoid bodies [122]. This ability of the TGF-β family members to commit mESCs toward a mesodermal fate is thought to be due to Smad-mediated regulation of the Oct4 promoter, further implicating a role for Smad signaling in the regulation of the core self-renewal network in ESCs [123], [124]. Consistent with this notion, it is found that several Smad target genes overlap with genes bound by the key pluripotency factors, for example, Smad4-regulated genes have a substantial overlap with those of Sox2, NR0B1/Dax1, and Klf4 [116]. In addition, another study demonstrated that several Smad targets were mapped to Nanog, Oct4, and TCF3-bound genes [116].

Jak/Stat3 pathway The self renewal and pluripotency of mESCs are initially maintained by an extrinsic factor, leukemia inhibitory factor (LIF) [7]. The key downstream mediators of LIF are the Jak/Stat pathway [7]. Stat3 has multiple roles in the regulation of mESC pluripotency including gene activation, cell cycle regulation, and inhibition of differentiation pathways [35], [125]. The activation of the Stat3 pathway by LIF induces transcription of self-renewal and pluripotency genes such as Nanog [35],[125]. Furthermore, constitutively active Stat3 promotes mESC self-renewal in the absence of LIF [35],[125]. Stat3 has also been reported to function through the regulation of c-Myc and Klf family proteins [126],[127], although these target genes have not been shown to be completely sufficient to replace the effect of LIF. This ability of LIF-mediated activation of Stat3 to support the long-term self-renewal of mESCs *in vitro* has been supported *in vivo* by the requirement of this pathway in gp130-deficient blastocysts, an embryonic diapause case [128].

In addition to Stat3 homodimers, Stat1 is able to heterodimerize with Stat3 in mESCs. However, Stat1 is unlikely to be required for self-renewal and pluripotency as LIF still maintains undifferentiated growth of Stat1-deficient cells [129].

Although it is sufficient to maintain the pluripotency of mESCs, the Jak/Stat3 pathway does not appear to maintain pluripotency of hESCs, which are possibly in the primed state of pluripotency [90], indicating that the signaling pathways responsible for maintaining pluripotency is species (or pluripotency state)-specific.

5.3. Fgf and the MAPK pathway

Fgf2 (or basic Fgf) is the first growth factor identified as being crucial for hESC pluripotency maintenance and self renewal. It is widely accepted that a serum-free culture of hESCs on mouse feeder cells requires soluble Fgf2 [88],[107],[130],[131]. In hESCs, exogenous Fgf2 activates the ERK/MAPK pathway, which is thought to be necessary for the maintenance of pluripotency, although the mechanism of action is still unclear [130],[132],[133]. In contrast to hESCs, mESCs and miPSCs do not require the Fgf2 or the ERK/MAPK pathway for pluripotency and self-renewal (Table 1) [5],[11]. Actually, ERK signaling triggers mESCs to differentiate towards the primitive endoderm lineage (Table 1) [1],[134]. Interestingly, inhibition of ERK activity has been shown to enhance the efficiency of mESC derivation from mouse embryos [135]. The mechanism underlying this seems to be that ERK1/2 activation triggers mESCs to exit the self-renewal program and enter lineage differentiation [136]. As mentioned earlier, the direct consequence of this is that blocking the ERK/MAPK-mediated differentiation pathway can help the derivation and maintenance of naive state PSCs, such as mESCs.

6. Epigenetic factors regulating stem cell pluripotency

Apart from the aforementioned transcription factors, epigenetics factors have also been found to play a vital role in stem cell pluripotency. These mechanisms include covalent modification of histone, DNA methylation and acetylation, and non-coding RNAs [31],[62],[137],[138]. Here we will discuss the functions of noncoding RNAs and chromatin remodeling factors in stem cell pluripotency.

6.1. Noncoding RNAs

The best understood class of noncoding RNA is the family of microRNAs (miRNAs), short RNAs capable of destabilizing and repressing specific target RNAs. These miRNAs are generally generated by the enzymes Dicer and Dcgr8 [139]-[141]. As for their function in stem cell pluripotency, it has been shown that genetic ablation of these enzymes affects the cell cycle and differentiation of ESCs [139]-[141]. Furthermore, some specific miRNAs are involved in pluripotency regulation. For example, mir-302 and mir-290–295 bind directly to and modulate the core pluripotency factors Oct4, Sox2, and Nanog [6]. miR-145 represses the 3′ untranslated regions of Oct4, Sox2, and Klf4, thus increasing the amount of mir-145 leads to loss of pluripotency [142]. It is further demonstrated that Oct4 also binds to the promoter of mir-145 and

suppresses its expression, forming a negative feedback loop involving mir-145, Oct4, Nanog, and Klf4 [142].

Another very important member of the miRNA family is let7 which has differentiation promoting activities itself, and also targets some of the pluripotency-associated genes [143], [144]. Let7 expression is negatively regulated by the RNA binding protein Lin28 [145]. Upon differentiation of pluripotent cells, Lin28 is down-regulated, resulting in stabilization and increase in the level of let7 [146]. This in turn provides the basis for establishment of negative feedback loops in which let7 expression is negatively regulated by the RNA binding protein Lin28 [145].

Recently, Oct4 has been shown to control and activate the expression of another type of noncoding RNA, the large intergenic noncoding RNAs [147]. Interestingly, knockdown of the expression of such RNAs caused growth defects and apoptosis, implying that these noncoding RNAs are involved in self-renewal and reprogramming of stem cells [147],[148].

6.2. Chromatin remodeling factors

Chromatin remodeling factors are recruited to the DNA to modify the density of the nucleosomes, thereby affecting gene expression [149],[150]. Some of these factors are essential for PSC viability, stability, and differentiation [31],[151],[152]. The ones known to have the most profound impact on ESC pluripotency are histone-modifying enzymes, such as Polycomb group (PcG) protein complexes, SetDB1, and Tip60-p400 [153],[154]. These enzymes repress genes that encode lineage-specific differentiation regulators by catalyzing methylation or ubiquitination of the histones in their promoters [153]-[155].

It is demonstrated that sumoylated SetDB1 binds to Oct4 and represses its expression [153], [156]. Loss of the Tip60-p400 complex affects ESC morphology and state [154]. The Tip60-p400 complex is shown to associate with active promoters in ESCs and appears to be recruited directly by the H3K4me3 mark and indirectly by Nanog [154]. Interestingly, the complex is also associated with nucleosomes with H3K4me3 at PcG-occupied genes encoding lineage specific regulators, where it apparently facilitates repression of these poised genes [154].

7. Conclusion

Our understanding of the nature of pluripotency has been formulated extensively by the recent development of different lines of PSCs, especially the iPSCs. Although differences exist between them, the naïve and primed PSCs share certain similarities. For example, they both express the core pluripotency factors, Oct4, Nanog, and Sox2. The core transcription factors frequently share enhancers and autoregulate themselves. They also collectively bind to the promoters of an expanded network of proteins, including pluripotency-associated factors and lineage-specific factors, to enhance or repress their gene expression, through which the fate of the cells is determined. The epigenetic studies have added another layer of complexity of the regulation of these core pluripotency factors and hence

pluripotency. In addition, a recent study of our/my laboratory shows that Hsp90 maintains stem cell pluripotency by associating with and sustaining the cellular levels of Oct4 and Nanog, implying that the maturation or stability of these core pluripotency factors are crucially important for stem cell pluripotency [21].

Many of the methodologies to induce or convert somatic cells into PSCs involve using chemical inhibitors targeting specific pathways. This highlights the importance of understanding the roles of signaling pathways in stem cell pluripotency and self-renewal.

Furthermore, an in-depth understanding of pluripotency is highly applicable to regenerative medicine. Knowledge of their culture condition, state of pluripotency, and signal transduction pathways could greatly facilitate *in vitro* culture, manipulation, and differentiation, either from autologous or allogeneic sources. This knowledge will also guide a more effective generation of iPSCs, which will ultimately lead to individualized regenerative medicine.

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