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Overexpression of SOX9 in mouse embryonic stem cells directs the immediate chondrogenic commitment

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Abbreviations: HMG, high mobility group; LIF, leukemia inhibitory factor; mES cell, mouse embryonic stem cell; MSC, mesenchymal stem cell; SOX, sry-type HMG box

Abstract

Mouse embryonic stem (mES) cells are capable of undergoing chondrogenesis *in vitro*. To enhance this process, the human SOX9 (hSOX9) cDNA was delivered into mES cells and the clones overexpressing hSOX9 (denoted as mES-hSOX9 cells) were verified by Western blot analysis. The transcripts of collagen IIA (a juvenile form), aggrecan and Pax1 were expressed in mES-hSOX9 cells grown on feeder layers, suggesting the immediate effect of exogenous SOX9 on chondrogenesis. However, SOX9 overexpression did not affect the cell cycle distribution in undifferentiated mES cells. Upon differentiation, collagen IIB (an adult form) was detected in day 3 immature embryoid bodies. In addition, the overexpression of exogenous SOX9 significantly induced transcriptional activity driven by SOX9 binding site. Taken together, we for the first time demonstrated that constitutive overexpression of exogenous SOX9 in undifferentiated mES cells might have dual potentials to induce both chondrogenic commitment and growth capacity in the undifferentiated status.

Keywords: cell differentiation; chondrogenesis; sox9 transcription factor; stem cells; transfection

Introduction

Articular cartilage is a complex of avascular tissues containing plenty of extracellular matrices including the molecules of collagens II, IX, XI and aggrecan, which functions as a shock absorber and provides a smooth joint movement. Once articular cartilage is damaged by trauma or degenerative arthritis, the complete repair of damaged cartilage is highly problematic mostly due to avascularity and low cellularity (Tsuchiya *et al.,* 2003). Currently, the reconstitution of damaged cartilage is achieved by injection of growth factors attached in biodegradable matrices, tissue transplantation and cellular engraftment (Buckwalter, 2002; Tanaka *et al.,* 2004). Recently, it has been interested in bone marrow-derived mesenchymal stem cells (MSC) as a good candidate for the source of articular cartilage repair because of their potentials to differentiate into various cell types of mesenchymal lineages such as chondrocytes, osteoblasts and adipocytes (Pittenger *et al.,* 1999; Tanaka *et al.,* 2004). However, MSC is a very limited source for tissue engineering due to the impaired growth potentials followed by aging (Tanaka and Liang, 1995; Tanaka *et al.,* 1999).

Mouse embryonic stem (mES) cells, first isolated from the inner cell mass of blastocysts (Martin, 1981), are defined as a stem cell line characterized by infinite proliferation capacities and pluripotentials to differentiate into various cell lineages (Wiles and Keller, 1991; Bain *et al.,* 1995; Tanaka *et al.,* 2004). Because of their characteristics, mES cells have been a good model system to study on *in vitro* differentiation into various cell types, such as cardiomyocytes, neurons, epithelial cells, and so on (Guan *et al.,* 1999). It has been also reported that mES cells undergo chondrogenic differentiation by cytokine stimulation or various culture conditions (Kramer *et al.,* 2000; Hegert *et al.,* 2002; Tanaka *et al.,* 2004) under a long-term culture system. Moreover, to enhance the efficiency of specified differentiation and obtain the homogenous cell populations, genetic manipulation has been accomplished by introducing exogenous genes into mES cells such as neurons or hepatocytes (Gratsch *et al.,* 2002; Ishizaka *et al.,* 2002). As the key regulators for chondrogenesis, several transcription factors are expressed in mesenchymal cells during early chondrogenesis, such as high-mobility group (HMG) box protein SOX9, and the paired-box-gene Pax-1 (Wallin *et al.,* 1994; Wright *et al.,* 1995). SOX9,

as a good candidate to enhance chondrogenic differentiation through genetic modification, is the member of the SOX (Sry-type HMG box) family and predominantly expressed during mesenchymal condensation and cartilage formation in mouse embryos (Wright *et al.,* 1995). In addition, SOX9 is known to be a key regulator for the expression of collagen II and aggrecan, the major matrix proteins specific for chondrocytes through binding to the enhancer region (Bell *et al.,* 1997; Lefebvre *et al.,* 1997; Sekiya *et al.,* 2000). In this study, to enhance the chondrogenic differentiation through genetic modification, we for the first time established the stable cell line overexpressing SOX9 in undifferentiated mES cells and analysed the expression of chondrogenic marker genes.

Materials and Methods

Cell culture

Mouse ES (129/Sv, AB2.2) cells on STO feeder cells were cultured in DMEM supplemented with 15 % FBS (Hyclone, Logan, UT), 1 mM β-mercaptoethanol, 0.1 mM Non-Essential Amino Acid, 100 IU penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and 1,000 U/ml mouse leukemia inhibitory factor (LIF; Chemicon, Temecula, CA). STO and NIH313 cells were cultured in DMEM supplemented with 10% FBS, 100 IU penicillin and 100 μg/ml streptomycin. For the formation of embryoid bodies for 3 days, STO feeder cells were removed by plating on gelatin-coated tissue culture dish for 30 min twice and then on bacteriological grade petri dish for another 30 min at 37°C incubator.

Plasmid constructs

A full-length human SOX9 cDNA amplified from human SOX9 clone (ATCC) was inserted into pIRES2-EGFP vector (Clontech, Palo Alto, CA) and the hEF1α promoter region obtained from the pTracer-EF/V5-His A vector (Invitrogen) was replaced with the CMV promoter region of pIRES2-EGFP. Flag epitope tag was added to the N-terminus of the hSOX9 open reading frame. A minimal promoter construct, pGL3ti (Jonk *et al.,* 1998), was prepared from pGL3-basic (Promega, Madison, WI) by inserting the adenovirus major late promoter TATA box and the mouse terminal deoxynucleotidyl transferase gene initiator sequence into the multiple cloning site. SOX9 binding site-driven luciferase reporter construct was prepared by inserting a 48 bp mouse collagen II intron 1 sequence containing SOX9 binding site (CTG TGA ATC GGG CTC TGT ATG CGC TTG AGA AAA GCC CCA TTC ATG AGA; Zhou *et al.,* 1998) into the upstream of the minimal promoter in pGL3ti vector (pGL3ti-SOX9 BS). All plasmid constructs were verified by DNA sequencing.

Establishment of stable cell lines in mES cells

Mouse ES cells were transfected with linearized pIRES2-hEF1α-EGFP or pIRES2-hEF1α-hSOX9-EGFP expression vector by electroporation with a single pulse of 270 V/500 μF and selected with 400 μg/ml G418 treatment (Invitrogen). Each independent clone of transfected mES cells was initially cultured in 48-well plates and propagated in 100 mm tissue culture dish for further analysis. To obtain neomycin-resistant feeder cells, STO cells were transfected with linearized pcDNA 3.0 (Invitrogen) by using FuGene 6 transfection reagents (Roche, Mannheim, Germany) and selected with 800 μg/ml G418.

Western blot analysis

The selected clones of transfected mouse ES cells were cultured in a 100 mm tissue culture dish and then washed twice with PBS and solubilized in extraction buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS, 0.5 % sodium deoxycholate, 1 mM DTT and 1X protease inhibitor cocktails. 20 μg whole lysates were separated on 8% SDS-polyacrylamide gels and transferred into polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins bound to anti-Flag monoclonal antibody (Sigma, St. Louis, MO) were detected with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) and visualized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) substrates.

Cell cycle analysis

Either empty vector- or hSOX9-transfected mES cells grown on STO feeder cells in complete ES growing medium were rinsed once in chilled PBS, then trypsinized briefly and resuspended in 10 ml ES growing media. Feeder cells were removed by plating the resuspended cells on tissue culture dish for 30 min twice and then on petri dish for another 30 min in 37°C incubator. The collected cells were pelleted and resuspended in iced-cold PBS. The resuspended cells were fixed by adding 100% ethanol while vortexing. The cells were kept on 4°C for 1 h, pelleted, and then resuspended with PBS containing 50 μg/ml RNase A. Followed by the incubation at room temperature for 5 min, the cells were further incubated in PBS containing 50 μg/ml propidium iodide at 37°C incubator for 30 min. Nuclei were analysed in a FACSVantage SE (BD Biosciences, Palo Alto, CA).

RT-PCR analysis

Total RNAs were extracted from transfected mouse ES cells by Trizol reagents (Invitrogen) and treated with DNase (Promega). Reverse transcription was carried out using the Superscript II (Invitrogen) and oligo-dT primers (Promega) under the condition of incubation at 42°C for 1 hr and 72°C for 15 min. The PCR reactions were performed by using DNA Taq polymerase (Promega) with the following conditions: 1 cycle at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, 70°C for 1 min and a final extension at 72°C for 15 min. The PCR products were electrophoresed in a 1.2% agarose gel and visualized by ethidium bromide staining. The primer sequences and product sizes were described in Table 1.

Table 1. Oligonucleotide primers used for reverse-transcription-polymerase chain reaction and expected product sizes.

Targets	Sequences	Product sizes (bp)
hSOX9		
	F: 5'-CCCGATCTGAAGAAGGAGAGC-3'	
	R: 5'-GTTCTTCACCGACTTCCTCCG-3'	381
mCollagen II A/B		
	F: 5'-CTGCTCATCGCCGCGGTCCTA -3'	432/
	R: 5'-AGGGGTACCAGGTTCTCCATC -3'	225
mPax1		
	F: 5'-GATGGAAGACTGGGCGGGTGTGAA-3'	318
	R: 5'-TTCTCGGTGTTTGAAGGTCATTGCCG-3'	
mAggrecan		
	F: 5'-CCAAGTTCCAGGGTCACTGTTACCG-3'	270
	R: 5'-TCCTCTCCGGTGGCAAAGAAGTTG-3'	
mβ-Actin		
	F: 5'-AGGCTGTGCTGTCCCTGTATGC-3'	373
	R: 5-ACCCAAGAAGGAAGGCTGGAAA-3	

Reporter assays

NIH3T3 cells were cotransfected with 0.5 μg pGL3ti or pGL3ti-SOX9 BS vector and 1 μg hSOX9 expression vector by using lipofectamine plus transfection reagent (Invitrogen) in 6-well tissue culture dish. The luciferase activity was normalized to the cotransfected internal control plasmid pcDNA3.1/hygro/LacZ (Invitrogen). Cells were harvested 48 h after transfection and luciferase activities were measured by using firefly luciferase assay system (Promega). Transfection and reporter assay experiments were carried out in duplicates and independently replicated at least twice.

Results

Establishment of stable cell lines overexpressing SOX9 in mouse ES cells

It has been reported that the hEF1α promoter robustly

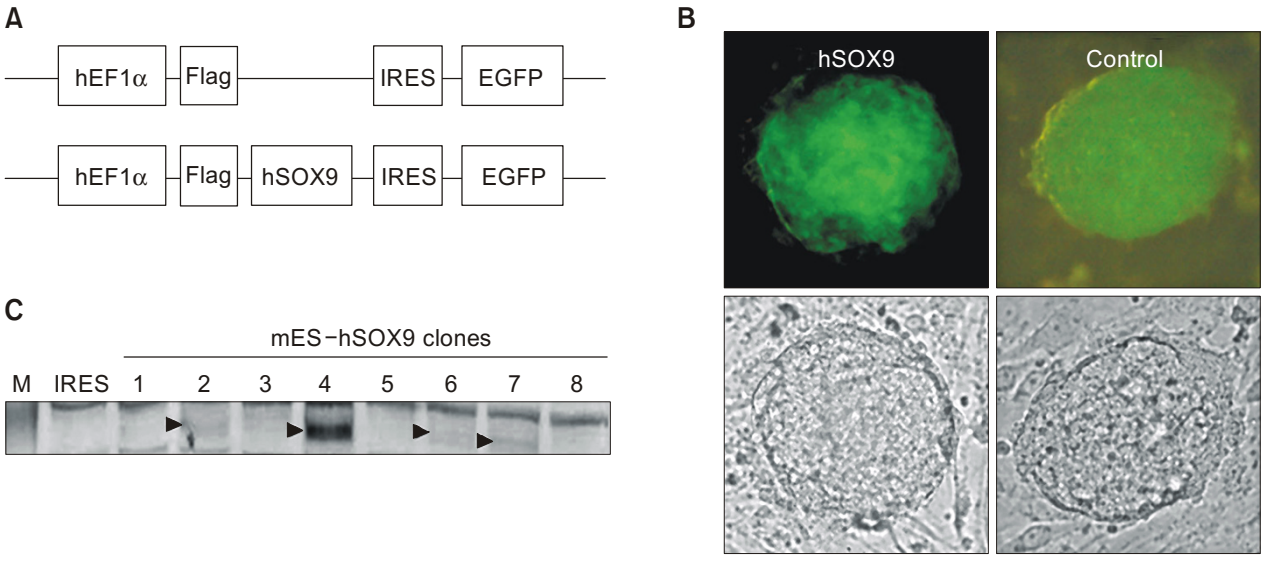

Figure 1. Establishment of stable cell lines overexpressing hSOX9 in mouse ES cells, (A) Vector constructs hSOX9 cDNA was inserted to pIRES2-EGFP and Flag epitope tag was added to the N-terminus of the hSOX9 open reading frame, followed by the modification of promoter system with hEF1α (B) Neomycin-resistant clones were selected with G418 treatment and fluorescent microscopic observation of enhanced green fluorescence protein (EGFP) expression; (C) Western blot analysis of mouse ES cells expressing hSOX9 by using anti-Flag antibody. The arrowheads indicate hSOX9 detected with anti-Flag antibody and only 4 out of 8 clones expressed hSOX9 proteins.

drove the reporter activity while the CMV promoter was inactive in undifferentiated mES cells (Chung *et al.,* 2002). In this study, hSOX9 cDNA was cloned into a bicistronic expression vector pIRES2-EGFP under the control of hEF1α promoter in order to induce hSOX9 expression effectively and for efficient selection. To enhance chondrogenic differentiation, the cloned hSOX9 expression vector was delivered into mES cells by electroporation with a single pulse of 270 V/500 μF. The hSOX9-containing clones were selected with 400 μg/ml G418 treatment and fluorescence microscopic observation (Figure 1A and 1B). Each independent clone was carefully detached by a pasteur glass pippet under a dissect microscopy and propagated for the further analysis. The expression of hSOX9 proteins was confirmed by Western blot analysis with anti-Flag antibody (Figure 1C). Despite of dual selection of SOX9-overexpressing clones by G418 treatment and fluorescence observation, only 4 out of 8 clones expressed hSOX9 proteins. We chose two clones with differential expression level of hSOX9 for further experiments.

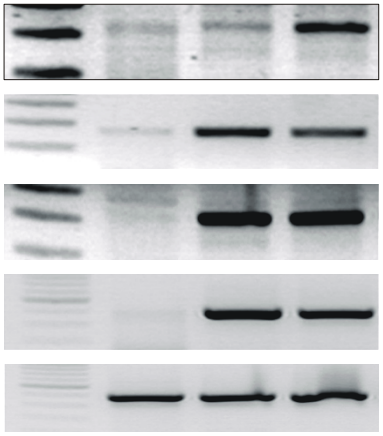

Figure 2. Expression analysis of SOX9 mRNA and chondrogenic marker genes in mES-IRES and mES-hSOX9 cells by RT-PCR.

Expression of chondrogenic marker genes in mES-hSOX9 cells

First of all, we reconfirmed the strong expression of hSOX9 mRNAs in hSOX9-overexpressing mES (designated as mES-hSOX9) cells by RT-PCR analysis (Figure 2). Thereafter, to examine the immediate effect of exogenous SOX9 overexpression on chondrogenesis, we analysed the representative chondrogenic markers by RT-PCR (Figure 2). The transcripts of aggrecan, collagen IIA, and Pax1 were strongly expressed in mES-hSOX9 cells, compared to the control (designated as mES-IRES) cells. However, faint expression of SOX9 transcripts was also observed in mES-IRES due to the cross-reactivity of SOX9 primers between human and mouse.

Exogenous SOX9 does not alter cell cycle pattern in the undifferentiated mES cells

We examined whether the delivery of exogenous SOX9 and the expression of chondrogenic marker genes might affect the changes in cell cycle pattern of undifferentiated mES cells. Flow cytometric analysis revealed that the mES-hSOX9 cells did not undergo the changes in cell cycle distribution, compared to mES-IRES cells (Figure 3). However mild increase of G1 (~3-5%, respectively) was observed in mES-hSOX9 clones.

Collagen IIB, an adult form was expressed during embryoid body formation

When we examined the immediate effect of exogenous SOX9 expression on chondrogenesis, relatively higher expression level of juvenile form, collagen IIA, was observed in mES-hSOX9 cells, compared to

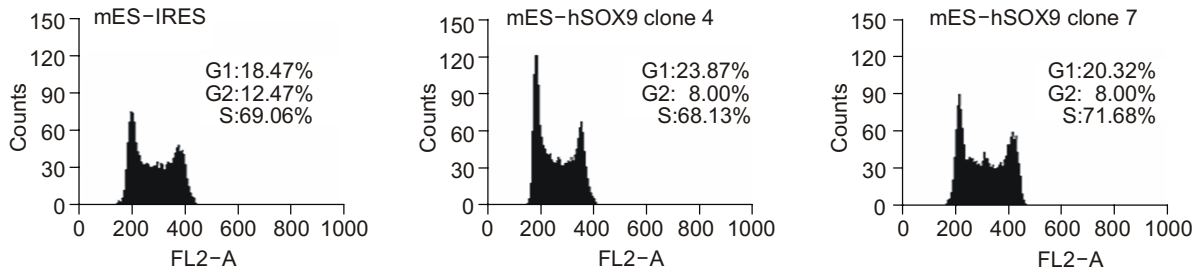

Figure 3. Cell cycle analysis of mES-IRES and mES-hSOX9 cells by flow cytometry. Cells were cultured for 2 days in complete mES growing media and analysed for cell cycle distribution. The percentage of cells in the G1, S and G2 phases of the cell cycle were calculated from three independent experiments, using the ModFit LT software.

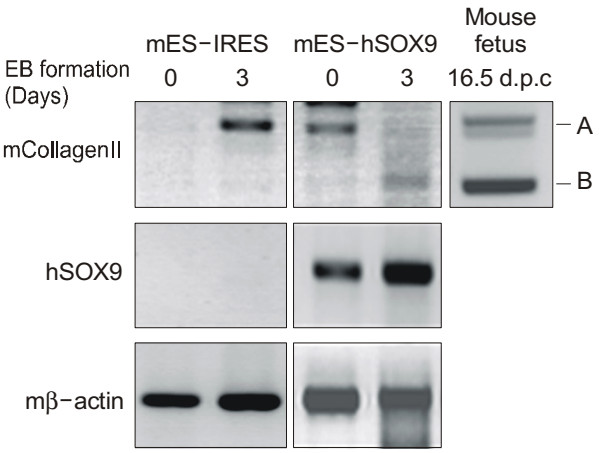

Figure 4. Expression analysis of collagen II in mES-IRES and -hSOX9 cells during embryoid body formation by RT-PCR. The forms of collagen II were indicated with an arrowhead (type A, a juvenile form) and an arrow (type B, an adult form).

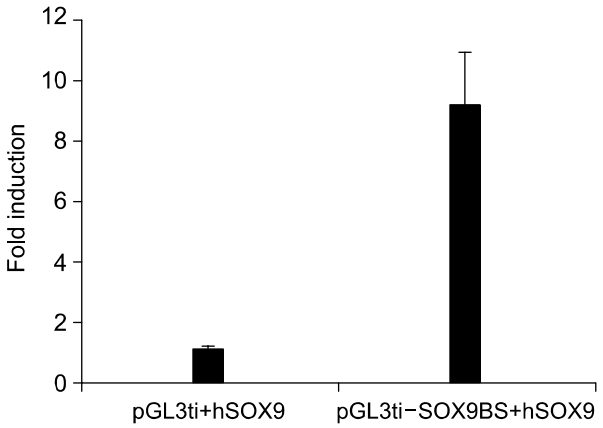

Figure 5. SOX9-mediated stimulation of SOX9 binding site-driven transcription. NIH3T3 cells were cotransfected with 1 μg hSOX9 expression vector and 0.5 μg reporter plasmid containing 48-bp SOX9 binding site (pGL3ti-SOX9 BS) or no SOX9 binding site (pGL3ti). The data shown are the means and SD of duplicate measurements obtained from one representative transfection. This experiment was performed at least twice in duplicate.

mES-IRES cells. However, the expression of adult form, collagen IIB was not detected in either of them (Figure 2). To examine whether overexpression of SOX9 could further induce the expression of collagen IIB, an adult form important for functional chondrocyte differentiation, we induced embryoid body formation of the transfected mES cells. RT-PCR analysis revealed that the expression of collagen IIA was significantly decreased and collagen IIB was detected in as early as day 3 embryoid bodies of mES-hSOX9 cells (Figure 4).

Exogenous SOX9 induces the transcriptional activity through binding to SOX9 binding site derived from mouse collagen II enhancer sequences

To verify whether the expression of collagen II could be induced by overexpression of SOX9, an SOX9 binding site-driven luciferase reporter construct was transiently cotransfected with hSOX9 expression vector in NIH3T3 cells. As a control, the reporter construct without the SOX9 binding site was used. Reporter assay revealed that cotransfection of hSOX9 induced higher transcriptional activity of the reporter containing SOX9 binding site (pGL3ti-SOX9 BS), compared to that of pGL3ti reporter being devoid of SOX9 binding site (Figure 5). This result suggests that overexpression of SOX9 may induce the expression of collagen II by binding to SOX9 binding site within the collagen II gene in mES cells.

Discussion

In this study, we presented that mES-hSOX9 cells grown on feeder cells could express chondrogenic marker genes such as collagen IIA, aggrecan, and Pax1 without exogenous differentiational stimulation. Moreover, the expression of exogenous SOX9 and chondrogenic markers did not affect the cell cycle pattern of undifferentiated mES cells. However, during differentiation process, collagen IIB, an adult form was detected in as early as day 3 embryoid bodies of mES-hSOX9 cells.

Extensive studies on *in vitro* chondrogenic differentiations and the molecular mechanisms have been performed in mesenchymal cells (Ahrens *et al.,* 1993; Erlebacher *et al.,* 1995; Mackay *et al.,* 1998; Bai *et al.,* 2004; Ikeda *et al.,* 2004). Recently, it has been reported that even primary mouse embryonic fibroblasts can enter and complete the program of chondrogenic differentiation under the appropriate conditions (Lengner *et al.,* 2004). However, to date, mES cells are obviously good source for cartilage regeneration due to the characteristics of immortality and pluripotentials. mES cells undergo chondrogenic differentiation by cytokine stimulation or different culture conditions (Kramer *et al.,* 2000; Hegert *et al.,* 2002; Tanaka *et al.,* 2004) under a long-term culture system. However, the prolonged differentiation systems would delay the treatment to patients or could alter the immunogenicity of cultured autologous cells, ultimately leading to immunorejection upon transplantation (Hodgetts *et al.,* 2000; Smythe *et al.,* 2000; Heng *et al.,* 2004). To enhance the chondrogenic differentiation through genetic modification, we successfully established stable cell lines constitutively expressing exogenous SOX9 in mES cells. The ex-

pression of exogenous SOX9 was confirmed by western blot analysis with anti-Flag antibody (Figure 1C).

SOX9 is the member of the SOX (Sry-type HMG box) family and predominantly expressed during mesenchymal condensation and cartilage formation in mouse embryos (Wright *et al.,* 1995). In addition, SOX9 is known to be a key regulator for the expression of collagen II, IX, XI, and aggrecan, the major matrix proteins specific for chondrocytes through binding to the enhancer region (Bell *et al.,* 1997; Lefebvre *et al.,* 1997; Bridgewater *et al.,* 1998; Sekiya *et al.,* 2000; Bernard *et al.,* 2003). SOX9 has been also reported to be required for chondrogenesis by the generation of SOX9-mutant ES cells and chimeric mice (Bi *et al.,* 1999). SOX9-mutant cells showed the different expression patterns of cartilage-specific genes upon embryonic development stage, indicating that SOX9 functions as an early marker to activate the genes during cartilage formation. The establishment of mES-hSOX9 cell lines may provide a good source to study on molecular mechanism of chondrogenesis and ultimately to regenerate functional chondrocytes for clinical applications. In this study, the higher expression of cartilage-specific marker genes, collagen IIA and aggrecan was detected in mES-hSOX9, compared with mES-IRES cells (Figure 2). However, the expression of collagen IIA was detected at relatively basal levels, suggesting other cofactors and optimal differentiation culture conditions may be needed to activate the gene sufficiently. In fact, SOX9 interacts with other cofactors, such as L-Sox5 and Sox6 for dimerization and subsequently activates cartilage-specific marker genes more effectively (Bernard *et al.,* 2003; Ikeda *et al.,* 2004).

mES cells exhibit the very unique distribution of cell cycle: 15% in G1, 75% in S, and 10% in G2/M, respectively, supporting their infinite proliferation potentials *in vitro* without differentiation under LIF signals (Savatier *et al.,* 2002). Our data revealed the similar cell cycle pattern in mES-IRES and -SOX9 cells to the undifferentiated mES cells (Figure 3), suggesting the unique cell cycle distribution of undifferentiated mES cells might be not interfered by the integration of exogenous SOX9 gene as well as drug selection process and long-term culture even if the mild increase of G1 was observed in mES-hSOX9 cells (3-5%, respectively), compared to mES-IRES cells (Figure 3). Moreover, mES-hSOX9 cells might not only have more directed and specified differentiation potentials into chondrocytes expressing chondrocyte-specific markers, but also still may have the growth capacities despite of genetic modification through exogenous gene delivery. However, further investigation is needed to understand the molecular and signaling mechanisms of genetically modified mES cells.

It has been reported that the entire nucleotide sequences of mouse collagen II gene were determined by restriction mapping and the two forms of collagen II (A and B) were generated by alternative splicing process (Metsaranta *et al.,* 1991). Collagen IIA is broadly expressed in embryonic or nonchondrogenic tissues as well as prechondrocytes and mesenchymal cells. The expression of collagen IIB, an isoform lacking a 69 amino acid cysteine-rich domain encoded by exon 2 is specifically localized in differentiating and adult chondrocytes, indicating the differentiation into functional chondrocytes (Ng *et al.,* 1993; Sandell *et al.,* 1994; Valcourt *et al.,* 2003). Moreover, the high expression level of total collagen II and an isoform IIB in a primary culture of chondrocytes dramatically decreased within a few days followed by the first passage, whereas the expression level of collagen IIA relatively increased (Valcout *et al.,* 2003). mES cells has been reported to undergo chondrogenic differentiation and express collagen IIA in as early as day 5 embryoid body. However, the expression of collagen IIB was detected as early as 14 days after replating day 5 embryoid body (Tanaka *et al.,* 2004). Our study for the first time showed that the expression of collagen IIB was detected as early as day 3 since forming the embryoid bodies of mES-hSOX9 cells, suggesting the successful shift from collagen IIA to IIB gene expression might indicate functional chondrocyte differentiation (Figure 4).

Forty eight bp enhancer sequences in the first intron of mouse collagen II have been shown to strongly increase the promoter activity and minimal 18-bp enhancer sequences within the 48 bp were sufficient to direct cartilage-specific expression (Lefebvre *et al.,* 1996). In addition, it has been reported that the collagen II expression was closely related to SOX9 expression and the minimal enhancer sequences directly bound to the HMG domain binding site of SOX9 (Lefebvre *et al.,* 1997). Our study also confirmed that hSOX9 expression vector sufficiently drove the reporter promoter activity containing mouse collagen II 48-bp enhancer sequences (Figure 5), supporting the expression of collagen II is stimulated by exogenous SOX9 expression in mES cells.

In conclusion, we for the first time established the stable cell lines constitutively overexpressing SOX9 and could observe the expression of chondrocyte differentiation marker genes in mES-hSOX9 cells without specified exogenous stimulation such as growth factors or differentiation culture conditions. However, other cofactors and optimal differentiation culture conditions may be needed to induce sufficient amount of collagen II expression and subsequent chondrogenic differentiation. Further investigation is also required to understand the molecular and signaling

mechanisms regarding of specified differentiation through genetic modification in pluripotent cells.

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