A preclinical study on a murine model associated with LMS

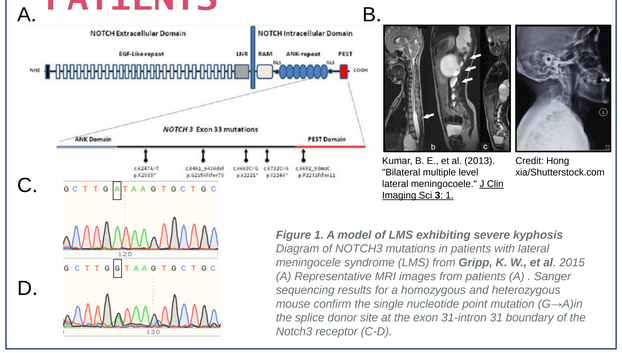
H.M. TORRES[1,2], A. VANCLEAVE[1], T. RODEZNO[1], Y. CAO[1] , Y. TECLEAB[3], M. MUKHERJEE[3], J. WESTENDORF[4,5] and J. TAO[1,2,6,7]

1 Cancer Biology & Immunotherapies Group at Sanford Research, 2 Department of Chemistry and Biochemistry, South Dakota State University, 3 Functional Genomics and Bioinformatics Core at Sanford Research, 4 Department of Orthopedic Surgery, Mayo Clinic, Rochester, MN, USA, 5 Department of Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN, USA, 6 Department of Biomedical Engineering, University of South Dakota, 7 Department of Pediatrics at Sanford School of Medicine at the University of South Dakota

INTRODUCTION

Lateral meningocele syndrome (LMS), also known as Lehman syndrome, is a rare hereditary musculoskeletal disorder with vertebral anomalies and familial osteosclerosis. Recently, whole exome sequencing of unrelated LMS patients revealed truncating mutations in the last exon of NOTCH3. Notch signaling is an evolutionarily conserved signaling pathway involved in cell fate decisions and stem cell renewal in the musculoskeletal system. To understand the gap in our knowledge of LMS and NOTCH3, we are characterizing a mouse model (also known as Humpback (hpbk)) associated with kyphosis and developmental disorders. The hpbk mutant mice harbor a pathogenic variant in the Notch3 gene that contains a G to A point mutation in the splice donor site at the exon 31-intron 31 boundary, resulting in a mutant protein product, which mimics the human NOTCH3 truncating protein found to occur in LMS patients. In order to study the hpbk mouse line at the younger age, we first designed and applied a multiplex tetra-primer amplification-refractory mutation system (ARMS-PCR) to precisely and swiftly genotype the mice. We then performed systematic studies on their phenotypes and pathological mechanisms. The homozygous hpbk mice typically develop kyphosis by 5 weeks of age, and we were able to observe a change in their weights at postnatal day 14. MicroCT analysis of homozygous mice indicates that female mutant mice exhibit osteosclerosis by 2 months, and both male and female mutant mice have a severe muscle phenotype evident by a decrease in TA muscle weight and lower gripping strength in our behavior analysis. We have also performed RNA-seq on calvaria and skeletal muscle samples of WT and homozygous mice. We are investigating the use of an HDACi epi-drug to treat this model, where we show promising data revealing rescue of kyphosis and trabecular bone volume. Ongoing studies include bone histomorphometry of wildtype, mutant, and SAHA treated mutant mice, and a single cell sequencing analysis of wildtype versus mutant mice. Because there is currently no treatment available, a thorough biological understanding of this novel mouse model may point to new treatments to reduce the severity of the disease in LMS patients.

A NOTCH3 POINT MUTATION MIMICS TRUNCATING NOTCH3 MUTATIONS IN HUMAN LMS PATIENTS



Kumar, B. E., et al. (2013). "Bilateral multiple level lateral meningocoele." J Clin Imaging Sci 3: 1.

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Figure 1. A model of LMS exhibiting severe kyphosis *Diagram of NOTCH3 mutations in patients with lateral meningocele syndrome (LMS) from* ***Gripp, K. W., et al****. 2015 (A) Representative MRI images from patients (A) . Sanger sequencing results for a homozygous and heterozygous mouse confirm the single nucleotide point mutation (G→A)in the splice donor site at the exon 31-intron 31 boundary of the Notch3 receptor (C-D).*

RESULTS

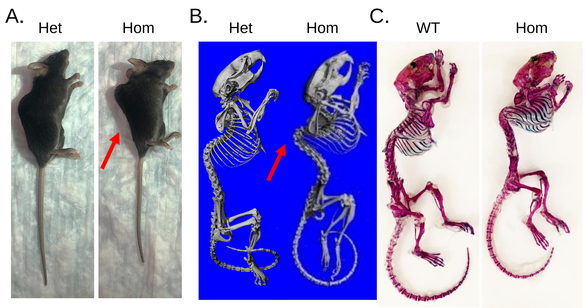


Figure 2. Humpback (hpbk) mice harbor a pathogenic mutation in the Notch3 gene. *Mice homozygous (hpbk/hpbk, Hom) for the mutation are comparable in appearance to wild-type mice (+/+, WT) when born, but develop musculoskeletal phenotypes such as a kyphotic curved spine soon after weaning. They become easily identifiable at approximately 5 weeks of age and are infertile. Heterozygote mice (hpbk/+, Het) are indistinguishable from their wild-type littermates (A). Skeletal and bone defects for Hom instead of Het) mice (not distinguishable from WT) include kyphosis or excessive outward curvature of the spine (red arrow) (B). Skeletal preparation of representative WT and Hom mice*

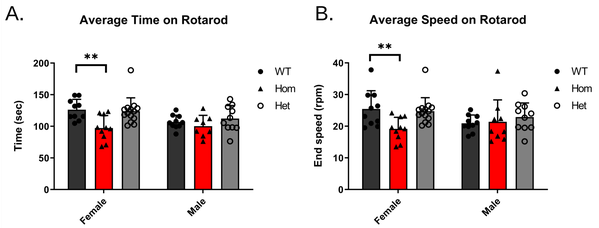


Figure 3. Accelerating Rotarod studies identifies early locomotor deficits in female Hom mice. *At 2 months of age, mice were tested on an accelerating Rotarod test and female homozygous mice performance was significantly poorer (i.e. less time spent on Rotarod (A) and slower speed (B)) than control (Wild type, WT and Heterozygous, Het) mice. Het mice results are indistinguishable from WT mice (A,B). Student's t-test **p<0.01. Bar graphs indicate SD.*

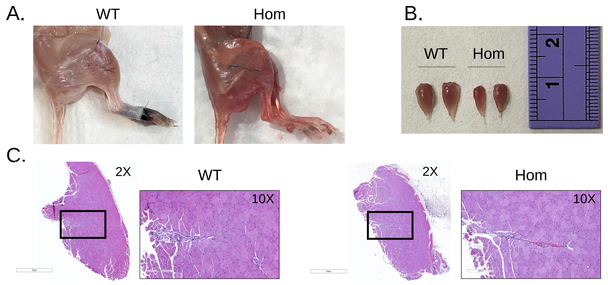


Figure 4. Muscle growth defects are present in homozygous mice, but no obvious signs of muscle pathology. *At 2 months of age, Hom mice appear to have weaker muscle than WT mice (A) Freshly isolated tibialis anterior (TA) skeletal muscle is smaller and weighs less than WT (see fig. 4) (B). However, a closer look at muscle histology indicates that there are no obvious signs of muscle pathology between two WT (left) and Hom (right) H&E sections (n=3). There is no indication of fiber degeneration, such as centralized nuclei, inflammatory infiltrates, fibrosis, or abnormal fiber size distribution (C).*

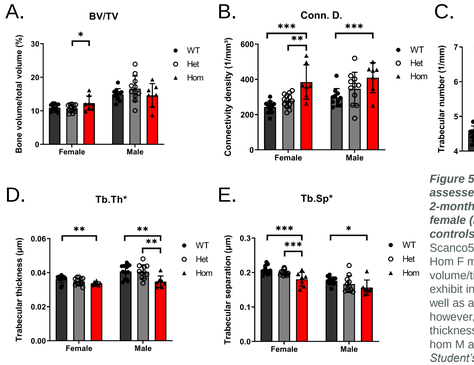


Figure 5. Femoral microarchitecture assessed by microcomputed tomography of 2-month-old Notch3 hom male (M) and female (F) mice, and sex/age matched controls. *μCT analysis was performed on a Scanco50 in distal femurs for trabecular bone. Hom F mice exhibit a slight increase in bone volume/tissue volume (A). Both M and F mice exhibit increased connectivity density (B) as well as an increase in trabecular number (C); however, there is a decrease in trabecular thickness (D) and trabecular separation (E) in hom M and F mice when compared to control. Student's t-test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bar graphs indicate mean±SD.*

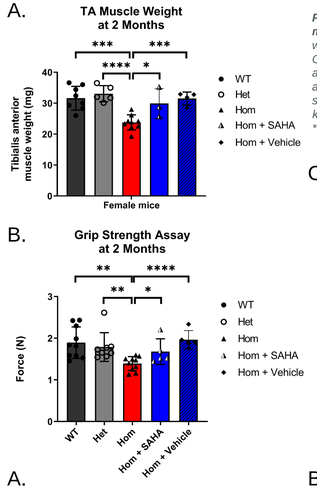
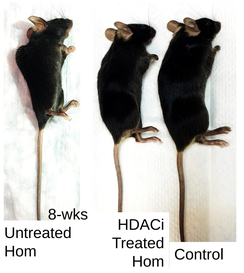


Figure 6. HDACi daily treatment rescues kyphosis and muscle function defects . *Hom mice were treated daily with SAHA from P10 until they reached two months old. Compared to controls (WT and Het), Hom mice tibialis anterior muscle is smaller by two months old. Daily SAHA and Vehicle treatment rescues muscle mass (A) and grip strength (M+F) (B), but only SAHA treatment rescues the kyphosis phenotype (C). Student's t-test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bar graphs indicate SD.*



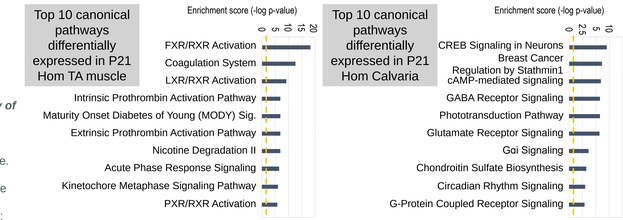


Figure 7. RNA-seq analysis of primary skeletal tissue in male and female Hom vs WT. *Ingenuity pathway analysis (IPA) of canonical signaling pathways associated with significantly regulated genes (p<0.05) in Tibialis anterior muscle (a total of 6 hom samples (3 F, 3 M) and 5 WT samples (3 F, 2 M) (A) and calvaria samples (a total of 6 hom samples (3 F, 3 M) and 6 WT samples (3 F, 3 M)) (B).*

CONCLUSIONS AND FUTURE DIRECTIONS

Humpback (hpbk) mice can be used as precious animal models for investigating developmental disorders related to myopathy and kyphosis. By 2-months-old homozygous mice exhibit locomotor defects, defects in muscle growth and motor function, and changes in femoral microarchitecture. RNA-seq analysis identified dysregulation in muscle associated with Retinoid X receptors and dysregulation of cAMP signaling and G-protein coupled receptors in calvaria. Daily treatment with an HDACi was able to prevent kyphosis in Hom mice. We are currently completing a RNA-scope study on primary samples, as well as an *in vitro* BioID proteomics analysis on homozygous mutant N3ICD constructs compared to wildtype N3ICD in order to better understand the proteomic profile of wildtype and mutant N3ICD. Additional ongoing studies include bone histomorphometry and single cell sequencing on TA muscle samples.

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CONTACT INFORMATION

Jianning.Tao@sanfordhealth.org

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