

# JAK-STAT3 and somatic cell reprogramming

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**Keywords:** JAK, STAT3, LIF, embryonic stem cells, reprogramming, iPSC, epigenetics

**Abbreviations:** BMP, bone morphogenetic protein; CHIP, chromatin immunoprecipitation; CLC-1/NNT-1/BSF-3, cardiotrophin-like cytokine-1/novel neurotrophin-1/B-cell stimulating factor-3; CNTF, ciliary neurotrophic factor; CT-1, cardiotropin-1; Dnmt, DNA methyltransferase; EpiSC, epiblast stem cell; Esrrb, estrogen-related receptor beta; ESC, embryonic stem cell; Erk, extracellular-signal-regulated kinases; FGF, fibroblast growth factor; GCSF, granulocyte colony stimulating factor; gp130, glycoprotein 130; GSK3, Glycogen synthase kinase 3; HDAC, histone deacetylase; iPSC, induced pluripotent stem cell; IL, interleukin; JAK, Janus kinase; Klf4, Krüppel-like factor 4; LIF, leukemia inhibitory factor; LIFR, LIF receptor; Nr5a2, nuclear receptor subfamily 5, group A, member 2; Oct4, Octamer-binding transcription factor 4; OSM, oncostatin M; PI3K, phosphatidylinositol 3-kinase; PKB/Akt, protein kinase B; PRC2, Polycomb repressive complex 2; SH2, sarcoma (Src)-homology-2; SHP2, SH2-domain containing tyrosine phosphatase; SOCS3, suppressors of cytokine signaling 3; Sox2, sex determining region Y-box 2; STAT3, signal transducer and activator of transcription 3; STAT3ER, a fusion protein of STAT3 with estrogen receptor; Tbx3, T-box transcription factor 3; Tcf3, transcription factor 3; Tgfb $\beta$ , transforming growth factor beta receptor; Tyk2, tyrosine kinase 2; Tyr, tyrosine

Reprogramming somatic cells to pluripotency, especially by the induced pluripotent stem cell (iPSC) technology, has become widely used today to generate various types of stem cells for research and for regenerative medicine. However the mechanism(s) of reprogramming still need detailed elucidation, including the roles played by the leukemia inhibitory factor (LIF) signaling pathway. LIF is central in maintaining the ground state pluripotency of mouse embryonic stem cells (ESCs) and iPSCs by activating the Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) pathway. Characterizing and understanding this pathway holds the key to generate naïve pluripotent human iPSCs which will facilitate the development of patient-specific stem cell therapy. Here we review the historical and recent developments on how LIF signaling pathway regulates ESC pluripotency maintenance and somatic cell reprogramming, with a focus on JAK-STAT3.

## Introduction

The importance of leukemia inhibitory factor (LIF) stimulated signaling in mouse embryonic stem cell (ESC) culture in vitro has long been recognized. By activating Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3), LIF plays essential roles in the generation and maintenance of mouse pluripotent embryonic stem cells which are capable of infinite self-renewal and differentiation to any cell type of the three-germ layers of an embryo.<sup>1,2</sup> Over the past two decades, much information has been obtained regarding the LIF signaling pathway

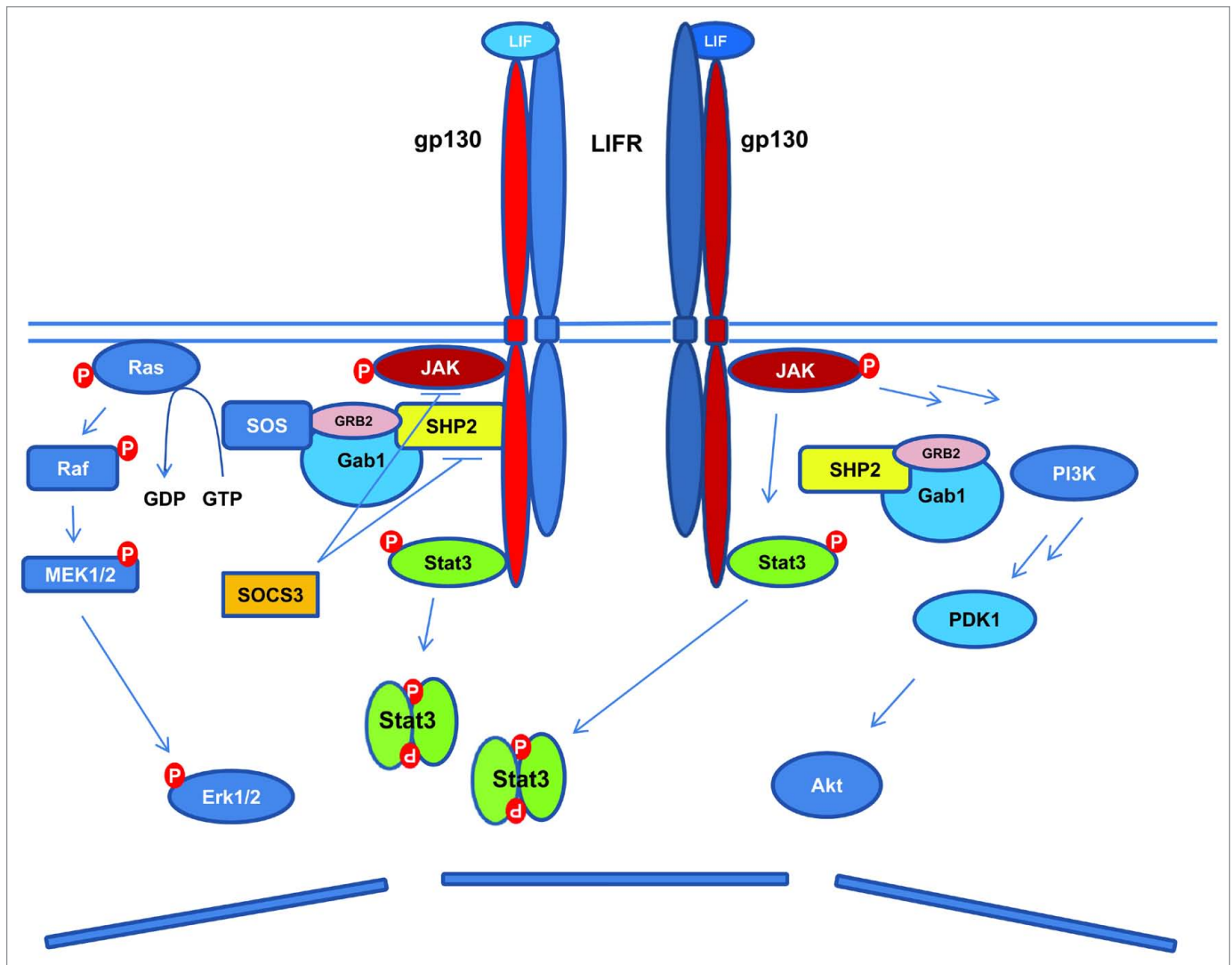
through studying mouse ESCs. However, the exact mechanism by which activated STAT3 controls pluripotency still remains to be elucidated.

The induced pluripotent stem cell (iPSC) technology<sup>3-5</sup> provides a powerful tool to reprogram somatic cells such as skin fibroblasts to a pluripotent state, and brings us much closer to the establishment of personalized, cell-based therapy for the treatment of currently incurable diseases such as diabetes and neural degenerative diseases.<sup>6-8</sup> In many aspects, somatic cell reprogramming represents a reversed cell differentiation process, by epigenetically resetting the cell nuclei back to a pluripotent state. At the same time reprogrammed cells turn on a cellular signal network capable of both robust cell division and fending off intra- and extra-cellular differentiation stimuli. Recently, the LIF-JAK-STAT3 axis was demonstrated by a number of studies to be essential for the naïve state pluripotency establishment during somatic cell reprogramming.<sup>9-11</sup> In addition, these studies provided important insight for further understanding of the mechanism behind LIF regulated ESC pluripotency. In this review, we will summarize the current knowledge on the role of LIF and especially JAK-STAT3 signaling pathway in ESC pluripotency maintenance and in somatic cell reprogramming.

## Historical Understanding of LIF

Leukemia inhibitory factor (LIF) is a member of the interleukin (IL)-6-type cytokine family, which includes IL-6, IL-11, IL-27, LIF, ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1), oncostatin M (OSM), and cardiotrophin-like cytokine-1/novel neurotrophin-1/B-cell stimulating factor-3 (CLC-1/NNT-1/BSF-3).<sup>12,13</sup> LIF was initially discovered to be pleiotropic in different mouse leukemia cell lines, by exerting either an inhibitory or promotional role on cell proliferation.<sup>14,15</sup> The same cytokine was also isolated from culture medium conditioned with Buffalo rat

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**Figure 1.** Schematic diagram of LIF signaling pathways. LIF binds to LIFR, which leads to the heterodimerization of LIFR and gp130. This is followed by the activation of JAK-STAT3, PI3K/Akt, and Erk1/2 signaling pathways. The activated STAT3 leads to increased expression of SOCS3, which serves as a negative feedback signal to LIF stimulated activation of STAT3 and Erk1/2.

liver cells,<sup>16</sup> which can promote the self-renewal and sustain the pluripotency of mouse ESCs in the absence of fibroblast feeder cells.<sup>16,17</sup> LIF binds to the low-affinity cell surface LIF receptor (LIFR),<sup>18</sup> which stimulates the hetero-dimerization of LIFR with the signal transducer glycoprotein 130 (gp130) (Fig. 1).<sup>19,20</sup> This triggers the activation of gp130-associated JAK kinases, and the subsequent phosphorylation of tyrosine residues in the gp130 cytoplasmic domain. These phosphor-tyrosines then serve as the docking sites to recruit the Src-homology-2 (SH2) domain containing STAT proteins (STAT1 and 3), which are phosphorylated by activated JAK kinase. Once phosphorylated, the STAT proteins dimerize and enter the cell nucleus to regulate the expression of their target genes.<sup>21</sup> Although the JAK kinases including JAK1, JAK2, and Tyk2 can all be phosphorylated following stimulation by LIF as well as other IL-6-type cytokines, JAK1-mediated IL-6/IL-11 signaling cannot be substituted by JAK2 and Tyk2.<sup>22,23</sup> Additionally, while RNA inhibition of JAK1

induces mouse ESC differentiation,<sup>24</sup> JAK2-deficient ESCs are responsive to LIF stimulation.<sup>25</sup> It was also found that in mouse ESCs, the activity of STAT3, but not STAT1, is responsible for pluripotency maintenance,<sup>2,26</sup> and artificially activated STAT3 can sustain ESC self-renewal in the absence of LIF.<sup>27</sup> These lines of evidence suggest that the prevailing “stemness” signaling is mediated by JAK1 activated STAT3 dimerization in mouse ESCs.

All IL-6-type cytokines share the common signal transducer gp130. Likewise, LIFR can serve as the receptor to mediate signals of several ligands including LIF, CNTF, CT-1, OSM, and CLC-1 (Table 1).<sup>28-30</sup> IL-6-type cytokines induced gp130 homo- or heterodimerization with LIFR also activates the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and the SH2-domain containing tyrosine phosphatase (SHP2)/extracellular-signal-regulated kinases 1/2 (Erk1/2) pathways.<sup>31-35</sup> Activated gp130 recruits SHP2 through its tyrosine residue (Tyr<sup>759</sup> in humans,

equivalent to Tyr<sup>757</sup> in mice), where SHP2 is phosphorylated by JAK kinases and interacts with the growth-factor receptor bound protein 2 (Grb2) and son of sevenless (SOS) complex to activate Erk1/2.<sup>31-35</sup> The activation of STAT3 also triggers the expression of the suppressors of cytokine signaling 3 (SOCS3), which competes with SHP2 for the cytoplasmic domain of gp130 phosphorylated at tyrosine residue Tyr<sup>759</sup> (human) or Tyr<sup>757</sup> (mouse), and inhibits the JAK-STAT3 and Erk1/2 activation (Fig. 1).<sup>24,36</sup> The negative regulation of JAK kinases by SOCS3 involves both direct inhibition of the JAK catalytic domain and the promotion of proteasomal degradation of JAK.<sup>13,37-39</sup> While constitutively activated Akt was reported to sustain mouse ESC pluripotency independent of STAT3,<sup>40</sup> signaling from activated Erk1/2 promotes the neural commitment of ESCs.<sup>41,42</sup> As the JAK-STAT3, PI3K/Akt and Erk1/2 pathways are simultaneously activated by LIF, it obviously contributes to the pleiotropic roles that LIF plays in different cell types, and complicates the understanding of LIF signaling regulated pluripotency.

### JAK-STAT3 Signaling and ESC Maintenance

It is well-known that JAK-STAT3 signaling is important in maintaining mouse ESC pluripotency and propagation. Unfortunately, thorough elucidation has not achieved despite the numerous attempts made to uncover the mechanism behind JAK-STAT3 mediated ESC self-renewal. STAT3 is a transcription factor whose amino acid sequence is highly conserved across species among humans, cattle, pigs, rats, and mice.<sup>43-47</sup> Its DNA binding domain recognizes the consensus sequence of TTCC(C/G)GGAA which is present in the regulatory regions of many genes.<sup>48,49</sup> The phosphorylation of Tyr<sup>705</sup> of STAT3 is important for STAT3 homo-dimerization and nuclear translocation following gp130 activation.<sup>45,50-52</sup> By mutating the STAT3 binding tyrosine residues in the cytoplasmic domain of gp130, it was shown that STAT3 activity was necessary for gp130 signaling mediated mouse ESC self-renewal.<sup>2</sup> Expressing a mutant form of STAT3 (STAT3F, tyr<sup>705</sup> to phenylalanine) leads to ESC differentiation even in the presence of LIF,<sup>2,53</sup> while dimerization of STAT3 fused with estrogen receptor (STAT3ER) upon 4-hydroxytamoxifen (4-HT) treatment promoted mouse ESC self-renewal in the absence of LIF.<sup>27</sup> Thus it became clear that STAT3 is the key downstream mediator of LIF stimulated pluripotent signal.

STAT3-deficient mouse embryos (*Stat3*<sup>-/-</sup>) die on E7.0 upon gastrulation in vivo. These embryos can form embryonic ectoderm and visceral endoderm but not the mesoderm.<sup>54</sup> In culture of wild-type ESCs, LIF prevents their differentiation into mesoderm and endoderm but needs either serum supplement or bone morphogenetic proteins (Bmps) to suppress the neuronal differentiation.<sup>55</sup> Bmp signaling induces the expression of inhibition of differentiation (Id) proteins, and mouse ESCs transfected with Ids remain undifferentiated in the presence of LIF without serum.<sup>55</sup> A number of functional genomic studies have identified many STAT3 target genes.<sup>48,49,56-58</sup> Microarray analysis using STAT3ER transfected ESCs identified 58 STAT3 targets, among which 22 are responsible for inhibiting the differentiation

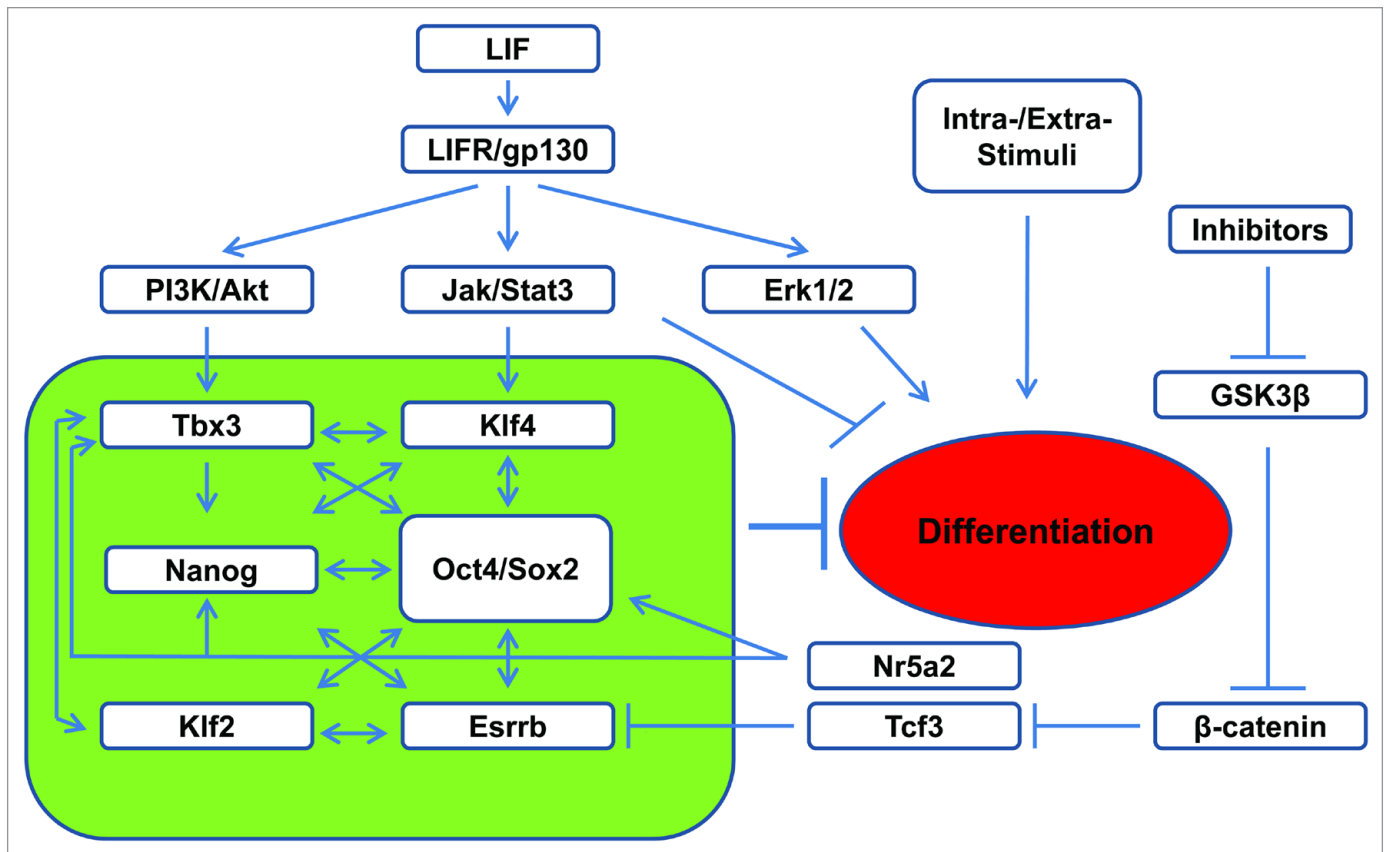
**Table 1.** Summary of IL-6-type cytokines and their receptor complexes

Cytokine	Receptor complex subunits		
	α-receptor	β-receptor	No. of gp130 involved in an induced receptor/ligand complex
IL-6	IL-6R	-	2
IL-11	IL-11R	-	2
IL-27	IL-27R	-	2
LIF	-	LIFR	1
CNTF	CNTFR	LIFR	1
CT-1	?	LIFR	1
OSM	-	LIFR or OSMR	1
CLC-1	CNTFR	LIFR	1

of either mesoderm (Ccm4l, Cyr61, Dact1, etc.), endoderm (Krüppel-like factor 4 [Klf4], Gbx2, Pim1, Pim3, Sall4, Smad7, etc.), or both (Klf5).<sup>58</sup> Interestingly, 14 of these genes, including Klf4, Klf5, Pim1, Dact1, and Smad7, are co-regulated by Nanog, indicating a partially functional redundancy between STAT3 and Nanog in pluripotency maintenance.<sup>58</sup> This is also in line with the finding that overexpression of either Nanog or STAT3 maintains ESC self-renewal in the presence of reduced signaling from one another.<sup>58,59</sup>

In mouse ESCs, the global DNA binding sites of 13 pluripotency-, cell cycle-, DNA binding-, or reprogramming-related transcription factors were evaluated using chromatin immunoprecipitation coupled with high-throughput sequencing (CHIP-seq). STAT3 occupies an impressive 2546 genomic loci.<sup>12,48</sup> Also, a total of 718 genomic loci were identified to be co-bound by STAT3 and at least another 3 of the 13 factors, and STAT3 clusters with Nanog, Oct4 (also known as Pou5f1), or sex determining region Y-box 2 (Sox2) among 56.8% of these loci, clearly demonstrating that STAT3 shares many common targets with the core pluripotent Oct4-Sox2-Nanog circuitry.<sup>48,60</sup> Interestingly, STAT3 were found to co-bind with Oct4, Sox2, Nanog, and Smad1 to the regulatory region of key pluripotent genes which were also found to be reprogramming factors such as Nanog, Oct4, Sox2, estrogen-related receptor β (Esrrb), Klf4, c-Myc, T-box transcription factor 3 (Tbx3), Sall4, etc.<sup>48</sup>

c-Myc had been previously identified as a STAT3 target and overexpression of constitutively active c-Myc mutant maintains mouse ESC pluripotency in the absence of LIF.<sup>61</sup> It was postulated that the LIF/STAT3 signaling promotes ESC self-renewal by stimulating c-Myc mRNA expression, and by stabilizing c-Myc protein levels through inhibiting glycogen synthase kinase 3β (GSK3β) mediated threonine 58 (Thr<sup>58</sup>) phosphorylation.<sup>61</sup> However, mouse ESCs with c-Myc disruption were reportedly normal in self-renewal,<sup>62</sup> presumably due to the redundant function from N-Myc.<sup>63,64</sup> Using CHIP and microarray analysis (CHIP-chip), 948 genes and 1459 genes were found bound by STAT3 and c-Myc in mouse ESCs, respectively, of which STAT3 and c-Myc co-occupy the promoters of 218 genes, including N-Myc, Rest, STAT3, Mbd3, and Jmjd3.<sup>49</sup> Interestingly, it was shown that while STAT3 binds to genes that are either transcriptionally



**Figure 2.** Schematic representation of LIF regulated pluripotency circuit in ESCs. Upon LIF activation, JAK-STAT3 promotes the expression of the core pluripotency circuit (green box) together with PI3K/Akt for pluripotency maintenance. Activated STAT3 also directly suppresses differentiation by inhibiting the expression of lineage commitment genes.

activated or repressed, *c-Myc* mainly occupies actively transcribed genes in ESCs.<sup>49</sup> These, together with other studies described above, indicate that while STAT3 functions synergistically with the Oct4-Sox2-Nanog pluripotent circuitry and *Myc* by co-regulating their common target gene expression, unique function by STAT3 through regulating its specific targets not bound by these factors may exist for the pluripotency maintenance.

### Current Understanding of LIF/STAT3 Regulated ESC Pluripotency

Similar to Nanog, overexpression of *Klf4* or *Tbx3* gene confers LIF-independent mouse ESC self-renewal.<sup>33,59,65</sup> It was elegantly demonstrated that upon LIF stimulation, *Klf4* expression in mouse ESCs is preferentially stimulated by JAK-STAT3 activation, whereas *Tbx3* expression is stimulated by PI3K/Akt pathway upon the inhibition of Erk activities.<sup>33</sup> Based on their finding, Niwa et al.<sup>33</sup> proposed a model where LIF regulates pluripotency maintenance by simultaneous stimulation of JAK-STAT3 and PI3K/Akt pathways in ESCs. This leads to the activation of *Klf4* and *Tbx3* expression, and the subsequent expression of Sox2 and Nanog, respectively. Sox2/Nanog cooperate to maintain Oct4 expression, while Nanog also reinforces the established Oct4-Sox2-Nanog pluripotency network in the absence of *Klf4* and *Tbx3* (Fig. 2).<sup>33</sup>

This model of parallel axes by LIF/JAK-STAT3/Sox2 and LIF/PI3K/Nanog to maintain pluripotency are supported by multiple lines of evidences. First, selective activation of *Klf4* by STAT3 was reported to synergize with *Klf2*, activated by Oct4, to ward off the differentiation stimuli to ESCs.<sup>66</sup> STAT3 was also reported to directly upregulate Sox2 during neural progenitor cell differentiation from ESCs.<sup>67</sup> Second, upregulation of Nanog through PI3K activation or GSK3β inhibition has been reported to contribute to ESC maintenance.<sup>68-70</sup> Using PI3K inhibitor LY294002, 646 downstream targets of PI3K pathway were identified in mouse ESCs by microarray analysis.<sup>71</sup> These include some key pluripotent genes such as Nanog, *Esrrb*, *Tbx3*, and *Tcl-1*, as well as *Zscan4c*, one of the *Zscan4* family of zinc finger proteins. Interestingly, it was found that inhibition of *Zscan4c* reduces the proportion of ESCs expressing high levels of Nanog,<sup>71</sup> and *Zscan4c* was found to regulate telomerase elongation and chromosome stability in ESCs.<sup>72</sup> In addition, although it is unclear why the constitutively activated Akt confers mouse ESC self-renewal,<sup>40</sup> Akt stabilizes *Myc* by inhibiting GSK3β-mediated phosphorylation and degradation,<sup>61,73</sup> and Akt also phosphorylates Sox2, thus enhancing its stability and transcriptional activity in ESCs.<sup>74</sup> However, in addition to the PI3K/Nanog axes in ESCs, STAT3 also binds to Nanog promoter with Brachyury which co-activates Nanog

expression in early mesoderm progenitor cells derived from ESCs.<sup>74</sup>

The activation of canonical Wnt pathway leads to phosphorylation and inhibition of GSK3 $\beta$  and the subsequent nuclear accumulation of  $\beta$ -catenin to regulate cell fate and many other biological events. Constitutive activation of the Wnt pathway results in differentiation defects of mouse ESCs.<sup>75</sup> It was reported that inhibition of GSK3 $\beta$  using 6-bromoindirubin-3'-oxime promotes mouse ESC self-renewal in the absence of LIF,<sup>76</sup> and that activated  $\beta$ -catenin upregulates Nanog expression by interacting with Oct4.<sup>77</sup> However, it was also reported that Wnt signaling alone is not sufficient for the maintenance of mouse ESC self-renewal but can cooperate with LIF for this action.<sup>78</sup> Part of this synergistic effect of Wnt could be through increasing STAT3 mRNA level in ESCs.<sup>79</sup> A more recent study indicates that Wnt regulated expression of orphan nuclear receptor Nr5a2 (also known as LRH-1) is significant in augmenting PI3K/Akt pluripotent signaling through regulating Tbx3, Nanog, and Oct4 expression independent of JAK-STAT3.<sup>80</sup> However, unlike Nanog and Tbx3, overexpression of Nr5a2 cannot sustain ESC pluripotency in the absence of LIF.<sup>81</sup>

The culture of mouse ESCs under the dual inhibition of Erk and GSK3 $\beta$  (2i) ensures ESC self-renewal and pluripotency maintenance without LIF.<sup>82</sup> STAT3 null ESCs can be derived under the 2i or triple inhibition of FGF receptor, Erk1/2, and GSK3 $\beta$  culture condition.<sup>66,82</sup> The main effect of GSK3 $\beta$  inhibition is the nuclear accumulation of  $\beta$ -catenin. Although  $\beta$ -catenin was found dispensable for ESC self-renewal,<sup>83,84</sup> its overexpression enhances pluripotent gene expression and delays the mouse ESC differentiation.<sup>85</sup> The pluripotency maintenance effect of GSK3 $\beta$  inhibition was recently found to be through inhibiting transcription factor 3 (Tcf3) protein by activated  $\beta$ -catenin,<sup>84</sup> which relieves the suppressive effect of Tcf3, leading to the expression of pluripotent gene Esrrb.<sup>86</sup> Smith's group therefore proposed a model of ESC signal network in which pluripotency is supported by the inhibition of GSK3 $\beta$  and the activation of STAT3 which in turn activate the expression of Tcf3 and Klf4, respectively, and ESC is maintained by an expanded core pluripotent circuitry including Oct4/Sox2, Nanog, Klf4, Tbx2, and Tcf3 (Fig. 2).<sup>86</sup> However, Esrrb is also dispensable for ESC maintenance in the presence of LIF,<sup>86</sup> which highlights the parallel compensatory capacity by the core pluripotent circuitry for ESC self-renewal. A complementary model was proposed by Savatier's group which describes the synergistic effect of STAT3 with Nanog in preventing mesoderm and endoderm differentiation, as well as STAT3's direct effect in preventing endoderm differentiation through regulating its specific target genes in mouse ESCs.<sup>58</sup> Together, these studies contribute a comprehensive depiction to our understanding of LIF signal network (Fig. 2).

A few studies also investigated the STAT3 regulated ESC pluripotency and gene expression at the epigenetic level. One study discovered that in mouse ESCs, STAT3, and Oct4 co-bind to the promoter region of embryonic ectoderm development (Eed) gene and promote its expression.<sup>87</sup> As Eed is a core component of Polycomb repressive complex 2 (PRC2), this stabilizes the histone 3 lysine 27 tri-methylation (H3K27me3) at the promoter regions

of several lineage commitment genes, therefore suppresses their expression.<sup>87</sup> The pluripotent cell-specific ATP-dependent SWI/SNF chromatin remodeling helicase complex (esBAF) is essential for ESC pluripotency and self-renewal, with Brg1 (also known as Smarca4) as a key esBAF component.<sup>88</sup> In mouse ESCs, Brg1 was shown to be required to prepare the chromatin access for STAT3 to activate many of its target genes, whose promoters are otherwise occupied by PRC2 complex.<sup>89</sup> Other studies also demonstrated that Brg1 binding to STAT3 is necessary to allow STAT3 to access the promoter region of INF regulatory factor 1 to control its expression, as well as the promoter of the p21waf1 gene followed by STAT3-cdk9 interaction promoted transcriptional elongation.<sup>90,91</sup>

## JAK-STAT3 in Somatic Cell Reprogramming

iPSCs are ESC-like cells reprogrammed from somatic cells by overexpression a set of reprogramming factors including Oct4, Klf4, Sox2, and c-Myc (OKSM) in the mice and humans, or Oct4, Sox2, Nanog, and Lin28 in humans.<sup>3,5,92</sup> This technology represents a powerful way for regenerative medicine in terms of massively producing patient-specific pluripotent cells within a short time. However, many technical issues still remain unsolved, including the efficiency of reprogramming, the development of integration-free reprogramming method, the quality and safety of induced iPSCs for clinical application, and lastly but probably most importantly, the understanding of the molecular mechanisms of reprogramming. Such information is required to ensure a proper translation of the iPSC technology to human medicine.

Many major differences exist between the mouse ESCs/iPSCs and human ESCs/iPSCs. For example, human ESCs/iPSCs are flat in colony morphology; depend on FGF/Activin signaling for their pluripotency maintenance; and have completed X chromosome inactivation. They divide slowly (36 h doubling time), and survive poorly after single cell dissociation—a property essential for gene targeting.<sup>93-96</sup> On the contrary, mouse ESCs/iPSCs are dome-shaped, depend on LIF signaling for pluripotency, divide rapidly (16 h doubling time), and are amenable for single-cell colonization and gene targeting.<sup>2,16</sup> The human ESCs/iPSCs are in fact similar to mouse epiblast stem cells (mEpiSCs) derived from pre- or post-implantation mouse embryos which have limited *in vivo* differentiation capacity,<sup>97-99</sup> and these cells are collectively referred to as being in the “primed” pluripotent state, while the mouse ESCs/iPSCs from pre-implantation embryos are referred as in the “naïve” state.<sup>100</sup>

So far numerous studies have focused on the role of JAK-STAT3 in the maintenance of the steady-state pluripotency of ESCs; however, until recently little had been known whether LIF signaling is essential for iPSC induction, despite that LIF was preferably included in the induction medium. Interestingly, withdrawing LIF and supplementing the culture medium with FGF/Activin A results in the transition of naïve mouse ESCs to primed EpiSC state.<sup>101</sup> At the same time, EpiSCs can be reprogrammed into naïve mouse iPSCs by overexpression of Klf4, Klf2, or Nanog.<sup>66,101-103</sup> Using this EpiSC-ESC reprogramming system, Yang et al. in Smith's group<sup>9</sup> studied the effect of LIF

signaling in the conversion of primed to naïve state pluripotency. They first found that LIF is necessary for EpiSC-ESCs transition induced by Klf4 or Nanog overexpression under the 2i condition.<sup>9</sup> Previously a chimeric receptor was described (GY118F),<sup>2</sup> in which the ligand binding domain of the granulocyte colony stimulating factor (GCSF) receptor was fused to the trans-membrane and cytoplasmic domains of gp130 modified at residue Tyr<sup>118</sup> (corresponding to the Tyr<sup>757</sup> of the wild-type mouse gp130) to phenylalanine. This ensures constitutive activation of STAT3 upon GCSF stimulation.<sup>2</sup> By expressing the GY118F receptor in EpiSCs with GCSF stimulation, Yang et al. discovered that activation of STAT3 can convert EpiSCs directly to naïve iPSCs in 2i medium. This was also confirmed with overexpression of STAT3ER activated by 4-HT.<sup>9</sup> They further demonstrated that STAT3 activation in EpiSCs does not stimulate Klf4 or Nanog expression. Rather, STAT3 acts synergistically with either of them to dramatically improve the reprogramming efficiency. Finally, they demonstrated that activated STAT3 also improves the reprogramming of mouse neural stem cells and partially reprogrammed cells (pre-iPSCs) to naïve state iPSCs induced by Oct4, Klf4, c-Myc (OKM), or OKSM, respectively.<sup>9</sup>

The study by Smith's group for the first time demonstrated that STAT3 activation is necessary for the reprogramming between mouse EpiSCs to iPSCs. A later study also demonstrated that enhanced STAT3 activation can reprogram mouse EpiSCs to naïve pluripotent cells even in the presence of FGF/Activin differentiation signals.<sup>10</sup> Thus the STAT3 activity becomes essential for converting the primed state to naïve state pluripotency in the mouse. However, it remained unclear whether the LIF signaling is necessary for reprogramming of terminally differentiated mouse somatic cells, which bypasses the EpiSC stage during reprogramming with LIF containing medium. Furthermore, the molecular mechanism of STAT3 induced reprogramming remains elusive, albeit much information has been obtained by studying the steady-state pluripotency maintenance.

We addressed these questions by studying the role of STAT3 during the reprogramming of terminally differentiated mouse embryonic fibroblasts (MEFs). Using STAT3C, a constitutively activated STAT3 mutant with two residues in its SH2 domain mutated to cysteine (A662C and N664C),<sup>104</sup> we discovered that enhanced STAT3 activation significantly promotes the reprogramming from MEF cells to iPSCs by Oct4, Klf4, Sox2 (OKS), or OKSM overexpression.<sup>11</sup> This result was doubly confirmed by expressing a mutant gp130 with Tyr<sup>757</sup> converted to phenylalanine, as well as by deprivation of LIF during reprogramming.<sup>11</sup> We further found that the complete MEF reprogramming, as indicated by expression of GFP transgene driven by the Oct4 distal enhancer,<sup>105</sup> was completely blocked in the presence of JAK inhibitor 1 (JAKi) but not the PI3K inhibitor, LY294002. However, the total number of induced colonies was not affected by the JAKi treatment. These observations demonstrated that STAT3 activation plays a vital role in the late-stage somatic cell reprogramming, i.e., the activation of the endogenous Oct4 gene.<sup>11</sup>

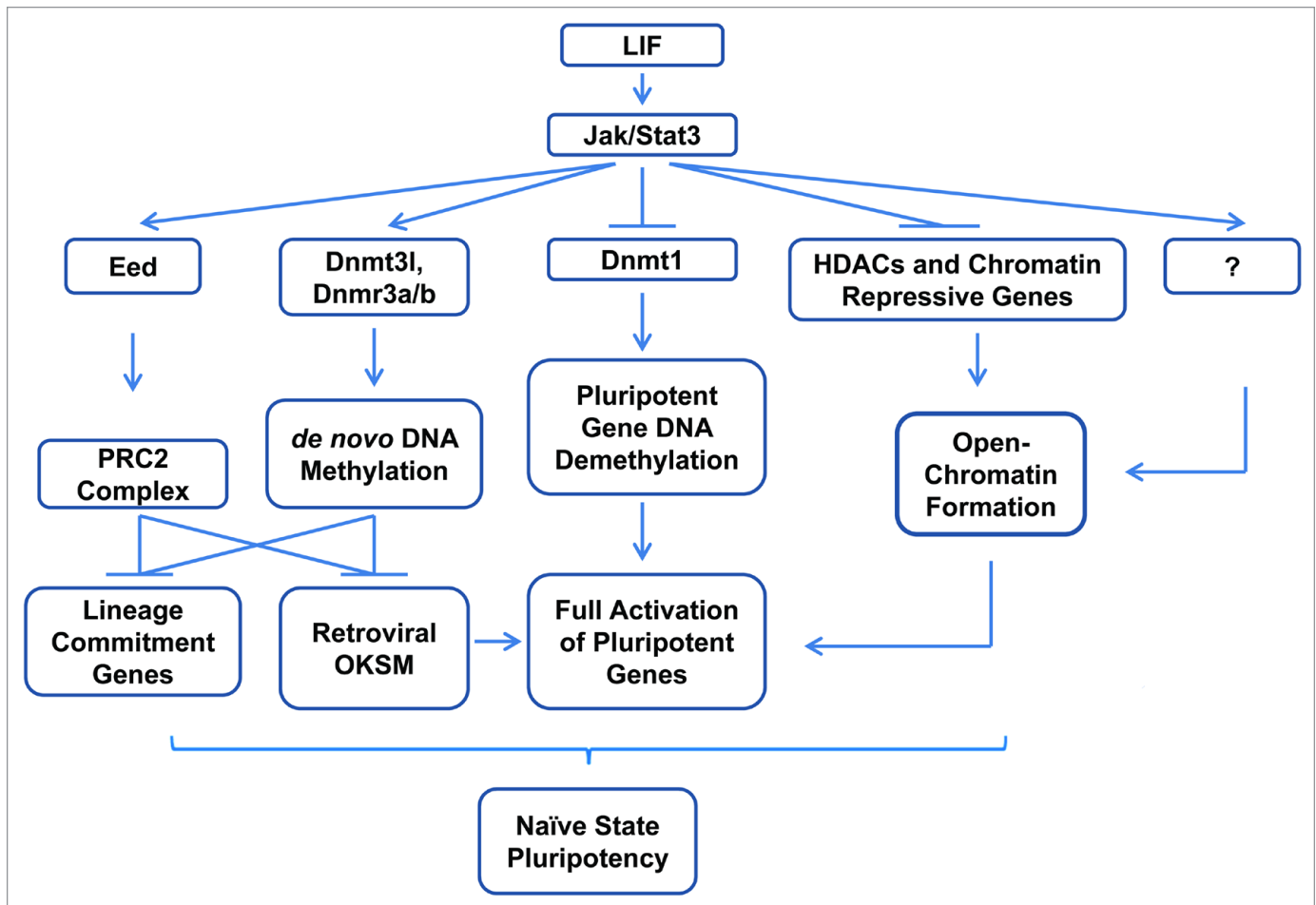
We also studied the role of STAT3 activation in the complete reprogramming of pre-iPSCs induced by OKM. These cells form

colonies but do not express endogenous Oct4, and can be completely reprogrammed by the expression of Sox2 or the addition of RepSox, an inhibitor of transforming growth factor  $\beta$  receptor (Tgfbr).<sup>106</sup> Again our results show that STAT3 activation is necessary for Sox2 or Tgfbr-signaling pathway inhibitor promoted reprogramming of pre-iPSCs.<sup>11</sup>

Examining the promoter DNA methylation status revealed that JAK-STAT3 inhibition prevents the demethylation of endogenous Oct4 and Nanog during reprogramming. This is associated with increased expression of DNA methyltransferase 1 (Dnmt1), class I histone deacetylases (HDACs), and other chromatin-repressive genes.<sup>11</sup> Inhibition of Dnmt1 and HDACs has been shown to significantly improve somatic cell reprogramming.<sup>107-110</sup> In order to test whether the STAT3 induced inhibition of Dnmt1 and HDAC expression is important for complete reprogramming, we applied inhibitors for Dnmt1 and HDACs<sup>107-110</sup> together with the inhibition of STAT3 during reprogramming. Indeed we found that inhibiting either Dnmt1 or HDACs but not the overexpression of Nanog can overcome the halted reprogramming by STAT3 inactivation.<sup>11</sup>

During this study, we also observed that STAT3 inhibition resulted in continued expression of the retroviral transgenes which are otherwise gradually silenced at late-stage reprogramming. This lack of external gene silencing is tightly associated with the diminished expression of de novo DNA methyltransferase Dnmt3l.<sup>11</sup> It has previously been demonstrated that expression of Dnmt3l in mouse ESCs is crucial to the activation of de novo DNA methylation which silences the viral transgenes.<sup>111</sup> Our finding therefore reveals a new role of STAT3 in regulating de novo DNA methylation for epigenetic gene silencing during reprogramming. Taken together, our work demonstrated that (1) JAK-STAT3 activation is essential for the reprogramming of murine somatic cells, (2) STAT3 activity is crucial for the pluripotent gene promoter demethylation, and (3) STAT3 is important for retroviral transgene silencing during the reprogramming.

The discovery that STAT3 activity is essential for the reprogramming from mouse EpiSCs and somatic cells to naïve iPSCs, as demonstrated by others and us, respectively,<sup>9-11</sup> is significant for the understanding of reprogramming and pluripotency. The continued high-level expression of ectopic transgenes in pre-iPSCs and in reprogrammed cells caused by STAT3 inhibition<sup>9,11</sup> strongly indicates the existence of a reprogramming barrier which cannot be simply overcome by a greater dosage of reprogramming factors in the absence of STAT3 activity, even with the overexpression of Klf4 and Nanog which can sustain ESC self-renewal in the absence of LIF.<sup>33,59,65</sup> A passive model of genomic DNA demethylation as a result of inhibited methylation maintenance has been proposed to explain the somatic cell reprogramming.<sup>112,113</sup> It was also found that transient STAT3 activation poises the EpiSCs for complete reprogramming by Klf4 or Nanog.<sup>9</sup> These are in accordance with our finding that JAK-STAT3 functions epigenetically to inhibit the expression of Dnmt1 and other heterochromatin-promoting genes, which in turn promotes the demethylation of pluripotent loci during reprogramming. Together, our results strongly suggest a central role of STAT3 during reprogramming, by orchestrating the



**Figure 3.** Schematic representation of STAT3 regulated somatic cell reprogramming via epigenetic mechanisms. During late-stage reprogramming, STAT3 orchestrates the epigenetic changes leading to complete pluripotency, by ensuring full activation of core pluripotency genes through promoting DNA demethylation and open chromatin formation, while suppressing the viral transgenes and lineage commitment genes by promoting de novo DNA methylation and probably PRC2 mediated histone modifications.

establishment of open-chromatin to completely activate the pluripotent genes, and by promoting the de novo DNA methylation activity to silence the expression of viral transgenes and possibly the lineage commitment genes, in addition to the STAT3 promoted suppression through PRC2 (Fig. 3).<sup>87</sup>

### Perspective

The JAK-STAT3 pathway has been identified as the core signaling pathway for pluripotency maintenance, and was also demonstrated recently to be essential for the complete reprogramming of mouse somatic cells. Although a number of STAT3 downstream target genes had been identified, a thorough understanding of the STAT3-mediated reprogramming still remains unachieved. An emerging area of study on STAT3 mechanism is to investigate the epigenetic roles of STAT3 in the maintenance of steady-state

pluripotency and during the dynamic changes of somatic cell reprogramming. Special emphasis may be placed on the STAT3 induced formation of euchromatin for the pluripotent gene activation, and formation of heterochromatin for lineage commitment gene silencing. Interestingly, recently several groups reported the generation of naïve state human iPSCs, which also rely on LIF signaling for their self-renewal.<sup>114-116</sup> Together, these series of studies have brought JAK-STAT3 to the central stage of iPSCs research.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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