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TITLE: Mechanisms and Consequences of Hedgehog Signaling and Immune Infiltration in Schwannoma

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13. SUPPLEMENTARY NOTES

N/A

14. ABSTRACT

Schwannomas are common tumors of the peripheral nervous system that arise from Schwann cells. Although schwannomas have excellent outcomes after resection or radiosurgery1-4, over 75% of patients develop permanent and debilitating pain from nerve injury or cytokine activation of nociceptors in the tumor microenvironment. Existing treatments of surgery and radiotherapy rarely improve cancer-associated pain from schwannomas and often exacerbate neuropathic conditions. Although radiotherapy-induced peripheral neuropathy is rare, complications after surgery for schwannomas is common. Indeed, between 30% and 90% of schwannoma patients develop postoperative complications, most commonly in the form of new or worse sensory deficits or pain. Thus, there is an urgent and unmet need for new strategies to treat schwannomas patients.

Malignant transformation of peripheral nerve tumors, such as schwannomas, is associated with misactivation of the Hedgehog pathway, a conserved gene expression program that is essential for development and adult stem cell homeostasis. Primary cilia are required for Hedgehog signal transduction, and Hedgehog ligands such as Sonic hedgehog (SHH) bind to Patched1 (PTCH1) to relive inhibition of Smoothened (SMO), allowing SMO to accumulate in primary cilia and activate Glioma-associated oncogene (GLI) transcription factors. Out lab has shown that cilia-associated sterol lipids bind to SMO and drive Hedgehog signaling, and that GLI transcription factors promote oncogenic Hedgehog signaling by activating the cell cycle. Indeed, misactivation of the Hedgehog transcriptional program can cause inherited and sporadic cancers, and our preliminary data indicate that misactivated Hedgehog signaling underlies schwannoma. We additionally find that schwannomas are comprised of a diversity of cancer cell states and immune cell types that resemble different stages of peripheral nerve injury and regeneration. The objective of this proposal is to validate Hedgehog signaling and the immune microenvironment as targets to treat schwannoma.

15. SUBJECT TERMS

None listed.

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INTRODUCTION: Tumors of the peripheral nervous system are a significant cause of chronic neuropathic pain, but the mechanisms underlying pain from peripheral nervous system tumors are poorly understood. Schwannomas are the most common cancer of the peripheral nervous system and arise from the neural crest, a multipotent embryonic cell population with remarkable molecular and functional diversity. Although schwannomas are slow growing and can be cured with surgery or radiotherapy, there are no effective systemic or molecular treatments for schwannoma patients, and existing therapies for schwannomas often exacerbate chronic pain conditions. *Can we design effective cancer treatments that prevent the development or progression of cancer-associated neuropathic pain?* Our preliminary data presented in this application reveal that schwannomas are comprised of 2 molecular subgroups distinguished by enrichment of neural crest genes or immune infiltration. Neural crest enriched schwannomas are associated with misactivation of the Hedgehog pathway and express primary cilia, an antenna-like projection on the surface of cells that is required for Hedgehog signal transduction. In contrast, immune enriched schwannomas misactivate a diversity of schwannoma cell states resembling different stages of peripheral nerve injury and regeneration that indelibly recruit immune cells to the tumor microenvironment. These data illuminate mechanisms underlying schwannoma and establish a framework for understanding how developmental pathways and the tumor microenvironment influence cancer growth and cancer-associated pain. The *objective* of this proposal is to determine if inhibiting the biologic pathways underlying schwannoma is an effective treatment for cancer or cancer-associated neuropathic pain. We *hypothesize* that inhibiting Hedgehog signaling or tumor immune infiltration will block schwannoma growth and cancer-associated neuropathic pain.

KEYWORDS: Schwannoma, Hedgehog, pain, immune microenvironment

ACCOMPLISHMENTS:

- **What were the major goals of the project?**
    - **Major Task 1:** Determine if cilia or the Hedgehog pathway are necessary for schwannoma cell proliferation or tumorigenesis.
    - **Major Task 2:** Determine if pharmacologic inhibition of Hedgehog signaling blocks schwannoma growth or cancer-associated pain.
    - **Major Task 3:** Define the impact of tumor-associated immune cells on schwannoma growth and cancer-associated pain.
    - **Major Task 4:** Determine if immunosuppression blocks schwannoma growth or cancer-associated pain.
- **What was accomplished under these goals?**
    - We focused our efforts on Major Task 1 and Major Task 2 during our initial reporting period. In brief, we suppressed *PTCH1, SMO* and *IFT88* in human HEI-193 primary schwannoma cells stably expressing the CRISPR interference components dCas9-KRAB. Suppresion was validated compared to HEI-193$^{dCas9\text{-}KRAB}$ cells expressing non-targeting control sgRNAs (sgNTC) using qRT-PCR for Hedgehog target genes and immunofluorescence for markers of Hedgehog pathway activation. Using these reagents, we defined the impact of Hedgehog signaling and cilia on schwannoma cell proliferation using HEI-193$^{dCas9\text{-}KRAB}$

cells, clonogenic assays, and Ki67 immunofluorescence. These experiments validated our hypothesis that ciliary Hedgehog signaling was important for schwannoma cell proliferation *in vitro*. Next, using these and wild type HEI-193 schwannoma cells, we established *in vivo* xenograft models and found that like *in vitro* results, genetic inhibition of Hedgehog signaling blocked schwannoma growth *in vivo*.

- **What opportunities for training and professional development has the project provided?**
    - Nothing to report
- **How were the results disseminated to communities of interest?**
    - Nothing to report
- **What do you plan to do during the next reporting period to accomplish the goals?**
    - In the next reporting period we intent to turn our attention to Major Tasks 3 and 4, focusing on associations between tumor-associated immune cells, schwannoma growth, and cancer-associated pain. These investigations will primarily consist of *in vivo* studies in immunocompetent mouse models as *in vitro* or computational systems lack the necessary complexity to adequately model the microenvironment/cancer cell interactions and the impact of those interactions of tumor growth or cancer-associated pain. As described in our statement of work, we have been crossing mouse genetic models to create animals for these experiments during our first year of funding.

IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**
    - Our findings suggest pharmacologic inhibition of the Hedgehog pathway may indeed be a tractable therapeutic strategy for human schwannoma patients.
- **What was the impact on other disciplines?**
    - Nothing to report.
- **What was the impact on technology transfer?**
    - Nothing to report.
- **What was the impact on society beyond science and technology?**
    - Nothing to report.

CHANGES/PROBLEMS

Nothing to report.

PRODUCTS

Nothing to report.

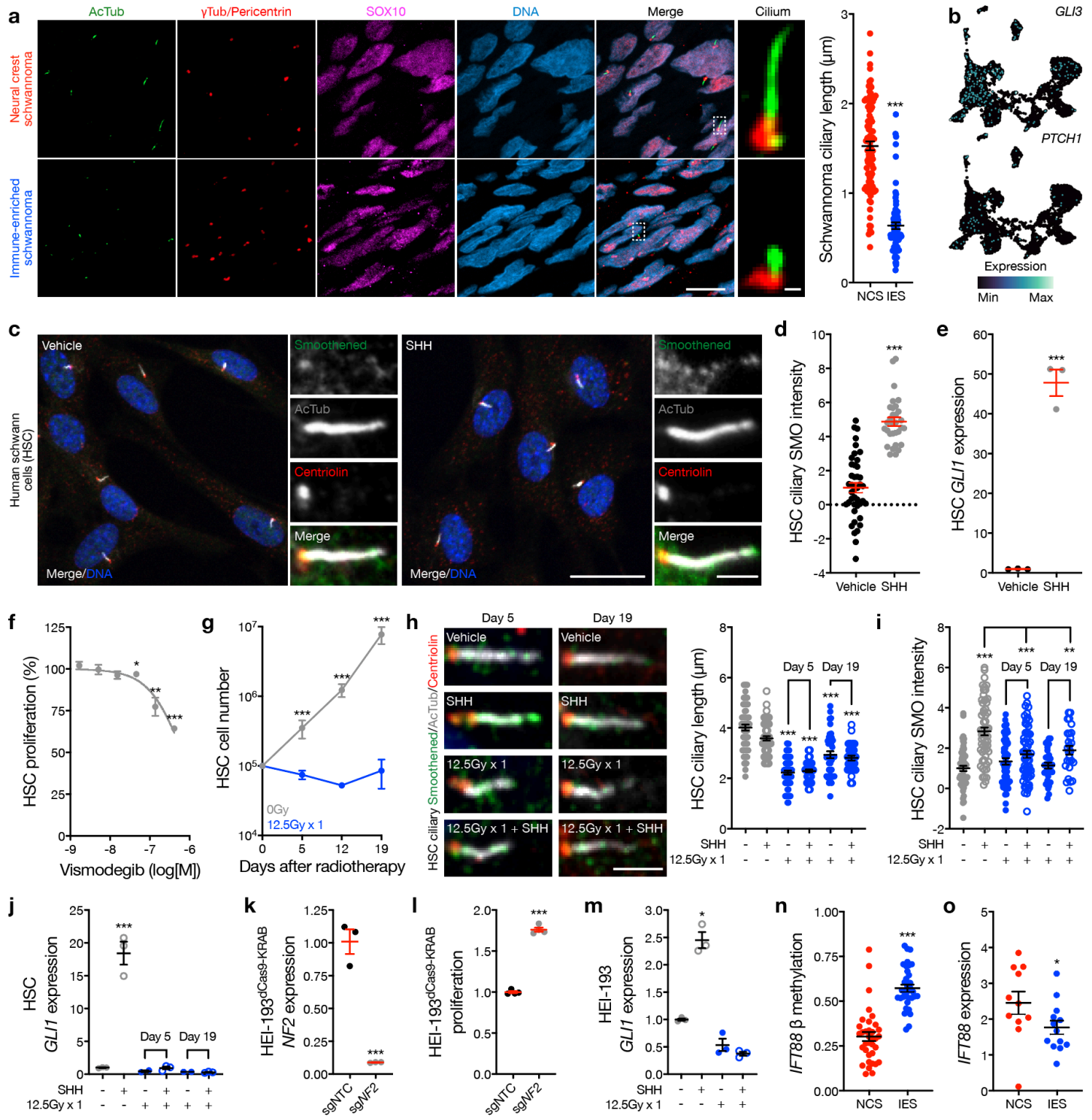
PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
    - **David Raleigh, M.D., Ph.D., Principal Investigator** No change
    - **Zhonghui Guan, M.D., Co-Investigator** No change
    - **Abrar Choudhury, Graduate Student** No change
    - **Sydney Lastella, Technician** No change
- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - Nothing to report.
- **What other organizations were involved as partners?**
    - Nothing to report

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** N/A
- **QUAD CHARTS:** N/A

APPENDICES: N/A



Radiotherapy inhibits Schwann and schwannoma cell Hedgehog signaling. **a**, Quantitative immunofluorescence microscopy for the ciliary axoneme marker acetylated tubulin (AcTub), the ciliary base markers gamma tubulin (γtub) and pericentrin (red), and the schwannoma cell marker SOX10 (purple) in 174 cilia from 6 schwannomas. Scale bars, 10μm and 1μm. **b**, Feature plot of integrated UMAP from harmonized schwannoma single-nuclei and single-cell RNA sequencing (Fig. 1b) showing expression of the Hedgehog target genes *GLI3* or *PTCH1* in schwannoma cells. **c** and **d**, Quantitative immunofluorescence microscopy for AcTub, the ciliary base marker Centriolin, and the Hedgehog pathway activator Smoothened in immortalized human Schwann cells (HSCs) treated with recombinant sonic Hedgehog (SHH) or vehicle control. Scale bars, 20μm and 2μm. **e**, QPCR for the Hedgehog target gene *GLI1* in HSC cultures treated with SHH or vehicle control. **f**, Quantification of HSC proliferation after treatment with the Smoothened antagonist vismodegib for 72h. Of note, vismodegib does not reduce ciliary length[118]. **g**, HSC proliferation after treatment with radiotherapy compared to control. **h** and **i**, Quantitative immunofluorescence imaging of primary cilia in HSC cultures 5 days or 19 days

after treatment with radiotherapy or control, and SHH or vehicle control. **j**, GLI1 QPCR in HSC cultures 5 days or 19 days after treatment with radiotherapy or control, and SHH or vehicle control. **k**, QPCR validation of *NF2* CRISPRi suppression in HEI-193 schwannoma cells compared to non-targeting control sgRNAs (sgNTC). **l**, Quantification of HEI-193 cell proliferation following CRISPRi suppression of *NF2* compared to sgNTC. **m**, GLI1 QPCR in HEI-193 cells 5 days after treatment with radiotherapy or control plus SHH or vehicle control. **n**, DNA methylation β values for the ciliary axoneme gene *IFT88* in NCS compared to IES. IFT88 is associated with the centrosome throughout the cell cycle and control cells proliferation by regulating the G1-S transition. As such, downregulation of *IFT88* in cultured human and mouse cells induces mitotic defects *in vitro*[45,46]. **o**, RNA sequencing transcripts per million expression of *IFT88* in NCS compared to IES. Lines represent means and error bars represent standard error of means (Student's t tests, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.0001$).