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Identification of Jmjd1a as a STAT3 Downstream Gene in mES Cells

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ABSTRACT. **Mouse embryonic stem (mES) cells can be maintained in undifferentiated state in the presence of a cytokine, leukemia inhibitory factor (LIF). Many investigators found that STAT3 activation is important for the maintenance of pluripotency by LIF. However, the downstream pathways of STAT3 activation are still unknown. To look for STAT3-downstream target genes, we performed DD-RT PCR in the presence or absence of LIF. Through further confirmation, we finally selected 8 genes whose expressions were significantly dependent upon the presence of LIF. Among them, Jmjd1a was down-regulated after LIF withdrawal, and it was selected for further investigation. Its expression started to decrease 1 day after the removal of LIF, and disappeared on day 3. It was also shown that STAT3 could bind to the promoter region of Jmjd1a gene. These data demonstrate that Jmjd1a might be a critical signaling molecule underlying the maintenance of pluripotency in mES cells.**

Key words: embryonic stem cells/LIF/STAT3/DD-RT PCR/differentiation

Introduction

Mouse embryonic stem (mES) cells are pluripotent cell lines derived from the inner cell mass of the pre-implantation mouse embryo (blastocyst stage). ES cells have the ability to divide (self-renewal) for indefinite periods and they can also give rise to many different cell types (differentiation) that make up the organism under the appropriate conditions. Several researchers reported methods for growing mouse and human ES cells in the laboratory (Evans and Kaufman, 1981; Thomson *et al.*, 1998). Undifferentiated human ES cultures require feeder cells to maintain the undifferentiated state, whereas the feeder layer can be replaced by the addition of the leukemia inhibitory factor (LIF) to the growth media in the case of mouse ES cultures (Williams *et al.*, 1988). LIF seems to have no effect on human ES cultures in maintaining the pluripotency, and instead, basic fibroblast growth factor (bFGF) must be added to preserve the pluripotency of the human ES cells. Even if LIF is present in the culture media, human ES cells undergo differentiation without the feeder cells or bFGF.

The effect of LIF on ES cells is mediated through a cell surface receptor complex composed of the low affinity LIF receptor (LIFRβ) and gp130, a common receptor subunit of the IL-6 cytokine family (Gearing *et al.*, 1991). LIF binds with LIFRβ, which then forms a high affinity heterodimer complex with gp130. Both LIFRβ and gp130 apparently have no intrinsic protein kinase domains, but constitutively associate with the JAK (Janus kinase) family of non-receptor cytoplasmic protein kinases (Stahl *et al.*, 1994). Binding of LIF with the LIFRβ/gp130 heterodimer results in the rapid activation of JAKs and subsequent phosphorylation of LIFRβ/gp130 on their cytoplasmic tyrosine residues (Stahl *et al.*, 1994). In ES cells, three JAK family kinases, Jak1, Jak2 and Tyk2, are reported to be activated upon LIF-stimulation (Ernst *et al.*, 1996). After tyrosine phosphorylation of LIFRβ/gp130, SH2 domain-containing signaling molecules such as STATs (signal transducers and activators of transcription) are recruited to the receptors and then phosphorylated by JAKs on their tyrosine residues (Niwa *et al.*, 1998). STATs are the transcription factors which form SH2 domain-mediated dimers after tyrosine phosphorylation (Becker *et al.*, 1998). Dimerized STATs are then translocated into the nucleus where they bind to DNA followed by direct specific transcriptional modulation (Darnell, 1997).

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Abbreviations: mES cells, mouse embryonic stem cells; LIF, leukemia inhibitory factor; bFGF, basic fibroblast growth factor; LIFR, LIF receptor; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; DD-RT PCR, differential display-reverse transcription polymerase chain reaction; Jmjd1a, jumonji domain containing 1a; 4HT, (Z)4-hydroxytamoxifen; ER, estrogen receptor; EMSA, electrophoretic mobility shift assay; X-ChIP, chromatin cross-linking and immunoprecipitation.

Among the seven members of the STAT family, STAT3 is believed to be the major mediator of LIF-LIFRβ/gp130 signals in mES cells (Matsuda *et al.*, 1999; Niwa *et al.*, 1998).

STAT3 was first described as a DNA-binding protein from IL-6-stimulated hepatocytes, capable of selectively interacting with an enhancer element of acute-phase genes, known as the acute-phase response element (Heinrich *et al.*, 1998). As with other STAT proteins, STAT3 is activated by tyrosine phosphorylation at a single site close to the carboxy-terminus (Y705), as well as by serine phosphorylation at a site within the trans-activation domain (S727) (Raz *et al.*, 1994). STAT3 activation is required and sufficient to maintain the undifferentiated state of mES cells. Dominant-negative forms of STAT3 lead to the differentiation of ES cells (Niwa *et al.*, 1998), and conditional activation of STAT3 can keep ES cells at undifferentiated state without further addition of LIF (Matsuda *et al.*, 1999). However, the downstream signals of LIF-STAT3 pathway are still largely unknown.

In order to fish out the STAT3 downstream signaling molecules and look at their roles in early differentiation of mES cells, DD-RT PCR (differential display-reverse transcription PCR) was carried out in mES cells. After the primary screening, about 180 genes that showed differential expression level according to the presence or absence of LIF were selected. The expression levels of these genes were further confirmed by Northern blot analysis. After this second round of screening procedure, eight clones whose expressions were significantly dependent upon the presence of LIF were selected and subjected to further analyses. Among them, one clone was down-regulated after the LIF withdrawal whereas the others were up-regulated upon removal of LIF in the medium. The former was previously reported as Jmjd1a (jumonji domain containing 1a), a testis-specific putative zinc-finger protein whose practical function is unknown. Among the other seven clones that were up-regulated after the LIF withdrawal, four clones were unknown genes and three others were reported as ubiquitin ligase NEDD4, topoisomerase I, and TAFII30, respectively.

In this study, among the eight genes selected as STAT3 downstream target genes, Jmjd1a gene, whose expression was down-regulated after the LIF withdrawal, was chosen for further analyses. Through the various experiments carried out in this report, we tried to interpret the relationship between STAT3 and Jmjd1a gene, and, furthermore, to understand the mechanism(s) through which STAT3 regulates the differentiation and self-renewal of ES cells.

Materials and Methods

Mouse ES cell culture

The mouse ES cell line A3-1 derived from embryos of 129/SvJ inbred strain (Azuma and Toyoda, 1990) was cultured on gelatin-coated dishes in the absence of feeder cells. A3-1 cells were grown in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (ES grade, HyClone, Logan, UT), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acid (Gibco-BRL), 2 mM L-glutamine, 3.3 mM nucleoside (Sigma-Aldrich, St. Louis, MO), 100 mg/ml streptomycin (Sigma-Aldrich), and 100 units/ml penicillin G (Sigma-Aldrich). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Recombinant human LIF (hLIF) was supplemented 10^3 U/ml in the media. A synthetic (Z)4-hydroxytamoxifen (4HT), purchased from Sigma-RBI, was dissolved in ethanol (5 mM stock).

Human ES cell culture

The human ES cell line Miz-hES1 (from MizMedi Hospital, Korea) was cultured on gelatin-coated dishes in the presence of STO cells as feeder layer. Miz-hES1 cells were grown in same culture media as mouse ES cell with the exception of knockout DMEM (Gibco-BRL) and 20% knockout serum replacement (Gibco-BRL). Recombinant human bFGF (4 ng/ml, Invitrogen, Carlsbad, CA) and feeder cells were supplied in order to maintain undifferentiated state. Feeder cells were grown in DMEM supplemented with heat-inactivated 10% FBS, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acid, 2 mM L-glutamine, 3.3 mM nucleoside, 100 mg/ml streptomycin and 100 units/ml penicillin G. One day before the subculture of human ES cells, feeder cells were treated with 10 mg/ml mitomycin C for 1.5 hr and then the cells were seeded at a density of 7.5×10^4 cells/cm^2.

Electrophoretic mobility shift assay (EMSA)

Harvested ES cells were resuspended in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1 mM DTT, and 0.1 mM EDTA) and then incubated on ice for 25 min. Then, 5 μl of 10% NP-40 was added and incubated on ice for 2 min. After centrifugation (at 500 x*g* for 1 min), supernatants were removed and 50 μl of buffer C (10 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) was added and mixed vigorously. After centrifugation (at 14,000 x*g* for 10 min), the supernatants were collected as nuclear extracts. For the annealing of single strand oligomers (consensus STAT3 binding site, 5'-agtcttcccagaaatttcccagaaagtc-3'; STAT3 binding site of Jmjd1a promoter, 5'-gaagaatctcacgggaaaggggcg-3', STAT3 binding sites are underlined), each sense and antisense oligomers were mixed and heated to 90°C for 3 min, then cooled slowly to below 45°C. The annealed double-stranded oligonucleotides were mixed with γ-^{32}P-ATP (1 μCi) and T4 PNK (polynucleotide kinase), and then incubated at 37°C for 1 hr. Radioactivity of end-labeled oligomers was measured using liquid scintillation counter. Five fmol of end-labeled oligonucleotides were mixed with 2 μg poly dI-dC, 3 μg nuclear extracts and EMSA buffer (20 mM HEPES pH 7.9, 40 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 5% glycerol). After incubation on ice for 20 min, additional incubation was carried out

at 25°C for 30 min. The sample was then electrophoresed on 5% non-denaturating polyacrylamide gel using 0.5×TBE as running buffer. The gel was then dried and subjected to autoradiography. For the competition assay, 500-fold excess cold oligomer or 3 μg of STAT3 antibody was pre-incubated with nuclear extracts at 25°C for 20 min.

DD-RT PCR (differential display-reverse transcription PCR)

Total RNA was isolated from A3-1 cells cultured for 0, 1.5, or 3.5 days after the removal of LIF. According to the manufacturer's protocol (Delta Differential Display kit, Clontech, Palo Alto, CA), 2 μg of total RNA was converted into first strand cDNA with oligo $dT_{15}G$ or anchoring primer. The cDNA was then amplified with oligo $dT_{15}G$ or anchoring primer and the arbitrary primers, and the PCR products were labeled using α-^{35}S-dATP. The PCR products were then resolved on a 6% polyacrylamide gel containing urea in 1×TBE buffer. The gel was dried and exposed to X-ray film. After developing the film, the differentially displayed bands were cut out from the dried gel. The DNA was eluted and reamplified using the same combination of primers according to the protocol of the supplier. The reamplification product was cloned into pGEM-T Easy vector (Promega, Madison, WI) and their identities were further investigated through the nucleotide sequence determination and analysis.

RT-PCR

Two micrograms of total RNA from ES cell were primed with the mixture of 0.25 μg random primer or 0.25 μg oligo-dT primer, incubated at 70°C for 10 min, and then cooled on ice. Reverse-transcription was carried out in the presence of 10 mM DTT, 0.5 mM dNTP, 1X first strand buffer, and Superscript II (Invitrogen) reverse transcriptase in a total volume of 20 μl at 42°C for 50 min. The reaction mixture was heated to 75°C for 10 min, and then cooled on ice. Using these mixtures as templates, PCR was carried out with appropriate primer sets. The primers used in this study are listed in Table I.

Western blot analysis

Harvested ES cells were suspended in lysis buffer (20 mM Tris-Cl pH 7.4, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml each of aprotinin, pepstatin A, antipain, and leupeptin), and incubated on ice for 15 min. After the centrifugation at 14,000 x*g* for 15 min at 4°C, supernatant was collected. The extracts were separated on a 10% SDS/PAGE and transferred onto PVDF membrane. Protein-bound membrane was incubated with appropriate antibodies, followed by horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Bio-Rad). The relevant protein bands were then visualized using Enhanced Chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ). Antibodies for phospho-STAT3 and STAT3 were purchased from Cell Signaling Technology (Beverly, MA). ERα (estrogen receptor α) and actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Table I. LIST OF PRIMERS USED IN THE RT-PCR EXPERIMENTS

Genes	Forward primers	Reverse primers
STAT3	5'-gtatgcccataatcacgc-3'	5'-agctccggaaaccgctgaat-3'
GAPDH	5'-ggtgaaggtcggagtcaacgg-3'	5'-ggtcatgagtccttccacgat-3'
Oct3/4 (POU5F1)	5'-ggcgttctctttggaaaggtgttc-3'	5'-ctcgaaccacatccttctct-3'
COUP-TF1	5'-agccatcgtgctattcacg-3'	5'-ttctcaccagacacgaggtc-3'
vHNF	5'-gaaagcaacgggagatcctccgac-3'	5'-cctccactaaggcctccctctcttcc-3'
#101 (Jmjd1a)	5'-gaaagacttgaaggtgtgtgt-3'	5'-gggagagacctcagagcaagatttt-3'
Cardiac actin	5'-tatttgctcccttgcttgga-3'	5'-cctaccccaaaaacaaacga-3'
αFP	5'-tgaaaaccctcttgaatgcc-3'	5'-tcttgcttcatcgtttgcag-3'
Renin	5'-gctttctcagccaggacatc-3'	5'-tattctttgcctcccaggtg-3'
Enolase	5'-gttcatgtcatcaatggcg-3'	5'-gtgaacttctgccaagctcc-3'
β-actin	5'-tggcaccacaccttctacaatgagc-3'	5'-gcacagcttctccttaatgtcacgc-3'
Human Jmjd1a	5'-gaaagacttgaaggtgtgtgt-3'	5'-gggagagacctcagagcaagatttt-3'

X-ChIP (chromatin cross-linking and immunoprecipitation)

A3-1 cells were trypsinized and the DNA and bound proteins were cross-linked using 1% formaldehyde for 1 hr. Cells were washed twice with TBS and harvested in SDS buffer (50 mM Tris-Cl pH 8.1, 5 mM EDTA, 100 mM NaCl, 0.2% NaN_3, and 10% SDS) containing protease inhibitors. The cells were then resuspended in cold IP buffer (1 volume SDS buffer: 0.5 volume Triton dilution buffer [100 mM Tris-Cl pH 8.6, 100 mM NaCl, 5 mM EDTA, 0.2% NaN_3, and 5% Triton X-100]) and sonicated until DNA was fragmented to the size of 500~1000 bp. Antibody (against STAT3 N-terminus) was added to the chromatin extract and incubated overnight at 4°C with continuous mixing. The supernatant was collected after centrifugation (at 14,000 x*g* for 20 min) and incubated for 2 hr at 4°C after the addition of protein A beads. These mixtures were washed using mixed micelle wash buffer (20 mM Tris-Cl pH 8.1, 150 mM NaCl, 5 mM EDTA, 5% sucrose, 0.2% NaN_3, 1% Triton X-100, and 0.2% SDS), buffer 500 (50 mM HEPES pH 7.5, 1 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.2% NaN_3, and 0.1% deoxycholic acid), LiCl/detergent wash buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.2% NaN_3, 0.5% NP-40, and 0.5% deoxycholic acid) and TE, and further incubated at 65°C overnight to reverse the crosslinking after adding 1% SDS and 0.1 M $NaHCO_3$. Proteinase K was then added to remove the STAT3 proteins and incubated at 37°C for 30 min. Remained DNA solution underwent phenol extraction and ethanol precipitation. Using this DNA as template, PCR was carried out with appropriate STAT3 binding site primer sets (forward primer, 5'-gctccattcttccatttgttgcc-3'; reverse primer, 5'-tagatggtttgcctctagatttaa-3'). Sonicated total DNA without IP reaction was used as positive control, and DNA sample not incubated with STAT3 antibody within IP procedure was used as negative control.

Results

A3-1 mES cells were maintained at undifferentiated state through STAT3 activation in LIF containing media

In order to look at the plausible roles of the STAT3 downstream genes in the maintenance of pluripotency and/or differentiation of mES cells, most of the experiments were performed using A3-1 mES cell line (Azuma and Toyoda, 1990). First of all, it was needed to confirm whether A3-1 cells differentiate in the culture condition lacking LIF, and whether STAT3 activation is the critical point in this procedure. Undifferentiated mES cells formed compact and round colonies (multilayer) (Fig. 1A, +LIF), whereas differentiated mES cells stretched horizontally out and made flattened colonies (–LIF). From 3 days after the LIF withdrawal, this morphological change was evident in the mES cell culture.

To verify that A3-1 cells in our culture system act correctly as previously reported, expression levels of marker genes were tested using RT-PCR in native A3-1 cells as well as STAT3ER-transfectant (Fig. 1B). A3-1 cells transfected with STAT3ER construct were previously used to elucidate that STAT3 activation is sufficient to maintain an undifferentiated state of mES cells (Matsuda *et al.*, 1999). This construct codes a fusion protein composed of the entire coding region of mouse STAT3 and the modified ligand binding domain of mouse estrogen receptor (ER) which binds with the synthetic steroid ligand 4-hydroxytamoxifen (4HT). Accordingly, STAT3 function is activated through 4HT stimulation without LIF in A3-1 cells transfected with STAT3ER construct and undifferentiated state of mES cells is maintained when 4HT is provided even though LIF is absent.

For the RT-PCR, RNA was extracted from native A3-1 cells (±LIF) and STAT3ER-transfectant (±4HT). The genes tested were well known markers that showed differential expression patterns during ES cell differentiation (Fig. 1C). Oct3/4 was reported as an important molecule during early embryo differentiation (Minucci *et al.*, 1996). COUP-TF1 is a primitive endoderm specific marker (Power and Cereghini, 1996) and vHNF is an extra embryonic endoderm marker (Cereghini *et al.*, 1992). As expected, the expression level of Oct3/4 was down-regulated when differentiation was induced, whereas those of COUP-TF1 and vHNF, expressed in specific differentiated states, were up-regulated (GAPDH was used as an internal control).

As reported before (Matsuda *et al.*, 1999), STAT3 activity continuously decreased after the induction of ES cell differentiation. STAT3 phosphorylation, an indicator of STAT3 activation, was abated by LIF withdrawal (Fig. 2A). In STAT3ER cells, phosphorylation of STAT3-ER fusion protein was also decreased after the removal of 4HT (Fig. 2B). Actually, STAT3 mRNA and protein expression was reduced after the removal of LIF in A3-1 cells (Fig. 2A, C), which was already reported by Matsuda *et al.* (1999). Also, STAT3 lost its DNA-binding activity after the LIF removal, which was analyzed by EMSA (data not shown). Based on these results, it was confirmed that A3-1 and STAT3ER-transfectant in our culture system showed the characteristics of ES cells, and STAT3 exerts crucial effects during the differentiation of ES cells.

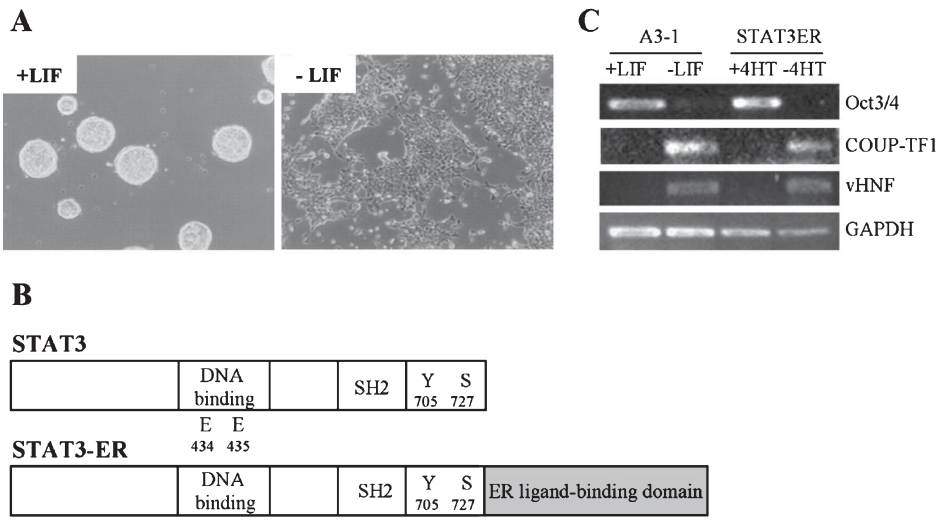

Fig. 1. Differentiation of mouse ES cell line, A3-1. (A) Undifferentiated mouse ES cell colonies cultured in the media containing LIF (+LIF) and differentiated colonies cultured for 4 days after the removal of LIF (–LIF). (B) Domain structure of STAT3 and a conditionally active form of STAT3 (STAT3ER). STAT3ER was constructed by fusing the entire coding region of STAT3 to modified ligand binding domain (G525R) of mouse estrogen receptor (Matsuda *et al.*, 1999). (C) Confirming the expression of marker genes by RT-PCR in undifferentiated or differentiated mouse ES cells. RNA samples used in RT-PCR were extracted from native A3-1 mouse ES cells cultured in the presence and absence of LIF, and STAT3ER transfectants cultured with or without 4HT for 5 days.

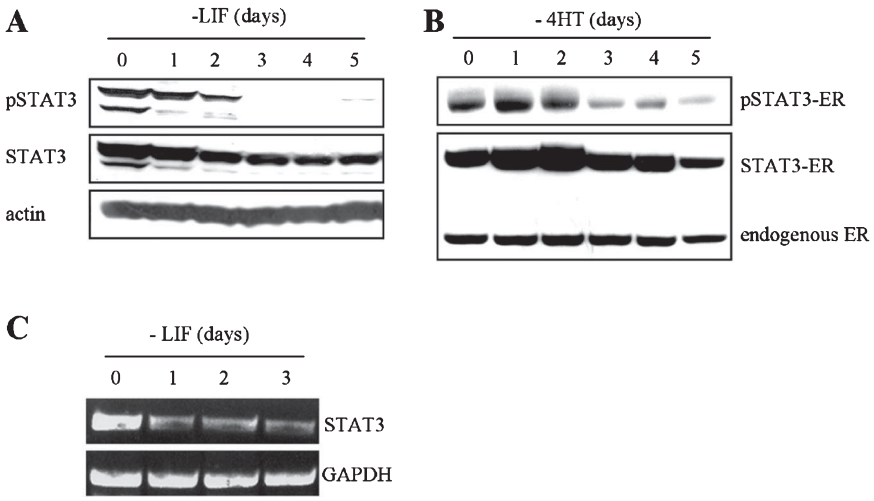

Fig. 2. Alteration of STAT3 activity during differentiation in mouse ES cell line, A3-1. (A) Western blot analysis of STAT3. After the removal of LIF for the indicated times, Western blot was carried out using total STAT3 or phosphorylated STAT3 (pSTAT3) specific antibody. pSTAT3 decreased from 2 days after the LIF removal. Actin was used as control. (B) Western blot analysis of STAT3-ER fusion protein. After the withdrawal of 4HT for the indicated times, Western blot was performed using pSTAT3 (upper) or ER (estrogen receptor; lower) specific antibody. (C) RT-PCR analysis of STAT3 mRNA. A3-1 cells were cultured without LIF for the indicated days, and then mRNA levels were compared. STAT3 mRNA decreased time-dependently after LIF removal. GAPDH was used as control.

DD-RT PCR and Northern blot analysis were carried out to identify STAT3 downstream genes

Because STAT3 has been known as a critical regulator of self-renewal and differentiation in ES cells, we tried to fish out the STAT3 downstream target genes in the ES cells using DD-RT PCR (differential display-reverse transcription PCR) analysis. Using this method, mRNA expression levels were compared after the LIF withdrawal (day 0, 1.5, and 3.5). About 180 genes that showed differential expression level according to the presence or absence of LIF in A3-1 cells were initially selected (about 10 genes were down-regulated after the removal of LIF and the others were up-regulated). To eliminate pseudo-signals in DD-RT PCR, the expression levels of these genes were further confirmed by Northern blotting and RT-PCR analysis in A3-1 and STAT3ER transfectant (Fig. 3). About 500 bp cDNA fragments cloned through DD-RT PCR were used as radioactive probes for Northern blot analysis. After this second round of screening procedure, eight clones were finally chosen whose expression levels were significantly dependent upon the LIF/STAT3 signaling pathway (Table II).

Among them, one clone (#101) was down-regulated after the LIF withdrawal (Fig. 3B) whereas all the others (seven clones) were up-regulated (Fig. 3A). The identities of the eight selected clones were pursued through nucleotide sequence determination. Clone #101 turned out to be Jmjd1a (jumonji domain containing 1A), which was previously reported in rat as a testis-specific putative zinc-finger protein whose practical function is unknown (Hoog *et al.*, 1991). The other seven clones were up-regulated by LIF withdrawal. The identities of the four clones (#39, #125, #127 and #128) were unknown and the other three clones (#25, #102 and #154) were previously reported. mRNA sizes of unknown clones (#39, #125, #127 and #128) were shown to be about 6.5 kb, 6 kb, 6.5 kb, and 6.5 kb, respectively, on Northern blot analysis (Fig. 3A).

Clone #25 was previously reported as TATA box binding protein (TBP)-associated factor (TAFII30), which was first cloned in mES cells as a human TAFII30 homolog (Metzger *et al.*, 1999). TAFII30-null embryonic carcinoma (EC) cells were not viable and it was shown to be required for cell cycle progression (G1-S phase progression and cyclin E expression). It was also suggested that TAFII30 participated in specific cellular differentiation program in EC cells (Metzger *et al.*, 1999).

Clone #102 is ubiquitin protein ligase Nedd-4 (neuronal precursor cell expressed, developmentally down-regulated), whose mRNA level was regulated during developmental stages and highly expressed in many embryonic tissue. Actually it was also confirmed in this study that its mRNA is also ubiquitously expressed in mouse adult tissues (data not shown). Recently, several proteins have been identified that interact with WW domain of NEDD4 protein (Jolliffe *et al.*, 2000).

Clone #154 was shown to be topoisomerase I gene and its mRNA size was 3.5 kb. DNA topoisomerases are ubiquitous enzymes that resolve torsional and topological hindrance generated during DNA replication and transcription. Although topoisomerase I was not essential in yeast, it was shown to be essential in both *Drosophila* and mice (Morham *et al.*, 1996). It was recently reported that mice

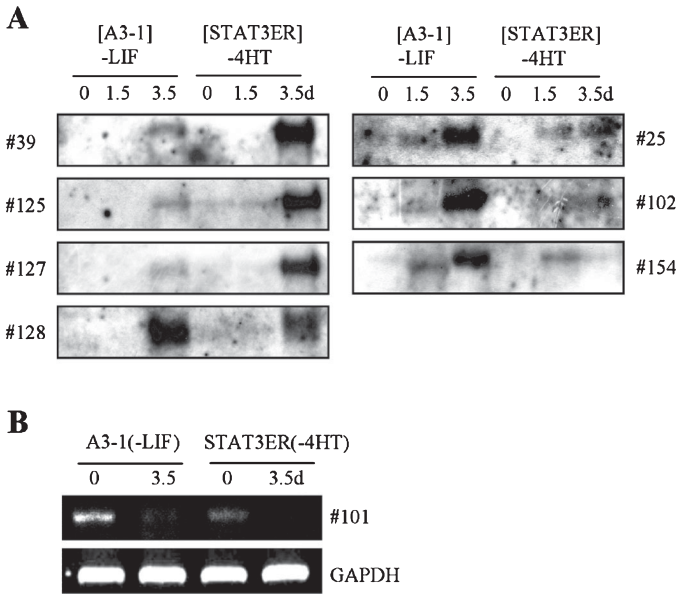

Fig. 3. Search for the STAT3 downstream genes using DD-RT PCR in mouse ES cells. (A) Northern blot analysis. Clones isolated using DD-RT PCR were further tested by Northern blot analysis. RNA samples were extracted from native A3-1 mouse ES cells cultured after the removal of LIF and STAT3ER transfectants cultured after the removal of 4HT for the indicated days. Radioactive probes for Northern blot analysis were prepared by random primer labeling method using the DD-RT PCR clones as template. (B) RT-PCR of clone #101. mRNA level of #101 clone decreased after the removal from the media of LIF (A3-1) and 4HT (STAT3ER). GAPDH was used as control.

Table II. SUMMARY OF STAT3 DOWNSTREAM GENES IDENTIFIED BY DD-RT PCR AND NORTHERN BLOT ANALYSIS

Clone with decreased expression level when LIF is removed	
reported gene (1)	#101 [Jmjd1a]
Clones with increased expression level when LIF is removed	
reported genes (3)	#154 [topoisomerase I] #102 [NEDD4] #25 [TAFII30]
unknown genes (4)	#39, #125, #127, #128

DD-RT PCR and Northern blot analysis were carried out to identify STAT3 downstream target genes. After the second round of screening procedure, eight clones whose expressions were significantly dependent upon the presence of LIF, that is, STAT3 activation, were finally selected. Among them, one clone (#101) was down-regulated after the removal of LIF whereas the others were up-regulated.

deficient in *top-1* gene die at early embryonic stages (between the 4- and 16-cell stages). This suggests that topoisomerase I has critical roles in mouse early embryogenesis.

Among the eight genes selected as STAT3 downstream genes after the DD-RT PCR analysis, the Jmjd1a gene, whose expression was down-regulated after the LIF withdrawal, was chosen to examine the function in ES cells. The mRNA expression level of Jmjd1a decreased after the LIF withdrawal and its expression disappeared from 3 days after the LIF withdrawal in A3-1 cells (Fig. 4A). In adult mouse tissues, Jmjd1a was expressed only in testis in parallel with its rat homolog; however, its message was much larger than the mRNA expressed in ES cell (Fig. 4B, arrowhead). It suggests that there exist alternative forms of Jmjd1a gene in testis, which may require further investigation.

Jmjd1a is also down-regulated during differentiation in human ES cells

To determine whether this transcriptional control in Jmjd1a gene by STAT3 shown in mouse ES cells also holds for human Jmjd1a during human ES cell differentiation, the expression of human Jmjd1a gene was examined using RT-PCR method. Unlike mouse ES cells, even though LIF was present in the media, human ES cells differentiated if the feeder layer or bFGF was absent (Fig. 5A).

Four culture conditions were set for human ES cells, where the cells were cultured for 12 days in the presence or absence of feeder cells and/or bFGF (Fig. 5A). Relatively speaking, human ES cells cultured without feeder cells differentiated more rapidly than cells without bFGF. Some marker genes were tested to confirm the culture condition (Fig. 5B). αFP (human alpha-fetoprotein) gene is an endoderm specific marker, cardiac actin is a heart (mesoderm) specific marker and enolase is known as a muscle (mesoderm) specific marker. POU5F1 is a down-regulated gene during development, renin is a kidney (mesoderm) specific

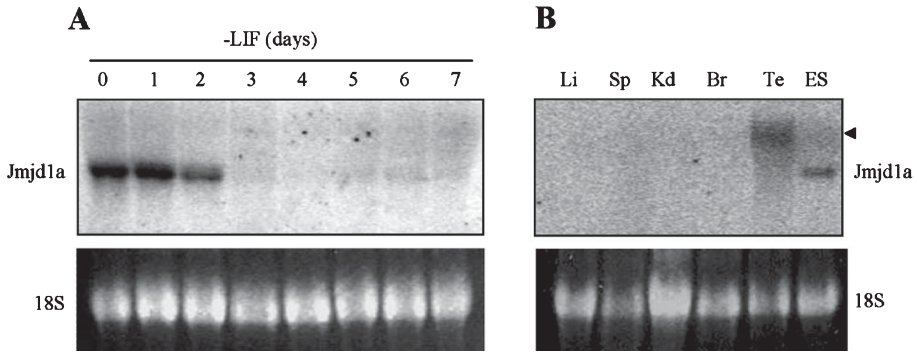

Fig. 4. Expression patterns of Jmjd1a gene. (A) Northern blot analysis of Jmjd1a gene expression in the absence of LIF. mRNA expression of Jmjd1a gene in mouse A3-1 ES cells was down-regulated after the removal of LIF in a time-dependent manner and disappeared on day 3. (B) Tissue-specific expression of Jmjd1a gene in mouse. Jmjd1a mRNA was specifically detected in testis; however, it was much larger in size than that expressed in ES cells (arrowhead). Li, liver; Sp, spleen; Kd, kidney; Br, brain; Te, testis; ES, ES cells. 18S rRNA was used as control in both A and B.

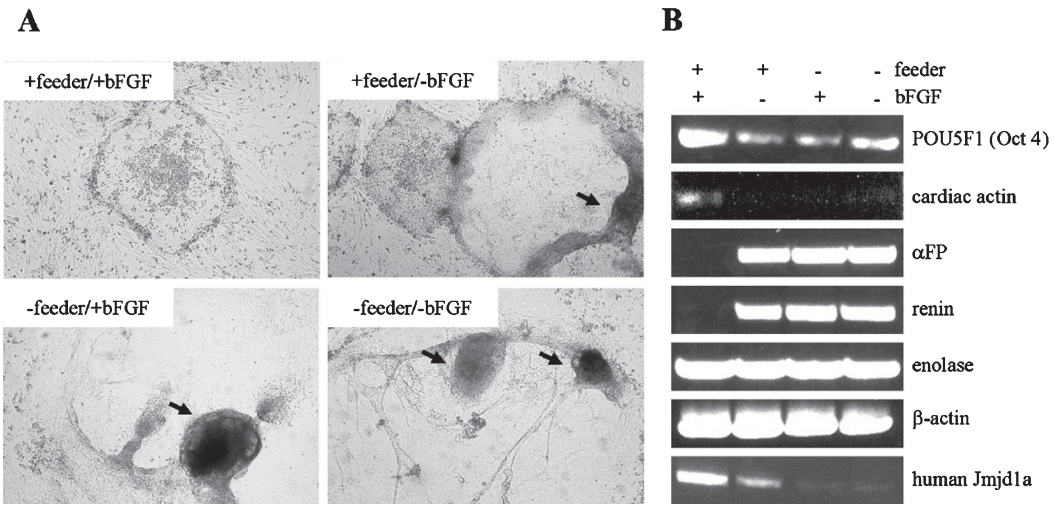

Fig. 5. Changes in the expression of marker genes and human Jmjd1a in human ES cells after the induction of differentiation. (A) Morphological changes when differentiation was induced for 7 days in human ES cells. Differentiation of human ES cells was induced by the removal of feeder cells and/or bFGF. When cultured without the feeder cell layer, ES cells showed more differentiated phenotypes than cells cultured without bFGF (arrows: embryoid bodies). (B) RT-PCR analysis of marker genes and human Jmjd1a. Human ES cells were induced to differentiate for 12 days and then RT-PCR was performed. mRNA expression of human Jmjd1a gene was diminished when human ES cells underwent differentiation. Expression patterns of the marker genes were as expected. POU5F1, a down-regulated gene during development; cardiac actin, a heart (mesoderm) specific marker; αFP (human alpha-fetoprotein) gene, an endoderm specific marker; renin, a kidney (mesoderm) specific marker; enolase, a muscle (mesoderm) specific marker; β-actin, a house keeping gene, was used as control.

marker and β-actin is a housekeeping gene used as control. As expected, the expressions of αFP and renin were up-regulated, POU5F1 and cardiac actin were down-regulated and enolase did not change in differentiated human ES cells as previously reported (Schuldiner *et al.*, 2000). With this condition, it was observed that the expression level of Jmjd1a was down-regulated in differentiated human ES cells in accordance with the mouse ES cells (Fig. 5B).

STAT3 regulates the expression of Jmjd1a by direct binding to the promoter region

The expression of Jmjd1a was down-regulated after the removal of LIF from the culture medium. Therefore, it can be expected that STAT3 might up-regulate the expression of Jmjd1a. To elucidate the relationship between Jmjd1a and STAT3, the promoter activity of the Jmjd1a gene was monitored at the molecular level. The promoter region of the Jmjd1a gene was pursued from the mouse genomic sequence database (from Mouse Genome Resources in

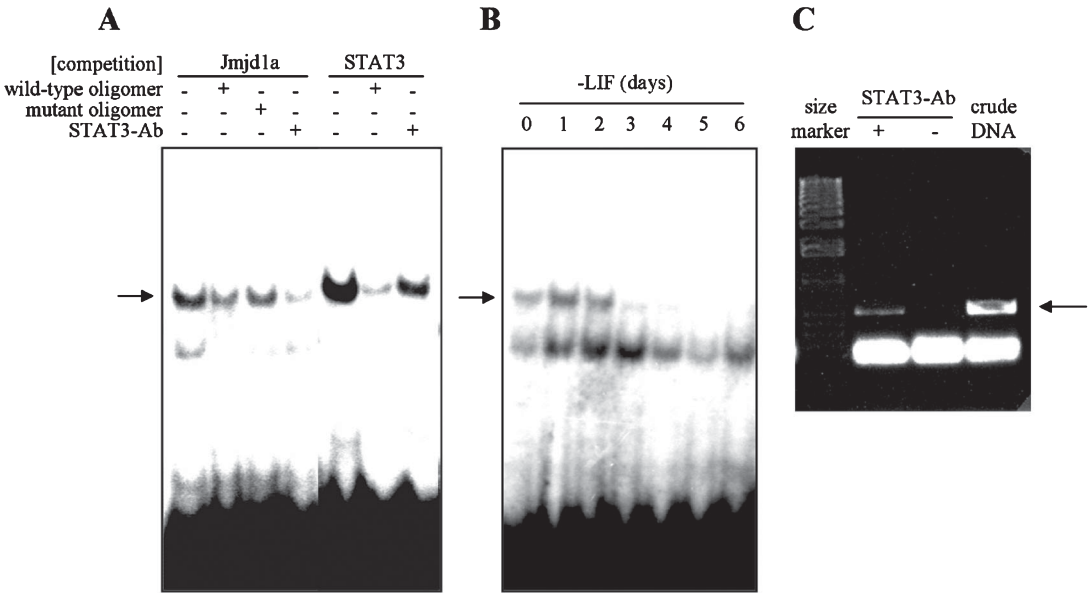

Fig. 6. Promoter activity assay of the Jmjd1a gene. (A) EMSA (electrophoretic mobility shift assay) of STAT3 binding site within Jmjd1a promoter. Nuclear extract used in EMSA was isolated from A3-1 cells cultured in the presence of LIF. Excess (500-fold) cold native oligomer (5'-gaagaatctcacgg-gaaaggggcg-3', STAT3 binding site is underlined), cold mutant oligomer (5'-gaagaatcgagtataggaggggcg-3', mutated at conserved STAT3 binding sequences) and STAT3 antibody were used as competitors. Previously reported STAT3-responsive element (5'-agtcttcccagaaatttcccagaaagtc-3') was used as positive control. (B) STAT3 binding in the promoter region of Jmjd1a gene is dependent on LIF. The STAT3 binding activity continuously decreased and disappeared from 3 days after the removal of LIF. The arrow indicates expected band shift by the binding of STAT3 or STAT3-containing complex to the oligomer. (C) Promoter activity assay of the Jmjd1a gene by X-ChIP. X-ChIP performed with STAT3 antibody successfully produced PCR product of expected size (arrow). Sonicated total crude DNA without immunoprecipitation was used as a positive control, and DNA sample not incubated with STAT3 antibodies was the negative control.

NCBI, http://www.ncbi.nlm.nih.gov/genome/guide/mouse). A region about 2 kb upstream from the transcription start site was investigated with computational prediction (using the TRANSFAC database at GenomeNet, http://motif.genome.ad.jp). It was found that a conserved STAT3 binding site existed within this region, about 650 bp-upstream (–648~–659 bp) from the transcription start site. To determine if this is the real STAT3 binding site, X-ChIP (chromatin immunoprecipitation) and EMSA (electrophoretic mobility shift assay) analyses were performed.

For the EMSA analysis, oligomer set (26 bp) corresponding to the STAT3 binding site of Jmjd1a was designed, end-labeled, and then mixed with nuclear extracts of A3-1 cells. After electrophoresis, labeled oligomer showed slow mobility, which was hindered by STAT3 competition, *i.e.*, wild-type oligomer and STAT3 antibody diminished the complex (Fig. 6A). The STAT3 binding activity after the LIF withdrawal was then monitored (Fig. 6B). As expected, this STAT3 binding activity was continuously decreased in a time-dependent manner and disappeared from 3 days after the LIF withdrawal, which coincided with the disappearance of the Jmjd1a transcript. All these results demonstrated that STAT3 might control the expression of Jmjd1a gene by direct binding to the promoter region in mES cells.

Similar results could be obtained using X-ChIP method, which is a powerful approach to define the interaction of certain factors (or proteins) with specific chromosomal DNA sequence in living cells. Sonicated total crude DNA without immunoprecipitation was used as positive control, and DNA sample not incubated with STAT3 antibody during immunoprecipitation procedure was used as negative control. The expected PCR product was present in positive control, but not in negative control confirming that the X-ChIP worked as expected. The X-ChIP performed with binding site primer set successfully produced the PCR product of expected size (Fig. 6C, arrow). This result shows that STAT3 can bind to the promoter region of Jmjd1a gene.

Discussion

ES cells derived from inner cell mass of mammalian blastocysts grow rapidly and infinitely while maintaining pluripotency, the ability to differentiate into all types of cells (Evans and Kaufman, 1981). These properties of ES cells are maintained by symmetrical self-renewal, producing two identical stem cell daughters upon cell division. These properties have raised the hope that ES cells could be used to treat a host of degenerative diseases such as Parkinson's

disease and diabetes. This hope was accelerated by the generation of pluripotent ES cells from human blastocysts (Thomson *et al.*, 1998). However, human ES cells also raised substantial ethical issues since human embryos must be destroyed to generate ES cells (Colman and Burley, 2001). One solution to avoid such ethical issues is to generate pluripotent cells directly from somatic stem and other cells. The first step toward this goal is to understand molecular mechanisms underlying pluripotency of ES cells.

Multiple lines of evidence indicated that STAT3 is the key downstream transcription factor of the LIF/gp130 signaling pathway in mES cells. Forced expression of a dominant-negative STAT3 mutant caused differentiation of ES cells even in the presence of LIF (Niwa *et al.*, 1998), and point mutation of the tyrosine residue of gp130 responsible for the STAT binding abrogated the ability of LIF to maintain self-renewal (Ernst *et al.*, 1999; Matsuda *et al.*, 1999). These results strongly suggest that STAT3 activation is required and sufficient to maintain the undifferentiated state of ES cells.

STAT3 seems to play essential roles in ES cell pluripotency, differentiation and self-renewal, but possibilities still exist that other pathways rather than LIF/gp130-STAT3 signaling might also be important for these procedures. Oct3/4 is a POU family of transcription factor specifically expressed in ES cells, preimplantation embryos, epiblast, and germ cells (Okamoto *et al.*, 1990). Inactivation of Oct3/4 in embryos and ES cells resulted in the loss of pluripotency and spontaneous differentiation into trophoblast lineage (Niwa, 2001). However, ES cells constitutively expressing Oct3/4 still required LIF for self-renewal (Niwa *et al.*, 2000). Overexpression of Oct3/4 induced differentiation similar to that caused by STAT3 inactivation, indicating a crosstalk between the two transcription factors. Two other transcription factors, Sox-2 (Avilon *et al.*, 2003) and FoxD3 (Hanna *et al.*, 2002), were recently reported as essential factors for pluripotency in embryos. Since both Sox2 and FoxD3 cooperatively function with Oct3/4 (Guo *et al.*, 2002), it is likely that they also could crosstalk with STAT3.

STAT3 plays an important role in a wide variety of physiological pathways such as cell proliferation, differentiation and apoptosis. How can a single transcription factor mediate these various processes? It is probably related to a specific set of genes whose activities are regulated by STAT3. Some genes regulated by STAT3 were previously reported in various cell systems. For example, STAT3 activation leads to the down-regulation of myc gene and induction of junB and IRF1, thereby inducing cell-cycle arrest of monocytic cells (Levy and Lee, 2002). Inhibition of melanoma cell proliferation also relies on STAT3 activity, and, importantly, STAT3 activates the expression of the cell-cycle inhibitor $p21^{wafl}$ (Giraud *et al.*, 2002). Recently, microarray analysis was carried out to isolate the LIF/STAT3 transcriptional targets in mES cells, and Aes (amino-terminal enhancer of split) gene, one of the targets, was found to be down-regulated by LIF removal (Sekkaï *et al*., 2005).

In order to clone and analyze the STAT3 downstream signaling molecules, we carried out DD-RT PCR. Compared to the gene microarray, DD-RT PCR method has the advantage of discovering novel genes which show differential expression. Using this method, eight clones whose expressions were significantly modulated upon STAT3 inactivation were selected. Among these eight genes, the relationship between STAT3 and Jmjd1a, whose expression was down-regulated after the LIF withdrawal, was further investigated. The presence of a zinc finger motif in Jmjd1a suggests that Jmjd1a protein might bind to DNA and regulate the transcription of other genes. This possibility is supported by the recent identification of a protein which possesses zinc finger motifs very similar to those of Jmjd1a and binds to transcriptional regulatory elements in ES cells. This gene, Rex-1, is a developmentally regulated acidic zinc finger gene that is detected in a limited range of cells and tissues: undifferentiated embryonic stem (ES) and EC cells, mouse embryos at the blastocyst stage, trophectoderm and meiotic germ cells of the adult mouse testis (Rogers *et al.*, 1991). This gene is regulated via Oct-3/4 and Oct-6 binding, which are also known to participate in ES cell differentiation (Ben Shushan *et al.*, 1998). Just as Rex-1 regulates ES cell differentiation via Oct-3/4 signal, it is plausible to assume that Jmjd1a could also exert some important effects on the fate of ES cells via STAT3 signaling. That is, Jmjd1a in the STAT3 pathway could be the counterpart of Rex-1 in Oct-3/4 pathway during mES cell differentiation. At this moment, transfection of Jmjd1a into mES cells is being carried out to see whether Jmjd1a has some important roles in ES cell differentiation.

In summary, Jmjd1a which has a putative zinc finger motif was expressed in undifferentiated ES cells and disappeared after the induction of differentiation. In mES cells, STAT3 up-regulated Jmjd1a by direct binding to the promoter of Jmjd1a. We hope that these new findings on the STAT3 downstream genes will provide further insights into understanding the regulatory mechanisms involved in the pluripotency of ES cells.

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