CONDITIONAL INACTIVATION OF CXCR4 IN OSTEOPRECURSORS REDUCES POSTNATAL BONE FORMATION DUE TO IMPAIRED OSTEOBLAST DEVELOPMENT

Wei Zhu12*, Gang Liang1, Zhiping Huang1, Stephen B. Doty1, Adele L. Boskey13,
1 Musculoskeletal Integrity Program, Hospital for Special Surgery, New York, NY 10021.
2 Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY 10021.
3 Department of Biochemistry, Weill Cornell Medical College, New York, NY 10021.

Running Title: CXCR4 in bone development

*Address correspondence to: Wei Zhu PhD, Room 624, Caspary Research Building, Hospital for Special Surgery, 535 East 70th Street, New York, NY, 10021, Tel. 2127747017; Fax. 2127747877; E-mail: zhuw@hss.edu.

Abstract
Cystine (C)-X-C motif chemokine receptor 4 (CXCR4), the primary receptor for stromal derived factor-1 (SDF-1), is involved in bone morphogenic protein 2 (BMP2)-induced osteogenic differentiation of mesenchymal progenitors. To target the in vivo function of CXCR4 in bone and explore the underlying mechanisms, we conditionally inactivated CXCR4 in osteoprecursors by crossing osterix (Osx)-Cre mice with floxed CXCR4 mice (CXCR4fl/fl) to generate knockouts with CXCR4 deletion driven by the Osx promoter (Osx::CXCR4fl/fl). The Cre-mediated excision of CXCR4 occurred exclusively in bone of Osx::CXCR4fl/fl mice. When compared to littermate controls, Osx::CXCR4 fl/fl mice developed smaller osteopenic skeletons, evidenced by reduced trabecular and cortical bone mass, lower bone mineral density, and a slower mineral apposition rate. In addition, Osx::CXCR4fl/fl mice displayed chondrocyte disorganization in the epiphyseal growth plate, associated with decreased proliferation and collagen matrix syntheses. Moreover, mature osteoblast-related expression of type I collagen α1 (Col1α1) and osteocalcin (OCN) was reduced in bone of Osx::CXCR4fl/fl mice versus controls, suggesting that CXCR4-deficiency results in arrested osteoblast progression. Primary cultures for osteoblastic cells derived from Osx::CXCR4fl/fl mice also showed decreased proliferation and impaired osteoblast differentiation in response to BMP2 or BMP6 stimulation, and suppressed activation of intracellular BMP receptor-regulated Smads (R-Smads) and Erk1/2 was identified in CXCR4-deficient cells and bone tissues. These findings provide the first in vivo evidence that CXCR4 functions in postnatal bone development via regulating osteoblast development in cooperation with BMP signaling. Thus, CXCR4 acts as an endogenous signaling component necessary for bone formation.

Introduction
Cystine (C)-X-C motif chemokine receptor 4 (CXCR4) is the primary transmembrane receptor for signaling chemokine stromal derived factor 1 (SDF-1, also named CXCL12 or pre B cell stimulating factor (PBSF)) (1-4). Both CXCR4 and SDF-1 are highly conserved in various mammalian cell types, and have broad functions in cell proliferation, morphogenesis and migration. The CXCR4-/- and SDF-1 -/- mice die in utero or perinatally due to multiple defects in developing brain, heart, vasculature, intestine and hematopoietic tissues (5-7). Mutations at the carboxyl terminus of CXCR4 gene also lead to the WHIM syndrome in humans, a complex immunodeficient disease associated with neutropenia and defective B cell development (8). The binding of SDF-1 to CXCR4 induces cytoskeleton rearrangement and integrin activation, and eventually results in the migration of CXCR4-expressing cells towards high gradients of SDF-1 (9-16). This SDF-1/CXCR4-mediated chemotaxis is involved in a variety of physiological and pathological events including the blood homeostasis (9), cellular inflammatory and immune response (10), bone remodeling (11), homing of stem/progenitors to bone marrow reservoir (17), tumor metastasis to bone or other organs containing high levels of SDF-1 (18), and cell recruitment in injured tissues (15, 19-24).
Previous studies suggested the expression of CXCR4 and SDF-1 in bone; however, their direct function as well as underlying mechanisms remained poorly defined. This is probably due to the absence of demonstrable reports about skeletal abnormalities in patients with CXCR4 mutations or mice lacking CXCR4 or SDF-1. In fact, accumulating evidence has suggested the intimate association of the SDF-1/CXCR4 pathway with progenitor cells that have potentials to become bone-producing osteoblasts or form bone. Expression of SDF-1 and CXCR4 is found in both mesenchymal cell cultures (15, 25-27) and bone sections (28-32), with greater levels in less differentiated cells or immature osteoblasts relative to mature osteoblasts and osteocytes. High levels of SDF-1 are also present in regions of perichondrium of embryonic bones and periosteum of injured bones, where osteoprogenitors arise and differentiate (15, 28-32). In addition, retrovirus-mediated over expression of SDF-1 in human MSCs was found to enhance the MSCs’ induction of ectopic bone formation in nude mice (27). In mesenchymal cultures, such as the human and mouse bone marrow-derived stromal cells (33), C2C12 and ST2 cells (25), we demonstrated that blocking of the SDF-1/CXCR4 signal axis inhibits the differentiation of these cells towards the osteoblastic lineage in response to bone morphogenic protein 2 (BMP2) stimulation. This reveals the direct involvement of the SDF-1/CXCR4 pathway in osteogenic differentiation in vitro. Moreover, recent evidence showing the effect of CXCR4 and SDF-1 on enhancing chondrocyte hypertrophy at the chondro-ossseous junction of long bone further implicates the involvement of SDF-1 signaling in endochondral ossification (34). In light of these findings, we hypothesized that SDF-1 signaling functions in bone formation via affecting osteoblast development.

To address the direct role of SDF-1 signaling in bone formation in vivo, in this study, we used the Cre/loxP genetic approach to conditionally remove CXCR4 from osteoblastic lineage precursors under the control of an osterix (Osx) promoter. Osx (35) is a zinc-finger domain-containing transcription factor, downstream of Runx2, mainly expressed by osteoprecursors during early phases of osteogenic differentiation. Osx is considered one of the “master” regulators for bone formation, because Osx<sup>−/−</sup> mice (35), like Runx2<sup>−/−</sup> (36, 37), develop no bone in their skeletons. Via this Osx-controlled conditional deletion of CXCR4, we demonstrated the requirement for CXCR4 in bone formation of the mouse skeleton. Moreover, we identified the function of CXCR4 in regulating osteoblast activities, and the interaction of CXCR4 with BMP signaling in this process.

**Experimental Procedures**

**Antibodies and reagents:**
Anti-CXCR4 and anti-Osx antibodies were purchased from eBioscience (San Diego, CA) and Abcam (Cambridge, MA), respectively. Antibodies for type X collagen α1 (Col10α1), ProCol1α1, and proliferating cell nuclear antigen (PCNA) were purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-osteocalcin (OCN) was purchased from Millipore (Billerica, MA). Antibodies for Smad1/5/8, phosphorylated Smad1/5, total Erk, phosphorylated Erk1/2, and β-Tubulin were obtained from Cell Signaling (Danvers, MA). Anti-phosphorylated Smad2 was purchased from Rockland Immunochemicals (Gilbertsville, PA). Antibodies for SDF-1, Col2α1, isotype-matched control antibodies and secondary goat, sheep or rabbit IgGs were from Santa Cruz (Santa Cruz, CA). Recombinant human (rh) BMP2 and BMP6 proteins were purchased from R&D Systems (Minneapolis, MN). General chemicals were from Sigma (St. Louis, MO). All cell culture media and supplements were from Gibco (Invitrogen, Carlsbad, CA).

**Animals and genotyping:**
All animal experiments were carried out following review and approval by IACUC at The Hospital for Special Surgery. CXCR4<sup>fl/fl</sup> mice (38-40), in which CXCR4 gene is flanked by loxP sequences, were provided by Dr. Yong-Rui Zou (Columbia University, New York, NY). Osx-Cre mice (41, 42), in which the expression of a tetracycline (Tet)-off regulatable GFP/Cre fusion protein is transcriptionally controlled by an Osx promoter, were purchased from The Jackson Laboratory (Bar Harbor, Maine). These two strains were crossed and maintained on a C57BL/6J background. We first generated double heterozygous mice for Cre and floxed CXCR4
(Osx-Cre;CXCR4fl/+) mice, which were then bred to CXCR4fl/fl mice via a back-mating strategy to generate excised floxed CXCR4 homozygous (Osx::CXCR4fl/fl, used as conditional knockouts), along with heterozygous for floxed CXCR4 (Osx-Cre;CXCR4fl/+ ) and Cre-null mice (CXCR4fl/+ and CXCR4fl/fl, used as littermate controls throughout the study). These mice were born at the expected Mendelian frequency. As additional controls, Osx::CXCR4fl/fl mice were fed with 200µg/ml doxycycline (Tet analog, Sigma) in drinking water, which prevents the Osx promoter from driving Cre expression, as suggested by the manufacturer.

For genotyping, genomic DNA was isolated from tail tips using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). PCR reactions were performed using PCR Master Mix (Fermentas, Glen Burnie, MI) with primer sequences for Cre transgene and floxed CXCR4 gene relative to wild type CXCR4 as shown in Table 1.

X-ray and micro-computed tomography (µCT) analyses:
For skeletal analysis, mice were sacrificed by CO₂ inhalation, and x-rays (40Kv, 4mAS, SID130, Faxitron, Lincolnshire, IL) were obtained in both lateral and anterior-posterior views to assess the spinal length and the femoral/tibia length, respectively, in the mouse skeleton.

µCT (Scanco Medical Micro CT 35) was performed on both calvarias and tibias of mice. Entire calvarias and tibias were scanned (isotropic resolution of 15-20µm) to obtain grayscale images, which were Gaussian-filtered and globally thresholded (15.2% of maximum gray value) to form binarized images for morphological analyses. Quantitative volumetric analyses of cortical and trabecular microstructures were performed on entire parietal calvarias and on a region of 200 micro tomographic slices (2.1mm) at the proximal tibia with an isotropic resolution of 6µm. These analyses measured cortical and trabecular bone volume fraction, thickness, and bone mineral density, as well as trabecular number, separation and connectivity density.

Double fluorochrome labeling and mineral apposition rate (MAR):
To measure bone mineralization in vivo, mice were subcutaneously and sequentially injected with xylene orange (orange red) and tetracycline (green) at 20µg/g (body weight) each in a 4-day time interval. One day after the second injection, tibias were collected and fixed in 80% ethanol, and then subjected to polymethyl methacrylate (PMMA) embedding and sectioned to 8-10µm.

Histomorphometry analysis using OsteoI software (Bioquant, Nashville, TN) was performed on tibia sections to measure the distance between two fluorochrome-labeled mineralization fronts at the midshaft of tibia. Periosteal MAR was calculated by dividing the measured distance with the time interval.

Histology, immunohistochemistry, and histomorphometry:
For phenotypic analysis, tibias including knee joints or calvarias were fixed in sodium phosphate-buffered 4% paraformaldehyde at 4°C for 2-4 days, decalcified in 5% EDTA, and transferred to 70% ethanol until paraffin-wax embedding. These tissues were sectioned to 4-5µm and subjected to alcian blue staining; and PMMA-sections were subjected to von Kossa staining following the standard histology protocols.

To reveal patterning changes in cartilage and bone of the entire skeleton, E18.5 whole embryos of mice were sequentially stained with alcian blue (for cartilage) and alizarin red (for bone) as described in previous studies (43). In short, after fixation with 95% ethanol for 48-72 hours, embryos were stained with 0.03% (w/v) alcian blue in 80% ethanol and 20% acetic acid solution for 1-3 days, followed by 0.03% (w/v) alizarin red in 1% KOH solution for another 12-24 hours, and then maintained in a solution of 2% KOH:glycerol (20:80) until analysis.

For immunohistochemistry, deparaffinized sections were treated with antigen retrieval in heated 10mM citrate buffer (pH6.0), and then incubated with the primary antibody at 4°C overnight. On the second day, sections were incubated with appropriate secondary antibodies for 30-60 minutes at room temperature, and developed with DAB or TMB as the chromogen, following the manufacturer’s instructions (Vector Laboratories, Burlingame, CA).

After histology or immunohistochemistry, histomorphometric analysis (Bioquant) was performed on tissue sections to quantify the number of positively stained cells per region of
interest or per bone surface and the fraction of mineralized bone, following the manufacturer’s protocols.

**RT-PCR, real time PCR, and Western blotting:**
Total RNA of mouse tissues was extracted using the RNeasy Extraction Kit (Qiagen), and the equal amount of 1μg of total RNA per sample was reverse-transcribed (First strand RT kit, Fermentas). Regular PCR or quantitative real time PCR (qPCR) was performed using PCR Master Mix (Fermentas) or SYBR Green Supermix (Bio-Rad, Hercules, CA) with primer sequences listed in Table 1. The level of target gene expression was normalized to the level of the housekeeping gene, GAPDH (25, 33).

Western blotting was performed as previously described (25, 33). In short, whole cell lysates were assayed for the amount of total cellular protein (Pierce bicinchoninic acid assay, Thermo Scientific, Rockford, IL). Equal loading of 20μg aliquots of total protein from each sample were fractionated on 12% Bis–Tris gels, following the manufacturer’s protocol (Invitrogen, Carlsbad, CA). After incubating with appropriate antibodies, immunoreactive bands were visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Piscataway, NJ). The intensities of bands were measured using gel image analysis software (ImageJ_1.32, NIH).

**Primary calvaria culture, proliferation, and differentiation assay:**
Calvarias were dissected from newborn (1-3 days after birth) mice, rinsed with PBS, and digested in freshly made 1mg/ml Collagenase/Dispase mixture (Roche, South San Francisco, CA) in αMEM medium at 37°C for 20 minutes and repeated three times. After digestion, supernatants were combined and centrifuged to pellet cells. Cells were then cultured in αMEM supplemented with 10% FBS and 1% penicillin/streptomycin (all from Gibco). Medium was replaced every 2-3 days.

Proliferation of calvaria cells was measured by evaluating cell cycle progression using bromodeoxyuridine (BrdU) incorporation assays (Chemicon, Billerica, MA) as the manufacturer describes.

To induce osteoblast differentiation, calvaria cells were stimulated with rhBMP2 or rhBMP6 at 100ng/ml for 4 and 7 days. ALP activity and OCN synthesis were evaluated in these cells as markers for immature and mature osteoblasts, respectively. As previously described (25, 33), ALP activity was measured in cell lysates using a colorimetric assay (Sigma), and was normalized to the level of total cellular protein. OCN synthesis was measured in culture medium, using a commercially available ELISA kit (Biomedical Technologies, Stoughton, MA).

**Serum OCN and tartrate-resistant acid phosphatase (TRAP) assays:**
OCN levels in mouse serum were also measured using ELISA kits from Biomedical Technologies. Serum TRAP, a marker of bone resorption, was measured using the ELISA-based mouse TRAP assay kit from Immunodiagnostic Systems (Fountain Hills, AZ).

**Statistical analysis:**
Experiments were repeated two to three times independently, with five to six samples included in each experimental group. All quantitative measurements were reported as the mean ± standard deviation (SD) calculated using SigmaPlot8 or Instat statistical software. Differences between two experimental groups were compared by One Way ANOVA or Student’s *t*-test, with significance accepted at *p*<0.05.

**Results**

1) **Bone-specific deletion of CXCR4 in Osx::CXCR4 floxed (f/f) mice:**
Osx::CXCR4 f/f mice and littermates were genotyped for the presence of Cre transgene and floxed CXCR4 gene relative to wild type CXCR4 (Figure 1A). To validate the Osx-controlled CXCR4 deletion, we performed RT-PCR to examine CXCR4 expression in several tissues derived from newborn Osx::CXCR4 f/f mice and Cre-null littermate controls (equivalent to the use of wild type). When compared to control mice, loss of CXCR4 gene was restricted to long bones of Osx::CXCR4 f/f mice that expressed Osx; while no CXCR4 deletion relative to the housekeeping gene, GAPDH, was observed in soft organs (kidneys and livers) of Osx::CXCR4 f/f mice that did not express Osx (Figure 1B).
We also performed Western blotting to compare the protein level of CXCR4 in osteoblastic cells derived from calvarias of newborn Osx::CXCR4^{fl/fl} mice with that of littermate controls. Similar Osx expression was detected in calvaria cells of Osx::CXCR4^{fl/fl} mice as that of controls; however, reduced CXCR4 expression relative to the β-Tubulin control was observed in calvaria osteoblasts of Osx::CXCR4^{fl/fl} mice versus cells of control mice (Figure 1C). Collectively, these data suggest the bone-specific deletion of CXCR4 in Osx::CXCR4^{fl/fl} mice.

2) Skeletal alterations in Osx::CXCR4^{fl/fl} mice:

To evaluate the effect of CXCR4 deficiency on the development of mouse skeleton, we compared Osx::CXCR4^{fl/fl} mice with their sex-matched littermate controls by high resolution x-ray and quantitative µCT. A gross assessment for reduced skeletal size in growing Osx::CXCR4^{fl/fl} mice versus controls was obtained by lateral and anterior-posterior view x-ray radiographs (Figure 2A). When compared to control mice at same ages, the spinal length of Osx::CXCR4^{fl/fl} mice was reduced 13% at 1wk and 28% at 4wks (Figure 2B). Reduced femoral length (20% at 1wk; 30% at 4wks) and femoral midshaft diameter (13% at 1wk; 22% at 4wks) were also observed in Osx::CXCR4^{fl/fl} mice versus controls (Figure 2B). Moreover, comparisons between Osx::CXCR4^{fl/fl} mice and control mice for skeletal growth from 1wk to 4wks old suggested growth retardation in Osx::CXCR4^{fl/fl} mice, since the increases in spinal length, femoral length, and midshaft diameter were 19%, 15%, and 14% less, respectively, in Osx::CXCR4^{fl/fl} mice versus controls (Figure 2C).

µCT analysis (Figures 3A-C) further revealed microstructural changes in calvarias and tibias of Osx::CXCR4^{fl/fl} mice, where bone forms via intramembraneous and endochondral ossification, respectively. When compared to control mice of same ages, a slightly decreased Ct.Th was observed in 1 wk old Osx::CXCR4^{fl/fl} mice, and a 24% decrease in Ct.Th was detected in Osx::CXCR4^{fl/fl} mice at 4wks old (Figure 3C). Cortical BMD was also reduced by 11% in tibias of 4-wk old Osx::CXCR4^{fl/fl} mice versus that of controls (Figure 3C). Similar cortical porosities (1-BV/TV) were found in tibias of Osx::CXCR4^{fl/fl} mice as that of littermate controls at both ages (data not shown).

Markedly decreased trabeculation was detected in the proximal tibia in Osx::CXCR4^{fl/fl} mice. When compared to littermate controls at both ages, lower BV/TV (44% decrease, 1wk; 32% decrease, 4wks), decreased trabecular thickness (Tb.Th; 18% decrease, 1wk; 17% decrease, 4wks), lower numbers of trabeculae (Tb.N; 21% decrease, 1wk; 25% decrease, 4wks) accompanied by higher trabecular separations (Tb.Sp; 37% increase, 1wk; 30% increase, 4wks) were found in tibias of Osx::CXCR4^{fl/fl} mice (Figure 3C). Moreover, the trabecular BMD was lower in tibias of Osx::CXCR4^{fl/fl} mice at both ages (9% decrease at 1wk; 13% decrease at 4wks), as well as the connective tissue density (Conn.D) of trabeculae (49% decrease at 1wk; 33% decrease at 4wks) (Figure 3C). Collectively, these µCT quantitative results were in agreement with qualitative images, suggesting reduced cortical and trabecular bone mass and BMD in endochondral bones of Osx::CXCR4^{fl/fl} mice.

To determine whether the postnatal skeletal changes originated prenatally, we examined the developing skeletons of E18.5 embryos by sequential alcian blue and alizarin red staining. Our data confirmed a smaller size of newborn and E18.5 embryos of Osx::CXCR4^{fl/fl} mice than that of littermate controls; while no significant patterning changes were seen in cartilage or bone in E18.5 embryos of Osx::CXCR4^{fl/fl} mice (Figure 4A). In addition, we observed less well developed calvarias in E18.5 embryos of Osx::CXCR4^{fl/fl} mice (Figure 4A). In addition, we observed less well developed calvarias in E18.5 embryos of Osx::CXCR4^{fl/fl} mice than that of littermate controls, evidenced by a loose mineralized bone structure along with delayed suture closure (Figure 4B). Moreover, when compared to controls, Osx::CXCR4^{fl/fl}
embryos lacked sufficient development of nasal bone around nasal capsule (Figure 4B). This prenatal calvaria defect correlates to our µCT detections of decreased BV/TV and Ct.Th in postnatal calvarias of Osx::CXCR4f/f mice.

3) Decreased bone mineralization rate in Osx::CXCR4f/f mice:

To further understand the effect of CXCR4 deficiency on reducing bone mass and BMD, we compared in vivo mineral apposition in Osx::CXCR4f/f mice with their sex-matched littermate controls via double fluorochrome-labeling. A smaller distance between xylene orange- and tetracycline- labeled mineralization fronts at the midshaft of tibia was observed in Osx::CXCR4f/f mice versus controls (Figure 5A), suggesting a lesser amount of new bone formed in Osx::CXCR4f/f mice. Histomorphometric measurements showed that the periosteal MAR of Osx::CXCR4f/f mice was 33% lower than that of littermate controls (Figure 5B).

Decreased mineralization in Osx::CXCR4f/f mice was also suggested by von Kossa staining on the above described fluoro-labeled tibias (Figure 5C). When compared to control mice, we detected a 22% decrease in BV/TV of trabecular bone; and a 14% decrease in BV/TV of cortical bone in Osx::CXCR4f/f mice (Figure 5D). These results in combination with our µCT measurements of decreased trabeculation and cortical thickening in tibias of Osx::CXCR4fl/fl mice suggest that a lower bone mineralization rate contributes, at least in part, to lower bone mass in these mice.

4) Growth plate disorganization and abnormal osteoblast development in Osx::CXCR4f/f mice:

To understand molecular and cellular changes associated with bone defects in Osx::CXCR4f/f mice, we examined the epiphseal growth plate, where bone formation occurs on a cartilaginous template (44, 45). In both Osx::CXCR4f/f mice and littermate controls, SDF-1 expression was detected mainly in prehypertrophic and hypertrophic chondrocytes and sporadically in bone marrow cells (Figure 6A), and similar numbers of SDF-1-positive cells were found between Osx::CXCR4fl/fl mice and controls (Figure 6B). In littermate control mice, CXCR4 expression was detected in chondrocytic cells across the proliferative and hypertrophic zone and also continued in the adjacent primary spongiosa by osteoblastic cells and marrow cells (Figure 6A). However, in both regions of Osx::CXCR4fl/fl mice, CXCR4 expression was markedly reduced (Figure 6A). Histomorphometric analysis showed that, when compared to controls, the number of cells positive for CXCR4 staining was 82% less in a chondrocytic region, and 70% less in a region of primary spongiosa in Osx::CXCR4fl/fl mice (Figure 6B). This pattern of CXCR4 deletion indicated that Osx promoter-driven Cre activities occurred not only in osteoblastic cells as expected, but also in chondrocytes of Osx::CXCR4fl/fl mice. In support of this notion, our immunohistochemistry results showed the expression of Osx itself in some chondrocytic cells in the growth plate (Figure 6C). Moreover, in Osx::CXCR4f/f mice that were treated with Dox to inactivate the Osx-controlled Cre transcription and recombination (41, 42), we found that CXCR4 loss in chondrocytes was rescued (Figure 6C).

No positive staining for SDF-1 or CXCR4 was observed when using isotype-matched control antibodies (Figure 6A). This expression pattern of CXCR4 and SDF-1 implicates a role of the SDF-1/CXCR4 signaling in regulating the transition of cartilage to bone. Next, we examined chondrocyte and osteoblast activities in the growth plate under the influence of CXCR4 deficiency. When compared to littermate controls, as shown by alcian blue staining that columnar chondrocytes in the proliferative zone of Osx::CXCR4fl/fl mice appeared to be disorganized with mixed cell size and shape, and no smooth progression in cell size occurred when moving from proliferative to prehypertrophic and hypertrophic zone (Figure 7A). Immunostaining for Col2α1 and Col10α1, specific collagens produced by proliferative and hypertrophic chondrocytes, respectively, further suggested reduced cartilaginous matrix syntheses in Osx::CXCR4fl/fl mice versus controls (Figure 7A). The height of the Col2α1-stained proliferative zone and Col10α1-stained hypertrophic zone was 27% and 23% shorter, respectively, in Osx::CXCR4fl/fl mice than that of controls (Figure 7B). Moreover, PCNA-immunohistochemistry (Figure 7A) indicated reduced proliferation in growth plate chondrocytes, as cells positive for PCNA staining were 36% less in Osx::CXCR4fl/fl mice than those of controls (Figure 7C). These observations suggest that...
reduced proliferation and size of a cartilaginous template in Osx::CXCR4 fl/fl mice correlate to the reduced long bone size in these mice.

CXCR4 deficiency also affected osteoblast activities in the primary spongiosa, where osteoblasts differentiate and lay down new bone matrix on the hypertrophic chondrocyte residues (44, 45). Our immunohistochemistry showed strong Osx expression in osteoblastic cells of the primary spongiosa in both Osx::CXCR4 fl/fl and control mice (Figure 8A), and no significant difference in the number of Osx-expressing cells was observed between Osx::CXCR4 fl/fl mice and controls (Figure 8B). We further detected the expression of ProCol1α1, a marker of initial collagen synthesis in relatively mature osteoblastic cells, along with OCN, a protein typically synthesized by mature osteoblasts, in the primary spongiosa by osteoblastic cells lining the trabecular bone surfaces (Figure 8A). However, as histomorphometric analysis showed that the number of cells stained positively for ProCol1α1 and OCN per bone surface was 62% and 56% less, respectively, in Osx::CXCR4 fl/fl mice than that of control mice (Figure 8B). This suggests an arrested osteoblast progression towards mature osteoblasts in the growth plate of Osx::CXCR4 fl/fl mice.

Additional evidence for impaired osteoblast development was obtained in long bones derived from newborn Osx::CXCR4 fl/fl mice (proved to be CXCR4-deficient, Figure 1C) and littermate controls for cell proliferation and differentiation in response to BMP stimulation. A 43% less BrdU incorporation was detected in cells of Osx::CXCR4 fl/fl mice versus cells of control mice, suggesting a lower proliferation rate in CXCR4-deficient osteoblasts (Figure 9A). Moreover, similar to our detections of arrested osteoblast progression in long bones, a 32% lower in the level of ALP expression and a 70% lower in the level of OCN expression relative to GAPDH control were detected in calvaria cells of Osx::CXCR4 fl/fl mice versus cells of control mice (Figure 9B). After stimulation with BMP2, a member of the BMP family, in calvaria cells of control mice, both levels of ALP activity (Figure 9C) and OCN synthesis (Figure 9D) were significantly increased (2.4-fold for ALP at d4; 4-fold for OCN at d7) over baseline levels in cultures maintained in medium only. In contrast, there was no increase in either ALP (Figure 9C) or OCN (Figure 9D) in BMP2-stimulated calvaria cells of Osx::CXCR4 fl/fl mice versus medium controls. Similarly, stimulation with BMP6, another member of the BMP family, significantly increased the level of ALP (1.8-fold at d4) and OCN (2.5-fold at d7) in calvaria cells of control mice; while no response to BMP6 stimulation was detected in calvaria cells of Osx::CXCR4 fl/fl mice (Figures 9E-F). These data suggest that CXCR4-deficiency inhibits osteoblastic differentiation of calvaria cells induced by two members of the BMP family.

Our Western blotting results further showed impaired BMP signaling cascades in CXCR4-deficient calvaria cells (Figures 10A-B). While BMP2 stimulation rapidly induced phosphorylation of intracellular R-Smads (Smad1/5/8) and Erk1/2, the key downstream targets of BMP signal transduction, in calvaria cells derived from control mice; no BMP2-stimulated phosphorylations of R-Smads and Erk
were observed in CXCR4-deficient calvaria cells derived from Osx::CXCR4fl/fl mice (Figure 10A). Gel image analysis showed that, when compared to medium control cells, the intensities of immunoreactive bands for phosphorylated (p) Smad1/5 and pErk1/2 were increased 5-fold and 4-fold, respectively, in BMP2-stimulated calvaria cells derived from control mice; as opposed to no significant increases in band intensities of pSmad1/5 and pErk1/2 after BMP2-stimulation were detected in calvaria cells of Osx::CXCR4fl/fl mice (Figure 10B).

Moreover, we compared Osx::CXCR4fl/fl mice with control mice for Smad1/5 phosphorylation in both calvaria (Figure 10C) and the epiphyseal growth plate (Figure 10D) by immunohistochemistry. In calvarias, the number of cells positive for pSmad1/5 staining was reduced by 72% in Osx::CXCR4fl/fl mice versus controls (Figure 10E). In tibias, pSmad1/5 expression was detected in pre- and hypertrophic chondrocytes in the growth plate (Figure 10D), where the number of cells expressing pSmad1/5 was reduced by 76% in Osx::CXCR4fl/fl mice when compared to that of control mice (Figure 10E). Other than BMP-regulated Smads, we also examined Smad2, a key intracellular transducer for TGFβ1/2/3 signaling, in the growth plate. Our data showed weak pSmad2 expression in Osx::CXCR4fl/fl mice similar as that in control mice (Figure 10D), confirming the specific involvment of BMP signaling rather than other members of the TGFβ super family in this case. Collectively, these data showed impaired BMP signaling in CXCR4-deficient cells and bone tissues, and thus suggest that CXCR4 functions in regulating osteoblast activities in cooperation with BMP signaling.

**Discussion**

Current literature illustrates multiple functions of CXCR4 in embryonic organ development, HIV or tumor invasion, and various tissue homeostasis and repair processes that require the chemotactic interaction of CXCR4 with its ligand, SDF-1 (1-7, 9-16). However, it remains unknown whether CXCR4 has a direct functional role in bone formation. In this study, we addressed this question by demonstrating the effect of bone-specific deletion of CXCR4 on postnatal skeletal development of the mouse, and identified the function of CXCR4 in regulating osteoblast development. This finding enhances the current understanding of CXCR4 with a novel concept that CXCR4, the essential signaling component for SDF-1, is required for proper bone formation.

Via the transcriptional control of Osx, we were able to target CXCR4 deletion in osteoprecursor cells and thus focused on analyzing the role of CXCR4 during bone formation. However, we noticed that CXCR4 was not completely removed with this approach, evidenced by trace CXCR4 expression remained in the Osx-expressing cells and bones (Figures 1B-C). This might have lessened the severity of bone defects in Osx::CXCR4fl/fl mice resulting from CXCR4 deficiency. Although Osx is considered as one of the osteoblastic lineage-specific transcription factors, we observed CXCR4 deletion in both osteoblasts and growth plate chondrocytes of Osx::CXCR4fl/fl mice (Figures 6A-C), suggesting that broader Cre activities occurred in both osteoblastic and chondrocytic cells. We further approved this point by shutting down the Osx-driven Cre activity with Dox administration, which restored CXCR4 expression in chondrocytes of Osx::CXCR4fl/fl mice (Figure 6C). In agreement with our findings, several previous studies also reported Osx expression in chondrocytes (35, 46). Moreover, when using R26LacZ reporter mice, the Osx-controlled Cre activity was originally noticed in chondrocytic cells in the Osx-Cre strain (41, 42). Similar to previous findings of CXCR4 and SDF-1 expression in the growth plate of a long bone (28, 47, 48), we also detected their expression in the growth plate of Cre-null littermate controls for Osx::CXCR4fl/fl mice. The pattern of CXCR4 expression in the growth plate and adjacent primary spongiosa and SDF-1 expression in pre- and hypertrophic chondrocytes at the chondro-osseous junction indicates that SDF-1 may function in both chondrocytes and osteoblasts via signaling through CXCR4. Moreover, this pattern of SDF-1 expression may serve to recruit CXCR4-expressing progenitor cells at blood vessel invasion to lay down new bone on the hypertrophic chondrocyte residues. Our detection of SDF-1 expression pattern in 4wk-old mice is different from that reported by Wei et al (34) in newborn (d1) mice, in which SDF-1 was predominantly expressed in bone marrow next to
hypertrophic chondrocytes, suggesting an age-related change in cellular distribution of SDF-1 that may be associated with SDF-1 function at different stages of bone development. This hypothesis remains to be tested.

A growth plate controls the size and shape of a long bone, therefore a shorter cartilaginous template with decreased chondrocyte proliferation and collagen matrix synthesis in the growth plate of Osx::CXCR4^fl/fl^ mice correlates to the reduced long bone size in these mice. Lower BMD, reduced cortical thickness and trabecular bone volume in tibias of Osx::CXCR4^fl/fl^ mice (Figures 3A-C) suggest that CXCR4 is required as an endogenous regulator for skeletal size and bone architecture. Moreover, a slower bone mineralization rate (Figures 5A-B) further correlates to growth retardation in the skeletal development of Osx::CXCR4^fl/fl^ mice (Figure 2C). Although we also detected decreased bone mass in calvarias of Osx::CXCR4^fl/fl^ mice, their BMD was not significantly reduced as that we found in tibias. This suggests differential roles of CXCR4 in intramembranous and endochondral ossification alone or via interacting with other signaling pathways that participate in these processes. When looking into mechanisms underlying bone defects in Osx::CXCR4^fl/fl^ mice, we identified reduced Coll1a1 and OCN expression relative to unreduced Osx expression in osteoblastic cells adjacent to growth plate (Figures 8A-B), suggesting an arrested osteoblast progression towards mature osteoblasts in these mice. Concomitantly, a lesser expression of mature osteoblast markers was found in long bones of newborn mice and serum of adult mice (Figures 8C-E). Taken together, these findings suggest that arrested osteoblast development contributes, at least in part, to the reduced bone formation in Osx::CXCR4^fl/fl^ mice.

Decreased proliferation and blunted response to BMP2- or BMP6-osteoblast differentiation in primary calvaria cells of Osx::CXCR4^fl/fl^ mice add additional evidence to CXCR4 function in osteoblast activities. The finding that CXCR4-deficient cells did not express sufficient levels of ALP activities nor adequately synthesized OCN protein after BMP stimulation (Figures 9C-F) is consistent with our previous detections in cultures for bone marrow stromal cells, C2C12 and ST2 cells, where blocking of SDF-1/CXCR4 by siRNA, antibody or antagonist against SDF-1 or CXCR4 inhibits the BMP2-induced cell differentiation towards the osteoblast lineage (25, 33). Moreover, suppressed BMP signaling cascades, the activation of key intracellular components R-Smads and Erk1/2, were detected in calvaria-derived cells as well as in CXCR4-deficient long bones and calvarias of Osx::CXCR4^fl/fl^ mice (Figures 10A-E). Furthermore, no suppression of pSmad2 in CXCR4-deficient tibias excluded the involvement of other members of TGFβ super family. These findings demonstrated an in vivo interaction between CXCR4 and BMP in bone, and suggest that CXCR4 regulates osteoblast development in postnatal bone formation involving its cooperation with BMP signaling. Future studies are required to determine roles of different members of the BMP family in CXCR4-regulated bone formation.

In conclusion, our data suggest that loss of CXCR4 gene in the Osx-expressing osteoprecursors results in impaired bone development and growth in the mouse skeleton, and this is associated with the regulation of CXCR4 on osteoblast development. These findings established a direct functional role of CXCR4 in bone formation in vivo. Targeting the SDF-1/CXCR4 pathway will deepen our understanding of molecular regulation of osteogenesis, and will help to develop new therapeutic targets to treat defective bone formation and regeneration ranging from metabolic bone diseases to fracture non-unions.

Acknowledgements

We thank Dr. Yong-Rui Zou at Columbia University for kindly providing CXCR4^fl/fl^ mice. This work was supported by grants from Arthritis Foundation, Beatrice and Samuel A. Seaver Foundation, and the NIAMS-funded (AR046121) Musculoskeletal Repair and Regeneration Core Centers (MRRCC).
References

43. McLeod, M.J. (1980) Teratology 22(3), 299-301

Footnotes
The abbreviations used in this study are: cystine (C)-X-C motif chemokine receptor 4 (CXCR4), stromal derived factor-1 (SDF-1), bone morphogenetic protein 2 (BMP2), BMP receptor-regulated Smad proteins (R-Smads), osterix (Osx), pro form of type I collagen α1 (ProCol1α1), type II collagen α1 (Col2α1), type X collagen α1 (Col10α1), proliferating cell nuclear antigen (PCNA), alkaline phosphatase..
(ALP), and osteocalcin (OCN). Author contributions: Zhu W designed the project, collected and analyzed data, and wrote the paper. Liang G and Huang Z assisted in conducting experiments. Doty SB and Boskey AL assisted research design and data interpretation.

Table 1. Primer sequences used in RT-PCR and qPCR reactions.

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<th>Target gene</th>
<th>Accession no.</th>
<th>Sequences (5’-3’)</th>
<th>Product size (bp)</th>
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| Cre recombinase   | HQ335171      | Forward: GCGGTCTGGCAGTAAAAACTATC  
                      |                  | Reverse: GTGAACAGCATTGCTGTCATT        | 102              |
| Floxed CXCR4 vs.  | U65580        | Forward: CACTACGCATGACTCGAAATG  
                      |                  | Reverse: GTGTCGGTGTTATCCAGC        | 434 vs. 334      |
| CXCR4             | NM_009911     | Forward: GATAGCCTGTGGATGGTGGT       
                      |                  | Reverse: GCAGGCAAAGAAAGCTAGGA    | 180              |
| Osx               | NM_130458     | Forward: CCCCCCTCAAGC ACCAATGG  
                      |                  | Reverse: ACGGTGGGTAGTCATTGCATAG        | 85               |
| ALP               | NM_007431     | Forward: CTTACTGTGTG TACTGCTG       
                      |                  | Reverse: GACGTAATCTACCATGGAG        | 200              |
| Col1α1            | NM_007742     | Forward: GCCGAAGAATGGGATGTGAGG     
                      |                  | Reverse: CTTAGGACCAGCAGGACCAG        | 180              |
| OCN               | NM_007541     | Forward: CCGGGACAGTG TGAGCTTA       
                      |                  | Reverse: AGGGCGTCTTCAAGCCCATACT       | 69               |
| GAPDH             | NM_008084     | Forward: GGTTGTAACCACGAGAAAT       
                      |                  | Reverse: CTTCCACAATGCCAAAGTT        | 120              |
Figure Legends

Figure 1. Bone specific-deletion of CXCR4 in Osx::CXCR4fl/fl mice. (A): Representative genotyping PCR for Cre transgene, floxed (fl) CXCR4 relative to wild type CXCR4 in Osx::CXCR4fl/fl mice (KO), littermate heterozygous (hz) for Cre and fl-CXCR4 (Osx-Cre;CXCR4fl/+), and Cre-null littermates (CXCR4fl/+ and CXCR4fl/fl), which were used equivalent to wild type (wt) controls. (B): RT-PCR detection of CXCR4, Osx and GAPDH control in kidneys, livers and long bones (femurs and tibias) derived from newborn (d1-3 after birth) KO and wt mice. (C): Western blotting for basal expression of CXCR4, Osx, and β-Tubulin control in calvaria cells derived from newborn KO or wt mice. Cells were pooled from N=3 wt or KO mice, experiments were repeated twice using mice from different litters.

Figure 2. Reduced skeletal size in Osx::CXCR4fl/fl mice. (A): Representative x-ray lateral and anterior-posterior view radiographs of Osx::CXCR4fl/fl (KO) mice and Cre-null littermate controls equivalent to wild type (wt) at 1wk and 4wks old, all females. (B): Fold changes of skeletal size parameters when compared to wt control at 1wk old, which was set as 1. (C): Fold increases in skeletal size parameters at 4wks versus those of 1wk in wt or KO mice. N=5 female wt or KO mice at 1wk or 4wks old. Measurements were presented as mean ± standard deviation (SD). *, p<0.05 vs. 1wk old wt (B) or vs. respective 1wk old mice (C).

Figure 3. Structural alterations in Osx::CXCR4fl/fl mice. (A): Representative μCT images of entire parietal calvarias (1wk old, females) and tibia cortical and trabecular bone (4wks old, females) in Osx::CXCR4fl/fl mice (KO) and Cre-null littermate controls (wt). Scale bar equals to 1mm in calvarias or 100µm in tibias. (B): Quantitative μCT analysis of calvaria cortical (Ct.) bone volume fraction (BV/TV) and thickness (Th). (C): Quantitative μCT analysis of cortical (Ct.) and trabecular (Tb.) structure on 200 micro tomographic slices (2.1mm) at the proximal tibia. Measurements were Ct.Th, Ct. bone mineral density (BMD), Tb.BV/TV, Tb.Th, Tb. number (N), Tb. separation (Sp), Tb.BMD, and connective tissue density (Conn.D). N=5 female wt or KO mice at 1wk or 4wks old. Measurements were presented as mean ± SD. *, p<0.05 vs. respective wt control.

Figure 4. Prenatal skeletal changes in Osx::CXCR4fl/fl mice. (A): Representative images of newborn (d1) Osx::CXCR4fl/fl mice (KO) and Cre-null littermates used as wild type (wt) controls (upper panel); alcian blue / alizarin red staining of E18.5 embryos (lower panel). (B): Alcian blue / alizarin red staining of entire heads (upper panel), and amplified regions of calvaria (framed areas) and nose (arrow indicated) of E18.5 embryos (lower panels). wt and KO from two different litters were observed.

Figure 5. Decreased mineral apposition rate (MAR) in Osx::CXCR4fl/fl mice. (A): Double fluorochrome-labeling in tibias of Osx::CXCR4fl/fl mice (KO) and Cre-null littermate controls (wt) by xylenol orange (orange red) and tetracycline (green) in a 4-day time interval. Scale bar equals to 100µm. (B): The distance between two fluorochrome-labeled mineralization fronts (arrow indicated) at the midshaft of tibia was quantified using OsteoII software (Bioquant). The MAR was calculated by dividing the measured distance with time interval. (C): von Kossa staining of the fluorochrome-labeled tibias (upper panel, scale bar equals to 500µm). Mineralized trabecular or cortical bone (amplification of framed areas) was stained black (lower panels, scale bar equals to 500µm). (D): Histomorphometric analysis for mineralized bone in von Kossa-stained tibias. The trabecular bone volume fraction (BV/TV) was determined by dividing the area of mineralized trabecular bone with the total area from the epiphyseal growth plate to the midshaft of tibia. The cortical BV/TV was determined at the midshaft of tibia by dividing the cortical thickness with the total width of tibia. Five measurements per section and three consecutive sections were taken to average each sample. N=5 (females, 2wks old) wt or KO mice. Measurements were presented as mean ± SD. *, p<0.05 vs. wt control.
Figure 6. Decreased CXCR4 expression in the epiphyseal growth plate of Osx::CXCR4 floxed/floxed (KO) versus Cre-null littermate controls (wt). (A): Immunohistochemistry for SDF-1 expression in pre- and hypertrophic chondrocytes, and CXCR4 expression in chondrocytic cells and in primary spongiosa (scale bar equals to 50µm). Negative (-) controls used isotype-matched control antibodies, scale bar equals to 100µm. (B): Histomorphometric analysis (OsteoII software, Bioquant) for the number of cells positive for SDF-1 or CXCR4 expression. The number of cells positive for SDF-1-immunostaining was measured from the pre- to hypertrophic zone. The number of cells positive for CXCR4 was measured in three regions (each at 185x250 µm²) in the middle of the growth plate or the primary spongiosa. Five measurements per section and three consecutive sections were taken to average each sample. (C): Immunohistochemistry for Osx and CXCR4 expression in growth plate of KO mice and control KO mice that were treated with Dox (200µg/ml in drinking water) after birth (Dox-KO). N=5 (3 females and 2 males, 4 wks old) wt or KO mice. Measurements were presented as mean ± SD. *, p<0.05 vs. respective wt control.

Figure 7. Epiphyseal growth plate disorganization in tibias of Osx::CXCR4 floxed/floxed mice. (A): Representative alcian blue staining, immunohistochemistry for Col2α1, Col10α1 and PCNA, or negative (-) controls using isotype-matched control antibody. Scale bar equals to 50µm in PCNA-stained sections, or 100µm in rest of sections. (B): Histomorphometric analysis (OsteoII, Bioquant) for the height of the proliferative (PZ) and hypertrophic zone (HZ) in the middle of the growth plate, indicated by right brackets. (C): Histomorphometric measurements for the number of PCNA-stained cells from the proliferative to hypertrophic zone. Five measurements per section and three consecutive sections were taken to average each sample. N=5 (3 females and 2 males, 4wks old) wt or KO mice. Measurements were presented as mean ± SD. *, p<0.05 vs. wt control.

Figure 8. Arrested osteoblast progression in Osx::CXCR4 floxed/floxed mice. (A): Representative immunohistochemistry for Osx, ProCol1α1, and OCN in the primary spongiosa of Osx::CXCR4 floxed/floxed (KO) mice and Cre-null littermate controls (wt). Isotype-matched control IgGs were used in negative (-) controls. Scale bar equals to 50µm. (B): Histomorphometric analysis (OsteoII, Bioquant) for the number of positively stained cells per bone surface in three regions (each at 185x250 µm²) in the middle of the primary spongiosa. Five measurements per section and three consecutive sections were taken to average each sample. N=5 (3 females and 2 males, 4wks old) wt or KO mice. (C): Representative RT-PCR detection for OCN, Col1α1, OCN, and GAPDH control in long bones derived from newborn (d1-3 after birth) KO or wt mice. (D): qPCR quantification of the expression level of Ocn, Col1α1 and OCN relative to GAPDH control in long bones derived from newborn KO or wt mice. N=5 wt or KO. (E): Serum OCN level in KO mouse versus wt controls. N=5 (3 females and 2 males, 12 wks old). All measurements were presented as mean ± SD. *, p<0.05 vs. wt control.

Figure 9. CXCR4-deficiency inhibits cell proliferation and osteoblast differentiation induced by BMP. Primary osteoblastic cells were derived from newborn (d1-3 after birth) calvarias of Osx::CXCR4 floxed/floxed (KO) mice and Cre-null littermate controls (wt). (A): BrdU incorporation for 6 hours in wt or KO calvaria cells. (B): qPCR measurements of baseline mRNA levels of ALP and OCN relative to GAPDH control in calvaria cells of wt or KO mice. (C-F): Calvaria cells were stimulated with rhBMP2 (C-D) or rhBMP6 (E-F) protein at 100ng/ml for 4 or 7 days, or as controls, maintained in culture medium without any stimulation. ALP activities were measured in cell lysates and normalized to the level of total cellular protein. OCN levels were measured in cell medium using ELISA. Cultures were pooled from calvarias of N=3 wt or KO mice and triplicate wells of cells were used for each sample. Experiments were repeated twice using mice from two different litters. Measurements were expressed as mean ± SD. *, p<0.05 vs. respective medium only control.

Figure 10. Impaired BMP signaling in CXCR4-deficient cells and bone tissues. Calvaria osteoblasts were derived from newborn (d1-3 after birth) Osx::CXCR4 floxed/floxed (KO) mice or Cre-null littermate controls (wt).
Cells were stimulated with rhBMP2 at 100ng/ml for 15 minutes, or as controls, maintained in culture medium (Med) only. (A-B): Western detection of Smad1/5 and Erk1/2 phosphorylation relative to unphosphorylated total Smads and Erk (A); and gel image analysis for band intensities of phosphorylated (p) Smad1/5 and Erk1/2 compared to that of medium controls cells (B). Cultures were pooled from calvarias of N=3 wt or KO mice and triplicate wells of cells were used for each sample. Experiments were repeated twice using mice from two different litters. Measurements were expressed as mean ± SD. *, p<0.05 vs. respective medium only control. (C-E): Immunohistochemistry detection of pSmad1/5 expression in calvaria (C); pSmad1/5 and pSmad2 expression in tibia growth plate (D); followed by histomorphometric analysis (OsteoII, Bioquant) for the number of positively stained cells in three regions of calvaria (each at 185x250 µm²) and from the pre- to hypertrophic zone in the growth plate of tibia (E). Isotype-matched control IgGs were used in negative (-) controls. Scale bar equals to 50µm in calvaria or growth plate sections. Five measurements per section and three consecutive sections were taken to average each sample. N=5 (3 females and 2 males, 4wks old) wt or KO. All measurements were expressed as mean ± SD. *, p<0.05 vs. respective wt control.
FIGURE 1

A

200 100

wt KO/hz

500 400 300

wt hz KO

Cre (100bp)

fl-CXCR4 (434bp)

CXCR4 (334bp)

B

Kidney Liver Long Bone

wt KO wt KO wt KO wt KO

300 200 100

CXCR4 (180bp)

Osx (85bp)

GADPH (120bp)

C

wt KO

50 37

50 37

50 37

CXCR4 (42kDa)

Osx (46kDa)

β-Tubulin (50kDa)
FIGURE 2

A

1wk

4wks

wt KO

wt KO

1wk

4wks

Femoral length

Spinal length

Midshaft diameter

B

Fold change of 1wk old wt

0.0

0.5

1.0

1.5

2.0

2.5

Spinal length

Femoral length

Midshaft diameter

C

Fold increase of 1wk

wt KO

wt KO

wt KO

wt KO

wt KO

wt KO

wt KO
FIGURE 3

A) Calvaria

B) Tibia

C) Graphs showing Ct.Th (mm*10^-2), Ct.BMD (10^2*mm^-3), Tb.BV/TV, Tb.Th (mm*10^-3), Tb.N (mm^-1), Tb.Sp (mm*10^-2), Tb.BMD (10^2*mm^-3), Conn.D (10^2*mm^-3) for wt and KO groups at 1 wk and 4 wks.
FIGURE 4
FIGURE 5

A

wt  KO

B

MAR (μm/day)

0  2  4  6  8

wt  KO

C

wt  KO

D

BV/TV

0.0  0.1  0.2  0.3  0.4  0.5

wt  KO  wt  KO

Trabecular Cortical

* *
FIGURE 7

A

Alcian blue  Col2α1  Col10α1  PCNA  (-)

wt  KO

wt  KO

B

Height (µm)

wt  KO  wt  KO

PZ  HZ

C

PCNA+ cells / mm²

wt  KO
**FIGURE 8**

A

<table>
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<th>Osx</th>
<th>ProCol1α1</th>
<th>OCN</th>
<th>(-)</th>
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<td></td>
<td></td>
</tr>
<tr>
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B

Graph showing the comparison of N/mm between wt and KO for Osx, ProCol1α1, and OCN.
FIGURE 8

C

wt KO

GADPH (120bp)

OCN (69bp)

Col1α1 (180bp)

Osx (85bp)

GADPH (120bp)

D

E

Relative expression

wt KO

wt KO

wt KO

Osx

Col1α1

OCN

OCN (ng/ml)

wt KO

wt KO

*
FIGURE 9

A

BrdU incorporation 
(OD450)

wt KO

0.0 0.5 1.0 1.5 2.0 2.5

B

Relative expression

wt KO ALP wt KO OCN

0.0 0.2 0.4 0.6 0.8 1.0

C

ALP activity 
(μmol p-np/mg protein)

Medium BMP2 Medium BMP2

wt KO

0.0 0.5 1.0 1.5 2.0

D

OCN (ng/ml)

Medium BMP2 Medium BMP2

wt KO

0.0 0.5 1.0 1.5 2.0

E

ALP activity 
(μmol p-np/mg protein)

Medium BMP2 Medium BMP2

wt KO

0.0 0.5 1.0 1.5 2.0

F

OCN (ng/ml)

Medium BMP2 Medium BMP2

wt KO

0.0 0.5 1.0 1.5 2.0

*
FIGURE 10

A

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- pSmad1/5 (60kDa)
- Smad1/5/8 (52kDa)
- pErk1/2 (42-44kDa)
- Erk1/2 (42-44kDa)

B

Fold change of medium control

- pSmad1/5
- p-Erk1/2

* Indicates significant difference.
**FIGURE 10**

C  

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D  

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E  

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*Statistical significance indicated by *.
Conditional inactivation of CXCR4 in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development
Wei Zhu, Gang Liang, Zhiping Huang, Stephen B. Doty and Adele L. Boskey

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