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Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse

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ABSTRACT

MicroRNA attenuation of protein translation has emerged as an important regulator of mesenchymal cell differentiation into the osteoblast lineage. A compelling question is the extent to which miR biogenesis is obligatory for bone formation. Here we show conditional deletion of the *Dicer* enzyme in osteoprogenitors by *Col1a1*-Cre compromised fetal survival after E14.5. A mechanism was associated with the post-commitment stage of osteoblastogenesis, demonstrated by impaired ECM mineralization and reduced expression of mature osteoblast markers during differentiation of mesenchymal cells of ex vivo deleted Dicer$^{c/c}$. In contrast, in vivo excision of *Dicer* by *Osteocalcin*-Cre in mature osteoblasts generated a viable mouse with a perinatal phenotype of delayed bone mineralization which was resolved by 1 month. However, a second phenotype of significantly increased bone mass developed by 2 months, which continued up to 8 months in long bones and vertebrae, but not calvariae. Cortical bone width and trabecular thickness in Dicer$^{\Delta oc/\Delta oc}$ was twice that of Dicer$^{c/c}$ controls. Normal cell and tissue organization was observed. Expression of osteoblast and osteoclast markers demonstrated increased coupled activity of both cell types. We propose that Dicer generated miRs are essential for two periods of bone formation, to promote osteoblast differentiation before birth, and control bone accrual in the adult.

Introduction

For normal development of a mineralized skeleton and renewal of bone throughout life, membranous and endochondral bone formation is tightly regulated by osteogenic signaling pathways (Wnt, TGFβ/BMP, Notch) and transcription factors directing cell specification (Lian et al., 2006; Soltanoff et al., 2009). Following commitment of mesenchymal stem cells to osteoprogenitors, further differentiation requires the temporal activation of genes coding proteins responsible for forming the bone ECM, promoting mineralization and supporting the metabolic activities of osteoblasts. Gene regulatory mechanisms essential for bone formation have been elaborated in relation to epigenetic and chromatin alteration of the gene, post-transcriptional control of mRNA from splice variants and post-translational biochemical modifications of protein activity. MicroRNA (miR), are small non-coding RNA molecules that regulate gene expression through post-transcriptional degradation or translational inhibition by binding to their target mRNAs. The regulatory function of miRs in controlling levels and/or translation of target mRNAs during in vivo bone formation has not been examined.

Only a small number of miRs that function during skeletal development and in relation to disease have been identified (Kobayashi et al., 2008; Li et al., 2009; Luzi et al., 2008; Mizuno et al., 2008a; Nakasa et al., 2008). MicroRNAs have been characterized in bone and cartilage and in association with rheumatoid arthritis synovial tissue (Kobayashi et al., 2008; Li et al., 2008b; Luzi et al., 2008; Mizuno et al., 2008b; Nakasa et al., 2008; Tuddenham et al., 2006). Significantly, two studies identified miRs which were both modulated at the onset of induced osteoblastogenesis by BMP2 in human mesenchymal stromal cells and C2C12 myogenic cells (Li et al., 2008b; Oskowitz et al., 2008). Profiling of BMP2 during bone formation in vivo (Kobayashi et al., 2008) and during stages of osteoblast differentiation in vitro (Li et al., 2009) has also implicated mature miRs in the regulation of transcripts that contribute to skeletal activities. In addition to these studies in bone forming osteoblasts, miRs regulating the bone resorbing osteoclasts are now known (Sugatani and Hruska, 2009). These findings raise a compelling question as to the requirement for miRs contributing to activation of bone formation and regulating suppression of the osteoblast phenotype in non-osseous cells. Here we addressed whether

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miRs are required for activation of bone formation and maintenance of osteoblast function in vivo.

One approach for understanding tissue specific requirements for miRs is the conditional deletion of the enzymes Drosha and Dicer (Bernstein et al., 2003). Drosha processes the primary miRNAs encoded in genes to 60–70 nucleotide long precursor miRNA (pre-miRNA) in the cytoplasm which are recognized and cleaved by an RNase III endonuclease protein, Dicer, to generate 21–23 nucleotide mature miRNAs that assemble into a ribonucleoprotein silencing complex (RISC) to bind to 3′ untranslated region (UTR) of the target mRNAs (Jaskiewicz and Filipowicz, 2008). Dicer plays a critical role in a vast range of physiologic processes, including embryonic development, cell growth and phenotype differentiation through regulation of microRNA maturation (Blakaj and Lin, 2008). Dicer functions are essential for embryo development and survival as a Dicer-null mouse (Bernstein et al., 2003) and a hypomorphic Dicer mutant (Yang et al., 2005) results in lethality in the first week of embryogenesis. Thus, conditional ablation of Dicer activity has been studied in various tissues, demonstrating a pivotal role of Dicer-dependent processing of microRNAs for cell fate specification, differentiation and tissue morphogenesis in all the tissues examined, among which include skin follicles, heart, brain, immune cells, lung, spermatogenesis and female germline cells (Andl et al., 2006; Chen et al., 2008; Cobb et al., 2005; da Costa Martins et al., 2008; Damiani et al., 2008; Davis et al., 2008; Harris et al., 2006; Harvey et al., 2008; Hayashi et al., 2008; Kanellopoulou et al., 2005; Koralov et al., 2008; Muljo et al., 2005; Murchison et al., 2007; Nagaraja et al., 2008; O'Rourke et al., 2007; Schaefer et al., 2007; Yi et al., 2006).

Limited information is available for Dicer and microRNA requirements during skeletal development. Limb mesoderm-specific deletion of *Dicer* using *prx1*-Cre in mice resulted in massive cell death and reduction in size, but did not affect skeletal patterning (Harfe et al., 2005). However, the requirement for Dicer processed mature miRs in bone tissue has remained unexplored. In this study, excision of *Dicer* in mice was performed at two stages of bone formation using *Col1a1*-Cre and *Osteocalcin* (*OC*)-Cre which resulted in distinct phenotypes identifying different requirements for miR biogenesis. Ablation of Dicer in osteoprogenitors (using *Col1a1*-Cre for conditional deletion) prevents their differentiation and compromises fetal survival at E15.5. *OC*-Cre excision of *Dicer* delays perinatal bone formation which is resolved during post-natal growth. However, in $Dicer^{\Delta oc/\Delta oc}$ adult mice a striking increase in both trabecular and cortical bone mass was found. This deregulated increase in bone tissue formation was accompanied by a coupled increase in bone resorption to support vascularization of the thickened bone. A contributing mechanism to this anabolic phenotype is the increased synthesis of bone matrix proteins including collagen type I, a target of several microRNAs in osteoblasts. Our studies establish that miRs are required to initiate osteoblast maturation during development and to regulate bone mass in adult mice.

Materials and methods

Conditional excision of Dicer

For conditional deletion of *Dicer*, $Dicer^{c/c}$ mice (Mudhasani et al., 2008) were crossed with *Col1a1*-Cre (2.3 kb promoter of *collagen type I*) (Liu et al., 2004) and *OC*-Cre (*Osteocalcin*) (Chiang et al., 2009; Yuan et al., 2008) mouse line obtained from Dr. Thomas Clemens (University of Alabama at Birmingham, AL). $Dicer^{c/c}$ mice were crossed with $Ink4a/Arf^{-/-}$ mice for deletion of the *Ink4a/Arf* locus to obtain senescence-resistance (Serrano et al., 1996). The mice were maintained at the University of Massachusetts by IACUC approved procedures. Genotyping was carried out as previously described (Chiang et al., 2009; Liu et al., 2004; Mudhasani et al., 2008).

MicroCT (μCT) analysis

MicroCT analysis was performed by the University of Massachusetts Medical School Musculoskeletal Center for Imaging Core facility. Bones were fixed in periodate-lysine-paraformaldehyde (PLP) fixative (Miao and Scutt, 2002) from $Dicer^{c/c}$ and $Dicer^{\Delta oc/\Delta oc}$ mice. After dehydration to 70% alcohol, femurs were scanned at 10 μm voxel resolution (μCT 40; Scanco Medical AG, Bruttisellen, Wangen-Brüttisellen, Switzerland). Image reconstruction was performed by Scanco software version 5.0. For trabecular bone 100 contiguous slices below the growth plate were selected for contouring inside the endosteal edge for analyses of various bone parameters. Cortical parameters were analyzed from 50 cross-sectional slices at the mid-diaphysis region. Parameters were obtained using threshold range 220–1000.

Skeletal staining and histology

Whole skeletal staining was performed by standard procedures using Alcian blue and Alizarin red stains for cartilage and bone tissue (Lufkin et al., 1992). Separate embryos, day 2 pups of femurs from 2 mo old mice were fixed in PLP, paraffin-embedded, and sequential staining of sections was performed by von Kossa stain for mineral and Toluidine blue for cellular detail. Cytochemical detection of bone-specific alkaline phosphatase (Alk Phos) and osteoclast-specific tartrate resistant acid phosphatase (TRAP) was performed (Lengner et al., 2004). Images were captured using a Zeiss Axioskop 40 (Mikron, San Marcos, CA, USA) microscope with a CCD camera.

Ex vivo osteoblast differentiation

Bone marrow stromal cells (BMSC) were isolated from $Dicer^{c/c}$ mice in αMEM supplemented with 20% fetal bovine serum (Hyclone, Logan, UT, USA). Cells were transduced at 60% confluency with Ad-Cre or Ad-GFP (control virus) for 3 h for excision of *Dicer*, then cultured in αMEM with 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate (SIGMA-Aldrich, St. Louis, MO, USA) to induce the osteoblast differentiation. Calvarial osteoblasts were isolated from newborn $Dicer^{\Delta oc/\Delta oc}$ by collagenase P digestion, genotyped and cultured for osteoblast differentiation (Pratap et al., 2003). Cells were harvested at indicated time points for Alk Phos activity, mineral staining, and gene expression analyses. Staining for senescence-associated β-Galactosidase (SA-βGal) was performed by a standard procedure (Dimri et al., 1995). Excision of *Dicer* was detected by PCR with forward (5′-CCGACCAGCCTTGTTACCTG-3′) and reverse primers (5′-CGGTGTTTCCTTTGAATACTT-3′) using GAPDH as internal control (Applied Biosystems). Experiments were repeated at least twice with similar results.

RNA isolation and quantitative real time PCR

Cells were harvested in 300 μl TRIzol reagent (Invitrogen, Carlsbad, California, USA). Total RNA isolated as per the manufacturer's instructions (Invitrogen, Carlsbad, California, USA) and treated with RNase-free DNase. The reverse transcription reaction was performed on 1 μg of RNA using the First Strand Synthesis Kit (Invitrogen, Carlsbad, California, USA). Relative transcript levels were measured by quantitative PCR in 25 μl reaction volume using ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA), following the recommended protocol for SYBR-Green, and normalized with GAPDH levels (Applied Biosystems, Foster City, CA, USA). The primers used for amplification are described in Table 1.

For detection of let-7a and miR-29b, mirVANA qRT-PCR miRNA detection kit along with primer sets for each microRNA (Applied Biosystems/Ambion, Foster City, CA, USA) were used, following the manufacturer's procedure. The miR levels were normalized using U6 primers.