TrkA^{+} Neurons Trigger Bony Bar Formation Within the Growth Plate Injury Site

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INTRODUCTION: Injuries to the growth plate constitute a notable 18%-30% of all fractures in children, frequently leading to compromised bone growth. Once damaged, growth plate cartilage is usually replaced by unwanted bony tissue. This "bony bar" can impose limitations on bone growth in children, potentially resulting in disparities in limb length and angular deformities. The underlying mechanisms responsible for this unfavorable bony repair process remain inadequately understood. Previous studies have indicated that experimental growth plate injuries prompt the expression of neurotrophins such as Nerve growth factor (NGF). Recent studies from our laboratory have demonstrated the essential role played by NGF-TrkA (Tropomyosin receptor kinase A) signaling and skeletal innervation in response to experimental injuries of adult mouse bone, including fracture healing, and segmental bone defect repair. This work has led to the entirely new hypothesis that skeletal TrkA+ sensory neurons may regulate tissue response to growth plate injury.

METHODS: All experiments were conducted with the approval of IACUC at the Johns Hopkins University. A drill-hole growth plate injury model was created in the distal femur of 4-week-old male C57BL/6 and NGF-eGFP reporter mice to model bony bar formation at the site of growth plate injury. The evaluation of bony bar formation took place at 1- and 6-week intervals post-injury using high-resolution µCT scans and histological staining. Immunofluorescent staining (IF) for Beta III Tubulin (TUBB3) and CD31 was employed to assess innervation and angiogenesis within the experimental injury site of mouse growth plate, as well as in the human epiphysis fracture samples. Subsequently, a chemical-genetic approach to temporally inhibit TrkA signaling was achieved in 4-week-old male TrkA^{F592A} mice with 1NMPP1 treatment, and its effects on bony bar formation were evaluated by µCT analysis, histological and IF staining (TUBB3, CD31). Conditioned medium from dorsal root ganglia (DRG) was isolated and ex vivo epiphysis organ culture was conducted with or without DRG Conditioned medium. EdU incorporation assay and Runx2 immunostaining were performed at 1-week post-culture. Single-cell RNA sequencing (ScRNA-Seq) analysis of an established scRNA dataset of mouse long bone was performed to investigate inhibitors of peripheral neurogenesis in the physis, with subsequent verification by IF staining.

RESULTS SECTION: µCT analysis and histological staining revealed the progressive disruption of the growth plate longitudinally due to the drill hole injury, leading to a gradual augmentation in bone tissue formation at the injury site from day 7 to day 42 (**Fig 1A**). NGF expression, nerves, and blood vessels were found to be absent in the uninjured growth plate (day 0). Exuberant sprouting of nerves and vasculature into the growth plate injury site was identified at 1 week post-injury, which persisted above baseline at 6 weeks post-injury (**Fig 1A**). Among fractured human growth plates, the presence of TUBB3+ nerve fibers and CD31+ vasculature was observed as well (**Fig 1B**). Suppression of TrkA signaling resulted in a noteworthy reduction in bony formation (-17% BV/TV) and OCN expression (-2.03-fold) at 6 weeks post-injury, along with decreased innervation (-1.75-fold), and vascularization (-2.42-fold) at 1-week post-injury in 1NMPP1 treated animals (**Fig 1C**). Ex vivo organ culture experiments demonstrated that conditioned medium from DRG-derived sensory neurons resulted in a 1.82-fold increase in EdU-positive cells and a 4.48-fold increase in Runx2-positive cells in the injured growth plate (**Fig 1D**). Upon re-evaluation of a pre-existing scRNA-Seq dataset from mouse long bones, Semaphorin 3d (Sema3d) emerged as a significantly upregulated inhibitor of axon growth, particularly within hypertrophic and terminal growth plate cells. IF staining confirmed the presence of Sema3d expression in hypertrophic and terminal cells within the uninjured growth plate while demonstrating diminished expression at 1 and 6 weeks post-injury (**Fig 1E**).

DISCUSSION: In summary, we confirmed that experimental growth plate injury is associated with a brisk infiltration of skeletal nerves, which appears to precede injury site angiogenesis and bony bar formation. Depletion of TrkA+ skeletal-innervating neurons via a chemical-genetic approach remarkably resulted in a significant attenuation of growth plate injury site ossification and bony bar formation. Conditioned medium from sensory nerve promotes osteogenesis of injured growth plates in culture. Furthermore, re-analysis of scRNA-Sequencing datasets implicated the axon inhibitor Sema3d as a molecule whose loss after injury is permissive for pathologic innervation of the growth plate.

SIGNIFICANCE/CLINICAL RELEVANCE: This study will provide a comprehensive understanding of the role of sensory innervation in physis repair, thereby shedding light on novel therapeutic avenues aimed at averting the formation of pathological bony bars.

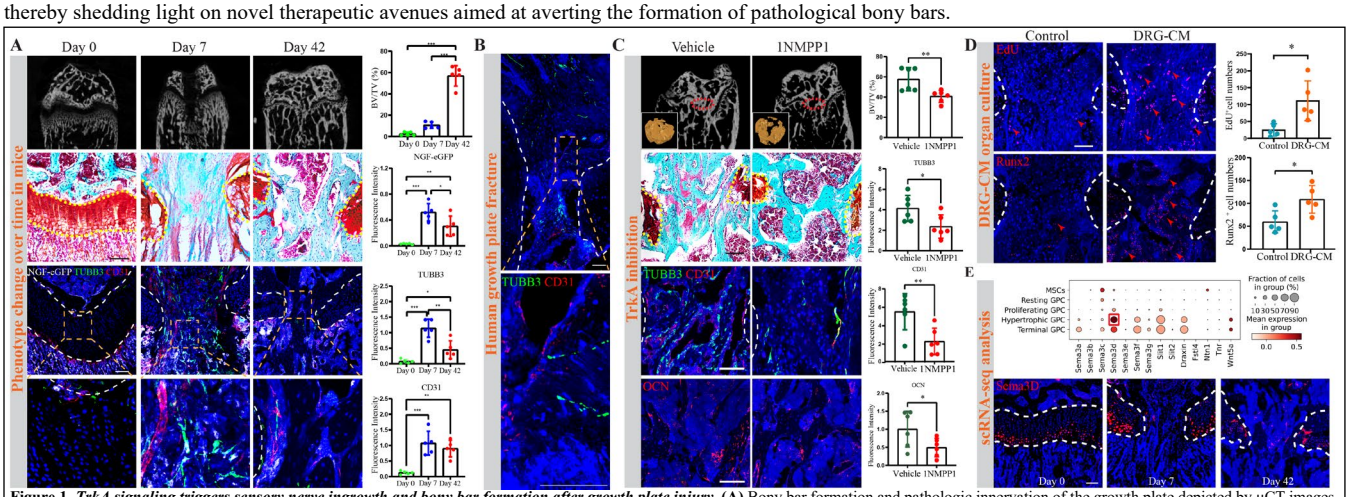

Figure 1. ***TrkA signaling triggers sensory nerve ingrowth and bony bar formation after growth plate injury.*** **(A)** Bony bar formation and pathologic innervation of the growth plate depicted by µCT images, Safranin O/Fast green staining, NGF-eGFP reporter activity, TUBB3, and CD31 immunofluorescent (IF) staining at day 0, 7, and 42. Quantitative analysis includes BV/TV measured by µCT and semiquantitative analysis of NGF-eGFP, TUBB3, and CD31 expression. N=5. **(B)** Representative images of TUBB3 and CD31 IF staining in a human epiphysis fracture sample. **(C)** Inhibition of TrkA+ sensory nerves reduce bony bar formation as observed in µCT images, Safranin O/Fast green staining, and Osteocalcin (OCN) IF staining of the injured growth plate in TrkA^{F592A} mice treated with 1NMPP1 or vehicle control at day 42 after injury. TUBB3 and CD31 IF staining was performed at day 7 after injury. Quantitative analysis includes BV/TV and semiquantitative analysis of TUBB3, CD31, and OCN expression. N=6. **(D)** Dorsal root ganglion-derived conditioned medium (DRG-CM) modulates the metabolism of in vivo cultured injured growth plate, as indicated by EdU incorporation, Runx2 IF staining, and quantitative analysis. N=5. The red arrowheads indicated marker-positive cells. **(E)** Dot plot of negative regulators of peripheral nerve growth across the different cell clusters within the mouse growth plate based on a re-analysis of an established scRNA-Seq dataset of mouse long bone and evaluation of Sema3d expression using IF staining in the uninjured and injured mouse growth plates at 7 and 42 days post-injury. MSC, mesenchymal stem cells; GPC, growth plate cells. Scale bars: 100 µm. Data presented as mean ± SD. *P<0.05, **P<0.01, and ***P<0.001.