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MiR-590-3p regulates osteogenic differentiation of human mesenchymal stem cells by regulating APC gene

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ABSTRACT

The present study aimed to investigate miR-590-3p's role in osteogenic differentiation of human mesenchymal stem cells (hMSC). HMSC were cultured and transfected with empty vector, miR-590-3p vector to construct control hMSC and miR-590-3p overexpression hMSC. MiR-590-3p suppressed hMSC was created with oligonucleotide transient transfection. All 3 groups of cells were analyzed for their osteogenic level as well as Wnt/β-catenin signaling pathway activities. The results of alizarin red staining indicated that overexpression of miR-590-3p significantly enhanced mineralized deposition. Osteogenic markers including ALP, OC and SOST were also up-regulated. The result of dual-luciferase and western blot analysis indicated that miR-590-3p bound to 3′UTR of APC mRNA selectively. By suppressing mRNA level of APC, over-expressed miR-590-3p stabilized β-catenin and increased the transcription activities of downstream target genes. These result suggested that miR-590-3p can promote osteogenic differentiation via suppressing APC expression and stabilizing β-catenin.

1. Introduction

Human mesenchymal stem cells (hMSC) are multi-potent cells that can differentiate into various lineages, including osteoblast, myoblasts and adipocytes [1]. Various signaling pathways control the cell fate of hMSC, including Wnt/β-catenin [2,3]. For hMSC, the activation of canonical Wnt signaling pathway would promote osteogenic differentiation [4], and suppression of this pathway would lead to adipogenic differentiation. In the absence of a Wnt signal stimulation, cytoplasmic β-catenin would be phosphorylated by a destruction complex consisting of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3β (GSK3β) and casein-kinase 1α (CK1α). The phosphorylation and degradation of β-catenin prevented its accumulation and nuclear translocation. As an integral part of the destruction complex, mutation of APC would lead to unchecked transcription of downstream target genes [5]. Recent study also suggested that delicate regulation of APC activities was mandatory for proper skeletal development [6]. These results indicated that a more comprehensive understanding of the regulating network involving APC is needed for a better understanding of osteogenic process.

MicroRNAs are a group of small non-coding RNA that can bind selectively to the 3′UTR of target mRNA, leading to its degradation [7]. Such post-transcriptional regulation activities were observed in stem cell differentiation [8], immune defense response [9], muscle development [9], etc. Recently, multiple miRNA were found to participate in osteogenic differentiation regulation [10], including miR-590-5p [11]. Bio-informatics investigation suggested that miR-590-3p could also be a potential factor in osteogenic regulation by targeting APC gene. In this study, we investigate the possible involvement of miR-590-3p in osteogenic differentiation of hMSC, and try to gain a better understanding of this post-transcriptional regulation network.

Abbreviation: hMSC, Human Mesenchymal Stem Cells; APC, adenomatous polyposis coli; miRNA, microRNAs; ALP, alkaline phosphatase; OC, Osteocalcin; HCO, Human Calvarial Osteoblasts; UTR, Untranslated Regions; GSK3β, glycogen synthase kinase 3β; CK1α, casein-kinase 1α; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; ARS, Alizarin Red Staining; CPC, hexadecylpyridinium chloride; OD, Optical Density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RIPA, RadioImmunoprecipitation Assay.

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2. Methods and materials

2.1. Cell culture

Human mesenchymal stem cells (HMSCs) were obtained from company (Cyagen Biosciences, Guangzhou, China) and cultured in low glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin. The third passage of hMSCs was used in our study.

2.2. miR-590-3p overexpression and interfering

MiR-590-3p gene was cloned into pSIN-EF2-IRES-GFP-puro lentivirus plasmid (Promega, Madison, USA). The cloned plasmids or the empty vector control plasmids were then co-transfected with virus packaging plasmid into hMSCs as previously described [12]. Stably transfected strains were selected with puromycin (0.5 mg/L) and confirmed with real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Oligonucleotide transfection for miR-590-3p inhibitor was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions.

2.3. Osteogenic differentiation

Vector control hMSCs, miR-590-3p hMSCs and miR-590-3p-inh hMSCs were seeded to 6 well plates at a density of 1.0×10^6 cells per well. Osteogenic induction medium was prepared per previous studies [13]. After culturing with osteogenic medium for 0, 15 and 25 days, mRNA was extracted to detect the expression level of miR-590-3p. Alizarin Red Staining (ARS) was conducted to visualize the mineral deposition in each group after exposure to osteogenic medium for 28 days. OD562 after hexadecylpyridinium chloride (CPC) was measured as mineralization quantification.

2.4. Western blot

Vector control hMSCs, miR-590-3p hMSCs and miR-590-3p-inh hMSCs were placed at a density of 1×10^6 cell per well. Total proteins were extracted using RIPA buffer per manufacturer's protocol. Nuclear protein was isolated with Nuclear Protein Extraction Kit according to manufacturer's instruction (Thermo Scientific™, Waltham, MA). Primary antibodies used were anti-cyclinD1 antibody, anti-p-pRb antibody, anti-pRb antibody, anti-β-catenin antibody and anti-p84 antibody. Anti-EF-1α antibody and anti-glyceraldehyde-3-phosphate dehydrogenase antibody were used as control. All assays were performed in triplicates.

2.5. qRT-PCR

Total RNA was extracted from 3 groups of hMSCs using Trizol (Invitrogen, Grand Island, NY) per manufacture's protocol. A total of 2-μg RNA per sample was used for cDNA synthesis primed with random hexamers. Target gene specific primers were used for PCR initial amplification. Real-time PCR was then employed to determine the fold of increase of target mRNA in each sample comparing with empty vector control group. All expression data were normalized to the geometric mean of housekeeping gene GAPDH. Reverse transcription-PCR and real-time PCR primers were designed using the Primer Express v2.0 software (Applied Biosystems) and the primer sequences are provided as follows:

GAPDH-up/dn: CGTCCCGTAGACAAAATGGT/TTGATGGCAACAATCTCCAC,
CyclinD1-up/dn: CATCTACACCGACAACTCCATC/TCTGGCATTTTGGAGAGGAAG,
ALP-up/dn: CTCAGGGGGCATAGACTTCA/CGTCAATAGCCAGGATGACA,
DMP1-up/dn: TTGTGAACTACGGAGGGTAGAG/ACCTGGTTACTGGGAGAGCA,
OC-up/dn: ATTGTGGCTCACCCTCCATCA/AGGGCTATTTGGGGGTCATC,
Runx2-up/dn: CTGTGGCATGCACTTTGACC/CTTTTCGGGGAGGAGAGCAG,
SOST-up/dn: CCCTGGGGTTTTAAGGGAGC/GAGGTGCAAGGGGGAATCTT,
OPN-up/dn: CAGAGCACAGCATCGTCGG/GAGTTTTCCTTGGTCGGCGT

2.6. Dual luciferase assay

PsiCHECK-2 plasmid were purchased from Promega (Promega,

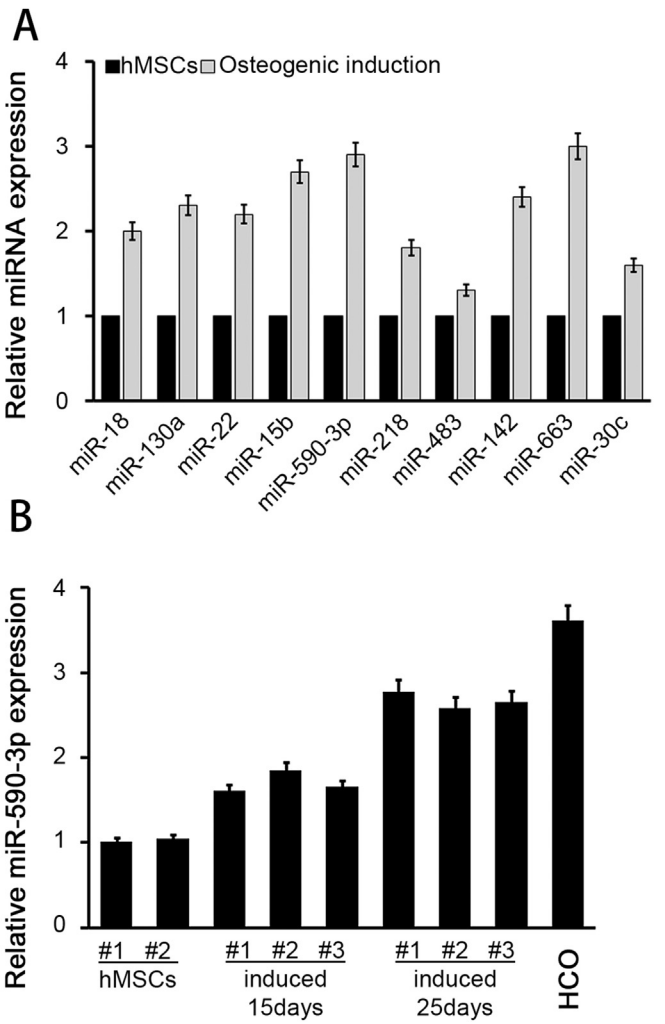

Fig. 1. Expression analysis of multiple miRNA after osteogenic induction A: Fold of increase in expression level for miRNAs after osteogenic induction. B: Expression level of miR-590-3p during osteogenic induction period. HCO: Human Calvarial Osteoblasts.

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