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Original Article

Lysine demethylase 3A promotes chondrogenic differentiation of aged human dental pulp stem cells

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KEYWORDS
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Abstract *Background/purpose:* Aging severely impairs the beneficial effects of human dental pulp stem cells (hDPSCs) on cartilage regeneration. Lysine demethylase 3A (KDM3A) is involved in regulating mesenchymal stem cells (MSCs) senescence and bone aging. In this study, we investigated the role of KDM3A in hDPSCs aging and whether KDM3A could rejuvenate aged hDPSCs to enhance their chondrogenic differentiation capacity.
Materials and methods: The cellular aging of hDPSCs was evaluated by senescence-associated β-galactosidase (SA-β-gal) staining. Protein levels were determined using Western blot analysis. KDM3A was overexpressed in aged hDPSCs by lentivirus infection. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) were used to determine the mRNA levels of stemness markers. Toluidine blue staining was used to evaluate the effect of KDM3A overexpression on the chondrogenic differentiation of aged hDPSCs.
Results: hDPSCs at passage 12 or treated with etoposide exhibited augmented cellular senescence as evidenced by increased SA-β-gal activity. KDM3A was significantly increased during senescence of hDPSCs. Overexpression of KDM3A did not affect the stemness properties but significantly promoted the chondrogenic differentiation of aged hDPSCs.
Conclusion: Our findings indicate that KDM3A plays an important role in the maintenance of

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the chondrogenic differentiation capacity of aged hDPSCs and suggest that therapies targeting KDM3A may be a novel strategy to rejuvenate aged hDPSCs.

Introduction

The high prevalence and limited treatment options of cartilage diseases produce significant clinical and economic burdens. Current clinical treatment of cartilage defects includes microfracture surgery, autologous chondrocyte transplantation, osteochondral autografts or allografts, and joint replacement, etc.[1–3] Despite their common uses in the clinic, notable limitations and drawbacks including shortage of chondrocyte source, long chondrocyte harvesting time, immunorejection, as well as limited life of the prosthesis still exist.[1,4,5] Over the last few decades, mesenchymal stem cell (MSC)-based therapy has emerged as a promising alternative treatment for cartilage regeneration.[6] Accumulating evidence has demonstrated that transplantation of MSCs can mediate cartilage regeneration and alleviate osteoarthritis symptoms through its self-renewal, immunomodulation and multipotent properties.[7,8] Human dental pulp stem cells (hDPSCs), isolated from the dental pulp of exfoliated deciduous teeth or discarded permanent teeth, are a promising source of stem cells for cartilage regeneration therapies because of their easy accessibility, plasticity, high proliferative ability and immunomodulatory properties.[9,10]

However, MSC-based tissue regeneration therapies often require extensive expansion *in vitro* to obtain sufficient numbers of stem cells. Cultured MSCs do not grown indefinitely but undergo only a limited number of cell divisions, so called cellular senescence.[11] Aging dramatically impairs the functions of MSCs, as evidenced by decreased multidifferentiation potential and increased cellular senescence, thereby severely reducing their capacity of cartilage regeneration.[12] It has been shown that the related biological mechanisms of cellular aging are related to cartilage degeneration.[13] Furthermore, the chondrogenic potential of chondrocytes derived from aged MSCs is comparatively lower, and they exhibit a hypertrophic phenotype.[14] This leads to the limited capacity of aged MSCs for regenerating hyaline cartilage, presenting a significant challenge for their application in cartilage repair and regeneration. Therefore, understanding the aging mechanisms of MSCs is pivotal to improving the efficiency of cartilage regeneration and identifying novel therapeutic targets.

As a member of histone demethylase family, lysine demethylase 3A (KDM3A) exerts demethylase activity by using mono- and di-methylated histone H3 lysine 9 as the preferred substrate.[15] Studies have reported that KDM3A plays key roles in metabolic modulation, promoting cancer progression, spermatogenesis and stem cell regulation.[15–17] Furthermore, KDM3A has been reported to regulate MSCs senescence and bone aging via condensin-mediated heterochromatin reorganization.[18] KDM3A depletion aggravates cellular senescence by promoting DNA damage response, whereas KDM3A overexpression blunts DNA damage response by promoting heterochromatin reorganization. In addition, KDM3A plays an important role in the maintenance of chondrocyte homeostasis in adult articular cartilage.[19] However, the precise function of KDM3A in relation to the chondrogenic differentiation of aged hDPSCs remains elusive.

In this study, we explored the role of KDM3A in replicative and etoposide (ETO)-induced cellular aging of hDPSCs. Meanwhile, we examined the effect of KDM3A on chondrogenic potential of aged hDPSCs.

Materials and methods

Cell culture

Human dental pulp stem cells (hDPSCs) were obtained from dental stem cell bank (Taisheng Biological Technology, Beijing, China). hDPSCs were cultured in α-minimum essential medium (α-MEM, Gibco, Grand Island, CA, USA) with 1% penicillin streptomycin (Gibco) and 10% fetal bovine serum (Procell, Wuhan, China) in a 5% CO_2 incubator at 37 °C.

Senescence-associated β-galactosidase staining

Senescent cells were stained by a senescence-associated β-galactosidase (SA-β-gal) kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. For spontaneous senescence, hDPSCs at passages 4 and 12 were harvested and fixed for SA-β-Gal staining. For induced senescence, hDPSCs were treated with 2 μM etoposide (ETO) for 48 h. Then, cells were cultured in normal medium for another 5 days before SA-β-gal staining was performed.

Western blot analysis

For Western blot analysis, hDPSCs were harvested, and lysed by vortexing in RIPA lysis buffer (Beyotime) containing a protease inhibitor cocktail. Lysate protein was quantified using the BCA method (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins were used for electrophoresis on 10% SDS-PAGE gels and transferred to PVDF membranes for 2 h at 200 mA. The membrane was blocked with 5% skim milk prepared in TBST and incubated overnight with primary antibodies at 4 °C. The membranes were then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The primary antibody against KDM3A and SOX9 were purchased from Zen-Bioscience, Inc.

(ZenBio, Chengdu, China). GAPDH or β-Actin was used as an internal control.

Viral infection

Adenoviruses expressing KDM3A and the control vector were constructed by GenePharma (GenePharma, Shanghai, China). The cells were cultured until confluence reached approximately 30—50%, and transfection was conducted. After 12 h, the fresh medium was replaced and cell growth was observed under a microscope. Blasticidin S (10 μg/mL) was added to the media the next day, and the screening medium was replaced every 2 days. When all cells in the untransfected group died, the Blasticidin S concentration was reduced to 1 μg/mL and the selection pressure was maintained.

CCK-8 assay

Cell survival rates were estimated by CCK-8 kit (Dojindo, Shanghai, China). Cells were infected with adenoviruses expressing KDM3A and the control vector. Then, 5000 cells were seeded in 96-well plates with 100 μl medium each well. Each well was incubated with CCK-8 reagent (10 μl) and α-MEM (90 μl) for 2 h. CCK-8 assay was performed at 1, 3, 5 and 7 days.

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

RNAs were extracted from the cultured cells using TRIzol. Full-length cDNA was synthesized using a cDNA synthesis kit (Transgen, Beijing, China). qPCR was performed according to the manufacturer's instructions (Transgen), using three independent RNA preparations as biological replicates. Human GAPDH gene transcripts were used as an internal control. The relevant primer sequences are given in Table 1.

Toluidine blue staining

hDPSCs at passage 12 were cultured in complete medium until they reached sub-confluence. The medium was then replaced by stem cell chondrogenic differentiation media (Cyagen, Guangzhou, China) and the medium were changed every 3 days. After 2 weeks of chondrogenic induction, cells were rinsed with PBS and fixed in 4% paraformaldehyde for 30 min. After fixation, cells were rinsed with PBS and stained with 1% toluidine blue (Solarbio, Beijing, China) for 30 min. Cells were rinsed with PBS to neutralize the acidity. After adequate washing, they were visualized under a light microscope, and images were captured for analysis. To quantify proteoglycan synthesis, toluidine blue was extracted using 4 M guanidine-HCl overnight 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 using a duplicate plate.

Statistical analysis

Statistical analysis was performed with SPSS 24.0. All results are presented as the mean and standard deviation (mean ± SD). Data were assessed via independent 2-tailed Student's t test. For all tests, $P < 0.05$ was considered as statistical significance.

Results

To characterize cellular senescence, we induced senescence of hDPSCs by repeated sub-culture. Senescence-associated-β-galactosidase (SA-β-gal) activity was then used to evaluate cellular senescence in early versus late passage hDPSCs. We found an increase SA-β-gal activity in hDPSCs at passage 12 (Fig. 1A and B) and subsequently performed a Western blot analysis to observe whether protein level of KDM3A changed during cellular aging in hDPSCs. Western blot results showed that the protein abundance of KDM3A significantly decreased after hDPSCs senescence, accompanied by the decreased expression of SOX9, the master transcription factor for MSC chondrogenesis (Fig. 1C). To comprehensively elucidate the expression of KDM3A in aged hDPSCs, we further employed ETO to induce cell senescence. SA-β-gal staining showed that ETO treatment successfully induced senescence of hDPSCs (Fig. 2A and B). Consistently, the expression of KDM3A and SOX9 were significantly decreased in ETO-induced hDPSCs (Fig. 2C).

To further explore the effect of KDM3A on MSC chondrogenesis, lentiviral KDM3A or the control vector was transduced into hDPSCs. CCK-8 assay revealed that the

Table 1 Sequences of primers used for qPCR.

Gene symbol	Forward primer (5′—3′)	Reverse primer (5′—3′)
NANOG[a]	CTCCATGAACATGCAACCTG	CTCGCTGATTAGGCTCCAAC
OCT4A[b]	AGTGAGAGGCAACCTGGAGA	GTGAAGTGAGGGCTCCCATA
OCT4B[c]	TATGGGAGCCCTCACTTCAC	CAAAAACCCTGGCACAAACT
SOX2[d]	TCCACACTCACGCAAAAACC	AGTCCCCCAAAAAGAAGTCCAG
GADPH[e]	AATTCCATGGCACCGTCAAG	ATCGCCCCACTTGATTTTGG

[a] NANOG, Nanog homeobox.
[b] OCT4A, Octamer-binding transcription factor 4A.
[c] OCT4B, Octamer-binding transcription factor 4B.
[d] SOX2, SRY-box transcription factor 2.
[e] GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

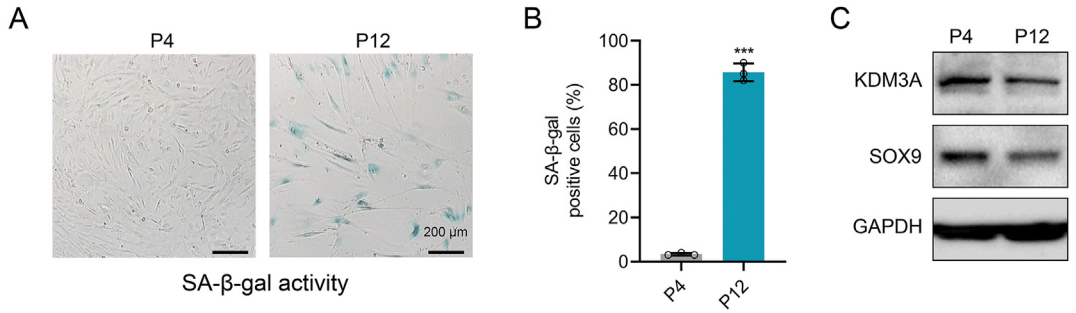

Figure 1 KDM3A is downregulated in replicative aging of hDPSCs. (A) Senescence-associated β-galactosidase (SA-β-gal) activity staining was carried out for assessing senescence of hDPSCs at passage 4 (P4) and passage 12 (P12). Scale bars: 200 μm. (B) Quantitative analysis of SA-β-gal positive cells in hDPSCs at P4 and P12. (C) Western blot analysis showing the total protein levels of KDM3A and SOX9 in hDPSCs at P4 and P12. Each bar represents mean ± SD; n = 3 per group; ***$P < 0.001$.

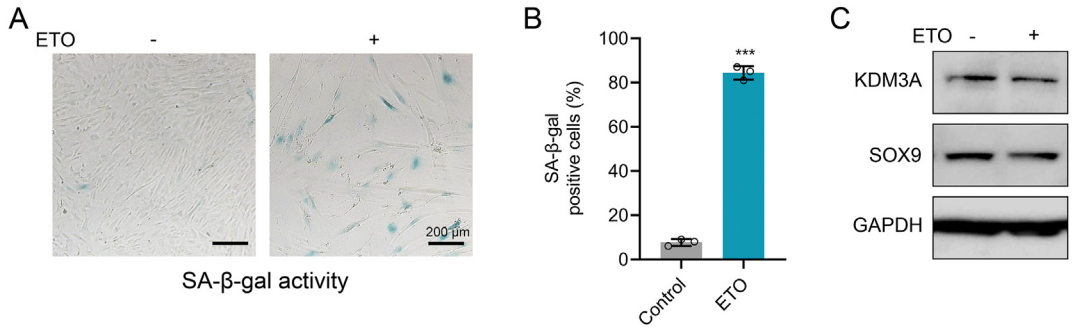

Figure 2 KDM3A is downregulated in etoposide (ETO)-induced senescence of hDPSCs. (A) Senescence-associated β-galactosidase (SA-β-gal) activity staining was carried out for assessing senescence of hDPSCs after ETO treatment. Scale bars: 200 μm. (B) Quantitative analysis of SA-β-gal positive cells in hDPSCs after ETO treatment. (C) Western blot analysis showing the total protein levels of KDM3A and SOX9 in hDPSCs after ETO treatment. Each bar represents mean ± SD; n = 3 per group; ***$P < 0.001$.

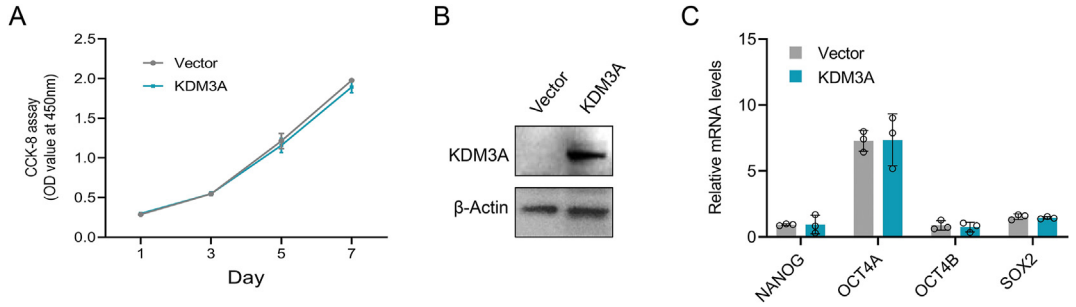

Figure 3 Effect of KDM3A overexpression on aged hDPSCs. (A) CCK-8 assay showing the cytotoxicity of KDM3A overexpression to hDPSCs. (B) Western blot analysis showing the enhanced expression of KDM3A after hDPSCs transduced with lentiviral-KDM3A. (C) RT-qPCR showing the expression of stemness markers in hDPSCs at passage 12. Each bar represents mean ± SD; n = 3 per group.

proliferation of hDPSCs was not affected by KDM3A overexpression (Fig. 3A). As demonstrated by immunoblot, KDM3A protein was greatly increased by lentivirus-mediated overexpression (Fig. 3B). Next, we investigated the effect of KDM3A overexpression on stemness properties of hDPSCs. RT-qPCR showed that the expression of three stemness markers, NANOG, OCT4 and SOX2, did not change significantly in KDM3A-overexpressed hDPSCs (Fig. 3C). Cultured under chondrogenic induction conditions, we found that ectopic expression of KDM3A enhanced chondrogenic differentiation capacity of aged hDPSCs, as indicated by the augmented proteoglycans (Fig. 4A). KDM3A-overexpressed cells produced approximately two times more proteoglycans than the negative control group (Fig. 4B).

Discussion

Aging is associated with a decrease in the capacity of stem cells to self-renew and regenerate,[20] so understanding the characteristics and potential molecular mechanisms of hDPSCs aging may help to improve the therapeutic effects of stem cells. This is the first study showing the involvement

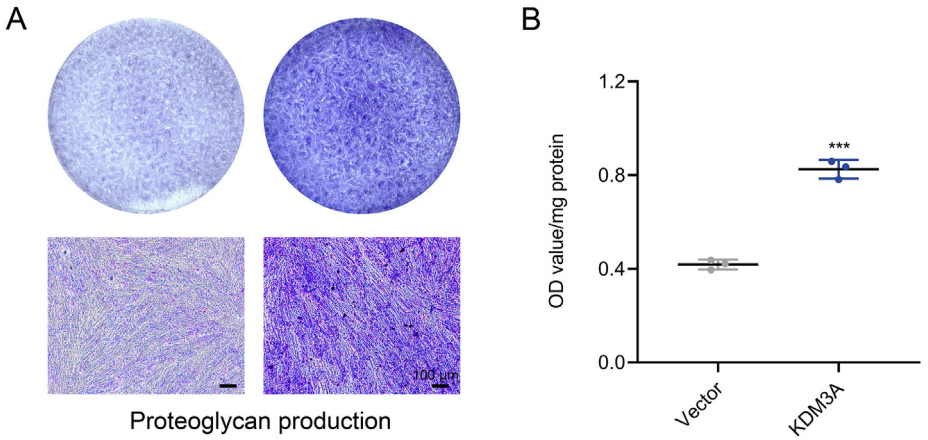

Figure 4 KDM3A promotes chondrogenic differentiation of aged hDPSCs. (A) Toluidine blue staining revealing proteoglycan production of replicative aged hDPSCs after induction with chondrogenic medium for 2 weeks. Scale bars: 100 μm. (B) Quantification of proteoglycans synthesis of toluidine blue staining. Each bar represents mean ± SD; n = 3 per group; ***$P < 0.001$.

of KDM3A in chondrogenic differentiation of aged hDPSCs. In this study, the epigenetic regulator KDM3A was found to be downregulated and drive chondrogenesis in aged hDPSCs. KDM3A overexpression enhanced the potential of aged hDPSCs to differentiate into chondrogenic cells, potentially leading to the development of future tissue engineering therapies for cartilage repair. This discovery highlights the importance of epigenetic regulation in stem cell differentiation and provides insight into strategies for enhancing the differentiation potential of stem cells.

Mounting evidence has shown that epigenetic regulation, including histone modifications, chromatin remodeling, and DNA methylation, are commonly associated with the MSC aging process and may contribute to MSC senescence-related manifestation.[21–23] For example, histone deacetylases (HDACs) mediate self-renewal of MSCs by balancing polycomb group genes and lysine demethylase 6B (KDM6B) expression.[24] It has been shown that upregulation of miR-155-5p lead to elevated cell senescence, while downregulation of miR-155-5p reduce MSC senescence.[25] Moreover, KDM4B ablation induces senescence-associated heterochromatin foci formation to hinder MSC self-renewal and exacerbate MSC exhaustion.[26] This provides a plausible mechanism to explain the phenomenon of stem cell exhaustion with aging. Moreover, DNA methyltransferases (DNMTs) isoforms DNMT1 and DNMT3B are significantly decreased during cellular senescence of human umbilical cord blood-derived multipotent stem cells.[27] Similarly, in this study, the expression of KDM3A is suppressed during senescence of hDPSCs. This finding provides crucial insights into the intrinsic relationship between epigenetic regulation and aging process of MSCs.

It is well-known that articular cartilage has a minimal ability for self-repair because of its avascular and aneural nature.[1,28] Over the past few decades, transplantation of hDPSCs has demonstrated promising results on cartilage regeneration in animal studies due to its easy accessibility, high proliferative ability, multi-lineage potential, and immunomodulatory properties.[29–31] However, MSCs cultured in replicative sub-culture or isolated from older patients undergo senescence, leading to a dramatic decline in cell homeostasis and tissue regeneration capacity.[32] Consistently, in the current study, hDPSCs in late passage or treated with ETO displayed an increased level of SA-β-gal activity and decreased chondrogenic differentiation capacity. These results suggested that cellular senescence impairs the multi-lineage potential. Therefore, it is of great significance to rejuvenate aged MSCs to increase their therapeutic efficacy for cartilage defects. Indeed, the aging of hDPSCs has attracted a lot of attention, and several preliminary research results have been reported. A study has reported that toll-like receptor 4 (TLR4) is involved in regulating cellular senescence in hDPSCs.[33] TLR4 is a molecular initiator of cellular senescence, and visfatin induces cellular senescence via TLR4 in hDPSCs. Moreover, serine metabolism and one carbon unit contribute to hDPSCs aging by providing less methyl donor to DNA methylation.[34]

In conclusion, the alteration of KDM3A expression may have implications for the aging process of hDPSCs and play a role in the maintenance of the multilineage differentiation capacity of MSCs. Consequently, therapies targeting KDM3A may help to increase the lifespan of hDPSCs and improve their cartilage regenerative potential in the context of aging. More importantly, further investigation is required to fully understand the role of KDM3A in cellular aging and determine whether epigenetic rejuvenation through activation of KDM3A can be used as a new strategy to prevent and treat MSC aging.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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