Transcriptional Properties of the BMP, TGF-β, RTK, Wnt, Hh, Notch, and JAK/STAT Signaling Molecules in Mouse Embryonic Stem Cells

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ABSTRACT

Major characteristics of embryonic stem cells (ESCs) are sustaining of stemness and pluripotency by self-renewal. In this report, transcriptional profiles of the molecules in the developmentally important signaling pathways including Wnt, BMP4, TGF-β, RTK, Hh, Notch, and JAK/STAT signaling pathways were investigated to understand the self-renewal of mouse ESCs (mESCs), J1 line, and compared with the NIH3T3 cell line and mouse embryonic fibroblast (MEF) cells as controls. In the Wnt signaling pathway, the expression of Wnt3 was seen widely in mESCs, suggesting that the ligand may be an important regulator for self-renewal in mESCs. In the Hh signaling pathway, the expression of Gli and N-myc were observed extensively in mESCs, whereas the expression levels of in a Shh was low, suggesting that intracellular molecules may be essential for the self-renewal of mESCs. IGF-I, IGF-II, IGF-IR and IGF-IIR of RTK signaling showed a lower expression in mESCs, these molecules related to embryo development may be restrained in mESCs. The expression levels of the Delta and HES5 in Notch signaling were enriched in mESCs. The expression of the molecules related to BMP and JAK-STAT signaling pathways were similar or at a slightly lower level in mESCs compared to those in MEF and NIH3T3 cells. It is suggested that the observed differences in gene expression profiles among the signaling pathways may contribute to the self-renewal and differentiation of mESCs in a signaling-specific manner.

(Key words : Embryonic stem cells, Signaling pathways, Self-renewal, Differentiation, Transcription)

INTRODUCTION

Although the "stem cell" concept was introduced many years ago, to date, stem cells can only be defined functionally, not morphologically or phenotypically. Two functions define stem cells. They can be self-renewing and are thus able to propagate and generate additional stem cells. They can also differentiate into various progenitor cells, which commit to further maturation along specific lineages. These functional properties of stem cells have attracted significant interest from both basic and clinical science researchers. The fundamental scheme of stem cells provides a model for basic science researchers to study developmental biology from a very early stage. Stem cell research has also presented opportunities for clinical science in developing new therapies through the functions of repair, replacement and regeneration. Since a definition of the stem cell is best given functionally, the identification and isolation of this unique cell population have become challenging tasks. Embryonic stem cells (ESCs), which are derived from the inner cell mass of preimplantation embryos (Evans and Kaufman 1981), have been recognized as the most pluripotent stem cell population. The cell fate of an ESC may be not governed by several factors or a simple mechanism but instead regulated by multiple factors - growth factors, serum, feeder cells, Oct3/4, Nanog of transcription factors - and complex mechanisms - cell cycle, signal networks, metabolisms, cell-to-cell contact. To understand the molecular mechanism regulating the self-renewal or cell fate decisions in mammalian embryonic stem cells, the characteristics of ESCs need to be elucidated above all. Recently, several research groups have characterized the properties of mouse embryonic stem cells (mESCs) from the viewpoint of the cell cycle and gene expression (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002; Stead *et al.*, 2002; Fortunel *et al.*, 2003; Kelly *et al.*, 2004). In addition, the leukemia

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inhibitory factor (LIF) / Stat3, bone morphogenetic protein (BMP) and wingless related (Wnt) signaling were reported to be involved in the self-renewal of mESCs (Matsuda *et al.*, 1999; Ying *et al.*, 2003; Sato *et al.*, 2004). Several studies have shown that the presence of LIF and the activation of STAT3 via the LIF receptor and gp130 signaling are sufficient for maintenance of mESCs pluripotency in the absence of feeders (Smith *et al.*, 1988; Williams *et al.*, 1988; Raz *et al.*, 1999).

Signaling between cells is commonly regarded as an important mechanism by which cellular and morphological diversities are generated during embryonic development. Indeed, the small number of growth factor/receptor families (including TGF-β/BMP, Wnt, Hedgehog, Notch, RTK, JAK/STAT) that orchestrate development by instructing different, uncommitted cell types to proliferate, differentiate and/or organize into specific tissue lineages. Implicit in the observation that a small number of signaling pathways regulate a large array of developmental processes is the notion that the cellular response to the same signals can vary and will depend on the nature of the recipient cell. Thus an outstanding challenge is to understand how signaling inputs are interpreted to generate cell type-specific patterns of gene expression and behavior. Generally, a signal transduction pathway is activated by the binding of extracellular ligands to specific membrane receptors, which in turn propagate the signal through signal transducing molecules. Ultimately, gene expression is altered by the transmitted signal. The signal specificity can be affected by the environment of cells, cross talk among signaling pathways and the intensity of signaling. Additionally, regulation of signal intensity or duration can occur anywhere in a signaling pathway. In other words, the control points of signaling can be determined by ligands, receptors, cytoplasmic signaling components, transcription factors and target genes. Looking at expression patterns of ligands, receptors, cytoplasmic signaling molecules, transcription factors and target genes which belong to the above-mentioned signaling pathways may provide insights into how the distinctive characteristics of embryonic stem cells can be generated by signaling pathways. Using reverse transcription-polymerase chain reaction (RT-PCR), the expression pattern of signaling molecules related to signal transduction pathways - Hh, Wnt, BMP, RTK, Notch, JAK/STAT pathways - in NIH-3T3 fibroblast, mouse embryonic fibroblast (MEF) and mESCs are investigated in this paper.

MATERIALS AND METHODS

Mouse Embryonic Stem Cell Culture

mESCs, J1 line (obtained from Dr. Dae-Yeol Yu, the Korea Research Institute of Bioscience and Biotechnology, Korea) cultured in the DMEM/F12 medium containing 15% serum, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and 1000 units/ml murine LIF (Chemicon, Temecula, CA, USA) on mytomycin C (Sigma, St. Louis, MO, USA)-treated MEF (mouse embryonic fibroblasts) feeders derived from E 13.5 day C57BL/6 mouse embryos. After 2 days culture, to remove MEF, cells were collected by trypsinization and plate on 10 cm dishes for 30 min. Nonadherent cells consisting mainly of mESCs was replated.

mRNA Extraction and RT-PCR

Total RNA was extracted from mESCs, NIH3T3 (Cat. CRL-1658, ATCC, Manassas, VA, USA) and MEF cells by using RNeasy Mini kit and poly (A^+) RNA was isolated by using Oligotex mRNA Mini kit according to the manufacture's protocol (Qiagen, Valencia, CA, USA). 100 ng of poly (A^+) RNA was used to generate the first strand cDNA by Superscript II reverse transcriptase (Invitrogen, Carlbad, CA, USA) and oligo (dT) primers. To perform RT-PCR, 0.25 μl of the first strands cDNA was used in the 20 μl PCR reaction mix (Bioneer, Daejeon, Korea). Beta-tubulin, a house-keeping gene, was used as the positive control. The PCR products were run in the 2% agarose gel. mRNA extraction and RT-PCR experiments were independently repeated three times. The primer sequences used, the sizes of expected PCR products and annealing temperature are presented in the supplementary data.

Protein Analysis

Antibodies against Oct3/4, β-catenin, Smad4, Gli1, Delta1, Smo, Stat3, ERK1, 2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GSK-3β and gp130 (Cell Signaling Technology, Danvers, MA, USA) were used for Western blot analysis. Proteins from mESCs were prepared with the cell extraction buffer (20 mM Hepes, 50 mM NaCl, 10% Glycerol, 0.5% Triton X-100 and 2% β -mercaptoethanol). Protein concentrations were determined by the Bradford method. 30 μg of protein was separated by the 10% SDS-PAGE and transferred to a nitrocellulose (NC) membrane (Schleicher & Schuell, Inc., Keene, NH, USA) with tris/glycine/methanol (25 mM Tris, 192 mM glycine, 20% methanol). After blocking with the TBS buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 5% non-fat dry milk and 0.1% Tween 20, the membrane was incubated with primary antibodies followed by HRP-conjugated antibody (Cell Signal Technology), and developed with the ECL reagent (Amersham, Piscataway, NJ, USA).

RESULTS

Mouse Embryonic Stem Cells (mESCs)

In order to confirm the undifferentiated state of the mESCsused in present study, the genes specifically expressed in undifferentiated mESCs, were investigated through the semi-quantitative RT-PCR analysis. NIH3T3 cells and mouse embryonic fibroblast (MEF) cells were used as controls for the differentiated cells. The expressions of specific maker genes of ESCs such as Oct3/4, Nanog, FGF4, Sox2, FoxD3, and Rex1 were detected in the mESCs, and suppressed with NIH3T3 and MEF (Fig. 1A). Oct3/4, Rex1 and FGF4 were expressed only in mESCs, whereas Nanog, Sox2 and FoxD3 were expressed strongly in mESCs compared to NIH3T3 cells and MEF cells (Fig. 1A).

Wnt Signaling Pathway in mESCs

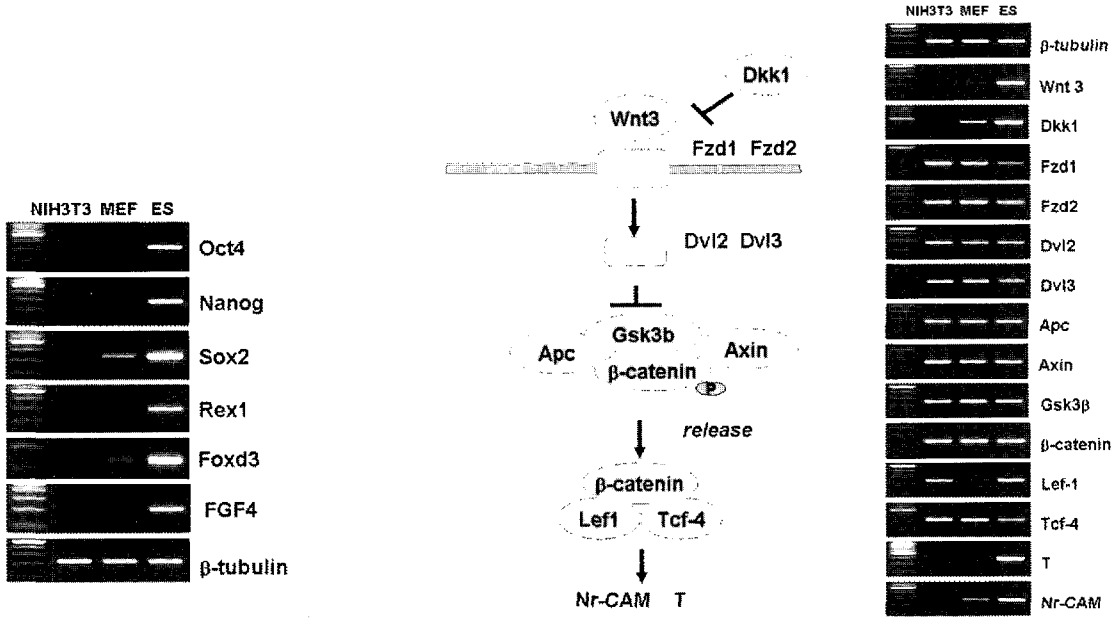
Fig. 1B demonstrates expression profiles of the Wnt signaling pathway in mESCs. Expression levels of the Wnt3 ligand and Dkk1 negative regulator were enriched to those of controls, which is consistent with the report in which the transcripts of Wnt3 and Dkk1 are abundant in mESCs (Fortunel *et al.*, 2003). There is no difference in the expression of dishevelled-2 (Dsh2), Dsh3, β-catenin, GSK-3b, adenomatous polyposis coli (APC) and axin between mESCs and those of controls. Furthermore, frizzled (Fzd) 1, 2 receptors and T cell-specific transcription factor 4 (TCF4), the β-catenin binding transcription factor, were slightly low in mESCs at the transcriptional level, whereas another binding transcription factor, the lymphoid enhancer factor (LEF) was at a similar transcriptional level in mESCs and NIH3T3 cells. A neuron glia-related cell adhesion molecule (Ng-CAM)-related cell adhesion molecule (Nr-CAM), a target gene of Wnt signaling (Conacci-Sorrell *et al.*, 2002), was highly expressed in mESCs, and the expression of the T (brachyury) gene, another target gene of Wnt signaling (Yamaguchi *et al.*, 1999), was expressedonly in mESCs (Fig. 1B).

Notch Signaling Pathway in mESCs

Expression profiles of Notch signaling in mESCs are described in Fig. 2A. The Delta 1 ligand was abundant in the mESCs. The expression of Notch 1 and 3 receptors were slightly more expressed in mESCs compared to NIH3T3 and MEF cells. There were no differences in the expression level of Numb and Presenlinin between the mESCs and controls. Transcripts of HES5, a target gene of Notch signaling, were enriched in mESCs.

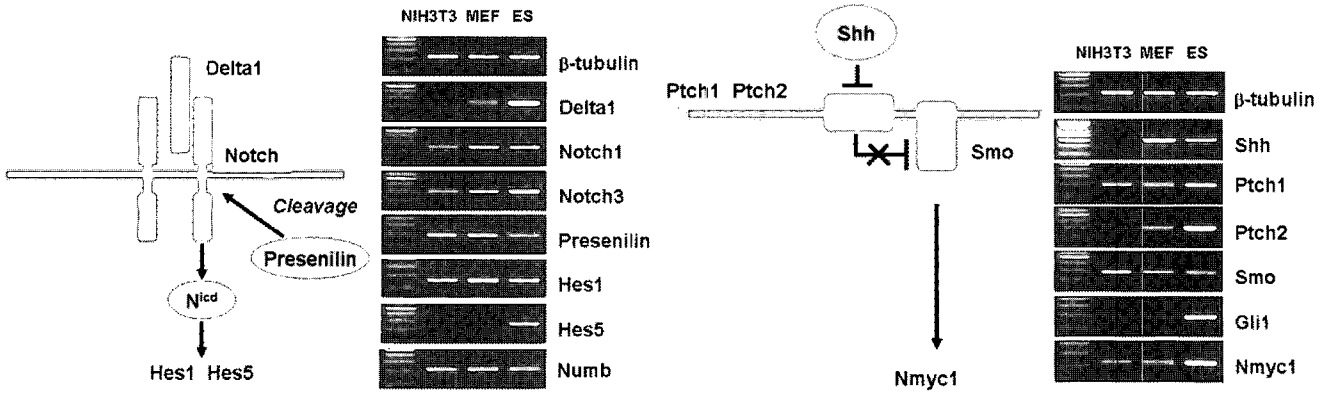
Hh Signaling Pathway in mESCs

Molecules of Hh signaling in mESCs were investigated at the transcriptional level (Fig. 2B). The expression of the Hh ligand was enriched in differentiating cells MEF. Despite a low expression level of the Hh ligand transcript, patched (Ptch) 1 and 2 receptors showed a high level of transcripts in mESCs. The expression of the Gli1 gene in mESCs washigher than it was in the controls. N-myc1, a target gene in Hh sig-



(A) ES cell specific genes (B) Wnt signaling

Fig. 1. (A) Expression of mESC specific genes by RT-PCR. (B) Expression profiles of Wnt signaling molecules in mESC. A schematic diagram of the Wnt signaling pathway (Nelson and Nusse 2004) and expression of various molecules in the Wnt signaling pathway. NIH3T3, NIH3T3 cell line; MEF, mouse embryonic fibroblast; ES, mouse embryonic stem cells.



(A) Notch-Delta signaling (B) Shh signaling

Fig. 2. Expression profiles of Notch and RTK signaling molecules in mESC. (A) A schematic diagram of the Notch signaling pathway (Iso *et al.*, 2003) and expression of various molecules in the Notch signaling pathway. (B) A schematic diagram of the RTK signaling pathway (Jones and Clemmons 1995; Dailey *et al.*, 2005) and expression of various molecules in the FGF4 signaling pathway. NIH3T3, NIH3T3 cell line; MEF, mouse embryonic fibroblast; ES, mouse embryonic stem cells.

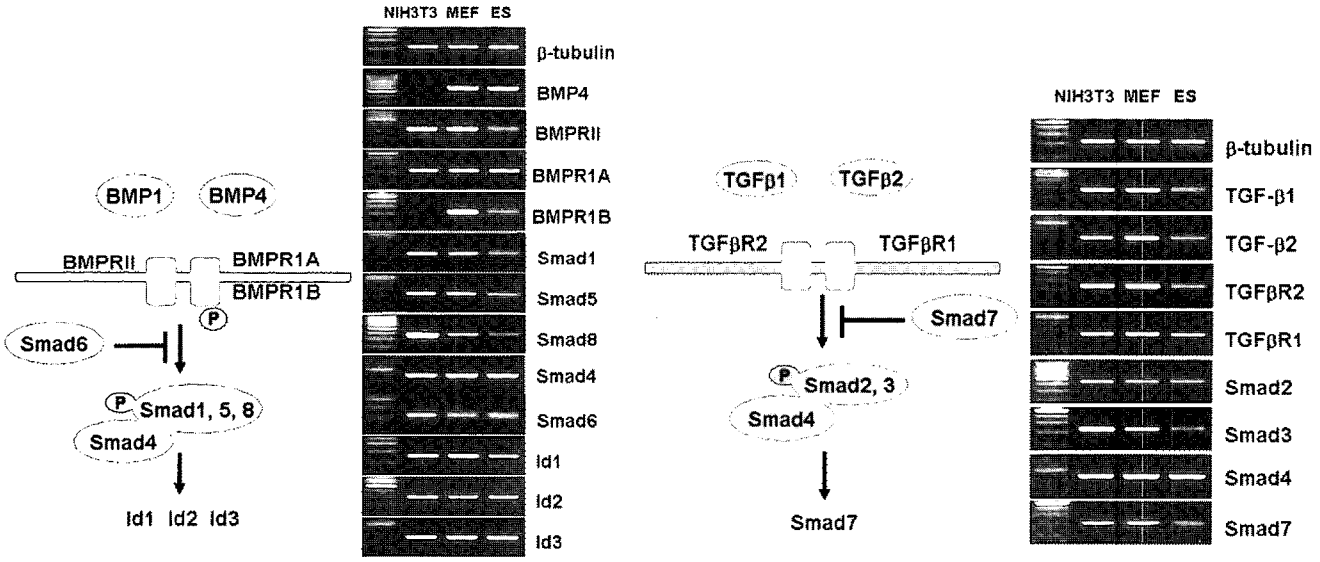
naling, was highly expressed in mESCs.

BMP4 Signaling Pathway in mESCs

In the present study, expression patterns of molecules involved in the BMP signaling pathway in mESCs were compared with those of NIH3T3 and MEF cells as controls (Fig. 3A). Interestingly, the BMP4 ligand was no differences between mESCs and MEF cells. BMPRIB and BMPRII were weakly expressed in mESCs compared with MEF cells. Receptors such as BMPR1A and intracellular molecules such as Smad 1, 4, 5 and 6 in mESCs were transcribed at a similar level compared to those in NIH3T3 and MEF cells. In addition, the expression level of Id genes, which encode the inhibitors of differentiation in mESCs and are one of target genes in BMP signaling (Ying *et al.*, 2003), in mESCs were similar to those of the NIH3T3 and MEF controls (Fig. 3A).

TGF-β Signaling Pathway in mESCs

A schematic diagram of the TGF-β signaling pathway and expression patterns of TGF-β signaling mo-



(A) BMP signaling (B) TGF-β signaling

Fig. 3. Expression profiles of BMP and TGF-β signaling molecules in mESC. (A) A schematic diagram of the BMP signaling pathway (Zwijsen *et al.*, 2003) and expression of various molecules in the BMP signaling pathway. (B) Expression of various molecules in the TGF-β signaling pathway (Zwijsen *et al.*, 2003). NIH3T3, NIH3T3 cell line; MEF, mouse embryonic fibroblast; ES, mouse embryonic stem cells.

lecules in mESCs are represented in Fig. 3B. TGF-β1, TGF-β2 ligands and TGF-βR2 were expressed less in mESCs. In cytoplasm, Smad 2 and 3 are directly phosphorylated by the receptor kinase and bind to Smad 4 to send the signal. There wasno difference in the expression levels of the Smad 4 intracellular molecule among mESCs, NIH3T3 and MEF cells. Smad4 in mESCs was transcribed at a similar level compared to those in NIH3T3 and MEF cells. Smad 7 works as an inhibitor for the signaling. The expression levels of Smad 2, 3 and 7 in mESCs were lower expressed than those of the control groups.

JAK/STAT Signaling Pathway in mESCs

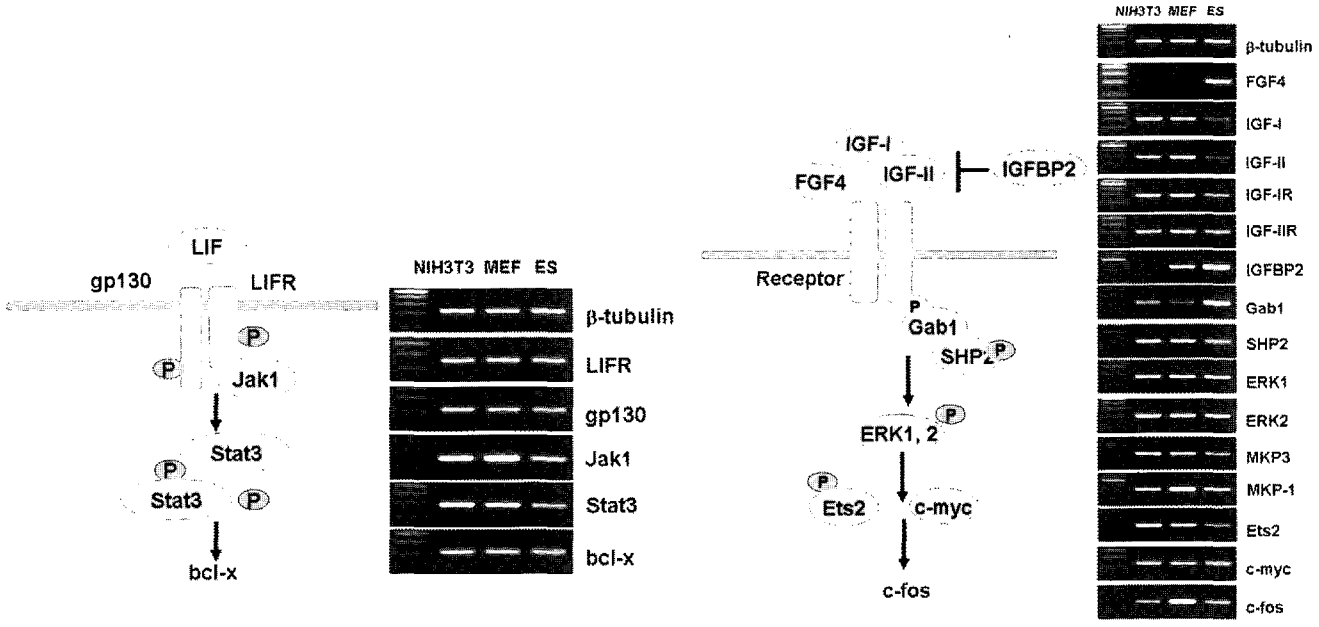
The expression levels of molecules in the JAK/STAT signaling such as LIFR and Bcl-xL, were not upregulated in the mESCs compared to those of the controls (Fig. 4A). The expression of gp130, JAK1 and Stat-3 genes were slightly down-regulated in mESCs compared to NIH3T3 and MEF cells.

RTK Signaling Pathway in mESCs

As shown in Fig. 4B, FGF4 was detected only in mESCs. IGF-I, IGF-II, IGF-IR, and IGF-IIR showed a lower expression in mESCs, indicating that ligands and receptors related to embryo development may be restrained in mESCs. IGFBP2, the inhibitory factor of IGF-I and II, was enriched in mESCs; the enhanced expression of IGFBP2 suggests that the inhibitory regulation of IGF signaling may be necessary for suppressing the differentiation of mESCs. The adaptor protein Gab1 was abundant in mESCs, it is expressed at an earlier stage of mouse embryo during development and trophoblast stem cells (*Xie et al.*, 2005). The SHP2 adaptor protein was expressed in mESCs, NIH3T3 and MEF cells. The transcripts and proteins of MAPKs (ERK1 and 2) were at identical levels in mESCs and the controls. The expression of MKP1 and 3, MAPK inhibitors showed similar transcriptional levels in m-ESCs, NIH3T3 and MEF cells. c-myc, transcriptional factors of RTK signaling, were observed as identical mRNA levels in mESCs, NIH3T3 and MEF cells. The expression of c-fos, a target gene of RTK signaling, was abundant in MEF.

Western Blot Analysis in hESC

It was investigated whether the translational patterns of signaling molecules follow the transcriptional patterns studied by RT-PCR (Fig. 5). The transcript and protein of the Oct3/4 gene, an undifferentiated mESCs marker, was observed only in mESCs. Highly expressed transcripts in mESCs such as Smad4, β-catenin, GSK-3β and Gli1 also have high protein productions. The transcripts and proteins of ERK1 and 2 were similar level in mESCs compared to those in MEF and NIH3T3 cells. Both transcript and protein of the gp130 receptor gene was expressed weekly in mESC (Fig. 5). These comparisons indicate that the expression pattern



(A) JAK/STAT signaling (B) RTK signaling

Fig. 4. Expression profiles of Hh and JAK/STAT signaling molecules in mESC. (A) A schematic diagram of the Hh signaling pathway (Ruiz i Altaba *et al.*, 2002) and expression of various molecules in the Hh signaling pathway. (B) A schematic diagram of the JAK/STAT signaling pathway (Burdon *et al.*, 2002) and expression of various molecules in the JAK/STAT signaling pathway. NIH3T3, NIH3T3 cell line; MEF, mouse embryonic fibroblast; ES, mouse embryonic stem cells.

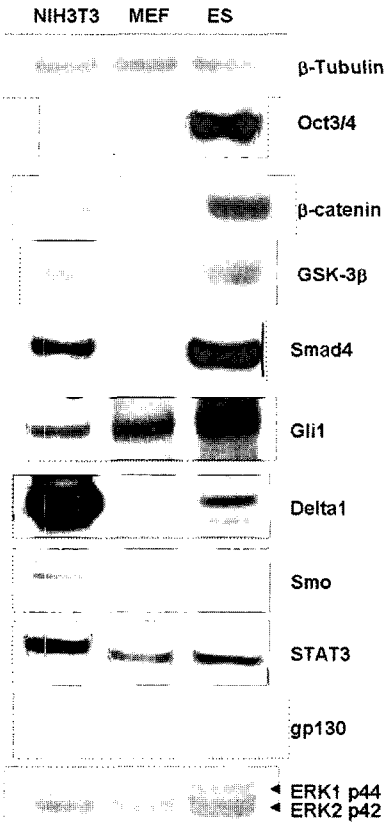


Fig. 5. Western blot analysis of Oct3/4, β-catenin, GSK-3β, Smad4, Gli1, Delta1, Smo, Stat3, ERK1, 2, and gp130 proteins in mESCs. NIH3T3, NIH3T3 cell line; MEF, mouse embryonic fibroblast; ES, mouse embryonic stem cells.

of the transcripts generally agrees with the pattern of protein productions in the signaling pathways of m-ESCs.

DISCUSSION

Mouse Embryonic Stem Cells (mESCs)

The most critical pathways regulating self-renewal in mouse embryonic stem cells (mESCs) are those mediated by Oct3/4 and the leukemia inhibitory factor (LIF). A model of their interactions has been proposed by Niwa (Niwa, 2001). LIF, a member of LIF-IL6 superfamily of cytokines, is critical for maintenance of ES lines. LIF activation of the JAK/STAT pathway via the gp130 receptor is required to maintain the pluripotency of mESCs (Yoshida *et al.*, 1994). Experiments have shown that it is critical for ESC self-renewal (Niwa *et al.*, 1998). However, recently, LIF is required, but not sufficient to expand the pluripotent mESCs in the absence of serum or feeder cells (Yamane *et al.*, 2005). The activation of STAT3 in the absence of LIF is sufficient for a prolonged self-renewal (Matsuda *et al.*, 1999) and the activation of a modified receptor, which acts to activate the MAPK pathway, is as efficient as the wild-type receptor in maintaining cells in an undifferentiated state (Burdon *et al.*, 1999; Burdon *et al.*, 1999). In the mouse, the expression of Oct3/4 is shown through the pluripotent cells during the embryonic development (Nichols *et al.*, 1998). The homeodomain transcription factor Nanog is essential for maintenance of pluripotentiality in mESCs (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). Sox2, a co-activator for Oct3/4 (Ambrosetti *et al.*, 1997), is expressed in pluripotent embryonic and extra-embryonic lineages (Avilion *et al.*, 2003). Other transcription factors such as FoxD3 and Rex1 are also involved in the maintenance of pluripotency in mESCs and in mouse embryonic development (Kola *et al.*, 1993; Ben-Shushan *et al.*, 1998; Hanna *et al.*, 2002). Oct3/4, Nanog, Sox2, FoxD3, Rex1, and Ets1 have been studied as representative transcription factors involved in maintenance of the pluripotent state of mESCs, although little is known about the upstream signals that regulate these molecules. In the self-renewal and the maintenance of pluripotency with mESCs, these genes are important. Oct3/4, one of the POU transcription factors, is expressed in embryonic stem and germ cells (Scholer *et al.*, 1989; Rosner *et al.*, 1990). A critical level of Oct3/4 expression is required to sustain stem cell self-renewal and pluripotency (Niwa *et al.*, 2000). Oct3/4 is not only a master regulator of pluripotency that controls lineage commitment, but is also the first and most widely recognized marker used for the identification of pluripotent ESCs.

Wnt Signaling Pathway in mESCs

Wnts, secreted glycoproteins that play key roles in carcinogenesis and embryogenesis, were identified as a proto-oncogene and expressed in nervous systems during early animal development (Cadigan and Nusse, 1997). The modules that transduce Wnt signals are highly conserved throughout evolution, and in the canonical Wnt pathway a central role is played by the β-catenin. Recently, it has been reported that Wnt signaling functions maintain pluripotency in human and mouse ESC, and in haematopoietic stem cells (Reya *et al.*, 2003; Sato *et al.*, 2003). In addition, the activation of Wnt signaling by the GSK-3 inhibitor facilitates self-renewal in both mESCs and hESCs (Sato *et al.*, 2004), suggesting that the role of Wnt is essential for maintaining the pluripotency of mESCs. Whereas T (brachyury) is known as a mesoderm marker gene, previous microarray data has reported that T (brachyury) is enriched in mESCs (Ramalho-Santos *et al.*, 2002; Fortunel *et al.*, 2003). These findings demonstrate that extracellular molecules such as Wnt3 and DKK-1 in Wnt signaling may shift the balance of agonists/

antagonists, as mESCs maintains the self-renewal and initiates differentiation.

Notch Signaling Pathway in mESCs

Notch signaling is evolutionarily conserved, and regulates cell fate decisions in a number of cell and tissue types (Harper *et al.*, 2003). Ligand binding by members of the Jagged or Delta-like families results in the proteolytic cleavage and the release of the intracellular fragment of the Notch heterodimer (Schweisguth, 2004). Translocation to the nucleus then allows for its regulation of gene expression. Notch signaling determines the neuronal cell fate via cell-cell interactions, functions actively in the differentiations of glial cells, keratinocytes, and endothelial cells (Artavanis-Tsakonas *et al.*, 1999) and regulates cell proliferation, differentiation, and apoptosis by the downstream target gene hairy/enhancer of split (HES) (Axelson, 2004). In a previous report, the inactivation of HES5, known as a Notch effector, accelerates cell differentiation and causes wide-ranging defects in the development of the brain. Therefore, the HES gene is indispensable to the structural generation of the brain to a proper size, shape, and cell arrangement according to the control of the timing of the cell differentiation. As for the Notch signaling pathway, mESCs seem to regulate differentiation and to maintain self-renewal as well as the central nervous system (Hatakeyama *et al.*, 2004). The present findings suggest that the Delta 1 ligand, Notch receptors and HES5 may play a role in the self-renewal of mESCs.

Hh Signaling Pathway in mESCs

The Shh signaling pathway functions throughout the development. Shh is involved in the determination of cell fate and embryonic patterning during early vertebrate development (McMahon *et al.*, 2003). Hh signaling is required for the differentiation of ESCs into neuroectoderm and the extra-embryonic endoderm, and ectoderm differentiation in EB (Maye *et al.*, 2000; Maye et al., 2004). An example of this activity is the patterning of the neural tube such that motor neurons are derived from the ventral region and sensory neurons are formed from the dorsal region (Goodrich and Scott, 1998). It should be noted that in some cases Shh works with other signaling factors such as FGFs, Wnts, and BMPs to mediate developmental processes. Vertebrate embryonic development utilizes both short- and long-range mechanisms of Shh signaling. Short-range signaling by Shh is apparent during floor plate induction by the notochord within the neural tube (Johnson and Tabin, 1995). Long-range signaling by Shh occurs during motor neuron formation in the neural tube, sclerotome induction and proliferation in the somites, and limb patterning along the anterior-posterior axis (Chuang and Kornberg, 2000). These developmental events were some of the first to be characterized for Shh signaling. The canonical Hedgehog signaling pathway is a tale of two transmembrane proteins. Patched (Ptc), a twelve-pass membrane protein binds Hedgehog ligand. Smoothened (Smo), a seven-pass membrane protein is a signal transducer. In the absence of the ligand, Ptc interacts with and inhibits Smo, either directly or indirectly. In vertebrates, three Gli proteins, Gli1, Gli2, and Gli3, are involved in the transcriptional control of Hh target genes. Mutant mouse analysis has revealed that Gli1 functions primarily as an activator (Park *et al.*, 2000; Bai *et al.*, 2002). The results suggest that Gli molecules in Hh signaling may behave as key factors in the self-renewal of mESCs. Moreover, the high expression of N-mycl, which is a neural ectoderm marker and related to cell proliferation (Oliver *et al.*, 2003), suggests that the Hh signal pathway may be involved in cell proliferation in mESCs.

BMP4 Signaling Pathway in mESCs

BMPs, involved in bone morphogenesis, have diverse functions including hematopoesis, mesoderm formation, and patterning (Johansson and Wiles, 1995; Winnier *et al.*,1995). BMPs are anti-neural factors in vertebrate embryos (Wilson and Edlund 2001) and they inhibit the neural differentiation of ESC (Ying *et al.*, 2003). Recently, it has been shown that in the absence of serum, BMPs can synergize with LIF to maintain the self-renewal of mESCs by inducing the expression of Id genes (Ying *et al.*, 2003). The expression levels of Id genes in mESCs were similar to those of the NIH3T3 and MEF controls, this is due to the fact that a critical level of transcriptional expression for maintaining the self-renewal of mESCs is required. In other words, a lesser or greater level of mRNA expression may disturb the self-renewal in mESCs, and even lead to differentiation.

TGF-β Signaling Pathway in mESCs

It is known that TGF-β signaling coordinates key roles in mammalian embryogenesis and organogenesis (Whitman, 1998; Goumans and Mummery, 2000; Schier and Shen, 2000; Tremblay *et al.*, 2000) and regulates cell proliferation and differentiation (Massague *et al.*, 2000). Recently, it was reported that TGF-β signaling contributed through Smad2/3 phosphorylation to self-renewal of mESCs (James *et al.*, 2005) however, similar to the BMP signaling, components of TGF-β signaling may not be highly expressed in the maintenance of the pluripotency in mESCs.

JAK/STAT Signaling Pathway in mESCs

The most critical pathways regulating self-renewal in mESCs arethose mediated by the Oct3/4 and leukemia

inhibitory factor (LIF), and a model of their interactions has been proposed by Niwa (Niwa, 2001). LIF, a member of LIF-IL6 superfamily of cytokines, is critical for the maintenance of ES lines. LIF activation of the JAK/STAT pathway via the gp130 receptor is required to maintain the pluripotency of mESCs (Yoshida *et al.*, 1994). Experiments have shown that it is critical for ESCs self-renewal (Niwa *et al.*, 1998). However, recently, LIF is required, but not sufficient to expand the pluripotent mESCs in the absence of serum or feeder cells (Yamane *et al.*, 2005). Activation of STAT3 in the absence of LIF is sufficient for a prolonged self-renewal (Matsuda *et al.*, 1999) and activation of a modified receptor, which has the ability to activate the MAPK pathway, and is as efficient as the wild-type receptor in maintaining cells in an undifferentiated state (Burdon *et al.*, 1999a,b).

RTK Signaling Pathway in mESCs

A major role for fibroblast growth factor (FGF) signaling has been demonstrated in embryonic development through mouse and human genetics (Muenke and Schell, 1995; Goldfarb, 1996; Martin, 1998; Naski and Ornitz, 1998; Ornitz and Itoh, 2001).

FGF4 is a signaling molecule that is related to growth and differentiation of the inner cell mass and required for post-implantation development in mice (Feldman *et al.*, 1995). Additionally, Sox2 and Oct3/4 bind to the FGF4 enhancer region in mESCs (Yuan *et al.*, 1995), indicating that FGF4 is one of downstream target genes functioning in the self-renewal of ESCs. Gab1is expressed at an earlier stage of mouse embryo during development and trophoblast stem cells (Xie *et al.*, 2005). Consistent with early and widespread expression during development, a targeted disruption in Gab1 at the embryonic stage is lethal in mice (Itoh *et al.*, 2000; Sachs *et al.*, 2000). The enriched Gab1 in mESCs might be considered that Gab1 stimulated through not only the MAPK signal pathway but also PI3K signaling. Similar to a previous report in which FGF4 was shown to be involved in self-renewal by the suppression of ERK in mESCs (Burdon *et al.*,. 1999b), the present results suggest that FGF4 may play an important role in the self-renewal of mESCs through the inhibition of MAP kinases.

Conclusion

Previously, we reported with regard to expression profiles of developmentally important BMP4, TGF-β, FGF4, Wnt, Hh, Notch, and JAK-STAT signal pathways in hESCs (Rho *et al.*, 2006). We compared our mESCs results with the enriched genes in other mESCs (Ramalho-Santos *et al.*, 2002; Fortunel *et al.*, 2003) and hESCs. These comparisons suggest that BMP4, FGF4, Wnt, and Shh signaling may be necessary for maintaining stemness in human and mouse ES cells even though specific gene expression levels may differ between mESCs and hESCs. In mESCs, several studies show that the presence of LIF signaling is sufficient for maintenance of mESCs pluripotency (Smith *et al.*, 1988; Williams *et al.*, 1988; Raz *et al.*, 1999). In contrast to mESCs, LIF does not maintain hESCs, and the fibroblast growth factor (FGF) signaling appears to be central importance to hESCs self-renewal (Amit *et al.*, 2004; Daheron *et al.*, 2004). It is the observed differences in gene expression profiles between mESCs and hESCs that may depend on different culture conditions. Here, the expression patterns of molecules involved in developmentally conserved signaling pathways in the self-renewal of mESCs is reported. The data from this study provide a basis for future research into ESCs in order to understand the molecular mechanisms of their self-renewal and differentiation. In addition, the identification of the genes which are differentially expressed between mESCsand differentiated cells will provide target genes whose functions can be modified by chemical inhibitors, knock-down or RNAi, thus helping with the study of the functions of signaling pathways in embryonic stem cells.

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*Supplementary Data

Table 1. Primers of ES specific mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
β-tubulin	Sense	GGA ACA TAG CCG TAA ACT GC	317	BC003825
	Antisense	TCA CTG TGC CTG AAC TTA CC		
Oct3/4	Sense	GCG TTC TCT TTG GAA AGG TG	313	NM_013633
	Antisense	ACT CGA ACC ACA TCC TTC TC		
Nanog	Sense	AAC GAT ATG GTG GCT ACT CTC	264	AY278951
	Antisense	TCG GTT CAT CAT GGT ACA GTC		
SOX2	Sense	TAG TGG TAC GTT AGG CGC TT	325	NM_011443
	Antisense	TCT TGC CAG TAC TTG CTC TC		
FoxD3	Sense	CTC TCT GAT CCT GGT CCA TC	128	NM_010425
	Antisense	GCT TAG GTG AGT GAG GGG AT		
Rex1	Sense	TGA GGA AGC ACA TGC TTG TCC A	287	NM_009556
	Antisense	TGC GTG GGT TAG GAT GTG AAT C		

Table 2. Primers of TGF-b signaling pathway related mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
BMP4	Sense	TGT GAG GAG TTT CCA TCA CG	566	NM_007554
	Antisense	TTA TTC TTC TTC CTG GAC CG		
BMPR Ia	Sense	ATC GTG TCT AAC CGC TGG AAC	208	NM_009758
	Antisense	TTG GGT GAA GAA GTT CTT GCA G		
BMPR Ib	Sense	ATG GAG CAG TGA TGA GTG TCT C	214	NM_007560
	Antisense	GTA GGC TAA CGT TCA AGG CTT G		
BMPR II	Sense	GCC ACG ACC ACA GTG TCT AAA G	323	NM_007561
	Antisense	GAG ACC ACT TTG GAT AAG CAC AC		
Smad1	Sense	AAA CGA CGG CTG CAA ATG TAG	189	NM_008539
	Antisense	CTC ATC TGC TCT CAC AGT TAG		
Smad5	Sense	TCA GCT CCA TGA GAG AGA TGT C	225	NM_008541
	Antisense	GTC CGA GAC CTA TGA CAT GAA G		
Smad6	Sense	GGA GAA ACA AGA AAG ACG CAC	117	NM_008542
	Antisense	GCT TTC CAC CTA GTT CTA CTG		
Smad8	Sense	CTA CCC TGA CTC TTT CCA GCA G	361	NM_019483
	Antisense	ACC CTT TCC AAT GTG CCT CCT G		
Smad4	Sense	GTA TGC CGC CCC ATC CTG	400	NM_008540
	Antisense	ACA GCG TCG CCA GGT GCT C		
Id1	Sense	GGG TTT GAT CAA CAG AGC CT	147	NM_010495
	Antisense	CAG AAA TCC GAG AAG CAC GA		
Id2	Sense	ATG ATC GTC TTG CCC AGG TGT C	292	NM_010496
	Antisense	CAG CAT TCA GTA GGC TCG TGT C		
Id3	Sense	TGA GCT TCG ATC TTA ACC CAG	260	NM_008321
	Antisense	AAC AGC TCT TAT GCT GCC TTG		
TGF-b1	Sense	AAA CTC CAC GTG GAA ATC AAC G	277	NM_011577
	Antisense	GAC AGA AGT TGG CAT GGT AGC		
TGF-b2	Sense	AAG GAG GTC ATA GTG GAT GAC	285	NM_009367
	Antisense	GTA GAT CAA CAG CCA CTT CAC		
TGF-bRI	Sense	TGT CAG CAT CCA CCA GGT TTG	257	NM_009370
	Antisense	TGC TTC TCT CTT CAC AGG TTT C		
TGF-bRII	Sense	TCA AGC AGA CGG ATG TCT ACT C	342	NM_009371
	Antisense	CAA AGT CTC ACA CAC GAT CTG G		
Smad2	Sense	GAC CCA GTA TTG CAG TAC TAT GC	430	NM_010754
	Antisense	CAT TCT GCT GTA CTG CTC TGA AC		
Smad3	Sense	GCC AAC AAG TCA ACA AGT GGT G	226	NM_016769
	Antisense	CTG GCT AAG GAG TGA CAA GAA C		
Smad7	Sense	ATG TGG AAA GTC AGC TCA GCA TC	197	NM_008543
	Antisense	AGT ATC ATA CGA GCG AGC GTA TG		

Table 3. Primers of receptor tyrosine kinase signaling pathway related mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
FGF4	Sense	AGC GAG GCG TGG TGA GCA TCT T	198	NM_010202
	Antisense	TGG TCC GCC CGT TCT TAC TGA G		
IGF-I	Sense	ACA GGC ATT GTG GAT GAG TGT TG	135	NM_184052
	Antisense	CTG AGT CTT GGG CAT GTC AGT G		
IGF-II	Sense	ACT CAC ACC ACA GGC ATT AAC AC	280	NM_010514
	Antisense	CTG AGA AAG ACA GAA CTA GCA GC		
IGF-IR	Sense	CAG GAC ACA AGG CTG AGA ATG G	273	NM_010513
	Antisense	TCT GAA GAT CCA CTG AGG TAC AG		
IGF-IIR	Sense	ACC CAT TCG ACC TAT AAG AAG C	192	NM_010515
	Antisense	GTG ACA AGT CAC TGA ACA CAT C		
IGFBP2	Sense	AGT GCA AGA TGT CTC TGA ACG	351	NM_008342
	Antisense	AAG GGA GGT TCA GCT TAA CAG		
Gab1	Sense	TCT CTG CAT TGA TCT GAG GCT C	122	NM_021356
	Antisense	GCA TTG TGT ACT CAG GCA ACA G		
SHP2	Sense	TCA GTG TTA TGG AAG GTG GTT GC	97	NM_011202
	Antisense	CGC TGT CTC AAA TCC ACA CCT C		
ERK1	Sense	CCA AAC AAG CGC ATC ACA GTA G	163	NM_011952
	Antisense	CTG TCT CCT GGA AGA TCA ACT C		
ERK2	Sense	GAA GTT GAA CAG GCT CTG GC	201	NM_011949
	Antisense	CAG TCC TCT GAG CCC TTG TC		
MKP3	Sense	GTT AGA CAA GGT TGC CAA GTG C	331	NM_026268
	Antisense	ACC GAT ACC GCA AAT ACA GAG C		
Ets2	Sense	ATG CTT TGT GGT TAA GCA CAG G	215	NM_011809
	Antisense	TGA GGA CTT CCA TGA CTG TTA G		
c-myc	Sense	ACT GAC CTA ACT CGA GGA GGA	122	NM_010849
	Antisense	AGC CAA GGT TGT GAG GTT AGG		
c-fos	Sense	CAG CGT CAA TGT TCA TTG TCA TG	184	NM_010234
	Antisense	TCC ACA TGT CGA AAG ACC TCA G		

Table 4. Primers of Wnt signaling pathway related mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
Wnt3	Sense	ACA CTT GAG CAG AAC GGA TAC A	207	NM_009521
	Antisense	TGG ATA CAG CAG GTT GGT AG		
DKK1	Sense	GAC ACT TCT GGT CCA AGA TCT G	423	NM_010051
	Antisense	TAC AGG TAA GTG CCA CAC TGA G		
Frizzled1	Sense	ACT CCT CAG CAG CAC ATT CTG	273	NM_021457
	Antisense	GAC TGC TTT TCT CCT CTT CTT C		
Frizzled2	Sense	CAC TGC AAG AGC CTA GCC ATC	180	NM_020510
	Antisense	GTT GGT GAG ACG AGT GTA GAA C		
Dishevelled2	Sense	GAG CTT TCT TCG TAC ACC TAT G	277	NM_007888
	Antisense	GAA GAG CTC GGA GAT TAG GAG		
Dishevelled3	Sense	AGG ACA CAC TGG CTC CTT TGC	163	NM_007889
	Antisense	CCT TCA CTG TGC TGA CTG CTG		
APC	Sense	AGG ATG AAA GGC ATG TGA GCT C	174	NM_007462
	Antisense	AGA TCA GAG GCT TGG ATT CAG C		
Axin	Sense	AGT GGA GAG GAT CGA CTG AGC	251	NM_015732
	Antisense	CAG GCT TTT CTC ATC TCG GTA C		
GSK-3b	Sense	TCT TGT TGG ATC CTG ATA CAG C	239	NM_019827
	Antisense	CAA CTG ATC CAC ACC ACT GTC		
β-catenin	Sense	GCC TGC AGA ACT CCA GAA AG	135	NM_007614
	Antisense	GTG GCA AAA ACA TCA ACG TG		
Lef1	Sense	TGA GAG CGA ATG TCG TAG CTG	236	NM_010703
	Antisense	ACC TGT ACC TGA AGT CGA CTC		
Tcf4	Sense	TAC GAG CTT GCG AAC CAA TCA C	313	NM_009333
	Antisense	ACG GAC ATA CAG GTA CAG CAA G		
Nr-CAM	Sense	CAT CTG CAG AGC TAA TGG CAA C	263	NM_176930
	Antisense	TGC AAT GAC CTG GTA CAG TGT G		

Table 5. Primers of Sonic hedgehog related mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
SHH	Sense	TCT GTA CTG CCT TCT TGG TTT G	214	NM_009170
	Antisense	TGT ACA AAC TAC AGG CTC CAT C		
Patched1	Sense	TGC TTC GGT GAC TGT TGC TG	288	NM_008957
	Antisense	CTC TCC TCA CAT TCC ACG TC		
Patched2	Sense	TTC ACA GTT CAC GTG GCT CTG	290	NM_008958
	Antisense	GGA CTC TCC TTG TAT ACC TGC		
Smoothened	Sense	TGT GGT GGT CTG TGA GGT AAC	276	NM_176996
	Antisense	GGA ACT GAG ATG TGA ATG TAG G		
Gli1	Sense	CTT TGT GGC TAT CCT AGA TGA G	162	NM_010296
	Antisense	TTG AGG AAT TGT GTC TCT CCA G		
N-myc1	Sense	TGT CGA GTC TGG ATC TGG GTA G	120	NM_008709
	Antisense	GCT GTC ACC AGG TGA TAT GGA G		

Table 6. Primers of notch signaling pathway related mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
Delta 1	Sense	CTA TGA GCC AGT CTT TCC TTG	235	NM_007865
	Antisense	TAG TCA CAT AGA CCC GAA GTG		
Notch1	Sense	CCT GTT GGA AGT CCT TTC CA	237	NM_008714
	Antisense	AAT CAA GGC TCT GAG AAC TAG		
Notch3	Sense	CGC TTT CTG CTT CTC ATT GTC	292	NM_008716
	Antisense	TGG CTA CTT GGT ACA TAC GAG		
Presenlinin	Sense	CAC ATC AGT GAC TCT GAC CAC	185	NM_008943
	Antisense	AGG ATG ACA GGG ACT GTT GAG		
HES1	Sense	CTC TCT CTT CTG ACG GAC AC	142	NM_008235
	Antisense	TGC AGT GCA TGG TCA GTC AC		
HES5	Sense	TCA GCA AGT GAC TTC TGC GAA G	257	NM_010419
	Antisense	CCA TGT GGA CCT TGA GGT GAG		

Table 7. Primers of JAK-STAT signaling pathway related mouse genes for RT-PCR

Transcript		Primer sequence (5'-3')	Product size (bp)	Accession No.
LIF receptor	Sense	TGC AAC TCA TCT CGG TCT GAG C	141	NM_013584
	Antisense	TCC CTG GTT AGT GCA CCC ATA G		
gp130	Sense	ACG AGT GGC TTC AGA TGA GA	103	M83336
	Antisense	TTC CAG CTA CTC TGG AAT GGA		
JAK1	Sense	GCT GCA TAG CAA AGG ACT GTG	133	NM_146145
	Antisense	CTA AGT GGT ATC TAC GTG CTT G		
STAT3	Sense	TGA GAG CAG AAG GGA GCA AG	178	NM_011486
	Antisense	AGC AAG GTT GAA AGT GCA GAG		
Bcl-xL	Sense	GTA TTG GTG AGT CGG ATT GC	105	NM_009743
	Antisense	GTA GAG ATC CAC AAA AGT GTC		