Chondrocyte FGFR3 Regulates Bone Mass by Inhibiting Osteogenesis*

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Chondrogenesis can regulate bone formation. Fibroblast growth factor receptor 3, highly expressed in chondrocytes, is a negative regulator of bone growth. To investigate whether chondrocyte FGFR3 regulates osteogenesis, thereby contributing to postnatal bone formation and bone remodeling, mice with conditional knock-out of Fgfr3 in chondrocytes (mutant (MUT)) were generated. MUT mice displayed overgrowth of bone with lengthened growth plates. Bone mass of MUT mice was significantly increased at both 1 month and 4 months of age. Histological analysis showed that osteoblast number and bone formation were remarkably enhanced after deletion of Fgfr3 in chondrocytes. Chondrocyte-osteoblast co-culture assay further revealed that Fgfr3 deficiency in chondrocytes promoted differentiation and mineralization of osteoblasts by up-regulating the expressions of Ihh, Bmp2, Bmp4, Bmp7, Wnt4, and Tgf-B1, as well as down-regulating Nog expression. In addition, osteoclastogenesis was also impaired in MUT mice with decreased number of osteoclasts lining trabecular bone, which may be related to the reduced ratio of Rankl to Opg in Fgfr3-deficient chondrocytes. This study reveals that chondrocyte FGFR3 is involved in the regulation of bone formation and bone remodeling by a paracrine mechanism.

Endochondral ossification includes two consecutive processes, cartilage formation and bone formation. During cartilage formation, mesenchyme differentiates into chondrocyte after condensation. After several rounds of proliferation, proliferating chondrocytes exit from the cell cycle and gradually undergo hypertrophy. Following calcification of the extracellular matrix (ECM) surrounding hypertrophic chondrocytes, hypertrophic chondrocytes die through apoptosis or transdifferentiate into osteoblasts (1–3). Simultaneously, blood vessels invade, bringing osteoclasts and osteoprogenitors to degrade the calcified cartilage ECM and to form bone tissue to replace the calcified cartilage template, respectively (1, 4). Endochondral ossification is a highly complicated process. The cross-talk between chondrocytes and other skeletogenic cells in growth plates could regulate trabecular bone modeling or remodeling (5). Numerous signaling molecules expressed by prehypertrophic or hypertrophic chondrocytes play essential roles in this cross-talk (6–13). For example, hypertrophic chondrocytes can secrete angiogenic and osteogenic factors such as VEGF, IHH, and BMPs to induce the vascular invasion and osteogenesis (6–13).

Fibroblast growth factor receptor 3 (FGFR3) is one of a family of four membrane-bound receptor tyrosine kinases (FGFR1–4) linked to downstream pathways including MAPK, PKC-γ, PI3K/AKT, and STAT signaling pathways (14). FGFR3 is highly expressed in chondrocytes of growth plates (15–18), and is a negative regulator of endochondral bone development (4, 14). Gain-of-function mutations in FGFR3 lead to chondrodysplasias including achondroplasia (ACH), hypochondroplasia (HCH), and thanatophoric dysplasia (TD). Mouse models with gain-of-function mutations in FGFR3 mimicking human ACH or thanatophoric dysplasia showed remarkably smaller body and skeleton size. Patients with down-regulated FGFR3 activity exhibit CATSLH syndrome (characterized with campodactyly, tall stature, scoliosis, and hearing loss) (19, 20). Fgr3 knock-out mice also showed overgrowth of skeleton (21).

FGFR3 inhibits both proliferation and hypertrophic differentiation of chondrocytes (22–24). Activation of STAT1-P21 pathway is considered as a potential mechanism for FGFR3-induced proliferation arrest (25–28). The inhibited hypertrophic differentiation of chondrocytes resulting from activated FGFR3 is related to ERK MAPK signaling (4). The

achondroplasia; Col10, collagen 10; BV/TV, bone volume/tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; Conn.D, connectivity density; Ob.S/BS, osteoblast surface/bone surface; N.Oc/B.Pm, osteoclast number/bone perimeter; N.Oc/B.Pm, osteoclast number/bone perimeter; Oc.S/BS, osteoclast surface/bone surface; BFR/BS, bone formation rate/bone surface; MAR, mineral apposition rate; SMI, structure model index; BMSC, bone marrow stromal cell; microCT, micro-computed tomography.
feedback loop IHH/PTHrP (PTH-related protein) pathway also participates in the inhibition of FGFR3 on the proliferation and differentiation of chondrocytes (30, 31).

Evidence suggests that FGFR3 also has an effect on bone formation and homeostasis. Activated mutations in FGFR3 (A391E and P250R) in human are responsible for Crouzon syndrome with acanthosis nigricans (A391E) and Muenke syndrome (P250R). Patients with either syndrome have pre-mature fusion of the cranial sutures (29, 30). Adult Fgfr3 and Fgfr3IIIc null mutant mice showed osteopenia (31, 32). However, mice with activated mutation of FGFR3, G369C (mimicking human ACH), also displayed decreased bone mass and increased differentiation of osteoblasts (25, 33). The seemingly contradictory data indicate that the underlying mechanisms for the effect of FGFR3 on bone formation are complicated.

FGFR3 is also expressed in mesenchymal stem cells and osteoblasts, indicating that FGFR3 mutations in these cells may directly affect bone formation (14). For example, activated mutation of FGFR3 (Y367C) in osteoblasts led to increased bone mass in mice (35). In addition, chondrogenesis can regulate bone formation as mentioned above. Highly expressed FGFR3 in chondrocytes may thus indirectly influence bone formation through its regulation of chondrogenesis. However, there is no genetic evidence, and there are no detailed mechanisms about the indirect influence of chondrocyte FGFR3 on bone formation. Because the effects of FGFR3 activity in global Fgfr3 knock-out or activation mice exist in all tissues, including chondrocytes, osteoblasts, and osteoclasts, it is difficult to distinguish the indirect effects of chondrocyte FGFR3 from the direct effects of FGFR3 on bone formation.

To explore the indirect effect of chondrocyte FGFR3 on osteogenesis and its underlying mechanism, we analyzed the bone phenotypes of mice with specific deletion of Fgfr3 in chondrocytes. Our data reveal that deletion of Fgfr3 in chondrocytes led to increased bone mass resulting from enhanced osteogenesis and decreased osteoclastogenesis via a paracrine mechanism.

Results

Deletion of Fgfr3 in Chondrocytes Leads to Increased Bone Growth in Mice—Longitudinal development of long bone is mainly determined by growth plate chondrocytes. Mice with targeted deletion of Fgfr3 in chondrocytes (Col2a1-Fgfr3fl/fl (MUT)) showed bone overgrowth similar to Fgfr3 knock-out mice (Fig. 1) (31). 7 days after birth, MUT mice showed bended tails (Fig. 1A). 1 and 4 months after birth, the lengths of whole body, tail, tibia, and femur in MUT mice were all longer than that in WT mice (Fig. 1C). The 4-month-old mice also showed curvature of the spine (Fig. 1B). These results indicate that deletion of Fgfr3 in chondrocytes affected bone development.

Ablation of Fgfr3 in Chondrocytes Results in Abnormal Growth Plates—To investigate the effect of Fgfr3 deficiency on chondrogenesis, we performed morphologic analyses of tibial growth plates from 1- and 4-month-old mice. The lengths of
proliferating zone and hypertrophic zone in MUT mice were increased by 25 and 45%, respectively, when compared with that in WT mice at 1 month after birth (Fig. 2, A and B). At 4 months of age, the proliferating zone was nearly absent in WT mice; however, it still existed in MUT mice (Fig. 2A). At this stage, there was a 2.3-fold increase in the length of the hypertrophic zone in MUT mice when compared with that in WT mice (Fig. 2C). These changes in growth plates are consistent with that in conventional Fgfr3 knock-out mice, which further demonstrates that both the proliferation and the differentiation of chondrocytes were increased by specific inactivation of Fgfr3 in chondrocytes.

**Bone Mass Is Increased in Mice with Targeted Deletion of Fgfr3 in Chondrocytes**—To determine whether bone formation was affected by deletion of Fgfr3 in growth plate chondrocytes, we first evaluated the bone volume of femoral bones by X-ray. The results showed that femurs of MUT mice were longer than WT mice as described above at 1 and 4 months of age (Fig. 3A). MUT mice also exhibited increased trabecular length when compared with WT mice (Fig. 3A). Micro-computed tomography (micro-CT) was used to analyze the structural parameters of trabecular bone in distal metaphysis of femurs. Three-dimensional images of the femur and femoral metaphysis were shown in Fig. 3, A and B. The trabecular number and length in MUT mice were significantly increased, which is consistent with the result of X-ray analysis at both 1 month and 4 months of age. Then we quantified structural parameters of trabecular bone including bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), structure model index (SMI), and connectivity density (Conn.D). At both 1 month and 4 months of age, MUT mice showed increased BV/TV, Tb.N, and Tb.Th, as well as decreased Tb.Sp. SMI was reduced in MUT mice at 1 month of age when compared with WT mice, as Conn.D of MUT mice was increased in both 1 month and 4 months of age. (Fig. 3C). However, there was no significant difference in cortical bone thickness between WT and MUT mice (supplemental Fig. S2). These results indicate that deletion of Fgfr3 in chondrocytes caused increased bone mass and architecture changes in mice, but did not affect cortical bone thickness.

**Bone Formation Is Enhanced after Targeted Deletion of Fgfr3 in Chondrocytes**—Osteoblasts are the major cells responsible for bone formation. To determine whether the increased bone mass in mice with Fgfr3 deleted in chondrocytes is due to increased bone formation, we performed histologic analysis of decalcified and undecalcified tibiae. von Kossa staining of undecalcified tibiae demonstrated that MUT mice had more trabecular bone and calcified ECM when compared with WT mice (Fig. 4A). The BV/TV, Tb.Th, and Tb.N in MUT tibiae were significantly increased by 30.7, 4.64, and 25.9% (p < 0.01), respectively (Fig. 4, C–E). Tb.Sp was significantly decreased by 12.3% in MUT mice (p < 0.01) (Fig. 4F). These changes are consistent with the results obtained by micro-CT examination.
Histomorphometric analysis showed that the percentage of bone surface occupied by osteoblasts (osteoblast surface/bone surface, Ob.S/BS) and the osteoblast number per length in trabecular bone (osteoblast number/bone perimeter, N.Ob/B.Pm) were significantly increased by 69.7 and 27.8%, respectively, in MUT mice when compared with WT mice (Fig. 4, G and H). The dynamics of bone formation at the metaphysis of tibiae were analyzed by double labeling of calcein. The distance between two labeling lines was increased in MUT mice (Fig. 4B). Histomorphometric measurements revealed that the bone formation rate/bone surface (BFR/BS) and mineral apposition rate (MAR), indexes of osteoblast activity (36), were all markedly increased in MUT mice (Fig. 4, I and J). These data suggest that ablation of Fgfr3 in chondrocytes promoted bone formation.

Chondrocytes with Fgfr3 Deficiency Promote Osteogenesis—During endochondral bone formation, cartilage formation precedes bone formation, and chondrocytes can provide inductive signals for osteogenesis (6, 37). As bone formation was increased in mice with chondrocyte-specific deletion of Fgfr3, we suspected that the Fgfr3-deficient chondrocytes may affect osteogenesis in these FGFR3 mutant mice. To test this hypothesis, primary WT osteoblasts were co-cultured with primary chondrocytes from WT or MUT mice. Osteoblast activity was evaluated by measuring the formation of alkaline phosphatase (ALP)-positive colonies, ALP activity, and mineralized nodules of co-cultured osteoblasts. The results showed that the numbers of crystal violet-staining (ALP-positive) cells and ALP activity of osteoblasts co-cultured with MUT chondrocytes were remarkably increased after co-culture for 14 days (Fig. 5, A and B), and the number of mineralized nodules was also increased in osteoblasts co-cultured with MUT chondrocytes (Fig. 5A, lower panels). These data suggested that the osteogenic differentiation and mineralization of osteoblasts co-cultured with MUT chondrocytes were improved. Osteogenesis was further examined by measuring the expressions of core binding factor α1 (Cbfa1), collagen1α1 (Col1a1), and osteocalcin (Oc) mRNAs. Expressions of these genes were all significantly enhanced in osteoblasts co-cultured with MUT chondrocytes for 14 days when compared with WT chondrocytes (Fig. 5C). These data illustrate that chondrocytes with Fgfr3 deficiency promoted osteogenesis.
As chondrocytes can secrete some important molecules regulating osteogenesis, such as Noggin, BMPs, IHH, etc. (38, 39), we asked whether the expressions of these osteogenesis-related molecules in Fgfr3-deficient chondrocytes are changed and subsequently affect osteogenesis. Our results showed that the expressions of Bmp2, Bmp4, and Bmp7, important molecules stimulating osteogenesis, were significantly up-regulated in MUT chondrocytes, whereas the expression of Nog, an inhibitor of BMP signaling, was apparently decreased (Fig. 5D). IHH is another factor molecule secreted by chondrocytes to promote osteogenesis differentiation (1). The mRNA level of Ihh in MUT chondrocytes was also remarkably elevated (Fig. 5D).

Wnt4, a prototypical non-canonical Wnt ligand, is able to promote osteoblast differentiation and bone formation in vitro and in vivo (40, 41). Wnt4 is also expressed in growth plates (42), and our results showed that the expression of Wnt4 was significantly increased in chondrocytes with Fgfr3 deficiency (Fig. 5D). TGF-β1, one of the three TGF-β isoforms, has a very important effect on bone formation (43–45). In MUT chondrocytes, Tgf-β1 expression was also higher than WT mice (Fig. 5D). These data showed that deletion of Fgfr3 in chondrocytes led to increased expression of Wnt4 and Tgf-β1, which may also participate in the regulation of chondrocyte FGF3 on osteogenesis.

Deletion of Fgfr3 in Chondrocytes Gives Rise to Decreased Osteoclastogenesis—Bone mass is determined by the tight coupling between bone formation and bone resorption. Because the MUT mice showed an increased bone mass, we examined whether the increased bone mass is also related to the changes of osteoclastogenesis. We found that the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in primary trabecular bone of MUT mice was significantly decreased when compared with those in WT mice (Fig. 6A). We further found that both the osteoclast number (osteoclast number/bone perimeter, N.Oc/B.Pm) and the osteoclast surface (osteoclast surface/bone surface, Oc.S/BS) in 1-month-old MUT mice were decreased (Fig. 6B), which suggests that osteoclastogenesis was inhibited.

RANKL and OPG are produced by both chondrocytes and osteoblasts, and play an important role in osteoclast formation and differentiation (46–49). We first detected the expressions of Rankl and Opg in osteoblasts co-cultured with chondrocytes. The expression levels of both Rankl and Opg were significantly up-regulated in osteoblasts co-cultured with MUT chondrocytes (Fig. 6C), so we further calculated the Rankl/Opg ratio, a
major determinant of osteoclastogenesis in osteoblasts (46–49). However, there was no remarkable difference between the ratios of Rankl/Opg in osteoblasts co-cultured with WT and MUT chondrocytes, which revealed that osteoblasts in these two groups had no significant difference in their effects on osteoclastogenesis. In chondrocytes, the mRNA expressions of both Rankl and Opg were also increased in MUT chondrocytes (Fig. 6D); moreover the ratio of Rankl to Opg was significantly decreased in MUT chondrocytes when compared with that of WT chondrocytes (Fig. 6D). These data suggest that the decreased osteoclastogenesis in MUT mice may be related to the down-regulated ratio of Rankl to Opg in Fgfr3-deficient chondrocytes.

Discussion

FGFR3 is a negative regulator of bone growth, and gain-of-function mutations in FGFR3 lead to chondrodysplasias with abnormal growth plates (14). In addition, FGFR3 also regulates bone mass. Previous studies showed that adult Fgr3 and Fgfr3IIIc knock-out mice displayed decreased bone mass (31, 32), and ubiquitous gain-of-function mutations in FGFR3 also led to decreased bone mass in mice (33, 35). FGFR3 can directly
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During endochondral bone formation, chondrocytes can provide inductive signals for osteogenesis (6, 37). Because of the important effect of FGFR3 on chondrocyte differentiation (52), we wondered whether chondrocyte FGFR3 has an indirect influence on osteogenesis. Chondrocyte-osteoblast co-culture experiments showed that osteoblasts co-cultured with Fgfr3-deficient chondrocytes exhibited enhanced differentiation and mineralization, which may be related to increased bone formation in vivo. A previous study also suggested that activating FGFR3 in chondrocytes affects trabecular bone formation (35), but the underlying mechanism was still unclear.

As hypertrophic chondrocyte can secrete osteoblastogenesis-related molecules (e.g. IHH, BMP, Noggin) (1, 51) to regulate osteogenesis, we wondered whether chondrocyte FGFR3 has an indirect influence on osteogenesis through these osteoblastogenesis-related molecules. The expressions of Ihh, Bmp2, Bmp4, and Bmp7 were significantly increased in Fgfr3-deficient chondrocytes, whereas the expression of Noggin, a BMP signaling inhibitor, was decreased.

In addition to IHH and BMP signaling, we also found two novel FGF3-regulated molecules regulated by FGFR3 in chondrocytes. Wnt4 and TGF-β1 play important roles in maintaining bone mass and promoting bone formation in vitro and in vivo (40, 41, 43–45). Wnt4 and TGF-β1 are notably expressed in growth plate (42, 44). Deletion of Fgfr3 in chondrocytes led to increased expression of Wnt4 and Tgf-β1. These data suggested that besides the role of enhanced activity of IHH and BMP signaling, the increased expression of Wnt4 and Tgf-β1 may also be involved in the regulation of osteogenesis by chondrocyte FGFR3 and may be responsible for increased bone mass in MUT mice. Our co-culture experiments, for the first time, directly demonstrate that deletion of Fgfr3 in chondrocytes can promote osteogenesis via a paracrine mechanism.

Recent studies showed that hypertrophic chondrocyte can directly transdifferentiate into osteoblasts during endochondral bone formation (2, 3, 53). In our mice with Fgfr3 deficiency in chondrocytes, the hypertrophic chondrocytes in the elongated hypertrophic zone may also transdifferentiate into osteoblasts and participate in bone formation, which needs to be further explored.

Furthermore, we found that BMSCs may also contribute to increased bone mass (33). Our data revealed that BMSCs from chondrocyte Fgfr3 deficiency mice exhibited enhanced mineralization and ALP activity (supplemental Fig. S1). As Col2 was not only expressed in chondrocytes, but also expressed in osteochondral progenitor (54), Fgfr3 may be deleted in osteochondral progenitors. Thus, the Col2-Cre-expressing cells in the bone marrow may also contribute to the increased bone mass of MUT mice.

Bone mass is regulated by the tight coupling between bone formation and bone resorption. We demonstrated that the increased bone mass in primary spongiosa in mice with deletion of Fgfr3 in chondrocytes was also related to decreased osteoclastogenesis. Osteoblasts were generally thought to be the major regulator of osteoclast differentiation through their secretion of RANKL and OPG. Recent studies demonstrate that chondrocytes also express RANKL and OPG to regulate osteoclast formation (47, 49, 55, 56). The ratio of Rankl/Opg was

regulate osteogenesis by promoting or inhibiting osteogenic differentiation and mineralization of bone marrow stromal cells (BMSCs) (31, 33). In this study, we further demonstrate that Fgfr3, in addition to its direct regulation of osteogenesis, can also regulate bone formation and bone mass indirectly through its regulation of chondrogenesis.

Long bone is formed through endochondral ossification (50). The hypertrophic zone of growth plates serves as a scaffold for bone formation (1, 51). The mineralized cartilage elements in growth plates are finally invaded by blood vessels accompanied by osteoclasts and osteoblasts, and then replaced by bone (1). FGFR3 is essential for chondrocyte hypertrophy (52). In this study, mice with deletion of Fgfr3 in chondrocytes showed an elongated hypertrophic zone with an increased number of hypertrophic chondrocytes in growth plates, which may be responsible for the increased bone volume of the primary spongiosa.
similar between osteoblasts co-cultured with Fgfr3-deficient or WT chondrocytes. However, the Rankl/Opg ratio was significantly decreased in Fgfr3-deficient chondrocytes when compared with that in WT chondrocytes, which indicated that Fgfr3 deficiency in chondrocytes could indirectly inhibit osteoclastogenesis.

In summary, we demonstrate that deletion of Fgfr3 in chondrocytes leads to increased bone mass by up-regulating osteogenesis and inhibiting osteoclastogenesis. Our study reveals that chondrocyte FGFR3, beside its well known regulation of chondrogenesis, also plays an important role in bone formation and bone remodeling by a paracrine mechanism, which further supports the concept that chondrogenesis regulates bone formation.

Materials and Methods

Generation of Mice with Deletion of Fgfr3 in Chondrocytes—Fgfr3<sup>lox/lox</sup> (Fgfr3<sup>0/0</sup>) mice were generated by our laboratory (57). Col2a1-Cre<sup>+</sup> mice were presented as a gift from Dr. Xiao Yang (58). Col2a1-Fgfr3<sup>0/0</sup> mice (MUT) with Fgfr3 deletion in chondrocytes were generated by breeding Fgfr3<sup>0/0</sup> mice (WT) with Col2a1-Cre transgenic mice. All protocols of the experiments were approved by the Institutional Animal Care and Use Committee of Daping Hospital.

X-ray Imaging and Micro-CT Analysis—Mice were sacrificed at 1 and 4 months after birth. Femurs were obtained and fixed in 70% ethanol at 4 °C. X-ray images of these bones were taken by an MX-20 system (Faxitron Bioptics, LLC, Tucson, AZ) (33). Distal metaphysis of femurs was scanned at 70 kV, 114 mA, and 8 watts by micro-CT (SCANCO vivaCT 40, Scanco Medical AG, Bassersdorf, Switzerland). The three-dimensional reconstructions and measurement were acquired to evaluate BV/TV, Tb.Th, Tb.N, Tb.Sp, SmI, and Conn.D as reported before (31).

Bone Histology and Immunohistochemistry—Adult mice were injected with calcein (25 mg/kg of body weight) (Sigma-Aldrich) at 10 and 3 days prior to sacrifice (33). Left and right tibiae were harvested and stored in 4% paraformaldehyde or 40% ethanol for 24 h, respectively. The left tibiae were decalcified in 15% EDTA for 2–3 weeks, and paraffin processing was performed. 5-μm-thick sections were used for Safranin O/Fast Green, TRAP (Sigma) staining, and immunohistochemical staining for Col10 (1:200; Abcam, Cambridge, MA). The right tibiae were dehydrated in a graded ethanol series and embedded in the methyl methacrylate and dibutyl phthalate mixture. The ten-micrometer-thick sections were prepared for fluorescence observation. 5-μm-thick sections were used for von Kossa staining. The BV/TV, Tb.Th, Tb.N, Tb.Sp, N.Oc/B.Pm, Ob.S/BS, BFR/BS, MAR, N.Oc/B.Pm, and Oc.S/BS of bone were analyzed using the OsteoMeasure system (OsteoMetrics, Inc., Atlanta, GA) as described previously (33).

Preparation of Primary Chondrocytes and Osteoblasts—Mouse primary chondrocytes were isolated from cartilage of knee joints of 3-day-old mice. After being treated by trypsin (Thermo Scientific HyClone), dissected cartilages were digested with 0.08% collagenase II (Gibco) overnight in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C (59, 60), and then cultured in DMEM/F12 with 10% FBS (HyClone) containing 100 units/ml of penicillin and streptomycin (HyClone). Primary osteoblasts were isolated from mouse calvaria (61). Briefly, calvaria from 3-day-old mice were dissected, isolated, and subjected to sequential digestions in collagenase I (1 mg/ml) (Gibco). Cells from the third digestion were collected, counted, and plated in α-MEM with 10% FBS (HyClone) containing 100 units/ml of penicillin and streptomycin (HyClone) (62). Passage 1 osteoblasts were used for all studies.

Chondrocyte-Osteoblast Co-culture Assay—Co-culture was carried out using 0.4-μm pore size and 6-well Transwell chambers (Corning Inc.) as described previously (63, 64). Passage 1 osteoblasts were seeded at 3.8 × 10<sup>4</sup> cells/well in 6-well plates in medium used above. The cultured medium was exchanged to osteoblastic cultures supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10<sup>-8</sup> M dexamethasone (Sigma) after 24 h. Primary chondrocytes were plated at 7.5 × 10<sup>4</sup> cells/well in the polycarbonate membrane insert and cultured in DMEM/F12 medium mentioned above. These media were changed every 3–4 days. After co-culture for 14 days, the osteoblasts were used for ALP activity and mineralization analysis.

ALP Staining and Activity Analysis—For ALP staining, osteoblasts were fixed with 4% paraformaldehyde for 10 min and stained with ALP detection solution (Sigma) for 30 min at 37 °C (33). For ALP activity analysis, osteoblasts were lysed with 10 mM Tris-HCl containing 2 mM MgCl<sub>2</sub> and 0.05% Triton X-100 (pH 8.2) at 4 °C. Lysates were incubated in ALP detection solution (Sigma) for 30 min at 37 °C. 0.1 M NaOH was used to stop the reaction and monitored at 405 nm. The stopped mixture and total protein were measured using a Micro BCA protein assay kit (Thermo Scientific Pierce) at 405 and 562 nm, respectively. The relative enzymatic activity of ALP was normalized to the total protein content of the sample (405 nm/562 nm).

Alizarin Red Staining of Mineralized Osteoblast Cultures—After co-culture for 14 days, mineralized osteoblast cultures were fixed in 70% ethanol for 1 h at 4 °C. The fixed cells were stained in 0.2% Alizarin red in 2% ethanol for 15 min, and then rinsed with distilled water twice to remove excess staining (65).

Quantitative PCR Analysis—The total RNA was extracted by TRIzol (Sigma), purified, and reverse-transcribed with PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer’s instructions (Takara Bio, Inc., Shiga, Japan). Analysis on a Mx3000P PCR machine (Stratagene Agilent Technologies Inc., Santa Clara, CA) was performed according to a previous study (33). The sequences of the primers of Fgfr3, Ihh, Oc, Col1a1, Col1b1, Bmp4, Rankl, Opg, and CypA (a housekeeping gene) were reported previously (33, 34, 65).

Other sequences of primers are as follows: Bmp2, 5'<GTGAGGGACTGCTGA-3' and 5'-TGGAAGCTGCAACCTTAC-3'; BMP7, 5'-GAGGGATCTTTACCGGCTCC-3' and 5'-GGTGGAGGAAAGACACGAC-3'; Nog, 5'-TGTAGTAACCTTTGTGCATGATG-3' and 5'-GTTTCGCGTGTGTGTTCG-3'; Wnt-4, 5'-AGACGTGCGAGAAGCTGAGGAA-3' and 5'-GGAACTGGTATTGCGACT-3'; and Tgf-B1, 5'-ATGTCACAGCTGGTGGCC-3' and 5'-GGTGGTGCACTGTGCTGTATA-3'. Each run was replicated three times.

Statistical Analysis—Data were evaluated statistically in SPSS Windows, version 13.0. Results are shown as mean ± S.D.
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Statistics were assessed using Student’s t test, assuming double-sided independent variance, and p values were considered significant at *, p < 0.05, **, p < 0.01, and ***, p < 0.001.

Author Contributions—X. W., N. S., and L. C. designed the study. X. W., X.L., Y. T., J. T., S. Z., Y. X., J. G., J. Y., and N. S. conducted the study. X. W. and N. S. collected data. N. S. and L. C. analyzed and interpreted data. X. W. and N. S. drafted the manuscript. X. D. and L. C. revised the manuscript.

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