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Award Number: W81XWH-11-1-0143

TITLE: SNF5 mutation leads to intractable pain in schwannomatosis patients

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REPORT DATE: July 2012

TYPE OF REPORT: Revised Annual report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> <i>OMB No. 0704-0188</i>	
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1. REPORT DATE July 2012		2. REPORT TYPE Revised Annual Report		3. DATES COVERED 15-June-2011 to 14-June-2012	
4. TITLE AND SUBTITLE SNF5 mutation leads to intractable pain in schwannomatosis patients				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Steven G. Matsumoto E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon Health and Sciences University Portland OR 97239				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Ablation of snf5, <i>in vivo</i> , results in an increase in the number and type of sensory neurons that express the capsaicin receptor, TRPV1. Using a culture system, we have determined that soluble factor is released by snf5-/- Schwann cells that acts on sensory neurons to induce the expression of TRPV1. This factor is greater than 10K molecular weight and does not affect neuron survival. The increase in the immunoreactivity of TRPV1 is correlated with an increase in the expression of functional capsaicin-sensitive ion fluxes.					
15. SUBJECT TERMS Capsaicin receptor expression, conditioned medium factor					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction.

Schwannomatosis does not affect longevity but it has profound effects on the patient's quality of life due to the presence of intractable pain. The cause of this pain is not known. Our hypothesis is that mutations in the SNF5 gene in Schwann cells and ganglionic satellite cells leads to enhanced pain sensitivity in peripheral sensory neurons (see Campana et al, 2007). Mutations in the human SNF5 gene are linked to schwannomatosis (Hulsebos et al., 2007; Boyd et al., 2008; Hadfield et al., 2008; Sestini et al., 2008; Patil et al., 2008). Mice homozygous for snf5 deletion are embryonic lethal while heterozygotes develop rhabdoid tumors and other malignancies (Roberts et al., 2000; Klochender et al., 2000). We are using a tamoxifen inducible Cre-mediated recombination system driven by a mouse proteolipid protein-1 (*Plp1*) promoter (Plp1-cre/ESR1; Jackson Labs) to target the KO to Schwann and satellite cells. When this mouse is crossed with a floxed snf5 mouse, gene activity is reduced by >80% in the peripheral nervous system. A description of our aims for this project and our preliminary data follows.

Project Proposed Aims and Results.

We proposed 2 specific aims: 1. **Test the hypothesis that the loss of *snf5* increases pain sensitivity by increasing the expression of the capsaicin receptor (TRPV1) in polymodal nociceptors and by inducing capsaicin-sensitivity in sensory neurons of other modalities.**

We have utilized a tamoxifen-inducible *plpCre* / *snf5-flox/flox* mouse to produce an *in vivo* targeted deletion of SNF5. We are using the expression of the capsaicin receptor, TRPV1, to estimate the number of nociceptors in sensory ganglia of these animals.

In experiments where we acutely ablated *snf5* in adult mice, we detect an increase in the number of TRPV1-expressing neurons in the trigeminal and dorsal root ganglia (**figure 1**).

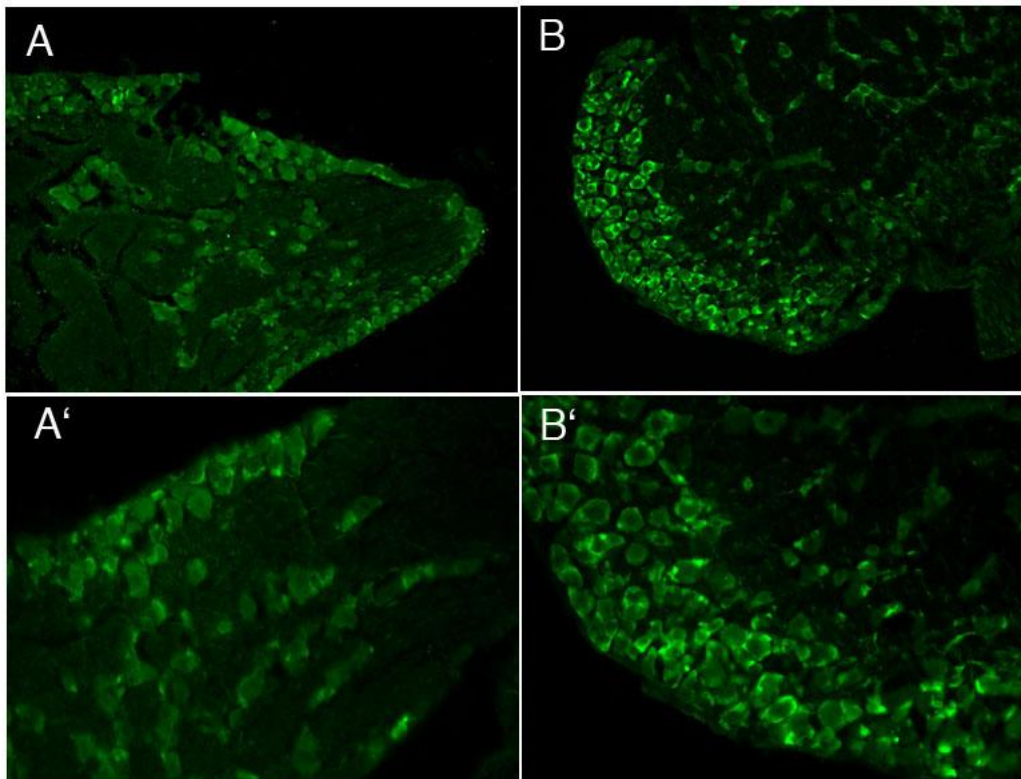


Figure 1. Trigeminal ganglion of oil injected (control; A,A') and tamoxifen injected (B, B') adult animals. Ganglia fixed 7 days after injections and labeled with anti-TRPV1. Panels are at 10X (A,B) and 20X (A', B').

To quantify the increase in TRPV1-IR neurons, we serially sectioned lumbar DRG (L1-5) and counted the neurons, $\pm$ TRPV1-IR, in every third section. The average percent positive neurons in 5 ganglia/animal were compared using a student's t-test. The results of 4 animals (2 tamoxifen-injected and 2 oil injected controls) were significantly different, 68 ± 9 vs $42 \pm 6\%$ respectively; $p < 0.01$

Strikingly, in preliminary cell counts, we observe an increase in large diameter ($>24\mu\text{m}$; 47% in tamoxifen- vs 8% oil injected controls) TRPV1-IR sensory neurons in lumbar DRGs (not shown) and trigeminal ganglia (**figure 1**). Since the small diameter ($<20\mu\text{m}$) neurons are the typical nociceptors, this result suggests that sensory neurons of non-pain modalities are being recruited to express a nociceptor property. Consistent with this *in vivo* result, we detect an increase in large diameter, TRPV1-IR sensory neurons in cultures treated with conditioned medium (see figure 2 and Aim 2 below).

2. **Test the hypothesis that the loss of *snf5* in Schwann and satellite cells results in an increase in their production and secretion of factors that increase the expression of TRPV1 in sensory neurons.**

In our second model we used an *in vitro* sensory neuron system to screen the biological activity of soluble factors released into the medium by *snf5*-knockout Schwann cells. Dorsal root ganglion (DRG) neurons of neonate to postnatal day 18 animals were dissociated and plated at a density of approximately 1000 neurons / cm^2 . The base culture medium consists of L15, modified for 5% CO_2 atmosphere, as described in Mains and Patterson, (1973) with 5% adult rat serum, antibiotics and 100 ng/ml of 7s NGF.

The base medium was supplemented with medium conditioned by Schwann cells (SC-CM) $\pm$ snf5 (ablated using cre-lentiviral infection). The SC-CM was collected from confluent Schwann cell cultures, and concentrated using Amicon Ultra centrifugal filters with a 10000 molecular weight cutoff (Millipore). The SC-CM $\pm$ snf5 was tested at 1-5 X concentration for 48-96 hours.

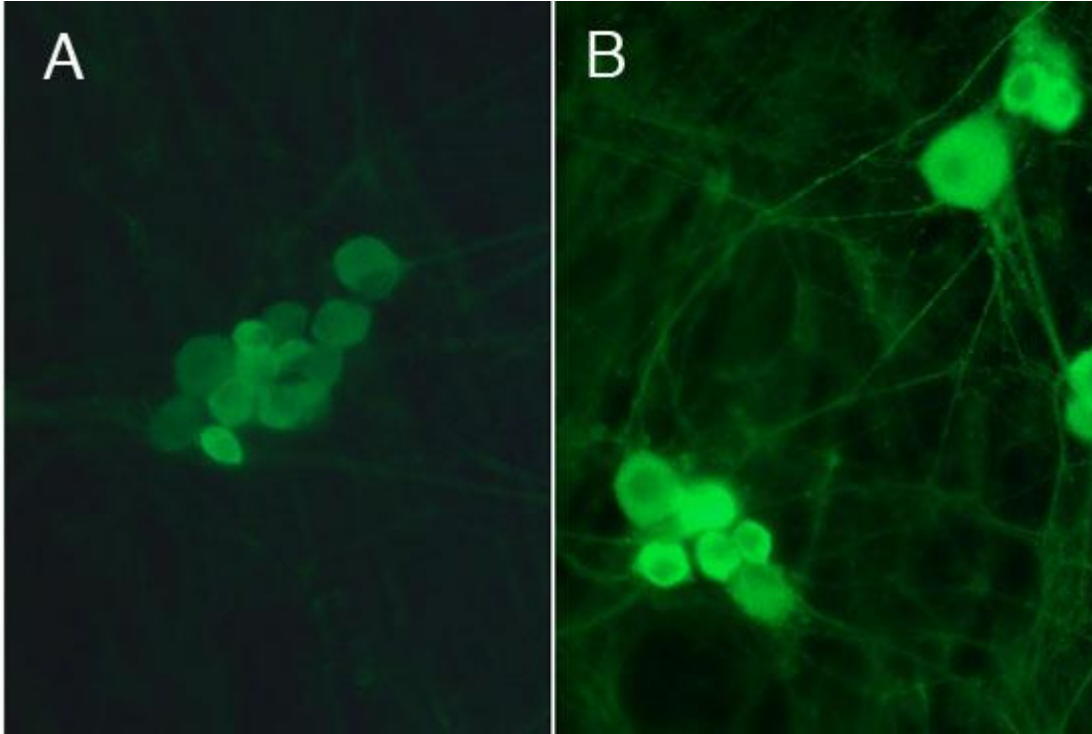


Figure 2. DRG culture treated with Control SC-CM (A) and $-/-$ snf5 SC-CM (B) for 48 hours and labeled with anti-TRPV1. Note the presence of large diameter TRPV1-IR neurons in (B)

In these experiments, dissociated neurons were plated onto laminin-coated, 1 cm diameter glass coverslips. After CM treatment, the cultures were fixed and labeled with anti-TRPV1 antibodies and the appropriate secondary antibodies. For cell counts, each coverslip was divided into 4 sections and 3 randomly selected 20X fields were counted.

After 48 hours in CM we observe an increase in the percentage of TRPV1-IR neurons in cultures treated with $-$ snf5 SC-CM ($73 \pm 7\%$) vs control SC-CM treatment ($52 \pm 2\%$). In addition, we observe both large and small diameter TRPV1-IR neurons in the snf5 $-/-$, SC-CM treatment but predominately small diameter neurons in the control SC-control CM, as we observed in the sensory ganglia *in situ* (not quantified at this time, figure 1)

We used a histochemical assay to determine the capsaicin sensitivity of sensory neurons co-cultured with $\pm$ SNF5 SC-CM. In this assay, DRG cultures were incubated in a saline solution containing 0 calcium and 5 mM cobalt $\pm$ capsaicin (50 μ M). After washing, intracellular cobalt was precipitated with ammonium sulfide. Following fixation, the cobalt precipitate was enhanced with silver using the Timm's intensification protocol (Matsumoto, 1994). Using this method, we detect a significant increase ($p < .01$) in capsaicin-sensitive neurons in $-$ snf5 SC-CM ($92 \pm 1\%$) vs $54 \pm 3\%$ in control SC-CM treated cultures (figure 3)

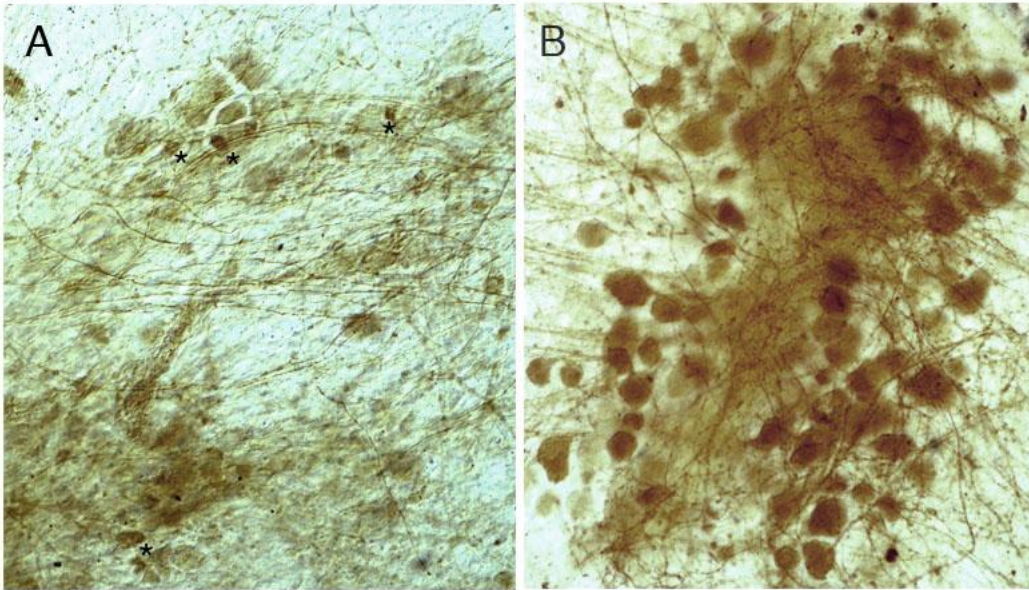


Figure 3. DRG culture, control SC-CM (A) and -snf5 SC-CM (B) treated with 50 μ M capsaicin in the presence of 5 mM cobalt and subsequently enhanced with Timm's silver intensification protocol. Note, small diameter neurons labeled in (*) in A) while many large and small diameter neurons labeled in (B).

We are currently preparing a microarray experiment to identify potential candidate molecules for the CM effect.

Key Research Accomplishments.

Target-deletion of SNF5 leads to increase TRPV1 expression in sensory neurons
SNF5 -/- Schwann cells secrete a factor that stimulates the expression of TRPV1
The conditioned medium factor increases the capsaicin sensitivity of multiple classes of sensory neuron.

Reportable Outcomes.

Manuscript in preparation

Conclusion.

The preliminary identification of a conditioned medium factor (CM) that is >10K mw is interesting and potentially very important towards advancing our understanding of this disease. We are preparing to run a microarray to identify potential candidate molecules mediating this activity. Our finding that the CM factor induces TRPV1 expression in large diameter sensory neurons is significant, since it suggests that one outcome of the mutation may be to convert non-pain sensory neurons to this phenotype.

References.

Boyd C, Smith M, Kluwe L, Balogh A, Maccollin M, Plotkin S. Alterations in the SMARCB1 (INI1) tumor suppressor gene in familial schwannomatosis. *Clin Genet*. 2008 Jul 21.

Campana WM. Schwann cells: activated peripheral glia and their role in neuropathic pain. *Brain Behav Immun*. 2007 Jul;21(5):522-7.

Hulsebos TJ, Plomp AS, Wolterman RA, Robanus-Maandag EC, Baas F, Wesseling P. Germline mutation of INI1/SMARCB1 in familial schwannomatosis. *Am J Hum Genet*. 2007 Apr;80(4):805-10.

Hadfield, KD, Newman, WG, Bowers, NL, Wallace, A, Bolger, C, Colley A, McCann, E, Trump D, Prescott T Evans, DG Molecular characterization of SMARCB1 and NF2 in familial and sporadic schwannomatosis. *J. Med. Genet*. 2008Jun;45(9):608

Klochender-Yeivin A, Fiette L, Barra J, Muchardt C, Babinet C, Yaniv M. The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression. *Page: 13*

Mains RE, Patterson, PH Primary cultures of dissociated sympathetic neurons. I Establishment of long-term growth in culture and studies of differentiated properties. 1973 *J. Cell Biol*. Nov; 59:329

Matsumoto, SG Neuronal differentiation in cultures of murine neural crest II. Development of capsaicin-sensitive neurons. 1994 *Brain Res. Dev. Brain Res*. Nov 83: 17-27

Patil S, Perry A, Maccollin M, Dong S, Betensky RA, Yeh TH, Gutmann DH, Stemmer-Rachamimov AO. Immunohistochemical Analysis Supports a Role for INI1/SMARCB1 in Hereditary Forms of Schwannomas, but Not in Solitary, Sporadic Schwannomas. *Brain Pathol*. 2008 Apr 15.

Roberts CW, Galusha SA, McMenamin ME, Fletcher CD, Orkin SH. Haploinsufficiency of Snf5 (integrator interactor 1) predisposes to malignant rhabdoid tumors in mice. *Proc Natl Acad Sci U S A*. 2000 Dec 5;97(25):13796-800.

Sestini R, Bacci C, Provenzano A, Genuardi M, Papi L. Evidence of a four-hit mechanism involving SMARCB1 and NF2 in schwannomatosis-associated schwannomas. *Hum Mutat*. 2008 Feb;29(2):227-31.