Identification and Characterization of MicroRNAs Controlled by the Osteoblast-Specific Transcription Factor Osterix

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Abstract

Osterix (Osx) is an osteoblast-specific transcription factor which is essential for bone formation. MicroRNAs (miRNAs) have been previously shown to be involved in osteogenesis. However, it is unclear whether Osx is involved in the regulation of miRNA expression. In this study, we have identified groups of miRNAs that are differentially expressed in calvaria of the E18.5 Osx−/− embryos compared to wild type embryos. The correlation between the levels of miRNAs and Osx expression was further verified in cultured M-Osx cells in which over-expression of Osx is inducible. Our results suggest that Osx down-regulates expression of a group of miRNAs including mir-133a and -204/211, but up-regulates expression of another group of miRNAs such as mir-141/200a. Mir-133a and -204/211 are known to target the master osteogenic transcription factor Runx2. Further assays suggest that Sost, which encodes the Wnt signaling antagonist Sclerostin, and alkaline phosphatase (ALP) are two additional targets of mir-204/211. Mir-141/200a has been known to target the transcription factor Dlx5. Thus, we postulate that during the process of Osx-controlled osteogenesis, Osx has the ability to coordinately modulate Runx2, Sclerostin, ALP and Dlx5 proteins at levels appropriate for optimal osteoblast differentiation and function, at least in part, through regulation of specific miRNAs. Our study shows a tight correlation between Osx and the miRNAs involved in bone formation, and provides new information about molecular mechanisms of Osx-controlled osteogenesis.

Introduction

Osteoblasts arise from mesenchymal precursors and play critical roles in osteogenesis. The progression through osteoblastic commitment, proliferation, and terminal differentiation integrates diverse signals and expression of differentiation marker genes. The marker gene expression and osteoblast differentiation are governed by several transcription factors, especially Osterix (Osx), runt-related transcription factor 2 (Runx2), and distal-less homeobox 5 (Dlx5) [1], [2], [3], [4], [5], [6]. In addition, several microRNAs (miRNAs) have recently been discovered as important regulators of osteoblast gene expression [7], [8], [9], [10].

Osx is a C2H2-type zinc finger-containing transcription factor that is specifically expressed in osteoblast lineage cells, and is essential for osteoblastogenesis and bone formation. In mice with deletion of the Osx gene (Osx−/−), formation of both endochondral and intramembranous bone was completely abolished [1], [2]. Primary culture of calvarial osteoblasts of the Osx−/− mice showed increased BrdU incorporation, indicating that those osteoprogenitors were less able to exit their cell cycle progression without Osx [11]. The normal progression of osteoblast differentiation is accompanied by expression of several markers, such as type I collagen (COL1), ALP, bone sialoprotein (BSP), and Osteocalcin (OC) [1]. In Osx−/− mice, since differentiation of osteoblasts was arrested, expression of these markers was dramatically decreased [1], [2], [12], highlighting an essential role of Osx in the differentiation of osteoprogenitors into mature and functional osteoblasts. Moreover, our in vitro study has recently shown that Osx can bind to and transactivate Bsp as well as Osteocalcin [13]. It is not yet clear whether Osx interacts with the ALP gene. In addition, our previous study showed that expression of Runx2 mRNAs was increased in calvaria of the Osx−/− mice [14].

Runx2 is a critical regulator of the osteogenic lineage. Runx2−/− mice did not form osteoblasts and failed to express osteoblast differentiation markers [3], [4]. Similar to Osx, Runx2 can also bind to and regulate expression of these marker genes [15]. Since expression of Osx mRNA in the Runx2−/− mice was undetectable, whereas expression of Runx2 mRNA in the Osx−/− mice was increased, it suggests that Runx2 acts upstream of Osx. In fact, Runx2 can directly bind to Osx gene and transactivate its promoter activity [16]. Presently, little is known about whether Osx regulates the level of Runx2 protein. Interestingly, in the Runx2 gene P1 promoter region, certain Dlx5-responsive elements have been...
identified [17]. Dlx5 is another transcriptional regulator of osteogenesis. Unlike the Osx−/− or Runx2−/− mice, Dlx5−/− mice showed less severe bone abnormalities [6], indicating that Dlx5 is not essential in osteoblastogenesis. Nevertheless, when over-expressed in chicken calvarial cells, Dlx5 accelerates osteoblast differentiation [10], suggesting that maintenance of an optimal level of Dlx5 is important for formation of normal bone tissue. So far, it is not clear whether Oss regulates Dlx5 expression.

Expression of Osx, Runx2 and Dlx5 is regulated by a broad signaling network including members of the Wnt family. The canonical Wnt/β-catenin signaling can promote osteoblastic fate determination, proliferation and survival [19], [20], [21]. A previous study showed that the interaction of Wnt ligands with Lrp5/6 co-receptors can be antagonized by Sclerostin (encoded by Sost gene) [22]. In Sost−/− mice, Wnt signaling and bone mass was increased [23], whereas transgenic mice over-expressing Sost in bone tissue had osteopenia [24], indicating that Sclerostin is an important negative regulator of bone formation. It has been known that Oss can directly bind to the Sost gene and transactivate its promoter activity [14], [25]. However, whether the control of Sost expression by Oss also involves miRNAs has not yet been examined.

MiRNAs, a form of non-coding RNAs (ncRNAs), have recently emerged as important regulators in diverse cell proliferation and differentiation processes. These endogenous ncRNAs are single-stranded small RNA molecules that consist of ~22 nucleotides and are evolutionarily conserved [26]. They regulate protein translation or mRNA stability by binding to the 3′-UTR of their target genes. Since miRNAs can bind to more than one target, it has been proposed that they regulate up to 30 percent of the protein-coding genes in the genome, highlighting their importance as regulators of gene expression. Several in vitro cell culture studies reported that many miRNAs, such as mir-23a, -30C, -34C, -93, -133, -137, -141/−200a, -204/−211, -2061 and -3960 are involved in osteogenesis [8], [9–10], [27], [28], [29], [30]. In particular, expression of mir-141 or its homologue -200a can repress BMP2-induced preosteoblast differentiation by the translational suppression of Dlx5 [28]. Mir-93 has been recently found to directly target Oss and inhibit osteoblast mineralization [10]. Mir-133, −204 and its homologue −211, were previously identified as Runx2-targeting miRNAs [27], [29]. Recently, a panel of 11 miRNAs, including mir-133a and -204, have been found to be expressed in a lineage-related pattern in mesenchymal cell types, and to directly target the 3′-UTR of the Runx2 gene [30]. When over-expressed in cultured MC3T3 cells, all of these miRNAs inhibited osteoblast differentiation as well as ALP production [30]. Moreover, a knockout study showed that conditional deletion of Dicer in cells of osteoblast lineage, which blocks formation of mature miRNAs, causes overt abnormality of bone formation [31], demonstrating a critical role of miRNAs in the regulation of osteoblast differentiation and bone formation.

To date, the physiological role of miRNAs in the regulation of osteoblast differentiation in vivo has not been well defined. In particular, we do not know whether deletion of the Osx gene, which results in a block in osteoblast differentiation and bone formation, causes changes in expression of specific miRNAs. To address this question, we first used miRNA array hybridizations comparing miRNA expression profiles between Osx−/− and wild-type calvaria of mouse embryos at E18.5. To identify the Osx-regulated miRNAs, we then used an inducible Osx-expressing cell line to further validate expression of several differentially expressed miRNAs with qPCR assays. Finally, using transfection assays we tested whether expression of selected miRNAs would interfere with osteoblast differentiation in vitro, and whether mir-204/211 was able to directly target the 3′-UTR of ALP or Sost gene and attenuate their protein synthesis.

Materials and Methods

Ethics Statement

All experimental procedures described in this study were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center (IACUC Protocol No: 108807638).

Animals

Homozygous Osx−/− mice were generated and described previously [2]. Genotyping was performed as described previously [2].

Total RNA Isolation

The total RNAs of cultured cells and RNAs of calvaria from E18.5 wild type or Osx−/− mouse embryos were isolated using TRIzol reagent (Invitrogen, 15596-018) [32]. The RNA quality control was performed using a Bioanalyzer 2100 (Agilent).

MiRNA Array, Data Collection and Analysis

MiRNA microarray processes, including probe library construction, printing, labeling, hybridization, image scanning, and initial data analysis, were conducted by the Center for Targeted Therapy (the Sequencing and Non-coding RNA Program) at The University of Texas MD Anderson Cancer Center, following an miRNA profiling protocol as described previously [32]. A 15K human/mouse miRNA/ncRNA chip was used. Within this chip, the mouse miRNA oligo probes were designed and derived from 720 Mus musculus miRNAs in the Sanger miRBase database.

### Table 1. Down-regulated miRNAs in E18.5 Osx−/− calvaria validated by qPCR.

| Name       | Wt (dCt) | Osx−/− (dCt) | p-Value | Ratio (Osx−/− vs Wt) |
|------------|----------|--------------|---------|----------------------|
| mmu-miR-141-3a | 5.67±0.31 | 7.34±0.39 | 0.01   | -3.14                      |
| mmu-miR-192  | 6.87±0.26 | 8.46±0.33 | 0.01   | -3.01                      |
| mmu-miR-200a | 7.89±0.65 | 9.49±0.67 | 0.01   | -3.03                      |
| mmu-miR-1194 | 6.66±0.39 | 8.69±0.58 | 0.01   | -4.08                      |

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### Table 2. Up-regulated miRNAs in E18.5 Osx−/− calvaria validated by qPCR.

| Name       | Wt (dCt) | Osx−/− (dCt) | p-Value | Ratio (Osx−/− vs Wt) |
|------------|----------|--------------|---------|----------------------|
| mmu-miR-133a | 4.50±0.25 | 2.85±0.24 | 0.01   | 3.14                      |
| mmu-miR-204  | 6.11±0.35 | 4.03±0.34 | 0.01   | 4.23                      |
| mmu-miR-211  | 6.29±0.23 | 4.63±0.45 | 0.01   | 3.16                      |
| mmu-miR-302a | 9.21±0.59 | 7.25±0.39 | 0.01   | 3.89                      |
| mmu-miR-433  | 5.54±0.86 | 3.14±0.50 | 0.01   | 5.27                      |
| mmu-miR-501  | 6.57±0.31 | 4.98±0.38 | 0.01   | 3.01                      |
| mmu-miR-544  | 8.91±0.45 | 7.09±0.41 | 0.01   | 3.51                      |

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The raw signal intensity data were generated using the software GenePix Pro 6.1 on the Axon 4000B scanner (Molecular Devices). The background corrected raw data (the “F635 Median - B635” column in the.gpr files) were checked for quality and filtered as follows: Buffers and blank spots were filtered first; the miRNA that signal intensity was low (i.e., a flag $\leq 50$ in the “Flags” column) in at least five out of the six samples was excluded from further analysis; the low-intensity spots above 50 were assigned a small positive value 1; the replicate spots were averaged. After log2-transformation, the raw data were normalized by quantile normalization [33] implemented in the R package limma [34–35], [36]. Differential expression was detected using limma. A ta false discovery rate (FDR) of 5, 10, or 20%, none of the miRNAs showed significant changes in expression between wild type and Osx2/2 sample groups by either Benjamini-Hochberg method [37] or q-value [38], [39]. MiRNAs with raw p value <0.1 and fold-change value of at least 1.5 in either direction were listed as candidate hits for experimental validation (Tables S1 and S2).

Real-time RT-PCR Quantification of miRNAs

To confirm the microarray results, expression levels of the selected miRNAs were quantified by real-time RT-PCR (qPCR) using the miScript PCR System (QIAGEN) according to vendor’s instructions. In brief, miRNAs were polyadenylated by poly (A) polymerase and subsequently converted into cDNA using reverse transcriptase with oligo (dT) priming. Reverse transcription reaction contained 1 µg of RNA template, 1 µl of miScript Reverse Transcripase Mix, and 4 µl of miScript RT Buffer, which includes Mg2+, dNTPs, and primers. Reaction mixture (20 µl) was incubated for 60 min at 37°C, then 5 min at 95°C. QPCR was performed using an Applied Biosystems 7500 sequence detection system; the reaction mixtures (50 µl) included 5 µl of cDNA template, 25 µl of SYBR Green PCR Master Mix, 5 µl of miScript Universal Primer, and 5 µl of miScript Primer Assay. Reactions were distributed into a 96-well optical plate and incubated at 95°C for 15 min in the real-time cycler, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The threshold cycle (Ct) was adjusted from a default “Manual Ct” threshold value of 0.2 to a lower value of 0.02. The Ct value was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. U6 small RNA was used as an internal control to normalize the miRNA input. The sequences for miScript Primer Assay are shown in Table S3. For each selected miRNA, the qPCR experiment was performed in triplicate with three independent batches of cDNAs. Changes (x-fold) in miRNA expression level were calculated by the equation $2^{\Delta\Delta \text{Ct}}$, where $\Delta\Delta \text{Ct} = (\text{Ct}_{\text{Target}} - \text{Ct}_{\text{U6}}) - (\text{Ct}_{\text{Control}} - \text{Ct}_{\text{U6}})$ Control.

Figure 1. Generation of M-Osx cell line and expression of miRNAs in those cells. A: Western blot using anti-Flag antibody: lanes 1–6, cell lysates from clones 24, 34 and 35 in the absence or presence of Tet. B: Northern blot of RNAs from cells of clone 35 cultured without (lane 1) or with (lane 2) BMP2 for 40 h, hybridized with mouse Osx, flag-HA, mouse Runx2, mouse Oc and mouse Bsp cDNAs. C, D: Expression of miRNAs in M-Osx cells, which were cultured in the absence or presence of Tet for 48 h. The reported values are relative to expression of miRNAs in cells plus Tet (set as 1). All data are means ± S.D. (n = 3). *p<0.05; **p<0.01. doi:10.1371/journal.pone.0058104.g001
All statistical analyses were performed with two-tailed Student’s t tests using Microsoft Excel software. Results are the means ± S.D. Data were considered to be significantly different for p<0.05.

**Generation and Culture of M-Osx Cells**
M-Osx cells have been generated by stably expressing inducible Osx expression plasmid vectors in mouse preosteoblast MC3T3 cells. Briefly, MC3T3 cells were first stably transfected with pTet-off (Clontech). The positive clones selected by G418 were then stably transfected with pTRE-Flag-HA-Osx and pTK-hyg plasmids [13]. After G418 and hygromycin selection, the positive clones were expanded and maintained in the presence of tetracycline (Tet) [13]. Osx expression was turned on or off by absence or presence of Tet in culture media. M-Osx cells were cultured in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS) in the absence or presence of Tet. After 48 hours (48 h), cells were harvested for total RNA isolation using TRIzol reagent.

**Generation and Culture of UMR-conRNA and UMR-mir204 Cells**
UMR-conRNA and UMR-mir204 cells have been generated by stably expressing inducible expression plasmids of control RNA or mir-204 in rat osteogenic UMR-106 cells. In brief, precursor of mir-204 was amplified by PCR using primers 5’-gttcatatactggcctactgtg/5’-gttatgggctcaatgatgg selected from intron 6 of Trpm3 gene. BAC clone R23-473O13 (BACPAC Resources Center) was used as the PCR template. The PCR fragment (~188-bp) was then inserted into the Xho1/EcoR1 site of pTRIPZ vector (Thermo Scientific Open Biosystems) to generate a mir-204-pTRIPZ plasmid. Non-silencing shRNA-pTRIPZ (conRNA-pTRIPZ) construct was obtained from Thermo Scientific Open Biosystems. The pTRIPZ vector has been engineered to be Tet-On and produce inducible expression of shRNAmir in the presence of doxycycline (Dox). It also contains a puromycin drug resistance gene for selecting stable cell lines, and a RFP (Red Fluorescence Protein) marker for tracking shRNAmir expression. Mir-204- or conRNA-pTRIPZ construct was transfected into the UMR-106 cells by use of Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol. The cells that incorporate conRNA- or mir-204-pTRIPZ construct were selected by puromycin treatment to generate two stable cell lines, which were named UMR-conRNA and UMR-mir204. When these cells are treated with Dox, expression of mir-204 or control RNA triggers expression of RFP, which shows red color under fluorescence microscope.

**Western Blot**
Western blot was performed as described previously [13]. For characterization of M-Osx cells, cells selected from three clones (number 24, 34 and 35) were cultured in α-MEM containing 10% fetal bovine serum (FBS) in the absence or presence of Tet. After 48 hours (48 h), cells were harvested for total RNA isolation using TRIzol reagent. Sample [40]. All statistical analyses were performed with two-tailed Student’s t tests using Microsoft Excel software. Results are the means ± S.D. Data were considered to be significantly different for p<0.05.
FBS in the absence or presence of Tet. For characterization of UMR-conRNA and UMR-mir204 cells, 1.5 \times 10^5 UMR-conRNA or UMR-mir204 cells were cultured in 6-well plate containing DMEM supplemented with 10% FBS in the presence or absence of 1.0 \mu g/ml Dox. After 40 h (for M-Osx) or 54 h (for UMR-conRNA or UMR-mir204) incubation, cells were collected and suspended with buffer containing 50 mM Tris-Cl (pH 8), 150 mM NaCl, and 1% NP-40 along with protease inhibitor cocktail, and further lysed by 1/6 SDS sample buffer. The total proteins in cell lysates were separated on SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted using anti-Flag (Millipore, MA), anti-Runx2 (MBL international), anti-Osx (Ambcan), or anti-Sost (R&D) antibody, respectively, followed by reaction with appropriate HRP-labeled secondary antibody. The signals were then detected by Super Signal chemiluminescence reagent (Pierce).

Northern Blot

After G418 and hygromycin selection, cells from the positive clone (number 35) were grown in α-MEM containing 10% FBS, Tet, and in the presence or absence of BMP2. After 40 h, cells were collected for total RNA isolation using TRIzol reagent. Northern blot was performed as previously described [41]. In brief, total RNA (15 \mu g) was electrophoresed in a 1.2% agarose-formaldehyde gel, transferred onto Hybond-N-membrane (Amersham International, Amersham, UK), and hybridized with (α-32P-dCTP)-labeled cDNA probes overnight at 42°C. After serial washing with 6 × and 2 × Standard Saline Citrate (SSC) plus 0.1% SDS, membranes were developed by auto-radiography. The cDNA probes used in this study are: mouse Osx cDNA containing 5' fragment, mouse Runx2, mouse Bp, and mouse Oste.

Figure 3. Expression of mir-204 inhibits ALP 3'-UTR reporter (p-ALP) activity. A: Schematic representation of wild-type and mutant ALP 3'-UTR reporter constructs with wild-type (p-ALP) or mutant (p-mALP) mir-204 seed sequence in the 3'-UTR of ALP gene. The mir-204 target sequence and its mutation in the 3'-UTR of the ALP gene were indicated in bold interface. B: Luciferase assay in 293T cells after transfection with different reporters and miRNAs as indicated in the panels. Mir-302a served as a control. C: Luciferase assay in MC3T3 cells after transfection with empty vector, p-ALP or p-mALP reporter with or without mir-204 or mir-204 inhibitor. All data represent means ± S.D. (n = 3). Statistical significance (p<0.05) was obtained by comparing with control cells treated with empty vector, mir-302a or mir-204 inhibitor.
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Reporter Constructs

Fragments containing different regions of the Sost 3′-UTR were generated by PCR amplification and were cloned into the Hind III/Spe I sites at the 3′ polylinker of pMIR-REPORT, a miRNA expression vector (Ambion). It contains a CMV promoter that directs expression of the firefly luciferase gene and a 3′-polylinker, which allows insertion of 3′-UTRs with miRNA seed sequences, followed by a SV40-derived polyadenylation site. Two 200-bp fragments spanning either a proximal (pSost1) or a distal (pSost2) 3′-UTR region, and one 700-bp fragment containing both proximal and distal 3′-UTR regions of Sost mRNA (pSost3) were inserted into the pMIR-REPORT vector to generate three reporters for miRNA luciferase assay. All three reporters (pSost1, pSost2, and pSost3) contain either the full mir-204 seed region (pSost3) or clusters of the mir-204 seed regions (pSost1 and pSost2). The primers used for PCR amplification are available on request. Each reporter construct was verified for correction by sequencing before use. For luciferase assay on mir-204 targeting ALP, a wild type (p-ALP) and a mutant (p-mALP) ALP reporter were generated. The oligonucleotides 5′-CTAG-TAATTTCCTTTTTGGGTGTTGTTAAAGGGAACAACAAGACATTTAAATAA and 5′-AGCTTTTATT-TAAATGTCTTGTTCCCTTTAAACCAACACAAAAAGAAATTA were annealed, followed by phosphorylation with T4 polynucleotide kinase, and then inserted in the Hind III/Spe I site of pMIR-REPORT to produce the p-ALP reporter. The underlined nucleotides are the mir-204 seed sequence (UUCCUUU). Mutagenesis of this fragment was achieved by annealing the oligonucleotides 5′-CTAG-TAATTTCCTTTTTGGGTGTTGTTATACTCATTACA-
CAAGACATTAAAATAAA and 5′- AGCTTTTATT- 
TAAATGTCTTGTG
AATGAGATAACCAACACCAAAAA-
GAGAAATTA. After T4 phosphorylation, the mutant fragment 
was inserted in the Hind III/Spe I site of pMIR-REPORT to 
generate the p-mALP reporter. In the mutated ALP fragment, the 
bases of mir-204 seed sequence AAAGGGA were replaced by 
TCTCATT to abolish the interaction between a specific binding 
site in the 3′-UTR and mir-204.

Transfection Assays

For an ALP activity assay, C2C12 cells were cultured in 
zMEM supplemented with 10% FBS, 300 ng/ml of BMP2, 50 μg/
ml of ascorbic acid, 10 mM β-glycerophosphate. After overnight 
incubation at 37°C, 50 nM control RNA, mir-204, -302a or
-544 was transfected into cells with Lipofectamine 2000 (Invitrogen) 
following the manufacturer’s protocol. Transfections were done in 
triple. At 72 h after transfection, cells were harvested to assess 
ALP production using an ALP staining kit (Sigma) following the 
manufacturer’s instructions.

For miRNA target gene reporter assays, 293T or MC3T3 cells 
were cultured in Dulbecco’s modified Eagle’s medium (DMEM) or
z-MEM supplemented with 10% FBS overnight at 37°C before 
transfection. For transient transfections, 50 ng of an empty pMIR-
REPORT vector (empty vector) or a target gene-reporter plasmid
was co-transfected with 25 ng of SV40-β-gal control plasmid plus
or minus mir-204 mimic or anti-mir-204 RNA oligos (50 nM) per
well of a 24-well tissue culture plate unless otherwise indicated.
Using Lipofectamine 2000 (Invitrogen), the DNA or DNA/RNA 
mixtures were prepared and added to the cells containing 0.5 ml 
of fresh DMEM or z-MEM supplemented with 10% fetal calf 
serum. After 48 h incubation, the cells were harvested and assayed 
for luciferase activity using a luciferase assay device (Promega). 
The β-gal activity was assayed using the Tropix β-galactosidase kit 
according to the manufacturer’s instructions (Tropix). Luciferase 
expression was normalized to the β-gal control. Transfections were 
done in triplicate, and results were calculated as means ± S.D. 
from three individual experiments. All statistical analyses were
performed with two-tailed Student’s t tests. Data were considered 
to be significantly different for p<0.05.

Results

MiRNA Array Profiling in Osx-null Calvaria

To establish a correlation between Osx and miRNA expression, 
we conducted miRNA expression profiling by miRNA-microarray 
analysis using total RNA isolated from calvaria of E18.5 wild-type
(WT) and Osx−/− mouse embryos. After background reduction 
and quantile normalization, we found that 40 mouse miRNAs had 
a 1.5-fold or greater change in expression in the 
Osx−/− mutants 
compared with their WT littermates. Among these miRNAs, 30
had increased expression and 10 had decreased expression in
Osx−/− calvaria relative to WT controls. Among the 30 up-
regulated miRNAs, 18 have a 2-fold or greater elevation in their 
expression level in the 
Osx−/− calvaria and are listed in Table S1. 
The 10 down-regulated miRNAs are shown in Table S2. The
changes in expression of these miRNAs are also shown in a heat
map (Figure S1). About 60% of miRNAs listed in Tables S1 and
S2 were verified by qPCR using total RNAs from calvaria of the
Osx−/− and WT mouse embryos at E18.5. MiRNAs with a 3-fold
or greater change in their expression level are shown in Tables 1
and 2.

Generation and Characterization of M-Osx Cell Line

To further test whether expression of miRNAs listed in Tables 1
and 2 is regulated by Osx expression during osteogenesis, we
engineered mouse preosteoblast MC3T3 cells by inducible 
expression of Flag-HA-tagged Osx (Flag-HA-Osx), which we
named M-Osx. The positive clones were selected by G418 and
hygromycin, and expression of the Flag-HA-Osx was verified by
Western blot using anti-Flag antibody. As shown in Figure 1A,
Flag-HA-Osx was highly expressed in positive clones number 34
and 35 in the absence of tetracycline (Tet) (Fig. 1A, lanes 3 and 5),
but was not expressed in the presence of Tet (Fig. 1A, lanes 4 and
6). As a control, Flag-HA-Osx was not expressed in clone number

Figure 5. Generation of UMR-conRNA and UMR-mir204 stable cell lines. A–D: UMR-conRNA (A, C) and UMR-mir204 (B, D) cells were 
cultured in the absence of doxycycline (Dox). After 54 h, the cultured cells were examined under light microscope (A, B) and fluorescence microscope 
(C, D). E–H: The UMR-conRNA (E, G) and UMR-mir204 (F, H) cells were cultured in the presence of Dox. After 54 h, the cultured cells were examined 
under light microscope (E, F) and fluorescence microscope (G, H). doi:10.1371/journal.pone.0058104.g005
24 in the presence or absence of Tet (Fig. 1A, lanes 1 and 2). Therefore, cells from either clone 34 or 35 were expanded and maintained in the presence of Tet as the M-Osx cell line. To test whether M-Osx cells express the osteoblast differentiation markers Bsp and Oc under BMP2 treatment, cells from positive clone number 35 were cultured in the absence of Tet with or without BMP2 (Fig. 1B). After 40 h, cells without BMP2 treatment expressed a low level of endogenous Osx, but high levels of exogenous Flag-HA-Osx and endogenous Runx2 (Fig. 1B, lane 1). However, expression of the osteoblast differentiation markers Bsp and Oc was still undetectable in those cells (Fig. 1B, lane 1). It suggests that when MC3T3 cells are cultured in the absence of BMP2 for 40 h, over-expression of Osx is not sufficient to turn on expression of its target genes, Bsp and Oc. In contrast, after cells were treated with BMP2, expression of Bsp and Oc was markedly increased, meanwhile, the levels of endogenous Osx and Runx2 were also higher than those in cells without BMP2 (Fig. 1B, lane 2). By comparison, expression of Flag-HA-Osx was not affected by the BMP2 treatment (Fig. 1B). These results indicate that M-Osx cells have the capacity to express the osteoblast differentiation markers Bsp and Oc in response to BMP2 treatment.

**MiRNAs Positively Regulated by Osx Expression**

In mouse calvarial tissue, after qPCR validation we found that expression of mir-141, -192, -200a, and -1194 was significantly down-regulated in the Osx^−/−^ calvaria compared to their levels in WT littermates, indicating a strong negative correlation between Osx expression and expression of this group of miRNAs. Mir-141 and -200a are homologous. Since mir-141/200a has been known as Dlx5-targeting miRNAs [28], these findings also imply that Osx may negatively regulate Dlx5 protein synthesis via, at least in part, up-regulation of mir-141/200a. However, the role of mir-192 and mir-1194 in osteogenesis is yet to be known.

**MiRNAs Negatively Regulated by Osx Expression**

As shown in Table 2, expression of mir-133a, -204, -211, -302a, -433, -501, and -544 was significantly up-regulated in the Osx^−/−^ calvaria compared to their levels in WT littermates, indicating a strong negative correlation between Osx expression and expression of this group of miRNAs. To further confirm this negative correlation, we used qPCR to measure expression of these miRNAs in the aforementioned M-Osx cells cultured with or without Tet. Mir-204 and -211 are homologous. A recent study indicates that mir-133a and -204/211 are moderately expressed in mouse preosteoblastic MC3T3 cells [30]. When M-Osx cells were cultured for 48 h in the presence of Tet, which turns off expression of Flag-HA-Osx (Fig. 1A), we noticed that mir-133a and -204/211 were all expressed in these cells. However, when M-Osx cells were cultured for 48 h in the absence of Tet to turn on the high level expression of Flag-HA-Osx (Fig. 1B), expression of these miRNAs was significantly down-regulated (Fig. 1D, p<0.05 or 0.01). These results suggest that
during the early-stage differentiation of preosteoblasts, over-expression of Osx may negatively regulate (either directly or indirectly) transcription of this group of miRNAs. Since mir-133a and -204/211 are known as Runx2-targeting miRNAs [29], [30], these observations also imply that Osx may modulate the cellular level of Runx2 protein via in part down-regulation of mir-133a and -204/211.

Expression of mir-302a and -544 was not Sufficient to Inhibit BMP2-induced Alkaline Phosphatase Activity

Among the miRNAs that were negatively correlated with Osx expression (Table 2; Fig. 1D), mir-302a, -433, -501, and -544 have not been reported to be involved in osteogenesis. Using miRDB, an online database (http://mirdb.org/miRDB/index.html) for analysis of miRNA targets, we noticed that mir-302a might target the 3'-UTR of Tgfbr2 and Smad2, whereas mir-544 might target the 3'-UTR of the BMP2 and Smad4/9 genes, suggesting that they might be involved in the TGF-β/BMP signaling pathway. To test whether over-expression of mir-302a or -544 inhibits the BMP2-induced ALP production during osteoblast differentiation, mouse C2C12 cells were used for RNA transfection and assay for ALP activity. We found that ALP activity was significantly reduced in mir-204-transfected cells treated with BMP2 for 72 h (Fig. 2A, compare well No.4 with wells No.5 and No.6; Fig. 2B), consistent with the previous study [27]. By comparison, there was no significant change in ALP staining in cells transfected with either mir-302a or -544 when compared to cells transfected with scrambled RNA or no RNA control (Fig. 2A, compare wells No.2 and No.3 with wells No.5 and No.6; Fig. 2B). These results indicate that over-expression of mir-302a or -544 in C2C12 cells, is not sufficient to repress the BMP2-induced ALP activity.

Alkaline Phosphatase (ALP) is a Direct Target of mir-204

Runx2 is essential for bone formation and ALP production. Since over-expression of mir-204/211 in cultured MC3T3 or BMP2-treated C2C12 cells reduces Runx2 protein level and decreases the ALP activity, it has been suggested that inhibition of ALP by mir-204/211 is Runx2-dependent [29], [30]. To date, it is not clear whether these miRNAs can directly interfere with ALP protein synthesis. From our miRNA target analysis using miRDB, we found that ALP was a putative target of mir-204. To confirm this, we fused a ~100-bp 3'-UTR segment of the ALP gene, which contains a mir-204 seed sequence, into the pMIR-REPORT vector to generate a wild-type ALP 3'-UTR reporter construct (p-ALP) for luciferase assay (Fig. 3A). The mutant reporter construct (p-mALP) was generated by modifying 7 nucleotides of the mir-204 seed sequence in the 3'-UTR of the ALP gene (Fig. 3A). 293T cells were first used for transfection with plasmid DNA of empty vector, p-ALP, or p-mALP, in the absence or presence of mir-204 or mir-302a (which served as a control). At 48 h after transfection, we found that co-transfection of mir-204 with empty vector did not alter its luciferase activity, whereas co-transfection of mir-204 with wild-type p-ALP significantly reduced its luciferase activity (Fig. 3B; p<0.01). By comparison, control mir-302a, which presumably does not target ALP gene, did not affect the activity of p-ALP (Fig. 3B). In addition, co-transfection of either mir-204 or -302a with the mutant p-mALP reporter did not inhibit its luciferase activity (Fig. 3B). These results suggest that mir-204 targets the 3'-UTR of ALP gene. To further confirm these observations in osteogenic cell line, we then transfected MC3T3 cells with the empty vector, p-ALP or p-mALP, in the presence or absence of mir-204 or mir-204 inhibitor. After 48 h, we noticed a significant down-regulation of luciferase activity when expressing p-ALP, but not empty vector or p-mALP alone, in MC3T3 cells (Fig. 3C, p<0.05), implicating an inhibitory effect of endogenous mir-204 on p-ALP. Co-transfection of p-ALP with mir-204, but not mir-204 inhibitor, further decreased its luciferase activity (Fig. 3C; p<0.01). By comparison, co-transfection of p-mALP with mir-204 or mir-204 inhibitor did not significantly affect its luciferase activity (Fig. 3C). These results indicate that mir-204 directly targets the 3'-UTR of ALP gene. The direct interaction between mir-204 and ALP gene may contribute to the mir-204-induced inhibition of osteoblast differentiation in MC3T3 as well as BMP2-treated C2C12 cells.

Sost, which Encodes a Wnt Signaling Antagonist, is also a Direct Target of mir-204

The Wnt/β-catenin signaling pathway plays a major role in osteoblast proliferation and differentiation [19], [20], [21], [42], [43]. Since the Sost gene which encodes sclerostin, an important antagonist of the Wnt signaling, was listed among the predicted targets of mir-204, we wanted to investigate whether the 3'-UTR of Sost is also a direct target of mir-204. Three PCR fragments, containing the mir-204 seed sequence in either the proximal or distal or both regions of the Sost 3'-UTR, were fused to the pMIR-REPORT vector to generate three Sost 3'-UTR reporter constructs, pSost1, pSost2 and pSost3, for luciferase assays (Fig. 4A). Empty vector or each Sost 3'-UTR reporter plasmid was transfected with or without mir-204 into the cultured 293T cells. At 48 h after transfection, we did not observe significant change in luciferase activity when expression of empty vector or each reporter in the absence of mir-204 (Fig. 4B). Co-transfection of mir-204 with empty vector did not affect its luciferase activity. However, co-expression of mir-204 with any of those three reporters significantly decreased their luciferase activity (Fig. 4B; p<0.01). By comparison, co-transfection of control mir-302a with pSost1 did not affect its activity. These observations suggest that the 3'-UTR of Sost is a direct target of mir-204. To confirm these results in osteoblastic cell line, we transfected the MC3T3 cells with empty vector or pSost1 reporter in the presence or absence of mir-204 or mir-204 inhibitor. After 48 h, we observed a significant reduction in luciferase activity of pSost1 reporter alone when compared to empty vector alone (Fig. 4C; p<0.05), suggesting an inhibitory effect of endogenous mir-204 on pSost1 reporter. Co-transfection of pSost1 with mir-204 further decreased the reporter activity (Fig. 4C; p<0.01). In contrast, co-transfection of either empty vector or pSost1 with mir-204 inhibitor did not significantly alter their luciferase activity (Fig. 4C). These data demonstrate that mir-204 directly targets the 3'-UTR of Sost gene.

All data represent means ± S.D. (n = 3). Statistical significance (p<0.05) was obtained by comparison with control cells treated with empty vector, mir-302a or mir-204 inhibitor.further examine whether expression of mir-204 directly attenuates Sost protein in osteoblasts, we used the osteogenic UMR-106 cells for this study. The UMR-106 cell line is a clonal derivative of a transplantable rat osteosarcoma and shares several phenotypic features of mature osteoblasts. Therefore, Runx2, Oxs and Sost are simultaneously expressed in these cells [13]. The UMR-106 cells were first engineered by stably expressing non-silencing control RNA (conRNA) or mir-204 plasmid vector to generate two stable cell lines, UMR-conRNA and UMR-mir-204. In both cell lines, expression of conRNA or mir-204, followed by expression of Red Fluorescent Protein (RFP), is inducible. As shown in Figure 5, in the absence of doxycycline (Dox) expression of conRNA or mir-204 was off, therefore, no red color was seen under fluorescence microscope (Fig. 5C, D). However, when cells were treated with Dox, expression of conRNA or mir-204 was on, and subsequently triggered expression of RFP, which shows red color under
Osx, mir-133a and -204/211, which target Runx2, was up-cultured cells. Among those miRNAs differentially regulated by levels of these miRNAs and Osx expression in compared to wild type embryos, and verified the correlation adipocytes, these previous studies suggest an inverse correlation The up-regulation of these miRNAs in Osx experimental system is different, it is challenging to provide a increases, and that in the cultured M-Osx cells, increased level of in vivo data is that mir-133a and -204/211 may be part of a physiological feedback system that coordinates the levels of Osx and Runx2 protein in osteoblasts to optimize the transcriptional efficiency of these two transcription factors at their target gene promoters. Indeed, Runx2 and Osx have common target genes in osteoblasts [15]. Since we know that the level of Runx2 mRNA increases in Osx-carrivia [14], we speculate that mir-133a and -204/211 mRNA transcripts may be regulated in calvaria of the E18.5 embryos, and these miRNAs to maintain an appropriate level of Runx2, master osteogenic transcription factor Osx and miRNAs involved expression and expression of mir-133a and -204/211. The up-regulation of these miRNAs in Osx-carrivia is not inconsistent with the results in C2C12 cells in vitro. However, inhibitor of ALP has been thought to be dependent on attenuation of Runx2 protein by these miRNAs. Here, we provide in vitro evidence showing that mir-204/211 can also directly target the 3' UTR of ALP (Fig. 3). Since Osx expression in cultured MC3T3 or BMP2-treated C2C12 cells is often induced concurrent with osteoblast differentiation and ALP production, the negative regulation of mir-204/211 by Osx implies that Osx may play a role in maintaining ALP protein level via in part down-regulation of mir-204/211 (Fig. 7). Mir-302a and -544 have not been reported to be involved in osteogenesis. Target analysis showed that mir-302a might target the 3' UTR of Tgfb2 and Smad2, whereas mir-544 might target the 3' UTR of Bmp2 and Smad4/9 genes. Although we did not test this target prediction, our finding that over-expression of either mir-302a or -544 was unable to modulate the BMP2-induced osteoblast differentiation marked by ALP production (Fig. 2), implies that the inhibition of target genes by mir-302a or -544, if any, may be compensated by other members of Smad family or Bmpr1. In addition, our study shows that Osx can up-regulate expression of mir-141/200a. Since mir-141/200a directly targets Dlx5, we hypothesize that during osteogenesis Osx may have the ability to prevent excessive production of Dlx5 protein via in part up-regulation of mir-141/200a. However, a previous study showed that MC3T3 cells were treated with BMP2, expression of Dlx5 along with Osx was increased, whereas expression of the endogenous mir-141/200a was down-regulated [28], implicating a negative correlation between Osx and expression of mir-141/200a. This seems inconsistent with our current finding. It is likely that exogenous BMP2 in cultured cells may inhibit the transcription of mir-141/200a.

Taken together, our data show a tight correlation between the master osteogenic transcription factor Osx and miRNAs involved in bone formation. It will be interesting to know how Osx coordinates to regulate these miRNAs to maintain an appropriate level of Runx2.

Figure 7. Schematic model for depicting a role of Osx in regulation of Runx2, Sclerostin and ALP through inhibition of miRNA expression. The solid lines with bars show inhibition; the dashed lines with bars show inhibition with unclear mechanisms; the arrow represents activation; the new findings of this study are shown in thicker lines, whereas the known information is shown in thinner lines. doi:10.1371/journal.pone.0058104.g007
Sclerostin, ALP and Dlx5 proteins for optimal differentiation and function of osteoblasts.

Supporting Information

Figure S1 A representative heat map. This heat map shows expression of the selected miRNAs (listed in Tables S1 and S2) in each miRNA chip hybridized with calvarial RNAs of three individual wild type (WT, A597.13, A548.11, A600.17) and three individual Osx−/− (KO, A599.15, A596.12, A598.14) mouse embryos at E18.5.

Table S1 Up-regulation of miRNAs in miRNA array chips.

Table S2 Down-regulation of miRNAs in miRNA array chips.

References

1. Nakashima K, de Crombrugge B (2005) Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet 19: 458–466.
2. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, et al. (2002) The novel zinc finger-containing transcription factor osteo is required for osteoblast differentiation and bone formation. Cell 108: 17–29.
3. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, et al. (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 90: 755–769.
4. Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, et al. (1999) Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 214: 279–290.
5. Zhao GQ, Zhao S, Zhou X, Eberspaecher H, Solursh M, et al. (1994) Dlx5, a novel distal-less-like homeoprotein is expressed in developing cartilages and discrete neuronal tissues. Dev Biol 166: 37–51.
6. Acampora D, Merlo GR, Palaei L, Zerrega B, Postigione MP, et al. (1999) Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. Development 126: 3753–3769.
7. Hu R, Li H, Liu W, Yang L, Tan YF, et al. (2010) Targeting miRNAs in osteoblast differentiation and bone formation. Expert Opin Ther Targets 14: 1109–1120.
8. Li H, Xie H, Liu H, Hu R, Huang B, et al. (2009) A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. J Clin Invest 119: 3666–3677.
9. Hu R, Liu W, Li H, Chen C, Xia ZY, et al. (2011) A Runx2/miR-3960/miR-2861 regulatory feedback loop during mouse osteoblast differentiation. J Biol Chem 286: 12323–12339.
10. Yang L, Cheng P, Chen C, He BB, Xie GQ, et al. (2012) miR-93/Sp7 function loop mediates osteoblast mineralization. J Bone Miner Res 27: 1594–1606.
11. Zhang C, Cho K, Huang Y, Lyons JP, Zhou X, et al. (2008) Inhibition of Wnt signaling by the osteoblast-specific transcription factor Osterix. Proc Natl Acad Sci USA 105: 6936–6941.
12. Backer WV, Lee MA, Junw JW, Kim SY, Akirama H, et al. (2009) Positive regulation of adult bone formation by osteoblast-specific transcription factor osterix. J Bone Miner Res 24: 1055–1065.
13. Sinha KM, Yasuda H, Coombes MM, Dent SY, de Crombrugge B (2010) Regulation of the osteoblast-specific transcription factor Osterix by NO66, a Junonji family histone demethylase. EMBO J 29: 69–79.
14. Zhou X, Zhang Z, Feng JQ, Dusevic VM, Sinha K, et al. (2010) Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. Proc Natl Acad Sci USA 107: 12919–12924.
15. Dusevic V, Zhang Z, Greulich V, Rahill AL, Kasemay G (1997) Odo2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 90: 747–754.
16. Nisho Y, Dong Y, Paris M, O’Keefe RJ, Schwarze EM, et al. (2006) Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. Gene 372: 62–70.
17. Lee MH, Kim YJ, Yoon WJ, Kim JJ, Kim BG, et al. (2006) Dlx5 specifically regulates Runx2 type II expression by binding to homeodomain-response elements in the Runx2 distal promoter. J Biol Chem 281: 35579–35587.
18. Tadic T, Dodig M, Erceg I, Marijanovic I, Mina M, et al. (2002) Overexpression of Dlx5 in chicken calvarial cells accelerates osteoblastic differentiation. J Bone Miner Res 17: 1008–1014.
19. Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ (2012) Update on Wnt signaling in bone cell biology and bone disease. Gene 492: 1–18.
20. Hu H, Hilton MJ, Tu X, Yu K, Omarza DM, et al. (2005) Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development 132: 49–60.
21. Day TF, Yang Y (2008) Wnt and hedgehog signaling pathways in bone development. J Bone Joint Surg Am 90 Suppl 1: 19–24.
22. Senemou M, Tamai K, He X (2005) SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem 280: 26770–26775.
23. Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, et al. (2008) Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res 23: 860–869.
24. Winkler DG, Sutherland MK, Georgiades JC, Yu C, Hayes T, et al. (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J 22: 6267–6276.
25. Yang F, Tang W, So S, de Crombrugge B, Zhang C (2010) Sclerostin is a direct target of osteoblast-specific transcription factor osterix. Biochem Biophys Res Commun 400: 684–688.
26. Stefani G, Shark FF (2008) Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol 9: 219–230.
27. Li Z, Hassan MQ, Volinia S, van Wijnen AJ, Stein JL, et al. (2008) A microRNA signature for a BMP2-induced osteoblast lineage commitment program. Proc Natl Acad Sci U S A 105: 13906–13911.
28. Rohi T, Nozawa Y, Akao Y (2009) MicroRNAs-141 and -200a are involved in bone morphogenic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homebox 5. J Biol Chem 284: 19272–19279.
29. Huang J, Zhao L, Xing L, Chen D (2010) MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. Stem Cells 28: 357–364.
30. Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, et al. (2011) A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. Proc Natl Acad Sci USA 108: 9865–9868.
31. Guir T, Hussain S, Mudhasilai R, Parulkar I, Collly JL, et al. (2010) Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse. Dev Biol 340: 10–21.
32. Liu CG, Calma GA, Volinia S, Croce CM (2008) MicroRNA expression profiling using microarrays. Nat Protoc 3: 563–578.
33. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185–193.
34. Smyth GK, Michaud J, Scott HS (2005) Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21: 2057–2058.
35. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article 3.
36. McCarthy DJ, Smyth GK (2009) Testing significance relative to a fold-change threshold is a TREAT. Bioinformatics 25: 765–771.
37. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57: 289–300.
38. Storey JD, Siegmund D (2001) Approximate p-values for local sequence alignments: numerical studies. J Comput Biol 8: 549–556.
39. Storey JD, Tibshirani R (2003) Statistical methods for identifying differentially expressed genes in DNA microarrays. Methods Mol Biol 224: 149–157.
40. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.
41. Iguradu XL, Kaminos N, Hasegawa M, Kasuya T, Takahashi T, et al. (2004) Inductive effects of dexamethasone on the gene expression of Cbfa1, Osterix and bone matrix proteins during differentiation of cultured primary rat osteoblasts. J Mol Endocrin 35: 3–10.

Author Contributions

Conceived and designed the experiments: QC HY BdC. Performed the experiments: QC HY KS. Analyzed the data: WL. Contributed reagents/materials/analysis tools: WL. Wrote the paper: QC HY BdC.
42. Hill TP, Später D, Takeo MM, Birchmeier W, Hartmann C (2005) Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell 8: 727–738.

43. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, et al. (2005) Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci USA 102: 3324–3329.