

Original Article

Histone demethylase KDM2B inhibits the chondrogenic differentiation potentials of stem cells from apical papilla

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Abstract: Mesenchymal stem cells (MSCs) are a reliable resource for tissue regeneration, but the molecular mechanism underlying directed differentiation remains unclear; this has restricted potential MSC applications. Histone methylation, controlled by histone methyltransferases and demethylases, may play a key role in MSCs differentiation. Previous studies determined that KDM2B can regulate the cell proliferation and osteo/dentinogenic differentiation of MSCs. It is not known whether KDM2B is involved in the other cell lineages differentiation of MSCs. Here we used the stem cells from apical papilla (SCAPs) to study the role of KDM2B on the chondrogenic differentiation potentials in MSCs. In this study, Gain- and loss-of-function assays were applied to investigate the role of KDM2B on the chondrogenic differentiation. Alcian Blue Staining and Quantitative Analysis were used to investigate the synthesis of proteoglycans by chondrocytes. Real-time RT-PCR was used to detect the expressions of chondrogenesis related genes. The Alcian Blue staining and Quantitative Analysis results revealed that overexpression of *KDM2B* decreased the proteoglycans production, and real-time RT-PCR results showed that the expressions of the chondrogenic differentiation markers, *COL1*, *COL2* and *SOX9* were inhibited by overexpression of *KDM2B* in SCAPs. On the contrary, depletion of *KDM2B* increased the proteoglycans production, and inhibited the expressions of *COL1*, *COL2* and *SOX9*. In conclusion, our results indicated that KDM2B is a negative regulator of chondrogenic differentiation in SCAPs and suggest that inhibition of *KDM2B* might improve MSC mediated cartilage regeneration.

Keywords: Histone demethylase, KDM2B, chondrogenic differentiation, stem cells from apical papilla (SCAPs)

Introduction

Mesenchymal stem cells (MSCs) were originally isolated from bone marrow. They are multipotent cells that differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes. Increasing evidence indicates that MSCs are also present in non-bone marrow tissues [1, 2]. Recently, a new population of MSCs has been isolated from dental and craniofacial tissues on the basis of their stem cell properties, including but not limited to periodontal ligament stem cells (PDLSCs), dental pulp stem cells (DPSCs), and

stem cells from apical papilla (SCAPs) [3-8]. They are multipotent, destined for osteogenic lineages, dentinogenic lineages, and other lineages such as chondrocytes, melanocytes, endothelial cells, and functionally active neurons; and capable of self-renewal [3-13]. Although MSCs are a reliable resource for tissue regeneration, the molecular mechanism underlying directed differentiation remains unclear; this restricts their potential applications. The establishment of specific gene expression patterns during stem cell differentiation is a result of the subtly elaborated control of activation/silencing of large numbers of

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Table 1. Primers sequences used in the real-time RT-PCR

Gene Symbol	PRIMER SEQUENCES (5'–3')
<i>GAPDH-F</i>	CGAACCTCTCTGCTCCTCCTGTTCG
<i>GAPDH-R</i>	CATGGTGTCTGAGCGATGTGG
<i>18S-F</i>	CGGCTACCACATCCAAGGAA
<i>18S-R</i>	GCTGGAATTACCGCGGCT
<i>COL1-F</i>	ACAGGGCTCTAATGATGTTGAA
<i>COL1-R</i>	AGGCGTGATGGCTTATTTGT
<i>KDM2B-F</i>	GTTAGTGGTAGTGGTGTTTTGG
<i>KDM2B-R</i>	AGCAGATGTGGTGTGGTC
<i>COL2-F</i>	CATCCACCCTCTCACAGTT
<i>COL2-R</i>	TCTGCCAGTTCAGGTCTCT
<i>SOX9-F</i>	CCCTCAACCTCCACACTA
<i>SOX9-R</i>	TGGTGGTCGGTGTAGTCGTA

genes [14-17]. Covalent histone modifications play an important role in regulating chromatin dynamics and functions [18]. One type of histone modification, methylation, occurs on both lysine and arginine residues. This modification is involved in a diverse range of biological processes, including heterochromatin formation, X-chromosome inactivation, and transcriptional regulation [19-21]. To date, histone modification profiles have not been extensively studied during the stem cell differentiation process, although they may be tightly associated with gene expression patterns.

The histone demethylase, lysine (K)-specific demethylase2B (KDM2B), is evolutionarily conserved and ubiquitously expressed members of the JmjC-domain-containing histone demethylase family. This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination. It also has Trimethyl Lys4 histone H3 (H3K4me3) and dimethyl Lys36 histone H3 (H3K36me2) demethylase activity [22]. Functionally, the demethylase KDM2B has been implicated in cell-cycle regulation and tumorigenesis [23]. In addition, KDM2B promotes transcriptional repression via de-repressing the transcripts of *let-7b/EZH2* [24]. Previous research discovered that KDM2B restrains the osteo/dentinogenic differentiation potentials in MSCs [25]. However, its role on chondrogenic differentiation in MSCs is unclear.

In this study, we used SCAPs to investigate the function of KDM2B on chondrogenic differentiation potentials. Our results showed that overexpression of *KDM2B* repressed the chondrogenic differentiation potential and depletion of *KDM2B* enhanced the chondrogenic differentiation potentials in SCAPs.

Materials and methods

Cell cultures

Human impacted third molar with immature roots were collected from 3 healthy patients (16-20 years old) under approved guidelines set by Beijing Stomatological Hospital Capital Medical University (Ethical committee agreement is Beijing Stomatological Hospital Ethics Review No. 2011-02), with informed patient consent. Wisdom teeth were first disinfected with 75% ethanol and then washed with phosphate buffered saline (PBS). SCAPs were isolated, cultured, and identified, as previously described [6]. Briefly, SCAPs were gently separated from the apical papilla of the root and then digested in a solution of 3 mg/ml collagenase type I (Worthington Biochem, USA) and 4 mg/mL dispase (Roche, Germany) for 1h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 µm strainer (Falcon, BD Labware, USA). Then they were inoculated and grown in a humidified, 5% CO₂ incubator at 37°C in DMEM alpha modified Eagle's medium (Invitrogen, USA), supplemented with 15% fetal bovine serum (FBS; Invitrogen), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). The culture medium was changed every 3 days. Cells at passages 3-5 were used in subsequent experiments.

Plasmid construction and viral infection

Plasmids were constructed by standard methods; all structures were verified by appropriate restriction digest and/or sequencing. The plasmid for *HA-KDM2B* in pQCXIH retroviral vector (BD Clontech, USA) was a kind gift from Dr. Cun-Yu Wang, University of California, Los Angeles, School of Dentistry. The short hairpin RNAs (shRNA) of *KDM2B* was subcloned into the pLKO.1 lentiviral vector (Addgene, USA). Viral packaging was prepared according to the manufacturer's protocol by using 293T cells (Clontech or Addgene). For viral infection, SCAPs were plated overnight and then infected with

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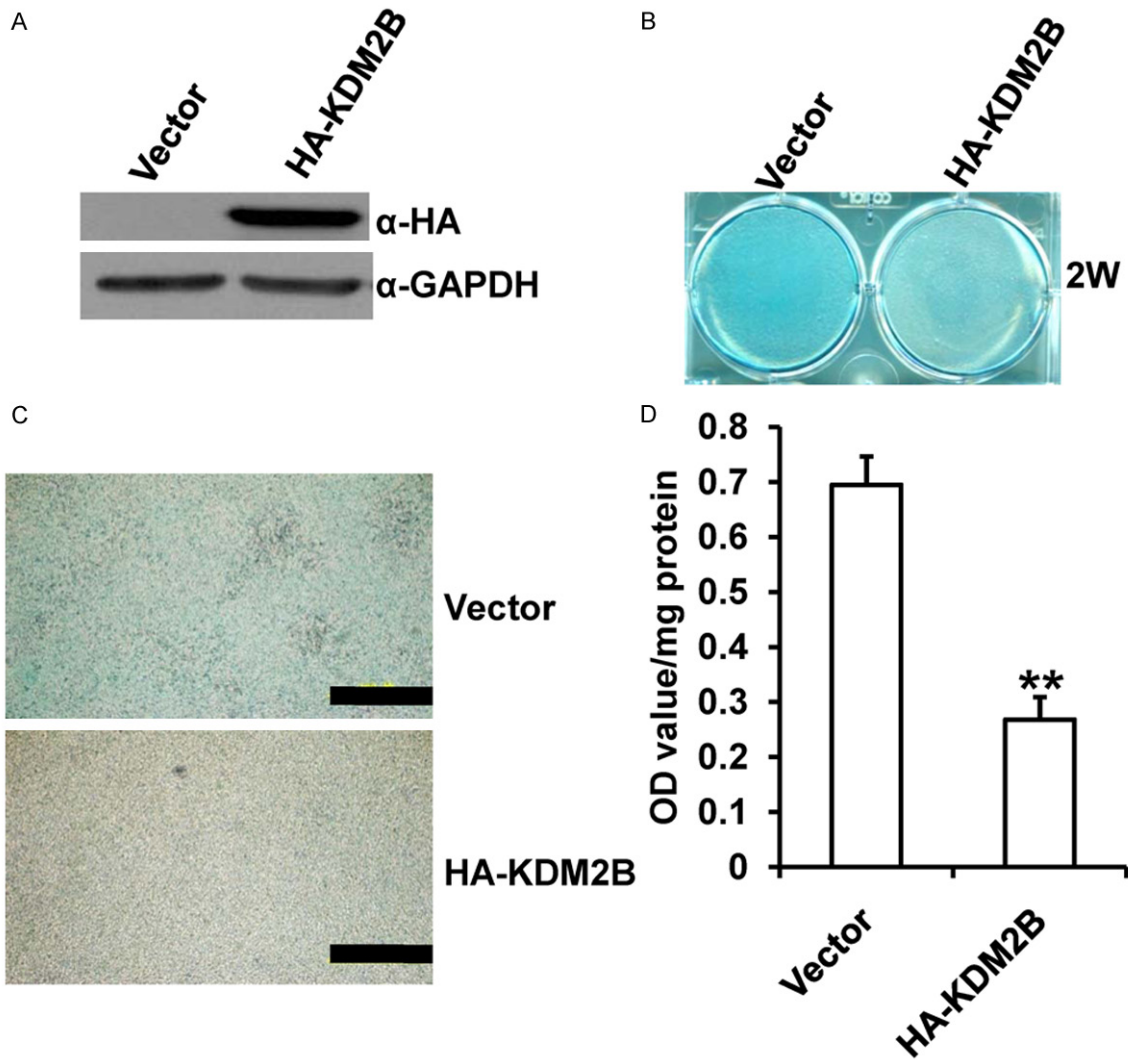


Figure 1. *KDM2B* overexpression repressed proteoglycans synthesis in SCAPs. (A) *KDM2B* overexpression in SCAPs. SCAPs were infected with retroviruses expressing wild-type *KDM2B* or empty vector. After selection with 200 $\mu\text{g}/\text{mL}$ hygromycin for 7 days, wild-type *KDM2B* was ectopically expressed in SCAPs, as confirmed by Western blot analysis. GAPDH was used as an internal control. (B-D) SCAPs were cultured with chondrogenic medium for the indicated time periods. Cells were stained with 1% Alcian Blue at 2 weeks (B, C). Scale bar: 100 μm . Quantification of proteoglycans synthesis at 2 weeks (D). Statistical significance was determined by Student T-test. All error bars represent s.d. (n = 3). ** $P < 0.01$.

retroviruses or lentiviruses in the presence of polybrene (6 $\mu\text{g}/\text{mL}$, Sigma-Aldrich, USA) for 6 h, and then after 48 h, selected by antibiotics. A scramble shRNA (*Scramsh*) was purchased from Addgene. The target sequences for *KDM2B* shRNA (*KDM2Bsh*) was, 5'-ATTTGACGGGTGGATAATCTG-3'.

Alcian blue stain analysis

Chondrogenic differentiation was induced by using the STEMPRO Chondrogenesis Differentiation Kit (Invitrogen). SCAPs were grown in the

chondrogenic medium for 2 weeks. For Alcian Blue staining, cells were rinsed once with DPBS, and fixed with 4% formaldehyde solution for 30 min. After fixation, rinsed wells with DPBS and stained cells with 1% Alcian Blue solution prepared in 0.1 N HCL for 30 min. And then rinsed wells 3 times with 0.1 N HCL, added distilled water to neutralize the acidity, visualized under light microscope, and captured images for analysis. Blue staining indicated synthesis of proteoglycans by chondrocytes. To quantify proteoglycans synthesis, Alcian Blue was extracted by 4 M guanidine-HCl overnight

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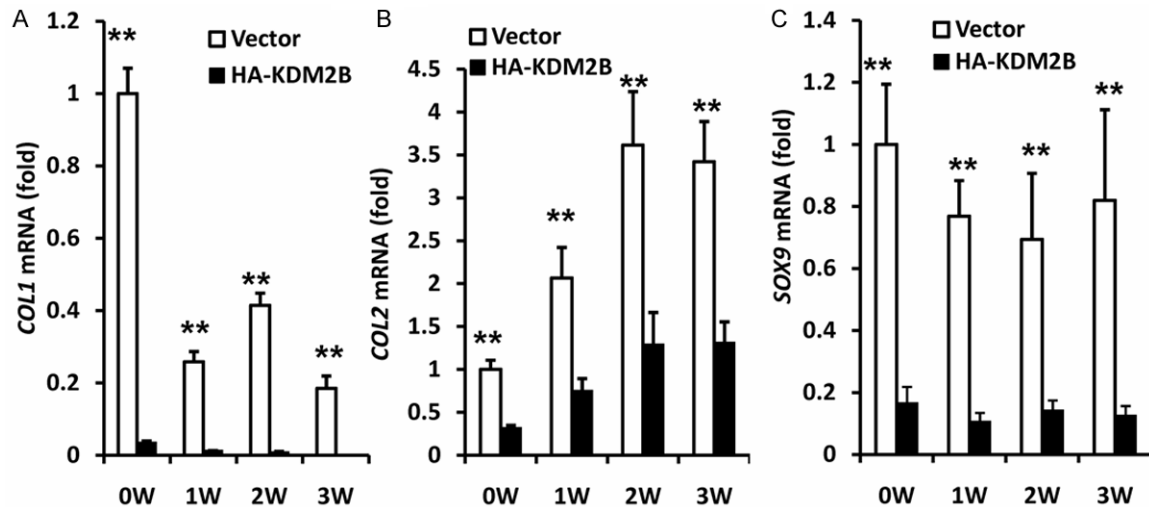


Figure 2. Overexpression of *KDM2B* decreased the expressions of *COL1*, *COL2* and *SOX9*. Real-time RT-PCR results showed that the expressions of *COL1* (A), *COL2* (B) and *SOX9* (C) were decreased in *KDM2B* overexpressed SCAPs compared with empty vector group at time points 0, 1, 2, and 3 weeks after chondrogenic induction. *GAPDH* and *18S* were used as an internal control. Statistical significance was determined by Student T-test. All error bars represent s.d. (n = 3). **P < 0.01.

at 4°C. Absorbance values were read at 600 nm after temperature equilibration. The final OD value in each group was normalized with the total protein concentrations prepared from a duplicate plate.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR

Total RNA was isolated from SCAPs with Trizol reagents (Invitrogen). We synthesized cDNA from 2 mg aliquots of RNA, random Hexamers or oligo (dT), and reverse transcriptase, according to the manufacturer's protocol (Invitrogen). Real-time RT-PCR reactions were performed with the QuantiTect SYBR Green PCR kit (Qiagen, Germany) and an Icyler iQ Multi-color Real-time PCR detection system. The expression of genes was calculated by the method of $2^{-\Delta\Delta CT}$ as described previous [26]. The primers for specific genes are shown in **Table 1**.

Western blot analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCl, 1 Mm EDTA, 1% sodium dodecyl sulfate [SDS], 1% NP-40, 1:100 proteinase inhibitor cocktail, 50 mM β -glycerophosphate, 50 mM sodium fluoride). The samples were separated on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes with a semi-dry transfer apparatus (Bio-Rad).

The membranes were blotted with 5% dehydrated milk for 2 h and then incubated with primary antibodies overnight. The immune complexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, USA) and visualized with Super Signal reagents (Pierce, USA). Primary monoclonal anti-HA (Clone No. C29F4, Cat No. 3724, Cell Signaling Technology, USA) was used. We also used a primary monoclonal antibody to detect the house keeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Clone No. GAPDH 71.1, Cat No. G8795, Sigma-Aldrich).

Statistics

All statistical calculations were performed with SPSS10 statistical software. The student's T-test was performed to determine statistical significance. A P-value ≤ 0.05 was considered significant.

Results

Overexpression of *KDM2B* repressed chondrogenic differentiation potential of SCAPs

To test the function of *KDM2B* in MSCs, the construct overexpressed ectopic *KDM2B* when transduced into SCAPs via retroviral infection. After antibiotics selection, Ectopic *KDM2B* expression was confirmed by Western Blot

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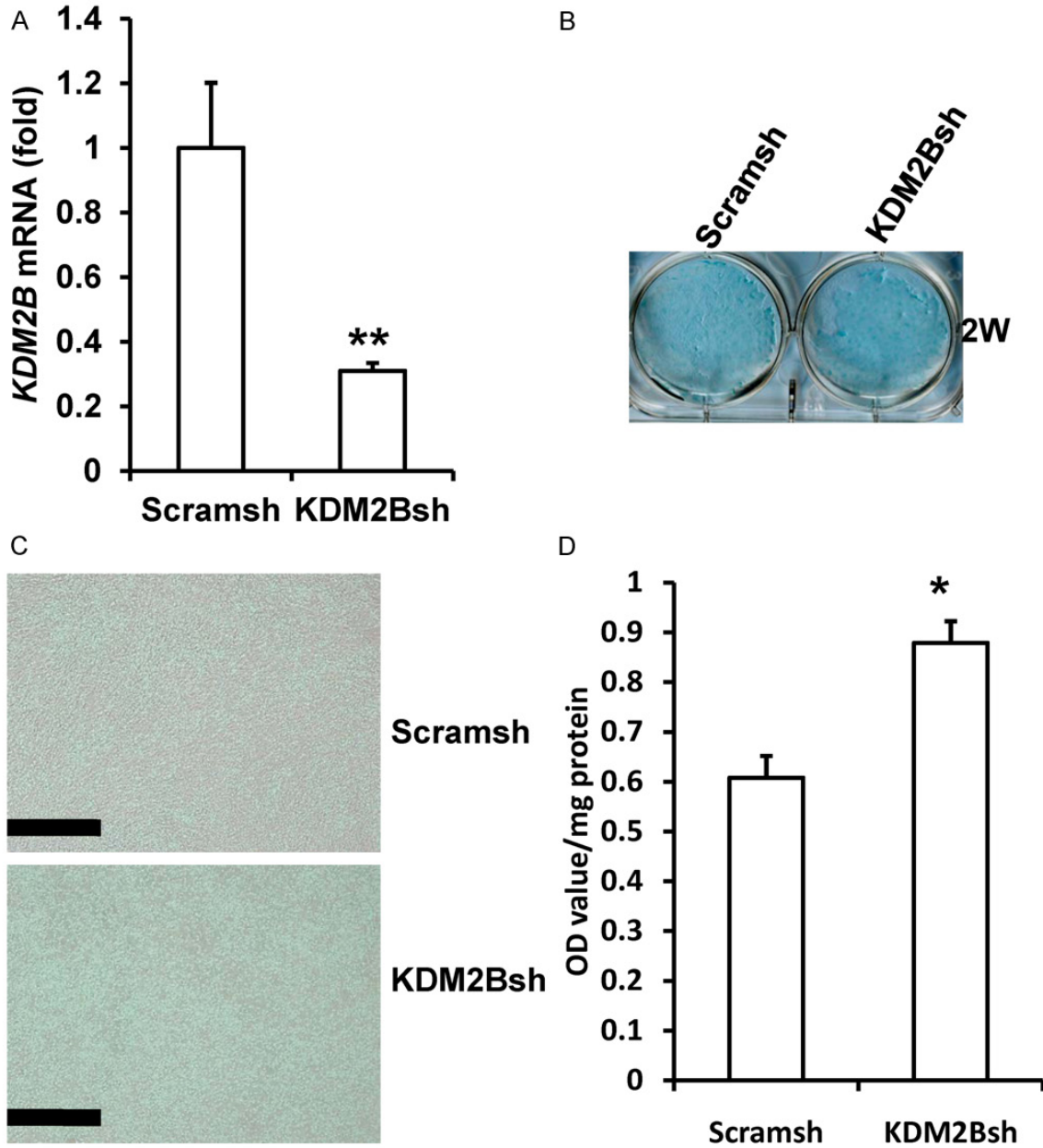


Figure 3. Depletion of *KDM2B* enhanced proteoglycans synthesis in SCAPs. (A) The knock-down of *KDM2B* in SCAPs. SCAPs were infected with lentiviruses expressing *KDM2B* shRNA (*KDM2Bsh*) or Scramble shRNA (*Scramsh*). After selection with 2 $\mu\text{g}/\text{mL}$ puromycin for 7 days, *KDM2B* expression was determined by real-time RT-PCR. The real-time RT-PCR results showed that *KDM2B* was 70% knocked down by *KDM2Bsh* compared with *Scramsh* in SCAPs. *GAPDH* was used as an internal control. (B-D) SCAPs were cultured with chondrogenic medium for the indicated time periods. Cells were stained with 1% Alcian blue at 2 weeks after induction with chondrogenic medium (B, C). Scale bar: 100 μm . Quantification of proteoglycans synthesis at 2 weeks (D). Statistical significance was determined by Student T-test. All error bars represent s.d. ($n = 3$). * $P < 0.05$. ** $P < 0.01$.

analysis (Figure 1A). Next, transduced SCAPs were cultured in chondrogenic medium to investigate the chondrogenic differentiation potential. After induction with chondrogenic medium for 2 weeks, the Alcian Blue staining and quantitative analysis results revealed that

proteoglycans production was decreased in SCAPs that overexpressed *HA-KDM2B* compared with cells infected with the empty vector (Figure 1B-D). We further examined the chondrogenic differentiation markers, *COL1*, *COL2* and *SOX9* by real-time RT-PCR. The results

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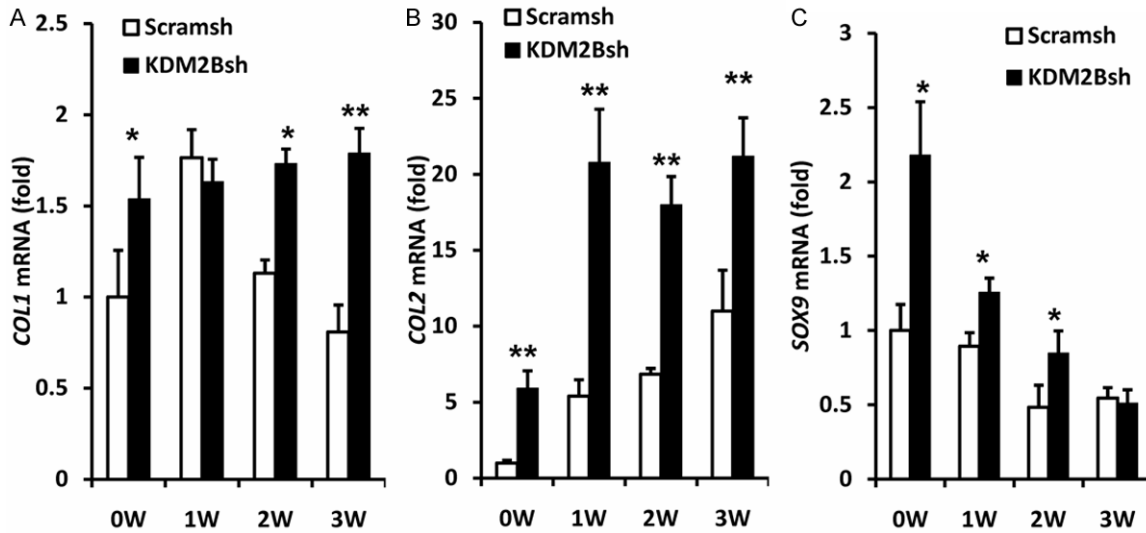


Figure 4. Depletion of *KDM2B* enhanced the expressions of *COL1*, *COL2* and *SOX9* in SCAPs. Real-time RT-PCR results showed that the expressions of *COL1* (A), *COL2* (B) and *SOX9* (C) were increased in SCAP-*KDM2Bsh* compared with SCAP-*Scramsh*. *GAPDH* and *18S* were used as an internal control. Statistical significance was determined by Student T-test. All error bars represent s.d. (n = 3). **P* < 0.05. ***P* < 0.01.

showed that the mRNA levels of *COL1*, *COL2*, *SOX9* were significantly decreased in SCAPs that overexpressed *HA-KDM2B* compared with cells infected with the empty vector at time points 0, 1, 2, and 3 weeks after chondrogenic induction (Figure 2).

Depletion of *KDM2B* enhanced chondrogenic differentiation potential of SCAPs

To confirm the function of *KDM2B* in MSCs, we designed a short hairpin RNA (shRNA) to target *KDM2B* and introduced it into SCAPs with lentiviral infection (SCAP-*KDM2Bsh*). After antibiotics selection, the knockdown efficiency (70%) in SCAP-*KDM2Bsh* compared with SCAPs infected with Scramble shRNA (SCAP-*Scramsh*) was verified by real-time RT-PCR (Figure 3A). Next, we compared the chondrogenic differentiation potential of SCAPs after knock-down of *KDM2B*. After induction with chondrogenic medium for 2 weeks, the Alcian Blue staining and quantitative analysis results revealed that proteoglycans production was increased in SCAP-*KDM2Bsh* compared with SCAP-*Scramsh* (Figure 3B-D). We further examined the chondrogenic differentiation markers, *COL1*, *COL2* and *SOX9* by real-time RT-PCR. The results showed that the mRNA level of *COL1* were increased in SCAP-*KDM2Bsh* compared with SCAP-*Scramsh* at time points 0, 2, and 3 weeks after chondrogenic induction (Figure 4A), the

expression of *COL2* were enhanced in SCAP-*KDM2Bsh* compared with SCAP-*Scramsh* at time points 0, 1, 2, and 3 weeks after chondrogenic induction (Figure 4B), while the expression of *SOX9* was increased in SCAP-*KDM2Bsh* compared with SCAP-*Scramsh* at time points 0, 1 and 2 weeks after chondrogenic induction (Figure 4C).

Discussion

MSCs have the potential to repair and regenerate damaged tissues, making them attractive candidates for cell-based therapies. Currently, MSC-mediated tissue regeneration has become a hot topic. The use of autologous and allogeneic MSCs from different tissues has successfully regenerated some tissues in large animal models or human [27-31]. Although MSC-mediated tissue regeneration has made surprising progress, some key issues remain to be resolved, including the molecular mechanism of directed differentiation and proliferation, etc. The differentiation of cells is accompanied by drastic changes in the epigenetic profiles of cells, which is determined by the unique pattern of DNA methylation and histone modifications [32, 33]. By now, few studies have been conducted with regards to epigenetic regulation of gene expression during cartilage differentiation in MSCs. Ezura et al. investigated the CpG methylation status in human synovium-

derived MSCs during *in vitro* chondrogenesis and found that DNA methylation levels of CpG-rich promoters of chondrocyte-specific genes were mostly maintained at low levels [34]. Histone acetylation is among the epigenetic mechanisms that have been reported to be involved in cartilage-specific gene expression. In this context the role of p300, an enzyme possessing a histone acetyltransferase (HAT) activity, was observed in several studies. Tsuda et al. have shown that Sox9 associates with CREB-binding protein (CBP)/p300 as an activator for cartilage tissue-specific gene expression during chondrocyte differentiation [35]. Other studies also reveal that histone acetylation favors cartilage differentiation which has been shown in both *in vivo* and *in vitro* studies [36, 37]. In contrast to acetylation, histone deacetylation blocks cartilage differentiation. Histone deacetylation by HDAC1 has been reported to have a critical inhibitory role in cartilage matrix deposition during cartilage differentiation [38]. Vega et al. have found that HDAC4 regulates chondrocyte hypertrophy and endochondral bone formation by inhibiting the activity of *Runx2* which is a transcription factor necessary for chondrocyte hypertrophy [39]. Others reported that histone deacetylase inhibitor trichostatin A (TSA) enhanced cartilage matrix formation and chondrogenic structure [40].

Histone demethylases play an important role in methylation which is one type of histone modification. Hata et al. identified AT-rich interactive domain 5b (Arid5b) as a transcriptional co-regulator of Sox9 [41]. Arid5b physically associates with Sox9 and synergistically induces chondrogenesis [41]. Arid5b recruits Phf2, a histone lysine demethylase, to the promoter region of Sox9 target genes and stimulates H3K9me2 demethylation of these genes [41]. Our previous study also indicated that depletion of KDM2A could enhance chondrogenic differentiation potentials in SCAPs. The demethylase KDM2B is a member of the KDM2 Lys histone demethylase family, which was previously identified as a H3K4me3 and H3K36me2 histone demethylase [22]. The most important functional domain, the Jmjc domain, has 79% homology in KDM2A and KDM2B; JmjC domain-containing proteins are predicted to be metalloenzymes that regulate chromatin function [17-21]. Thus, presumably, the histone demethylation functions should be highly similar in KDM2A and KDM2B. Previous researches dis-

covered that KDM2B and KDM2A both inhibited the osteo/dentinogenic differentiation function in MSCs [25, 42]. However, the function of KDM2B on chondrogenic differentiation in MSCs is unknown. Here, in a gain of function study, we discovered that overexpression of *KDM2B* significantly decreased the chondrogenic differentiation potentials in SCAPs based on the results of the Alcian Blue staining and quantitative analysis. In addition, in a loss of function study, the Alcian Blue Staining and quantitative analysis indicated that silencing of *KDM2B* possessed stronger chondrogenic differentiation potentials in SCAPs. Furthermore, we tested the chondrogenic differentiation markers including *COL1*, *COL2* and *SOX9*. *COL1* and *COL2* are usually considered to be significant to the formation of bone and cartilage. Sox9 proteins expressed in the cartilage of the stages before the differentiation of cells and chondrocytes are important in regulating chondrocyte differentiation and cartilage formation [43]. With decreased expressions of *COL1*, *COL2*, *SOX9* in SCAPs that overexpressed KDM2B, and enhanced expressions of *COL1*, *COL2*, *SOX9* after depletion of KDM2B in SCAPs, we concluded that KDM2B can inhibit the chondrogenic differentiation potential of SCAPs through regulate and control the expression of chondrogenic differentiation related genes such as *COL1*, *COL2* and *SOX9*. Previous studies also showed that KDM2B were BCL6 co-factors, and can associate with BCOR and exert histone demethylase functions on target genes [25]. Further study is required to determine whether BCL6 or BCOR involved in this procession.

In conclusion, these results indicated that KDM2B is a negative regulator of chondrogenic differentiation in SCAPs, suggest that inhibition of KDM2B might improve MSC mediated cartilage regeneration and provided useful information to understand the molecular mechanism underlying directed differentiation in MSCs derived from dental tissues.

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Disclosure of conflict of interest

None.

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