Timing Of Systemic Hedgehog Agonist Delivery Differentially Modulates Progenitor Proliferation and Fibrocartilage Formation During Tendon-To-Bone Healing

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INTRODUCTION: Tendon and ligament injuries affect nearly 30% of U.S. adults and often involve the enthesis, a graded fibrocartilaginous interface essential for load transfer. Surgical repair, such as rotator cuff repair, frequently fails to restore the native zonal architecture, leading to fibrous scar and high re-tear rates. Notably, ACL reconstruction (ACLR) models can recapitulate zonal enthesis-like attachments between the tendon graft and bone in the bone tunnels, largely driven by αSMA+ bone marrow–derived progenitors, which give rise to these zonal fibrocartilaginous tendon-to-bone attachments[1]. Hedgehog (Hh) signaling is a critical regulator of fibrocartilage formation during enthesis development, and similarly, it enhances zonal tendon-to-bone attachment formation in the bone tunnels following ACLR[2,3]. However, the specific mechanism for this enhancement is unknown; specifically, whether these benefits were due to Hh promoting expansion of the progenitor pool or promoting fibrocartilage differentiation. Here, we test the temporal role of Hh signaling by activating the pathway during the early proliferative or later fibrocartilage maturation phases following ACLR.

METHODS: All animals and procedures were IACUC approved. Experimental Design: We used a murine ACLR model to examine the temporal role of Hedgehog (Hh) signaling. αSMACre^{ERT2};R26R-tdTomato mice received systemic Smoothened agonist (SAG, 20 mg/kg) or vehicle at defined post-surgical intervals. Early treatment targeted the proliferative phase (days 0, 2, 4, 6), while late treatment targeted fibrocartilage mineralization (days 14, 16, 18, 20). To label αSMA+ progenitors, tamoxifen (90 mg/kg) was given on days 0, 2, and 4, and EdU (3 mg/kg) was administered on days 0, 2, 4, and 6 to map the fate of these proliferating cells. All mice were euthanized at day 28 post-ACLR. ACL Reconstruction: Unilateral ACL reconstructions were performed on a total of 40 mice (mean ± SD age, 13 ± 1.3 weeks old; 22 males and 18 females) as previously described[1,3]. Multiplexed mineralized cryohistology to assess tunnel integration: Tissue sections underwent three sequential rounds of staining and imaging on a Zeiss Axio Scan.Z1. First, calcein blue (CB, 10 min) and Sytox Green (SG) were applied, with imaging for deposited mineral (CB), αSMA-lineage tdTom+ cells, nuclei, and polarized light for collagen. Second, the sections were decalcified and stained for alkaline phosphatase (ELF97) and EdU (Alexa Fluor 647), and imaged. Finally, sections were stained with toluidine blue (0.025%) and imaged under brightfield for morphology. Bone tunnel histomorphometry: Images were analyzed in FIJI (ImageJ). ROIs encompassing the bone tunnel (tendon graft + mineralized tissue, excluding adjacent bone) were segmented using polarized light and toluidine blue to define graft–bone boundaries. Percent area positive for calcein blue (CB) and alkaline phosphatase (AP) was quantified, and mineralized fibrocartilage (MFC) was segmented from CB-positive regions within the tunnel (excluding mineral in the bone). For cell quantification, a nuclear mask was applied with the "Analyze Particles" function, redirecting to RFP (tdTomato) and Cy5 (EdU) channels using a common threshold. Statistics: Statistical analyses were performed in GraphPad Prism 9.2.0. Outliers were removed using the ROUT method (Q=1%). Group differences were assessed by one-way ANOVA with Tukey's post-hoc tests ($p<0.05$).

RESULTS: Early delivery of SAG does not impact MFC formation, whereas late delivery results in a trending decrease in MFC formation. While MFC area (CB) was unchanged in most groups, a trend toward reduction was observed in the SAG late group versus control (**Fig. 1B**, p=0.066). AP intensity remained unchanged across groups (**Fig. 1C**). Early SAG delivery leads to an increased number of EdU+ cells in the tunnel and the MFC at day 28. EdU+ cell density was significantly higher in the SAG early group compared to control early, control late, and SAG late ($p<0.05$) (**Fig. 2B**). Within the MFC, EdU+ density was likewise increased in SAG early relative to all groups (**Fig. 2C**). Early SAG leads to an increased number of EdU+ cells within the αSMA-lineage in the tunnel as well as in the MFC at day 28. In the tunnel, EdU+/tdTomato+ cell density was significantly higher in SAG early versus all other groups (**Fig. 2D**, p=0.024 vs control early, p=0.015 vs control late, p=0.006 vs SAG late). In the MFC, double-positive cell density was also elevated in SAG early vs all other groups (**Fig. 2E**, p=0.044 vs control early, p=0.004 vs control late, p=0.039 SAG late). Late SAG did not alter the fate of EdU+ cells labeled in week 1.

DISCUSSION: Our findings demonstrate that Hedgehog pathway activation exerts distinct, time-dependent effects on the cell populations contributing to the zonal tendon-to-bone attachments following ACLR. Early SAG delivery resulted in an increased percentage of EdU+ cells in both the bone tunnels and MFC at day 28. Given that this was also true in the αSMA-lineage, these results suggest that Hh activation during the early stages of the tunnel integration process increases proliferation of the progenitor populations that give rise to MFC at later stages of the integration process. On the other hand, later delivery of SAG resulted in a trending decrease in MFC deposition, suggesting that this delivery dose and timing may actually impair the healing response. In fact, delayed SAG delivery may disrupt the progression from proliferation to differentiation needed for tendon-to-bone integration, which is contrary to our hypothesis. Despite robust cellular effects, neither treatment altered mineral deposition or alkaline phosphatase activity, likely due to the short dosing windows compared to our previous studies[3]. These results underscore the importance of temporal regulation of Hh signaling and point to sustained-release strategies as a means to optimize progenitor expansion and matrix production.

SIGNIFICANCE/CLINICAL RELEVANCE: This work further defines the therapeutic potential of Hh signaling agonism in promoting tendon-to-bone integration. In particular, these findings identify a key therapeutic window for SAG delivery that we can further optimize to improve surgical repair outcomes.

REFERENCES: 1. Kamalitdinov, JOR, 2020; 2. Dyment, Dev Biol, 2015; 3. Kamalitdinov, OAC, 2023.

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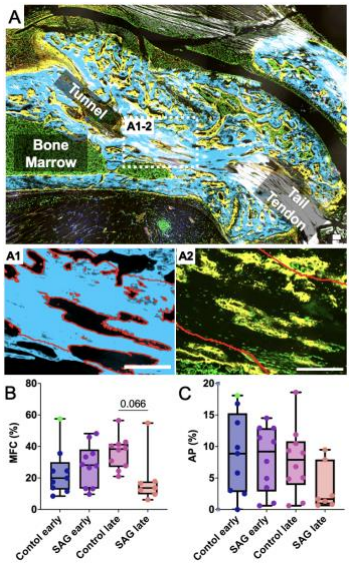

Figure 1. (A) Calcein blue (mineral deposition) and AP (mineral activity) staining. (B) MFC area showed no significant differences, with a trend toward reduction in SAG late. (C) AP intensity was unchanged across groups.

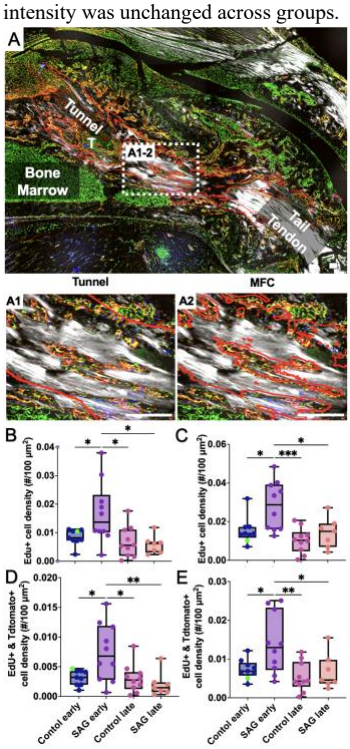

Figure 2. (A) Example AP and EdU-stained image (green dot in plots) with tunnel (A1) and MFC (A2) ROIs outlined. (B–C) SAG early increased EdU+ cell density in both tunnel and MFC ($p<0.05$). (D–E) Double-positive (EdU+/tdTomato+) density was also elevated in SAG early versus all other groups in both regions ($p<0.05$).