Runx2 Protein Expression Utilizes the Runx2 P1 Promoter to Establish Osteoprogenitor Cell Number for Normal Bone Formation*5

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The Runt-related transcription factor, Runx2, is essential for osteogenesis and is controlled by both distal (P1) and proximal (P2) promoters. To understand Runx2 function requires determination of the spatiotemporal activity of P1 and P2 to Runx2 protein production. We generated a mouse model in which the P1-derived transcript was replaced with a lacZ reporter allele, resulting in loss of P1-derived protein while simultaneously allowing discrimination between the activities of the two promoters. Loss of P1-driven expression causes developmental defects with cleidocranial dysplasia-like syndromes that persist in the postnatal skeleton. P1 activity is robust in preosteogenic mesenchyme and at the onset of bone formation but decreases as bone matures. Homozygous Runx2−P1lacZ/lacZ mice have a normal life span but exhibit severe osteopenia and compromised bone repair in adult mice because of osteoblastic defects and not increased osteoclastic resorption. Gene expression profiles of bone, immunohistochemical studies, and ex vivo differentiation using calvarial osteoblasts and marrow stromal cells identified mechanisms for the skeletal phenotype. The findings indicate that P1 promoter activity is necessary for generating a threshold level of Runx2 protein to commit sufficient osteoprogenitor numbers for normal bone formation. P1 promoter function is not compensated via the P2 promoter. However, the P2 transcript with compensatory activity is expressed in mature osteoblasts and is similarly capable of transactivating target genes in bone morphogenetic protein (BMP) and Wnt signaling is adequate for mineralization of the bone tissue that does form. We conclude that selective utilization of the P1 and P2 promoters enables the precise spatiotemporal expression of Runx2 necessary for normal skeletogenesis and the maintenance of bone mass in the adult.

Runx2 is the master regulator of both osteoblast and terminal chondrocyte differentiation and is essential for in vivo bone formation and mineralization (1, 2). Runx2 is strongly expressed in mesenchymal condensations of the developing skeleton (2) during endochondral bone formation (3). A large number of bone-related genes are regulated by Runx2 including Runx2 and its targets that contribute to the bone matrix: osteocalcin (OC), osteopontin, bone sialoprotein, and alkaline phosphatase (AP). Runx2 also contributes to bone turnover through regulation of osteoprotegerin and receptor activator of nuclear factor κ-B ligand (RANKL) and maturation of the growth plate by expression of vascular endothelial growth factor (VEGF) and collagen type X (4–10).

Promoter switching is a common developmental mechanism used to control the gene expression levels and the functional activities of several genes in osteoblasts (e.g. collagen type I (Col1a1) and parathyroid hormone-related protein) (11–13). The two distinct promoters of Runx2 may specifically regulate the dynamic process of bone development by controlling spatiotemporal expression of Runx2. The proximal P2 promoter (Runx2 P2) regulates the type I isoform (designated Runx2-I), which begins with the amino acids MRIPV and is the first Runx2 discovered in the thymus (14). The distal P1 promoter (Runx2 P1) regulates the type II isoform (Runx2-II), which has an MASNS start sequence (15). The two proteins, which differ only in 19 amino acids at the N terminus, share the same functional domains and are similarly capable of transactivating target genes (16–18). The P2 promoter is active at a basal level in a broad number of cells and tissues, including the thymus, cartilage, periosteum, and suture tissue of the calvarium (17, 19–23), whereas the P1 promoter is considered to be more bone-related and is active in hypertrophic chondrocytes and mature osteoblasts (22, 24, 25). During cranial suture morphogenesis, the P2 transcript is highly expressed in undifferentiated

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6 The abbreviations used are: OC, osteocalcin; AP, alkaline phosphatase; TRAP, tartrate-resistant acidic phosphatase; RANKL, receptor activator of nuclear factor κ-B ligand; CCD, cleidocranial dysplasia; β-gal, β-galactosidase; microCT, micro-computed tomography; qRT-PCR, quantitative RT-PCR; Osx, osterix.
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mesenchymal cells, whereas the P1 transcript is present in the osteogenic fronts (20, 26). Taken together, the apparent functional equivalence of the two Runx2 isoforms, coupled with differential spatiotemporal expression, suggests that Runx2 gene structure has evolved for a more precise control of Runx2 protein during skeletal development and maintenance.

Several mouse models have been developed to better understand Runx2 function. The earliest null models demonstrated that mice deficient in Runx2 die at birth and lack a mineralized skeleton (1, 2). Deletion of the C terminus, including the nuclear matrix targeting signal region in exon 8, revealed the importance of appropriate subnuclear localization for full functionality of Runx2, as mice homozygous for this deletion displayed a phenotype resembling that of Runx2 null mice (27). Recent studies have revealed the significance of Runx2 dosage in determining the severity of pleiotropic skeletal defects analogous to cleidocranial disorder in humans, caused by mutations in Runx2 (28–30). To probe the roles of the Runx2 isoforms, Xiao et al. (31, 32) created a mouse model (Runx2-II−/−) in which the P1 promoter and exon 1 were deleted, thus abrogating expression of Runx2 P1 but allowing expression of Runx2 P2-driven Runx2-1 protein. Homozygous mice (Runx2-II−/−) formed skeletons but did not survive after 6 weeks, and defects in endochondral bone formation were observed, but intramembranous bone was not severely affected (31,32).

None of these mouse models, however, has addressed the fundamental contribution of the intact P1 and P2 promoters in maintaining proper spatiotemporal expression of Runx2 protein. We have therefore developed a mouse model (Runx2-P1lacZ/lacZ) that maintains a fully functional P1 promoter that drives expression of the lacZ reporter gene while simultaneously eliminating the Runx2 P1-derived protein isoform, Runx2-II. In contrast to previous mouse models (31, 33), our mutant homozygous mice exhibited a life span equivalent to the wild type (WT) but displayed skeletal defects resembling cleidocranial dysplasia (CCD), including defects in calvarial sutures, hypoplastic or aplastic clavicles, and dental abnormalities consistent with human CCD disorders. Our studies have established distinct spatial and temporal activities of the P1 and P2 promoters that regulate developmental expression in prenatal mice and provide normal cellular levels of Runx2 protein to support bone formation in adult mice. The data identify a mechanism for the bone defects and establish a critical role for Runx2 P1 promoter activity as a determinant of adequate numbers of osteoprogenitors cells to form normal bone tissue.

EXPERIMENTAL PROCEDURES

Mice—Genomic DNA for use in the Runx2 P1 targeting vector was isolated from AB2.2 murine embryonic stem cells. Target DNA for generating arms of homology was amplified using Pfu Ultra DNA polymerase (Invitrogen). The lacZ reporter gene was ligated immediately downstream of the Runx2 P1 promoter (included in the 5′ targeting arm), replacing the Runx2-II transcriptional start site and the first exon. A PGK-neomycin-positive selection cassette was inserted after the lacZ reporter allele in reverse orientation followed by the 3′ targeting arm of homology. A PGK-thymidine kinase negative selection marker was finally inserted downstream of the 3′ targeting arm to select against random integrants (Fig. 1A). The final targeting vector was constructed in the pBluescript backbone (Stratagene, La Jolla, CA). Targeting vector (10 µg) was linearized and electroporated into 1 × 10⁷ AB2.2 murine embryonic stem cells followed by positive and negative selection. The resulting clones were screened for successful homologous recombination by Southern blot using an external probe downstream of the 3′ arm of homology and PCR-based analysis. Properly targeted clones were expanded and used in blastocyst injections performed by the University of Massachusetts Medical School Gene Targeting Core Facility. Resulting chimeric mice were back-crossed to wild-type C57BL/6 mice until germ-line transmission was achieved. These crosses yielded one female heterozygous mouse (Runx2-P1−/lacZ). This founding female was back-crossed to wild-type C57BL/6 males to expand the colony.

Runx2-P1lacZ/lacZ females are unable to carry a pregnancy to full term. To maintain the colony, either heterozygous mice were crossed or homozygous mutant male mice were crossed with heterozygous female mice. Animals were maintained at the University of Massachusetts following procedures approved by the Institutional Animal Care and Use Committee (IACUC). Routine genotyping of offspring was carried out using two PCR primer sets. The first primer set amplified the targeted exon-1 in the wild-type allele (Fwd, 5′-agt ctt cat tcg cct cac aaa c-3′; Rev, 5′-ccc aaaaga aga gcc ttt gct g-3′), generating a product in both the wild type and heterozygotes. The second set amplifies the lacZ coding region (Fwd, 5′-cca act taa tccg cct tgg agc aca tca-3′; Rev, 5′-cgg gac aaa cgg cgg att gcc-3′), generating a product in both the heterozygotes and nullizygotes.

Cell Culture—Adherent cells from freshly harvested bone marrow were cultured in α-MEM medium supplemented with 20% fetal bovine serum (FBS) (Hyclone, Waltham, MA), 1-glutamine, penicillin, and streptomycin. After 7 days of culture, cells were fixed in 4% paraformaldehyde in 0.1 M cacodylic acid buffer. AP activity was detected using naphthol AS-MX phosphate and Fast Red salt as described previously (34). The area of AP-positive cells was quantified using ImageJ software.

Calvarial osteoblasts were isolated from wild-type and homozygous 1-day-old mice and maintained as described previously (35, 36). Cells were plated at a density of 8 × 10⁴ cells/well in a 6-well plate. At confluence, regular growth medium (α-MEM supplemented with 10% FBS) was replaced with osteogenic medium (BGlb supplemented with 10% FBS, 10 mM β-glycerophosphate, and 25 µg/ml ascorbic acid for the first feeding and 50 µg/ml ascorbic acid for subsequent feedings) for differentiation assays. Cells were stained for AP activity, as described above, at days 7, 14, and 28. For stimulation response assays to osteogenic factors, isolated calvarial osteoblasts were driven to differentiation with osteogenic medium for 14 days. Media supplemented with 100 ng/ml BMP-2 or 10% Wnt3a conditioned medium (collected from L-Wnt3a cells (ATCC, Manassas, VA) as recommended) were added for 48 h.

RT-PCR Analysis—Using TRIzol reagent (Invitrogen), total RNA was isolated from cultured cells, calvarial bone, or femur after the removal of the cartilage cap. One microgram of RNA was treated with RNase-free DNase I (Zymo Research, Orange,
CA) and reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). Relative transcript levels were measured by real-time PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) and normalized to mitochondrial cyclooxygenase levels. RANKL, matrix metalloproteinase-9 (MMP-9), and rodent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were purchased from Applied Biosystems. All other primer sequences used for amplification are listed in supplemental Table 1.

Histology and Skeletal Staining Procedures—For β-galactosidase (β-gal) staining, the mice were fixed in 2% paraformaldehyde containing 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), and 2 mM MgCl₂, all in 1× PBS (pH 7.4) at 4 °C (37). E13.5 embryos were fixed for 2–3 h. E16.5 embryos and postnatal mice were fixed overnight. The standard staining reagent was used (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide (Sigma), 2 mM MgCl₂ (Fisher Scientific), 0.2% Nonidet P-40 (U. S. Biological, Swampscott, MA), 0.01% sodium deoxycholate (Sigma), and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Gold Biotechnology, St. Louis, MO), made up in 1× PBS (pH 7.4)). Embryos and dissected tissue were rinsed in and equilibrated in a PBS solution containing 3% dimethyl sulfoxide and 3.7% EDTA to prevent nonspecific staining from developing upon storage in PBS. Photographs were taken using a stereoscope (CLS 150, Leica, Wetzlar, Germany) with a digital camera (Axiocam HRC, Zeiss, Thornwood, NY).

Frozen sections of Runx2-P1lacZ/lacZ mice were obtained after β-gal staining by equilibrating the bones in 30% sucrose (pH 7.4) and embedding in OCT (Triangle Biomedical Sciences, Durham, NC). Sections of 8–10 μm were obtained with a Bright/Hacker (model OTF) Instrument (Fairfield, NJ). The Cryoflake (Hackensack, NJ) tape transfer system was used on bone sections. The sections were refixed in 0.5% glutaraldehyde for 5–10 min, retracted for β-gal for 4–6 h at 37 °C, and then counterstained with eosin. For Alizarin red/Alcian blue staining, newborn mice were eviscerated and fixed in 100% ethanol. The mice were sequentially stained with Alcian blue and Alizarin red followed by tissue clarification with KOH by standard procedures (38).

Postnatal bones were fixed in 4% paraformaldehyde and embedded in paraffin or plastic. Bones from adult mice were decalcified in 18% EDTA (pH 7.4) for 3–4 weeks prior to embedding them in paraffin. Toluidine blue and von Kossa staining were performed by equilibrating the bones in 30% sucrose (pH 7.4) and embedding in OCT (Triangle Biomedical Sciences, Durham, NC). Sections of 8–10 μm were obtained with a Bright/Hacker (model OTF) Instrument (Fairfield, NJ). The Cryoflake (Hackensack, NJ) tape transfer system was used on bone sections. The sections were refixed in 0.5% glutaraldehyde for 5–10 min, retracted for β-gal for 4–6 h at 37 °C, and then counterstained with eosin. For Alizarin red/Alcian blue staining, newborn mice were eviscerated and fixed in 100% ethanol. The mice were sequentially stained with Alcian blue and Alizarin red followed by tissue clarification with KOH by standard procedures (38).

RESULTS

Loss of Runx2 Expression from the P1 Distal Promoter Results in Viable Mice—To retain Runx2 P1 promoter activity while eliminating Runx2 expression from the P1 promoter, exon 1 was replaced by the lacZ gene and PGK-neomycin cassette in reverse orientation (Runx2-P1lacZ allele, Fig. 1A). This targeting strategy allowed for retention of the 70-kb intron downstream of P1 and upstream of the intact Runx2 P2 promoter that drives the type I protein isoform. Successful specific deletion of exon 1 was confirmed by Southern blot (Fig. 1B) and PCR analysis (Fig. 1C). Homozygous and heterozygous mice were viable and had a normal life span. This result is in contrast to the Runx2-Δ2 mouse model reported by Xiao et al. (32), which was highly penetrant (~80%) with inexplicable postnatal lethality of homozygotes beginning the first week. Notably, mice characterized by Xiao et al. (32) and in this study were on similar genetic backgrounds. Our Runx2-
P1/lacZ/lacZ mice displayed a reduced weight compared with WT during the period of rapid postnatal growth (Fig. 1D; males, weeks 3–25, 78.8 ± 2.7%; females, weeks 3–18, 79.1 ± 3.3%). We also observed abnormalities in tooth shape and underlying jaw tissue (supplemental Fig. S1) that were not explored further.

To validate that the Runx2 P1-targeted knock-in reporter allele specifically inactivates P1-derived Runx2 expression, we examined the relative levels of Runx2 P1- and P2-derived transcripts as well as lacZ transcripts in calvaria, a tissue known to express high levels of both Runx2 mRNAs (20). lacZ transcripts were not detected in WT mice but were present in both Runx2-P1/lacZ and Runx2-P1/lacZ/lacZ mice. As expected, Runx2-P1/lacZ mice had a 50% reduction in levels of the P1 transcript, and Runx2-P1/lacZ/lacZ mice displayed a complete absence of this transcript (Fig. 1E). The Runx2 P2-driven mRNA was equivalently expressed in all genotypes, demonstrating that the P2 promoter was not affected by the gene targeting. Total Runx2 mRNA levels were reduced accordingly in Runx2-P1/lacZ and Runx2-P1/lacZ/lacZ mice. The ~50% reduction in the total Runx2 transcript from the homozygous mutant neonatal calvarial bones indicates that the P1 promoter is normally responsible for half of the total Runx2 activity. Interestingly, elimination of Runx2 P1-driven expression results in coordinate down-regulation of Runx1 and Runx3 transcripts during membranous bone formation of the calvaria from Runx2-P1/lacZ/lacZ mice compared with WT (Fig. 1F). This finding suggests that the loss of Runx2 P1-expressed protein leads to a decrease in cell populations such as mesenchymal stem cells (MSCs) or later osteochondroprogenitor cells expressing Runx1 and maturing chondrocytes in the growth plate that express Runx3 (41, 42).

FIGURE 1. Generation of a Runx2 P1-targeted knock-in reporter allele. A, targeting vector designed to replace exon 1 with a lacZ reporter upon homologous recombination. EP, external probe used for confirmation of recombination. B, Southern blot confirming recombination using the external probe shown in A after digestion of genomic DNA with BamH1. C, PCR verifying deletion of exon 1 (top) and insertion of lacZ (bottom) using primer sets depicted in A. D, weight of male mice (n = 3) over time. Inset shows females at 6 weeks. Scale bar represents 1 cm. E, Runx2 and lacZ gene expression from day 2 calvaria. F, Runx1 and Runx3 at neonatal day 2 and postnatal (6 weeks) ages in calvaria. Values are shown for Runx2-P1/lacZ (black bars) mice. Values are mean ± S.E. of independent samples. A one-tailed t test assuming equal variances was performed: *, p < 0.05; **, p < 0.01; and ***, p < 0.001, compared with Runx2-P1/+ mice.

Spatiotemporal Utilization of the Runx2 P1 and P2 Promoters
Normal Bone Formation Requires P1 Promoter Activity in Osteoprogenitor Cells—The P1 promoter remains physiologically responsive, as evidenced by expression of the lacZ reporter gene. Detection of β-gal activity driven by the P1 promoter should predict sites of potential osteogenic defects in Runx2-P1lacZ/lacZ and Runx2-P1lacZ/lacZ mice. Robust β-gal activity was detected beginning in E13.5 Runx2-P1lacZ/lacZ embryos but not in control embryos (supplemental Fig. S2, A–D). The majority of the β-gal staining was seen in the mesenchyme of the developing craniofacial, axial, and appendicular skeleton. In E16.5 mice, lacZ expression remained elevated in the craniofacial skeleton and was observed in the ribs, ossifying vertebral bodies, and the bone-forming fronts of the limbs (supplemental Fig. S2, E–I).

Strong Runx2 P1 promoter activity continued in the newborn calvaria, the phalanges, and the ribs (Fig. 2, left panels), as well as vertebral bodies (supplemental Fig. S2, J–M). Whole tissues from the homozygote were slightly smaller in size than those from control mice. To gain insight into the localization of Runx2 P1 expression in chondrogenic and osteogenic lineage cells, β-gal staining was performed in frozen sections (Fig. 2, right panels). In calvaria of the homozygous mice, peristeal osteoprogenitor cells and bone surface osteoblasts were positive for β-gal staining. Coronal sections (Fig. 2A, right panels) revealed the highest P1 promoter expression in differentiating osteoblasts and mesenchymal cells within the suture tissue at the bone-forming front. Osteocytes in the ossified bone (under the surface osteoblasts) showed few β-gal-positive cells. In the delayed developing phalanges of the homozygotes, activity of the P1 promoter was robust in three defined anatomical domains (Fig. 2B): the expanding bone collar, in chondrocytes in the hypertrophic zone, and in the primary spongiosa trabeculae. Similarly, in the ribs, the peristeum and bone collar, hypertrophic zone cells and the primary spongiosa bone under the growth plate exhibited Runx2 P1 activity (Fig. 2C). In sections of the long bones (Fig. 2, B (digits) and C (ribs)), Runx2 P1 activity

![FIGURE 2. Activation of the Runx2 P1 promoter in developing bone. β-Gal staining of WT is shown and either Runx2-P1lacZ/lacZ or Runx2-P1lacZ/lacZ newborn whole bone tissues (left) and histology (right panels, with eosin counterstaining) to show location of Runx2 P1 promoter-expressing cells. A, calvarium, whole tissue (+/+); B, +/+, hand with metacarpals; C, +/+, rib. LacZ expression was detected in periosteum/bone collar and osteoblasts in all growth plate regions. The boxed area (inset) is magnified. C, rib cage is β-gal-positive in the bone portion of sternum bodies and ribs. Rib sections show β-gal staining in peristeum and bone collar. Hypertrophic chondrocytes are also β-gal-positive at higher magnification. Tissues and growth plate zones are indicated. PO/BC, periosteum/bone collar; B, bone trabecular; PZ, proliferating zone; HZ, hypertrophic zone; M, marrow; C, cartilage; m, muscle. Scale bars for magnifications are indicated in all panels.](image-url)
activity was not detected near the articular surface or underlying hyaline cartilage. However, β-gal staining of the vertebral bodies after birth revealed numerous Runx2 P1-active cells throughout the tissue surrounding the core bone (supplemental Fig. S2, L and M). In contrast, there is a clear absence of β-gal-positive cells in the adjacent permanent cartilage of the intervertebral disc that separates each vertebral body. These findings show: 1) a temporal function of the Runx2 P1 promoter active at all stages of osteoblast lineage commitment for both intramembranous and endochondral processes; 2) that Runx2 P1 promoter activity is maximal at the onset of bone formation and becomes attenuated as bone matures; and 3) that Runx2-P1 driven lacZ gene expression in mesenchymal cells may be indicative of an epigenetic Runx2 spatial function by epigenetic control of cells destined for bone formation.

To identify potential defects in skeletal development resulting from loss of the Runx2 P1 protein, we examined selected bone tissues of newborn mice by Alizarin red and Alcian blue staining; supplemental Fig. S3A shows whole skeletal Alizarin red/Alcian blue staining, and Fig. 3 displays individual tissues. The strong P1 promoter activity observed in various bone tissues (Fig. 2) was reflected in abnormalities of ossification (Fig. 3, left panels). Staining of histological sections of calvarium, phalanges, and ribs for cartilage, bone, and mineral (by toluidine blue) further identified the bone defects (Fig. 3, right panels). The frontal, parietal, and interparietal bones of the calvaria in Runx2-P1lacZ/lacZ mice were not ossified, and heterozygous mice had an intermediate phenotype. Fibrous tissue represented the wide suture gap with disorganized connective tissue at the bone-forming front (Fig. 3A, right panels). The delay in bone formation was most apparent in the phalanges; the distal and proximal phalanges lacked ossification centers, and the metacarpal bones were just beginning to ossify in Runx2-P1lacZ/lacZ mice compared with WT (Fig. 3B). The ribs had a marked irregular curvature (Fig. 3C, arrow) and a lower ratio of bone to cartilage (1.8 ± 0.6) compared with WT (3.3 ± 1.3) or heterozygote (2.6 ± 0.9) mice (Fig. 3C), suggesting that production of Runx2 through the P1 promoter normally contributes to regular growth of the ribs. The vertebral bone bodies in Runx2-P1lacZ/lacZ mice were smaller and underdeveloped with respect to endochondral bone formation found in controls (Fig. 3C, right panels). At a higher magnification (supplemental Fig. S4A), the growth plates of the vertebral are expanded and trabecular bone has not formed in the homozygous mice, further supporting delayed ossification. Other severe skeletal defects were identified at birth in the hyoid bone and clavicles of Runx2-P1lacZ/lacZ mice (supplemental Fig. S3, B and C). Thus, examination of promoter activity through β-gal expression at the cellular level suggests that the endochondral and intramembranous bone abnormalities are due to defects in cells beginning at the earliest stages of osteoblast lineage commitment and differentiation. Taken together, these results suggest that expression from the Runx2 P2 promoter (type I) alone can sustain survival and growth to mature ages but is not sufficient to prevent CCD-like skeletal defects.

**FIGURE 3. Bony defects in neonatal Runx2 P1-deficient mice.** Alizarin red (bone) and Alcian blue (cartilage) staining of day 2 mice (left panels at ×5 magnification) and corresponding histological sections (right panels) are shown. The sections are stained with toluidine blue for bone and muscle (blue) and for cartilage (purple), combined with von Kossa stain for mineral (black). Left panels are top views of the calvaria (A). The dotted lines in WT and Runx2-P1lacZ/lacZ designate the area from which coronal sections are derived. Bone forms toward the midline suture in WT mice, but in Runx2-P1lacZ/lacZ mice, a thin layer of connective tissue is found. B, phalanges. The Runx2-P1lacZ/lacZ mice show fewer bony regions in the phalanges (arrowheads). Stained longitudinal sections of phalanges from 1-day-old pups are shown at left at ×2.5 and at right at ×10 magnification. C, rib cage with vertebral bodies. The bony areas of the vertebrae are outlined (white dashed line). The Runx2-P1lacZ/lacZ mouse shows irregular curvature in the ribs (arrow). Histological sections at ×5 magnification show underdeveloped vertebrae bone in Runx2-P1lacZ/lacZ mice.
FIGURE 4. Cellular defects contributing to delayed bone formation in Runx2 P1-deficient neonatal pups (2-day-old). Growth plate maturation and endochondral bone abnormalities in Runx2-P1 lacZ/lacZ mice are revealed by longitudinal sections of femur. A, demineralized bone stained with toluidine blue (purple, cartilage; blue, other tissues). The growth plate is outlined in red. B, mineralized bone stained with toluidine blue and von Kossa for mineral (black). C, alkaline phosphatase activity in hypertrophic chondrocytes and osteoblasts. D, TRAP staining, a marker of osteoclasts. E, Runx2 immunohistochemical staining of femurs with methyl green counterstain. Top panel, day 2 at \( \times 2.5 \); middle panel, day 9 at \( \times 10 \); bottom panel, day 14 at \( \times 20 \). PZ, proliferating zone; HZ, hypertrophic zone. F, growth plate area quantitation of WT versus Runx2-P1 lacZ/lacZ mice (n = 5 sections of n = 3 mice/group). G–I, gene expression profiles by qRT-PCR analysis of metaphysis and diaphysis from day 2 pups. G, growth plate markers; H, osteoclast markers; I, osteoblast markers. Values are mean ± S.E. of independent samples. A one-tailed t test assuming equal variances was performed, and p values are indicated.
Cellular and Molecular Basis for Defective Growth Plate Maturation and Reduced Cortical and Trabecular Bone Formation in Runx2 P1lacZ/lacZ Mice—To identify mechanisms contributing to the phenotype of these mice, we investigated the cellular basis for the delay in endochondral bone formation in the absence of Runx2 P1-derived protein expression (Fig. 4). Neonatal Runx2P1lacZ/lacZ pups exhibited normal cellular organization of both the hyaline cartilage in the epiphysis and the proliferating zone of the growth plate when compared with WT mice (Fig. 4A). However, the hypertrophic chondrocyte zone was consistently elongated in homozygotes (Fig. 4, A–E). The extended hypertrophic zone was 30% greater in the homozygous mice compared with WT (Fig. 4F, quantitation of all toluidine blue-stained sections). Although a calcified cartilage zone remained, there were very few trabecular representing the primary spongiosa where osteoblasts form bone on top of calcified cartilage. In contrast, the medullary cavity of WT mice had an abundance of trabecular bone (Fig. 4B). Similar to the long bones, the Runx2P1lacZ/lacZ vertebrae at birth have an extended hypertrophic zone (supplemental Fig. S4A). The functional activities of the osteoblasts and hypertrophic chondrocytes were revealed by AP activity, an early marker of cells is found in prehypertrophic and hypertrophic zones cells with AP activity, an early marker of cells that supports a calcifying matrix (Fig. 4C). Osteoclast activity was identified by histochemical staining for TRAP (Fig. 4D). On a per cell basis, the activity of both enzymes appears similar between WT and Runx2P1lacZ/lacZ tissue sections. However, there is a striking reduction of AP-positive cells in the marrow of Runx2P1lacZ/lacZ mice due to the absence of bone trabeculae. In Fig. 4D, osteoclast resorbing activity is abundant just below the growth plate and surrounding trabeculae in the marrow of WT mice. In contrast, few osteoclasts were found in Runx2P1lacZ/lacZ mice in the medullary cavity, a finding consistent with markedly reduced bone trabeculae number.

Because P1-driven β-gal activity is robust in developing growth plates, we investigated how loss of Runx2 P1-driven protein expression influences the observed phenotype during long bone formation. To probe this mechanism for the inability to complete the endochondral bone formation process to primary bone, we examined Runx2 protein localization in the Runx2P1lacZ/lacZ mouse in association with the growth plate abnormality observed in the neonatal femur (Fig. 4E, top panel). In the WT, total Runx2 protein (the sum of P1 and P2 activity) is found in prehypertrophic and hypertrophic zones cells with robust protein surrounding all trabeculae in the marrow and appears as well in osteoblasts along the cortical bone surfaces. In contrast, in Runx2P1lacZ/lacZ mice, where Runx2 protein can be attributed entirely to activity of the P2 promoter, there is a striking reduction of Runx2 protein in growth plate cells. However, on day 9 (Fig. 4E, middle panels) the proliferating chondrocytes and pre-hypertrophic zone cells exhibit Runx2 protein in the Runx2P1lacZ/lacZ mice. The primary spongiosa (calcified cartilage spicules) in the marrow under the growth plate now have detectable Runx2 protein, but bone trabeculae are not forming. By 14 days the robust Runx2-positive cells observed around trabeculae in the WT are clearly lacking around the trabeculae in the Runx2P1lacZ/lacZ mice. Thus, Runx2 P1 promoter activity appears to be responsible for the vast majority of Runx2 protein production at the growth plate for endochondral bone formation from osteoprogenitor cells in bone marrow. Together, these findings suggest that expression from the P1 promoter is required for the appropriate transition from mineralized cartilage to primary bone spongiosa.

Molecular defects in Runx2 P1-mediated bone formation were revealed by expression of Runx2 target genes. Fig. 4G shows increased levels of collagen type X in Runx2P1lacZ/lacZ mice, which correlates with the extended prehypertrophic zone of the growth plate that was reported previously in Runx2 null mice (43). We also found that Runx2P1lacZ/lacZ mice have reduced levels of VEGF, which can retard maturation of the growth plate due to decreased vascularization (44, 45). RANKL, which is up-regulated by Runx2 and produced by osteoblasts to activate bone resorption (46), was reduced, consistent with the reduced osteoclasts at the growth plate and primary spongiosa region (Fig. 4H). Notably, osteoprotegerin, also an osteoblast product and an inhibitor of osteoclast differentiation, was modestly reduced in Runx2P1lacZ/lacZ mice. Thus, the decreased trabecular bone mass in Runx2P1lacZ/lacZ mice is not due to increased bone resorption by osteoclasts. There was a significant reduction in the expression of osteoblast lineage-specific genes including Osterix, Col1a1, and OC in Runx2P1lacZ/lacZ mice compared with WT (Fig. 4I). Osterix (Osx), a transcription factor also essential for bone formation that is expressed developmentally after Runx2 (47), was reduced by 32% in the bones of homozygotes when compared with WT mice. These findings suggest that the absence of Runx2 expression from the Runx2 P1 promoter results in a decreased pool of osteoprogenitors that will mature into osteoblasts.

Physiological Consequences of Loss of Runx2 P1 Protein Expression on Bone Structure—Because these mice lived a normal life span, we determined whether the cellular and molecular defects in bone formation at birth persisted in the adult mouse (Fig. 5). Indeed, histologic sections of mineralized bone show that less trabecular bone is present and a thinner cortex is found in the mutant homozygous skeleton (Fig. 5A, top panel), but the osteoblasts and osteoclasts show similar enzymatic activity per cell in the Runx2P1lacZ/lacZ mice compared with WT (Fig. 5A, middle and bottom panels). Thus, analogous to the newborn, and due to the fewer numbers of trabeculae in Runx2P1lacZ/lacZ mice, there were fewer AP- and TRAP-positive cells throughout the bone sections. Therefore, to address a mechanism contributing to this persistent osteopenia related to bone formation, dynamic measurements of the rate and amount of newly synthesized bone were performed by calcein labeling studies in adolescent 5-week-old mice (Fig. 5B). This analysis revealed a striking 75–80% decrease in trabecular and cortical bone formation in homozygotes on the endosteal and periosteal surfaces of the cortical bone (membranous bone formation process) (Fig. 5C). Thus, the reduced amount of bone trabeculae and cortical thickness in homozygous mice is due to an impaired rate of bone formation. This finding further suggests that defects in recruitment of MSCs from the marrow and peristium into the osteoblast lineage in the postnatal skeleton are a result of Runx2 P1 loss of function.

We qualitatively and quantitatively assessed the structure of bone that is formed by microCT three-dimensional reconstructions of the vertebrae and femurs (Fig. 5, D–F). The femur
length was shorter and the cortical thickness and segmented bone area were significantly reduced (Fig. 5G). The tissue density measurement, which reflects the hydroxyapatite content of the mineralized bone, did not differ between WT and homozygous mice for both cortical bone and trabecular bone (data not shown). These results indicate that normal mineral content was formed in the bone tissue but that less bone formed in Runx2-P1lacZ/lacZ mice. However, the apparent bone density, which
reflects the amount of mature bone, was reduced in the Runx2-P1\textsuperscript{lacZ/lacZ} mice in the trabecular bone of the femur (Fig. 5\textit{H}) as well as in the cortical bone (data not shown). There was a 22\% reduction in trabecular number, complemented by a 30\% increase in trabecular spacing, in Runx2-P1\textsuperscript{lacZ/lacZ} mice (Fig. 5\textit{H}). The vertebrae also exhibited this osteopenia (supplemental Fig. S4\textit{B}), and significantly, an extended growth plate was observed in vertebral bodies of the 5-week-old mice, which is consistent with newborns (supplemental Fig. S4\textit{C}). At 5 months of age, microCT showed that the Runx2-P1\textsuperscript{lacZ/lacZ} mice continued to have osteopenia and decreased bone volume measurements (data not shown).

Taken together, the quantitative bone parameters demonstrated that the osteopenia defect, first observed in perinatal Runx2-P1\textsuperscript{lacZ/lacZ} mice, is not corrected during adolescent and adult growth. The results indicate a retarded bone formation rate that reduces bone mass, not from increased osteoclastic resorption of bone but from loss of bone accrual as a consequence of the absence of the Runx2 P1 transcript.

**Decreased Osteoprogenitor Numbers and Cell-autonomous Defects in Osteoblast Maturation Occur in Runx2-P1\textsuperscript{lacZ/lacZ} Mice**—Calvarial bone is formed directly by the membranous bone process and thus enriched in osteoblast subpopulations. To provide direct evidence that defects in osteoblast functions are related to Runx2 P1 loss of function, we examined molecular markers of osteoblast differentiation at two ages (Fig. 6, day 2 and week 6). Measurement of each Runx2 transcript and total Runx2 mRNA levels revealed that total levels are reduced by 50\% in perinatal mice and by 75\% at 6 weeks in homozygotes compared with WT (Fig. 6\textit{A}). This decrease results in a more porous, thinner calvarium with total bone mass reduced by 40\% (supplemental Fig. S5). At both time points, several markers of committed osteoblasts, including AP and osterix (Osx), remained strikingly reduced (Fig. 6\textit{B}). With the exception of \textit{Col1}a1, which was not significantly altered at day 2 among WT, heterozygous, and homozygous mice, the matrix proteins that accumulate in mineralized bone (bone sialoprotein, osteopontin, and osteocalcin) were significantly reduced in the Runx2-P1\textsuperscript{lacZ/lacZ} neonates. However, at 6 weeks, these markers of mature bone were present at more normalized levels. These findings indicate that the few osteoblasts present in bone, despite reduced synthesis of matrix proteins initially, ultimately produce sufficient extracellular matrix for mineralized tissue formation.

To further characterize a cell-autonomous defect in Runx2-deficient cells, we first evaluated the osteoprogenitor representation in bone marrow cells isolated from 6-week WT and Runx2-P1\textsuperscript{lacZ/lacZ} mice by examining the number of osteoblast colony-forming units (CFUs) they produced (Fig. 7). Although the majority of CFUs from WT became positive for AP by day 7 after plating, cells from Runx2-P1\textsuperscript{lacZ/lacZ} mice were compromised (Fig. 7\textit{A}). A 50\% reduction in osteoprogenitor cells was quantitated (Fig. 7\textit{A}, right panel). This finding further supports an early lineage effect in that mice lacking the Runx2 P1 transcript have an overall reduced number of bone marrow-derived stromal cells capable of commitment to the osteogenic lineage.
Osteoblast differentiation to the final stage of mineralized matrix formation was further examined by ex vivo differentiation of calvarial osteoblasts from newborn WT and Runx2-P1lacZ/lacZ mice. Runx2-P1lacZ/lacZ cultures displayed a decreased number of AP-positive cells (Fig. 7B) and no evidence of multilayered nodules as seen in WT. At three time points representing proliferation (day 4), matrix maturation (day 10, peak AP), and mineralization (day 19, peak OC), all markers in Runx2-P1lacZ/lacZ cells were decreased relative to WT (Fig. 7C). This trend was particularly prominent for Runx2 and AP on day 10 and for OC on day 19. However, in Runx2-P1lacZ/lacZ cells, there was a 2–3-fold increase of total Runx2 and P2-driven Runx2 levels on day 19 compared with day 10. WT osteoblasts show the expected peak expression of Runx2 on day 10 and attenuation on day 19. This finding reflects a delay in bone formation, with increased total Runx2 transcripts equivalent on day 19 in the homozygous cells to the day 10 levels in the WT. This delay is confirmed by decreased expression of early and late bone markers (Fig. 8C). Taken together, the inability to generate sufficient numbers of committed osteoprogenitors along with the reduced maturation of osteoblasts in the absence of Runx2 P1 expression is concordant with our in vivo observations.

Because the Runx2-P1lacZ/lacZ bone can eventually mineralize (normal bone density by microCT), we addressed a contributing compensatory mechanism by examining whether Runx2-P1lacZ/lacZ cells are able to respond to osteogenic signals. Calvarial osteoblasts were treated with BMP-2 or Wnt3a after 14 days of differentiation. In response to BMP-2, expression of Osterix, AP, and Dlx5 mRNAs were all increased severalfold in both WT and homozygous cells (Fig. 7D). These results indicate that, in Runx2-P1lacZ/lacZ cells, BMP-2-responsive proteins that are linked to osteogenesis contribute to osteoblast differentiation. As expected, adding BMP-2 did not increase the gene expression levels of osteocalcin, which requires Runx2 for activation. Runx2-P1lacZ/lacZ cells also remained responsive to Wnt signaling, as evidenced by the increase in TCF4 levels. This study thus demonstrates that Runx2-P1lacZ/lacZ calvarial osteoblasts exhibit a cell-autonomous developmental defect in osteoblast differentiation but that the cells are competent to respond to external osteogenic signals.

Both in vitro and in vivo data demonstrated that membranous bone formation did not return to normal in postnatal mice. To probe whether bone renewal was indeed defective in adult mice, we examined a well established model of membranous bone repair by introducing a defect into the cortical bone.
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FIGURE 8. In vivo model of bone repair recapitulates delayed intramembranous bone formation in the absence of the Runx2 P1 isoform. Longitudinal sections stained with toluidine blue are shown from the tibia of Runx2-P1+/+ and Runx2-P1lacZ/lacZ female mice at 7 (A) and 14 (B) days after drill hole defect. Scale bars represent 1 mm. C, qRT-PCR analysis of drill hole site 7 days after defect in Runx2-P1+/+ (white bars) and Runx2-P1lacZ/lacZ (black bars) mice. Error bars represent S.E. A one-tailed t test assuming equal variances was performed. *, p ≤ 0.05; and **, p ≤ 0.01, when compared with Runx2-P1+/+ mice.

A +/+ lacZ/lacZ

B +/+ lacZ/lacZ

C

DISCUSSION

Here we have shown that expression of the Runx2 transcript from the P1 promoter is essential in mesenchymal cells for commitment of multipotent skeletal cells to osteoblasts. This function cannot be compensated for by the P2-driven transcript, which functions to support low levels of Runx2 expression prior to the appearance of Runx2 P1 promoter activity. As a result of this spatiotemporal function of the P1 and P2 promoters, loss of Runx2 P1 protein results in a CCD phenotype with severe osteopenia due to decreased osteoblast lineage cells throughout the life span of the homozygous mouse.

This phenotype is caused by a combination of factors including: loss of Runx2 P1-driven protein in preosteogenic mesenchyme where the Runx2 P1 promoter exhibits robust expression; the inability of Runx2-P1lacZ/lacZ mice to generate sufficient committed osteoprogenitor cells to support normal bone formation; cell-autonomous defects in differentiation of ex vivo calvarial osteogenic cells isolated from Runx2-P1lacZ/lacZ mice; and the reduced expression of Runx2 target genes. These mechanisms both contribute to and reflect the phenotype of the developing Runx2-P1lacZ/lacZ mice and persistence of the phenotype in adult mice. All of this evidence points to an inability of Runx2-P1lacZ/lacZ mice to produce an adequate number of osteoblasts for normal bone formation. This conclusion is consistent with a role for activation of the Runx2 P1 promoter early in development, “marking” cells in the mesenchyme for osteogenesis for spatiotemporal events of bone formation. Novel epigenetic roles for Runx2 have been established, including the retention of Runx2 on mitotic chromosomes to maintain phenotype stability of osteoprogenitors during cell proliferation (48). Thus, a specific and primary function of Runx2 P1 that cannot be compensated for by Runx2 P2 protein is the requirement for early recruitment of MSCs into the osteoblast lineage.

Monitoring the spatial and temporal activation of the P1 promoter through β-galactosidase staining revealed that the P1 promoter is active in areas where progenitor cells must commit to the osteogenic lineage, such as the precartilaginous mesenchymal anlagen of skeletal elements in E13.5 embryos and the perichondrium of the developing bone collar in postnatal growth and periosteum, as well as the hyaline cartilage of the developing vertebral body that becomes endochondrally-formed bone. Strong activation of the P1 promoter corresponded to sites of delayed development (e.g., the developing bone collar, hypertrophic zone, and primary spongiosa). The mouse model described by Otto et al. (2, 33) allowed for visualization of the combined P1 and P2 promoter activities. A comparison of their findings with our findings in the P1Runx2lacZ/lacZ mouse suggests that the P2 promoter is active earlier than the P1 promoter in the developing embryo (E10.5), hence supporting the epigenetic functions of Runx2 in the skeleton. However, by E12.5–E13.5 the two mouse models both show promoter activation in similar bone tissues (33). In contrast, a transgenic mouse model expressing only the Runx2 P1 proximal promoter (3 kb) fused to the lacZ gene was found to have very limited expression, restricted to undifferentiated mesenchymal condensations of the axial skeleton (49). Therefore, the 3-kb P1 promoter does not share the same spatiotemporal regulation as the full-length promoter, and thus important regulatory elements essential for expression of Runx2 P1-driven protein in osteoblast lineage cells are located upstream beyond the 3-kb promoter.

One key finding of this study is that the loss of Runx2 P1 mRNA reduces osteogenic activity at precise temporal intervals required for commitment to osteoblasts. This molecular defect of the tibia (40). Seven days after bone injury, WT mice showed a rapid healing response with a large callus at the defect site that consisted primarily of woven bone (Fig. 8A). The bone continued to remodel after 14 days (Fig. 8B), and by day 28, the site was completely healed (data not shown). In contrast, Runx2-P1lacZ/lacZ mice showed a delayed and less robust healing response. The callus was smaller in size at 7 and 14 days after defect (Fig. 8C) and contained a small amount of woven bone and mainly cartilage tissue (which is unusual for this model of bone repair). However, at 28 days, the site had healed (data not shown). Molecular markers at the defect site were analyzed after 7 days (Fig. 8C). As expected, the Runx2 P1 transcript was not detected in Runx2-P1lacZ/lacZ mice. The Runx2-P1lacZ/lacZ mice showed increased expression of markers of remodeling and vascularization (MMP-9 and VEGF) and a reduction in the expression of the late bone marker, OC. These changes identify a delayed healing response. Taken together, the histological and molecular analyses demonstrated that defects in intramembranous bone formation persist in adult Runx2-P1lacZ/lacZ mice.

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reduces tissue formation and causes delays in osteoblast maturation and bone formation. In addition, the phenotype is compounded by low levels in vivo of the transcription factor Osterix, a downstream target of Runx2, which is also essential for completion of bone formation. Although there is a defect in recruiting a sufficient number of committed osteoblasts from MSCs through P1 promoter activity, cells that successfully commit to the osteogenic lineage can produce a matrix, albeit at a slower rate, that is competent to mineralize. Interestingly, osteoblasts do remain responsive to osteogenic signals such as Wnt and BMP-2 signaling. The latter strongly induces Osterix in Runx2 P1 null cells. Expression of Runx2 protein through the P2 promoter may also contribute to osteoblast maturation. As a result, these pathways can contribute to the formation of bone tissue in vivo, although the amount of bone is clearly diminished.

Although the P2-driven protein is sufficient for the formation of a mineralized skeleton, Runx2-P1lacZ/lacZ mice have developmental defects that lead to a CCD-like phenotype that persists in the adult. Human CCD is characterized by defects in cranial development, small or missing collar bones, dental abnormalities, and a shorter stature (33, 37, 50–53). The CCD-like phenotype we observed in the Runx2-P1lacZ/lacZ mouse is more closely related to abnormalities in the clinical disorder than other mouse models of Runx2 genetic inactivation that result in embryonic or early postnatal lethality. The bone formation we found in our Runx2-P1lacZ/lacZ mouse may result from the lack of Runx2 P1-driven expression, the haploinsufficiency of total Runx2 levels or some combination of these two possibilities. In fact, the phenotype of the Runx2-P1lacZ/lacZ mouse, in which we observed a 50% reduction in total Runx2 transcripts, is reminiscent of the heterozygotes of the pan-Runx2 null mouse models (1, 2). The similarities include a lack of ossification centers in the phalanges, defects in cranial bone formation resulting in an open anterior region, and delays in hyoid bone formation (2). A distinguishing feature is the absence of clavicles in the pan Runx2+/- mice, whereas Runx2-P1lacZ/lacZ mice have defective clavicles, analogous to a Runx2 haploinsufficient mouse model that exhibits similarly distorted and underdeveloped clavicles (28).

A comparison of our model with those of previous studies is instructive in understanding the biological roles of P1 and P2 promoters in their in vivo native context. Interestingly, our findings of P1 promoter activation in the calvarial mesenchyme appear to contrast with the studies of Park et al. (20), who examined the mRNA levels of the isoforms by in situ hybridization. Their results show the P2-derived transcript as the predominant form present in mesenchymal sutures of the calvaria, with the P1-derived mRNA completely absent. However, our mutant mice had very little bone tissue and undefined suture lines. Thus, our β-gal staining in developing calvarial tissue and during intramembranous bone repair suggests that Runx2 P1-driven protein is critical not only for intramembranous bone formation but also for the development of normal tissue organization of bone and suture tissue in the calvarium.

This study investigated the specific consequences of the loss of Runx2 P1 protein function in a biological context in which the P1 promoter was still subject to regulation by factors that influence its transcription. Based on β-gal staining that is driven by the P1 promoter, we have demonstrated the unique expression patterns of the P1 and P2 promoters driving expression of Runx2 type II and type I, respectively. Significantly, there are striking contrasts between the phenotype of our mouse model and the model described by Xiao et al. (31, 32) in which the P1 promoter was deleted and expression of the type II isoform was inactivated but no reporter expression was studied. We did not find compensation of Runx2-II loss by increased levels of Runx2-I or other Runx factors as reported (32). In the Runx2-P1lacZ/lacZ mice, Runx1 and Runx3 expression was reduced by ~50%, indicating that subpopulations of skeletal lineage cells expressing Runx1 and Runx3 are not present in adequate numbers (41, 54, 55). Further, our study found that loss of Runx2 protein expression through the P1 promoter does not impair survival of homozygous mice, whereas Xiao et al. (32) found that more than 90% of the mutant mice died within 6 weeks after birth, a result that could not be explained. Xiao et al. (31, 32) propose that the phenotype of their mouse lacking Runx2-II is due to differential functions of the two Runx2 isoforms. It is conceivable that the promoter deletion they genetically engineered may have generated an epigenetic alteration in the Runx2 locus. This idea is supported by our finding that mice survive when the Runx2 P1 promoter remains active in its endogenous context. Further, there is no conclusively published evidence to indicate that the 19-amino acid difference between the two proteins changes Runx2 function through gain or loss of a biochemical activity or unique protein/protein interaction. The remaining residues may be a minor vestige of the two promoters producing Runx2 protein. We propose a different conclusion based on the lacZ reporter expression in our model. Rather, it is the timing, location, and level of expression of Runx2-II and -I specifically driven by the P1 and P2 promoters, respectively, that regulate bone formation.

In conclusion, these studies have revealed the distinct role of the Runx2 P1 promoter in osteoblast commitment and maturation. Our findings provide compelling evidence that the structure of the Runx2 gene, with two promoters driving expression of transcripts encoding highly similar proteins, exists not to provide proteins with individualized function but rather to support distinct spatial and temporal patterns of Runx2 with appropriate expression levels in specific populations of osteogenic lineage cells.

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