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miR-195 inhibited abnormal activation of osteoblast differentiation in MC3T3-E1 cells via targeting RAF-1

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ABSTRACT

Background: Recent reports have demonstrated that RAF-1^{L613V} (a mutant of RAF-1) mutant mice show bone deformities similar to Noonan syndrome. It has been suggested that RAF-1^{L613V} might abnormally activate osteoblast differentiation of MC3T3-E1 cells.

Methods: To demonstrate that RAF-1 is associated with bone deformity and that RAF-1^{L613V} dependent bone deformity could be inhibited by microRNA-195 (miR-195), we first investigated the amplifying influence of wild-type RAF-1 (WT) or RAF-1^{L613V} (L613V) on the viability and differentiation of MC3T3-E1 cells induced by bone morphogenetic protein-2 (BMP-2) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining, quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis. Subsequently, we investigated the blocking effect and its mechanism of miR-195 for abnormal activation of osteoblast differentiation of MC3T3-E1 cells via targeting RAF-1.

Results: RAF-1, especially RAF-1^{L613V}, abnormally activates osteoblast differentiation of MC3T3-E1 cells induced by BMP-2. Meanwhile, miR-195 could inhibit the cell viability and differentiation of MC3T3-E1 cells. Transfection of miR-195 largely suppressed the L613V-induced viability and osteoblast differentiation of MC3T3-E1 cells and attenuated the accelerative effect of L613V on runt-related transcription factor-2 (Runx2), Osterix (OSX), alkaline phosphatase (ALP), osteocalcin (OCN), and distal-less homeobox 5 (DLX5) osteogenic gene expressions. In addition, miR-195 decreased the expression of RAF-1 mRNA and protein by directly targeting the 3′-untranslated regions (3′-UTR) of RAF-1 mRNA in MC3T3-E1 cells.

Conclusions: Our findings indicated that miR-195 inhibited WT and L613V RAF-1 induced hyperactive osteoblast differentiation in MC3T3-E1 cells by targeting RAF-1. miR-195 might be a novel therapeutic agent for the treatment of L613V-induced bone deformity in Noonan syndrome.

1. Introduction

The development of vertebrate endoskeletons initially began with osteoblast differentiation, which is mediated by such factors as extracellular signalling molecules (e.g., bone morphogenetic proteins (BMPs)) [1], topography [2], and bioactive elements [3]. These regulatory factors are crucial components of the extracellular matrix (ECM). Previous studies demonstrated that their unusual activities could induce various bone deformities and related orthopaedic disease [4,5]. Mutations in genes were more likely to induce bone deformities in certain cases. For example, Noonan syndrome is an autosomal dominant disorder caused by dysfunction of the rat sarcoma (RAS)/mitogen-activated protein kinase (MAPK) signalling pathway with clinical features of skeletal dysplasia followed by many other diseases, including characteristic facial features, pectus abnormalities, cryptorchidism, lymphatic abnormalities and cardiac defects [6]. The pathway is essential for regulating a series of cellular responses (e.g., migration, proliferation, and differentiation) of osteogenic-related cells (e.g., osteoblasts and mesenchymal stem cells (MSCs)) [7,8], thereby sustaining bone homeostasis. However, there is limited information regarding abnormal osteoblast differentiation in Noonan syndrome.

RAF-1 is a pivotal intermediate for the cellular responses of osteoblast differentiation [9,10]. Yang et al. confirmed that sprouty homolog 2 (Spry2) expression was an early response to stimulation by fibroblast growth factor 1 (FGF1) in MC3T3-E1 cells and acted as a feedback inhibitor of FGF1-induced osteoblast responses, possibly through interaction with RAF-1 [9]. Thereafter, Li et al. applied integrated proteomics, statistical and network biology techniques to study proteome-level changes in bone tissue cells in response to two different conditions, normal loading and fatigue loading [10]. The study showed that

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the combination of a down-regulated anti-apoptotic factor, RAF-1, and an up-regulated pro-apoptotic factor, programmed cell death 8 (PDCD8), significantly increased the number of apoptotic osteocytes following fatigue loading [10]. Therefore, RAF-1 has the ability to abnormally activate osteoblast differentiation of MC3T3-E1 cells. Mutations in human RAF-1 that lead to the substitution of valine for leucine at amino acid 613 are associated with Noonan syndrome, which is characterized in part by cardiac hypertrophy [11]. A RAF-1 mouse mutant had enhanced mitogen-activated protein kinase kinase 1 (MKK1) and extracellular signal-regulated kinase 1 (ERK1) and ERK2 signalling [12]. Treatment of these mice with MAPK/ERK kinase (MEK) inhibitors attenuated many phenotypic abnormalities, including bone deformities [12]. Based on these findings, it is suggested that L613V might also abnormally activate osteoblast differentiationin Noonan syndrome. In short, RAF-1, especially RAF-1^{L613V}, induced bone deformity upon the occurrence of Noonan syndrome. Therefore, it is necessary to seek a novel therapeutic agent for RAF-1 or L613V-induced bone deformity in Noonan syndrome.

RAS can activate RAF-1 in the RAS/MAPK signalling pathway [13]. RAF-1 directly contributes to ERK activation but not to c-Jun N-terminal kinase (JNK) activation, whereas MEK kinase (MEKK) participates in JNK activation but causes ERK activation only after overexpression [13]. In MC3T3-E1 cells, after being activated by bone morphogenetic protein-2 (BMP-2) signalling, RAS becomes involved in osteoblastic determination, differentiation, and transdifferentiation under p38 MAPK and JNK regulation [14]. Therefore, we can hypothesize that RAF-1 has an amplification effect on osteoblast differentiation of MC3T3-E1 cells induced by BMP-2 because the RAS/MAPK signalling pathway has the same downstream signalling proteins as the BMP-2 signalling pathway, such as ERK and MEK.

As small non-coding RNAs, microRNAs (miRNAs) can control gene expression at the post-transcriptional level by binding to the 3′-untranslated regions (3′-UTR) of messenger RNAs (mRNA) [15,16]. miRNAs have been anticipated to regulate virtually all cellular mechanisms [17] and to play an important role in cellular responses, including cell proliferation, apoptosis and differentiation [18,19]. An increasing number of miRNAs have recently been identified to strongly regulate the osteoblast differentiation and bone formation, such as microRNA-194 (miR-194) [20] and microRNA-195 (miR-195) [15,16]. These findings suggest that it is feasible to develop methods for improving osteoblast differentiation by targeting critical miRNAs. Recent reports have demonstrated that the exogenous overexpression of miR-195 significantly inhibits the protein expression of RAF-1 and blocks thyroid cancer cell proliferation [21]. The expression of miR-195 was inversely associated with RAF-1 expression in breast cancer cell lines and tissue specimens [22]. Furthermore, the expression of miR-195 or knockdown of RAF-1 can similarly reduce tumour cell survival but increase apoptosis through the down-regulation of RAF-1 [22]. Therefore, it could be seen that RAF-1 is the target gene of miR-195. Whether miR-195 could inhibit osteoblast differentiation of MC3T3-E1 cells abnormally activated by RAF-1 or RAF-1^{L613V}, however, remains largely unknown.

In this study, we first identified that RAF-1, especially RAF-1^{L613V}, has an amplification effect on osteoblast differentiation of MC3T3-E1 cells induced by BMP-2. Next, the inhibitory role of miR-195 in regulating cell activity and differentiation of MC3T3-E1 cells was also confirmed. Further study showed that miR-195 largely suppressed the wild-type RAF-1 (WT) or RAF-1^{L613V} (L613V)–induced viability and osteoblast differentiation of MC3T3-E1 cells by targeting RAF-1 3′-UTR. Our findings would provide a novel therapeutic agent for the treatment of RAF-1 or RAF-1^{L613V}–induced bone deformity in Noonan syndrome.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), paraformaldehyde, Triton X-100, BMP-2, streptomycine, penicillin and L-glutamine (L-Gln) were provided by Sigma Aldrich Co. (St. Louis, MO, USA). Alizarin Red S sodium salt was provided by Alfa Aesar Co. (Tianjin, China). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), p-nitrophenyl phosphate assay kit, bicinchoninic acid (BCA) assay kit, the mammalian protein extraction kit, peroxidase-conjugated goat anti-rabbit antibody, BCIP/NBT alkaline phosphatase staining kit and RIPA lysis buffer were obtained from Beyotime Biotechnology Co. (Jiangsu, China). Phosphate buffer solution (PBS) was provided by Dingguo Biotechnology Co. (Beijing, China). Foetal bovine serum (FBS) was from Gibco Inc. (Waltham, MA, USA). Mouse anti-goat fluorescein isothiocyanate (FITC)-conjugated secondary antibody was supplied by ZSGB-BIO Co. (Beijing, China). The specific primary antibodies (anti-RAF-1,anti-p-ERK, anti-ERK, anti-p-p38, anti-p38, anti-p-MEK, anti-MEK, anti-p-Smad1/5 and anti - glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) used in the western blot analysis were provided by Abcam (Cambridge, MA, USA). The other chemicals were purchased from Oriental Chemical Co. (Chongqing, China).

2.2. Cell culture

MC3T3-E1 cells were cultured at 37 °C under 5% CO_2 atmosphere with normal medium (without adding BMP-2), i.e., high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 0.1 mg/mL streptomycin, 100 U/mL penicillin, 1.5 mg/mL sodium bicarbonate ($NaHCO_3$) and 0.33 mg/mL L-Gln. The cell culture medium was refreshed on the first day and then every 2 days. When the cell confluence reached approximately 90%, they were detached with 0.25% trypsin in 0.01% ethylene diamine tetraaceticacid disodium salt ($EDTANa_2$)/PBS solution, and re-suspended into new medium for subculture. The cells with better cellular growth behaviour would be used for *in vitro* studies. To sufficiently induce osteogenic differentiation, the cells were seeded and cultured onto tissue culture plates with 300 ng/mL BMP-2 for inducing. For the calcification assay, two groups of transduced MC3T3-E1 cells were cultured in β-glycerophosphate and ascorbic acid added full growth medium followed by BMP-2 treatment or not.

2.3. Plasmids construction and cell transfection

To construct a RAF-1 overexpressing plasmid, the cDNA of WT and L613V were obtained and inserted into the pLenti-Ubi-EGFP plasmids (Genechem Technology Co., Shanghai, China). The empty plasmid without any insertion was logged as Vector and served as the control. The transfection was conducted in Opti-MEM (Invitrogen, Carlsbad, CA, USA), and the transfection mix was composed of 3 mg of each plasmid and an optimal volume of Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). Two groups of MC3T3-E1 cells were individually treated through transfection processing for 24 h and respectively cultured with BMP-2 or without BMP-2 at 37 °C in a constant humidity environment containing 5% CO_2 for 7 days. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were used to verify the results of the transfection. miR-195 mimic, miR-195 inhibitor and respective negative control (NC) were purchased from Genechem Technology Co. The transfection was performed in accordance with the above steps.

2.4. MTT assay

The proliferation of MC3T3-E1 cells was measured via an MTT assay according to a previous study [2]. Briefly, after culturing for a specific