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14. ABSTRACT The purpose of this study was to determine the role of Notch signaling in lymphatic endothelial cell (LEC) behavior and to determine the effects on tumor vasculature upon Notch inhibition. We hypothesized that disrupting Notch activity may interfere with tumor (lymph)angiogenesis by disrupting expression and activity of EC genes. To that end, we have created a treatment agent known as Notch1 decoy (hN1DFc). Activation or inactivation of Notch changes the gene profile of LEC and changes their in vitro behavior. An orthotopic model of human breast cancer was established. These tumors are rich in Notch-positive vasculature, and reliably metastasize to the lungs and/or lymph nodes, making this a good model for future studies of Notch inhibition on tumor vasculature.					
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Introduction

Tumor size, lymph node involvement, and distant metastases are all important prognostic factors in breast cancer. Interestingly, angiogenesis and lymphangiogenesis are involved in each of these factors. In order for a tumor to grow, invade nearby lymph nodes, and spread to distant parts of the body, it needs blood and lymphatic vessels. The Notch signaling pathway is a cell-fate determining pathway that consists of 4 receptors (Notch 1, 2, 3, 4) and 5 ligands (Delta-like 1, 3, 4 and Jagged 1, 2). When a Notch receptor interacts with a ligand and is activated, a series of proteolytic cleavages release the intracellular domain of the receptor from the cell membrane, allowing it to translocate to the nucleus and act as a transcriptional regulator. It has been established that Notch is present and active in the vasculature. Our lab has demonstrated that Notch can regulate endothelial cell (EC) genes. We hypothesize that disrupting Notch activity may interfere with tumor angiogenesis and lymphangiogenesis by disrupting expression and activity of EC genes. To that end, we have created a treatment agent known as Notch1 decoy (hN1DFc).

Body

Task 1. Study how Notch signaling functions in primary lymphatic endothelial cells

Notch function in the behavior of primary blood endothelial cells (BEC) has been established in human umbilical venous endothelial cells (HUVEC). Notch family receptors and ligands (Notch 1, Notch 4, Dll-4, Jag1) are present in HUVEC. Activation of Notch in HUVEC inhibits *in vitro* proliferation, migration, and network formation, presumably by Notch's ability to repress VEGFR-2 (unpublished data).

In order to study Notch function in the behavior of primary lymphatic endothelial cells (LEC) *in vitro*, we established a method of isolating human dermal lymphatic endothelial cells (HDLEC) from neonatal foreskins. Notch family receptors and ligands (Notch 1, Notch2, Notch 4, Dll-4, Jag1) were present in our isolated HDLEC on the transcript (**Figure 1a**) and protein level (**Figure 1b**).

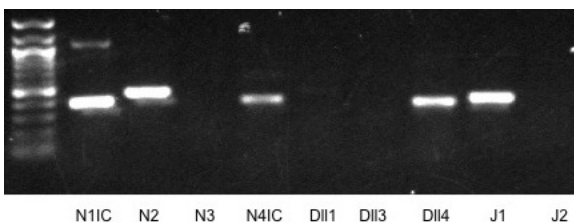


Figure 1a

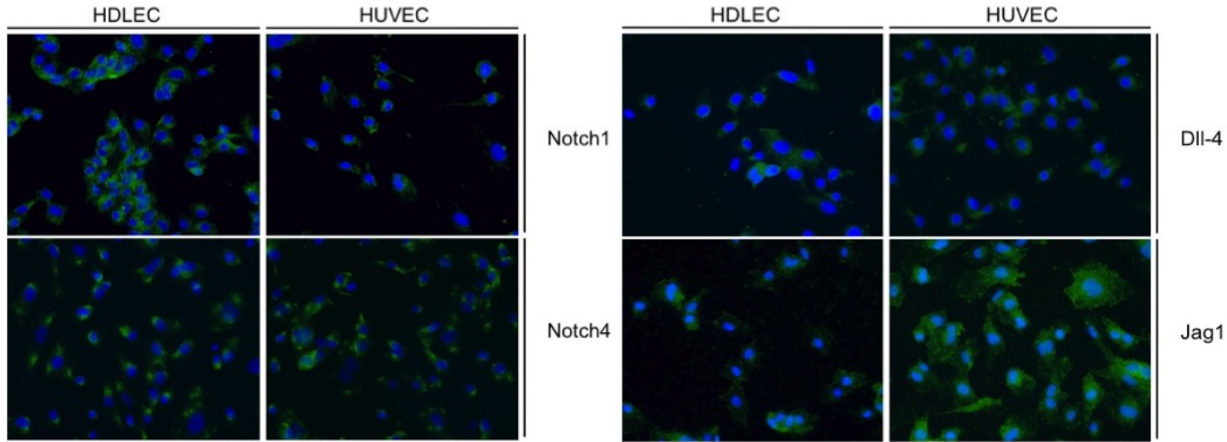


Figure 1b

To activate Notch in HDLEC, lentiviral constructs expressing activated Notch 1 (N1IC) or activated Notch 4 (N4int3) were used. As expected, Notch activation (with either N1IC or N4int3) significantly induced transcripts for direct targets such as Hey1 and Hey2 (data not shown), as well as the LEC gene VEGFR-3 (**Figure 2a**). Interestingly, Notch activation significantly repressed transcripts for most LEC genes (e.g., Prox1, LYVE1, and podoplanin) (**Figure 2b**), as well as for VEGFR-2 (a BEC-associated gene, which is also repressed in HUVEC upon Notch activation) (data not shown).

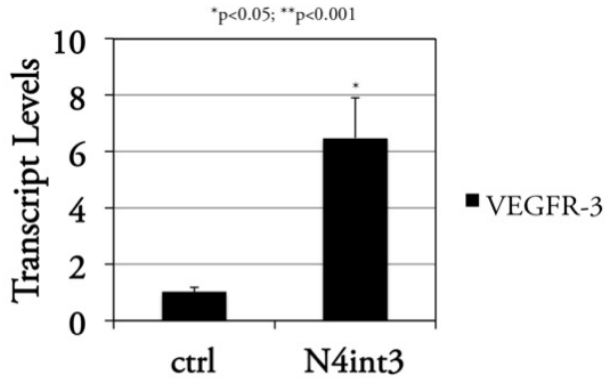


Figure 2a

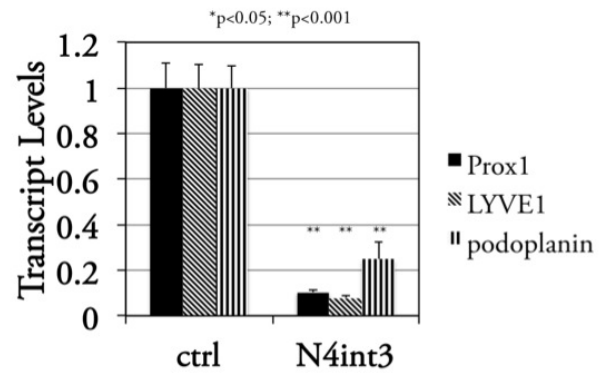


Figure 2b

Upon observation that Notch activation repressed many LEC-associated genes, namely Prox1 (which has been shown to be necessary to maintain LEC identity), we set out to determine whether Notch activation in HDLEC reverts LECs to BECs. Notch activation did not appear to revert LECs to BECs, as CD34 (a BEC marker) was repressed and Nrp1 (another BEC marker) was unaffected (**Figure 3**).

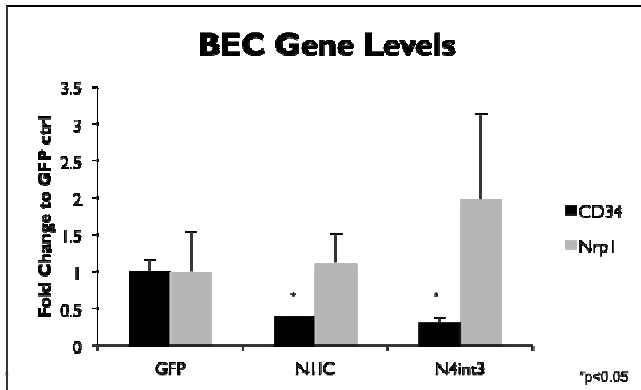


Figure 3

To inactivate Notch in HDLEC, lentiviral constructs expressing activated human Fc (hFc) or Notch1 decoy (1-36) were used. Unexpectedly, Notch inactivation did not have any effect on LEC genes, but repressed VEGFR-2 (**Figure 4**). These results need to be repeated, however, as this was only observed from a single experiment.

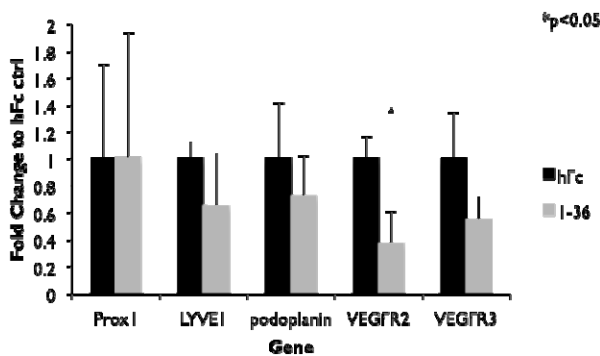


Figure 4

Effects of Notch activation on HDLEC behavior were tested in various different *in vitro* assays. Briefly, proliferation was tested by seeding equal numbers of cells for all conditions being tested, then quantifying cell number after 4 days of culture. Migration was observed by using a pipet tip to make equal-sized scratches in cell monolayers, then observing the closing of the scratch over a period of 24hrs. Network formation was observed by plating equal numbers of cells in between two collagen gel layers and observing the formation of networks over 4 days of culture. All assays were performed in serum-free endothelial media supplemented with EGF and VEGF-C. Preliminary data suggest that Notch activation in HDLECs inhibits *in vitro* proliferation, migration, and network formation (**Figure 5a, b, c**).

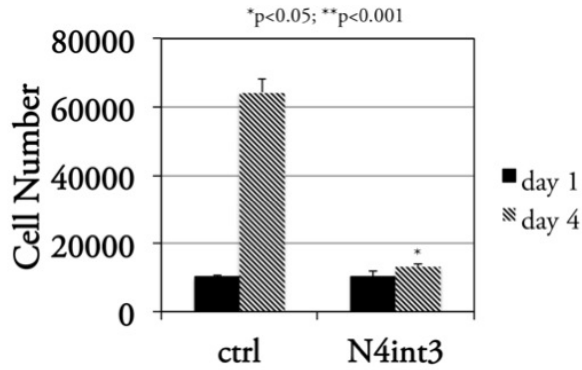


Figure 5a

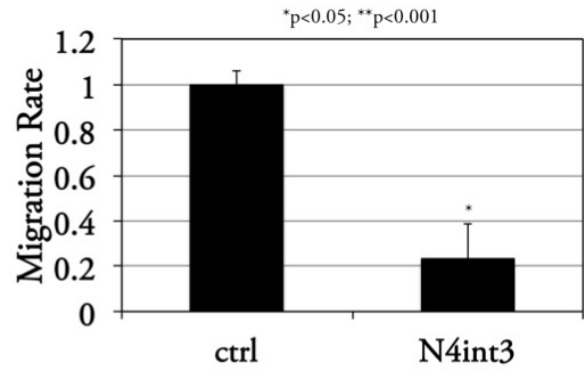


Figure 5b

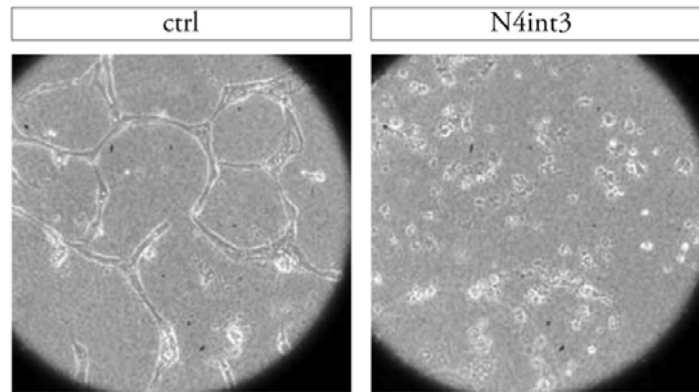


Figure 5c

Tasks 2 and 3. Study Notch function in pathological angiogenesis and lymphangiogenesis

To study the effects of Notch inhibition in breast tumor vasculature *in vivo*, the MDA-MB-231 human breast cancer cell line was used for xenograft studies. An MDA-MB-231 line that stably expresses luciferase (231-luc) was used. Luciferase activity was useful for live imaging of tumor progression throughout the course of tumor studies. A survey revealed that transcripts for both Notch and VEGF receptor family members were present in cultured 231-luc cells (data not shown). Notches 1, 2, and 3; Dll-1 and Dll-4; Jag1; and VEGFR-2 were present. Immunohistochemistry of pilot 231-luc tumors grown in the mammary fat pads of female nude mice demonstrated that Notch family receptors and ligands were indeed expressed by the tumors themselves, as well as in the blood (yellow arrows) and lymphatic (white arrows) vasculature (**Figure 6**).

The luciferase-expressing cell line was used to generate the following cell lines:

1. GFP
2. VEGF-C_cys156ser (V_c156s)
3. hFc+GFP
4. hFc+V_c156s
5. 1-36+GFP
6. 1-36+V_c156s

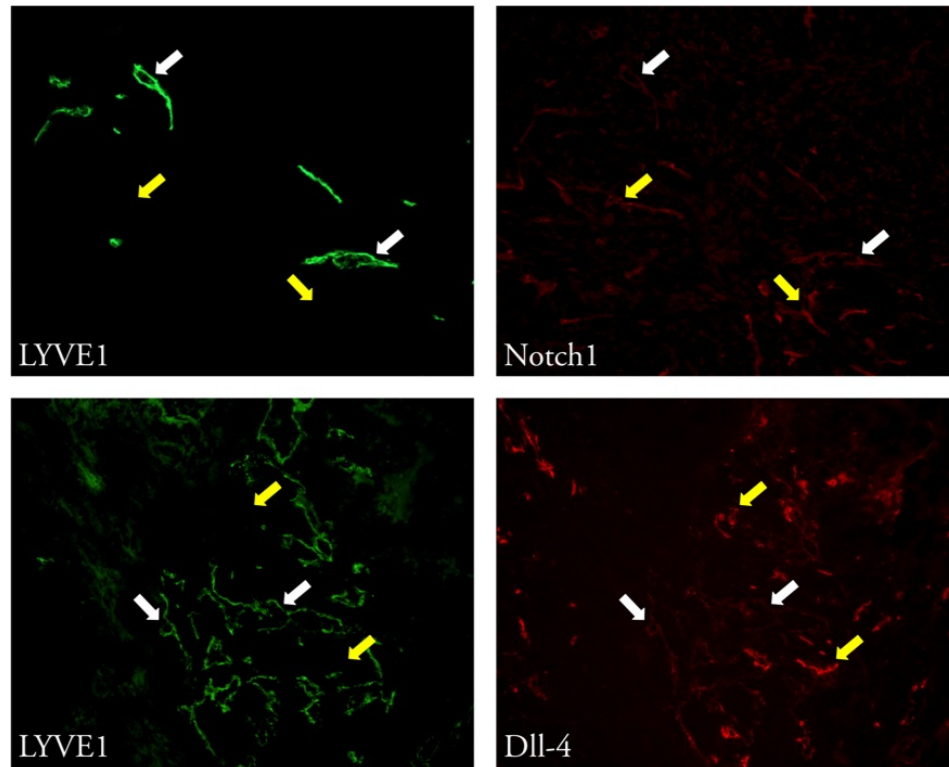


Figure 6

VEGF-C_{cys156ser} (V_{c156s}) is a mutant VEGF-C that binds only VEGFR-3. This mutant VEGF-C was used to focus more closely on effects of Notch inhibition on VEGFR-3⁺ vasculature (tumor lymphatics and blood vessels both express VEGFR-3). 1-36 is Notch1 decoy (consisting of the 36 EGF-like repeats in the extracellular region of human Notch 1, fused to an Fc tag) that was created in our lab as a pan-Notch inhibitor. Western blot demonstrated that the cell lines expressed and secreted the appropriate proteins (data not shown).

These cell lines were tested to determine whether *in vitro* proliferation, migration, or growth in soft agar was affected. Although 231-luc express Notch receptors and ligands, there were no significant differences in proliferation or migration of the 6 cell lines (data not shown). We were unable to compare colony formation, as the 231-luc cell line does not grow well in soft agar.

Subsequently, pilot tumor studies were performed. In the first pilot study, 231-luc/GFP and 231-luc/V_{c156s} were used to determine whether V_{c156s} secretion induces tumor (lymph)angiogenesis and tumor growth/progression. 2×10^6 cells mixed with Matrigel were orthotopically implanted into the right 4th mammary fat pad of 4-6 week old female nude mice. Tumors were allowed to progress for 10 weeks before mice were

sacrificed, tumors resected, and lungs as well as lymph nodes were imaged *ex vivo* for metastases. We found that V_c156s conferred a slight growth advantage over GFP (**Figure 7a**), and tumor weights at the end of the study were found to be approximately 1.5x greater in V_c156s mice (**Figure 7b**).

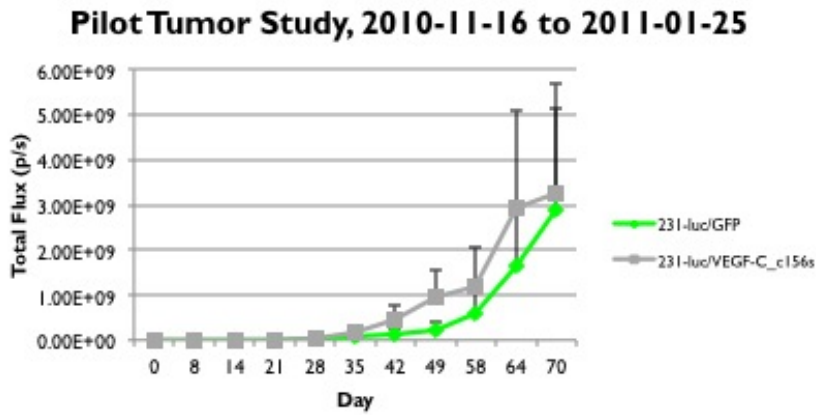


Figure 7a

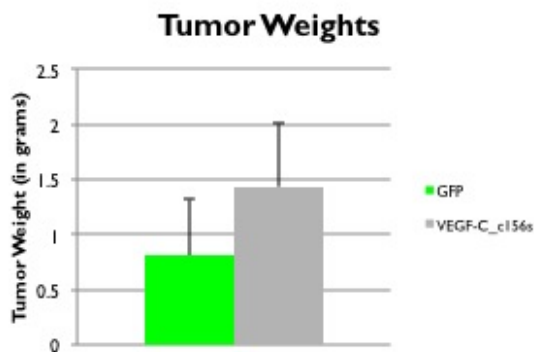


Figure 7b

Ex vivo imaging of lungs and lymph nodes found no difference between groups for lung metastases (33% of mice had lung metastases, data not shown), but a significant difference between groups for metastases to axillary lymph nodes (4 of 9 mice in the V_c156s had axillary lymph node metastases, while 0 of 9 mice in the GFP group had axillary lymph node metastases, data not shown). Quantification of immunostaining for CD31 (a general vessel marker that stains both blood and lymphatic vasculature) revealed that V_c156s tumors had approximately a 1.5-fold increase in CD31⁺ vasculature, which was not significant (data not shown). Immunostaining for LYVE1 (a lymphatic vessel marker) revealed that V_c156s tumors had an increase in tumor lymphatics, as well as an increase in invasion of lymphatics into the tumor (**Figure 8, not yet quantified**). This correlates with our axillary lymph node *ex vivo* imaging data, suggesting that the increase in tumor lymphangiogenesis in V_c156s tumors may be contributing to increased metastasis to lymph nodes.

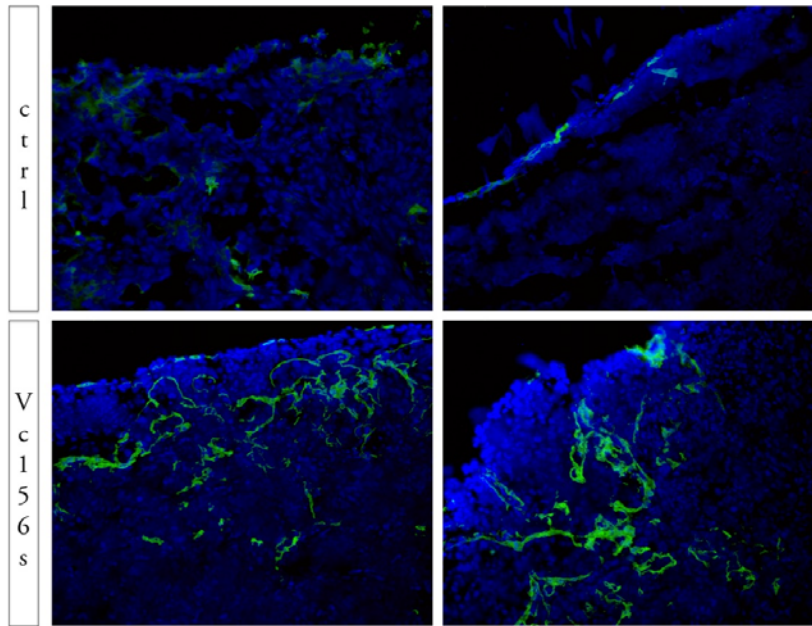


Figure 8

In our second pilot study, 231-luc/Vc156s and 231-luc/V_c156s+hFc were used to confirm that hFc (which is used as a control for our 1-36 line) has no effect on tumor vasculature, progression, or growth. The tumor study was performed in the same manner as described for the first pilot study. We found that hFc did not affect tumor growth (**Figure 9a**) or tumor weight (**Figure 9b**).

Pilot Tumor Study, 2011-07-05 to 2011-09-14

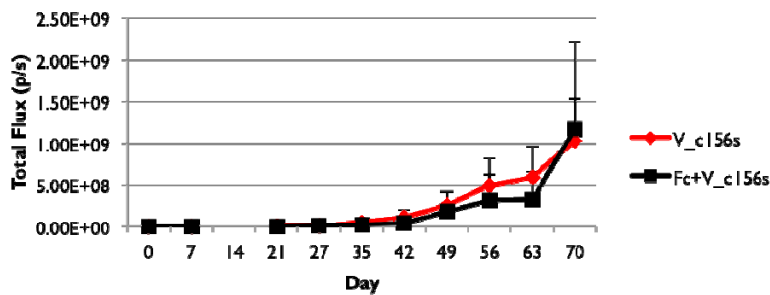


Figure 9a

Tumor Weights

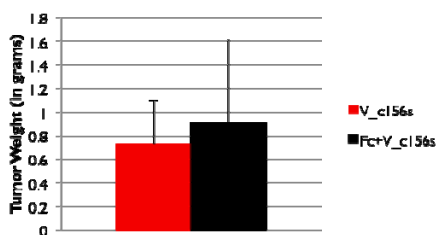


Figure 9b

Ex vivo imaging of lungs and lymph nodes found no difference between groups for lung metastases (4/10 Vc156s mice had lung metastases, while 3/10 Vc156s+Fc mice had lung metastases, data not shown), or metastases to axillary lymph nodes (3/10 Vc156s mice had axillary lymph node metastases, while 4/10 Vc156s+Fc mice had axillary lymph node metastases, data not shown). Immunostaining for LYVE1 and Prox1 (two lymphatic vessel markers) revealed that there was no difference in tumor lymphatics between the two groups (**Figure 10, not yet quantified**). This correlates with our axillary lymph node *ex vivo* imaging data.

Prox1/LYVE1

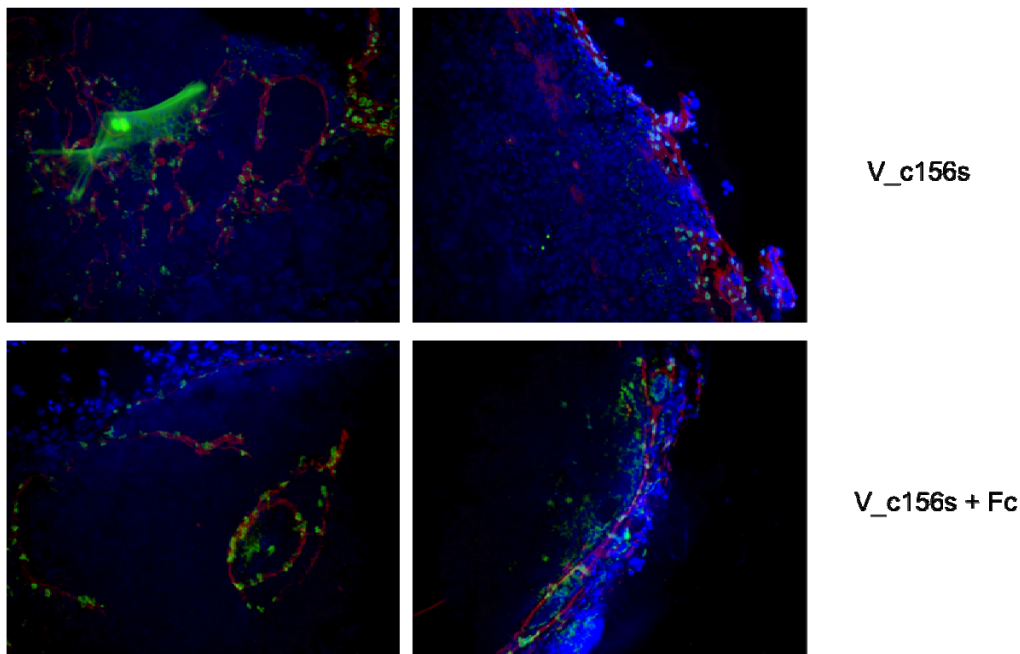


Figure 10

Our first two pilot studies allowed us to establish a tumor model that is rich in vasculature, particularly in lymphatics, and that reliably metastasizes via both the blood and lymphatic vasculature. Subsequently, for our first full tumor study, we implanted 231-luc/Vc156s tumors. After 31 days, the mice were randomized into two treatment groups – hFc, or N1 decoy. Treatment was administered via injection of adenovirus into the bloodstream. Injection of 5×10^8 ffu/mouse allowed for infection of the liver, resulting in the continued expression and secretion into the bloodstream of our treatment agents. Every other aspect of the tumor study was performed in the same manner as described for the first pilot study.

N1 decoy treatment did not significantly affect tumor progression (**Figure 11a**). However, average tumor weight of treated tumors trended towards being lower (**Figure 11b**).

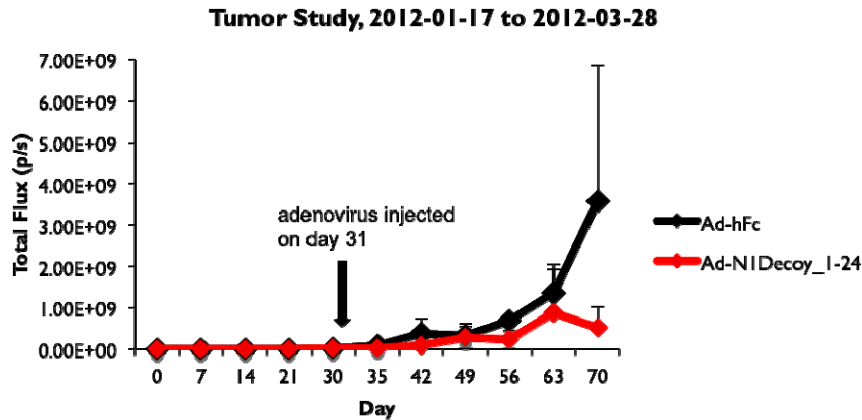


Figure 11a

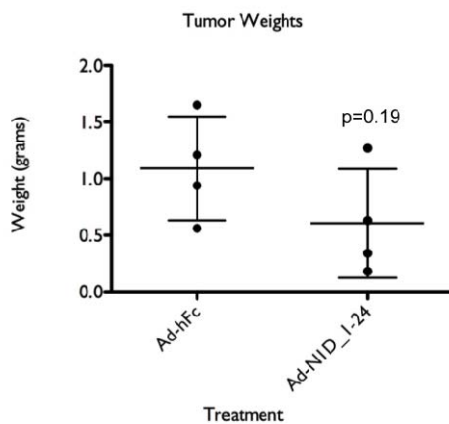


Figure 11b

Immunostaining for LYVE1 (a lymphatic vessel marker) on a subset of tumors suggested that N1 decoy treatment reduced tumor lymphatic vessel density (**Figure 12a, not yet quantified**). Immunostaining for endomucin (a blood vessel marker) on the same subset of tumors suggested that N1 decoy treatment reduced tumor blood vessel density (**Figure 12b, not yet quantified**). Metastasis detection in lungs and lymph nodes is currently being performed but has proved to be challenging. We believe our current method of *ex vivo* metastasis imaging (by dropping luciferin directly on top of excised lymph nodes and lungs) is not a very sensitive method, and are working to increase sensitivity in detection. Further analysis of all tumors included in the study needs to be performed in order to draw conclusions, but we expect that treatment with our N1 decoy will be able to suppress tumor blood and lymphatic vasculature, tumor growth, and metastasis to lymph nodes and lungs.

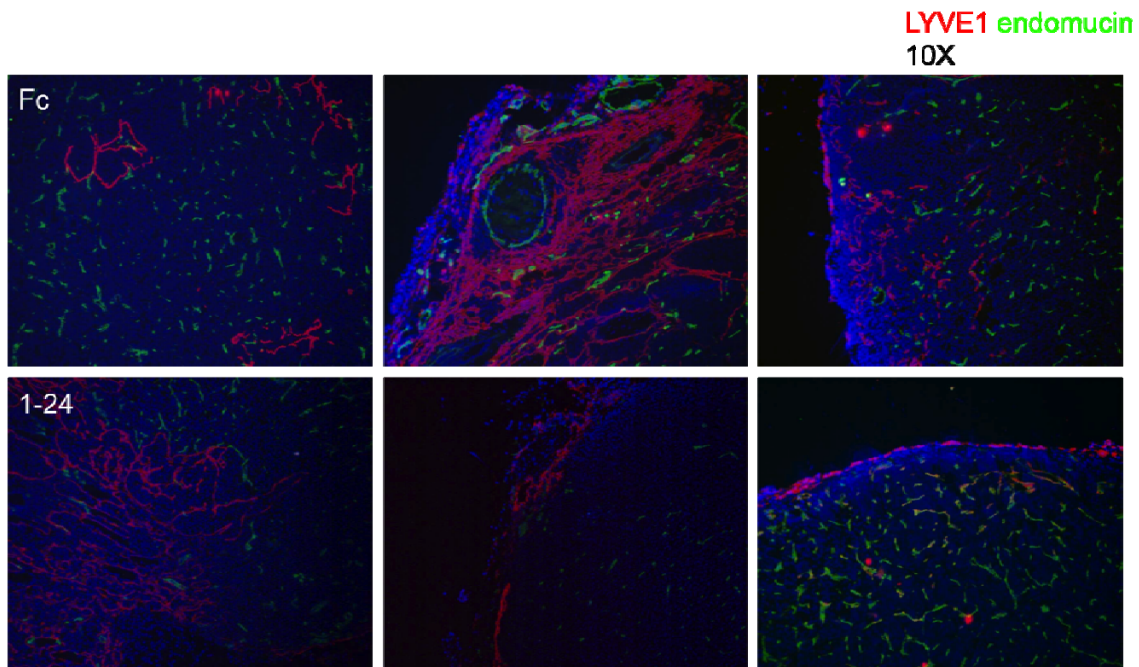


Figure 12a

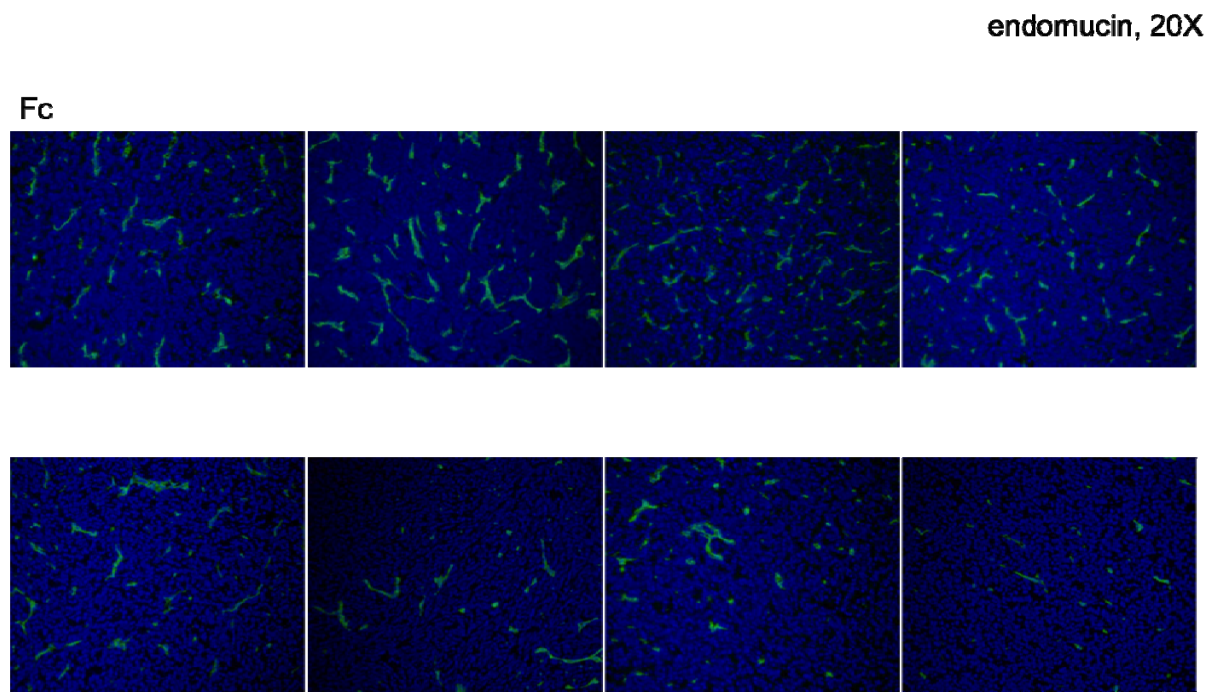


Figure 12b

Key Research Accomplishments

- Established a method of isolating primary human dermal lymphatic endothelial cells
- Demonstrated that Notch receptors and ligands are present in lymphatic endothelial cells (LEC)
- Demonstrated that perturbation of Notch signaling in LEC changes the gene profile of LEC
- Demonstrated that perturbation of Notch signaling in LEC inhibits proliferation, migration, and network formation *in vitro*
- Established an orthotopic model of human breast cancer that recruits blood and lymphatic vasculature and metastasizes to lungs and lymph nodes (231-luc). This will be a good model to study how Notch inhibition affects tumor blood and lymphatic vessels.
- Demonstrated that Notch receptors and ligands are present both in 231-luc tumors, as well as in their vasculature
- Demonstrated that secretion of a mutant VEGF-C (V_c156s) by 231-luc induces tumor lymphangiogenesis and increases metastasis to lymph nodes
- Working to refine metastasis detection
- Preliminary data suggests that N1 decoy is able to reduce tumor blood and lymphatic vasculature

Reportable Outcomes

- Poster presentation for the Era of Hope DOD Breast Cancer Research Program Meeting 2011
- Master of Philosophy, Columbia University, awarded in October 2010

Conclusion

I have demonstrated that Notch is expressed by isolated HDLEC *in vitro*. I have also shown that Notch plays a role in LEC behavior, as inducing Notch signaling affects HDLEC behavior *in vitro*. Induction of Notch signaling also changes the endothelial cell gene profile of HDLEC on the transcriptional level. Though the link between the changes in HDLEC gene profile and changes in *in vitro* activity has not yet been shown, my data suggests that induction of VEGFR-3 by Notch is not the sole factor in Notch's effects on HDLEC *in vitro* behavior. Notch's ability to repress other EC genes such as Prox1, LYVE1, podoplanin, and VEGFR-2 may also play an important role. In future studies, it will be important to more thoroughly study the effects of Notch

inhibition in HDLEC. It is interesting to note that preliminary results show that Notch inhibition does not have the opposite effects of Notch activation. This suggests that perturbation of Notch signaling in either direction (exogenously activating or inactivating signaling) may have similar effects on HDLEC gene expression and *in vitro* behavior.

Additionally, I was able to establish an orthotopic model of human breast cancer that recruits both blood and lymphatic vasculature and metastasizes to lungs and lymph nodes. I have shown that these tumors have Notch-positive blood and lymphatic vasculature. Initial studies using adenovirus expressing N1 decoy suggests that N1 decoy treatment may suppress tumor vascularization and metastasis in this model. Additionally we are working to refine our method of metastasis detection, both *in vivo* and *ex vivo*.

The goal of this project is to understand the mechanisms involved in a tumor's ability to recruit vasculature, grow, and metastasize. If we are able to consistently show that Notch1 decoy treatment is able to significantly inhibit these processes, we may be able to translate these results into the clinical setting. Therefore, this research is highly relevant to breast cancer and potential treatment.