The Potential Role of TGF-β Signaling on the Skeletal Stem Cell and Tendon Enthesis Healing

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INTRODUCTION: Tendons are important musculoskeletal tissues which function to transfer forces from muscle to bone, allowing movement of the skeleton. One critical component of the tendon is the enthesis, or tendon-to-bone interface (TBI) which specifically transmits forces between the bone and tendon. Following injury, the TBI will not be able to fully regenerate, and the tendon will never be able to restore the mechanical and structural properties which existed pre-injury. The mouse skeletal stem cell (mSSC) and its downstream progenitor the bone cartilage and stromal progenitor (mBCSP) have previously been shown by our group to participate in TBI healing and downregulation of TGF-β signaling pathway elements after injury in wild-type C57BL/6 mice[1]. To further investigate this finding, we interrogated if functional modulation of TGF-β via a transgenic mouse with a *Fibrillin-1* mutation alters mSSC response and TBI healing. This mouse was chosen because it is known to have endogenously increased levels of active TGF-β, as a result of a mutation of the *Fibrillin-1* protein which normally binds TGF-β and controls its bioavailability. This mouse is additionally clinically relevant as mutations in the fibrillin protein lead to Marfan's Syndrome, a heritable, autosomal dominant connective tissue disorder leading to cardiovascular, ocular, and musculoskeletal defects. Understanding the mSSC response and role of TGF-β can provide novel approaches for regenerative tendon healing.

METHODS: 24 transgenic *Fibrillin-1* mice were used in this study. To test Achilles healing, we created a partial laceration at the Achilles TBI. Sham surgery, consisting of skin incision and closure without disruption of tendon, was performed contralaterally. Mice were treated via local injection adjacent to the TBI with DMSO (vehicle control) or SB43154 (a TGF-β inhibitor). Treatment was given at the time of surgery and then every 48 hours until euthanasia. Mice were euthanized 7 days or 14 after injury (POD7, POD14). Gross evaluation was performed to determine the quality of healing, while mSSC and mBCSP profiles in the Achilles TBI were measured using fluorescence-activated cell sorting (FACS). Results were reported as fold-change of mSSC and mBCSP cell counts when compared to sham surgery. Statistical analysis was performed using GraphPad Prism v.6. Data analysis was performed using unpaired t-test assuming 2-tailed distribution. Statistical significance was assigned for $P \leq 0.05$. All mouse procedures were approved by Stanford's APLAC.

RESULTS: Using 24 transgenic *Fibrillin-1* mice, we observed a repressed mSSC and mBCSP injury response and poor wound healing, similar to that previously seen in TBI injuries treated with exogenous TGF-β in wild-type mice[1]. In terms of cell frequency counts, *Fibrillin-1* mice treated with DMSO (n = 2 pooled per datapoint, with 3 replicates per timepoint) did not mount a meaningful mSSC response, with a fold change of 0.5 ± 0.3 at POD7 and 1.0 ± 0.4 at POD14 compared to sham TBI. mBCSP frequency counts after treatment with DMSO are similarly low, with a fold change of 0.8 ± 0.9 at POD7 and 1.3 ± 0.5 at POD14. However, this phenotype is rescued by post-injury application of the small molecule TGF-β inhibitor SB43154 (n = 2 pooled, with 3 replicates). Both the mSSC and mBCSP populations experience a frequency increase after injury in the SB43154-treated *Fibrillin-1* mice when compared to the untreated mice (Figure 1A and 1B). In SB43154-treated mice, mSSC counts significantly increased from POD7 to POD14 (1.0 ± 0.5 and 3.0 ± 1.4, respectively) (*P < 0.05) (Figure 1A). mBCSP frequency significantly increased in the treated condition between POD7 and POD14 (0.6 ± 0.5 and 4.5 ± 2.1, respectively) (*P < 0.05). The difference between mBCSP frequency in mice treated with DMSO and SB43154 was also significantly increased in the SB43154 condition at POD14 (1.3 ± 0.5 DMSO, 4.5 ± 2.1 SB43154) (*P < 0.05) (Figure 1B).

DISCUSSION: Studies of TBI injury in a *Fibrillin-1* mouse support the hypothesis that fibrillin's interference with TGF-β, and the resultant increase in TGF-β bioavailability, leads to a decrease in mSSC and mBCSP response. FACS analysis shows that this reduction in response is rescued when *Fibrillin-1* mice are treated with small molecule TGF-β inhibitor SB43154 after injury. This study provides key insight into the potential role of TGF-β in TBI healing. Upregulating the mSSC and mBCSP response by modulating TGF-β signaling could improve the speed and quality of healing from TBI injuries clinically.

SIGNIFICANCE/CLINICAL RELEVANCE: This study builds on our group's previous work to further elucidate the role of TGF-β signaling on TBI injury response and the specific contribution of the mSSC and mBCSP to TBI healing. This information can be further used to design therapeutics to promote tendon regeneration.

REFERENCES:

1. Titan, A. L. *et al.* Partial Tendon Injury at the Tendon-to-Bone Enthesis Activates Skeletal Stem Cells. *Stem Cells Transl Med* **11**, 715 (2022).

IMAGES AND TABLES:

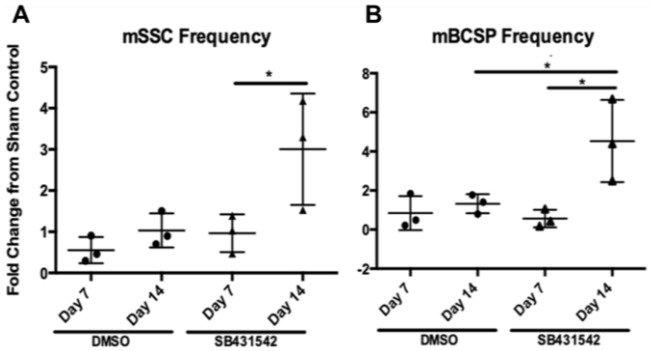

Figure 1. Quantification of cellular frequency of FACS isolated-mSSC (A) and mBCSP (B) demonstrating a significantly increased response with SB431542 treatment in *Fibrillin-1* mice. Each datapoint is 2 mice pooled, mSSC and mBCSP for corresponding conditions were FACS-isolated from the same mouse (n = 24 mice total). Data and error bars shown as mean ± STD. *P < 0.05, unpaired two-tailed t-test.