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# Jak2 and Tyk2 are necessary for lineage-specific differentiation, but not for the maintenance of self-renewal of mouse embryonic stem cells

Bo Mee Chung [a,1], Ho Chul Kang [a,1], Su Youne Han [a,1], Hyen Seok Heo [a], Jong Joo Lee [a], Jinseon Jeon [a], Ji Young Lim [a], Incheol Shin [a], Seung Hwan Hong [b], Yoon Shin Cho [a], Chul Geun Kim [a,*]

[a] *Department of Life Science, College of Natural Sciences, and Stem Cell Research Center, The Research Institute of Natural Sciences, Hanyang University, Seoul 133-791, Republic of Korea*
[b] *Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Republic of Korea*

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**Abstract**

As the LIF-induced Jak1/STAT3 pathway has been reported to play a crucial role in self-renewal of mESCs, we sought to determine if Jak2, which is also expressed in mESCs, might also be involved in the pathway. By employing an RNAi strategy, we established both Jak2 and Jak2/Tyk2 knockdown mESC clones. Both Jak2 and Jak2/Tyk2 knockdown clones maintained the undifferentiated state as wild-type controls, even in a very low concentration of LIF. However, we observed not only faster onset of differentiation but also differential expression of tissue-specific lineage genes for ectodermal and mesodermal, but not endodermal origins from embryoid bodies generated from both types of knockdown clones compared to the wild-type. Furthermore, the reduced level of Jak2 caused differentiation of mESCs in the presence of LIF when the Wnt pathway was activated by LiCl treatment. Taken together, we demonstrated that Jak2 and Tyk2 are not involved in LIF-induced STAT3 pathway for self-renewal of mESCs, but play a role in early lineage decision of mESCs to various differentiated cell types.


*Keywords:* mESCs; Self-renewal; LIF-induced Jak1/STAT3 signaling pathway; Jak2; Tyk2; RNAi; Microarray; Differentiation; Lineage specification

Embryonic stem (ES) cells derived from the inner cell mass of pre-implantation embryos retain the ability to undergo self-renewal and to differentiate into all cell types simultaneously [1–3]. These unique properties of ES cells make them a fascinating potential tool for cell therapy in regenerative medicine. Although the molecular mechanisms involved in their self-renewal and pluripotency are not fully understood, several factors that maintain the ES cell state have been identified from the current remarkable progress on ES cell biology. Stemness factors identified so far include transcription factors such as Oct3/4 and Nanog, and signaling pathways such as LIF, BMP, FGF, and Wnt [4]. Pathways mediated by LIF and Oct3/4 are known as the most critical ones regulating self-renewal in mouse ES cells (mESCs) [5].

LIF binding to the heterodimer of LIFRβ and gp130 rapidly activates Janus kinase (Jak) which phosphorylates IFRβ and gp130 on their tyrosine residues [6]. Phosphorylated LIFRβ and gp130 recruit SH2 domain containing signaling molecules such as signal transducer and activator of transcription (STATs) and SHP2 to their phosphorylated regions, and Jaks phosphorylate both STATs and SHP2 [7]. Phosphorylation of STATs induces formation of STAT dimers, and subsequently dimerized STATs control the expression of target genes in the nucleus as transcription factors [8]. Among the seven mammalian STAT genes, STAT3 is known to be important for the maintenance of the undifferentiated state of mESCs [9,10]. In mESCs, Jak1, Jak2,

* Corresponding author. Fax: +82 2 2296 5996.
*E-mail address:* cgkim@hanyang.ac.kr (C.G. Kim).
[1] These authors contributed equally to this work.

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and Tyk2 are expressed. Although activation of STAT3 by Jak1 in mESCs has been elucidated [11], the functions of the two other isoforms, Jak2 and Tyk2, are not clear. To gain insight into the functional roles of Jak2 in mESCs, we established in this study nine Jak2 knockdown cell lines using a Jak2 RNAi strategy on the basis of mESCs that express the estrogen receptor fusion form of STAT3 (STAT3ER) [10], and tested their STAT3 mediated self-renewal activity and lineage-specific differentiation potential.

## Materials and methods

*Cell culture.* Undifferentiated STAT3ER expressing cells [10] were maintained as previously described elsewhere [12]. To induce EB formation, dissociated ES cells were cultured in hanging drops in the absence of LIF [13]. The number of undifferentiated EBs was scored by monitoring expression of alkaline phosphatase with NBT/BCIP staining. Cell signaling inhibitors were dissolved in dimethyl sulfoxide (DMSO) at the following concentrations: LY294002 (50 μM), U0126 (10 μM), PD098059 (50 μM), chelerythrin (10 μM), genistein (100 μM), and LiCl (20 mM). The final concentration of DMSO was less than 0.01%. The differentiation state of mESCs was determined by monitoring stem cell colony morphology. The proportion of undifferentiated colonies to the total number of colonies was calculated after scoring the morphology of 300 randomly chosen colonies in triplicate dishes.

*Plasmid construction, transfection, and viral infection.* Two target sequences for Jak2 RNAi (#1, 5′-TCC TTG ACG GAG AGC AAG T-3′; #2, 5′-GAG CAA CGG AAG ATT GCC A-3′) were designed as previously described elsewhere [14,15]. The first synthesized DNA oligonucleotide targeting Jak2 was inserted into the *Apa*I/*Eco*RI linearized pSilencer 1.0 vector (Ambion) to construct pSilencer-Jak2i. To generate the retroviral vector with the U6 promoter and target sequence for Jak2 RNAi, DNA fragments were liberated from pSilencer-Jak2i with *Bam*HI/*Pst*I and subcloned into the *Nsi*I/*Bgl*II sites of the pSuper.Retro.Hygro vector (Fig. 1A, left panel). The second DNA oligonuleotide targeting Jak2 was inserted into the *Bgl*II/*Hin*dIII linearized pSuper vector (Oligoengine) to generate pSuper.Retro.Puro-Jak2i. To create the lentiviral vector pLV-Jak2i containing the H1 promoter and target sequence for Jak2 RNAi, DNA fragments liberated from pSuper.Retro.Puro-Jak2i with *Eco*RI/*Cla*I were subcloned into the *Eco*RI/*Cla*I sites of the pLV-TH vector [16] (Fig. 1A, right panel). The linearized pSilencer-Jak2i (15 μg) and the neo$^r$ gene (3 μg) were cotransfected into $5 \times 10^6$ STAT3ER cells by electroporation, and the cells were selected with 125 μg/ml of G418 for 14 days. For transient transfection, mESCs ($2 \times 10^4$) were plated in 12-well dishes, and 0.6 μg DNA was transiently transfected using Effectene (Qiagen). To produce retrovirus or lentivirus, 1.5–2.0 μg of the retroviral or lentiviral plasmids was transfected into the pT67 virus package cell line (Clontech) using calcium phosphate precipitation. Eight micrograms per milliliter of polybrene (Sigma) was added to the virus preparation before

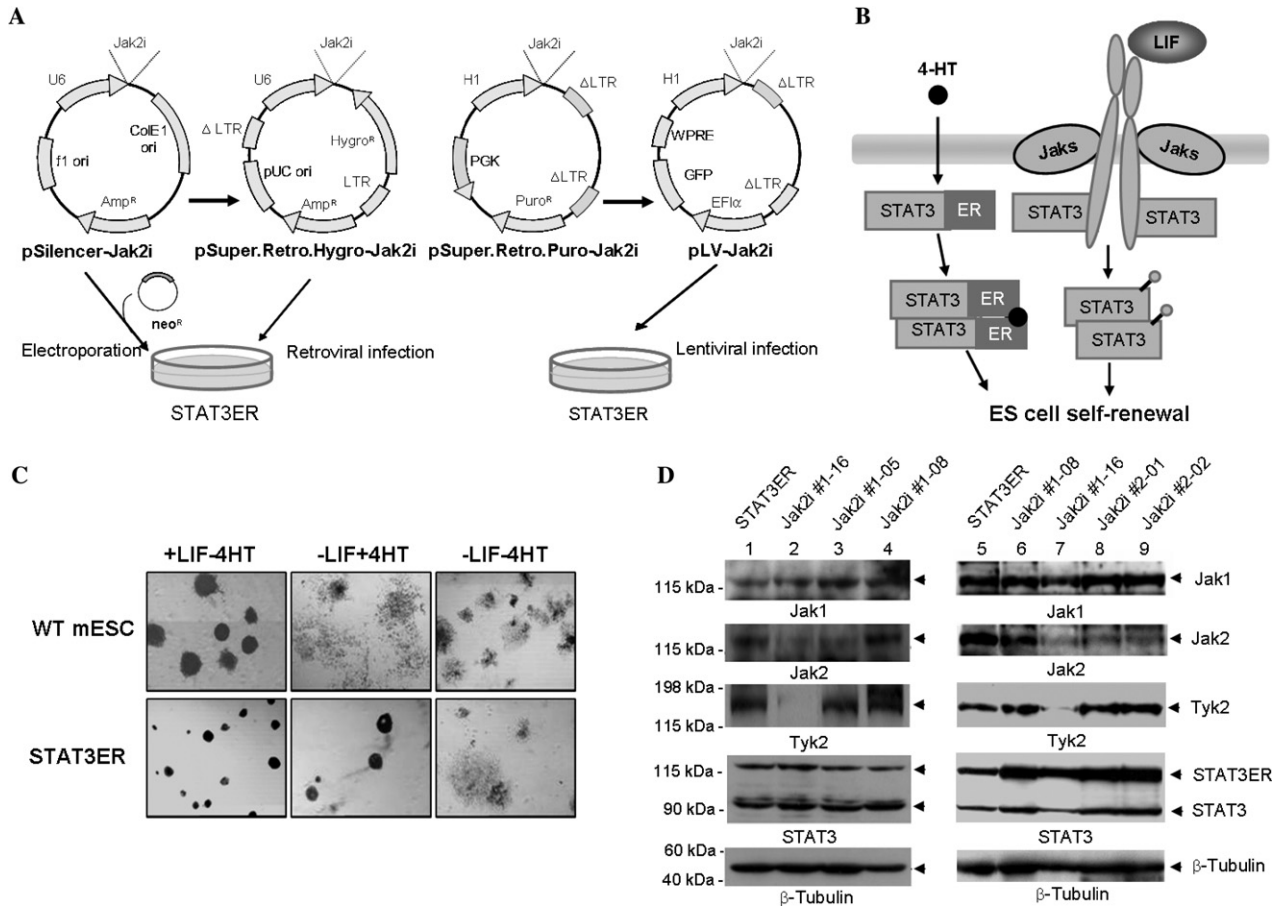


Fig. 1. Establishment of Jak2 knockdown clones using a vector-based RNAi system. (A) A schematic diagram showing Jak2i vector constructs and establishment of RNAi expression cell lines. (B) Schematic presentation of the STAT3ER cell line to maintain its self-renewal in the presence of 4-HT. STAT3ER can form dimers in the presence of 4-HT, resulting in the maintenance of undifferentiated state without LIF signals. (C) mESC colonies maintained in various combination of LIF (500 pM) and 4-HT (1 μM). Undifferentiated mESCs were detected by alkaline phosphatase staining on the fourth day of culture (40× magnification). (D) Western blotting showing the expression of Jaks and STAT3 in the selected Jak2i knockdown clones.

the mESC transduction. Twenty-four hours after infection, the cells were selected with 100 μg/ml of hygromycin for 7 days or by GFP fluorescence, respectively.

*Western blotting.* Protein extracts were prepared using a lysis buffer (10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 20% glycerol, 1 mM EDTA, 50 mM NaCl, 1 mM $Na_3VO_4$, and 1 mM PMSF). Ninety micrograms of protein was loaded onto SDS–polyacrylamide gels and subjected to electrophoresis, and transferred onto nitrocellulose membrane by semi-dry electrotransfer. Blotted membranes were then incubated with a 1:200 dilution of the anti-Jak1, Jak2, Tyk2, STAT3, or phospho-STAT3 antibody (Santa Cruz) in 1% milk/PBST for 2 h. Following three 10-min washes in PBST, the membranes were incubated with a 1:1000 dilution of the rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) in 1% milk/PBST for 2 h. Finally, membranes were subjected to three 10-min washes in PBST, and then immunocomplexes were visualized using an enhanced chemiluminescence system (ECL; Amersham).

*RNA preparation and semi-quantitative RT-PCR.* Total RNA was isolated from differentiating mESCs using Trizol (Invitrogen). cDNA synthesis reactions were performed as described previously [17]. PCR primer sequences are as follows: HPRT (5′-GCT GGT GAA AAG GAC CTC T-3′ and 5′-CAC AGG ACT AGA ACA CCT GC-3′); Nestin (5′-GGA GTG TCG CTT AGA GGT GC-3′ and 5′-TCC AGA AAG CCA AGA GAA GC-3′); βH1 globin (5′-GGA AAC CCC CGG ATT AGA GC-3′ and 5′-CAG CCT GCA CCT CTG GGG TG-3′); β-major globin (5′-GCC CTG GGC AGG CTG CTG GT-3′ and 5′-GGT GGT GGC CCA GCA CAA TCA CG-3′); and GATA4 (5′-GCG TCG TAA TGC CGA GGG TG-3′ and 5′-AGT CTG GGA CAT GGA GCT GC-3′).

*cDNA microarray and data analysis.* RNA amplification was performed as previously described [18,19], and labeling efficiency was determined using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies). Cy-dye-labeled reference and sample probes were cohybridized overnight at 65 °C to the TwinChip™ Mouse 7.4 K cDNA microarray (Digital Genomics) as described previously [17]. It was considered as significant when logarithmic gene expression ratios in the four independent hybridizations were more than 1 or less than −1, i.e., a 2-fold difference in the logarithmic expression level, and when the $q$ value was <0.1.

## Results and discussion

### *RNAi induced downregulation of Jak2 in mouse ES cells*

To investigate the functional role of Jak2 in LIF-dependent self-renewal activity of mESCs, we generated stable Jak2 knockdown mESC lines using an RNAi strategy. To avoid unexpected differentiation by down-regulation of Jak2, the mESC line expressing STAT3ER [10] was employed in this study. Treatment of this cell line with 4-hydroxytamoxifen (4-HT) induces dimerization of STAT3, which allows STAT3ER-expressing cells to the undifferentiated state without LIF-induced Jak signaling (Fig. 1B). Indeed, STAT3ER cells were able to be maintained in the undifferentiated state with either 4-HT or LIF (Fig. 1C).

By transfecting STAT3ER cells with the pSuper.Retro.hygro-Jak2i retroviral vector or the pLV-Jak2i lentiviral vector (Fig. 1A), we obtained total 9 clones of Jak2 knockdown candidate clones. The expression level of Jak2 protein in clones was monitored by Western blotting. From electroporation or retroviral infection using RNAi targeting #1 Jak2 sequence (see Materials and methods), we selected two clones (Jak2i #1-05 and Jak2i #1-16) showing significantly reduced expression of Jak2 (Fig. 1D). From lentiviral infection using RNAi targeting #2 Jak2 sequence (see Materials and methods), we also selected two clones (Jak2i #2-01 and Jak2i #2-02) showing significantly reduced level of Jak2 protein (Fig. 1D). Interestingly, Western blotting results detected with antibodies for Jak/STAT pathway proteins (such as Jak1, Tyk2, and STAT3) revealed concomitant loss of Tyk2 along with Jak2 in one of Jak2i clones (Jak2i #1-16) (Fig. 1D), while other proteins were not changed in their levels in the investigated clones.

### *Self renewal activity is maintained in both Jak2 single and Jak2/Tyk2 double knockdown mESCs*

To elucidate the roles of Jak2 and/or Tyk2 in LIF-mediated signaling to promote self-renewal in mESCs, we investigated LIF dependency of Jak2 single and Jak2/Tyk2 double knockdown clones in various concentrations of LIF in the absence of 4-HT. The number of undifferentiated colonies was scored by monitoring the expression of alkaline phosphatase with NBT/BCIP staining or counting mESC colonies that consisted entirely of densely packed cells 4 days after LIF treatment (Fig. 2A). The Jak2 and Jak2/Tyk2 knockdown colonies were able to maintain the undifferentiated state even at LIF concentration as low as 8 pM, like the parental STAT3ER cell line or the wild-type control clone (Fig. 2B). Indeed, we were able to maintain these knockdown clones in the undifferentiated state in the presence of LIF without addition of 4-HT for more than 10 passages (data not shown). These results indicate that both Jak2 and Tyk2 are not crucial components of Jak1/STAT3 signaling pathway that mediates LIF dependent self-renewal of mESCs.

### *Fast onset of differentiation occurs in the Jak2 single and Jak2/Tyk2 double knockdown mESCs in the absence of both LIF and 4-HT*

To investigate the effect of Jak2 and Tyk2 down-regulation on the differentiation of mESCs, we generated hanging-drop embryoid bodies (EBs) of Jak2 single and Jak2/Tyk2 double knockdown clones, and cultured EBs for 10 days. Although there was no considerable difference detected in morphology among clones, the initiation of bursting down of EBs to the bottom of culture dish was observed in Jak2 single and Jak2/Tyk2 double knockdown clones from day 5, while EBs of the control clone remained intact until day 10 (Fig. 3A). These results suggest that reduction of Jak2 or Jak2/Tyk2 might have caused early differentiation of mESCs.

To test this possibility, we compared the expression level of three germ layer marker genes in all Jak2 RNAi clones including the control clones at different time points using semi-quantitative RT-PCR (Fig. 3B). Expression of an ectoderm marker Nestin initiated and reached maximum level in both Jak2 and Jak2/Tyk2 knockdown clones one or two days earlier than in the control clone. Both Jak2 and Jak2/Tyk2 knockdown clones also showed similar expression patterns for a mesoderm marker βH1 and an

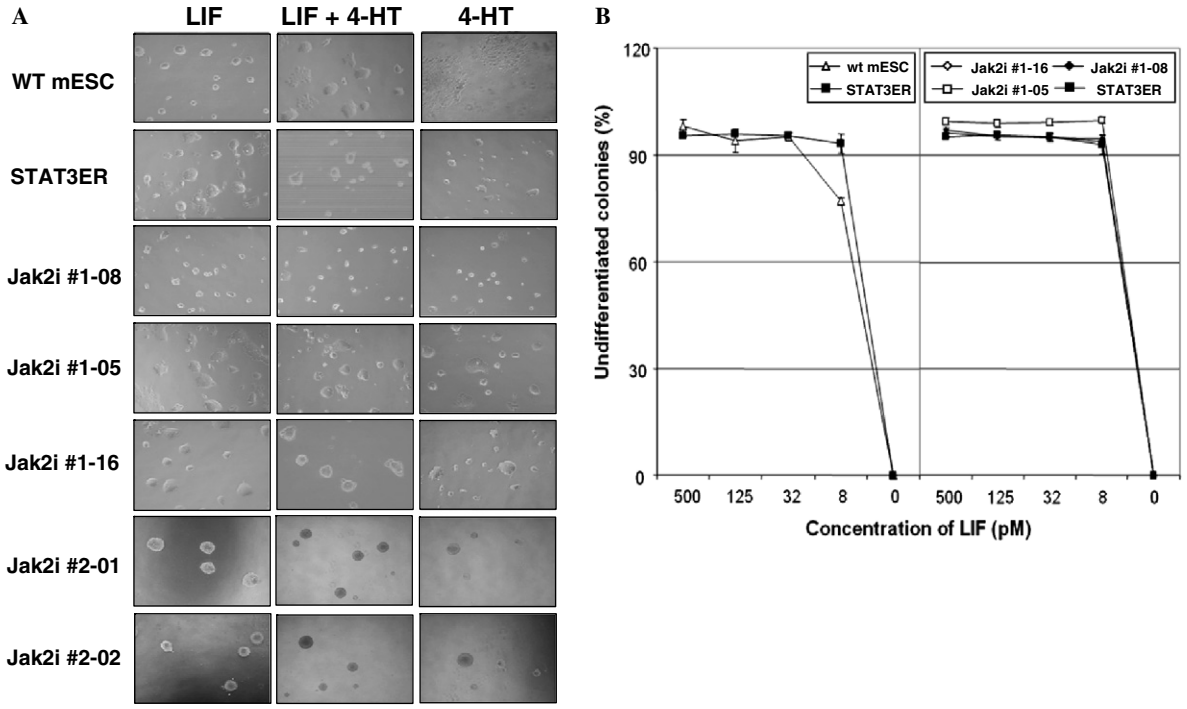


Fig. 2. Effect of Jak2 and Jak2/Tyk2 knockdown on the differentiation of mESCs. (A) The undifferentiated state of mESC clones was maintained regardless of Jak2/Tyk2 expression in the presence of LIF or 4-HT. (B) LIF dependency of the Jak2i knockdown clones. Cells were maintained in their undifferentiated state in various concentrations of LIF for 4 days. Undifferentiated colonies were monitored by alkaline phosphatase staining. The fraction of undifferentiated colonies to the total number of colonies was calculated and plotted against concentration of LIF.

endoderm marker GATA4 in comparison to the control clone (Fig. 3B). Considering almost identical morphological phenotype and three germ layer marker gene expression patterns observed in both Jak2 single and Jak2/Tyk2 double knockdown clones, it is speculated that Jak2 is a sole player to induce early onset of mESC differentiation when its level is reduced.

### *Differential gene expression patterns during mESC differentiation suggest that Jak2 or Jak2/Tyk2 knockdown causes cell fate changes during mESC differentiation*

To understand the role of Jak2 or Tyk2 in mESC differentiation, we compared the gene expression patterns of differentiating Jak2 and Jak2/Tyk2 knockdown clones with control STAT3ER cells by cDNA microarrays. Our microarray results showed that many genes of different lineages were differentially expressed during differentiation in Jak2 single knockdown and Jak2/Tyk2 double knockdown clones (Table 1). At day 0 of differentiation, nearly none of the genes changed significantly in their expression in both knockdown clones as compared to STAT3ER cells. However, many differentially expressed genes conspicuously appeared from day 2 of differentiation. Both up- and down-regulated genes increased gradually during differentiation (10 up- and 18 down-regulated genes at day 2, and 56 up- and 191 down-regulated genes at day 5, data not shown). These results reflect dynamic knockdown effects of one gene (Jak2) and two genes (Jak2/Tyk2) on mESC differentiation.

Several up-regulated genes in Jak2 single knockdown clones included genes involved in the inhibition of hematopoietic or lymphoid cell maturation (Table 1). Down-regulated genes were involved in maturation and proliferation of hematopoietic or lymphoid cells. In the Jak2/Tyk2 double knockdown clone, down-regulated genes are also known to be involved in maturation and proliferation of hematopoietic or lymphoid cells (Table 1).

In addition to the genes involved in hematopoiesis, a number of signaling pathway-related genes were also found to be expressed differentially in knockdown clones compared to STAT3ER control cells (Table 1). A number of marker genes for lineage specifications were also differentially expressed in Jak2 single and Jak2/Tyk2 double knockdown clones as compared to STAT3ER control cells (Table 1). Taken together, these results indicate that Jak2 and/or Tyk2 might induce various downstream signal transducing pathways causing cell fate changes. It is also noteworthy that the differentially expressed genes are of mesodermal and ectodermal origins, but not endodermal, suggesting a role of Jak2 and/or Tyk2, possibly as early as in germ layer specification.

### *Potential Jak2/Tyk2-mediated pathways revealed by inhibitor studies*

It has been increasingly clear that in addition to STATs, Jak proteins may regulate other signaling proteins for self-renewal in mESCs [4,20]. Therefore, in search of the potential epigenetic pathways for Jak2 and Tyk2 of mESCs, we

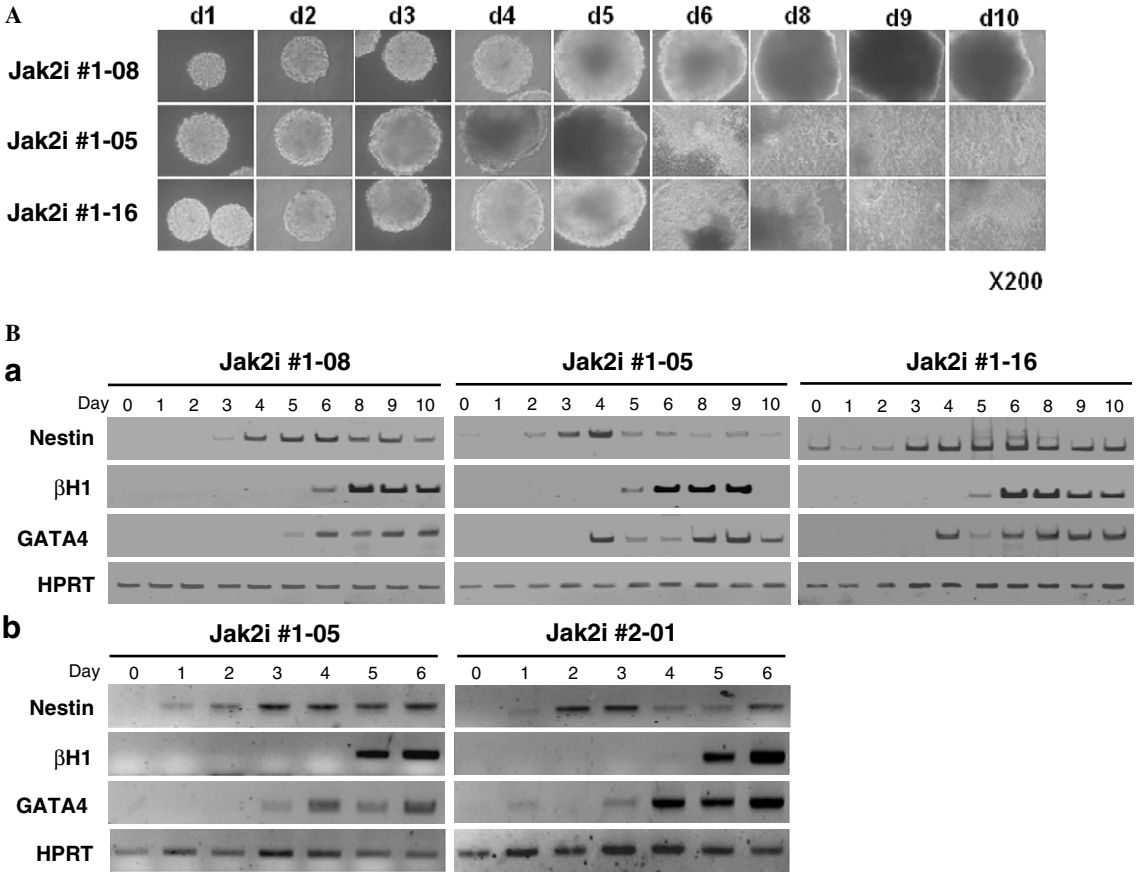


Fig. 3. Effect of Jak2/Tyk2 knockdown on mESC EB differentiation. (A) Differentiating EBs of Jak2 single knockdown (Jak2i #1-05), Jak2/Tyk2 double knockdown (Jak2i #1-16), and control (Jak2i #1-08) clones. Jak2i #1-08 that shows no down-regulation of Jak2 expression was used as a differentiation control of mESCs in vitro. (B) Expression patterns of three germ layer marker genes as determined by the semi-quantitative RT-PCR assays. Expression levels of each gene were determined after normalizing by that of HPRT.

treated Jak2 single and Jak2/Tyk2 double knockdown clones with various inhibitors for different signaling pathways in the presence of LIF. LiCl is known to activate Wnt signaling by blocking glycogen synthase kinase-3β (GSK3β), a negative regulator of β-catenin in the Wnt signaling pathway [20]. Wnt signaling has been known to be involved in pluripotency of mESCs and hESCs [4,21]. When GSK-3β was inhibited by 6-bromoindirubin-3′-oxime (BIO), the Wnt pathway was activated, by which the undifferentiated states in the both types of ESCs were maintained and expression of the pluripotent state-specific transcription factors such as Oct3/4, Rex1, and Nanog was sustained [21]. In control clones of mESCs (STAT3ER, and Jak2i #1-08), inhibition of GSK-3β by LiCl treatment did not affect self-renewal of mESCs. On the other hand, LiCl treatment induced differentiation in Jak2 and Jak2/Tyk2 knockdown clones (Fig. 4). These results suggest that Jak2 might be the downstream of Wnt-mediated self-renewal of mESCs. The extent of how much Wnt signaling interacts with LIF-induced Jak1/STAT3 pathway and the specific role of Jak2 in Wnt-mediated self-renewal remain largely unknown at this point.

Treatment of MEK inhibitor U0126 [22], ERK inhibitor PD098059 [23], and protein tyrosine kinase (PTK) inhibitor genistein [24] did not affect LIF-dependent self-renewal of mESCs (Fig. 4), suggesting that Jak2/Tyk2, Ras/Raf/MEK/ERK, and PTK signalings are not necessary for LIF-induced self-renewal. On the other hand, when we added a selective phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 [25] to different mESC cultures including wild-type mESCs, STAT3ER, Jak2i #1-08, Jak2i #1-05, and Jak2i #1-16, all mESCs did not maintain the undifferentiated state (Fig. 4), indicating that the PI3K pathway is crucial for mESC self-renewal in addition to the Jak/STAT pathway. Almost identical results were also observed with chelerythrin, the protein kinase C (PKC) inhibitor [26] (Fig. 4). It is likely that Jak1, not Jak2 or Tyk2, is directly involved in the activation of PI3K for mESC self-renewal as the case of STAT3 activation because Jak2/Tyk2 knockdown clone was able to maintain undifferentiated state even in the absence of PI3K inhibitor. This idea is further supported by the observation in which the activation of Akt, a downstream molecule of PI3K [27], is dependent on Jak1 in v-Abl-mediated transformation [28].

We demonstrated in this study that Jak2 and Tyk2 are not involved in LIF-induced Jak1/STAT3 pathway for self-renewal of mESCs, but play a role during early lineage

Table 1
Lineage-specific genes that are differentially expressed over 2-fold in both Jak2 (Jak2i #1-16) and Jak2/Tyk2 (Jak2i #2-02) knockdown clones at differentiation day 2 and day 5

| Origin | Lineage | Jak2i #1-16 | | Jak2i #2-02 | |
|---|---|---|---|---|---|
| | | d2 | d5 | d2 | d5 |
| *Up-regulated genes* | | | | | |
| | Eye | — | Ttbk1, Cryab | — | — |
| | Neuron | — | Prph1 | — | — |
| Mesoderm | Erythrocyte | — | Add2, Spna1 | — | — |
| | Lymphocyte | — | Ly75 | — | — |
| | Muscle | — | Myl3, Tncc, Tagln, Anxa6 | — | Fhl1, Tpm1 |
| | Neutrophile | — | Cxcl7 | — | — |
| | Smooth muscle | — | Hand1 | — | — |
| Endoderm | — | — | — | — | — |
| *Down-regulated genes* | | | | | |
| Ectoderm | Brain | Atp2b2 | Pcdhb22, Nap1l1, Gprin1 | — | — |
| | Eye | — | — | Rpgrip1, Guca1a | Guca1a |
| | Neural tube | Ptk7 | — | — | — |
| | Neuron | — | Pafah1b1, Prps1, Plxnb1, Plxnb2 | Anln | — |
| | Keratinocyte | — | Sprr2a | Trp63 | — |
| Mesoderm | Bone | — | Spp1 | Spp1 | Spp1 |
| | Erythrocyte | — | — | Tfrc | — |
| | Heart | — | Nfatc1 | — | — |
| | Kidney | — | Aqp3 | — | — |
| | Liver | — | Hrg, 1300017J02Rik | Fabp1 | — |
| | Muscle | — | — | Capza1 | — |
| | Osteoclast | — | — | Chuk | — |
| | Pancreas | Nr5a2 | — | — | — |
| | Platelet | — | F11 | F5 | — |
| | Smooth muscle | — | Myh11 | — | — |
| | T cell | — | Ccl5 | Tmpo | — |
| Endoderm | — | — | — | — | — |

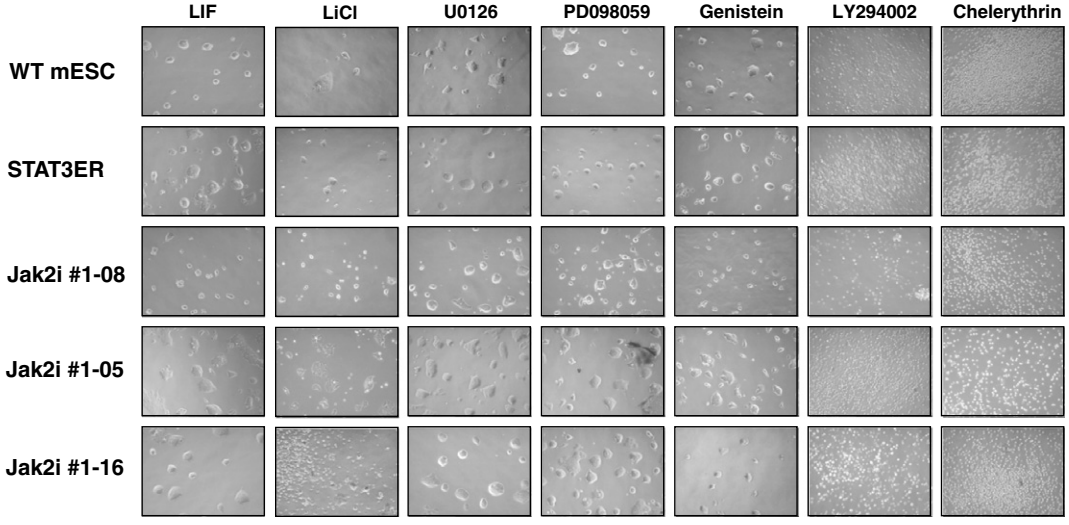


Fig. 4. Effects of various signaling molecule inhibitors on mESC differentiation. Various cell signaling inhibitors dissolved in DMSO were applied along with LIF to different mESC cultures. The differentiation state of mESCs was monitored by mESC colony morphology.

decision of mESC leading to various differentiated cell types such as erythrocyte, lymphocyte, keratinocyte, lens, muscle, bone, cartilage, and neuron. We also identified the potential interrelation between Jak2/Tyk2 and other signaling pathways for pluripotency of mESC including PI3K and Wnt signaling.

## Acknowledgments

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