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Vascular Tissues Are a Primary Source of BMP2 Expression during Bone Formation Induced by Distraction Osteogenesis

Hidenori Matsubara¹, Daniel E. Hogan¹, Elise F. Morgan², Douglas P. Mortolock³, Thomas A. Einhorn¹, and Louis C. Gerstenfeld^{1,*}

¹Orthopaedic Research Laboratory, Boston University School of Medicine, Boston, MA, USA

²Department of Mechanical Engineering, Boston University, Boston, MA, USA

³Department of Molecular Physiology and Biophysics, Center for Human Genetics Research, Vanderbilt University School of Medicine, Nashville, TN, USA

Abstract

Prior studies showed that bone regeneration during distraction osteogenesis (DO) was dependent on vascular tissue development and that inhibition of VEGFR signaling diminished the expression of BMP2. A combination of micro-computed tomography (μ CT) analysis of vascular and skeletal tissues, immunohistological and histological analysis of transgenic mice containing a BAC transgene in which β -galactosidase had been inserted into the coding region of BMP2 and qRT-PCR analysis, were used to examine how the spatial temporal expression of the morphogenetic signals that drive skeletal and vascular tissue development are coordinated during DO. These results showed BMP2 expression was induced in smooth muscle and vascular endothelial cells of arteries and veins, capillary endothelial cells, hypertrophic chondrocytes and osteocytes. BMP2 was not expressed by lymphatic vessels or macrophages. Separate peaks of BMP2 mRNA expression were induced in the surrounding muscular tissues and the distraction gap and corresponded first with large vessel collateralization and arteriole remodeling followed by periods of angiogenesis in the gap region. Immunohistological and qRT-PCR analysis of VEGF receptors and ligands showed that mesenchymal cells, lining cells and chondrocytes, expressed VEGFA, although PlGF expression was only seen in mesenchymal cells within the gap region. On the other hand VEGFR2 appeared to be predominantly expressed by vascular endothelia and hematopoietic cells. These results suggest that bone and vascular tissue formation is coordinated via a mutually supporting set of paracrine loops in which blood vessels primarily synthesize the morphogens that promote bone formation while mesenchymal cells primarily synthesize the morphogens that promote vascular tissue formation.

Keywords

distraction osteogenesis; bone morphogenetic protein 2; angiogenesis; vascular endothelial growth factor; placental growth factor

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***Corresponding Author:** Department of Orthopaedic Surgery Orthopaedic Research Laboratory Boston University School of Medicine 72 E. Concord Street, E-243 Boston, MA 02118, USA Phone : 617-414-1658 Fax : 617-414-1661 lgersten@bu.edu. Hidenori Matsubara: ortho331@yahoo.co.jp; Daniel E. Hogan: d.hogan24@gmail.com Elise F. Morgan : efmorgan@bu.edu Thomas A. Einhorn : Thomas.Einhorn@bmc.org

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Introduction

It is well established that vascularization plays an important role in bone development and *post-natal* bone repair [1,2,3]. Numerous studies have shown that bone formation is preceded by vascular formation, which suggests that angiogenesis is essential for bone repair [4,5]. During fracture healing, angiogenesis has been shown to be vitally important and intricately involved in multiple stages of the repair process, including the inflammatory response [6], formation of the soft callus, and the transition from cartilaginous callus to bone [7,8]. Functional studies have also shown that disruption of angiogenesis during fracture healing [8] and during bone regeneration that is facilitated by distraction osteogenesis [9,10] impairs bone formation and leads to a non-union. While the importance of vascularization to bone healing has been recognized, the molecular and cellular mechanisms regulating angiogenesis and their relationship to the mechanisms of bone repair are not fully understood. Prior studies from our laboratory and a number of other studies have shown the essential nature of VEGFR-mediated signaling in the regulation of both vascular and osseous tissue formation [8,10,11].

Inadequate vascularization during skeletal tissue repair and regeneration is often thought of in terms of the lack of appropriate nutrition and oxygenation that are believed to provide important environmental signals for promoting osteogenesis and inhibiting chondrogenesis [12, 13]. However, vascular morphogenesis is itself the structural template around which bone morphogenesis takes place. Cortical bone formation is patterned around the Haversian system, and trabecular bone formation is patterned around the vascular structures that infiltrate the empty lacunae left after chondrocyte apoptosis during endochondral bone formation. In this context, recent studies have shown that both vascular and skeletal morphogenesis are interdependent on each other: development of vascular tissue precedes bone cell differentiation in BMP2-induced ectopic bone formation [14]; and during development of the appendicular skeleton, the skeletal tissues serve as a signaling center that directs patterning of the limb vasculature [15]. These phenomena suggest that there is a reciprocal co-dependency between vascular and skeletal tissues in which each tissue provides morphogenetic signals or environmental cues that are crucial for the other's development.

The role of the TGF- β morphogenetic factors in skeletal tissue development has been extensively documented, and numerous studies have specifically shown that BMP2 is a central and essential regulator during *post natal* bone formation and fracture healing [16,17]. We have shown that BMP2 is produced autogenously as mesenchymal stem cells (MSCs) progress to terminal osteogenic differentiation, which feeds back to further promote differentiation. We also showed that BMP2 regulates the expression of a number of other BMPs during MSC osteogenic differentiation [17,18]. To date, there have been many studies both *in vivo* and *in vitro* that have shown the importance of BMP2 in osteogenic differentiation (16-18). Early immunological studies of BMP2 expression during fracture healing showed that BMP2 was most strongly expressed in periosteal cells [19], while more recent immunohistological studies showed that the most intense BMP expression was found in both periosteal cells and hypertrophic chondrocytes [20]. Such findings have led to general assumption that the cells within the mesenchymal lineages that give rise to osteogenic and chondrogenic lineages are the primary cells in the skeletal environment that express BMP2 during *post-natal* bone growth and healing [21].

Recently, however, a number of studies have now shown that BMP2 is associated with the vascularization of tumors [22,23] and the progression of cardiovascular disease (24). Still other studies suggest that BMP2 is an important regulator of endothelial function and proliferation of smooth muscle cells in pulmonary vascular tissues [24-27]. Most recently

we showed in our analysis of the transcriptome of fracture healing that gene ontologies associated with BMP canonical signaling were equally associated with vascular and skeletal cell development [28]. Interestingly, *in vitro* experiments have shown that BMP2 stimulates proliferation of human aortic endothelial cells, promotes tube formation of human umbilical endothelial cells, and stimulates migration, chemotaxis and survival of circulating endothelial cell precursors [26,27,29]. BMP2 has been shown to be important in controlling the expansion of smooth muscle cells in pulmonary arterioles by acting as a negative regulator of the proliferation of these cells [25]. Up-regulation of BMP2 in vascular smooth muscle cells has also been found to be associated with the development of atherosclerotic vascular calcification [30,31]. Finally, BMP2 was shown to carry out an essential role in the in the crosstalk between endothelial progenitor cells (EPCs) and MSCs [32].

These prior studies suggested to us that BMP2 might carry out important roles not only in osteogenesis, but also in angiogenesis, during bone formation. In order to study the relationship between BMP2 expression and formation of vascular tissue, we examined the temporal, spatial, and molecular aspects of BMP2 expression during distraction osteogenesis. Using a combination of micro-computed tomography (μ CT) analysis of vascular and skeletal tissues both immunohistological, and histological analysis using transgenic mice containing ~238 kilobase BAC transgene in which β -galactosidase has been inserted into the coding region of BMP2 [33], and qRT-PCR analysis, we examined spatial temporal expression patterns of BMP2 during DO. In conjunction with these studies we also examined the spatial temporal expression of the VEGF morphogenetic family. We show in our studies that a major source of BMP2 expression during bone regeneration is the vascular tissue both within the regions of new bone formation and in the surrounding vascular bed of the adjacent muscular tissues while VEGF ligands appear to be predominantly expressed by mesenchymal cells and cells within the skeletal lineage.

Material and Methods

Materials

All studies were performed with male mice at 9-12 weeks of age. Micro-computed tomography (μ CT) and qRT-PCR analysis were carried out in C57BL/6J mice purchased from Jackson Laboratories. Transgenic mice containing two separate BAC transgenes were examined in this study. One transgenic line contained ~185 kb 5' to the BMP2 gene, the entire ~11 kb coding region of the gene, as well as ~42 kb of 3' sequence. The second BAC transgene contained ~193 kb of 3' flanking sequence as well as the ~11 kb of the coding sequence and ~3kb of 5 prime flanking region [33]. All transgenic mice were bred on site, and both heterozygotes and homozygotes containing the transgenes were used for our studies. DO devices were obtained from KLS Martin L.P. All reagents for the PCR analysis were from Applied Biosystems, and plate assays were read on an ABI 7700 Sequence Detector (Applied Biosystems).

Experimental Design

All animal studies were carried out under a protocol that was approved by Institutional Animal Care and Use Committee (IACUC). All mice enrolled in the study weighed 25 to 35 g and underwent DO on their left femur. The total time course for the experiment was 31 days. The distraction protocol consisted of three phases: 1) latency phase of 7 days' duration; 2) active distraction phase of 10 days; and 3) and a consolidation phase of 14 days. Animals were sacrificed and tissue was harvested at five postoperative days (PODs): day 7 (end of latency period), day 14 (seven days into active distraction), day 17 (end of active distraction), day 20 (three days into consolidation), and day 31 (end of consolidation). A group of control mice that received no surgery was collected at day 0 at the time the surgical

enrollment was initiated. The rate and rhythm of distraction were 0.15 mm once a day and active distraction was for 10 days, leading to a total distraction length of 1.5 mm. Four controls were assessed in this analysis: un-operated femurs at day 0; and three surgical controls (osteotomy surgery with no distraction) that were harvested at PODs 10, 17, and 31. For the μ CT analysis, $N=2$ samples were collected for each group. The mRNA assessments were carried out on two separate pools of total RNA, with each pool prepared from $N=3$ mice for each time point. For x-ray and histological assessments, $N=2$ (12 BMP2 / β -galactosidase mice total) were used.

Surgical Procedure

The surgical procedure was carried out following the methods similar to our previous study [10], but modified for the femur. The switch to the femur was made due to considerations of better anatomical configuration for reproducibility of μ CT analysis and better muscle coverage to support bone repair. A ~1.5 cm of incision was made longitudinally along the axis of femur extending from the greater trochanter to the knee joint. The underlying muscle was split along the intramuscular plane, and the femur was elevated and carefully separated from the surrounding musculature so as not to damage the periosteum. The distraction device was then attached to the femur. The arms of the device were opened a distance of 4 mm and were then secured to the bone using two sets of 0.01-inch ligature wire (Dentsply International) for each arm. A low-speed, rotary, diamond disk (Benco Dental) was then used to perform a transverse osteotomy in the mid-diaphysis of the femur, centered between the arms of the distraction device. Constant saline irrigation was used to prevent thermal damage. The muscle and skin were sutured to each layer with 5-0 plain gut resorbable sutures (Benco Dental). A subcutaneous 0.5 mg/kg dose of Buprenorphine was given into the left thigh. All animals tolerated the procedure well and were able to walk with fully weight bear on awakening from anesthesia. No systemic effects on the overall health of the mice were observed. Euthanasia of the mice was by CO₂ asphyxiation.

Radiographic Evaluation

High-resolution radiographs (Faxitron X-ray Corporation) were taken with the leg intact. The images were taken at 3X magnification with 25 KV for 60 seconds exposure on XAR-5 film (Kodak).

Microcomputed Tomography and Image Registration

Vasculature and bone tissue were assessed by μ CT [10]. After euthanasia the mice were pinned in a supine position and a shallow incision to the skin was made from the mid-region of the abdomen to neck. The thorax was opened just right of the sternum and then straight through to the jugular notch. Diagonal incisions were made on both sides of diaphragm along its attachment to the thoracic wall. The lower part of sternum was pinned to the side to expose the heart. A 23G butterfly needle was inserted into the left ventricle. A cut through the inferior vena cava (IVC) was made to drain the re-circulating blood and perfusate. Ten ml of 10% buffered formalin was perfused to fix the vasculature after which the silicone rubber polymer, containing lead chromate contrast agent (Microfil MV 122 yellow, Flow Tech) was perfused. Perfusion with contrast agent was carried out using a 1ml syringe using a total of ~5 ml of agent per mouse. The perfused animals were stored at 4°C overnight for completion of the polymerization of the contrast agent. The legs were then disarticulated and stored in 4% buffered paraformaldehyde for 24 hours. The fixative was changed with fresh reagent and fixation was continued for three additional days after which the tissues were then stored until use in neutral buffered PBS at 4°C.

Legs from animals that had undergone the vessel perfusion protocol were scanned using a Scanco μ CT40 system (Scanco Medical) both before and after decalcification. In order to

provide support to the regenerate during scanning, specimens were embedded in 1% agarose made in buffered saline. Scans were carried out at a resolution of 10 $\mu\text{m}/\text{voxel}$, with voltage set at 70 kVp and current set at 114 μA . The integration time for scanning was 300 ms, and scans generally lasted 60 to 130 minutes. Decalcification was carried out for two weeks in 14x EDTA (Boston BioProducts) at 4°C. The post-decalcification images for a specimens were registered against the corresponding pre-decalcification images (Amira 5.2, Visage Imaging) in order to remove the deformation of the tissues that occurred during decalcification. The registration process involved placing ~100 pairs of landmarks on matching features of the vasculature in the two sets of images and then applying a deformation to the post-decalcification images, according to the Bookstein algorithm, such that the location of each landmark in those images coincided with that of the corresponding landmark in the pre-decalcification images.

RNA Isolation and Quantitative Real-Time RT-PCR

The RNA isolation and quantitative real-time RT-PCR were carried out as previously described [10]; however, in the studies reported here L32 ribosomal protein RNA was used for normalization of each target mRNA. Analysis of mRNA expression was carried out on replicate pools (N=3 mice per pool) of mRNAs and individual assessments were done three times on each pool. The two arms of the distraction fixator were used to define the anatomical regions of interest that were dissected for mRNA isolation. The cortical bones were used as the margins by which the intra-osseous regions were defined. The entire surrounding mass of muscle was used for the mRNA from the muscle. The expression values of target genes were normalized to bone and muscle tissues from unoperated controls that are collected at the start of the study. Comparable anatomical intraosseous and muscular regions, were used for the control tissues.

MSC Culture

Marrow stromal cell preparations were made as previously reported [17,18]. All MSC preparations were from C57BL/6J (B6) male mice of 8–10 weeks of age (Jackson Laboratories). Medium was replaced every two days from day 6 to the appropriate time point. One group of cultures received 100ng/ml rhBMP2 starting on day 6 for the remainder of the experiment. Messenger RNA was isolated for real-time RT-PCR at 6, 10 and 16 days after plating.

Immunohistochemistry and Staining for β -Galactosidase

The femur was disarticulated from the acetabulum of the pelvis. X-Gal staining was performed as described previously [33,34]. Stained specimens were then placed into fresh 4% paraformaldehyde for 48 hours for fixation. Samples were decalcified in 0.048M (14% W/V), ethylene diamine tetra acetic acid (EDTA) (Boston, BioProducts) for two weeks. Once the specimens were decalcified, they were embedded in paraffin and sectioned at 5 μm in the sagittal plane. Sections for plain histology were cleared and stained with hematoxylin and eosin. The slides that were processed for immunohistochemistry after normal processing and rehydration were washed for five minutes in PBS and incubated in 3% H_2O_2 for 30 minutes to eliminate any endogenous peroxidase activity. Slides were then incubated in 2% horse serum for ten minutes to block non-specific binding and washed again in PBS. For immunohistochemistry, sections were incubated with primary antibodies against CD31 (1:200, 4°C, overnight) (Abcam), smooth muscle α -actin (1:400, 4°C, overnight) (Sigma Aldrich), placental growth factor (1:200) (Abcam), LYVE-1:lymphatic vessel marker (1:1000, 4°C, overnight) (Abcam), BMP2 (1:1000, 22°C, 2hr), (Abcam), mouse macrophage surface marker (1:100, 22°C) (Santa Cruz Biotechnology) and VEGFA (1:200 (1:100 22°C) (Santa Cruz Biotechnology) and VEGFR2 (1:100 22°C) (Abcam). Appropriate specie specific secondary antibodies were used with each primary antibody. Each of these was

obtained complexed with biotin from Vector Laboratories. Secondary immunoreactions were for 1 hour at antibody dilutions of 1:1000 at 22°C. After which the slides were washed with PBS and then reacted with avidin conjugated peroxidase using a Vectastain *Elite* ABC kit (Vector Laboratories). A Vectastain DAB substrate kit (Vector Laboratories) was used for visualization of the binding interactions. All counter staining was with a short 30 second wash with only hematoxylin.

Results

Radiographic Assessment of the Structural Morphogenesis of Vascular and Skeletal Tissues

While our previous studies were carried out in the tibia, the current study was performed in the femur since its unbowed anatomical structure provides a higher degree of uniformity to obtain reproducible morphometric assessments. A representative series of x-rays across the time-course of DO are presented to show the general progression of healing in Figure 1A. Very little new bone was observed in the osteotomy gap at the end of the latency phase (POD 7), and only a small increase in x-ray opacity were observed during the period of active distraction (POD 10-17). In contrast, the majority of mineralized tissue formation took place during the consolidation phase (POD 20 and 31).

Figure 1B presents a series of representative μ CT reconstructions of the structural morphogenesis of vascular and osseous tissues over the time course of distraction osteogenesis. The upper series of panels shows the series of images of the femur and supporting vasculature in the surrounding muscle at 3 days after distraction is initiated (POD 10), at the end of the distraction period (POD17), and at two weeks of consolidation (POD 31). The lower panels are a series of controls showing the native vasculature in an unoperated femur (Day 0) and at 17 and 31 days after osteotomy with no distraction. The osteotomy alone produced a strong angiogenic response in the vasculature within the surrounding musculature, resulting in increased size and number of vessels as compared to the unoperated limb. However, active application of mechanical strain by distraction osteogenesis produced an even greater and profound effect on the existent vasculature. This effect was seen initially as a massive increase in the size of the existent vessels that is most easily observed for the femoral artery during the active distraction period. Formation of smaller vessels was primarily seen during the consolidation period and was observed both within the developing bone and the surrounding muscular space. In contrast, the bones that had undergone osteotomy and no distraction showed an extensive amount of vascular remodeling had occurred by day 31 and actually exhibited a reduction in both the number and size of vessels in the surrounding tissues. Within this control osteotomy group, the bone was completely bridged by 17 days after surgery, whereas the distraction samples still showed a large unmineralized gap which only showed completion of bridging by day 31.

Histological Characterization of BMP2 Expression during DO

Previous studies examining the effects of VEGFR 1 and 2 blockade on bone and vessel formation during DO had shown that BMP2 expression was inhibited by approximately 50% over the time course of DO, commensurate with the inhibition of vessel formation (10). In order to determine if MSCs and skeletal cell types are the only cells expressing BMP2 during DO, BMP2 expression was analyzed in two different mouse lines containing BAC transgenes encompassing ~200 Kb of either the 5' or 3' flanking regions to the coding sequences [33]. Only the strain containing genomic sequences 5' to the BMP2 coding region (chondrogenic preference strain) showed expression of the β -galactosidase gene during the DO bone healing although β -galactosidase activity was examined during DO for both strains of mice. Figure 2 shows both the extent of the induction of BMP2 at a gross level in

response to DO as well a series of micrographs of the β -galactosidase staining at the tissue and cellular level across the time-course of DO. The top two panels (Figure 2A) shows the gross comparison of the β -galactosidase activity in the operated and distracted limb versus contralateral unoperated limb from the same mouse at 20 days post surgery. DO induced an extensive amount of staining throughout the entire vascular bed of the operated limb in comparison to the unoperated limb in which only a small number of vessels showed reactivity. In panel B of this figure the staining pattern at day 10 (left set of panels) and day 20 (right set of panels) within the distraction gap and the surrounding muscular tissues are depicted. At day 10 a small number of chondrocytes within the gap and cells on the surface of host bone close to the gap were positive for β -galactosidase staining. Interestingly, many small vessels adjacent to the gap and in the surrounding muscular tissues were also positive. At day 20, the cells on the surface of host bone and in small vessels close to gap were positive. The small focal areas of cartilage cells could still be seen in the gap with numerous chondrocytes still staining positive. Higher magnifications of these regions (Panel C) showed the presence of intensely staining positive osteocytes that are fully embedded in mature trabeculae that were now forming in the gap (Region 1) in the 20 day specimens. Higher magnification (400X) of chondrocytes in the gap region are compared to staining seen in control mice from the epiphyseal growth plates of the distal femur. This comparison would suggest that the BMP2 expression was restricted primarily to hypertrophic cells since only cells towards the bottom of the normal growth plates were positive. Higher magnifications of the vessels in the surrounding tissues showed both the lining cells on the inner vessel walls and the medial layers of the vessels appeared to be stained positively for BMP2 expression. The bottom set of panels of Figure 1C were reacted with a polyclonal antibody to BMP2 in order to provide independent confirmation that the transgene expression was showing fidelity in both intensity and cellular localization of BMP2 expression. These results showed very high degree of overlap between those cells that were reactive with the antibody and those that showed expression of the transgene. It is interesting to note in this regard that while the cells themselves showed very strong immunoreactivity, that the surrounding matrix also showed diffuse and low levels of antibody staining in areas in which cell are expressing high levels BMP2. This pattern of antibody staining would be consistent with the BMP2 protein being actively secreted and sequestered into the surrounding extracellular matrix. Such finding would also suggest that while the transgene is very sensitive at detecting those cells that are most actively expressing BMP2 that the transgenic protein does not become secreted and is not accumulated but is rapidly turned over.

Several controls are shown in Figure 3 that both address the specificity of the transgenes expression and its relationship to the DO process. In the upper panels of the figure patterns of the transgene's expression are presented for the 31 day specimens of the DO time-course (Figure 3A left hand panel). Two control specimens (osteotomy with no distraction) specimens from 20 and 31 days after surgery are also shown (Figure 3A middle and right panels). These data demonstrate that maximal BMP2 expression was seen during the period of active distraction and while at 31 days there were still a small number of blood vessels in the muscular tissues that showed β -galactosidase activity, there were no cells that were actively expressing BMP2 in the control tissues that had not been distracted at days 20 and 31. The data in Figure 3B shows DO specimens from 20 days post surgery that were isolated from mice containing the BAC transgene containing the 3' BMP2 flanking sequence. In these mice no β -galactosidase activity was observed in the distraction gap after active distraction. Analysis of the femoral artery from these distraction specimens showed only diffuse areas of β -galactosidase activity, although when an adjacent tissue section containing the femoral artery was reacted with antibody for BMP2, it showed strong immunoreactivity, thereby further validating the fidelity of the expression pattern of the 5' BAC transgene.

In order to elucidate which cell types (endothelial cells or smooth muscle cells) and types of vessels (arteries, veins, capillaries or lymphatics) that are expressing BMP2, a series of immunohistochemical co-localization studies with three antibodies specific for smooth muscle cells (α -Smooth Muscle Actin), vascular endothelial cells (CD31) and lymphatic endothelial cells (Lyve1) were performed. We also assessed in this set of studies whether macrophages would show BMP2 expression. Representative pictures of large and small arteries and veins and capillaries are seen in Figure 4A. In all cases smooth muscle cells in the vascular medial layer of both large- and medium-sized vessels stained positive for β -galactosidase and co-stained with α -SMA. Eosin-stained capillaries were surrounded by β -galactosidase positive cells, although it could not be definitively determined if these cells were endothelial cells or pericytes. However, given that immunohistochemistry for α -SMA did not stain these cells (data not shown), and that pericytes would show expression of α -SMA, it was concluded that these cells were endothelial cells. In smallest, capillary-sized vessels in the gap region, endothelial cells also showed intense β -galactosidase and CD31 staining. In panel B of Figure 4 two separate sets of immunological reactions were carried out to identify if lymphatic endothelial cells or macrophages also expressed BMP2 in the DO tissues. These data clearly show that while lymphatic tissues were abundantly present in the gap region, were immediately peripheral to the bone, and formed in proximity to small and intermediate-sized arteries and veins, these tissues did not express BMP2. It is interesting to compare these results with those seen in the immunolocalization studies carried out with the anti BMP2 antibody in Figure 2, since while the BMP2 antibody clearly reacted with small arteries and veins seen in this figure it did react with the adjacent small vessel structures, which presumably are lymphatic vessel. This result would again suggest that BMP2 transgene's expression showed a high degree of fidelity in its patterns of expression. In sections containing well developed trabecular bone and hematopoietic cells only osteocytes showed transgene expression while none of the hematopoietic cells showed reactivity. In contrast when marrow elements and cells in the gap region were reacted with a macrophage specific antibody many positive cells were observed but none of these cells showed either transgene expression or reacted with the BMP2 antibody verifying that during DO that macrophages that are present in this tissue do not express BMP2.

Spatial-Temporal Coordination of the Signaling Processes that Drive Vascular and Bone Morphogenesis

We next assayed the temporal and spatial expression of various sets of mRNAs that drive vessel formation (Figure 5). We first determined if unique morphogenetic mechanisms were separately driving vascular tissue formation in the bone as compared to the surrounding soft tissues by examining the levels of mRNA expression of the three major VEGFRs and ligands that interact with these receptors in the surrounding musculature and in the bone and mesenchymal tissues within distraction gap (Figure 5A). The upper four panels present the expression of ligands and receptors that primarily drive blood vessel formation (VEGFR1 and 2 and VEGFA, VEGFB, PIGF), while the lower four panels show the results for those associated with lymphangiogenesis (VEGFR3 and VEGFD and VEGFC). The ligands that interact with VEGFR1 and 2 and that were induced within the intraosseous region of the distraction gap were PIGF, VEGFA, and, to a much lesser degree, VEGFB. Both PIGF and VEGFA showed approximately a ~2- and 2.5-fold induction, respectively, by the end of the latency period (POD 7), while PIGF showed a sharp ~4-fold induction during the consolidation period. In contrast, in the muscular tissues, no induction of PIGF was seen, while both VEGFA and VEGFB showed strong induction during the period of active distraction after which their expression flattened out but did not fall off during the consolidation period. Interestingly, VEGFD, which primarily interacts with VEGFR3, was found to have the greatest levels of induction overall and was upregulated in both the distraction gap and musculature. The expression pattern for this ligand showed peak levels

by the end of the latency period and throughout the distraction period after which its relative expression decreased markedly. The expression pattern of all three VEGF receptors paralleled each other within the distraction gap, displaying two peaks of expression at days 10 and 20. In muscle the expression of VEGFR1 and 2 rose during the consolidation period, while VEGFR3 was downregulated over the time-course of DO.

We then examined the spatial patterns of expression of the two most prevalently expressed ligands (VEGFA and PIGF) and the primary receptor (VEGFR2/KDR) that would interact and mediate their activities. VEGFR2 was primarily detected in endothelia cells lining both arteries and veins and on the surface of hemopoietic cells in the marrow space. It was also seen reacting within vessels that did not show expression of the BMP2 transgene. Such a finding is consistent with location and appearance of the lymphatic vessels observed in Figures 2 and 3 and would corroborate recent studies that show that VEGFR2 is expressed by lymphatic endothelia cells [35]. Interestingly while no mesenchymal or osteoblast cells in any of the tissue regions appeared to be positive for VEGFR2/KDR, hypertrophic chondrocytes did appear to express this receptor. An examination of PIGF showed that only mesenchymal appearing cells in the gap region expressed this ligand. While VEGFA showed a similar localization as PIGF in the gap region, unlike PIGF it was observed in lining cells on trabecular surfaces, non-hypertrophic chondrocytes and small satellite cells between muscle fibers.

We next assessed the temporal progression of skeletal tissue development relative to that of vascular tissue development in the intraosseous and muscular compartments. These data were considered in context with the expression of both BMP2 and two specific antagonists of Wnt signaling, Sost and DKK (Figure 6). Both transcription factors that drive osteogenic differentiation, Runx2 and Osterix, showed a 2.5-fold induction throughout the active distraction period. Interestingly, osterix expression increased almost 6-fold during consolidation, while Runx2 showed a more tempered peak during this period, consistent with the suggestion that osterix acts downstream of Runx2 to further control the progression of osteogenic differentiation. During the period of active distraction, very high levels of RANKL were induced and were matched temporally with a smaller level of induction of BSP. The late markers of osteoblast progression to the osteocyte stage were shown to peak during the consolidation period. The extremely high levels of RANKL expression and the prolonged period of its expression relative to the when the terminal osteocyte markers showed peak expression suggests that large numbers of osteoprogenitors are recruited during active distraction but do not progress to terminal differentiation until very late in the distraction period.

The progression of vascular tissue development was made by assessing two genes associated with endothelial cells Ve-Cadherin [36] and arteriole development and remodeling, Ephrin B2 [37]. These marker genes showed differing, out-of-phase patterns of expression in the two tissue compartments with a biphasic pattern of expression in the muscle compartment and a single sharp peak of induction during the distraction period in the bone. It is interesting to note that these markers in part parallel the expression of VEGFR2, which has been shown to be closely co-regulated with ephrin B2 in endothelial cells [38], but that in the gap region the receptor showed a second peak during the consolidation period. In this regard it has been shown that hematopoietic cells including osteoclasts also express VEGFR2 [39]. The expression of the surface marker found on endothelial cells lining lymph vessels LYVE1 showed very strong induction in both compartments with a very sharp spike at the end of the consolidation period in the muscle compartment that paralleled the patterns seen for VEGFD; however, these changes did not follow the pattern of the expression of the VEGFR3 since it showed only very low levels of induction in the muscle compartment.

In the final panels of this figure the expression of three separate morphogenetic regulators, BMP2, Sost and DKK, are shown. Because we observed that the distraction period is characterized by little tissue mineralization and prolonged, very elevated levels of RankL expression, we speculated that one or both of the Wnt antagonists that are known to regulate mineral deposition and to control terminal osteoblast to osteocyte transition [40], might play a functional role in the osteogenic processes of DO. BMP2 expression almost perfectly matched the expression patterns seen for the two sets of endothelial cell markers that tracked vessel development. The one difference between these data and those for the endothelial markers was that a second smaller peak of BMP2 expression was seen in the tissues from the gap region during the consolidation period. In this regard, the second peak would be consistent with our data depicted in Figure 2 showing that mature osteocytes and chondrocytes expressed BMP2 in the gap region during the consolidation period. Perhaps the most interesting aspect of these data was those assessing Dkk and Sost expression. These genes appeared to be expressed later in each tissue compartment relative to BMP2. Interestingly, in the muscle compartment, Sost and DKK exactly paralleled each other, while in the gap region Sost showed no induction during the distraction period and downregulation in the consolidation period, while DKK was upregulated during consolidation.

In the last experiments that are presented we use MSC culture experiments, both with and without BMP2 treatment, to further define the nature of morphogenetic signaling processes that occur between the mesenchymal and vascular tissues during DO, by defining the nature of the expression of the various VEGFR and their ligands across the time course of in vitro mesenchymal stem cell differentiation in control cultures, and those in which MSC differentiation was enhanced by BMP2 treatment (Figure 7). These data show that as MSC differentiation proceeded, expression of multiple ligands for the VEGFRs increased, and that BMP2 treatment augmented and in some cases accelerated this upregulation. This assessment demonstrated that PlGF was one of the major VEGFR ligands whose induction during osteogenic differentiation of MSCs was enhanced by BMP2 treatment. Interestingly, the expression of both VEGFR1 and VEGFR2 showed an 80% decrease in expression as the MSCs differentiated, suggesting that the MSCs themselves are not a target of the VEGF or PlGF but that they produce these morphogenetic proteins only as paracrine factors.

Discussion

Our previous studies showing that VEGFR blockade during DO caused commensurate failures in both vascular and bone tissue formation that were associated with a ~60% loss in BMP2 expression [10], led us to further investigate the nature of the tissues that would express BMP2 during DO. While it is generally believed that BMPs, which promotes bone repair, are either produced locally by MSCs and osteogenic cells or are proteolytically released from sequestered stores in the ECM [21], our data challenges these assumptions. Using several different approaches to address this question, we found that both vascular smooth muscle and endothelial cells were one of the primary cell types in the regenerative environment that expressed BMP2. Although the use of the transgene's expression may not fully identify all of the cells that express BMP2 the comparable cell distribution of BMP2 expression that was observed using the BMP2 antibody localization was strong evidence that the transgene did provide a faithful representation of BMP2 expression. While these findings were unexpected, the role that BMP signaling plays in vascularization has recently begun to be more fully investigated. Studies showing that BMP2 was expressed during tumor induced angiogenesis [22,23] were some of the first to intimate that BMPs might carry out functional roles in normal vascular tissue formation. Early studies of vascular endothelial cells in vitro showed that these cells produce BMP2 [41] in response to both hypoxic conditions as well as VEGFA treatment. Other studies have further shown that BMP2 stimulates proliferation, tube formation, migration and chemotaxis of endothelial

progenitor cells [26,29,42]. Our own studies of the transcriptome of fracture healing have shown that expressed gene ontologies associated with BMP signaling are associated with both vascular and skeletal tissue development [28]. Recent studies of two genetic vascular diseases (hereditary hemorrhagic telangiectasia (HHT) and pulmonary arterial hypertension (PAH) have clearly defined specific roles of BMP signaling in post natal vascular tissue development and homeostasis and have implicated functional roles for BMP signaling in both vascular endothelia and smooth muscle cell [43]. Numerous studies have now been carried out that also suggest that BMP signaling prevents pathological smooth muscle cell proliferation in pulmonary arterioles while promoting endothelial survival within these smaller arteries [44-47]. It has also been suggested that BMP signaling plays a much broader role in the systemic homeostatic maintenances of all vascular tissues [42,45]. Finally it is interesting to note the recent findings showing that endothelial cells expressing the mutant form of BMP type 2 receptor Alk2 associated with Fibrodysplasia Ossificans Progressiva (FOP) [46], undergo transdifferentiation to take on an osteogenic phenotype [47]. The suggestion that the extraosteal bone that forms during FOP is due to endothelia cells taking on an osteogenic phenotype, begs the question as to whether the local vascular induction of BMP2 in response to injury may contribute to the progression of this disease process.

In regard to ectopic mineralization associated with vascular tissues, there is an emerging set of data that suggest that BMP2 expression in smooth muscle cells is associated with pathological calcification of arterial medial tissues [30,31]. In our model of DO, smooth muscle cells produce BMP2 without the resulting pathology, and Li *et al.*, [48] showed that treatment of SMCs with BMP2 did not induce calcification under normal phosphate conditions. Moreover, inhibition of phosphate uptake by a competitive inhibitor of sodium-dependent phosphate co-transport, phosphonoformic acid, abrogated BMP2-induced calcification [49]. These results indicate that phosphate transport is crucial in BMP2-regulated SMC calcification. Other studies as well have shown that a variety of endogenous BMP2 antagonists, such as noggin, chordin, and matrix gamma-carboxyglutamic acid protein regulate the BMP2 expression [49] in smooth muscle cells. More recent studies have also shown that there is considerable crosstalk between BMP2 and Wnt signaling. Prolonged BMP signaling can negatively regulate bone mass through the upregulation of sclerostin and inhibition of canonical Wnt signaling in osteoblasts [50]. In vascular tissues, Wnt/BMP crosstalk appears to be a central mechanism by which BMP2 can facilitate SMC motility while simultaneously suppressing growth through the activation of the Wnt- β -catenin (β C) and Wnt-planar cell polarity (PCP) signaling pathways [25].

One of the most interesting biological features of DO relative to other processes of bone formation is that although extensive numbers of MSCs are recruited into the gap region, they do not undergo terminal differentiation and mineralization until the distraction period is completed. This delay in the osteogenic progression may be evidence of a mechanism that coordinates the processes of vascular morphogenesis and bone morphogenesis, because if the connective tissue were to mineralize prematurely, the blood vessels would be unable to grow into the tissue. It was in this context that we specifically examined the two Wnt antagonists Sost and DKK. We found that these antagonists are induced in each tissue compartment after each peak of BMP2 induction, consistent with emerging indications that they are downstream targets to BMP signaling through the BMPRII receptor [50]. It is also of interest that there are low but detectable levels of the induction of these genes in the muscle compartment, since a number of studies have now shown that these proteins may function in development and remodeling of vascular tissue. Recent data has suggested that BMP signaling in both vascular tissues [51-55] and developing bone tissues [56,57] regulate Wnt signaling by controlling the expression of DKK and Sost. For example, DKK regulates neoangiogenesis [29,52,53] within vessels, while Sost serves to the control osteoblast to

osteocyte differentiation and mineralization in bone [57]. Thus BMP signaling is at the apex of regulatory control of the formation of both vascular and bone tissues, in part through its additional function in the regulation of Wnt activity.

These prior studies are also all consistent with our observations that BMP2 showed alternating temporal expression patterns—induction first in the surrounding musculature, then with in the gap region. We hypothesize that BMP2 levels rise first in the surrounding muscle tissues for two reasons: first, to promote endothelial cell survival, proliferation, and subsequently, migration into the regenerate where they are needed to form new vessels; and second, to keep in check the proliferation of smooth muscle cells during this period of nascent vascularization. BMP2 levels in the musculature then fall during the distraction period when structural morphogenesis and increases in vessel thickness occur. BMP2 levels concurrently rise within the gap region as invading smaller vessels grow into the regenerate, thus prompting further growth of capillaries and other small-size vessels and promoting MSC commitment to undergo osteogenic differentiation. In this regard, the observed increases in expression of both VEGF and BMP2 that are seen in the gap region, which would lead to both proliferative expansion of endothelial cells and MSC commitment to the osteogenic lineage, are also be consistent with the hypoxic state of the gap region during the period of active distraction and the known of effects of hypoxia on VEGF and BMP2 expression [41,58]. Finally, during consolidation BMP2 levels again rise in the muscle space, concurrent with small vessel formation and neoangiogenesis in the extraosteal space.

The other component of morphogen signaling that we examined in this study was focused on VEGF. The VEGFs, VEGFRs, and marker genes for vessel development in the gap region and in the muscle also showed a biphasic and alternating pattern of expression in the two tissue compartments, with muscle compartment showing an initial earlier induction than the gap region. Interestingly the expression of these genes in the gap region appeared to begin to return to their baseline expression as osteogenesis progressed through day 31, while their expression in muscle continued to be elevated. These results are consistent with the persistence of vascular formation in the muscle even after 14 days of consolidation. A comparison of these findings to our previous studies carried out in a murine model of DO in the tibia, indicates that the femur exhibits a stronger, earlier, and longer induction in the expression of these genes and also heals faster [10]. A similar contrast has been observed for fracture healing [59], suggesting that bone healing in the femur is more robust than the tibia.

In regards to the nature of the morphogenetic factors that drives vessel formation, the increased expression of PIGF, all four VEGF ligands, and all three VEGFRs that was seen in the gap region are consistent with our own published data and those of others [10, 6,7,60,61]. Although expression of PIGF was first shown in umbilical vein endothelial cells and placental tissues [62], more recent studies have shown that PIGF plays an important role in the recruitment of both endothelial and hematopoietic stem cells (HSCs) in the marrow microenvironment [63,64]. Our observation that PIGF expression increased during osteogenic differentiation of MSCs and was induced by BMP2 in vitro is consistent with the results of other studies [65]. Our findings that VEGFR3 was also expressed during DO and that abundant lymphatic tissues formed in the gap region and immediately peripheral to the new bone are further supported with by the known role of this VEGF receptor in lymphoangiogenesis [66,67]. While the expression of LYVE1, a known marker of lymphatic tissues, was also seen in the muscle tissues, we did not see strong induction of VEGFR3 in the muscle tissues. This apparent discrepancy is most likely due to the approach of assaying mRNA levels relative to their baseline levels in the tissue. There may in fact be only a very small number of lymphatic endothelial cells in the muscle, and their presence is more easily seen through the induction of their surface marker LYVE1 than through changes in the overall low levels of expression of VEGFR3. The lack of overlap between cells expressing

LYVE1 with β -galactosidase suggests that the mechanisms of lympho-angiogenesis and the role that BMP2 plays in this process is intrinsically different from those that control arteriogenesis and angiogenesis. Such findings also suggest that the formation of vessels in lymphatic tissue is primarily associated with bone development and does not occur in conjunction with the formation vascular tissue observed in the muscle.

While previous clinical studies have shown that vessels in soft tissue are essential for bone repair, and that the extent of soft tissue injury affects the ability of bones to undergo repair, the extent of soft tissue damage has traditionally been thought of in the context of compromised tissue nutrition and oxygenation. The current data provides strong evidence that the surrounding vasculature outside the bone also provides an essential source of morphogens and a source from which regenerative cells may be recruited during injury-induced bone regeneration. The close proximity of MSCs producing angiogenic growth factors, such as VEGFa and PIGF, in the gap region to the vessels that grow into the gap would suggest that factors produced by MSCs promote local angiogenesis at the bone repair site via chemotactic effects on SMCs and EPCs that originate from the existent vasculature or the marrow space. Furthermore, the expression of BMP2 by the vessels suggests that the VEGF ligands and BMP2 act in a paracrine manner to form a reciprocal, self-sustaining, positive feedback loop by which angiogenesis and osteogenesis are maintained and balanced during bone healing. In summary, we have shown in these studies that the cellular origin of BMP2 during distraction osteogenesis is intimately and extensively associated with vascular tissues at the site of bone injury, and we suggest mechanisms by which BMP2 might mediate the crosstalk between angiogenesis and osteogenesis during bone repair.

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Highlights

Vascular Smooth Muscle and Endothelia Cells Express BMP2 during Osteogenesis

Mesenchymal Cells and Skeletal Cells Express VEGFA and PlGF during Osteogenesis

Lymphatic vessels and macrophages do not express BMP

Figure 1. Progression of Osteogenesis and Vasculogenesis across the Time-Course of Distraction Osteogenesis

A) Radiographic assessment of bone formation. Representative series of plain-film images obtained from a Faxitron X-ray machine. All images are oriented with the proximal end of the bone at the top. Post-operative days (PODs) are indicated in the figure. **B)** Representative reconstructions of the structural morphogenesis of vascular tissues and co-registered vasculature and bone tissues. Vascular tissues are false-colored red. Bone is false-colored yellow. All images are oriented with the proximal aspect at the top. Post-operative days are denoted in the figure. The upper half of panel B depicts reconstructions obtained from animals that underwent distraction. The bottom half depicts three control specimens: an un-operated femur and two femora in which the osteotomy was performed and was stabilized with the distraction device but in which no distraction was performed. The arrows point to the femoral artery.

Figure 2. Tissue-Specific Expression of BMP2 Over the Time-Course of Distraction Osteogenesis
 Expression of β -galactosidase transgene inserted into the coding region of BMP2 within a ~238 kilobase BAC transgene. **A)** Gross tissue level assessment of the induction of BMP2 expression in response of DO. Appearance of freshly dissected operated distracted leg at 20 days post surgery and the contralateral leg from the same mouse after fixation and staining for β -galactosidase. **B)** Top panels show panoramic 40x overviews of BMP2 expression in the region of the distraction gap and in the surrounding muscular tissues at post-operative days 10 and 20. Arrow denotes the site of the distraction gap. Numbered boxed areas within the micrographs show selective regions that were examined at higher magnification (200x) as shown immediately below each 40x image. Arrows denotes the distal edge of the osteotomy gap. Each panel shows positive reactions in one of three cell types: osteocytes, chondrocyte-appearing cells, and vascular tissues. **C)** These higher magnification depicting 400x micrographs showing cellular details for BMP2 expression for each of the individual cell types. The upper panels of the figure show β -galactosidase positive reactions in mature osteocytes and in occasional cells on the surface of bone. The middle two panels show β -galactosidase positive reactions in the chondrocytes seen in the gap region (DO) and in the growth plate (GP) of the same femur. Right panels show β -galactosidase positive reactions in large vessels. The lower set of panels depicts co-localization between areas that were reactive with Anti-BMP2 antibodies and β -galactosidase positive cells. Comparable regions highlighting the cells types depicted in the upper panels are shown in this series of micrographs.

Figure 3. MP2 Expression is Associated Only With Vascular Tissues During Active Distraction and Is Specific to the Transgene Containing the 5' Genomic Region to the BMP2 Gene

A) Top panels show panoramic 40x overviews of 5' BMP2 transgene expression in the region of the distraction gap at 31 days post surgery (14 days after end of distraction). Insert shows a 100x magnification of a selected region in the muscular tissues that still shows reactive vessels. Middle panel and right panels show panoramic 40x overviews of 5' BMP2 transgene expression at 20 days and 31 days post surgery respectively in the region of the osteotomy gap from animals that have not been distracted. **B)** Bottom Panels show selected samples from distracted tissue specimens at 20 days from mice containing the BAC transgene containing the 3' BMP2 flanking sequence. Left panel is a panoramic 40x overviews of the region of the distraction gap. Middle panel shows 400x micrographs showing cellular femoral artery from these mice at the same time point. Right panel shows an adjacent tissue section of the femoral artery reacted with Anti-BMP2 antibodies.

Figure 4. Characterization of Cell-Specific Expression of BMP2 during Distraction Osteogenesis
A) Immunohistological confirmation that smooth muscle cells and endothelial cells in large, medium, and small vessels express BMP2. Selected micrographs showing β -galactosidase enzyme activity in large, medium, and small blood vessels. Validation that both smooth muscle cells and endothelial cells were expressing BMP2 was achieved by demonstration of co-localization of β -galactosidase reactivity with smooth muscle actin and CD31, respectively. **B)** Immunohistological confirmation that lymphatic vessels and macrophages do not express BMP2. Sections from tissues reacted to show β -galactosidase activity were separately reacted with antibodies for lymphatic vascular endothelial cell marker LYVE1, and macrophage selective surface marker B220. Separate areas within the gap region as indicated by the double headed arrow and in the surrounding muscle were examined for LYVE1. The boxed area highlights the low magnification seen in the center panel. The bottom panels depict the marrow space between newly formed trabeculae were examined for macrophage staining.

Figure 5. Differential Temporal Spatial Expression of VEGF Ligands and Receptors in Muscle and Bone Tissues during Distraction Osteogenesis

A) Temporal mRNA expression of VEGF ligands and VEGF receptors in the surrounding muscle tissue (“Muscle”) or within the distraction gap (“Gap Region – Bone”) across the time-course of distraction osteogenesis. The upper panels present the expression profiles for those receptors and ligands primarily associated with formation of blood vessels. The lower panels present the expression profiles for those receptors and ligands primarily associated with formation of lymphatic vessels. The mRNA expression is normalized to L32 of each target sequence and is expressed as a fold change relative to day 0 (control, no surgery), i.e. $2^{-\Delta\Delta CT(\text{day } 0)} = 1$. The three phases of the time-course of DO are: latency phase (days 0-7); active distraction (days 8-17); and consolidation (days 18-31). All values are representative of three to four replicates from two pools of mRNAs. Error bars are the S.D. of each set of measurements. **B)** Spatial localization of VEGFR2, PlGF and VEGFA reactivity in various cell types within the DO tissues. Nature of the tissues examined and that of the reactive antibody are indicated in the figure.

Figure 6. Comparison of Temporal mRNA Expression Profiles for Marker Genes Associated with the Progression of Osteogenic Differentiation and Vascular Development and for Morphogenetic Proteins

Left panel depicts marker genes associated with the progression of osteogenesis. Middle panel shows selected set of markers genes associated with either vascular endothelial cells (Ve-Cadherin and ephrin B2) or lymphatic endothelial cells (Lyve1). Right panel shows the expression of three different morphogenetic proteins (BMP2, Sost and DKK). In the left panels, the data correspond to mRNA isolated from the gap region. In the middle and right panels the expression profiles are separately presented for these marker genes in the surrounding muscle tissues (M) and in the gap region (B). The mRNA expression is normalized to L32 and is expressed as a fold change relative to the expression value in the corresponding tissue (bone or muscle) at day 0 (control, no surgery), i.e., $2^{-\Delta\Delta CT(\text{day } 0)} = 1$. All values are representative of three to four replicates from two pools of mRNAs. Error bars are the S.D. of each set of measurements

Figure 7. Effect of BMP2 on Expression of VEGF Ligands and VEGFR in MSCs
mRNA expression was profiled over the time course of in vitro osteogenic differentiation. Left panels in the figure present the steady-state mRNA expression levels of endogenous BMP2 and of the primary transcription factors that control osteogenesis. Data are presented as a relative change of expression compared to day 6, when the cultures were switched to osteogenic media. Middle panel shows the effect of BMP2 treatment on three specific VEGF ligands while the right panel shows the three VEGFR receptors. Mean values are measurements made from three separate measurements. Error bars represent standard deviation from the replicates measurements from three experiments.