Selective Deficiency of the “Bone-related” Runx2-II Unexpectedly Preserves Osteoblast-mediated Skeletogenesis*

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Zhou-Sheng Xiao¶§, Anita B. Hjelmeland¶, and L. D. Quarles¶¶¶

From the ¶Department of Medicine and the ¶¶Center for Bone and Mineral Disorders, Duke University Medical Center, Durham, North Carolina 27710

Runx2 (runt-related transcription factor 2) is a master regulator of skeletogenesis. Distinct promoters in the Runx2 gene transcribe the “bone-related” Runx2-II and non-osseous Runx2-I isoforms that differ only in their respective N termini. Existing mutant mouse models with both isoforms deleted exhibit an arrest of osteoblast and chondrocyte maturation and the complete absence of mineralized bone, but they do not distinguish the separate functions of the two N-terminal isoforms. To elucidate the function of the bone-related isoform, we generated selective Runx2-II-deficient mice by the targeted deletion of the distal promoter and exon 1. Homozygous Runx2-II-deficient (Runx2-II−/−) mice unexpectedly formed axial, appendicular, and craniofacial bones derived from either intramembranous ossification or direct mesenchymal conversion of the bone collar, but they failed to form the posterior cranium and other bones derived from endochondral ossification. Heterozygous Runx2-II-deficient mice had grossly normal skeletons, but were osteopenic. The commitment of mesenchymal cells ex vivo to the osteoblast lineage occurred in Runx2-II−/− mice, but osteoblastic gene expression was impaired. Chondrocyte maturation appeared normal, but the zone of hypertrophic chondrocytes was not transformed into metaphyseal bone, leading to widened growth plates in Runx2-II−/− mice. Compensatory increments in Runx2-I expression occurred in Runx2-II−/− mice but were not sufficient to normalize osteoblastic maturation or transcriptional activity. Our findings support distinct functions of Runx2-II and -I in the control of skeletogenesis. Runx2-I is sufficient for early osteoblastogenesis and intramembranous bone formation, whereas Runx2-II is necessary for complete osteoblastic maturation and endochondral bone formation.

The Runx family of transcription factors consists of three highly conserved mammalian genes, designated Runx1, Runx2, and Runx3 (1–5), that regulate, respectively, hematopoiesis (6), skeletogenesis (7–10), and neuronal development (11–12). RUNX2 is the master transcription factor essential for skeletogenesis. Targeted disruption of the Run domain of Runx2 results in the complete lack of both intramembranous and endochondral bone formation due to an arrest in osteoblast and chondrocyte maturation (8, 10, 13–19). Haplosufficiency of RUNX2 causes cleidocranial dysplasia in humans, and heterozygous Runx2 deficient mice show a similar phenotype, suggesting that the expression level of Runx2 influences the skeletal phenotype (10).

Separate promoters control the expression of Runx2 isoforms with different 5′-UTRs and N-terminal sequences that bind to the same consensual DNA cis-acting elements, interact with similar co-factors, and demonstrate overlapping transactivation potential in vitro (17–24). The Runx2-II isoform (also called Pebp2a, major til-1 and Cbfal.1, Osp2) is derived from the distal ‘bone-related’ promoter (P1) and encodes a 518-amino acid protein that begins with the 19 amino acids MASNLFSATPCQPSFW derived from exon 1 (18, 22–25). The Runx2-II isoform (also called Cbfal.org and Cbfal/p56) is derived from the more primitive non-osseous proximal promoter (P2), utilizes a translation start site in exon 2, and encodes a 514-amino acid protein that begins with the five amino acids MRIPV (8, 19, 21). Thus, Runx2-II and I differ only in their 5′-UTRs and N-terminal amino acids and are identical with respect to their runt domains and C termini. The functional consequences of these apparently redundant Runx2 gene products, which differ only in their N termini, are poorly understood.

Runx2-II and Runx2-I are presumed to have distinct roles based on the predominant expression of the type II isoform in bone (13, 16) and the type I isoform in non-osseous tissues (21, 26), but their separate functions have not been experimentally established. Existing mouse knock-out models, generated by either the targeted disruption of the common Runt domain (8, 10) or the overexpression of dominant negative constructs (7), disrupt the function of both isoforms and, therefore, do not permit the separate assessment of Runx2-II and -I. Transgenic mouse studies overexpressing Runx2 isoforms by using heterologous promoters also fail to identify important differences between Runx2-I and II (15–16, 27). These forced expression studies are limited by high levels of expression and alterations in the temporal-spatial expression of the isoforms that may lead to erroneous conclusions about the physiological differences in function of these isoforms. In vitro studies that evaluate the function of transfected Runx2-I and -II have also produced mixed results (23, 28), with most studies demonstrating similar transactivation potential for both isoforms (29–30). The selective deletion of Runx2-I or Runx2-II in vivo is necessary to understand their separate functions.

In the present study, we generated selective Runx2-II-deficient mice to assess the role of the “bone-specific” isoform in skeletogenesis. We show an unexpectedly mild phenotype in the skeletal system of selectively Runx2-II−/− mice. Rather
than the complete absence of osteoblasts and mineralized skeleton that occurs with the deletion of both Runx2-I and II, selective Runx2-II-deficient mice have limited impairment of osteoblast maturation and form an almost complete mineralized skeleton characterized by greater defects in endochondral than in intramembranous bone formation.

EXPERIMENTAL PROCEDURES

Generation of Runx2-II Mutant Mice—We isolated Runx2-II genomic clones from a 129/SVJ genomic library (Stratagene). We prepared a targeting construct in which the Runx2-II sequence between NheI and XbaI, including the P1 promoter and exon1, was removed and replaced with a Neo cassette. We linearized the targeting plasmid with NolI and transfected it into embryonic stem cells. Targeted clones were identified by PCR. We used two embryonic stem cell lines with disruptions in Runx2-II to generate Runx2-II-deficient mice. The correctly mutated embryonic stem clones were injected into blastocysts of C57BL/6J and implanted into 2.5-day-old pseudopregnant females by the Duke Transgenic Facility. The resulting chimeric animals were backcrossed to C57BL/6J, and heterozygous mutants were genotyped by PCR using tail-tip DNA. Brother-sister mating was then carried out to generate homozygous mutants.

Skeletal and Histological Preparations—Whole mouse carcasses were collected from newborn mice after euthanasia, defatted for 2–3 days in acetone, stained sequentially with Alcian blue and Alizarin red S in 2% KOH, cleared with 1% KOH and 20% glycerol, and stored in 50% EtOH and 50% glycerol (31). Tibias from newborn mice were decalcified at 4°C in 12.5% EDTA and 2.5% paraformaldehyde in phosphate-buffered saline. Sections were stained with hematoxylin and eosin to assess the histology of the growth plate in tibias.

Real Time Reverse Transcription PCR—For quantitative real time reverse transcription PCR, 2.0 μg of total RNA isolated from the long bone of newborn mice was reverse transcribed as described (32). PCR reactions contained 100 ng of template (cDNA or RNA) and 300 ng each forward and reverse primer and 1X iQ™ SYBR® Green Supermix (Bio-Rad) in 50 μl. Samples were amplified for 40 cycles in an iCycler iQ™ real time PCR detection system with an initial melt at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to double-stranded DNA. The threshold cycle of tested gene product from the indicated genotype was normalized to the threshold cycle by measuring the increase in fluorescence caused by the binding of PicoGreen® with a PicoGreen double-stranded DNA quantitation reagent and kit (Molecular Probes, Eugene, OR).

Electromobility Shift Assay and Reporter Assay—Nuclear extracts from osteoblasts of newborn mice were incubated with 10 fmol of γ-32P-labeled double-stranded OSE2 oligonucleotides, and electromobility shift assays (33) were performed in the presence and absence of a 100-fold molar excess of unlabeled OSE2 oligonucleotides. For supershift experiments, polyclonal Runx2 antibody was added. Osteoblasts were transfected with the following: 1) pcDNA 3.1 expression empty vector or pcDNA 3.1 expression vector containing the Runx2-I or II isoform (0.25 μg); 2) 1.3-kb mouse osteocalcin promoter-luciferase fusion plasmid (p1.3OC-luc, 1.0 μg); and 3) a pCMVβ-gal plasmid (0.1 μg) using a Lipofectin (Invitrogen) protocol. After 48 h, we assayed luciferase activity in cell lysates as described previously (35). Relative luciferase activity was normalized for transfection efficiency by quantifying the β-galactosidase activity and dividing by the value obtained with cotransfection of the pCDNA 3.1 empty expression vector and p1.3OC-Luc in wild-type osteoblasts.

Statistics—We evaluated differences between groups by one-way analysis of variance. All values are expressed as means ± S.E. All computations were performed using the Statgraphics statistical graphics system (STSC Inc.).

RESULTS

Creation of Selective Runx2-II-deficient Mice—Our targeting strategy (Fig. 1A) resulted in selective deletion of the P1 promoter and exon 1 encoding the N terminal and the first 19 amino acids (i.e. MASNSLFSAVTPCQQSFF) of the Runx2-II isoform, respectively. Wild-type, Runx2-II+/−, and Runx2-II−/− mice were genotyped by PCR (Fig. 1, B and C). Mice were born at the expected Mendelian frequency. The gross appearance of wild-type and Runx2-II−/− mutant mice were indistinguishable, whereas the Runx2-II+/− mutants were smaller in size (Fig. 2A) and had significantly (p < 0.05) lower body weight (1.2 ± 0.05 g) and length (3.6 ± 0.07 cm) compared with the respective values for wild-type litter mates (1.7 ± 0.05 g and 4.5 ± 0.04 cm) (Fig. 2, B and C). The Runx2-II+/− mice had an intermediate phenotype (Fig. 2, A–C) with body weight (1.5 ± 0.03 g) and length (4.3 ± 0.4 cm) also significantly less than those of wild-type mice. Approximately 20% of the selective Runx2-II−/− mice survived for at least 1 week (Fig. 2D); death appeared to be due to respiratory failure. The survival of Runx2-II−/− mice was not different from that of wild-type litter mates.

Skeletal Phenotype in Runx2-II-deficient Mice—Selective Runx2-II−/− mice formed a nearly intact mineralized skeleton (Fig. 3A) characterized by severe abnormalities of endochondral bone and grossly intact intramembranous bone (Fig. 3, B–G). This skeletal phenotype was much less severe than the complete absence of mineralized bone in the previously described Runx2 null mice lacking both Runx2-I and II (8, 10). In the skull, selective Runx2-II−/− mice have absent occipital
bones, which are formed by endochondral bone formation (Fig. 3B), and posterior zygomatic arches (Fig. 3B), as well as impaired formation of nasal bones and wider anterior fontanelle as compared with wild-type litter mates (Fig. 3B). In the rest of the skeleton, Runx2-II−/− mice have abnormalities of the hyoid bone (Fig. 3B), distal clavicles (Fig. 3B), and distal ribs (Fig. 3E), as well as shorter long bones, absent ossification centers in phalangeal bones with absent ossification centers (Fig. 3, A and F), and delayed endochondral ossification of the caudal spine (Fig. 3G). In contrast, cranial and cortical bones, which are derived from mesenchymal precursors, were present in Runx2-II−/− mice (Fig. 3, A–G). There were no apparent skeletal abnormalities in heterozygous selective Runx2-II-deficient mice (Fig. 3, A–G). This is in contrast to the previously reported skeletal abnormalities observed in heterozygous Runx2-I- and Runx2-II-deficient mice (8, 10), which exhibit delayed ossification of cranial, nasal, clavicular, hyoid bone, pubic, and ischial bones.

Long bones from Runx2-II−/− mice consisted of two cartilaginous epiphysis-containing growth plate cartilage and a diaphysis-containing cortical bone and a medullary cavity (Fig. 4A); however, the zone of hypertrophic chondrocytes was wider in Runx2-II−/− mice (Fig. 4B), an observation that contrasts with the inhibition of chondrocyte differentiation before prehypertrophic chondrocytes in combined Runx2-I and II null mice (8, 10) and correlates with the broader expression of Runx2-I in chondrocytes and the predominant expression of Runx2-II in terminal hypertrophic chondrocytes (36). In addition, the primary trabeculae in the metaphyseal region were diminished in Runx2-II−/− mice (Fig. 4C). Three-dimensional μCT analysis of the entire skeleton confirmed the skeletal defects in selective Runx2-II−/− mice (Fig. 4D) as well as the reductions in trabecular bone in the metaphyseal region (Fig. 4, E and F) and the thinner and more porous cortical bone of the mid-shaft region of the tibia (Fig. 4F). Calculated three-dimensional parameters of bone volume/tissue volume, trabecular thickness, trabecular number, and cortical thickness were significantly decreased in selective Runx2-II−/− mice (Table I). Analysis of heterozygous Runx2-II−/+− deficient mice revealed significant reductions in trabecular bone volume/tissue volume and trabecular thickness but less severe decrements in cortical thickness (Figs. 3B and 4, D–F; also see Table I). The preserved cortical bone formation in Runx2-II−/− and Runx2-II−/− mice correlates with the predominant expression of Runx2-I isoform in perichondrial/periosteal bone formation (37).
Differential Actions of Runx2 Isoforms

Gene Expression Profiles in Runx2-II-deficient Mice—Selective Runx2-II−/− mice had undetectable levels of Runx2-II, but compensatory increases in other transcription factors that may account for the mild skeletal phenotype (Table II). In this regard, Runx2-II−/− mice had a 2-fold increase in the Runx2-I isoform, consistent with autoregulation of Runx2 expression (24), but there were no significant changes in Runx1, Runx3 or Chf8 expression. c-jun, which can form a ternary complex with Runx2, and Mxs2, a homeodomain regulator of osteoblasts, were increased, whereas Dlx5, a transcription factor involved in patterning and osteoblast differentiation, was unchanged in Runx2-II−/− mice (38). Osterix, a down-stream transcriptional regulator of Runx2 in osteoblast-mediated bone formation (38–39), was decreased in selective Runx2-II−/− mice, along with a significant decrease in osteoblastic markers such as in alkaline phosphatase, osteocalcin, osteopontin, matrix extracellular phosphophylcoprotein (MEPE), and collagenase 3 (MMP13). Rank ligand (RANKL) was decreased, whereas osteoprotegerin, the soluble inhibitor of RANKL, was increased. Consistent with a ratio of osteoprotegerin/RANKL that favors decreased osteoclastic coupling, the tartrate-resistant acid phosphatase (TRAP), an osteoclast marker, was decreased in Runx2-II−/− mice. MMP-9, which is expressed in osteoclasts and chondroclasts, was also decreased. There was no evidence for impaired chondrogenesis in selective Runx2-II−/− mice, which is in contrast to the arrest of chondrocytes development that was reported for the original Runx2 null mice lacking both Runx2-I and II (40). Indeed, we observed significant increments in chondrocyte markers, including Type X and Type II collagen, VEGFA, and Sox9 (38) in selective Runx2-II−/− mice, possibly reflecting the preponderance of hypertrophic chondrocytes observed histologically (Fig. 4). The peroxisome proliferator-activated receptor γ (PPARγ), an adipocyte transcription factor, was also increased in Runx2-II−/− mice, but not adipocyte markers lipoprotein lipase (Lpl) and adipocyte fatty acid-binding protein 2 (aP2). Heterozygous Runx2-II-deficient mice had levels of Runx2-II message, ~60% of normal, and no significant alterations in Runx2-I, Runx1, Runx3, Mxs2, or Osterix, but did have a significant increase in PPARγ, intermediate to that observed in selective Runx2-II−/− mice. Unlike the reduction in osteoblastic markers found in homozygous mice, there were no significant alterations in cell type markers in heterozygous Runx2-II-deficient mice.

Assessment of Osteoblast Maturation ex Vivo—Consistent with the impaired expression of osteoblast markers in vivo, we also observed abnormalities of osteoblast maturation ex vivo using calvaria-derived osteoblasts. Osteoblasts derived from Runx2-II-deficient mice had lower levels of alkaline phosphatase (Fig 5A) and impaired mineralization (Fig. 5B) compared with osteoblasts from wild-type litter mates. In addition, the compensatory increase in the Runx2-I message expression in bone tissue (Table II) was not sufficient to normalize Runx2 binding to cis-acting elements in the osteocalcin promoter (Fig. 5C). Consistent with the decrease in Runx2-II expression and impaired osteoblasts differentiation, osteocalcin promoter activity was significantly less in Runx2-II−/− osteoblasts, and co-expression of either Runx2-I or Runx2-II into Runx2-II−/− osteoblasts was sufficient to normalize osteocalcin promoter activity (Fig. 5D), consistent with other in vitro studies indicating that both isoforms have similar transactivating potential (30).

**D I S C U S S I O N**

In the present study we created a mouse selectively deficient in Runx2-II, the bone-related isoform, by deleting the P1 promoter and exon 1, while leaving intact the P2 promoter and exon 2 encoding the more generally expressed Runx2-I gene product. Prior studies showed that mutant mice completely lacking both Runx2-I and Runx2-II gene products show a complete absence of osteoblasts and mature chondrocytes and fail to form a mineralized skeleton (8, 10). Our results provide the first in vivo evidence that Runx2-II and Runx2-I have separate roles in controlling intramembranous, cortical, and enchondral bone formation. In this regard, mice selectively lacking the Runx2-II gene product had limited skeletal abnormalities, predominantly of enchondral bone (Fig. 1 and 2) but with a surprisingly preserved formation of intramembranous and cortical bone, a finding that is inconsistent with the original concept that the bone-related isoform is essential for skeletogenesis as well as osteoblast and chondrocyte maturation. In addition, we observed no gross abnormalities in either enchondral or intramembranous bone development in selective heterozygous Runx2-II+/− mice (Figs. 1–4), which are haploinsufficient for the Type II isoform and have normal expression of Runx2-I (Table II). This contrasts with the original Runx2 heterogeneous mice, which were haploinsufficient for both type I and II isoforms and showed defects of intramembranous bone formation (10).

Osteoblast function was impaired in both homozygous and heterozygous Runx2-II-deficient mice, as evidenced by significant reductions in trabecular and cortical bone volume (Table I). Selective Runx2-II deficiency resulted in a dose-dependent effect on osteoblastic gene expression (Table II). Indeed, selective Runx2-II−/− mice had significant decrements in Oex, a down-stream effector of Runx2 required for osteoblasts development (39), as well as concomitant reductions in bone expression of alkaline phosphatase, osteocalcin, osteopontin, and MEPE, whereas these markers were normal or only slightly reduced in heterozygous mutant mice (Table II). Both homozygous and heterozygous Runx2-II-deficient mice showed increments in the adipocyte differentiation factor PPARγ, which can also act as a negative regulator of osteoblast differentiation (41–42). Interestingly, Runx2-I c-jun, which can form a ternary complex with Runx2 (43), and Mxs2, a homeodomain regulator of osteoblasts (38), were increased in Runx2-II null mice. Thus, selective absence of Runx2-II resulted in compensatory increases in factors that may preserve osteoblastic function and limit the severity of the skeletal phenotype. Nevertheless, gel mobility shift assays demonstrated reduced binding of Runx2 to a consensus cis-acting element, which is consistent with

**TABLE I**

Micro-CT analysis of tibias in wild-type and mutant mice

| Genotype      | BV/TV | Th.N | Th.Th | Th.Sp | Ct.Th(mm) |
|---------------|-------|------|-------|-------|-----------|
| Runx2-II+/−   | 9.6 ± 0.6 | 12.0 ± 0.3 | 0.022 ± 0.001 | 0.084 ± 0.002 | 0.041 ± 0.001 |
| Runx2-II−/−   | 6.2 ± 0.4± | 11.3 ± 0.4 | 0.019 ± 0.001 | 0.089 ± 0.003 | 0.037 ± 0.001 |
| Runx2-II+/−   | 1.3 ± 0.2± | 6.1 ± 0.3± | 0.014 ± 0.001 | 0.166 ± 0.007 | 0.034 ± 0.001 |

* a Difference from wild-type Runx2-II+/− mice at p < 0.05 by one-way analysis of variance.

b Difference from heterozygous Runx2-II+/− mice at p < 0.05 by one-way analysis of variance.

Data are mean ± S.E. from nine newborn mice. Abbreviations are: BV/TV, bone volume/tissue volume; Th.N, trabecular number; Th.Th, trabecular thickness; Th.Sp, trabecular separation; and Ct.Th, cortical thickness.
Differential Actions of Runx2 Isoforms

Data are mean ± S.E. from 6–10 newborn mice and expressed as the fold changes relative to the housekeeping gene cyclophilin A subsequently normalized to wild-type mice. Abbreviations used are: ALP, alkaline phosphatase; aP2, adipocyte fatty acid-binding protein 2; Col I/II/X, collagen type I, II, and X; and Lpl, lipoprotein lipase.

| Gene-expression profiles in mutant mice |
|----------------------------------------|
| Gene                                | Accession no. | Runx2-II−/− | Runx2-I/II−/− |
|---------------------------------------|---------------|-------------|--------------|
| Transcriptional Factors               |               |             |              |
| Runx2-II                              | NM_009820     | 0.65 ± 0.10a | 0.86 ± 0.06a |
| Runx2-I                               | D14636        | 1.26 ± 0.06 | 1.94 ± 0.26b |
| Runx1                                 | NM_009821     | 1.28 ± 0.22 | 1.51 ± 0.32  |
| Runx3                                 | NM_017322     | 1.51 ± 0.30 | 1.47 ± 0.27  |
| Cbfα                                  | NM_022309     | 1.02 ± 0.06 | 1.03 ± 0.09  |
| c-fos                                 | NM_010234     | 1.14 ± 0.26 | 1.49 ± 0.20  |
| e-jun                                 | BC02081       | 0.98 ± 0.09 | 2.49 ± 0.33b |
| Max2                                  | NM_013601     | 1.46 ± 0.10 | 2.90 ± 0.51b |
| Sox9                                  | NM_011446     | 1.35 ± 0.10 | 1.69 ± 0.19b |
| PPARγ                                 | NM_011146     | 1.96 ± 0.24 | 2.35 ± 0.28a |
| Dlx5                                  | NM_010056     | 1.11 ± 0.12 | 1.29 ± 0.24  |
| MyoD1                                 | NM_018866     | 1.07 ± 0.13 | 1.16 ± 0.13  |
| Osterix                               | AF184902      | 0.99 ± 0.16 | 0.58 ± 0.10b |
| Cell-type Markers                     |               |             |              |
| ALP                                   | NM_007431     | 0.90 ± 0.07 | 0.61 ± 0.05b |
| Osteocalcin                           | NM_007541     | 0.78 ± 0.13 | 0.22 ± 0.01b |
| Osteopontin                           | AF515708      | 0.71 ± 0.10 | 0.50 ± 0.09a |
| MEF2                                  | NM_005372     | 0.84 ± 0.11 | 0.28 ± 0.03b |
| ColI                                  | NM_007742     | 0.97 ± 0.19 | 1.39 ± 0.11b |
| Osteoprotegerin                      | MMU94331      | 0.87 ± 0.07 | 1.51 ± 0.21b |
| RANKL                                 | NM_011613     | 0.95 ± 0.11 | 0.59 ± 0.08b |
| TRAP                                  | NM_007358     | 0.70 ± 0.10 | 0.51 ± 0.07b |
| MMP9                                  | NM_008607     | 0.87 ± 0.06 | 0.63 ± 0.09a |
| MMP13                                 | NM_001599     | 0.96 ± 0.07 | 0.72 ± 0.07b |
| ColII                                 | NM_031163     | 1.11 ± 0.07 | 1.37 ± 0.16a |
| ColIX                                 | NM_009975     | 1.19 ± 0.21 | 1.64 ± 0.25a |
| VEGFA                                 | NM_009505     | 2.04 ± 0.23 | 2.98 ± 0.61a |
| eP2                                   | NM_024406     | 1.25 ± 0.11 | 1.21 ± 0.15  |
| Lpl                                    | NM_008509     | 1.25 ± 0.22 | 1.05 ± 0.15  |

a Significant difference from wild-type Runx2-II−/− mice at p < 0.05.

b Significant difference from heterozygous Runx2-II−/− mice at p < 0.05.

decreased total Runx2 transactivation potential in Runx2-II null mice (Fig. 5C). Finally, cultures of calvaria-derived osteoblasts from Runx2-II−/− also demonstrated delayed maturation as measured by alkaline phosphatase activity and mineralization of extracellular matrix (Fig. 5, A and B).

We also found evidence for decreased osteoblastic recruitment/activity in Runx2-II null mice, as evidenced by the high osteoprotegerin/RANKL ratio and decreased osteoblast markers TRAP and MMP-9 (44). These findings are consistent with reported effects of Runx2 to control osteoclastogenesis in early skeletal development (45–46). Because decreased osteoclastogenesis is typically associated with increased bone mass, the marked reductions in bone volume in Runx2-II-deficient mice provides further evidence that the osteopenia is due to diminished osteoblastic activity.

Runx2-II null mice also had mild growth plate abnormalities. Rather than the inhibition of chondrocyte differentiation before the prehypertrophic stages as was observed in the original Runx2 null mice lacking both isoforms (8, 10), selective Runx2-II−/− mice had chondrocytes representing resting, prehypertrophic, and hypertrophic stages in the growth plate. The major abnormality was a widened hypertrophic chondrocyte zone and impaired remodeling of the metaphyseal region into trabecular bone (Figs. 3 and 4). The associated decrements in the interstitial collagenases MMP13 and MMP9 raises the possibility that the growth plate abnormalities are due to impaired matrix remodeling in selective Runx2-II−/− mice, similar to that observed in MMP9-null mice (44). Although Runx2 has been implicated in angiogenesis by regulating vascular endothelial growth factor (VEGF) (47), we found no evidence for alterations in VEGF in Runx2 null mice. The expression of type X collagen, a marker of hypertrophic chondrocytes (36, 48), as well as the expression Sox9 and type II collagen in Runx2-II null mice, indicates that chondrocytes progressed through the various stages of maturation in the growth plate in Runx2-II mutant mice.

We also can infer the function of Runx2-1 by comparing the limited abnormalities in the selective Runx2-II-deficient mice with the global defects in osteoblast and chondrocyte function originally reported in the Runx2 null mice lacking both isoforms (8, 36, 40). From this analysis, the Runx2-1 gene product appears to be sufficient to control the differentiation of mesenchymal precursors to cells within the osteoblast lineage that underlies intramembranous and cortical bone formation (49–50) and for the maturation of the chondrocyte lineage to the stage of hypertrophic chondrocytes necessary for forming cartilage anlagen and growth plates.

Our study did not investigate the mechanisms whereby the N-terminal Runx2 isoforms exert differential effects on intramembranous and endochondral bone formation. Possible explanations include distinct functions imparted by the N-terminal sequences, differential actions arising from differences in spatial and temporal expression of the two isoforms, and/or potential dose-dependent effects derived from the sum effect of the Runx2 gene products. Similarly as in other in vivo or in vitro studies that failed to identify distinct functions of these two isoforms (15–16, 29–30), we found no evidence for differential function of Runx2-1 and Runx2-II in cultured osteoblasts (Fig. 5D), although functional differences between Runx2-I and II have been reported under some experimental conditions (23, 28, 36, 51). Prior in situ analysis of Runx2 isoform expression demonstrated that Runx2-1 is expressed predominantly in undifferentiated mesenchymal cells, preosteoblasts, and chondrocytes, as well as during later stages of development within these lineages, whereas Runx2-II expression is limited to later stages of osteoblastic differentiation and
terminal hypertrophic chondrocytes (36–37, 40, 48). In addition, Runx2-II is differentially regulated by bone morphogenetic proteins 2 and 7 (BMP-2 and BMP-7), transforming growth factor β1 (TGF-β1), and 1,25(OH)₂D₃ in osteoblasts (26, 29, 35, 52–57), and tumor necrosis factor α (TNFα) selectively suppresses Runx2-I (58). Such differences in temporal and spatial expression patterns and regulation imply specialized functions of the isoforms. Finally, the severity of the skeletal phenotype in various mouse models is proportionate to the gene dose (27). Thus, the amount of Runx2, transcriptionally controlled by alternative promoters, as well as the amount translationally controlled by internal ribosome entry site (IRES)-mediated mechanisms inherent to the 5'-UTRs (32), might also permit fine tuning of expression levels with different functional consequences across a wide range of cellular conditions.

In conclusion, Runx2-II, which is increased during differentiation of primary osteoblasts and hypertrophic chondrocytes (13, 16), is necessary for complete skeletogenesis and osteoblast maturation, especially in the later stages of endochondral bone formation, including the normal development of the primary and secondary spongiogenesis in the metaphysis of long bone. Control of skeletogenesis by Runx2 gene products may arise from yet to be discovered differential functions imparted by their respective N termini or may be proportional to the amount of Runx2-I and II, which is controlled temporally and spatially by the distinct promoters (26, 37) and post-translational mechanisms imparted by the 5'-UTRs of these genes (32). Runx2-I, which is constitutively expressed in non-osseous mesenchymal tissues (26) and in osteoblast progenitor cells (16, 21) appears to be sufficient for the formation of both intramembranous and cortical bone and likely plays a role in the early stages of mesenchymal cell development. Further studies that selectively delete the P2 promoter and the Runx2-I isoform or efforts to replace the Runx2-I gene product by Runx2-II cDNA under the control of the P1 promoter will be necessary to confirm the differential function of the two Runx2-II isoforms.

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