Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
C M B Association Publisher

Original Research

Effect of SOX2 on osteogenic differentiation of dental pulp stem cells

J. Yuan[1,4], X. Liu[2], Y. Chen[2], Y. Zhao[2], P. Liu[2], L. Zhao[1,4*], W. Han[3*]

[1] Department of Anesthesiology, Second Clinical Medical College of Jinan University, Shenzhen People's Hospital, Shenzhen, Guangdong, 518020, China
[2] Department of Regenerative Medicine, School of Pharmaceutical Science, Jilin University, Changchun, Jilin, 130021, China
[3] Department of Pain, The Fourth People's Hospital, Shenzhen, Guangdong, 518020, China
[4] Shenzhen Anesthesiology Engineering Center, Shenzhen, Guangdong, 518020, China

Correspondence to: leizhao16@sina.com or lulong125@cntv.cn

Received June 21, 2016; **Accepted** January 25, 2017; **Published** January 30, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.1.8

Abstract: To explore the effect of SOX2 on osteogenic differentiation of dental pulp stem cells (DPSCs) and to develop a new method for facilitating osteogenic differentiation of DPSCs. SOX2-overexpressing human DPSCs (DPSCs-SOX2) were established through retrovirus infection. Differentiation was induced with osteogenic differentiation medium and further evaluated by alkaline phosphatase (ALP) staining and qPCR. Following the differentiation procedure, the mechanism of SOX2 on osteogenic differentiation of DPSCs was analyzed using genome RNA microarray and flow cytometry. The normal DPSCs, DPSCs-vector, and DPSCs-SOX2 were capable of osteogenic differentiation after 3-4 weeks of introduction and positive for ALP staining. Compared to DPSCs and DPSCs-vector, DPSCs-SOX2 exhibited higher levels of osteogenic gene expression (ALP, collagen I, Runx2, and osterix), indicating the superiority of DPSCs-SOX2 in osteogenic differentiation. Moreover, SOX2 overexpression resulted in the activation of the Hippo signal pathway and increased expressions of the BMPs family. SOX2 promotes the osteogenic differentiation of DPSCs by regulating the osteogenic genes and BMPs family, suggesting the therapeutic potential in bone repair.

Key words: Dental pulp stem cells; SOX2; Osteogenic differentiation; BMP.

Introduction

SOX2 is an important transcription regulator that plays a role in maintaining the multipotency of stem cells (1-4). SOX2 is also required for neuronal differentiation and maintains normal functioning of neural precursor cells. According to recent research, SOX2 can bind to several transcription factors, thus transforming adult cells back to neural cells. This SOX2 function offers a new pathway in the development of cell therapy for neurologic diseases (5, 6).

Dental pulp stem cells (DPSCs) are undifferentiated mesenchymal cells located in dental pulp. The isolation and establishment of DPSCs are important for providing a new source of cells for research on adult stem cells. Indeed, shed or extracted teeth are considered an abundant source of DPSCs for use in the treatment of diseases. DPSCs exhibit proliferative and differentiation capacities *in vitro* and maintain neural differentiation and odontoblast differentiation. Therefore, DPSCs have been used to treat bone injuries (7-10). Our preliminary study showed that overexpressed SOX2 promotes the proliferation, migration, and adhesion of DPSCs (11). Whether or not SOX2 has an effect on osteogenic differentiation of DPSCs is unknown. This study aimed to explore the role of SOX2 on the osteogenic differentiation of DPSCs.

Materials and Methods

Reagents and equipment

DMEM/F12 medium and fetal bovine serum (FBS) were purchased from Gibco (USA). β-GP, dexamethasone, and vitamin C (Vc) were purchased from Sigma (St. Louis, MO, USA). An alkaline phosphatase (ALP) staining kit was purchased from Beyotime Institute of Biotechnology. Trizol RNA extraction buffer was purchased from Invitrogen (USA). RNA reverse transcription and real-time qPCR kits were purchased from TaKaRa (Japan). All primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Mouse anti-human BMP6 monoclonal antibody was purchased from Santa (USA). Goat anti-mouse IgG-FITC polyclonal antibody was purchased from Invitrogen. A human genome microarray was purchased from Agilent (USA).

An IX-70 inverted phase-contrast microscope was purchased from Olympus (Japan). A PCR instrument was purchased from Eastwin Life Sciences, Inc. A CFX96 real-time PCR detection system was purchased from BIO-RAD (USA). A FACS Calibur Flow Cytometry System was purchased from BD (USA).

Experiments

Isolation and identification of DPSCs and establishment of SOX2-overexpressing DPSCs

Isolation and identification of DPSCs were performed according to the extant literature (11). Dental pulp

was collected from human third molars and crushed before twice-washing with PBS. Then, DPSCs were digested with 3 mg/mL of type I collagenase at 37°C for 1h and filtered through a 70-mm membrane to collect single cells. The cells were twice-washed with PBS and cultured with DMEM/F12 containing 10% FBS. Then, the first generation cells were harvested and cultured. The medium was replaced every 2 days for 4-6 days before passage and third or fourth generation cells were used for subsequent experiments. The levels of expression of DPSC markers (CD29, CD44, and CD73) were detected by flow cytometry. Multi-potency of DPSCs was evaluated by adipogenic and osteogenic differentiation tests

The pMX-SOX2 overexpressing vector was provided by Bioengineering Laboratory (College of Pharmacy, Jilin University). The retrovirus was prepared using 293T cells as the packaging cells. SOX2-overexpressing DPSCs (DPSCs-SOX2) were prepared by retroviral infection, while DPSCs transfected with empty vector (DPSCs-vector) served as a control, as previously described (11).

Induction and evaluation of osteogenic differentiation of DPSCs

DPSCs in the log phase were harvested and inoculated in 24-well plates at a density of 2×10^5 cells/well. Differentiation was induced with osteogenic differentiation medium containing 10% FBS, 10^{-3} mM dexamethasone, 10 mM β-GP, and 50 mg/L Vc for 3-4 weeks.

After induction, the ALP kit was used for staining to evaluate osteogenic differentiation in each group. Expression of genes associated with osteogenic differentiation was detected by real-time quantitative PCR (qPCR). First total RNA extraction was performed using the Trizol method and genomic cDNA was obtained by reverse transcription. Using genomic cDNA as a template, qPCR was performed for ALP, collagen I, RunX2, and osterix genes. β-actin was used as an internal reference gene and normal DPSCs were used as a blank control group, with the levels of expression of genes in the blank control group set to 1. Thus, the expression of relevant genes was compared between the DPSCs-SOX2 and DPSCs-vector groups. The primers used for qPCR were as follows: ALP, forward (F) 5'-AAATACGAGATCCACCGAGACTCCA-3' and reverse (R) 5'-GATGCGACCACCCTCCACGAAG-3'; collagen I, forward (F) 5'-TGGTGGTTATGACTTTGGTTACGAT-3' and reverse (R) 5'-TGTGCGAGCTGGGTTCTTTCTA-3'; Runx2, forward (F) 5'-CCTCAGGCATGTCCCTCGGTAT-3' and reverse (R) 5'-TGGCTTCCATCAGCGTCAACAC-3'; osterix, forward (F) 5'-GCTCCTCCTGCGACTGCCCTAA-3' and reverse (R) 5'-GCTCATCCGAACGAGTGAACCTC-3'; and β-actin, forward (F) 5'-CCCAGAGCAAGAGAGG-3' and reverse (R) 5'-GTCCAGACGCAGGATG-3'.

Mechanism of SOX2 effect on the osteogenic differentiation of DPSCs

Genomic RNA was extracted from DPSCs and DPSCs-SOX2. Changes in the expression of genomic RNA were detected using a genome microarray. The mechanism by which SOX2 influenced osteogenic differentiation of DPSCs was discussed from the perspective of signaling pathways [These experiments were performed by Kang Chen Bio-tech, Inc. {city, country}. Additional details are described in the literature (11)]. The results of microarray analysis were uploaded to the Gene Expression Omnibus database (No.: GSE73548).

The results of microarray analysis were verified by performing qPCR and flow cytometry. The expression of genes in the Hippo signaling pathway were detected by qPCR according to Section 1.2.2. The primers used were as follows: BMP2, forward (F) 5'-ACTACCAGAAACGAGTGGGAA-3' and reverse (R) 5'-GCATCTGTTCTCGGAAAACCT-3'; BMP4, forward (F) 5'-TAGCAAGAGTGCCGTCATTCC-3' and reverse (R) 5'-GCGCTCAGGATACTCAAGACC-3'; and BMP6, forward (F) 5'-AGCGACACCACAAAGAGTTCA-3' and reverse (R) 5'-GCTGATGCTCCTGTAAGACTTGA-3'.

Flow cytometry was performed using the following steps. The cells were fixed, transparentized, and incubated with mouse anti-human BMP6 monoclonal antibodies (1:50 dilution) at room temperature for 30 min. Next, the cells were twice-washed with PBS and further incubated with goat anti-mouse IgG-FITC polyclonal antibodies (1:400 dilution) at room temperature for 30 min. The cells were washed with PBS before loading onto the flow cytometry system.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. All data are expressed as X±S and a t-test was used for intergroup comparisons. A $P<0.05$ indicated a significant difference.

Results

Results of DPSC osteogenic differentiation in each group

Osteogenic differentiation of DPSCs was compared among normal DPSCs, DPSCs-vector, and DPSCs-SOX2. There were no significant morphologic differences and the cells were all spindle-like and adherent to the walls (Figure 1A). After induction of osteogenic differentiation, cells of the three groups were ALP-positive. Some regions of the cells were deeply stained and presented with a nodular structure (Figure 1B). To further evaluate the osteogenic differentiation capacity, the levels of ALP, collagen I, Runx2, and osterix gene expression associated with osteogenic differentiation were detected by qPCR. The results showed that after induction for 3-4 weeks, these genes were not particularly up-regulated in DPSCs-vector compared to normal DPSCs. In contrast, the genes were significantly up-regulated in DPSCs-SOX2 ($P<0.05$). This finding indicated that SOX2 overexpression promoted osteogenic differentiation of DPSCs (Figure 1C).

Working mechanism of the promoting effect of SOX2 on osteogenic differentiation of DPSCs

Microarray analysis was performed to detect the expression of genes in SOX2-overexpressing DPSCs, which showed that in DPSCs-SOX2, the Hippo signaling pathway was activated. Compared to normal DPSCs, several key genes (TGF-β, APC, and PP2A) in the Hippo signaling pathway were significantly up-

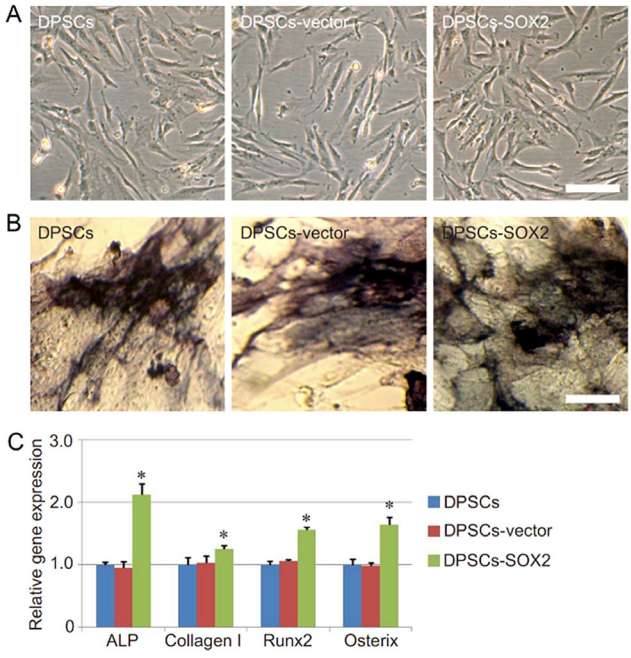

Figure 1. Evaluation of osteogenic differentiation of DPSCs. A. Morphologic characteristics of DPSCs in each group (scale bar=200 μm); B. ALP staining for detecting osteogenic differentiation of DPSCs (scale bar=100 μm); C. Expression of genes associated with osteogenic differentiation of DPSCs in each group.

regulated. The bone morphogenic protein (BMP) gene, one of the promoters of the Hippo signaling pathway, was up-regulated considerably in DPSCs-SOX2; this further led to activation of the Hippo signaling pathway.

The expression of BMP family genes (BMP2, BMP4, and BMP6) was further detected by qPCR and flow cytometry to verify the results of microarray analysis. qPCR showed that BMP2, BMP4, and BMP6 were up-regulated in DPSCs-SOX2 compared with normal DPSCs and DPSCs-vector ($P<0.05$). The expression of BMP6 was much higher in DPSCs-SOX2 than normal DPSCs and DPSCs-vector; however, no significant differences existed in the expression of BMP2, BMP4, and BMP6 between normal DPSCs and DPSCs-vector (Figure 2A). As indicated by subsequent flow cytometry for BMP6, the results were consistent with the results of qPCR. Thus, the efficiency of BMP4 expression in DPSCs-SOX2 (64.5%) was clearly higher than DPSCs

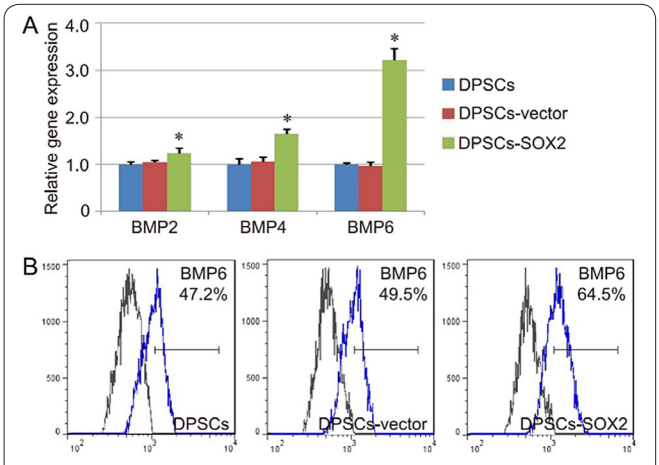

Figure 2. Expression analysis of genes in the BMP family. A. qPCR detection of genes in the BMP family; B. FACS analysis of BMP6 expression.

(47.2%) and DPSCs-vector (49.5%; Figure 2B).

Discussion

DPSCs are now widely known for osteogenic differentiation capacity, which make DPSCs eligible for bone repair (8). It is still uncertain about the effect of SOX2 as a regulator of multi-potent and neural stem cells on the osteogenic differentiation of DPSCs. To clarify this problem, SOX2-overexpressing DPSCs were obtained by retroviral infection, followed by induction of osteogenic differentiation. In the present study, overexpression of SOX2 can promote osteogenic differentiation of DPSCs. Many studies have demonstrated that the transcription regulator, SOX2, is critically involved in ectoderm and neural differentiation of human pluripotent stem cells (hPSCs; 1,2,12,13). Han et al. (14) showed that co-transfection of SOX2 and Oct4 can induce proliferation and differentiation in mesenchymal stem cells. Kopp et al. (15) also showed that that an increase < 2-fold in the levels of Sox2 triggers embryonic stem cell differentiation.

In the current study, SOX2-induced differentiation in DPSCs was further evaluated by qPCR analysis of ALP, collagen I, Runx2, and osterix genes which are associated with osteogenic differentiation. It is known that collagen I is expressed in the growth and mature-stage osteoblasts, and Runx2 and osterix genes are required during osteoblast formation (16-18). The mRNA levels of ALP, collagen I, Runx2, and osterix in DPSCs-SOX2 were promoted significantly. Hence, SOX2 overexpression up-regulated the capacity of DPSCs-SOX2 for differentiation towards osteogenesis by increasing osteogenic differentiation genes in this way.

With respect to the working mechanism, activation of the Hippo signaling pathway and up-regulation of the BMP family genes in DPSCs-SOX2 may play a role. According to the previous literature, the Hippo signaling pathway is important for osteogenic differentiation of mesenchymal cells and the expressions of several key genes in this pathway will determine the results of osteogenic differentiation (19). In the current study, microarray analysis indicated that several key genes in the Hippo signaling pathway were significantly up-regulated in DPSCs-SOX2, which may be the underlying reason for the increased osteogenic differentiation of DPSCs. The up-regulation of BMP family genes (BMP2, BMP4, and BMP6) was directly related to activation of the Hippo signaling pathway and other signaling pathways, thus facilitating osteogenic differentiation (20). Therefore, up-regulation of BMP family genes is another mechanism for the promotion of osteogenic differentiation of DPSCs-SOX2.

To conclude, SOX2-overexpressing DPSCs established by retroviral infection were shown to exhibit a higher level of osteogenic differentiation capacity and could be a novel form of cell therapy for bone repair. Because the experiments were conducted *in vitro*, the osteogenic differentiation capacity of DPSCs-SOX2 *in vivo* will be explored in a corollary study.

Author Contributions

Jing Yuan and Xiaobo Liu carried out the study concepts and design, participated in experimental studies, data

analysis, statistical analysis. Yaoyu Chen and Yifan Zhao carried out the definition of intellectual content, data acquisition and literature research. Pengfei Liu participated in clinical studies and experimental studies. Lei Zhao participated in data acquisition and analysis. Weidong Han helped to clinical studies and drafted the manuscript. All authors read and approved the final manuscript.

Funding

This project was supported by the Knowledge Innovation Program of Shenzhen City (JCYJ20140416122812052), Graduate Innovation Fund of Jilin University (2015009), the Scientific Research Project of Shenzhen Health and Family Planning System (201401010), and the Science and Technology Development Project of Jilin Province (20120955).

References

1. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126(4): 663-76.
2. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131(5): 861-72.
3. Cai J, Li W, Su H, Qin D, Yang J, Zhu F, et al. Generation of human induced pluripotent stem cells from umbilical cord matrix and amniotic membrane mesenchymal cells. J Biol Chem 2010; 285(15): 11227-34.
4. Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, et al. Vitamin c enhances the generation of mouse and human induced pluripotent stem cells. Cell Stem Cell 2010; 6(1): 71-9.
5. Feng RP, Wen JH. Overview of the roles of sox2 in stem cell and development. Biol Chem 2015; 396(8): 883-91.
6. Thier M, Worsdorfer P, Lakes YB, Gorris R, Herms S, Opitz T, et al. Direct conversion of fibroblasts into stably expandable neural stem cells. Cell Stem Cell 2012; 10(4): 473-9.
7. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (dpscs) in vitro and in vivo. Proc Natl Acad Sci U S A 2000; 97(25): 13625-30.
8. Tatullo M, Marrelli M, Shakesheff KM, White LJ. Dental pulp stem cells: Function, isolation and applications in regenerative medicine. J Tissue Eng Regen Med 2015; 9(11): 1205-16.
9. Paino F, La Noce M, Tirino V, Naddeo P, Desiderio V, Pirozzi G, et al. Histone deacetylase inhibition with valproic acid downregulates osteocalcin gene expression in human dental pulp stem cells and osteoblasts: Evidence for hdac2 involvement. Stem cells 2014; 32(1): 279-89.
10. Chang CC, Chang KC, Tsai SJ, Chang HH, Lin CP. Neurogenic differentiation of dental pulp stem cells to neuron-like cells in dopaminergic and motor neuronal inductive media. J Formos Med Assoc 2014;113(12): 956-65.
11. Liu P, Cai J, Dong D, Chen Y, Liu X, Wang Y, et al. Effects of sox2 on proliferation, migration and adhesion of human dental pulp stem cells. PloS one 2015; 10(10): e0141346.
12. Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S.Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 2011; 145(6): 875-89.
13. Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, et al. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell 2008; 133(6):1106-17
14. Han SM, Han SH, Coh YR, Jang G, Chan Ra J, Kang SK, et al. Enhanced proliferation and differentiation of Oct4- and Sox2-overexpressing human adipose tissue mesenchymal stem cells. Lee HW, Youn HY. Exp Mol Med 2014; 46: e101.
15. Kopp JL, Ormsbee BD, Desler M, Rizzino A. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells 2008; 26: 903-911.
16. Vater C, Kasten P, Stiehler M. Culture media for the differentiation of mesenchymal stromal cells. Acta Biomater. 2011;7:463–477.
17. Weitzmann MN. T-cells and B-cells in osteoporosis. Curr Opin Endocrinol Diabetes Obes. 2014;21:461–467.
18. Wang L, Zhou GB, Liu P, Song JH, Liang Y, Yan XJ, Xu F, Wang BS, Mao JH, Shen ZX, Chen SJ, Chen Z. Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. Proc Natl Acad Sci U S A. 2008;105:4826–4831.
19. Byun MR, Hwang JH, Kim AR, Kim KM, Hwang ES, Yaffe MB et al. Canonical wnt signalling activates taz through pp1a during osteogenic differentiation. Cell Death Differ 2014; 21(6): 854-63.
20. Jain AP, Pundir S, Sharma A. Bone morphogenetic proteins: The anomalous molecules. J Indian Soc Periodontol 2013; 17(5): 583-6.