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Expression of MicroRNA-30c and its Target Genes by nano-Bioglass Ceramic-treatment in Human Osteoblastic Cells

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Abstract

Osteoblast differentiation is tightly regulated by transcriptional regulators such as microRNAs (miRNAs). Several bioactive materials including nano-bioglass ceramic particles (nBGC) influence differentiation of the osteoblasts, but the molecular mechanisms of nBGC-stimulation of osteoblast differentiation via miRNAs are not yet known. In this study, we identified that nBGC-treatment stimulated expression of miR-30c in human osteoblastic cells (MG63). The bioinformatics tools identified its regulatory network, molecular function, biological process and its target genes involved in negative regulation of osteoblast differentiation. TGIF2 and HDAC4 were found to be its putative target genes and they were down regulated by nBGC-treatment in MG63 cells. Thus, this study advances our understanding of nBGC action on bone cells and supports utilization of nBGC in bone tissue engineering.

Keywords

MicroRNA; nano-Bioglass Ceramic (nBGC); Osteoblast; miR-30c; HDAC4; DIANA-mirPath; ToppCluster

Introduction

MicroRNAs (miRNAs), single stranded RNAs (~23 nt), act as transcriptional regulators thus, they are involved in various biological and pathological process. Altering the expression profile of miRNAs can unexceptionally modulate the expression of proteins. In response to different stimuli, the basal copy of miRNAs could either increase or decrease reasonably thus, the level of expression of targets altered moderately. miRNAs mediated physiological process are in huge extremities, because the mechanism of miRNAs action is quicker than the mechanism of transcription of targets [1–2]. The external stimulations such as environmental, physical and chemical factors indirectly perform the physiological role

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through regulating the expression of numerous miRNAs [3–5]. BMPs (Bone morphogenetic proteins) and osteogenic stimulants (dexamethasone, ascorbic acid and β -glycerophosphate) have been reported to regulate several miRNAs [6–8]. Organic and inorganic materials which are used for tissue engineering have been documented for the regulation of miRNAs expression during osteoblast differentiation [9–13]. Several bioactive materials influence differentiation and mineralization of the osteoblasts by increasing transcription factors followed by principle phenotypic markers, but the exact mechanism of up and down regulation of genes via miRNAs remains unclear.

Bioglass ceramics, a bioactive material, is widely used for bone tissue engineering. The nano bioglass ceramic particles (nBGC) are nanoscaled bioglass ceramics composed of $\text{SiO}_2\text{Na}_2\text{O}$, CaO and P_2O_5 . The enhanced properties of proliferation and differentiation of osteoblast by nBGC have been investigated in osteogenesis associated transcript and transcriptome expression [14–15]. However, the nBGC responsibilities in molecular mechanisms that regulate osteoblast differentiation are still indescribable and they warrant further analysis. miRNAs are considered to be as a potential candidate for determining the molecular mechanisms of osteoblast associated genes regulation by nBGC particles. The aim of the present study was to determine the expression of nBGC-stimulation of miRNA and to predict its putative target genes by computational analysis. The target genes of nBGC-stimulated miRNA were also validated using real time RT-PCR and Western blot analyses.

Materials and methods

Cell culture

MG63, human osteoblast-like cells were seeded at a density of 5×10^4 cells per well in the 12-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplementing with 100 nM dexamethasone, 0.2 mM L-ascorbic acid and 10 mM β -glycerophosphate (Sigma aldrich), 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. The sterilized nBGC particles (0.1 mg/ml) were suspended in the medium and a control without nano particles. The medium was renewed for every 48 hrs. The cells were maintained for 24 hrs, 7 and 21 days in a 5% CO_2 humid environment at 37°C.

Real-time RT-PCR analysis

Total RNA was isolated from cultured cells with Trizol reagent (Invitrogen) according to the manufacturer's instructions and quantitatively evaluated by Qubit 2.0 Fluorometer (Invitrogen), and qualitatively evaluated by 2% agarose gel electrophoresis. The cDNA was synthesized using Reverse Transcriptase kit according to the manufacturer's protocol (Invitrogen). The real-time PCR analysis was performed using SYBR green reagents (Invitrogen). Precursor miRNAs specific oligonucleotide primers/mRNA specific oligonucleotide primers and internal controls (GAPDH or U6) primers (Table 1) were added to the same PCR reaction tubes and were co-amplified. The fold increase of each miRNA/mRNA of interest was normalized against internal control U6 or GAPDH. The thermal cycling conditions were as follows: 95°C for 5 mins as initial denaturing, followed by 35

cycles of 95°C for 30s, 58°C for 30s and 72°C for 30s, with a final extension at 72°C for 5 mins.

Western Blot Analysis

Whole cell lysates were collected and they were subjected to Western blot analysis [15]. The antibody α -tubulin antibody and the secondary antibody conjugated with horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology. The primary HDAC4 antibody was obtained from Cell Signaling, USA.

Bioinformatics analysis

MiRanda (<http://www.microrna.org/microrna/home.do>) and TargetScan 6.2 (<http://www.targetscan.org/>) tools were used to predict miRNAs' putative target genes. DIANA-mirPath, a bioinformatics tool was used to find the signaling pathways targeted by single miRNA or multiple miRNAs on a given pathway with DIANA-MicroT-4.0 [16]. This tool was also used to perform an enrichment analysis of input datasets by contrasting each set of targeted genes to all available biological pathways which is provided by Kyoto Encyclopedia of Genes and Genomes (KEGG). ToppCluster (<http://toppcluster.cchmc.org>) and Cytoscape (<http://www.cytoscape.org/>) were used to analysis the regulatory network of miRNAs. The miRNAs predicted targets were subjected to generate regulatory network, molecular function, biological process and interactions in Toppcluster [17]. The resulted files (xgmml) were then subjected to Cytoscape for the preparation of visualisable networks.

Statistical analysis

Student's *t*-test was used to analyze significant differences between groups ($P < 0.05$). All data were shown as mean $\pm$ standard deviation (SD) from at least three separate experiments.

Results and Discussion

Identification of nBGC-stimulation of expression of miRNAs in Osteoblast Differentiation

We previously reported that nBGC particles stimulated proliferation of osteoblastic cells. They also enhanced osteoblast differentiation in the presence of osteogenic environment [15]. In this study we wanted to more precisely the molecular targets responsible for mediating nBGC effect during osteoblast differentiation. mir-130a and mir-30c have been reported as osteoblast specific miRNAs [18] but their expression and putative or validated target genes expression were not reported. mir-30c also regulates functions of several cancerous cells [19], thrombosis [20] and angiogenesis [21]. In order to identify the expression of miRNAs by nBGC-treatment, human osteoblastic cells (MG63) were treated with nBGC particles for 24 hrs, 7 days, and 21 days in the presence of osteogenic medium. Total RNA was isolated and subjected to real time RT-PCR analysis using the primers for the above miRNAs as shown in Table 1. The result showed that there was no significant change in the expression level of mir-130a; whereas, the expression level of mir-30c was significantly up regulated during 24 hrs and 7 days of BGC-treatment. The interaction of biomaterial with cell is essential for cell surface and/or intracellular signaling [22] and as a result, there might be differential expression of miRNAs mediating osteoblast

differentiation. For instance, BMP-2 down regulates miR-206 expression by its intracellular signal transducer, Smad1 and Smad4 which are known to interrupt the microprocessor complex [7]. Differential expression of miRNAs upon treatment with organic and inorganic materials used in bone tissue engineering has been reported [9–13]. Our result suggested that nBGC-treatment stimulates expression of miR-30c and that could directly or indirectly alter expression of its target genes responsible for osteoblast differentiation.

Putative target genes prediction for mir-30c

The up regulation of mir-30c expression by nBGC-treatment was subjected to predict its putative targets and it was analyzed using DIANA-mirPath and ToppCluster which are represented in Table 2 and Figure 2, respectively. In DIANA-mirPath analysis, miR-30c was predicted to target most of the genes in ubiquitin mediated proteolysis, regulation of actin cytoskeleton, JAK-STAT signaling pathway etc (Table 2). Putative targets of mir-30c was incorporated together to generate biological regulatory network using ToppCluster tool (Fig. 2). The network revealed the information about its target genes and its interactive genes involved in the negative regulation of osteoblast differentiation (Fig. 2). Simultaneously, a few of the predicted target genes of the mir-30c was validated in this study. Using miRanda, target prediction of the 3'untranslated region of TGIF2 and HDAC4 containing mir-30c seed sequence of at least 7-mer have been found (Fig. 3A). To determine the expression of miR-30c target genes, real time RT-PCR analysis was carried out. The result indicated that the transcript levels of TGIF2 and HDAC4 were significantly decreased during 24 hrs and 7 days of nBGC-treatment in MG63 cells (Fig. 3B).

Runx2 is a bone transcription factor responsible of activation of osteoblast differentiation marker genes [23–15]. HDAC4 represses activation of Runx2 target genes by preventing binding of Runx2 to its response element in DNA [26]. To determine the expression levels of HDAC4 and Runx2 proteins in response to nBGC-treatment, we next carried out Western blot analysis. HDAC4 protein expression was decreased in MG63 cells treated with nBGC for 24 hrs; whereas Runx2 expression was not altered (Fig. 4). The result suggested that down regulation of HDAC4 by nBGC-treatment would allow Runx2 to bind its responsive region of the osteoblast differentiation gene(s) DNA, thus enhancing osteoblast differentiation. Thus, expression of mir-30c pattern is associated with expression of its target genes, i.e. up regulation of miR-30c can down regulate expression of TGIF2 and HDAC4, resulting in enhanced osteoblast differentiation.

TGIF (TG Interacting Factor) is the first identified transcriptional co-repressor of Smads [27]; in addition it inhibits Smad dependent transcription by recruiting HDACs to the complex of Smad2/3 [28]. TGIF regulates ubiquitin mediated degradation of Smad2 and it has the ability to directly bind with DNA resulting blocks the other transcription factors bindings [29]. Thus, up regulation of miR-30c by nBGC-treatment could prevent the Smads degradation by down regulating expression of TGIF. These results propose that up regulation of mir-30c potentially down regulate its targets TGIF2 and HDAC4, resulting promotion of osteoblast differentiation.

Over all, we identified that miR-30c is up regulated by nBGC-treatment in human osteoblastic cells. The bioinformatics analysis revealed its regulatory network, molecular

function, biological process and its target gene(s) interaction and associated genes involved in negative regulation of osteoblast differentiation. Further experimental screening showed down regulation of its target genes such as TGIF2, HDAC4. Thus, this study reveals the physiological role played by nBGC-treatment by stimulating expression of miR-30c followed by its targets genes during osteoblast differentiation.

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Figure 1.

Real time RT-PCR analysis of nBGC-stimulation of miR-30c. Human osteoblastic cells (MG63) were incubated in osteogenic media in the presence or absence of nBGC particles for 24 hrs, 7 and 21 days. Total RNA was isolated and subjected to real time RT-PCR analysis using the primers as indicated. * indicates significant up regulation compared to control.

Figure 2. Functional enrichment analysis of regulatory network influenced by mir-30c. The cytoscape graph networks were generated from ToppCluster network analyzer.

A **B**

Figure 3. nBGC-stimulation of mir-30c targets genes, TGIF2 and HDAC4. (A) Predicted targeted sequences of TGIF2 and HDAC4 with miR-30c. (B) MG63 cells were incubated in osteogenic media in the presence or absence of nBGC particles for 24 hrs, 7 and 21 days. Total RNA was isolated and subjected to real time RT-PCR analysis using the primers as indicated. ** indicates significant down regulation compared to control.

Figure 4.

Expression of miR-30c targeted HDAC4 protein. Rat osteoblastic cells (UMR106-01) were incubated in osteogenic media in the presence or absence of nBGC particles for 24 hrs. Whole cell lysates were prepared and subjected to Western blot analysis using the antibodies as indicated.

Table 1

The primers used for real time PCR studies in this study.

Name of the miRNA/Gene		Sequence (5'→3')
mir-30c	Forward	TGTGTAAACATCCTACACTCTCAG
	Reverse	GAGTAAACAACCCCTCTCCCA
mir-130a	Forward	GCTGGCCAGAGCTCTTTTCACA
	Reverse	CACTACACGGCCAATGCCCTTT
TGIF2	Forward	GGGAGGAGAGGGGATCTGAC
	Reverse	TACCTGCCGGGTCTGACA
HDAC4	Forward	GCCCTCACCGTCCCGGTACTT
	Reverse	GTGGTTCACGCGGCAGGAT
Smurf1	Forward	CGGTGGAGACCTTCGATGAG
	Reverse	CAGAGCCTTGAAGCCTTGGA
GAPDH	Forward	GAGAGACCCCACTTGCTGCCA
	Reverse	CTCACACTGCCCTCCCTGGT
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

Table 2

Pathways affected by miR-30c putative targets.

KEGG Pathway and ID	miR-30c		
	Gene name	found genes	-ln(p-value)
Ubiquitin mediated proteolysis (hsa04120)	UBE2D1, RNF7, SOCS1, UBE2D2, UBE2G1, HERC2, UBE3C, UBE2R2, HERC3, MAP3K1, BIRC6, UBE2J1, UBE2I, WWP1, CUL2, SOCS3, CBLB, NEDD4L, UBE2O, NEDD4, UBE2F, UBE2D3	22	14.88
Axon guidance (hsa04360)	SRGAP3, PLXNA2, NCK2, GNAI2, DPYSL2, ITGB1, UNC5C, EFNA3, PLXNA1, EPHB2, SEMA6D, KRAS, SEMA6B, CFL2, UNC5D, PPP3CA, RASA1, SEMA3A, NFAT5, PPP3CB	20	12.36
Regulation of actin cytoskeleton (hsa04810)	MYH9, ITGB1, SSH2, WASL, ACTN1, ARHGEF6, KRAS, RRAS2, PIP5K1B, PIP4K2B, CRKL, CFL2, PIP4K2A, ITGA6, SOS1, VAV3, ITGB3, PIK3R2, GNAI3, ACTC1, PIP5K3, MYH10, PDGFRB, PIK3CD, PFN2	25	8.15
ErbB signaling pathway (hsa04012)	CAMK2D, NCK2, MAPK8, NRG3, KRAS, CRKL, MAP2K4, SOS1, ABL2, PIK3R2, CBLB, PLCG1, PIK3CD	13	6.62
Cell cycle (hsa04110)	CCNE2, DBF4, E2F3, SMAD2, BUB3, CDC14A, YWHAZ, ORC2L, CDC7, TFDP1, YWHAG	11	1.91
PPAR signaling pathway (hsa03320)	ME1	1	1.8
Jak-STAT signaling pathway (hsa04630)	PRLR, SOCS1, LIFR, LEPR, SOS1, IFNAR2, SOCS3, PIK3R2, CBLB, JAK1, IL28RA, CLCF1, PIK3CD	13	1.38
VEGF signaling pathway (hsa04370)	KRAS, PPP3CA, PIK3R2, NFAT5, PLCG1, PIK3CD, PPP3CB	7	1.3
Adipocytokine signaling pathway (hsa04920)	PPARGC1A, IRS2, MAPK8, LEPR, IRS1, SOCS3, JAK1	7	1.3
Adherens junction (hsa04520)	IGF1R, SNAIL1, WASL, ACTN1, SMAD2, SSX2IP, NLK	7	1.18
p53 signaling pathway (hsa04115)	CCNE2, SERPINE1	2	0.93
Natural killer cell mediated cytotoxicity (hsa04650)	KRAS, SOS1, VAV3, IFNAR2, PPP3CA, PIK3R2, NFAT5, PLCG1, PIK3CD, PPP3CB	10	0.88
Gap junction (hsa04540)	GNAI2, GJA1, KRAS, SOS1, ADRB1, GRM5, PLCB4, PDGFRB	8	0.81
ECM-receptor interaction (hsa04512)	ITGB1, DAG1, ITGA6, SV2B, FNDC3A, THBS2, ITGB3	7	0.77
mTOR signaling pathway (hsa04150)	RPS6KA2, PIK3R2, DDIT4, PIK3CD	4	0.31