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14. ABSTRACT The *overall objective* is to define the interaction between Notch and VEGFR-3 signaling in breast cancer. We are examining a role for Notch in breast tumor vessels and attempting to block Notch and VEGFR-3 activity in breast tumors grown in mice. We proposed two aims: 1) studies of Notch/Dll4 function in murine mammary tumorigenesis and 2) studies of the inhibitory effects of a Notch antagonist (Notch decoy) in a murine mammary tumor model. To study the role for notch in murine mammary tumorigenesis, progress has been made in developing two new transgenic lines that will allow for conditional activation or inactivation of Notch specifically within the endothelium. In addition, we have begun an assessment of mammary cancer cell growth in Notch4 mutant mice. We have also initiated experiments to test if circulating Notch antagonists can inhibit tumor growth and angiogenesis. To achieve this, we have developed a system that uses injection of Notch antagonist expressing adenoviruses into immunocompromised mice. This leads to expression of the antagonist in the circulation and inhibition of tumor xenografts. We found that Notch decoy blocked tumor growth, presumably by blocking tumor angiogenesis. If this approach inhibits mammary tumor growth, the ectodomains of Notch will be further pursued as candidates for breast cancer therapy.

15. SUBJECT TERMS
Notch, VEGF, Breast Cancer, Angiogenesis

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TABLE OF CONTENTS

INTRODUCTION

Expression studies have shown that the angiogenic/lymphangiogenic factor VEGFR-3, its ligand VEGF-C and the Notch ligand, Dll4 are up-regulated in the invading blood and lymphatic vessels in human breast cancer (1, 2). Using mouse models, Notch signaling has been found to be essential for angiogenesis to progress to completion in the developing embryo (3). However, the exact mechanism(s) by which Notch signaling regulates angiogenesis and/or lymphangiogenesis is not well understood. Though, it is known that Notch modulates cell-fate decisions by regulating the expression of tissue specific genes. As many angiogenic regulators have been identified, we have focused on defining the interactions between Notch and that of known angiogenic regulators. Using quantitative RT-PCR analysis, we found that both VEGFR-3 and Dll4 were induced in response to an activated form of Notch4 (Notch4/int-3) in three human primary endothelial cells (HUVEC-umbilical vein, HUAEC-umbilical artery, and HMVEC-dermal microvascular). Taken together these data suggested a relationship between Notch signaling and VEGFR-3. Thus, we ***hypothesized*** that Notch may regulate blood and/or lymphatic vessel development via its induction of VEGFR-3 during physiological angiogenesis, as well as pathological angiogenesis and/or lymphangiogenesis in breast cancer. The ***overall objective*** of this proposal is to define the interaction between Notch and VEGFR-3 signaling in breast cancer.

Our original study design was as follows:

We will investigate the relationship between Notch and VEGFR-3 signaling in gain-of-function and loss-of-function mouse models. We have developed two mouse models in which Notch signaling can be specifically induced in the vascular endothelium. Murine mammary tumor cells will be implanted into these mice and the affect(s) of Notch signaling on tumor angiogenesis/lymphangiogenesis determined. Expression of Dll4 and VEGFR-3 and its ligands, VEGF-C and VEGF-D will also be monitored. We will also determine if blocking endogenous Notch signaling will perturb breast tumor angiogenesis/lymphangiogenesis. We have generated Notch antagonists that encode the extracellular domains of the four Notch proteins but not the signaling domain. Human mammary tumor cells lines will be generated that express the different Notch antagonists and xenografted into immuno-compromised mice. The affects of the different Notch antagonists on tumor angiogenesis and/or lymphangiogenesis will be examined, as well as the affects on Dll4 and VEGFR-3 expression.

In year one we made progress on three of our designated tasks. In **Task 1** (Months 1-3) we set out to optimize conditions for tamoxifen treatment of mice to conditionally activate Notch or Dll4 expression. We have made progress in establishing that NotchIC can be conditionally activated but have been unable to detect Dll4 expression or function in our conditional Dll4 mice. Thus, we have altered our strategy to develop a new transgenic NotchIC line and a transgenic Notch decoy (inhibitor) line and our progress will be described. In **Task 2** (Months 4-24) we set out to induce Notch activity in adult mice and determine the consequences for breast tumor cell growth. We describe progress to this goal and present a new model to address this question, which is to evaluate breast tumor xenografts in Notch4 nullizygous mice. In **Task 4** (Months 6-36) analysis of VEGFR-3 expression in tumors expressing Notch antagonists. An initial goal in this task was to develop breast cancer cell lines over-expressing NotchIC or Notch decoy in order to manipulate Notch activity in the cancer cell. Ultimately, we wanted to create line that secrete decoy to assess the affects on tumor cell/endothelial interaction. What we found was that Notch activity affected the cancer cell. Activation of Notch suppressed breast cancer cell growth and expression of Notch decoy promoted growth. This result was unexpected and will be discussed in the body, below.

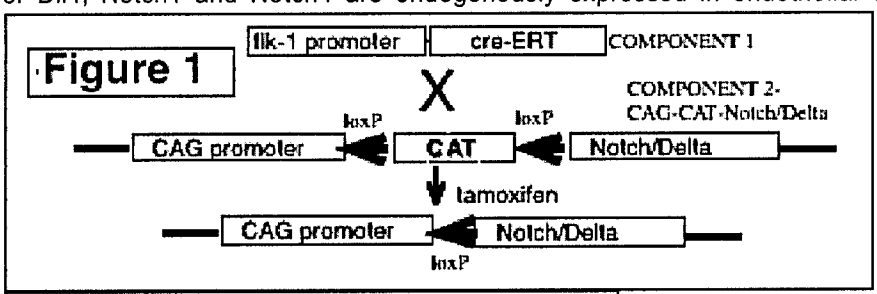
BODY

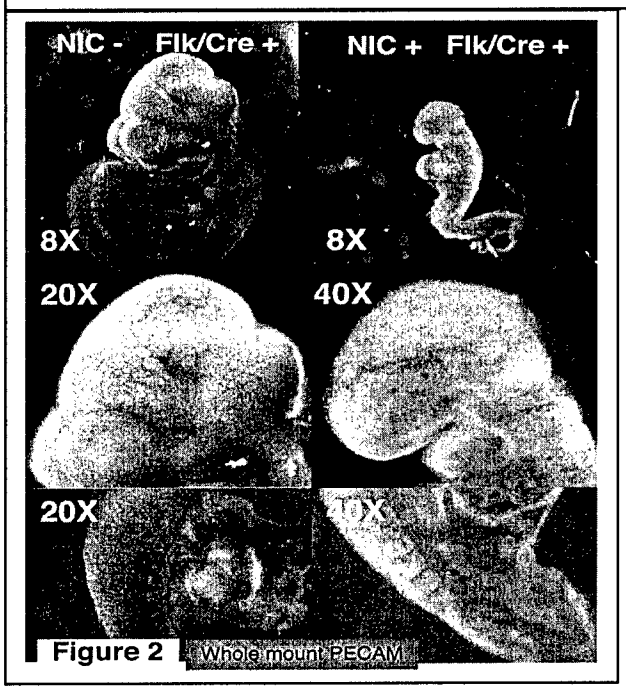
Specific Aims 1: Notch and Dll4 function in murine mammary tumorigenesis.

Task 1 (Months 1-3) Optimizing conditions for TM treatment of N1ICD and Dll4 double transgenic mice

We set out to optimize conditions for tamoxifen treatment of mice to conditionally activate Notch or Dll4 expression. We have made progress in establishing that NotchIC can be conditionally activated but have been unable to detect Dll4 expression or function in our conditional Dll4 mice. Thus, we have altered our strategy to develop a new transgenic NotchIC line and a transgenic Notch decoy (inhibitor) line and our progress will be described.

In our original proposal, we pursued a system to conditionally express Dll4 and N1ICD, an activated form of Notch1, in the adult vasculature. We have generated mice doubly transgenic for a Flk1-Cre-ERT transgene and either a N1ICD or Dll4 CAG-CAT transgene in the C57BL/6J background. The Flk1-Cre-ERT transgene encodes the Flk1 (VEGFR-2) regulatory region upstream of a Cre-estrogen receptor fusion gene. In the adult mouse, Flk1 expression is restricted the vascular endothelium. Thus, this transgene limits the expression of the Cre-ERT fusion protein to the vascular endothelial cells. The Cre-ERT fusion protein has a tamoxifen(TM)-responsive estrogen receptor binding component and is latent until TM is administered. The CAG-CAT transgene contains a non-functional gene (CAT) with its associated stop codon, flanked by lox P sites followed by either N1ICD or Dll4 gene. In the absence of Cre expression, the CAT gene is expressed. When the CAT gene is excised by Cre, the N1ICD or Dll4 gene is placed behind the CAG promoter and expressed. The CAG promoter consists of a CMV enhancer-chicken β-actin hybrid protomoter that is strongly expressed in endothelial cells. The receptors for Dll4, Notch1 and Notch4 are endogenously expressed in endothelial cells (4, 5) and should be activated by the exogenous Dll4. Thus, when treated with tamoxifen these double transgenic mice activate Notch signaling specifically within the vascular endothelium (Figure 1).





Original conditional mouse models to regulate Notch activity (CAG-CAT)

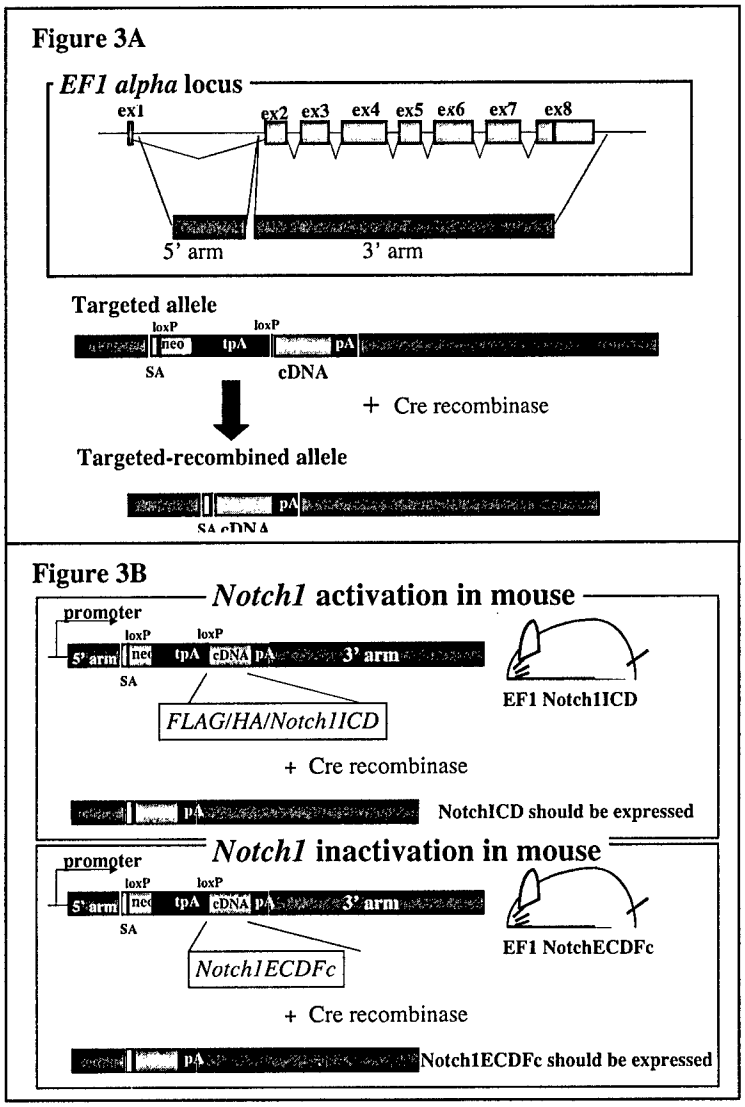
The CAG-CAT-NotchIC has been largely validated as crossing Flk1-creERT to CAG-CAT-NotchIC and treating pregnant mothers with tamoxifen leads to embryonic lethality due to a vascular remodeling defect (Figure 2). This is the previously described phenotype for Notch activation in embryonic endothelium (6). In addition, we have generated doubly transgenic Flk1-creERT / CAG-CAT-NotchIC offspring in the absence of tamoxifen. If these mice are now treated with tamoxifen, NotchIC expression can be detected in liver, a highly vascularized tissue (data not shown).

In contrast, we have not seen any affects from similar experiments done with the two CAG-CAT-Dll4 transgenic lines that we developed. That is, Flk1-creERT / CAG-CAT-Dll4 doubly transgenic embryos treated with tamoxifen show no phenotype. We believe this

indicates that the Dll4 transgenic does not express sufficient levels of Dll4 in response to cre to allow for alteration of vascular development. We made several unsuccessful attempts to document transgene expression and conclude that these two transgenic lines are not useful for the proposed studies.

New conditional mouse models to regulate Notch (EF-1-flox)

We have initiated the development of another type of conditional models that will be used in these studies and is described here. As discussed above, we have successfully developed one transgenic line to conditionally regulate NotchIC expression (CAG-CAT-NotchIC). To enhance our chances of success in conditionally manipulating Notch in tumor endothelium, we have pursued another model for conditional Notch regulation, which we will refer to as EF1-flox-Notch (Figure 3A, 3B). In this new strategy, we also include a mouse line designed to inactivate Notch signaling in tumor endothelium that incorporates the Notch decoy. Although these approaches were not originally proposed, we feel that inclusion enhances our ability to understand Notch function in tumor endothelium by either activating or inactivating Notch in tumor vessels.



The EF1-flox-Notch constructs use the strong promoter from the eukaryotic Elongation Factor 1 (EF1) gene to drive expression (Figure 3A). Using homologous recombination, we have inserted a flox-stop-NotchIC construct and a flox-stop-NotchECDFc (Notch decoy) construct into the EF1 locus (Figure 3B). The proper targeting of ES cells has been confirmed and a mouse has already been generated for expression of NotchIC (EF1-flox-stop-Notch1IC). This will be used in the same fashion as CAG-CAT-NotchIC. To date, we have crossed EF1-flox-stop-Notch1IC mice with Rosa-cre mice and this leads to embryonic lethality, as expected. The mice will next be used to determine the consequences of activation in endothelium, which we predict will lead to a vascular remodeling defect. Subsequently, the tumor studies will be initiated, see below. The ES cells with the EF1-flox-stop NotchECDFc are currently being used to generate mouse lines. In the next year, we will thus have two gain-of-function mouse models and one loss of function.

Task 2 (Months 4-24) Induction of N1IC/Dl4 in adult mice and tumor cell implantation.

We set out to induce Notch activity in adult mice and determine the consequences for breast tumor cell growth. We describe progress to this goal and present a new model to address this question, which is to evaluate breast tumor xenografts in Notch4 nullizygous mice.

Experiments using both gain-of-function (described above) and loss-of-function mouse breast tumor models will aid our understanding of the role of Notch in tumor angiogenesis. Notch4 nullizygous mice are viable and fertile (7). However, we noticed that the adults have vascular patterning defects. We have begun to perform "proof of principle" tumor xenograft experiments in Notch4 nullizygous mice with

promising results. Notch4 is primarily expressed within the vascular endothelium during development and this expression is maintained in the adult (8). In a preliminary study, Colon-38 tumor cells were implanted into the abdominal cavity of two wildtype and two Notch4 nullizygous C57Bl mice. Colon-38 tumor cells are a murine colon cancer cell line, chosen because they promote both angiogenesis and

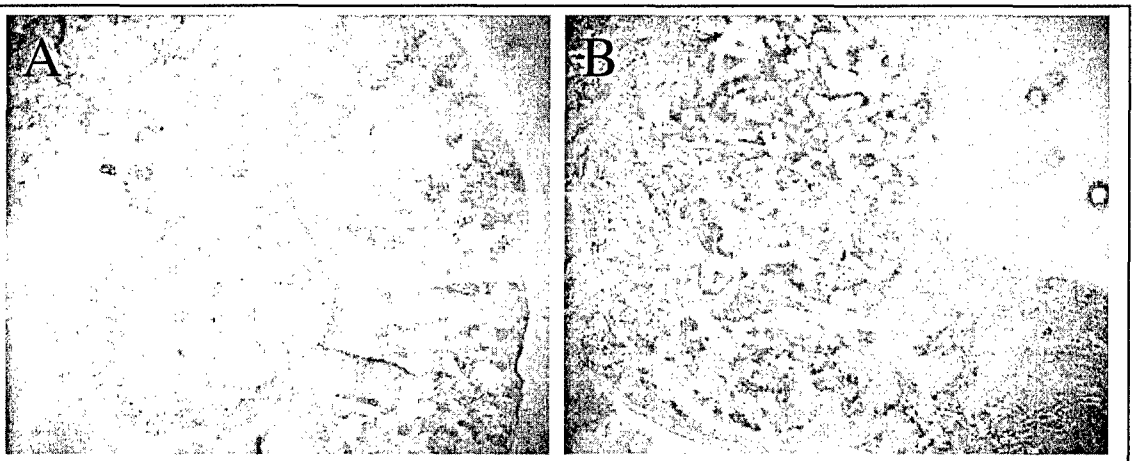


Figure 4. Hematoxylin and eosin staining of colorectal tumor xenografts in A) wildtype and B) Notch4 nullizygous mice.

lymphangiogenesis when xenografted. Twenty-eight days post-implantation tumors were removed, weighed and embedded in OCT. Although a difference in the tumor size between wildtype and Notch4 nullizygous tumors was not observed, an increase in tumor necrosis was observed in the Notch4 nullizygous tumors as seen by hematoxylin and eosin staining (Figure 4).

PECAM staining for endothelial cells also revealed differences between the tumors grown in the wildtype and Notch4 mutant mice (Figure 5). In the tumors grown in the Notch4 nullizygous mice, the vessels were often dilated and tortuous (Figure 5B) as compared to the vessels of the tumors from the wildtype mice (Figure 5A). At high magnification, PECAM staining was often observed within the necrotic tissue, whereas PECAM staining in the control tumors was usually several cells distant from the necrotic regions. This observation suggests that the increase in tumor necrosis was due to collapsed vessels in which blood is not circulating. To evaluate this hypothesis, we will perform fluorescent angiographies to determine the functionality of the vessels. We plan to continue these studies with the syngenic murine mammary tumor cell line, CCL-51 that will be implanted subcutaneously and into the mammary fat pads of the Notch4 nullizygous and wildtype littermates. Tumors will be extracted and evaluated for tumor growth, angiogenesis, lymphangiogenesis and immunostained for VEGFR-3, VEGF-C, VEGF-D, podoplanin, and CD34. To determine endothelial cell proliferation and apoptosis, co-immunostaining with PECAM and H3 or tunnel will be performed, respectively.

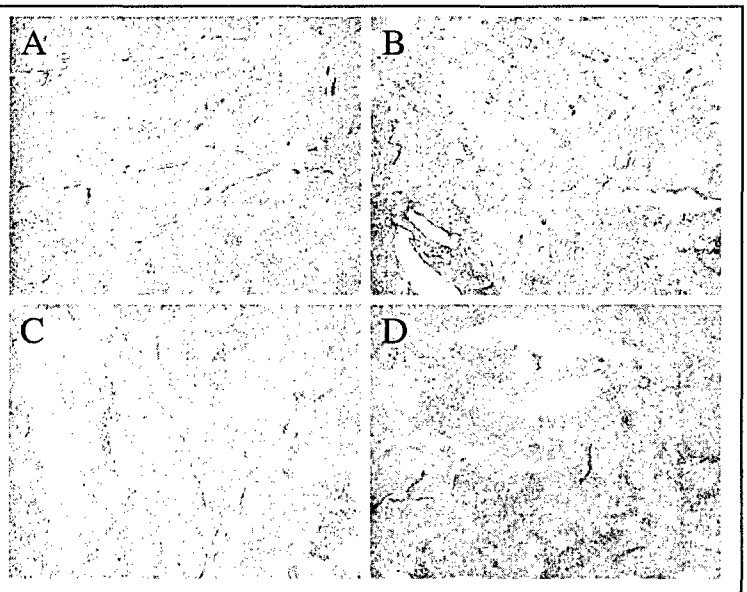


Figure 5. PECAM staining colorectal tumor xenografts in A & C) wildtype and B &D) Notch4 nullizygous mice. A &B) 10x magnification and C & D) 25x magnification.

Specific Aims 2: Analysis of Notch antagonists in a murine mammary tumor model

Task 4 (Months 6-36) Analysis of VEGFR-3 expression in tumors expressing Notch antagonists

In previous studies of murine xenografts of human mammary tumor cells, Dll4 expression was found to be enhanced within the tumor vessels (2). Moreover, ectopic expression of the VEGFR-3 ligand, VEGF-C, in mammary xenografts induced lymphangiogenesis and nodal spread of the breast cancer cell (9, 10). Taken together with the observation that Notch signaling induces VEGFR-3 expression in cultured endothelial cells, we want to test whether blocking Notch activity suppressed tumor growth and pathological angiogenesis/lymphangiogenesis. We have generated Notch antagonists that are composed of the signal peptide and EGF-like repeats of Notch1 and Notch2 fused in frame with Fc fragment of human IgG. Using an *in vitro* co-culture assay, we have found that the Notch antagonists N1ECDFc and N2ECDFc perturb ligand-activated signaling of Notch1, Notch2 and Notch4 (data not shown). Thus, we proposed to generate mammary tumor cell lines expressing these Notch antagonists and transplant them into immunocompromised mice.

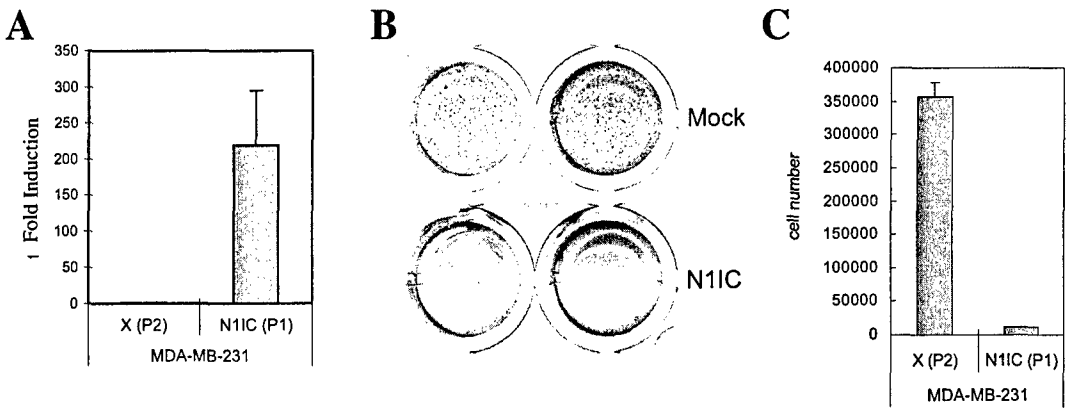


Figure 6. Notch signal activation inhibits adhesion independent growth of human MDA-MB-231 breast cancer cell line. A. N1IC transactivate a CSL luciferase reporter in retroviral MDA-MB-231 cell lines. **B.** Soft agar growth of Mock and N1IC MDA-MB-231 cell lines. **B.** Quantification of cell viability of Mock (X) and N1IC MDA-MB-231 in soft agar.

Our expression studies of human mammary tissue demonstrated that Notch1, Notch4 and Dll4 were expressed in the normal and malignant ductal epithelial cells (data not shown). This suggests that Notch signaling functions in normal as well as malignant ductal epithelium. Therefore, we decided to determine the affects of Notch signal activation on growth and tumorigenicity of human MDA-MB-231 breast cancer cell line (Figure 6) and FGF expressing mouse Mm5MT mammary tumor cell line (Figure 7). MDA-MB-231 and FGF expressing Mm5MT cells were retrovirally transduced with empty virus or viruses expressing activated forms of Notch1 (N1IC) or Notch4 (N4/int-3). Cell populations were selected in hygromycin. These constitutively activated forms of Notch encode the cytoplasmic domains of Notch free of the regulatory extracellular domain. Consistent with Notch signaling being activated, cell populations expressing N1IC or N4/int-3 transactivated luciferase reporters encoding 6 Notch/CSL binding sites relative to the mock infected controls (Figure 6A & 7A). Next, we determined the ability of the mock and Notch expressing mammary tumor populations to form colonies in soft agar (Figure 6B & 7B). Notch signal activation inhibited adhesion independent growth of the transformed mammary tumor lines. In MDA-MB-231, Notch1 signaling suppressed cell growth greater than 20 fold relative to the mock-infected cells (Figure 6C). The inhibition of cell growth was also observed in monolayer cultures and thus, they could not be subcutaneously transplanted in immunocompromised mice. The Notch-dependent growth suppression of the FGF-expressing Mm5MT cells was not as severe and appropriate cell numbers were achieve to perform subcutaneous xenografts in immunocompromised mice. 1 x 10^6 mock or N1IC expressing cells were injected into 5 immunocompromised mice each (Figure 7C). FGF

expressing Mm5MT cells grew to a tumor volume of 4 cm^2 within 20 days. In contrast, the growth of the N1IC expressing mouse mammary tumor cells was suppressed 60% relative to the mock controls.

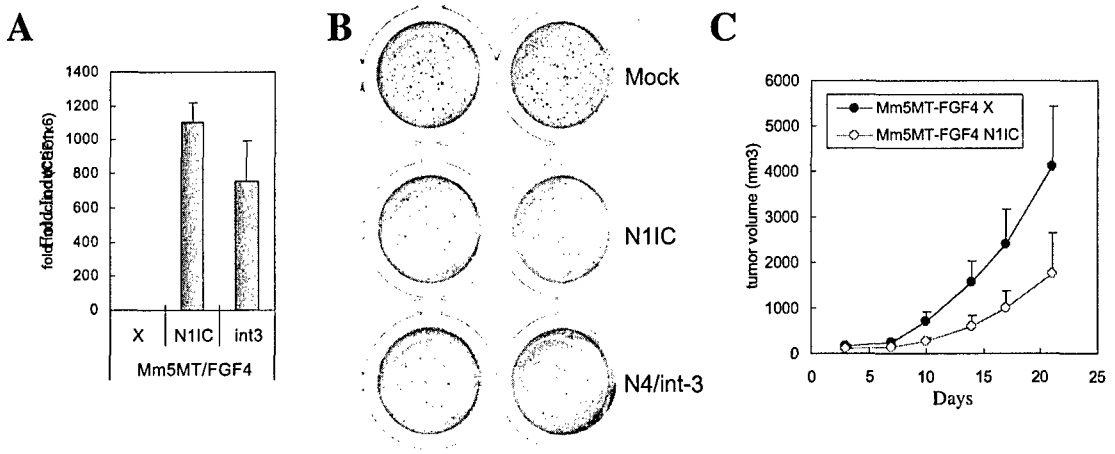


Figure 7. Notch signal activation inhibits adhesion independent growth and tumor xenografts of FGF4 expressing Mm5MT cells. A. N1IC and N4/int-3 transactivate a CSL luciferase reporter in retroviral Mm5MT lines. **B.** Notch1 and Notch4 signaling inhibits FGF4 expressing Mm5MT growth in soft agar. **C.** Notch1 inhibits subcutaneous FGF4 expressing Mm5MT tumor xenografts in mice.

Next, we determined the affect of expressing the Notch decoys, N1ECDFc and N2ECDFc, on the tumorigenicity of mammary tumor cells. FGF-expressing Mm5MT cells were retrovirally infected with empty virus, N1ECDFc or N2ECDFc expressing viruses and cell populations generated by hygromycin selection. In soft agar assays, expression of the Notch antagonists slightly promoted soft agar growth as seen by a 1.7-2 fold increase in the number of viable cells as determined by WST-8 assay (Figure 8A & B). 1 x 10^6 mock, N1ECDFc, or N2ECDFc expressing cells were injected subcutaneously into 5 immunocompromised mice each. In contrast to the results observed in the soft agar assay, we observed a decrease in FGF-expressing Mm5MT tumors expressing either Notch antagonist. However, the results did not achieve significance and there was no obvious difference in vessel density (data not shown).

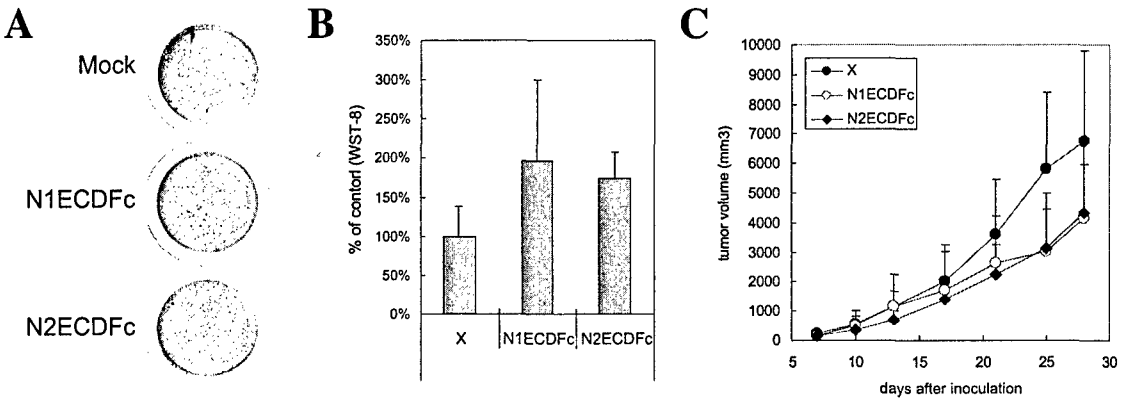
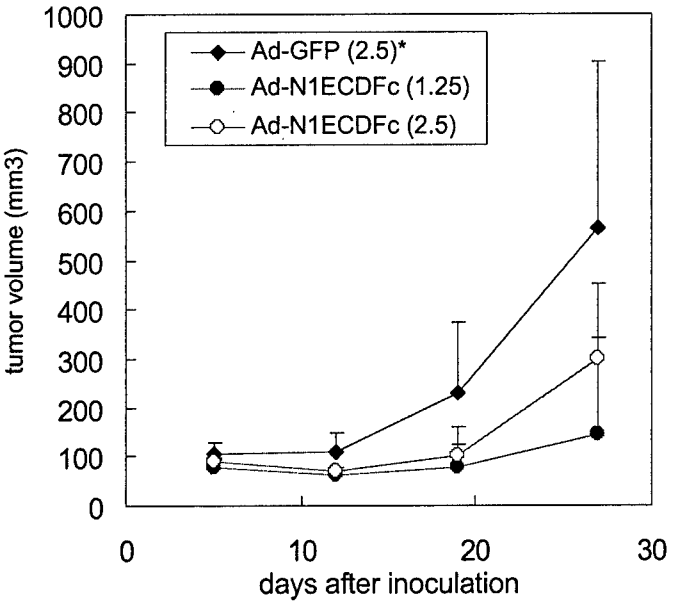


Figure 8. Expression of Notch decoys N1ECDFc and N2ECDFc in FGF4 expressing Mm5MT cells promotes soft agar growth and has no affect on subcutaneous xenografts in mice. A. Soft agar growth of Mock, N1ECDFc and N2ECDFc Mm5MT/FGF4 cells. **B.** Quantification of cell viability of Mock (X), N1ECDFc and N2ECDFc Mm5MT/FGF4 cells in soft agar. **C.** Expression of Notch decoys does not significantly alter tumor growth of FGF4 expressing Mm5MT subcutaneous xenografts in mice.

Due to these conflicting results, we have begun an alternate approach in expressing the Notch antagonists in the murine tumor model. Our initial experiment used a human pancreatic tumor cell line. We chose to tail vein inject an adenovirus encoding either GFP or N1ECDFc into immunocompromised mice. Five immunocompromised mice for each were injected with 2.5 x10^9 Ad-GFP or Ad-N1ECDFc, or 1.25 x 10^9 Ad-N1ECDFc. The following day 1 x 10^6 tumor cells were subcutaneously injected. After 20

days, a 30% suppression of tumor growth was observed in the mice that were injected with Ad-N1ECDFc (Figure 9). The animals were sacrificed at 27 days due to the large tumor volume in the Ad-GFP injected controls. The livers of the Ad-GFP injected mice expressed GFP demonstrating that the adenoviral infection of the liver was successful (data not shown). We also collect serum from the mice were able to detect N1ECDFc within the serum by immunoblotting (data not shown). Thus, we believe that circulating N1ECDFc suppressed tumor growth. In the following year, we intend to continue these experiments with mammary tumor cell lines xenografted into nude mice and to evaluate the lymphatic and blood vasculature by immunohistochemistry of tumor sections.



* x10^9 pfu per mouse

Figure 9. Tail vein injection of Ad-N1ECDFc suppresses growth of subcutaneous pancreatic tumors.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a Flk1-CreERT/CAG-CAT NotchIC line that expresses an activated form of Notch1 within the murine endothelium using the CAG-CAT promoter in response to Tamoxifin treatment.
- Developed a EF-1-flox-NotchIC mouse line that expresses an activated form of Notch1 with within the murine endothelium when crossed with an endothelial specific driven Cre.
- Generated EF-1-flox-Notch decoy murine ES cells for constructing conditional loss-of-function Notch mice.
- Found a tumor vascular remodeling defect in Notch4 deficient mice.
- Found that the expression of activated Notch constructs or Notch antagonists altered the growth of murine and human mammary tumor cell lines in soft agar.
- Developed a system to generate circulating Notch antagonists in mice using tail vein injects of adenovirus.
- Demonstrated that circulating Notch antagonist inhibited tumor growth of subcutaneous xenografts in immunocompromised mice.

REPORTABLE OUTCOMES (PUBLICATIONS/ABSTRACTS)

Publication:
Shawber, C.J., Kandel, J.J., and Kitajewski, J. (2004) Notch: cell fate determination from vascular development to human vasculopathy. *Drug Discovery Today: Disease Models.* 1: 351-358.

Abstract:
Shawber, C.J., Funahashi, Y., Franscisco, E., Podgrabinska, S., Kitamura, Y., Vorontchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and Kitajewski, J. (2005) *VEGFR-3, a direct endothelial cell specific target of Notch signaling in vitro and in vivo.* Vascular Cell Biology. Gordon Research Conference. Ventura, California

CONCLUSIONS

The proposal ***objective*** is to define the interaction between Notch and VEGFR-3 in breast cancer. To study the role for notch in murine mammary tumorigenesis, progress has been made in developing two new transgenic lines that will allow for conditional activation or inactivation of Notch specifically within the endothelium. These will be used to manipulate Notch activity in tumor endothelium of mice. Second, tumor vasculature grows aberrantly in Notch4 mutant mice, establishing that Notch signaling plays a role in tumor angiogenesis. Finally, Notch activity can alter the growth of human breast cancer cells, unexpectedly in an inhibitory fashion. This result means that we must interpret in vivo experiments with an appreciation of the role of Notch in both the cancer cell and tumor endothelium in order to understand the role in tumor growth. Thus, we have initiated an alternative method to test if circulating Notch antagonists can inhibit tumor growth and angiogenesis. To achieve this, we have developed a system that uses injection of Notch antagonist expressing adenoviruses into immunocompromised mice. This leads to expression of the antagonist in the circulation and inhibition of tumor xenografts. We found that Notch decoy blocked tumor growth, presumably by blocking tumor angiogenesis. Future experiments are aimed at focusing on the potential anti-tumor activity of the Notch decoy, an inhibitor with potential to block tumor angiogenesis in breast cancers.

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