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## VEGFA from Osteoblasts Is Not Required for Lamellar Bone Formation Following Tibial Loading

Jennifer McKenzie<sup>1</sup>, Ian M. Galbreath<sup>1,2</sup>, Andre F. Coello<sup>1,3</sup>, Katherine R. Hixon<sup>1,4</sup>, Matthew J. Silva<sup>1,3</sup>

<sup>1</sup>Department of Orthopaedic Surgery and Musculoskeletal Research Center, Washington University in St. Louis, St. Louis, MO

<sup>2</sup>St. Louis University, St. Louis, MO

<sup>3</sup>Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, MO

<sup>4</sup>Dartmouth Engineering, Dartmouth College, Hanover, NH

### Abstract

The relationship between osteogenesis and angiogenesis is complex. Normal bone development requires angiogenesis, mediated by vascular endothelial growth factor A (VEGFA). Studies have demonstrated through systemic inhibition or genetic modification that VEGFA is indispensable for several types of bone repair, presumably via its role in supporting angiogenesis. But a direct role for VEGFA within osteoblasts, in the absence of angiogenesis, has also been suggested. To address the question of whether VEGFA from osteoblasts supports bone formation directly, we applied anabolic loading to induce lamellar bone formation in mice, a process shown to be independent of angiogenesis. We hypothesized that VEGFA from osteoblasts is required for lamellar bone formation. To test this hypothesis, we applied axial tibial compression to inducible Cre/LoxP mice from three lines. Vegfa<sup>fl/fl</sup> mice were crossed with Ubiquitin C (UBC), Osterix (Osx) and Dentin-Matrix Protein 1 (DMP1) Cre-ERT2 mice to target all cells, (pre)osteoblast-lineage cells, and mature osteoblasts and osteocytes, respectively. Genotype effects were determined by comparing control (Vegfa<sup>fl/fl</sup>) and Cre+ (Vegfa<sup>fl/fl</sup>) mice for each line. At 5 months of age tamoxifen was injected for 5 days followed by a 3-week clearance prior to loading. Female and male mice (N = 100) were loaded for 5 days to peak forces to engender ~3100  $\mu$ e peak compressive strain and processed for dynamic histomorphometry (day 12). Percent MS/BS increased 20–70% as a result of loading, with no effect of genotype in Osx or Dmp1 lines. In contrast, the UBC groups had a significant decrease in relative periosteal BFR/BS in Vegfa<sup>fl/fl</sup> vs. Vegfa<sup>fl/fl</sup> mice. The UBC line did not have any cortical bone phenotype in non-loaded femurs. In summary, dynamic histomorphometry data confirmed that tibial loading induces lamellar bone formation. Contrary to our hypothesis, there was no decrease in loading-induced bone formation in the Osx or Dmp1 lines in the absence of VEGFA. There was a decrease in bone formation in the UBC line where all

Correspondence to: Matthew Silva, silvam@wustl.edu.

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Appendix A. Supplementary Data

Supplementary Tables can be found in the excel file.

cells were targeted. This result indicates that VEGFA from a non-osteoblast cell source supports loading-induced lamellar bone formation, although osteoblast/osteocyte VEGFA is dispensable. These findings support a paracrine model whereby non-osteoblast VEGFA supports lamellar bone formation, independent of angiogenesis.

## 1. Introduction

Vascular endothelial growth factor A (VEGFA), produced by osteoblasts, leukocytes, and endothelium, is a key biological growth factor in angiogenesis and normal bone development (Hu and Olsen, 2016)(Neufeld et al., 1999)(Lee et al., 2007)(Street et al., 2002). A role for VEGFA in bone repair has also been demonstrated. Following full femur fracture, mice treated with a soluble VEGF receptor exhibited decreased angiogenesis, callus mineralization, and bone formation (Street et al., 2002). In the same study, the addition of VEGF improved healing of radius segmental defects in rabbits (Street et al., 2002). These results demonstrate that modulation of VEGF can alter bone repair outcomes.

More recent studies have used inducible Cre-LoxP mouse models to examine the role of VEGFA from different cell types in bone repair. Using a mouse tibial defect model, Hu et al. showed that both osteogenesis and angiogenesis was compromised when VEGFA was knocked out with *Osx* Cre (targeting the osteoblast lineage) but not with *VECAD* Cre (targeting vascular endothelial cells), indicating that VEGFA from osteoblasts is critical for defect repair (Hu and Olsen, 2016). They further showed that optimal levels of VEGFA are required for coupling of angiogenesis and osteogenesis during defect healing. Buettmann et al. evaluated healing of full femur fractures and ulnar stress fractures in mice carrying VEGFA floxed alleles (*Vegfa<sup>fl/fl</sup>*) crossed with tamoxifen inducible Cre-ERT2 lines driven by Ubiquitin C (*Vegfa<sup>UBC</sup>*), Osterix (*Vegfa<sup>Osx</sup>*), and Dentin matrix protein 1 (*Vegfa<sup>Dmp1</sup>*) (Buettmann et al., 2019). This allowed for global (UBC), early osteoblast lineage (*Osx*), or late osteoblast lineage (*Dmp1*) conditional deletion of VEGFA at 12-weeks of age. Following fracture, knockout mice had varying amounts of impaired healing that directly correlated to the broadness of Cre expression within the osteoblast lineage. Fractures in *Vegfa<sup>UBC</sup>* mice did not heal, while fractures in *Vegfa<sup>Osx</sup>* mice had moderately impaired healing and *Vegfa<sup>Dmp1</sup>* mice exhibited normal healing (Buettmann et al., 2019). Collectively, these findings support that VEGFA from early osteoblasts is crucial for several types of bone repair.

In studies of osteogenesis in the setting of bone injury and repair, it is difficult to decouple angiogenesis from osteogenesis because the two processes are so tightly linked (Brandi and Collin-Osdoby, 2006; Diomedea et al., 2020; Hu and Olsen, 2016; Maes et al., 2008; Matsuzaki et al., 2007). For example, Buettmann et al. used endomucin immunostaining to mark vessels, and found that vessel density in fracture callus of VEGFA knockout mice was reduced in proportion to the reduction in bone formation (Buettmann et al., 2019). Thus, it remains unclear if VEGFA from osteoblasts acts directly (autocrine or intracrine) to promote bone formation, or if it acts indirectly (paracrine) by promoting angiogenesis which in turn supports bone formation by secretion of other factors such as PDGF (Böhm et al., 2019). There is evidence supporting both possibilities (Hu and Olsen, 2016; Liu et al., 2012).

To address the question of whether VEGFA from osteoblasts supports bone formation independent of angiogenesis (i.e., a direct effect), we turned to a model of non-damaging, anabolic loading to induce lamellar bone formation in adult mice. We have previously reported that lamellar bone formation in this context is independent of angiogenesis (Tomlinson et al., 2014), which is in contrast to the vascular-dependent formation of intramembranous (woven) bone that occurs with bone injury and repair (Hausman et al., 2001; Tomlinson et al., 2013).

We hypothesized that VEGFA from osteoblasts is required for lamellar bone formation in adult mice. To test this hypothesis, we performed axial tibial loading in inducible Cre/LoxP mice (UBC, *Osx*, and *Dmp1* Cre-ERT2 drivers crossed with *Vegfa<sup>fl/fl</sup>* mice) and evaluated loading-induced bone formation in the absence of VEGFA broadly (*Vegfa<sup>UBC</sup>*), from (pre)osteoblast-lineage cells (*Vegfa<sup>Osx</sup>*), and from mature osteoblasts and osteocytes (*Vegfa<sup>Dmp1</sup>*). At 5 months of age, mice were given tamoxifen followed by loading and subsequent assessment of dynamic histomorphometry outcomes. We demonstrated that loading-induced lamellar bone formation was decreased in *Vegfa<sup>UBC</sup>* mice, but was not affected in *Vegfa<sup>Osx</sup>* mice or *Vegfa<sup>Dmp1</sup>* mice.

## 2. Methods

### 2.1. Study Design

Three separate tamoxifen inducible Cre-ERT2 mouse lines (UBC (Ruzankina et al., 2007), *Osx* (Maes et al., 2007), *Dmp1* (Kim et al., 2012)) were crossed to mice carrying floxed VEGFA (*Vegfa<sup>fl/fl</sup>*) alleles. Previous work with reporter versions of mouse lines confirmed targeting of the cell types of interest (Buettmann et al., 2019). UBC Cre-ERT2 mice (broad targeting of all cells) were purchased from Jackson Laboratories (catalog #007001, Bar Harbor, ME, USA). *Osx* Cre-ERT2 mice (targeting osteoblast lineage cells from pre-osteoblast through osteocyte) were shared from the laboratory of Dr. Henry Kronenberg (Mass General). *Dmp1* Cre-ERT2 mice (targeting late osteoblast lineage cells including osteocytes) were shared by Dr. Paola Pajevic (Boston University). *Vegfa<sup>fl/fl</sup>* mice were provided by Dr. Bjorn Olsen (Harvard) with permission from Genentech (South San Francisco, CA, USA). In all breeding pairs both male and female mice contained two floxed VEGFA alleles. Male breeders also had a single copy of either UBC-, *Osx*-, or *Dmp1*-CreERT2. Cre-positive (Cre+) and Cre-negative (Control) littermates were used for experiments. Throughout this paper, conditional knockout experimental groups are labeled *Vegfa<sup>UBC</sup>*, *Vegfa<sup>Osx</sup>*, and *Vegfa<sup>Dmp1</sup>*, while littermate controls are labeled *Vegfa<sup>fl/fl</sup>* or Control. Mice were group-housed (maximum five per cage) under a standard 12-hour light/dark cycle and given access to food (Purina LabDiet 5053; Purina) and water *ad libitum*. All mice at 5 months of age (male and female, N = 128) were given tamoxifen for 5 days (100 mg/kg/day PO; Sigma-Aldrich) for Cre activation followed by a 3-week clearance (Figure 1). Tamoxifen-induced recombination was confirmed by PCR of genomic DNA from tissues of interest. In brief, femurs from Cre+ mice of each line (*Vegfa<sup>UBC</sup>*, *Vegfa<sup>Osx</sup>*, and *Vegfa<sup>Dmp1</sup>*) showed recombination, while kidneys from *Vegfa<sup>UBC</sup>* and *Vegfa<sup>Dmp1</sup>* mice also showed recombination (Supplemental Figure 1). Kidneys from Cre+ *Vegfa<sup>Osx</sup>* did not show recombination, and likewise recombination was not seen in tissues from Cre- mice. All

experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at Washington University in St. Louis School of Medicine in accordance with the Animal Welfare Act and PHS Policy on Humane Care and Use of Laboratory Animals. Mice were euthanized by CO<sub>2</sub> asphyxiation.

## 2.2. Bone Strain Analysis

Prior to *in vivo* loading, a subset of mice was used for *ex vivo* strain gauge analysis (n = 2–4/sex/genotype, N = 28 total, Supplemental Table 1) in order to determine forces for strain-matched loading of male and female mice from each Cre line. These mice were euthanized 28 days after the first tamoxifen dose (corresponding to the first day of *in vivo* loading for ‘Anabolic Tibial Loading’). Strain data was captured from a single element gauge (FLK-1-11-1LJC, Tokyo Measuring Instruments Lab) placed on the anteromedial (tensile) surface 5 mm proximal to the tibial-fibular junction (corresponding to the section where bone formation was measured). A preload (–0.5 N, Dynamight 8841, Instron) was applied and the right tibias were cyclically loaded (12 cycles, 4-Hz) to compressive forces of 2, 4, 6, 8 and 10 N with 3 minutes of rest between each loading force. In some cases (at high forces) the anterior cruciate ligament was torn during loading and for these samples strain data after the tear was omitted. Compressive strains were extrapolated to the posterolateral apex (site of peak compression) (Patel et al., 2014). Force-strain relationships for both tensile and compressive strains were determined using linear regression. A preliminary study using 8 N loading force in *Osx* and *Dmp1* lines demonstrated lamellar bone formation in male and female *Osx* mice, with woven bone formation in female *Dmp1* mice and no bone formation in male *Dmp1* mice. Based on these observations, peak compressive loading forces were selected to induce an anabolic response with minimal woven bone formation (approx. –3100  $\mu\epsilon$ ).

## 2.3. Anabolic Tibia Loading

The axial tibial compression model induces bone formation without bone injury. To induce bone formation, the right hind limbs from 100 mice were cyclically loaded at a frequency of 4 Hz with a 7–10 N peak force (Supplemental Table 1) for 60 cycles. The loading was repeated for 5 consecutive days. Mice received buprenorphine (0.1 mg/kg s.c.) following each loading bout for analgesia, were returned to their cages to resume normal activity, and were monitored daily. The left tibias were not loaded and served as contralateral controls. Calcein (10 mg/kg, i.p., Sigma) was administered on day 5 and alizarin red (30 mg/kg i.p., Sigma) on day 10 to label newly forming mineralizing bone. Following loading, mice were sacrificed on day 12, allowing time for post-loading bone formation to occur. After dissection from soft tissues, bilateral tibias were embedded for plastic histology.

## 2.4. Dynamic Histomorphometry

Following axial tibial compression loading, bilateral mouse tibias (N = 200 combined loaded and non-loaded) were embedded in methylmethacrylate plastic (Sigma). Transverse sections (100- $\mu\text{m}$ -thick) were cut from the diaphysis, 5 mm proximal to the tibial-fibular junction on a saw-microtome (Leica 1600SP) and imaged using a confocal microscope (Leica TCS SPE). Dynamic histomorphometric analysis was performed on sections (one section/bone) using calcein and alizarin red labeling analyzed with commercial software

(Osteo II, BIOQUANT). Following published guidelines (Dempster et al., 2013), standard lamellar and woven bone formation indices were determined for the entire periosteal (Ps) and endocortical (Ec) surfaces separately. Primary outcome variables were 1) mineralizing surface per bone surface (MS/BS): percentage of the perimeter that has new bone formation (an indication of the number of active osteoblasts), 2) mineral apposition rate (MAR): the amount of space between labels (a surrogate for rate of osteoblast output), and 3) bone formation rate (BFR/BS): a multiplication of MS/BS and MAR (total osteoblast output). There were small amounts of woven bone on some samples, and to include that, *total* MS/BS was computed as  $(0.5sLS + dLS + Wo.S)/BS$ . Measurements of mineral apposition rate (MAR) and bone formation rate per bone surface (BFR/BS) including woven bone are denoted MAR+ and BFR/BS+, respectively (De Souza et al., 2005). Some samples had no visible double-labeled surface, so a minimum value of  $0.3 \mu\text{m}/\text{day}$  was assigned for MAR and was used to calculate BFR/BS (Dempster et al., 2013); this approach avoids excluding samples with no double-label from data analysis.

## 2.5. Bone phenotyping and mechanical testing of Vegfa<sup>UBC</sup>

Right femurs were harvested at euthanasia (post loading day 12) from male and female Vegfa<sup>UBC</sup> and Vegfa<sup>fl/fl</sup> mice for cortical bone analysis and three-point bending tests ( $n = 8-10/\text{group}$ , 38 total). Spinal segments (L5) were harvested for cancellous bone analysis from the same mice. After dissection and muscle removal, bones were wrapped in saline-soaked gauze and stored frozen at  $-20^\circ\text{C}$  until use. Bones were scanned by microCT (vivaCT 40, Scanco Medical AG,  $10.5 \mu\text{m}$ , 1000 projections, 300 ms, 70 kVp,  $114 \mu\text{A}$ , high resolution). The femoral mid-diaphysis (1.05 mm, 100 slices) was analyzed using the Scanco software with contours drawn on the periosteal surface of the bone (analysis parameters: sigma 0.4, support 1, threshold  $350 \text{ mg HA}/\text{cm}^3$ ). Cortical analysis parameters included total area (Tt.Ar), bone area (Ct.Ar), bone area fraction (Ct.Ar/Tt.Ar), medullary area (Ma.Ar), cortical bone thickness (Ct.Th), polar moment of inertia (pMOI), minimum moment of inertia (Imin), maximum moment of inertia (Imax), and tissue mineral density (TMD). For cancellous analysis, the analysis region was defined as the entirety of L5 vertebral body (average length 2.4 mm). Contours were drawn on the endosteal surface of the cortical bone (analysis parameters: sigma 0.4, support 1, threshold  $200 \text{ mg HA}/\text{cm}^3$ ). Cancellous analysis parameters included total volume (TV), bone volume (BV), bone volume fraction (BV/TV), connectivity density (Conn-Dens), structure model index (TRI-SMI), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and volumetric bone mineral density (vBMD). After microCT, femur lengths were measured with a digital caliper and the midpoint marked with pencil. Following one additional freeze/thaw cycle, the right femurs were subjected to three-point bending according to published guidelines (Jepsen et al., 2015) (span = 7 mm, monotonic ramp,  $0.1 \text{ mm}/\text{s}$ ; Dynamight 8841, Instron). Force-displacement plots were analyzed to determine structural properties: stiffness, yield load, maximum (ultimate) load, post-yield displacement, and work to fracture. Material properties were estimated using simple beam theory (Jepsen et al., 2015): yield stress, ultimate stress, and Young's modulus.

## 2.6. Statistics

Data are presented as means and standard deviation with individual data points plotted. For periosteal dynamic histomorphometry outcomes (total MS/BS, MAR, MAR+, BFR/BS, and BFR/BS+), each parameter was initially analyzed using a repeated measures 3-way analysis of variance (ANOVA) with factors of loading (loaded vs non-loaded limb), sex, and genotype (Vegfa<sup>fl/fl</sup> vs Vegfa<sup>-/-</sup>). Due to significant sex effects and sex-loading interactions, males and females were subsequently analyzed separately using repeated measures 2-way ANOVA with factors of limb (loaded vs non-loaded) and genotype with Tukey post hoc tests. In addition, the data were analyzed using relative values (loaded minus non-loaded) with 1-way ANOVA. Endocortical results were analyzed using 2-way ANOVA (factors of genotype and sex). Bone phenotyping data from microCT and mechanical testing was analyzed using 2-way ANOVA. Significance was defined at  $p < 0.05$ . Analysis was performed with Prism (GraphPad, La Jolla, CA).

## 3. Results

### 3.1. Load-strain relationships differed between Cre lines

*Ex vivo* bone strain measurements were taken from the region of peak tensile strain and extrapolated to the peak compressive region (Figure 2). Each Cre line was analyzed independently. For all mouse lines, genotypes were pooled between sexes, as force-strain relationships were not significantly different between Vegfa<sup>-/-</sup> and Vegfa<sup>fl/fl</sup>. In both the UBC and Osx-Cre lines there were no sex differences in the load-strain relationships and data were pooled. In the Dmp1-Cre line there were significant differences between sexes. Loading parameters were strain-matched for each Cre-line (approx.  $-3100 \mu\epsilon$  compression,  $+1800 \mu\epsilon$  tension) (Supplemental Table 1) following a pilot study to verify loading-induced bone formation at these strains. Accordingly, UBC and Osx-Cre lines (both males and females) were loaded to  $-10$  N and  $-8$  N, respectively. Male Dmp1 mice were loaded to  $-10$  N while females were loaded to  $-7$  N. Importantly, within each sex, control (Vegfa<sup>fl/fl</sup>) and Vegfa<sup>-/-</sup> mice received similar bone strain stimuli. This allowed for a direct comparison to see the effect of loading-induced bone formation with reduced VEGFA expression in specific cell types.

### 3.2. Body weight was moderately influenced by VEGFA loss in UBC mice

The tamoxifen injections and loading protocols (Figure 1) were well tolerated. All three Cre mouse lines demonstrated similar changes in body weight throughout the experiment with no major weight losses as a result of tamoxifen (TMX) administration or loading (Supplemental Table 2, Supplemental Figure 2). In the UBC mouse line there was moderate weight loss in three of four groups in the 24 days between the end of TMX administration and loading ( $<5\%$ ). There were no differences in weight loss between Vegfa<sup>fl/fl</sup> and Vegfa<sup>Osx</sup> mice; however, there was a sex effect with females losing weight ( $-1.5\%$ ) and male mice gaining weight ( $1.5\%$ ). There were no sex or genotype differences between Vegfa<sup>fl/fl</sup> and Vegfa<sup>Dmp1</sup> mice related to body weight.

### 3.3. Loading-induced bone formation was impaired in mice with global (UBC) deletion of Vegfa

To investigate the role of VEGFA from all cells in the anabolic effect of skeletal loading, 5-month-old male and female mice were subjected to 5 bouts of axial compression on the right tibia while contralateral left tibias served as controls. Cre expression for global deletion (UBC) was induced by tamoxifen administration 4 weeks prior to loading (Figure 1). Tibial loading stimulated periosteal bone formation in both control (Vegfa<sup>fl/fl</sup>) and conditional knockout (Vegfa<sup>UBC</sup>) mice, as evidenced by significantly greater indices of bone formation in loaded versus contralateral non-loaded tibias (Figure 3, Supplemental Tables 3–4). However, the periosteal loading response was slightly diminished in conditional knockout mice. For female mice, Ps.MS/BS (indication of number of active osteoblasts) of loaded tibias was 18% higher in Vegfa<sup>UBC</sup> mice relative to Vegfa<sup>fl/fl</sup> control mice ( $p < 0.05$ , Figure 3B, Supplemental Table 4), whereas Ps.MAR (spacing between labels) and Ps.BFR/BS (total osteoblast output) were 13% and 15% lower, respectively. In male mice, Ps.MS/BS of loaded tibias was 12% lower in Vegfa<sup>UBC</sup> mice relative to Vegfa<sup>fl/fl</sup> control mice, and Ps.MAR and Ps.BFR/BS were 18% and 28% lower, respectively (Figure 3C, Supplemental Table 4). Importantly, a significant loading-genotype interaction was detected for Ps.BFR/BS in both sexes, indicating that the effect of loading on periosteal bone formation differed between control and knockout mice with a global deletion of VEGFA ( $p < 0.05$ ). Similarly, when the effect of loading was expressed as a relative measure (i.e., loaded minus non-loaded control), periosteal bone formation rate was reduced by 22% ( $p < 0.05$ ) and 28% ( $p < 0.05$ ) in female and male mice, respectively, in the Vegfa<sup>UBC</sup> group compared to Vegfa<sup>fl/fl</sup>.

While the goal of tibial loading was to only stimulate lamellar bone formation, results show that loading did occasionally produce woven bone in the area of peak compression. There was evidence of periosteal woven bone formation in 7/38 samples from the UBC line, with 6 of those samples in the female Vegfa<sup>fl/fl</sup> group. Data trends were similar when evaluating bone measurements including or excluding the contribution of woven bone (both are provided in Supplemental Table 4; data in Figure 3 do not include woven bone). Endocortical measurements were also analyzed, but were not significantly different for any outcome between genotypes for non-loaded or loaded limbs. In summary, the anabolic response to loading was significantly impaired in the periosteum of female and male mice by the absence of VEGFA globally.

### 3.4. Loading induced bone formation was not significantly impacted by VEGFA deletion in osteoblast lineage (Osx+) cells

Similar to the UBC line, 5 days of tibial loading of mice from the Osx line stimulated periosteal bone formation in both control (Vegfa<sup>fl/fl</sup>) and conditional knockout (Vegfa<sup>Osx</sup>) mice (Figure 4, Supplemental Tables 3, 5). The periosteal loading response was less in male compared to female mice, and only in females did we observe any significant effects of knockout. For female mice, in loaded tibias Ps.MS/BS was 19% lower in Vegfa<sup>Osx</sup> mice relative to Vegfa<sup>fl/fl</sup> control mice ( $p < 0.05$ , Figure 4B, Supplemental Table 4), whereas Ps.MAR was increased 60% ( $p = 0.07$ ) and Ps.BFR/BS was not different between genotypes. For male mice, in loaded tibias Ps.MS/BS, Ps.MAR and Ps.BFR/BS were not

different in Vegfa<sup>Osx</sup> mice relative to Vegfa<sup>fl/fl</sup> control mice (Figure 4C). Moreover, a significant loading-genotype interaction was not detected for Ps.BFR/BS in either sex, indicating that the effect of loading on periosteal bone formation was not impacted by osteoblast lineage deletion of VEGFA. Similarly, when the effect of loading was expressed as a relative measure (i.e., loaded minus non-loaded control), there were no genotype differences.

Some woven bone formation occurred in Osx mice in the area of peak compression. Periosteal woven bone formation was observed in 6/36 samples, with 5 of those samples in the female Vegfa<sup>Osx</sup> group. Data trends were similar for bone measurements including or excluding the contribution of woven bone (both are provided in Supplemental Table 5; data in Figure 4 do not include woven bone). Endocortical measurements were also analyzed, but were not significantly different for any variable between genotypes for non-loaded or loaded limbs. Overall, the anabolic response to tibial loading was not impaired by the absence of VEGFA from osteoblast lineage (Osx+) cells.

### 3.5. Loading induced bone formation was not altered by VEGFA deletion in mature osteoblasts and osteocytes (Dmp1+)

As with the other Cre lines, 5 days of tibial loading in mice of the Dmp1 line stimulated periosteal bone formation in both control (Vegfa<sup>fl/fl</sup>) and conditional knockout (Vegfa<sup>Dmp1</sup>) mice (Figure 5, Supplemental Tables 3, 6). Unlike the other Cre lines, there were no genotype or sex related differences in Vegfa<sup>Dmp1</sup> mice relative to Vegfa<sup>fl/fl</sup> control mice (Figure 5B,C), indicating that loading-induced periosteal bone formation was unaffected by deletion of VEGFA in mature osteoblasts and osteocytes. Similarly, when the effect of loading is expressed as a relative measure (i.e. loaded minus non-loaded control), there were no genotype or sex differences for the Vegfa<sup>fl/fl</sup> and Vegfa<sup>Dmp1</sup> groups.

Tibial loading produced a small amount of woven bone in the area of peak compression in 3/26 Dmp1 mice. Data trends were similar for bone measurements including or excluding the contribution of woven bone (both are provided in Supplemental Table 6; data in Figure 5 do not include woven bone). Endocortical measurements were also analyzed but were not significantly different for any variable between genotypes for non-loaded or loaded limbs. In summary, the anabolic response to tibial loading was not impaired by the absence of VEGFA from late-stage osteoblast lineage cells.

### 3.6. Baseline bone size or strength was not altered in Vegfa<sup>UBC</sup> mice

To assess the possible effects of global VEGFA deletion on bone homeostasis, we evaluated phenotypes of Vegfa<sup>fl/fl</sup> and Vegfa<sup>UBC</sup> bones not subjected to mechanical loading. Vertebral segments (L5) were removed to analyze cancellous bone using microCT, and right femurs were harvested for cortical bone midshaft analysis by microCT and three-point bending. There were some differences in cancellous bone parameters by 2-way ANOVA with respect to sex and genotype (Supplemental Table 7). Female Vegfa<sup>UBC</sup> mice had significantly lower BV, BV/TV, Tb.N, and vBMD (12 to 20%) compared to controls (Vegfa<sup>fl/fl</sup>). There were no differences between male Vegfa<sup>UBC</sup> mice and Vegfa<sup>fl/fl</sup> mice. For cortical bone analysis, there were some modest differences attributed to sex; female



femurs had slightly lower total area, but increased cortical thickness compared to males (Supplemental Table 7). Nonetheless, in both males and females, there were no significant differences in post-hoc testing comparing control versus conditional knockout mice for any of the six cortical parameters evaluated (T.Ar, B.Ar, B.Ar/T.Ar, C.Th, pMOI, and TMD). Mechanical testing of femurs revealed no genotype differences in whole-bone (structural) properties and only modest sex differences. In post-hoc testing there were no significant differences in any femur structural or material property between Vegfa<sup>UBC</sup> mice in comparison to Vegfa<sup>fl/fl</sup> mice. These results indicate that short-term global deletion of VEGFA had a mild effect on cancellous bone in female mice, but overall did not impact cortical bone morphology or mechanical properties.

#### 4. Discussion

VEGF is essential to blood vessel formation, and promotes normal bone development accompanied by angiogenesis (Hu and Olsen, 2016; Lee et al., 2007; Neufeld et al., 1999; Street et al., 2002). Following skeletal injury, VEGFA in bone is upregulated (McKenzie et al., 2011; Wohl et al., 2009) and its expression by (pre)osteoblasts (Osx+) is required for intramembranous and endochondral healing exhibited in a range of bone injuries, from small drill holes, to non-displaced stress fractures, to full fractures (Buettmann et al., 2019; Hu and Olsen, 2016). However, because bone formation during healing depends on, and is tightly coupled to angiogenesis (Hausman et al., 2001; Jacobsen et al., 2008; Maes et al., 2010; Street et al., 2002; Tomlinson et al., 2013), it has been difficult to determine whether osteoblast VEGFA stimulates bone formation directly or does so by promoting angiogenesis.

In a previous study, we showed that inhibition of angiogenesis blocked woven bone formation after stress fracture but did not inhibit lamellar bone formation induced by non-injurious, anabolic bone loading even though lamellar bone formation was associated with modest angiogenesis (Tomlinson et al., 2014). In the current study, we turned to non-damaging axial tibial loading to induce lamellar bone formation in adult mice with inducible deletion of VEGFA in different cell types; we hypothesized that VEGFA from osteoblasts is required for lamellar bone formation. Results indicated that mice with conditional deletion of VEGFA in (pre)osteoblasts (Vegfa<sup>Osx</sup>) and mature osteoblasts and osteocytes (Vegfa<sup>Dmp1</sup>) had comparable loading-induced lamellar bone formation as control (Vegfa<sup>fl/fl</sup>) mice, which does not support our hypothesis. On the other hand, loading-induced bone formation was decreased in mice where VEGFA was deleted from all cells (Vegfa<sup>UBC</sup>). Our findings indicate that VEGFA from a non-osteoblast cell source supports lamellar bone formation in a setting where angiogenesis is not required, whereas osteoblast/osteocyte derived VEGFA is not required. This supports a model whereby osteoblasts are responding in a paracrine manner to VEGFA from local non-osteolineage cells. Identifying the cell type responsible for this effect will require additional studies.

We used strain gauge analysis of the different mouse genotypes to estimate forces needed for strain-matched mechanical loading. Despite being on similar genetic backgrounds, the different Cre lines required different forces to strain match, and there was a sex effect in one of the three lines. Specifically, UBC- and Osx-Cre lines (both male and female) were loaded to -10 N and -8 N, respectively, while male Dmp1 mice were loaded to

–10 N and females were loaded to –7 N. The overall goal of this approach is to deliver a comparable mechanical stimulus across gene and sex groups (Main et al., 2020), and the consistent formation of lamellar bone observed by dynamic histomorphometry indicates that strain-matched loading induced a comparable anabolic response. However, there were modest differences between the different genetic backgrounds most notably that the UBC line had the greatest loading response (based on Ps.rBFR/BS). In addition, female mice from the UBC and *Osx* lines had a greater response compared to the respective males. Importantly, the strain analysis indicated that there were no differences between control (*Vegfa<sup>fl/fl</sup>*) and conditional knockout (*Vegfa<sup>-/-</sup>*) mice of the same sex and Cre line, consistent with the lack of a cortical bone phenotype caused by short-term deletion of VEGFA in our study. Thus, for the primary comparison of interest (control vs cKO) mice were loaded to equal forces.

This study has several limitations. First, it was observed that 16% of loaded tibias responded to loading by producing some woven bone. Specifically, 7/38 samples from UBC mice, 6/36 samples from *Osx* mice, and 3/26 samples from *Dmp1* mice had small amounts of woven bone at the region of peak compressive strain. The presence of woven bone indicates that a more robust bone formation process has occurred, and indicates that our loading stimulus was near the woven-lamellar transition (Turner et al., 1994). Woven bone formation has been linked to angiogenesis (Tomlinson et al., 2014). Because we wanted to test the role of VEGFA independent of angiogenesis, the parameters shown in Figures 3–5 are based solely on the lamellar bone contributions to bone formation. Nonetheless, data analysis including woven bone contributions leads to similar overall conclusions about the effect of VEGFA conditional deletion (Suppl. Tables S3–S5). Second, when mice were exposed to tamoxifen, weight was found to vary based on genotype where the UBC line lost slightly more weight than their control counterparts, the *Osx* line had modest effects of tamoxifen on weight when stratified by sex, and the *Dmp1* line were found to have a slight difference in weight stratified by genotype. While differences in body weight were observed, the tamoxifen effects appeared to be modest. The UBC line underwent additional phenotyping analysis and found that baseline cortical bone morphology and mechanical properties were not affected in knockout mice.

In summary, axial tibial loading induced lamellar bone formation in 5-month-old mice from three Cre/*Vegfa* floxed lines. Only *Vegfa<sup>UBC</sup>* mice had diminished loading-induced bone formation, indicating that VEGFA from a non-osteoblast cell source supports osteoblast bone formation in a context (lamellar bone formation) where angiogenesis is not required. In contrast, VEGFA derived from osteoblasts and osteocytes (*Osx<sup>+</sup>* or *Dmp1<sup>+</sup>*) is not necessary for lamellar bone formation to occur in response to an anabolic loading stimulus. This latter result is different from prior findings that VEGFA from osteoblasts (*Osx<sup>+</sup>*) contributes to woven bone formation during fracture healing. Taken together, the data support a model whereby VEGFA from osteoblasts supports bone formation indirectly by coupling osteogenesis and angiogenesis, and thus does not play a role in settings where bone formation is independent of angiogenesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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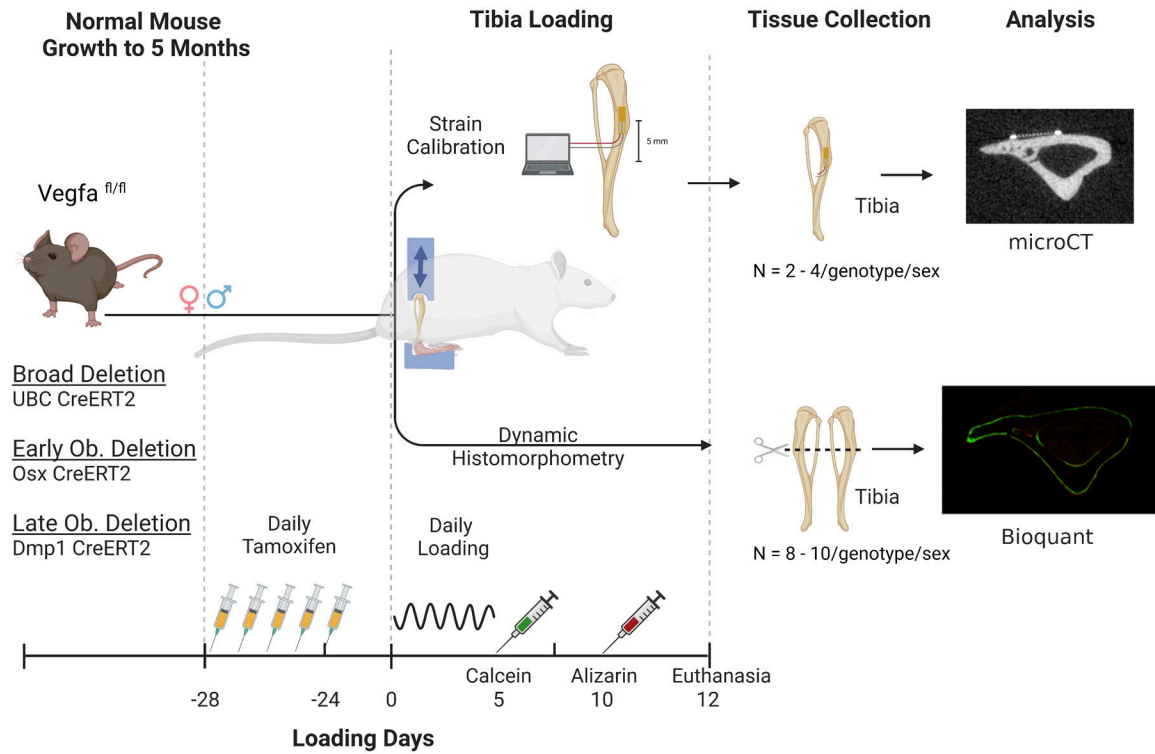
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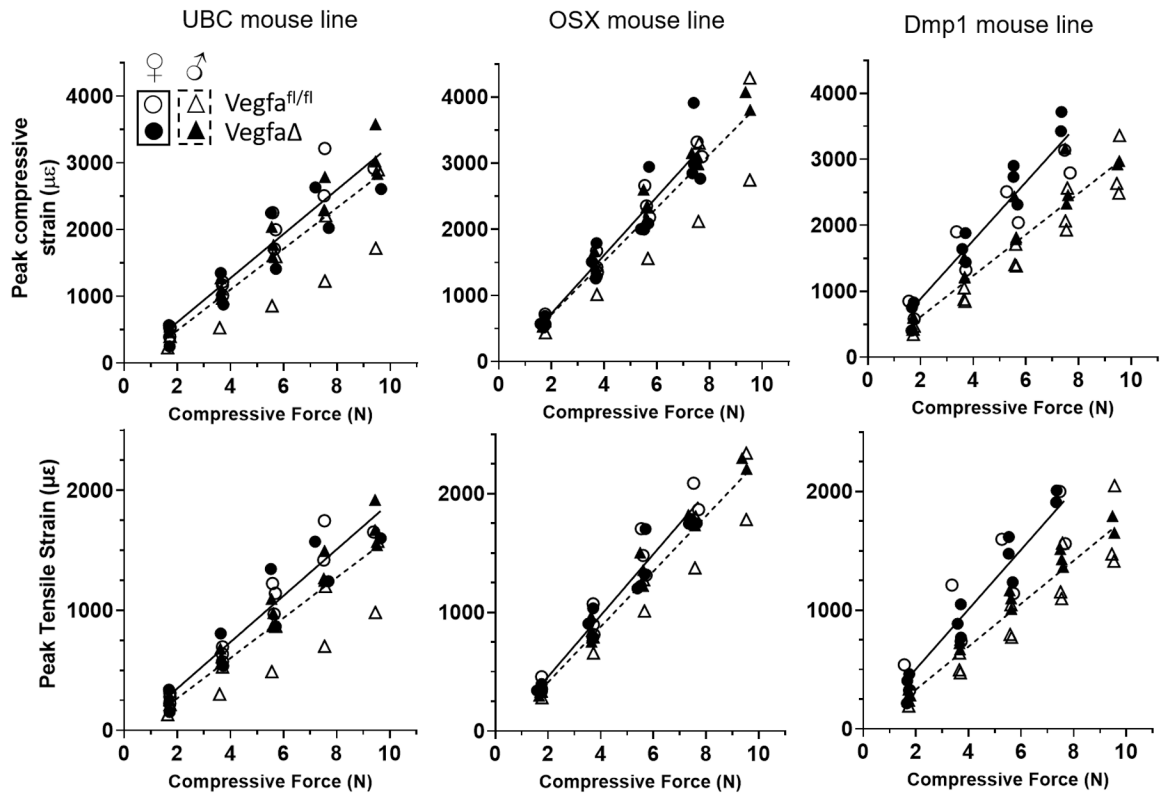
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### Highlights

- Tibial loading stimulated lamellar bone formation in mice with inducible deletion of VEGFA
- Loading-induced bone formation was unaffected by loss of VEGFA in osteoblasts/osteocytes (Osx+, Dmp1+)
- Loading-induced bone formation was diminished by global loss of VEGFA (UBC+)
- Lamellar bone formation in adult mice does not require VEGFA from osteoblasts/osteocytes

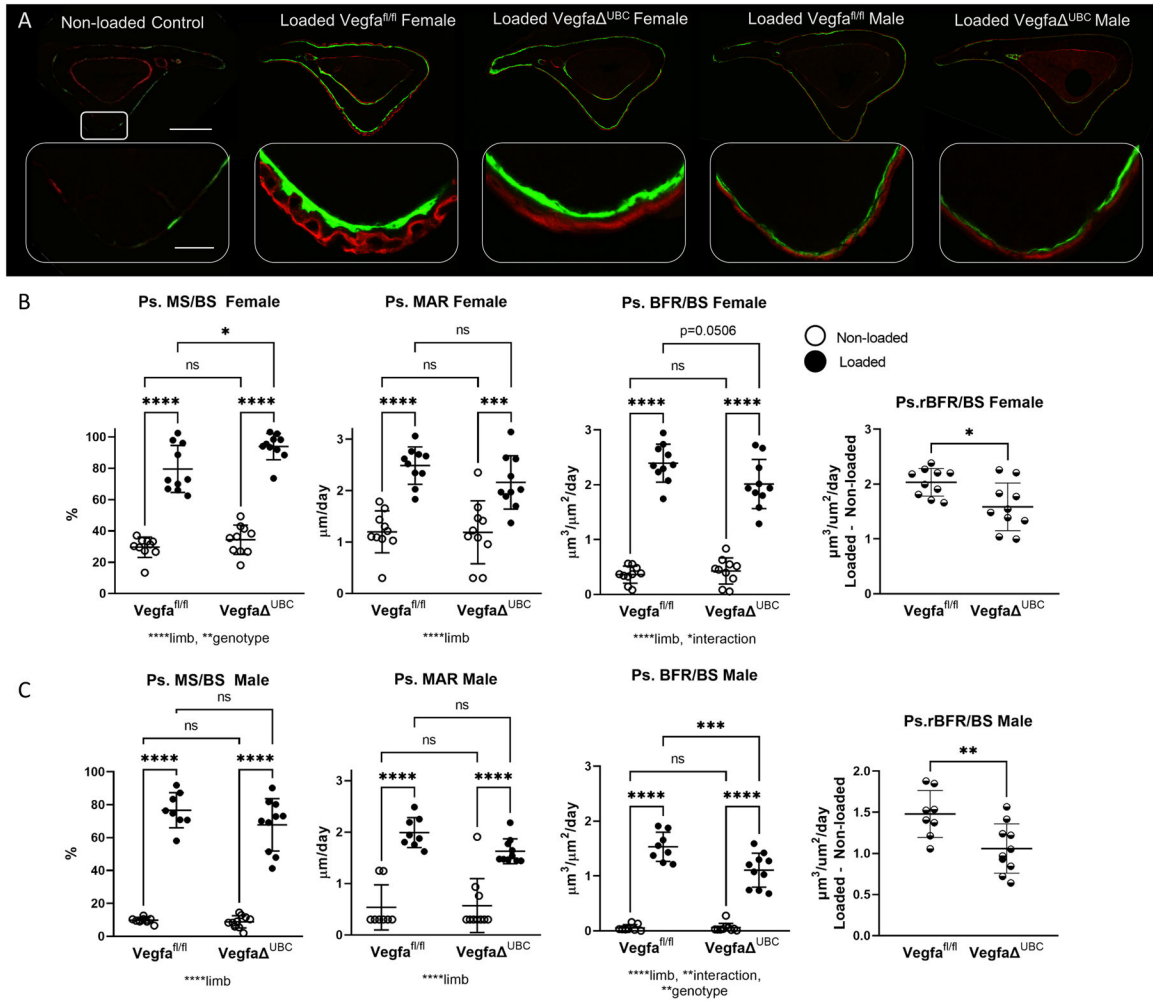


**Figure 1.** Overview of study design. Three separate tamoxifen inducible Cre-ERT2 mouse lines (UBC, Osx, or Dmp1) were crossed to mice carrying floxed VEGFA (Vegfa<sup>fl/fl</sup>) alleles. At 20 weeks of age (5 mo) mice received 5 daily doses of tamoxifen followed by a 3-week clearance. An initial group was euthanized at 24 weeks of age (day 0) and tibia were used for strain calibration. Separate cohorts of mice were loaded daily for 5 days and euthanized 12 days after the start of loading for dynamic histomorphometry outcomes. (Created with [BioRender.com](https://www.biorender.com))



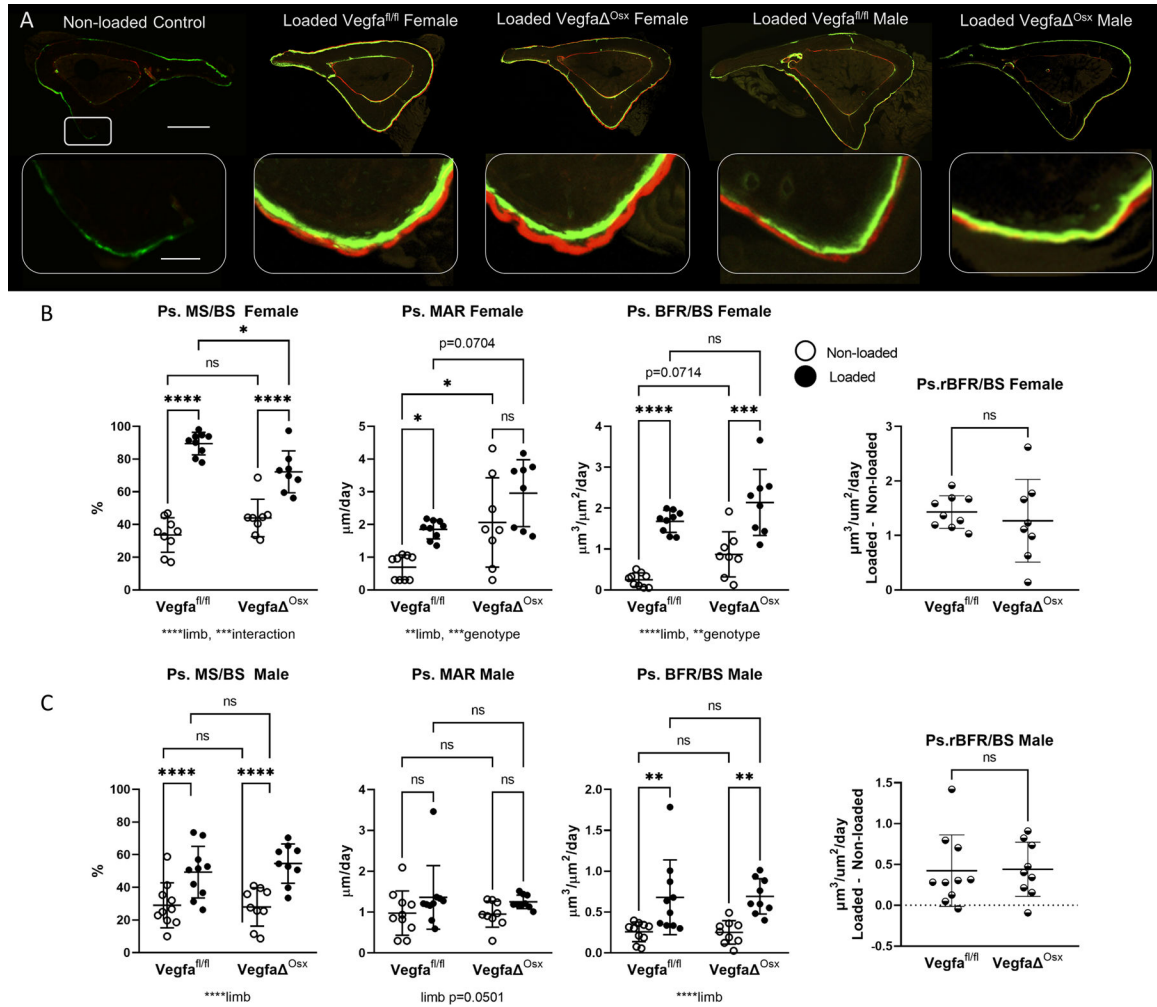
**Figure 2.**

Strain gauge data from loaded tibia. Peak tensile strain was collected and peak compressive strain was calculated after microCT imaging. In all lines of mice, there were no genotype (Vegfa<sup>fl/fl</sup> (control) vs Vegfa<sup>Δ</sup> (Cre+)) related differences within each sex, indicating that short-term deletion of Vegfa did not alter force-strain relationships. For linear regression analysis, data for the two genotypes of each mouse line were pooled and analyzed separately for females (solid line) and males (dashed line). In the UBC line, the strain profiles were similar for all mice. Trends for the Osx line matched those of UBC, with the exception that the slope of the curve fit was higher. In the Dmp1 line there were sex differences. In all groups of mice a target peak compressive strain of  $-3100$  microstrain was used for *in vivo* loading.



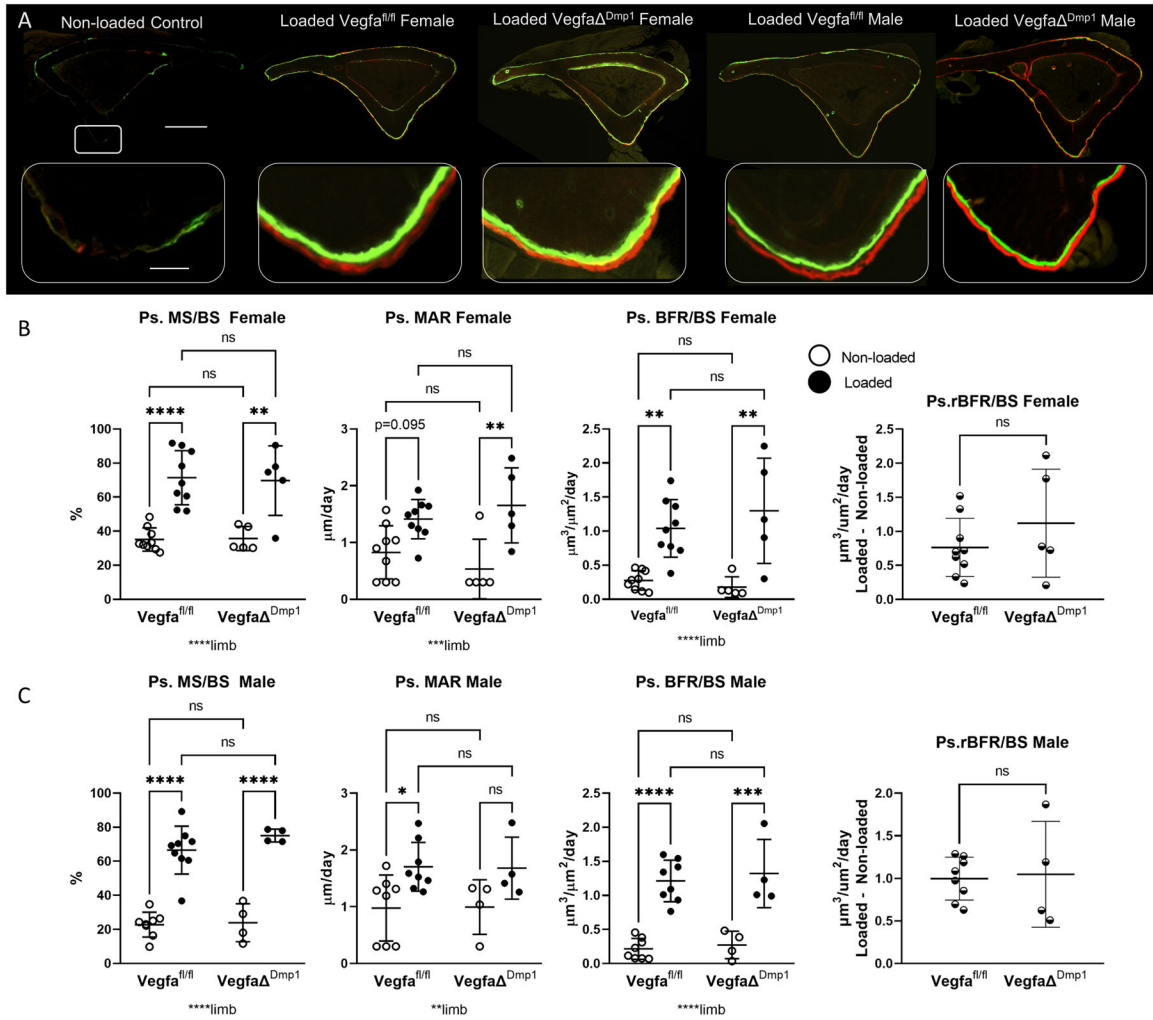
**Figure 3.** Loading-induced periosteal bone formation is impaired in mice with a global deletion of VEGFA using UBC Cre. A) Representative images from control and loaded groups demonstrate increased periosteal label around the entire tibia after loading (scale bar 500 micron). Magnified images of the area of peak compression demonstrate increased label and spacing between labels on loaded bones (scale bar 50 micron). B) Indices of periosteal bone formation in female control (Vegfa<sup>fl/fl</sup>) and conditional knockout (Vegfa<sup>UBC</sup>) mice (n = 10). C) Indices of periosteal bone formation in male control (Vegfa<sup>fl/fl</sup>) and conditional knockout (Vegfa<sup>UBC</sup>) mice (n = 8–10). Two-factor ANOVA was used to evaluate the effects of limb (loading) and genotype, with Tukey post hoc comparisons. One-way ANOVA was used to compare relative bone formation rate (BFR/BS Loaded minus BFR/BS Non-loaded), which is an index of loading-induced bone formation. Bars depict mean ± SD, with individual data points shown. Parameters shown do not include woven bone. \* p<0.05 \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001.





**Figure 4.**

Loading-induced periosteal bone formation is maintained in mice with osteoblast lineage deletion of VEGFA using *Osx* Cre. A) Representative images from control and four loaded groups demonstrate increased periosteal label around the entire tibia after loading (scale bar 500 micron). Magnified images of the area of peak compression demonstrate increased label and spacing between labels on loaded bones (scale bar 50 micron). B) Indices of periosteal bone formation in female control (*Vegfa*<sup>fl/fl</sup>) and conditional knockout (*Vegfa*<sup>Osx</sup>) mice (n = 8–9). C) Indices of periosteal bone formation in male control (*Vegfa*<sup>fl/fl</sup>) and conditional knockout (*Vegfa*<sup>Osx</sup>) mice (n = 9–10). Two-factor ANOVA was used to evaluate the effects of limb (loading) and genotype, with Tukey post hoc comparisons. One-way ANOVA was used to compare relative bone formation rate (BFR/BS Loaded minus BFR/BS Non-loaded), which was used as an index of loading-induced bone formation. Bars depict mean  $\pm$  SD, with individual data points shown. Parameters shown do not include woven bone. \*  $p < 0.05$



**Figure 5.**

Loading-induced periosteal bone formation is maintained in mice with mature osteoblast lineage deletion of VEGFA using *Dmp1* Cre. A) Representative images from control and four loaded groups demonstrate increased periosteal label around the entire tibia after loading (scale bar 500 micron). Magnified images of the area of peak compression demonstrate increased label and spacing between labels on loaded bones (scale bar 50 micron). B) Indices of periosteal bone formation in female control (*Vegfa<sup>fl/fl</sup>*) and conditional knockout (*Vegfa<sup>Dmp1</sup>*) mice (n = 5–9). C) Indices of periosteal bone formation in male control (*Vegfa<sup>fl/fl</sup>*) and conditional knockout (*Vegfa<sup>Dmp1</sup>*) mice (n = 4–7). Two-factor ANOVA was used to evaluate the effects of limb (loading) and genotype, with Tukey post hoc comparisons. One-way ANOVA was used to compare relative bone formation rate (BFR/BS Loaded minus BFR/BS Non-loaded), which was used as an index of loading-induced bone formation. Bars depict mean ± SD, with individual data points shown. Parameters shown do not include woven bone. \* p<0.05