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Stress Fracture Healing: Fatigue Loading of the Rat Ulna Induces Upregulation in Expression of Osteogenic and Angiogenic Genes that Mimic the Intramembranous Portion of Fracture Repair

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Abstract

Woven bone is formed in response to fatigue-induced stress fractures and is associated with increased local angiogenesis. The molecular mechanisms that regulate this woven bone formation are unknown. Our objective was to measure the temporal and spatial expression of osteo- and angiogenic genes in woven bone formation in response to increasing levels of fatigue-induced damage. We used the rat forelimb compression model to produce four discrete levels of fatigue damage in the right ulna of 115 male Fischer rats. Rats were killed at 0 (1 hr), 1, 3 and 7 days after loading. Using qRT-PCR, we quantified gene expression associated with osteogenesis (BMP2, *Msx2*, *Runx2*, *Osx*, *BSP*, *Osc*), cell proliferation (*Hist4*), and angiogenesis (VEGF, PECAM-1) from the central half of the ulna. The spatial distribution of BMP2, *BSP* and *PCNA* was assessed by immunohistochemistry or *in situ* hybridization in transverse histological sections 1, 4, and 7 mm distal to the ulnar mid-diaphysis. One hour after loading, BMP2 was significantly upregulated in neurovascular structures in the medial ulnar periosteum. Expression of angiogenic markers (VEGF, PECAM-1) increased significantly between Day 0 and 1 and, as with BMP2 expression, remained upregulated through Day 7. While *Osx* and *BSP* were upregulated on Day 1, the other osteogenic genes (*Msx2*, *Runx2*, *Osx*, *BSP* and *Osc*) were induced on Day 3 in association with the initiation of periosteal woven bone formation and continued through Day 7. The magnitude of osteogenic gene expression, particularly matrix genes (*BSP*, *Osc*) was significantly proportional the level of fatigue damage. The woven bone response to fatigue injury is remarkably similar to the “intramembranous” portion of fracture repair — rapid formation of periosteal woven bone characterized by early BMP2 expression, cell proliferation, and upregulation of osteogenic genes. We speculate that woven bone repair of fatigue damage may be an abbreviated fracture response without the requirement for endochondral repair. We conclude that bone fatigue repair is a process similar to intramembranous fracture repair characterized by increases in the expression of genes associated with angiogenesis, cell proliferation and osteoblastogenesis, and that the response from the local vasculature precedes the osteogenic response to fatigue loading.

Keywords

rat ulna; woven bone; osteogenesis; angiogenesis; gene expression

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INTRODUCTION

Woven bone forms during fracture healing, distraction osteogenesis and in response to stress fractures engendered by bone fatigue [1,2]. Stress fractures occur commonly in people who engage in rigorous physical activities, such as military recruits and runners [3]. While a number of studies have explored the changes in gene expression associated with fracture healing [4–9] and distraction osteogenesis [10–12], the molecular events that occur during woven bone formation in response to fatigue injury are unknown.

Fatigue loading of the rat ulna by cyclic compression of the forelimb results in the formation of stress fractures [13–15] and leads to a repair response characterized by rapid, non-endochondral woven bone formation [16]. We recently developed a protocol whereby the level of ulnar fatigue damage can be modulated [13]. Using this model we observed that the amount of woven bone formed [16] and the magnitude of associated vascular changes [17,18] are proportional to the level of fatigue damage. There is also a strong spatial correspondence between the formation of woven bone and the location of fatigue-induced bone damage. Fatigue cracks form within the medial ulnar cortex [14,15] and are centered near the mid-diaphysis [13]. Maximal woven bone formation occurs at the same location, and diminishes equally in the proximal and distal directions [16,19].

In the “intramembranous” period of fracture repair, within the first seven days after fracture, there is a similar symmetric formation of woven bone on either side of the fracture site [20]. Contributions to the fracture repair process are thought to derive from four main areas: the periosteum, the original bone cortex, external soft tissues, and the bone marrow, with much of the chondrogenic response derived from the marrow component and surrounding soft tissues [20]. The early intramembranous response is also thought to be initiated by endothelial cells within the bone marrow that take on an osteoblastic phenotype within 24 hours after fracture [20,21]. Periosteal stress fractures resulting from fatigue damage do not typically involve direct contact with the endosteum. Nevertheless, the woven bone response to fatigue damage and the intramembranous portion of fracture repair share morphological and temporal similarities.

The formation of new blood vessels (vasculogenesis or angiogenesis) plays an important role during osteogenesis and bone healing [22]. In fracture repair [23,24] and distraction osteogenesis [11,25,26], vascular changes precede bone formation. In the rat ulnar fatigue model, the spatial pattern of maximal vascular changes along the ulna is identical to the pattern of woven bone formation [18]. Moreover, we observed increases in vessel size in the periosteum on day 1 after fatigue loading [17] and measured significant increases in vessel size and number on day 3 [18], whereas significant increases in woven bone area were not evident until after day 3 [17,18]. We therefore expect that the upregulation of genes associated with angiogenesis precedes the upregulation of genes associated with osteogenesis following fatigue loading.

Our objective was to extend prior histological descriptions of stress fracture healing by characterizing the temporal-spatial pattern of the expression of bone formation and angiogenic genes. We used quantitative real-time polymerase chain reaction (qPCR) along with immunohistochemistry and in situ hybridization to assess gene expression following fatigue loading and creation of an ulnar stress fracture in rats.

Materials and Methods

Experimental Design

Male Fischer/NHsd rats (N=115; 4.5–5.5 months old; 339 ± 29 g; Harlan) were assigned randomly to one of four loading groups according to the level of imposed fatigue damage and one of four survival time points. As described below, right forelimbs were loaded cyclically to a prescribed displacement level corresponding to 30, 45, 65 or 85% of the average displacement to fracture (2.0 ± 0.2 mm). We have shown previously that these displacement levels correspond to four discrete levels of ulnar damage, with increasing size and extent of cracks in the medial ulnar cortex at the mid-diaphysis, and progressive reductions in whole-bone strength [13]. Left forelimbs were not loaded and were used as controls. Rats were killed 0 (1 hr), 1, 3, or 7 days after loading. Eighty-seven rats were loaded for quantification of gene expression by real time polymerase chain reaction. Samples from seven rats were not used because of technical problems during RNA extraction or sample processing for qRT-PCR. Thus, we analyzed RNA from 80 rats ($n = 5$ per group). Samples from an additional 28 rats were used for immunohistochemistry and in situ hybridization to localize the expression of selected genes (or their proteins) at two levels of displacement (30% and 65%; $n = 2$ –4 per group). All procedures were approved by our institutional Animal Studies Committee.

In Vivo Mechanical Loading

The fatigue loading protocol has been described in detail recently [13]. Briefly, with the animals anesthetized (1–3% isoflurane), right forelimbs were positioned in a loading fixture connected to a servohydraulic materials testing machine (Instron 1331/8500R) and a 0.3 N compressive pre-load applied. The forelimbs were cyclically loaded using a 2 Hz haversine waveform to a peak compressive force of 0.055 N/g BW (average 18.7 N) until the actuator reached the prescribed increase in axial displacement (0.6, 0.9, 1.3 or 1.7 mm). The average number of loading cycles ranged from 1477 to 6236 (Table 1). After loading the animals received analgesia (i.m., 0.05 mg/kg buprenorphine) and were returned to their cages. All animals recovered well from anesthesia and mechanical loading. They were allowed normal cage activity and ad libitum access to water and chow. At 1 hr, 1 day, 3 days, or 7 days after loading the rats were killed individually by carbon dioxide asphyxia.

qRT-PCR

Immediately *post mortem*, right and left ulnae were dissected (periosteum intact) and rapidly frozen in liquid nitrogen. The ends of each ulna were removed and the central half stored (-80° C) until analysis by quantitative reverse transcription–real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using standard protocols [27]. Each piece of ulna was pulverized to powder in a liquid nitrogen-cooled stainless steel shaking flask (B. Braun Biotech Inc.). TRIzol reagent (1 mL, Invitrogen) was added to the sample and allowed to thaw. The mixture was transferred to a polypropylene tube and incubated (room temperature, 5 min) on a nutator. Chloroform (0.2 mL, Sigma) was added, mixed vigorously, and the mixture was transferred to a 2 mL Phase Lock Gel tube (PLG-heavy, Eppendorf) and centrifuged (4° C, 5 min). The nucleic acid phase was decanted and an equal volume of RNase-free 70% ethanol was added. Total RNA was isolated from the aqueous ethanol mixture (RNeasy Mini Kit, QIAGEN). Potential DNA contamination was removed by RNase-free DNase (QIAGEN). RNA integrity was verified by RNA gel electrophoresis and spectrophotometry.

Nine genes of interest (Table 2) were selected representing anabolic processes associated with osteogenesis and/or angiogenesis. For each gene, rat specific primers were designed (Integrated DNA Technologies, <http://scitools.idtdna.com/Primerquest/>) for real time PCR around exon junctions where possible. To create standards for the real-time PCR primers, larger oligonucleotide sequences were primed from rat bone RNA, sequenced, and cloned. All real-

time primers were initially tested against standards and a standard curve was generated. Standards (1 pg) were included in duplicate with all subsequent experimental reactions.

First strand cDNA was synthesized (M-MLV RT, Invitrogen) from total RNA (1 µg). qRT-PCR reactions were carried out at 50 µL total volume. Following an initial denaturing step of 95°C for 10 min, genes of interest were amplified through 40 cycles of denaturing (95°C, 15 sec) and annealing/elongation (60°C, 40 sec) (7300 Real-Time PCR System, Applied Biosystems). Gene amplification was measured by SYBR green (Applied Biosystems) fluorescence during the annealing/elongation phase. All samples were run in duplicate and the average was used for further analysis.

Measures of real-time PCR cycle to threshold were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each ulna. Within each group, the GAPDH-normalized gene expression for the loaded ulnae was compared to contralateral non-loaded control ulnae (paired t-test, $p < 0.05$). For comparison between experimental groups, GAPDH-normalized expression from each loaded ulna was divided by the normalized gene expression from the non-loaded contralateral control to obtain a fold increase in gene expression of loaded over non-loaded for each animal. Two-way ANOVA was used to assess the effect of displacement level and time. Post hoc, multiple comparisons were made using Fisher's protected least significant difference tests with statistical significance defined as $p < 0.05$.

Histology — In Situ Hybridization and Immunohistochemistry

Of the nine genes of interest assayed by qRT-PCR, three (BMP2, PCNA/Hist4, and BSP) were selected for histological characterization. In situ hybridization and immunohistochemistry protocols were performed as described [28,29]. Immediately post mortem, the forelimbs (ulna and radius with some muscle tissue left intact) were dissected, fixed overnight in 4% paraformaldehyde at room temperature and then decalcified in 14% EDTA. All solutions were nuclease-free to maintain RNA integrity. Specimens were embedded in paraffin and 5 µm sections were cut at three locations distal to the midpoint of the ulna [Figure 1]: Dist1 (1 mm distal to the midpoint), Dist4 (4 mm distal), and Dist7 (7 mm distal). The Dist1 location experiences the greatest woven bone formation in response to fatigue injury while Dist7 shows little or no woven bone response [16,18].

Primary antibody for BMP2 (Santa Cruz #sc-6895) was used to localize the protein at a dilution of 1:100. Sections were deparaffinized and rehydrated. Antigen retrieval was performed using a decloaking chamber (Biocare Medical). Slides were incubated (95°C, 10 min) in coplin jars containing 10 ml Sodium Citrate buffer (pH 6.0). Specimens were cooled (room temperature, 20 min) then washed in dH₂O, treated with H₂O₂ (10 min), and washed in PBS. Sections were blocked with 10% of the appropriate serum (room temperature, 1 hr) and then incubated overnight (4°C) with the optimized dilution of the primary antibody. The next day, slides were washed, incubated with a 1:500 dilution of Streptavidin (Sigma), washed, incubated with 1:200 of secondary antibody, washed, then visualized using 3,3'-diaminobenzidine (DAB) for a predetermined time. Slides were dehydrated, coverslipped, counterstained lightly with Mayer's hematoxylin, and examined under light field microscopy. Proliferating cell nuclear antigen (PCNA) immunohistochemistry was done according to the manufacturer's protocol (Zymed) to measure cell proliferation. The antibody was visualized with DAB after exposure for 5 minutes. Slides were then dehydrated and coverslipped.

For in situ hybridization, the BSP sequence generated as a PCR standard was used as a probe [Table 2]. The probe was cloned in the pGEM T-easy vector in DH5α *E. coli*, and then re-sequenced to verify the orientation and identity. Sense and Antisense probes were linearized and labeled by *in vitro* transcription in the presence of [³⁵S] UTP (PerkinElmer). After the

generation of the probes, paraffin sections were rehydrated, post-fixed in 4% paraformaldehyde, digested with proteinase K (10µg/ml), acetylated, and dehydrated. Sections were hybridized with a diluted probe in a hybridization buffer in a humidified chamber (55°C, 16 hr). Sections were then washed, treated with RNase A (10µg/ml), and dehydrated. Slides were exposed to ISO-MAX x-ray film (Scimart) for 3 days and developed to verify the efficiency of the hybridization. Specimens were then processed for standard autoradiography in a dark room. Slides were dipped in Ilford K5 emulsion (Polysciences), diluted 1:1 with 2% glycerol, dried at room temperature overnight, and stored at 4°C for the appropriate incubation time. Slides were incubated in a black slide box, double-wrapped in foil, and stored to prevent any exposure to radioactivity, and then developed (Kodak D-19 developer) and fixed (Kodak). To visualize histological features, all sections were stained with 0.1% toluidine blue, dehydrated, and coverslipped. Analysis was carried out using both light- and dark-field optics on an Olympus BX51 microscope.

RESULTS

Day 0: BMP2 is rapidly upregulated in extra-osseous structures of fatigue-loaded ulnae

Of the nine genes reflecting osteogenic-angiogenic coupling potential in this model, only *BMP2* was consistently and significantly upregulated at all time points with all extents of loading. At Day 0, *BMP2* expression was significantly elevated, up by an average of 3-fold in loaded compared to non-loaded control ulnae in all displacement groups [Table 3]. *VEGF* and *PECAM-1* were also upregulated in loaded versus control ulnae in the 45, 65, and 85% groups. Other genes did not show a significant change, and there was no significant effect of displacement level on gene expression.

There was evidence of an early increase in BMP2 staining in non-osseous structures in the medial periosteum as soon as 1 hr after loading. Histological sections at Dist1 (1 mm distal to the mid-diaphysis) showed cracking in the medial half of the ulnar cortex in both the 30% and 65% displacement groups. In the loaded ulnae there was positive BMP2 staining of extra-osseous structures on the medial periosteal surface, most prominently at Dist4, whereas these structures were not BMP2-positive in non-loaded controls [Figure 2a,b]. Histoanatomically these appear to be neurovascular bundles. Importantly, none of the other osteogenic markers studied – including the osteoblast transcription factors (*Msx2*, *Runx2*, and *Osx*) – were significantly induced at any degree of loading at this immediate early point. Osteoblast matrix proteins and proliferation markers were also not altered.

Day 1: Periosteal angiogenic responses follow fatigue load-induced BMP2 induction

One day after loading, an early repair process was initiated in loaded ulnae as evident by cell proliferation, early osteogenesis, and vascular changes. As on Day 0, BMP2 expression was significantly elevated in loaded ulnae from all displacement groups versus controls [Table 3]. The cell proliferation marker, histone-H4 (*Hist4*) was upregulated, and there was now consistent and significant upregulation of the angiogenic genes *VEGF* and *PECAM-1* – a response that persisted throughout the remainder of the time-course. An early osteogenic response was also evident, with significant upregulation of the BMP2 target gene, *Osx*, as compared to non-loaded ulnae. Similarly, expression of the matrix protein gene *BSP* – a critical stimulus for osteogenic differentiation and mineralization [30] – was induced by loading at all extents. The expression of both *Osx* and *BSP* displayed a “dose response” with proportionally greater expression in ulnae subject to greater fatigue damage. The remaining genes — *Hist4*, *Msx2*, and *Runx2* — were induced to a level that consistently reached significance only at the 65% loading displacement. Expression of the late osteoblast phenotypic marker, *Osc*, did not significantly differ between loaded and non-loaded ulnae on day one.

Histologically, the periosteum on the medial surface of loaded ulnae was thickened in the area of Dist1 and Dist4 [Figure 3a], but not Dist7 (furthest removed from the site of crack formation [13]). Blood vessels in the thickened medial periosteum were enlarged and were positive for BMP2 [Figure 2c, d]. There was also some positive BMP2 staining of cells lining the medial surface of the ulnae at Dist1 and Dist4 [Figure 3b]. The increase in cell proliferation was evident through PCNA staining of cells in the thickened periosteum primarily on the medial aspect of loaded ulnae at Dist1 and Dist4 [Figure 3c]. BSP expression [Figure 3d] was restricted to cells on the periosteal surface of loaded ulnae, both medially and laterally. Unlike the localization of BMP2 and PCNA only at Dist1 and Dist4, BSP expression extended into segment Dist7.

Day 3: Osteogenic gene expression is robustly upregulated in fatigue-loaded bone

On Day 3 there was clear evidence of osteogenesis both molecularly and histologically. All three of the critical osteoblast transcriptional regulators studied (*Msx2*, *Runx2*, and *Osx*) were significantly upregulated in loaded versus control ulnae [Table 3]. BMP2, BSP, and angiogenic markers (VEGF, PECAM-1) were again uniformly elevated in loaded versus non-loaded bone, and cell proliferation (Hist4+) was readily detected. As on Day 1, the expression of *Osx* and BSP was proportionally greater in ulnae subject to greater fatigue damage. The late-stage osteoblast phenotypic marker, *Osc*, was also upregulated in loaded vs. non-loaded ulnae. Histologically, the periosteum was dramatically thicker on the medial aspect of loaded ulnae at Dist1 and Dist4, but not Dist7 [Figure 4a]. There was also a notable difference in periosteal thickness and woven bone extent between the displacement groups; thickness in the 30% group (~150 μ m) was half that of the 65% group (~300 μ m). In loaded ulnae, BMP2 immunostaining was still positive in extra-osseous structures [Figure 2e, f] — and now additionally observed in osteoblast-like cells within the new woven bone forming at Dist1 and Dist4 [Figure 4b]. As predicted by the increased Hist4 mRNA, fatigue loading induced a sharp increase in PCNA positive cells throughout the periosteum at Dist1 and Dist4; these proliferating cells localized to the outer periosteal layer and cells juxtaposed to the new woven bone buds [Figure 4c]. The expression of BSP varied along the bone length corresponding with the amount of new woven bone, greatest at Dist1 in osteoblastic cells with the newly forming woven bone [Figure 4d], and less so at Dist4. Periosteal BSP expression was also detectable at low levels at Dist7.

Day 7: The exuberant proliferative responses begin to decline in the periosteum as woven bone formation progresses

On day 7, the woven bone reached its greatest extent and osteogenesis continued actively in all displacement groups, with evidence of a clear loading dose-response. BMP2 expression remained significantly upregulated in all displacement groups [Table 3]. Hist4 expression returned to control levels in all groups except the 85% displacement group, indicating reduced cell proliferation between Day 3 and 7. *Msx2*, *Runx2*, *Osx*, BSP and *Osc* continued to be significantly upregulated compared to control in most or all displacement groups, indicating persistent osteoblast differentiation and bone formation. While gene expression in the two higher displacement groups was maintained or increased between Day 3 and Day 7, in the 45% displacement group there was a significant drop in the expression of several genes. As a result, the two higher displacement groups (65, 85%) had significantly elevated expression of BMP2, VEGF, PECAM-1, *Runx2*, *Osx*, BSP and *Osc* compared the two lower groups (30, 45%).

Histologically, porous woven bone occupied the entire space that had been fibrous tissue on Day 3, and the periosteum at the outer surface of the woven bone was no longer “thickened” [Figure 5]. In comparison to the 30% group, the amount of woven bone area in the 65% group was greater but the woven bone was more porous. BMP2 staining was positive in osteocytes and osteoblasts within pores of the woven bone [Figure 5a]. PCNA staining was localized to only a few cells within the woven bone pores in sections of Dist1 and Dist4 [Figure

5b). BSP was localized to osteoblasts in pores of the woven bone [Figure 5c]; greatest at Dist1 where there was still active bone formation.

The temporal pattern of induction is different between growth factors, angiogenic genes, and osteogenic genes in fatigue-loaded ulnae

The temporal pattern of expression after fatigue loading was different for growth factors, angiogenic genes, and osteogenic genes. The expression of VEGF was significantly upregulated in the 45 and 85% displacement groups in comparison to non-loaded controls 1 hr after loading, while PECAM-1 was significantly upregulated in the 45 and 65% groups [Table 3]. The expression of VEGF and PECAM-1 increased significantly between Day 0 and 1, but did not change at subsequent time points [Figure 5]. The temporal pattern of BMP2 expression more closely matched that of the angiogenic genes than the osteogenic genes; BMP2 expression was significantly upregulated at 1 hr and did not change significantly over time. In contrast, there was no upregulation in osteogenic markers on Day 0. From Day 0 to 1, expression of *Osx* and *BSP* in loaded ulnae increased significantly, but *Osc* expression did not increase. From Day 1 to 3, while expression of the angiogenic genes remained unchanged, there was a significant increase in expression for *Osx*, *BSP* and *Osc*. *BSP* expression peaked on Day 3 and tended to decline on Day 7, but *Osc* expression increased significantly between Day 3 and Day 7.

DISCUSSION

Our objective was to characterize the effects of increasing fatigue damage on the expression of osteogenic and angiogenic genes using an established model of fatigue loading that creates a rat ulnar stress fracture [14,15]. We recently documented that our loading protocol induces woven bone formation at the ulnar mid-diaphysis in proportion to the level of fatigue damage, leading to recovery of ulnar strength two weeks after loading [16]. While a number of studies have explored molecular responses associated with fracture repair [6,7] and distraction osteogenesis [10–12], to date we are aware of no studies that examined gene expression related to woven bone formation after a stress fracture. Our results demonstrate that stress fracture repair is characterized by early response from growth factors and the vasculature followed by a response of cell proliferation and osteogenesis that increases significantly with the temporal requirements for tissue repair. We observed that as early as 1 hour after loading BMP2 was upregulated and localized to neurovascular structures at the medial periosteal surface, and genes associated with angiogenesis (VEGF, PECAM-1) were also significantly upregulated, whereas osteogenic-related genes (*Osx*, *Runx2*, *BSP*, *Osc*) were not upregulated until 1 – 3 days after fatigue. We also observed that the expression of genes supporting the repair response was localized primarily to the region of greatest bone formation near to the mid-diaphysis. Similar to our previous observation that the magnitude of the tissue-level response correlates with the magnitude of bone damage [16], our current results indicate that there is a proportional increase in the magnitude of the osteogenic molecular response to increasing damage. However, the effect of damage level was much less apparent in the expression of the osteogenic growth factor, BMP2.

The woven bone response to fatigue injury is remarkably similar to the “intramembranous” portion of fracture repair in terms of both histological features and patterns of gene expression [4,7–9,20]. Similar to the process described in the current study, the intramembranous portion of fracture repair features a rapid thickening of the periosteum followed by the formation of woven bone symmetrically within a few millimeters proximal and distal to the fracture site [7,20]. One day after fracture, there is an upregulation of BMP2 [5] and cells in the thickening periosteum are PCNA-positive [8]. On day 3 after fracture, cell proliferation in the intramembranous portion of the repair reaches its peak [4] and periosteal woven bone is

initiated with in situ measures of Osc detectable in the new matrix on day 4 [8]. On day 7 the processes of fatigue damage repair and fracture repair diverge as the endochondral portion of fracture healing becomes more prominent and cell proliferation shifts toward the fracture site [4,8]. Thus, the healing response to fatigue damage appears to be a similar process as the initial stages of fracture repair without the endochondral component.

BMP2 expression is prominent in the early stages of both the response to fatigue damage and fracture repair. In the mouse tibial fracture model, BMP2 and GDF8 are the only members of the transforming growth factor (TGF) super-family to upregulate one day after fracture [5]. Two days after fracture, BMP signal is localized to cells in the periosteum (no earlier time point has been reported) [9], and then, on day 6, the BMP signal is detected in cells of the newly formed woven bone [9]. Recently, Rosen and colleagues [31] reported that fracture repair is clearly abnormal in mice with conditional deletion of BMP2. Thus, similar to our fatigue model, BMP2 is involved early on in periosteal woven bone formation in fracture healing. Our observation that BMP2 was localized to neurovascular structures adjacent to the periosteum in loaded ulnae is consistent with several reports of BMPs in nerve. BMP signaling regulates sensory neuron number and spatial patterning in the developing face [32,33]. Notably, BMP2 expression has been localized in peripheral nerves and was increased after crush injury [34, 35]. There is also evidence for neural responses to mechanical loading. A recent study using the rat forelimb loading model reported that temporary nerve blockade led to a diminished bone formation response after loading and that loading led to altered neuropeptide levels [36]. Whether or not neural-derived BMP2 plays a role in loading-induced bone formation remains to be determined, but our findings suggest the possibility.

The temporal patterns of gene expression during stress fracture repair suggest functional roles for several genes in the repair process. The early upregulation of growth factors (BMP2, VEGF) indicates their roles in initiating the repair process via cell recruitment, proliferation, and differentiation. The subsequent upregulation on day 1 of Msx2, Runx2, and Osx is consistent with in vitro evidence that BMP2 regulates these osteogenic transcriptional regulators [37–39]. Interestingly, expression levels of Hist4 and the transcriptional regulators paralleled the histological pattern of progressively increasing cell proliferation and osteoblastogenesis, respectively, from day 1 and day 3, whereas BMP2 and VEGF expression remained relatively constant [Figure 6]. Notably, in a mouse tibial model of fracture repair [5], BMP2 expression peaked at 3.6–4 fold over basal expression at 1 and 20 days after fracture – similar to the level of expression (2.5–5 fold) we measured for the woven bone response in the rat ulna. Taken together, these findings support the notion that the magnitude of BMP2 expression is relatively consistent during bone healing, regardless of the repair process. In sharp contrast, the strong upregulation of matrix protein expression (BSP, Osc) was in sync with the timing of bone matrix formation (days 3–7) and showed a strong dose-response between loading groups.

Our findings point to a critical role for the vasculature in stress fracture repair, consistent with the documented role of angiogenesis in other bone formation scenarios. Angiogenesis plays an important role during skeletal development [40,41] and fracture healing [23,24]. Treadmill running in rats stimulates a VEGF-dependent increase in vessel formation and bone remodeling [42]; and the formation of non-endochondral bone during distraction osteogenesis is intimately coupled to angiogenesis [25,26]. The expression of the angiogenic genes (VEGF, PECAM-1) was upregulated early in the fatigue response, which may be a response to cyclic deformation (or damage) of the vasculature itself. Our observation of BMP2 localized to periosteal and intracortical vessels is consistent with findings that endothelial cells express BMP2 in response to mechanical stimulation [43,44] and hypoxia [45]. The expression of BMP2 in vessels may play a role in cell recruitment and lineage allocation. Pre-myogenic cells are capable of differentiating into chondrogenic or osteogenic cells when exposed to BMPs [7], and signaling of BMP2 initiating from the vasculature may attract circulating cells of osteoblastic lineage

[46]. During distraction osteogenesis, the spatial patterns of VEGF and BMP2 expression are tightly linked [12], and there is a temporal correspondence between BMP2 expression and the amount of vascularized connective tissue in the distraction gap during the first two weeks of distraction [47]. Moreover, inhibition of angiogenesis during distraction osteogenesis [11] and fracture repair [23] prevents bone formation and leads to fibrous non-union. While our findings suggest a similar dependence, it remains to be determined if angiogenesis is required for successful woven bone repair following bone fatigue.

A number of the genes of interest demonstrated a “dose response” and were upregulated in proportion to the level of induced fatigue damage in the 30–65% displacement groups. The increased gene expression, particularly for the matrix genes (BSP, Osc) is consistent with our previous findings that the amount of woven bone formed is proportional to the level of fatigue damage [16]. An unexpected finding was the relatively diminished osteogenic gene expression in the highest displacement group (85%). Expression patterns in the 85% group may be delayed by excessive damage and cracks in the ulnar cortex. Histology from Day 3 shows that woven bone formation is absent at locations where a cortical crack intersects the periosteum [Figure 4a]. In fracture repair, the magnitude of the endochondral response is related to the mechanical stability of the fracture site – greater motion leads to a greater cartilaginous response while greater stability limits the amount of endochondral repair [48]. Similarly, regions adjacent to the cracks from the greatest fatigue damage may experience micro-motion during healing that impairs direct osteogenesis, and rather results in a more “chondroid bone formation” [18]. Even in the 85% damage group, however, the response is different from fracture repair. There is no fracture callous, and beyond the small amount of chondroid tissue at the crack-periosteum interface, the process does not require chondrogenesis for successful repair. Rather, the loss of structural strength after fatigue loading is fully recovered, or exceeded by day 7 due to rapid woven bone formation [16]. Whereas in fracture repair, recovery of mechanical integrity requires calcification of the cartilaginous callous [20]. The woven bone response to fatigue injury may represent an abbreviated fracture response with pockets of chondroid tissue in the highest (85%) damage group representing a transition between the intramembranous repair response and endochondral repair response.

There are limitations to our study. First, the histological assays of osteogenic gene expression and the quantification of gene expression were performed on separate animals. We were unable to directly correlate the quantitative upregulation in gene expression with localized distribution. Nevertheless, the qualitative assessment of gene expression from histological sections corresponded well with the magnitude and temporal pattern of qRT-PCR results. Further, as a first characterization of the gene response in woven bone repair, we quantified expression of only nine genes, chosen for their known role in skeletal anabolism. However, woven bone repair to fatigue damage likely involves many genes associated with other pathways and functions including mechanotransduction, endochondral repair, and catabolism. Using the temporal pattern of gene expression from the current study as a guide, future studies will make use of microarrays to characterize the expression of other genes during the fatigue repair process. Lastly, Clemens and colleagues recently established the critical role for hypoxia-inducible factor (HIF)-1 α signaling in osteogenic-angiogenic coupling during bone formation [49]. We have not evaluated the spatial patterns of HIF-1 α protein accumulation in this model, and have not characterized whether periosteal hypoxia contributes to the osteogenic responses observed.

In summary, we have characterized the expression of osteogenic and angiogenic genes in the rat ulna following fatigue injury (stress fracture). The temporal and spatial pattern of osteogenic gene upregulation is similar to expression during the intramembranous portion of fracture repair. There is a significant increase in BMP2 expression as early as 1 hour after fatigue loading, independent of damage level and localized to extraosseous structures including

neurovascular bundles and periosteal vessels. While the upregulation of a number of the osteogenic genes is significantly influenced by the amount of fatigue damage, the expression of growth factors (BMP2, VEGF) is relatively independent of temporal requirements. We conclude that bone fatigue repair is a process similar to intramembranous fracture repair characterized by increases in the expression of genes associated with angiogenesis, cell proliferation and osteoblastogenesis, and that the response from the local vasculature precedes the osteogenic response to fatigue loading.

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Figure 1.

Low power photomicrographs of transverse histological sections of the rat ulna 1 day after loading (65% displacement level, stained with toluidine blue, scale bar = 500 μ m). Sections were taken from 1 mm (Dist1), 4 mm (Dist4), and 7 mm (Dist7) distal to the mid-diaphysis. In all sections the medial ulnar surface is toward the top (U = ulna, R = radius, M = muscle).

Figure 2.

High power photomicrographs (scale bar = 100 μ m) stained for immunohistochemical probe against BMP2. (a,b) Comparison of medial periosteum from (a) loaded ulna (65% of fracture) and (b) non-loaded ulna, both 1hr after loading. Neurovascular structures in the medial periosteum of loaded ulnae were positive for BMP2 (black arrows) while non-loaded control ulnae showed no BMP2 staining. (c,d) Comparison of BMP2-positive staining with negative control (no primary antibody) in sections from loaded (65% of fracture) ulna on day 1 after loading. (c) BMP2 positive vessels (black arrows) adjacent to medial periosteum. (d) No staining evident in same vessels (white arrows) in negative control. (e,f) Sections from loaded (65% of fracture) ulna, on day 3 after loading. (e) BMP2 positive vessel (black arrows)

traversing bone near radio-ulnar interosseous ligament. (f) No staining evident in same vessels (white arrows) in negative control (no primary antibody).

Figure 3.

Photomicrographs of transverse histological sections Day 1 after loading to 65% of fracture displacement. In all panels the medial ulnar surface is toward the top (M = muscle, B = bone, P = periosteum). (a) Low power images (stained with toluidine blue) showing thickened medial periosteum at Dist1, and Dist4, but not Dist7 in comparison to non-loaded control ulna. (b–d) High power photomicrographs (scale bar = 100 μ m) comparing loaded to non-loaded ulnae at Dist1. (b) Immunohistochemical staining for BMP2. Enlarged blood vessels (white arrows) populated the thickened periosteum on the medial cortex in loaded ulnae and, in addition to BMP2-positive vessels [Figure 2b], lining cells (black arrows) were positive for BMP2. (c) PCNA and (d) BSP (in situ hybridization) were localized to the periosteum in loaded ulnae (black arrows), but not in non-loaded control ulnae.

Figure 4.

Photomicrographs of transverse histological sections Day 3 after loading to 65% of failure displacement. In all panels the medial ulnar surface is toward the top (M = muscle, B = cortical bone, P = upper periosteum, W = woven bone). Sections from non-loaded control ulnae were similar to control sections at Day 1 [see Figure 3]. (a) Low power images (stained with toluidine blue with in situ hybridization probe for BSP). In section Dist1 the periosteum was dramatically thickened – mostly on the medial aspect; the upper two thirds [P] was composed of loose fibrovascular tissue, the lower third contained buds of woven bone [W]. At locations where a crack intersected the periosteum (*) there was no woven bone formation and no BSP expression. In section Dist4 the periosteum was not as thick as in Dist1; a narrow strip of woven

bone was adjacent to the original bone surface. In section Dist 7, the periosteum was not thickened, but some of the lining cells were positive for BSP signal. (b–d) High power photomicrographs (scale bar = 100 μ m). (b) Immunohistochemical staining for BMP2. In addition to BMP2-positive staining in periosteal vessels (white arrow, and Figure 2e), BMP2 was positive in numerous cuboidal, osteoblastic cells within the newly formed woven bone (black arrows). (c) PCNA was positive in numerous periosteal cells (white arrows) of Dist1, Dist4, and Dist7, and in cells within newly formed bone (black arrows) at Dist1 and Dist4. (d) BSP (black grains) was strongly localized in the newly forming woven bone at Dist1 and Dist4.

a – BMP2 **b – PCNA** **c – BSP** **Figure 5.**

Photomicrographs of transverse histological sections Day 7 after loading to 65% of failure displacement (scale bar = 100 μ m). In all panels the medial ulnar surface is toward the top (M = muscle, B = cortical bone, P = periosteum, W = woven bone). Sections from non-loaded control ulnae were similar to control sections at Day 1 [see Figure 3a]. (a–c) What was previously thickened periosteum at Day 3 was replaced by woven bone on Day 7 and there were no enlarged blood vessels in the thinner periosteal layer at the edge of the woven bone. (a) Immunohistochemical staining for BMP2 was positive in osteocytes (white arrows) and osteoblastic cells (black arrows) of the woven bone. (b) PCNA staining was not as strong as in previous time points and was limited primarily to the osteoblastic cells within pores of the

woven bone (black arrows). (c) BSP signal (in situ hybridization) was also localized to osteoblastic cells in the woven bone pores.

Figure 6.

Temporal changes in upregulation of gene expression for the 30 and 65% groups (mean $\pm$ SD fold increase of loaded over non-loaded control) for BMP2, VEGF, PECAM-1, Osx, BSP, and Osc. The dotted line in each plot indicates no change in expression (loaded/non-loaded = 1). BMP2 and PECAM-1 expression are up early (Day 0); VEGF is also up early in the 45% and 85% groups (not shown). After Day 1 BMP2, PECAM-1 and VEGF stay relatively constant. There is no upregulation in osteogenic gene expression until Day 1 and then there is a significant increase in osteogenic expression from Day 1 to Day 3. Osc expression continues to increase between Day 3 and Day 7 while BSP peaks on Day 3. * –Significant increase vs. non-loaded control ($p < 0.05$). ^ - 65% group significantly greater than 30% group for the specific time point.

Table 1

Number of in vivo loading cycles applied to the right forelimb

Fatigue Displacement (% of fracture displacement [2 mm] as determined previously[13])	Mean $\pm$ SD	Min	Max
30	1477 $\pm$ 1102	109	3869
45	6236 $\pm$ 7612	561	21600
65	5881 $\pm$ 3963	999	14809
85	3873 $\pm$ 3313	632	11186

Table 2

Genes of interest and supporting rationale.

Gene	Accession#	Primers for Standards	Real Time PCR Primers
BMP2	L20678	5' – 343 Clone Accession#: BF549588 3' – 901 ESTname: UI-R-C2-mt-h-03-0-UI.r1 Position: 343–901	5' – 379 CACCGTGCTCAGCTTCCA 3' – 447 CGGGAAGTTTCCCACCTCATT
BSP	AB001383	5' – 15 AATCTTGCTCTGCATTTTGG 3' – 771 ACTCAACCGTGCTGCTCTTT	5' – 118 CGGCCACGCTACTTTCTTTA 3' – 220 CCCTCCTCCTCCGAACATC
Hist4	NM_022686	5' – 17 ATGCTGGACGTGGTAAAGGTGGT 3' – 328 TTAACCAACCGAAGCCGTAGAGTGT	5' – 88 CGACAACATCCAGGGCATCACAAA 3' – 210 TTCTCCAGGAACACCTTCAGCACA
Msx-2	U12514	5' – 11 CGTCCAAAGGCGGTGACTTGT 3' – 747 GATGGGAAGCACAGGTCTATGGAA	5' – 283 CTCGTCAAGCCCTTCGAGACC 3' – 431 TTCCGGTTGGTCTTGTGTTCTCTC
Osc	M11777	5' – 9 Previously sequenced OC (bone gla protein - BGP)[29] 3' – 376	5' – 99 AAGCCCAGCGACTCTGAGTCT 3' – 173 CCGGAGTCTATTACACCTTACT
Osx	NM_181374	5' – 136 AAGCCATACACTGACCTTTCAGCC 3' – 1107 ATGTTTGCTCAAGTGGTCGCTTCG	5' – 345 TTCTGACTGCCTGCCTAGTGTCT 3' – 422 TGGATGCCCGCCTTGTA
PECAM-1	U77697	5' – 30 GTGCAAAGTGGAATCGAACC 3' – 620 ACCGGGGCTATTACCTTGAC	5' – 424 AATGGGACTGCACCCATCACTT 3' – 529 TGGTGGGCTTGTCTGTGAATGT
Runx2	XM_346016	5' – 529 ACCATGGTGGAGATCATCGC 3' – 934 CATCAAGCTTCTGTCTGTGC	5' – 697 ATGGCCGGGAATGATGAGAA 3' – 925 TCTGTCTGTGCCTTCTTGGT
VEGF-A	NM_031836	5' – 39 TTTACTGCTGTACCTCCACCATGC 3' – 636 TGGCTTGTACATCTGCAAGTACG	5' – 209 ATATCTTCAAGCCGTCCTGTGTGC 3' – 353 TCTCTATGTGCTGGCTTTGGTGA

Angiogenesis: **VEGF-A**, vascular endothelial growth factor-A: prototypic angiogenic and osteogenesis-angiogenesis coupling factor [40,50]; produced by endothelial cells and osteoblasts [51,52]. **PECAM-1**, platelet-endothelial cell adhesion molecule-1 (CD31): endothelial cell adhesion molecule essential to angiogenesis; endothelial cell marker [53]; putative mechanosensory gene [54]. Osteogenesis: **BMP-2**, bone morphogenetic protein 2: osteogenesis-angiogenesis coupling factor; osteoinductive factor [55]; responsive to mechanical stimuli [56]; synthesized by endothelial cells [45]; stimulates osteoblasts to produce VEGF [51]. Cell Proliferation: **Hist4**, histone H4: Cell cycle protein; marker for cell proliferation [57]. Osteoblast Transcriptional Regulators: **Msx2**, homeobox msh-like 2: Important for osteoprogenitor proliferation and differentiation [37,38]. **OSX**, Osterix: required for osteoblast formation [58], stimulated by BMP-2 [39]. **Runx2**, Runt related transcription factor 2 (Cbfa1, Osf2): transcription factor required for osteogenesis [59] and angiogenesis during skeletal development [60]. Osteoblast Differentiation Markers/Matrix Proteins: **OSC**, osteocalcin: matrix protein expressed by mineralizing osteoblasts and odontoblasts; marker for fully differentiated osteoblast [29]. **BSP**, bone sialoprotein: matrix glycoprotein mediates bone cell adhesion [61]; promotes angiogenesis in vitro [62]; enhances osteoblast differentiation and matrix mineralization [30].

Table 3

Fold change in gene expression of loaded compared to non-loaded ulnae by qRT-PCR (mean $\pm$ SD). Gene expression within each ulna was normalized to the expression of GAPDH before comparing loaded to non-loaded expression.

Day	Displ. [%]	N	BMP2	Hist4	VEGF	PECAM-1	Msx2	Runx2	Osx	BSP	Osc
0	30	4	2.4 $\pm$ 0.4 [*]	2.1 $\pm$ 1.2	1.1 $\pm$ 0.5	1.2 $\pm$ 0.2	1.8 $\pm$ 0.6	1.8 $\pm$ 1.1	1.4 $\pm$ 0.6	1.1 $\pm$ 0.1	1.3 $\pm$ 0.5
	45	5	3.3 $\pm$ 1.0 [*]	0.9 $\pm$ 0.4	1.9 $\pm$ 0.7 [*] _a	1.2 $\pm$ 0.2 [*]	1.1 $\pm$ 0.6	1.6 $\pm$ 0.5	1.8 $\pm$ 1.2	1.2 $\pm$ 0.3	0.8 $\pm$ 0.4
	65	5	3.8 $\pm$ 1.1 [*]	1.7 $\pm$ 1.6	1.4 $\pm$ 0.5	1.4 $\pm$ 0.4 [*]	2.2 $\pm$ 2.8	1.6 $\pm$ 1.1	1.5 $\pm$ 1.3	1.6 $\pm$ 0.8	1.6 $\pm$ 0.9
	85	5	2.7 $\pm$ 0.9 [*]	1.2 $\pm$ 0.8	1.6 $\pm$ 0.3 [*]	1.0 $\pm$ 0.4	1.0 $\pm$ 0.5	1.0 $\pm$ 0.6	1.1 $\pm$ 0.8	1.0 $\pm$ 0.4	0.8 $\pm$ 0.2
1	30	5	2.5 $\pm$ 1.0 [*]	2.3 $\pm$ 1.8	1.6 $\pm$ 0.3 [*]	1.9 $\pm$ 0.4 [*]	2.3 $\pm$ 1.1 [*]	2.0 $\pm$ 0.6 [*]	2.5 $\pm$ 0.9 [*]	2.7 $\pm$ 1.3 [*]	1.6 $\pm$ 0.7
	45	4	2.9 $\pm$ 1.0 [*]	3.1 $\pm$ 3.2	1.8 $\pm$ 0.4 [*]	2.4 $\pm$ 0.4 [*]	1.7 $\pm$ 1.3	2.3 $\pm$ 1.6	3.2 $\pm$ 1.4 [*]	4.1 $\pm$ 1.9 [*]	0.6 $\pm$ 0.1
	65	5	4.4 $\pm$ 1.2 [*] _{a,b,c}	5.8 $\pm$ 4.9 [*] _{a,b,c}	2.3 $\pm$ 0.6 [*]	3.2 $\pm$ 0.2 [*] _{a,b,c}	2.5 $\pm$ 1.6 [*]	3.0 $\pm$ 1.6 [*]	4.6 $\pm$ 2.6 [*]	7.9 $\pm$ 1.5 [*]	0.7 $\pm$ 0.3
	85	5	2.4 $\pm$ 0.8 [*]	2.4 $\pm$ 1.4	2.1 $\pm$ 0.9 [*]	2.2 $\pm$ 0.7 [*]	1.8 $\pm$ 1.2	1.5 $\pm$ 0.7	2.7 $\pm$ 0.7 [*]	4.8 $\pm$ 0.8 [*]	0.5 $\pm$ 0.4
3	30	5	2.5 $\pm$ 0.5 [*]	2.0 $\pm$ 0.7 [*]	1.8 $\pm$ 0.4 [*]	2.3 $\pm$ 0.4 [*]	1.8 $\pm$ 1.1	3.0 $\pm$ 1.1 [*]	4.8 $\pm$ 1.0 [*]	8.5 $\pm$ 2.4 [*]	3.0 $\pm$ 1.1 [*]
	45	5	3.2 $\pm$ 1.0 [*]	2.6 $\pm$ 1.3 [*]	2.2 $\pm$ 0.4 [*]	2.8 $\pm$ 0.8 [*]	3.1 $\pm$ 1.0 [*]	3.7 $\pm$ 1.4 [*]	9.7 $\pm$ 3.4 [*] _a	19.1 $\pm$ 11.4 [*] _a	3.5 $\pm$ 2.1 [*]
	65	4	3.4 $\pm$ 0.7 [*]	3.4 $\pm$ 0.9 [*]	3.3 $\pm$ 1.2 [*] _{a,b,c}	3.1 $\pm$ 0.4 [*] _a	4.6 $\pm$ 1.2 [*] _a	4.1 $\pm$ 0.6 [*]	8.3 $\pm$ 0.7 [*] _a	29.2 $\pm$ 8.1 [*] _{a,b,c}	2.6 $\pm$ 1.5 [*]
	85	4	2.4 $\pm$ 0.7 [*]	1.7 $\pm$ 0.6	2.1 $\pm$ 0.5 [*]	2.9 $\pm$ 0.1 [*]	5.0 $\pm$ 4.4 [*] _a	3.5 $\pm$ 1.3 [*] _a	7.7 $\pm$ 1.6 [*] _a	17.8 $\pm$ 3.7 [*] _a	2.0 $\pm$ 1.2
7	30	5	2.8 $\pm$ 0.5 [*]	1.6 $\pm$ 0.9	1.6 $\pm$ 0.2 [*]	1.8 $\pm$ 0.4 [*]	2.0 $\pm$ 0.7 [*]	2.5 $\pm$ 0.6 [*]	4.1 $\pm$ 1.2 [*]	5.7 $\pm$ 3.0 [*]	4.7 $\pm$ 1.8 [*]
	45	5	2.4 $\pm$ 0.9 [*]	0.9 $\pm$ 0.4	1.4 $\pm$ 0.5	1.9 $\pm$ 0.5 [*]	1.9 $\pm$ 1.5	2.2 $\pm$ 1.3	5.8 $\pm$ 2.9 [*]	5.4 $\pm$ 2.9 [*]	3.4 $\pm$ 1.8 [*]
	65	5	4.8 $\pm$ 1.8 [*] _{a,b}	2.0 $\pm$ 1.2	2.7 $\pm$ 0.7 [*] _{a,b}	3.5 $\pm$ 1.0 [*] _{a,b}	2.5 $\pm$ 0.7 [*]	5.9 $\pm$ 2.3 [*] _{a,b}	11.4 $\pm$ 3.6 [*] _{a,b}	23.2 $\pm$ 11.1 [*] _{a,b}	6.8 $\pm$ 3.3 [*] _b
	85	5	4.5 $\pm$ 2.4 [*] _{a,b}	1.9 $\pm$ 0.5 [*]	2.2 $\pm$ 0.6 [*]	3.3 $\pm$ 1.0 [*] _{a,b}	3.0 $\pm$ 1.0 [*]	5.9 $\pm$ 3.4 [*] _{a,b}	9.4 $\pm$ 5.9 [*] _{a,b}	16.5 $\pm$ 11.1 [*] _{a,b}	6.9 $\pm$ 4.7 [*] _b

* –Significant increase vs. non-loaded control ($p < 0.05$).

^a Different from 30% group at same time,

^b different from 45% group at same time,

^c different from 85% group at same time. Red shading – the gene of interest is significantly upregulated in all displacement groups vs. non-loaded control for the time point. Blue shading – the gene of interest is not upregulated for any displacement group vs. non-loaded control for the time point.