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Runx2 activity in committed osteoblasts is not essential for embryonic skeletogenesis

Mitra D. Adhami, Harunur Rashid, Haiyan Chen, and Amjad Javed

Department of Oral and Maxillofacial Surgery, Institute of Oral Health Research, School of Dentistry, University of Alabama, Birmingham, AL, USA

Abstract

Runx2 transcription factor is essential for the development of mineralized tissue, and is required for osteoblast commitment and chondrocyte maturation. Mice with global deletion of Runx2 exhibit complete failure of bone tissue formation, while chondrocyte-specific Runx2-deficient mice lack endochondral ossification. However, the function of Runx2 after commitment of mesenchymal cells to the osteoblast lineage remains unknown. Here, we elucidate the osteoblast-specific requirements of Runx2 during development of the tissue. Runx2 was deleted in committed osteoblasts using Cre-recombinase driven by the 2.3kbCol1a1 promoter. Surprisingly, Runx2^{E8/E8} mice were born alive and were essentially indistinguishable from wild-type littermates. At birth, we failed to detect any alterations in skeletal patterning or extent of bone development in homozygous mutants. However, by 4 weeks of age, mutant mice showed obvious growth deficiencies, and weighed 20–25% less than sex-matched wild-type littermates. Micro-CT analysis of the hindlimb revealed a dramatic decrease of 50% in both cortical and trabecular bone volume compared with wild-type mice. Consistent with this observation, trabecular number and thickness were decreased by 51% and 21%, respectively, and trabecular space was increased by 2-fold in limbs of Runx2^{E8/E8} mice. In addition to poor acquisition of bone mass, the average density of hydroxyapatite was markedly decreased in bone of Runx2^{E8/E8} mice. Together, these findings demonstrate that loss of Runx2 activity in committed osteoblasts impairs osteoblast function, and that Runx2 is critical for postnatal, but not embryonic endochondral ossification.

Keywords

Bone development; Osteoblast; Runx2

Introduction

Expression of Runx2 transcription factor is essential for mesenchymal cell commitment and differentiation to the osteoblast and chondrocyte lineages (1–4). Mice with global deletion of Runx2 exhibit a complete absence of skeletal development, while those with chondrocyte-

Corresponding author: Amjad Javed, Department of Oral and Maxillofacial Surgery, School of Dentistry SDB 714, University of Alabama at Birmingham, 1720 2nd Ave South, Birmingham, AL 35294-0007, Tel: (205) 996 5124, Fax: (205) 934-1560, javeda@uab.edu.

Declaration of interest

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specific deletion lack endochondral ossification (5,6). Homozygous mutants in both models exhibit perinatal lethality. *Ex vivo* studies have shown that Runx2 induces expression of a large number of genes involved in bone synthesis and homeostasis, and continues to promote skeletogenesis after commitment to the osteoblast lineage (3,4,7). Consistent with this notion, overexpression of a dominant-negative form of Runx2 in osteoblasts by the 2.3kbCol1a1 promoter results in low rate of mineralization and decreased number of osteoblasts (8). Importantly, overexpression of the full-length Runx2 protein also results in low bone mass. These contradictory observations bring into question the physiologic role of Runx2 in osteoblasts.

The skeletal phenotype resulting from overexpression of Runx2 varies significantly among independent transgenic lines. For example, mice overexpressing Runx2 under the 2.3kbCol1a1 promoter exhibit osteopenia due to impaired osteoblast function and maturity (9). An independent study employing the same promoter to overexpress Runx2 also reported osteopenia in transgenic mice (10). Surprisingly, this transgenic model exhibited an increase in both bone formation and resorption, indicating that the osteopenic phenotype was a result of high bone turnover. These findings suggest that Runx2 regulates osteoblast and/or osteoclast activity in postnatal skeletogenesis. However, the precise function of Runx2 in osteoblasts remains unclear. The purpose of this study was to understand the physiologic role of Runx2 in committed osteoblasts during skeletal development.

Here, we conditionally deleted Runx2 in committed osteoblasts by using a novel floxed model. We generated Runx2-floxed mice by inserting loxP sites around exon 8 of the Runx2 gene, which encodes the biologically active C-terminus of the Runx2 protein. We have previously shown that Cre-mediated recombination results in a truncated, non-functional Runx2 protein (5). Surprisingly, deletion of Runx2 from committed osteoblasts did not cause perinatal lethality, and mutant mice were indistinguishable from wild-type littermates at birth. However, Runx2-deficient mice exhibited low bone mass within 1 month of postnatal life. These results demonstrate that Runx2 activity is required for postnatal bone synthesis after osteoblast commitment.

Materials and Methods

Generation of osteoblast-specific knockout mice

Osteoblast-specific Runx2 knockout mice were established by employing a previously described Runx2-floxed line (5). Runx2-floxed mice were bred with 2.3kbCol1a1-Cre transgenic mice (11). Heterozygous mice were interbred to obtain homozygous and wild-type littermates. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at UAB and conformed to federal guidelines and regulations.

Osteoblast-specific expression of Cre

To confirm tissue and cell specificity of 2.3kbCol1a1-Cre transgene, long bones of R26R littermates with and without the Cre transgene were harvested at 3 months of age and fixed in ice-cold 4% paraformaldehyde overnight. De-skinned limbs were placed in freshly

prepared X-gal reaction buffer (5mM potassium ferricyanide and ferrocyanide, 2mM MgCl₂, 0.2% NP40, 0.1% sodium deoxycholate, 1 mg/ml X-gal) for 24 h at 37°C in the dark. X-gal-stained tissues were then decalcified in 0.27M EDTA and dehydrated with serial solutions of increasing concentrations of ethanol for histological analysis. Tissues were embedded in paraffin and sectioned at 5µm thickness. Sections were cleared of paraffin by xylene, and analyzed by digital microscopy using Nikon 80i Eclipse microscope.

Histologic Analysis

To understand the cellular phenotype, histological analysis was performed on hindlimbs harvested from wild-type and Runx2^{E8/E8} newborn mice. Limbs were fixed and prepared for histology as above. Sections were stained with H&E according to standard protocol. Briefly, sections were cleared in xylene, rehydrated to 70% ethanol, and placed in Hematoxylin for 8 min. Sections were then rinsed under running tap water for 10 min, and counterstained with Eosin Y for 1 min. Finally, tissues were dehydrated to 100% ethanol, cleared in xylene and mounted with xylene-based medium.

Micro-CT Analysis

To assess three-dimensional bone structure and mineral density, limbs were dissected from littermates at 1 month of age. Undecalcified mouse bones were scanned by a cone beam microcomputed tomography system µCT40 (Scanco Medical AG, Brüttisellen, Switzerland).

Results

Loss of Runx2 in osteoblasts does not arrest embryonic skeletogenesis

To understand the role of Runx2 in committed osteoblasts during skeletogenesis, we crossed our Runx2-floxed model with mice expressing Cre recombinase (Figure 1). Osteoblast-specific activity of the 2.3kbColla1-Cre transgene was first confirmed with the R26R mice. We observed Colla1-Cre activity only in osteoblasts and osteocytes lining the cortical and trabecular bone of all limbs. We did not detect X-gal staining in chondrocytes or extra-skeletal tissues. Absence of X-gal stain in control mice confirmed the osteoblast specificity of Colla1-Cre (Figure 1A). Runx2^{+/-} E8 mice were interbred, and we retrieved homozygous Runx2^{E8/E8} mice with expected mendelian frequency. Surprisingly, homozygous mutants were born alive and were indistinguishable from wild-type littermates. Size and weight were comparable among wild-type, Runx2^{+/-} E8 and Runx2^{E8/E8} littermates (Figure 1B). Moreover, we were unable to identify any disruptions in skeletal development. Histological analysis revealed no difference in length or width of hindlimbs among littermates. We observed a well-defined primary center of ossification, which was appropriately filled with bone marrow and trabecular spicules (Figure 1C). Moreover, the number of chondrocytes within each chondrocytic zone of the epiphysis was similar among all genotypes (Figure 1C). Taken together, these data indicate that after mesenchymal cell commitment to the osteoblast lineage Runx2 function is not essential for embryonic skeletal development.

The Carboxy-terminus of Runx2 is critical for bone synthesis in postnatal limb development

Mice undergo rapid skeletal development and postnatal growth during the first month of life. Therefore, we monitored the weight of four independent pairs of sex-matched littermates for a period of 5 weeks (Figure 2A). Wild-type mice exhibited robust growth in length and weight in the first month of postnatal life. Interestingly, Runx2^{E8/E8} mice showed poor growth and by 1 month of age, weighed 20–25% less than wild-type littermates. To determine whether deficiencies in growth were related to the skeletal development in Runx2^{E8/E8} mice, we examined hindlimbs by micro-CT (Figure 2B). We observed a dramatic decrease in both cortical and trabecular BV in Runx2^{E8/E8} mice. This decrease was consistently noted in multiple litters and both sexes, indicating that impaired bone synthesis is not gender-specific. Moreover, we observed a 51% decrease in trabecular number, and a 21% decrease in trabecular thickness, resulting in a 2-fold increase in trabecular space (Figure 2D). Furthermore, the average density of hydroxyapatite per BV was decreased in both cortical and trabecular bone, indicating disrupted mineralization in mutant mice (Figure 2E). Taken together, these results demonstrate that Runx2 continues to function in committed osteoblasts and positively regulates bone acquisition and mineralization during early postnatal development.

Discussion

Osteoblasts are derived from mesenchymal precursors, which undergo sequential stages of differentiation prior to becoming functional bone-synthesizing cells (4). Previous *in vitro* studies have shown that Runx2 is both necessary and sufficient for osteoblast differentiation, as cells lacking Runx2 are unable to undergo osteoblastogenesis (7). Furthermore, its overexpression drives non-osteoblastic mesenchymal cells toward the osteoblast lineage (7). These studies indicate that Runx2 is essential for osteoblast function. Consistent with this notion, the expression of Runx2 increases throughout osteoblast differentiation (4,7). Runx2-null mice completely lack osteoblasts and mineralized tissue; thus, it is well accepted that Runx2 is critical for commitment to the osteoblast lineage (1–3). However, its role in osteoblast differentiation after the initial commitment step remains largely unknown.

Runx2 is a multi-partite transcription factor, with a DNA-binding domain in its N-terminus and a transactivation/repression domain in its C-terminus. The DNA-binding domain is highly conserved among all Runx family members. In contrast, the C-terminus of Runx2 is distinct, and has been shown to participate in protein–protein interactions that synergistically induce the expression of osteoblast-specific genes (4). To understand the unique functions of Runx2, we generated Runx2-floxed mice by insertion of loxP sites around exon 8 of the Runx2 gene, which encodes over one-third of the Runx2 protein, including the C-terminus (5). Here, we employed mice expressing the 2.3kbColl1a1-Cre transgene to conditionally delete Runx2 C-terminus after osteoblast commitment. In stark contrast to mice with specific deletion of Runx2 in chondroblasts, mice with osteoblast-specific deletion were born alive with no overt skeletal phenotype. The viability of Runx2^{E8/E8} provided a unique opportunity to understand the postnatal functions of Runx2.

Mice undergo a period of rapid growth and development after birth. We determined that loss of Runx2 function in osteoblasts caused growth deficiencies in the first month of postnatal life. Analysis of the skeleton revealed a dramatic decrease in bone acquisition of mutant mice. Moreover, a severe decrease in average density of hydroxyapatite was noted in both cortical and trabecular bone in homozygous mice, indicating impaired mineralization. These data confirm that Runx2 activity is critical for bone synthesis during postnatal skeletal development. In sharp contrast, a recent study that deleted the Runx2 gene with the 2.3kbCol1a1-Cre line showed no disruption in skeletogenesis (6). Micro-CT analysis at 6 weeks of age revealed no difference in the trabecular BV/TV ratio between the femurs of wild-type and mutant mice. However, in our model, a 30–50% decrease in both cortical and trabecular BV/TV is evident by 4 weeks of age. These differences could be related to the strain of mice, analytic approaches, or the region of Runx2 gene targeted.

Variations in bone acquisition are well known among different strains of mice. Unlike a mixed background, our model is established on a pure C57/BL6 background. Moreover, the short report contained limited analysis of the skeletal phenotype. Finally, the model reported by Takarada et al. floxed exon 4 of the Runx2 gene. Exon 4 encodes the DNA binding Runt Homology Domain and also contains a nuclear import signal. The exon 4 deleted Runx2 protein is not imported into the nucleus, and is degraded (6). We deleted exon 8 of the Runx2 gene. In our model, a mutant Runx2 protein is expressed that does not function as a dominant negative, and retains nuclear import, DNA binding, and protein–protein interactions in the N-terminus. However, exon 8 deleted Runx2 protein has reduced transcriptional activity, and lacks protein–protein interactions that occur in the C-terminus. Future studies with detailed analyses may better define the underlying reason for the lack of phenotype in exon 4 floxed Runx2 model.

Conclusions

We demonstrate that Runx2 continues to play a critical role in bone synthesis after commitment to the osteoblast lineage. However, embryonic skeletogenesis proceeds even with the absence of functional Runx2 in osteoblasts.

Abbreviations

Runx2	Runt-related transcription factor 2
BV	Bone Volume
BMD	Bone Mineral Density
R26R	Rosa-26 Reporter
H&E	Hematoxylin and Eosin
Micro-CT	Micro Computed Tomography
Tb.N	Trabecular number
Tb.Th	Trabecular thickness

Tb.Sp

Trabecular space

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Figure 1. Runx2 activity in committed osteoblast development is not essential for development of embryonic bones

(A) Hindlimbs from 3-month-old R26R mice with and without Cre transgene were processed for X-gal staining. The mid-diaphyseal region of both littermates are shown at 4X magnification. No β -galactosidase activity was detected in control R26R mice without Cre. R26R mice positive for 2.3kbCol1a1-Cre revealed LacZ expression only in osteoblasts and osteocytes of the limbs. (B) Gross images of newborn pups show no overt difference among wild-type, heterozygous, and homozygous littermates. (C) Histological sections from epiphysis through diaphysis of proximal femurs of wild-type and homozygous newborn littermates stained with H&E are shown at 4X magnification. Similar number and distribution of cells in the growth plate, cortical and trabecular bone, and bone marrow were noted. CB, cortical bone; BM, bone marrow.

Figure 2. Low bone mass in Runx2^{E8/E8} hindlimbs

(A) Weight of sex-matched littermates was monitored weekly through 4 weeks of age, beginning at birth. Runx2 deletion in osteoblasts leads to low bone mass in postnatal limbs. Pooled body weight data from four female and four male mice is shown. Runx2^{E8/E8} mice weighed 20–25% less than control by 4 weeks of age. (B) 3D reconstruction of femurs from 4-week-old wild-type and Runx2^{E8/E8} littermates showing obvious decrease in both cortical (top) and trabecular (bottom) bone mass. Representative data set quantifies the decreases in cortical and trabecular BV (C), trabecular parameters (D), and average density of hydroxyapatite (E). BV, bone volume; TV, total volume. Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular space.