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Epigenetic remodeling and modification to preserve skeletogenesis *in vivo*

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Abstract

Current studies offer little insight on how epigenetic remodeling of bone-specific chromatin maintains bone mass *in vivo*. Understanding this gap and precise mechanism is pivotal for future therapeutic innovation to prevent bone loss. Recently, we found that low bone mass is associated with decreased H3K27 acetylation (activating histone modification) of bone specific gene promoters. Here, we aim to elucidate the epigenetic mechanisms by which a miRNA cluster controls bone synthesis and homeostasis by regulating chromatin accessibility and H3K27 acetylation. In order to decipher the epigenetic axis that regulates osteogenesis, we studied a drug inducible anti-miR-23a cluster (miR-23a Cl^{ZIP}) knockdown mouse model. MiR-23a cluster knockdown (heterozygous) mice developed high bone mass. These mice displayed increased expression of Runx2 and Baf45a, essential factors for skeletogenesis; and decreased expression of Ezh2, a chromatin repressor indispensable for skeletogenesis. ChIP assays using miR-23a Cl knockdown calvarial cells revealed a BAF45A-EZH2 epigenetic antagonistic mechanism that maintains bone formation. Together, our findings support that the miR-23a Cl connection with tissue-specific RUNX2-BAF45A-EZH2 function is a novel molecular epigenetic axis through which a miRNA cluster orchestrates chromatin modification to elicit major effects on osteogenesis *in vivo*.

Keywords

BAF; osteoblast differentiation; miR-23a cluster; chromatin remodeling; chromatin modification; PRC2

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

The differentiation of osteoblast-progenitors to mature osteoblasts is substantially controlled via various epigenetic mechanisms (1,2). One of these processes involves the remodeling of nucleosome organization through N-terminal modification (acetylation—ac or methylation—me) of histone 3 lysine 27 (H3K27) at the transcriptional start site of osteoblast specific genes. This process regulates promoter accessibility and transcription of key osteoblast genes required for the differentiation process (1). Recently, we have found that osteoblast promoters undergo dynamic changes from a repressive state (H3K27me3) to an active state (H3K27ac) during differentiation. Two remodeling complexes orchestrate this process: the Polycomb Repressive Complex (PRC2) via EZH2, and the Brg1 associated complex (BAF) via BAF45A (3).

The PRC2 complex is primarily responsible for the deposition of H3K27me3, while BAF is linked to the H3K27ac modification through chromatin remodeling (3). Our study reveals that the miR-23a CI regulates the balance between these opposing modifying complexes, which is conducive to bone formation (Figure 1).

Materials and methods

We developed a doxycycline (Dox) inducible single copy anti-miR-23a CI knock-in mouse model (Het miR-23a CI^{ZIP}). This cassette was targeted downstream of the Collagen 1a1 locus and is driven by the CMV minimal promoter containing tetracycline responsive operator binding sequences (4). Additionally, these mice are heterozygous for a Dox inducible Reverse Tet Trans-Activator (rtTA) targeted to the Rosa26 Locus. Treatment of doxycycline induces the whole body knockdown of the miR-23a CI (miRs -23a, -27a and -24-2) *in vivo*, serving as a model to study the epigenetic balance which drives bone formation. Two-month-old femurs were analyzed by microCT to determine the bone volume to tissue volume ratio (BV/TV). Calvarial cells were isolated from Het miR-23a CI^{ZIP} mice and alkaline phosphatase staining, Mrna isolation for RT-qPCR, and ChIP-PCR were performed at day twelve of differentiation using anti-BAF45A (Thermofisher: PA5-30678), anti-EZH2 (Active Motif: 39933), anti-H3K27ac (Abcam: AB4729), anti-H3K27me3 (Abcam: AB6002), and normal mouse IgG (Millipore: 12-371). Animal work completed in this study was approved via the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC).

Results

miR-23a CI knockdown increases osteogenesis *in vivo*

In vitro 2-fold knockdown of the miR-23a CI by doxycycline induction in calvarial cells (Figure 2A) resulted in a robust increase in osteoblast differentiation as characterized by increased alkaline phosphatase staining at twelve days of differentiation (Figure 2B). Additionally, *in vivo* knockdown of the miR-23a CI showed an increase in bone formation as seen by increased trabecular bone volume to tissue volume of 2-month-old Het miR-23a CI^{ZIP} femurs +dox/-dox (Figure 2C & D). Gene expression analysis from 2-month-old femur RNA revealed an approximate 16-fold increase in Baf45a expression, increased

Runx2 expression, decreased Ezh2 expression, and a greater than 8 fold increase in Ocn expression in osteoblasts isolated from miR-23a CI^{ZIP}+Dox as compared to untreated (Figure 2E). These findings show that miR-23a CI knockdown results in increased osteogenesis and bone formation associated with increased Baf45a and decreased Ezh2 expression.

miR-23a CI knockdown promotes BAF45A recruitment and active chromatin formation

We next examined chromatin modification on the osteocalcin (Ocn) proximal promoter (Figure 3A). ChIP experiments in miR-23a CI knockdown calvarial cells revealed an approximate 2-fold increase in BAF45A occupancy with an approximate 2-fold decrease in EZH2 occupancy (Figure 3B). Furthermore, analysis of H3K27 modification status showed that the occupancy of H3K27ac increased by approximately 8-fold with an approximate 4-fold decrease in H2K27me3 in miR-23a CI^{ZIP} calvarial cells differentiated treated with doxycycline and to day twelve (Figure 3C). Hence, our findings revealed that knockdown of the miR-23a CI results in activation of the Ocn promoter through a switch in histone modification from the repressive H3K27me3 to activating H3K27ac.

Discussion

Modulating the epigenetic status of specific Genes between repressive H3K27me3 and activating H3K27ac is essential in facilitating osteoblast differentiation (3). The PRC2 complex and the BAF complex play opposing roles in the process, with PRC2 responsible for H3K27me3 deposition and BAF responsible for increasing chromatin accessibility followed by H3K27ac deposition. In this study we show that the miR-23a CI plays a critical role in balancing PRC2 and BAF, ultimately resulting in physiological gene expression promoting osteoblast differentiation.

We have demonstrated that the miR-23a CI knockdown results in increased expression of Baf45a and Runx2 and decreased expression of Ezh2. This results in an increase in BAF45A occupancy at the osteoblast specific promoter osteocalcin, while EZH2 occupancy decreases. By modulating these key subunits of BAF and PRC2 respectively, the epigenetic homeostasis shifts to a state favorable for osteoblast differentiation with increased H3K27ac and decreased H3K27me3. Importantly, knockdown of the miR-23a CI results in a robust increase in osteoblast differentiation and bone formation *in vivo*. Hence, miR-23a CI is a key non-coding miRNA (5) which controls skeletogenesis at the epigenetic level.

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Figure 1.

Osteoblast differentiation program and epigenetic homeostasis. Stages of differentiation from pre to mature osteoblasts. Peak expression of Runx2 and osteocalcin (Ocn) genes which serve as markers for the induction and maintenance of osteoblasts are shown. Chromatin remodelers and associated histone modifications controlling osteoblast differentiation are presented. The miR-23a Cl controls Runx2, Baf45a and Ezh2 expression to modulate PRC2 and BAF complexes and balance the histone modification status of osteoblast gene promoters. This results in the physiological levels of osteoblast gene expression for bone formation.

Figure 2.

miR-23a CI knockdown promotes osteogenesis in vivo A) Expression of miR-23a CI in differentiating Het miR-23a CI^{ZIP} calvarial cells (+Dox indicates miR-23a CI Knockdown). B) Alkaline phosphatase staining of Het miR-23a CI^{ZIP} calvarial osteoblasts at day 12. C) μ CT analysis of 2-month-old Het miR-23a CI^{ZIP} femur. D) Bone volume to tissue volume ratio (BV/TV) computed from μ CT as described above. E) Baf45a, Runx2, Ezh2, and Ocn Mrna expression in 2-month-old Het miR-23a CI^{ZIP} mouse femur bone.

Figure 3.

Knockdown of miR-23a CI favors active osteoblastic chromatin A) Proximal mouse Ocn promoter, nucleosome positioned at -0.2 to -0.4kb flanked by two RUNX2 binding sites. ChIP primers depicted. B) ChIP analysis on Ocn proximal promoter using BAF45A and EZH2 antibodies in Het miR-23a CI^{ZIP} calvarial cells (+Dox indicates miR-23a CI Knockdown). C) ChIP of Ocn proximal promoter using anti-H3K27ac and anti-H3K27me3 antibodies in Het miR-23a CI^{ZIP} calvarial cells. Normal IgG was used as a control. Binding and modifications were assayed with the amplification of the promoter fragment using two flanking primers spanning the transcriptional start site for Ocn gene.