Endothelial Cells Are the Primary Cells that Express BMP2 during Distraction Osteogenesis

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INTRODUCTION:
Distraction osteogenesis (DO) is a surgical procedure by which new bone is induced through the application of gradual mechanical force across an osteotomy. Many studies have demonstrated that angiogenesis is essential for successful induction of new bone formation during DO. Other studies have definitively shown that BMP2 is a central and necessary regulator during postnatal bone formation and fracture healing. While it has been generally assumed that cells within the mesenchymal lineages that give rise to osteogenic and chondrogenic lineages express BMP2 during postnatal bone growth and healing, the cellular origins of BMP2 during these processes has not been fully defined. In the current study transgenic mice containing a BAC transgene carrying ~180Kb of the loci encompassing the BMP2 gene into which the β-galactosidase gene had been inserted were used to identify the cellular origins of BMP2 expression across the time course of DO.

METHODS:
All animal research was performed under an approved IACUC protocol. All research was performed on male (9–12 wk old) mice. BMP2 β-galactosidase mice were kindly provided by Dr. Motolock (1) and bred on site. Control C57B6 mice were purchased from Jackson Laboratories. Five time points were assayed for the DO studies: 7 days post operative day POD (end of latency); POD 10 (3 days of DO); POD 17 (10 days DO) the last day of distraction; POD 20 (3 days consolidation); and POD 31, (14 days consolidation) the end of the experiment. Two types of controls were used. No surgery or osteotomy no distraction. The surgery was performed as previously described accept with application of the device on the femur instead of the tibia (2).

RNA isolation and real-time qRT-PCR
RNA was separately isolated from the site of the distraction in the gap, and from the surrounding muscle from the operated leg (2). Real-time qRT-PCR was performed for BMP2, BSP, Osterix, and Runx2 as indices of bone formation and for specific angiogenic markers VEGFA-D, PIGF, and VEGF receptor 1, 2, 3. The values were normalized to the value at day 3.2 for each time point. The fold change in mRNA expression for each time point was plotted in a graph using unoperated bone as a reference.

Histology and immunohistochemistry
The operated legs were harvested and fixed, and β-galactosidase activity was detected by soaking the intact tissue in X-gal followed by decalcification and standard methods of embedding in paraffin and thin sectioning. Vessel formation was evaluated by immunohistochemistry staining for VEGFR2, PECAM (CD31), smooth muscle α-actin and the lectin binding of Griffonia simplicifolia.

Assessments of bone and vascular formation by µCT
After euthanasia the vasculature was injected with a radiopaque silicone rubber compound containing lead chromate and contrast MicroCT was performed as previously described (2).

RESULTS:
Real-time qRT-PCR
BMP2 mRNA was first examined in both the gap tissues at the osteotomy site and in the surrounding muscle around the bone. Two peaks of expression were observed in both the bone at d10 and d20, and in muscle at d7 and d20 (Fig. 1). Surprisingly BMP2 had certain amount of expression in muscle, although BMP2 is believed to be predominantly expressed in skeletal tissues. Other bone markers were induced throughout the DO period with almost no expression in the muscle. The expression of all four VEGFs except VEGF-C plus PIGF mRNA was induced throughout the period in bone. In muscle, VEGF-A and B were the predominant angiogenic ligands, while in the bone PIGF had high expression and was not expressed only in muscle. The ligands receptor mRNA expression had two peaks in bone at d10 and d20, and in muscle the highest expressions are at d31.

Histology and immunohistochemistry
The positive cells for LacZ were seen at the chondrocytes of the knee and hip joints, with low levels of expression in small vessels in muscle near joints in control (no surgery). At d20 of DO, many endothelial cells in the gap, and muscle around gap, were positive (Fig. 2a,b). The closer to the gap, the smaller the vessels were and the more positive cells that were observed which were expressing BMP2. Upper front of lining cells and chondrocyte in the gap were positive (Fig.2c). At d31 of DO, after remodeling of the gap has been initiated, very few positive cells were seen in bone. In osteotomy, no distraction controls at day 20 and day 31 had similar patterns of BM2 expression that were observed. Immunohistochemistry for vasculature specific surface markers validated that vascular endothelia cells were expressing BMP2.

Assessments of bone and vascular formation by µCT
Micro CT showed vessel formation increased from day 20 in DO model. New vessels were created first in surrounding muscles, then bone in the gap in DO. Most of newly formed vessels were observed in the surrounding muscles rather than bone in the gap in DO. DO had extensively more angiogenesis than osteotomy alone.

DISCUSSION
We show that cellular origin of BMP2 production in vivo for the first time is extensively associated with vascular tissue at the site of bone repair. It is interesting to note that endothelial cells of mainly small arteries produced BMP2. This fact is supported by our result of LacZ histology and real-time qRT-PCR that BMP2 expression exists not only in bone, but soft tissue. It is reported that EPCs and MSCs circulate in bone repair and BMP2 can promote angiogenesis by enhancing the recruitment of EPCs, and plays an important role in the regulation of differentiation and functions of MSCs. From these facts, it is speculated that BMP2 produced by endothelial cells promotes EPCs and MSCs recruitment and their differentiation, which leads to the new vessel formation and subsequent bone formation. Our micro CT result showed neovascularization preceded osteogenesis and most new vessels were first formed in muscle in DO. This suggests that angiogenesis in surrounding tissue is crucial for bone repair because the vessels supply various growth factor including BMP2 and progenitor/stem cells. Therefore it is important to consider the extent of soft tissue damage in the treatment of bone repair. In conclusion, we elucidated cellular origin and possible role of BMP2 in the crosstalk between angiogenesis and osteogenesis.

Fig.1 : BMP2 expression

Fig.2a-c : day 20 DO
2) Jacobsen et al., JBMR. 2008; 23(5):596-609