The role of Semaphorin 3A in neurovascular exclusion during postnatal enthesis development

Jonovan J. Osorio, Stavros Thomopoulos
Columbia University, New York, NY
jonovan.osorio@columbia.edu

Disclosures: The authors have no disclosures.

INTRODUCTION: Proper innervation is essential for regulating sensory and motor function throughout the musculoskeletal system. However, in adults, the attachment between tendon and bone (the enthesis) resides within a largely aneural and avascular niche.[1] The formation of this specialized microenvironment suggests the presence of local mechanisms that actively restrict neurovascular ingrowth during development. While nerves and blood vessels are known to play critical roles in the endochondral ossification of developing bone,[2] the biological link between innervation, vascularization, and enthesis formation remains poorly defined. One possibility is that, similar to articular cartilage, the absence of nerves and vasculature at the enthesis is necessary to minimize pain signaling or vascular-mediated mineralization in a region subjected to high mechanical stress. Therefore, the overall objective of this study was to characterize the spatial and temporal patterning of nerves and blood vessels during postnatal enthesis maturation and to identify molecular mechanisms that govern neurovascular exclusion. We used histological and transcriptional analyses to define the cellular communication networks that maintain the aneural, avascular niche at the developing tendon-to-bone interface.

METHODS: Mouse models: To monitor the expression of key collagen types during fibrocartilaginous enthesis maturation, triple-transgenic ColI/II/X[3] reporter mice were used for histological analysis (equal numbers of male and female). All protocols were approved by Columbia University's Institutional Animal Care and Use Committee (IACUC). Cryohistology: Mouse shoulders were harvested and fixed at postnatal days 11, 14, 18, and 56 (n = 4/age). To visualize the supraspinatus tendon-to-bone insertion, shoulders were sectioned in the coronal plane. All sections were made from undecalcified shoulders using cryofilm tape.[4] Immunofluorescence: Samples were incubated with anti-beta III tubulin (TUBB3) or anti-α smooth muscle actin (αSMA) antibody, then incubated in secondary antibody conjugated to the appropriate fluorophore, and mounted with DAPI mounting medium. Single-cell RNA sequencing data analysis: Data was acquired as described from a previous study.[5] The R package Seurat (v4.4.0) was used to load data and build Seurat objects, which were processed for controlling data quality, filtering, clustering, visualizing data, and examining differential expression analysis. Shared patterns of transcriptional profiles were highlighted by canonical correlation analysis and visualized by heatmaps and dot plots. To investigate nerve-associated signaling, the expression of neuronal genes was analyzed. The R package CellChat (v1.5.0) was utilized to investigate cell-cell communication.[6] Fluorescence *in situ* hybridization: Expression of *Sema3a* mRNA was probed following established manufacturer protocols (RNAscope, ACDBio) with minor modifications.

RESULTS: Immunofluorescence staining revealed that the tendon-to-bone interface was aneural and avascular throughout postnatal development. Prior to enthesis mineralization, at postnatal day 11, nerves and blood vessels were localized with ColX-expressing, mineralizing chondrocytes at the secondary ossification center of the humeral head (**Fig. 1A**). At postnatal days 14 and 18, corresponding to the early stages of enthesis mineralization, nerves and blood vessels were present exclusively within the trabecular bone (**Fig. 1B-C**). Similarly, at postnatal day 56, nerves and blood vessels were restricted to the trabecular bone (data not shown). Single-cell RNA sequencing of mouse enthesis tissue collected at postnatal day 11, 18, and 56 revealed that tendon progenitor and tenogenic cell populations expressed high levels of *Sema3a*, a nerve exclusion factor. High-affinity Sema3A co-receptors, neuropilins (Nrp1/2) and plexins (Plxnd1/Plxna2),[7] were differentially expressed within the endothelial cell population (**Fig. 2A**). CellChat analysis demonstrated robust Sema3A-Neuropilin/Plexin pathway activity between tendon and endothelial cells at early (P11, **Fig. 2B**) and mature (P56) stages, but not at P18 (data not shown). Fluorescence *in situ* hybridization confirmed *Sema3a* mRNA localization within the supraspinatus tendon at P11 (**Fig. 3**), P14, and P56.

DISCUSSION: Findings from histological and single-cell transcriptional analyses establish a spatiotemporal framework for neurovascular exclusion at the developing enthesis. Throughout postnatal maturation, nerves and blood vessels were restricted to the secondary ossification center and trabecular bone, coincident with endochondral bone formation at the humeral head epiphysis. In contrast, the supraspinatus tendon enthesis remained avascular and aneural, reflecting the establishment of its mature phenotype. At the transcriptional level, single-cell RNA sequencing and CellChat analyses identified Sema3A signaling as a potential mechanism by which the developing enthesis maintains an aneural and avascular niche. At postnatal days 11 and 56, tenogenic progenitors and tendon cells expressed *Sema3a*, while endothelial populations expressed its high-affinity receptors, suggesting paracrine communication between tendon and endothelial cell populations. This signaling pattern supports a model in which tendon-derived Sema3A restricts innervation and angiogenesis, preserving the aneural and avascular microenvironment of both developing and mature entheses. Previous studies have shown that Sema3A regulates both axonal guidance and endothelial patterning, supporting its role as a dual modulator of neurovascular organization.[8] Consistent with these functions, our findings suggest that tendon-derived Sema3A may act through Neuropilin/Plexin signaling to maintain the aneural and avascular niche of the developing enthesis. The observed changes in Sema3A-mediated crosstalk during postnatal maturation further imply that this pathway is dynamically regulated as the enthesis mineralizes. Together, these findings position Sema3A as a key regulator of neurovascular exclusion and provide a framework for investigating how its modulation could enhance tendon-to-bone healing. Future studies will aim to validate Sema3A-Neuropilin/Plexin signaling dynamics across postnatal and adult stages using spatial transcriptomics and targeted genetic perturbations *in vivo*.

SIGNIFICANCE/CLINICAL RELEVANCE: This study investigates a potential mechanism by which the tendon-to-bone interface maintains an aneural microenvironment during enthesis formation and maturation. Defining how Sema3A signaling regulates neurovascular exclusion provides insight into the molecular regulators of enthesis development and may inform therapeutic approaches to improve tendon-to-bone healing and repair strategies.

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ACKNOWLEDGEMENTS: This study was funded by NIH R01 AR080717 and T32 AR080744.

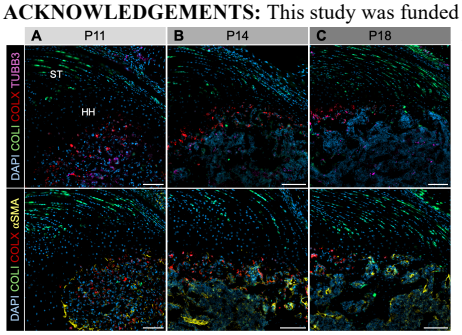

Figure 1: COLI, COLX, TUBB3, and αSMA expression at postnatal days (A) 11, (B) 14, and (C) 18. (ST: supraspinatus tendon, HH: humeral head). Scale bars = 100μm.

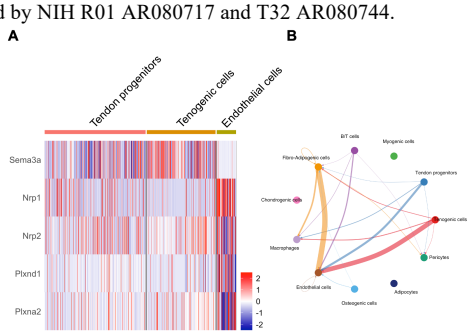

Figure 2: Transcriptomes of supraspinatus enthesis cells by scRNA-seq. (A) Heatmap of *Sema3a* and its receptors among tendon progenitors, tenogenic, and endothelial cell populations and (B) CellChat analysis of the SEMA3 signaling pathway at postnatal day 11.

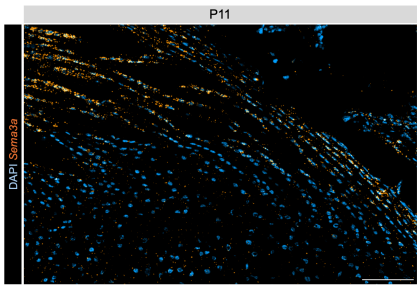

Figure 3: Spatial distribution of *Sema3a* at postnatal day 11 using fluorescence *in situ* hybridization. Scale bar = 100μm.