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Chondrocyte BMP2 signaling plays an essential role in bone fracture healing

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Abstract

The specific role of endogenous *Bmp2* gene in chondrocytes and in osteoblasts in fracture healing was investigated by generation and analysis of chondrocyte- and osteoblast-specific *Bmp2* conditional knockout (cKO) mice. The unilateral open transverse tibial fractures were created in these *Bmp2* cKO mice. Bone fracture callus samples were collected and analyzed by X-ray, micro-CT, histology analyses, biomechanical testing and gene expression assays. The results demonstrated that the lack of *Bmp2* expression in chondrocytes leads to a prolonged cartilage callus formation and a delayed osteogenesis initiation and progression into mineralization phase with lower biomechanical properties. In contrast, when the *Bmp2* gene was deleted in osteoblasts, the mice showed no significant difference in the fracture healing process compared to control mice. These findings suggest that endogenous BMP2 expression in chondrocytes may play an essential role in cartilage callus maturation at an early stage of fracture healing. Our studies may provide important information for clinical application of BMP2.

Keywords

BMP2; Fracture healing; Fracture callus; Chondrocyte; Osteoblast; Conditional knockout

1. Introduction

Bone fracture healing relies on the coordinated actions of numerous growth factors and cytokines (Bertone et al., 2004; Borovecki et al., 2007; Cook et al., 1994; Einhorn et al.,

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1995; Govender et al., 2002; Simic and Vukicevic, 2007; Yasko et al., 1992). Among them, bone morphogenetic proteins (BMPs) play the most important role in fracture healing. BMP2 is a member of the transforming growth factor (TGF) β superfamily and induces bone and cartilage formation in vivo (Hogan, 1996; Huang et al., 2008; Otsuka et al., 2000; Wozney et al., 1988). BMP2 was one of the crucial components of BMP signaling cascade in human fracture callus, and there is a significant reduction in BMP2 expression in the cartilaginous areas of human nonunion fractures compared to normal healing fractures (Kloen et al., 2003; Kwong et al., 2009). BMP2 has been used clinically in the treatment of bone defects, non-union fractures and spinal fusion. BMP2 seems to play a key role in fracture repair, especially at the early stage of fracture healing (Cheng et al., 2003; Tsuji et al., 2008; White et al., 2007). The role of BMP2 in bone formation and fracture repair has been extensively studied by in vitro, ex vivo and in vivo studies. In vitro studies showed that BMP2 promotes differentiation of periosteal-derived cells and mesenchymal stem cells (MSCs) into chondrocytes and osteoblasts (Hanada et al., 1997, 2001; Kawabata and Miyazono, 1999; Lou et al., 1999; Thies et al., 1992; Zachos et al., 2006). Ex vivo studies in which MSCs were genetically engineered to overexpress *Bmp2* demonstrated that BMP2 improves the healing of segmental defects and induces spinal fusion by triggering a cascade of BMP expression through autocrine and paracrine regulatory mechanisms (Gazit et al., 1999; Hasharoni et al., 2005; Sheyn et al., 2008; Turgeman et al., 2001). In vivo approaches include application of exogenous BMP2 protein locally with or without carriers (Baltzer et al., 2000; Lieberman et al., 1999; Turgeman et al., 2001; Zegzula et al., 1997) and generation of genetically engineered mouse models to achieve *Bmp2* gene knockout or overexpression.

In clinical practices, most fractures receive a relatively stabilized fixation with applications of plaster cast, external fixator or intramedullary needle. During the healing process, both periosteal and endochondral ossification processes are involved in new bone formation and the latter process plays a more important role during fracture healing. In this process, cartilaginous callus formation is detected at the early stage of fracture healing (Bolander, 1992; Simmons, 1985), followed by callus maturation, ossification and remodeling. The critical role of the endogenous *Bmp2* gene in initiation of bone fracture healing was demonstrated in limb mesenchymal cell-specific *Bmp2* cKO mice (Tsuji et al., 2006). However, the role of endogenous *Bmp2* gene in chondrocytes or in osteoblasts at the specific stage of fracture healing remains to be defined. Our recent studies demonstrated that *Bmp2* is required for chondrocyte maturation and endochondral ossification during early skeletal development (Shu et al., 2011). In the present studies, we generated chondrocyte- and osteoblast-specific *Bmp2* cKO mice and determined the role of BMP2 expressed in specific cell population in cartilage callus formation, maturation and ossification during the fracture healing process.

2. Materials and methods

2.1. Experimental animals

To generate chondrocyte- and osteoblast-specific *Bmp2* cKO mice, *Bmp2^{flx/flx}* mice (obtained from Dr. Steve Harris, University of Texas Health Science Center at San Antonio, San Antonio, Texas) (Shu et al., 2011) were bred with either *Col1Cre* or *Col2Cre* transgenic mice. The *Col1Cre* and *Col2Cre* transgenic mice could efficiently target mature osteoblasts and growth plate chondrocytes (Dacquin et al., 2002; Ovchinnikov et al., 2000). In our previous studies, we demonstrated that chondrocyte-specific deletion of the *Bmp2* gene leads to severe defects in chondrocyte development and endochondral bone formation (Shu et al., 2011). All mice were under C57BL/6 background and mouse genotyping was determined by PCR using DNA extracted from tail biopsy tissues 1 month after birth. PCR primer sequences for genotyping were as follows, MP1-600F(A) 5'-AGGGTTT

CAGGTCAGTTTCCG, MP1-Hap-rv2(B) 5'-GATGATGAGGTTCTTGCGG, and MP1-1615RW(C) 5'-TCCGAAGGTAAGTGTGCTTGG. Cre-positive and *Bmp2^{fx/fx}* littermates were designated as experimental groups while Cre-negative littermates were used as controls. Only male mice were used in this study (mean body weight 22±2 g). All mice had free access to food and water during the entire study. All procedures conducted in this study were approved by the IACUC committee of the University of Rochester.

2.2. Fracture model

A unilateral (right side) open transverse tibial fracture with intramedullary needle fixation was selected as the bone fracture model in this study. 2-month-old mice were anesthetized with ketamine (60 mg/kg) by intraperitoneal injection. An electric hair shaver was used to make the operation field free of covering fur. Surgeries were then performed under aseptic conditions. A 1.5 cm-long skin incision was made along the antero-medial surface of the tibia. At the medial side of the patellar ligament, a 27 gage syringe needle was inserted into the bone marrow cavity of the tibia through the tibial plateau, by which a pin canal was well established and the marrow cavity was suitably enlarged. The needle is then removed, and a no. 11 surgical blade was used to transect the diaphysis of the tibia at the middle point of the diaphysis shaft, followed by selecting and inserting a syringe needle varying from 25 to 27 gages, whichever fits the bone marrow cavity well to simulate fixation of intramedullary needle. The distal tip of the syringe needle was ensured to reach the narrowest point of the marrow cavity of tibia. The periosteum remained intact and soft tissues were well protected and the fibula was carefully preserved to be unabridged. The pin beyond the cortex of the tibial plateau was cut off within 2 mm by a wire-cutter. A 4-0 silk suture was used to close the wound and buprenorphine was administered in drinking water for pain relief for the first three days after the operation.

2.3. Skeletal preparation

Both *Bmp2* cKO mice and Cre-negative mice were sacrificed post-operatively by time points, days 7, 10, 14, 21 and 28 to obtain tibial samples from the operation side. During the procedure, callus tissue was carefully protected and the intramedullary needle was removed. Samples harvested were analyzed by X-ray, micro-CT, histology, mechanical testing and mRNA expression.

2.4. X-ray and micro-CT analysis

X-ray radiographic analysis was performed on fracture samples with both antero-posterior and lateral directions immediately after operation to ensure the fracture pattern and the position of the fixation needle. After the mice were sacrificed, the fracture healing quality (n=10 in each time point, with 5.0-kV for 6.0 s, Faxitron X-ray, Wheeling, IL) was examined. The extent of bridging between the fracture gap was determined blindly by two independent investigators by evaluation of healing quality including clear gap (either no indication of bridging or some indications of bridging), blurred gap (either more consolidated bridging or almost completely mineralized bridging) and no gap (completely mineralized bridging). Micro-CT analysis was performed on harvested samples. Callus mineralized volume fraction (BV/TV) (%) and connectivity density (Conn.D, 1/mm³), which describe the porosity of bone sample and show the branching of bone tissue structure (Kallai et al., 2010), were analyzed with 10.5-μm isotropic resolution (n=5 in each time point, Scanco VivaCT 40, Scanco Medical AG, Switzerland).

2.5. Histology and histomorphometry

Bone samples harvested for histological analysis were serially fixed in 10% normal buffered formaldehyde for 3 days, decalcified in 14% EDTA solution for 14 days and then embedded

in paraffin. Tissue sections at the fracture site were cut longitudinally by microtome at a thickness of 3- μ m and prepared for Alcian blue/H&E staining. Histomorphometric analysis (n=5 in each time point) was conducted using Image-Pro Plus 4.5 software (Media Cybernetics). The tibial diameter (baseline) was used to define the size of the region of interest (ROI) (Fig. 3B). The callus was divided into the proximal and distal part and a 1.5 length of the baseline was used to define the ROI of the proximal and distal callus halves. The total diameter of the callus was included in the ROI. The mineralized volume of the cortices, the area of the periosteal calluses, and the mineralized and cartilaginous volume of the calluses were measured and compared between groups (Parfitt et al., 1987; Schmidmaier et al., 2002). The structural indices were calculated as follows: 1) tibial diameter (Ti.Dm) at the fracture gap: including Ct.Wi and Ma.Dm (mm); 2) mineralized area of the corticalis (Ct): Md.Ar/Ct.B.Ar (%); 3) periosteal callus area: Ps.Cl.Ar (mm²); 4) mineralized area of the periosteal callus area: Md.Ar/Ps.Cl.Ar (%); and 5) cartilage area of the periosteal callus area: Cg.Ar/Ps.Cl.Ar (%). Abbreviations used include: Ar, area; B, bone; Cg, cartilage; Ct, corticalis; Dm, diameter; Ps, periosteal; Ma, marrow; Md, mineralized; Wi, width; and Cl, callus.

2.6. Biomechanical torsion testing

The proximal and distal points of harvested soft tissue-free bone samples (n=5 in each time point for time points day 10, 14, 21, and 28) were firmly fixed in two aluminous square tubes (0.5 cm) filled with bone cement to make sure that the fracture line was at the middle point of the interval of two tubes. The samples then underwent torsion testing at a rate of 1°/s until callus disruption, during which a recording curve was obtained (EnduraTec TestBench™ system, Bose Corp., Minnetonka, MN), and the ultimate torque, stiffness and toughness of callus tissues were determined.

2.7. Quantitative gene expression analysis

The callus of the fracture site with 1 mm of adjacent bone tissue of both proximal and distal sides was harvested and total RNA was extracted using the Qiagen RNeasy Minikit (n=5 in each time point). cDNA was synthesized using 1 μ g of RNA per callus with the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR analysis was performed with murine specific primers for chondrocyte and osteoblast marker genes, such as *Runx2*, *Col2a1*, *Col10a1*, and *osteocalcin (OC)*. The expression levels of these genes were normalized by the level of β -actin.

2.8. Statistical analysis

Results were presented as the mean $\pm$ SE. Statistical analyses were performed with the two-way ANOVA followed by Dunnett's test. A *p* value<0.05 was considered as significant.

3. Results

3.1. X-ray radiographic evaluation

X-ray images showed no malformation of skeletons of mice from all groups. The fracture pattern from all groups was similar with consistent transverse gap at the middle of the diaphysis of tibia. The mouse tibiae with fracture operation received anatomical reduction and intramedullary needles were at the right position. No displacement was observed and no newly-formed fractures were detected at both tibiae and fibulae. For chondrocyte-specific *Bmp2* cKO mice (*Col2Cre;Bmp2^{flx/flx}*), the fracture gap remained clear without indication of bridging by day 7 post-operatively and some indications of bridging by days 10 and 14. By day 21 the blurred gap with more consolidated bridging was observed and almost completed bridging on the last time point (day 28) in this study. Osteoblast-specific *Bmp2* cKO (*Col1Cre;Bmp2^{flx/flx}*) mice and Cre-negative control mice had similar image patterns with

clear gap by day 7 and day 10 postoperatively, but more blurred gap with more consolidated bridging by day 14 and almost completely mineralized bridging by day 21. In addition, completely mineralized replacement was observed by day 28 in these mice (Fig. 1).

3.2. Micro-CT analysis

Micro-CT data showed that both BV/TV (%) and Conn.D ($1/\text{mm}^3$) at fracture sites were significantly decreased in chondrocyte-specific *Bmp2* cKO mice compared to Cre-negative control mice by days 7, 10, 14 and 21 post-operatively ($p < 0.05$). At the last time points (day 28), similar BV/TV (%) and Conn.D ($1/\text{mm}^3$) were detected in chondrocyte- and osteoblast-specific *Bmp2* cKO mice and Cre-negative control mice. Osteoblast-specific *Bmp2* cKO mice and Cre-negative control mice showed no significant difference during the entire fracture healing process. The micro-CT images showed similar patterns, structures and the degree of mineralization of the total callus in these mice (Fig. 2).

3.3. Histological and histomorphometric analyses

Histologic and histomorphometric analyses showed that the morphology and the sizes of cartilage-like or trabecular bone-like callus formed between the fracture gap and periosteum were similar in all groups of mice by day 7 post-operatively. The cartilage area (Cg.Ar/Ps.Cl.Ar) (%) was enlarged by days 10 and 14 and the mineralization bone area was reduced (Md.Ar/Ps.Cl.Ar) (%) by days 10–21 postoperatively ($p < 0.05$) in chondrocyte-specific *Bmp2* cKO mice compared to Cre-negative control mice. At day 28, the cartilage area was no longer detectable in both Cre-negative control and chondrocyte-specific *Bmp2* cKO mice. Mineralized bone volume (Md.Ar/Ps.Cl.Ar) (%) was slightly increased in Cre-negative control mice (74.70 ± 4.00) in the last time point compared to chondrocyte-specific *Bmp2* cKO mice (70.82 ± 4.16). There was no significant difference observed in osteoblast-specific *Bmp2* cKO mice compared to Cre-negative control mice in any time points (Fig. 3).

3.4. Biomechanical testing analysis

Results of biomechanical torsion testing showed that all parameters including ultimate torque, stiffness and toughness were significantly decreased in samples of day 10, 14 and 21 in chondrocyte-specific *Bmp2* cKO mice compared to Cre-negative control mice ($p < 0.05$). Differences of ultimate torque and toughness remained obvious by day 28 ($p < 0.05$) with stiffness level catching up close to the Cre-negative control mice. Osteoblast-specific *Bmp2* cKO mice did not show significant changes in parameters of torsion test during the entire fracture healing period (Figs. 4A–C).

3.5. Gene expression analysis

Gene expression analysis showed that levels of *Col2a1* and *Col10a1* were significantly reduced by days 7 and 10 in chondrocyte-specific *Bmp2* cKO mice compared to those of Cre-negative control mice. There was no significant difference observed in other time points. In contrast, no significant changes in *Col2a1* and *Col10a1* expression were found in osteoblast-specific *Bmp2* cKO mice (Figs. 5A and B). *Runx2* and *osteocalcin* (OC) levels were significantly decreased by days 7–21 in chondrocyte-specific *Bmp2* cKO mice compared to those of Cre-negative control mice, although the difference was reduced by days 14 and 21 between chondrocyte-specific *Bmp2* cKO mice and control mice. No significant changes in *Runx2* and OC expression were found in all time points in osteoblast-specific *Bmp2* cKO mice (Figs. 5A–D).

4. Discussion

Several different techniques have been utilized in recent years to investigate the biological functions of BMP2. Among them, the *Bmp2* cKO approach is the most powerful method to

determine the function of *Bmp2* gene in vivo. Although local application of recombinant BMP2 has been used to analyze the in vivo function of BMP2, potential risks may exist considering improper dosage and incorrect placement of application devices leading to unpredicted bone formation in non-targeting tissues. In addition, the short half-life of recombinant protein is also a concern (Parfitt et al., 1987; Zapf et al., 1986). In this study, we investigated the role of endogenous *Bmp2* gene in chondrocytes and in osteoblasts during bone fracture healing using the *Bmp2* cKO approach. In a previous study it was demonstrated that endogenous *Bmp2* is required for chondrocyte maturation and endochondral ossification during early embryonic skeletal development (Shu et al., 2011). *Bmp2* global KO mice are embryonically lethal (Zhang and Bradley, 1996). In this study we have bred *Col2Cre* or *Col1Cre* transgenic mice with *Bmp2^{flx/flx}* mice to generate chondrocyte-or osteoblast-specific cKO mice to delete *Bmp2* gene in a specific cell population. This approach enables us to avoid embryonic lethality and bone abnormalities at an early embryonic stage. The *Bmp2* cKO mice survived at birth and bone tissue grew properly at the postnatal stage. The animal size and body weight of the male *Bmp2* cKO mice and Cre-negative control mice were used in this study and no skeletal defect was detected in any of these *Bmp2* cKO mice at the time of fracture studies.

In clinical practice, transverse fractures of long bone diaphysis shaft are most commonly seen. In this study, we chose a unilateral open transverse tibial fracture with intramedullary needle fixation as a fracture healing model to investigate the role of *Bmp2* in fracture healing. An open fracture model allows the fracture operation procedure to be observed under naked eyes to accurately locate the fracture site and ensure if the fracture pattern is as expected. Although no displacements are expected to occur during the fracture procedure, for an individual long bone, spiral displacement could occur even if the needle goes through both ends of the proximal and distal cortices. We selected tibia rather than femur for creating fractures since with the fibula support from the posterior side, a triangle fixation pattern could be formed to make bone fracture more stable and reduce the possibility of displacement. With the puncture at the tibial plateau, the needle is firmly fixed in the cortices at both ends of the bone longitudinally. Periosteum contains mesenchymal progenitor cells which are involved in both chondrogenesis and osteogenesis during the fracture healing process (Yu et al., 2010). The integrity of the periosteum has been carefully protected. Since there is hardly any subcutaneous tissue covering the anteromedial surface of the tibia, the surrounding muscles were protected during surgery.

In this study, we classified the degree of bridging between fracture gaps on X-ray images into three levels. 1) Clear gap indicates that there is no indication or only some indications of mineralized bridging. At this stage, the callus consisted mostly of hematoma, collagen fibers and cartilage and few mineralized bone could be detected. 2) Blurred gap represents more consolidated bridging or almost completely mineralized bridging, indicating a more mineralized tissue of callus, and less cartilage remained, which links the proximal and distal ends of fracture sites. 3) No gap indicates completely mineralized bridging, possibly with osteogenesis close to the peak level and the bone remodeling has been initiated. Significant differences were shown by day 14 with clear gap and day 28 with blurred gap for chondrocyte-specific *Bmp2* cKO mice compared to Cre-negative mice, suggesting that there may be a delayed osteogenic initiation by day 14 and delayed mineralization by day 28. We then utilized micro-CT to analyze the fracture healing in more detail. The use of micro-CT to assess the callus properties has been well adopted in many bone fracture studies (Morgan et al., 2009). In our study, we measured the mineralized bone volume fraction in callus and connectivity density, two parameters indicating bone-like callus formation. Significant decreases in bone volume and connectivity density were detected in chondrocyte-specific *Bmp2* cKO mice from days 7–21 compared to Cre-negative control mice, suggesting a delayed osteogenic initiation in callus in which there was less newly-formed trabecular bone

in callus tissue. At the last time point, both bone volume and connectivity density were similar between chondrocyte-specific *Bmp2* cKO mice and Cre-negative control mice.

We noticed that in X-ray analysis, the healing of the fracture gaps at day 28 was blurred for chondrocyte-specific *Bmp2* cKO mice and no gaps for control mice. The slight difference of these results between X-ray and micro-CT may be attributed to the distribution of mineralized bone tissue in the entire callus and there may be less bone density in the area between or surrounding the fracture gaps where cartilage callus was formed and then replaced by bone tissue. If cartilage callus was removed by a delayed process, the bone density for the area previously occupied by cartilage may be lower to some extent, and blurred gaps could be observed, although the entire bone tissue was similar to that of control mice. This correlated to the results of biomechanical testing, in which ultimate torque, stiffness, and toughness were decreased in samples of chondrocyte-specific *Bmp2* cKO mice at all time points with only the exception of stiffness which is similar in chondrocyte-specific *Bmp2* cKO mice compared to the control mice at day 28. Stiffness reflects the elasticity of bone while ultimate torque and toughness reflect the forces and energy required to disrupt the callus, which were more closely associated with the quantity and quality of newly-formed mineralized bone. The results suggest that although the elasticity of callus of chondrocyte-specific *Bmp2* cKO mice was similar to that of control mice, the newly-formed mineralized bone in the callus remains less than that of control mice, suggesting that the callus could be disrupted with less force and energy. The histology and histomorphometry analyses further support the radiographic findings. The cartilage volume was similar in 3 groups of mice by day 7, suggesting that there may be no significant difference in the initiation process and cartilage callus formation during fracture healing. However, large cartilage volume and smaller bone volume were detected in chondrocyte-specific *Bmp2* cKO mice compared to the Cre-negative control mice by days 10–21, suggesting that *Bmp2* deficiency in chondrocytes may lead to a prolonged cartilage phase and delayed osteogenic phase in fracture callus.

The mechanisms of the prolonged cartilage phase and a delayed osteogenic phase were further confirmed by gene expression analysis. *Runx2* is a transcription factor controlling chondrocyte- and osteoblast-specific gene expression. *Col2a1* is expressed in proliferating chondrocytes and *Col10a1* is expressed in hypertrophic chondrocytes which could further undergo mineralization and apoptosis, linking to subsequent angiogenesis and endochondral bone formation (Chung et al., 2001; Kugimiya et al., 2005; Takeda et al., 2001). *Osteocalcin* is mainly expressed in mature osteoblasts although it is also detected in hypertrophic chondrocytes. In this study, we found that in chondrocyte-specific *Bmp2* cKO mice, *Runx2* and *OC* expression was reduced at days 7–21. *Col2a1* and *Col10a1* expression was reduced at days 7 and 10, indicating that there were impediments for chondrocyte proliferation and hypertrophy at the early stage of fracture healing, which resulted in the delay of maturation of the cartilaginous callus and osteogenesis. Consequently, the *osteocalcin* levels remained at lower levels at days 14 and 21. The detailed mechanisms of a delayed osteogenesis in an immature cartilage callus were not fully understood and the possible explanation may include lack of sufficient gene expression to stimulate MSCs to differentiate into osteoblasts.

In this study, we did not observe a significant difference in fracture healing in osteoblast-specific *Bmp2* cKO mice, suggesting that BMP2 expression in osteoblasts may not be essential for callus formation, osteogenic initiation and mineralization during the fracture healing process. The lack of *Bmp2* expression in osteoblasts may be compensated by other BMP family members or other growth factors. Among BMP family members, BMP4, BMP6 and BMP7 have been shown to have similar function with BMP2 in bone formation and bone repair (Kloen et al., 2002; Kugimiya et al., 2005; Kwong et al., 2009; Spector et al.,

2001). In order to determine the compensatory mechanism in osteoblast-specific *Bmp2* cKO mice, a compound cKO approach may be required.

In conclusion, we investigated the role of endogenous *Bmp2* in bone fracture healing with a unilateral open transverse tibial fracture model using *Bmp2* cKO approach and demonstrated that endogenous *Bmp2* expression in chondrocytes plays an essential role in cartilage callus maturation during the early stage of bone fracture healing. The findings obtained from this study may help us apply our knowledge in the clinical application of BMP2 with more precise guidance for the time window of treatment for bone defect-related diseases.

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Abbreviations

BMP2	bone morphogenetic protein 2
cKO	conditional knockout
ROI	region of interest
OC	osteocalcin
BV	bone volume
TV	tissue volume

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Fig. 1.

Fracture healing was delayed in chondrocyte-specific *Bmp2* cKO mice (Ch *Bmp2* cKO). X-ray radiographic evaluation of the fracture sites showed that there were clear gaps with no indications of bridging by days 7 and 10 in both *Col2Cre;Bmp2^{fx/fx}* (Ch *Bmp2* cKO) mice (indicated by red arrows). In contrast, blurred gaps with more consolidated bridging was detected in Cre-negative mice by day 14, but for *Col2Cre;Bmp2^{fx/fx}* (Ch *Bmp2* cKO) mice, the gaps remain clear (red arrows). Similar fracture healing status was observed in *Col1Cre;Bmp2^{fx/fx}* (Ob *Bmp2* cKO) mice compared to Cre-negative control mice.

Fig. 2.

Fracture healing was delayed in chondrocyte-specific *Bmp2* cKO (Ch *Bmp2* cKO) mice. (A) Micro-CT analysis of fracture callus showed that the morphological structure and mineralization of callus tissue were disorganized or delayed in *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice. (B–C) Both bone volume (BV/TV, %) and connectivity density (Conn.D, 1/mm³) of *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice at the fracture sites were significantly decreased in comparison to Cre-negative control mice by days 7–21 post-operatively ($p < 0.05$) and these two groups reached similar bone volume and connectivity density at the last time point. No significant difference in bone volume and connectivity density was observed between *Col1Cre;Bmp2^{flx/flx}* (Ob *Bmp2* cKO) mice and Cre-negative control mice. * $p < 0.05$, two-way ANOVA followed by Dunnett's test.

Fig. 3.

Fracture healing was delayed in chondrocyte-specific *Bmp2* cKO (Ch *Bmp2* cKO) mice. (A and B) Histological analysis demonstrated that the cartilage-like or bone-like callus tissues that formed between fracture gap and periosteum were similar in Cre-negative and *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice by day 7 post-operatively; the cartilage-like callus lasted longer and remained large in *Col2Cre;Bmp2^{flx/flx}* mice compared to Cre-negative mice by days 10 and 14 (indicated by blue arrows, magnification 100×). (C and D) Large cartilage volume (Cg.Ar/Ps.Cl.Ar, %) while smaller bone volume (Md.Ar/Ps.Cl.Ar, %) were detected in *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice compared to Cre-negative control mice by days 10–21 post-operatively. No significant difference in cartilage and bone volume was observed between *Col1Cre;Bmp2^{flx/flx}* (Ob *Bmp2* cKO) mice and Cre-negative control mice. **p* < 0.05, two-way ANOVA followed by Dunnett's test.

Fig. 4.

Mechanical strength was reduced in chondrocyte-specific *Bmp2* (Ch *Bmp2* cKO) cKO mice. (A and B) Ultimate torque and toughness were significantly decreased in bone samples of *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice compared to Cre-negative control mice by days 10–28. (C) Stiffness was significantly decreased in bone samples of days 10, 14 and 21 of *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice compared with Cre-negative control mice by days 10–21 but was similar by day 28. No significant difference in ultimate torque, toughness and stiffness was observed between *Col1Cre;Bmp2^{flx/flx}* (Ob *Bmp2* cKO) mice and Cre-negative control mice. * $p < 0.05$, two-way ANOVA followed by Dunnett's test.

Fig. 5.

Chondrocyte and osteoblast marker gene expression was reduced in chondrocyte-specific *Bmp2* cKO mice (Ch *Bmp2* cKO). (A–D) *Col2a1* and *Col10a1* levels were significantly reduced by days 7 and 10 compared to those in Cre-negative control mice and there was no significant difference in other time points. *Runx2* and *osteocalcin* (OC) levels were significantly reduced by days 7–21 in *Col2Cre;Bmp2^{flx/flx}* (Ch *Bmp2* cKO) mice compared to Cre-negative control mice. No significant difference in expression of *Col2a1*, *Col10a1*, *Runx2* and *OC* was observed between *Col1Cre;Bmp2^{flx/flx}* (Ob *Bmp2* cKO) mice and Cre-negative control mice. * $p < 0.05$, two-way ANOVA followed by Dunnett's test.