Single cell RNA Sequencing Identifies Influence of Sensory Nerves on Osteosarcoma Disease Progression

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INTRODUCTION: In epithelial malignancies, literature suggests that surgical or pharmacologic methods to denervate tumors impedes cancer progression[1,2]. Nevertheless, the role of peripheral neurons in sarcomas remains essentially unknown, and by extension neural-based therapies to improve sarcoma disease progression remain unexplored. Here, by performing osteosarcoma (OS) orthotopic implants in combination with single cell RNA sequencing (scRNA-Seq) we describe potential nerve-to-sarcoma interactions in a murine OS xenograft model and document the importance of sensory neural regulation of OS using an established knock-in chemical-genetic mouse model. Next and in a translational effort of drug repurposing (off-label use), we leveraged an FDA-approved analgesic drug[3] to perturb the neuron-to-sarcoma interaction.

METHODS: All experiments were conducted under IACUC approval. We utilized two different approaches to curb pathological axonal invasion in OS. *(i) Chemical-genetic inhibition of TrkA neuronal signaling* -TrkA^{F592A} mice with impaired TrkA signaling were crossbred with Thy1-YFP; *Scid* mice to allow xenograft transplantation. Human 143B OS cells (1x10^6) were orthotopically inoculated into the mouse tibia (TrkA^{WT} or TrkA^{F592A}) and assayed for tumor growth. For survival curves, animals were monitored until they showed signs of morbidity or reached the planned endpoint. Immunostaining for the pan-neuronal marker Beta III Tubulin (TUBB3), neurofilament 200 (NF200), and vascular marker Endomucin (EMCN) was performed. Data was compared using the unpaired Student's t-test. Local xenograft tumors were harvested from TrkA^{WT} and TrkA^{F592A} animals and processed scRNA-Seq using 10X Genomics (Chromium platform). CellChat 1.0.0, was used to infer and quantify the neuron-sarcoma at single-cell level. (ii) *To pharmacologically target nerve-to-sarcoma interactions*, bupivacaine liposomes (L-Bup, Exparel™), FDA approved long-acting local analgesic was tested as a neurotoxic agent (off-label use). Following OS inoculation in *Nod/Scid* animals, the treatment group received intratumoral injection of 10mg/kg L-Bup while the sham group received saline (PBS) injection every 3 days for up to 4 weeks. Mechanical sensitivity of hind paws was assessed using a von Frey filament (0.4 g). Data were analyzed using a one-way analysis of variance, $p<0.05$ was considered significant.

RESULTS SECTION: TrkA inhibition in mutant animals led to an overall reduction in tumor size (60% reduction, **Fig. 1A**), and prolonged overall survival of mice ($p<0.0001$, **Fig. 1B**). The observed reduction in sarcoma-associated sensory nerves in TrkA mutant animals led to prominently reduced tumor vasculature (47% reduction assessed by EMCN staining, **Fig 1C**), suggesting disruption of neurovascular coupling. This was further corroborated using scRNA-Seq analysis where tumor-associated endothelial cells among TrkA^{F592A} showed reduced CGRP signaling ($p<0.05$, **Fig. 1D**), both by gene module score and network centrality score. To recapitulate the neuron-to-sarcoma interactions, scRNA-Seq data from human lumbar dorsal root ganglion neurons[4] were integrated along with TrkA OS datasets. Using CellChat global communication analysis, we found an overall reduction in the number of interactions as well as reduced neuron (ligand) to human OS (receiver) interactions among the TrkA^{F592A} OS **(Fig. 1E)**. To dissect this global alteration, we calculated the information flows for each signaling pathway among all pairs of cell groups in the communication network **(Fig. 1F)**. Among the denervated OS, NGF, CALCR, and VEGF signaling pathways were the most downregulated. Further, network centrality analysis of the inferred signaling network identified reduced VEGF ligands from the neuronal cluster acting onto human OS cells (**Fig. 1G**). Finally, and in a translational effort, we found that use of bupivacaine lipid nanoparticles (L-Bup) peri-tumorally has a dual effect of inhibiting the progression of 143B OS xenograft growth (59.51% reduction, $p<0.01$, **Fig. 2A**) while also providing pain relief ($p<0.01$, **Fig. 2B**). Immunostaining revealed in L-Bup OS implants a 15-fold reduction in TUBB3$^+$ nerve density **(Fig. 2C)**, 10-fold reduction in EMCN vessel density **(Fig. 2D)**, and a 2-fold reduction in tumor proliferative index KI67 ($p<0.05$, **Fig. 2E**), when compared to PBS-treated implants.

DISCUSSION: Our data provide evidence that hyperinnervation of OS tumors secondarily incites tumor vascularization, and that targeting axons using pharmacological approaches have dual beneficial effects – both as an analgesic and negative regulator of OS disease progression. Sequencing analysis revealed several intercellular signaling pathways, including growth factors, neurotrophic factors, neuropeptides, and angiogenic pathways altered in denervated OS implants, further suggesting the biological importance of peripheral sensory neurons in sarcoma disease progression.

SIGNIFICANCE/CLINICAL RELEVANCE: Targeting nerves within the tumor microenvironment using FDA-approved non-opioid anesthetic delivery system (off-label use) may serve as a novel adjunctive therapy in OS patients.

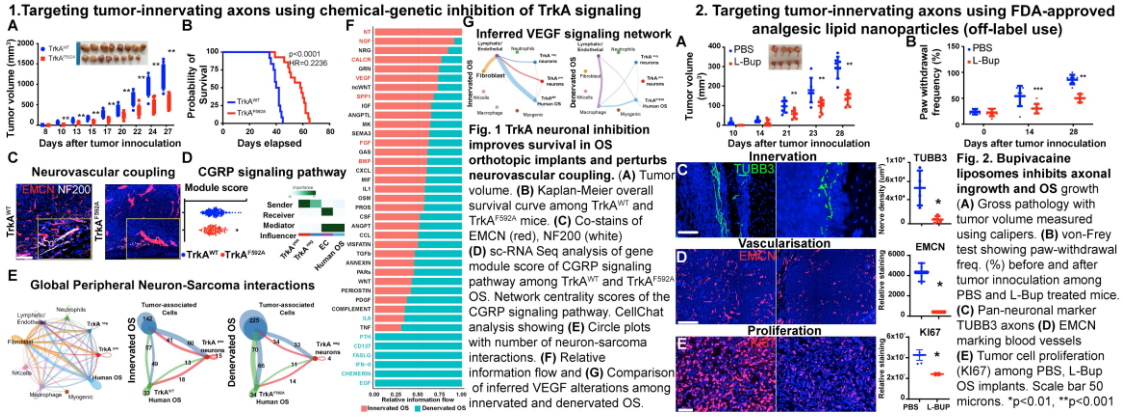

Fig. 1 TrkA neuronal inhibition improves survival in OS orthotopic implants and perturbs neurovascular coupling. **(A)** Tumor volume. **(B)** Kaplan-Meier overall survival curve among TrkA^{WT} and TrkA^{F592A} mice. **(C)** Co-stains of EMCN (red), NF200 (white) **(D)** sc-RNA Seq analysis of gene module score of CGRP signaling pathway among TrkA^{WT} and TrkA^{F592A} OS. Network centrality scores of the CGRP signaling pathway. CellChat analysis showing **(E)** Circle plots with number of neuron-sarcoma interactions. **(F)** Relative information flow and **(G)** Comparison of inferred VEGF alterations among innervated and denervated OS.

Fig. 2. Bupivacaine liposomes inhibits axonal ingrowth and OS growth **(A)** Gross pathology with tumor volume measured using calipers. **(B)** von-Frey test showing paw-withdrawal freq. (%) before and after tumor innoculation among PBS and L-Bup treated mice. **(C)** Pan-neuronal marker TUBB3 axons **(D)** EMCN marking blood vessels **(E)** Tumor cell proliferation (KI67) among PBS, L-Bup OS implants. Scale bar 50 microns. *p<0.01, **p<0.001