Thrombospondin 1 (TSP1) Is Necessary for Fracture Healing Under Normoxic and Ischemic Conditions

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INTRODUCTION: Although there has been extensive research into the family of five glycoproteins known as thrombospondins, the role of thrombospondin 1 (TSP1) in fracture healing has been largely neglected[1]. TSP1 protein is present during the early stages of wound healing, deposited by platelets and expressed by monocytes. TSP1 is implicated in regulation of angiogenesis as well as extracellular matrix organization, fibrosis, and endothelial cell senescence[2-6]. Our lab has previously demonstrated that an absence of the TSP1 receptor, CD47, results in decreased callus formation and disturbances in mesenchymal progenitor cell (MSC) proliferation[7]. We hypothesize that TSP1 binding to CD47 during early callus formation is necessary for proper fracture healing and the absence of TSP1 will similarly result in decreased callus size and mesenchymal progenitor cell (MSC) function.

METHODS: With proper animal regulatory approval, wildtype (WT) and TSP1-null mice on a C57Bl/6 background underwent closed-stabilized bilateral femoral fractures or ischemic left tibia fractures, where the femoral artery was ligated. All fractures were generated using a three-point-bend apparatus. Femurs were harvested at 10- and 20-days post-fracture for bilateral femoral fractures and at 15 days post-fracture for ischemic tibia fractures. Limbs were fixed in 4% paraformaldehyde and intramedullary pins were removed. Limbs were scanned using µCT and reconstructed. A callus region of interest (ROI) was created every 10 slices and interpolated at 15- and 20-days post-fracture. Callus mineralization was analyzed using well-established protocols[8]. At 10 days post-fracture, a 3x3x5mm cylindrical ROI was generated around the fracture site. Primary bone marrow-derived MSCs (BMSC) were isolated from the long bones of WT and TSP1-null mice. Osteogenic differentiation was performed after passage 1 and Alkaline Phosphatase and Alizarin Red S staining were analyzed as well as gene expression analysis of osteogenic markers - Osterix, Runx2, and ALP (data not shown). Colony formation assays were performed on primary BMSCs seeded at 2 million cells/60mm dish. Plates were fixed, stained, and colonies counted. Cell clusters >30 cells were considered to be a colony.

RESULTS: TSP1 depletion results in diminished femoral callus formation by 10 days post-fracture. (Fig A) Through µCT analysis, we observed reductions in bone volume at 10 days post fracture. By 20 days post fracture, we observe significant decreases in bone volume as well as callus volume, bone mineral content and tissue mineral content emphasizing a disruption in osteogenesis in the absence of TSP1 (Fig A). Previous studies as well as our own work have demonstrated that in the absence of TSP1, mesenchymal stem cells (MSCs) are unable to undergo robust osteogenesis. The absence of TSP1 results in reduced calcium deposition through Alizarin red S staining (Fig B) as well as reduction in Osterix, Runx2, and Alkaline Phosphatase gene expression (data not shown). We further observed decreased ability of TSP1-null MSCs to form colonies, suggesting a potential decrease in proliferative capacity when TSP1 is absent (Fig C). Due to TSP1's known antiangiogenic role we interrogated the effect of a TSP1 knockout on fracture repair under ischemic conditions. Here we revealed that TSP1 depletion exacerbates the impaired healing response of ischemic fracture, resulting in significantly decreased callus formation (Fig D). Bone volume fraction and bone mineral density demonstrate significant decreases in mineralization (Fig E).

DISCUSSION: Our findings reveal that TSP1 is necessary for proper fracture healing to occur. Although previous studies have demonstrated a role for TSP1 in bone homeostasis and osteogenesis, this has not been previously linked to impaired fracture callus formation. Our findings support reduced osteogenic potential of MSCs in the absence of TSP1 and further demonstrates a reduced ability to form colonies. TSP1 has been shown to modulate senescence in endothelial cells and this suggests that this role may apply in the context of MSCs as well. Despite TSP1's anti-angiogenic role, decreased callus formation in the absence of TSP1 suggests a potential compensatory mechanism and could emphasize the importance of TSP1 as a mediator of matrix organization in the context of fracture healing.

SIGNIFICANCE/CLINICAL RELEVANCE: Our findings indicate that TSP1 plays an important role in fracture repair. Ongoing studies seek to interrogate the vascularization of the TSP1-null fracture callus and the interplay between TSP1 and its binding partners, TGFβ, CD47 and CD36, to modulate the fracture environment and assist in healing.

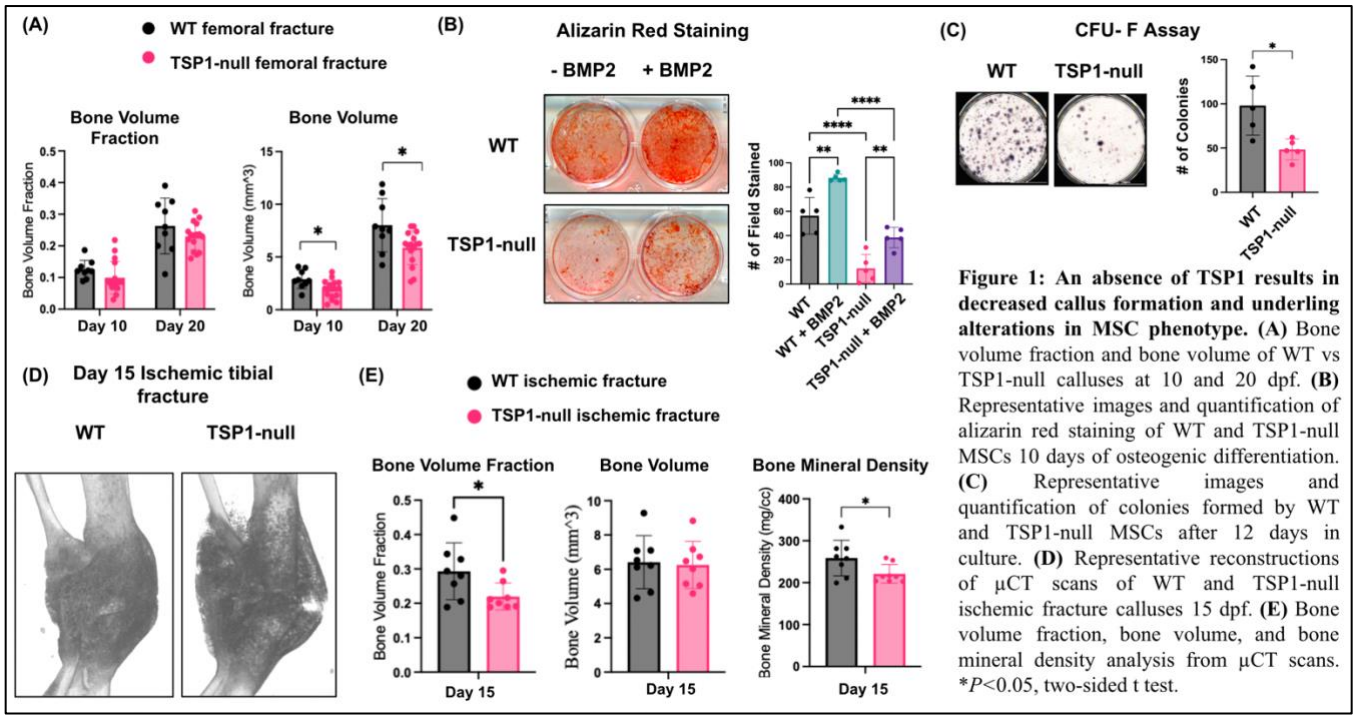

Figure 1: An absence of TSP1 results in decreased callus formation and underling alterations in MSC phenotype. (A) Bone volume fraction and bone volume of WT vs TSP1-null calluses at 10 and 20 dpf. **(B)** Representative images and quantification of alizarin red staining of WT and TSP1-null MSCs 10 days of osteogenic differentiation. **(C)** Representative images and quantification of colonies formed by WT and TSP1-null MSCs after 12 days in culture. **(D)** Representative reconstructions of µCT scans of WT and TSP1-null ischemic fracture calluses 15 dpf. **(E)** Bone volume fraction, bone volume, and bone mineral density analysis from µCT scans. *$P<0.05$, two-sided t test.

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