Cellular and Molecular Biology

Xiao *et al. Cell. Mol. Biol.*2016, 62 (3): 95-99
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.3.16

Original Research

Role of microRNA-129-5p in osteoblast differentiation from bone marrow mesenchymal stem cells

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Abstract: Mesenchymal stem cells derived from bone marrow have the capacity to differentiate into osteoblast, chondrocyte, nerve cell and myocardial cell *in vitro*, which are an ideal engraft in tissue-engineered repair. Osteoblast differentiation is a vital process in maintaining bone homeostasis in which various transcriptional factors, including signaling molecules, and microRNAs (miRNAs). In this research, human bone marrow mesenchymal stem cells (hBMSCs) were induced differentiation into osteoblast *in vitro* after over-expression of miR-129-5p. The results showed that the hBMSCs could induce differentiation into osteoblast under the special condition medium, but when the miR-129-5p was over-expressed in hBMSCs, the differentiated efficiency and induced time of osteoblast from hBMSCs could be promoted. This reason was demonstrated that signal transducer and activator of transcription 1 (STAT1) was a transcriptional repressor of osteoblast gene (Runx 2) expression during osteoblast differentiation, miR-129-5p reduced STAT1 levels, leading to the accumulation of correctly spliced Runx 2 mRNA and a dramatic increase in Runx 2 protein.

Key words: Bone marrow, Mesenchymal stem cells, Osteoblast differentiation, microRNA.

Introduction

MicroRNAs (miRNAs) are a fundamental class of biological molecules that play a crucial role in development (1). miRNA dysfunction has been linked to cancer (2), as well as other biological processes. And miRNAs are small noncoding RNAs that are cleaved from 70-100 nucleotide (nt) hairpin pre-miRNA precursors in the cytoplasm by RNaseIII Dicer into their mature form of 19-25 nt. Single-stranded miRNAs bind messenger RNAs of potentially hundreds of genes at the region of 3'UTR with perfect or near perfect complementarity, resulting in degradation or inhibition of the target messenger RNA (3). Genes encoding miRNAs, which are found in most eukaryotes, produce short RNAs that bind to mRNA transcripts and downregulate expression either through mRNA destabilization or translational repression (4,5). miRNAs regulate gene expression by guiding the RNA-induced silencing complex to a target sequ ence, which is usually located at the 3′untranslated region (UTR) of mRNAs (6). The widespread involvement of miRNAs in regulating developmental processes, physiological responses, and pathological conditions in animals has been amply demonstrated (7,5,8). Nonetheless, the specific functions of each miRNA in the various contexts in which it is expressed are only beginning to be discovered. Mesenchymal stem cells (MSCs) are promising candidates for cellular therapies and tissue engineering strategies. They are a population of adult stem cells and derive their name from an intrinsic ability to differentiate into multiple cell lineages of mesodermal origin, such as bone. Bone marrow mesenchymal stem cells (BMMSCs) are easy to isolate, culture, and manipulate in vitro and have great plasticity, for these reasons they have become an important tool in cell replacement therapy and are considered as candidates for different clinical applications (9,10). Osteoblasts are cells which originate from bone marrow and contribute to the production of new bone. These cells build up the matrix of bone structure and play a role in the mineralization of bone matrix. In previously report, signal transducer and activator of transcription 1 (STAT1) has been found to play an important role in regulating osteoblast differentiation, which was a transcriptional repressor of osteoblast differentiation (11). We found that STAT1 had a target site for microRNA-129-5p after bioinformatics analysis. Therefore, this study aims to research regulated mechanism of microRNA-129-5p in osteoblasts differentiation from human bone marrow MSCs (hBMSCs), which can be used for repairing bone injury.

Materials and Methods

Isolation and culture of hBMSCs

hBMSCs were collected from donor bone marrow, fractionated on density gradient (Percoll, 1.077 g/ml, Amersharm Biosciences, Sweden) for 30 min at

Received January 9, 2016; **Accepted** March 24, 2016; **Published** March 31, 2016

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400g. The interface was collected and seeded at 1×10^4 cells/well in six-well culture plates with 1-ml medium containing L-DMEM (Gibco, USA) + 10% (v:v) fetal bovine serum (FBS) (Gibco, USA) + 10 U/ml penicillin + 10 ug/ml streptomycin. Cultures were maintained at 37.5°C in a humidified atmosphere containing 95% air and 5% CO_2. The culture medium was changed every three days and the non-adherent cells were removed after three days. hBMSC morphology was observed by phase-contrast microscopy and photographed (Olympus, Japan). When the cultures became 80-90% confluence, the cells were detached with 0.25% trypsin and 0.02% EDTA for 3 min at room temperature, and reseeded at 1×10^4 cells/well in six-well culture plates with 1-ml L-DMEM for serial passaging.

Expression of Pre-miR-129-5p by recombinant adenoviruses

Recombinant adenoviruses were generated by using AdEasy technology (12,13). The sequence of pre-miR-129-5p was synthesized by Sangon Biotech (Shanghai, China), cloned into an adenoviral vector and then used to recombine adenoviruses in HEK293 cells. The adenoviruses designated as AdBMP9 also express GFP as a marker for monitoring infection efficiency. Analogous adenovirus only expressing monomeric GFP (Ad-GFP) was used as a control. Real time PCR was used to evaluated the expression level of miR-129-5P after virus infection. miRNAs were isolated from cells using microRNA isolation kit (Applied Biosystems, USA) according to the manufacturer's instructions. cDNA synthesis was carried out with the High Capacity cDNA synthesis kit (Applied Biosystems, USA) using 2 ng of RNA as template. The miRNA sequence-specific reverse transcription PCR primers for miR-129-5p and endogenous control U6 were purchased from Ambion (USA). Real-time PCR analysis was carried out using Applied Biosystems 7500 real-time PCR system. The gene expression threshold cycle (CT) values of miRNAs from each sample were calculated by normalizing with internal control U6 and relative quantitation values were plotted. The target genes STAT1 and Runx 2 were detected by western blotting.

Osteogenic differentiation

Osteogenic differentiated cells were divided into three groups, hBMSCs (Control, group A), hBMSCs within osteogenic medium (group B) and hBMSCs after over-expressed miR-129-5p within osteogenic medium (group C). Osteogenic medium: DMEM medium containing 0.5 nmol/L FGF 2 and 100 μg/L BMP 2 (Peprotech, US) (14,15,16). hBMSCs were plated to 24-well plates for osteogenic differentiation, After 14 days, the cells were harvested and tested alkaline phosphatase (ALP) concentration using alkaline phosphatase assay kit (Abcam, US). And the expressions of osteoblasts specific genes, Runx 2, collage type I and Osteopont were analyzed using real time PCR in differentiated time of osteoblasts.

Assay of luciferase activity

The 3′UTR fragments for STAT 1 were generated by PCR using the following primers: 5′- TGAGTGGATGATGTTTCGTGA-3′ and 5′-AGCAGTATGTAAAAGGGAAGAACC-3′and cloned into the psiCHECK-2 vector (Promega) downstream from the Renilla luciferase cassette. The predicted miR-149-5P binding site was mutated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). HEK293T cells were grown in a 96-well plate and co-transfected with the luciferase reporter vector together with a miRNA precursor or a negative control (20 nM; Ambion) using Attractene (Qiagen) according to the manufacturer's instructions. Activities of firefly and Renilla luciferase were analyzed using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

Western blotting

STAT1, the target gene of miR-149-5p, was detected by western blot analysis following overexpression of miR-149-5p. Cells were lysed using M-PER Protein Extraction Reagent (Pierce, USA) supplemented with a protease inhibitor cocktail (DMSF). Protein concentrations of the extracts were measured using the BCA assay (Pierce, USA) and equalized with extraction reagent. Equal amounts of extracts were loaded and subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes. Specific antibodies and horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz, USA. Membranes were probed using ultra-enhanced chemiluminescence western blotting detection reagents. GAPDH was used as an internal control.

PCR

RNA was extracted from BMMSCs using Trizol reagent (Invitrogen, USA). Total RNA was reverse transcribed, followed by 35 PCR cycles using RNA PCR kit ver 3.0 (TARAKA, China). PCR was performed in 50 μl of mixture containing 10μl of 5×PCR Buffer (TARAKA, China), 28.5μl of ddH_2O, 0.25μl of Ex-Taq (TARAKA, China), 0.5μl of forward and reverse primers, and 1.5μl of template cDNA. The cycling conditions consisted of one initial 2-min cycle at 94°C, followed by 30 30-s cycles at 94°C (denaturation), one 30-sec cycle at 50-60 °C (annealing), and one 2-min cycle at 72°C (extension). PCR products were detected by 2.5% agarose gel electrophoresis. Real-time PCR was carried out using a DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The primer of miR-129-5p and U6 were purchased in Tangen (China), PCR mixture, which contained 20 pmol of forward and reverse primers and 2 μl of cDNA, was subjected to amplification with a DNA Engine Opticon 1 (MJ Research, San Francisco, CA, USA). The cycles were set at 95°C for 10 min for preheating, followed by 40 cycles at 94°C for 15 sec, at 55°C for 30 sec and at 72°C for 30 sec. The amplicons were detected directly by measuring the increase in fluorescence caused by the binding of the SYBR Green I dye to gene-specific and amplified double-strand DNA using a DNA Engine Opticon 1. Following the completion of the PCR reaction, the temperature was raised from the annealing temperature to 95°C for melting curve analysis. The expression level was calculated by the $2^{-\Delta\Delta Ct}$ method and compared with the relative expression.

Statistical Analysis

Statistical analyses of the data were performed with a one-way ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) test for the three sets of results. A P-value of less than 0.05 was considered significant. Statistical analyses were done with a JMP® Statistical Discovery Software (SAS Institute, Cary, NC).

Results

miR-129-5p over-expression in hBMSCs after transfection

To determine the functions of miR-129-5p in hBMSCs, we transfected the pre-miR-129-5p into hBMSCs for over-expression. The expression of miR-129-5p was quantified by real time-PCR at 72 hr after transfection. As shown in Fig. 1, miR-129-5p levels were significantly elevated by the pre-microRNA transfection. We then analyzed the miR-129-5p sequence and its target gene sequence (using TargetScan and miRanda tools), confirmed that STAT 1 was target genes for miR-129-5p. STAT1 is an important signaling molecule in the interferon signaling pathway, has been widely studied in regulating osteoblast differentiation, and then STAT1 is a transcriptional repressor of osteoblast gene -Runx 2 expression during osteoblast differentiation (17,18). Fig. 1 showed that miR-129-5p targeted STAT 1 at the 3'-UTR in hBMSCs to regulate expression. Protein expression of STAT 1, putative target genes was performed on miR-129-5P transfected cells according to Image J tools comparative method.

STAT1 is a target gene of miR-129-5p

miRNA target genes are likely to have relatively long and conserved 3′UTR (5). We noticed that STAT 1 has a long evolutionarily conserved 3′UTR, so we used the TargetScan algorithm (19) to search for miRNAs that could potentially regulate STAT 1. The miR-129-5p has the same putative target binding sites in STAT 1 in the human genome. To directly test whether miR-129-5p targets STAT 1, we cloned the 3′UTRs of STAT 1 downstream of a luciferase reporter, and co-transfected these reporter constructs along with miRNA precursors into the human cell line HEK293T. Co-expression of miR-129-5p was found to effectively downregulate luciferase expression in constructs with these 3′UTRs (Fig. 2). Mutations in the seed sequence of the predicted miR-129-5p binding sites within STAT 1 abolished the inhibitory effects of miR-129-5p on luciferase expression.

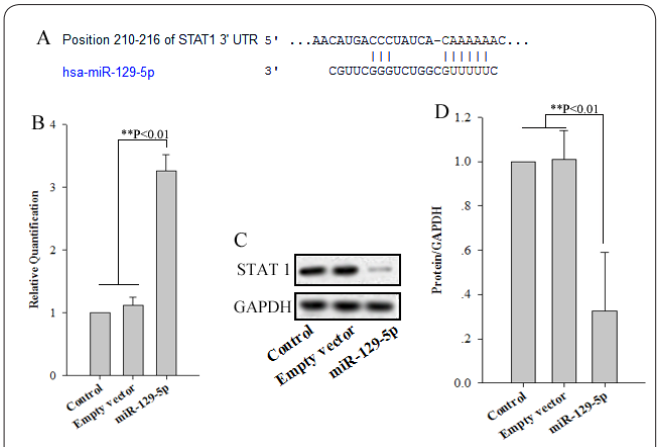

Figure 1. The target site of miRNA-129-5p and up-regulation of miR-129-5p in pre-miR-129-5p transfected hBMSCs. A The miR-129-5p complementary sites with 3'UTR of STAT 1. B hBMSCs were transfected with adenoviruses as described in the Methods, and the expression of miR-129-5p was quantified by real time PCR. C Effect of miR-129-5p on protein level of STAT 1. D Quantification of STAT 1 in hBMSCs transfected with miR-129-5p, empty vector or Control for 72 h.

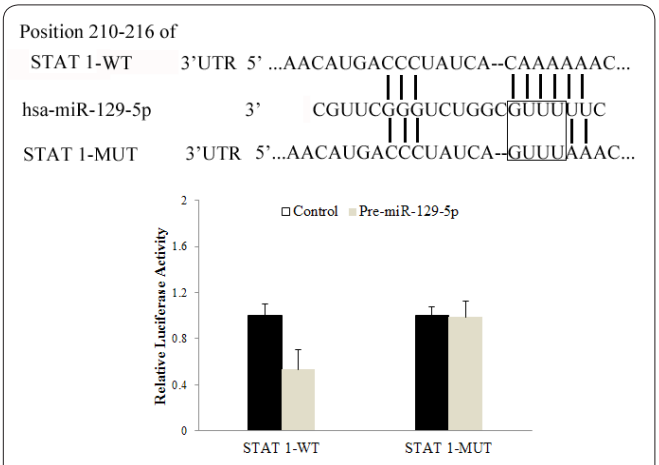

Figure 2. STAT 1 is target of miR-129-5p. miR-129-5p complementary sites with 3′UTR of STAT 1. The mutant sequence (STAT 1-MUT) is identical to STAT 1-WT construct except for five point mutations disrupting base-pairing at the 5′end of miR-129-5p. Mutating the miR-129-5p target site in the 3′UTR of STAT 1 abolishes inhibition of luciferase activity by endogenous miR-129-5p in 293 cells.

Osteogenic differentiation of hBMSCs

After the induction with osteoblast inducers, hBMSCs had apparent changes in appearance. From the 4th day after the induction, MSCs in group C changed from fusiform to three-dimension, becoming larger and changing into polygon. As time went by, triangle or polygonal cells increased, and then grew into multilayers, and many crystal particles could be observed. The cell morphology of group B changed at the 7th day, but group A was no significant change. Reverse transcription (RT)-PCR indicated that the special genes, Runx 2, Collage type I and Osteopont were positive in group B and C after osteogenic induction 14 day, but expression level of special genes in group C were dramatically higher than group B after relative quantitation (Fig.3). Real time PCR indicated that after osteogenic incubation, the specific genes, including Runx 2, collage type I and osteopontin were detected in different induction time point, these gene expression level showed a time-lapse increase, however special gene were found at 4th day after the induction in the group C (Fig.3). The ALP concentration assay of medium showed that ALP concentration increased with induced time extension in group B and C (Fig.3), but ALP concentration of group C was dramatically higher than group B ($P<0.05$).

Discussion

Cell therapy has emerged as a strategy for the treatment of many human diseases, especially bone injury.

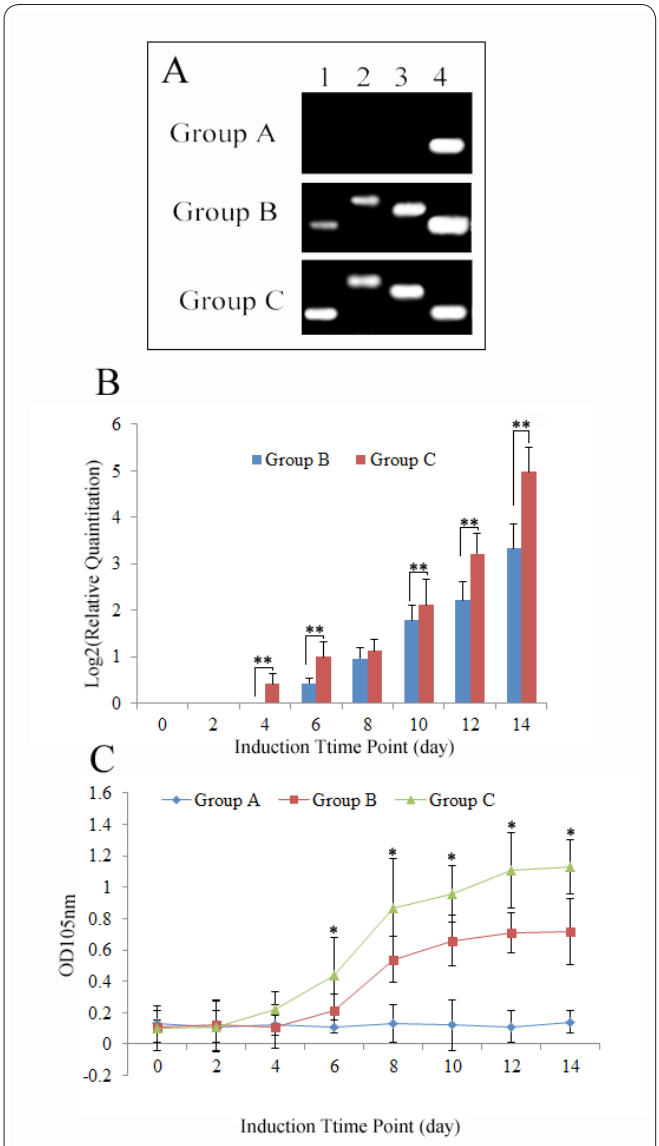

Figure 3. Osteogenic differentiation of hBMSCs. A RT-PCR assays revealed expression of osteoblast-specific genes, including collagen type I, Runx 2, and osteopontin, at day14 post-induction. These genes were not expressed in the control group (group A), 1 collagen type I, 2 Runx 2, 3 osteopontin, 4 GAPDH. B Osteoblast-specific gene expressions, Runx 2, collage type I and osteopontin were detected in different induction time point, these gene expression level showed a time-lapse increase, however special gene were found at 4th day after the induction in the group C. C Detection of ALP concentration in inducer medium. The ALP concentration assay of medium showed that ALP concentration increased with induced time extension. Group C are significantly different ($P<0.05$) then other groups.

At present, tissue stem cells were tentatively expanded and orientationally induced *in vitro* to some needed cells, which are then implanted into patients to repair damage, to replace regressive tissues and to improve the function of hereditarily defective tissues. It was reported that BMMSCs could be used to repair and reconstruct some tissues such as bone cartilage, lung, brain and liver (20,21,22,23). MiRNAs are fundamental biological molecules that have been shown to play important roles in biological development (24). Role of signal transducer and activator of transcription 1 (STAT1), which is an important signaling molecule in the interferon signaling pathway (17), has been widely studied in regulating osteoblast differentiation (18). Although, it has been found that mice lacking STAT1 showed increased osteoclasts and bone resorption (25), the mice also have increased bone formation and bone mass in vivo. Generally, it is suggested that STAT1 interacts with and impedes nuclear translocation in Runx2, thus leading to a blockage of osteoblast differentiation. Runx2 is an important transcription factor for osteoblastogenesis, and mice lacking Runx2 completely abrogate bone formation (26,27). Previously study found that lack of STAT1 resulted in an enhanced activation of Runx2 and osteoblast differentiation by disrupting the interaction between STAT1 and Runx2, which leads to an accumulation of Runx2 nuclear translocation. In contrast, overexpression of STAT1 significantly impeded Runx2 nuclear translocation (28). Consistent with the findings, our data demonstrated that inhibition of STAT1 by miR-129-5p significantly promoted osteoblast differentiation by upregulation of Runx2 nuclear translocation. Inhibiting STAT1 using miRNAs has been demonstrated by several studies. For instance, miR-146a inhibits anti-hepatitis B virus immune response via downregulating STAT1 in hepatocytes (29). In chronic hepatitis B patients, miR-146a is found to be highly upregulated, which inhibits T cell immune function by targeting and inhibiting STAT1(30). MiR-145 inhibits colon cancer by directly suppressing gene expression of STAT1(31). Other research also demonstrated that miR-194 regulates osteoblast differentiation through modulating STAT1-mediated Runx2 nuclear translocation in stem cells (11). Therefore, these reports hint that inhibiting STAT1 expression with miRNAs is a feasible method, our data suggest that suppressing STAT1 expression with miR-129-5p accelerates osteoblast differentiation from hBMSCs, thus implying that miR-129-5p could be used as a potential molecular target for improving bone homeostasis.

In this study, we detected the function of miR-129-5p in differentiation of osteoblasts from hBMSCs and performed the hBMSCs into osteoblasts *in vitro*. The results demonstrated that miR-129-5p could promote the productivity of osteoblasts from hBMSCs and increased the ALP concentration *in vitro*.

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