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Mechanical stimulation promote the osteogenic differentiation of bone marrow stromal cells through epigenetic regulation of Sonic Hedgehog

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ABSTRACT

Mechanical unloading leads to bone loss and disuse osteoporosis partly due to impaired osteoblastogenesis of bone marrow stromal cells (BMSCs). However, the underlying molecular mechanisms of this phenomenon are not fully understood. In this study, we demonstrated that cyclic mechanical stretch (CMS) promotes osteoblastogenesis of BMSCs both *in vivo* and *in vitro*. Besides, we found that Hedgehog (Hh) signaling pathway was activated in this process. Inhibition of which by either knockdown of Sonic hedgehog (Shh) or treating BMSCs with Hh inhibitors attenuated the osteogenic effect of CMS on BMSCs, suggesting that Hh signaling pathway acts as an endogenous mediator of mechanical stimuli on BMSCs. Furthermore, we demonstrated that Shh expression level was regulated by DNA methylation mechanism. Chromatin Immunoprecipitation (ChIP) assay showed that DNA methyltransferase 3b (Dnmt3b) binds to Shh gene promoter, leading to DNA hypermethylation in mechanical unloading BMSCs. However, mechanical stimulation down-regulates the protein level of Dnmt3b, results in DNA demethylation and Shh expression. More importantly, we found that inhibition of Dnmt3b partly rescued bone loss in HU mice by mechanical unloading. Our results demonstrate, for the first time, that mechanical stimulation regulates osteoblastic genes expression *via* direct regulation of Dnmt3b, and the therapeutic inhibition of Dnmt3b may be an efficient strategy for enhancing bone formation under mechanical unloading.

1. Introduction

Bone has the unique ability to modify its structure in response to changes in skeletal loading [1,2]. Previous reports show that skeletal loading is a key regulator of bone metabolism controlling bone turnover, growth and mineralization [3–5]. Unloading (reduced mechanical stress on bone) leads to significant bone loss, as evidenced by disuse osteoporosis, which is a critical issue for bedridden patients and astronauts [6,7]. Osteoporosis occurs in part when BMSCs fail to produce sufficient numbers of osteoblasts to counteract bone resorption by osteoclasts. The fate of BMSCs is determined through integration of chemical (including BMPs, Wnt, Notch and Hedgehog signaling), spatial, and physical signals [8,9]. *In vitro* studies have shown that mechanics play a key role in the regulation of BMSC differentiation into osteoblastic lineage, as well as metabolic activity of osteoblasts [10,11]. Although rapid progress has recently been achieved, the cellular and molecular mechanisms underlying the response of bone to unloading are just starting to emerge.

BMSC differentiation into certain lineage is strictly controlled by particular protein expression patterns resulting from specific patterns of gene expression [9]. Epigenetic modifications, one of the most important gene expression regulation mechanisms, alter gene expression in an inherited fashion by modulating transcription factor accessibility [12,13]. The main epigenetic mechanisms of gene regulation are DNA methylation and histone modification [14]. Specifically, DNA methylation at CpG dinucleotide either directly blocks transcription factor binding or binding of proteins that induce a more transcription-resistant condensed chromatin [15]. However, relatively

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little is understood about the regulatory role of DNA methylation during mechanical stimuli mediated osteogenesis of BMSCs.

In this study, we investigated the regulatory mechanisms of mechanical stimuli on osteogenic differentiation of BMSCs at cellular and molecular level. We identified Hedgehog (Hh) signal, which can be regulated by DNA methylation mechanism by mechanical stimuli, as an important regulator that mediated extracellular mechanical stimuli signal on osteogenic differentiation. Specifically, mechanical loading on BMSCs down regulates of Dnmt3b, leading to upregulation of Sonic hedgehog (Shh) and activation of Hh signal through DNA demethylation of Shh gene promoter. Our studies further demonstrate that targeting Dnmt3b partly rescued bone loss in mechanical unloading mice. This study may provide a novel mechanism and potential therapeutic target for enhancing bone formation under mechanical unloading conditions.

2. Material and methods

2.1. Hindlimb-unloading (HU) mice

6-month-old male C57BL/6 J mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd, and were individually caged under standard conditions (12 h light/12 h dark cycle, 21 °C controlled temperature). The animals were suspended from the hindlimb for a period of 28 days as described previously [16]. After euthanasia, bilateral femurs were dissected and processed for μCT examination and bone histomorphometric analysis. All the experimental procedures were approved by the Committees of the Shanghai Jiao Tong University School of Medicine (SJTUSM).

2.2. Cell culture

Bone marrow stromal cells from tibia and femur of mice were flushed out with DMEM and cultured in growth medium (DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (all from hyclone, Logan, UT, USA)) at 37 °C culture conditions in the presence of 5% CO_2, following lysis of red blood cells. Non-adherent cells were removed by replacing the medium after 3 days. The attached BMSCs were not used beyond passage 5.

2.3. Cyclic mechanical stretch application

BMSCs were seeded on six-well BioFlex™ culture plates (Flexcell International Corporation, Hillsborough, USA). Cells were cultured for 48–72 h to reach 90% confluency, at which time the cyclic mechanical stretch at 0.5 Hz sinusoidal curve at 10% elongation was applied (10% elongation, 80000 με, Sin, 0.5 Hz, CMS) using an FX-5000T™ for 12 h per day Flexercell Tension Plus™ unit (Flexcell International Corporation, Hillsborough, USA). Cells were harvested immediately when CMS stimulation finished.

2.4. Cell staining

For alkaline phosphatase (ALP) staining, the cultured cells were rinsed with PBS three times and fixed with 4% paraformaldehyde for 10 min at room temperature. Then cells were soaked in 0.1% naphthol AS-TR phosphate (Sigma Aldrich, St. Louis, MO) and 0.1% fast violet B salt (Sigma-Aldrich) in 56 mM 2-amino-2-methyll,3-propanediol (pH 9.9, Sigma-Aldrich) for 10 min at room temperature, washed with PBS, and were then observed under an digital camera. For ALP activity assay, BMSCs were scrapped from the dishes and suspended in ddH_2O before freezing and thawing for three times. ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP) (Sigma Aldrich) as the substrate. A 50 μl of sample was mixed with 50 μl of pNPP (1 mg/mL) in 1 M diethanolamine buffer containing 0.5 mM $MgCl_2$ (pH 9.8) and incubated at 37 °C for 15 min on a bench shaker. The reaction was stopped by adding of 200 mL of 2 M NaOH per 200 μl of reaction mixture. Total protein content was determined by the BCA method with protein assay kit (PIERCE, Rockford, IL). ALP activity was calculated as nmol p-nitrophenol per minute per mg protein, and presented as fold changes over control group. For Alizarin red staining, cells were fixed in 70% ice-cold ethanol for 1 h and rinsed with double-distilled H_2O (ddH_2O). Cells were stained with 40 mM Alizarin red S (Sigma-Aldrich), pH 4.0, for 15 min with gentle agitation. Cells were rinsed five times with ddH_2O and then rinsed for 15 min with 1× PBS while gently agitating. For the quantitative assessment of the degree of mineralization, the red stain was eluted by 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich) for 1 h and quantified *via* spectrophotometric absorbance measurements of optical density at 570 nm.

2.5. RNA isolation and quantitative real-time PCR

Total RNA from cells was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA by incubating for 1 h at 42 °C with Superscript III reverse transcriptase (Invitrogen, Mulgrave, Australia) following oligo (dT) priming. After reverse transcription reaction, quantitative realtime PCR (qRT-PCR) was performed by LightCycler480 system (Roche, Mannheim, Germany) using SYBR Premix (Takara, Dalian, China) according to the manufacturer's instructions. All amplifications were normalized by GAPDH. Data were analyzed using the comparison Ct ($2^{-\triangle\triangle Ct}$) method and expressed as fold change compared to respective control. Each sample was analyzed in triplicate. The primer sequences used in this study were as follows: GAPDH: forward, 5'-AGGTCGGTGTGAACGGATTTG-3'; reverse, 5'-GGGGTCGTTGATGGCAACA-3'; Runx2 forward, 5'-GACTGTGGTT ACCGTCATGGC-3'; reverse, 5'- ACTTGGTTTTTCATAACAGCGGA-3'; Col1a1: forward, 5'-GCTCCTCTTAGGGGCCACT-3'; reverse, 5'-ATTGGGGACCCTTAGGCCAT-3'; Ocn: forward, 5'-GACAAGT CCCACACAGCAACT-3'; reverse, 5'- GGACATGAAGGCTT TGTCAGA-3'; Shh: forward, 5'-AAAGCTGACCCCTTTAGCCTA-3'; reverse, 5'-TTCGGAGTTTCTTGTGATCTTCC-3'; Gli1: forward, 5'-CCAAGCCAACTTTATGTCAGGG-3'; reverse, 5'-AGCCCGCTT CTTTGTTAATTTGA-3'; Ptch1: forward, 5'-AAAGAACTGCGG CAAGTTTTTG-3'; reverse, 5'-CTTCTCCTATCTTCTGACGGGT-3'; Dnmt3a: forward, 5'- GATGAGCCTGAGTATGAGGATGG-3'; reverse, 5'- CAAGACACAATTCGGCCTGG-3'; Dnmt3b: forward, 5'-CTGCGTGTAATTCAGAAGGCT-3'; reverse, 5'- CGTTAATGGGAAC TTCAGTGACC-3'.

2.6. Western blot analysis

For Western blot analysis, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1 mM PMSF and protease inhibitor cocktail (10 mg/mL leupeptin, 10 mg/mL pepstatin A, and 10 mg/mL aprotinin) on ice for 30 min. Protein fractions were collected by centrifugation at 15,000*g* at 4 °C for 10 min and then subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% BSA and incubated with specific antibodies overnight at 4 °C. A horseradish peroxidase–labeled secondary antibody was added and visualized using the enhanced chemiluminescence detection system (Millipore, Billerica, MA) as recommended by the manufacturer. Immunoreactive bands were quantitatively analyzed in triplicate by normalizing the band intensities to GAPDH on scanned films with Alpha Image software. Primary antibodies used in this study were: anti-Shh, anti-Ptch1, anti-Gli1, anti-Runx2, anti-Ocn, anti-Col1a1 mAb (1:1000, Abcam, Cambridge, UK), anti-Dnmt3a, anti-Dnmt3b, anti-GAPDH mAb (1:1000, Cell Signaling Technology, Inc).