Brittleness in Osteoblast-derived BMP2 Knockout Bones is Due to Increased Porosity & Cellularity

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Disclosures:  Z. Toth: None. S.H. McBride: None.

Introduction: Fragility fractures, fractures occurring from normally innocuous loading events (e.g. fall from standing, coughing), can result in decreased quality of life, chronic disabilities, and even mortality in otherwise healthy patients. Traditionally, an individual’s fragility fracture risk is assessed by bone mineral density (BMD), risk increases with decreasing BMD (i.e. osteopenia or osteoporosis). However, many people with normal BMD experience fragility fractures. This indicates that other factors are important to consider when assessing an individual’s fragility fracture risk. To date, one of the best indicators is a family history of fragility fractures. This signals that genetics, rather than environmental factors, are playing a large part in regulating bone quality independent of BMD. One of the candidate genes is BMP2[1], a protein well known to induce bone formation. However, it is difficult to elucidate possible mechanisms in human samples. Recent studies with BMP2 conditional/tissue-specific knockout mice appear to mimic the human condition. Knockout bones have altered elastic modulus [2,3], are more brittle [2,3], and will spontaneously fracture[4]. The cause of the defect has not been conclusively identified. There are no apparent differences in mineral on the molecular level[3]. Mice lacking osteoblast Smad4, a key protein in the BMP2 signaling pathway, have defects in the production of collagen, the factor responsible for bone’s toughness[5]. Our goal is to investigate collagen and bone architecture at multiple length scales to determine if the increased brittleness is due to defects in collagen production or assembly. We hypothesize that lack of osteoblast derived BMP2 leads to a misregulation of collagen production similar to that seen in Smad4 knockout.

Methods: All procedures were done with the approval of Washington University’s animal review board (20110209). All mice had two BMP2 floxed alleles. Knockout was targeted to osteoblasts using an osterix promoted Cre (OSX-Cre, B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J). Littermates lacking OSX-Cre served as controls. Two small groups of animals were used. Timing of the Cre expression was not controlled in the first group (n=3-4/group). In the second group, Cre expression was delayed with doxycycline administration until weaning (P21, n=2-4/group). Two-way ANOVA (doxycycline treatment and genotype) showed no significant effect of doxycycline treatment for any reported factor. So the two groups were pooled for analysis to increase the power. Future work will focus on group 1 animals. At 12 to 14 weeks old the mice were euthanized and the femora were harvested, fixed in 10% neutral buffered formalin, decalcified, and processed for paraffin sectioning. Serial cross sections of the midshaft were treated with one of three stains: picrosirius red (collagen alignment and woven bone studies), H&E (porosity and lacunae area), or DAPI (cell density). Then they were imaged under polarized (picrosirius red, Olympus BX51) or fluorescent (H&E, DAPI, Leica DMI4000) light. The images were processed using custom MatLab programs or ImageJ to determine collagen alignment (angular deviation of a well aligned region in the posterior lateral area), fibril size (polarized light hue in the same region), percent woven bone (woven bone/total bone area), and percent porosity (void area/total bone area).
Because of the results from these studies we included cell density (DAPI stained nuclei/total bone area) and lacunae size as well. One-way ANOVA was used to determine statistical significance (Statview).

**Results:** At the fibril length scale, collagen fibrils in the knockouts had a higher angular deviation (18±7°) than controls (14±3°), but this did not reach significance. The hue, an indirect measure of fibril size or packing, and its variation was not significantly different (0.19±0.06 vs 0.22±0.11 - red/orange color, 0.02±0.005 vs 0.02±0.006, KO vs WT). This indicates that the fibrils are approximately the same size and are similarly well aligned. At the tissue length scale, the percent woven bone was similar (36±10% vs 30±13%, KO vs WT). However, knockout bones are more porous (10±3% vs 6±2%, Figures 1 & 2). To determine if the increased porosity was due to increased cellularity the cell density was calculated. BMP2 knockout bones have significantly more cellularity (1350±100cell/mm2 vs 1100±40cells/mm2, Figure 3) without a concurrent change in lacunae size (18±4um2 vs 20±2um2). However, when the percent porosity was recalculated considering only small voids (<100um2) the porosity was the same in both groups (3±1%). Together these indicate that most of the difference in void space is at the tissue length scale in the bone architecture rather than on the cellular level.

**Discussion:** Based on this preliminary data we conclude that the brittleness of BMP2 knockout bones is due to a dramatic increase in porosity rather than an obvious change in collagen production or assembly. Increased cellularity, thus increased cellular-scale voids, are partially contributing to the increased porosity. However, the larger scale bone architecture is altered as well. BMP2 knockout bones have an extensive number of large cavities compared to controls which decrease the overall toughness. The collagen fibrils themselves are similar in size for both groups, but the knockout fibrils trend towards a less uniform orientation. This could be an indication of differences at lower length scales (i.e. collagen bonds, d-spacing) than covered by the current work.


**Significance:** Understanding the genetic factors controlling bone quality will aid in better identifying people at risk for fragility fractures and the development of preventative treatments.
Figure 1. Pictures of H&E sections imaged under fluorescent light (A,B) were processed in MatLab (C,D) to count the number and size of void areas to calculate porosity. Note the larger more numerous void areas in KOs.
Figure 2. BMP2 knockout bones had significantly higher porosity than controls.
Figure 3. DAPI stained sections from control (A) and knockout (B) animals. Cross sections from the medial femoral midshaft demonstrate the increased cellularity in knockout bones. Analysis of the whole bone cross sections demonstrated an average cell density increase of 22% (C).