Bone morphogenetic proteins (BMPs) are potent bone inducers used clinically to enhance fracture repair. BMPs have been shown to be produced in the fracture callus; however, the comparative expression of BMPs and BMP signaling components has only been partially examined at the cellular level. The aim of the present study was to establish a detailed spatiotemporal localization of BMPs and BMP signaling components in mouse models of stabilized and nonstabilized fractures. During healing of nonstabilized fractures, which occurs via endochondral ossification, BMP2, 3, 4, 5, and 8, noggin, BMPRIA, BMPRII, and pSmad 1/5/8 were immunolocalized in the activated periosteum as early as 3 days after fracture. BMP2, 4, 5, 6, 7, and 8 and noggin were also found in isolated inflammatory cells within granulation tissue during the early stages of repair, but not BMP receptors and effectors. During the soft callus phase of repair, all BMPs and BMP signaling components were detected in chondrocytes with various intensities of staining depending on the stage of chondrocyte differentiation and their location in the callus. The strongest staining was observed in hypertrophic chondrocytes with decreased intensity during the hard callus phase of repair. All BMPs and components of the BMP pathway were detected in osteoblasts and osteocytes within new bone, with strongest intensity of immunoreaction reported during the early soft callus phase followed by decreasing intensity during the hard callus phase of repair. Most components of the BMP pathway were also detected in endothelial cells associated with new bone. In stabilized fractures that heal strictly via intramembranous ossification, BMPs and BMP antagonists were detected in isolated inflammatory cells and BMP signaling components were not detectable in osteoblasts or osteocytes within new bone. In conclusion, the BMP signaling pathway is primarily activated during fracture healing via endochondral ossification, suggesting that this pathway may influence the mode of healing during the recruitment of skeletal progenitors.

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Introduction

Fracture healing involves the synchronized effects of many local and systemic regulatory factors including growth and differentiation factors, and extracellular matrix components [1–3]. The interaction of these factors causes undifferentiated mesenchymal cells to migrate, proliferate, and differentiate at the fracture site. The recruitment of stem cells in response to these growth factors is a key step to assure the success of bone regeneration. In a significant amount of cases, however, impaired healing requires surgical intervention combined with the implantation of osteoconductive materials and osteoinductive factors [4,5]. Bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily, play important roles in skeletogenesis and are now used as therapeutic agents for treating skeletal diseases and injuries [6–8]. Since their clinical efficacy is sometimes limited, we would benefit from a better understanding of BMP functions during fracture healing. Functional and expression analyses of BMPs and their inhibitors have been reported [9–12], but we still lack a detailed localization of BMP signaling components during fracture repair. BMP signaling is mediated through serine–threonine transmembrane receptors (BMPR) type I and type II, which subsequently phosphorylate the receptor-regulated Smads (R-Smads) 1, 5, and 8 [13–15]. Once activated, the R-Smads undergo heterodimerization with Smad 4 (co-Smad) in the cytoplasm and the complex translocates into the nucleus, where it regulates gene transcription. BMP signal transduction is regulated by the inhibitory Smads, which are potent intracellular antagonists that inhibit BMP signaling at different levels in the cell [13,14,16]. BMP signaling is also influenced by extracellular antagonists such as noggin, which bind to BMPs and prevent interaction with receptors [17,18]. Alternatively, BMP3 is an antagonist, which inhibits BMP2 activity by activating TGF-β/activin pathway [19,20]. To better relate the in vivo functions of BMPs during fracture repair with the cellular localization of BMPs and BMP signaling components during the course of healing, we performed a detailed spatiotemporal localization of BMPs (2, 4, 5, 6, 7, 8), BMP extracellular antagonists (noggin, BMP3), BMP type I (BMPRIA, BMPRIIB), and BMP type II (BMPRII) receptors, as well as the activated form of BMP receptor-regulated Smads (pSmad 1–5–8) during fracture...
healing in mice. Since the balance between chondrogenesis and osteogenesis in the fracture callus depends on the mechanical environment, we also examined if activation of the BMP pathway differs in mechanically stable and unstable fractures.

Materials and methods

Animals

Adult C57/B6 wild type mice aged 12–16 weeks were used in this study. The animals were housed in a light- and temperature-controlled room and given unrestricted access to food and water during the experimental period. All procedures followed protocols approved by the University of California at San Francisco Animal Care and Use Committee.

Nonstabilized and stabilized fractures

The animals were anesthetized with an intraperitoneal injection of 50 mg/ml ketamine mixed with 0.5 mg/ml medetomidine (0.03 ml/mouse total). Closed, standardized nonstabilized tibial fractures were produced via the three-point bending as described [21]. For stabilized tibial fractures, external fixators were placed before creating the fracture [22].

Sample preparation

Callus tissues were collected at days 3, 5, 7, 10, 14, and 21 after fracture (2–3 mice per time point). Callus tissues were fixed overnight at 4 °C in 4% paraformaldehyde, decalcified at 4 °C in 19% EDTA (pH 7.4) for 10–14 days, then dehydrated in a graded ethanol series and embedded in paraffin. Tissues were sectioned longitudinally using a Leica microtome at 10 μm, mounted on glass slides coated with poly-I-lysine (Fisher Scientific, USA), and dried at 37 °C overnight before immunohistochemical and histology staining.

Antibodies

Affinity-purified goat polyclonal antibody against BMP2 (sc-6895), BMP3 (sc-7404), BMP4 (sc-6896), BMP5 (sc-7405), BMP6 (sc-7406), BMP7 (sc-9305), BMP8 (sc-6900), noggin (sc-16627), BMPRIA (sc-5676), BMPRIB (sc-5679), and BMPRII (sc-5683) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified rabbit polyclonal antibody against phospho-Smad 1/5/8 was purchased from Chemicon (Millipore, Billerica, MA). The specificity of each antibody has been previously confirmed by Western blotting and immunostaining [23–25]. Affinity-purified anti-mouse F4/80 and anti-mouse Ly-6G antibodies were purchased from eBioscience (San Diego, CA) for detecting macrophages and neutrophils, respectively.

Immunohistochemistry and histology

Immunohistochemistry staining was performed for each antibody on 2–3 center sections per callus. After deparaffinization and hydration, sections were washed in PBS (pH 7.4) containing 0.3% Triton X-100 for 15 minutes. Antigen retrieval was performed by heating slides at 37 °C for 20 min in 0.05% trypsin and 0.1% CaCl₂ dissolved in distilled water followed by 20 min of cooling without changing buffer. Slides were washed twice with PBS for 3 min and were immersed for 15 min in 3% H₂O₂ in methanol to inhibit endogenous peroxidase activity. After two washes with PBS, slides were treated with 1.5% donkey serum or 1.5% goat serum (goat or rabbit ABC staining kit; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were incubated with primary antibodies diluted (1:50) in the blocking serum in a humidified chamber at 4 °C overnight. Control sections were incubated with normal goat IgG or normal rabbit IgG serum. Detection of primary antibody binding was done using goat or rabbit ABC staining kit (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were developed with DAB and counterstained with 1% Fast Green. Adjacent sections were stained with Safranin-O/Fast Green (SO) to detect cartilage formation or a modified Milligan’s Trichrome (TC) staining using Aniline Blue to visualize bone [22].

Evaluation of immunohistochemistry staining

Two to three sections per callus were evaluated for each antibody. Sections were visualized using a Leica DM 5000 B light microscope (Leica Microsystems GmbH, Wetzler, Germany) and images were captured with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Multiple fields throughout the callus were examined on each section using ×20 and ×40 objective lenses to identify positive staining in osteoblasts, osteocytes, inflammatory cells, and proliferative, prehypertrophic, and hypertrophic chondrocytes. Cell types were determined based on their morphology and their location within the callus. Hypertrophic chondrocytes exhibit a characteristic enlargement in size compared to proliferative or prehypertrophic chondrocytes and are located near sites of vascular invasion and new bone deposition.

The intensity of immunostaining varied among antibodies, cell types, and time points. To reflect these variations, we established a scoring system based on qualitative evaluation (− = no staining (negative), + = weak, ++ = moderate, and +++ = strong). The scores listed in Table 1 were obtained by consensus of two independent researchers.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Immunoreactivity for BMPs, BMP antagonists, receptors, and effectors during nonstabilized fracture healing.</th>
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<td>pSmad1/5/8</td>
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Intensity of staining was graded on a scale of − to ++++, with − representing negative staining, + weak staining, ++ moderate staining, and +++ strong staining.
Fig. 1. Immunodetection of BMPs and components of the BMP pathway during the inflammatory phase of nonstabilized fracture healing. (A, B) Milligan's Trichrome staining (top) and (C-Q) immunohistochemistry at days 3 (left) and 5 (right) after fracture. (C) Weak intracellular immunostaining for BMP2 (and BMP4 and 5 as observed on adjacent sections) in the activated periosteum (arrow). (D) Moderate immunostaining for BMP2, 5, and 6 in the cytoplasm of isolated cells in granulation tissue (arrow). (E) Moderate intracellular immunostaining for BMP8 in the activated periosteum (arrow). (F) Weak immunostaining for BMP4 (and BMP7 and 8 on adjacent sections) in the cytoplasm of isolated cells (arrow) in granulation tissue. Weak intracellular immunostaining for (G) BMP3 and (H) noggin in the activated periosteum (arrows). (I) Negative immunostaining for BMP3 in granulation tissue. (J) Moderate immunostaining for noggin in the cytoplasm of isolated cells (arrow) in granulation tissue. (K) Weak intracellular immunostaining for BMPRIA (and II, arrow), (L) but not BMPRIB in the activated periosteum. (M) Negative immunostaining for BMPRIA (as well as IB and II on adjacent sections) in granulation tissue. (N) Moderate immunostaining for pSmad 1/5/8 in the nucleus of periosteal cells (arrow). (O) Negative staining in periosteal cells in negative controls. (P) Negative immunostaining for pSmad 1/5/8 in granulation tissue. (Q) Negative staining in granulation tissue in negative controls. Scale bars (A, B) = 100 μm, (C–Q) = 10 μm.
Results

Immunostaining for BMPs, BMP antagonists, receptors, and effectors during the inflammatory phase of nonstabilized fracture healing

Nonstabilized fractures heal via endochondral ossification, which allowed us to assess expression of components of the BMP pathway during both osteogenesis and chondrogenesis [22]. In this mouse fracture model, healing follows the classical phases of repair with an initial inflammatory phase followed by the soft callus, hard callus, and remodeling phases [21,26]. The inflammatory phase is a critical step during which mesenchymal cells are recruited to the injury site [1,27]. As early as 3 days after fracture, activated periosteum can be observed at histological and molecular levels (Fig. 1A and Table 1) and by 5 days, the callus is filled with granulation tissue (Fig. 1B). At 3 days, we observed weak intracellular immunohistochemical staining for BMP2, 4, and 5 in activated periosteal cells near the fracture site (Fig. 1C). Moderate BMP8 immunoreactivity was detected in the activated periosteum, while BMP6 and 7 were undetectable (Fig. 1E and Table 1). By day 5, the activated periosteum did not show any BMP2, 4, and 8 immunostaining but BMP5 immunostaining was still present (Table 1). Similarly, weak immunostaining for the BMP antagonists, BMP3 and noggin was seen in activated periosteal cells at day 3 (Figs. 1G and H and Table 1) after fracture but not at day 5 (Table 1). Periosteal cells near the fracture site also exhibited weak immunoreactivity for BMP receptors IA and II, but not IB at both days 3 (Figs. 1K and L Table 1) and 5 (Table 1). This correlated with moderate and weak immunoreactivity for pSmad 1/5/8 at days 3 (Figs. 1N and Table 1) and 5 (Table 1), respectively.

Simultaneously, weak to moderate immunoreactivity was detected for BMP2, 5, and 6 in isolated cells within the granulation tissue at the fracture site at days 3 (Table 1) and 5 after fracture (Fig. 1D and Table 1). Adjacent sections stained with inflammatory cell markers such as macrophages or neutrophils markers indicated that these stained isolated cells are inflammatory cells (data not shown). In these cells, weak signals for BMPF were found at both days 3 and 5 (Fig. 1F and Table 1), but immunoreactivity for BMP4 and 8 were only found at day 5 after fracture (Fig. 1F and Table 1). Weak to moderate immunoreactivity for noggin, but not BMP3, was detected at days 3 (Table 1) and 5 (Figs. 1I and J and Table 1). Immunoreactivity for BMP receptors and effectors pSmad 1/5/8 was undetectable in the granulation tissue at both days 3 (Table 1) and 5 after fracture (Figs. 1M and P and Table 1). Controls without primary antibodies did not show any immunoreactivity in cells within the activated periosteum and in the granulation tissue (Figs. 1O and Q).

Distribution of BMPs, BMP antagonists, receptors, and effectors in chondrocytes during the soft callus phase of nonstabilized fracture healing

Following the inflammatory phase, the soft callus phase of repair is characterized by the differentiation of mesenchymal cells into chondrocytes and osteoblasts, which deposit cartilage and bone in the callus. During repair of nonstabilized fractures, a large amount of cartilage forms within the callus, which becomes hypertrophic and is gradually replaced by bone. Typically, in this mouse model, cartilage is detected histologically by day 5, peaks at day 10, and is removed by day 21 after fracture (Figs. 2–5) [21,26]. We observed immunoreactivity for all BMPs in cartilage within the fracture callus, but the signals varied in intensity depending on the stage of chondrocyte differentiation and the location of chondrocytes within the callus. At day 5 after fracture, we observed weak to moderate immunostaining for BMP2, 6, 7, and 8 in the cytoplasm of chondrocytes located near the periosteum within the fracture callus (Fig. 2D, E, P and Table 1). BMP2, 6, and 7 immunostaining was apparent in prehypertrophic chondrocytes, while BMP8 immunostaining was apparent in proliferative chondrocytes. Immunoreactivity for BMP4 and BMP5 was not detected in chondrocytes at this time point (Figs. 2 and K and Table 1). The immunoreactivity for all BMPs gradually increased in chondrocytes between days 5 and 10. By day 7, proliferative, prehypertrophic, and hypertrophic chondrocytes exhibited strong staining for BMP2, 5, 6, 7, and 8 (Figs. 2F, G, and M and Table 1). BMP8 immunoreactivity was particularly strong in hypertrophic chondrocytes adjacent to the periosteum (Fig. 2Q and Table 1). Only BMP4 showed a weak staining in prehypertrophic chondrocytes at day 7 (Fig. 2L and Table 1). At day 10, hypertrophic chondrocytes showed strong immunoreactivity for BMP2, 4, 5, 6, 7, and 8 (Figs. 2H, I, N, O, and R and Table 1). BMP immunoreactivity then decreased at day 14 in the remaining hypertrophic chondrocytes in the callus (Table 1).

The general distribution of BMP antagonists, receptors, and effectors in cartilage was similar to that of BMPs. The intensity of BMP3 immunostaining was weak at day 5 in the cytoplasm of prehypertrophic chondrocytes, increased at days 7 and 10 in hypertrophic chondrocytes, and decreased at day 14 after fracture (Figs. 3D, F, and H and Table 1). Noggin immunostaining was moderate at days 5, 7, and 10 in hypertrophic chondrocytes and abruptly disappeared at day 14 (Figs. 3E, G, and I and Table 1). Moderate immunoreactivity for BMP receptors was detected in the cytoplasm of proliferating and prehypertrophic chondrocytes near the periosteum at day 5 (Figs. 3J and K and Table 1). The intensity of immunostaining in proliferating, prehypertrophic, and hypertrophic chondrocytes increased from day 7 to day 10 and decreased by day 14 (Figs. 3L–Q and Table 1). Moderate immunoreactivity for BMP effectors pSmad 1/5/8 was observed in the nucleus of proliferating and prehypertrophic chondrocytes at day 5 (Fig. 3P). The staining increased from day 5 to day 7 after fracture and was localized both in the nucleus and in the cytoplasm of prehypertrophic and hypertrophic chondrocytes (Figs. 3Q and Table 1). By days 10 to 14, a moderate to weak pSmad 1/5/8 immunoreactivity was detected in the nucleus and cytoplasm of hypertrophic chondrocytes (Figs. 3R and Table 1). The specificity of immunostaining for all BMPs, receptors, and effectors was confirmed by the negative staining in the chondrocytes of control samples (Fig. 3S).

Immunoreactivity for BMPs, BMP antagonists, receptors, and effectors in osteoblasts and osteocytes during the soft and hard callus phases of healing

At 7 days after fracture, new bone is deposited near the periosteum within the fracture callus. New bone formation increases during the soft callus phase of repair, peaks during the hard callus phase of repair from day 14 until day 21, and is subsequently remodeled [21,26]. Immunoreactivity for BMPs and components of the BMP pathway was observed mainly in osteocytes but also in osteoblasts within new bone. Overall, the staining in osteoblasts and osteocytes was not as intense as that in chondrocytes. At both day 7 and day 10, osteocytes within new bone near the periosteum and in the bone marrow cavity presented a weak to strong immunoreactivity for all BMPs with a stronger immunoreactivity at day 7 (Figs. 4D–E, H–I, K–M, P–Q and Table 1). By day 14, osteoblasts and osteocytes did not exhibit any immunostaining for BMPs except for BMP2 and BMP6 (Figs. 4F, J, N, O, and R and Table 1). In addition, endothelial cells along the woven bone showed weak to strong immunoreactivity for BMP4 at day 10 (Fig. 4I, arrowhead) and BMP5, 6, 7, and 8 at both days 10 and 14 (Figs. 4L, M, O, Q, and R, arrowheads). By day 21, only BMP5 and BMP7 exhibited a weak immunostaining in endothelial cells along the woven bone (data not shown).

The distribution of immunoreactivity for BMP antagonists in new bone did not exactly parallel that of BMPs. At day 7, noggin and BMP3 immunostaining was restricted to osteoblasts and osteocytes that were found near hypertrophic chondrocytes in the callus (Fig. 5D and Table 1). At day 10, weak to moderate immunoreactivity for noggin and BMP3 was detected in osteoblasts and osteocytes along and within new woven bone.
Endothelial cells did not exhibit any immunostaining for BMP antagonists during osteogenesis (Figs. 5D and E). Consistent with BMP immunostaining, moderate to strong immunoreactivity for BMP receptors and effectors pSmad 1/5/8 was detected in osteoblasts and osteocytes within the new woven bone near the periosteum at day 7 (Figs. 5G, J, and M and Table 1), while weak immunoreactivity was detected in other areas of the callus. The intensity of immunostaining in osteoblasts and osteocytes gradually decreased from day 10 to day 14 after fracture (Figs. 5H, I, K, L, N, and O and Table 1). Endothelial cells along the new bone were immunostained for BMP receptors and effectors at days 10 and 14, and the staining persisted for BMPRIA at day 21 (Figs. 5H, L, arrowhead and data not shown). Immunostaining for pSmad 1/5/8 was also found in endothelial cells along the woven bone at day 10 and decreased at day 14 (Figs. 5N and O, arrowhead).

Immunoreactivity for BMPs, BMP antagonists, receptors, and effectors during stabilized fracture healing

Since the BMP pathway was active throughout the process of endochondral ossification in nonstabilized fractures, we wondered if it was also active in stabilized fractures, which heal via intramembranous ossification [21,22]. In mouse tibial fractures that were rigidly stabilized with an external device, no cartilage formed during repair, and the callus was considerably smaller compared to the callus of nonstabilized fractures (Figs. 6A and B). By day 5 after injury, granulation tissue was present at the fracture site (Fig. 6A), and new bone matrix deposition was not evident via histology until 10 days after fracture (Fig. 6B). At 5 days, we observed a strong immunoreactivity for BMP2, 4, 5, 6, 7, and 8 in the cytoplasm of isolated cells within granulation tissue (Fig. 6C). These isolated cells also exhibited a moderate intracellular immunoreactivity for BMP3 and noggin (Fig. 6E), but no immunoreactivity for BMP receptors and effectors pSmad 1/5/8 (Figs. 6F and I). Throughout the process of stabilized fracture repair, we did not detect immunoreactivity for BMPs, BMP antagonists, receptors, and effectors in osteoblasts and osteocytes within new bone (Figs. 6D, G, H, and K and data not shown). Controls showed no immunoreactivity in granulation tissue, osteocytes, and osteoblasts (Figs. 6J and L).

Discussion

Extensive studies have shown important roles of BMP signaling components during skeletal development and regeneration. Deletions...
of BMPs, BMP receptors, and BMP antagonists in mice induce various
degrees of bone defects, ranging from mild changes in bone mass to
severe skeletal abnormalities [15,19,28–33]. Following bone fracture,
mice lacking BMP2 fail to heal, while healing appears normal in mice
lacking BMP4 or BMP7 [28,31,34]. The requirement of BMPs during
bone formation has been correlated with their expression in various
skeletal cell types in vivo [35]. Recent studies also showed decreased
immunoreactivity of BMP signaling components and noggin in
nonunions [10,11,36]. Our study provides a detailed description of
the cellular localization of BMPs and BMP signaling components
during the course of normal fracture repair in order to better relate
expression patterns with in vivo functions and with efforts to utilize
BMPs as diagnostic tools.

**BMP expression and BMP pathway activation in multiple cell types
during fracture repair**

Expression analyses during healing of nonstabilized fractures
revealed that BMPs are produced and BMP pathway is activated in
multiple cell types throughout the process of bone repair via
endochondral ossification. It has been shown that bone matrix is a
reservoir of BMPs, and broken bone cortex could be a source of BMPs
after bone injury [37,38]. Recent studies have shown that BMPs
regulate inflammatory processes by downregulating numerous genes
associated with the activity of cytokines and chemokines [39]. The
presence of BMPs in inflammatory cells suggests that inflammatory
cells could be another source of BMPs in the early stages of repair.
Early detection of BMP signaling components in the activated
periosteum, suggests that peristeal cells are one of the first targets
of BMPs in response to injury.

During the soft callus phase of repair, chondrocytes and osteo-
brasts start differentiating within the fracture callus. Strong BMP
expression and activation of the BMP pathway were detected in these
two cell types. Production of BMPs by chondrocytes and osteoblasts in
the early soft callus phase of repair may further stimulate the healing
process. The ability of BMPs to regulate chondrogenic and osteoblastic
differentiation has been extensively studied [39–42]. Here, we
observed stronger immunostaining for BMPs and BMP signaling
components in chondrocytes relative to osteoblasts and osteocytes in
correlation with previous reports [36]. The strong expression of BMPs
in cartilaginous area may contribute to amplify the BMP-mediated
response to injury.
Fig. 4. BMP proteins distribution in osteoblasts and osteocytes within new bone during the hard callus phase of nonstabilized fracture healing. (A–C) Milligan’s Trichrome staining (top) and (D–R) immunohistochemistry at days 7 (left), 10 (middle), and 14 (right) after fracture. (D–F) Weak to moderate immunostaining of BMP2 in osteocytes (arrows) within new woven bone near the periosteum at days 7 (D), 10 (E), and 14 (F). (G) Negative staining in osteoblasts and osteocytes in negative controls. (H–J) Weak immunostaining of BMP4 in osteocytes (arrow) within the new bone near the periosteum at day 7 (H), and moderate staining in osteocytes (arrow) within the new bone and in endothelial cells (arrowhead) along the new bone at day 10 (I). Negative BMP4 immunostaining at day 14 (J). (K–O) Strong immunostaining of BMP5 (and BMP6 and 7 on adjacent sections) in osteocytes (arrows) within new bone at day 7 (K). Weak staining of BMP6 in osteoblasts (open arrow) and in endothelial cells (arrowhead) along the new bone at day 10 (L). Weak staining of BMP5 (and BMP7 on adjacent sections) in osteocytes (arrow) within new bone and in endothelial cells (arrowhead) along the new bone at day 10 (M). Weak staining of BMP6 in osteoblasts (arrow) within new bone at day 14 (N). Strong staining of BMP5 (and BMP7 on adjacent sections) in endothelial cells (arrowhead) along the new bone at day 14 (O). (P–R) Strong immunostaining of BMP8 in osteocytes (arrow) within new bone at day 7 (P) and day 10 (Q). Strong staining of BMP8 in endothelial cells (arrowhead) along the new bone at day 10 (Q) and day 14 (R). Scale bars (A–C) = 100 μm, (D–S) = 10 μm.
During the hard callus phase of repair, BMP signaling components were still expressed in osteoblasts/osteocytes and chondrocytes with a decrease intensity of staining, suggesting a repression of the pathway when repair is well underway. At this stage, we also detected expression of BMP signaling components in endothelial cells adjacent to new bone, while expression was not detected in blood vessels during the early stages of repair. These expression data indicate that endothelial cells appear to be responsive to BMP signaling *in vivo* and that activation of the BMP pathway in blood vessels may depend on the close proximity of endothelial cells and cells adjacent to new bone, while expression was not detected in blood vessels during the early stages of repair. These expression data indicate that endothelial cells appear to be responsive to BMP signaling *in vivo* and that activation of the BMP pathway in blood vessels may depend on the close proximity of endothelial cells and
osteoblasts in new bone. Indeed, osteogenesis and angiogenesis have been shown to be closely linked and BMP can enhance angiogenesis by inducing vascular endothelial growth factor A (VEGF-A) expression in osteoblasts [43]. Interestingly, pericytes also closely interact with endothelial cells within blood vessels and have been suggested as a potential source of progenitor cells in bone [44,45]. Whether BMP signaling is specifically activated in pericytes has yet to be established. Finally, we did not observe expression of BMP pathway components in osteoclasts, which play an important role in bone resorption during the hard callus and remodeling phases of bone repair. Although BMPs can stimulate osteoclastic bone resorption, this function may also be an indirect effect of BMPs via osteoblasts [46,47].

**Redundant expression of BMPs during fracture repair**

We describe overlapping expression patterns of BMPs in the fracture callus suggesting complementary and/or redundant roles of BMPs during bone repair. We found that all BMPs analyzed were present in periosteal cells and inflammatory cells in the granulation tissue at the early stages of repair, except for BMP6 and 7, which were found only in granulation tissue. All BMPs were also produced by chondrocytes and osteoblasts/osteocytes during healing via endochondral ossification with notable differences in the intensity and distribution of the staining within the fracture callus. BMP2 exhibited the strongest expression level in chondrocytes at various stages of repair and BMP8 appeared to be the most strongly expressed BMP in osteoblasts and osteocytes. BMP8 expression in cartilage was stronger in chondrocytes adjacent to the periosteum, suggesting interactions between periosteal cells and cartilage. Finally, all BMPs except BMP2 were present in endothelial cells within new bone. These data suggest that BMPs may all potentially play roles at various stages of repair and in various cell types. However, in vivo studies have already demonstrated that BMP2 is indispensable while BMP4 or BMP7 are dispensable for fracture repair [48]. The functional roles of BMPs during bone repair may therefore depend on their biological activities in addition to regulation in their expression patterns. For example, in vitro
osteogenic activities vary among BMPs: while most BMPs can support osteoblast differentiation, BMP2, 6, and 9 can induce osteogenic differentiation of mesenchymal stem cells and may potentially act by inducing osteogenesis in the early stages of repair [49,50]. Indeed, BMP2 is not only strongly expressed in chondrocytes but also activated in the early stages of repair, where its role in recruiting skeletal precursor cells may be crucial to initiate repair.

Expression of BMP antagonists during fracture healing

BMP activity can be modulated by a number of antagonists [18,20,51]. Our expression analyses concentrate on two antagonists, BMP3 and noggin, which were found to coexpress with BMPs during fracture repair. Like all BMPs examined, BMP3 and noggin were detected in periosteal cells and inflammatory cells during the early stages of repair, indicating the regulation of BMP function by their antagonists in the initial healing response. BMP3 and noggin were also found in chondrocytes and osteoblast/osteocytes during the soft and hard callus phases of repair. In osteoblasts and osteocytes, however, BMP3 and noggin were first confined to new bone adjacent to hypertrophic cartilage, while BMPs were found throughout the callus. This observation suggests a tight regulation of BMP activity at the chondro-osseous junction, which is also the case at the growth plate during development [52,53]. Another difference between the expression patterns of BMPs and their antagonists was the absence of BMP3 and noggin in endothelial cells associated with new bone in the hard callus phase of repair. The expression of BMP antagonists is therefore more closely associated with BMP expression in cartilage and bone forming cells, where they may play important roles in modulating osteogenic and chondrogenic differentiation induced by BMPs. Several in vitro and in vivo studies indicate the inhibitory effects of the antagonists on cartilage and bone formation but more remains to be done in the context of the fracture callus in vivo [17,54,55].

Regulation of BMP pathway expression by the mechanical environment

Although the components of the BMP pathway are strongly expressed during healing of nonstabilized fractures, our immunohistochemical analyses show that they are minimally expressed in stabilized fractures. The mechanical environment clearly alters cartilage and bone formation during bone repair since stabilized fractures heal via intramembranous ossification and nonstabilized fractures heal via endochondral ossification [22,27,56,57]. Our findings indicate that biomechanical stimuli may activate the BMP signaling pathway. A previous report has shown enhanced expression of BMP signaling components during distraction osteogenesis in the mandible compared to normal healing in the absence of distraction [58]. These data also support the hypothesis that mechanical stress regulates BMP signaling. However, to what extent BMP pathway activation plays a role in the mechanically dependent cell fate decisions during the early stages of repair is unknown and remains to be tested functionally. Recent studies have demonstrated that exposure of osteoblastic cells to compressive forces upregulates osteogenesis–related factors including BMP2 [59,60]. Further studies will be required to assess the possible upstream regulation of BMP expression by mechanical forces and the consequences on cell differentiation.

In summary, we report a detailed description of the expression pattern of BMPs and BMP signaling components during the course of fracture healing. We reveal the presence of BMPs and activation of the pathway as early as day 3 in the activated periosteum and granulation tissue, and throughout repair via endochondral ossification in chondrocytes, osteoblasts/osteocytes, and endothelial cells. These data may serve as reference for functional analyses of BMPs and the BMP pathway in bone repair.

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