Leveraging Hedgehog Signaling to Improve Tendon-to-Bone Repair

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Introduction

- Pivotal growth and development studies have elucidated key mechanisms required for the formation of a zonal tendon-to-bone insertion site (i.e., enthesis) [1-4].
- Studying these mechanisms during repair in the adult has been difficult because traditional tendon-to-bone repair surgeries do not recreate a zonal insertion.
- Ligament injuries reconstructed with a tendon graft passing through a bone tunnel can result in zonal attachments at the tunnel-bone marrow interface [5,6].
- We recently demonstrated that bone marrow-derived mesenchymal progenitor cells, which express α-smooth muscle actin (αSMA) during the early stages of repair, are the primary contributors to tunnel integration following anterior cruciate ligament (ACL) reconstruction [5].
- Targeting key signaling pathways in these cells may provide insights into mechanisms of tendon-to-bone repair.
- Hedgehog (Hh) signaling regulates zonal enthesis formation during growth and development [1].
- We hypothesize that Hh signaling regulates zonal tendon-to-bone repair in ACL reconstructions.

OBJECTIVES

The objective of the current study is to genetically and pharmacologically modulate Hh signaling to measure the effect of hedgehog signaling on tunnel integration following ACL reconstruction.

Methods

Transgenic Mice

All animals and procedures were approved by UPenn's IACUC. Four transgenic mouse lines were used in this study:

- **αSMACreERT2** – Smooth muscle α-actin promoter driving Cre expression upon tamoxifen administration [7]. These mice target the amplifying progenitor pool that gives rise to zonal attachments [5].
- **Ai9 R26R-tdTomato** – Cre reporter construct with tdTomato expression in the Rosa26 locus [8].
- **RosaSmoM2** – Constitutive smoothened (Smo) expression is activated in Cre-expressing cells [9].
- **Smo^{flox}** – loxP sites flanking exon 1 of the Smo gene resulting in conditional deletion in Cre-expressing cells [10].

αSMACreERT2;R26R-tdTomato mice were crossed with either RosaSmoM2 (**SmoCA**) or Smo^{flox} (**SmoKO**) mice to yield triple transgenic mice. Cre recombination was induced by intraperitoneal tamoxifen injections every other day after surgery for 5 days. Demeclocycline was given 1 day before sacrifice to label depositing mineral and help define mineralized fibrocartilage (MFC) zones of attachments.

Experimental Design

ACL reconstructions (ACLR) were performed on 39 transgenic mice. *Pharmacologic Hh Activation Study*: CD1 mice received either Hh agonist (Hh-Ag1.5) or PBS injections intraperitoneally 5X per week and were assigned to cryohistology (n = 4/group) or biomechanics (n = 5-6/group) at 28 days post-surgery. *Genetic Hh Activation Study*: SmoCA mice with increased Hh signaling and Cre-negative control mice were sacrificed 28 days post-surgery and assigned to cryohistology (n = 4-6/group). *Genetic Hh Inhibition Study*: SmoKO mice with decreased Hh signaling and Smo$^{flox/flox}$ control mice were sacrificed at 28 days post-surgery and assigned to cryohistology (n = 5/group).

ACL Reconstruction

The ACL was excised near the femoral insertion. The tibial and femoral tunnels were drilled with 27G needles. A tail tendon autograft was passed through the tunnels and anchored to outer cortices with stainless steel washers [5,6].

Multiplexed Mineralized Cryohistology

Knees were fixed in formalin, embedded, sectioned undecalcified with cryofilm, and imaged on the Zeiss Axio Scan.Z1.

Tunnel Pullout Test

Femurs were potted in PMMA and gripped such that the tunnel was parallel to the linear actuator. A suture was passed through the washer and loaded to failure

Image Analysis

The area of MFC was normalized to tunnel length within the section using ImageJ to define the extent of zonal integration.

Statistics

Parameters were compared between experimental and control groups via Student's t-tests and presented as Mean±SD ($p < 0.05$).

Results

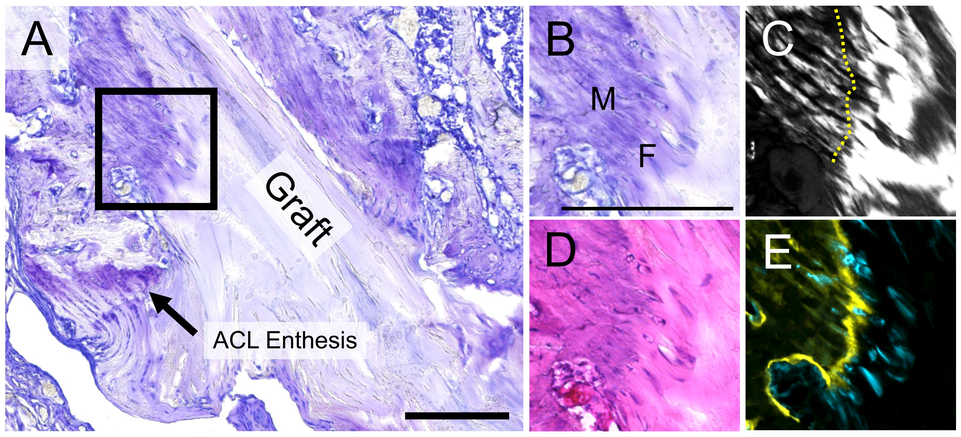

Fig. 1: Murine ACL reconstruction led to zonal attachment formation after 28 days. Toluidine blue staining of a femoral tunnel entrance with a zonal attachment (inset) and native transected ACL enthesis (A). The attachment had proteoglycan-rich mineralized (M) and unmineralized (F) fibrocartilage zones (B) with collagen fibers (C) spanning a tidemark (yellow dotted, E). Scale bars = 200μm.

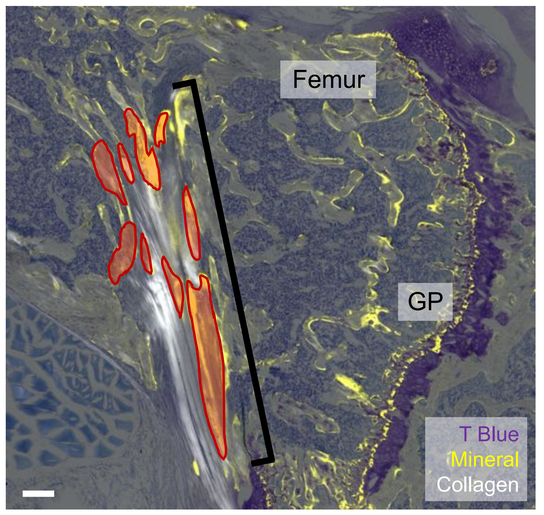

Fig. 2: Mineralized fibrocartilage quantification in bone tunnels. Sagittal sections of the femoral tunnel were used to quantify MFC area (red) based on mineral label (yellow) and aligned collagen fibers (white). Area measurements were normalized by the length of the tunnel (black). Scale bar = 200μm.

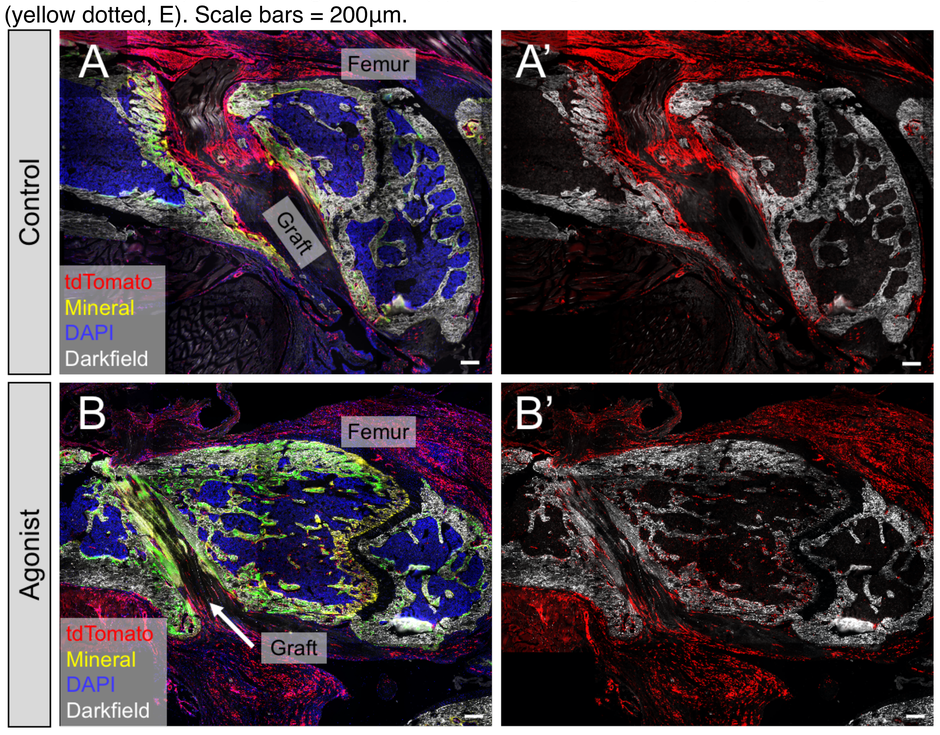

Fig. 3: Hh agonist treatment increased MFC formation in zonal attachments 28 days post-surgery. Sagittal femoral tunnel sections showed mineralization in the tunnels after 28 days of PBS (A) or Hh agonist (B) administration, with cells contributing to the repair process labeled in red (A' and B'). Agonist-treated mice had 70% greater MFC area than PBS controls ($p = 0.01$, C). Scale bars = 200μm.

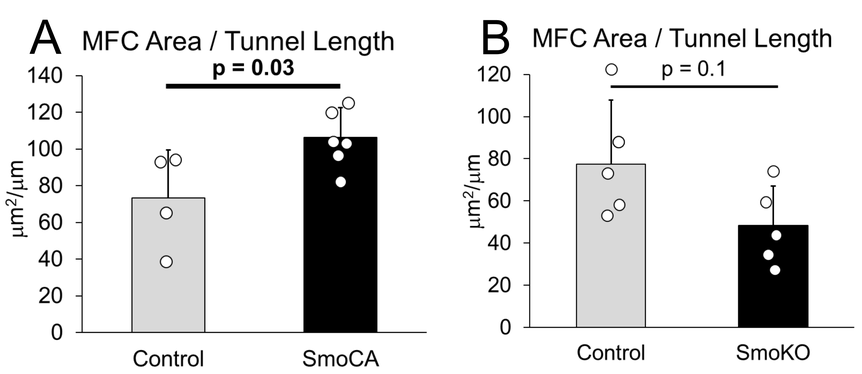

Fig. 4: Hh signaling within αSMACreERT2-lineage cells positively regulated MFC formation in zonal attachments 28 days post-surgery. Increased Hh signaling in αSMACreERT2-lineage cells led to 45% greater MFC area compared to control ($p = 0.03$, A) while decreased Hh signaling in these cells led to a trending 40% decrease in MFC area ($p = 0.1$, B).

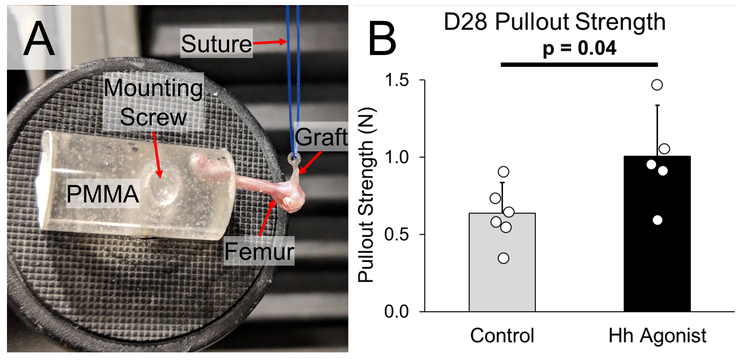

Fig. 5: Hh agonist treatment yielded improved tunnel pullout strength 28 days post-surgery. Femurs were mounted in PMMA while a suture was attached to the washer on the femur as well as the actuator of the testing machine (A). The washer was pulled until the graft was removed from the tunnel. Agonist-treatment yielded a 60% greater pullout strength than PBS controls ($p = 0.04$, B).

Discussion

- We demonstrated that Hh signaling positively regulated mineralized fibrocartilage formation during the tunnel integration process following ACLR (Figs. 3 and 4) and led to a better functional outcome with an increased pullout strength (Fig. 5).
- While Hh signaling improved fracture repair via improved osteogenesis and angiogenesis [11], here we found that Hh improved tendon-to-bone attachment formation with increased levels of mineralized fibrocartilage similar to enthesis development.
- Future studies will determine whether this Hh signaling-induced increase in mineralized fibrocartilage is due to an elevated expansion of the initial progenitor pool resulting in more cells capable to differentiate or if Hh signaling improves fibrochondrogenic differentiation of these progenitor cells.

References

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