The Role of Hedgehog Signaling in Enthesis Healing

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Introduction: The mature tendon-to-bone attachment (the “enthusis”) heals via a scar-mediated process that does not recapitulate the developmental program that produces enthesis fibrocartilage. In contrast, injuries sustained in utero heal through a regenerative process and musculoskeletal injuries in young animals and children heal more readily than in adults [1]. Enthesis injuries are typically accompanied by a significant decrease in the mineralized tissue within and underlying the tendon attachment site resulting in poor mechanical behavior of the healed tissue [2]. The Indian hedgehog signaling pathway is one of the master regulators of endochondral bone formation. We recently identified a population of cells positive for Gli1, a transcription factor that indicates activated Hedgehog signaling (Hh), in the neonatal enthesis that are required for the development of mineralized fibrocartilage in the enthesis [3]. The aims of the current study were to investigate potential differential regulation of Hedgehog signaling in enthesis injuries occurring in animals with immature entheses and mature entheses and to determine the possible involvement of Gli1+ progenitor cells in the healing process. To this end, we developed an enthesis defect model that was applied to neonatal through adult mice and used this model to probe enthesis healing in a Hh reporter mouse model.

Methods: The use of animals was approved by the animal studies committee at Washington University. To evaluate Hh pathway activity in tendon healing, Gli1-CreERT2 mice [4] were crossed with Rosa26-mT/mG mice (Jackson Labs) to enable fluorescent identification of Cre positive cells. Animals underwent a unilateral fibrocartilage injury at 1 wk (immature enthesis group, N=22) or 6 wks (mature enthesis group, N=23). A small incision was made in the right shoulder. The limb was externally rotated to bring the supraspinatus insertion into the surgical field. Using the acromial branch vessel as a landmark for the lateral acromion to locate the supraspinatus attachment, a 28 G needle was inserted into the humeral head to make a punch defect into the enthesis, which completely bisected the mineralized fibrocartilage into the marrow cavity. The skin incision was closed using a 6.0 proline suture and animals were allowed to heal for 1 or 3 weeks. Mice were injected with 100-200 μg/g body weight tamoxifen (TAM) to probe Hh activation in a temporally controlled manner. Injections were performed on P4 to label the Gli1+ enthesis progenitor population prior to injury or 3, 7, or 14 days post injury to see if Hh signaling was activated after injury. Animals were euthanized 1 or 3 weeks after injury. Enthesis healing was evaluated by micro-computed tomography (microCT) (Scanco) and histology.

Results: MicroCT was used to identify the bony defect and healing was visually evaluated at 3 weeks. The immature enthesis group resolved more of the bony defects than the mature group (Figure 1). To investigate the role of the Gli1+ cell population in enthesis healing, mice were labeled with TAM on P4 and injured on P7 (immature) or P42 (mature). Gli1+ (green) cells were observed lining the defect site in the immature group while very few cells from the original Gli1+ lineage were observed in the mature defect sites (Figure 2). Next, we used this model to investigate activation of Hh signaling during tendon healing. Immature and mature mice were labeled with TAM 3, 7 or 14 days post injury and tissues were harvested 3 weeks post injury. When immature entheses were labeled with TAM 3 days post injury,
Gli1+ cells were observed lining the defect site and throughout the enthesis (Figure 3A”, B”). In mature entheses, hardly any Gli1+ cells were evident in the injured enthesis relative to basal levels in the contralateral control limb (Figure 3C”, D”).

**Discussion:** These results suggest that the Gli1+ cell lineage critical for mineralized fibrocartilage development participates in enthesis remodeling when injury is sustained during early postnatal development. In the mature enthesis, this cell lineage is present but not Hh active, and these cells are no longer able to participate in repair and remodeling post injury. In immature entheses, the number of Hh-responsive cells is not affected during the healing process. In mature entheses, the number of Hh-responsive cells is reduced during the healing process. This suggests that healing occurs via different mechanisms in mature vs. immature entheses. The decrease in the size of the Gli1+ cell population at the mineralization front in mature entheses was associated with a decrease in mineralization and impaired healing the injury compared to younger animals.

**Significance:** Enthesis fibrocartilage does not readily regenerate or heal after rotator cuff injury or anterior cruciate ligament reconstruction and is prone to age-related degeneration. Understanding the biological factors involved in fibrocartilage healing and their differential responsiveness during development vs. maturity may inspire novel therapeutics. Delivery of relevant factors or cell types to the adult injury may improve tendon enthesis healing.
**Figure 1:** Enthesis healing evaluated 3 weeks post injury by microCT. “Not healed” indicates a visible bony defect. $p=0.006$, Fisher’s exact test.
Figure 2: Gli1CreERT2;Rosa-26 mTmG mice were injected with TAM on P4 and injured on P7 (A, A') or P42 (B, B'). Controls for each time point are shown in (C, C') and (D, D') respectively. (A-D) microCT reconstructions used to visualize enthesis mineral defects. The color scale indicates mineral density (red = high, blue = low). (A'-D') Frozen sections showing Hh-responsive cells (green), all remaining cells (red), and nuclei stained with DAPI (blue). Scale is 100 μm. Injury site is indicated with a white circle.

Figure 3: Gli1CreERT2;Rosa-26 mTmG mice were injured on P7 (A, A', A'') or P42 (C, C', C'') and injected with TAM 3 days after injury. Controls for each time point are shown in (B, B', B'') and (D, D', D'') respectively. (A-D) microCT reconstructions used to visualize enthesis mineral defects. The color scale indicates mineral density (red = high, blue = low). (A'-D') Cut planes from microCT reconstructions. (A''-D'') Frozen sections showing Hh-responsive cells (green), all remaining cells (red), and nuclei stained with DAPI (blue). Scale is 100 μm. Injury site is indicated with a white circle.