

## ORIGINAL ARTICLE

# KDM6B epigenetically regulates odontogenic differentiation of dental mesenchymal stem cells

Juan Xu<sup>1,2</sup>, Bo Yu<sup>2</sup>, Christine Hong<sup>2,3</sup> and Cun-Yu Wang<sup>2,4</sup>

Mesenchymal stem cells (MSCs) have been identified and isolated from dental tissues, including stem cells from apical papilla, which demonstrated the ability to differentiate into dentin-forming odontoblasts. The histone demethylase KDM6B (also known as JMJD3) was shown to play a key role in promoting osteogenic commitment by removing epigenetic marks H3K27me3 from the promoters of osteogenic genes. Whether KDM6B is involved in odontogenic differentiation of dental MSCs, however, is not known. Here, we explored the role of KDM6B in dental MSC fate determination into the odontogenic lineage. Using shRNA-expressing lentivirus, we performed KDM6B knockdown in dental MSCs and observed that KDM6B depletion leads to a significant reduction in alkaline phosphatase (ALP) activity and in formation of mineralized nodules assessed by Alizarin Red staining. Additionally, mRNA expression of odontogenic marker gene *SP7* (*osterix*, *OSX*), as well as extracellular matrix genes *BGLAP* (*osteocalcin*, *OCN*) and *SPP1* (*osteopontin*, *OPN*), was suppressed by KDM6B depletion. When KDM6B was overexpressed in KDM6B-knockdown MSCs, odontogenic differentiation was restored, further confirming the facilitating role of KDM6B in odontogenic commitment. Mechanistically, KDM6B was recruited to bone morphogenetic protein 2 (BMP2) promoters and the subsequent removal of silencing H3K27me3 marks led to the activation of this odontogenic master transcription gene. Taken together, our results demonstrated the critical role of a histone demethylase in the epigenetic regulation of odontogenic differentiation of dental MSCs. KDM6B may present as a potential therapeutic target in the regeneration of tooth structures and the repair of craniofacial defects.

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## INTRODUCTION

Human mesenchymal stem cells (MSCs) are multipotent progenitor cells with the capability to self-renew and differentiate into a variety of cell types including osteoblasts and adipocytes.<sup>1</sup> Additional characteristics of MSCs, including their ease of isolation and lack of immunogenicity, make them unique and indispensable tools in tissue engineering and regenerative therapy.<sup>2–4</sup> MSCs were originally discovered in the stroma of adult bone marrow. Soon after their multipotency became apparent, a search for other MSC subpopulations in different tissue organs uncovered stem cells in synovium, fat, muscle and umbilical cord.<sup>5</sup> Similarly, a number of different MSCs have been identified and isolated from the dental tissues, including dental pulpal stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells and stem cells from apical papilla.<sup>6</sup> Moreover, the pluripotency of these dental MSCs for odontogenic, osteogenic, chondrogenic and adipogenic potentials under different culture conditions *in vitro* has been demonstrated, suggesting that these dental MSCs hold significant promise for clinical applications in regenerative dental and craniofacial therapies.<sup>7</sup>

Cell fate determination requires intricate orchestration between genetic and epigenetic programs. In particular, epigenetic regulation has only recently emerged as an important mechanism of stem cell differentiation, and it does so by shaping the fate of MSCs through modifications in chromatin architecture and the accessibility of genes without changing the primary nucleotide sequence.<sup>8–10</sup> In particular, modifications at the N-terminal tails of histones such as acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation establish epigenetic signatures that either mark for gene activation such as trimethylated histone 3 lysine 4 (H3K4) or mark for gene repression, such as demethylated H3K9 and trimethylated H3K27 (H3K27me3). Epigenetic phenomena are largely responsible for the elaborate balance of gene activation and inhibition at specific time points directing towards terminally differentiated phenotypes such as osteoblasts or odontoblasts. Nonetheless, the epigenetic component in the context of odontogenic differentiation has not been identified previously, and very limited information is available for the epigenetic regulation of odontogenic differentiation.

Among different epigenetic machineries, histone demethylases, enzymes responsible for removing methyl groups from the histones,

<sup>1</sup>Department of Stomatology, Chinese People's Liberation Army General Hospital, Beijing, China; <sup>2</sup>Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, USA; <sup>3</sup>Section of Orthodontics, School of Dentistry, University of California, Los Angeles, USA and <sup>4</sup>Department of Bioengineering, Henry Samueli School of Engineering and Applied Science, University of California, Los Angeles, USA

Correspondence: Dr C Hong, School of Dentistry, University of California, Los Angeles, CHS 63-014, 10833 Le Conte Avenue, Los Angeles CA 90095-1668, USA

E-mail: chong@dentistry.ucla.edu

Dr CY Wang, Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CHS 33-030, 10833 Le Conte Avenue, Los Angeles CA 90095-1668, USA

E-mail: cwang@dentistry.ucla.edu

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have been demonstrated to influence cell fate decisions and subsequently, cell differentiation.<sup>10–12</sup> The existence of these demethylases is essential to facilitate rapid removal of methyl group from gene silencing epigenetic marks. The precise functions of histone demethylases in regulating osteogenic differentiation have only begun to be unraveled recently.<sup>13</sup>

We previously identified a specific histone demethylase, KDM6B (JMJD3) as a critical epigenetic regulator in bone marrow stem cell (BMSC) fate commitment by promoting osteogenic commitment while inhibiting adipogenic differentiation *in vitro* and *in vivo*.<sup>14</sup> KDM6B is a histone demethylase, which activates gene activation through the removal of H3K27me<sub>3</sub>, a repressive epigenetic mark.<sup>15</sup> Previous studies using BMSCs demonstrated that KDM6B plays an important role in neuronal differentiation by regulating neurogenic lineage genes such as *NES*, and through its interaction with Smad3 upon transforming growth factor  $\beta$  (TGF $\beta$ ) signaling initiation.<sup>16–18</sup> During the osteogenic differentiation of BMSCs, KDM6B occupies and demethylates histones in the promoters of *BMP2*, *BMP4* and *HOXC6-1* through which the expression of *RUNX2*, the master gene of osteogenesis, is regulated.<sup>14</sup> Because of the similarities between BMSCs and dental MSCs in differentiation capacity into mineralized tissues, these previous findings strongly support the notion that epigenetic regulation, histone demethylation in particular, may also play a key role in odontogenic differentiation of dental MSCs. In this paper, we investigated the role of KDM6B in odontogenic differentiation of dental MSCs.

## MATERIALS AND METHODS

### Cell isolation and culture

Primary dental MSCs used in this study were described previously.<sup>6</sup> Cells were grown in a humidified 5% CO<sub>2</sub> incubator at 37 °C in alpha modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), Gibco MEM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 2 mmol·L<sup>-1</sup> L-glutamate, 100 units·mL<sup>-1</sup> penicillin and 100 units·mL<sup>-1</sup> streptomycin each.

### Viral infection

Viral packaging was prepared as described previously.<sup>6,19</sup> For viral infection, MSCs were plated overnight and then infected with retroviruses or lentiviruses in the presence of polybrene (6  $\mu$ g·mL<sup>-1</sup>; Sigma-Aldrich, St Louis, MO, USA) for 24 h. The cells were then selected with puromycin for 3 days. Resistant clones were pooled and knockdown/overexpression was confirmed *via* reverse transcription-polymerase chain reaction (RT-PCR) analysis. In rescue experiments, the knockdown MSCs were transduced with retroviral constructs containing the Flag- or HA-tagged gene of interest. The target sequences for shRNA were: KDM6Bsh2, 5'-GCA GTC GGA AAC CGT TCT T-3'; KDM6Bsh3, 5'-GTG GGA ACT GAA ATG GTA T-3'. Flag-KDM6B full-length cDNA was cloned into retroviral construct by PCR.

### Alkaline phosphate and Alizarin Red staining

MSCs were grown in mineralization-inducing media containing 100  $\mu$ mol·L<sup>-1</sup> ascorbic acid, 2 mmol·L<sup>-1</sup>  $\beta$ -glycerophosphate and 10 nmol·L<sup>-1</sup> dexamethasone. For alkaline phosphate (ALP) staining, after induction, cells were fixed with 4% paraformaldehyde and incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.1 mol·L<sup>-1</sup> Tris buffer (pH 9.3). ALP activity assay was performed using an ALP kit according to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO, USA) and normalized based on protein concentrations. To

detect mineralization potential, cells were induced for 2–3 weeks, fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (Sigma-Aldrich, St Louis, MO, USA). To quantify the calcium mineral deposition, Alizarin Red was destained with 10% cetylpyridinium chloride in 10 mmol·L<sup>-1</sup> sodium phosphate for 30 min at room temperature. The concentration was determined by absorbance measurement at 562 nm on a multiplate reader using a standard calcium curve in the same solution. The final calcium level in each group was normalized with the total protein concentrations prepared from a duplicate plate.

### Real-time RT-PCR

Total RNA was isolated from MSCs using Trizol reagents (Invitrogen, Carlsbad, CA, USA). Two microgram aliquots of RNAs were synthesized using random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The real-time PCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and the iCycler iQ Multi-color Real-time PCR Detection System. The primers for 18S rRNA were: forward, 5'-CGG CTA CCA CAT CCA AGG AA-3'; reverse, 5'-GCT GGA ATT ACC GCG GCT-3'. The primers for *OSX* were: forward, 5'-CGC TTT GTG CCT TTG AAA T-3'; reverse, 5'-CCG TCA ACG ACG TTA TGC-3'. The primers for *OCN* were: forward, 5'-CAG ACA CCA TGA GGA CCA TC-3'; reverse, 5'-GGA CTG AGG CTC TGT GAG T-3'. The primers for *OPN* were: forward, 5'-ATG ATG GCC GAG GTG ATA GT-3'; reverse, 5'-ACC ATT CAA CTC CTC GCT TT-3'. The primers for *ALP* were: forward, 5'-GAC CTC CTC GGA AGA CAC TC-3'; reverse, 5'-TGA AGG GCT TCT TGT CTG TG-3'. The primers for *BMP2* were: forward, 5'-GTC AAC TCG ATG CTG TAC CTT GAC G-3'; reverse, 5'-CAA CCC TCC ACA ACC ATG TCC-3'. The primers for *BMP4* were: forward, 5'-CGG CGA AGA AGA ATA AGA ACT GCC G-3'; reverse, 5'-CCA GTC ATT CCA GCC CAC ATC-3'. The primers for *DLX2* were: forward, 5'-ACT CAA TAC TTG GCC TTG CC-3'; reverse, 5'-TTG ACC TGA GTC TGG GTG AG-3'. The primers for *KDM6B* were: forward, 5'-GCA CTA CTG GGA GAC CAT CA-3'; reverse, 5'-ACC AGG AAC CCG TCA AGT AG-3'.

### Chromatin immunoprecipitation assays

The assay was performed using a chromatin immunoprecipitation (ChIP) assay kit (Upstate/Millipore, Billerica, MA, USA) according to the manufacturer's protocol as described previously.<sup>6</sup> Cells were incubated with a dimethyl 3,3'-dithiobispropionimidate-HCl (Pierce/Thermo Scientific, Rockford, IL, USA) solution (5 mmol) for 10 min at room temperature, and then by formaldehyde treated for 15 min in 37 °C water bath. For each ChIP reaction, 2 $\times$ 10<sup>6</sup> cells were used. All resulting precipitated DNA samples were quantified with real-time PCR. Data are expressed as the percentage of input DNA. Antibodies for ChIP assays were purchased from the following commercial sources: rabbit polyclonal anti-KDM6B (Abgent, San Diego, CA, USA), rabbit monoclonal anti-H3K27me<sub>3</sub> (Millipore, Billerica, MA, USA). The primers for *BMP2* were: forward, 5'-CGT CTA GTA TTT TGG CAT AGC ATA GAC G-3'; reverse, 5'-ACT CAA TTT CCA GCC TGC TGT TT-3'. The primers for *BMP2-4 kb-down* were: forward, 5'-CAA TCA TAA GAA TTA CCT GTT GGG-3'; reverse, 5'-TGG TCG CAT TAT ACT CAT ATT GG-3'.

## RESULTS

### KDM6B promotes odontogenic potential of dental MSCs

Our previous study showed that osteogenic differentiation of BMSCs is epigenetically regulated by the histone demethylase, KDM6B.<sup>14</sup> Because certain dental MSCs are capable of differentiating into the odontoblast

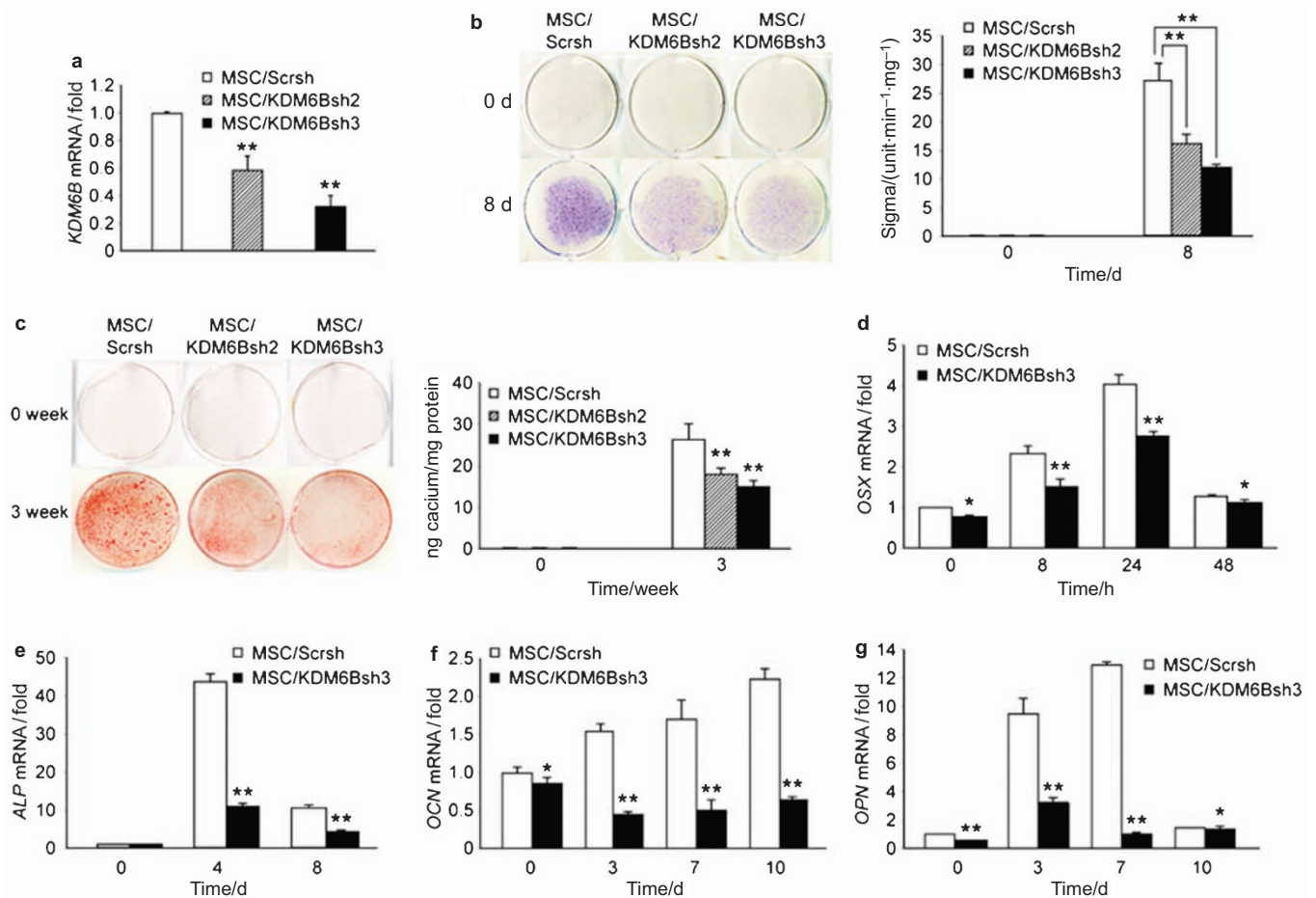
lineage through similar mechanisms as BMSCs,<sup>20</sup> we explored whether KDM6B plays a role in the fate determination of dental MSCs. To do so, we knocked down *KDM6B* in dental MSCs by transducing lentiviruses expressing *KDM6B* shRNAs. The knockdown of *KDM6B* by two different shRNAs was confirmed using real-time RT-PCR (Figure 1a). When these cells were induced to undergo odontogenic differentiation, MSC/KDM6Bsh2 and MSC/KDM6Bsh3 cells lost their capacity to differentiate as demonstrated by ALP staining and activity on the eighth day (Figure 1b). Furthermore, *KDM6B* knockdown suppressed the formation of mineralized nodules after prolonged treatment with odontogenic media for 3 weeks as demonstrated by Alizarin Red staining (Figure 1c). Of note, there was a dose-dependent reduction of odontogenic differentiation potential that reflected the efficiency of *KDM6B* knockdown in MSCs. Using MSC/KDM6Bsh3 cells, which exhibited more significant suppression of *KDM6B*, we further confirmed that *KDM6B* deficiency inhibited odontogenic potential by examining mRNA expression of several odontogenic marker genes at different time points after induction. In particular, *KDM6B* depletion significantly suppressed the expression of marker genes for early (*OSX*, Figure 1d), middle (*ALP*, Figure 1e) and late (*OCN* and *OPN*, Figure 1f and 1g) stages of odontogenic differentiation, indicating that *KDM6B* is required for the odontogenic differentiation of MSCs.

### KDM6B activates genes regulating odontogenic differentiation

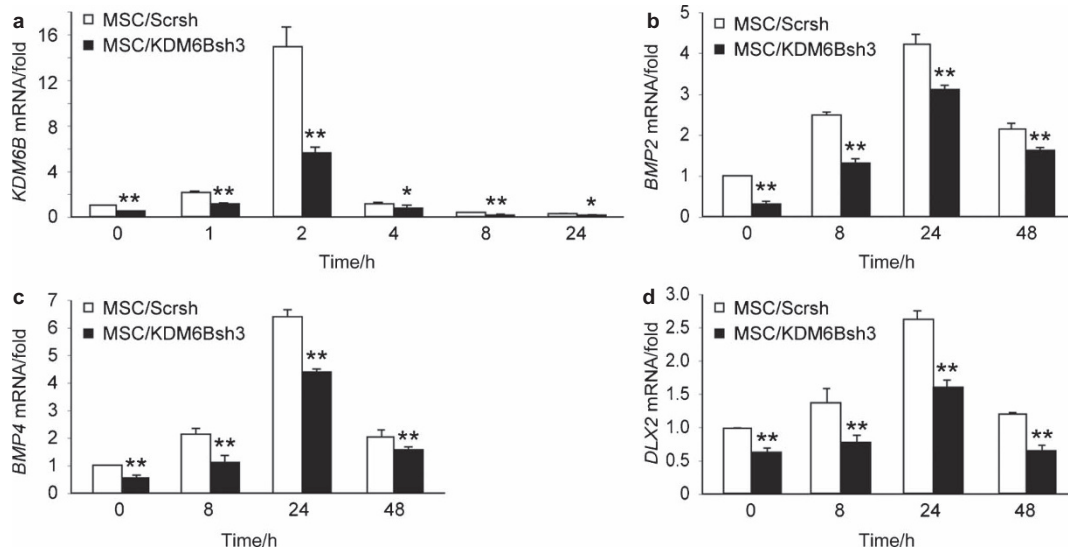
BMP2 and BMP4 play significant roles in odontogenic differentiation<sup>21–22</sup> and DLX family genes are suggested to be involved in dentin matrix mineralization.<sup>23</sup> To further explore whether *KDM6B* mediates odontogenic differentiation through these genes, we induced MSC/Scrsch and MSC/KDM6Bsh3 cells to undergo odontogenic differentiation and assessed mRNA expression of *BMP2*, *BMP4* and *DLX2*. Although *KDM6B* was strongly upregulated upon odontogenic differentiation in MSC/Scrsch cells, MSC/KDM6Bsh3 cells exhibited suppressed induction of *KDM6B* due to the presence of *KDM6B* shRNA (Figure 2a). Similarly, mRNA levels of *BMP2*, *BMP4* and *DLX2* were induced upon odontogenic differentiation in both MSC/Scrsch and MSC/KDM6Bsh3 cells. However, the degree of induction was significantly suppressed in MSC/KDM6Bsh3 cells when compared to MSC/Scrsch cells, indicating that *KDM6B* activates genes that regulate odontogenic differentiation.

### Rescue of KDM6B restores odontogenic potential of dental MSCs

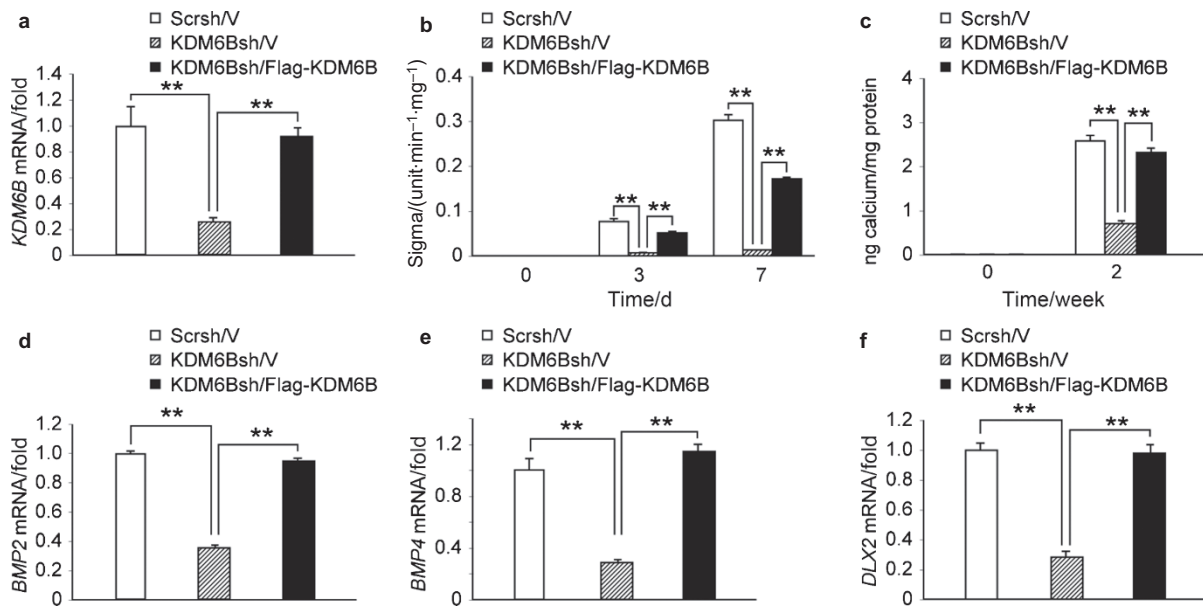
In order to mechanistically delineate whether *KDM6B* histone demethylase activity is required for odontogenic differentiation of MSCs, we strategically restored *KDM6B* expression in MSCs that already express *KDM6B* shRNA. We introduced Flag-tagged *KDM6B*



**Figure 1** KDM6B promotes odontogenic potential of dental MSCs. (a) Knockdown of *KDM6B* by shRNA. MSC/Scrsch, MSCs expressing scramble shRNA; MSC/KDM6Bsh2, MSCs expressing *KDM6B* shRNA2; MSC/KDM6Bsh3, MSCs expressing *KDM6B* shRNA3. (b) Knockdown of *KDM6B* inhibited ALP activities in MSCs. (c) Knockdown of *KDM6B* inhibited mineralization in MSCs. (d) Knockdown of *KDM6B* inhibited *OSX* expression in MSCs as determined by real-time RT-PCR. (e) Knockdown of *KDM6B* inhibited *ALP* expression in MSCs. (f) Knockdown of *KDM6B* inhibited *OCN* expression in MSCs. (g) Knockdown of *KDM6B* inhibited *OPN* expression in MSCs. ALP, alkaline phosphatase; MSC, mesenchymal stem cell; RT-PCR, reverse transcription-polymerase chain reaction.



**Figure 2** KDM6B activates genes regulating odontogenic differentiation. (a) Knockdown of *KDM6B* by shRNA suppresses *KDM6B* expression in MSC differentiation. MSC/Scrchsh, MSCs expressing scramble shRNA; MSC/KDM6Bsh3, MSCs expressing *KDM6B* shRNA. (b) Knockdown of *KDM6B* inhibited *BMP2* expression in MSCs as determined by real-time RT-PCR. (c) Knockdown of *KDM6B* inhibited *BMP4* expression in MSCs. (d) Knockdown of *KDM6B* inhibited *DLX2* expression in MSCs. BMP, bone morphogenic protein; MSC, mesenchymal stem cell; RT-PCR, reverse transcription-polymerase chain reaction.

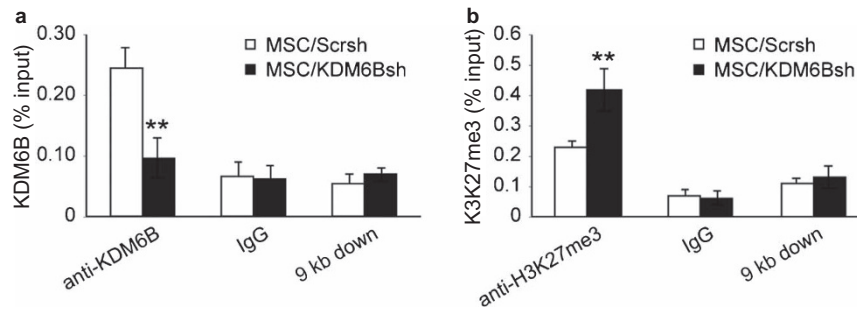


**Figure 3** Rescue of *KDM6B* restores odontogenic potential of dental MSCs. (a) Overexpression of *KDM6B* in MSCs that express *KDM6B* shRNA. Scrsh/V, MSCs expressing scramble shRNA and empty vector; *KDM6Bsh/V*, MSCs expressing *KDM6B* shRNA and empty vector; *KDM6Bsh/Flag-KDM6B*, MSCs expressing *KDM6B* shRNA and Flag-tagged *KDM6B*. (b) Overexpression of Flag-*KDM6B* rescued ALP activities in MSC differentiation. (c) Overexpression of Flag-*KDM6B* rescued mineralization in MSC differentiation. (d) Overexpression of Flag-*KDM6B* rescued *BMP2* expression in MSC differentiation as determined by real-time RT-PCR. (e) Overexpression of Flag-*KDM6B* rescued *BMP4* expression in MSC differentiation. (f) Overexpression of Flag-*KDM6B* rescued *DLX2* expression in MSC differentiation. ALP, alkaline phosphatase; BMP, bone morphogenic protein; MSC, mesenchymal stem cell; RT-PCR, reverse transcription-polymerase chain reaction.

in MSCs that harbor *KDM6B* shRNA targeting the 5' UTR region of *KDM6B* mRNA. By doing so, shRNA targets only the endogenous *KDM6B* but not ectopically introduced *KDM6B*. As expected, the control MSCs (Scrsh/V) exhibited significant amounts of *KDM6B*, which was suppressed upon introducing *KDM6B* shRNA (*KDM6Bsh/V*) (Figure 3a). When Flag-tagged *KDM6B* was ectopically introduced in these *KDM6Bsh* cells, *KDM6B* was re-expressed to the level similar to Scrsh/V (Figure 3a). When *KDM6Bsh/Flag-KDM6B* cells were induced

to undergo odontogenic differentiation, the inhibited odontogenic potential of MSCs due to *KDM6B* depletion was restored with the retrieval of functional *KDM6B* in *KDM6B* knockdown cells was demonstrated by ALP staining (Figure 3b) and Alizarin Red staining (Figure 3c). Similarly, the suppressed expression of *BMP2*, *BMP4* and *DLX2* as a result of *KDM6B* depletion was rescued as well when *KDM6B* was re-introduced, indicating that *KDM6B* specifically regulates odontogenic differentiation.





**Figure 4** KDM6B epigenetically regulates BMP2 expression. (a) Knockdown of KDM6B reduced KDM6B binding to the promoter of BMP2 in MSCs. The promoter of BMP2 was ChIPed with anti-KDM6B antibody or IgG control. The 9 kb downstream of the transcriptional start site was also ChIPed with anti-KDM6B antibody. (b) Knockdown of KDM6B increased the level of H3K27me3 at the BMP2 promoter. BMP, bone morphogenetic protein; ChIP, chromatin immunoprecipitation; MSC, mesenchymal stem cell.

### KDM6B epigenetically regulates BMP expression in dental MSCs by removing the H3K27me3 mark

KDM6B is a histone demethylase that activates the targeting gene expressions by removing the methyl groups in H3K27me3.<sup>24</sup> Previously, it was shown that KDM6B regulates BMP expression in human BMSCs.<sup>14</sup> To examine whether KDM6B is directly involved in the epigenetic regulation of genes associated with odontogenic differentiation by demethylating H3K27me3, we performed the ChIP assay to assess the physical occupancy of KDM6B and the changes in the histone methylation status at the *BMP2* promoter region. We confirmed the direct binding of KDM6B to the promoter region of *BMP2* as shown previously.<sup>14</sup> *KDM6B* knockdown diminished the binding pattern of *KDM6B* in the *BMP2* promoter regions (Figure 4a), and the loss of *KDM6B* occupancy was mirrored by an increase in the amount of H3K27me3 (Figure 4b). Therefore, our data suggest that the recruitment of KDM6B to BMP promoters and removal of H3K27me3 is an important mechanism by which KDM6B epigenetically regulates expression of odontogenic-mediating genes such as *BMP2*.

### DISCUSSION

One of the most important goals of dental tissue engineering utilizing dental MSCs is to regenerate forms and functions of a tooth. Understanding the underlying mechanisms of odontogenic differentiation potential in these cells is therefore of significant interest in regenerative dental medicine. While dental MSCs resemble BMSCs in their potent ability to differentiate into mineralized tissues *in vitro* and *in vivo*, MSCs of different origins exhibit tissue-specific mechanisms of osteogenic differentiation at the transcriptional level.<sup>25–26</sup> Developmentally, dental MSCs are derived from neuroectoderm while BMSCs are derived from mesoderm. Indeed, direct comparisons between dental MSCs and BMSCs from the same donor revealed increased mineralization potential, a faster proliferation rate and a higher number of stem/progenitor cells in dental MSCs.<sup>27</sup> These disparities in stem cell properties suggest possibly similar but nuanced molecular mechanisms underlying cell fate decision depending on the sources of the cell origins.

KDM6B is a histone demethylase that has been shown to epigenetically regulate osteogenic differentiation of MSCs by removing H3K27me3 and subsequently activating genes associated with osteogenesis.<sup>14</sup> However, the role of KDM6B in the epigenetic regulation of dental MSCs has not been studied. Our present study showed that depletion of KDM6B inhibited odontogenic differentiation of dental MSCs and restoration of KDM6B in the knockdown cells abrogated the suppression of odontogenic potential. Mechanistically, upon induction, KDM6B removes H3K27me3 silencing marks at *BMP2* promoters, thus activating this odontogenic gene. Our findings confirmed that in dental MSCs, KDM6B is also an important epigenetic link in their differentiation towards odontoblasts.

In both BMSCs and dental MSCs, the osteogenic/odontogenic commitment was mediated by KDM6B activating *BMP2* which is primarily known to regulate cartilage and bone formation.<sup>28–29</sup> We also discovered distinct molecular mechanisms that reflect tissue specificity of the impact from KDM6B. While *Hox* family genes were activated by KDM6B in BMSCs, they were not expressed in dental MSCs (data not shown). Previously, we reported that in BMSCs, KDM6B knockdown did not affect *DLX* genes,<sup>14</sup> transcription factors that are well known to play especially important roles in craniofacial development.<sup>30–31</sup> Instead, *DLX2* expression was significantly altered in dental MSCs, an observation that is consistent with other reports that *DLX2* is essential for initial patterning of dentition as well as for biomineralization by regulating expression of ECM proteins including *OCN*.<sup>32–33</sup> *DLX2* is necessary in tooth development and odontogenic differentiation such that its inactivation resulted in dental defects and failure of normal dental development.<sup>34–35</sup> Recent study showed that odontoblast-specific terminal differentiation genes, dentin sialophosphoprotein and dentin matrix protein are induced by *BMP* signaling by stimulating the activity of several transcription factors including *DLX* gene.<sup>23</sup> We did not observe as significant induction of *DLX5* as in *DLX2* and this finding is consistent with greater expression of *DLX2* than *DLX5* in dental pulp clonal cell lines.<sup>36</sup> Therefore, it may be possible that, unlike in BMSCs, *DLX2* is the downstream target gene of KDM6B and plays a critical role in odontogenic differentiation of dental MSCs. Other *DLX* family members such as *DLX3* and *DLX5* found to be involved in osteogenic commitment of BMSCs were epigenetically regulated by KDM4B to mediate *BMP2* signaling, activate *RUNX2* and regulate *OCN* expression.<sup>37–38</sup> However, in dental MSCs, neither *DLX3* nor *DLX5* genes were suppressed by KDM6B depletion (data not shown), thus implicating that finer regulatory mechanisms exist to ensure that varied sets of genes are switched on in different tissue microenvironments.

The histone demethylase KDM6B has also been reported to play critical roles in controlling cell fate of stem cells. Recent reports have linked KDM6B to the key epigenetic factor facilitating differentiation of progenitor cells into epidermal,<sup>39</sup> endodermal,<sup>40–41</sup> as well as mesodermal lineages.<sup>14,17–18</sup> In human embryonic stem cells, KDM6B is temporally associated with activation of *WNT3* and *DKK1* to drive differentiation from pluripotent cells to mesendoderm, then to definitive endoderm.<sup>40</sup> In addition, KDM6B associates with *Tbx-3* at the enhancer of *EOMES* gene to allow chromatin reconfiguration, which further stimulates *EOMES* expression to drive the stem cells towards definitive endoderm.<sup>41</sup> Similarly, during neuronal and epidermal differentiation, KDM6B occupancy at the promoters of lineage-specific transcription factors increases when stem cells exit the poised state, and the accompanying removal of H3K27me3 repressing marks leads to

transcriptional activation of related genes. This mechanism holds true for osteogenic/odontogenic differentiation of MSCs as well. Of note, KDM6B could also achieve chromatin reconfiguration independent of its demethylase activity in Th1 differentiation.<sup>42</sup> However, in our case, we find the demethylase activity to be essential,<sup>14</sup> consistent with KDM6B activity in other lineage programs of stem cell differentiation.

The therapeutic function of MSCs relies on the intricate mechanisms that control the fate and delineation of these multipotent cells.<sup>43–46</sup> MSC commitment to different lineages is controlled by transcriptional and epigenetic regulatory factors; thus, understanding the pathways that MSCs undergo when differentiating into odontoblasts may guide MSCs to make lineage-specific decisions for therapeutic applications. Here, we provide evidence that differentiation of dental MSCs to odontogenic lineages is epigenetically regulated by the histone demethylase, KDM6B. Our finding of this novel epigenetic approach to direct dental MSC fate into odontoblasts can have potentially innovative clinical implications in regenerative dentistry. With a better understanding of factors influencing differentiation potential of dental MSCs, dental MSCs may be the key to the repair of craniofacial defects and therapeutic regeneration of a living and functioning tooth to replace a damaged or decayed one.<sup>47</sup>

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