Are Endogenous BMPs Necessary for Bone Healing during Distraction Osteogenesis?

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Abstract Previous reports suggest the application of exogenous BMPs can accelerate bone formation during distraction osteogenesis (DO). However, there are drawbacks associated with the use of exogenous BMPs. A possible alternative to the use of exogenous BMPs is to upregulate the expression of endogenous BMPs. Since DO results in spontaneously generated de novo bone formation in a uniform radiographic, histological, and biomechanical temporal sequence, a genetically engineered model lacking endogenous BMP2 should have measurable deficits in bone formation at different time points. We performed DO on BMP2^{fl/+} and BMP2^{fl/+ cre} mice using a miniature Ilizarov fixator. Distracted samples were collected at various time points and analyzed using Real Time-quantitative PCR, μ CT, radiology, immunohistochemistry, histology,

and biomechanical testing. Immunohistochemical studies of 34-day heterozygous samples showed reduced expression of BMP2, BMP7, BMPR1a, ACTR1, and ACTR2b. μ CT analysis of 51-day heterozygous samples revealed a decrease in trabecular number and increase in trabecular separation. Biomechanical testing of 51-day heterozygous samples revealed decreased stiffness and increased ultimate displacement. Radiological analysis showed the heterozygotes contained a decreased bone fill score at 17, 34, and 51 days. These data suggest endogenous BMPs are important for bone healing and manipulating endogenous BMPs may help accelerate bone consolidation during DO.

Introduction

Distraction osteogenesis (DO) is used to treat limb-length discrepancies, bone deformities, and bone loss secondary to trauma, infection, or tumors [3, 15, 16]. In this technique, an osteotomy of the bone to be lengthened is performed, followed by the application of an external fixator to the proximal and distal ends of the bone. After a short latency period, gradual and controlled distraction of the two bone fragments is applied until the desired amount of lengthening is obtained. Following completion of distraction, the

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newly formed bone is allowed to heal until full consolidation. One of the main drawbacks of DO is the long period of time the external fixator must be kept on until the newly formed bone is consolidated [25]. Thus, different methods are currently being studied to accelerate the consolidation phase of DO including the local application of growth factors such as transforming growth factor β (TGF- β) [24], platelet-derived growth factors (PDGFs) [21], insulin-like growth factors (IGFs) [6], fibroblast growth factors (FGFs) [1], vascular endothelial growth factors (VEGFs) [4], and bone morphogenetic proteins (BMPs) [14, 18, 20].

The application of BMP2 and BMP7 has been reported to stimulate bone healing in many clinical cases [7, 9, 12, 26, 31]. We previously showed local application of exogenous BMP7 enhanced bone healing in a rabbit model of DO [20]. A limitation of using exogenous BMPs in humans compared to animals is that much larger doses of BMPs must be used to accelerate bone consolidation. Such large doses are costly and may trigger the onset of unknown side effects, such as bone resorption [8, 23] and heterotopic ossification [2, 32]. Thus to avoid such potential problems associated with the use of exogenous BMPs, a logical alternative would be to upregulate the expression of endogenous BMPs in order to produce similar effects on bone consolidation during DO. Our group [10, 11], as well as another [17], previously analyzed the expression patterns of various members of the endogenous BMP signaling pathway in mouse and rabbit models of DO. These studies demonstrated the importance of endogenous BMPs in bone formation during DO, as evidenced by an increased expression of various BMP ligands, BMP receptors, and related transcription factors during the different phases of DO. Since DO results in spontaneously generated de novo bone formation in a uniform radiographic, histological, and biomechanical temporal sequence, a genetically engineered model lacking endogenous BMP2 should have measurable deficits in bone formation at different time points.

The purpose of this study was therefore to answer two key questions: First, do conditional BMP2 knockout mice have reduced BMP2 expression in the limbs? Second, are endogenous BMPs important for bone healing during DO?

Materials and Methods

We generated a colony of conditional BMP2 knockout mice by intercrossing mice containing a floxed *Bmp2* allele with *Prx1::cre* transgenic mice. Upon breeding these mice, the *cre* recombinase enzyme recognizes the loxP sites flanking exon 3 of the *Bmp2* gene and excises this portion of the gene out. The remaining ends of the cleaved DNA segment are ligated back together forming one continuous

strand [33]. Under the control of the paired-related homeobox-1 (*Prx1*) enhancer, *Bmp2* is inactivated in the conditional BMP2 knockout mice early during limb bud development [30]. Wild-type and heterozygous mice were genotyped using PCR. Primers used for PCR: *bmp2* primers (AHP2-9: 5'-GTGTGGTCCACCGCATCAC-3' and AHP2-35: 5'-GGCAGACATTGTATCTCTAGG-3') and *cre* primers (Forward: 5'-GCCTGCATTACCGGTGCA zzzzTGCAACGA-3' and Reverse: 5'-GTGGCAGATGG CGCGGCAACACCATT-3'). PCR conditions included heating for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds, with further extension at 72°C for 7 minutes. All experimental procedures including the operative protocol and handling of mice were approved by the McGill University Animal Care Committee.

We studied a total of 63 mice that were divided into two groups. The first group of mice included nine nonoperated, 1-week-old mice (three wild-type *BMP2*^{fl/+} mice, three heterozygous *BMP2*^{fl/+ cre} mice, and three homozygous *BMP2*^{fl/fl cre} mice). mRNA was collected from these mice and analyzed for BMP2 gene expression using Real Time-quantitative PCR (RT-qPCR). The three wild-type mice were used as a positive control that contained full BMP2 expression in the limbs. The three heterozygous mice were used as the test group that should contain reduced BMP2 expression. Lastly, the three homozygous mice were used as a negative control that should completely lack BMP2 expression in the limbs. RNA isolation was performed according to an Invitrogen RNA isolation protocol (Invitrogen, Carlsbad, CA). RNA samples were reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Reverse-transcribed mRNA collected from the nonoperated samples were loaded onto a 96-well plate and mixed with Universal Master Mix (Applied Biosystems, Foster City, CA), a TaqMan probe for BMP2 (Applied Biosystems, Foster City, CA), and RNase free water (Ambion, Austin, TX). The 96-well plate was placed into a 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) for RT-qPCR analysis. GAPDH was used as an endogenous control to normalize all samples used in the RT-qPCR reaction. Relative quantification of the target cDNA was performed using Applied Biosystems' comparative C_t method (ABI Prism 7700 Sequence Detection System User Bulletin #2, 2001).

The second group of mice included 54 adult mice aged 2 to 3 months old that underwent DO (27 wild-type and 27 heterozygous mice). DO was performed on these mice using a miniature Ilizarov fixator as previously reported by our group [10] and Tay et al. [28]. We were unable to perform DO on the homozygous mice as these mice suffered from multiple spontaneous fractures during surgery.

Fig. 1 Experimental design of the 54 operated mice. Distracted tibial samples were collected from wild-type and heterozygous mice at various time points: 11, 17, 34, and 51 days post-surgery. These samples were analyzed using RT-qPCR, μ CT, Faxitron xray, immunohistochemistry, Goldner-Trichrome staining (histology), and biomechanical testing.

Abbreviations: μ CT; Micro-computed tomography, FX; Faxitron x-ray, IHC; Immunohistochemistry, GTS; Goldner-Trichrome staining (histology), BT; Biomechanical testing, WT; Wild-type, Het; Heterozygous

The mice were anesthetized using isoflurane throughout the surgery. A set of two 0.25-mm pins were drilled 90° apart into the proximal and distal metaphysis of the right tibia. The pins were secured into position using two rings and eight hexagonal nuts. Three screws were used to connect the two parallel rings. A transverse osteotomy was then performed along the diaphysis of the right tibia using a no.11 surgical scalpel (Fish Scientific, Osaka, Japan). The mice were subcutaneously injected with 0.1 mL of (1 mg/kg-Sigma) buprenorphine before and after surgery for pain management and monitored regularly throughout the duration of surgery until sacrifice. After a 5-day latency period, distraction was started at a rate of 0.2 mm every 12 hours for 12 days, followed by 34 days of consolidation.

Mice were sacrificed by CO₂ asphyxia under general anesthesia at four time points: 11 days (mid-distraction), 17 days (end of distraction), 34 days (mid-consolidation), and 51 days (end of consolidation) after surgery (Fig. 1). Twelve mice (six wild-type and six heterozygous mice) were sacrificed at each of the four time points for μ CT, Faxitron xray, immunohistochemistry, and histology, with the exception of the 51-day time point where an additional six mice (three wild-type and three heterozygous mice) were euthanized for biomechanical testing; thus, 18 mice (nine wild-type and nine heterozygous mice) were euthanized at 51 days post-surgery. We dissected all the callus located between the distracted bone fragments of the operated mice for subsequent analysis. Cuts were made proximal and distal to the distracted region to avoid disturbing the bony callus.

Immediately following sacrifice, μ CT and Faxitron xray analysis were performed on all specimens (12 mice at each time point; except for biomechanical testing samples). Samples of the operated tibiae were immersed in 4% paraformaldehyde, washed with 1× phosphate buffer saline (PBS), and dehydrated in 50% and 70% ethanol. Soft tissues were not removed from the distracted bone

specimens. The samples were then taken to the McGill Centre of Bone and Periodontal Research for μ CT and radiological assessment. μ CT analysis was completed using the SkyScan 1072 (Aartselaar, Belgium). The distracted tibiae were scanned at 45 KeV/222 μ A with 25× magnification (11.25- μ m pixel size). Image reconstruction was performed using NRecon (1.4.4, SkyScan, Aartselaar, Belgium). Static histomorphometric parameters analyzed included tissue volume (mm³), bone volume (mm³), bone volume/tissue volume (BV/TV %), trabecular number (1/mm), trabecular separation (mm), and trabecular thickness (mm). The region of interest for static histomorphometric analysis was defined as the distracted area located between the two ends of the fractured bone. These parameters were measured using the CT Analyser (1.8.0.2, SkyScan). Average scores of each parameter were recorded (Table 1). The Faxitron MX-20 (Faxitron X-Ray Corporation, Wheeling, IL) was used to produce radiographs of the distracted samples. Unlabeled radiographs were then graded by three blinded observers using a four-point bone fill score in which 0 = no bone, 1 = 0% to less than 50% bone fill, 2 = 50% to less than 100% bone fill, and 4 = complete bone fill [13, 29]. The average bone fill scores were recorded (Table 2).

After μ CT and radiological analysis were completed, the same samples were subdivided into two equal groups for immunohistochemical (n = 6 mice/group × four time points) and histological analysis (n = 6 mice/group × 4 time points). For immunohistochemistry, samples were fixed in 4% paraformaldehyde overnight, decalcified in 20% ethylene diamine tetra-acetic acid for 3 weeks, embedded in MMA, and sectioned using a Leica RM 2255 microtome (Leica Microsystems, Richmond Hill, ON). Following deparaffinization and hydration, endogenous peroxidase activity was blocked using 10% hydrogen peroxide for 10 minutes. Nonspecific binding was blocked by incubating samples in phosphate-buffered saline containing

Table 1. μ CT analysis of wild-type and heterozygous distracted limbs at various time points of DO

Variable	11 days		17 days		34 days		51 days	
	WT	Het	WT	Het	WT	Het	WT	Het
Tissue volume (mm ³)	9.371085	7.797865	24.35504	19.98558	10.06985	14.74117	25.23202	19.45774
Bone volume (mm ³)	0.160123	0.11889	0.486595	0.447992	0.456673	0.936365	3.015048	2.27589
BV/TV (%)	2.54188	2.6729	1.58292	1.685033	4.527028	6.674693	12.08007	8.066395
Trabecular thickness (mm)	0.105992	0.099138	0.12197	0.131212	0.141132	0.169688	0.174585	0.224465
Trabecular number (1/mm)	0.23387	0.253553	0.116877	0.13147	0.303213	0.366708	0.647168	0.34042
Trabecular separation (mm)	0.742892	0.699758	1.687852	1.745193	1.336975	1.623127	0.720015	0.996188

Table 2. Radiological results of distracted limbs from wild-type and heterozygous conditional BMP2 knockout mice

Time point of sacrifice (days after surgery)	Number of samples	Average bone fill score	
		WT	Het
11 days	6	0	0
17 days	6	0.8	0.2
34 days	6	0.9	0.8
51 days	6	1.7	1.1

10% normal goat serum (same species as secondary antibody) for 20 minutes. For immunostaining, commercially available polyclonal goat antibodies were used to detect BMP ligands: BMP2 and BMP7, BMP receptors (BMPR1a, BMPR1b, and BMPR2) and activin receptors (ACTR1 and ACTR2b) (Santa Cruz Biotechnology, Santa Cruz, CA; 1/100 dilution in 1% normal goat serum). These ligands and receptors are key members involved in the BMP signaling pathway. Distracted tissue sections were probed with the polyclonal goat antibody overnight at 4°C in a humidified chamber. For negative controls, we omitted the primary antibody. We then incubated the sections with a biotinylated mouse antigoat secondary antibody (Santa Cruz Biotechnology; 1/400 dilution in 1% normal goat serum) for 30 minutes at room temperature in a humidified chamber. Sections were stained using the avidin-biotin complex method for 30 minutes, followed by DAB-peroxidase revelation. Finally, we counterstained sections with hematoxylin and mounted with Permount (Fisher Scientific, Montreal, QC). Photomicrographs of the distracted regions were taken under 10 $\times$ and 40 $\times$ magnification using a Leica microscope (Leica Microsystems, Richmond Hill, ON) attached to a Q-Imaging camera (Olympus DP70, Japan). We analyzed the entire tissue sections of the distracted callus using a semiquantitative method for grading positive cell staining that we have used in previous studies [11]. The grading scheme included: + represents > 25% of the cells stained positively for the gene of interest, ++ represents 25% to 50% of cells stained positive, +++ represents 25% to 75%

Table 3. Average immunohistochemical results for BMP members present in chondrocytes of the distracted limbs of wild-type and heterozygous conditional BMP2 deficient mice

Gene	Consolidation			
	34 days		51 days	
	WT	Het	WT	Het
BMP ligands				
BMP2	++	+	++	+
BMP7	++	+	+	++
Receptors				
BMPR1a	++	+	-	+
BMPR1b	++	++++	+	++
BMPR2	+++	+++	+	+
ActR1	++++	+	++	++
ActR2b	+++	++	+	++
Inhibitors				
BMP3	+	+	+	+

Staining of chondrocytes in distracted zone, -: no positive staining, +: less than ¼ of cells stained positive, ++: ¼ to ½ of cells stained positive, +++: ½ to ¾ of cells stained positive, and ++++: more than ¾ of cells stained positive.

of cells stained positive, ++++ represented more than 75% of cells stained positive, lastly - denoted no cells stained positively. Sections were analyzed blindly in triplicates by a single immunohistochemistry specialist (Dominique Lauzier, Shriners Hospital, Montréal, QC). The averages of all results were recorded (Table 3).

Histological analysis was performed on the second group of samples (n = 6 mice/group $\times$ 4 time points) following μ CT and radiological analysis. Samples were embedded in MMA, sectioned at 0.5 μ m, deplastified, and stained using the Goldner-Trichrome technique. The stained sections were mounted using Permount (Fisher Scientific, Montreal, QC). Photographs of the distracted zones were taken under 25 $\times$ and 100 $\times$ magnification. Histological analysis involved the detection of mineralized (green) and nonmineralized (red) areas of the distracted tissue.

Biomechanical testing was performed on six samples (three wild-type mice and three heterozygous mice) collected only at 51 days post-surgery and analyzed at the McGill Centre for Bone and Periodontal Research of McGill University. A three-point bending test was conducted using the Mach-1™ Micromechanical Systems device (Bio Syntech Canada, Inc., Laval, QC). The three-point bending test was chosen over other methods of biomechanical testing based on previously reported studies in mice [5, 19, 22]. The distracted bone was placed on its posterior surface, resting on two supports of a bending apparatus that lie 7 mm apart. A bending load was applied downwards on the midshaft of the lengthened tibia at a rate 50 $\mu\text{m/s}$, until failure. Failure loads were analyzed using the Mach-1™ Motion and Analysis software (version 3.0.2, Bio Syntech Canada). A load-displacement curve was generated using this software to measure biomechanical parameters including stiffness (N/mm), ultimate force (N), ultimate displacement (μm), and work to ultimate failure (N*mm).

For statistical analysis, we determined differences in relative BMP2 gene expression between wild-type and homozygous samples and between heterozygous and homozygous samples using a one-way nonparametric ANOVA test and Newman-Keuls multiple comparison post-test. We determined differences in trabecular separation and trabecular number between wild-type and heterozygous distracted samples using an unpaired t-test. All statistical tests for this study were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA).

Results

In the first group of nine nonoperated mice, RT-qPCR analysis (Fig. 2) we observed differences in BMP2 expression between all three groups of nonoperated mice. BMP2 expression was reduced ($p = 0.0195$) in the homozygous mice compared to that in the wild-type mice and reduced ($p = 0.0195$) in the homozygous mice compared to that in the heterozygous mice.

In the second group of 54 mice that underwent DO, μCT results showed no apparent changes in osteogenic patterns between wild-type and heterozygous samples at 11, 17 (distraction phase; Fig. 3A–D), and 34 days (mid-consolidation phase; Fig. 3E, F). However, at 51 days (end of consolidation phase; Fig. 3G, H) results showed reduced bone formation within the distracted gap of the heterozygous samples compared to the wild-type controls (Table 1) as evidenced by an increase ($p = 0.0093$) in trabecular separation (Fig. 4A) and decrease ($p = 0.0091$) in trabecular number (Fig. 4B) between the heterozygous and wild-

Fig. 2 Nonoperated mice contain reduced BMP2 expression in the limbs. mRNA was collected from the limbs of non-operated 1-week-old wild-type, heterozygous and homozygous mice and analyzed using RT-qPCR. GAPDH was used as an endogenous control. Decreased BMP2 levels were expressed in the heterozygous and homozygous mice compared to the wild-type mice. No change in BMP2 levels was detected between the wild-type and heterozygous mice.

type samples. Radiological analysis (Table 2) showed the heterozygous mice had a reduced average bone fill score compared to the wild-type controls at 17, 34, and 51 days. The immunohistochemical analysis suggested that mostly chondrocytes and fibrocartilaginous cells stained positively for the BMP ligands and related receptors (Table 3). At 34 days (mid-consolidation phase), the heterozygous mice exhibited decreased expression of BMP2 (Fig. 5), BMP7, BMPR1a, ACTR1, ACTR2b, and increased BMPR1b levels compared to the control group. There was no difference in BMPR2 levels between the control and heterozygous mice at 34 days. At 51 days (end of consolidation phase), the heterozygous mice had an increased expression of BMP7, BMPR1b, and ACTR2 and a reduction of BMP2 expression. We observed no change in BMPR2 and ACTR1 levels during late consolidation between any of the groups. Histological sections of 51 days specimen showed varying amounts of mineralized (green) and nonmineralized (red) tissues, chondrocytes, and fibrous tissues in wild-type and heterozygous mice (Fig. 6). We observed decreased stiffness ($p = 0.3228$) and increased ultimate displacement ($p = 0.6540$) in the heterozygous mice compared to the controls (Fig. 7).

Discussion

The literature suggests that the application of exogenous BMPs can accelerate bone formation during DO. However, there are a number of drawbacks associated with the use of exogenous BMPs in humans. Thus, a possible alternative to

Fig. 3A–P μ CT and radiological images of distracted limbs collected from wild-type and heterozygous BMP2 knockout mice. Distracted tibial samples were collected at 11 days (mid-distraction phase), 17 days (end of distraction phase), 34 days (early consolidation phase), and 51 days (end of consolidation phase) post-surgery and analyzed using (A–H) μ CT and (I–P) Faxitron xray.

Fig. 4A–B Static histomorphometric parameters revealed heterozygous mice contained less bone formation at 51 days. Distracted samples collected at 51 days post-surgery revealed an increase ($p = 0.0093$) in (A) trabecular separation and decrease ($p = 0.0091$) in (B) trabecular number, indicating there was less bone formation in the heterozygous mice during the end of consolidation compared to the controls.

the use of exogenous BMPs would be to upregulate the expression of endogenous BMPs. In this study, we raised two questions: First, do conditional BMP2 knockout mice contain reduced BMP2 expression in the limbs? Second, are endogenous BMPs important for bone healing during DO?

There are limitations to this study. First, μ CT, radiological and biomechanical testing results showed varied patterns of bone regeneration within samples of the same group. Gender differences in mice and incomplete penetrance of *cre*-mediated *Bmp2* excision may lead to inconsistent levels of BMP2 expression that may account for the variability in bone formation. Second, the instability of the external fixator may have potentially contributed to the discrepancy of the observed results. Third, biomechanical testing using the three-point bending test may have been an unreliable method to assess bone quality of samples, especially using a small sample size.

To answer the first question, our RT-qPCR results indicated that there was reduced BMP2 expression in the limbs of heterozygous and homozygous conditional BMP2 knockout mice. These results are supported by the findings of Tsuji et al.[30] who have used a fracture healing model of conditional BMP2 deficient mice.

To address the second question, our μ CT, radiological and biomechanical testing showed there was less bone formation in the heterozygotes compared to the wild-type controls at 51 days post-surgery (end of consolidation phase). We were unable to find any similar studies in the literature analyzing DO in conditional BMP2 knockout mice. Therefore, we could not compare our results to those of other authors. However, Tsuji et al. [30], using the same conditional BMP2 knockout mice, demonstrated that BMP2 does play a role in early fracture healing. Our data could be explained by our immunohistochemical observation of a decrease in BMP2, BMP7, BMPR1a, ActR1, and ActR2b expression in chondrocytes in the distracted callus

Fig. 5A–H BMP2 expression in chondrocytes present in the distracted limbs of BMP2 knockout mice. Immunohistochemical images of (A, B, E, F) 34 and (C, D, G, H) 51 day heterozygous and wild-type distracted samples revealed that the heterozygotes contained

decreased BMP2 expression at 34 days (early consolidation) that gradually leveled off by 51 days (late consolidation). BMP2-stained chondrocytes are indicated by the arrows in the diagram. (Immunohistochemistry, BMP2; original magnification, $\times 10$ and $\times 40$).

Fig. 6A–D Histological images of distracted limbs of wild-type and heterozygous mice at 51 days post-surgery are shown. MMA-embedded tibial sections of (A, C) wild-type and (B, D) heterozygous mice were stained using the Goldner-Trichrome technique. At 51 days (end of consolidation phase), both groups contained varying levels of mineralized (green) and non-mineralized (red) tissue. Chondrocytes and fibrous tissue were also present in the distracted samples, as indicated by the arrows in the diagram. (Stain, Goldner-Trichrome; original magnification, $\times 25$ and $\times 100$).

of the heterozygotes at 34 days post-surgery. However, we observed an upregulation of BMP7 and ActR2b at 51 days post-surgery. Whether this upregulation of BMP7 at 51 days was a response triggered by decreased BMP2 levels at 34 days (acting as a compensatory mechanism) remains yet to be determined. One study suggests that BMP7 uses ActR2 as its Type II receptor [27]. Therefore, ActR2b expression may have been upregulated by

increased BMP7 expression at 51 days in the heterozygous mice. The effect of this BMP7 upregulation was not, however, sufficient enough to compensate for the decreased BMP2 activity.

In conclusion, our observations demonstrate endogenous BMPs may be necessary for bone healing during DO. Therefore, manipulating the endogenous BMP pathway is a plausible alternative to the use of exogenous BMPs that

Fig. 7A–B Biomechanical testing results of distracted wild-type and heterozygous BMP2 knockout mice at 51 days post-surgery are shown. The three-point bending test was performed on distracted wild-type and heterozygous samples collected at 51 days (end of consolidation phase) post-surgery. A decrease ($p = 0.323$) in (A) stiffness and increase ($p = 0.654$) in (B) ultimate displacement was observed in the heterozygous mice compared to the wild-type controls.

may accelerate bone consolidation during DO. Further research is required to investigate the methods for manipulating the BMP pathway.

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