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Autophagy activation facilitates mechanical stimulation-promoted osteoblast differentiation and ameliorates hindlimb unloading-induced bone loss

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ABSTRACT

Autophagy has been indicated to be involved in regulating bone metabolism. However, little is known about the role of autophagy in mechanical stimulation-influenced osteoblast differentiation and bone formation. In the present study, we first demonstrated that autophagy activation was essential for cyclic mechanical stretching-promoted osteoblast differentiation of bone marrow mesenchymal stem cells. To explore the in vivo role of autophagy in osteoblast differentiation, the hindlimb unloading-induced disuse osteoporosis model was used. Compared to the normal controls, hindlimb unloading led to abundant bone loss as well as lessened autophagy activation of osteoblasts. However, the activation of autophagy by ULK1 overexpression or in the presence of rapamycin significantly increased osteoblast differentiation activity and restored the bone volume. The findings implicate autophagy as a novel mechanosensitive pathway that regulates osteoblast differentiation. The pharmacological activation of autophagy may be an interesting approach for the prevention and treatment of disuse osteoporosis.

1. Background

Bone is a remarkable tissue that can respond to external stimuli, and the importance of mechanical stimuli on the mass and structural development of bone has long been accepted [1]. Decreased mechanical stimuli due to prolonged bed rest or hindlimb unloading (HU) could ultimately lead to the development of disuse osteoporosis and an increased risk of fractures [2–4]. The role of mechanical stimuli in promoting the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) has been reported [5–7]. However, the precise molecular events that govern the transformation of the mechanical signals into biochemical responses remain poorly understood.

Autophagy is an evolutionarily conserved physical molecular process that allows cells to degrade unnecessary or dysfunctional cellular organelles, which is essential for cell survival, differentiation, and development, and homeostasis [8,9]. During this process, the cytoplasmic components targeted for degradation are delivered to lysosomes upon sequestration within double-membrane vesicles termed autophagosomes [10]. Autophagy occurs at low levels in all cells to ensure the homeostatic turnover of long-lived proteins and organelles and is upregulated by various stress conditions [11,12]. Deregulation of autophagy is associated with diverse disease states, such as cancer, neurodegeneration, microbial infection, and with aging [13]. In addition, autophagy-related proteins have been implicated as critical mediators of bone cell function in normal physiology and pathology [14]. Emerging evidence suggests that autophagy has a profound effect on osteoblast differentiation and is involved in both bone mineralization and homeostasis [15–19]. However, whether autophagy is involved in mechanical stretching-mediated osteoblast differentiation and bone metabolism is unclear.

Given the knowledge that autophagy has a profound effect on osteoblast differentiation and bone homeostasis, and that mechanical stretching promotes the differentiation of BMSCs to osteoblasts, we investigated whether autophagy is activated during the differentiation of BMSCs to osteoblasts that is promoted by mechanical stimuli, and to determine whether autophagy has potential functional relevance in this setting. Our findings uncover a vital facilitating role of autophagy in osteoblast differentiation in vitro and in vivo.

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2. Materials and methods

2.1. Animals and treatments

Six-month-old male C57BL/6J mice were purchased from Shanghai SIPPR-BK Laboratory Animal Co. Ltd (Shanghai, China) and reared under standard environment (12-h light/dark cycle, 22 °C controlled temperature, and free food and water). The HU mice were suspended for 28 days by the tail using a strip of adhesive surgical tape attached to a chain hanging from the top of the cage to reduce mechanical loading as previously described [20]. To induce autophagy, the HU mice were treated with rapamycin (3 mg/kg/day) during the period of tail suspension and the mice in control group were treated with vehicle for a total of 4 weeks. All the animal care procedures were conducted in accordance with the guidelines of our University Committee on Animal Use and Care.

2.2. Cell culture

The mouse BMSCs were purchased from Cyagen Biosciences (Guangzhou, China, MUBMX-01001). BMSCs were maintained in special C57BL/6 Mouse Mesenchymal Stem Cell growth medium (Cyagen Biosciences) with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. The cells were cultured at 37 °C in a 5% CO_2 humidified incubator and not used beyond passage 10. For the experiments, confluent cells were removed using 0.25% trypsin, resuspended in growth medium and passaged 1:3 onto 10-cm dishes. To induce osteoblastic mineralization, BMSCs were seeded onto six-well plates at a density of 2.0×10^5 cells per well and cultured in specialized osteogenic medium (Cyagen Biosciences) containing 10% FBS, 1% penicillin-streptomycin, 1% glutamine, 0.2% ascorbate, 1% β-glycerophosphate and 0.01% dexamethasone.

2.3. CMS application

BMSCs were seeded on six-well BioFlex culture plates carried flexible and collagen-I coated silicone rubber membranes (Flexcell International Corporation, Burlington, NC, USA). The cells were cultured for 24–48 h to reach 90% confluency, at which time the growth medium was replaced by osteogenic medium. Then, the cells were subjected to cyclic mechanical stretching (CMS) with a 0.5 Hz sinusoidal curve at 5% elongation using an FX-5000T Flexercell Tension Plus unit (Flexcell International Corporation). The cells were incubated in a humidified atmosphere at 37 °C and 5% CO_2 while stretching. When the loading ended, the cells were immediately harvested.

2.4. Adenovirus and GFP-LC3 transfection

BMSCs were seeded onto six-well plates, and adenoviral vector (containing GFP, ULK1 overexpression or ULK1 knockdown) was added at an MOI (multiplicity of infection) of 10. After 2 h, the medium containing adenovirus was changed with normal fresh medium. This same technique is used for autophagy-adenovirus of LC3 infection. At 48 h post-transfection, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, and then GFP puncta were detected with a fluorescence microscope (Leica, Germany). For the in vivo study, HU mice were anesthetized and their knees were sterilized with alcohol and flexed to 90°. A 25-μL microsyringe (Hamilton, Reno, NV, USA) was inserted carefully through the femoral notch and into the bone marrow of the femur [21]. The bilateral femurs were both accepted adenovirus injection (5×10^8 pfu per limb) once two weeks. The adenovirus was purchased from Hanbio Biotechnology Co., Ltd. (Shanghai, China).

2.5. ALP and alizarin red staining

BMSCs under osteogenic induction were rinsed with PBS for 3 times and fixed with 4% paraformaldehyde for 10 min. The ALP staining was conducted in the dark and assayed with the BCIP/NBT regent kit (Beyotime, Jiangsu, China). For the Alizarin Red staining, cells were stained with Alizarin Red solution (Cyagen Biosciences, Shanghai, China) for 5 min, rinsed 5 times with PBS and then imaged.

2.6. Quantitative real-time PCR

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. qRT-PCR was performed in a 20-μL reaction system by using SYBR Premix Ex TaqTM (TaKara, Dalian, China) and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). All genes were normalized to β-actin and performed in triplicate. The experimental results were analyzed using the method of $2^{-\Delta\Delta CT}$ (cycle threshold). The primer sequences used in this study were as follows: Ocn: forward, 5′- CCATCTTTCTGCTCACTCTGC-3′; reverse, 5′-ACCTTATTGCCCTCCTGCTT-3′; Runx2: forward, 5′-AGAACCCAGCACCTTGGAC-3′; reverse, 5′-CACAGGACGAGGATGGAGAT-3′; Osx: forward, 5′-AGGCACAAAGAAGCCATACG-3′; reverse, 5′-GGGAAGGGTGGGTAGTCATT-3′; β-actin: forward, 5′-CCTGGCACCCAGCACAAT-3′; reverse, 5′-GGGCCGGACTCGTCATAC -3′.

2.7. Western blotting analysis

Total proteins from bone tissues or cells were extracted by using the RIPA (Radio-Immunoprecipitation Assay) Lysis Buffer (Beyotime, Jiangsu, China). Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with specific primary antibodies and visualized using the enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA). Protein expression was quantified and normalized to GAPDH by using Image J software. The primary antibodies used in this study that were p62 (1:1000), ULK1 (1:1000), LC3 (1:1000) and GAPDH (1:1000) from Cell Signaling Technology (San Antonio, TX, USA) and Runx2 (1 μg/ml) from Abcam (Cambridge, MA, USA).

2.8. Micro-computed tomography analysis

Mice bilateral femurs were obtained, dissected to free them from the soft tissue, fixed overnight in 70% ethanol and analyzed by using a Scanco μCT 40 (Scanco Medical AG, Zurich, Switzerland) at a resolution of 18-μm. Three-/two-dimensional images were obtained to analyze various parameters of trabecular bone in the distal femur included the following: bone mineral density (BMD), bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp). The region of interest (ROI) was selected and originated 2.0 mm below the epiphyseal growth plate.

2.9. In situ hybridization

The femurs were treated and fixed for 1 h in freshly prepared 4% paraformaldehyde in 0.1 M PBS (pH 7.4), containing 1/1000 DEPC. The sections (4 μm) were prepared and utilized for hybridization by using a DIG RNA labeling and detection kit (Boster Biological Technology, Wuhan, China) following the manufacturer's protocol. The mRNA probe sequences targeting map1lc3b included 5′-AAAGAGTGGAAGATGTCCGGCTCATCCGGGAGCAGCACCC-3′and 5′-TCCCATCTCCGAAGTGTACGAGAGTGAGAGAGAGTAAGAC-3′. To

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