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The epigenetic promotion of osteogenic differentiation of human adipose-derived stem cells by the genetic and chemical blockade of histone demethylase LSD1

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ABSTRACT

Human adipose-derived stem cells (hASCs) are a highly attractive source in bone tissue engineering. It has become increasingly clear that chromatin regulators play an important role in cell fate determination. However, how osteogenic differentiation of hASCs is controlled by epigenetic mechanisms is not fully understood. Here we use genetic tools and chemical inhibitors to modify the epigenetic program of hASCs and identify lysine-specific demethylase 1 (LSD1), a histone demethylase that specifically catalyzes demethylation of di- and mono- methyl histone H3 lysine 4 (H3K4me2/1), as a key regulator in osteogenic differentiation of hASCs. Specifically, we demonstrated that genetic depletion of LSD1 with lentiviral strategy for gene knockdown promoted osteogenic differentiation of hASCs by cell studies and xenograft assays. At the molecular level, we found that LSD1 regulates osteogenesis-associated genes expression through its histone demethylase activity. Significantly, we demonstrated LSD1 demethylase inhibitors could efficiently block its catalytic activity and epigenetically boost osteogenic differentiation of hASCs. Altogether, our study defined the functional and biological roles of LSD1 and extensively explored the effects of its enzymatic activity in osteogenic differentiation of hASCs. A better understanding of how LSD1 influences on osteogenesis associated epigenetic events will provide new insights into the modulation of hASCs based cell therapy and improve the development of bone tissue engineering with epigenetic intervention.

1. Introduction

Human adipose-derived stem cells (hASCs), one type of mesenchymal stem cells, possess a high plasticity to differentiate into multiple lineages including cartilage, bone, muscle and adipose in the presence of appropriate signaling factors and culture conditions [1–5]. Currently, hASCs have become a highly attractive source in tissue engineering and cell-based therapy of damaged bone defects, spinal fusion and skeletal reconstruction bone [2,6,7]. The main reason lies in the fact that hASCs can be obtained easily from adipose tissues carrying a more abundant and accessible pool of mesenchymal stem cells with a less invasive and less expensive procedures [8–10].

The critical issue for application of stem cells in tissue engineering is the initiation and control of cellular differentiation in a precise and appropriate manner. Recent evidence suggests that epigenetic regulation including DNA methylation and histone modification plays a key role in fate maintenance and lineage commitment of embryonic stem cells as well as mesenchymal stromal/stem cells [11–16]. Slight variations of these epigenetic

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components might result in the changes of local chromatin configuration or nuclear architecture, impose flexible but precise control over the expression of the important regulatory genes, and eventually influence on cell fate determination without changes of the DNA sequences. Unlike genetic alterations, epigenetic changes are reversible and accessible to be regulated, and as such, drugs that target critical epigenetic regulators in cell commitment or differentiation are being overwhelmingly investigated [17,18].

Lysine-specific demethylase 1 (LSD1/KDM1A) is a flavin adenine dinucleotide (FAD)-dependent amine oxidase that catalyzes mono- and di-methyl moieties removal from histone H3 lysine 4 (H3K4) [19–21]. Despite progress in understanding the dynamic histone methylation regulation and in revealing the diverse molecular interactions for LSD1, the biological function of LSD1 is just beginning to be uncovered. Recent studies have linked LSD1 to certain high-risk tumors [22–30]. Moreover, LSD1 has been identified as one of the chromatin regulators implicated in the control of early embryogenesis [15,31–33]. In addition, it was reported that LSD1 represses hematopoietic stem and progenitor cell signatures during blood cell maturation [34]. Indeed, within the framework of the so-called epigenetic therapies, there is a growing interest in LSD1 as a potential drug target [26,35–38]. However, whether histone methylation associated epigenetic events impacted by LSD1 and currently developed LSD1 inhibitors can contribute to mesenchymal stem cells differentiation as well as bone tissue engineering are largely unknown.

Here, our study focused on investigating the functional role and the molecular mechanism of histone H3K4 demethylase LSD1, especially its catalytic activity in osteogenic differentiation of hASCs.

2. Materials and methods

2.1. Cell culture, osteogenic induction and LSD1 inhibitors

Human adipose-derived stem cells (hASCs) were purchased from ScienCell Research Laboratories (San Diego, CA). Stem cells from 3 donors with different lot numbers of the third passage were used for the *in vitro* and *in vivo* experiments. All cell-based *in vitro* experiments were repeated in triplicate. Osteogenic differentiation was induced with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 IU/ml penicillin/streptomycin, 100 nM dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerophosphate. Pargyline (Sigma, St. Louis, MO) at the concentration of 3 mM, and CBB1007 (Millipore, Billerica, MA) at the concentration of 10 μM, 20 μM and 50 μM were used to induce osteogenic differentiation of hASCs. The hASCs with vehicle or LSD1 inhibitor treatment were cultured for 14 days before collection.

2.2. Plasmid constructions

Wild-type human LSD1 (LSD1-wt) was amplified from pcDNA3-LSD1, a gift from Dr. Yan Wang (Tianjin Medical University). The amplicons were then digested using NotI and BsrgI endonuclease (Fermentas, Hanover, MD) and incorporated into the PITA lentiviral vector. The LSD1-mt (K661A) was created following standard point mutation procedures with PITA-LSD1-wt as template using the following primers (F: GATTTGGCAACCTTAACGCGGTGGTGTTGTGTTTTG; R: CAAAACACAACACCACCGCGTTAAGGTTGCCAAATC). LSD1 siRNA Sequence (GAGACAGACAAATACTTG) was designed by White Head Institute Online Server and the DNA oligoes (F: TGAGACAGACAAATACTTGATTCAAGAGATCAAGTATTTGTCTGTCTCTTTTTTC; R: TCGAGAAAAAA-GAGACAGACAAATACTTGATCTCTTGAATCAAGTATTTGTCTGTCTCA) were cloned into the pLL3.7 shuttle vector with an independent cassette encoding enhanced green fluorescent protein (EGFP). The control siRNA sequence is GATATGGGCTGAATACAA and the corresponding DNA oligoes sequences are F: TGATATGGGCTGAATACAAATTCAAGAGATTTGTATTCAGCCCATATCTTTTTTC; R: TCGAGAAAAAAGATATGGGCTGAATACAAATCTCTTGAATTTGTATTCAGCCCATATCA.

2.3. Lentiviral production and infection

The recombinant overexpression construct or shRNA construct, as well as three helper vectors (pMDLg/pRRE, pRSV-REV and pVSVG) were transiently transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The viral supernatants were collected 48 h later, clarified by filtration and concentrated by ultracentrifugation. The concentrated viruses were then used to infect hASCs of passage 3 at a multiplicity of infection (MOI) of 100 in the presence of 8 μg/ml polybrene (Sigma). Twenty four hours later, the lentiviral containing medium were removed and replaced with fresh growth medium. The proliferation medium was replaced with differentiation medium when cells grew up to 80%–90% of confluence and the differences of osteogenic differentiation ability between the experimental group and control group were examined.

2.4. Alkaline phosphatase (ALP) activity of hASCs

The hASCs were seeded in 6-well plates, and ALP activity was determined by staining with nitro blue tetrazolium (NBT) and 5-bromo-4-chroro-3-indolyl phosphate (BCIP). For quantification of ALP activity, cells seeded in 6-well plates were rinsed two times with phosphate-buffered saline (PBS), followed by trypsinization and then scraping in distilled water. This was followed by three cycles of freezing and thawing. ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP) as the substrate. Total protein contents were determined with the BCA method using the Pierce (Thermo Fisher Scientific, Rockford, IL, www.piercenet.com) protein assay kit in aliquots of the same samples, which were read at 562 nm and calculated against a series of bovine albumin (BSA) standards. Relative ALP activity to the control treatment was calculated after normalization to the total protein content.

2.5. Mineralization assays for hASCs

The hASCs were seeded in 6-well plates, and mineralization was determined by staining with Alizarin red S. To quantify matrix mineralization, Alizarin red S-stained cultures were incubated in 100 mM cetylpyridinium chloride for 1 h to solubilize and release calcium-bound Alizarin red S into the solution. The absorbance of the released Alizarin red S was measured at 562 nm. Relative Alizarin red S intensity to the control treatment was calculated after normalization to the total protein content.

2.6. In vivo implantation of hASCs and Bio-Oss collagen scaffold hybrids

The hASCs (1×10^6) infected with lentivirus carrying control siRNA or LSD1 siRNA, were trypsinized and re-suspended directly into DMEM. The cells were then incubated with 7 mm × 4 mm × 2 mm Bio-Oss Collagen® (Geistlich, GEWO GmbH, Baden–Baden, Germany) scaffolds for 1 h at 37 °C with gently shaking followed by centrifugation at 150 g for 5 min. The collected hASCs-seeded scaffolds were implanted into the dorsal subcutaneous space of the 4–6-weeks old, BALB/c homozygous nude (nu/nu) mice (Peking University Experimental Animal Center) that had been randomly divided into two groups ($n = 5$ per group). One transplantation site was prepared in each mouse and transplanted with either scaffolds carrying control hASCs or LSD1 knockdown hASCs. This study was approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China (PKUSSIRB-2013023) and all animal experiments were performed in accordance with the institutional animal guidelines.

2.7. Analyses of bone formation in vivo

Specimens were harvested at eight weeks after implantation, and animals were sacrificed by CO_2 asphyxiation. The specimens taken as a whole were radiographed with digital radiographic apparatus (GE Senograph 2000D, USA). Gray scales of five specimens in each group were then analyzed with medical image analyzing software (Image J, NIH). The mean density of hASCs-scaffold complex was presented as mean ± S.D. The bone constructs were fixed in 4% paraformaldehyde and then decalcified for 10 days in 10% EDTA (pH 7.4). After decalcification, the specimens were dehydrated and subsequently embedded in paraffin. Sections (5 mm thickness) were stained with hematoxylin and eosin (H&E) and Masson's trichrome. Meanwhile, osteogenesis was evaluated with immunohistochemical analysis for osteopontin (OPN), osteocalcin (OC) and GFP (sp kit, Vector, Burlingame, CA, primary antibodies were purchased from Santa Cruz Biotechnology, CA). Specimens were processed using identical protocols. For quantification of bone-like tissue, 3 images of each sample (15 images for each group) were taken randomly by microscope (Olympus, Tokyo, Japan) and Image-Pro Plus software (Media Cybernetics, Rockville, MD) was used to measure the area of new bone formation (osteoid or organized extracellular matrix) versus total area or mean density (total density of positive staining/cell containing tissue area) of immunohistochemical staining. Box-plot was used to exhibit the semi-quantitative results.

2.8. RNA extraction, reverse transcription, and quantitative real-time PCR

Total cellular RNAs from hASCs cultured in proliferation or differentiation medium for 14 days were isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and used for first strand cDNA synthesis with the Reverse Transcription System (Promega, Madison, WI). Quantifications of all gene transcripts were performed by real-time RT-PCR using a Power SYBR Green PCR Master Mix and an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) with the expression of *GAPDH* detected as the internal control. The primers used were: *ALP*, (forward) 5′-ATGGGATGGGTGTCTCCACA-3′ and (reverse) 5′-CCACGAAGGGGAACTTGTC-3′; *OC*, (forward) 5′-CACTCCTCGCCCTATTGGC-3′ and (reverse) 5′-CCCTCCTGCTTGGACACAAAG-3′; *OSX*, (forward) 5′-CCTCTGCGGGACTCAACAAC-3′ and (reverse) 5′-TAAAGGGGGCTGGATAAGCAT-3′; RUNX2, (forward) 5′-CCGCCTCAGTGATTTAGGGC-