rhBMP-2 Modulation of Gene Expression in Infected Segmental Bone Defects

Katherine E. Brick BS, Xinqian Chen MD,
Jamie Lohr MD, Andrew H. Schmidt MD,
Louis S. Kidder PhD, William D. Lew MS

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Abstract The osteoinductive capability of BMPs appears diminished in the setting of acute infection. We applied rhBMP-2 to a segmental defect in a rat femur and measured the expression of key bone formation genes in the presence of acute infection. Types I and II collagen, osteocalcin, and BMP Type II receptor mRNA expression were characterized in 72 Sprague-Dawley rats, which received either bovine collagen carrier with 200 µg rhBMP-2 plus *Staphylococcus aureus*, carrier with bacteria only, carrier with rhBMP-2 only, or carrier alone. Six animals from each group were euthanized at 1, 2, and 4 weeks. Total RNA was isolated and extracted, and mRNA was determined by

real-time comparative quantitative PCR. Infected defects had little expression of collagen I and II and osteocalcin mRNAs, while BMP receptor II expression with infection was greater than carrier-only controls at Weeks 2 and 4. Notably, all four genes were upregulated in infected defects in the presence of rhBMP-2. Thus, in a clinical setting with a high risk of infection and nonunion, such as a compound fracture with bone loss, rhBMP-2 may increase the rate and extent of bone formation. Even if infection does occur, rhBMP-2 may allow a quicker overall recovery time.

Introduction

Deep infection is one of the most difficult complications encountered after the surgical management of fractures. When infection occurs after internal fixation, further surgery is almost always required, and the infection threatens both fracture healing and retention of the associated implant. The presence of an orthopaedic implant complicates the treatment of osteomyelitis by serving as a site for bacterial glycocalyx formation [18]. Yet, it is clear maintaining fracture stability is important for obtaining fracture union and decreasing the clinical progression of infection [35]. The clinician therefore faces a difficult decision regarding the merits of implant removal versus maintaining fracture fixation.

Our previous research has shown BMP stimulates healing of a critical defect in the rat femur in the presence of both acute and chronic infection [4–7]. Both fracture healing and the host response to infection involve complex temporal and spatial interactions among various cytokines and other cell-signaling molecules. Genetic mechanisms underlying the host response to infection at the site of a healing fracture are poorly understood, as are

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K. E. Brick, X. Chen, L. S. Kidder, W. D. Lew
Midwest Orthopaedic Research Foundation,
Minneapolis, MN, USA

J. Lohr
Division of Pediatric Cardiology, Department of Pediatrics,
University of Minnesota School of Medicine,
Minneapolis, MN, USA

A. H. Schmidt
Orthopaedic Surgery Department, Hennepin County Medical
Center, Minneapolis, MN, USA

L. S. Kidder (✉)
Department of Radiology, University of Minnesota School of
Medicine, B292 Mayo, 420 Delaware Street SE,
Minneapolis, MN 55455, USA
e-mail: kidde001@umn.edu

the changes in gene regulation potentially induced by growth factors.

Although there are numerous genes involved in the repair of noninfected and infected fractures, we focus on four due to the essential roles they play in different stages of endochondral ossification and bone defect healing [42]. Types I and II collagen are frequently used as markers of bone growth, osteocalcin reflects matrix mineralization [26], and BMP Type II receptor was chosen to identify potential changes in expression due to the exogenous rhBMP-2 application [31]. By understanding how the regulation of genes involved in infected fracture healing is affected favorably by BMP application, we hope to clarify evidence for the use of BMPs in clinical settings where infection is likely.

We hypothesized rhBMP-2 addition in the setting of an acutely infected fracture would increase mRNA expression of the four selected genes relative to an infected defect without rhBMP-2. In addition, we hypothesized rhBMP-2 would accelerate the expression of bone formation genes in the setting of infection, as shown by an earlier peak in the level of gene expression at the time points of 1, 2, and 4 weeks.

Materials and Methods

A 6-mm mid-diaphyseal defect was surgically created under aseptic conditions and stabilized with a polyacetyl plate and six Kirschner wires in the left femur of 72 Sprague-Dawley rats (350–399 g) [4]. The animals were divided into four treatment groups of 18 animals (Table 1). The first group received a 1- × 1- × 0.4-cm segment of Type I bovine collagen sponge (absorbable collagen sponge [ACS]; Medtronic Sofamor Danek, Memphis, TN) wetted with 0.1 mL sterile water containing 200 µg rhBMP-2 (Medtronic Sofamor Danek), which was allowed to bind to the ACS during a 15-minute soak period at room temperature. Then, 0.1 mL normal saline containing 5×10^5 colony-forming units (CFUs) of *Staphylococcus aureus* was added to the sponge, and the wetted sponge was

packed into the defect. This group is referred to as the rhBMP-2/infection group. The second group received an ACS wetted with 0.1 mL sterile water alone (no rhBMP-2), followed 15 minutes later by addition of a 0.1-mL suspension of 5×10^5 CFUs of *S aureus* (infection group). Bacterial introduction at the time of fixation was chosen in an attempt to minimize confounding variables that may affect the gene expression profile and time course during fracture healing and to simulate a clinical scenario in which there is a higher probability of infection despite débridement (eg, open fractures or revision of infected internal fixation devices). The third group received an ACS with 200 µg rhBMP-2 in sterile water and normal saline without bacteria (rhBMP-2 group). The fourth group received an ACS wetted only with 0.1 mL sterile water and 0.1 mL normal saline (carrier-only group). The carrier-only and rhBMP-2 only conditions were included as controls to verify bone growth would occur without the presence of infection, as would be expected [55]. Six animals from each of the four groups were euthanized at 1, 2, or 4 weeks after surgery. The ACS is the commercially available inert carrier for rhBMP-2 with a previously characterized rhBMP-2 release pattern [5] and also served to retain the bacteria within the defect in the short term. The pathogenic isolate of *S aureus* used in this study was previously obtained from a patient with an infected THA and is penicillin sensitive. All procedures in this study were approved by our Institutional Animal Care and Use Committee.

A single surgeon (XC) performed all surgeries using a previously described procedure [4]. Animals were anesthetized with an intraperitoneal injection of ketamine (80–120 mg/kg) and xylazine (2–3 mg/kg). A lateral approach to the femur was performed, and muscles and periosteum were stripped from the diaphysis. A predrilled, six-hole polyacetyl plate (Midwest Orthopaedic Research Foundation, Minneapolis, MN) was fixed to the femur bicortically with 0.9-mm threaded Kirschner wires. A full-thickness 6-mm defect was then created with a small pneumatic-powered oscillating saw. The collagen sponge and its contents were placed inside the defect and the wound was closed. An analgesic (buprenorphine, 0.013–0.026 mg/kg) was administered intramuscularly immediately after surgery. The animals were allowed full activity postoperatively, and their activity level, food intake, and surgical wound were monitored daily.

To minimize RNase activity before tissue procurement (which would confound our results), each rat was anesthetized and the femur exposed under sterile conditions and harvested in vivo. All animals with previously inoculated defects showed signs of a localized infection, which included a well-defined pus-filled fibrous membrane encasing the defect and fixation, and no apparent infection of the surrounding tissues. The fibrous membrane was

Table 1. Experimental design and numbers of animals

Treatment		Number of animals		
<i>S aureus</i> (CFUs)	rhBMP-2 (µg)	1 week after contamination	2 weeks after contamination	4 weeks after contamination
5×10^5	200	6	6	6
5×10^5	0	6	6	6
0	200	6	6	6
0	0	6	6	6

isolated and opened in these animals; cultures were obtained and subsequently inoculated on plated media to verify bacterial growth. The femoral defect was exposed, and the new bone bridging the defect was removed for gene expression evaluation. Care was taken to exclude muscle tissue from the sample. After being harvested, the tissue sample was immediately placed in a vial and snap-frozen in liquid nitrogen. The animals were euthanized by an intraperitoneal barbiturate injection. Tissues obtained from the femoral defects were handled and processed for RNA isolation, cDNA synthesis, and real-time comparative quantitative PCR according to generally accepted techniques (Appendix 1).

A three-way analysis of variance was used to determine the effect of the independent variable of rhBMP-2 (200 µg) in the setting of infection (5×10^5 CFUs) and also the effect of time (1, 2, and 4 weeks) on the dependent expression of each of the four genes (SigmaStat[®]; Systat Software, Inc, San Jose, CA). Pairwise comparisons were made with the Student-Newman-Keuls method between the independent variable of rhBMP-2 treatment in the setting of infection and the dependent variable of mRNA expression of the four selected genes. Post hoc analysis was performed for each of the bone formation genes between the conditions of rhBMP-2 plus infection and infection alone at each of the three time points studied.

Results

Visual assessment of the newly mineralized callus at the time of harvest for RNA isolation revealed treatment with rhBMP-2 alone produced exuberant callus within and around the defect by 4 weeks, while defects filled with the collagen sponge alone exhibited minute amounts of callus formation at 2 and 4 weeks. This confirmed the efficacy of rhBMP-2 treatment in the absence of infection. The infected animals treated with rhBMP-2 also had extensive callus despite the obvious infection. In contrast, the infected animals without rhBMP-2 exhibited essentially no bone formation. Swab samples and subsequent bacterial cultures revealed all contaminated defects became infected. For the four treatment groups in this study, amplification efficiencies for all genes were similar, ranging from 97% to 100%. While β -actin expression increased slightly with infection when evaluated with BMP receptor II mRNA, the magnitude of change for the BMP receptor II gene was over twofold greater than that of β -actin. β -Actin expressed no interactions as the normalizer for the other three genes.

Overall, gene expression was greater in infected defects receiving rhBMP-2 than in infected defects without rhBMP-2 for all four genes. Although rhBMP-2 treatment did not reveal any difference in collagen I expression at

Fig. 1 Expression of Type I collagen mRNA from segmental defects with each of the four treatment conditions at 1, 2, and 4 weeks after surgery is presented in terms of fold change relative to the expression of the normalizing gene β -actin (BA). The p values (< 0.05) comparing magnitude of gene expression between the rhBMP-2/infection and infection conditions are shown.

Fig. 2 Expression of Type II collagen mRNA from segmental defects with each of the four treatment conditions at 1, 2, and 4 weeks after surgery is presented in terms of fold change relative to the expression of the normalizing gene β -actin (BA). The p values (< 0.05) comparing magnitude of gene expression between the rhBMP-2/infection and infection conditions are shown.

1 week (Fig. 1), addition of rhBMP-2 to an infected defect elicited greater ($p = 0.010$) collagen I mRNA expression at 2 and 4 weeks, compared with the carrier/infection group. Addition of rhBMP-2 to an infected defect resulted in greater collagen II expression at 2 weeks ($p = 0.019$) and 4 weeks ($p < 0.001$), compared with the carrier/infection group (Fig. 2). Concurrently, peak osteocalcin expression in the rhBMP-2/infection group occurred at 4 weeks (Fig. 3). Addition of rhBMP-2 to an infected defect resulted in greater osteocalcin message at 2 and 4 weeks ($p = 0.002$), compared with infection defects alone. Interestingly, animals with infected defects with and

Fig. 3 Expression of osteocalcin mRNA from segmental defects with each of the four treatment conditions at 1, 2, and 4 weeks after surgery is presented in terms of fold change relative to the expression of the normalizing gene β -actin (BA). The p values (< 0.05) comparing magnitude of gene expression between the rhBMP-2/infection and infection conditions are shown.

Fig. 4 Expression of BMP Type II receptor mRNA from segmental defects with each of the four treatment conditions at 1, 2, and 4 weeks after surgery is presented in terms of fold change relative to the expression of the normalizing gene β -actin (BA). The p values (< 0.05) comparing magnitude of gene expression between the rhBMP-2/infection and infection conditions are shown.

without rhBMP-2 exhibited a similar time course of BMP receptor II expression, while the two uninfected groups exhibited a different temporal pattern (Fig. 4). Treatment of infected defects with rhBMP-2 increased BMP receptor II expression at Week 1 ($p = 0.014$), compared to defects with carrier/infection. Addition of bacteria to rhBMP-2-treated animals resulted in greater ($p = 0.021$) BMP receptor II expression at Week 2.

Peak gene expression occurred sooner in infected defects with rhBMP-2 than in infected defects without the growth factor only for the BMP Type II receptor gene. The peak occurred 2 weeks earlier (Fig. 4). Gene expression of Type I collagen peaked at 2 weeks for both infection alone and rhBMP-2 with infection (Fig. 1). Type II collagen achieved its highest level at 1 week for infection but

reached its much higher peak at 2 weeks with rhBMP-2/infection (Fig. 2). While osteocalcin peaked at 2 weeks for the infection condition, it was at a very low level in comparison to the later peak at 4 weeks for the rhBMP-2/infection condition (Fig. 3).

Unexpectedly, expression of BMP receptor II mRNA was greater ($p = 0.027$) in animals with infection than without infection over all time points. This is in contrast to the other three genes where expression was greater in the uninfected conditions in comparison to the infected conditions. Another surprising finding was the greater Type I collagen expression in the uninfected carrier only condition versus rhBMP-2 treatment.

Discussion

Fracture healing proceeds through consistent spatial and temporal patterns regulated by complex relationships between the local fracture environment, vascularity [14], growth factors [12], cellular elements [19], presence of internal fixation [51], and infection [1, 13]. Many studies have demonstrated the ability of BMPs to promote fracture healing within a critical defect [10, 25, 53, 55] and to heal nonunions in humans [17]. In a series of previous animal studies, we used histology, biomechanical testing, radiography, and high-resolution CT to document BMPs promote healing of an infected critical defect and the amount of bone formed is also influenced by antibiotic therapy [4–7]. For this study, we hypothesized the differing patterns of bone formation observed may be due to changes in levels of gene activation in response to BMP administration, modulated by the effects of the infection. Our first hypothesis examined whether rhBMP-2 would upregulate the expression of four genes (osteocalcin, Types I and II collagen, and BMP Type II receptor) associated with bone formation in the presence of an acute infection, in comparison to an infected defect not receiving rhBMP-2. To examine the hypothesis regarding the timing of changes in gene activation, mRNA expression of these genes was determined at 1, 2, and 4 weeks postfracture.

The major limitations of this study include the use of an animal model, an acute versus chronic infection, gene expression profiling of only four genes, and the fact that we did not directly measure bone formation in these same animals. We chose a well-defined animal model to have a repeatable, safe, in vivo environment in which to measure the effect of rhBMP-2 application [13]. We chose to use an acute infection without antibiotic therapy because such a model provided a more controlled infected environment than a chronic infection model [7]. The four genes studied have well-characterized temporal patterns of expression in noninfected defect healing [3, 37, 54], which we believed

would be helpful for comparison to rhBMP-2 application. Although we could have used a gene microarray to study many more genes, we chose to use PCR because it is a more targeted and quantitative approach to further characterize genes involved in callus formation. Finally, we were unable to measure bone growth directly or perform high-resolution imaging because the callus had to be harvested from the femoral defects in situ before the animal was euthanized and immediately snap-frozen in liquid nitrogen to prevent RNA degradation. However, since the experimental techniques and model used in this study are the same as used in our previous study [4], we consider the differences in bone formation we previously observed are the result of the differences in gene activation shown in this study.

The results reported herein confirmed upregulation of all four genes in animals receiving rhBMP-2, most notably at 2 and 4 weeks. As expected, Type I collagen mRNA was highly expressed in all experimental conditions, indicating bone matrix formation [19, 42]. Application of rhBMP-2 to infected defects induced greater Type I collagen expression than the infected condition alone. Maximal expression of Type I collagen message at 2 weeks is consistent with previous research of *de novo* osteogenesis [42, 54], fracture repair [26, 37], and bone formation within a critical defect [47]. We expected carrier/rhBMP-2 treatment alone would elicit the greatest Type I collagen signal. However, carrier-only controls expressed greater message at 2 weeks. One possible explanation for this is that BMP accelerates remodeling wherein bone formation is preceded by bone resorption, consistent with an earlier reported observation [46]. Type II collagen message was also increased due to rhBMP-2 in the infected conditions, but its expression was generally at low levels and essentially nonexistent for all conditions by 4 weeks, consistent with endochondral ossification [16, 42] by chondrocytes and osteoblasts [45]. Compared to Type I collagen, the concurrent lack of Type II collagen expression at 4 weeks may reflect synthesis of a Type I collagen-rich fibrous tissue in the carrier-only and rhBMP-2/carrier defects in response to inflammation from surgical trauma. Osteocalcin levels peak between 1 to 4 weeks in uninfected fracture models [23, 26, 36, 37, 49], agreeing with our finding of peak expression at 2 weeks for the carrier/rhBMP-2 group. We observed increased osteocalcin mRNA in the rhBMP-2 plus infection condition versus infection alone at 4 weeks, consistent with our hypothesis. Additionally, we found greater BMP Type II receptor message expression in the infected groups after rhBMP-2 treatment. BMP Type II receptor mRNA expression has not been previously examined in a critical defect model with exogenous rhBMP-2 but has been reported to exhibit BMP-2 dose-dependent upregulation in vitro [30, 38]. In

response to osteoblastic signaling pathways induced by fracture [20–22, 28, 29, 39, 43], BMP Type II receptor is known to colocalize with the BMP Type I receptor [27, 31, 44], forming a heterodimeric complex with endogenous BMP-2 [40, 48]. Thus, it is not surprising the expression profile observed shows a similar pattern as that reported for the Type IA BMP receptor, which peaked at low levels [52] at 1 week in a noninfected defect [37], consistent with our carrier-only controls.

We were able to demonstrate earlier peak expression in the setting of infection for the BMP Type II receptor, confirming our second hypothesis for this gene in particular. Both infected groups followed markedly different patterns of BMP Type II receptor expression than the noninfected groups. Because there is little *in vivo* experimental data with which to compare our results, the novel trends observed in the context of infection may help clarify the role of BMP Type II receptor action with rhBMP-2. The other genes showed no evidence of accelerated expression, but rhBMP-2 did maintain upregulation of Type I collagen mRNA out to 4 weeks. The Type II collagen message peaked at 1 week in all conditions; this was previously observed [9, 26, 37, 45]. Since osteocalcin mRNA is only expressed during the later stages of fracture healing [3, 26, 33, 34], our data indicate bacterial infection delayed the BMP-promoted matrix mineralization. It is possible early proliferation of bacteria in the infected defect may have inhibited neovascularization [14], delaying osteogenesis. Local hypoxia may also contribute since decreased oxygen tension reduces osteoblastic differentiation and expression of osteocalcin mRNA in vitro [50]. The application of a BMP may encourage neovascularization of the site [11, 12, 41] and the differentiation of fibroblastic and angiogenic elements within the defect [24].

We have shown increased expression for an extended period of time of four genes important for fracture healing within an infected critical defect when stimulated by rhBMP-2. Previous studies suggest BMP treatment stimulates new bone formation in an infected critical defect [2, 4–7, 34], but this osseous response is delayed. Our data suggest callus formation differs in a critical defect, depending on BMP treatment, and this differential bone growth is associated with an increased expression of genes involved in fracture healing. It appears the inhibitory effects of infection could be partially overcome with rhBMP-2 application in appropriate clinical settings.

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Appendix 1

Detailed Description of Gene Expression Methodologies

RNA Isolation and cDNA Synthesis

Frozen samples were weighed and processed for total RNA isolation by freezer mill pulverization under liquid nitrogen (6750 Freezer Mill; SPEX SamplePrep LLC, Metuchen, NJ) with guanidine isothiocyanate (TRIZOL[®]; Life Technologies, Grand Island, NY) and subsequent phenol extraction using a previously described method [8]. The RNA pellet formed was dissolved in approximately 40 μ L Tris-EDTA (TE) (pH 7.25) and stored at -80°C . RNA purity was periodically assessed visually by 1.5% agarose gel electrophoresis, revealing both 18S and 28S bands. Total RNA concentration was obtained through ultraviolet spectrophotometry in TE solution. The RNA was then diluted to 1 $\mu\text{g}/\mu\text{L}$ using nuclease-free H_2O . First strand cDNA synthesis was done by reverse transcription using 1 $\mu\text{g}/\mu\text{L}$ total RNA and oligo $\text{p}(\text{dT})_{15}$ primers (Roche Molecular Biochemicals, Basel, Switzerland).

Primer Design and Real-time Quantitative PCR Optimization

Forward (F) and reverse (R) primers were designed for β -actin, Types I and II collagen, osteocalcin, and BMP Type II receptor with MacVector software (Accelrys Software, Inc, San Diego, CA) using established cDNA sequences for *Rattus norvegicus*. The primers were synthesized by the Biomedical Genomics Center at the University of Minnesota (Minneapolis, MN). Their inherent characteristics are shown (Appendix Table 1).

The optimal F:R ratio for each primer set was determined by testing 5:1, 3:1, 1:1, 1:3, and 1:5 ratios on a control cDNA sample. Real-time annealing time and temperature, magnesium concentration, and amplification efficiency were independently determined using this sample. The control cDNA sample was further used as an internal calibrator for each real-time run to control for differences in reverse transcription and amplification of the cDNA, demonstrating an interrun variability from 1% to 6% among all five genes. The passive reference dye was used to account for minute interwell differences due to potential pipetting errors. No-template controls were also used to evaluate product specificity and monitor dimerization in conjunction with the dissociation curve. Magnesium chloride concentration was tested at 3, 4, and 5 mmol/L of the total 25- μL reaction volume, eliciting the lowest cycle threshold fluorescence value (Ct) at the default amount of 2.5 mmol/L. The amplification efficiencies for all genes were determined to be approximately equal and were 97.6% or greater using a 10-fold dilution series over 4 orders of magnitude. Comparable efficiencies close to 1.0 validated the use of the primer sets and ratios.

Real-time Comparative PCR

Real-time comparative quantitative PCR was performed using the Mx3000P[™] Quantitative PCR System thermal cycler and MxPro[™] software (Stratagene Corp, La Jolla, CA). SYBR[®] Green dye detection (Invitrogen, Carlsbad, CA) with an accompanying dissociation curve was used to detect the comparative amounts of β -actin, Type I collagen, osteocalcin, Type II collagen, and BMP Type II receptor mRNAs in samples from the newly mineralized callus harvested from the femoral defects. β -Actin served as the comparative normalizer gene. Samples were analyzed in

Appendix Table 1. Characteristics of primers

Primer name	Sequence 5' to 3'	F:R ratio	Product temperature ($^{\circ}\text{C}$)	Product size (bp)	Accession number
β -Actin F	atggtgggtatgggtcagaagg	1:1	80.3	234	NM031144
β -Actin R	ctgggtcatcttttcacgggtg				
Collagen I F	gttcgtggctctcagggtag	3:1	80.7	123	NM053356
Collagen I R	ctacctgagagccacgaac				
Collagen II F	agaaaggcgaacctggagat	1:3	79.2	122	L48440
Collagen II R	atctccagggttcgctttct				
Osteocalcin F	aataactccggcgctacct	1:3	77.5	90	X04141
Osteocalcin R	aggtagcgcggagtctatt				
BMP Type II receptor F	tacaacaccactcagtcgcg	1:1	71.8	133	AB073714
BMP Type II receptor R	cctgtctcgtcaacattctg				

F = forward; R = reverse.

triplicate with a final reaction volume of 25 μ L. Master mixes were made for each primer set and included 8.125 μ L nuclease-free H₂O, 12.5 μ L SYBR[®] Green dye, 2 μ L (400 nmol/L of total reaction) of the appropriate F:R primer ratio, and 0.375 μ L 1:500 reference dye as a fluorescence control. The optimal real-time thermocycler conditions were 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds to denature the cDNA, 56°C for 45 seconds for annealing, and 72°C for 30 seconds for extension. The cycle ended with a dissociation step to examine product quality.

Gene Expression

The Ct expression of each gene was subtracted relative to the Ct of the normalizer β -actin for each animal. The fold change from β -actin was computed using the formula $2^{-\Delta C_t}$, and the average fold change was found for the six animals in each treatment/time point [15, 32].

References

- Bolander ME. Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med*. 1992;200:165–170.
- Bost KL, Ramp WK, Nicholson NC, Bento JL, Marriott I, Hudson MC. Staphylococcus aureus infection of mouse or human osteoblasts induces high levels of interleukin-6 and interleukin-12 production. *J Infect Dis*. 1999;180:1912–1920.
- Bostrom MP. Expression of bone morphogenetic proteins in fracture healing. *Clin Orthop Relat Res*. 1998;355(Suppl):S116–S123.
- Chen X, Kidder LS, Lew WD. Osteogenic protein-1 induced bone formation in an infected segmental defect in the rat femur. *J Orthop Res*. 2002;20:142–150.
- Chen X, Schmidt AH, Mahjour S, Polly DW Jr, Lew W. Union of a chronically infected internally stabilized segmental defect in the rat femur after debridement and application of BMP-2 and systemic antibiotic. *J Orthop Trauma*. 2007;21:693–700.
- Chen X, Schmidt AH, Tsukayama DT, Bourgeault CA, Lew WD. Recombinant human osteogenic protein-1 induces bone formation in a chronically infected, internally stabilized segmental defect in the rat femur. *J Bone Joint Surg Am*. 2006;88:1510–1523.
- Chen X, Tsukayama DT, Kidder LS, Bourgeault CA, Schmidt AH, Lew WD. Characterization of a chronic infection in an internally-stabilized segmental defect in the rat femur. *J Orthop Res*. 2005;23:816–823.
- Chomczynski P, Sacchi N. Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156–159.
- Clancy BM, Johnson JD, Lambert AJ, Rezvankhah S, Wong A, Resmini C, Feldman JL, Leppanen S, Pittman DD. A gene expression profile for endochondral bone formation: oligonucleotide microarrays establish novel connections between known genes and BMP-2-induced bone formation in mouse quadriceps. *Bone*. 2003;33:46–63.
- Cook SD, Salkeld SL, Patron LP, Sargent MC. Healing course of primate ulna segmental defects treated with osteogenic protein-1. *J Invest Surg*. 2002;15:69–79.
- Dai J, Kitagawa Y, Zhang J, Yao Z, Mizokami A, Cheng S, Nör J, McCauley LK, Taichman RS, Keller ET. Vascular endothelial growth factor contributes to the prostate cancer-induced osteoblast differentiation mediated by bone morphogenetic protein. *Cancer Res*. 2004;64:994–999.
- Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE, Löwik CW. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology*. 2002;143:1545–1553.
- Einhorn TA. The cell and molecular biology of fracture healing. *Clin Orthop Relat Res*. 1998;355(Suppl):S7–S21.
- Emslie KR, Fenner LM, Nade SM. Acute haematogenous osteomyelitis: II. The effect of a metaphyseal abscess on the surrounding blood supply. *J Pathol*. 1984;142:129–134.
- Gentle A, Anastasopoulos F, McBrien NA. High-resolution semi-quantitative real-time PCR without the use of a standard curve. *Biotechniques*. 2001;31:502–508.
- Gibson G. Active role of chondrocyte apoptosis in endochondral ossification. *Microsc Res Tech*. 1998;43:191–204.
- Govender S, Csimma C, Genant HK, Valentin-Opran A, Amit Y, Arbel R, Aro H, Atar D, Bishay M, Börner MG, Chiron P, Choong P, Cinats J, Courtenay B, Feibel R, Geulette B, Gravel C, Haas N, Raschke M, Hammacher E, van der Velde D, Hardy P, Holt M, Josten C, Ketterl RL, Lindeque B, Lob G, Mathevon H, McCoy G, Marsh D, Miller R, Munting E, Oevre S, Nordsletten L, Patel A, Pohl A, Rennie W, Reynders P, Rommens PM, Rondia J, Rossouw WC, Daneel PJ, Ruff S, Rüter A, Santavirta S, Schildhauer TA, Gekle C, Schnettler R, Segal D, Seiler H, Snowdowne RB, Stapert J, Taglang G, Verdonk R, Vogels L, Weckbach A, Wentzensen A, Wisniewski T; BMP-2 Evaluation in Surgery for Tibial Trauma (BESTT) Study Group. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred fifty patients. *J Bone Joint Surg Am*. 2002;84:2123–2134.
- Gristina AG, Costerton JW. Bacterial adherence to biomaterials and tissue: the significance of its role in clinical sepsis. *J Bone Joint Surg Am*. 1985;67:264–273.
- Hadjiargyrou M, Lombardo F, Zhao S, Ahrens W, Joo J, Ahn H, Jurman M, White DW, Rubin CT. Transcriptional profiling of bone regeneration. *J Biol Chem*. 2002;33:30177–30182.
- Hassel S, Schmitt S, Hartung A, Roth M, Nohe A, Petersen N, Ehrlich M, Henis YI, Sebald W, Knaus P. Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes. *J Bone Joint Surg Am*. 2003;85(Suppl 3):44–51.
- Hay E, Hott M, Graulet AM, Lomri A, Marie PJ. Effects of bone morphogenetic protein-2 on human neonatal calvaria cell differentiation. *J Cell Biochem*. 1999;72:81–93.
- Heldin CH, Miyazono K, ten Dijke P. TGF-beta signaling from cell membrane to nucleus through SMAD proteins. *Nature*. 1997;390:465–471.
- Hirakawa K, Hirota S, Ikeda T, Yamaguchi A, Takemura T, Nagoshi J, Yoshiki S, Suda T, Kitamura Y, Nomura S. Localization of the mRNA for bone matrix proteins during fracture healing as determined by in situ hybridization. *J Bone Miner Res*. 1994;9:1551–1557.
- Hoshi K, Amizuka N, Sakou T, Kurokawa T, Ozawa H. Fibroblasts express BMP-R and differentiate into chondroblasts with enhanced BMP-R expression. *Bone*. 1997;21:155–162.
- Hyun SJ, Han DK, Choi SH, Chai JK, Cho KS, Kim CK, Kim CS. Effect of recombinant human bone morphogenetic protein-2, -4, and -7 on bone formation in rat calvarial defects. *J Periodontol*. 2005;76:1667–1674.

26. Jingushi S, Joyce ME, Bolander ME. Genetic expression of extracellular matrix proteins correlates with histologic changes during fracture repair. *J Bone Miner Res*. 1992;7:1045–1055.
27. Kawabata M, Chytil A, Moses HL. Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor- β receptor. *Biol Chem*. 1995; 270:5625–5630.
28. Kloen P, Doty SB, Gordon E, Rubel IF, Goumans MJ, Helfet DL. Expression and activation of the BMP-signaling components in human fracture nonunions. *J Bone Joint Surg Am*. 2002;84:1909–1918.
29. Lai CF, Cheng SL. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor- β in normal human osteoblastic cells. *J Biol Chem*. 2002;277:15514–15522.
30. Lecanda F, Avioli LV, Cheng SL. Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *J Cell Biochem*. 1997;67:386–396.
31. Liu F, Ventura F, Doody J, Massagué J. Human type II receptor for bone morphogenetic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol Cell Biol*. 1995; 15:3479–3486.
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. 2001;25:402–408.
33. Marinova-Mutafchieva L, Taylor P, Funa K, Maini RN, Zvaifler NJ. Mesenchymal cells expressing bone morphogenetic protein receptors are present in the rheumatoid arthritis joint. *Arthritis Rheum*. 2000;43:2046–2055.
34. Marriott I, Gray DL, Tranguch SI, Fowler VG Sr, Strykowski M, Scott Levin L, Hudson MC, Bost KL. Osteoblasts express the inflammatory cytokine interleukin-6 in a murine model of *Staphylococcus aureus* osteomyelitis and infected human bone tissue. *Am J Pathol*. 2004;164:1399–1406.
35. Melcher GA, Hauke C, Metzendorf A, Perren SM, Printzen G, Schlegel U, Ziegler WJ. Infection after intramedullary nailing: an experimental investigation on rabbits. *Injury*. 1996;27(Suppl 3):SC23–SC26.
36. Meyer RA, Meyer MH, Phieffer LS, Banks DM. Delayed union of femoral fractures in older rats: decreased gene expression. *BMC Musculoskelet Disord*. 2001;2:2.
37. Meyer RA, Meyer MH, Tenholder M, Wondracek S, Wasserman R, Garges P. Gene expression in older rats with delayed union of femoral fractures. *J Bone Joint Surg Am*. 2003;85:1243–1254.
38. Nakamura Y, Wakitani S, Saito N, Takaoka K. Expression profiles of BMP-related molecules induced by BMP-2 or -4 in muscle-derived primary culture cells. *J Bone Miner Metab*. 2005;23:426–434.
39. Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J Biol Chem*. 2002;277:5330–5338.
40. Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, Kato M, Sampath TK, ten Dijke P, Sakou T. Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. *Bone*. 1998;22:605–612.
41. Raida M, Clement JH, Leek RD, Ameri K, Bicknell R, Niederwieser D, Harris AL. Bone morphogenetic protein 2 (BMP-2) and induction of tumor angiogenesis. *J Cancer Res Clin Oncol*. 2005;131:741–750.
42. Reddi AH. Initiation of fracture repair by bone morphogenetic proteins. *Clin Orthop Relat Res*. 1998;355(Suppl):S66–S72.
43. Reddi AH, Gay R, Gay S, Miller EJ. Transitions in collagen types during matrix-induced cartilage, bone, and bone marrow formation. *Proc Natl Acad Sci USA*. 1977;74:5589–5592.
44. Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K. Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proc Natl Acad Sci USA*. 1995;92:7632–7636.
45. Sandberg M, Aro H, Multimaki P, Aho H, Vuorio E. In situ localization of collagen production by chondrocytes and osteoblasts in fracture callus. *J Bone Joint Surg Am*. 1989;71:69–77.
46. Soballe K, Jensen TB, Mouzin O, Kidder L, Bechtold JE. Differential effect of a bone morphogenetic protein-7 (OP-1) on primary and revision loaded, stable implants with allograft. *J Biomed Mater Res A*. 2004;71:569–576.
47. Tcacencu I, Calsoo B, Stierna P. Effect of recombinant human BMP-2 on the repair of cricoid cartilage defects in young and adult rabbits: a comparative study. *Int J Pediatr Otorhinolaryngol*. 2005;69:1239–1246.
48. ten Dijke P, Miyazono K, Heldin C-H. Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. *Curr Opin Cell Biol*. 1996;8:139–145.
49. Thompson Z, Miclau T, Hu D, Helms JA. A model for intramembranous ossification during fracture healing. *J Orthop Res*. 2002;20:1091–1098.
50. Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J, Arnett TR. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. *Exp Cell Res*. 2006; 312:1693–1702.
51. Wang JY, Wicklund BH, Gustilo RB, Tsukayama DT. Prosthetic metals impair murine immune response and cytokine release in vivo and in vitro. *J Orthop Res*. 1997;15:688–699.
52. Yaoita H, Orimo H, Shirai Y, Shimada T. Expression of bone morphogenetic proteins and rat distal-less homolog genes following rat femoral fracture. *J Bone Miner Metab*. 2000;18:63–70.
53. Yasko AW, Lane JM, Fellingner EJ, Rosen V, Wozney JM, Wang EA. The healing of segmental bone defects, induced by recombinant human bone morphogenetic protein (rhBMP-2). *J Bone Joint Surg Am*. 1992;74:659–670.
54. Yu YM, Becvar R, Yamada Y, Reddi AH. Changes in the gene expression of collagens, fibronectin, integrin and proteoglycans during matrix-induced bone morphogenesis. *Biochem Biophys Res Commun*. 1991;177:427–432.
55. Zachos TA, Shields KA, Bertone AL. Gene-mediated osteogenic differentiation of stem cells by bone morphogenetic proteins-2 or -6. *J Orthop Res*. 2006;24:1279–1291.