MiR-133a Modulates Osteogenic Differentiation of Vascular Smooth Muscle Cells

Xiao-Bo Liao,* Zhi-Yuan Zhang,* Ke Yuan, Yuan Liu, Xiang Feng, Rong-Rong Cui, Ye-Rong Hu, Zhao-Shun Yuan, Lu Gu, Shi-Jun Li, Ding-An Mao, Qiong Lu, Xin-Ming Zhou, Vinicio A. de Jesus Perez, and Ling-Qing Yuan

Departments of Cardiothoracic Surgery (X.-B.L., Z.-Y.Z., X.F., Y.-R.H., Z.-S.Y., L.G., X.-M.Z.) and Pediatrics (S.-J.L., D.-A.M.) and Institutes of Metabolism and Endocrinology (Y.L., R.-R.C., L.-Q.Y.) and Clinical Pharmacy and Pharmacology (Q.L.), The Second Xiangya Hospital of Central South University, Changsha, 410011, People's Republic of China; and Department of Medicine (K.Y., V.A.d.J.P.), Stanford University, Stanford, California 94305;

Arterial calcification is a key pathologic component of vascular diseases such as atherosclerosis, coronary artery disease, and peripheral vascular disease. A hallmark of this pathological process is the phenotypic transition of vascular smooth muscle cells (VSMCs) to osteoblast-like cells. Several studies have demonstrated that microRNAs (miRNAs) regulate osteoblast differentiation, but it is unclear whether miRNAs also regulate VSMC-mediated arterial calcification. In the present study, we sought to characterize the role of miR-133a in regulating VSMC-mediated arterial calcification. Northern blotting analysis of VSMCs treated with β-glycerophosphate demonstrated that miR-133a was significantly decreased during osteogenic differentiation. Overexpression of miR-133a inhibited VSMC transdifferentiation into osteoblast-like cells as evidenced by a decrease in alkaline phosphatase activity, osteocalcin secretion, *Runx2* expression, and mineralized nodule formation. Conversely, the knockdown of miR-133a using an miR-133a inhibitor promoted osteogenic differentiation of VSMCs by increasing alkaline phosphatase activity, osteocalcin secretion, and *Runx2* expression. *Runx2* was identified as a direct target of miR-133a by a cotransfection experiment in VSMCs with luciferase reporter plasmids containing wild-type or mutant 3′-untranslated region sequences of *Runx2*. Furthermore, the pro-osteogenic effects of miR-133a inhibitor were abrogated in *Runx2*-knockdown cells, and the inhibition of osteogenic differentiation by pre–miR-133a was reversed by overexpression of *Runx2*, providing functional evidence that the effects of miR-133a in osteogenic differentiation were mediated by targeting *Runx2*. These results demonstrate that miR-133a is a key negative regulator of the osteogenic differentiation of VSMCs. **(*Endocrinology* 154: 3344–3352, 2013)**

MicroRNAs (miRNAs) are an abundant class of short (~19–25 nucleotides), noncoding RNAs that negatively regulate gene expression and play a pivotal role in several physiological and pathological processes, including cell proliferation, differentiation, and apoptosis (1, 2). The impact of miRNAs on cardiovascular diseases has been investigated, suggesting that cardiovascular development (3), heart failure (4), and acute myocardial infarction (MI) (5–7) are regulated by miRNAs. Therefore, miRNAs are under consideration as attractive new drug targets.

Among cardiovascular disorders, arterial calcification is prevalent among patients with end-stage renal disease (8, 9) and diabetes (10, 11), for whom it is associated with a high index of mortality (12). Previously, arterial calcification was thought to be a passive, degenerative, end-stage process involving the precipitation of calcium-phosphate minerals in vascular tissue. However, vast evidence

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received December 18, 2012. Accepted May 29, 2013.
First Published Online June 24, 2013

* X.-B.L. and Z.-Y.Z. contributed equally to this work.

Abbreviations: ALP, alkaline phosphatase; β-GP, β-glycerophosphate; miRNA, microRNA; qRT-PCR, quantitative RT-PCR; Runx2, runt-related transcription factor 2; siRNA, small interfering RNA; UTR, untranslated region; VSMC, vascular smooth muscle cells; WT, wild-type.

has demonstrated that arterial calcification is an active, cell-regulated process, which shares numerous similarities with osteogenesis at the cellular level (13–16). This is based on the discovery that vascular smooth muscle cells (VSMCs) can undergo transdifferentiation into osteoblast-like cells, as evidenced by an increase in osteoblast-related markers, such as runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and osteocalcin.

Among the proposed mechanisms involved in transdifferentiation of VSMCs, several investigations have suggested roles for miRNAs, given the accumulating evidence of their involvement in osteoblast differentiation (17, 18). Recent studies have demonstrated that miR-125b (accession number MIMAT0000423), miR-204 (accession number MIMAT0000237), and miR-29a/b (accession numbers MIMAT0000802 and MIMAT0000801) are involved in regulating calcification of VSMCs (19–21). Among known miRNAs, miR-133a (accession number MIMAT0000145) is preferentially expressed in cardiac and skeletal muscle cells and has been shown to regulate their differentiation (22). More recently, miR-133a has been shown to oppose osteoblastic differentiation of C2C12 mesenchymal cells by directly suppressing Runx2, a key transcription factor involved in differentiation and maintenance of osteoprogenitor cells (23–25). Whether miR-133a is also involved in VSMC osteogenic differentiation has not yet been explored.

In the present study, we tested the hypothesis that miR-133a protects against the osteogenic differentiation of VSMCs by targeting Runx2. We demonstrated that the level of miR-133a was reduced in VSMCs undergoing osteogenic differentiation. MiR-133a was shown by both gain-of-function and loss-of-function studies to function as a negative regulator in the osteogenic differentiation of VSMCs. Moreover, Luciferase reporter assays demonstrated that miR-133a directly targeted the 3′-untranslated region (UTR) of Runx2. Finally, we demonstrated that the knockdown of Runx2 abrogated the effects of miR-133a inhibitor on osteogenic differentiation in VSMCs, whereas conversely, the expression of Runx2 plasmid abrogated the effects of pre–miR-133a. These results implicated that miR-133a is an important regulator in the osteogenic differentiation of VSMCs through targeting of Runx2.

Materials and Methods

Reagents

β-Glycerophosphate (β-GP) was purchased from Sigma (St Louis, Missouri). DMEM and fetal bovine serum was purchased from Gibco BRL Co (Grand Island, New York). Lipofectamine 2000 was purchased from Invitrogen Co (Carlsbad, California). Antibodies for Runx2 and β-actin were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California). Runx2 small interfering RNA (siRNA) oligos and control siRNA oligos were purchased from QIAGEN (Hilden, Germany).

Cell culture

The Second Xiangya Hospital of the Central South University Ethics Review Board approved the experiments (2010-S163). Primary VSMCs were isolated from C57BL/6 female mice by an explant method as previously described (26, 27). All experiments were performed using cells from passages 3 to 6. VSMCs were cultured in DMEM (high glucose, 4.5 g/L) supplemented with 15% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). To induce calcification, the cells were incubated in DMEM containing 15% fetal bovine serum supplemented with 10mM β-GP.

Measurement of ALP activity, osteocalcin secretion, and mineralized matrix formation in cultured VSMCs

ALP activity, osteocalcin secretion assay, Alizarin Red S staining and quantification of calcium deposits were performed as previously described (13, 14). Briefly, the cells were washed with PBS, and the cell layers were scraped into solution. The lysates were homogenized. ALP activity was assayed by the spectrophotometric measurement of *p*-nitrophenol release at 37°C. Osteocalcin release into the culture media was measured using a specific RIA kit (DiaSorin, Stillwater, Minnesota). A fraction of the lysate was used in a Bradford protein assay to normalize protein expression to total cellular protein.

For Alizarin Red S staining, cells were fixed in 70% ethanol for 1 hour at room temperature and then stained with 40mM Alizarin Red S for 10 minutes. The stained matrix was assessed and photographed using a digital microscope. Alizarin Red S stain was released from the cell matrix by incubation in cetylpyridinium chloride for 15 minutes. The amount of released dye was quantified by spectrophotometry at 540 nm. Results were then normalized to total cellular protein values.

Plasmid constructs and transfections

To construct an miR-133a expression vector, an 80-bp genomic sequence of the miR-133a-1 precursor (pre–miR-133a, Table 1, accession number MI0000159) was inserted into pSi-

Table 1. Nucleotide Sequences of Primers for Constructing Pre-miRNA Plasmids

Pre-miRNA	Primer Sequences (5′–3′)
Pre--miR-133a	
Primer 1	GATCCTGCTAAAGCTGGTAAAATGGAACCAAATCGCCTCTTCAATGGATTTGGTCCCCTTCAACCAGCTGTAGCTGGAAA
Primer 2	AGCTTTTCCAGCTACAGCTGGTTGAAGGGGACCAAATCCATTGAAGAGGCGATTTGGTTCCATTTTACCAGCTTTAGCAG

lencer 4.1-CMV vector expressing puromycin (Ambion Inc, Austin, Texas). Oligonucleotide pairs were constructed to anneal to the linearized vector and were subsequently ligated into pSilencer 4.1-CMV vector with T4 DNA ligase (Invitrogen) according to the manufacturer's instructions. For stable transfection of pre–miR-133a, VSMCs were seeded in 6-well plates at a density of 2×10^5 cells per well and transfected with pre–miR-133a plasmid using Lipofectamine 2000 (Invitrogen). Stably transfected cells were clonally selected using puromycin (1 μg/mL). Empty vector (miR-C) was also stably transfected into VSMCs as a control.

For the functional analysis of miR-133a, a fragment of the 3′-UTR of Runx2 containing the previously identified miR-133a binding site was cloned into *Pme*I and *Xba*I restriction sites of the single luciferase reporter vector pmirGLO (Promega, Madison, Wisconsin), which encodes both firefly luciferase for reporter signal and renilla luciferase for assay normalization. For studies of the wild-type (WT) Runx2 3′-UTR, 3 point mutations in the Runx2 3′-UTR containing the miR-133a target site were generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California) to produce a mutant Runx2 3′-UTR (the primer is listed in Table 2).

To construct the Runx2 expression plasmid, pcDNA3.1-Runx2, a DNA fragment containing Runx2 coding sequence was amplified from mouse mRNA using specific primers (Table 3). The PCR products were digested with *Bam*HI-*Xho*I and subcloned into pcDNA3.1 vector. All plasmids mentioned above were sequenced to ensure authenticity.

The miRNA inhibitor 2′-*O*-methyl antisense oligonucleotides targeted toward miR-133a (miR-133a inhibitor) and the miRNA inhibitor negative control (miR-inhibitor control) were purchased from RiboBio (Guangzhou, China). For transient transfection, complexes of Lipofectamine 2000 and miRNA inhibitors were directly mixed with cells in 6-well cell culture plates at a density of 2×10^5 cells per well. Cells were harvested 48 hours after transfection.

Luciferase reporter assay

VSMCs were cotransfected with luciferase reporter carrying WT Runx2 3′-UTR (WT-Runx2–3′UTR) or mutant Runx2 3′-UTR (MUT-Runx2–3′UTR) and pre–miR-133a or empty vector (miR-C). After 48 hours, luciferase activity was quantified with the Luciferase Assay System (Promega).

Northern blotting and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated, and Northern blotting was performed as described previously (28). Briefly, 20 μg total RNA was separated on 15% urea-PAGE gels and transferred onto Hybond-N+ nylon membranes (GE Healthcare, Piscataway, New Jersey). ^{32}P-labeled oligonucleotide probe corresponding to mature miR-133a was hybridized to the membranes. U6 was reprobed as a loading control.

For qRT-PCR, RNA was extracted from VSMCs, and cDNA was prepared. miRNAs were quantified using SYBR-Green (Bio-Rad, Hercules, California) and the Bulge-Loop miRNA qPCR primer set for miR-133a and U6 small nuclear RNA (RiboBio). Relative quantification was calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 3. Primer Sequences for *Runx2* Plasmids

Name	Primer Sequences (5′–3′)
Runx2 sense	CGGGATCCTTTACAACAGAGGGCACAA
Runx2 antisense	CGCTCGAGCACAGCCAACTCAAACACTA

Western blotting

To investigate Runx2 protein expression, Western blotting was performed. Total protein was separated using 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts). Primary antibodies against mouse Runx2 or β-actin (Santa Cruz Biotechnology, Santa Cruz, California) was incubated with the polyvinylidene difluoride membrane in PBS for 3 hours. Then the membranes were incubated with appropriate secondary antibodies for 1 hour, detected with an ECL kit (Promega Biosciences, San Luis Obispo, California), and exposed to film.

Statistical analysis

SPSS version 13.0 for Windows (SPSS, Chicago, Illinois) was used for all statistics. Data are presented as means ± SD. Comparisons were made using 1-way ANOVA and the least significant difference (LSD) post hoc test for multiple comparisons. Differences of $P < .05$ were considered significant.

Results

MiR-133a levels are reduced in VSMCs undergoing osteogenic differentiation

It has previously been suggested that miR-133a is specifically expressed in skeletal muscle and cardiac myocytes (29). However, recent studies revealed that it also might be expressed in smooth muscle cells (30, 31). The presence of miR-133a in mouse aortic VSMCs was determined by Northern blotting. Our results confirmed that miR-133a was expressed in VSMCs, although its abundance was lower than in heart and skeletal muscle (Figure 1A). To determine whether miR-133a expression was affected by

Table 2. Primer Sequences for 3′-UTR of WT and Mutant *Runx2* Plasmids

Name	Primer Sequences (5′–3′)
WT *Runx2* sense	GTTTAAACCCAGAATGATGGTGTTGACG
WT *Runx2* antisense	GCTCTAGAGCCTGCCTCTTGTCCCTTTCTG
Mutant *Runx2* sense	GGCCCAGTGGCATGGTCGACACATCCCGCATGTG
Mutant *Runx2* antisense	CACATGCGGGATGTGTCGACCATGCCACTGGGCC

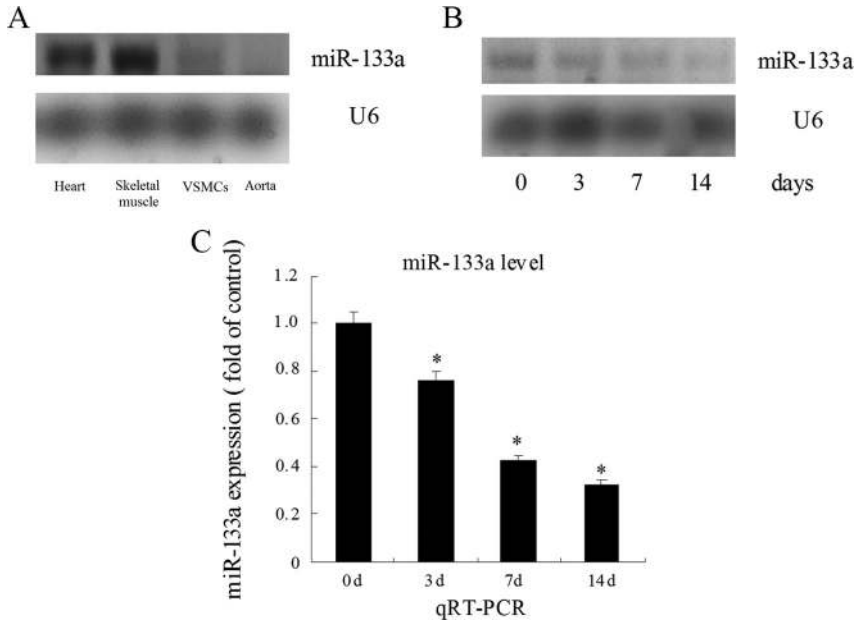

Figure 1. MiR-133a expression in VSMCs. A, MiR-133a expression in mouse heart, skeletal muscle, VSMCs, and aorta was detected by Northern blotting. B and C, MiR-133a expression during osteoblastic differentiation of VSMCs is induced by β-GP at the indicated time points as determined by Northern blotting (B) and qRT-PCR (C). Data are presented as means ± SD. *, $P < .05$ vs day 0; n = 3.

osteogenic differentiation, VSMCs were treated with β-GP, a reagent known to induce osteogenic differentiation of VSMCs. Compared with unstimulated cells, miR-133a levels were significantly reduced after β-GP treatment as measured by both Northern blotting (Figure 1B) and qRT-PCR (Figure 1C). Taken together, these results demonstrate that osteogenic differentiation of VSMCs is associated with a significant reduction in miR-133a levels, suggesting that miR-133a might play an important role in the osteoblastic differentiation of VSMCs.

Osteogenic differentiation of VSMCs is regulated by miR-133a

To test whether miR-133a directly regulated the osteogenic differentiation of VSMCs, we overexpressed miR-133a by transfecting an expression construct containing miR-133a (pre–miR-133a). Compared with cells transfected with the empty vector (miR-C), VSMCs transfected with pre–miR-133a produced significantly higher levels of miR-133a, as demonstrated by Northern blotting analysis (Figure 2A) and real-time PCR (Figure 2B).

To characterize the effects of miR-133a overexpression during the osteogenic differentiation of VSMCs, we measured ALP activity, osteocalcin secretion, and Runx2 expression in miR-133a–overexpressing cells treated with β-GP for 72 hours. Our results demonstrated a significant decrease in ALP activity (Figure 2C), osteocalcin secretion (Figure 2D), and Runx2 protein expression (Figure 2E) in miR-133a–overexpressing cells compared with control cells. The miR-133a–overexpressing VSMCs also had decreased mineralized nodule formation and Alizarin Red S staining after 12 days in culture (Figure 2, F–H), providing additional evidence for an inhibitory role of miR-133a in osteogenic differentiation.

Given that miR-133a overexpression prevented VSMC transdifferentiation, we sought to determine whether reducing miR-133a levels could facilitate osteogenic differentiation. To test this, VSMCs were transfected with a specific miR-133a inhibitor, which achieved significant knockdown as measured by qRT-PCR (Figure 3B). Consistent with a role of miR-133a in inhibiting osteogenic differentiation, we found that suppression of miR-133a resulted in increased ALP activity (Figure 2C), osteocalcin secretion (Figure 2D), and Runx2 protein expression (Figure 2E). Taken together, these studies demonstrated a direct role of miR-133a in the regulation of osteogenic differentiation of VSMCs.

Runx2 is a direct target of miR133a in VSMCs

We sought to explore how miR-133a influenced the transcriptional mechanisms involved in osteogenic transdifferentiation of VSMCs. A previous study reported that miR-133a directly targeted the 3′-UTR of Runx2 and inhibited the expression of Runx2 protein (23). Consistent with these results, luciferase reporter construct containing the WT or mutant 3′-UTR coding sequences for Runx2 confirmed miR-133a directly targeted Runx2. Overexpression of miR-133a significantly repressed the luciferase activity of reporter constructs containing the WT-Runx2-3′-UTR, but not the MUT-Runx2-3′-UTR. The miR-C did not affect the WT or mutant constructs, confirming the specificity of action (Figure 3A). Furthermore, our results showed that overexpression of miR-133a promoted a decrease in Runx2 protein levels and that miR-133 inhibitor promoted an increase in Runx2 (Figure 2E). These results provide direct evidence for the miR-133a–mediated regulation of Runx2.

To obtain further evidence for a functional connection between miR-133a and Runx2, we tested whether Runx2 deregulation was required for the effects of miR-133a in regulating the osteogenic differentiation of VSMCs. First, we determined the effect of Runx2 siRNA on the miR-

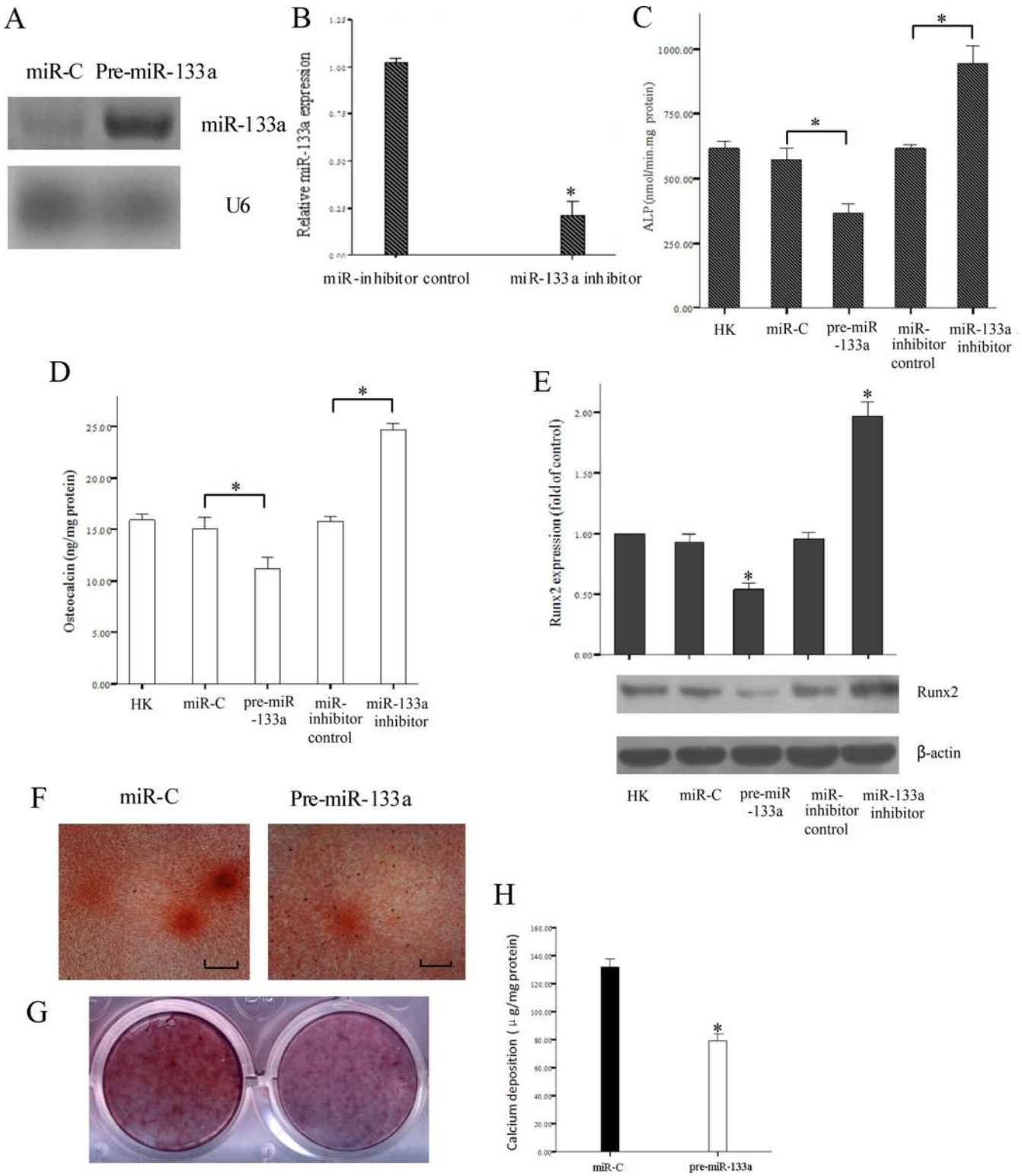

Figure 2. MiR-133a inhibits β-GP–induced osteoblastic differentiation of VSMCs. A, Northern blotting analysis of miR-133a levels in VSMCs transfected with pre–miR-133a or miR-C. U6 was tested as a loading control. B, qRT-PCR analysis of miR-133a expression in VSMCs transfected with miR-133a inhibitor and its control. C–E, VSMCs were transfected with pre–miR-133a, empty vector (miR-C), miR-133a inhibitor, or control oligos. Cells were then treated with β-GP, and after 72 hours transfection, ALP activity was determined using spectrophotometric methods (C), osteocalcin secretion was determined using RIA (D), and Runx2 expression was measured by Western blotting (E). F, Microscopic view of the effects of miR-133a on matrix mineralization in VSMCs induced by β-GP. Scale bar, 200 μm. G, A representative microscopic view (magnification, ×200) of cells transfected with pre–miR-133a or miR-C after 12 days of culture. H, Quantification of Alizarin Red S stain via extraction with cetylpyridinium chloride. Data are shown as means ± SD. *, $P < .05$; n = 3. HK, housekeeping (control).

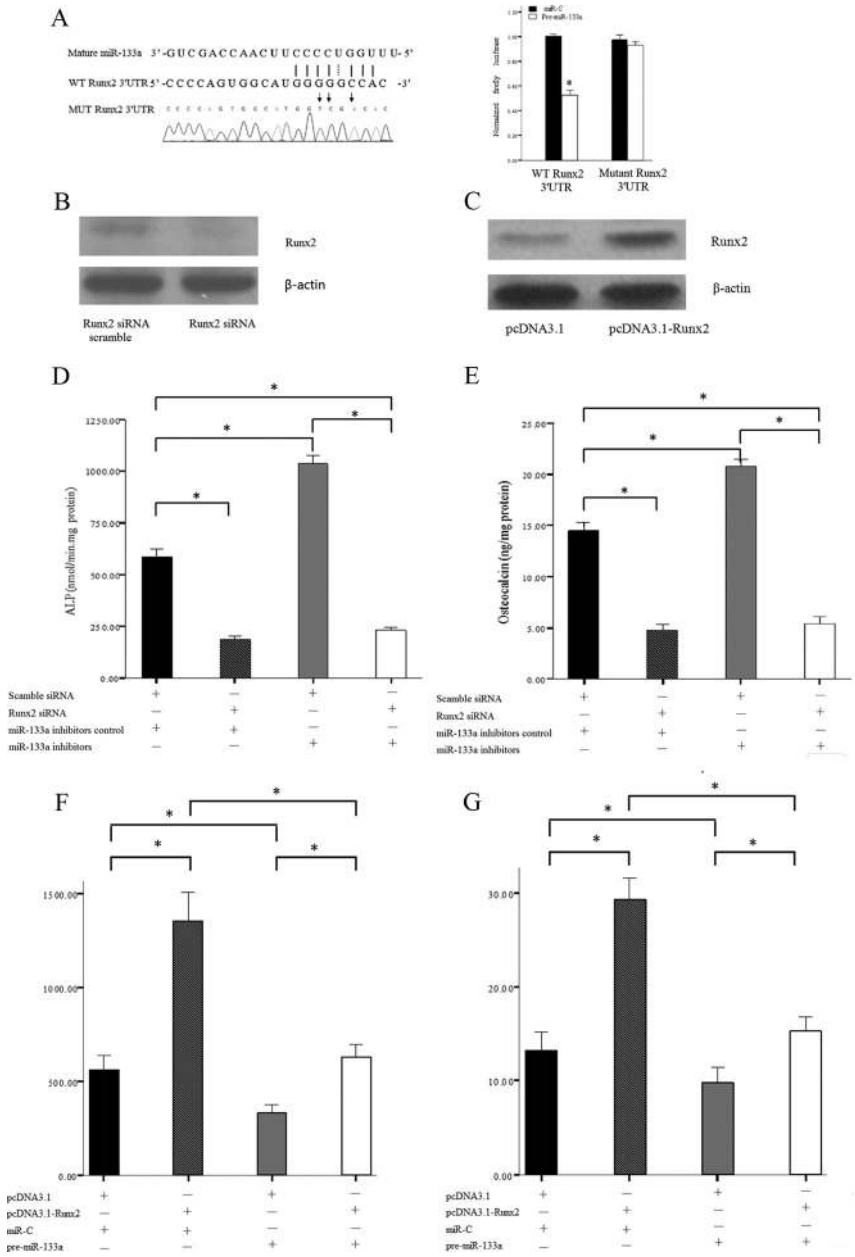

Figure 3. Runx2 plays a pivotal role in the miR-133a–regulated osteoblastic differentiation of VSMCs. A, Luciferase reporter assays were performed using luciferase constructs carrying a WT or mutant Runx2 3′-UTR cotransfected into VSMCs with a pre-miR133a–expressing construct as compared with an empty vector control. The luciferase activity of the WT-Runx2-3′-UTR construct was reduced by 40% in the presence of pre-miR133a as compared with the empty vector control. The reduction was abolished after the introduction of 3 point mutations in the seed region of the miR-133a target site as shown in the left panel. Firefly luciferase activity was normalized to renilla luciferase activity. Error bars represent SD. B, Runx2 expression was successfully knocked down using siRNA in VSMCs. Runx2 expression in VSMCs transfected with scrambled siRNA or Runx2 siRNA was analyzed by Western blotting. C, Runx2 expression was achieved using pcDNA3.1-Runx2 in VSMCs as determined by Western blotting. D and E, Osteogenic differentiation was determined using ALP activity (D) and osteocalcin secretion (E) after 3 days of osteogenic induction. β-GP–induced VSMCs were transfected with scramble siRNA or Runx2 siRNA and miRNA control inhibitor or miR-133a inhibitor. F and G, osteogenic differentiation was determined by ALP activity (F) and osteocalcin secretion (G) after 3 days of osteogenic induction. β-GP–induced VSMCs were transfected with pcDNA3.1-Runx2 or pcDNA3.1 and pre–miR-133a or miR-C. Protein expression was normalized to total cellular protein by Bradford protein assay. The data are expressed as means $\pm$ SD. *, $P < .05$; n = 5.

133a–regulated osteogenic process in mouse VSMCs. As shown in Figure 3B, Runx2 siRNA successfully knocked down the expression of Runx2 protein in VSMCs. miR-133a inhibitor increased ALP activity and osteocalcin secretion in control cells. However, this effect was reversed by transfection of Runx2 siRNA (Figure 3, D and E), suggesting that the effects of miR-133a inhibitor on ALP activity and osteocalcin secretion were Runx2-dependent. To further test the role of Runx2 in miR-133a–mediated suppression of osteogenic differentiation, we transfected VSMCs with pcDNA3.1-Runx2, which carries the entire Runx2 coding sequence with miR-133a target on the 3′-UTR. As shown in Figure 3C, transfection of pcDNA3.1-Runx2 significantly upregulated Runx2 protein expression in VSMCs. Consistent with previous reports (32), ALP activity and osteocalcin secretion were dramatically increased after transfection with pcDNA3.1-Runx2. In the absence of exogenous Runx2, pre–miR-133a significantly decreased the ALP activity and osteocalcin secretion. However, this decrease was attenuated by transfection of pcDNA3.1-Runx2 (Figure 3, F and G). Taken together, our experiments demonstrated that Runx2 is a critical target of miR-133a in osteogenic differentiation.

Discussion

In the present study, we identified miR-133a as a negative regulator of osteogenic differentiation of VSMCs via direct suppression of Runx2, a transcription factor involved in osteogenesis. Recently, several studies suggested that miRNAs were involved in osteoblast differentiation. These studies demonstrated that miR-26a (accession number MI-

MAT0000082) was involved in osteoblastic differentiation of adipose tissue-derived mesenchymal stem cells by targeting SM-actin mothers against decapentaplegic family member 1 (SMAD1), a translation of the osteogenic marker (33), whereas miR-196a (accession number MIMAT0000226) directly targeted homeobox protein Hox-C8 and inhibited the osteoblastic differentiation of adipose tissue-derived mesenchymal stem cells (34). Additionally, miR-29b (accession number MIMAT0000127), miRNA-204/211, miR-141, and miR-200a (accession number MIMAT0000519) were recently suggested to be regulators of osteoblast differentiation (35, 36). Several experiments demonstrated that a series of miRNAs could negatively regulate the expression of Runx2 via direct targeting. Runx2 in mesenchymal progenitor cells and bone marrow stromal cells was shown to be negatively regulated by miR-204/211 (accession number MIMAT0000237/ MIMAT0000668), which induced adipocyte differentiation and inhibited osteoblastic differentiation (37). Furthermore, miR-196a has been suggested to regulate osteogenic differentiation in mesenchymal stem cells by targeting homeobox protein Hox-C8 (34). These results suggest that miRNAs play an important role in regulating osteogenesis.

In addition to these miRNAs, miR-133a attracted our interest because it has been reported to be specifically expressed in cardiac and skeletal muscle and might play important roles in skeletal and cardiac muscle development, physiology, and disease pathogenesis (38, 39). However, a recent study demonstrated that miR-133 inhibited osteoblastic differentiation of C2C12 mesenchymal cells by directly targeting Runx2 (23). Data from Wang et al (40) verified that levels of circulating miR-133a negatively correlated with bone mineral density, which suggested that miR-133a may be a key clinical regulator of bone metabolism. Moreover, Torella et al (31) identified a potent inhibitory effect of miR-133 on VSMC phenotypic switch in vitro and in vivo. These data suggested that miR-133 might play a role in osteogenic differentiation of VSMCs. In the present study, we tested the hypothesis that miR-133a was involved in the osteogenic differentiation of VSMCs.

MiR-133 can be reconstituted using 3 miRNAs: miR-133a-1 (accession number MI0000159), miR-133a-2 (accession number MI0000820), and miR-133b (accession number MI0000821). The mature sequence of miR-133a-1 and miR-133a-2 is identical and is different from miR-133b by only 2 nucleotides at the 3′ terminus. Numerous studies have demonstrated that miR-133 plays an important role in skeletal muscle and cardiac muscle development and function, and several proteins have been suggested to be the targets of miR-133, including Ras homolog gene family member A (RhoA) (30), cell division control protein 42 homolog (Cdc42), negative elongation factor A (Nelf-A/WHSC2) (31), serum response factor (SRF) (41), specificity protein 1 (SP-1) (31) and Runx2 (23). Here, we focused on miR-133a, a muscle-specific miRNA that has been considered to be exclusively expressed in skeletal muscle and cardiac myocytes. However, recent studies demonstrated that miR-133a can be detected in bronchial smooth muscle cells (30) and VSMCs (31). Consistent with those results, our experiments demonstrated that miR-133a could be detected in VSMCs. During the process of osteogenic differentiation of VSMCs induced by β-GP, the expression of miR-133a decreased significantly. These results suggested that miR-133a might have a functional role in the osteogenic differentiation of VSMCs.

To further determine the effect of miR-133a in osteogenic differentiation of VSMCs, we performed gain-of-function studies using pre–miR-133a, which significantly increased the expression of miR-133a. Pre–miR-133a expression decreased ALP activity, osteocalcin secretion, the markers of osteogenic differentiation, the expression of Runx2 (a pivotal osteogenic transcription factor in osteogenesis), and mineralization of matrix (the phenotype hallmark of osteogenic differentiation). These results demonstrated that the osteogenic differentiation of VSMCs was inhibited by pre–miR-133a. Conversely, loss of function by transfection of miR-133a inhibitor in VSMCs enhanced ALP activity, osteocalcin secretion, and Runx2 expression, which supported the hypothesis that miR-133a was a negative regulator of the osteogenic differentiation of VSMCs.

To investigate the molecular mechanism by which miR-133a inhibits osteogenic differentiation, we evaluated the target genes of miR-133a. Previous studies reported that miR-133a can act as a negative regulator of Runx2 in mesenchymal cells, resulting in decreased osteoblast differentiation (23). In the present study, we observed that miR-133a overexpression inhibited the expression of Runx2 protein in VSMCs. Furthermore, our luciferase experiments confirmed that miR-133a directly targeted the Runx2 3′-UTR. Moreover, the knockdown of Runx2 using siRNA attenuated the ability of miR-133a inhibitor to stimulate ALP activity and osteocalcin secretion. Conversely, overexpression of Runx2 using pcDNA3.1-Runx2 reversed the inhibiting effect of pre–miR-133a on ALP activity and osteocalcin secretion. Thus, these results directly demonstrated that miR-133a attenuated the osteogenic differentiation of VSMCs by negatively regulating Runx2.

Taken together, our results suggest an inhibitory role of miR-133a in the osteogenic differentiation of VSMCs by targeting Runx2. This raises an interesting mechanism of

the regulation of osteogenic differentiation in VSMCs. Although further studies are necessary to test whether miR-133a might be a useful treatment for arterial calcification in vivo, the data presented here suggest that miR-133a could be a potent therapeutic agent. Investigating the role of miRNAs on osteogenic differentiation of VSMCs will give us a better understanding of vascular calcification, and based on this understanding, future miRNA drugs targeting specific genes may be developed to treat cardiovascular diseases.

Acknowledgments

Address all correspondence and requests for reprints to: Ling-Qing Yuan, Institute of Metabolism and Endocrinology, the Second Xiang-Ya Hospital, Central South University, Changsha, Hunan, 410011, People's Republic of China. E-mail: allenylq@hotmail.com.

This work was supported by The National Natural Science Foundation of China (81270962, 30900622, 81000313, 81070159, and 81070246), China Postdoctoral Science Foundation (2012M521568) and a C.J. Huang Medical Fellowship of Stanford University.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Mendell JT, Olson EN.** MicroRNAs in stress signaling and human disease. *Cell*. 2012;148:1172–1187.
2. **Filipowicz W, Bhattacharyya SN, Sonenberg N.** Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 2008;9:102–114.
3. **Boettger T, Braun T.** A new level of complexity: the role of microRNAs in cardiovascular development. *Circ Res*. 2012;110:1000–1013.
4. **Tijsen AJ, Creemers EE, Moerland PD, et al.** MiR423-5p as a circulating biomarker for heart failure. *Circ Res*. 2010;106:1035–1039.
5. **Zhu W, Yang L, Shan H, et al.** MicroRNA expression analysis: clinical advantage of propranolol reveals key microRNAs in myocardial infarction. *PLoS One*. 2011;6:e14736.
6. **D'Alessandra Y, Devanna P, Limana F, et al.** Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J*. 2010;31:2765–2773.
7. **Adachi T, Nakanishi M, Otsuka Y, et al.** Plasma microRNA 499 as a biomarker of acute myocardial infarction. *Clin Chem*. 2010;56:1183–1185.
8. **Goodman WG, Goldin J, Kuizon BD, et al.** Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med*. 2000;342:1478–1483.
9. **Verbeke F, Van Biesen W, Honkanen E, et al; CORD Study Investigators.** Prognostic value of aortic stiffness and calcification for cardiovascular events and mortality in dialysis patients: outcome of the calcification outcome in renal disease (CORD) study. *Clin J Am Soc Nephrol*. 2011;6:153–159.
10. **Qasim AN, Martin SS, Mehta NN, et al.** Lipoprotein(a) is strongly associated with coronary artery calcification in type-2 diabetic women. *Int J Cardiol*. 2011;150:17–21.
11. **Schauer IE, Snell-Bergeon JK, Bergman BC, et al.** Insulin resistance, defective insulin-mediated fatty acid suppression, and coronary artery calcification in subjects with and without type 1 diabetes: The CACTI study. *Diabetes*. 2011;60:306–314.
12. **Nicoll R, Henein MY.** Arterial calcification: Friend or foe? *Int J Cardiol*. 2013;167:322–327.
13. **Shan PF, Lu Y, Cui RR, Jiang Y, Yuan LQ, Liao EY.** Apelin attenuates the osteoblastic differentiation of vascular smooth muscle cells. *PLoS One*. 2011;6:e17938.
14. **Yuan LQ, Zhu JH, Wang HW, et al.** RANKL is a downstream mediator for insulin-induced osteoblastic differentiation of vascular smooth muscle cells. *PLoS One*. 2011;6:e29037.
15. **Son BK, Akishita M, Iijima K, et al.** Adiponectin antagonizes stimulatory effect of tumor necrosis factor-α on vascular smooth muscle cell calcification: regulation of growth arrest-specific gene 6-mediated survival pathway by adenosine 5′-monophosphate-activated protein kinase. *Endocrinology*. 2008;149:1646–1653.
16. **Du Y, Wang Y, Wang L, et al.** Cartilage oligomeric matrix protein inhibits vascular smooth muscle calcification by interacting with bone morphogenetic protein-2. *Circ Res*. 2011;108:917–928.
17. **Eskildsen T, Taipaleenmäki H, Stenvang J, et al.** MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. *Proc Natl Acad Sci U S A*. 2011;108:6139–6144.
18. **Zhang J, Tu Q, Bonewald LF, et al.** Effects of miR-335-5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1. *J Bone Miner Res*. 2011;26:1953–1963.
19. **Cui RR, Li SJ, Liu LJ, et al.** MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo. *Cardiovasc Res*. 2012;96:320–329.
20. **Goettsch C, Rauner M, Pacyna N, Hempel U, Bornstein SR, Hofbauer LC.** miR-125b regulates calcification of vascular smooth muscle cells. *Am J Pathol*. 2011;179:1594–1600.
21. **Du Y, Gao C, Liu Z, et al.** Upregulation of a disintegrin and metalloproteinase with thrombospondin motifs-7 by miR-29 repression mediates vascular smooth muscle calcification. *Arterioscler Thromb Vasc Biol*. 2012;32:2580–2588.
22. **Liu N, Bezprozvannaya S, Williams AH, et al.** microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. *Genes Dev*. 2008;2:3242–3254.
23. **Li Z, Hassan MQ, Volinia S, et al.** A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proc Natl Acad Sci U S A*. 2008;105:13906–13911.
24. **Zhang Y, Xie RL, Croce CM, et al.** A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proc Natl Acad Sci U S A*. 2011;108:9863–9868.
25. **Zhang Y, Xie RL, Gordon J, et al.** Control of mesenchymal lineage progression by microRNAs targeting skeletal gene regulators Trps1 and Runx2. *J Biol Chem*. 2012;287:21926–21935.
26. **Liang QH, Jiang Y, Zhu X, et al.** Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the ERK pathway. *PLoS One*. 2012;7:e33126.
27. **Liao XB, Zhou XM, Li JM, et al.** Taurine inhibits osteoblastic differentiation of vascular smooth muscle cells via the ERK pathway. *Amino Acids*. 2008;34:525–530.
28. **Lee RC, Ambros V.** An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*. 2001;294:862–864.
29. **Carè A, Catalucci D, Felicetti F, et al.** MicroRNA-133 controls cardiac hypertrophy. *Nat Med*. 2007;13:613–618.
30. **Chiba Y, Tanabe M, Goto K, Sakai H, Misawa M.** Down-regulation of miR-133a contributes to up-regulation of Rhoa in bronchial smooth muscle cells. *Am J Respir Crit Care Med*. 2009;180:713–719.
31. **Torella D, Iaconetti C, Catalucci D, et al.** MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo. *Circ Res*. 2011;109:880–893.
32. **Byon CH, Javed A, Dai Q, et al.** Oxidative stress induces vascular

calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. *J Biol Chem*. 2008;283:15319–15327.
33. **Luzi E, Marini F, Sala SC, Tognarini I, Galli G, Brandi ML.** Osteogenic differentiation of human adipose tissue-derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. *J Bone Miner Res*. 2008;23:287–295.
34. **Kim YJ, Bae SW, Yu SS, Bae YC, Jung JS.** miR-196a regulates proliferation and osteogenic differentiation in mesenchymal stem cells derived from human adipose tissue. *J Bone Miner Res*. 2009;24:816–825.
35. **Inose H, Ochi H, Kimura A, et al.** A microRNA regulatory mechanism of osteoblast differentiation. *Proc Natl Acad Sci U S A*. 2009;106:20794–20799.
36. **Li Z, Hassan MQ, Jafferji M, et al.** Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. *J Biol Chem*. 2009;284:15676–15684.
37. **Huang J, Zhao L, Xing L, Chen D.** MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. *Stem Cells*. 2010;28:357–264.
38. **Liu N, Olson EN.** MicroRNA regulatory networks in cardiovascular development. *Dev Cell*. 2010;18:510–525.
39. **Latronico MV, Catalucci D, Condorelli G.** Emerging role of microRNAs in cardiovascular biology. *Circ Res*. 2007;101:1225–1236.
40. **Wang Y, Li L, Moore BT, et al.** MiR-133a in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. *PLoS One*. 2012;7:e34641.
41. **Chen JF, Mandel EM, Thomson JM, et al.** The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet*. 2006;38:228–233.