Original Paper

Hypoxia-Induced MicroRNA-429 Promotes Differentiation of MC3T3-E1 Osteoblastic Cells by Mediating ZFPM2 Expression

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Key Words

Hypoxia • MiR-429 • Differentiation • ZFPM2

Abstract

Background/Aims: Hypoxia has been reported to regulate osteoblastic differentiation of bone cells and cartilage development. However, information concerning the molecular mechanisms remains largely unknown. **Methods:** The expression of miR-429 was evaluated by quantitative real-time PCR analysis. To test whether miR-429 directly regulate the expression level of ZFPM2 at transcription level, dual-luciferase reporter gene assay was performed. Western blotting was performed to detect osteogenesis related protein expression. The cell proliferation, apoptosis, alkaline phosphatase activity and matrix mineralization were performed to assess the functions of miR-429 *in vitro* and *in vivo* the effects of miR-429 on fracture healing. **Results:** Expression of miR-429 was increased in MC3T3-E1 cells treated with 200 μ M CoCl₂ by qRT-PCR, and overexpression of miR-429 promoted cell differentiation, and enhanced alkaline phosphatase activity and matrix mineralization. Luciferase reporter assays suggested that miR-429 directly targets the 3'UTR of ZFPM2. In addition, knockdown of ZFPM2 could phenocopy the effects of miR-429 expression. Furthermore, overexpression of ZFPM2 in miR-429-expressing MC3T3-E1 cells suppressed cell differentiation. **Conclusions:** Our results provide valuable insight into the potential role of hypoxia in regulation of osteoblastic cell differentiation.

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Introduction

Tibial pilon fractures are a major health problem worldwide. The major causes of fractures are motor vehicle accidents and falls from a height [1, 2]. Despite effective therapeutic strategies, such as intramedullary nails and plates, used to improve healing

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potential both for acute fractures and nonunions, high rates of delayed union or nonunion have been reported [3]. Our previous study showed that hypoxia could accelerate bone union and acute tibial fracture healing [4]. However, the precise mechanisms of hypoxia and its involvement in osteoblastic differentiation are still poorly understood.

MicroRNAs (miRNAs) are small endogenous non-coding RNAs, which bind to target mRNAs with complementary sequences to suppress the translation and stabilization of the target mRNA [5, 6]. Mature miRNAs derive from a complex process, where a primitive form of miRNA (pri-miR) is transcribed by RNA pol II and cleaved by the ribonuclease DROSHA in the nucleus to originate a precursor miRNA (pre-miR), which is exported to the cytoplasm or the endoplasmic reticulum and undergoes a second cleavage by a RNase III endonuclease, Dicer, to become approximately 22 nucleotides length miRNA duplex [7]. Emerging evidence suggests that miRNAs play critical roles in many cellular processes, including cell growth, invasion, metabolism and differentiation [8-11]. It was recently revealed that miRNAs have been involved in mediating the osteogenic differentiation of osteoblasts and mesenchymal stem cells (MSCs). MiR-143 and miR-145 were decreased in osteogenic differentiation, and their overexpression suppressed osteogenic differentiation by targeting Osterix and Sp7 transcription factor 7 (Sp7) *in vitro*, respectively [12, 13]. MiR-155 is induced by Interferon- β and inhibits osteoclast differentiation by targeting suppressor of cytokine signaling 1 (Socs1) and microphthalmia-associated transcription factor (Mitf), two essential regulators of osteoclastogenesis [14]. In addition, miR-29b was increased in osteoblast differentiation, and ectopic expression of miR-29b promoted osteogenesis by directly down-regulating the expression of collagen type I alpha 1 (COL1A1), collagen type V alpha 3 (COL5A3), and collagen, type IV, alpha 2 (COL4A2) [15].

In this study, we focused on miR-429 that is significantly upregulated under hypoxia in human endothelial cells [16]. To examine osteoblast differentiation in MC3T3-E1 cells, we found that miR-429 was upregulated under hypoxia. Furthermore, gain-of-function experiments indicated that miR-429 promoted osteogenic differentiation by negatively regulating the expression of ZFPM2. Our results suggest that miR-429 is a novel positive regulator of osteogenesis and provide a new mechanism for understanding accelerated fracture healing under hypoxia.

Materials and Methods

Cell culture and drug treatment

The murine pre-osteoblastic cell line MC3T3-E1 was obtained from ATCC (Manassas, VA, USA). The cells were cultured in α -minimum essential medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies), 100 units/ml of penicillin and 100 μ g/ml of streptomycin. HEK293T cells were purchased from the Institutes of Biochemistry and Cell Biology (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies), 100 units/ml of penicillin and 100 μ g/ml of streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂.

To mimic the effects of hypoxia, 200 μ M CoCl₂ was added to the medium. Subsequently, the RNA and protein were harvested at the specified times. Osteogenic differentiation of MC3T3-E1 cells was induced by osteogenic medium (complete medium supplemented with 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid) that was refreshed every 2 days.

Quantitative RT-PCR

Total RNA from tissues and cells was extracted using Trizol as previously described [4]. To measure the mRNA levels of ZFPM2, 500 ng of total RNA was reverse-transcribed into cDNA and then quantified on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described [4]. The following primers were used for mRNA detection: ZFPM2: (forward) 5'-ACC GAA GGG ATG TAC CCTG-3'; (reverse) 5'-TCG TTG CCT CCC ACT ACAGT-3'. The relative expression level of ZFPM2 mRNA was normalized against β -actin. Mature miR-429 expression was measured by qRT-PCR using the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan® MicroRNA Assays (Applied Biosystems, Foster City,

CA, USA) and normalized to U6 according to the manufacturer's instructions. All reactions were performed in triplicate, and three independent assays were performed.

Plasmid construction and lentivirus packaging and infection

A fragment encoding the pre-miR-429 sequence plus 150 bp flanking both ends was PCR amplified from mouse genomic DNA using the forward primer 5'-AAA GAA TTC AGT CTG CTG TGG CTG TAA CCG-3' and the reverse primer 5'-AAA GGA TCC CCA AAG AGG TGC TAT GAC GAGC-3'. The PCR product was then cloned into the EcoRI/BamHI sites of the pCDH-CMV-MCS-EF1-puro vector (System Biosciences, Mountain View, CA, USA).

The ZFPM2 3' UTR (untranslated region) was PCR amplified from MC3T3-E1 cDNA using the forward primer 5'-AAA AAG CTT TGA GTT ACT AAA GAA AGC AGT CACC-3' and the reverse primer 5'-AAA ACG CGT CGT CTC CAA CAA TTT GGAAT-3'. The PCR product was then cloned into the HindIII/MluI site of pMIR-REPORT™ vector (Ambion, Austin, TX, USA). Mutations of the miR-429 binding sites in the ZFPM2 3' UTR sequence were performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. All plasmids were confirmed by restriction enzyme digestion and sequencing.

Dual luciferase reporter

The Lentivirus-mediated miR-429 packaging system was co-transfected into HEK-293T cells with the pPACKH1™ Lentivector Packaging Kit (System Biosciences) using Lipofectamine™ 2000 (Invitrogen). The supernatant was collected 48 hours post-infection and concentrated by ultracentrifugation. MC3T3-E1 cells were infected with 1×10^7 lentivirus particles in the presence of 8 µg/ml polybrene (Sigma-Aldrich).

Cell proliferation and apoptosis assay

Cell viability was determined by Cell Counting Kit-8 assay (Dojindo, Japan) according to the manufacturer's protocol. In brief, 3×10^3 cells in 100 µl of cell suspensions were seeded into each well of 96-well plates and cultured overnight. 10 µl of CCK-8 solution was added to each well at 0 h, 24 h, 48 h and 72 h, respectively. The optical density (OD) was measured by a microplate reader (Bio-Rad Laboratories) at 450 nm. Apoptosis was evaluated by Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) following the manufacturer's instructions.

Alkaline phosphatase activity assay

Alkaline phosphatase activity was determined using the WAKO LabAssay™ ALP Alkaline Phosphatase Assay Kit (LabAssay™ ALP, Wako, Japan) according to the manufacturer's instructions. Alkaline phosphatase activity was normalized to total protein concentration as assessed by BCA protein assay kit (Thermo Scientific Pierce, USA).

Alizarin Red S staining

To detect the effects of miR-429 and ZFPM2 in mineralization of MC3T3-E1 cells, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were stained with 1% Alizarin Red S solution (Sigma-Aldrich). Pictures were taken of five random non-overlapping fields with a light microscope (Olympus, Tokyo, Japan). Quantification of Alizarin Red S stain was assessed via extraction with Image J software (NIH, Bethesda, MD, USA).

Bioinformatics methods and Luciferase reporter assay

The potential targets of miR-429 were obtained using computer-aided algorithms from TargetScan and DIANA-TOOLS. MC3T3-E1 cells were transfected with the luciferase reporter plasmids, phRL-TK, together with control or pre-miR-429 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was determined 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western blotting

Western blotting was performed as previously described [4]. Briefly, tissues and cells were lysed with RIPA buffer supplemented with complete protease inhibitors (Roche, Mannheim, Germany). Proteins (50 µg)

were separated on a 10% SDS-polyacrylamide gradient gel (Bio Rad Laboratories, Hercules, CA, USA) and transferred to PVDF membrane (Millipore, Billerica, MA). After blocking, blots were incubated with Rabbit polyclonal ZFPM2 antibody (1:200, Sigma-Aldrich), Rabbit Polyclonal GATA4 antibody (1:1000, Abcam), Rabbit polyclonal ALP antibody (1:100, Abcam), Rabbit polyclonal OC antibody (1:100, Thermofisher), or Rabbit polyclonal β -actin antibody (1:1000, Santa Cruz Biotechnology). Blots were visualized using a chemiluminescence reagent (Millipore, Temecula, CA, USA) in a LAS4000 Luminescent Image Analyzer (GE Healthcare Japan, Japan).

Animal experiments

This study was performed in accordance with the Capital Medical University Xuanwu Hospital Affiliated with the Capital Medical University with the permit number of 20141126FR. All 5-day-old Swiss mice were obtained from the Laboratory Animal Center of Capital Medical University. The mouse model (8 per group) of bone fracture was performed with a fracture device as previously described [4]. One hundred microliters of lentivirus (1×10^9 TU/ml) were directly injected into the subcutaneous region of a local fracture. Healing parameters were measured using a Faxitron X-ray machine (MX-20 Specimen Radiography System, Faxitron X-ray Corp) at 28 days as previously described [4].

Statistical analysis

All experiments were run in triplicate and carried out three independent times. Quantitative data were expressed as the mean $\pm$ standard deviation (SD) and were evaluated using two-tailed t-tests. Data were considered significant at a value of $p < 0.05$.

Results

MiR-429 is induced by CoCl₂ in vitro and in vivo

It was previously shown that miR-429 was induced by hypoxia [16], and our *in vivo* experiments indicated that CoCl₂ could promote fracture repair [4]. To investigate the expression of miR-429 under hypoxic conditions in MC3T3-E1 cells, 200 μ M CoCl₂ was added to the medium. HIF-1 α were detected to be induced by CoCl₂ (Fig. 1A). The expression of miR-429 was detected at the specified times, and our results exhibited miR-429 upregulation by CoCl₂ in MC3T3-E1 cells, with the peak value at 4 hours (Fig. 1B). However, CoCl₂ treatment had no significant effect on cell proliferation and apoptosis (Fig. 1C and D). To further confirm the *in vitro* data, we detected the expression of miR-429 in the fractured bone tissues of mice treated with CoCl₂. Consistent with the results *in vitro*, miR-429 was significantly upregulated at 7 days and remained increased at 28 days (Fig. 1C), suggesting that miR-429 might be involved in fracture healing.

Effects of miR-429 on osteoblast differentiation in MC3T3-E1 cells

To explore the possible biological functions of miR-429, MC3T3-E1 cells were infected with control or miR-429 expressing lentivirus. As shown in Fig. 2A, miR-429 expression levels were significantly increased in cells infected with the miR-429 lentivirus 72 h later, confirming successful expression of miR-429 by lentivirus in MC3T3-E1 cells. To investigate the effect of miR-429 on osteoblastic differentiation of MC3T3-E1 cells, we measured alkaline phosphatase (ALP) activity after induction with differentiation medium. Our results showed that miR-429 greatly increased ALP activity, with approximately 1.6- and 1.9-fold increased activity at 21 days and 28 days, respectively, compared to the control virus (Fig. 2B). Furthermore, mineralization analysis showed a significant increase in mineralized nodule formation in miR-429 overexpressing cells compared to controls (Fig. 2C). In addition, we detected the effect of miR-429 on ALP, osteocalcin (OC), and Runt-related transcription factor 2 (RUNX2) by Western blot. As shown in Fig. 2D, ALP, OC and RUNX2 expression levels were significantly increased with overexpression of miR-429. Therefore, miR-429 expression promotes osteogenic differentiation in MC3T3-E1 cells.

Fig. 1. (A) The HIF-1 α expressions in MC3T3-E1 cells were treated without or with CoCl₂ treatment at the indicated times. β -actin was used as a loading control. (B) qRT-PCR analysis of miR-429 expression in MC3T3-E1 cells treated with 200 μ M CoCl₂ at the indicated times. (C) Relative expression of miR-429 in fractured bone tissues from mice treated with CoCl₂ or without CoCl₂. (D) Growth curves of MC3T3-E1 cells were treated without or with 200 μ M CoCl₂ treatment. (E) Apoptosis of MC3T3-E1 cells treated without or with 200 μ M CoCl₂ treatment was analyzed by flow cytometry. **, $P < 0.01$.

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ZFPM2 is a direct target of miR-429

To identify the molecular mechanisms of miR-429-mediated regulation of osteoblast differentiation, TargetScan and miRBase were used to predict the potential targets of miR-429. We found two potential binding sites in the 3' UTR of ZFPM2 at -224 to -230 (sequence 1) and -763 to -769 (sequence 2) (Fig. 3A). To test whether ZFPM2 was a direct target of miR-429, luciferase reporter assays were performed in MC3T3-E1 cells. As shown in Fig. 3B, miR-429 markedly reduced firefly luciferase activity of reporter plasmids containing the wild-type ZFPM2 3' UTR or ZFPM2-MUT2, whereas luciferase activity was not significantly changed in ZFPM2-MUT1. Furthermore, endogenous ZFPM2 expression at both the mRNA and protein levels was reduced upon miR-429 overexpression in MC3T3-E1 cells (Fig. 3C and D). Moreover, the real-time PCR assay showed that the mRNA level of ZFPM2 was decreased and inversely correlated with miR-429 expression in MC3T3-E1 cells treated with CoCl₂. These results suggested that miR-429 binding site 1 played a functionally significant role in the regulation of ZFPM2 expression.

ZFPM2 silencing mimics the effects of miR-429

In order to confirm whether miR-429-mediated-osteoblast differentiation is directly related to downregulation of ZFPM2, cells were transfected with ZFPM2 small interfering RNA (siRNA). As shown in Fig. 4A, the effects of ZFPM2 knockdown was confirmed by Western blot, and siRNA2 exhibited the best knockdown efficiency (Fig. 4A). The siRNA2 targeting ZFPM2 was named siZFPM2 and used for subsequent experiments. Knockdown of ZFPM2 in MC3T3-E1 cells increased the ALP activity compared to the negative control (NC) (Fig. 4B). Mineralization was significantly higher at 21 days in the ZFPM2 knockdown cells

Fig. 2. MiR-429 promotes mineralization of osteoblast cells. (A) qRT-PCR analysis of miR-429 expression in MC3T3-E1 cells transduced with control vector or miR-429. (B) Relative ALP activity was evaluated at the indicated times. (C) The images show matrix mineralization in MC3T3-E1 cells transduced with control vector or miR-429 at 21 days. Bar represents the means $\pm$ SD. (D) Western blot analysis of ALP, OC and RUNX2 expression in MC3T3-E1 cells transduced with control vector or miR-429 at the indicated time. **, $P < 0.01$.

Fig. 3. The negative regulation of ZFPM2 by miR-429. (A) The sequences of the putative miR-429 binding sites in wild type and mutant ZFPM2 3' UTR. (B) Luciferase activity analysis in the presence of wild type or mutant ZFPM2 3' UTR with control vector or miR-429. (C and D) The mRNA and protein levels of ZFPM2 were measured in miR-429-stably expressing MC3T3-E1 cells. (E) The relative expression of ZFPM2 mRNA was analyzed in MC3T3-E1 cells treated with 200 μ M CoCl_2 at the indicated time. **, $P < 0.01$.

Fig. 4. ZFPM2 silencing promotes osteoblast differentiation. (A) Western blot analysis of ZFPM2 in MC3T3-E1 cells transfected with siRNA targeting ZFPM2. (B) Detection of ALP activity in MC3T3-E1 cells transfected with NC or siZFPM2 at the indicated time. (C) Matrix mineralization was examined by Alizarin red S staining in MC3T3-E1 cells transfected with NC or siZFPM2 at 21 days. (D) Analysis of GATA4, ALP, OC and RUNX2 expression in MC3T3-E1 cells transfected with NC or siZFPM2 by western blotting. **, $P < 0.01$.

Fig. 5. MiR-429 promotes fracture healing *in vivo*. (A) Radiographic images of the fractured callus at 28 days in mice treated with or without miR-429. (B) qRT-PCR analysis of miR-429 expression in bone tissues from mice. (C) Analysis of ZFPM2, OC and RUNX2 expression in bone tissues from mice. **, $P < 0.01$.

compared to the NC cells (Fig. 4C). It has been reported that ZFPM2 interacts with GATA4 to regulate heart development, and GATA4 has been reported to negatively regulate osteoblast differentiation by downregulation of Runx2 [17, 18]. Subsequently, the expression of GATA4 and osteogenic marker genes (ALP, OC and RUNX2) were determined in ZFPM2 knockdown cells. ZFPM2 silencing strongly induced the expression of ALP, OC and RUNX2 and resulted in a decrease of GATA4. These findings suggested that ZFPM2 was involved in regulating osteoblast differentiation through regulation of GATA4 expression.

MiR-429 accelerates the formation and remodeling of bone during fracture repair in vivo

To determine whether miR-429 could trigger fracture repair *in vivo*, as observed in cultured cells, Lentivirus was injected into the subcutaneous region of local fracture, and radiographic examination was taken after 28 days. MiR-429 was found to remarkably

enhance new bone formation during the course of fracture repair compared to control vector (Fig. 5A). qRT-PCR was performed to detect the expression of miR-429 in the bone tissue from mice infected with miR-429 or control lentivirus. As we expected, the expression of miR-429 was significantly increased in mice infected with the miR-429 lentivirus (Fig. 5B). These data imply that miR-429 could accelerate fracture healing *in vivo*.

Discussion

It has been shown that many molecules can inhibit bone resorption and enhance bone formation and repair [19–21]. Previous studies have shown that the activated Hypoxia Inducible Factor-1 α (HIF-1 α) pathway plays an important role in regulating osteoclast differentiation [22, 23]. In addition, our recent study reported that a rat model of bone fracture treated with CoCl₂ could augment fracture healing [4]. However, the underlying mechanisms have not been fully elaborated. In our present study, we focus on miR-429, a hypoxia-regulated gene, involved in CoCl₂-mediated activation of cell differentiation in preosteoblastic cells.

The hypoxia-inducible factor (HIF) pathway has been considered a powerful stimulus under a variety of pathological conditions by influencing angiogenesis and cellular metabolism [24, 25]. A set of hypoxia-regulated microRNAs were identified under hypoxic stress and provided an approach to understand the relation between stress factors and gene expression control [16, 26, 27]. MiR-429 was increased under hypoxic stress, and miR-429 negatively regulated HIF-1 activity through a negative feedback loop in human endothelial cells [16]. In the present study, we demonstrate that miR-429 was also increased in response to hypoxia in osteoclast differentiation *in vitro* and *in vivo*. Importantly, overexpression of miR-429 was able to increase ALP activity and osteoblast mineralization in MC3T3-E1 osteoblasts, indicating that the positive effects of miR-429 cannot be explained by the CoCl₂-mediated fracture healing. In keeping with its role in regulation of osteoclast differentiation, overexpression of miR-429 could induce osteogenic marker genes that were increased by CoCl₂ *in vivo* [4]. Overexpression of miR-429 *in vivo* also showed that it could enhance fracture healing. Of course, it was synergetic that miR-429 regulated the expression of HIF1A and VEGFA to promote angiogenesis [16].

ZFPM2, a multi-zinc finger protein, has been found to play important roles in cardiac development [17, 28]. The interaction of ZFPM2 with GATA4 leads to the transcriptional repression of target genes, and a recent study has shown that GATA4 negatively regulates osteoblast differentiation by downregulation of Runx2 [18]. Our present report indicated that ZFPM2 silencing stimulated osteoclast differentiation as well as the upregulation of osteogenic marker expression and downregulation of GATA4 expression. In addition, we confirmed the direct interaction of miR-429 and the 3' UTR of ZFPM2 using a luciferase assay and showed that overexpression of miR-429 could effectively suppress the endogenous expression of ZFPM2.

In conclusion, we found that miR-429 was induced by CoCl₂, and it promoted preosteoblastic cell differentiation, thus providing an explanation for hypoxia as a major stimulator of osteoclast differentiation. We additionally identified ZFPM2 as a direct target of miR-429 involved in osteoclast differentiation. Though miR-429 was induced by hypoxia or CoCl₂, CoCl₂ was just a tools drug to mimic hypoxia to activate HIF1 pathway. Further studies to identify key target genes of miR-429 in osteoblasts will advance our understanding of the bone formation process under hypoxic conditions.

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Disclosure Statement

We confirm that the manuscript has been read and approved by all named authors.

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