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14. ABSTRACT

We have developed a novel mouse model that carries a conditional allele for Notch4 ($N4^{ex1}$). In preliminary studies, we demonstrated that $N4^{Ex1}$ developed many of the hallmarks of patients with Hereditary Hemorrhagic Telangiectasia, while lymphatic endothelial cell deletion of *Notch4* ($N4^{ex1:LEC}$) resulted in lymphatic defects in the dermis, liver, and lungs consistent with human lymphatic malformations (LM). Whole exome sequencing of LM patient cells identified 4 variants of uncertain significance in *Notch4*. ***We hypothesize that Notch4 functions in vascular maturation and homeostasis, and that its disruption contributes to the development of vascular malformations.*** In year 1 studies, we demonstrate that $N4^{ex1}$homozygous mice are embryonic lethal. $N4^{ex1}$ and $N4^{ex1:LEC}$ males, but not females, mice develop hepatic liver lymphatic defects associated with increased stellate cell activation, collagen deposition, and immune cell inflammation. We also found microbleeds in heterozygous $N4^{ex1}$ brains, and progressive dermal lymphatic defect in $N4^{ex1:LEC}$ mice. The latter was associated with reduced LEC proliferation and VECADHERIN expression. Analysis of LM tissues and cells showed that DLL4 expression is increased the LECs carrying common pathogenic *Pik3ca* variants. Finally, analysis of LEC Jag1/Notch4 signaling suggests that it regulates inflammation, immune cell recruitment/activation and cellular locomotion which is distinct from Dll4/Notch signaling in LECs. Consistent with this, we find that Jag1 is expressed in the lymph node LECs and that there are lymph node lymphatic defects in $N4^{ex1:LEC}$ mice.

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1) Introduction:

The Notch signaling pathway is an essential regulator of vascular development, and its dysfunction has been suggested to contribute to the development of a number of vascular malformations. Multiple Notch proteins are expressed and required dynamically in the developing and homeostatic vasculature. Loss of endothelial Notch1 or ectopic endothelial expression of activated Notch1 or Notch4 lead to arteriovenous malformations (AVMs) in mice, suggesting that functions of Notch signaling are dose dependent in the vasculature. Consistent with this, both reduced and increased expression of NOTCH1 and NOTCH4 in vascular malformation has been reported. Numerous therapies that target Notch proteins, and ligands, as well as proteases are in clinical development or clinical trials and their use for vascular malformations has been proposed. Thus, it is essential to understand the role of the distinct receptors and ligands of Notch in vascular development and how their misregulation contributes to vascular malformations.

We have very recently developed a mouse that carries a novel conditional allele for Notch4 ($N4^{Ex1}$). By six weeks of age, mice haploinsufficient for $N4^{Ex1}$ developed many of the hallmarks of patients with Hereditary Hemorrhagic Telangiectasia (HHT), including telangiectasias in the feet, tongue, and intestines, defects in sinusoidal, venous, lymphatic vessels and coagulopathies in the liver, as well as pulmonary arteriovenous shunts. When Notch4 was conditionally deleted in the lymphatic endothelium, we observed that lymphatic endothelial cell (LEC) deletion of *Notch4* resulted in lymphatic vessel dilation and patterning defects in the dermis, liver, and lungs consistent with human lymphatic malformations (LM). In humans, NOTCH4 is expressed in the normal adult dermal lymphatics which we found to be downregulated in the lymphatic endothelium of LM tissues. Finally, whole exome sequencing and variant analyses of prenatal and postnatal LM specimens identified 4 rare Notch4 missense variants of unknown significance predicted to disrupt protein function.

We hypothesize that Notch4 functions in vascular maturation and homeostasis, and that its disruption contributes to the development of vascular malformations.

2) Keywords:

Notch4, Dll4, Jag1, blood endothelial cells, lymphatic endothelial cells, liver, skin, lungs

3) Accomplishments:

A) What were the major goals of the project?

SPECIFIC AIM 1: DETERMINE ENDOTHELIAL NOTCH4 FUNCTIONS IN MICE

- Major Task 1: Obtain appropriate regulatory approval (months 0-3)
- Major Task 2: Evaluation of $N4^{Ex1}$ mice vascular phenotypes (months 3-12)
- Major Task 3: Evaluation of LEC specific $N4^{Ex1}$ mice vascular phenotypes (months 6-18)
- Major Task 4: Evaluation of EC specific $N4^{Ex1}$ mice vascular phenotypes (months 12-24)

SPECIFIC AIM 2: CHARACTERIZATION OF PUTATIVE LYMPHATIC MALFORMATION NOTCH4 VARIANTS

- Major Task 1: Structure function studies of human Notch4 variants (months 2-18)
- Major Task 2: Determine the effects of Notch4 variants on EC specification and gene expression (months 8-24)

SPECIFIC AIM 3: DETERMINE THE ENDOTHELIAL NOTCH4 TRANSCRIPTIONAL PROFILE

- Major Task 1: Gene expression studies of murine ECs from $N4^{ex1/+}$ mice (months 12-24)
- Major Task 2: Ligand activation studies of ECs with Notch4 knockdown (months 8-20)

B) What was accomplished under these goals?

SPECIFIC AIM 1: DETERMINE ENDOTHELIAL NOTCH4 FUNCTIONS IN MICE

Major Task 1: Obtain appropriate regulatory approval

IACUC and ACURO approval was obtained and has been kept up to date.

Major Task 2: Evaluation of N4^{ex1} mice vascular phenotypes

We have continued to cross *N4$^{ex1/+}$* males and females to generate *N4$^{ex1/ex1}$* nullizygous mice. We have found that *N4$^{ex1/ex1}$* mice are mostly embryonic lethal (Fig. 1A). Using lung tissue collected from the 3/4 viable *N4$^{ex1/ex1}$*, we found that NOTCH4 was not express consistent with its complete deletion (Fig. 1B). Analysis of 6-week ears from *N4$^{ex1/+}$* littermates demonstrated both dermal blood and lymphatic defects relative to wild-type littermates (Fig. 1C). Strikingly, the dermal lymphatics of *N4$^{ex1/+}$* ears had decreased in VECADHERIN expression. In addition, we found that 6-weeks, *N4$^{ex1/+}$* mice developed dermal microbleeds (n=17/28), foot edema (n=10/12), tongue telangiectasias (n=3/21) and brain microbleeds (n=2/4) (Fig. 2). One *N4$^{ex1/+}$* brain had developed supernumerous blood vessels at the base of the brain consistent with an AVM nidus (Fig. 2B).

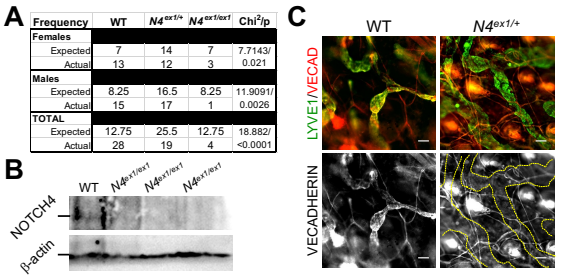

Fig 1. *N4$^{ex1/ex1}$* mice are embryonic lethal and viable *N4$^{ex1/+}$* mice display altered blood and lymphatic vessels. A) Expected vs actual frequency of viable mice. **B)** NOTCH4/β-ACTIN Western blot of lung lysates from wild-type and *N4$^{ex1/ex1}$* littermates. **C)** LYVE1/VECADHERIN staining of 6-week ears. Dotted line marks *N4$^{ex1/+}$* lymphatics with reduced VECADHERIN. Scale bars 50μm.

In preliminary studies, we showed that 6-week *N4$^{ex1/+}$* livers had thrombi in the portal and central veins, sinusoidal vessel dilation, and supernumerous portal lymphatics. To determine if the phenotype progresses, we analyzed the 6-month liver phenotype. This timepoint was chosen as it is equivalent to the age when humans begin to develop liver fibrosis[1]. *N4$^{ex1/+}$* male livers (n=2/4) were enlarged and reddened consistent with liver congestion (Fig. 3A). Sirius red staining revealed increased collagen deposition surrounding the portal triad vasculature and some enlarged sinusoidal vessels (Fig. 3B). We next analyzed where Notch signaling was active in adult livers and found that it is enriched in the hepatic lymphatic endothelium, as well as the portal epithelium (Fig. 4). As hepatic portal lymphatic hyperplasia is observed in NASH and Notch activity is enriched in the hepatic lymphatic endothelium, we analyzed the hepatic lymphatics of wild-type mice fed a NASH diet and treated with a gamma-secretase inhibitor (GSI) that suppresses canonical Notch signaling. In this model, 3 weeks of GSI treatment decreased fibrogenic gene expression and collagen deposition in the liver[2]. Consistent with prior publications[3,4], the NASH diet led to hepatic lymphatic defects in the most affected portal triads (Fig. 5A). These lymphatics expressed JAG1 further supporting a role for Notch signaling in the NASH (Fig. 5B). Moreover, GSI treatment decreased the abnormal hepatic portal triad lymphatic density (Fig. 5C,D). Together these data support a role for Notch4 and Notch signaling in the hepatic lymphatic homeostasis and liver fibrosis.

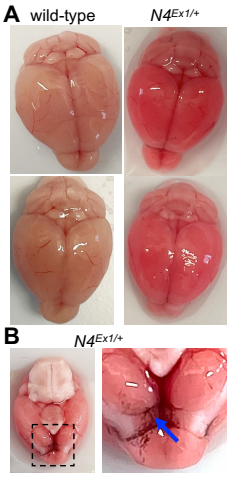

Fig 2. *N4$^{ex1/+}$* mice developed cranial microbleeds. A) 6-week N4$^{ex/+}$ brains (n=2/4) were reddish consistent with microbleeds. Cranial view. **B)** 6-week N4$^{ex/+}$ brains (n=1/4) developed supernumerous vessels (blue arrow). Boxed area enlarged to right. Caudal view.

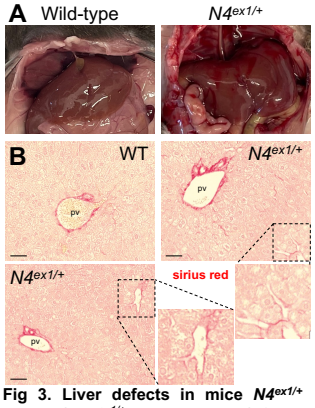

Fig 3. Liver defects in mice *N4$^{ex1/+}$* mice. A) *N4$^{ex1/+}$* enlarge liver. **B)** Sirius red staining. Enlarged boxed areas show dilated sinusoidal vessels with increased collagen. Scale bars 50μm

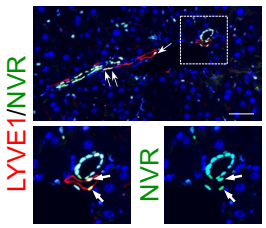

Fig 4. Notch activity in hepatic portal lymphatics. LYVE1 staining of *NVR* liver sections. Arrows mark LEC nuclei with Notch activity. Boxed area enlarged below. Scale bars 50μm

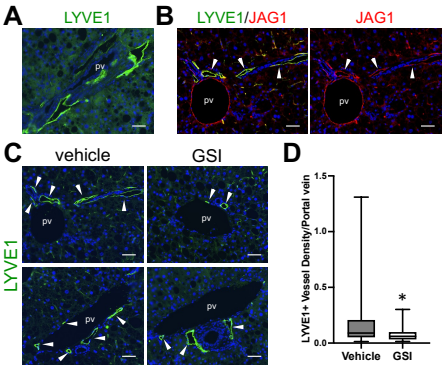

Fig 5. GSI suppressed lymphatic hyperplasia in a NASH mouse model. Wild-type mice fed a NASH diet provided vehicle or GSI every other day for three weeks. **A)** LYVE1 staining. Example of increased lymphatics around a severely affected portal triad in NASH. **B)** JAG1/LYVE1 staining of NASH liver. Arrow mark JAG1 expressing lymphatics. **C)** LYVE1 staining. Arrows mark lymphatics within the portal triads. **D)** LYVE1+ vessel density within the portal triad normalized to portal vein area (min/max). Vehicle (n=6); GSI (n=9) **A-C)** Scale bars 50μm

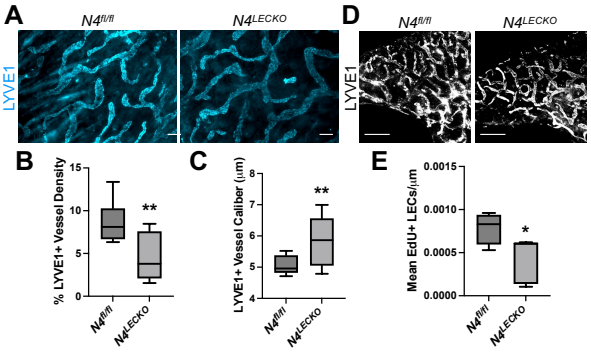

Fig 6. Dermal lymphatic defects *N4^{LECKO}* mice. TM: P1, P2, P3 **A)** LYVE1 whole-mount staining of 6-week ears. Scale bars 100μm **B)** Mean LYVE1+ vessel density (min/max). **C)** Mean LYVE1+ vessel caliber (min/max). **D,E)** 6-week mice ear-punched, 19 hours later EdU injected then euthanized after 6 hours. **D)** LYVE1 whole-mount staining. Scale bars 500μm. **E)** Mean EdU+/LYVE1+ LECs normalized to vessel area (min/max). T-Test: *p<0.05, **p<0.006 **A-C)** *N4$^{fl/fl}$* (n=3), *N4^{LECKO}* (n=10) **D,E)** *N4$^{fl/fl}$* (n=4), *N4^{LECKO}* (n=5).

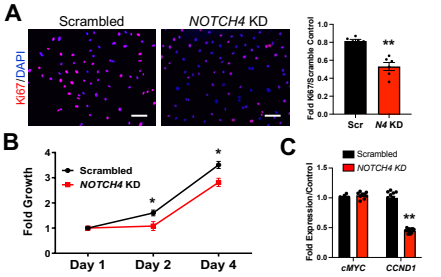

Fig 7. *NOTCH4* knockdown reduced LEC proliferation. **A)** KI67 staining and quantification of HdLECs expressing either *Notch4* shRNA or scrambled sequence. Scale bars 50μm **B)** Cell growth normalize to cell number on Day 1 for each. **C)** *cMYC* and *CCND1* qRT-PCR normalized to *β-ACTIN*. **A-C)** Data for 3 technical replicates each for 2 transductions presented relative to scrambled ± s.e.m. T-Test: *p<0.005; **p<0.0001

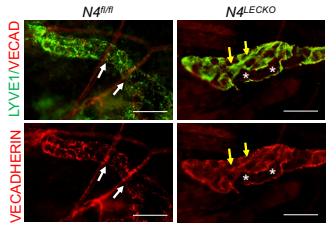

Fig 8. Dermal lymphatic VECADHERIN defects in *N4^{LECKO}* mice. TM:P1, P2, P3. VECADHERIN/LYVE1 whole-mount staining of 6-week ears. White arrows point to button-junctions. Yellow arrows point to zipper-junctions. Asterisk mark loss of expression. Scale bars 50μm.

Major Task 3: Evaluation of LEC specific N4^{ex1} mice vascular phenotypes

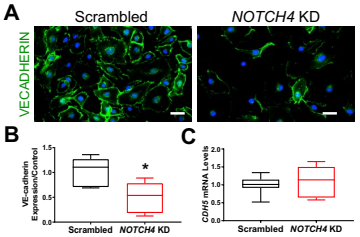

Fig 9. Reduced VECADHERIN in *NOTCH4* knockdown HdLECs. A) VECADHERIN staining of HdLECs expressing either *Notch4* shRNA or scrambled sequence. Scale bars 50μm **B)** Mean VECADHERIN intensity normalized to control. **C)** *CDH5* qRT-PCR normalized to *β-ACTIN*. **B,C)** Data for 3 technical replicates for 3 transductions relative to scrambled controls (min/max). *p<0.02

To delete *Notch4* in the postnatal LECs, we generated *Prox1CreER^{T2};N4$^{fl/fl}$* (*N4^{LECKO}*) and control *N4$^{fl/fl}$* mice and induced recombination at postnatal day 1 (P1), P2, and P3. At 6-weeks of age, dermal lymphatic density was reduced and vessels tortuous with an increased caliber relative to *N4$^{fl/fl}$* controls (Fig. 6A-C). To evaluate dermal LEC proliferation, an ear-punch model was used to promote inflammation-induced lymphangiogenesis around the wound. LEC proliferation was significantly reduced in the ears of 6-week *N4^{LECKO}* mice with a nearly 50% reduction in dermal LEC proliferation relative to *N4$^{fl/fl}$* mice (Fig. 6D,E). HdLECs with NOTCH4 knockdown (*NOTCH4KD)* had decreased LEC proliferation (Fig. 7A,B) which correlated with a downregulation of the cell cycle gene, *CCND1,* but not *cMYC* (Fig. 7C). Analysis of VECADHERIN expression revealed a change in its expression pattern from a button-like morphology to zipper-like morphology in *N4^{LECKO}* dermal lymphatics, with some LECs loosing VECADHERIN expression entirely (Fig. 8). The loss of the button-like VECADHERIN expression is similar to a lacteal phenotype observed in mice with LEC *Dll4* deletion[5, 6]. Next, we examined VECADHERIN expression in *NOTCH4KD* and control HdLECs. *NOTCH4*KD reduced VECADHERIN protein expression (Fig. 9A,B), but not transcripts levels (Fig. 9C), suggesting Notch4 regulates VECADHERIN expression post-translationally.

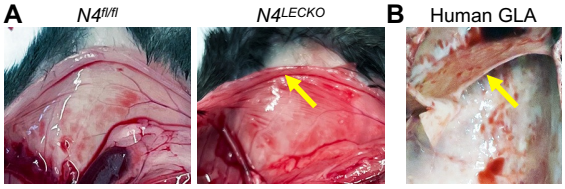

Fig 10. Thickening of the peritoneum in 6-month *N4^{LECKO}* mice. Arrow points to peritoneal thickening **A)** in mice and **B)** in a human case of GLA.

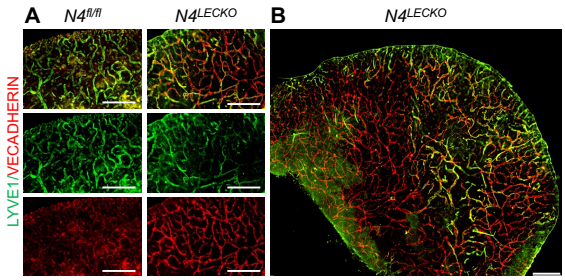

Fig 11. Dermal lymphatic defects in 6-month *N4^{LECKO}* mice. LYVE1 and VECADHERIN whole-mount staining. A) Comparison of the dermal vascular phenotype in *N4^{LECKO}* and *N4$^{fl/fl}$* mice. **B)** Low magnification image of *N4^{LECKO}* dermal lymphatic phenotype. Scale bars 1mm.

To determine if the dermal lymphatic phenotype was progressive, we have begun to assess 5/6-month *N4^{LECKO}* and control *N4$^{fl/fl}$* mice provided tamoxifen at P1, P2, and P3. Two of *N4^{LECKO}* mice had a significant thickening of the peritoneal lining (Fig. 10A). We have observed a similar phenotype in the peritoneum of a generalized lymphatic anomaly patient (Fig. 10B). Initial analysis of the dermal lymphatic in the ear revealed a torturous lymphatic vasculature and in one case a complete absence of dermal blood capillaries and a mosaic lymphatic vasculature suggesting the blood and lymphatic system had merged (Fig. 11). Analysis of the 5/6-month timepoint is ongoing.

To evaluate LEC Notch4 functions in the liver, 6-week and 5-month *N4^{LECKO}* mice were compared to *N4$^{fl/fl}$* controls. Surprisingly, we observed a liver phenotype in male *N4^{LECKO}*, but not females. At 6 weeks, the lymphatics were dilated and supernumerous in the *N4^{LECKO}* portal triads (Fig. 12A,B). This *N4^{LECKO}* lymphatic phenotype was associated with increased αSMA and CD45 expression, indicating stellate cell activation and immune cell infiltration, two hallmarks of liver fibrosis (Fig. 12C-F). Sirius red and trichrome staining revealed an increase in collagen deposition surrounding the hepatic portal triads consistent with increased fibrosis

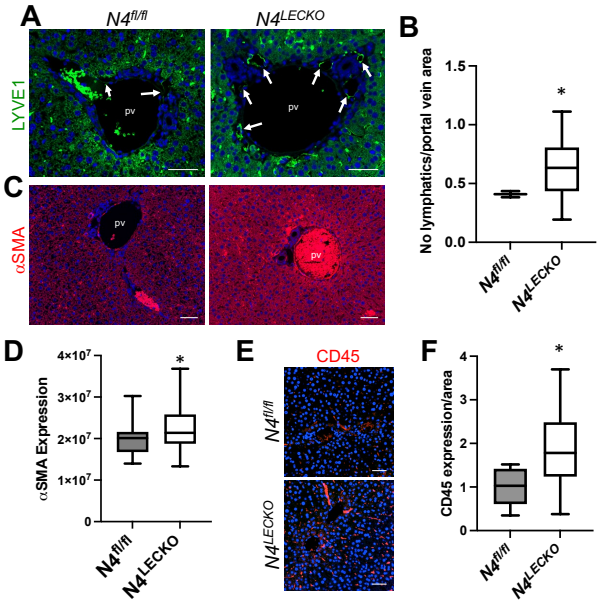

Fig 12. Liver defects in 6-week *N4^{LECKO}* males. *N4$^{fl/fl}$* and *N4^{LECKO}* livers. TM:P1, P2, P3. **A)** LYVE1 staining. Arrows mark portal lymphatics. **B)** Number of LYVE1+/Prox1+ portal lymphatics normalized to portal vein area (min/max). **C)** αSMA staining and **D)** mean hepatic signal intensity (min/max). **E)** CD45 staining and **F)** mean signal intensity relative to litter controls (min/max). Scale bars 50μm. T-test: *p<0.05. **B,D**) *N4$^{fl/fl}$* (n=3); *N4^{LECKO}* (n=10). **F**) *N4$^{fl/fl}$* (n=3); *N4^{LECKO}* (n=3).

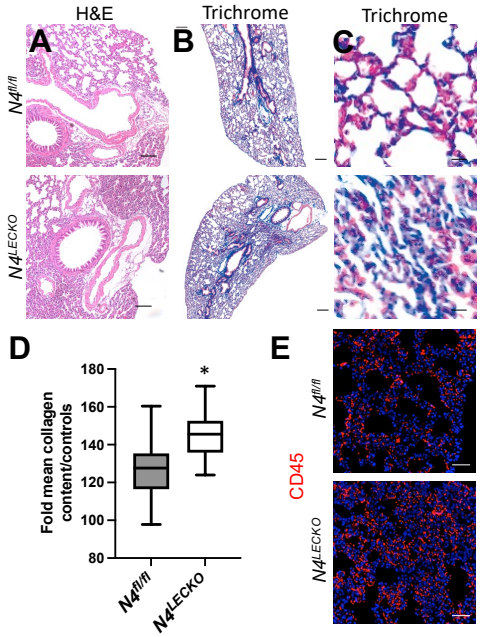

Fig 14. Increased ECM deposition and immune cell infiltration in the lungs of mice with postnatal loss of LEC *Notch4*. **A)** H&E of lung cross-sections. **B,C)** Trichrome staining. **D)** Quantification of collagen content normalized to area (min/max). T-test: *p = 0.012 (n=2 *N4$^{fl/fl}$*; n=2 *N4^{LECKO}*). **E)** CD45 staining. Scale bars **A, E)** 100μm, **B)** 200μm, **C)** 20μm

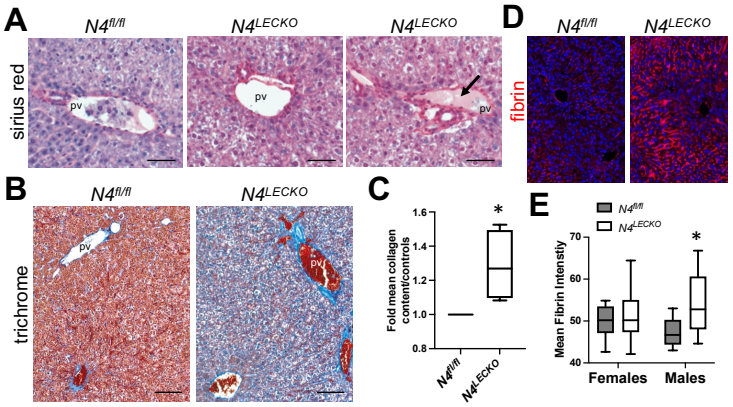

Fig 13. Collagen and fibrin accumulation in *N4^{LECKO}* male livers. TM:P1, P2, P3. **A-C)** 6 weeks. **D,E)** 5 months. **A)** Sirius red and hematoxylin staining. Scale bars 50μm **B)** Trichrome staining. Scale bars 100μm **C)** Quantification of collagen content for trichrome staining normalized to area (min/max). *N4$^{fl/fl}$* (n=3); *N4^{LECKO}* (n=3). **D)** Fibrinogen staining and **E)** mean signal intensity per field of view. T-test: *p<0.005 females *N4$^{fl/fl}$* (n=3); *N4^{LECKO}* (n=5); males *N4$^{fl/fl}$* (n=2); *N4^{LECKO}* (n=5).

(Fig. 13A-C). Like *N4$^{ex1/+}$* livers, thrombi were observed in *N4^{LECKO}* portal veins at 6-weeks (Fig. 13A), which was associated with increased fibrin deposition throughout liver observed at 5-months (Fig. 13D,E). Our studies suggest a role for LEC Notch4 in liver fibrosis in male mice.

Similar to the liver, we showed in preliminary studies that there were lymphatic defects in *N4^{LECKO}* lungs. Further analysis of the 6-week lung phenotype demonstrated an increase in collagen deposition in the alveolar region and CD45+ immune cells in *N4^{LECKO}* mice relative to *N4$^{fl/fl}$* controls (Fig. 14). Lymphatic defects are associated with idiopathic pulmonary fibrosis in humans[7]. Similar to the liver, VEGFC treatment ameliorated the extent of lung fibrosis in a bleomycin mouse model[7]. Thus, we assessed the expression of the vascular Notch ligands, DLL4 and JAG1 in the lymphatics of bleomycin treated mice, a mouse model of pulmonary fibrosis. DLL4 was expressed in both normal and pathological lymphatics, whereas JAG1 expression was upregulated in a subset of the lymphatics in bleomycin-treated lungs (Fig. 15).

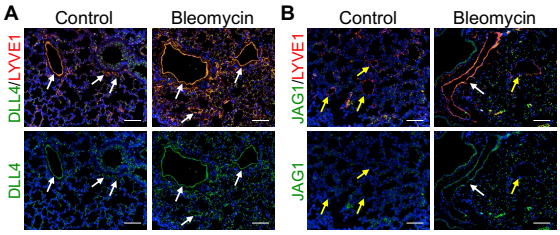

Fig 15. DLL4 and JAG1 expression in control and bleomycin treated murine lungs. Mice were given bleomycin via the airway and tissues collected at Day 8. LYVE1 and **A)** DLL4 or **B)** JAG1 staining of lung sections from control and bleomycin treated mice. White arrows mark expressing lymphatics and yellow arrows non-expressing lymphatics. Scale bars 100μm

Major Task 4: Evaluation of EC specific N4Ex1 mice vascular phenotypes

The EC *Cdh5-CreER^{T2}* driver mice for these studies have been maintained and studies are planned for year 2 of the grant.

SPECIFIC AIM 2: CHARACTERIZATION OF PUTATIVE LYMPHATIC MALFORMATION NOTCH4 VARIANTS

Major Task 1: Structure function studies of human Notch4 variants

Major Task 2: Determine the effects of Notch4 variants on EC specification and gene expression

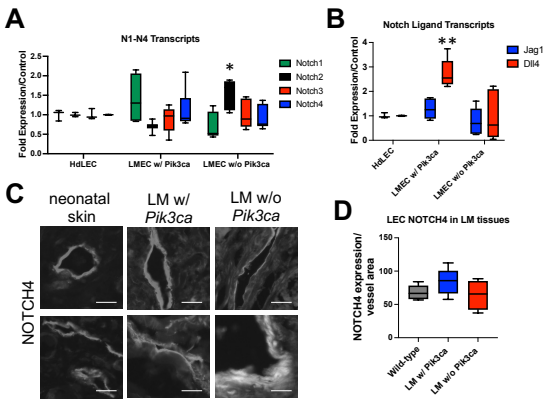

Fig 16. Notch receptor/ligand expression in LMs. A) *Notch1-4,* **B)** *Jag1* and *Dll4* qRT-PCR normalized to *β-ACTIN*. LMEC w/ *Pik3ca* n=3; LMEC w/out *Pik3ca* n=3. Two-way-ANOVA p<0.05. Dunnett's multiple comparisons test versus HdLEC (min/max). *p<0.05; **p<0.0001. **C)** NOTCH4 staining of control neonatal and LM tissues. Images presented for two individuals. Scale bars 25μm. **D)** Quantification of NOTCH4 expression normalized to lymphatic vessel area. Control n=5, LM w/ *Pik3ca* n=8; LMEC w/out *Pik3ca* n=4.

Due to success of studies in Specific Aim 1 these studies were not initiated in year 1. We did begin studies to determine if there is a relationship between the presence of *Pik3ca* variants in LMs and Notch receptor/ligand expression. To do this, we determined their transcript levels in LECs isolated from LMs (LMECs) that had undergone whole exome sequencing and variant calling. The expression of the majority of Notch family members was unchanged in all LMECs, except for *Notch2* transcripts which were significantly increased in LMECs without *Pik3ca* variants (Fig. 16A). Analysis of the endothelial ligands of Notch revealed *Dll4* and not *Jag1* transcript levels was increased in LMECs with *Pik3ca* variants (Fig. 16B). In studies of blood and lymphatic endothelial cells increased *Dll4* expression is associated with increased Notch signaling[5, 8-10].

As *Pik3ca* signaling has been shown to directly promote Notch4 protein degradation[11], it is possible that protein levels may be altered in the absence of changes in transcript levels. Thus, we stained control neonatal skin and LM tissues from patients with and without *Pik3ca* variants for NOTCH4 and PODOPLANIN. Both controls and LMECs expressed NOTCH4 (Fig. 16C). In all LM tissues, NOTCH4 was inconsistent and always overlapped with LECs that expressed PODOPLANIN. Although variable, LEC NOTCH4 expression in LM tissues was similar to that of control lymphatics (Fig. 16D). As the number of samples queried in the RT-PCR for each group was small, the studies may not have been sufficiently powered at this time. We have RNA collected from additional LMEC populations and plan on assessing transcript and protein expression of Notch components in the coming year. Assessment of LEC expression of NOTCH1, DLL4 and JAG1 in control and LM tissues is currently ongoing.

SPECIFIC AIM 3: DETERMINE THE ENDOTHELIAL NOTCH4 TRANSCRIPTIONAL PROFILE

Major Task 1: Gene expression studies of murine ECs from Notch4 heterozygous mice

These studies were not initiated in year 1 and are planned for year 2.

Major Task 2: Ligand activation studies of ECs with Notch4 knockdown

HdLECs express the Notch ligands, DLL4 and JAG1, while the murine lymphatic endothelium expresses DLL4 and JAG1[12]. To understand JAG1/Notch signaling in LECs, we seeded HdLECs on JAG1FC- or FC-coated plates. Seeding on the immobilized ligands robustly activates Notch signaling in a majority of cells. Surprisingly, most DLL4FC-induced genes including direct targets of canonical Notch signaling[8] were not induced upon JAG1 stimulation. Instead, JAG1 upregulated genes that function in inflammation, immune cell recruitment/activation and cellular locomotion (Fig. 17A-C). When JAG1-induced genes were assessed in HdLECs with Notch1 or Notch4 activation, most were induced by Notch4, and not Notch1 (Fig. 17D). Taking advantage of recent single cell (sc)RNA sequencing of murine dermal LECs[13], we determined the expression of Notch pathway genes in the 5 dermal LEC subtypes (Fig. 18). Notch genes were enriched in capillary LEC (capLEC) subtypes. Quiescent capLECs expressed *Notch4*, *Notch1*, and *Dll4*, while proliferating capLECs expressed *Notch1*, *Jag1* and *Dll1*. The newly identified immune capLECs weakly co-expressed Notch1 and Notch4. Consistent with the observed Notch activity, several

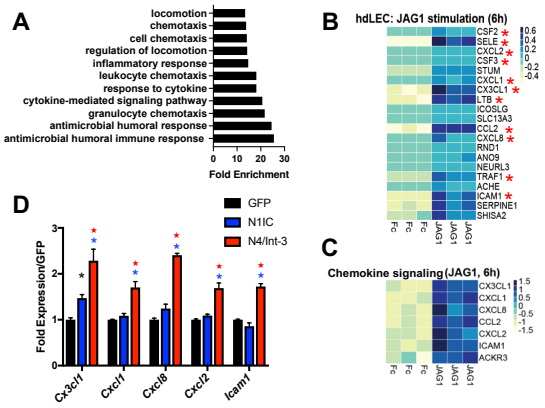

Fig 17. Jag1/Notch4 signaling in unique in LECs. HdLECs seeded on JAG1FC- or FC-coated plates, and RNA isolated after 6 hours, followed by mRNA sequencing performed in triplicate. **A)** Enriched Panther GO Biological Processes **B)** Top 20 genes induced by JAG1FC. Red asterisks mark genes involved in immune cell recruitment and immune cell/LEC interactions. **C)** Chemokine genes induced by JAG1FC. **D)** qRT-PCR of JAG1FC-induced genes in GFP, N1IC or N4/Int-3 expressing HdLECs. Data presented for 2 transductions done in duplicate and relative to GFP ± s.e.m. Two-way ANOVA: p<0.0001; T-test: *p<0.05; *p<0.003 N4/Int-3 or N1IC relative to GFP controls. ★p<0.0003 N4/Int-3 relative to N1IC.

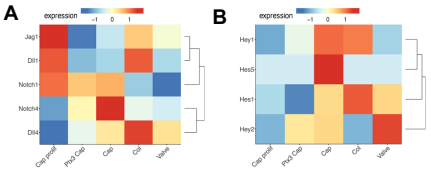

Fig 18. Notch pathway components in murine dermal LECs. **A)** Heatmap of murine dermal LEC scRNA sequencing for Notch and its ligands, or **B)** Notch effectors.

Notch effectors, *Hes1*, *Hes5*, *Hey1* and *Hey2* were expressed in the Notch4/Notch1-expressing capLECs (Fig. 18). *Hes1/Hey1* were also highly expressed in the *Jag1/Dll4/Dll1*-expressing collecting LECs, while valve LECs co-expressed Hes1/Hey2 with the ligands, *Dll4/Jag1*. Thus, not only was Notch active, but JAG1/NOTCH4 signaling was unique from DLL4/NOTCH1/4 signaling in the dermal LECs.

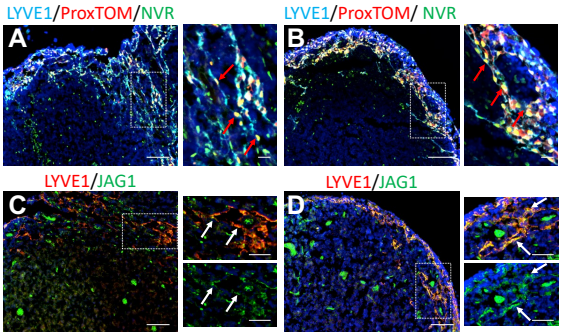

Fig 19. Notch was active and JAG1 expressed in the lymph node LECs. A, B) LYVE1 staining of 6-week *ProxTOM;NVR* axial lymph node sections. **C, D)** LYVE1 and JAG1 staining of 6-week axial lymph node sections. **A, C)** Shows efferent medullary lymphatics and **B, D)** lymphatics lining the subcapsular space. Red arrows mark LECs with canonical Notch activation (GFP). White arrows mark JAG1-expressing lymphatics. Scale bars: Low mag images 100μm. Boxed areas enlarge to the right **A, B)** 20μm, **C, D)** 50μm

Outside of the dermal lymphatics, single cell sequencing has shown that *Jag1* is expressed in a subset of LECs in the murine lymph nodes[14, 15]. This latter observation is consistent with the genes induced by JAG1/Notch4 signaling (Fig. 17). Immunostaining of lymph nodes from 6-week mice revealed that Notch signaling is active and JAG1 expressed in lymph node lymphatics (Fig. 19), suggesting a role for Jag1 in the lymphatics. Gene expression studies in HdLECs suggested that JAG1/NOTCH4 signaling induces multiple cytokines in LECs, including CSF2 (GM-CSF), CSF3 (G-CSF), and CCL2 (MCP-1) (Fig. 17) that modulate monocytes/macrophages. To assess the macrophage phenotype in control and mutant lymph nodes, LYVE1 and F4/80 staining was done. Postnatal deletion of LEC *Notch4* was associated with a significant increase in the macrophage density in the region of the efferent lymphatics (Fig. 20).

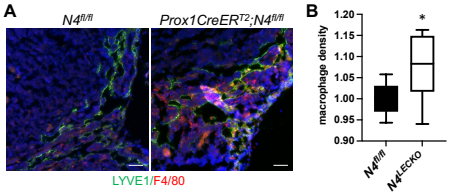

Fig. 20. Increased macrophage density around the efferent lymphatic vessels in the lymph node of mice with postnatal LEC Notch4 deletion. A) F4/80 and LYVE1 staining of 6-week lymph node of *N4$^{fl/fl}$* and *Prox1CreER^{T2};N4$^{fl/fl}$* (*N4^{LECKO}*) mice provided TM at P1, 2 & 3. Scale bars 50 μm. **B**) Macrophage density determined as F4/80 signal intensity normalized to area. T-test: *p = 0.0167.

C) What opportunities for training and professional development has the project provided?

Dr. Glicella Salazar-De Simone is an Associate Research Scientist in the laboratory who has driven the project described in this report. Training of Dr. Salazar-De Simone included re-evaluation of her formal IDP plan. From these discussions, we refined her training plan which has included the submission and presentation of these studies at the upcoming Lymphatic Forum and Columbia University Digestive and Liver Disease Research Center yearly retreat. She also expressed interest in becoming involved in promoting science to underrepresented Hispanic women. Glicella continues to serve on the Education Committee for North American Vascular Biology Organization, and I have encouraged her to apply for other appropriate funding. In addition to my guidance, Columbia University has an office of Postdoctoral Affairs with programs on career development, counseling, networking, expert guidance, postdoctoral training, finding funding opportunities, and increase postdoc visibility which I encourage Glicella to attend. Finally, Glicella is currently enrolled in an applied statistics course at Columbia University.

D) How were the results disseminated to communities of interest?

Studies related to the DOD project were presented at the International Society for the Study of Vascular Anomalies World Congress 2022 in Vancouver Canada and Lymphatics Gordon Conference 2022 in Lucca Italy. Refer to section 6A for details.

E) What do you plan to do during the next reporting period to accomplish the goals?

SPECIFIC AIM 1: DETERMINE ENDOTHELIAL NOTCH4 FUNCTIONS IN MICE

Major Task 1: Obtain appropriate regulatory approval

We plan to maintain regulatory approval.

Major Task 2: Evaluation of N4Ex1 mice vascular phenotypes

We will continue these studies in year 2. We have submitted an abstract to the Lymphatic Forum 2023 to present these studies. We also plan to prepare a manuscript describing the liver phenotype with the $N4^{LECKO}$ liver studies.

Major Task 3: Evaluation of LEC specific N4Ex1 mice vascular phenotypes

We plan to continue the studies of the dermal and liver lymphatic phenotypes. The dermal phenotypes may be included in a paper describing Notch4 function in postnatal dermal lymphatics. We also plan to prepare a manuscript describing the liver phenotype with the $N4^{ex1/ex1}$ liver studies.

Major Task 4: Evaluation of EC specific N4Ex1 mice vascular phenotypes

We plan to start these experiments in year 2 with a goal in determining if blood endothelial Notch4 contributes to the liver phenotype we observed in the $N4^{ex1/ex1}$ mice.

SPECIFIC AIM 2: CHARACTERIZATION OF PUTATIVE LYMPHATIC MALFORMATION NOTCH4 VARIANTS

Major Task 1: Structure function studies of human Notch4 variants

We plan to perform these studies in year 2.

Major Task 2: Determine the effects of Notch4 variants on EC specification and gene expression

We plan to perform these studies in year 2.

SPECIFIC AIM 3: DETERMINE THE ENDOTHELIAL NOTCH4 TRANSCRIPTIONAL PROFILE

Major Task 1: Gene expression studies of murine ECs from Notch4 heterozygous mice

Mice have been generated for these studies and we are in the process of optimizing LEC isolation. We plan to perform these studies in year 2.

Major Task 2: Ligand activation studies of ECs with Notch4 knockdown

We plan to continue with these studies in year 2.

4) Impact:

A) What was the impact on the development of the principal discipline of the project?

These studies revealed the first sex specific difference in a lymphatic phenotype in mice. We found that only mice with LEC *Notch4* develop hallmarks of liver fibrosis.

B) What was the impact on other disciplines?

The finding that *Notch4* haploinsufficiency or loss of LEC *Notch4* leads to progressive liver fibrosis will increase our understanding of fibrotic liver diseases such as NASH, cirrhosis, liver failure, and portal hypertension. These studies also have the potential to explain why men are more affected by these diseases.

C) What was the impact on technology transfer?

Nothing to report.

D) What was the impact on society beyond science and technology?

Nothing to report.

5) Changes/Problems:

A) Changes in approach and reasons for change?

We plan to focus on liver and dermal phenotypes in the $N4^{ex1/+}$ and $N4^{LECKO}$ studies for year 2 with a goal of completing these studies and preparing manuscripts. This will also provide us time to start the endothelial specific knockout studies which will increase the impact of future publication of this work.

B) Actual or anticipated problems or delays and actions or plans to resolve them.

The only problem that we have had is the over ambitiousness of our original proposal which was commented on by reviewers. We have made tremendous progress in our Aim 1 studies which we plan on continuing to focus on with a goal of preparing 1-2 manuscripts by the end of year 2. We will also begin the studies described in Aims 2 and 3. I've hired a part-time student, who volunteered my lab for a year, to support the senior scientist on the project and increase productivity.

C) Changes that had a significant impact on expenditures

None to report

D) Significant changes in use or care of human subjects, vertebrate animals, biohazard, and/or selected agents.

Nothing to report.

6) Products

A) Publications, conferences, and presentations

Conferences

- (October 2022) Notch4 Functions in Organ-Specific Lymphatic Vessel Maintenance. Lymphatics Gordon Conference Lymphatic Vessels as Multifaceted Regulators of Health and Disease, Lucca, Italy. *Invited Speaker*

- Salazar-De Simone, G., McCarron, J., Muley, A., Wu, J.K., and **Shawber C.J**. (May 2022) Novel murine model of Notch4 haploinsufficiency develops HHT-like and LM-like phenotypes. International Society for the Study of Vascular Anomalies World Congress 2022. Vancouver, Canada. *Selected for oral presentation*. (Abstract provided in Appendix)

B) Website or other internet sites:

Nothing to report

C) Technology or Techniques:

Nothing to report

D) Inventions, patent applications and/or licenses

Nothing to report.

E) Other products:

Nothing to report

7) Participants and Other Collaborating Organizations:

A) What individuals worked on the project?

Name: Carrie Shawber, PhD
Project Role: Principal Investigator
Researcher Identifier: 0000-0003-2654-3559 (ORCID ID)
Nearest person month worked: 1.20 calendar months
Contribution to Project: Dr. Shawber led the overall project and was responsible for all phases of the work including analysis and interpretation of data, and preparation and editing of reports and abstracts. Dr. Shawber was also responsible for day-to-day lab management and scientific oversight.
Funding Support: Not Applicable

Name: Glicella Salazar-DeSimone, PhD
Project Role: Postdoctoral Scientist/Associate Research Scientist
Researcher Identifier: 0000-0002-5380-9562 (ORCID ID)
Nearest person month worked: 6 calendar months
Contribution to Project: Dr. Salazar-De Simone performed and led the studies at the bench and worked closely with the two technicians to perform the studies described in this report.
Funding Support: Not Applicable

Name: Joseph McCarron
Project Role: Technician
Researcher Identifier:
Nearest person month worked: 2.5 calendar months
Contribution to Project: Mr. McCarron helped to oversee the colony, genotype mice, collect tissues, immunostaining, image and analyze data. He was also responsible for ordering and overseeing supplies for the project. As Valeriya Borisenko retired, Mr. McCarron took over her responsibilities until Hannah was hired.
Funding Support: Not Applicable

Name: Hannah Fruitman
Project Role: Technician
Researcher Identifier:
Nearest person month worked: 0.9 calendar months
Contribution to Project: Ms. Fruitman started in the Shawber Lab in July 2022 and replaced Valeriya Borisenko. She was responsible for overseeing the colony, genotype mice, immunostaining, imaging and analyzing data.
Funding Support: Not Applicable

B) Has there been a change in the active support of the PD/PIs or senior/key personnel since the last reporting period?

Nothing to report.

C) What other organization were involved as partners?

N/A

8) Special Reporting Requirements: N/A

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ISSVA Abstract 2022

Novel Murine Model of Notch4 Haploinsufficiency Develops HHT-Like and LM-Like Phenotypes

Glicella Salazar-De Simone, Joseph McCarron, Ajit Muley, June K. Wu, and Carrie J. Shawber

Purpose:
Notch1 and Notch4 have overlapping and dynamic expression in the developing and homeostatic vasculature. In mice, loss of endothelial *Notch1*, or ectopic expression of constitutively active *Notch1* or *Notch4* leads to vascular malformations. In humans increased and reduced NOTCH4 expression has been described in vascular malformations. We hypothesized that Notch4 is necessary for proper vascular development and that reduced Notch4 signaling would lead to vascular malformation development.

Methods:
We developed a novel conditional *Notch4* mouse in which lox-P sites are flanking exon 1. Mice with heterozygous deletion of $Notch4^{Ex1}$ ($N4^{Ex1/+}$) were bred, and the resulting 5 litters of mice assessed for blood and lymphatic defects. Wholemounts and sections were stained for the lymphatic marker, LYVE1, and assessed by microscopy.

Results:
Analysis of 2-week old pups generated from $N4^{Ex1/+}$ and $N4^{Ex1/+}$ matings revealed a significant loss of homozygous $N4^{Ex1/Ex1}$ mice (n=1/30, Chi-Squared; TTEST $p<0.0136$). At 2 weeks, 57% of $N4^{Ex1/+}$ male and female mice had redder skin than control littermates. At 6-week mice, some $N4^{Ex1/+}$ mice developed telangiectasias on the tongue, feet, mouth and intestinal walls, and mild limb edema. Blood and lymphatic vessels in $N4^{Ex1/+}$ lungs and liver were dilated. $N4^{Ex1/+}$ lungs also has arteriovenous shunts and blood-filled lymphatics associated with alveolar thickening and immune cell infiltration. In $N4^{Ex1/+}$ livers, venous enlargement, lymphatic vessel dilation with increased density surrounding the portal triads, and a reduction of LYVE1+ sinusoidal vessels were observed. Congestion in the sinusoidal vessels was associated with fibrin clots in the portal and central veins, that was also seen in the lungs.

Conclusions:
Notch4 is necessary for development of the blood and lymphatic vasculature and that decreased Notch4 signaling contributes to the development of vascular malformations in mice similar to HHT and LMs. Taken together the data suggest that vascular Notch4 functions are dose-dependent.