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Cryptochrome 1 promotes osteogenic differentiation of human osteoblastic cells via Wnt/β-Catenin signaling

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ABSTRACT

Aims: The exact mechanism underlying osteoblast differentiation and proliferation remains to be further elucidated. The circadian clock has been universally acknowledged controls behavioral activities and biological process in mammals. Cryptochrome 1 (Cry1), one of the core circadian genes, is associated with bone metabolism. However, the exact role and potential mechanism of Cry1 in regulating osteogenesis are still unclear.

Main methods: Western blotting and qRT-PCR were applied to detect Cry1 expression levels, molecules in osteogenesis related signaling pathways and osteogenic transcriptional markers. The ALP staining and Alizarin red S staining were performed to weigh osteogenic state, while CCK8 assay was used to detect cell growth rates. Osteogenic capability of osteoblasts was determined using an ectopic bone formation assay.

Key findings: Cry1 was upregulated in the process of osteoblast differentiation, along with osteogenic transcriptional factors. Then, Cry1 upregulation and knockdown cell lines were established and we found Cry1 overexpression promoted osteogenesis and proliferation of osteoblasts both *in vitro* and *in vivo*. Besides, the canonical Wnt/β-Catenin signaling was increasingly activated by Cry1 overexpression, whereas inhibition of β-Catenin restrained enhanced osteogenic capability of Cry1 upregulated osteoblasts.

Significance: In conclusion, these results suggest that Cry1 promotes osteogenic differentiation of human osteoblasts through the canonical Wnt/β-Catenin signaling.

1. Introduction

Bone is a skeleton and renewable tissue mainly composed of mineral and collagen, with balanced osteogenesis and bone resorption [1]. However, cases of failed bone repair happen sometimes because of limited regenerative ability, especially in large segmental bone defects as a result of trauma, tumor or congenital diseases [2,3]. Tissue engineering is a promising method for treating large segmental bone defects [4]. Meanwhile, several attempts have been made to promote osteogenic capability of osteoblasts, so as to improve therapeutic effect of tissue engineering [5,6]. For enhancing the osteogenic capability, it is of great importance to have an insight into molecule mechanism and relevant regulatory factors underlying the osteogenesis. The complex process of osteogenic differentiation is mainly composed of three steps (proliferation, maturation and mineralization), accompanied by the expression of a series of canonical osteogenic transcriptional factors, including runt-related transcript factor 2 (Runx2), alkaline phosphatase (ALP), Osterix (OSX), Collagen type I alpha 1 (Col1α1) and Osteocalcin (OC) to insure a well-balanced osteoblast development [7,8].

The circadian clock controls the intrinsic rhythmicity of behavioral activities and biological process in nearly all organisms, which is driven by the clock genes in both central (suprachiasmatic nucleus, SCN) and peripheral clock. Clock genes, mainly include Cryptochromes (Cry1 and Cry2), Periods (Per, Per2 and Per3), Bmal1 and Clock, form the oscillator timing system with a negative feedback loop to control daily rhythms [9]. Many metabolic diseases happen once the clock genes are abnormally expressed due to disruption of circadian rhythm, especially bone metabolic disorders [10]. For example, night shift workers showed decreased bone mineral density and high probability of osteoporosis as a result of circadian disruption [11]; conditional osteoclast Bmal1 knockout mice showed a high bone mass phenotype [12]; overexpression of Rev-erbα in BMSCs inhibited cell proliferation and osteogenic differentiation [13]. What's more, it was reported that parathyroid hormone (PTH) administration was effective in treating bone fracture and osteoporosis by regulating circadian clock oscillation [14]. In general, the close relationship between circadian clock and

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bone metabolism exists for a certainty, but further researches are also needed to figure out the regulatory mechanism of circadian genes in regulating bone remodeling. Cry1, known as one of the master regulator of circadian rhythm, was found accumulated in nucleus of MSCs after blue laser irradiation, along with increased extracellular calcification [15,16]. Besides, Crys knockout mice exhibited lower bone mass [9]. However, how Cry1 controls osteoblast differentiation and mineralization is as yet undermined.

Therefore, we assumed that Cry1 plays an important role in bone remodeling and designed a series of *ex vivo* and *in vivo* experiments with Cry1 overexpression and knock-down Mg63 and Saos2 cells, which were derived from primary osteosarcoma and well proved for the natural manner of osteogenic differentiation. Our data showed that after Cry1 was upregulated, proliferation and osteogenesis were promoted and a series of changes in canonical Wnt/β-Catenin signaling had taken place. In addition, accelerated proliferation and differentiation in overexpression group were reversed when Cry1 was knocked-down or treated with KY1220. Taken together, these findings suggest that Cry1 exerts its osteoinductive ability through activation of the canonical Wnt/β-Catenin signaling pathway.

2. Materials and methods

2.1. Reagents and drug preparation

Dexamethasone (DXMS, D4902), Ascorbic acid (AA, A4403), β-Glycerophosphate (β-GP, G9422) and Alizarin red S (AR-S, A5533) were purchased from Sigma Aldrich. The stock solutions of DXMS was dissolved in dimethyl sulfoxide (DMSO), while AA and β-GP were dissolved in PBS at the concentration of 1 mM, 10 mM and 1 M respectively, stored at −20 °C, then diluted in growth medium immediately before osteogenic induction experiments. AR-S was dissolved in PBS at the concentration of 40 mM (pH 4.2). KY1220 is a Wnt/β-Catenin specific inhibitor, which was obtained from MedChem Express (Princeton, USA) and prepared in DMSO at the concentration of 10 mM, stored at −80 °C and worked at the concentration of 25 μM.

2.2. Culture of cells and osteogenic induction

MG63, Saos2 and 293 T cell lines were purchased from Cell Bank of the Chinese Academy of Science, Shanghai. MG63 cells were cultured in MEM medium with 10% FBS, Saos2 cells were grown in Mc Coy's 5A medium with 15% FBS. 293 T cells were cultured in DMEM with 10% FBS. Osteogenic induction medium was made from fresh growth medium containing 100 mM DXMS, 50 μM AA and 10 mM. The osteogenic induction medium was changed every 2 days.

2.3. Viral infection

PLKO.1-EGFP-puromycin, psPAX2 and pMD2.G (GeneChem, CHN) were involved in the lentivirus packing system for short hairpin RNA (shRNA) expression. Three CRY1 RNAi sequences were chosen to knock down the expression of CRY1 on the basis of hCRY1 (NM_004075) from GenBank. As a result, three shRNA (Cry1-shRNA1, 5′-ATTGAGTTCTA TGATCTTGTC-3′; Cry1-shRNA2, 5′-TACTGTCTGT.

GTTAACAGAGG-3′, Cry1-shRNA3, 5′-ATCAGTGTTTGATCTA ATT-3′) targeting human CRY1 were designed, annealed and inserted into the lentivirus. Full-length coding DNA sequence of human Cry1 was amplified using the forward primer 5′-CGGGATCCACCGGTCGCC ACCATGGGAGCAAGGGCGAG-3′ and the reverse primer with flag tag: 5′-ATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAA.

GCTTCGAATTCCGCCACC-3′. The PCR product was cloned into the lentiviral vector CMV-MCS-EGFP-SV40-Neomycin. Then, the above lentiviral vectors were mixed with 30% 293T cells with 20 μL LipoFiter (HanBio, CHN). 6 h to 12 h later, the medium was replaced by DMEM with 10% FBS. Supernatants containing lentivirus were collected at 48 h and 72 h, and filtered by 0.45 μm cellulose acetate filter (Milipore, USA) and stored at −80 °C.

Osteoblasts were seeded into 6 cm culture dishes and infected with lentivirus with the help of 6 μg/mL polybrene (Sigma, USA) for 48 h. At last, puromycin (Sigma, USA) was used to screen out stable transfected cells at the concentration of 3 μg/mL for 3 days and 1 μg/mL for 2 weeks.

2.4. Alkaline phosphatase staining and Alizarin red staining

Cells were rinsed in PBS buffer solution twice and fixed with 4% polyformaldehyde for 10 min at 37 °C. Then, alkaline phosphatase (ALP) staining kit (Leagene, Beijing) was applied according to the manufacturer's protocol. About Alizarin red staining, cells were stained with AR-S solution (pH 4.2) for 60 min at 37 °C to reflect the calcium salt deposition. When staining finished, rinsed with PBS to take residual dyeing solution away, air dried and photographed.

2.5. Alkaline phosphatase activity assay

Cells were scratched from culture dishes, and destructed with lysis buffer to extract total protein. The concentration of total protein was detected using a BCA kit (23228, Thermo Scientific, USA). Then, the ALP activity was detected by a commercial kit (Jiancheng Biotechnology Institute, Nanjing), and the relative ALP activity was normalized by the total protein concentration.

2.6. CCK-8 assay

The CCK-8 kit was bought from Dojindo (JPN) and applied to evaluate the effect of CRY1 on cell proliferation of MG63 and Saos2 cells. MG63 and Saos2 cells were digested and seeded at $3 * 10^3$ and $1.5 * 10^3$ cells/well respectively into 96-well plates with three replicates. After culturing for 0, 24, 48, 72, 96 h, 10 μL CCK-8 regent was mixed with 100 μL fresh medium and was added into each well. Then the plates were incubated for 2 h at 37 °C, and detected the absorbance of 450 nm by a Tecan Infinity 200Pro multi-well plate reader (Switzerland). The experiment was repeated three times.

2.7. In vivo ectopic bone formation assay

Ectopic bone formation assay was performed to test whether CRY1-overexpression promotes osteogenic differentiation *in vivo*. $2 * 10^6$ control cells or CRY1-overexpressed MG63 cells was injected subcutaneously to skin under the left front leg of female BALB/c nude mice aged 4 weeks. Each group set 7 replicates. After six weeks' observation, the implants were harvested and fixed in 10% formaldehyde for HE, Masson's staining and Immunohistochemistry (IHC) of osteocalcin (OCN) to evaluate osteogenesis. All animal care and experimental protocols were complied with the Animal Management Guidelines of China and approved by the Animal Care and Use Committee of Fudan University.

2.8. Quantitative real-time reverse transcription PCR

Quantitative real-time reverse transcription PCR (qRT-PCR) was performed to compare the relative expression of genes at RNA level. Exponential phase cells were rinsed with PBS twice and mixed with RNAiso Plus (Takara, JPN) to extract total RNA on the basis of manufacturer's instruction. The concentration and quality of total RNA were determined by the Tecan Infinity 200Pro multi-well plate reader to measure the ratio of absorbance at 260 nm to 280 nm. The PrimeScript™ RT Master Mix (Takara, JPN) was used to reverse transcript the total RNA to cDNA at 37 °C for 15 min and 85 °C for 5 s. qRT-PCR was performed on ABI 7500 Real-time PCR system (USA) using the SYBR Premix Ex Taq™ (Takara, JPN). Reactions were implemented in a