A New Mouse Model to Understand Pathogenesis of Heterotopic Ossification in Tendon

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Introduction: Heterotopic Ossification (HO) is a common post-surgical or post-traumatic complication, causing abnormal ectopic formation of bone in extraskeletal soft tissues.1-2 The exact mechanism governing HO process is complex, involving the dysregulation of multiple molecular and biological processes.1 Our previous studies have shown that transplantation and then retention of cells with activated hedgehog (Hh) signaling leads to HO in tendon, which indicates the potential role of Hh signaling in driving HO formation.3-5 To further understand whether or how Hh signaling as a molecular mechanism regulates HO initiation and progression, the objective of this study is to build a new mouse model to induce HO in tendon by activating Hh signaling and track HO growth. We hypothesize that Hh signaling drives tendon HO by directing tendon cell transdifferentiation.

Methods: Through the Icahn School of Medicine at Mount Sinai IACUC, all animal procedures were approved. Animal Models: We used C57BL/6J mice to determine the effectiveness of Hedgehog Agonist (Hh-Ag1.5, Cellagen Technology) in inducing HO via over-activation of Hh signaling.3 Inducible Gli1CreERT2:Ai14 mice (Gli1, a Hh effector) were bred to determine the role of Hh-responsive cells during HO development.5 Heterotopic Ossification Model: HO was induced by injecting 7.6 mM Hh-Ag1.5 into the right Achilles tendons twice per week. The left Achilles tendons were injected with saline and served as controls. For the C57BL/6J mice, injections were performed from 9 weeks to 11 weeks, and were sacrificed at 14 weeks. For Gli1CreERT2:Ai14 mice, Hh-Ag1.5 injection was performed from 7 weeks to 9 weeks, and the mice were sacrificed at 14 weeks. Tamoxifen was administered for lineage-tracing right after Hh activation.3 µCT Analysis: Hindlimbs from the mice were dissected and analyzed by microCT (ScancoMed). The samples were imaged at a voltage of 70 kVp, current of 114 μA, voxel size of 4.9 μm per voxel, and a low resolution. The images were reconstructed and analyzed to determine bone volume and tissue mineral density. Paired t-tests were used to compare parameters for the control and Hh-activated groups. Histology: Tissue specimens were fixed overnight, decalcified, and embedded in OCT. The samples were sectioned and stained with Safranin O-Fast Green and imaged on a Zeiss AxioImager microscope. Immunohistochemistry (IHC). The sample sections were incubated with hyaluronidase, blocked with goat serum, and incubated with Sox9 antibody and corresponding secondary antibody, followed by image collection on a Leica DM6 microscope and analysis using Image J.

Results: In the C57BL/6J mice, mineralized tissue was found within all the samples that received Hh-Ag1.5 injection (Figure 1A). The average bone volume of the mineralized tissue was 0.2536 mm³ (Figure 1B). The average tissue mineral density of the mineralized tissue was 603.05 mgHA/cm³. There was a statistically significant difference in bone volume, as well as tissue mineral density between the control and Hh activation. Based on these results, we successfully created a Hh-induced HO model in vivo. The role of Hh-responsive cells during HO progression was evaluated using Gli1CreERT2:Ai14 lineage-tracing reporter mice. Consistently, mineralized tissue was found within tissue volume in 75% of the samples that received Hh-Ag1.5 injection (Figure 2A). There was no statistically significant difference in bone volume between the control and Hh activation, potentially due to insufficient sample size (Figure 2B). The average tissue mineral density of the mineralized tissue was 672.80 mgHA/cm³, which was significantly different from the control. Once the HO was located and quantified via µCT, histology was used to further confirm HO. The Safranin O-Fast Green stain showed presence of cartilaginous tissues within the right Achilles tendons of the mice after Hh activation that appeared to have come from µCT scans (Figure 3A). Gli1-lineage cells (red, Figure 3B) were identified in HO tissues. Additionally, Sox9 (green), a marker for chondrogenesis, was expressed within the HO region of Achilles tendons. Also, the HO regions had overlapped red and green signals, indicating that Gli1-lineage cells, expressing Sox9, contribute to chondrogenesis during HO pathogenesis.

Discussion: This study created a new in vivo animal model to demonstrate that Hh signaling plays an essential mechanistic role in HO progression. The advantage of this in vivo model is that no injury or trauma is involved in HO. However, surgical or post-traumatic complications can cause abnormal ectopic bone formation. In our model, the HO regions were formed within the Achilles tendon. This implies potential therapeutic targets for inhibiting heterotopically chondrogenic and ossified tissues in the context of post trauma or surgery.

Significance: Activation of Hh signaling induced HO tissue formation in the Achilles tendon, illustrating the importance of Hh signaling in HO pathogenesis. Our study supports that Hh signaling is a potential therapeutic target for inhibiting heterotopically chondrogenic and ossified tissues in the context of HO.