Repressing of SOX6 and SOX9 in Situ Chondrogenic Differentiation of Rat Bone Marrow Stromal Cells

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Article info:
Received: 27 Dec. 2014
Accepted: 06 Mar. 2015

Key Words:
SOX6, SOX9, Bone marrow stromal cells, Differentiation

ABSTRACT

Introduction: SOX9 is a transcriptional activator which is necessary for chondrogenesis. SOX6 are closely related to DNA-binding proteins that critically enhance its function. Therefore, to carry out the growth plate chondrocyte differentiation program, SOX9 and SOX6 collaborate genome-wide. Chondrocyte differentiation is also known to be promoted by glucocorticoids through unknown molecular mechanisms.

Methods: We investigated the effects of asynthetic glucocorticoid, dexamethasone (DEX), on SOX9 gene expression in chondrocytes.

Results: SOX9 mRNA was expressed at high levels in these chondrocytes. Treatment with DEX resulted in enhancement of SOX9 mRNA expression. The DEX effect was dose dependent (0·5 nM and 1 nM).

Conclusion: RT-PCR analysis revealed that DEX also enhanced the levels of SOX9 expression. It was observed that DEX had enhancing effect only on SOX9; the expression level was low for SOX6. It can thus be concluded that chondrocyte differentiation can be promoted by DEX via SOX9 enhancement.

1. Introduction

The genesis of the chondrocyte lineage during evolution had profound contribution to the advent of the vertebrate phylum. During vertebrate embryogenesis, chondrocytes build up hundreds of cartilaginous anlagen. These structures serve as morphological and mechanical body support, and encourage body growth by their unique potentials to elongate fast. Cartilage is either preserved during lifetime in articular joints and airways or is remodeled progressively during fetal and postnatal development in order to develop most craniofacial, axial, and appendicular bones [1–3]. Human cartilage disorders present themselves in a variety of forms. Chondrodysplasias can affect skeleton pattern-

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ing, growth, and ossification [4]. The sequence CCTTGAG together with the other members of the HMG-box class DNA-binding proteins is recognized by SOX9. It acts during chondrocyte differentiation and regulates transcription of the anti-Müllerian hormone (AMH) gene with steroidogenic factor 1 [2]. SOX9 has a fundamental role in male sexual development, as well; by working with Sf1, it can produce AMH in Sertoli cells so as to prevent creating a female reproductive system [3]. Moreover, SOX9 interacts with a few other genes to promote the development of male sexual organs. It also activates FGF9 and forms feed forward loops via FGF9 [5] and PGD2 [4]. Complex molecular networks regulate the lineage commitment, differentiation status, and activity of chondrocytes. Ranking high in these networks is a factor of three transcription factors including SOX5, SOX6, and SOX9. SOX5 and SOX6, in the SOX family, are members of the SOXD group [5].

SOX9, as a SOXE group member, has only half the identity similarity with SOX5 and SOX6 in the SOX domain and possesses a various type of homodimerization domain [6]. Moreover, contrary to SOX5 and SOX6, SOX9 carries a potent transactivation domain. Chondrogenesis occurs only after SOX9 expression in mesenchymal progenitors and the latter increases when SOX5 and SOX6 expression begin in early chondrocytes [7]. The three SOX genes expression increases in growth plate and prehypertrophic chondrocytes, and is rapidly turned off when chondrocytes undergo hypertrophy. SOX9 is an essential element for chondrogenesis. The significance of SOX9 was not understood until it was observed that heterozygous mutations within and around its gene cause campomelic dysplasia, i.e. a human syndrome with severely malformed cartilage [8]. It is observed that conditional inactivation of SOX9 in mouse embryo mesenchymal progenitors prevents precartilaginous condensation while inactivation at the onset of chondrocyte differentiation deters overt chondrogenesis [9].

Inactivated SOX9 in fully differentiated chondrocytes stimulates growth plate and articular cartilage failure [10]. Unlike SOX9, chondrogenesis does not need SOX5 and SOX6. Yet, for efficient chondrogenesis, these cells lines are required. Global inactivation of either SOX5 or SOX6 results in mild defects in skeletogenesis [11]. So far, however, SOX5 and SOX6 have not been studied in chondrocytes. The SOX trio proteins, besides transactivating genes, are suggested as directly repressing genes in chondrocytes and other cell types. For instance, it is observed that SOX9 might collaborate with GLI factors in order to repress hypertrophic chondrocyte markers. SOX5 and/or SOX6 compete with SOX9 for DNA binding and thus block oligodendrocyte and melanocyte differentiation [12]. It was also revealed that SOX6 represses specific gene sets in erythroid cells and in skeletal myoblasts, as well [13]. The present study was an attempt to repressing SOX6 and SOX9 in situ chondrogenic differentiation of rat bone marrow stromal cells by DEX.

2. Materials and Methods

Bone marrow stromal cell extraction and culturing

Vistar female rats, weighing 200-250 g (Razi Institute, Karaj, Iran), were kept under a controlled light/dark cycle (lights on at 6 a.m. and off at 6 p.m.) at a temperature of 18-25°C. All the animal studies were carried out in line with the principles and procedures approved by the Ethical Committee of the School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The bone marrow was extracted from the rats' long bones and cultured in DMEM/F12 (Stem Cell Technology Company, Tehran, Iran), supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM/ml L-glutamine incubated in a humidified incubator with 5% CO_2 at 37°C. Next, the cells were immunostained for fibronectin (Fn) and CD106. The stemness genes (Oct-4) were evaluated via RT-PCR.

Osteogenic and adipogenic differentiation

The BMSCs were differentiated into osteogenic and lipogenic phenotypes in vitro by means of induction media according to Abbaszadeh et al. 2014 [14]. After 3 weeks of culturing, Alizarin red stain was used to stain the bone ossicle. The adipogenic differentiation was carried out via incubating the cells in the differentiation medium for 3 weeks and then staining them with oil red stain.

Chondrogenic differentiation

When the cultures were close to confluence, the cells were detached via treatment with trypsin and EDTA and rinsed three times using serum-free complete chondrogenic medium. A total of 200'000 cells were put in a 15-ml conical polypropylene tube, centrifuged at 500g for 5 min at 20°C, and suspended in serum-free CCM consisting of 1 mM sodium pyruvate, 0.1 mM ascorbic acid-2-phosphate, 0.5 and 1 nM dexamethasone, 1% ITS+(Collaborative Biomedical Products), and 10 ng/ml TGF-β1 dissolved in DMEM-LG. The cells were

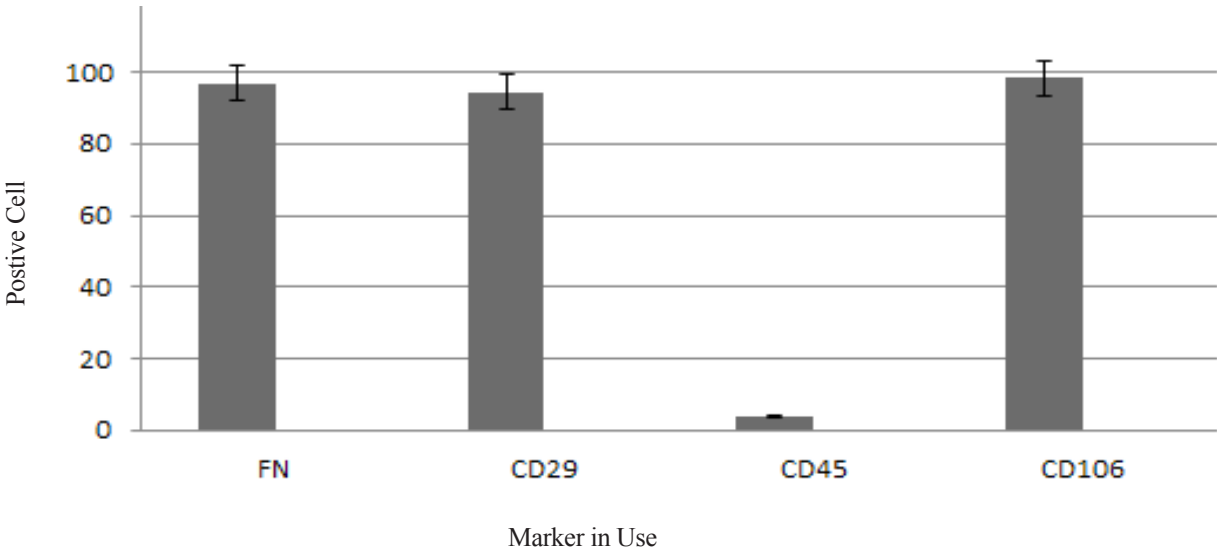

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Figure 1. The immunocytochemistry representation of different cell markers. After treating bone marrow stromal cells with Dexamethasone at induction stage, the cells were labeled with primary antibodies, followed by incubation of FITC-conjugated.

then recentrifuged and later maintained in culture for up to 35 days with one pellet/tube and 0.5 ml CCM/tube. The medium was changed every 2–3 days.

MTT Assay

BMSCs cells were cultured in 96-well plates. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and then incubated for 4 h. Next, supernatant was removed and dark blue crystals of formazan were dissolved in dimethyl sulfoxide. Absorption of the suspension was read at 630 nm and the measurements were reported as percentage of control.

Immunocytochemical method

The cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. Cells were blocked by 5% bovine serum albumin for 30 min after permeabilization. Immunostaining was carried out on BMSC, pre-induced, and induced cells. Mouse anti-fibronectin monoclonal antibody (1:100), mouse anti-CD45 monoclonal antibody (1:100), and mouse anti-CD90 monoclonal

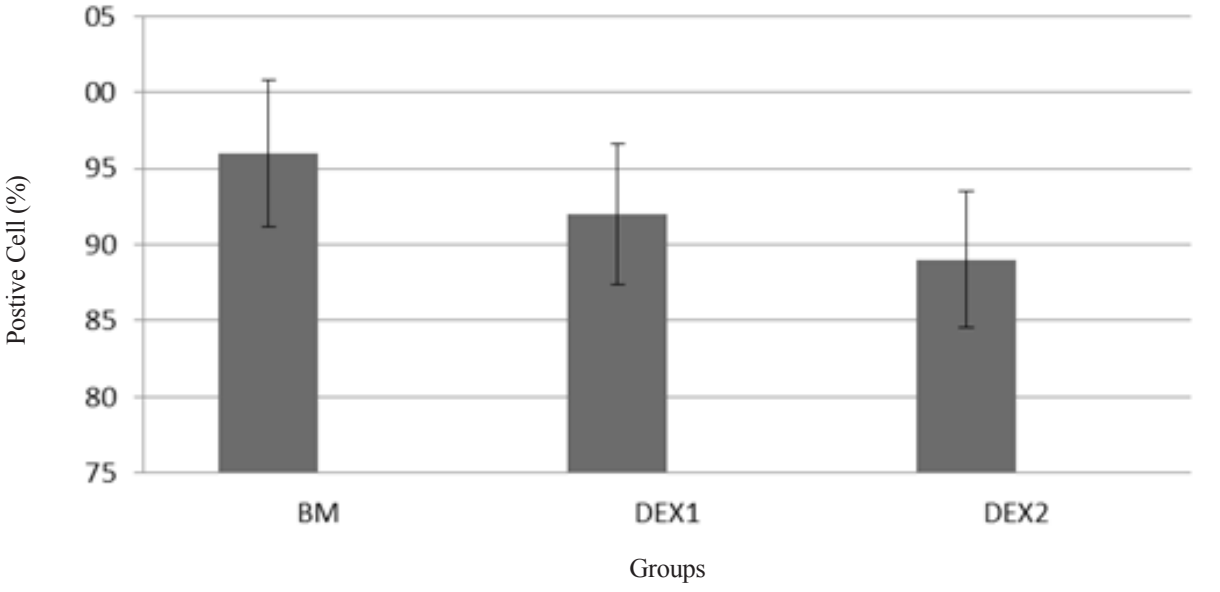

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Figure 2. Cell viability measured by MTT assay for BMSC treated by dexamethasone 0.5 nM (DEX1) and 1 nM (DEX2) was observed to be statistically lower as compared with untreated BMSC.

antibody (1:200) specific markers were implemented for mesenchymal stem cells. Next, the cells were incubated via FITC conjugated rabbit anti-mouse secondary antibody (1:100, EMD Millipore Corporation, Billerica, MA and USA) for 2 hours at room temperature. The cells were counterstained with 1:10,000 ethidium bromide (Sigma-Aldrich, St. Luis, MO, USA) for 1 min [15].

RT-PCR

The BMSC at the end of the fourth passage and chondrogenic cells were evaluated for the expression of SOX2, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), SOX9, and SOX6 genes. The RNX-Plus Kit (Hybrid-r miRNA) was used to treat 2 µg of the total RNA from each sample via DNase I (Thermo, Maryland, USA). To evaluate the purity and integrity of the extracted RNA, optical density measurements and electrophoresis on 1% agarose gel were used. Extracted RNA (1 µg) was converted to cDNA via the First Strand cDNA Synthesis Kit (Thermo, INC, Maryland and USA). A total of 50 ng of cDNA was added to the PCR reaction for 35 cycles with denaturation at 95°C for 45 seconds, annealing at 58°C for 45 seconds, and then elongation at 72°C for 30 seconds. When amplification was completed, the products were separated on 2% agarose gel and visualized making use of ethidium bromide under UV light. To make sure about the reproducibility, each experiment was repeated at least 3 times.

Primer sequences (forward and reverse), the size of the product, and PCR conditions were as follows: expression of rat SOX2 gene (a marker for stemness) was carried out via the 5′ AAGCTGCTGAAACAGAAGAGG 3′ SOX2 forward primer and the 5′ACACGGTTCTCAATGCTAGTC3′, forward and backward primers, (annealing at 62ºC). GAPDH has served as an internal control gene: 5′ CCACAACTC TTCCATTCT C 3′ and 5′ CCAAGATTCACGGTAGATAC 3′, forward and backward primers, respectively (400 bp, accession number: NP_002037.2, annealing at 58ºC), SOX9 has served as a chondrogenic gene: 5′ CCACAACTC TTCCATTCT C 3′ and 5′ CCAAGATTCACGGTAGATAC 3′, forward and backward primers, respectively (annealing at 55ºC), and SOX6 has served as an chondrogenic gene: 5′ CGACAAGCCTGCTACTCTC 3′ and 5′ CCTACAGTCCCAGTAGATAC 3′, forward and backward primers, respectively (annealing at 65ºC).

Statistical analysis: To carry out the statistical analysis, SPSS, version 16.0, was used. To be accurate, statistical comparison of the multiple means in the groups and one-way ANOVA followed by Tukey's post hoc test were run. The data in the histogram were presented as mean±SEM. Values of $P<0.05$ were considered statistically significant.

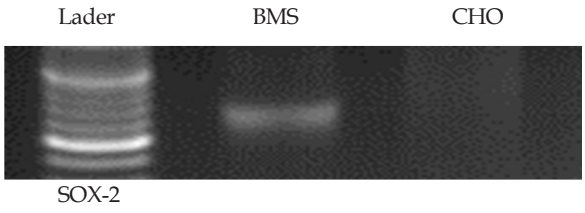

Figure 3a. SOX2 (190 bp) was expressed in BMS and was not expressed in CHO using RT-PCR. BMS: Bone Marrow Stromal Cells. CHO: Chondrogenic Cells.

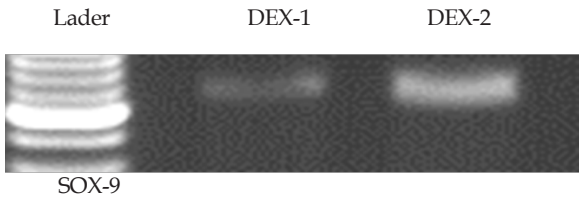

Figure 3b. The electrophorogram illustrates that SOX9 was expressed in both groups using RT-PCR. The expression was found to be higher in DEX2.

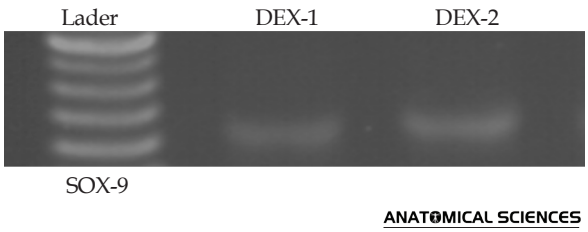

Figure 3c. This electrophorogram demonstrates that SOX6 was expressed identically in both DEX1 and DEX2 using RT-PCR.

3. Results

Immunocytochemistry

The immunocytochemistry pattern of the third passage isolated rat BMSCs revealed high purity of the cells. Immunoreactivity of CD29, CD45, CD106, and fibronectin markers were 98.3±0.9%, 4±0.1%, 94.5±0.3%, 96.8±0.4%, respectively (Figure 1).

MTT Assay

The viability of BMSC treated with dexamethasone 0.5 nM (92.36±4.82%), 1 nM (87.86±9.63) was found to be significantly lower comparing with that of the untreated BMSC (96.84±2.36%).

RT-PCR

The expression of SOX2 was not detected in condrogenic cells, whereas untreated BMSC expressed this gene (Figure 3a). Also, RT-PCR analysis revealed that DEX enhanced the

levels of SOX9 expression (Figure 3b). SOX6 was expressed in both groups of DEX1 and DEX 2, but the expression level was similarly low in the two groups (Figure 3c).

4. Discussion

The chondrogenic differentiation of MSCs taken from adult bone marrow involves rapid biosynthesis of glycosaminoglycan and deposition of an integrated extracellular matrix along with a dramatic alteration in cell morphology. Many significant factors influence this process, including a three dimensional culture format [13], low oxygen tension, and the presence of appropriate growth factors. This response can be triggered by three isoforms of TGF-β, and under the conditions of culture described here, the initial appearance of mRNA coding for cartilage matrix components becomes evident within 24 h. Compared with TGF-β 1, both TGF-β2 and TGF-β3 are more effective in promoting chondrogenesis, causing a two-fold greater accumulation of glycosaminoglycan and earlier and more extensive deposition of type II collagen. A promising strategy for cartilaginous pathologies is gene-enhanced tissue engineering cartilage. Yet, what makes the problem is the difficulty in acquiring seed cells with sufficient biological activity [13].

SOX family was originally identified in Sry proteins, the male sex-determination transcription factor, a gene localized on the Y chromosome. It includes 12 groups, and belongs to group E, together with SOX8, SOX10, and SOX9 while SOX5, SOX6, and Sox13 belong to group D (15). The role of SOX transcription factors is that of architecture organizers. SOX5, SOX6, and SOX9, known as SOX trio, working together during the chondrogenesis [16]. According to Venkatesan et al. [17], SOX9 gene transfer via replication-defective recombinant adeno-associated virus (rAAV) vectors can trigger human MSCs chondrogenic differentiation and reduce the expression of osteogenic differentiation markers for 21 days. Also, Cucchiarini et al. [18] used rAAV, as a gene transfer tool, to show a process of cartilage defect repair in rabbits' knee joints. Cao, L et al. [19] revealed that implantation of SOX9 modifying MSCs in a polyglycolic acid (PGA) scaffold led to better repair of knee osteochondral defect in rabbit via recombinant adenovirus mediated gene transfer. Yet, it is also reported that, to induce MSCs chondrogenic differentiation, SOX9 alone is not enough and it requires other growth factors, including SOX5, SOX6, IGF1, FGF or TGF-β [20].

Articular cartilage is an avascular, aneural tissue and lacks lymphatic drainage, which is composed of chondrocytes and cartilage matrix. It is necessary for cartilage tissue engineering to preserve the hyaline cartilage phenotype. However, chondrogenesis and endochondral ossification are tightly coupled and work closely together during bone and cartilage formation [21]. When endochondral ossification is activated, maintenance of the hyaline cartilage phenotype fails. The TGF-β, BMPs, and FGFs have been reported to have the ability to direct MSCs towards the chondrocyte lineage.

Nonetheless, these growth factors lead to undesirable endochondral ossification or ectopic ossification [22]. As a master transcription factor for chondrogenesis, SOX9 too delays BMP2-induced bone formation of MSCs and endochondral ossification through repressing Runx2 expression, and thus plays a significant role in cartilage formation and cartilaginous pathologies healing [23].

The findings of the current study confirm that SOX9 mediated inhibition of osteogenic differentiation plays an important role in BMP2-induced cartilage formation and retains hyaline cartilage phenotype. In assays of chondrogenic differentiation in 14-day cell aggregate cultures, the FGF-2-treated hMSCs already contained higher expression of SOX9 and SOX5 prior to the assay. The expression of these genes and the other transcription factor SOX6 increased throughout culture and was higher than that in non-FGF-2-treated hMSCs. Gene expression of COL2A1 and COLXA1 also increased throughout the culture and there was an increase in glycosaminoglycan accumulation and the histological evidence of collagen type II and collagen type X protein deposition. Cellular proliferation showed a main increase in DNA during the first 7 days of culture, prior to the major weight gain, which clearly occurred through deposition of cartilage-specific ECM proteins and proteoglycans and the retention of water [24].

The accumulation of a glycosaminoglycan-rich ECM and up-regulation of the chondrocyte-specific ECM genes COL2A1, COLXA1, and aggrecan chiefly occurred from day 7 onwards. The thousand-fold changes in COL2A1 and COLXA1 gene expression were substantially greater than those in aggrecan expression, which consistently showed only small changes. Increased expression of these genes, as well as that of minor cartilage proteins involved in regulating the macromolecular assemblies within the ECM, including cartilage oligomeric protein (COMP), decorin and fibromodulin, are reported to increase from day 3 and 8 of chondrogenic pellet culture [25].

Immunolocalization data have also shown the initial deposition of aggrecan, link protein, COMP, decorin, biglycan, KS, and chondroitin-4-sulphate from between day 3 and 5 of Chondrogenic pellet culture [26]. Radiolabelled sulphate incorporation, which is a measure of glycosaminoglycan biosynthesis, was also reported to be the greatest from day 5

onwards [27]. All these findings prove that signaling events are likely during the first few days of the culture previous to a cartilage ECM deposition and organization.

It was found that DEX enhanced the levels of SOX9 expression. Moreover, the enhancing effect of DEX was witnessed to be specific to SOX9; DEX did not alter the levels of SOX6 mRNA expression. Data obtained in the present study suggest that, through enhancement of SOX9, DEX promotes chondrocyte differentiation.

Acknowledgement

The present project was sponsored by Shahid Beheshti University of Medical Sciences (Grant#94-N-6102), Tehran, Iran.

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