Hedgehog signaling promotes meniscus growth and accelerates meniscus repair after injury

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INTRODUCTION: Meniscal tears are the most common injuries of the knee affecting young and aged populations. Gli1, an integral effector of the Hedgehog (Hh) signaling pathway, was recently identified as a marker of mesenchymal progenitors in bone and cartilage. We previously identified a Gli+ mesenchymal progenitor population in the meniscus and demonstrated their critical role in meniscus development and tissue repair using a lineage tracing approach (1). However, whether Hh signaling pathway is involved in meniscus health and regeneration is largely unknown. Hh ligands bind to the receptor Patched1 and release its inhibition on another membrane protein, Smoothened (Smo). Active Smo then promotes the downstream signaling through the Hh pathway. Here, we constructed two mouse models possessing either constitutive activation of Smo *(Smo CA*) or depletion of Smo (*Smo CKO*) specifically in Gli1+ cells. With these novel tools, we carried out complementary studies to query the role of Hh signaling in meniscus development and injury repair.

METHODS: *Animals*- All animal work was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. *Gli1-CreER Rosa-tdTomato (Td)*, *Gli1-CreER Rosa-Smo/Td*, and *Gli1-CreER Smoflox/flox Td* mice were independently bred as *WT, Smo CA, and Smo CKO, respectively*. Both males and females were used because of same phenotypes. Mice received tamoxifen (Tam) injections (75 mg/kg/day) for 5 days to modulate Hh signaling. Meniscus injury was performed on the right knees of 2-month-old mice. The joint capsule was opened, and the anterior horn of medial meniscus was bisected using microsurgical scissors. *RNA analysis*- Menisci were homogenized in Tri reagent for RNA purification and real-time RT-PCR. *Histology*- For fluorescence imaging, knee joints were processed for frozen sections using a cryofilm approach. For morphological imaging, knee joints were processed for paraffin sections and Safranin O/fast green staining. Sections corresponding to the center of anterior horn of medial meniscus were selected to measure meniscus size and count cells. Meniscus repair scores were blindly calculated according to the connection between two ends, existence of fibrochondrocytes in the repair tissue, and intensity of Safranin O staining (2). *CFU-F analysis*- Menisci were digested in 0.25% Trypsin-EDTA for 0.5 hr and 300 U/mL Collagenase Type I (Worthington Biochemical) for 2 hr. Cells collected from the second digestion were seeded in growth medium at 10,000 cells per well in a 6-well plate for 2 weeks. *Statistics*- Data are expressed as means ± SEM and analyzed by t-tests or one-way ANOVA using Prism.

RESULTS: At 2 weeks post Tam injections, real-time RT-PCR confirmed that expression of Hh signaling genes, including *Smo*, *Gli1*, and *Ihh*, was significantly increased in *Smo CA* meniscus and decreased in *Smo CKO* meniscus (Fig. 1A). To study the role of Hh signaling in development, we subjected 1-month-old *WT* and *Smo CA* mice to Tam injections. Four weeks later, the anterior horn area of *Smo CA* meniscus was 60% larger than that of *WT* meniscus, due to extended meniscus width (Fig. 1B). This change corroborates with an increase in meniscal cell number (Fig. 1C). A CFU-F assay of meniscal cells isolated 1 week after Tam injections showed a 35% increase in *Smo CA* mice compared to *WT* (Fig. 2A). Interestingly, at 2 weeks post Tam injections, *Gli1*/Td+ cells at the meniscus surface were expanded by 65% in *Smo CA* mice but reduced by 42% in *Smo CKO* mice (Fig. 2B, C), suggesting that Hh signaling regulates the proliferation of meniscal mesenchymal progenitors and controls meniscus tissue size. To study the role of Hh signaling in tissue repair, we subjected 2-month-old mice to Tam injections followed by meniscus injury at the anterior horn (Fig. 3A). In *WT* mice, transected meniscus ends showed partial synovial hyperplasia at 2 weeks post-injury (Fig. 3Ba, b). By 4 weeks, the gap had narrowed but still remained open (Fig. 3Ca, b). In *Smo CA* mice, the gap was filled with dense reparative tissue and many more Td^+ cells at 2 weeks (Fig. 3Bc, d), and the two ends were nearly connected by 4 weeks (Fig. 3Cc, d), resulting a highest repair score (Fig. 3D). In contrast, *Smo CKO* mice had the largest gap between two injury ends at 2 weeks (Fig. 3Be, f). By 4 weeks, the newly formed tissue at the gap site appeared more fibrous than in *WT* mice (Fig. 3Ce, f) leading to a lowest repair score (Fig. 3D). At both times, their repair site had fewer Td+ cells compared to *WT*. Furthermore, CFU-F assay revealed the highest total and Td+ colonies from *Smo CA* meniscus and the least total colonies and amost no Td+ colonies from *CKO* meniscus (Fig. 3E). These data suggest that Hh signaling amplifies *Gli1*+ meniscus progenitors to promote repair.

DISCUSSION: Meniscal repair in clinical practice still relies predominantly on surgical intervention. Procedures such as rasping and bone marrow stimulation can enhance repair by modulating progenitor cell behavior; however, the signaling pathways that regulate the key meniscus progenitor population remain poorly defined. Using both gain- and loss-of-function mouse models, our complementary data demonstrate for the first time that Hh signaling plays a regulatory role in meniscus mesenchymal progenitors, thereby promoting meniscus growth and injury repair. While promising, the current findings are based on limited sample size and time points. Future work will increase the number of animals and examine mice at later stages of meniscus development and tissue regeneration, as well as query the structure and mechanical properties of the forming repair tissue.

SIGNIFICANCE: Our studies uncover a critical role of Hh signaling in regulating meniscus progenitors.

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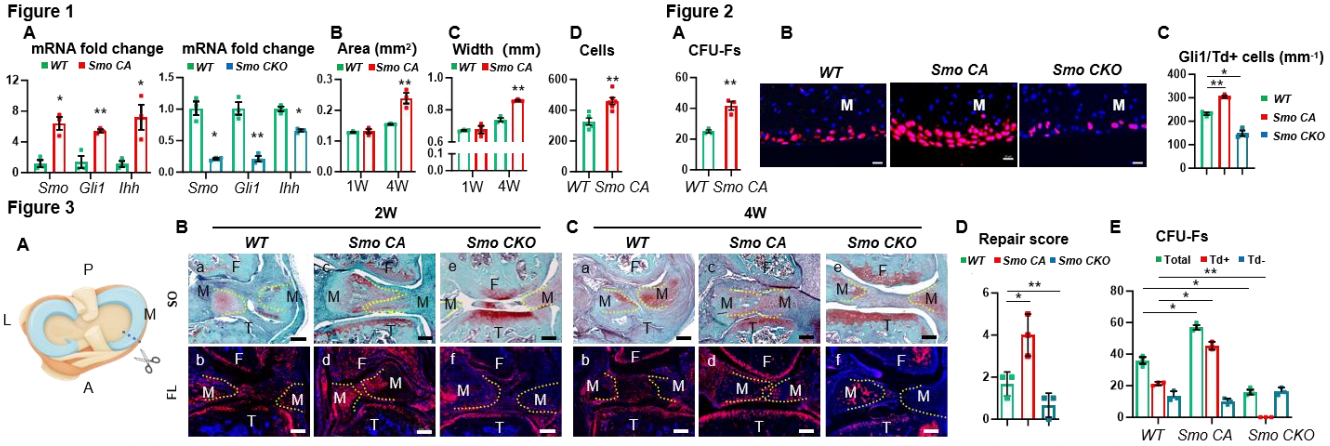

Figure 1. Hedgehog signaling is critical for meniscus development. (A) qRT-PCR analysis of Hh signaling genes in meniscus tissues from *Smo CA* and *Smo CKO* mice. Data are calculated from 3 independent experiments with n=5 mice/group. (B) Quantification of tissue area and width at the anterior horn of medial meniscus from *WT*, and *Smo CA* mice. Mice received Tam injections at 1 month of age and their knees were collected 1 and 4 weeks later for Safranin O staining. n=3-4 mice/group. (C) Joints from additional mice were collected at 4 weeks after Tam injections for DAPI staining to count meniscal cells at a similar location. n=3-4 mice/group. **Figure 2.** Hh signaling drives the expansion of Gli1+ progenitors at the meniscus surface. (A) CFU-F assay of meniscus cells from mice at 1 week post Tam injections. n=3 mice/group. (B) Fluorescence images of the anterior horn of medial meniscus from mice at 2 weeks after Tam injections. M: meniscus. Scale bar, 20 μm (C) Td+ cells along the meniscus surface were counted and normalized by surface length. n=3 mice/group. **Figure 3.** Hh signaling accelerates meniscus injury repair. (A) Schematic representation of meniscus injury. (B, C) Safranin O/Fast green staining (top) and fluorescence images (bottom) of mouse knee joints harvested at 2 (B) and 4 (C) weeks after injury. Dashed lines outline the meniscus. Scale bar, 200 μm. (D) The repair score was evaluated 4 weeks after injury. n=3 mice/group. (D) CFU-F assay of meniscus at 2 weeks after injury. Total colonies, Td+ colonies, and Td- colonies were counted. n=4 mice/group. *p<0.05; **: p<0.01; ***: p<0.001 vs *WT*.