Thrombospondins 1 and 2 Deletion Mitigates Injury-Induced Heterotopic Ossification

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INTRODUCTION: Previous studies have shown that mice with both thrombospondin-1 (TSP1) and thrombospondin-2 (TSP2) germ-line deletions (double knockout, DKO) develop quadricep tendon exostoses and early femorotibial (knee) joint degeneration (osteoarthritis) (Lammlin et al. 2023 ORS). TSP1 and TSP2 are homologous large extracellular matrix proteins that are characterized as matricellular proteins, regulating structural matrix composition and assembly, angiogenesis, and cell proliferation and differentiation (Alford & Hankenson, 2015). Considering the development of extraosseous bone in the quadriceps tendon of the DKO mice, we were interested in exploring whether the absence of TSP1 and TSP2 would result in more pronounced bone development in injury-induced heterotopic ossification (HO). We hypothesized that TSP1 and TSP2 serve as inhibitors of extraosseous bone formation.

METHODS: All experiments were completed with institutional animal care and use approval. To longitudinally assess quadricep tendon ossification, uninjured C57BL/6 mice (wildtype, WT) and DKO mice were longitudinally radiographed using a Kubtec Xpert40 imaging system. Mice were anesthetized with 2-4% isoflurane, radiographed in a fixed prone position, and scored for extraosseous ossification proximal to the patella by a blinded observer scoring system. A score of 0 to 3 was given based on size and density of exostosis and statistical significance determined using a Mann-Whitney nonparametric test. To assess induced HO formation, age-matched male and female WT (N=19) and DKO (N=12) mice were injured with burn tenotomy at an average age of 38 weeks. Immediately prior to surgery, mice were injected with subcutaneous Buprenorphine XR (3.25 mg/kg) and anesthetized using 2-4% isoflurane. Burn tenotomy injuries were performed as previously described (Peterson et al. 2015). The left hind limb was harvested 6 weeks after surgery, fixed using 4% paraformaldehyde, and stored in 70% ethanol following fixation. Microcomputed tomography (μCT) images were obtained of the hindlimbs at 9 microns using a Bruker Skyscan 1176 imaging system. The images were then analyzed using Dragonfly analysis software, in which HO was manually identified and quantified for total volume at four regions **(Fig. 1A)** distal to the femorotibial joint: S1 represents floating HO bone proximal to the fibula-tibial junction; S2 represents anterior tibial HO bone; S3 represents floating HO bone distal to the fibula-tibial junction; S4 represents calcaneal associated HO bone. Statistically significant differences were assessed using two-way ANOVA.

RESULTS: We characterized quadricep tendon exostoses using a blinded observer-based scoring system in WT and DKO using longitudinal x-rays. Over time WT mice also developed exostoses; however, DKO developed exostoses in the tendon much earlier (at 8 weeks) and to a greater extent in comparison to the WT, which did not develop quadricep exostoses until 20 weeks of age (data not shown). DKO mice had a statistically higher exostosis score than the WT at 60 weeks (.75 WT vs 1.5 DKO), 72 weeks (1.0 WT vs 2.0 DKO), and 84 weeks (.75 WT vs DKO 2.0) (data not shown). To understand injury induced HO bone formation, we compared WT and DKO mice using a well-characterized burn tenotomy model. Neither WT nor DKO mice showed appreciable S1 or S2 HO bone. Both had S3 and S4 bone present. The amount of S3 HO bone did not differ between WT and DKO, but WT showed much greater S4 bone formation, in both males and females **(Figure 1**).

DISCUSSION: Surprisingly, considering that mice lacking both TSP1 and TSP2 develop idiopathic extraosseous bone in the quadricep tendon, the DKO mice were less prone to developing injury-induced HO associated bone. This data suggests that TSPs may play a protective role in the development of injury-induced HO associated bone. One possible explanation for this is that TSPs are anti-angiogenic and perhaps in the absence of TSP1/2 there is increased vascularity which contributes to decreased HO. However, this is not consistent with Cocks et al. (2017) and Lin et al. (2022) in which increased blood vessel number and angiogenic factors were associated with more HO. Another potential explanation for the reduced HO in the absence of TSP1/2 is the role of TSPs in regulating inflammation. Inflammation is a hallmark of HO (Ranganathan et al., 2016). TSPs play an immunomodulatory role through interactions with the CD47 receptor, and could promote an inflammatory state that supports HO. Therefore, in the absence of TSPs there could be an altered inflammatory composition, decreasing HO. This will require further investigation and currently we are using TSP1 and TSP2 floxed mice to explore HO development in a temporal and cell-type specific manner.

SIGNIFICANCE/CLINICAL RELEVANCE: HO is a painful and intractable clinical problem for individuals that experience musculoskeletal polytrauma. These results support the development of new therapeutics. For instance, inhibitors of TSP1/2, may be efficacious at limiting HO formation.

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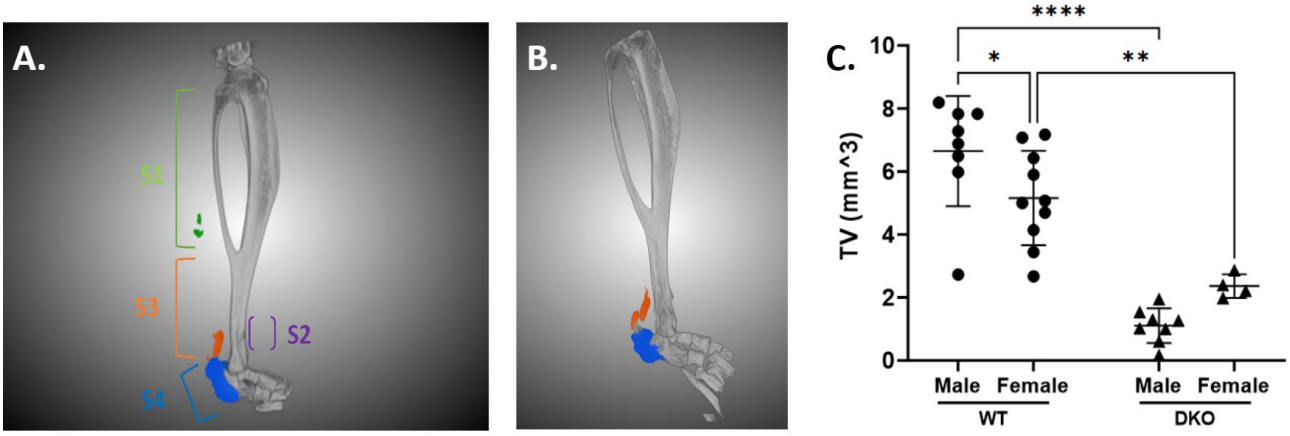

Figure 1. In the absence of TSP1/2 there is a reduction in HO following burn tenotomy. A and B. μCT reconstructions of representative limbs from (A) WT with burn tenotomy injury showing the different Segments used for analysis (S1-S4) **(B**) DKO with burn tenotomy. (**C**) total volume of S4 region bone in WT vs DKO male and female mice following burn tenotomy. *=p<0.05; **=p<0.01; ****=p<0.0001