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MiR-25 promotes proliferation, differentiation and migration of osteoblasts by up-regulating Rac1 expression

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ABSTRACT

According to many studies, miRNAs are involved in the control of bone cell differentiation and function. Hence, an understanding of the pathways regulated by miRNAs involved in skeletal function is essential for the development of miRNA-based therapeutic strategies for bone diseases. In this study we evaluated the role of miR-25 in osteoblast differentiation by examining the expressions of key osteoblast differentiation markers like Runx2 and Ocn and also evaluated the effects of miR-25-Rac1 axis on PI3K/AKT and JNK pathways. MC3T3-E1 osteoprogenitors were treated with osteogenic differentiation media which was refreshed every 48 h after the initial differentiation treatment and were then quantified for total miRNA content. The viability, migration as well as Runx2 and Ocn expressions in cells transfected with miR-25 mimic, miR-25 inhibitor, and si-Rac1 were evaluated by CCK-8 assay, wound migration assay, qRT-PCR and Western blot. Finally, the effects of miR-25-Rac1 axis on PI3K/AKT and JNK pathways were studied. MiR-25 was found to significantly enhance cell viability and migration and up-regulate the expressions of Runx2 and Ocn. MiR-25 was also found to enhance the expression levels of Rac1, which contributed to the effects of miR-25 on osteoblastic cell lines. MiR-25 activated PI3K/AKT and JNK pathways possibly by up-regulation of Rac1. Enhanced expression of miR-25 led to the promotion of cell viability and migration, as well as up-regulation of Runx2 and Ocn markers by enhancing Rac1 expression.

1. Introduction

Bone fracture is a common medical condition, which occurs due to traumatic injury or disease-related bone fragility. Bone fracture leads to disability and morbidity, specifically in elderly people [1]. Bone healing process is started after the fracture. Bone healing is a complex regenerative process that restores the skeletal function. However, studies show that approximately 5–10% of fractures are not healed properly, which include delayed union or nonunion [2,3]. Moreover, the rate of bone repair declines with age [4]. Delayed healing usually leads to an increased duration of immobilization and increased risk of joint stiffness. During bone healing process, maintenance of osteoblast differentiation is vital [5]. Bone formation in fractured areas involves proliferation of osteogenic mesenchymal stem cells and subsequently their differentiation to mature osteoblasts, which is accompanied by synthesis of osteoid extracellular matrix proteins such as collagen type I, bone sialoprotein, osteocalcin, and osteopontin, followed by matrix mineralization [6].

MicroRNAs (miRNAs or miRs) are endogenous, non-coding, single-stranded RNAs made up of approximately 22 nucleotides. They regulate hundreds of gene expressions via RNA-induced silencing complexes by targeting mRNAs, thereby inhibiting translation or inducing direct destructive cleavage [7]. A recent report suggested that miRNAs regulate almost 60% of all genes in the human genome [8]. MiRNAs exert their biological functions by directly suppressing the translation and also by deadenylation and subsequent degradation of various mRNA targets. MiRNAs regulate the activation or suppression of many osteoblast-related genes. There are some miRNAs promoting osteoblast differentiation. MiRNA (MiR)-15b enhances osteoblast differentiation of human MSC (hMSC)s by inhibiting BMP inhibitor, BMPER (BMP binding endothelial regulator) [7,9].

Many transcription factors are involved in skeletal development, amongst which runt-related transcription factor 2 (Runx2) and osterix are key factors for osteoblast-specific transcription that activates a repertoire of genes during the differentiation of preosteoblasts into mature osteoblasts and osteocytes. Runx2 (known as core binding factor alpha 1 (Cbfa1), osteoblast specific transcription factor 2 (Osf2) and acute myeloid leukemia 3 protein (AML3)) is a bone restricted transcription factor needed for osteoblast differentiation and bone formation and mutations in Runx2 lead to cleidocranial dysplasia [10,11].

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Runx2 synergistically enhances osteogenic gene expression, which includes osteocalcin (Ocn), osteopontin (OPN), type I collagen, bone sialoprotein (BSP), and alkaline phosphatase (ALP). Additionally, it also enhances biological mineral deposition in primary dermal fibroblasts [12]. MiRNAs interact with Runx2 by directly targeting its gene or by affecting other genes that enhance or inhibit Runx2 expression level. Rac1 is a vital regulator needed for the activation of NFATc1. GTPase is an activated downstream enzyme of RANKL (receptor activator of NF-κB ligand) and produces reactive oxygen species (ROS), which stimulate PLCγ to induce sustained calcium oscillations which leads to NFATc1 activation [13,14] and those bone marrow macrophage which lack Rac1 show an impaired osteoclastogenesis and are unable to generate RANKL-dependent ROS [15].

Studies have shown that miRNAs regulate bone cell differentiation, lineage commitment and cell progression of mesenchymal stem cells [16]. Understanding of miRNA-regulated pathways and identification of miRNAs involved in skeletal function are important for the development of miRNA-based therapeutic strategies for bone diseases. In the present study, we have investigated the role of miR-25 in osteoblast differentiation by evaluating the expressions of key markers – Runx2 and Ocn. The role of Rac1 in the function of miR-25 was also investigated.

2. Material and methods

2.1. Cell culture and differentiation induction

MC3T3-E1 osteoprogenitors were plated in 100 mm dishes and incubated in α-MEM with 10% FBS (Atlanta), 100 units/ml of penicillin, and 100 g/ml of streptomycin. At confluence (day 0), these cells were treated with osteogenic differentiation media containing 10 mM glycerophosphate and 50 g/ml of ascorbic acid. The differentiation media was refreshed every 48 h after the initial differentiation treatment.

2.2. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. For the measurement of expression levels of miR-25 in the cells, TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II were used; U6 (Applied Biosystems, Foster City, CA, USA) was used as an internal control.

2.3. miRNAs transfection

MiR-25 mimic, miR-25 inhibitor, si-Rac1 and negative control (NC) were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were done using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

2.4. Cell counting kit-8 (CCK-8) assay

Cell viability was assessed using CCK-8 assay (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Cells were seeded in a 96 well plate with 5000 cells/well. After stimulation, CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37 °C in humidified 95% air and 5% CO_2. Absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

2.5. Migration assay

Cells were plated in 60 mM dishes until confluence. After the pretreatment of cells with 50 μM mytomicin C for 3 h, wounds were created by scratching cell sheets with a sterile 200 μl pipette tip. The culture medium was replaced with a fresh medium. The pictures of a specific position on the scratched areas were taken by an inverted microscope (Leica, Germany) every 24 h. The wound widths were measured and the relative wound widths in terms of percentage were calculated.

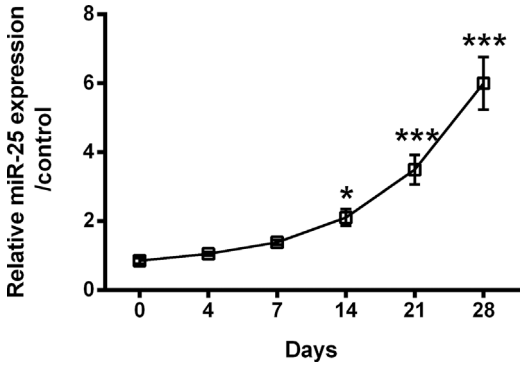

Fig. 1. MiR-25 expression in MC3T3-E1 osteoprogenitor cells was up-regulated during the period of osteoblast differentiation. Relative miR-25 expressions were evaluated by quantitative real-time PCR (qRT-PCR). Each bar represents the mean ± SD (n = 3). $^{*}P < 0.05$ and $^{***}P < 0.001$ (compared with 0 day).

2.6. Alkaline Phosphatase (ALP) activity

Osteoblasts were cultured for up to 21 days in osteogenic media containing 10 mM ascorbic acid and 50 mM b-glycerolphosphate. For ALP staining, cells were fixed in 10% formalin and stained.

2.7. Western blot

The proteins used for Western blotting were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride membrane-carried blots and antibodies were transferred into Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

2.8. Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. P-values were calculated using one-way analysis of variance. P-value of < 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. Expressions of miR-25 during osteoblast differentiation

MiR-25 expression in MC3T3-E1 osteoprogenitors treated with osteogenic differentiation media containing 10 mM glycerophosphate and 50 g/ml of ascorbic acid were detected by qRT-PCR through 28 days. The significant increase of miR-25 expression was observed after 14,