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REVIEW

Molecular basis of embryonic stem cell self-renewal: from signaling pathways to pluripotency network

Guanyi Huang · Shoudong Ye · Xingliang Zhou · Dahai Liu · Qi-Long Ying

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Abstract Embryonic stem cells (ESCs) can be maintained in culture indefinitely while retaining the capacity to generate any type of cell in the body, and therefore not only hold great promise for tissue repair and regeneration, but also provide a powerful tool for modeling human disease and understanding biological development. In order to fulfill the full potential of ESCs, it is critical to understand how ESC fate, whether to self-renew or to differentiate into specialized cells, is regulated. On the molecular level, ESC fate is controlled by the intracellular transcriptional regulatory networks that respond to various extrinsic signaling stimuli. In this review, we discuss and compare important signaling pathways in the self-renewal and differentiation of mouse, rat, and human ESCs with an emphasis on how these pathways integrate into ESC-specific transcription circuitries. This will be beneficial for understanding the common and conserved mechanisms that govern self-renewal, and for developing novel culture conditions that support ESC derivation and maintenance.

Keywords Embryonic stem cells · Stem cell self-renewal · Pluripotency · LIF/Stat3 signaling pathway · Wnt/β-catenin signaling pathway

G. Huang and S. Ye contributed equally to this work.

G. Huang · S. Ye · D. Liu
Center for Stem Cell and Translational Medicine, School of Life Sciences, Anhui University, Hefei 230601, PR China

G. Huang · S. Ye · X. Zhou · Q.-L. Ying (✉)
Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research at USC, Department of Stem Cell Biology and Regenerative Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA
e-mail: qying@med.usc.edu

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the pre-implantation blastocyst. Under appropriate in vitro culture conditions, ESCs proliferate indefinitely without differentiation, a property hereinafter referred to as "self-renewal", and at the same time retain the developing potential to generate cells of all three primary germ layers, termed "pluripotency" [1]. Mouse ESCs were firstly established in 1981 (refs [2, 3]), followed by the isolation of human ESCs in 1998 (ref [4]), and rat ESCs in 2008 (refs [5, 6]). Although ESC-like cells derived from other species, such as fish, monkey, dog and chicken, have also been reported, only mouse and rat ESCs possess the germline transmission ability and have been used to create genetically modified animals. Mouse, rat, and human ESCs require distinct culture conditions for the maintenance of their pluripotent state. Different growth factors, cytokines, and small molecules have been used to promote ESC self-renewal by activating or suppressing a variety of intracellular signaling pathways. In this review, we summarize important molecular characteristics and signaling pathways involved in the self-renewal of mouse, rat, and human ESCs.

The developmental origin of ESCs

Embryogenesis is often accompanied with a progressive loss of developmental capacity from a totipotent zygote. The zygote undergoes a series of cleavage divisions to give rise to a cluster of cells known as blastomeres, which further differentiate and rearrange to form the blastocyst. The blastocyst is characterized by the presence of a fluid-filled cavity and an ICM, which are together surrounded by

the trophectoderm (TE). In this developmental process, two morphogenetic events occur: compaction and cavitation. The blastomeres form tight junctions with one another during compaction and form the morula from the eight-cell stage embryo. Then, cells located inside of the morula, after subsequent cell divisions, become the ICM of the blastocyst; while cells forming the outer layer of the morula develop into an epithelial layer, the TE. TE cells (trophoblasts) transfer fluid into the blastocyst to form the cavity. After cavitation, ICM cells that are exposed to the fluid cavity develop into hypoblast [primitive endoderm (PrE)], while the remaining cells become epiblast surrounded by the TE and the PrE. The TE mediates implantation of the embryo into the uterus and placenta formation for further maternal sustenance of embryonic development. The PrE develops into the visceral endoderm (VE) and the parietal endoderm (ParE) after implantation [7–9] (Fig. 1).

ESCs are isolated from the pre-implantation blastocyst and can be maintained in a pluripotent state when cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) and in medium supplemented with cytokines, growth factors, chemicals, and/or serum. Interestingly, human ESCs are similar to mouse post-implantation epiblast-derived stem cells (EpiSCs) in growth requirements, morphology, clonogenicity, and gene expression patterns [10, 11]. Thus it has been proposed that there are two successive yet distinct states of pluripotency as embryonic development proceeds: naïve (mouse and rat ESCs) and primed (human ESCs and mouse EpiSCs) [12]. Although human, mouse, and rat ESCs differ in many aspects (Table 1), they all self-renew and stay pluripotent under their respective culture conditions. It remains unclear, however, how their transcriptional and epigenetic status result in the phenotypic differences and whether they share common and conserved mechanisms that govern self-renewal.

The molecular foundation of pluripotency

Mouse, rat and human ESCs share a common subset of transcription factors specifying "stemness", among which Oct4, Sox2, and Nanog are considered to be the key factors that constitute the core pluripotency circuitry [20]. Oct4 is expressed in the embryo throughout the pre-implantation period and re-appears in germ cell precursors of adult mice [21]. *Oct4*-deficient embryos can survive the morula stage, but fail to form the ICM in vivo and ESC colonies in vitro, indicating the essential role of Oct4 in ESC maintenance [22]. Oct4 binds to the octamer motif (5′-ATGCAAAT-3′) of DNA to control the expression of a number of genes involved in pluripotency, and in many cases works in partnership with Sox2 (ref [23]). Oct4 is also one of the transcription factors used to generate induced pluripotent stem cells (iPSCs) in mouse, rat, and human, demonstrating its capacity to induce an ESC-like state [24–27]. Interestingly, Oct4 expression level must be precisely regulated, as either too much or too little of Oct4 causes ESC differentiation [28]. Karwacki-Neisius et al. also found that mouse ESCs with reduced Oct4 expression showed increased genome-wide binding of Oct4, particularly at pluripotency-associated enhancers, leading to homogeneous expression of pluripotency factors and improved self-renewal [29]. In addition, Oct4 is not expressed exclusively in the epiblast and therefore itself alone does not suffice pluripotency specification [12]. These observations together bring more complexity to the exact role of Oct4 in self-renewal.

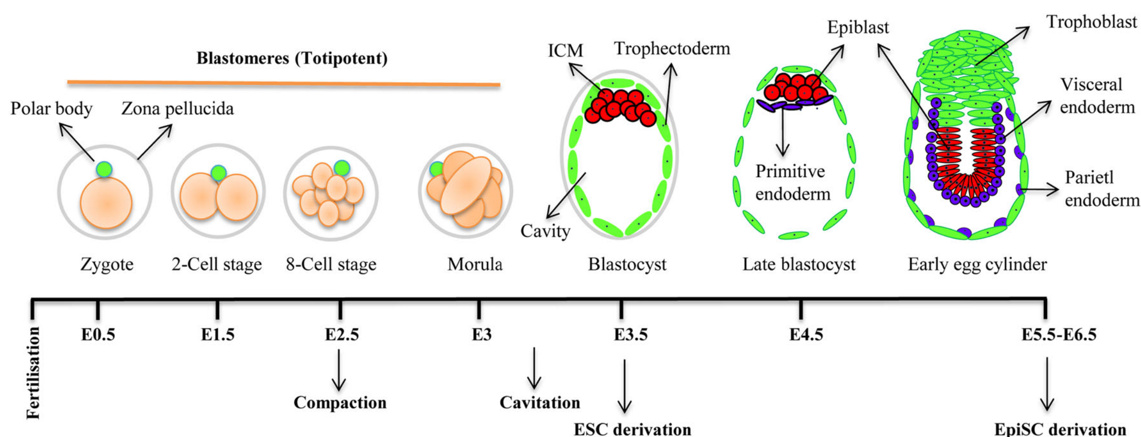

Fig. 1 Origin of ESCs in the mouse. After fertilization, the totipotent zygote develops into the blastocyst stage embryo (E3.5) through a series of cleavage division, compaction and finally cavitation of morula. The blastocyst further develops into egg cylinder to prepare for germ layer specification. ESCs are derived from ICM cells in the blastocyst at E3.5, while epiblast-derived stem cells (EpiSCs) are derived at E5.5–6.5

Table 1 Comparison among mouse, human and rat ESCs

	Mouse ESCs	Human ESCs/mouse EpiSCs	Rat ESCs	References
Type of pluripotency	Naïve	Primed	Naïve	[2–6, 13]
Colony morphology	Dome	Flat	Dome	[4–6, 14]
Clonogenicity	Good	Poor	Good	[5, 6, 14, 15]
Pluripotency Markers	*Oct4, Sox2, Nanog, Klf2/4, Esrrb*	*Oct4, Sox2, Nanog*	*Oct4, Sox2, Nanog*	[4–6, 16]
X chromosome inactivation	XaXa	XaXi	XaXa	[5, 6, 13]
Teratoma formation	Yes	Yes	Yes	[4, 5, 13–15]
Germline contribution	Yes	Unknown/No	Yes	[5, 6, 14]
Culture Condition	Serum/LIF, N2B27/LIF + BMP4 N2B27/2i	KSR/Activin + bFGF, Serum/ CHIR + IWR1	N2B27/2i	[4–6, 14, 15, 17–19]

Sox2 belongs to Sox family of transcription factors that have a highly conserved HMG (high-mobility group) DNA-binding domain. Sox2 expression is widely distributed in the developing embryo, including ICM, epiblast, neural tissues, and extra-embryonic ectoderm. *Sox2*-null embryos die immediately after implantation [30, 31]. Sox2 is essential for ESC self-renewal and pluripotency, as knockdown or conditional deletion of Sox2 results in trophoblast differentiation [32, 33]. This phenotype is similar to that caused by Oct4 deletion because Sox2 often acts as a heterodimer with Oct4 to regulate transcription of important genes such as *Fgf4* (ref [34]), *Nanog [*35], *Lefty1* (ref [36]) as well as *Oct4* and *Sox2* themselves [37, 38].

Nanog is a homeodomain-containing protein that functions in coordination with Oct4 and Sox2 to establish the ESC identity. Nanog expression level fluctuates greatly in mouse ESCs to contribute to population heterogeneity [39, 40]. Over-expression of Nanog in mouse ESCs stabilizes an undifferentiated state by constitutively conferring self-renewal independent of growth factors or small molecules [17, 41, 42], while in human ESCs allows feeder-free propagation for multiple passages [43]. *Nanog*-null embryos appeared to be able to initially give rise to pluripotent cells, yet these cells immediately differentiated into the extra-embryonic endoderm lineage [44]. Nanog knockdown assay in mouse and human ESCs resulted in similar phenotypes [41, 42, 45], which could partially be explained by a negative regulation on primitive endoderm-inducer Gata6 (ref [42]). Genome-wide mapping of Nanog binding sites has identified many pluripotency genes, including *Esrrb*, *Rif1*, *Foxd3* and *REST [*23]. For example, *Esrrb* has been proved to be a direct Nanog target [46]: over-expression of Esrrb in *Nanog*$^{-/-}$ ESCs led to cytokine-independent self-renewal, while its deletion abolished the effect of Nanog over-expression. Interestingly, Nanog is not strictly required for the maintenance or establishment of pluripotency, as suggested by the derivation of *Nanog*$^{-/-}$ ESCs [44] and iPSCs from *Nanog*$^{-/-}$ somatic cells [47, 48].

Table 2 Transcriptional factors associated with ESC fate regulation

Factor	Function	References
Oct4	Core factor	[22]
Sox2	Core factor	[30]
Nanog	Core factor	[41, 42]
Klf2/4/5	Self-renewal	[54]
c-Myc	Self-renewal	[56]
Tbx3	Self-renewal	[58]
Esrrb	Self-renewal	[16]
Tfcp2l1	Self-renewal	[61, 62]
Prdm14	Self-renewal	[64]
Tfe3	Self-renewal	[66]
YAP	Self-renewal	[67, 68]
CBX7	Self-renewal	[70]
Gbx2	Self-renewal	[52]
Pim1/3	Self-renewal	[53]
Id1/2/3,Sall4	Self-renewal	[17, 72]
Zscan4	Genomic stability	[55]
Mbd3	Differentiation	[57]
Tcf3	Differentiation	[59]
Otx2	Differentiation	[60]
Cdx2	Differentiation	[63]
Gata4/6	Differentiation	[65]
Fnip1/2	Differentiation	[66]
Tfap2c	Differentiation	[69]
Sox17	Differentiation	[71]

Recent studies have identified many additional transcription factors in ESC regulatory network (Table 2). Importantly, many of the self-renewal factors work in cooperation with each other to maintain pluripotency. For example, Sall4 and Esrrb have been shown to interact with Nanog physically and co-occupy *Nanog* genomic sites in mouse ESCs [49, 50]. These factors also serve as hubs between extrinsic signaling pathways and intrinsic pluripotency determinants. Using high-throughput ChIP-

seq technologies, Chen and colleagues attempted to map the genomic occupation of 13 sequence-specific pluripotency factors, and identified a protein cluster containing Nanog, Oct4, Sox2, SMAD1 and STAT3 (ref [51]). The readouts show that 87.4 % of SMAD1 and 56.8 % of STAT3-binding sites are associated with the Oct4–Sox2–Nanog core factor-binding loci; they also share many common regulatory coordinators including Klf4, Esrrb, c-myc, and Tcfcp2l1. Given that mouse ESCs can be maintained under LIF/BMP condition that enables SMAD1 and STAT3 activation and binding to genomic sites, this observation provided direct evidence that LIF/BMP signaling supports self-renewal by strengthening core pluripotency circuitry.

Signaling pathways in pluripotency regulation

Biomedical applications of ESCs depend on the ability to freely manipulate ESC fates. Although many intrinsic factors are essential determinants for the ESC identity, it is very difficult to perform direct regulation on the "transcription factor" level without using genetic methods. Instead, researchers have launched intensive efforts to control ESC self-renewal and differentiation by applying different culture conditions. Therefore, identification of signaling pathways involved in ESC fate determination and their downstream effectors is of great significance. So far, several signaling pathways have been reported associated with pluripotency, including LIF/STAT3, Wnt/β-catenin, FGF/ERK, TGF/SMAD and PKC signaling.

LIF/JAK/STAT3 signaling pathway

Historically, mouse ESCs were maintained in co-culture with mitotically inactivated feeder fibroblasts [2, 3] or in buffalo rat liver cell-conditioned medium [73], yet later efforts in pinpointing the active component(s) in conditioned medium identified a single cytokine, leukemia inhibitory factor (LIF), which supported self-renewal of ESCs derived from 129 strain of mice in the absence of feeder cells [18, 19]. LIF now is routinely used in the culture of mouse ESCs and its withdrawal leads to rapid differentiation into a mixed population of mesoderm and endoderm cells [74]. Interestingly, LIF is not an ESC-specific signal molecule, but belongs to the well-characterized IL-6 family of cytokines that mediate inflammation, immune responses, hematopoiesis, neuronal regeneration and embryonic development [75]. LIF initiates signaling cascade by binding to a low-affinity LIF receptor (LIFR) in association with a common IL-6 family co-receptor subunit glycoprotein 130 (gp130). LIFR and gp130 form heterodimers and activate associated tyrosine kinases such as family of Janus kinases (JAKs). JAKs subsequently phosphorylate the tyrosine residues within the cytoplasmic tail of the cytokine receptor, which in turn provides the critical docking site for recruitment of cytoplasmic STAT3 (signal transducer and activator of transcription 3) monomer via its

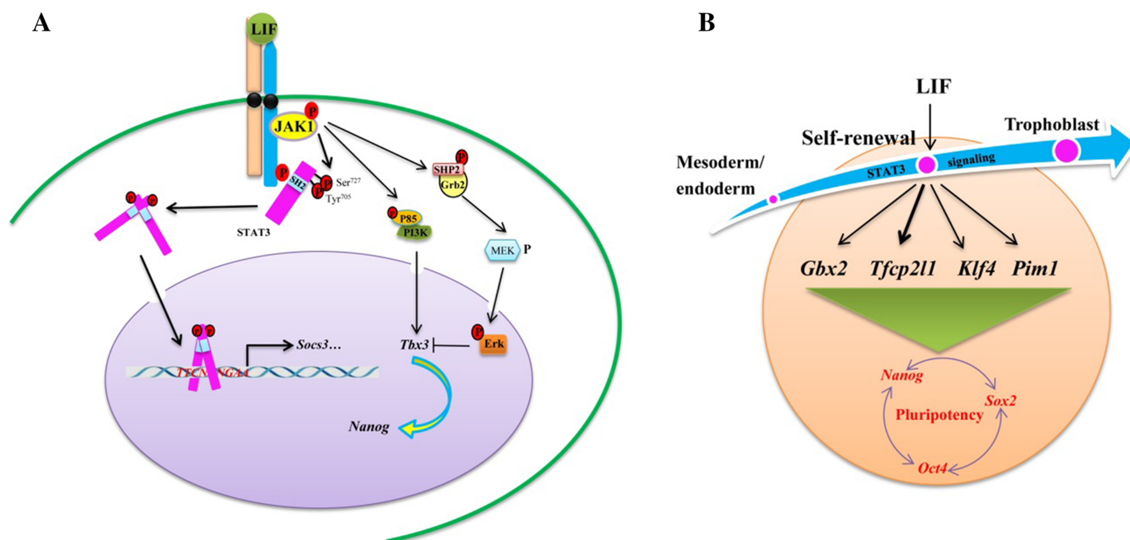

Fig. 2 LIF/JAK/STAT3 signaling pathway in mouse ESC self-renewal. **a** Binding of LIF to its membrane receptor results in recruitment of JAKs and phosphorylation of STAT3 at Tyrosine 705. Activated STAT3 dimerizes and translocates into nucleus to activate transcription. LIF also activates PI3K/AKT and SHP2/MAPK pathways that are not essential for mouse ESC self-renewal. **b** STAT3 activation level is critical for maintaining mouse ESC self-renewal. Multiple downstream target genes have been identified to connect STAT3 signaling to core pluripotency network

SH2 domain. Recruited STAT3 molecules become themselves substrates for JAK-mediated phosphorylation (at tyrosine 705) [76]. After phosphorylation, STAT3 dimerizes through reciprocal SH2 interaction and translocates into the nucleus, where the homodimers activate target gene transcription [77] (Fig. 2a). JAK-STAT3 canonical pathway represents one of the common mechanisms of how extracellular signaling proteins regulate gene transcription and control cell behaviors [78].

It is worth mentioning that LIF triggers at least three different signaling pathways in mouse ESCs: the JAK/STAT3 pathway; the PI3K (phosphoinositide 3-kinase)/AKT pathway; and the SHP2 (SH2 domain-containing tyrosine phosphatase 2)/MAPK (mitogen-activated protein kinase) pathway [79, 80] (Fig. 2a). LIFR/gp130 receptor dimerization leads to phosphorylation of p85 (ref [81, 82]) and SHP2 (ref [83]) for activation of PI3K/AKT and SHP2/MAPK pathways, respectively. It has been shown that only STAT3 pathway is essential for LIF-mediated mouse ESC self-renewal, as constitutive activation of STAT3 renders mouse ESC self-renewal independent of LIF [74, 84]. This notion has been further confirmed by the observation that selective stimulation of JAK/STAT3 alone using chimeric receptor GCSF-R-gp130-Y118F is sufficient to support mouse ESC self-renewal without LIF [85].

Niwa et al. found that a dominant-negative mutant of STAT3, in which tyrosine 705 (Tyr^{705}) is replaced with a phenylalanine residue, blocked the activation of endogenous STAT3 and abrogated mouse ESC self-renewal even in the presence of LIF [74]. Consistently, LIF failed to support $STAT3^{-/-}$ mouse ESC self-renewal [14, 54]. Studies using fusion protein containing full-length STAT3 and the ligand-binding domain of the estrogen receptor (STAT3-ER) demonstrated that ER ligand 4-hydroxytamoxifen (4-OHT) could substitute LIF to maintain mouse ESCs in an undifferentiated state by bringing STAT3 into the nucleus [84]. Besides Tyr^{705} phosphorylation, STAT3 can also be phosphorylated on Ser^{727} by extracellular signal-regulated kinase (ERK) in ESCs. We found that these two phosphorylation sites differentially regulate mouse ESC fates: Tyr^{705} phosphorylation is absolutely required for STAT3-mediated self-renewal, while Ser^{727} phosphorylation is dispensable and only promotes proliferation and optimal pluripotency by enhancing transcription activity of STAT3. Furthermore, Ser^{727} phosphorylation is crucial in transition from self-renewal to neural commitment, suggesting a dynamic equilibrium of STAT3 Tyr^{705} and Ser^{727} phosphorylation in the control of mouse ESC fate [86]. Additional complexity was also realized when a recent study suggested the importance of STAT3 activation level in mouse ESC maintenance: insufficient STAT3 activation failed to prevent ESC differentiation into meso/endoderm cells, yet STAT3 signaling overload also led to ESC crisis and differentiation into the trophoblast lineage [87].

A number of studies have been carried out to identify STAT3 downstream target genes as potential candidates for key pluripotency factors [88–92]. For example, STAT3 directly regulates the expression of Myc transcription factor and sustained expression of Myc supports mouse ESC self-renewal in the absence of LIF [56]. Myc and STAT3 also co-occupy the promoter regions of many genes that are highly enriched in mouse ESCs, suggesting the existence of feed-forward loops for signal amplification [93]. Additional factors identified include Klf4, Pim1/3, Prr13, Gbx2, Pramel7, Pem/Rhox5, Jmjd1a and Tfcp2l1 whose overexpression in mouse ESCs are able to recapitulate certain effects of LIF [52–54, 56, 61, 62, 91, 94–96]. Among them, Klf4 is one of the four canonical Yamanaka factors that direct somatic cell reprogramming [24] and is also sufficient to reprogram post-implantation EpiSCs to naïve pluripotency [97]. However, forced expression of Gbx2 or Tfcp2l1, but not Klf4, suffices to sustain $STAT3^{-/-}$ ESC propagation [52, 54, 61, 62]. Only knockdown of Tfcp2l1 was able to impair the self-renewal and reprogramming ability rendered by STAT3 activation [62, 95]. Tcfcp2l1 is hardwired into core pluripotency factor network through activation of *Nanog* and *Tbx3* and its function is further enhanced by Oct4, Sox2 and Esrrb. This observation provided a major connection between LIF/STAT3 signaling and intrinsic pluripotency factors as LIF does not directly regulate them (Fig. 2b).

LIF/STAT3 signaling fails to support self-renewal of human and rat ESCs [5, 6, 98]. Interestingly, hyper-activation of STAT3 has been shown to convert mouse EpiSCs, which share many features with human ESCs, into naïve pluripotency [99, 100], and the very recently isolated naïve human ESCs exhibit high level of LIF/STAT3 activation [101, 102]. It is thus generally believed that LIF/STAT3 is a hallmark of naïve pluripotency. Tfcp2l1 is also highly expressed in the ICM of human blastocysts, but is significantly down-regulated during derivation of human ESCs [103] and up-regulated during generation of naïve state human ESCs by introducing Klf2 + Klf4 or Klf4 + Oct4 (ref [13]). Moreover, depletion of *Tfcp2l1* results in the collapse of the naïve-like state in conventional human pluripotent stem cells [104]. Tfcp2l1 may thus play an important role in establishing and maintaining naïve pluripotency by acting downstream of LIF/STAT3.

Additional studies have suggested that the role of LIF/STAT3 signaling in mouse ESC derivation and maintenance is closely related to diapause, a naturally occurred stage identified by arrested embryonic development and delayed implantation of mouse late blastocyst [12]. Maternal estrogen induces trophectoderm secretion of LIF to sustain ICM cell self-renewal during diapause [105] and

embryos lacking gp130, one component of LIF co-receptor, showed significant ICM cell death and failed to resume from diapause and implant [106]. This mechanism partially explains the increased efficiency of ESC derivation when blastocysts enter diapause [107]. Importantly, LIF signaling is not required during normal blastocyst development without diapause [12]. This notion is also supported by the fact that human ESCs do not exhibit diapause and are non-responsive to LIF/STAT3.

Canonical Wnt/β-catenin signaling pathway

Signaling pathways other than LIF/STAT3 started to attract attention during the attempts to further improve the established "serum + LIF" culture condition for mouse ESCs. One of the motivations came from an urgent need for directed differentiation of mouse ESCs for research and therapeutic purposes [108]. Therefore, it is essential to establish a refined serum-free, as opposed to a complex multi-factorial, condition in which the role of each component is clarified [109]. Ying et al. [14] argued that self-renewal could be achieved by blocking intrinsic differentiation-inducing momentum rather than introducing extrinsic signal stimuli. For example, the addition of small molecular inhibitors against FGF/ERK signaling (SU5402 and PD184352, or PD0325901 alone) resulted in suppressed differentiation of mouse ESCs, suggested by their continued self-renewal for several passages after LIF withdrawal [14]. However, those cells showed poor clonogenicity due to compromised growth and viability, a common side effect caused by FGF/ERK inhibition. This drawback was eventually compensated by using a glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021, which consolidates pluripotency by enhancing metabolic and biosynthetic ability and thus overall viability of mouse ESCs [14]. In addition, optimization in basal culture medium led to the application of the serum-free N2B27 medium to best support mouse ESC growth nutritionally. Together, a chemically defined culture condition (3i: CHIR99021, SU5402 and PD184352 or 2i: CHIR99021 and PD0325901, in N2B27 medium) was developed [14]. Notably, 2i or 3i bypasses the otherwise obligatory LIF/STAT3 signaling for self-renewal and therefore enables the establishment of STAT3$^{-/-}$ mouse ESCs [14]. 2i/3i culture represents a true "stemness" condition that not only supports derivation of ESC lines from completely recalcitrant mouse strains, but also from the species of rat [5, 6, 110], suggesting a conserved mechanism governing pluripotency in rodents.

Inhibition of GSK3 by CHIR99021 promotes ESC self-renewal through stabilizing cytoplasmic β-catenin, an essential component of canonical Wnt signaling pathway [14, 59, 111]. In the absence of Wnt ligand, GSK3 forms a so-called destruction complex with casein kinase 1 (CK1), adenomatous polyposis coli (APC) and Axin, and phosphorylates β-catenin. Phosphorylated β-catenin becomes susceptible to ubiquitination- and proteasome-mediated protein degradation. Conversely, upon Wnt ligand stimulation, signal is passed down through Frizzled and LRP (low-density lipoprotein receptor protein) 5/6 receptors to inhibit the assembly of destruction complex, leading to the accumulation of unphoshporylated β-catenin in the cytoplasm. Accumulated β-catenin then enters the nucleus, interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) family member of transcription factors, and binds to a consensus motif AGATCAAAGG to activate the transcription of target genes such as *Axin2*, *Cdx1* and *T* [112, 113] (Fig. 3a). In addition to its function as a transcription regulator, β-catenin may also stay in the cell membrane, where it is immobilized by E-cadherin and α-catenin to form adherent junctions that modulate cytoskeletal re-arrangement and cell adhesion [113]. However, E-cadherin has been proven dispensable for mouse ESC maintenance, as E-cadherin$^{-/-}$ ESCs remained undifferentiated and proliferated rapidly in 2i [14].

β-catenin is required for ESC self-renewal promoted by CHIR99021 as CHIR99021 fails to maintain β-catenin$^{-/-}$ ESC self-renewal [114]. Over-expression of β-catenin can recapitulate the effect of CHIR99021 in mouse and rat ESCs [59, 111, 115]. Several studies have shown that stabilized β-catenin directly abrogates TCF3-mediated transcriptional repression by stimulating TCF3 degradation [116–118]. Other supporting evidence includes the observation that over-expression of TCF3 promoted differentiation while TCF3$^{-/-}$ ESCs showed delayed differentiation, a phenotype similar to that caused by inhibition of GSK3. Genome-wide analysis suggested that TCF3 serves as a limiting factor for high expression of pluripotent factor, including Oct4, Nanog, Tfcp2l1 and Esrrb (Fig. 3a) [59, 119–122]. Indeed, ablation of *Esrrb* neutralized the self-renewal effect provided by CHIR99021, indicating Esrrb as the main downstream effector through which the GSK3/β-catenin/TCF3 axis modulates mouse ESC self-renewal [16, 61, 62]. β-catenin/TCF3 also directly activates the orphan nuclear receptor Lrh-1/Nr5a2 transcription and stabilizes Oct4, Nanog, and Tbx3 expression in a Nr5a2-dependent manner [123, 124]. Nr5a2 can replace Oct4 in iPSC reprogramming and convert EpiSCs to naïve pluripotency, although it is not required for the maintenance of ESC self-renewal [125, 126]. In contrast, another nuclear receptor, Nr6a1, serves as Nr5a2 antagonist to repress Oct4 expression during differentiation [127, 128]. It should be noted that excessive β-catenin activity, usually caused by over-inhibition of GSK3, can induce differentiation in mouse and rat ESCs [14, 115]. One possible explanation is that over-inhibition

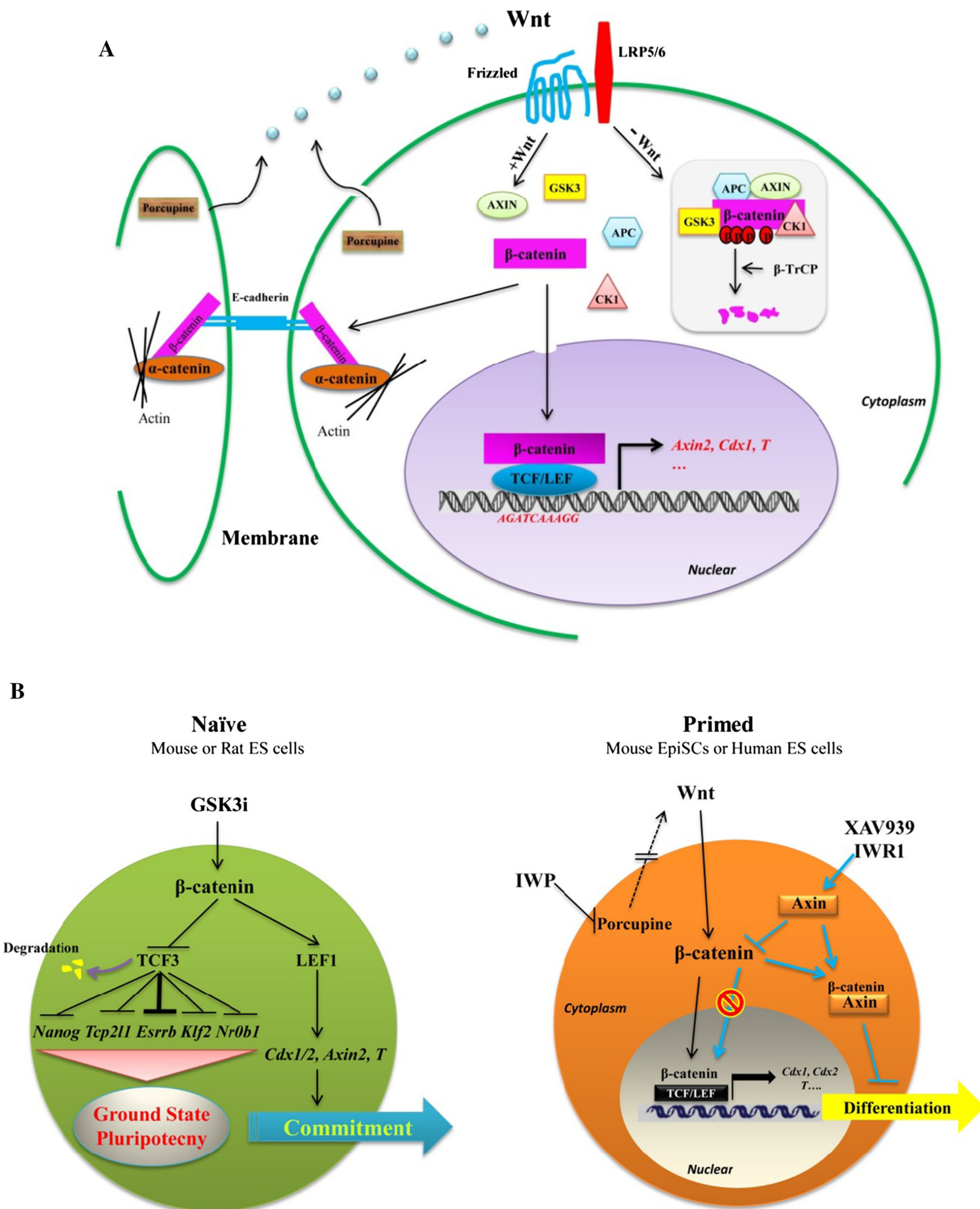

Fig. 3 Canonical Wnt/β-catenin signaling pathway in mouse, rat and human ESC self-renewal. **a** The presence of Wnt ligand prevents the formation of intracellular destruction complex (GSK3, CK1, APC and Axin) that phosphorylates β-catenin for its subsequent degradation. Thus, Wnt signaling stabilizes β-catenin and activates β-catenin-mediated transcriptional activation. **b** The complex role of Wnt/β-catenin signaling in self-renewal: in mouse/rat ESCs, β-catenin primarily supports ground-state pluripotency by removing TCF3-mediated transcription repression; yet in mouse EpiSCs or human ESCs, self-renewal is achieved when β-catenin transcriptional activity is blocked through its sequestration in the cytoplasm

of GSK3 increases the expression of the canonical Wnt pathway effector LEF1, and elevated LEF1 interacts with β-catenin and leads to activation of lineage specification genes *Cdx2* and *T* [129] (Fig. 3a).

The exact function of canonical Wnt/β-catenin pathway in human ESCs remains unclear. It was first reported that activation of the Wnt/β-catenin pathway using either Wnt3A or GSK3 inhibitor BIO can maintain human ESC

self-renewal [130–132]. Yet this conclusion was challenged later by a series of studies claiming that Wnt3A/BIO can only support self-renewal in short-term culture, but not in further expansion of pluripotent human ESCs [133–135]. Genetic strategies were then employed using a chimeric protein ΔNβ-catenin-ER consisting of the stabilized form of β-catenin (ΔNβ-catenin) fused with the ligand-binding domain of estrogen receptor (ER): addition of ER ligand 4-OHT induced rapid differentiation of human ESCs into primitive streak (PS)/mesoderm progenitors [15, 136]. Moreover, studies using other small molecules targeting different components of Wnt/β-catenin signaling pathway have also provided novel insights into the role of Wnt/β-catenin signaling in human ESCs. For instance, Wnt signaling could be blocked by inhibition of Porcupine, an enzyme essential for Wnt ligand secretion [137], or by stabilizing Axin1/2 to stop β-catenin nuclear translocation [15, 135]. The self-renewal and propagation of human ESCs were greatly improved under those conditions. Therefore, it appears that Wnt/β-catenin/TCF axis plays opposite roles in the self-renewal regulation of rodent and human ESCs: in mouse/rat ESCs, β-catenin/TCF binding and transcriptional activation are required for pluripotency maintenance; in human ESCs, self-renewal effects can only be realized when transcriptional activity is blocked, albeit the mechanistic details remain unknown (Fig. 3b).

FGF/MEK/ERK signaling pathway

FGF4 is one of the important growth factors expressed throughout early mouse embryonic development, from one-cell stage to blastocyst, egg cylinder, and primitive streak [138, 139]. FGF signaling regulates the early differentiation processes in the mouse blastocyst. *Fgf4*$^{-/-}$ embryos appear normal up to blastocyst stage, yet die after implantation [140], likely due to a primary defect in the formation of trophectoderm and primitive endoderm. Surprisingly, in E3.5 and E4.5 blastocysts, FGF4 is produced by ICM, but not by trophectoderm or primitive endoderm, which require FGF4 for growth and proliferation [138, 139]. FGF4 is also required for the derivation and maintenance of trophoblast stem cells (TSCs) from E3.5 blastocysts [141] and is routinely added to extra-embryonic endoderm (XEN) cell culture [142]. On the other hand, culture of blastocysts or isolated ICMs in the presence of exogenous FGF4 leads to an increased number of parietal endoderm-like cells. Over-expression of activated H-RAS, a downstream effector of FGF4 signaling, induces ESC differentiation toward primitive endoderm [138, 141, 143], whereas genetic ablation of Grb2, which couples the FGF receptor to the MEK-ERK pathway, results in blastocysts that lack hypoblast (primitive endoderm) [144], suggesting an essential role of FGF4 in specification of primitive endoderm. FGF signaling is triggered by a ligand–receptor interaction that leads to the auto-phosphorylation of tyrosine residues in the intracellular domain of FGF receptor (FGFR), followed by activation of several downstream intracellular pathways. Fibroblast growth factor receptor substrate 2 (FRS2) and Grb2 are the main mediators that activate PI3K–AKT and RAS–MEK–ERK pathways (Fig. 4). Phosphoinositide phospholipase C (PLC-Y) pathway is also activated by FGF signaling [145, 146].

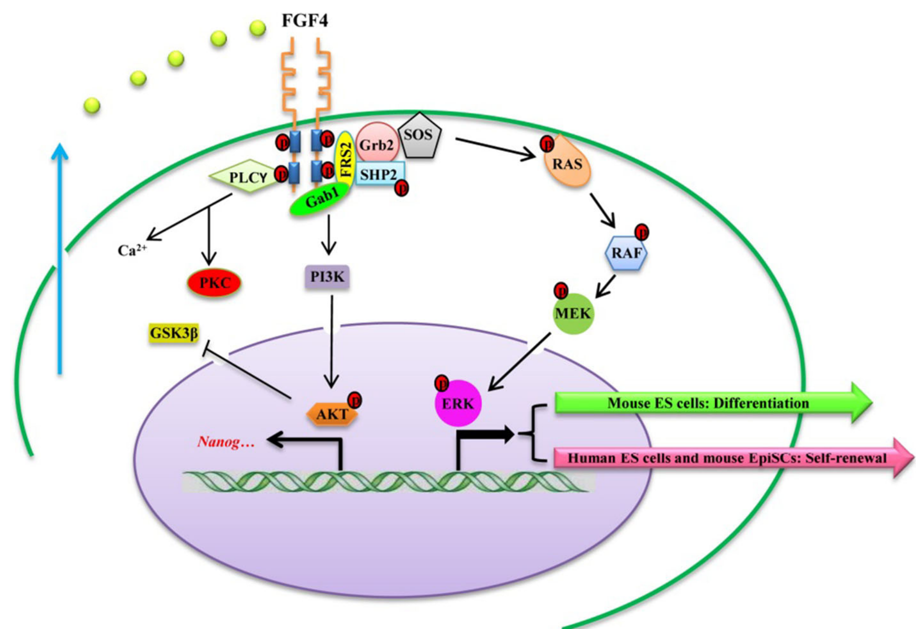

Fig. 4 FGF signaling pathway in mouse, rat and human ESC self-renewal. Autocrine FGF4 in ESCs primarily activate RAS–MEK–ERK signaling cascade that promotes differentiation of mouse/rat ESCs into endoderm lineages. Therefore, small molecule inhibitors that block FGF/ERK signaling promote pluripotency. In contrast, mouse EpiSCs and human ESCs require FGF signaling for self-renewal

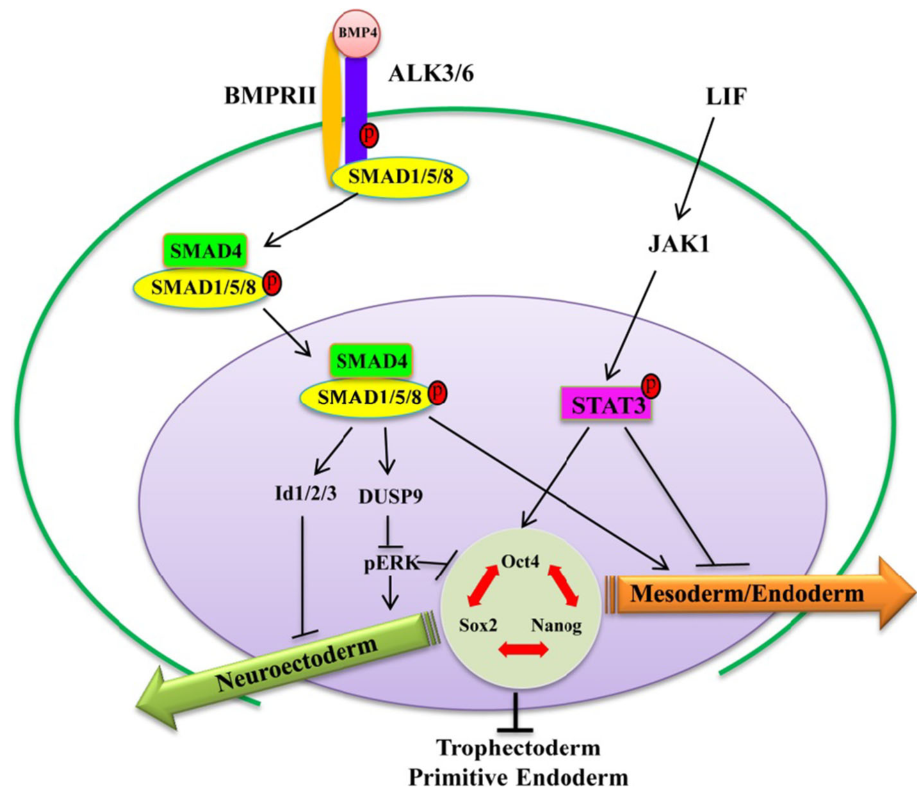

Fig. 5 BMP/SMAD and LIF/STAT3 signaling in mouse ESC self-renewal. BMP ligand-binding leads to homo-trimerization of SMAD1/5/8 and subsequent association with co-regulatory component SMAD4 to activate Id1/2/3 and DUSP9 expression, which inhibits neuroectoderm differentiation. LIF/STAT3, on the other hand, inhibits mesoderm and endoderm differentiation of mouse ESCs

Mouse ESCs produce FGF4, which activates FGF/MEK/ERK signaling in an autocrine manner. FGF4 is shown to be dispensable for mouse ESC maintenance as *Fgf4*-null cells, unlike *Fgf4*-null ICMs, do not display defect in proliferation in vitro under LIF condition [147]. However, *Fgf4*-null mouse ESCs resist neural and mesodermal induction, and addition of exogenous FGF4 could restore the lineage commitment potential, indicating that FGF/MEK/ERK acts as a differentiation cue and is essential for exiting from self-renewal [148]. *Fgf4* deletion leads to a massive reduction in steady-state ERK1/2 phosphorylation; $ERK2^{-/-}$ mouse ESCs also differentiate inefficiently in adherent culture [148]. Similar results were observed in FGFR and MEK inhibitor-treated ESCs [14, 148]. Inspired by these results, derivation of mouse ESC lines from recalcitrant C57BL/6 and CBA strains were achieved using the selective MEK inhibitor PD184352 in combination with LIF and BMP4 (ref [149]).

So far, some progress has been made toward understanding how FGF/MEK/ERK signaling guides ESCs to exit from pluripotency. Yang et al. performed a genome-wide siRNA screening in mouse ESCs and identified more than 400 genes involved in loss of pluripotency [150]. Many of the differentiation-associated genes, such as RAS downstream targets *Gmnn*, *Psmb3* and *Ifna14*, enhance ERK activation by down-regulating the expression level of the MAP kinase phosphatases Dusp1 and/or Dusp6. Dusp1/6 are negative regulators of FGF/MEK/ERK, thus inhibition of their activities promotes ESC differentiation [150]. MEK inhibition prevents emergence of Gata4-positive hypoblast cells during morula development, leading to an expanded pluripotent epiblast. Segregation of hypoblast from ICM depends on activation of FGF/MEK/ERK, and in its absence, the entire ICM acquires pluripotency [151]. Blockade of MEK activity leads to an increased expression of many pluripotency-associated genes in mouse ESCs, such as Nanog, Tfcp2l1 and Klf4 [62, 152, 153], and forced expression of Nanog or Tfcp2l1 can reproduce the self-renewal rendered by MEK inhibitor PD0325901. FGF/ERK phosphorylates STAT3 at Ser[727] to prime mouse ESCs for neural commitment and to secure a fate toward differentiation by inhibiting JAK/STAT3-induced reprogramming of mouse EpiSCs [86]. Kim et al. also found that ERK1/2 binds to the activation domain of Klf4 and directly phosphorylates Klf4 at Ser [123], which suppresses Klf4 activity and contributes to ESC differentiation [153].

Human ESCs and mouse EpiSCs differ from mouse ESCs in culture conditions in that human ESCs require FGF2 to support self-renewal [10, 11]. FGF has been shown to cooperate with Activin/SMAD2/3 signaling to maintain high level of Nanog expression in human ESCs and, at the same time, activate PI3K/AKT signaling pathway to enhance propagation and survival of human ESCs [154–157]. Inhibition of MEK/ERK by specific MEK inhibitors PD98059

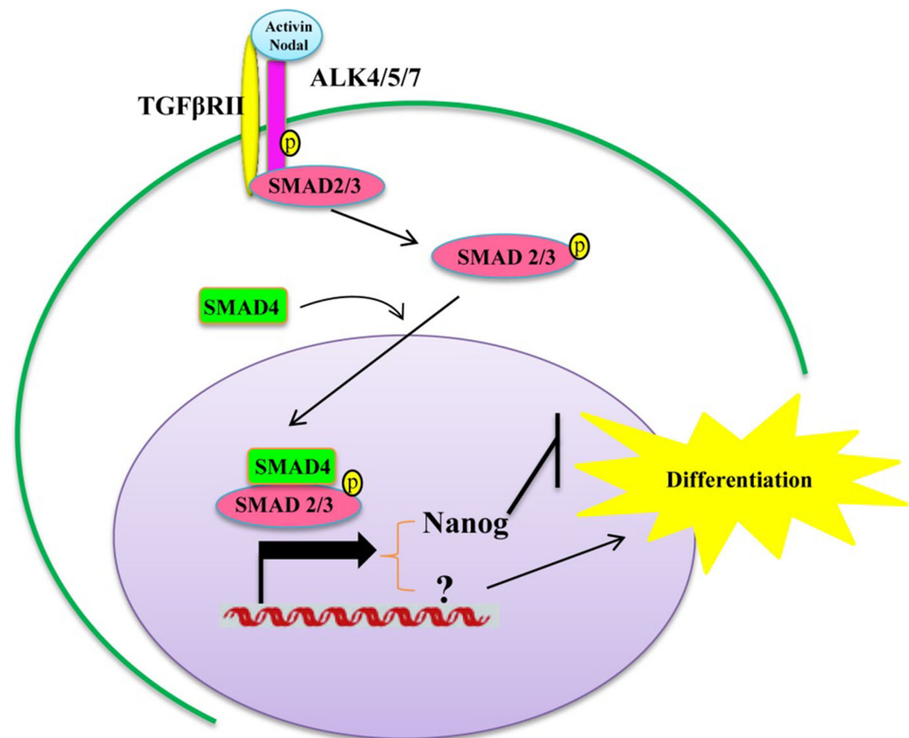

Fig. 6 Activin/Nodal signaling pathway in human ESC self-renewal. Activin/Nodal treatment results in SMAD2/3-mediated transcriptional activation of Nanog, which promotes pluripotency and, at the same time, antagonizes potential differentiation effects from other unknown downstream targets

and U0126 or by RNA interference severely impaired the self-renewal capacity of human ESCs, and blockade of PI3K/AKT signaling using LY294002 induced a significant decrease in cell proliferation and a markedly increased apoptosis [157]. FGF was reported to induce feeder cells to secrete IGF-2, and together they establish a regulatory niche for human ESC maintenance [158]. On the other hand, contradictory explanations regarding the underlying mechanisms were also presented: Singh et al. [159] found that high concentration of insulin and IGF-1 synergistically activate PI3K/AKT and suppress MEK/ERK, leading to high GSK3β activity and low β-catenin-mediated transcriptional activation, which prevents human ESC differentiation. In contrast to the FGF-SMAD2/3 pathway crosstalk in human ESCs [154–157], FGF2 does not cooperate with SMAD2/3 to regulate *Nanog* in mouse EpiSCs, but appears to stabilize the primed pluripotency state by dual inhibition of differentiation to neuroectoderm and of media-induced reversion to a mouse ESC-like state [156]. Therefore, FGF may activate multiple downstream cascades and work in cooperation with additional signaling effectors to collectively contribute to human ESC self-renewal.

TGF-β/SMAD signaling pathway

The TGF-β superfamily of growth factors is divided into two subgroups: the TGF-β/Activin/Nodal pathway and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Muellerian inhibiting substance) pathway. TGF-β superfamily exerts evolutionarily conserved functions in a wide variety of important biological processes including morphogenesis, cell fate choice, proliferation, differentiation, and apoptosis [160]. Both TGF-β signaling pathways are initiated by ligand-binding to the trans-membrane type I and type II receptors on the cell surface. Then the serine/threonine kinase activity at the intracellular domain of the type I receptors (ALK1-7) phosphorylates the mediators of TGF-β signaling: the SMAD proteins (SMAD1, 2, 3, 5, and 8). Phosphorylated SMADs undergo homo-trimerization and form heteromeric complexes with the co-regulatory SMAD molecule SMAD4. Activated SMAD complexes translocate into the nucleus, where they bind to DNA in a sequence-specific manner to control transcription of target genes [160].

BMP signaling maintains mouse ESC self-renewal in cooperation with LIF/STAT3 signaling by suppressing lineage commitment under serum and feeder-free condition: LIF/STAT3 activation primarily inhibits mesoderm and endoderm differentiation, while BMPs are well known as anti-neural factors in vertebrate pendent derivations and prevent neural differentiation of mouse ESCs through SMAD1/5/8 (ref [108, 161]) (Fig. 5a). Ying et al. further demonstrated that BMP/SMAD signaling activation induces expression of inhibitor of differentiation (Id) family members to suppress neural differentiation by inhibiting

pro-neural basic helix-loop-helix (bHLH) factors [17]. SMAD binding sites were recently identified in Id gene promoter regions in mouse ESCs [162]. Genome-wide occupancy analysis also revealed that SMAD1 shares many common targets with core pluripotency factors Oct4, Sox2 and Nanog [51]. In addition, Qi et al. [163] found that MEF feeder cells produce BMP4 to maintain the pluripotency of mouse ESCs through a novel mechanism: inhibition of ERK and p38 mitogen-activated protein. One recent study supported this finding by demonstrating that BMP4 localizes to the promoter region of *DUSP9* gene and up-regulates its expression through SMAD1/5 activation, which in turn leads to decreased phosphorylation of ERK. Forced expression of DUSP9 elevated the effect of BMP in repression of early neural differentiation and could substitute BMP4 to promote ESC self-renewal. Therefore, DUSP9 may strengthen BMP4 signaling by attenuating ERK activity and solidify the self-renewal status of mouse ESCs together with LIF [164] (Fig. 5).

In human ESCs, however, BMP treatment results in mesoderm and trophoblast induction, and BMP antagonist Noggin maintains human ESCs in an undifferentiated state [165–167]. As a matter of fact, human ESCs rely on Activin/Nodal/SMAD2/3 signaling pathway to self-renew and inhibition of SMAD2/3 signaling using Activin receptor inhibitor SB431542 leads to loss of pluripotency and rapid differentiation [168]. Similar results were obtained using Lefty or Follistatin to block this pathway [154, 169]. SMAD2/3 binds to the proximal promoter region of *Nanog* and directly up-regulates its expression in human ESCs. In turn, Nanog prevents the endoderm differentiation induced by SMAD2/3 to reinforce pluripotency [168, 170]. In addition, Nanog is not the only important target of Activin/Nodal signaling: ChIP-seq analysis revealed that SMAD2/3 participates in the control of the many pluripotency factors including Oct4 and Myc [171, 172]. Taken together, these results delineate a general picture of how Activin/Nodal/SMAD2/3 signaling cascades sustain human ESC pluripotency (Fig. 6).

Protein kinase C signaling pathway

The protein kinase C (PKC) family of serine/threonine kinases transduce signals mediated by phospholipid hydrolysis and can be subcategorized into three groups: conventional PKCs (α, βI, βII and γ) that are Ca^{2+}-sensitive and require lipid second messenger diacylglycerol (DAG) for their activation; novel PKCs (δ, ε, η and θ) that require DAG but not Ca^{2+}; atypical PKCs (ζ, λ and ι) that are activated independent of DAG and Ca^{2+} signal [173]. PKC isoenzymes are involved in a wide variety of biophysical processes due to its broad substrate specificity and are often intertwined with other signaling pathways to regulate cellular behaviors such as proliferation and differentiation.

In ESCs, early investigations suggested that activation of PKC pathway promotes cell proliferation together with PI3K/AKT and MAPK signaling [174, 175]. However, in order to precisely control stem cell self-renewal/proliferation, it is essential to distinguish the exact roles of individual PKC isoenzymes in this process. For example, Garavello and colleagues used peptide ψδRACK to specifically activate PKC δ in mouse ESCs and observed increased DNA synthesis and cell proliferation due to a transient activation of ERK1/2 (ref [176]). On the other hand, many studies also showed that PKC signaling, which can be activated by FGF stimulation [145, 146], promotes ESC differentiation. An atypical PKC isoform PKC ζ phosphorylates NF-κB and plays an important role in ESC differentiation, while a selective PKC inhibitor Gö 6983 can abrogate NF-κB activity, and is able to relieve LIF dependency of ESCs in serum condition, or 2i dependency in N2B27 medium [177, 178]. In human pluripotent stem cells, PKC δ activation leads to epithelial mesenchymal transition and thus extraembryonic endoderm differentiation [179], while PKC inhibition functions cooperatively with FGF-2 to promote self-renewal [180]. Importantly, one recent study explored the possibility to reset human ESC/iPSC self-renewal to the ground-state pluripotency by modulating PKC activity: a transient expression of Nanog and Klf2 was sufficient to initiate the generation of naïve human pluripotent stem cells, which can be stably maintained in LIF + 2i + Gö 6983 (ref [104]). Although high concentration of Gö 6983 (10 μM) was potentially toxic and might compromise serum-free cell growth, 2 μM of Gö 6983 addition suppressed differentiation and resulted in compact refractile human pluripotent stem cell colonies that could survive single cell passaging without ROCK inhibitor.

Naïve human ESCs

The crosstalk between different signaling pathways occurs constantly in ESCs and results in cooperative regulation of self-renewal and/or differentiation. This concept is best exemplified by recent efforts for the establishment or de novo derivation of naïve state human ESCs. Gafni and colleagues tested combined actions of 16 different factors that are known to be important for ESC pluripotency regulation and developed a new culture condition NHSM (LIF/2i with TGFβ1, FGF2, JNKi, p38i, ROCKi and PKCi) that supports ground-state human ESC establishment and maintenance [101]. On the other hand, screening experiments can also be performed to identify novel molecules and pathways that contribute to ESC self-renewal. For example, a chemical library screening was performed on

the hESC cell line bearing Oct4 distal enhancer activity reporter, a molecular signature of naïve pluripotency [181]. Two novel kinase inhibitors (BRAFi SB590885 and SRCi WH-4-023) were identified to support naïve human ESCs when used in combination with 2i/ROCKi/LIF/Activin A. Together, the manipulation of multiple signaling pathways in culture medium has presented new opportunities to isolate chimera-competent naïve ESCs from non-human primates.

Conclusions and prospects

In the past three decades of stem cell research, various combinations of feeder cells, conditioned media, cytokines, growth factors, hormones, sera and serum extracts as well as small molecules have been explored to facilitate de novo derivation and in vitro maintenance of ESCs in culture. Distilled from these empirical observations, several signaling pathways involved in ESC fate determination have been identified and intensively studied, with the hope of fully elucidating the molecular basis of ESC self-renewal. LIF/STAT3 is the first signaling pathway identified that can promote ESC self-renewal. The study of LIF/STAT3 signaling pathway not only provides fundamental understanding of proteins and genes that contribute to pluripotency, but also exemplifies how extrinsic stimulus can be converted into intrinsic responses to modulate ESC fate. TGFβ/SMAD signaling pathway, as inspired by the derivation and further investigation of human ESCs, serves as another example of the interaction between extrinsic stimuli and ESC fate choice, and establishes the important role of extracellular signals in promoting ESC self-renewal. Such point of view, however, has been challenged by the discovery of small molecule combinations that suppress the spontaneous differentiation of ESCs and therefore maintain pluripotency without extrinsic signals. Moreover, later screening assays and pharmacological studies on small molecules have uncovered several additional signaling pathways, such as Wnt/β-catenin, FGF/MEK/ERK and PKC signaling pathways, which could contribute to the maintenance of ESC pluripotency. In the effort to identify the target genes of known self-renewal signaling pathways, it is suggested that various signaling pathways form a regulatory network to control the fate of ESCs. Instead of being simply "switched on or off", the exact level on which a signaling pathway is activated or repressed, as well as how such fine tuning of signaling pathways integrate into each other, has also been shown to significantly affect its role in self-renewal.

Despite the rapid progress in the understanding of pluripotency and stem cell regulation in recent years, a number of important scientific questions and technical barriers remain to be addressed. To realize the full potential of stem cell therapies, especially ESC- and iPSC-based regenerative medicine, it is important to establish robust and reliable culture conditions for patient-derived pluripotent stem cells. To utilize animals for disease modeling studies, it is also critical to develop efficient and stable culture conditions for pluripotent stem cells from species such as livestock animals and non-human primates. So far, our understanding of ESC fate choice is insufficient and fractionized. Because pluripotency-related signaling pathways are mainly identified by phenotypical observations under certain culture conditions, the interaction between different stimuli as well as the relationship between different pluripotency factors are not yet clearly established. Moreover, a genome-wide view of the pluripotency network formed by transcription factors is limited. To fully dissect the complicated interactions within such network and recapitulate the mechanistic nature of ESC fate choice, large-scale high-throughput methods, such as screening of cDNA and chemical compound libraries, may help in discovering unforeseen factors and regulation patterns. Besides, ESC fate determination is a highly comprehensive process that is also regulated by many other elements, including epigenetic modulators [182] and non-coding RNAs (ncRNAs) [183]. A detailed comparison of these elements and key molecular events in signaling pathway-mediated self-renewal in mouse, rat, and human ESCs will be beneficial for future exploration of conserved self-renewal mechanisms, directed differentiation of ESCs, and future translational applications of ESCs for therapeutic purposes.

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