The microRNA-23a cluster regulates the developmental HoxA cluster function during osteoblast differentiation

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MicroRNAs (miRs) and Hox transcription factors have decisive roles in postnatal bone formation and homeostasis. In silico analysis identified extensive interaction between HOXA cluster mRNA and microRNAs from the miR-23a cluster. However, Hox regulation by the miR-23a cluster during osteoblast differentiation remains undefined. We examined this regulation in preosteoblasts and in a novel miR-23a cluster knockdown mouse model. Overexpression and knockdown of the miR-23a cluster in preosteoblasts decreased and increased, respectively, the expression of the proteins HOXA5, HOXA10, and HOXA11; these proteins’ mRNAs exhibited significant binding with the miR-23a cluster miRNAs, and miRNA 3′-UTR reporter assays confirmed repression. Importantly, during periods correlating with development and differentiation of bone cells, we found an inverse pattern of expression between HoxA factors and members of the miR-23a cluster. HOXA5 and HOXA11 bound to bone-specific promoters, physically interacted with transcription factor RUNX2, and regulated bone-specific genes. Depletion of HOXA5 or HOXA11 in preosteoblasts also decreased cellular differentiation. Additionally, stable overexpression of the miR-23a cluster in osteoblasts decreased the recruitment of HOXA5 and HOXA11 to osteoblast gene promoters, significantly inhibiting histone H3 acetylation. Heterozygous miR-23a cluster knockdown female mice (miR-23aGWT/ZIP) had significantly increased trabecular bone mass when compared with WT mice. Furthermore, miR-23a cluster knockdown in calvarial osteoblasts of these mice increased the recruitment of HOXA5 and HOXA11, with a substantial enrichment of promoter histone H3 acetylation. Taken together, these findings demonstrate that the miR-23a cluster is required for maintaining stage-specific HoxA factor expression during osteogenesis.

Mammalian homebox (Hox)3 developmental transcription factors have diverse roles in bone development and postnatal bone formation (1–3). These roles include skeletal element formation (4), anterior–posterior homeotic development (4–10), and limb and axial skeleton formation (11–15). In fact, cooperation among Hox genes is critical in skeletal development (2, 13, 16–18). Moreover, Leucht et al. (19) reported that the Hox gene expression status influences the process of adult bone regeneration. A high-throughput ChIP-sequencing study revealed that HOXD13 binds numerous genes that act in key pathways required for early limb and skeletal patterning (20). Furthermore, Wan and Cao (21) reported that a SMAD–HOX association is required to decipher the mechanism of bone morphogenetic protein signaling in osteoblast growth and differentiation. In previous studies, we demonstrated that selective recruitment of HOX transcription factors to bone-specific chromatin at specific stages of osteoblast maturation mediates gene activation (1, 22–24). Hence, multiple levels of transcriptional and epigenetic regulation by HOX proteins must be examined to define the complete mechanism(s) for Hox gene function in osteoblast differentiation.
likely to regulate vital cell processes in osteoblasts (58–60). Recently, in an osteoblast-specific loss-of-function transgenic model (61), it was shown that the miR-23a cluster regulates osteocyte differentiation by modulating the transforming growth factor-β signaling pathway through targeting of Prdm16. Additionally, findings from this study indicated that mice overexpressing the osteoblast-specific miR-23a cluster have low bone mass associated with fewer osteoblasts and more osteocytes. Previously, our group reported that the miR-23a cluster represses osteoblast differentiation by inhibiting the synergistic action of the transcription factors RUNX2 and SATB2 (58).

Notably, the factors mediating post-transcriptional control of Hox genes remain unclear. Genetic deletion studies recently revealed that miRNA embedded within the Hox clusters is important to refine Hox gene expression to ensure axial identity (62–64). Three miRNAs, miR-196a-1, miR-196a-2, and miR-196b, directly control Hoxb8 mRNA through 3′-UTR binding (65). These miRNAs are extensively conserved and potentially bind to the mRNAs of the Hox8 and Hox7 paralogues and thus regulate posterior limb bud patterning by preventing inappropriate Hox gene expression (66). Overall, however, very few miRNAs have been reported to target and repress Hox mRNA translation. Furthermore, the mechanism by which miRNA post-transcriptionally regulates the expression of stage-specific HOX proteins for commitment, growth, and differentiation of bone cells is not clearly understood.

Here, we investigated the mechanism by which the miR-23a cluster regulates Hox-mediated gene activation and identified epigenetic changes associated with mineralization and maturation of osteoblasts. Overall, our findings indicate that regulation of HoxA transcription factors by the miR-23a cluster is required to understand the miRNA-mediated epigenetic basis of bone formation.

Results
Potential HoxA class mRNA targets for the miR-23a cluster relevant to skeletogenesis

Bioinformatics analysis using three different programs (TargetScan version 6.2, DIANA-MICROT, and MICRORNA. ORG) revealed that 50% of the mouse and human Hox mRNAs are predicted potential targets for the miR-23a cluster (Table S1). A majority of the research on Hox loss of function in mice has unequivocally shown that these potential miRNA targets are critical regulators in skeletal pattern formation and differentiation of osteoblasts (Table S2). Among the HoxA genes, binding affinity scores from three *in silico* miRNA bioinformatics programs identified HoxA5, HoxA10, and HoxA11 as potential targets for miR-23a and miR-27a (Table 1). Each of these genes received high scores as potential targets for the miR-23a cluster in all three programs.

**miR-23a cluster binds the 3′-UTR of HoxA5, HoxA10, and HoxA11 mRNA**

miRNA–mRNA binding analysis showed that the 3′-UTRs of HoxA5, HoxA10, and HoxA11 contain three, one, and one putative binding site(s) for miR-27a. The 3′-UTR of HoxA11 also contains two binding sites for miR-23a (Fig. 1A). Stable lentivirus-mediated overexpression of the miR-23a cluster in mouse preosteoblast MC3T3-E1 cells significantly down-regulated HoxA5, HoxA10, and HoxA11 mRNA levels by 50–80%. However, HoxA1, HoxA2, and HoxA3, mRNA levels (also predicted as targets *in silico*; Table 1) did not change significantly (Fig. 1B). Conversely, knockdown of the miR-23a cluster in MC3T3-E1 cells significantly increased the expression of HoxA5, HoxA10, and HoxA11 mRNA by ≥1.7-fold. However, HoxA1, HoxA2, and HoxA3 mRNA levels did not alter significantly (Fig. 1C). Western blotting studies revealed that miR-23a cluster overexpression significantly decreased, and miR-23a cluster knockdown noticeably increased, the HoxA5, HoxA10, and HoxA11 protein levels, whereas no change was noticed in the protein expression of HoxA3 and HoxA13 (Fig. 1D) in MC3T3-E1 cells 72 h after infection. Unexpectedly, we found a significant increase in HoxA13 expression with miR-23a cluster overexpression, whereas there was a substantial decrease with miR-23a cluster knockdown in HoxA13 expression. These findings suggest that HoxA13 gene expression by miR-23a cluster may be an indirect, non-UTR-mediated regulation by an unknown mechanism.

Furthermore, we examined the effect of the individual miR-23a cluster members on the expression of HoxA5, HoxA10, and HoxA11 in MC3T3-E1 cells stably overexpressing miR-23a or miR-27a. Quantitative RT-PCR (RT-qPCR) analysis in these stable cells revealed that miR-27a significantly down-regulated HoxA5 and HoxA10 mRNA levels, whereas miR-23a and miR-27a each significantly down-regulated HoxA11 mRNA levels (Fig. 1E). To study the endogenous binding of miR-23a and miR-27a to the 3′-UTR of HoxA5, HoxA10, and HoxA11 mRNAs, we performed a ribonucleoprotein immunoprecipitation to isolate miR–RISC complexes (RISC-IP assay). In miR-23a– and miR-27a–overexpressing MC3T3-E1 cells, a 5-fold enrichment of bound HoxA5 and HoxA11 mRNA and an 8-fold enrichment in bound HoxA10 mRNA was revealed (Fig. 1F). To further confirm direct binding and regulation of miR-23a and miR-27a, we cloned a 100–120-bp fragment of the HoxA5, HoxA10, or HoxA11 3′-UTRs (Table S3 and S4), which contain WT and mutant miRNA binding sites into the 3′-UTR of a

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**Table 1**

*In silico* analysis of HoxA class mRNAs for miR-23a and -27a binding

| miRs (mouse) | HoxA targets | Diana MiCroT1 | miTG score | PicTar* | Score | Target scan† | Score |
|-------------|--------------|----------------|------------|--------|-------|--------------|-------|
| miR-23a     | HoxA1        | ✓              | 6.19       | 5.074  | ✓     |               | ×     |
|             | HoxA2        | ✓              | 3.90       | ×      |       |               | ×     |
|             | HoxA3        | ✓              | 7.80       | 0.12   | ✓     |               | ✓     |
|             | HoxA5        | ×              | ×          | ×      | ×     |               | ×     |
|             | HoxA10       | ×              | ×          | ×      | ×     |               | ×     |
|             | HoxA11       | ×              | ×          | ×      | ×     |               | ×     |
| miR-27a     | HoxA1        | ×              | 2.70       | 1.929  | 0.30  |               |       |
|             | HoxA2        | ×              | ×          | ×      | ×     |               | ×     |
|             | HoxA3        | ×              | ×          | ×      | ×     |               | ×     |
|             | HoxA5        | ×              | 18.50      | 12.88  | 0.80  |               |       |
|             | HoxA10       | ×              | 26.36      | 3.149  | 0.84  |               |       |
|             | HoxA11       | ×              | 2.00       | 0.63   | ×     |               | ×     |

1. Diana-MicroT version 3.0 ([http://diana.imis.athena-innovation.gr/DianaTools/index.php](http://diana.imis.athena-innovation.gr/DianaTools/index.php)). Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
2. PicTar ([http://pic tar.mdc-berlin.de/](http://pictar.mdc-berlin.de/)). Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
3. TargetScan version 6.2 ([http://www.targetscan.org/](http://www.targetscan.org/)) ([72]). Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

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miR-23a cluster regulates HoxA cluster function

Figure 1. Identification and validation of functional miR-23a cluster binding sites in the 3′-UTR of HoxA5, HoxA10, and HoxA11 mRNA 3′-UTR. A, putative binding sites for miR-23a cluster members were identified in the 3′-UTRs of HoxA5 (nucleotide positions 97–103, 558–564, and 704–710), HoxA10 (nucleotide positions 1032–1039), and HoxA11 (nucleotide positions 38–44, 496–503, and 525–531) mRNA using miRNA bioinformatics programs (Table 1). B and C, quantitative analysis of the total RNA to examine relative HoxA member mRNA expression levels, normalized to Gapdh mRNA levels, in MC3T3-E1 cells stably infected with control, miR-23a cluster, or anti-miR-23a cluster (MIR-ZIP)-expressing lentiviral particles, after 72 h. D, representative Western blotting of HOXA3, HOXA5, HOXA10, HOXA11, and HOXA13 protein levels, normalized to actin levels, in control, miR-23a cluster, or anti-miR-23a cluster (MIR-ZIP)-expressing lentiviral particles, detected by RT-qPCR after 72 h (C). Oligo(dT) primer was used to synthesize cDNA, and gene-specific primers were used to amplify the cDNA (Table S5, D). E, quantitative analysis of the total RNA to examine relative HoxA member mRNA expression levels, normalized to Gapdh mRNA levels, in MC3T3-E1 cells stably infected with control, miR-23a cluster, or anti-miR-23a cluster (MIR-ZIP)-expressing lentiviral particles, after 72 h. F, luciferase reporter activity. Lysates from HEK-293 cells transfected with WT (Wild type), miR-23a, miR-27a, synthetic miRNA (C), miR-23a–expressing, or miR-27a–expressing lentiviral vectors, detected by RT-qPCR after 72 h. Oligo(dT) primer was used to synthesize cDNA, and gene-specific primers were used to amplify the cDNA (Table S5). G, representative RISC-IP assay in polysome lysates of MC3T3-E1 cells overexpressing the miR-23a cluster immunoprecipitated with IgG (control) or anti-AGO2 antibody. Immunoprecipitated RNA was isolated using the TRIzol method. Hexamer oligonucleotide primers were used to synthesize cDNA, and gene-specific primers were used to amplify the cDNA (Table S5). Data are presented as the amount of miR-23a and miR-27a bound to the 3′-UTR of HoxA5, HoxA10, and HoxA11 (black bars) in the RISC relative to total HoxA (miR-23a cluster construct (miR-Cl), or anti-miR-23a cluster construct (miR-ZIP) for 48 h. Cells were lysed in Passive Lysis Buffer (Promega Corp.) and assayed (20 μl) for luciferase activity. Relative luciferase activity was normalized with Renilla luciferase activity and expressed in relative luminescence units. Statistical significance was determined by Student’s t test, *p = 0.05 versus matched control. Luc, luciferase. Error bars, S.E.

Luciferase reporter gene. Lysates from HEK-293 cells transfected with miRNAs along with WT or mutant 3′-UTR-luciferase plasmids were assayed for luciferase reporter activity. Compared with the effects of control miRNA, overexpression of miR-23a, miR-27a, or miR-23a + miR-27a significantly (2–6-fold) repressed luciferase activity from the WT HoxA5, HoxA10, and HoxA11 3′-UTR reporter plasmids (Fig. 1G). Conversely, overexpression of anti-miR-23a cluster significantly increased luciferase activity from the WT HoxA5, HoxA10, and HoxA11 3′-UTR reporter plasmids by 2–10-fold. However, luciferase activity did not change or slightly increased when the respective miRNA binding sites were mutated (Fig. 1, H and I). Taken together, these results indicate that the expression of HoxA5, HoxA10, and HoxA11 is post-transcriptionally controlled by the miR-23a cluster through 3′-UTR regulation. Hence, we conclude that the miR-23a cluster has a critical role in the regulation of HoxA cluster members.

miR-23a cluster expression correlates inversely with HoxA5 and HoxA11 expression during osteoblast differentiation and embryonic development

Our previous studies demonstrated that HOXA10 activates bone-specific genes and promotes osteoblast differentiation (1). However, the mechanisms by which other HoxA cluster members, including HOXA5 and HOXA11, regulate osteoblast differentiation remain unknown. Because the miR-23a cluster directly targets HoxA5, HoxA10, and HoxA11 (Fig. 1), we hypothesized that a miR-23a cluster regulatory network controls osteoblast maturation by targeting HoxA proteins. We compared the expression levels of the mRNA and proteins encoded by HoxA5, HoxA10, HoxA11, and the miR-23a cluster during preosteoblast differentiation and embryonic development. Temporal differences in HoxA5 and HoxA11 expression were found during osteoblast differentiation, with low expression in early osteoblasts (day 0–7), followed by peak expression levels in mature osteoblasts (day 10–12) and decreased expression during the mineralization stage (day 15–18) (Fig. 2A). We also found an analogous differential pattern of mRNA expression for HoxA1, HoxA2, HoxA3, HoxA10, and HoxA13 during MC3T3-E1 cell differentiation (Fig. S1B). Expression of the miR-23a cluster correlated inversely with the expression of HoxA5, HoxA10, and HoxA11 at each stage of osteoblast differentiation, with the exception of miR-24-2 during the mineralization stage (Fig. 2A and Fig. S1C). The initial increase of miRNA expression (days 5–7) also correlated with low expression of HOXA5 and HOXA11 proteins (Fig. 2B showing graphic quantification of Fig. S1D), indicating little or no induc-
miR-23a cluster regulates HoxA cluster function

Figure 2. Temporal expression of the miR-23a cluster and HoxA5 and HoxA11 maintains the physiology of osteoblast differentiation in vitro and in vivo. A, relative expression of the miR-23a cluster miRNA compared with HoxA5 and HoxA11 mRNA during MC3T3-E1 (preosteoblast) cell differentiation at the indicated days with n = 3 time course experiments, normalized to Gapdh levels. Oligo(dT) primer was used to synthesize cDNA, and gene-specific primers were used to amplify the cDNA (Table S5). B, densitometry quantitation (ImageJ) of Western blots (n = 3) showing total protein from MC3T3-E1 cells assayed for HOXA5 and HOXA10 expression at the indicated days with n = 3 time course experiments, normalized to Gapdh levels. Oligo(dT) primer was used to synthesize cDNA, and gene-specific primers were used to amplify the cDNA (Table S5). U6 expression was used as the experimental control. E, embryonic. Error bars, S.E.

tion of osteoblastogenesis. The in vitro differentiation of MC3T3-E1 cells was confirmed by the expression of osteoblast stage–specific marker genes Alp, Runx2, Col1A1, and Ocn (Fig. S1A). To understand the relationship between HoxA cluster genes and the miR-23a cluster during embryonic skeletogenesis, we analyzed bone RNA for the expression of the miR-23a cluster, HoxA5, and HoxA11 during skeletal development in utero (embryonic days 12–19) and found a similar inverse relationship in expression levels (Fig. 2C). These results indicate a requirement for biological regulation of HoxA cluster protein expression by the miR-23a cluster during osteogenesis.

HOXA5 and HOXA11 directly regulate bone-specific gene expression

The temporal expression patterns of different HoxA cluster genes during osteoblast differentiation suggested that HOX proteins might directly regulate bone-related gene promoter activity at different stages of maturation. In our previous report (1), we characterized HOX-responsive elements (ATTAT) in the promoters of bone-related Runx2 and Ocn, which are expressed in early and late stages of differentiation. However, evidence of HOXA5 and HOXA11 binding to such bone-specific promoters and modulation of gene expression has not yet been shown. We sought to determine whether HOXA5 and HOXA11 regulate the expression of Runx2 and Ocn through interaction with the putative Hox regulatory elements located in the proximal promoters of these genes. HoxA5- and HoxA11-targeting shRNA or overexpression plasmids were co-transfected with luciferase reporter constructs containing −600 bp of the Runx2 (−600 bp Runx2-Luc) or −285 bp of the Ocn (−285 bp Ocn-Luc) promoter region in HEK-293 cells. Several shRNA constructs were tested to achieve HOXA5 and HOXA11 protein knockdown; we identified two constructs that knocked down these proteins with ~90% efficiency (Fig. S2, A and B). The transcriptional activity of these overexpression and knockdown clones was validated with bone-specific Ocn and Runx2 proximal promoters (Fig. 3, A–D). The forced expression of HOXA5- and HoxA11-targeting shRNAs led to a 2-fold decrease in the promoter activity of the −600 Runx2 and −285 Ocn promoter fragments, suggesting that the proximal ATTAT binding site of Ocn and Runx2 is responsive to HOXA5 and HOXA11 regulation (Fig. 3, A and B), as we observed previously for HOXA10 (1). We next examined the specificity of HOXA5 and HOXA11 regulation by co-transfecting both overexpression and shRNA clones together with the −285 bp Ocn proximal promoter fragment (Fig. 3C). We found a 10- and 3-fold increase in the Ocn promoter activity when we overexpressed cytomegalovirus (CMV)-driven HOXA5 and HOXA11 cDNA, respectively. However, co-expression of the respective shRNAs decreased this HOXA5- or HOXA11-induced Ocn promoter activity by 50%. Taken together, these results confirm that HOXA5 and HOXA11 regulate the bone-specific Runx2 and Ocn genes through functional ATTAT sites present in the proximal promoter fragment. In additional experiments, overexpression of HOXA5 and HoxA11 substantially up-regulated luciferase activity of the −285 Ocn promoter (Fig. 3D), and an additive effect was observed when HOXA5 and HOXA11 were overexpressed with RUNX2 (Fig. 3D). No mechanistic interaction of RUNX2 with HOXA5 or HOXA11 in osteoblasts has been reported. Therefore, we focused on determining how HOXA5, HOXA11, and RUNX2 interact and mediate the transcriptional control of bone-specific genes. To study the endogenous physical interaction between HOXA5, HOXA11, and RUNX2, we immunoprecipitated whole-cell lysate at day 7
from MC3T3-E1 cells with anti-RUNX2 antibody overnight. Analysis of immunoprecipitates indicated a physical interaction between RUNX2 and each HOXA protein with weak interaction between RUNX2 and HOXA11 (Fig. 3E). This weak interaction profile of HOXA11 with RUNX2 may be due to stronger interaction of RUNX2 with HOXA5 compared with HOXA11 at day 7 of differentiation. Notably, in silico analysis of the Ocn promoter identified putative RUNX2 and HOXA5/10/11 binding sites within the 1-kb promoter region (Fig. 3F).

To gain further understanding of the in vivo regulation of osteoblast differentiation by HOXA5 and HOXA11, we performed ChIP (Fig. 3G) and sequential ChIP (re-ChIP) (Fig. 3H) assays. The ChIP assay revealed occupancy by HOXA5, HOXA10, HOXA11, and RUNX2, as well as activating histone H3 Lys-4 trimethylation mark (H3K4me3), in the Ocn proximal (−20 to −200 bp) and distal promoter (−400 to −600 bp). ChIP and re-ChIP assays further demonstrated the association of HOXA5 and HOXA11 with the Ocn promoter in a RUNX2-dependent manner (through protein–protein interactions) and in a RUNX2-independent manner (presumably through HOX-binding elements) (Fig. 3H). Taken together, these results indicate that HOXA5 and HOXA11 bind to the Ocn promoter, interact with RUNX2 as a part of activating complex, and functionally cooperate in regulating the Ocn promoter.
miR-23a cluster regulates HoxA cluster function

**HoxA5 and HoxA11 are required for osteogenesis**

To study the functional contribution of HOXA5 and HOXA11 in osteogenic differentiation, we determined the consequences of shRNA-mediated HOXA5 and HOXA11 knockdown on osteoblast activity and marker gene expression. For these studies, we selected the MC3T3-E1 cell line, which recapitulates in vitro differentiation in osteogenic medium containing β-glycerolphosphate and ascorbic acid. Cells were transfected with control, HoxA5, or HoxA11 shRNA and then allowed to differentiate for 12 days. Compared with control, nonspecific shRNA, the respective targeted shRNAs substantially decreased the expression of HoxA5- and HoxA11-encoded mRNA and protein during early differentiation (day 4) and matrix formation (day 12) (Fig. 4, A, B, D, and E). Furthermore, histological staining for alkaline phosphatase (ALP) activity, an early marker of bone matrix maturation (Fig. 4C), revealed that HoxA5 shRNA caused a complete block in ALP activity, and HoxA11 shRNA reduced ALP activity at day 12. HOXA5 or HOXA11 knockdown also decreased the mRNA expression of the Runx2 and osterix transcription factors (Runx2, osterix, Alp, Col1A1, osteopontin (Opn), and Ocn) after transient knockdown of HOXA5 (D) or HOXA11 (E) in MC3T3-E1 cells at days 4 and 12 of differentiation by real-time RT-qPCR. Gapdh expression was used as the experimental control to calculate ΔΔCT. Statistical significance was determined by Student’s t test. *p ≤ 0.05 versus matched control. Error bars, S.E.

**miR-23a cluster reduces HOXA5 and HOXA11 recruitment and epigenetically inhibits chromatin modifications for enhanced osteogenesis**

To understand the mechanism by which the miR-23a cluster regulates the chromatin modification program through HOXA5 and HOXA11 recruitment and inhibition, we next performed ChIP assays with control and miR-23a cluster–overexpressing MC3T3-E1 cells. ChIP assays for occupancy on promoters of osteogenic genes (Alp, Runx2, and Ocn) at day 12 of differentiation (Fig. 5, A–C) showed markedly lower occupancy of HOXA5 (3-fold for Alp, 4-fold for Runx2, and 4-fold for Ocn) and HOXA11 (2.5-fold for Alp, 6-fold for Runx2, and 4.5-fold for Ocn) in miR-23a cluster–overexpressing cells than in control cells. The lower occupancy of HOXA5 and HOXA11 correlates with lower expression of all marker genes required for osteoblastogenesis in HOXA5 and HOXA11 knockdown cells (Fig. 4, D and E). To test the hypothesis that lower recruitment of HOXA5 and HOXA11 reduces the histone H3 acetylation modification at lysine positions 18 (H3K18) and 27 (H3K27), we performed ChIP assays with antibodies against H3K18ac and H3K27ac in MC3T3-E1 cells at day 12 of differentiation. The levels of histone H3K27ac and H3K18ac modifications were 30–60% lower across the same osteogenic promoters in cells overexpressing the miR-23 cluster (Fig. 5, D and E). To test the hypothesis that lower recruitment of HOXA5 and HOXA11 reduces the histone H3 acetylation modification at lysine positions 18 (H3K18) and 27 (H3K27), we performed ChIP assays with antibodies against H3K18ac and H3K27ac in MC3T3-E1 cells at day 12 of differentiation. The levels of histone H3K27ac and H3K18ac modifications were 30–60% lower across the same osteogenic promoters in cells overexpressing the miR-23 cluster (Fig. 5, D and E), indicating that the miR-23a cluster–mediated decreases in HOXA5 and HOXA11 recruitment are associated with reduced H3K18 and H3K27 acetylation promoter modifications that favor closed chromatin and lead to decreased early and late marker gene synthesis during differentiation. Taken together, these ChIP assay results indicate that the miR-23a cluster represses HOXA5 and HOXA11 expression to reduce the formation of a transcriptional and epigenetic activating complex with RUNX2 and, finally, reduces the acetylation of H3 necessary for the promotion of osteogenic gene activation.
miR-23a cluster knockdown mouse

Because germ line deletion of the miR-23a cluster results in embryonic lethality, we have generated a single-copy transgenic mouse model in which miR-23a cluster function can be inhibited by the anti-miR-23a cluster in vivo by Dox induction. This strategy generates anti-miRNAs (22-nucleotide antisense) from a 160-bp anti-miR-23a cluster cassette (MIR-ZIP cassette) (Fig. 6) that completely block the function of all six mature miRNAs (a/b isoforms of miR-23 and -27 and 2/1 isoforms of miR-24) processed from chromosomes 8 and 13. Before developing the mouse lines, we verified the ability of the anti-miR-23a cluster transgene to knock down expression of the endogenous miR-23a cluster in MC3T3-E1 cells. Overexpression of the MIR-ZIP clone led to 50–60% reduction in the expression of miR-23a, miR-27a, and miR-24-2 in MC3T3-E1 cells (Fig. 6B). We next inserted the anti-miR-23a cassette under control of the tetracycline-responsive element (TRE) into safe-haven chromatin downstream of the Col1A1 locus in KH2 mouse ES cells using a Flp-FRT flp-in system developed by the Jaenisch laboratory (69) (Fig. 6C). These ES cells also harbor a modified reverse tetracycline transactivator (M2rtTA) targeted to and under the control of the ROSA26 locus, enabling Dox-inducible expression of the anti-miRNA cassette (Fig. 6C, bottom). Correct targeting of the TRE-anti-miR-23a cassette was validated by Southern blot analysis (Fig. 6D), and Dox-inducible expression of the anti-miRNAs was verified by RT-qPCR analysis (Fig. 6E). Importantly, treatment of the ES cells with 2 μg/ml Dox induced a 20-fold increase in the expression of all three anti-miRs in this system, and the expression levels could be tightly controlled by Dox titration. We then injected ES cell clones into blastocysts. Germ line transmission from chimeras to the F1 generation was verified by PCR genotyping (Fig. 6F) using WT and Flp-In allele-specific primers (Table S5), revealing the anticipated Mendelian inheritance (1:2:1).

miR-23a cluster knockdown mice (miR-23a ClZIP) results in high bone mass

The germ line knockout model we developed for the miR-23a cluster was embryonic lethal. On the other hand, a transgenic mouse model with random integration of multiple miR-23a cluster transgenes causes functional interference with the phenotype. Thus, our tetracycline-inducible miR-23a ClZIP mouse model is highly effective because a single copy, doxycycline-inducible anti-miR-23a cluster transgene is inserted into specific Col1A1 locus to eliminate interference of undesired expression. This mouse model is viable and allows us to study the different stages of bone development and remodeling by doxycycline induction. Additionally, homozygous miR-23a ClZIP mice (male as well as female) treated with doxycycline died postnatally because of higher knockdown of the miR-23a cluster.
miR-23a cluster regulates HoxA cluster function

Calvarial osteoblasts isolated from Dox-treated MIR-ZIP mice and induced to differentiate showed evidence of osteoblast differentiation, with a significant increase in ALP activity after day 12 (Fig. 8A). Analysis of miRNA expression by RT-qPCR revealed that miR-23a and miR-27a expression levels decreased ≥90% in calvarial osteoblasts from Dox-treated compared with untreated MIR-ZIP mice (Fig. 8B). In contrast, mRNA levels of HoxA5 and HoxA11 increased ≥4.0-fold in Dox-treated compared with untreated calvarial osteoblasts (Fig. 8C). These results suggest that blocking miR-23a cluster inhibition promotes HoxA5 and HoxA11 expression, which triggers activation of osteoblast differentiation. We next hypothesized that repression of the miR-23a cluster would promote osteoblast-specific gene expression through HoxA5 and HoxA11 recruitment and open chromatin formation. ChIP assays to determine the occupancy on Runx2 and Ocn promoters in primary mouse calvarial cells (MOBs) at day 12 of differentiation (Fig. 8, D and F) showed markedly higher occupancy of HOXA5 (6.5-fold for Runx2 and 2.5-fold for Ocn) and HOXA11 (5.5-fold for Runx2 and 2.5-fold for Ocn) in Dox-treated miR-23a cluster knockdown cells than in controls. The higher occupancy of HOXA5 and HOXA11 correlated with...
Higher recruitment of H3K27 acetylation for open chromatin (Fig. 8, D and F). Furthermore, these results were supported by a 3-fold increase of Runx2 and ≥4-fold increase in Ocn mRNA expression (Fig. 8, E and G). These findings indicate that the miR-23a cluster maintains tight post-transcriptional control over HOXA5 and HOXA11 recruitment and H3K27ac promoter modifications for the open chromatin of osteoblast-specific marker gene synthesis during differentiation.

**Discussion**

Hox gene expression is critical during skeletal patterning and postnatal bone formation (1–3). A majority of research in vertebrates has shown that Hox-regulating miRNAs are encoded within the four Hox clusters. Besides Hox-embedded miRNAs, many other miRNAs encoded outside the Hox clusters could have key roles in the regulation of Hox gene expression. This study provides evidence that regulation of Hox transcription factors by the miR-23a cluster is critical in osteoblasts, as the temporal expression pattern of this cluster is stage-specific and opposes Hox expression, and 50% of Hox mRNAs are predicted as potential targets. We observed that an increase in miR-23a cluster expression or a relative decrease in HoxA5, HoxA10, and HoxA11 expression inhibits bone formation. Thus, our research indicates that the miR-23a cluster controls 3 of 11 HoxA cluster members that are indispensable in transcriptional activation of osteoblast marker genes at specific stages of differentiation. Additional support for the significance of this finding in bone formation is that the miR-23a cluster targets three Hox factors as well as Runx2 to modify the coordinated transcriptional activation of all RUNX2-responsive osteoblast-specific gene promoters (58). Mechanistically, we also found that the inhibition of osteoblast differentiation by miR-23a cluster-mediated repression of these factors is associated with lower recruitment of H3K27 acetylation on the promoters of bone-specific genes.

Finally, our results provide novel molecular evidence that the miR-23a cluster and targets HOXA5 and HOXA11 function in an miRNA-epigenetic regulatory network to control osteogenesis. We propose that HOXA5, HOXA10, and HOXA11 activate osteoblast-specific promoter activity, which subsequently supports bone formation by direct regulation of osteoblast phenotypic genes.

**miR-23a cluster and HoxA class protein regulatory network**

HOXA5 and HOXA11 are members of the HoxA class homeobox family of transcription factors vital in bone tissue to regulate postnatal skeletal development (Table S2). Our results indicate an axis in which bone formation is controlled by the miR-23a cluster through the direct regulation of HOXA5, HOXA10, and HOXA11 expression within physiologic limits for maturation of preosteoblasts to mature osteoblasts. HoxA5 and HoxA11 were found to be directly targeted by 3′-UTR binding of the miR-23a cluster. The relatively low expression of the miR-23a cluster compared with the high expression of HoxA5 and HoxA11 mRNA during induction of differentiation or embryonic bone development (embryonic day 13) indicates that HOXA5, HOXA11, and the miR-23a cluster create an miRNA–transcription factor cross-regulatory network during bone development and differentiation. These data illustrate the fundamental epigenetic basis of the miR-23a cluster/HoxA gene axis. Loss of function of HOXA5 and HOXA11, mediated by the miR-23a cluster, blocked the differentiation program of osteoblasts. Our findings demonstrated that selective binding of HOXA5 and HOXA11 with RUNX2 on bone-specific promoters is critical at specific stages of osteoblast maturation. Together, these findings emphasize the importance of preserving the proper co-linearity of HoxA class protein levels at each stage of maturation.

**miR-23a cluster, HOXA5, HOXA11, and chromatin regulation**

This study revealed that, in addition to regulating the protein–protein interactions involving HOXA5 and HOXA11, by control of the post-transcriptional output of HoxA5 and HoxA11, the miR23a cluster may alter the chromatin status of Runx2 and Ocn gene transcription. Specifically, lower recruitment of HOXA5 and HOXA11 appears to decrease the formation of a HOX–RUNX2 activation complex that mechanistically primes the promoter for H3K27 acetylation. This suggests that the cellular physiology modulates miR-23a cluster levels to control Hox gene expression during differentiation and thereby regulates nucleosome organization and remodeling of osteoblast-specific markers for histone modification. Our findings propose mechanisms by which the miR-23a cluster regulates osteogenesis at multiple layers (Fig. 9). During osteoblast differentiation, the miR-23a cluster post-transcriptionally controls the stability of HoxA class transcription factors and inhib-
its recruitment to osteoblast marker gene (Runx2 and Ocn) promoters. Furthermore, the miR-23a cluster, through reduced recruitment of HoxA factors, reduces tissue-specific acetylation of histone H3 tails on Runx2 and Ocn chromatin. Taken together, our results provide the first molecular evidence for miR-23a cluster regulation of a major Hox cluster to control the osteoblast differentiation program. The multifunctional role of the miR-23a cluster in controlling osteoblast differentiation, including direct targeting of Hox mRNA and the modulation of epigenetic mechanisms through histone acetylation, defines it as a key regulator of osteoblast differentiation and bone tissue development. Overall, the linkage of the miR-23a cluster to Hox transcription factors and the RUNX2 transcription factor supports the emerging concept that miRNAs link transcriptional and epigenetic events that are indispensable for maintaining physiological tissue development. Hence, our discovery of the multiple mechanisms by which the miR-23a cluster regulates bone-specific gene expression suggests that this cluster regulates various aspects of bone development, formation, and homeostasis and may inform the design of therapeutics for osteoporosis and arthritis. Overall, our data reveal an original concept that may be useful in combating bone loss by promoting bone anabolic activity through blockade of miR-23 cluster expression.

**Experimental procedures**

**Cell culture**

HEK-293T and MC3T3-E1 cells were obtained from ATCC (Manassas, VA), cultured, and maintained in Dulbecco’s modified Eagle’s medium and minimum essential medium α (Invitrogen), respectively, supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. MOBs...
miR-23a cluster regulates HoxA cluster function

were isolated from the calvarium of neonatal mice (2–5 days old) as described (67). Cells were cultured in minimum essential medium α containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. To induce osteogenic differentiation, medium was further supplemented with 50 μg/ml ascorbic acid and 3–5 mM β-glycerophosphate (Sigma-Aldrich). Medium was replaced every 2 days for the duration of all experiments. All cells were maintained at 37 °C in a humidified 5% CO2 environment.

miRNA lentivirus generation and infection

CMV promoter–driven Lenti-miR and H1 promoter–driven anti-miR precursor clones (System Biosciences, Mountain View, CA) were used to produce mature miR/anti-miR-23a and -27a individually or miR-23a~27a~24-2/anti-miR cluster virus particles. Lentiviral clones containing respective precursor clone sequences were co-transfected along with pMD2.G and pCMVΔR-8.91 (Addgene plasmids 12259 and 12263) viral plasmids into HEK-293T cells. Cells were cultured for ~48 h after transfection, and the supernatant was collected, analyzed for titer, and subsequently used for infections. MOBs or MC3T3-E1 cells were infected at 60–70% confluence for 48 h. Co-expressed GFP reporter was monitored and used to sort miR-expressing or anti-miR–expressing cells. Cells were then seeded at 0.5–1.0 × 105 cells/10-cm plate to assay osteogenic functions of each miR following cell differentiation. Synthetic miRs, anti-miRs, and nonspecific miRs (Life Technologies, Inc.) at concentrations of 50 or 100 nM were transfected after transfection, and the supernatant was collected, analyzed for titer, and subsequently used for infections. MOBs or MC3T3-E1 cells were infected at 60–70% confluence for 48 h. Co-expressed GFP reporter was monitored and used to sort miR-expressing or anti-miR–expressing cells. Cells were then seeded at 0.5–1.0 × 105 cells/10-cm plate to assay osteogenic functions of each miR following cell differentiation. Synthetic miRs, anti-miRs, and nonspecific miRs (Life Technologies, Inc.) at concentrations of 50 or 100 nM were transfected into MC3T3-E1 cells at ~50% confluence using FuGENE 6 transfection reagent (Promega Corp.), following the manufacturer’s instructions, and harvested after 48 h for protein and RNA analysis.

3’-UTR cloning

The 3’-UTR (90–100 bp) for HoxA5, HoxA10, and HoxA11 genes were synthesized with flanking 5’ SpeI and 3’ MluI restriction sites. Double-stranded annealed WT (WT UTR) and mutant (MT UTR) DNA fragments were phosphorylated and ligated to the pMIR-REPORT™ miRNA reporter vector (Thermo Fisher Scientific). Transformants were grown, and plasmid DNAs were confirmed by sequencing.

Expression constructs and short hairpin RNA clones

Murine cDNA clones for HoxA5 (clone ID 3985274) and HoxA11 (clone ID 6504963) containing the CMV promoter were obtained from Open Biosystems (Thermo Fisher Scientific), as were HoxA5- and HoxA11-specific RNAi Consortium mouse short hairpin (shRNA) clones (TRCN0000012519, TRCN0000012521, and TRCN0000070754). All plasmids were either entirely or partially sequenced to ensure fidelity at the University of Alabama sequencing core facility (Birmingham, AL). miRNA and anti-miRNA precursor clones for miR-23a, miR-27a, miR-23a~27a~24-2 cluster, and anti-miR 23a~27a~24-2 cluster (MMIR-23a-PA-1, MMIR-27a-PA-1, MMIR-23a+27a-PA-CL, and MMIR-ZIP-23a+27a-PA-1) were obtained from System Biosciences.

Antibodies

The following antibodies were used for Western blotting: anti-HOXA5 (H-125 (sc-28599) and C-11 (sc-365784)), anti-HOXA10 (N-20, sc-17158), anti-HOXA11 (N-15 (sc-48542) and B-11 (sc-393440)), and anti-actin (I-19, sc-16161) from Santa Cruz Biotechnology, Inc. and anti-RUNX2 (monoclonal, clone 8G5) from MBL International (Woburn, MA). The following antibodies were used for immunoprecipitations and/or ChIP assays: anti-RUNX2 (M-70, sc-10758), anti-HOXA10 (N-20, sc-17158), anti-HOXA5 (H-125, sc-28599), and anti-HOXA11 (B-11, sc-393440) from Santa Cruz Biotechnology; anti-HOXA5 (ab103310), anti-HOXA13 (ab26084), anti-histone H3 (acetyl-Lys-27) (anti-H3K27ac, ab4729), anti-histone H3 (acetyl-Lys-18) (anti-H3K18ac, ab1191), anti-histone H3 (trimethyl-Lys-4) (anti-H3K4me3, ab8580), and anti-luciferase (ab16466) from Abcam (Cambridge, MA); and normal rabbit immunoglobulin G (IgG, 12-370) and anti-argonate 2 (anti-AGO2, 03-248) from MilliporeSigma (Burlington, MA).

RNA isolation, cDNA synthesis, and RT-qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s specifications. DNase I–treated total cellular RNA was primed with oligo(dT) or random hexamer and reverse-transcribed into cDNA using the SuperScript III first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. For miRNA detection, poly(A) tailing was performed using the Poly-A Polymerase Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions, and reverse transcription was carried out with Invitrogen’s SuperScript III RT kit following the manufacturer’s instructions. Gene expression was determined by RT-qPCR using Power SYBR green PCR master mix (Applied Biosystems Inc., Foster City, CA) and gene-specific primers (Table S5) in an ABI Prism 7000 thermocycler. For each gene, expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) or U6 RNA expression. Experiments were performed in triplicate, and results displayed as mean values ± S.E.

Reporter assay

MC3T3-E1 cells were co-transfected with ~285 osteocalcin or ~600 Runx2 promoter luciferase constructs (1 μg) along with 5 ng of pRL Renilla luciferase vector (vectors that contain WT Renilla luciferase for normalization in reporter assays; Promega, Madison, WI) and 500 ng of empty vector or 500 μg of HOXA5 and HOXA11 overexpression constructs for 24 h. Luciferase assays were performed with the Dual-Luciferase Assay Kit (Promega), and luminescence was measured using GloMax®-Multi Detection System (Promega). Three independent experiments were performed and assayed in triplicate per group. Data represent the mean value ± S.E. for three experiments and triplicate samples. Luciferase activity (firefly/Renilla) was expressed in relative luminescence units and plotted.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay lysis buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet
P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitor mixture (Roche Applied Science), and 25 μM MG132 (proteasome inhibitor; Sigma-Aldrich). Lysates were sonicated on ice at 2% power for 10 s three times. Samples (cleared lysates) were quantified, and equal amounts of protein were resolved by 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and were subjected to immunoblotting with the appropriate antibodies. Immunoreactivity proteins were detected using Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences) or the LI-COR Bio Imaging System (LI-COR Biosciences, Lincoln, NE).

**ChIP assays**

ChIP assays were performed as described previously (22). Briefly, formaldehyde cross-linking was performed for 15 min, and cells were collected in 1× PBS and then lysed in lysis buffer (25 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM EDTA, 1% SDS, 1% Triton X-100, 162.5 mM NaCl, 25 μM MG-132, and 1× complete protease inhibitor). Lysates were sonicated to obtain DNA fragments with an average size of 0.2–0.6 kb. Immunoprecipitations were performed with the appropriate antibodies. Immuno-complexes were washed, followed by recovery of DNA.

Re-ChIP assays were performed using the primary pulldown from one antibody, which was divided into equal aliquots for the second pulldown with antibodies specific for co-regulatory molecules. Instead of the elution step (1% SDS and 100 mM Na₂HCO₃ after washing, immunocomplexes were eluted in 10 mM DTT). The eluate was further diluted 1:40 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM Tris-HCl (pH 8.1), and 167 mM NaCl) and used for the secondary immunoprecipitations.

Aliquots of each recovered DNA sample were assayed by RT-qPCR to detect the proximal and distal mouse osteocalcin (Ocn) promoter region spanning bp −121 to −7 upstream and −620 to −459 upstream of the transcription start site. The primers for ChIP DNA amplification were as follows: proximal promoter, mOcn F (−121CGCAATCACTACACAGC−105) and mOcn R (−26CCGCTGCTGTTGCCTCTCG−39); distal promoter region, mOcn F (−620GTAGCTCCACAATGGCTA−596) and mOcn R (−479ATCCAGTGGGGGTGTTGTG−460). The proximal and distal sites are encompassed by Runx2 (TGTGGT or ACCACA) and HOXA5/HOXA10/HOXA11 (consensus YYNNATATGY) binding sites: proximal promoter region mRunx2 F (−282TTCTCTGATGGACTGTGTT−303) and mRunx2 R (−356GGCTCTGCCGTTAACATGTT−379). Samples were normalized to the initial input and expressed as percentage chromatin pulldown. Three independent ChIP experiments were performed and assayed in triplicate per group. Experiments were performed in triplicate, and results are displayed as mean values ± S.E.

**Co-immunoprecipitation**

Initially, protein A/G Plus–agarose beads (Santa Cruz Biotechnology) (100 μl; 1 bead/PBS) were noncovalently complexed with 10 μg of specific antibody (anti-Runx2, Santa Cruz Biotechnology catalog no. sc-10758; anti-HoxA5, Santa Cruz Biotechnology catalog no. sc-10758; anti-HoxA11, Abnova catalog no. H00003207-M08). Unbound antibodies were washed away with PBS, and antibody-bound protein A/G–agarose was resuspended in 100 μl of PBS. To detect endogenous interactions among RUNX2, HOXA5, and HOXA11 interaction, co-immunoprecipitation was performed on the lystate prepared from preosteoblast MC3T3 cells. Cells were grown in minimal essential medium α and differentiated for 7 days with osteogenic medium containing 5 mM β-glycerol phosphate and 0.1 mg/ml ascorbic acid. Approximately 10⁶ cells/immunoprecipitation from day 7 were lysed in 500 μl of FA lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitor mixture (Roche Applied Science), and 25 μM MG132 (proteasome inhibitor, MilliporeSigma) for 15 min at 4 °C. Cell lysates were sonicated, followed by centrifugation at 16,000 × g for 15 min at 4 °C. The supernatant was transferred to a clean microcentrifuge tube and precleared with 40 μl of protein A/G Plus–agarose beads at 4 °C for 30 min. To immunoprecipitate, 50 μl of antibody–protein A/G Plus–agarose complex was added and incubated at 4 °C with agitation overnight. Beads were washed three times with 50 mM HEPES, pH 7.5, 140 mM NaCl, 0.25 mM EDTA, pH 8.0, 20% glycerol, and 0.1% Tween 20 containing 1× complete protease inhibitors. The beads were finally eluted in 2× Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromphenol blue, and 0.125 mM Tris-HCl, pH 6.8). Samples were heat-denatured at 100 °C for 5 min and then analyzed by Western blotting.

**RISC-IP**

RISC-IP was conducted to isolate miR–RISC complexes, associated miRNAs, and target mRNAs. Polysomal extracts from MC3T3-E1 cells overexpressing the miR-23a−27a−24-2 cluster were immunoprecipitated with an affinity-purified silencing complex–specific anti-AGO2 antibody as described previously (68). The RNA isolated from the RISC-IP was subjected to cDNA synthesis using primers derived from specific seed sequences for miR-27a or miR-23a. The first-strand cDNA was further amplified with forward and reverse primers derived from the target mRNA (Table S3).

**ALP staining**

To detect ALP activity, osteoblast cells were fixed in cold, freshly made 10% neutral buffered formalin for 15 min. The cells were then incubated for 2 h at room temperature in 0.4 mg/ml naphthol AS-MX phosphate (catalog no. N5000, MilliporeSigma) and 0.08 mg/ml Fast Red TR (catalog no. F8764, MilliporeSigma) to develop the color. After 2 h, cells were washed with distilled water, air-dried, and photographed.

**Tetracycline-inducible Flp-in expression vector, Flp-in, and screening**

Germ line deletion of the miR-23a cluster results in embryonic lethality; therefore, we have generated a single-copy transgenic mouse model (MIR-ZIP mouse) in which miR-23a cluster
miR-23a cluster regulates HoxA cluster function

function can be inhibited by the anti-miR-23a cluster in vivo by feeding the mice a Dox diet (Envigo, Huntingdon, UK). The pBS31 flp-in vector used consists of the following five cassettes: the pgk promoter followed by an ATG translation initiation codon and a FRT site, the CMV minimal promoter containing tetacycline-responsive operator-binding sequences derived from pTETOP (69), a splice acceptor double poly(A) cassette derived from the ROSA26-targeting vector (70), the rabbit \(\beta\)-globin polyadenylation signal sequence, and a unique EcoRI site for cloning genes. Approximately 1.5 \(\times 10^7\) KH2 mouse ES cells were electroporated with 50 \(\mu\)g of flp-in vector and 25 \(\mu\)g of pCAGGS-FLPe-puro (71) at 500 V and 25 microfarads using two pulses in a Gene Pulser II (Bio-Rad). Hygromycin (Roche Applied Science) selection (140 \(\mu\)g/ml) was started after 24 h. DNAs from picked clones were digested with SpeI and analyzed by Southern blotting for correct targeting using the ColAl 3’ internal probe.

Northern blotting (Fig. S1)

Total RNA isolated from MC3T3-E1 osteoblasts at the indicated days using TRIzol was separated on a 6% acrylamide/urea gel and was transferred onto Hybond-XL membranes (GE Healthcare). The 5’-end-labeled miR-23a (5’-ATCACATTC-CCAGGGATTCC-3’), miR-27a (5’-AACACAGGCGCA-AGAACC GCC-3’), and miR-24-2 (5’-AGGCACAGAACA-GCAGGAACAG-3’) oligonucleotide probes were used to hybridize the membranes in Rapid-Hyb Buffer (GE Healthcare) following the manufacturer’s instructions. The blots were reprobed with labeled U6 oligonucleotide (ATATGGAAGCGT-TAGCGAATT) as a control for equal loading.

Genotyping

Animal experiments were accomplished with the approval of the institutional animal care and use committee of the University of Alabama at Birmingham and conformed to relevant federal and state guidelines and regulations. For genotyping of littermates, genomic DNA was extracted from the tail snips using DirectPCR® DNA extraction buffer (Viagen Biotech, CA). Genotyping of MIR-ZIP mice was performed according to the protocol developed by the laboratory of Rudolf Jaenisch (Whitehead Institute, Boston, MA). Sequences of three primers used to specifically amplify WT and flp-in alleles are as follows: Col/flt-B, 5’-CCCTCCCATGTGTGACCAAGG-3’/Col/flt-A1, 5’-GACACATTCGCGACATGC-3’; and Col/flt-C1, 5’-GCAGAAGCGCGCCGTCTGG-3’. The results were interpreted according to the size of the PCR product; the WT allele is 331 bases, and the flp-in allele is 551 bases.

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