The International Journal of Biochemistry & Cell Biology 85 (2017) 35–43

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

MicroRNA-467g inhibits new bone regeneration by targeting Ihh/Runx-2 signaling☆

Jyoti Kureel, Aijaz A John [1], Manisha Dixit [1], Divya Singh *

Division of Endocrinology and Centre for Research in Anabolic Skeletal Targets in Health and Illness (ASTHI), CSIR-Central Drug Research Institute, B.S. 10/1, Sector-10, Jankipuram Extension, Lucknow, India

ARTICLE INFO

Article history:
Received 6 September 2016
Received in revised form 10 January 2017
Accepted 29 January 2017
Available online 2 February 2017

Keywords:
MicroRNA
Runx-2
Hedgehog signaling
Osteoblast differentiation
bone regeneration
osteogenesis

ABSTRACT

MicroRNAs are important post transcriptional regulators of gene expression and play critical role in osteoblast differentiation. In this study we report miR-467g, an uncharacterized novel miRNA, in regulation of osteoblast functions. Over-expression of miR-467g inhibited osteoblast differentiation. Target prediction analysis tools and experimental validation by luciferase 3′ UTR reporter assay identified Runx-2 as a direct target of miR-467g. Over expression of miR-467g in osteoblasts down regulated Runx-2 and Ihh signaling components. Furthermore, silencing of miR-467g was done to see its role in Ihh and Runx-2 mediated bone healing and regeneration in a drill hole injury model in BALB/c mice. Silencing of miR-467g led to significant increase in new bone regeneration and Ihh and Runx-2 localization at injury site in a day dependent manner. In conclusion, miR-467g negatively regulates osteogenesis by targeting Ihh/Runx-2 signaling. We, thus, propose that therapeutic approaches targeting miR-467g could be useful in enhancing the new bone formation.

1. Introduction

MicroRNAs (miRNAs) are small (~22 nucleotides), single-stranded non-coding RNAs found in diverse organisms which have emerged as important post transcriptional regulators of gene expression (Dong et al., 2013; Eskildsen et al., 2011; Hassan et al., 2012; Inose et al., 2009; Li et al., 2009). MiRNAs negatively regulate translation of specific mRNAs by base pairing with partially or fully complementary sequences in target mRNAs and play a key role in various biological processes (Dong et al., 2013; Eskildsen et al., 2011; Hassan et al., 2012; Inose et al., 2009; Li et al., 2009). The expression of miRNAs is altered in various diseases like cancer, hepatitis C infection, myocardial infarction, and metabolic disease. For instance, miR-17–192 is significantly over expressed in lung cancer while let-7 is a tumor suppressor miRNA and aberrant expression of *let-7* results in oncogenic loss of differentiation (Christopher et al., 2016). MiRs also regulate several properties of cardiac physiology. The examples include miR-29, miR-30 and miR-133 which are down regulated in atrial fibrillation. On the other hand, miR-328 and miR-499 are up regulated in atrial fibrillation (Santulli et al., 2014). MiR-21 is substantially increased in response to cardiac injury in experimental murine models. Inhibition of miR-21 exhibits antihypertrophic and antifibrotic effects, which leads to a significant functional improvement (Thum et al., 2008). Other examples include miR-130a which is up regulated in hepatitis C virus infection. Introduction of anti-miR-130a in hepatocytes increased IFITM1 expression with concomitantly reduction in HCV replication (Shrivastava et al., 2013).

Many miRNAs have been identified which either negatively regulate osteoblast differentiation or bone formation by targeting osteogenic factors or positively by targeting negative regulators of osteogenesis (Bae et al., 2012; Dong et al., 2013; Eskildsen et al., 2011; Gao et al., 2011; Hassan et al., 2012; Inose et al., 2009; Li et al., 2009). These include miRNAs such as 133 and 204/211 which attenuate osteoblast differentiation by directly targeting Runx2 in C2C12 mesenchymal progenitor cells and MSCs respectively (Huang et al., 2010; Li et al., 2008) or miR-141 and miR-200a which target Dlx5 to inhibit osteoblast differentiation (Itoh et al., 2009). On the contrary, miR-335-5p directly targets and down-regulates Wnt inhibitor DKK1, enhances Wnt signaling and promotes osteogenesis (Zhang et al., 2011). Besides, several microRNAs including the *miR-34* family have been implicated in osteosarcoma tumorigenesis via their effects on notch signaling components (Nugent, 2015).

☆ CDRI Communication number: 9423.

* Corresponding author.
E-mail address: divya_singh@cdri.res.in (D. Singh).

[1] Authors have contributed equally.

http://dx.doi.org/10.1016/j.biocel.2017.01.018
1357-2725/

Previous studies by our group revealed that medicarpin, a natural pterocarpan, treatment to mice calvarial osteoblasts (MCO) led to differential regulation of several microRNAs (Kureel et al., 2014). About 80 miRNAs were down regulated by medicarpin. In the previous study we characterized the role of a well known tumor suppressor miR-542-3p in osteoblasts and found that it negatively regulated bone formation *in vivo* by targeting BMP-7(Kureel et al., 2014). Apart from miR-542-3p, several other candidates were robustly down regulated post medicarpin treatment and were of potential interest. In this study we report the functional characterization of miR-467g in osteoblast cells and how it suppresses new bone regeneration. Of importance was the fact, that it was a novel miRNA with no assigned role and was highly conserved across mammals like rat, chimpanzee, rhesus, humans, dog, cat and horse especially in the seed region through which miRNA recognize their target mRNAs.

2. Material and Methods

2.1. Reagents and chemicals

Fetal bovine serum (FBS), cell culture media, α-MEM, antibiotic solution, sodium pyruvate, trypsin and non-essential amino acid were purchased from sigma (St. Louis, MO, USA). Low serum media, opti-MEM, Taqman miRNA assays, miRNA mimic, miRNA inhibitor, in vivo ready miRNA, taqman universal master mix, taqman miRNA reverse transcriptase kit and mirVana miRNA isolation kit were purchased from ambion (81 Wyman Street Waltham, MA USA). Antibodies were purchased from Abcam (Cambridge Science Park, Cambridge, UK) and CST.

Dual-glow luciferase assay system was purchased from Promega (Madison, WI 53711 USA). MiRNA 3′UTR target expression clone was purchased from Genecopoeia (Medical Center Dr. Suite 101 Rockville, MD 20850 USA).

2.2. Calvarial osteoblast culture:

For mouse calvarial osteoblast (MCO) cell culture, 1-2 day old mouse pups were used and calvarial osteoblasts were harvested as per standardized protocol (Gautam et al., 2011; Kureel et al., 2014). Briefly, calvariae were surgically removed and subjected to five sequential digestions at 37 °C in 0.1% dispase and 0.1% collagenase P solution. Cells released from the second to fifth digestions were collected, pooled, centrifuged, resuspended and plated in T25 cm^2 flasks in α-MEM media containing 10% fetal bovine serum (FCS) and 1% penicillin/streptomycin (complete growth medium).

2.3. miRNA Target site prediction

MiRNA targeting genes were identified using the bioinformatics analysis programme, target scan (http://www.targetscan.org), PicTar (http://pictar.bio.nyu.edu) and miRanda (http://www.mirdb.org) in order to find out possible target genes for the differentially expressed miRNAs.

2.4. qRT-PCR analysis for miRNA

The mirVana qRT-PCR miRNA detection kit (Ambion, 81 Wyman Street Waltham, MA, USA) was used in conjunction with Taqman microRNA reverse transcription kit and a Taqman microRNA assay kit (Applied Biosystems, Foster City, CA, USA) for quantification of miR-467g according to previously published protocol (Kureel et al., 2014). Whole RNA was isolated from mice calvarial osteoblast cells and cDNA was constructed through reverse transcription and used as template for real-time PCR. Briefly, the reaction master mix containing 10X RT buffer, 5X RT primers, MultiScribe reverse transcriptase, RNase inhibitor, 100 mM dNTPs and nuclease-free water was mixed with 30 ng of total RNA. The mixture was incubated for 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C. The PCR was done using 10 μl of PCR master mix containing TaqMan 2X Universal PCR Master Mix, 20X TaqMan MicroRNA Assay Mix (Applied Biosystems) and the RT products in a volume of 20 μl. The reaction mixtures were incubated in a 96 well plate at 95 °C for 10 min followed by 40 cycles of 95 °C for 15s and at 60 °C for 1 min using the Step One plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The mean Ct values of each sample were determined from triplicate reactions. The relative expression level of miRNA examined was calculated by $\log_2 |2^{-DCt}|$, in which DCt was defined as the subtraction of the Ct value of the target miRNA from the Ct value of internal control U6.

2.5. ALP assay

For the measurement of ALP activity, MCO at 70-80% confluence were trypsinized and plated in 96 well (2000 cells/well) plates in growth medium with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. Cells were transfected with miR-467g, anti-miR-467g and negative control and incubated for 48 h. At the end of incubation period, total ALP activity was determined using p-nitrophenylphosphate as substrate and absorbance was read at 405 nm.

2.6. Mineralization assay

MCO cells were grown to ~80% confluence. Cells were trypsinized and plated at 2500-2000 cells/well in osteoblast differentiation media with 10% FCS. Cells were transfected with miC, miR-467g and anti-miR-467g and cultured for 18 days at 37 °C, and the medium was changed every 48 h. At the end of experiment, attached cells were washed with PBS and fixed in 4% formaldehyde for 20 min at room temperature. Fixed cells were stained with 40 mM alizarin red for 30 min. Stained calcium nodules were first photographed under a light microscope and then extracted by using 800 μl of 10% (v/v) acetic acid at room temperature for 30 min with gentle shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5 ml tube. After vortexing for 30 s, the slurry was overlaid with 500 μl mineral oil (Sigma-Aldrich, St. Louis, MO, USA), heated to exactly 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 g for 15 min and 500 μl of the supernatant was removed to a new tube. Then 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. O.D. (405 nm) of 150-ul aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent bottomed plates.

2.7. qRT-PCR analysis for osteogenic markers

For this experiment, total RNA was isolated from the miR-467g mimic and anti-miR transfected cultured cells using Trizol (Life Technologies, 81 Wyman Street Waltham, MA USA). Reverse transcription was performed using a Revert AidTM H Minus first strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) according to the manufacturer's protocol. SYBR green chemistry was used for quantitative determination of the various mRNAs for RUNX-2, BMP-2, Osterix, Osteocalcin and a housekeeping gene GAPDH. Primer sequences are listed in Table 1. For real-time PCR, cDNA was amplified with Light Cycler 480 (Roche Diagnostics, Indianapolis, IN, USA). The temperature profile of the reaction was 95 °C for 5 min, 40 cycles of denaturation at 94^0 C for 2 min and annealing and extension at 62^0 C for 30 s, extension at 72 °C for 30 s.