Transcriptional profiling of the developmentally important signalling pathways in human embryonic stem cells

Jeung-Yon Rho1,†, Kweon Yu1,†, Jee-Soo Han1, Jung-II Chae1, Deog-Bon Koo1, Hyun-So Yoon2, Shin-Yong Moon3, Kyung-Kwang Lee1 and Yong-Mahn Han1,4

1Laboratory of Development and Differentiation, Korea Research Institute of Bioscience and Biotechnology (KRIIBB), 52 Eoeun-dong Yuseong-gu, Daejeon 305-806, 2Department of Anatomy and Cell Biology, College of Medicine, Hanyang University, Seoul and 3Department of Obstetrics and Gynecology, College of Medicine, Seoul National University, Seoul, Korea

†These authors contributed equally to this work

4To whom correspondence should be addressed. E-mail: ymhan@kribb.re.kr

BACKGROUND: Embryonic stem cells (ESC) maintain their ‘stemness’ by self-renewal. However, the molecular mechanisms underlying self-renewal of human embryonic stem cells (hESC) remain to be elucidated. In this study, expression profiles of the molecules of developmentally important signalling pathways were investigated to better understand the relationships of the signalling pathways for self-renewal in hESC. METHODS: Two human ESC lines were cultured on mouse embryonic fibroblast (MEF) feeder cells. Gene expression was analysed by RT-PCR, real-time RT-PCR and Western blotting. RESULTS: In the bone morphogenetic protein (BMP4), transforming growth factor (TGF-β) and fibroblast growth factor (FGF4) signalling pathways, ligands and antagonists were highly expressed in hESC compared with human embryoid body (hEB). Human ESC showed abundant transcripts of intracellular molecules in the Wnt, Hh and Notch signalling pathways. No difference was detected in the expression level of the JAK/STAT signalling molecules between hESC and hEB. Western blot analysis showed that the transcriptional levels of the signalling molecules in hESC were consistent with translational levels. From the real-time PCR analysis, expression levels of some genes, such as Oct3/4, Nodal and β-catenin, were different between two hESC lines. CONCLUSION: The self-renewal of hESC is probably maintained by coordinated regulation of signalling-specific molecules and in a signalling-specific manner.

Key words: human embryoid body/human embryonic stem cells/self-renewal/signalling pathways/transcription level

Introduction

Embryonic stem cells (ESC) derived from preimplantation embryos are able to maintain indefinitely their ‘stemness’ by self-renewal and differentiate into all cell types of embryo proper (Ramalho-Santos et al., 2002). The self-renewal of ESC is affected by many factors, such as growth factors and feeder cells in culture (Nichols et al., 1998). Transcriptional profiling has been performed from embryonic, neural and haematopoietic stem cells by microarray analysis (Ivanova et al., 2002; Ramalho-Santos et al., 2002), demonstrating that stem cells are different from differentiated cells at the transcriptional level. Some transcriptional factors, including Oct3/4, fibroblast growth factor 4 (FGF4), FoxD3, Sox2 and Nanog, play key roles in maintaining pluripotency in mouse ESC (mESC) (Yuan et al., 1995; Wilder et al., 1997; Nichols et al., 1998; Hanna et al., 2002; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003).

Although various signalling pathways are involved in the self-renewal of stem cells, little information is available regarding the expression of the developmentally important signalling pathways in human ESC (hESC). It is known that the Janus kinase (JAK)/signal-transduced and activator of transcription (STAT) signalling pathway is crucial for self-renewal in mESC, but it does not appear to play an important role in the self-renewal of hESC (Duheron et al., 2004). In mESC, bone morphogenetic proteins (BMPs) sustain self-renewal of ESC in combination with leukaemia inhibitory factor (LIF), the ligand of JAK/STAT signalling, by expression of the BMP signalling target gene, inhibitor of differentiation (Id) (Ying et al., 2003). Activation of Wnt signalling by the glycogen synthase kinase-3 (GSK-3) inhibitor BIO maintains the undifferentiated status in both hESC and mESC (Sato et al., 2004). The receptor tyrosine kinase (RTK) pathway promotes differentiation and the Hedgehog (Hh) signalling pathway functions on neuronal differentiation in mESC (Burdon et al., 1999a; Maye et al., 2004). In mouse haematopoietic stem cells, the Notch signalling pathway is involved in the maintenance of self-renewal (Stier et al., 2002).

In this study, transcriptional profiles of the BMP4, transforming growth factor-β (TGF-β), RTK, Wnt, Hh, JAK/STAT and...
Notch signalling pathways, which are conserved in animal development (Pires-daSilva and Sommer, 2003), were investigated to increase understanding of the self-renewal of hESC. These findings will provide fundamental information to elucidate the molecular mechanisms of self-renewal and differentiation in hESC.

Materials and methods

Human embryonic stem cell culture and embryoid body production

The cell lines Miz-hES1 (80–100 passages) and SNU-hES3 (70–90 passages) were maintained in an undifferentiated state, as described previously (Park et al., 2003; Oh et al., 2005). Human ESC were cultured in DMEM/F12 medium (Invitrogen, Carlsbad USA) containing 20% knockout serum replacement (Invitrogen, Carlsbad USA), 1% β-mercaptoethanol (Sigma, USA), 0.1 mmol/l penicillin–streptomycin (Invitrogen), 4 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen) on mitomycin C (Sigma St Louis, MO)-treated mouse embryonic fibroblast (MEF) feeders at 37°C, 5% CO₂ in air. Approximately three-quarters of the culture medium was exchanged daily. After 5 days of culture, the surrounding MEF feeder layers were carefully pushed away from the hESC colonies using a hand-made glass pipette. hESC colonies were detached mechanically by using the glass micropipette from the MEF feeders. Single hESC colony was mechanically divided into four or five parts and then the clumps were separately plated on a fresh MEF feeder layer. Human embryoid bodies (hEB) were generated as previously described (Sato et al., 2003). Briefly, to form hEB, clumps of hESC colonies were cultured under the differentiation condition without feeder cells for 3 days and further incubated at 37°C, 5% CO₂ in air for 3 weeks.

mRNA extraction and RT-PCR

Human ESC and hEB were homogenized in the TRIzol reagent (Invitrogen). Poly(A⁺) RNA was prepared from total RNA using the oligotex mRNA kit (Qiagen, Valencia, USA). Poly(A⁺) RNAs (100 ng) were used to generate the first-strand cDNA by using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) primers. In each PCR, 0.5 μl of the first-strand cDNA was used in the 20 μl PCR reaction mix (Bioneer, Daejeon, Korea). The PCR reaction consisted of an initial step at 95°C for 3 min, 30 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, and a final elongation step at 72°C for 10 min. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control. Experiments of mRNA extraction and RT-PCR were repeated three times. The PCR products were resolved by 2% agarose gel electrophoresis. The primer sequences used and the expected sizes of PCR products are shown in Tables 1–7 (Tables 1–7 are available as supplementary material at Human Reproduction online).

Real-time PCR

Real-time PCR was carried out with the DNA Engine Opticon 2 System (MJ Research, South San Francisco, CA USA) according to the manufacturer’s instructions. SYBR Green (Molecular Probes, Eugene, OR, USA) was used for quantitative PCR as a double-stranded DNA-specific fluorescent dye. The PCR was conducted by initial denaturation for 10 min followed by 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s using the SYBR Green qPCR kit (Finnzymes, Finland). To determine the specificity of PCR products, a melting curve was acquired by heating the product at 95°C, cooling at 65°C for 20 s and then remelting slowly (0.3°C/s) up to 95°C. The relative quantification of gene expression between multiple samples was achieved by normalization against endogenous GAPDH using the ΔCt method of quantification. Fold changes were calculated as 2−(ΔΔCt). The primers used for the real-time RT-PCR were the same as in RT-PCR.

Immunocytochemistry of hESC

Human ESC were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde at 4°C for 30 min. Alkaline phosphatase (AP) was stained using the AP staining kit according to the manufacturer’s protocol (Sigma). Briefly, the cells were washed three times in PBS, and the cells were then stained with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indorylphosphate (BCIP) (Sigma Roche Molecular Biochemicals, USA). To detect cell-surface markers, antibodies against stage-specific embryonic antigen (SSEA)-1, -3 and -4 (Chemicon International, Temecula USA) were used. The antibodies were diluted with PBS containing 1% bovine serum albumin to block non-specific reactivity. Then, cells were incubated with the corresponding biotinylated secondary antibody and stained following the avidin–biotin–peroxidase method (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was made visible using 0.01% (v/v) H₂O₂ and 0.05% (w/v) 3,3’-diaminobenzidine. Immunostained cells were observed on an inverted microscope (Olympus, Tokyo, Japan).

Western blot analysis

Antibodies against Oct3/4, Nanog, ERK1 and 2, β-catenin, Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA USA) and gp130 (Cell Signaling Technology, Beverley, MA USA) were used for Western blot analysis. To isolate proteins, hESC and HeLa cells were lysed in lysis buffer (20 mmol/l HEPES, 50 mmol/l NaCl, 10% glycerol, 0.5% Triton X-100 and 2% β-mercaptoethanol). Protein concentrations were determined by the Bradford method. The protein samples (30 μg) were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH, USA) with Tris/glycine/methanol (25 mmol/l Tris, 192 mmol/l glycine and 20% methanol). After blocking with the TBS buffer (10 mmol/l Tris–HCl pH 7.5, 150 mmol/l NaCl) containing 5% non-fat dry milk and 0.1% Tween20, the membrane was incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat antibody to mouse IgG (Cell Signaling Technology) or goat antibody to rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA USA), and developed with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ, USA).

Results and discussion

Human embryonic stem cells

The Miz-hES1 line showed typical undifferentiated morphology and round-shaped colonies with well-defined edges (Figure 1A). ESC-specific markers, such as AP, SSEA-3 and -4, were also positive for Miz-hES1 cells (Figure 1B), indicating that the hESC line retains the undifferentiated status. Transcripts of ESC-specific genes, such as Oct3/4, Nanog, Sox2, FoxD3, Rex1 and Ets1, were enriched in hESC compared with hEB (Figure 1C). In the mouse, Oct3/4 expression directs pluripotent cell lineages during embryonic development (Nichols et al., 1998). Nanog, a homeodomain transcription factor, is essential for self-renewal in mESC (Chambers et al., 2003; Mitsu, 2003), and Sox2, a co-activator for Oct3/4 (Ambrosetti et al., 1997), is expressed in multipotent embryonic and extraembryonic lineages (Avilion et al., 2003). Other transcription factors, such as FoxD3, Rex1 and Ets1, are required for the self-renewal of mESC and embryonic development in mice (Kola et al., 1993; Ben-Shushan et al., 1998; Hanna et al., 2002).
BMP4 signalling pathway in hESC

The BMP4 and TGF-β subfamilies belong to the TGF-β superfamily, which regulates a variety of cellular and physiological processes (Massague et al., 2000; Nakashima and Reddi, 2003). BMPs have diverse biological functions, including haematopoiesis, mesoderm formation and patterning (Johansson and Wiles, 1995; Winnier et al., 1995). BMPs as antineural factors inhibit neural differentiation in ESC and vertebrate embryos (Wilson and Edlund, 2001; Ying et al., 2003). In this study, BMP4, 7 and growth/differentiation factor 3 (GDF3) ligands showed a higher expression level in hESC than in hEB and HeLa cells, whereas expression of the BMP4 antagonists chordin and noggin was very low in hESC (Figure 2A). Transcripts of BMPRIA and BMPRII were slightly enriched in hESC, but no difference was observed in the expression level of BMPRIB between different cell types. Similarly, it was reported that BMP4, BMPRIA and GDF3 genes were highly expressed in mESC and hESC (Ramalho-Santos et al., 2002; Fortunel et al., 2003). Transcripts of the Smad1, 4 and 5 genes were slightly abundant in hESC and Id genes were transcribed at similar level in hESC, hEB and HeLa cells (Figure 2A). From our observations, we suggest that, in the BMP4 signalling pathway, extracellular molecules such as ligands and antagonists may play important roles in the self-renewal of hESC.

TGF-β signalling pathway in hESC

The TGF-β signalling pathway is involved in patterning of mammalian embryogenesis and organogenesis by the regulation of cell proliferation and differentiation (Whitman, 1998; Goumans and Mummery, 2000). Among ligands of this signalling pathway, transcripts of TGF-β1 and Nodal were enriched in hESC, whereas expression levels of TGF-β2 and TGF-β3 in hESC were similar to those in hEB and HeLa cells (Figure 2B). Likewise, the Nodal and TGF-β genes were highly transcribed in mESC and hESC (Ramalho-Santos et al., 2002; Fortunel et al., 2003; Sato et al., 2003). Follistatin, an antagonist of the TGF-β ligand, was highly expressed in hESC and hEB compared with the HeLa control (Figure 2B), suggesting that the inhibitory regulation of TGF-β signalling may help to maintain the pluripotency of hESC. In addition, there were no big differences at the expression level of TGF-β receptors among hESC, hEB and HeLa cells. Expression levels of Smad2, 3, 4 and 7 in hESC were also similar to those of control groups (Figure 2B). Recently, it has been suggested that the TGF-β signalling pathway takes part in the self-renewal of hESC (James et al., 2005). Our data also indicate that high expression of TGF-β1 and Nodal in the TGF-β signalling pathway may contribute to the self-renewal of hESC.

FGF4 signalling pathway in hESC

The FGF4 signalling pathway is related to cell growth and differentiation during early development in mammals (Feldman et al., 1995). FGF4 is expressed in the inner cell mass of blastocysts and mESC (Yuan et al., 1995). Microarray analysis shows that transcripts of FGF4 and FGFR1 genes are enriched in mESC and hESC (Ramalho-Santos et al., 2002; Fortunel et al., 2003; Sato et al., 2003; Brandenberger et al., 2004). In the present study, high expression levels of FGF4 and FGFR1 were also observed in hESC (Figure 2C). Transcripts of the E-Ras and K-Ras genes were enriched in hESC, whereas the expression level of H-Ras was similar among hESC, hEB and HeLa cells. It is known that E-Ras promotes tumour-like growth in mESC (Takahashi et al., 2003). Therefore, our results indicate that E-Ras may facilitate tumour-like growth in hESC. Similar levels of SH2 domain-containing phosphatase (SHP2) and signal-regulated kinase (ERK) transcripts were detected among hESC, hEB and HeLa cells (Figure 2C). In general, FGF4 signalling promotes self-renewal by the suppression of ERK expression in mESC (Burdon et al., 1999b). Intriguingly, expression of MKP3, a mitogen-activated protein kinase (MAPK) inhibitor, was enriched in hEB (Figure 2C), indicating that inhibition of MAPK may facilitate the differentiation of hESC. Target genes of FGF4 signalling, including c-kit, c-jun and c-fos, were highly expressed in hESC compared with hEB (Figure 2C). c-Kit is essential for the maintenance of pluripotent primordial germ cells and haematopoietic stem cells (Cairns et al., 2003). Thus, high expression of target genes may be required for maintaining the self-renewal of hESC.

Wnt signalling pathway in hESC

β-Catenin and Wnt3A are involved in the self-renewal of haematopoietic stem cells (Reya et al., 2003; Willert et al., 2003; Sato et al., 2004). Microarray analysis shows abundant transcripts of Wnt3 and Dkk1 in mESC (Fortunel et al., 2003). In contrast to mESC, expression levels of Wnt3 and Dkk1 in hESC were similar to those of the controls (Figure 3A). This result leads to speculation that the molecules involved in Wnt signalling may function differently in the self-renewal of ESC between species. The frizzled 1 (Fzd1), dishevelled-1 (Dsh1) and disabled-2 (Dab2) genes were expressed at similar levels in hESC, hEB and HeLa cells (Figure 3A). Among components
of the β-catenin complex, transcripts of adenomatous polyposis coli (APC) and β-catenin genes were enriched in hESC compared with hEB and HeLa cells (Figure 3A). Also, the GSK-3β transcript was abundant in hESC (Figure 3A). The T-cell-specific transcription factor 4 (Tcf4), a β-catenin-binding transcription factor, was highly expressed in hESC, whereas the expression of lymphoid enhancer-binding factor 1 (Lef1) was abundant in hEB (Figure 3A). These findings indicate that the β-catenin/Tcf4 complex may function in maintaining the self-renewal of hESC and that the β-catenin/Lef1 complex may be involved in the differentiation of cells such as hEB cells. Microarray analysis also showed abundant transcripts of APC, β-catenin and Tcf4 genes in hESC (Sato et al., 2003). Nr-CAM, a target gene of Wnt signalling (Conacci-Sorrell et al., 2002), was expressed in hESC and hEB, but not in HeLa cells (Figure 3A). In the Wnt signalling pathway, intracellular molecules such as β-catenin, APC and GSK-3β may play important roles in maintaining the self-renewal of hESC. Our data support a previous report that Wnt signalling is sufficient for maintaining the self-renewal in both mESC and hESC (Sato et al., 2004).

**Hh signalling pathway in hESC**

The hedgehog gene is expressed in the outer visceral endoderm and inner ectoderm of mouse embryos (Becker et al., 1997; Maye et al., 2000). The Hh signalling pathway is required for the differentiation of ESC into neuroectoderm and extraembryonic endoderm (Maye et al., 2000, 2004), and activated by the action of
Transcriptional profiling of signalling pathways in human ESC

Two membrane proteins, patched (Ptc) and smoothened (Smo) (McMahon, 2000). In the present study, expression of the Sonic hedgehog (Shh) gene was enriched in hEB but very low in hESC (Figure 3B), suggesting that Shh molecules facilitate the differentiation of hESC. As shown in Figure 3B, Ptc and Smo receptors showed high levels of transcripts in hESC. Expression levels of PKA-Cα, PKA-Cβ, PKA-R1 and PKA-R2 in hESC were similar to those in control groups, but the Gli1, 2 and 3 genes were up-regulated in hESC. Likewise, microarray experiments demonstrated that the Smo, tc and Gli1 genes were highly expressed in hESC and mESC (Ramalho-Santos et al., 2002; Fortunel et al., 2003; Sato et al., 2003). N-myc, a mediator of proliferation (Oliver et al., 2003), was highly expressed in hESC. This result indicates that the Hh signalling pathway may be involved in the cell proliferation of hESC.

Notch signalling pathway in hESC

The Notch signalling pathway controls cell fate decisions through cell–cell interactions (Egan et al., 1998; Greenwald, 1998;

Artavanis-Tsakonas et al., 1999). The Delta1 gene was expressed in both hESC and hEB but not in HeLa cells (Figure 4A). Expression of Notch 1 and 3 receptors was abundant in hESC compared with the controls, but the expression level of Notch 2 was similar among hESC, hEB and HeLa cells (Figure 4A). It has been reported that the Notch 3 gene is highly expressed in mESC and hESC (Ramalho-Santos et al., 2002; Fortunel et al., 2003; Sato et al., 2003). Transcripts of the HES1 gene, which is a target gene of Notch signalling, were enriched in hEB (Figure 4A). Hairy/enhancer of split (HES) functions as a transcriptional repressor, thereby leading to cell proliferation, differentiation and apoptosis (Axelson, 2004). Our results indicate that the Notch signalling pathway may be involved in the self-renewal of hESC by repression of HES.

JAK/STAT signalling pathway in hESC

The JAK/STAT signalling pathway is essential for maintaining the self-renewal of mESC (Niwa et al., 1998). However, LIF, LIF receptor (LIFR) and gp130 receptor were slightly down-regulated in hESC compared with HeLa cells (Figure 4B). Expression levels of JAK1 and 2 were low in hESC, but JAK3 transcripts were abundant in hESC, hEB and HeLa cells (Figure 4B). Expression levels of STATs and Bcl-xL in hESC were similar to those of the controls. These results are consistent with previous reports indicating that the JAK/STAT
signalling pathway is probably not critical in maintaining the self-renewal of hESC (Reubinoff et al., 2000; Daheron et al., 2004).

**Quantitative expression patterns in hESC**

Expression patterns of the genes obtained by semiquantitative RT-PCR analysis were confirmed by quantitative real-time RT-PCR analysis and Western blotting. As shown in Figure 5, expression of the Oct3/4 gene was significantly high in hESC. The Nodal, FGF4, β-catenin, Gli1 and STAT3 genes were up-regulated in hESC lines compared with hEB, while the Wnt3, Shh and Delta1 genes were down-regulated in hESC. However, the expression levels of Smad4 and gp130 genes in hESC were similar to those of hEB. These results indicate that the transcription patterns of the genes in hESC by real-time RT-PCR analysis were the same as those obtained in the semiquantitative RT-PCR analysis. Noticeably, expression levels of the Oct3/4, β-catenin and Nodal genes were different between Miz-hESC and SNU-hESC lines (Figure 5). This difference may due to inherent differences among hESC lines, although each hESC line is pluripotent and proliferates indefinitely with the normal karyotype during long-term culture in vitro (Hoffman and Carpenter, 2005). Understanding the differences in characteristics between hESC lines will be helpful for stem cell research in the future.

Next, protein levels of the important molecules in the signalling pathways were analysed by Western blotting (Figure 6).

Because of experimental limitations in preparing proteins from hEB, only HeLa cells were subjected to Western blot analysis as the control. In hESC, the Oct3/4 protein showed two bands. It has been reported that two forms of the Oct3/4 protein could be generated by alternative splicing (Takeda et al., 1992). Consequently, our results demonstrate that the transcriptional levels of these molecules in the signalling pathways in hESC are consistent with translational levels.

**Conclusion**

Transcriptional profiling of ESC provides fundamental data for understanding the molecular mechanisms regulating the self-renewal and differentiation of stem cells. For this reason, the molecular signatures of hESC, mESC and other adult stem cells have been studied by microarray analysis (Ivanova et al., 2002; Ramalho-Santos et al., 2002), but the data are insufficient for providing information on the direction of stem cell fate in either self-renewal or differentiation. Even though a variety of signalling pathways are involved in the self-renewal of mESC (Smith, 2001), their roles are poorly understood in hESC (Thomson et al., 1998; Reubinoff et al., 2000). Here, we highlight the expression patterns of molecules involved in the developmentally important signalling pathways in terms of the self-renewal of hESC. Transcriptional profiles of the developmentally important signalling pathways may have important implications for our understanding of the self-renewal of embryonic stem cells.

---

Figure 5. Relative expression levels of the genes involved in signalling evaluated by quantitative real-time RT-PCR. Numbers on the vertical axis in each graph denote relative expression level. 1, HeLa cells; 2, line Miz-hEB; 3, line Miz-hES1; 4, line SNU-hES3.


Fortunel NO, Ou HH, Ng HH et al. (2003) Comment on ‘Stemness’: transcriptional profiling of embryonic and adult stem cells’ and ‘a stem cell molecular signature’. Science 302,393; author reply 393.


Kola I, Brookes S, Green AR, Garber R, Tynns M, Papas TS and Seth A (1993) The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. Proc Natl Acad Sci USA 90,7588–7592.


Submitted on June 20, 2005; resubmitted on August 29, 2005; accepted on September 5, 2005