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REGULATION OF OSTEOBLAST DIFFERENTIATION BY MIR-148B, MIR-181B, AND MIR-181C

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Abstract

Mesenchymal stem cells are directed towards the osteoblast lineage and bone cell formation, regulated at transcriptional, post-transcriptional, and translational levels by various signaling pathways and transcription factors. Recently, a new level post-transcriptional regulation by microRNAs has been identified. MicroRNAs are small, non-coding RNA strands that regulate protein synthesis by binding to mRNA and inhibiting translation, and thus can control virtually all biological processes. The expression of many microRNAs have previously been shown to be modulated during osteoblast differentiation. We observed that expression levels of miR-148b, miR-181b, and miR-181c are upregulated during osteoblast differentiation of MC3T3 cells. To determine the role of these miRs in the differentiation process, we examined phenotypic changes in cells transfected for overexpression and inhibition of each miR. By qRT-PCR studies, we found that miR-181b and miR-181c induce the expression of differentiation markers genes Runx2, alkaline phosphatase and Col1a1, and support osteoblast differentiation, possibly by targeting the inhibitors of differentiation such as FGF21, Bcl2, Smad4 and Nlk. These findings suggest that miR-181b and miR-181c may be a potential therapeutic target for enhancing bone formation.

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BACKGROUND

MicroRNAs (miRs) are the emerging powerful tools for gene regulation, which can function to control biological processes such as apoptosis, growth, phenotype differentiation (muscle, hematopoietic, nerve) and are also associated with diseases such as cancer. MiRs are ~22nt long, non-coding, single-stranded RNA. MiRs are post-transcriptional regulators that bind to the 3' UTR of mRNA and inhibit translation or cause degradation of the transcript.[1] MiRs target over 60% of human protein-coding genes, as the miRs bind to any complementary site of an mRNA.[2] These miRs come from either their own genes or from introns, and are transcribed by the enzyme RNA polymerase II as primary microRNA, which consist of a hairpin loop with a 5' cap and a poly(A) tail.[3] The primary miR is recognized by DGCR8 (the nuclear protein DiGeorge Syndrome Critical Region 8) and is cleaved by Drosha into a precursor microRNA (pre-miR). This cleavage removes about eleven nucleotides from the hairpin structure, and leaves a two-nucleotide overhang on the 3' end.[4] The pre-miR is translocated to the cytoplasm by the transporter Exportin-5, where the ribonuclease III enzyme Dicer cleaves the loop of the structure, creating two strands that are 22 nucleotides long each. Either of the two strands can act as a functional mature miR.[5] Typically, one of the strands becomes incorporated into the RNA-induced silencing complex, or RISC.[6] The RISC can silence genes by preventing translation, or less commonly, by direction degradation of the mRNA.[7]

Bone formation and continuous repair is largely dependent on the activity of two cell types: osteoclasts and osteoblasts. The osteoblasts differentiate from mesenchymal stem cells (MSCs) after their commitment to osteoblast lineage and secrete bone matrix proteins. During

the process of osteoblast differentiation, the cells undergo three distinct stages: proliferation, extracellular matrix maturation, and mineralization. The process of osteoblast differentiation is highly regulated by various transcription factors and signaling pathways, directing the differentiation from precursor cells to mature osteoblasts. MSCs commit to an osteoblastic lineage with the help of bone morphogenetic protein (BMP) signaling, specifically with BMP2. [8] This signaling controls the expression of essential transcription factors Runx2 and Osterix, directing the osteoprogenitor cells to become preosteoblasts. [9] Preosteoblasts can be identified by high expression levels of histones, collagen, fibronectin, osteopontin, and TGF-β. Proliferation drops as the cells become mature osteoblasts, mediated by proteins required for extracellular matrix production. Osteoblasts in the matrix maturation stage show high levels of alkaline phosphatase, collagen, SPARC, and BSP. At the third stage of differentiation, the osteoblasts mineralize, forming the final bone tissue as osteocytes embedded in the matrix. The increased presence of osteocalcin, collagenase, and DMP1 mark this stage, as well as an increase in sclerostin, which regulates BMP2, preventing more MSCs from becoming osteoprogenitors.[8]

Many genes involved in the bone formation process also undergo post-transcriptional regulation, as miRs inhibit the translation of mRNAs during bone formation.[10] The effects of Dicer inactivation and subsequent loss of miRs on bone formation have been demonstrated through tissue-specific knockdown using Cre recombination. Using collagen type 1, which is expressed at high levels in osteoprogenitor cells, the inactivation of Dicer was found to be lethal in mouse embryos after E14.5. With the deletion of Dicer, the fetal skeletons were deformed and not mineralized, consisting of cartilage with no bone elements. Levels of osteocalcin and bone sialoprotein, both of which function in mineralization, were significantly reduced, leading to an absence of mineralized matrix. These findings indicate the importance of miR regulation of

differentiation early in skeletal development. In the same study, Dicer was also inactivated with osteocalcin-Cre, since osteocalcin is expressed during mineralization in mature osteoblasts. These mice were viable, but showed delayed bone maturation and reduced levels of Runx2 and Osterix at embryonic and post-natal stages. However, at two months of age, the conditional knockout mice had increased bone mass with thicker trabecular and cortical bone than the control mice. This bone mass continued to increase up to eight months, along with an upregulation in collagens Col1a1, Col1a2, and Col5. This data shows a reliance on miR regulation of growth factors found in the extracellular matrix for proper bone formation.[10]

There are a few miRs which have been reported to regulate osteoblast differentiation. Several miRs have been proposed to be inhibitory to osteogenic differentiation, whereas other miRs have been shown to promote osteogenesis (Table 1). Large-scale profiling studies in the fetal mouse calvaria preosteoblast cell line MC3T3 have reported upregulation of 58 miRs during differentiation and mineralization. Of the upregulated miRs, miR-29b was specifically shown to target several negative regulators of differentiation signaling pathways, positively affecting osteoblast differentiation.[11] In this study, we selected three miRs found to be upregulated during osteoblast differentiation: miR-148b, miR-181b, and miR-181c, and examined the expression and function of these miRs during osteogenesis using MC3T3 cells by employing overexpression and inhibition studies to understand the effect on differentiation markers. By histochemical staining and expression levels, we can determine the role these regulators play in osteoblast differentiation. We found that miR-181b and miR-181c have the potential to promote osteogenesis in MC3T3 cells, however, more studies are required to get a conclusive function for miR-148b.

METHODS

Cell Culture - MC3T3 cells, a cell line originated from fetal mouse calvaria cells, were plated as pre-osteoblasts and maintained in 6-well plates. The cells were fed with αMEM (α-minimum essential medium, Hyclone) with 10% fetal bovine serum, 10 units/mL Penicillin-Streptomycin, and 1x L-glutamine, replacing the medium every 2 days. Cell differentiation was induced at confluence by adding 10 mM β-glycerophosphate and 50 µg/mL ascorbic acid to the medium, continuing to replace the medium every 2 days. Cells were harvested for RNA and histological staining at days -2, 1, and 7.

For miR overexpression and inhibition studies, MC3T3 cells were transfected with mirVana miR mimics and inhibitors for miR-148b, miR-181b, and miR-181c, as well as mimic and inhibitor controls (Ambion) using Oligofectamine Reagent (Invitrogen), following the manufacturer's instructions. Cell differentiation was induced post-confluence and cells were harvested for RNA and histological staining at day 7 after induction. Cell images were captured at regular intervals during culture using a Spot camera.

Histological Staining – Cells harvested for histological staining were fixed in 2% paraformaldehyde. To stain for alkaline phosphatase, cells were washed with 0.2 M cacodylic buffer, pH 7.4 and stained with staining solution (0.5mg/mL Napthol As-Mx phosphate disodium salt, 28 µL/mL NN dimethyl formamide, 0.1 M Tris maleate buffer at pH 8.4, and 1mg/mL Fast Red TR salt). The cells were incubated at 37°C for 20 minutes and photographed.

RNA Isolation and qPCR –To isolate the total RNA, 1 mL of TRIzol (Invitrogen) was added to each sample to lyse the cells. For phase separation, 0.2 mL of chloroform was added for each 1 mL of TRIzol. The samples were mixed by vortexing and incubated at room temperature

for 3 minutes. The samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. To precipitate the RNA, the aqueous phase was carefully transferred to a new centrifuge tube with 0.6 mL isopropanol for every 1 ml TRIzol used. The samples were incubated on ice for 15 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. To wash the RNA, the supernatant was removed and 0.5 mL of 70% ethanol was added for each 1 mL TRIzol used. After mixing, the samples were centrifuged at 12,000 x g for 5 minutes at 4°C. The resulting pellets were dried and dissolved into 30 µL of DEPC treated water.

Possible genomic contamination was removed using the DNA-Free RNA kit (Zymo), following the manufacturer's procedure to recover RNA larger than 17 nt. The purified RNA was used for cDNA synthesis using First-Strand cDNA Synthesis kit (Invitrogen). The cDNA was diluted 10 times with nuclease-free water, and used for qRT-PCR using SYBR-Green master mix (BioRad). The primers used for qRT-PCR are listed in Table 2.

A set of samples were also prepared for detection of miR expression. One µg of RNA was processed through QuantiMir RT Kit (System Biosciences), and the diluted cDNA was used for qRT-PCR using miR-specific forward primers, universal reverse primer and SYBR-Green master mix (BioRad). The primers are listed in Table 2.

RESULTS

Osteoblast-Specific Marker Genes are Induced during MC3T3 Cell Differentiation

MC3T3-E1, a pro-osteoblast cell line, was studied to determine the expression pattern of markers for osteoblast differentiation. Cell images were taken at day 0, 1, 6, and 8 of plating, and differentiation was induced on day 4 to examine cell phenotypes over the course of differentiation (Figure 1A). The cells were actively proliferating on day 1, had reached confluence by day 4, and by day 8, had formed multiple layers of tightly packed cells. After differentiation was induced, osteoblasts begin producing extracellular matrix, as evidenced by an increase in enzyme activity of alkaline phosphatase. Alkaline phosphatase staining of the cells at day 1 shows low levels of alkaline phosphatase, as the cells are still in the proliferating stage (Figure 1B). By day 7, the alkaline phosphatase activity was significantly increased, indicating a progression in the osteoblast differentiation. The qRT-PCR analysis of RNA samples from the cells suggests that Runx2 is expressed at basal levels in proliferating cells (day 0 and day 1), but shows ~2.5-fold increase in the mRNA levels of Runx2 on day 7, which is essential to osteoblasts differentiation (Figure 1C). The mRNA levels of alkaline phosphatase showed an 8 to 9-fold increase shortly after induced differentiation, since alkaline phosphatase is important in the matrix maturation stage of differentiation, though the levels decreased earlier than expected at day 7.

Upregulation of Selected MircoRNAs during Osteoblast Differentiation

As osteoblasts undergo differentiation, many miRs are upregulated or downregulated to aid in controlling differentiation. Previous studies in the lab using microarray analysis have shown that a number of miR are upregulated during osteoblast differentiation.[11] Of these

upregulated miRs, 148b, 181b, and 181c were selected for further study, showing significant upregulation as early as day 7. The qRT-PCR analysis was used to validate the expression of miR-148b, 181b, and 181c over the MC3T3 differentiation time course (Figure 2). For all three miR, the expression increased significantly by day 7, suggesting an important role in promoting osteoblast differentiation. These findings are consistent with the expression analysis of Li *et al*. which saw a significant increase in expression of miR-148b up through day 21 and an early increase in miR-181b through the entire differentiation time course.

Overexpression of miR-181b and 181c Promotes Differentiation

MC3T3 cells were transfected with miR mimics (to overexpress the miR) and inhibitors (to inhibit the miR function)to understand the regulation of osteogenic differentiation by these miR. Cell images were taken at day 1 and 6 post-transfection to study morphological changes in the cells, and the extent of differentiation was monitored by alkaline phosphatase staining and expression analyses of marker genes on day 7 post-transfection. Overexpression and inhibition of miR-148b did not change the cell morphology or the extent of alkaline phosphatase staining over the controls (Figure 3A). Through qRT-PCR analysis, we find that miR-148b was expressed ~1000-fold in miR-148b mimic samples over the mimic control (Figure 3B, lower right panel). Overexpression of miR-148b showed reduced expression of Runx2 and Col1a1, indicating reduced differentiation of the MC3T3 cells at this early stage. It should be noted that the mimic control also downregulated Runx2 expression 0.6-fold, whereas inhibitor control displayed a non-specific upregulation for all of the genes. The inhibition of miR-148b also reduced the expression of these markers over the inhibitor control; however, the difference was not significant when compared with the expression levels in the mock or mimic control samples. For

alkaline phosphatase, it is difficult to interpret the changes in expression considering the larger error bars (Figure 3B).

For miR-181b, the overexpression did not change the cell morphology; however, the inhibitor samples had reduced cell numbers, either due to reduced proliferation or increased cell death (Figure 4A, upper and middle panels). There was no significant difference in staining in either overexpression or inhibition (Figure 4A, lower panels). Expression analysis showed that miR-181b was overexpressed up to 8-fold over the control (Figure 4B, lower right panel). Similar to miR-148b, the mimic control downregulated Runx2 expression, and inhibitor control increased the expression of all of the markers. For Runx2 and alkaline phosphatase, there appears to be a trend of increased expression upon overexpression, and downregulation upon inhibition of miR-181b. For Col1a1, the expression is consistent among treatments (Figure 4B).

The transfected cells showed little variation in phenotype through the proliferation stage for miR-181c (Figure 5A, upper and middle panels). The miR-181c mimic showed higher levels of staining, potentially promoting differentiation when overexpressed, though there was no visible difference detected for inhibition of miR-181c (5A, lower panels). MiR-181c was upregulated 28-fold in mimic-treated samples (Figure 5B, lower right panel), which provided slight upregulation of Runx2 over the mimic control. For alkaline phosphatase, again, the error bars are too large, preventing us from making any conclusions. We did not find any significant difference in the expression pattern of Col1a1 upon overexpression or inhibition of miR-181c (Figure 5B).

DISCUSSION

As osteoblasts differentiation progresses, expression of miRs 148b, 181b, and 181c significantly increases, indicating a role of these miRs in the differentiation process. While it is not clear what mechanism these miRs use for regulating differentiation, functional studies using overexpression and inhibition of these miRs can provide important insights on their effects on the cells. By examining extent of differentiation after manipulation of the miRs, the general effect of the miRs on the cells can be determined. To examine more specific function of a miR, these effects can be compared to the functions of target genes.

MC3T3 cells demonstrate a significant increase in miR-148b transcript levels by day 7, which would imply the miR plays an important role in differentiation regulation. However, the overexpression study revealed considerably lower levels of Runx2 and alkaline phosphatase transcripts than would be expected during differentiation, implying a suppression of differentiation. In contrast to miR-148b, the high levels of Runx2 and alkaline phosphatase seen in overexpression of miRs 181b and 181c indicate they target negative regulators of differentiation early in the matrix maturation stage. These miRs demonstrate a promotion of osteoblast differentiation by inhibiting genes that would normally attenuate the differentiation process. In addition to an indirect effect on Runx2 and alkaline phosphatase, both miRs-181b and 181c reduce transcript levels of Col1a1, which is required to promote further differentiation into the mineralization stage. This is supported by Li *et al*., who showed a considerable increase in miR-181b transcripts over the differentiation time course.[11]

Possible Mechanisms for miR-148b, miR-181b, and miR-181c in regulating Osteoblast Differentiation

Using the miRWalk database, predicted target genes that were common between miR-148b, 181b, and 181c were identified (Table 3).[12] One of the predicted targets, Dicer1, regulates miR biogenesis to control bone formation. Lowered levels of Dicer1 would lead to increased osteoblast differentiation, as demonstrated in mice carrying Dicer-inactivation in osteoblasts.[10] Another potential target, Fgf21 is a negative regulator of bone growth, and deletion of the gene has been shown to prevent bone loss in mice.[13] Bcl2 was identified as another target, whose expression has been shown to inhibit differentiation.[14] Smad4, which is an inhibitory Smad for osteoblast differentiation via BMP signaling pathways, is targeted. Inhibition of Smad4 would negatively affect the signaling and therefore differentiation.[15] Cyclin B2 is one of many cyclins that regulates the cell cycle through mitosis; inhibiting the gene would prevent mitosis and slow proliferation of the cells, negatively affecting differentiation.[16] In addition to these verified targets, TargetScan identified the gene nemo-like kinase (Nlk) as a predicted target for all three miRs. Nlk is a negative regulator of differentiation, inhibiting the Wnt signaling pathway as well as Smad-regulated BMP signaling.[17] Inhibition of Nlk would then promote differentiation in osteoblasts.

All the potential targets, Dicer1, Fgf21, Bcl2, Cyclin B2, Smad4, and Nlk, are inhibitory to bone growth and differentiation. Inhibition of any of these targets would yield the increased levels of differentiation markers seen in the overexpression studies. Very recently, ACVR1, a crucial activin receptor in BMP signaling pathways, has been shown to be a target of miR-148a. As a negative regulator of ACVR1, miR-148a acts as an inhibitor for osteoblast differentiation;

miR-148b could also target this receptor, as evident by the reduction in differentiation during miR-148b overexpression.

Future Studies

To gain a better understanding of the specific functions of miRs 148b, 181b, and 18lc, it would be beneficial to carry out overexpression and inhibition studies over the full differentiation time course for individual miRs as wells as the combinations of miRs. Further evidence of effect on differentiation could be obtained by examining alkaline phosphatase staining as well as gene expression studies up through matrix maturation and mineralization stages. It is also needed to validate the predicted targets for these miRs by examining their expression levels after overexpression or inhibition. Further, to confirm that these genes are directly targeted by miRs to regulation osteoblast differentiation, one needs to perform 3' UTR assays using WT and mutant constructs. If these miRs are validated to be truly promoting osteoblast differentiation, these can be overexpressed in vivo, as a therapy for bone-related diseases. It should also be noted that miR-181b and miR-181c are associated with cell differentiation and tumor suppression in cancers, which should be considered in future in vivo studies of these miRs.[19]

Table 1: Known miR Regulators of Osteoblast Differentiation[8, 9, 17]

miRs that Promote Osteogenesis	**Target Genes**	**miRs that Inhibit Differentiation**	**Target Genes**
miR-20a	BAMB1, CRIM, PPARγ	miR-23a-27a-24-2	SatB2, Runx2, Hoxa10
miR-27	APC	miR-26a	Smad1
miR-29b	HDAC4, DUSP2, TGFβ3, AcvR2b, CTNNIBP1	miR-29a,b	Col1A, Col3A, Col5
miR-29c	Dkk1, Kremen, SFRP2, Osteonectin	Panel of 10 miRs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-338)	Runx2
miR-196a	Hox8	miR-138	FAK
miR-210	AcvR1b	miR-211	Runx2
miR-218	Runx2	miR-335	Runx2
miR-335-5p	DKK1	miR-637	Osterix
miR-378	Nephronectin, GaINT-7		
miR-2861	HDAC5, Hoxa2		
miR-3960	HDAC5, Hoxa2		

Table 2: qRT-PCR Primers

Primer Name	Primer Sequence
Runx2	Forward 5’ CCGGCCACTTCGCTAACTT 3’
	Reverse 5’ TGGTGCTCGGATCTACAGGAA 3’
Alkaline Phosphatase	Forward 5’ TTGTGCGAGAGAAAGAGAGAGA 3’
	Reverse 5’ GTTTCAGGGCATTTTTCAAGGT 3’
Col1a1	Forward 5’ CCCAAGGAAAAGAAGCACGTC 3’
	Reverse 5’ AGGTCAGCTGGATAGCGACATC 3’
mcox	Forward 5’ ACGAAATCAACAACCCCGTA 3’
	Reverse 5’ GGCAGAACGACTCGGTTATC 3’
mmu-miR-148b Forward	5’ TCAGTGCATCACAGAACTTTGT 3’
mmu-miR-181b Forward	5’ AACATTCATTGCTGTCGGTGGGT 3’
mmu-miR-181c Forward	5’ AACATTCAACCTGTCGGTGAGT 3’
U6 Forward	5’ CGC TTC GGC AGC ACA TAT AC 3’
Universal Reverse miR	5’ GACGAGGACTCGAGCTCAAGCT 3’

Table 3: Predicted Target Genes of miR-148b, miR-181b, and miR-181c

	miR-148b	miR-181b	miR-181c
Dicer1	x	x	x
Fgf21	x	x	x
Bcl2		x	x
Cyclin B2	x	x	x
Smad4	x	x	x
Nlk	x	x	x

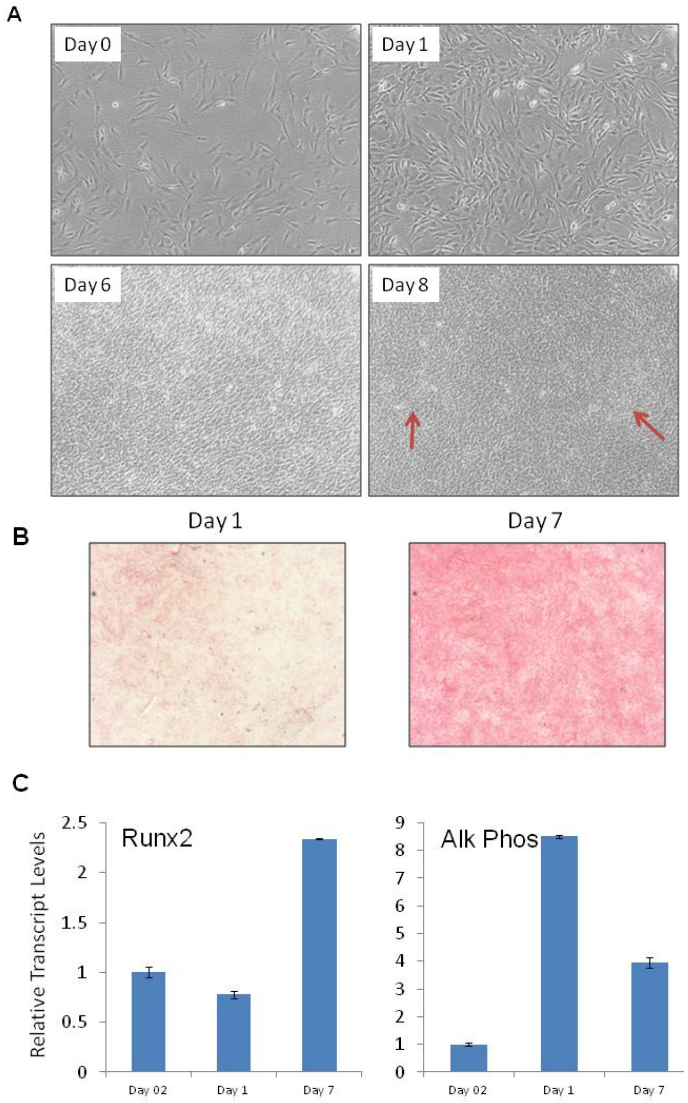

Figure 1: Expression Patterns of Osteoblast Differentiation Markers

A. MC3T3 cell images taken after day 0, 1, 6, and 8 after plating. Arrows demonstrate multiple layers of cells during proliferation. B. Alkaline phosphatase staining of MC3T3 cells on indicated days after induction of differentiation. C. qRT-PCR analysis of RNA samples collected at indicated time points for Runx2 and alkaline phosphatase transcripts.

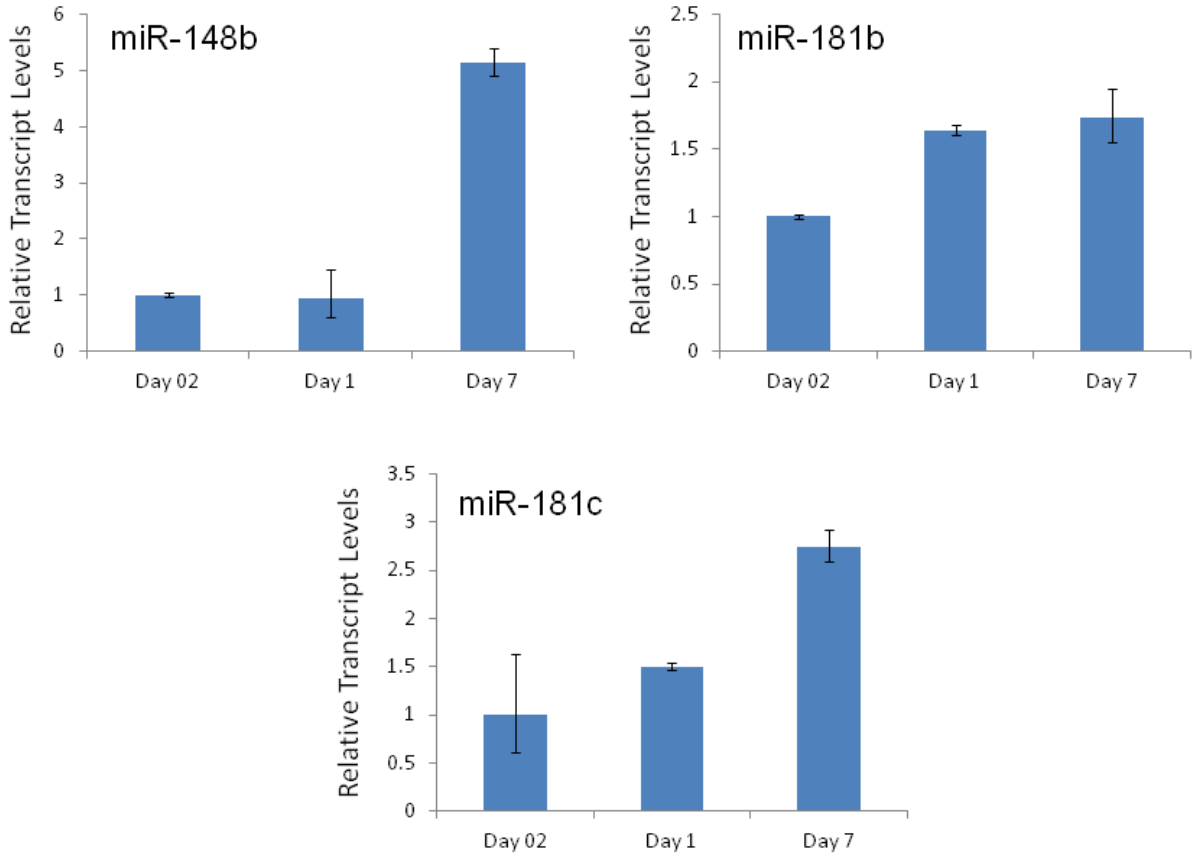

Figure 2: Expression of selected miRs during Osteoblast Differentiation

qRT-PCR analysis of RNA samples for expression of miR-148b, miR-181b, and miR-181c during osteoblast differentiation in MC3T3 cells.

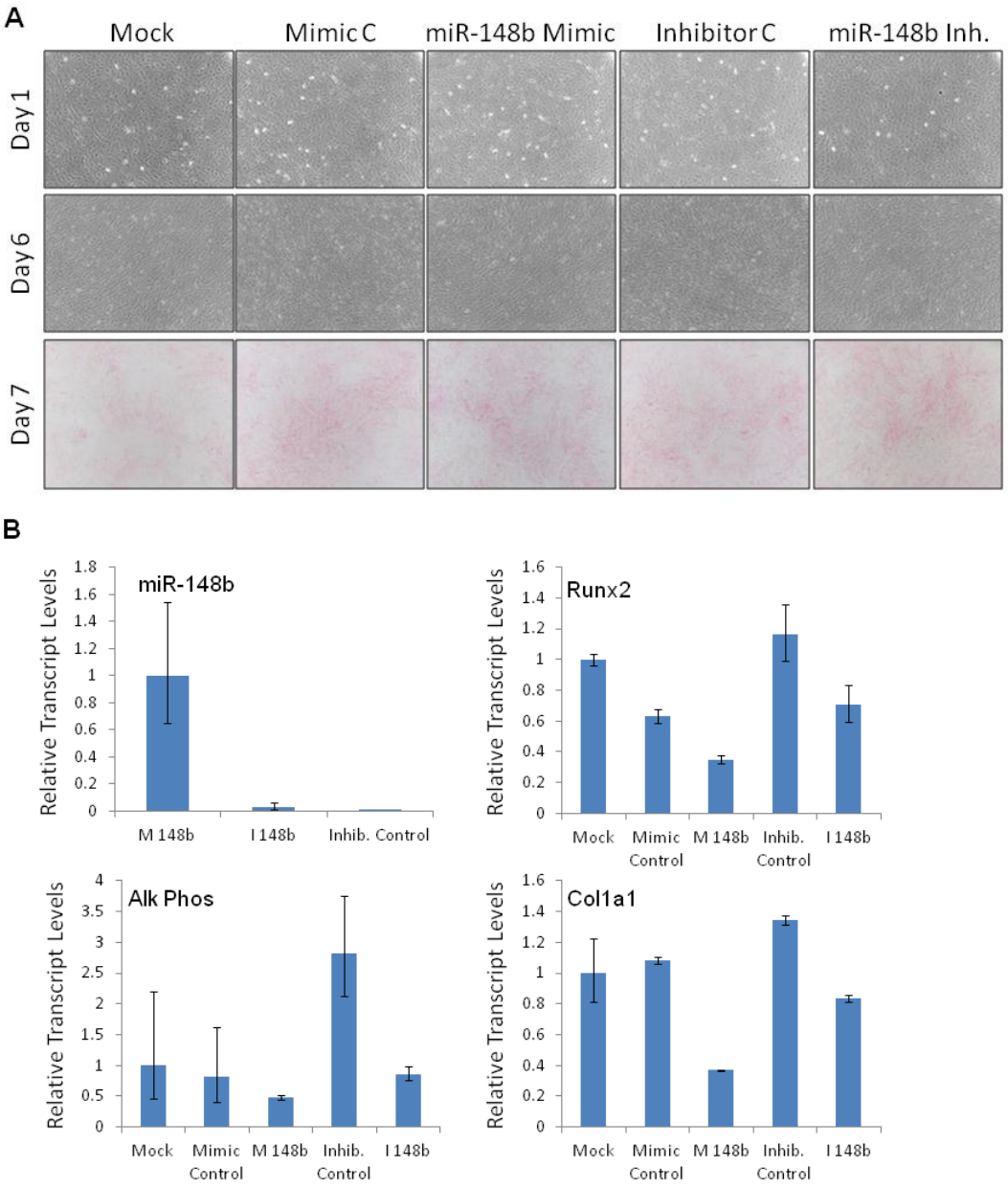

Figure 3: Overexpression and Inhibition of miR-148b

A. Cell images of MC3T3 cells on day 1 and 6 post-transfection with miR-148b mimic and inhibitor (upper and middle panels), alkaline phosphatase staining on day 7 post-transfection (lower panels). B. qRT-PCR analysis for differentiation markers Runx2, alkaline phosphatase, and collagen type 1, and overexpression of miR-148b (upper-left panel).

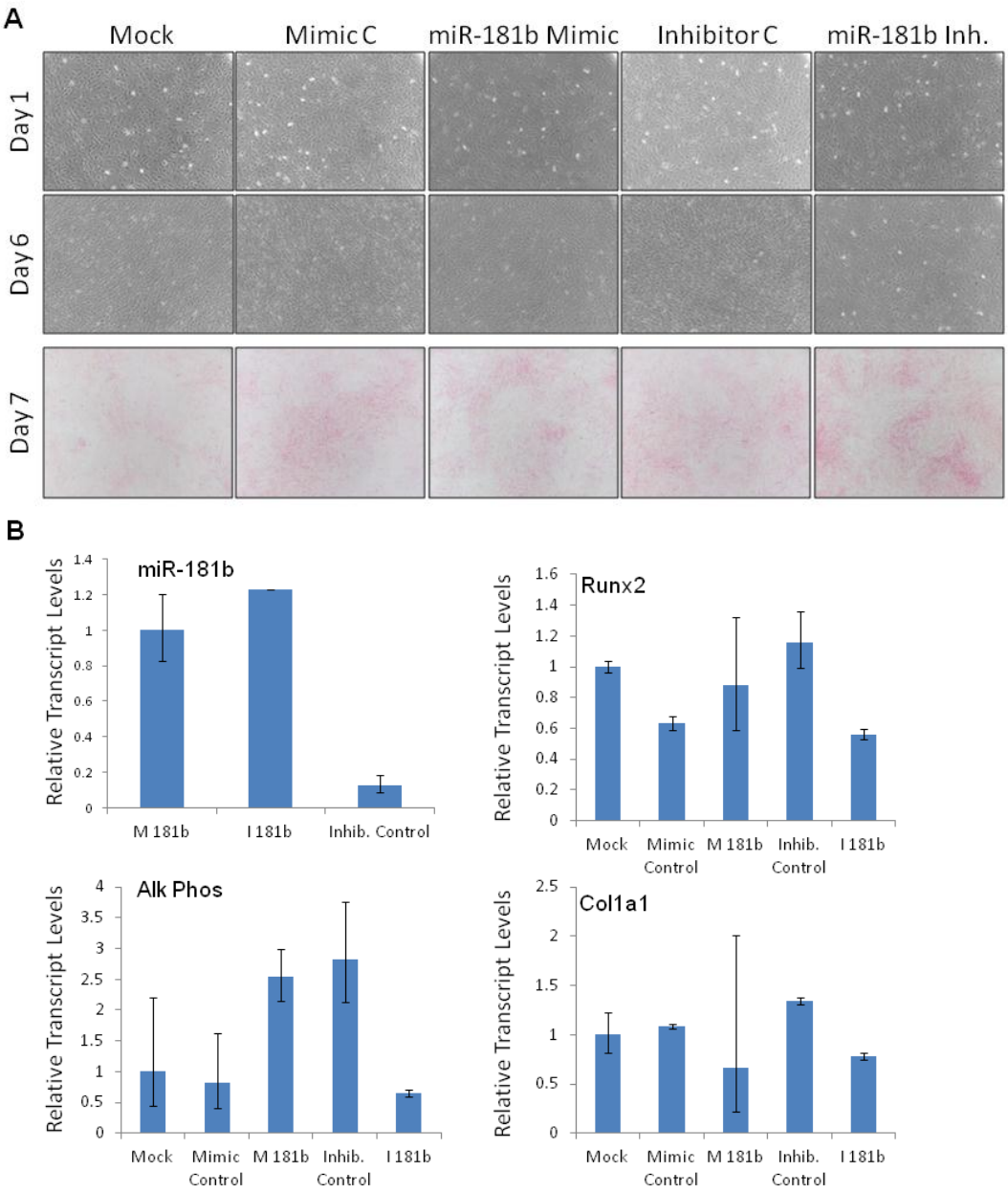

Figure 4: Overexpression and Inhibition of miR-181b

A. Cell images of MC3T3 cells on day 1 and 6 post-transfection with miR-181b mimic and inhibitor (upper and middle panels), alkaline phosphatase staining on day 7 post-transfection (lower panels). B. qRT-PCR analysis for differentiation markers Runx2, alkaline phosphatase, and collagen type 1, and overexpression of miR-181b (upper-right panel).

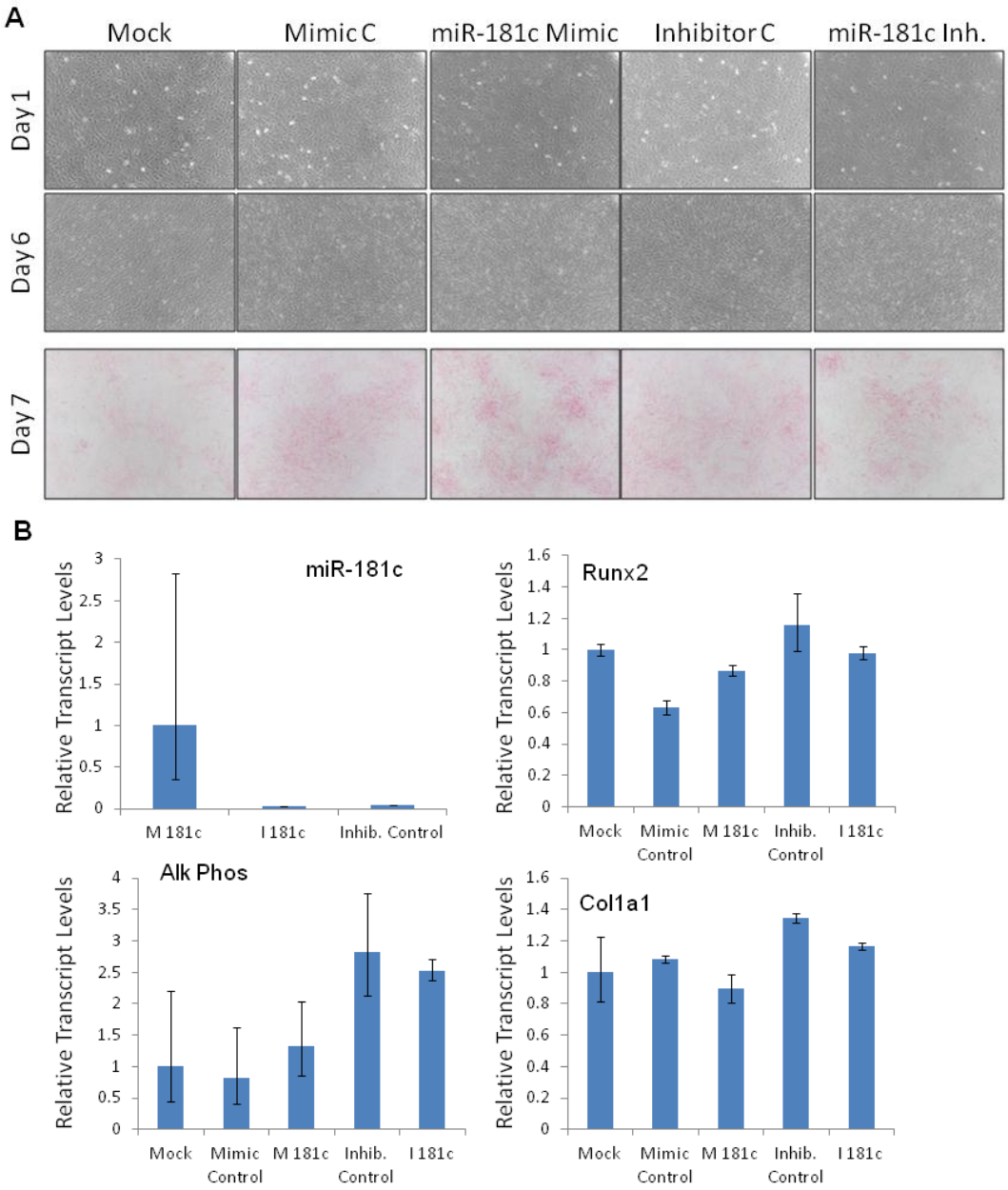

Figure 5: Overexpression and Inhibition of miR-181c

A. Cell images of MC3T3 cells on day 1 and 6 post-transfection with miR-181c mimic and inhibitor (upper and middle panels), alkaline phosphatase staining on day 7 post-transfection (lower panels). B. qRT-PCR analysis for differentiation markers Runx2, alkaline phosphatase, and collagen type 1, and overexpression of miR-181c (upper-left panel).

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