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ATP6V1H regulates the growth and differentiation of bone marrow stromal cells

Lin Li [a, b], Shaoqing Yang [a], Yanli Zhang [a], Dongrui Ji [a], Zuolin Jin [b, **], Xiaohong Duan [a, *]

[a] State Key Laboratory of Military Stomatology, National Clinical Research Center for Oral Diseases, Shaanxi Key Laboratory of Oral Diseases, Department of Oral Biology, School of Stomatology, The Fourth Military Medical University, 145 West Changle Road, Xi'an, Shaanxi, 710032, PR China
[b] State Key Laboratory of Military Stomatology, Department of Orthodontics School of Stomatology, The Fourth Military Medical University, 145 West Changle Road, Xi'an, Shaanxi, 710032, PR China

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ABSTRACT

ATP6V1H encodes subunit H of vacuolar ATPase (V-ATPase) and may regulate osteoclastic function. The deficiency of ATP6V1H caused bone loss in human, mouse and zebrafish. In this report, we identified the mechanisms by which ATP6V1H regulates proliferation and differentiation of bone marrow stromal cells (BMSCs). We found that ATP6V1H was expressed in BMSCs, and *Atp6v1h*$^{+/-}$ BMSCs exhibited the lower proliferation rate, cell cycle arrest and reduced osteogenic differentiation capacity, as well as the increased adipogenic potentials. Histologic analysis confirmed less bone formation and more fatty degeneration in *Atp6v1h*$^{+/-}$ mice in the different age groups. Q-PCR analysis revealed that loss of ATP6V1H function downregulated the mRNA level of TGF-β1 receptor, and its binding molecule, subunit β of adaptor protein complex 2 (AP-2), suggesting ATP6V1H regulates the proliferation and differentiation of BMSCs by interacting with TGF-β receptor I and AP-2 complex.

1. Introduction

Bone marrow stromal cells (BMSCs) are the progenitors of osteoblasts and have multi-potentials to differentiate into adipocytes, neural and myogenic cells. Plenty of research focus on therapeutic use of BMSCs in local and systemic transplantation [1]. By now, few reports explore the role of ion channels in BMSCs [2–6].

The vacuolar ATPase (V-ATPase) is a group of proteins that couples ATP hydrolysis to proton transport in intracellular compartments or across the plasma membrane. V-ATPase is important in maintaining the acidic environment of intracellular organelles which is necessary for protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation [7].

The mammalian V-ATPase proton pump is composed of peripheral V_1 component and membrane bound V_0 component with at least fourteen subunits. Subunit H is a small subunit of V-ATPase connects the V_1 and V_0 domains of V-ATPase. We previously reported that partial loss of ATP6V1H function results in osteoporosis/osteopenia in a population of 1625 Han Chinese individuals as well as in an Italian pedigree [8,9]. *Atp6v1h*$^{+/-}$ mice generated by the CRISPR/Cas9 technique had decreased bone remodeling and a net bone matrix loss. Similarly, *Atp6v1h*$^{+/-}$ osteoclasts showed impaired formation and bone resorption activity. The increased intracellular pH of *Atp6v1h*$^{+/-}$ osteoclasts downregulated the TGF-β1 activation, which may affect the differentiation of bone mesenchymal stem cells into osteoblasts.

To further understand the mechanisms by which subunit H regulates bone formation, we compared the characteristics of BMSCs from wild type and *Atp6v1h*$^{+/-}$ mice. And we found that ATP6V1H regulated the proliferation and differentiation of BMSCs.

2. Materials and methods

2.1. Animals

Atp6v1h knockout mice were used [9], and the animal protocols followed the ethical guidelines of the School of Stomatology, the Fourth Military Medical University (Xi'an, China).

* Corresponding author.
** Corresponding author.
E-mail addresses: zuolinj@fmmu.edu.cn (Z. Jin), xhduan@fmmu.edu.cn (X. Duan).

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2.2. BMSCs culture

6-week-old male *Atp6v1h*$^{+/-}$mice and their wild-type (WT) littermates were scarified by cervical dislocation. BMSCs were harvested from the femur and tibia. Primary BMSCs were cultured with α-MEM (Gibico, USA) medium containing 20% FBS (Hyclone, USA) in the condition of 95% humidity, 37 °C and 5% CO_2.

2.3. CCK8 assay of cell proliferation and cell cycle analysis

BMSCs from WT and *Atp6v1h*$^{+/-}$ group were seeded at the concentration of 5000 cells per well in 96-well plate and the medium were changed every two days. The cellular proliferation activities from 0 to 7 days were detected using cell counting Kit-8 (CCK8) (Enogene, China). Briefly, at each time point, 10 μl CCK-8 solution were added in the medium and incubated for 4 h at 37 °C. Then the amount of formazan dye was measured at 450 nm using a Biotek Synergy HTX (Biotek, USA).

The 80%–90% confluent two genotype BMSCs were harvested and fixed with ice-cold 70% ethanol (Fuyu, China) at 4 °C overnight. Then the cells were incubated in 100 μg/ml propidium iodide (Servicebio, China). The cell cycle distributions were analyzed by flow cytometry (Beckman, USA) using the ModFit 4.1 software.

2.4. Osteogenic and adipogenic induction of BMSCs

The 2nd passage BMSCs were seeded in the density of 5×10^5/well in 6-well plates. After 24 h, the osteogenic induction medium containing 10 mM sodium β-glycerophosphate, 1×10^{-7} M dexamethasone and 50 μg/mL ascorbate acid were added. For adipogenic induction, we treated cells with α-MEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μg/ml insulin, and 1 μM dexamethasone. The induction mediums were changed every two days. The cells were collected before the induction, seven days after being cultured in the induction medium or control medium for RNA extraction and Q-PCR analysis.

2.5. Quantitative real-time polymerase chain reaction (Q-PCR)

The total RNA were extracted using the regular TRIZOL method and the cDNA was synthesized using Prime Script TM RT Reagent Kit (Perfect RealTime) (TaKaRa, Japan). We used SYBR® Premix Ex Tag TM II kit (TaKaRa, Japan) for Q-PCR analysis with ABI 7500 RT-PCR system (ABI, USA) [10]. The primers were listed in Table 1.

2.6. Western blot

Total proteins of BMSCs were extracted as previously mentioned methods [9]. Antibodies included rabbit polyclonal IgG anti-ATP6V1H (1:1000, Origene, USA), mouse anti-GAPDH monoclonal antibody (1:1000, Cowbio, China), and HRP-labelled goat anti-mouse or goat anti-rabbit secondary antibody (1:10000, Cowbio, China).

2.7. Immunocytochemistry staining

The 2×10^4/ml BMSCs from two genotypic mice were seeded in the glass slides. The immunocytochemistry staining of ATP6V1H were carried out as described previously [9].

2.8. Aalizarin red S and oil red staining

The BMSCs were cultured in osteogenic and control medium in 24-well plate for 21 days and 1% Aalizarin red S staining kit (Solarbio, China) was performed to identify the formation of calcified nodules. 10% cetylpyridinium chloride was used to dissolve alizarin red staining nodules for quantitative analysis. The absorbance values of each well were obtained at 562 nm using a Biotek Synergy HTX(Biotek, USA).

After being cultured in adipogenic induction or control medium for 14 days, the BMSCs were stained with Oil red staining solution kit (Solarbio, China).

2.9. Histological analysis

Femur samples from 2-week-old, 6-week-old, and 3-month-old male *Atp6v1h*$^{+/-}$mice and their WT littermates were fixed with 4% paraformaldehyde and demineralized with 10% EDTA (pH 7.2–7.4) for 20–30 days at 37 °C, followed by gradient dehydration with ethanol, embedded in paraffin and sectioned at a thickness of 5 μm. Modified Ponceau red staining method was used to analysis the histological changes in bone, cartilage and bone marrows.

2.10. Statistical analysis

The GraphPad Prism 5 software was used to analyze the differences by unpaired *t*-test. The significant levels were $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, respectively.

3. Results

3.1. mRNA and protein expression of ATP6V1H in BMSCs

Q-PCR and western blot analysis showed a decreased mRNA and protein level of ATP6V1H in *Atp6v1h*$^{+/-}$ BMSCs (Fig. 1A and Fig. 1B). The protein level also presented weaker than that of brain tissue (Fig. 1B). Immunocytochemistry staining confirmed this difference (Fig. 1C).

Table 1
Q-PCR primers.

Gene Name	Forward primer (5′-3′)	Reverse primer (5′-3′)
Gapdh	CATGTTCCAGTATGACTCCACTC	GGCCTCACCCCATTTGATGT
Atp6v1h	GGATGCTGCTGTCCCAACTAA	TCTCTTGCTTGTCCTCGGAAC
Tgfb1	CCGCAACAACGCCATCTATG	CTCTGCACGGGACAGCAAT
Tgfbr1	GCTGACATCTATGCAATGGG	TTTCTTCAACCGATGGATCA
Tgfbr2	CCGCTGCATATCGTCCTGTG	AGTGGATGGATGGTCCTATTACA
Alp	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Bsp	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT
Runx2	AGTAGCCAGGTTCAACGATCTGA	GACTGTTATGGTCAAGGTGAAACTCTT
Pparg	CCCAATGGT TGCTGATTACAA A	AATAATAAGGTGGAGATGCAGGTTCT
Fabp4	CTTCAAACTGGGCGTGGAA	CCATCTAGGGTTATGATGCTCTTCA
Ap2b1	GCTTGGAGATTTCGGGGACG	GTGTTGAGCGGCAGAGAGACG
Ap2m1	GCAGTCACCAAGCAGAATGTCAA	CCAAAGTCCAGAATCTCATCCAG

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