Differential Expression of TPX2 upon Differentiation of Human Embryonic Stem Cells

Hye Min Noh[1], Seong-Jun Choi[1], Sehee Kim[1], Kye-Seong Kim[2] and Jin Kyeoung Kim[1,†]

[1]*Graduate School of Life Science and Biotechnology, College of Medicine, Pochon CHA University, Seongnam 463-836, Korea*

[2]*College of Medicine, Hanyang University, Seoul 133-791, Korea*

ABSTRACT

Embryonic stem (ES) cells are known to have an infinite proliferation and pluripotency that are associated with complex processes. The objective of this study was to examine expression of genes differentially regulated during differentiation of human ES cells by suppression subtractive hybridization (SSH). Human ES cells were induced to differentiate into neural precursor cells via embryoid body. Neural precursor cells were isolated physically based on morphological criteria. Immunocytochemical analysis showed expression of *pax6* in neural precursor cells, confirming that the isolated cells were neural precursor cells. Undifferentiated human ES cells and neural precursor cells were subject to the SSH. TPX2 (Targeting Protein for *Xklp2* (Xenopus centrosomal kinesin-like protein 2)) was identified, cloned and analyzed during differentiation of human ES cells into neural lineages. Expression of TPX2 was gradually down-regulated in embryoid bodies and neural precursor cells relative to undifferentiated ES cells. Targeting Protein for *Xklp2* has been shown to be involved in cell division by interaction with microtubule development in cancer cells. Taken together, result of this study suggests that TPX2 may be involved in proliferation and differentiation of human ES cells.

(Key words : Human ES cells, Suppression subtractive hybridization, TPX2, Up-regulated, Down-regulated, Differentiated stem cells)

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells that give rise to the entire tissues of body, including ectoderm, mesoderm and endoderm. Recently, human ES cells were isolated from inner cell mass of blastocyst (Thomson *et al.*, 1998; Rasmussen, 2003). Undifferentiated human ES cells expressed oct4 and cell surface markers, including SSEA-3, -4, TRA-1-60, TRA-1-81, and alkaline phosphatase. Human ES cells were able to differentiate into various tissues *via* formation of embryoid body (EB) *in vitro*. Human ES cells have been suggested to be a valuable resource for the degenerative diseases. In this study, differentially expressed gene was screened in human ES cells, TPX2 was selected.

Targeting protein for *Xklp2* is a microtubule associated protein. Targeting Protein for *Xklp2* has been shown to play a role on meiotic and mitotic spindle formation in vertebrates (Karsenti *et al.*, 2006), although detailed mechanism of action and regulation is not understood (Brunet, 2004; O'Brien, 2006). Targeting Protein for *Xklp2* was shown to bind to *Xklp2* in Xenopus laevis (Brunet, 2004). Xenopus centrosomal kinesin-like protein 2 was identified as an essential factor for construction of microtubules or interaction with dynein (Wittmann *et al.*, 1998). Xenopus centrosomal kinesin-like protein 2 was reported to be localized at nucleus during interphase and then migrated to spindle poles during mitosis (Garrett *et al.*, 2002; Schatz *et al.*, 2003). Targeting Protein for *Xklp2* is activated by binding to *Xklp2* that contains kinesin motor domain, KLP2-like subgroup. The subgroup has been suggested to play a role during mitosis and meiosis (Garrett *et al.*, 2002; Zhu *et al.*, 2005). Targeting Protein for *Xklp2* was also shown to be an essential protein for microtubule nucleation around chromatin (Garrett *et al.*, 2002).

Establishment of ES cell lines enabled notable scientific discoveries including insights into cell cycle regulation, spatial and temporal relationships during development, and the roles of transcription factors and homeobox genes along developmental pathways (Down-

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† Corresponding author : Phone: +82-31-725-8394, E-mail: kyeoung66@hanmail.net

ing *et al.*, 2004). However, regulation of genes during differentiation of human ES cells has not been studied well. In this study, expression of TPX2 was analyzed during differentiation of human ES cells into neural lineages.

MATERIALS AND METHODS

Culture of Human ES Cells

Two human ES cell lines (CHAhES4 and Miz-hES1) were used in this study (Park *et al.*, 2003; Kim *et al.*, 2007). Human ES cells were cultured on mitotically inactivated STO cells (ATCC CRL-1503) in ES cell medium that consisted of DMEM/F12 (1:1) supplemented with 100 mM MEM nonessential amino acid, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 55 mM β -mercaptoethanol, 20% Knockout Serum Replacement and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF; R&D systems, USA). Feeder cells were cultured in DMEM supplemented with 100 mM MEM nonessential amino acid, 100 U/ml penicillin, 100 μ g /ml streptomycin and 55 mM β -mercaptoethanol and 10% fetal bovine serum (FBS, Hyclone, Logan, UT). Media were changed daily. Human ES cells were manually subcultured by fire-thrown pasteur pipettes on freshly prepared STO cells at 7-day interval. All cultures were maintained at 37℃ and 5% CO_2 in atmosphere. All reagents were purchased from Gibco BRL (Gaithersburg, MD, USA), otherwise indicated.

Differentiation of Human ES Cells into Neural Precursor Cells

Differentiation of human ES cells into neural precursor cells was carried out according to the previous report (Kim *et al.*, 2004). Briefly, Formation of EB was carried out by suspension culture of ES cell clumps in bacteriological plate coated with F-127 (Sigma, St. Louis, MO, USA) in N2B27 medium (Ying *et al.*, 2003) in the presence of bFGF (10 ng/ml) that consisted of D-MEM/F12 (1:1) and Neurobasal medium (Gibco BRL) supplemented with N2 supplement (Gibco BRL), B27 supplement (Gibco BRL) and 2 mM L-glutamine (Gibco BRL). EBs were cultured for 14 days and medium was changed every other day. Fourteen-day old EBs were replated onto culture plates coated with 0.2 % gelatin and cultured for 14~21 days in N2B27 medium. Medium was changed every other day. Neuroepithelium cells with rosette structures appeared after 7 days in culture were manually isolated from the culture and transferred into bacteriological plate coated with F-127 in neurosphere (NS) medium (Zhang *et al.*, 2001). Neurospheres were formed in suspension culture within 24 hr and subcultured every 14 days. Neurosphere medium consisted of DMEM/F12 (1:1) supplemented with 2 mM L-glutamine, 25 μ g/ ml human insulin (Sigma), 100 μ g/ ml human transferrin (Sigma), 20 nM progesterone (Sigma), 60 μ M putrescine (Sigma), 30 nM sodium selenite (Sigma), 2 μ g/ ml heparin (Sigma), recombinant human epidermal growth factor (EGF; R&D Systems) and 20 ng/ ml FGF-2. Medium was changed every other day. Human ES cell was granted by No. 18 Research Proposal from Ministry of Health and Welfare.

Immunocytochemical Analysis

Cells were fixed in 4% formaldehyde overnight at 4℃ and incubated with primary antibodies overnight at 4℃ after treatment with 5% normal goat serum (Sigma) in PBS containing 0.01% Triton-X (Sigma). Cells were incubated with secondary antibodies in PBS (1:200) for 1 hr at room temperature. Cells were analyzed for expression of pax6 using anti-pax6 antibody (Chemicon, Temecula, CA, USA). Secondary antibody was goat anti-rabbit IgG (Chemicon) conjugated with rhodamine. Cells were visualized under the fluorescent microscope.

Extraction of RNA

Total RNA was extracted from undifferentiated human ES cells, EBs and neurospheres. Samples in 1.5 ml tube were mixed with 500 ml of TRI reagent (Molecular Research Center Inc., USA) by pipetting. One hundred milliliters of chloroform (volume proportion of tri reagent: chloroform=5:1) of chloroform (use for RNA) were added to the tube and shaken briefly. It was kept at room temperature for 5 min and subsequently done centrifugation at top speed for 10 min at 4℃. After centrifugation, separated supernatant was transferred into new tube. Equal volume of cool isopropanol was added and mixed. It was kept for 20 min at −20℃ and subsequently done centrifugation at top speed for 10 min at 4℃. After the centrifugation, supernatant was discarded and the remaining pellet was washed with 70% ethanol and centrifuged twice as the previous condition. Subsequently the RNA was thawed in DEPC-treated water, and analyzed on 1.2% agarose gel containing 0.1% ethidium bromide and measured its density through Nano-drop by spectrophotometry.

Synthesis of cDNA by Reverse Transcription

Total RNA from the previous step was prepared at 1.0 mg concentration, treated DNase I (Roche, Basel, Switzerland) and incubated at 37℃ for 20 min and 65 ℃ for 10 min to eliminate genomic DNA. Total RNA was reverse transcribed into cDNA by the Superscript™ First-strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA, USA) according to manufactu-

rer instructions. Reaction mixture supplemented with 0.25 ml of SuperScript II reverse transcriptase (200 units), 1 ml of oligo dT (0.5 μ g/ ml), 2 ml of 10x RT buffer, 2 ml of 0.1 M DTT and 1 ml of 10 mM dNTP was incubated at 42℃ for 50 min and then at 70℃ for 15 min. To avoid contamination of extra RNA, 1 ml of RNase H was treated to the cDNA samples for 10 min at 37℃ after completion of cDNA synthesis.

Suppression Subtractive Hybridization

The synthesized and amplified cDNA by long distance PCR (LD PCR) (BD biosciences clontech, CA, USA) were purified and collected through colomn-chromatography. Subsequently, samples were digested randomly using restriction enzyme, *Rsa* I, and purified for next step. For detection of differentially expressed gene fragments between human ES cell (tester) and neural precursor cell (driver), specific adaptors designed to match with *Rsa* I cutting sites for suppression subtractive hybridization (SSH) were ligated to tester sample that was hybridized with driver sample. Differentially expressed gene fragments were amplified by PCR using the ligated adaptor sequence primers and cloned in T easy vector (Promega, USA) for sequence and transformation. Gene fragments were transformed in competent cells on LB agar plate containing X-gal and ampicillin for approximately 17 hr at 37℃. A single colony was collected and amplified in LB broth for 17 hr at 37℃ in shaking incubator at the following day. Subsequently, amplified colonies were prepared for purification of plasmid. Extracted plasmids were confirmed by PCR analysis with T7 promoter primer.

Analysis of Gene Expression by Semi-Quantitative RT-PCR

Each cDNA was used as a template for PCR amplification (35 cycles) with specific primers designed 20 mers against TPX2 sequences (580 bases) (Table 1). PCR reaction was carried out for 40 sec at 94℃ for denature the template, 30 sec at 55℃ for annealing primers to template and 40 sec at 72℃ for target sequence extension of 35 times repeatedly. PCR products were analyzed by gel electrophoresis in 1% agarose and 0.1% ethidium bromide running on 100 V for 30 min.

Table 1. Specific primers for TPX2

Clone	Primer sequences	Size (bp)
TPX2	Sense:5'-CAGACTTTTACCTACCCGTG-3'	580
	Anti-sense:5'-GTGCAGTGTGCATTTTAAGA-3'	

RESULTS

Identification of Differentially Expressed Genes by SSH

Human ES cells were induced to differentiate into neural fates in stepwise processes. Differentiation of ES cells was initiated by formation of EB in suspension culture under the serum-free condition as shown in Fig. 1. At 7 to 10 days after replating EB, neuro-epithelial cells that were arranged in rosettes emerged from the center of the replated EB. Based on their distinctive morphology, the rosettes structures were physically purified by fire thrown pasteur pipettes and cultured in suspension to form neurospheres under the neurosphere (NS) medium supplemented with bFGF and EGF (Fig. 1C). Immunocytochemical analysis for pax6 confirmed the identity of the differentiated neural precursor cells (Fig. 1D). Differentially expressed genes were identified in human ES cells through SSH between undifferentiated ES and neural precursor cells. As shown in Fig. 2, specific gene fragments were more abundant in un-subtracted sample

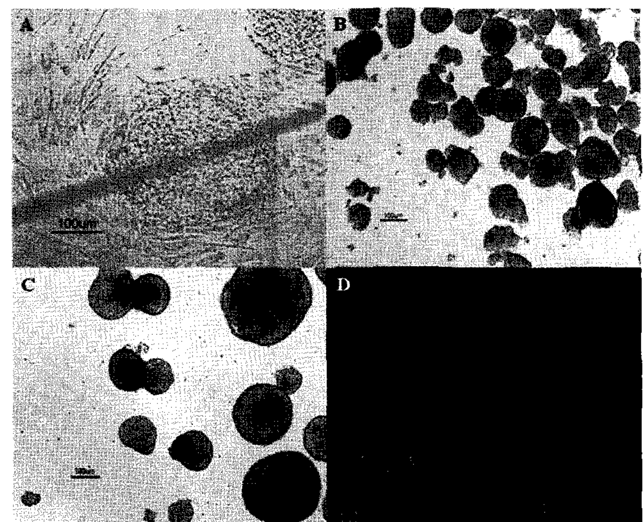

Fig. 1. Differentiation of human ES cells into neural precursor cells. A : Undifferentiated hES cells, B : Embryoid bodies, C : Neurospheres, D : Expression of *pax6* specific for neural precursor cells.

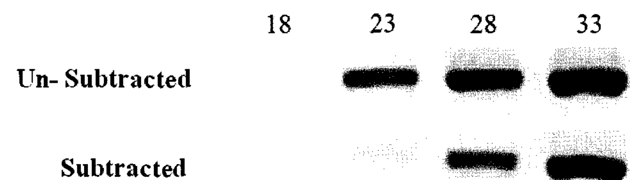

Fig. 2. Reduction of G3PDH abundance by PCR-select subtraction. Un-subtracted samples show more abundant expression compared with subtracted samples, demonstrating that suppression subtractive hybridization was performed completely. The numbers represent the cycles of PCR was performed.

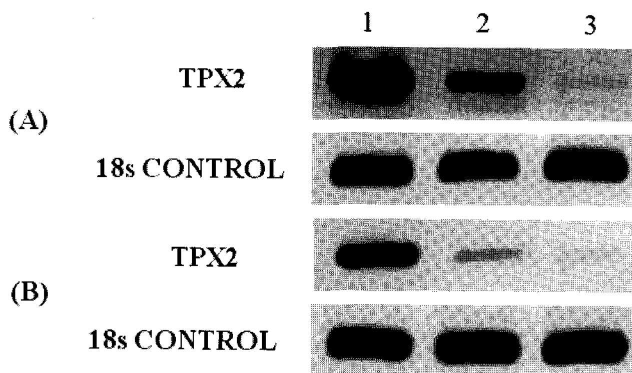

Fig. 3. Expression of TPX2 during differentiation of human ES cells into neural precursor cells. (A) Miz-hES1 cell line. (B) CHAhES4 cell line. ; Lane 1: undifferentiated human ES cells, lane 2: embryoid bodies, lane 3: neural precursor cells.

than in subtracted sample.

Down-Regulation of TPX2 Expression during Neural Differentiation

Among genes differentially expressed, TPX2 was identified, cloned and analyzed for expression pattern during differentiation processes of human ES cells. TPX2 was dramatically down-regulated in human EBs and neural precursor cells compared to undifferentiated human ES cells (Fig. 3). Gradual reduction of TPX2 expression was observed along the differentiation pathway of human ES cells into neural lineages. A similar pattern of TPX2 expression was shown from two lines of human ES cells. However, quantity of TPX2 expression along differentiation stages was different among two ES cell lines. Expression of TPX2 at EB stage was higher in Miz-hES1 cells than in CHA-hES4 cells.

DISCUSSION

ES cells can proliferate infinitely and differentiate to various types of cells in the body (Hansis, 2006). During normal development, pluripotency is acquired by the cells of the early embryo, which shortly thereafter undergo differentiation, whereas ES cells uniquely maintain pluripotency while undergoing extensive *in vitro* proliferation (Vallier *et al.*, 2005). Recently, ES cells have been suggested as useful resources for diverse degenerative diseases since they have unlimited capacities for proliferation and differentiation. Especially, human ES cells have been in the limelight for cell therapies. Directed differentiation of human ES cells into specific cell types *in vitro* has been a major goal to be used for therapeutic applications (Sikora *et al.*, 2004). Studies for molecular mechanisms on differentiation of ES cells are critical for the development of stem cell-based medical therapies (Bhattacharya *et al.*, 2005). Studies on differentiation of human ES cells to specific lineages of interests for medical therapies are carried out by various groups of scientists.

In this study, human ES cells were induced to differentiate to neuronal precursor cells. Neural precursor cells are the progenitors that have capacities to differentiate into neuronal and glial lineages of cells. For identification of genes differentially expressed in human ES cells, SSH was carried out using human ES and neural precursor cells differentiated from the corresponding ES cells. A gene, TPX2, was obtained as a down-regulated gene upon differentiation of human ES cells. Targeting protein for *Xklp2* is known as a microtubule associated protein, although its function is not well known yet. Targeting protein for *Xklp2* was shown to bind to *Xklp2* in Xenopus laevis and demonstrated to play a role on meiotic and mitotic spindle formation in vertebrates (Karsenti *et al.*, 2006). Xenopus centrosomal kinesin-like protein2 was identified as a kinesin-like protein that is an essential for construction of microtubules or interaction with dynein (Zhu *et al.*, 2005; Tulu *et al.*, 2006). Xenopus centrosomal kinesin-like protein2 was also reported to be localized in nucleus during interphase and then migrated to spindle poles during mitosis (Garrett, 2002; Mitchison *et al.*, 2004; O'Brien *et al.*, 2006). Targeting protein for *Xklp2* is activated by binding to *Xklp2* that contains kinesin motor domain, KLP2-like subgroup. The subgroup has been suggested to play a role during mitosis and meiosis (Pilling *et al.*, 2006). Targeting protein for *Xklp2* was also shown to be an essential protein for microtubule nucleation around chromatin (Schatz *et al.*, 2003; Wiese *et al.*, 2006). Recently, TPX2 was reported to be involved in progression of squamous cell lung cancer and malignant transformation of respiratory epithelium (Lin *et al.*, 2006; MA *et al.*, 2006). Drugs and diets have been shown to retard the progression of lung cancer by TPX2 (Jadad *et al.*, 1995).

Expression of TPX2 in human ES cells was regulated during differentiation processes. Expression of TPX2 was down-regulated along the differentiation stages of human ES cells, such as undifferentiated ES, EB and neural precursor cells. Targeting protein for *Xklp2* was expressed abundantly in undifferentiated ES cells, but down-regulated gradually in EB and neural precursor cells. The phenomenon can be explained by a fact that normal mature cells can have less proliferation capacity than tumor cells. Proliferation of cells is associated with increased expression of microtubules. Expression of microtubules is more abundant in actively dividing cells than in non-dividing cells as shown in tumor cells. Since TPX2 is associated with microtubule formation, its expression is lower in non-

dividing cells than in tumor cells (Ma *et al.*, 2006). Similarly, neural precursor cells differentiated from ES cells expressed a decreased level of TPX2 relative to undifferentiated ES cells, suggesting that proliferation capacity of ES cells was declined upon differentiation into neural precursor cells. In previous reports, various genes were differentially regulated as ES cells underwent differentiation (Bhattacharya *et al.*, 2005). For example, ES specific markers, nanog and oct 3/4, were down-regulated upon differentiation of ES cells (Bhattacharya *et al.*, 2005). On the contrary, markers for differentiation, GATA4 and nestin, were up-regulated in EB compared to undifferentiated ES cells (Bhattacharya *et al.*, 2005). Decreased expression of TPX2 in the differentiated progenies relative to that in undifferentiated ES cells seemed to be associated with differentiation processes. Taken together, results of this study suggest that TPX2 may be involved in proliferation and differentiation of human ES cells.

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