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14. ABSTRACT

The proposal objective is to define Notch and VEGFR-3 in breast cancer. We investigated this relationship in primary endothelial cell cultures, mouse embryos, human breast tumors, and mouse mammary tumor xenografts. We demonstrate that Notch signaling promotes VEGFR-3 messages and protein in both primary endothelial cell cultures and mouse embryos in which Notch4 signaling is activated within the endothelium. In normal breast tissue, Notch1, Notch4 and Dll4 are expressed in the ductal epithelium and vasculature, whereas Jagged1 is restricted to the vasculature. Moreover, Notch is actively signaling within a subset of normal ductal epithelial cells. In malignant mammary tissue, Notch1, Notch4, Dll4, and VEGFR-3 were expressed in both epithelial and endothelia cells. Though, there were differences in the patterns of expression that suggest Notch1 and Notch4 have different functions in tumor angiogenesis. We also show for the first time that Notch1 and Notch4 are expressed in breast tumor lymphatic endothelial cells and most likely actively signaling. We have developed a Notch1 antagonist that blocks ligand dependent Notch signaling using in vitro coculture assays. We found that this Notch1 antagonist inhibits mouse mammary tumor growth in a xenograft mouse model. Thus the Notch1 antagonist may make a good breast cancer therapeutic.

15. SUBJECT TERMS
Notch, VEGF, breast cancer, angiogenesis

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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 cancers [1, 2]. Using mouse models, Notch signaling has been found to be essential for angiogenesis in the developing embryo [3]. However, the exact mechanism(s) by which Notch signaling regulates vasculogenesis and/or lymphangiogenesis is not well understood. 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. 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. We proposed to study their relationship in four different settings: primary human endothelial cell cultures, mouse embryos, human breast tumors and breast tumors grown in mice.

Complication from metastatic disease is the leading cause of cancer-related deaths. In breast cancer, this spread of tumor cells throughout the body is in part dependent on their access to blood and lymphatic vessels. Thus, there is a crucial need to identify the genes and understand their function in blood and lymphatic vessel development. By studying the relationship between Notch and VEGFR-3 in both physiological and pathological vascular development, we will increase our understanding of these processes and may define new targets for potential cancer therapeutics.

BODY

Notch1 and Notch4 signal activation up-regulates VEGFR-3 at surface of endothelial cells. Previously, we had shown that the ectopic expression of a truncated and constitutively active allele of Notch4, N4/Int-3 strongly induced the expression of VEGFR-3 transcripts in three different human primary endothelial cell lines [human umbilical vein endothelial cells (HUVEC), human umbilical artery endothelial cells (HUAEC) and human neonatal dermal microvascular endothelial cells (HMVEC)]. HUVEC and HUAEC are endothelial cells isolated from specialized large vessels. Whereas, HMVEC are derived from dermal capillaries and are comprised of both blood and lymphatic endothelial cells. To further characterize the interaction between Notch and VEGFR-3, we determined if the induction of VEGFR-3 mRNA by Notch4 correlated with an up-regulation of VEGFR-3 protein. HUVEC, HUAEC and HMVEC were infected with adenoviruses encoding either N4/Int-3 or LacZ (control) at a moi of 25 pfu/cell and 48 hours later surface-biotinylated. The surface proteins were purified with streptavidin beads and Western analysis performed using an antibody against VEGFR-3

(Fig. 1). Western analysis was performed on total cell lysates using an antibody against α-tubulin to control for protein concentration of the lysates. All three endothelial cell lines infected with Ad-LacZ expressed a low level of VEGFR-3. Consistent with an increase in VEGFR-3 transcripts in response to Notch4 signal activation, Notch4 signal activation up-regulated the expression of VEGFR-3 at the cell surface of all three endothelial cell lines.

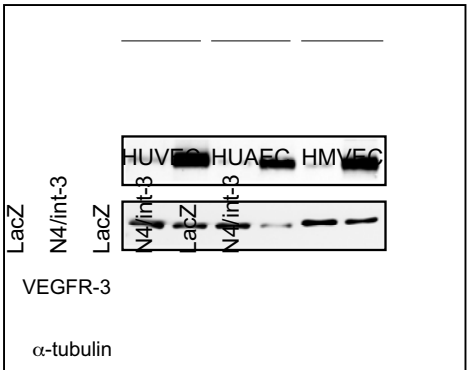

Figure 1. Notch4 signaling up-regulates VEGFR-3 expression on the surface of endothelial cells. HUVEC, HUAEC and HMVEC were infected with Ad-LacZ or Ad-N4/Int-3 and surface biotinylated. Western analysis of streptavidin purified proteins using an antibody against VEGFR-3 was performed. Total cell lysates using anti-α-tubulin as a control for protein concentration.

Since loss of function studies in mice suggested that both Notch1 and Notch4 have an overlapping role in embryonic angiogenesis, we wanted to determine if Notch1 signaling could also up-regulate VEGFR-3. To activate Notch1, we used a constitutively active human Notch1 adenoviral construct that encodes the cytoplasmic domain of Notch1 (N1IC). HUVEC and HMVEC were infected with Ad-N1IC, Ad-N4/Int-3 or Ad-LacZ and cell surface biotinylation performed 24 hours post-infection. Strept-avidin purified, and total cell lysates were analyzed by immunoblotting (Fig 2). Both Notch1 and Notch4 signal activation up-regulated VEGFR-3 to a similar level.

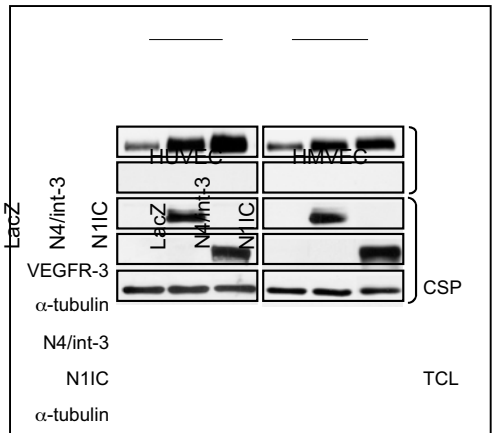

Figure 2. Notch4 and Notch1 both induce VEGFR-3 expression in HUVEC and HMVEC. HUVEC and HMVEC were infected with Ad-LacZ, Ad-N4/Int-3 or Ad-N1IC and surface biotinylated. Western analysis of streptavidin purified proteins using an antibody against VEGFR-3 and α-tubulin. Western analysis of total lysates using 12CA5 (an antibody against the HA tag of N4/int-3) and antibodies against Notch1 and α-tubulin.

Previous experiments utilized constitutively active forms of Notch1 and Notch4 to demonstrate that Notch signaling promotes VEGFR-3 expression in endothelial cells. We next wanted to show that ligand activated Notch signaling also induced VEGFR-3. HUVEC were infected adenoviruses encoding either LacZ, the ligands Jagged1 (J1), Dll4 or the full length Notch4 (N4) in suspension cultures. Control and ligand infected HUVEC were combined with Notch4 expressing HUVEC and co-cultured for 48 hours. Total RNA was isolated and quantitative RT-PCR performed for VEGFR-3 as well as the endothelial Notch target genes, Hey1 and Hey2 (Fig. 3)[4]. Jagged1 and Dll4 both induced VEGFR-3 via Notch4 similar to Hey1 and Hey2. Thus, ligand induced Notch signaling also up-regulates VEGFR-3 transcripts.

Notch does not affect the expression of VEGFR-3 ligands, VEGF-C and VEGF-D. We also wanted to ascertain if the Notch specific induction of VEGFR-3 corresponded with an up-regulation of the ligands for VEGFR-3, VEGF-C and VEGF-D. To determine their expression, the three primary endothelial cells lines were infected with Ad-LacZ or Ad-N4/int-3, and quantitative RT-PCR performed. VEGF-C was expressed by all three

primary endothelial cell lines, but was not affected by Notch4 activation (Fig. 4). In contrast, VEGF-D was not expressed by any of the primary endothelial cells, nor induced by Notch (data not shown)

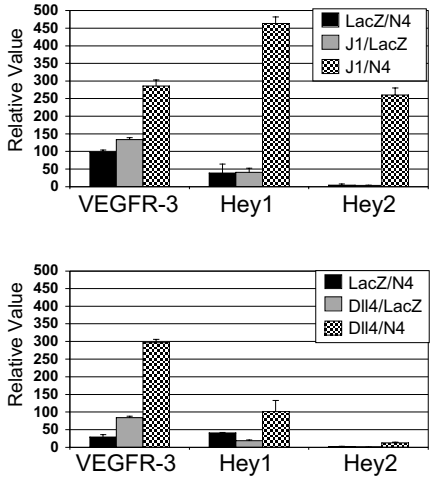

Figure 3. Jagged1 (J1) and Dll4 activation of Notch4 induces VEGFR-3. HUVEC were infected with Ad-LacZ, Ad-N4/Int-3, Ad-Dll4 or Ad-J1 and cocultured for 48 hours, RNA isolated and quantitative RT-PCR performed for VEGFR-3, Hey1 and Hey2. Values were normalized by β-actin expression.

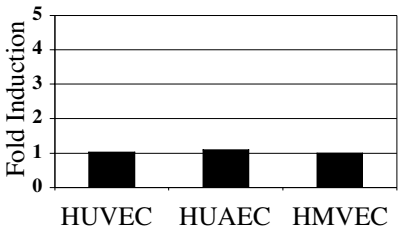

Figure 4. Notch4 signal activation does not alter VEGF-C expression. Quantitative RT-PCR of HUVEC, HUAEC, and HMVEC infected with either Ad-LacZ or Ad-N4/Int-3. Data represented as fold induction of VEGF-C in Ad-N4/Int-3 relative to Ad-LacZ Control.

Notch signaling up-regulates VEGFR-3 in blood endothelial cells

VEGFR-3 has been suggested function in both angiogenesis and lymphangiogenesis. HMVEC isolated from dermis are comprised of both blood (BEC) and lymphatic (LEC) endothelial cells [5]. Since HUVEC and HUAEC are comprised solely of BECs and Notch induced VEGFR-3 in these primary endothelial cell lines, this suggested that Notch was up-regulating VEGFR-3 in the BEC population of HMVEC. We further wanted to determine if Notch induced VEGFR-3 in a subset of cells or if Notch signaling increased the number of cells expressing high levels of VEGFR-3. BECs purified from HMVEC [5] were infected with adenoviruses for N1IC, N4/Int-3 or LacZ. The following day, Ad-infected BECs were analyzed by FACS for VEGFR-2 and VEGFR-3 expression (Fig. 5). The percentage of cells expressing VEGFR-2 remained unchanged between LacZ and Notch expressing BECs (Table 1). In contrast, Notch1 and Notch4 signal activation increased the number of VEGFR-3 expressing BECs. FACs analysis was also performed on LECs infected with adenoviruses encoding activated forms of Notch or LacZ. However, the percentage of parental LECs expressing VEGFR-3 is very high and was unaffected by Notch activation (data not shown). We plan on inhibiting endogenous Notch signaling by expressing a Notch antagonist in LECs to determine if VEGFR-3 expression is perturbed.

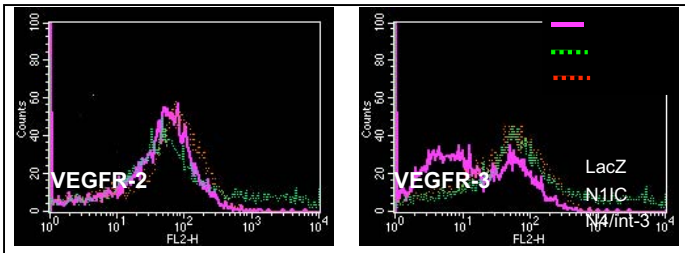

Figure 5. Notch1 and Notch4 up-regulate VEGFR-3 in blood endothelial cells. VEGFR-2 and VEGFR-3 FACS of Ad-LacZ, Ad-N1IC, and Ad-N4/int-3 BECs. VEGFR-2 expression is unaffected by Notch activation whereas Notch1 and Notch4 signal activation induces VEGFR-3 expression.

	LacZ	Notch1IC	Notch4/Int3
VEGFR-2	77.31%	78.34%	79.76%
VEGFR-3	40.08%	76.71%	70.61%

Table 1. Notch signaling up-regulates VEGFR-3 in BECs isolated from HMVEC. BECs were infected with either Ad-LacZ, Ad-N1IC or Ad-N4/int3 and 24 hours post infection FACS was performed using antibodies against VEGFR-2 and VEGFR-3. Data represented as percentage of cells expressing either receptor.

Notch signaling within the vascular endothelium induces VEGFR-3. Having established that Notch signaling induces VEGFR-3 expression *in vitro*, we wanted to confirm this observation *in vivo*. In collaboration with Janet Rossant at the Samuel Lunenfeld Research Institute, Toronto, we have previously demonstrated that transgenic embryos in which Notch4 has been constitutively activated specifically within the vascular endothelium under control of the VEGFR-1 promoter ($Tie1^{+/LacZ}$, $Flk1^{+/N4/int-3}$) die by 10 days of embryonic development due to hemorrhaging and vascular disorganization [6]. To determine if Notch4 activation within the endothelium correlated with an induction of VEGFR-3, wholemount immunohistochemistry of $Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$ and phenotypically normal $Tie1^{+/LacZ}$ e9.0 embryos was performed using antibodies against the vascular endothelial cell marker, PECAM and VEGFR-3 (Fig. 6 A-H). Less severely affect double transgenic embryos were analyzed so that differences in VEGFR-3 expression were due to Notch activation and not gross differences in the number of endothelial cells. As compared to $Tie1^{+/LacZ}$ controls, $Tie1^{+/LacZ}$, $Flk1^{+/N4/int-3}$ embryos have less PECAM staining in the head and heart region consistent with a loss of capillary vessels due to defects in angiogenesis (Fig. 6A, B). In contrast, the large intersomitic vessels do not appear to be as affected in the $Tie1^{+/LacZ}$, $Flk1^{+/N4/int-3}$ embryos (Fig. 6E, F). Consistent with the PECAM staining, more VEGFR-3 was found to be expressed in the head region of the control embryos than the Notch4 transgenic (Fig. 6C,D). VEGFR-3 was expressed more strongly in the intersomitic vessels of the $Tie1^{+/LacZ}$, $Flk1^{+/N4/int-3}$ embryos than the $Tie1^{+/LacZ}$ control embryos (Fig. 6G, H). We also performed quantitative RT-PCR to evaluate VEGFR-3 transcript levels in e9.5 control $Tie1^{+/LacZ}$ and gain of function $Tie1^{+/LacZ}$, $Flk1^{+/N4/int-3}$

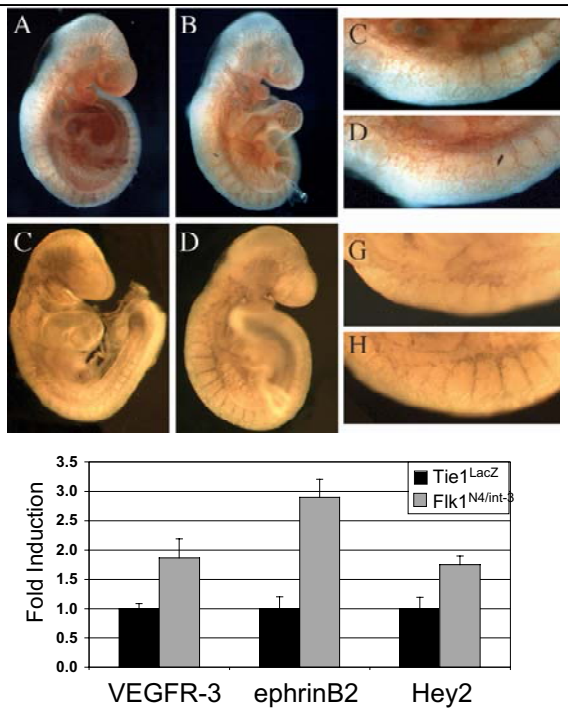

Figure 6. Notch4 signal activation in the embryonic endothelium results in precocious VEGFR-3 expression. Wholemount immunostaining of E9.0 $Tie1^{+/LacZ}$ **(A, C, E, G)** and $Tie1^{+/LacZ}$, $Flk1^{+/N4/int-3}$ **(B, D, F, H)** embryos using antibodies against the vascular endothelial cell marker, PECAM **(A, B, E, F)** and VEGFR-3 **(C, D, G, H)**. **E-H)** Magnified views of intersomitic vessels. **I)** Quantitative RT-PCR for VEGFR-3 and the Notch-target genes ephrinB2 and Hey2. Values normalized by β-actin and VE-Cadherin.

embryos (Fig. 6I). Values were normalized by VE-cadherin to adjust for differences in endothelial cell numbers between control and mutant embryos. Notch activation correlated with a nearly two-fold increase in VEGFR-3 transcripts, similar to the induction of the endothelial Notch target genes, EphrinB2 and Hey2.

Mice nullizygous for both Notch1 and Notch4 also die around e9.5 due to defects in vascular remodeling [7]. We have re-established these lines in our laboratory and generated double homozygous null embryos for immunohistochemical and quantitative RT-PCR analyses. However, the vasculature as well as the neuronal and somatic tissues is severely affected in these mutant embryos making it difficult to evaluate the immunostaining and RT-PCR results (data not shown). We are currently evaluating the yolk sacs isolated from e9.5 embryos for alteration in VEGFR-3 expression.

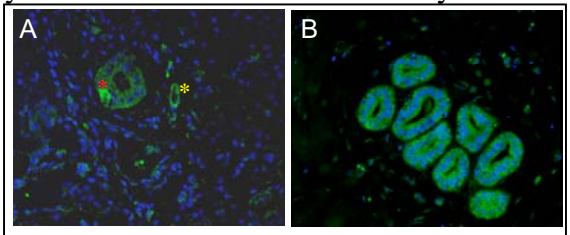

Figure 7. Notch1 and Notch 4 expression in normal breast tissue. A) Notch1 expression in both the ductal epithelium (*) and vasculature (*). **B)** Notch4 expression in the ductal epithelium.

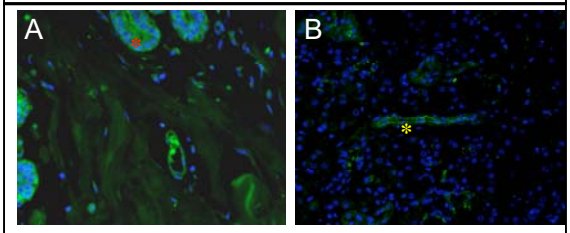

Figure 8. Dll4 and Jagged1 expression in normal breast tissue. A) Dll4 expression in both the ductal epithelium (*) and vasculature (*). **B)** Jagged1 expression is restricted to the vasculature (*).

Expression of Notch1, Notch4 and VEGFR-3 in normal breast tissue. Before we began our studies of Notch and VEGFR-3 expression in human breast cancer tissues, we determined the expression pattern for Notch1, Notch4, Dll4 and Jagged1 in normal human breast tissue. Immunohistochemistry was performed on 5μm section of normal breast tissue from using antibodies against the Notch proteins and ligands (Fig. 7 & 8). Notch1 was expressed in both the wildtype ductal epithlium as well as the vasculature (Fig. 7A). Notch4 shared a similar expression pattern to Notch1 (Fig. 7B & 10A). Though the expression of Notch4 within the epithelium was much stronger than that observed in the vascular endothelium. Dll4 was also expressed in the ductal epithelium and the vasculature (Fig. 8A), while the expression of Jagged1 was mainly restricted to a subset of large vessels (Fig. 8B).

The co-expression of Notch1, Notch4 and Dll4 within normal ductal epithelium suggests that Notch is actively signaling. However, Notch signaling is thought to occur between neighboring cells in which one cell is the ligand cell and the other the receptor cell that is actively undergoing Notch signaling. To determine whether Notch is actively signaling within all epithelial cells or a subset of cells, we have performed immunostaining with a cleavage specific Notch1 antibody that recognizes activated Notch1 (Notch1Val) or Hey1 antibody, a direct target of Notch transactivation (Fig. 9). Notch1 activation was only observed in

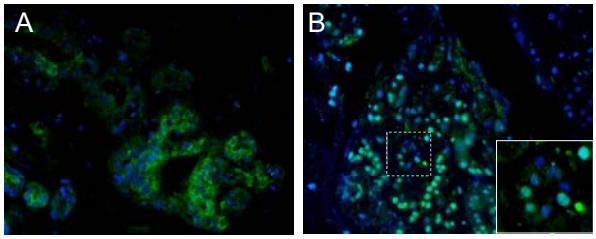

Figure 9. Notch1Val and Hey1 expression in normal breast tissue. A) Notch1 is activated in a subset of wildtype ductal epithelium. **B**) Hey2 is expressed in a subset of nuclei of the ductal epithelium.

a subset of the ductal epithelial cells near the end of the ducts (Fig. 9A). Hey1 expression was also observed in a subset of epithelial nuclei (Fig. 9B). This pattern is consistent with Notch being activated by ligand expressing neighboring cells.

Table 2. Expression of Notch1, Notch4, Dll4, and VEGFR-3 in human breast tissue

	Notch 1	Notch4	Dll4	VEGFR-3
Histologically Normal Tissue	Strong in ductal epithelial cells Moderate in endothelial cells	Strong in ductal epithelial cell Weak in endothelial cells	Moderate in aciner ductal epithelial cells Predominantly negative in terminal ductal epithelial cells Moderate endothelial cells	Strong in ductal epithelial cells Weak to absent in endothelial cells
Ductal Carcinoma In Situ	Strong in malignant epithelial cells Strong in endothelial cells Strong in lymphocytes	Weak in a subset of malignant epithelial cells Moderate in endothelial cells	Moderate in malignant epithelial cells Moderate in endothelial cells	Strong in malignant epithelial cells Moderate in endothelial cells
Invasive Breast Carcinoma	Strong in malignant epithelial cells Moderate in endothelial cells Strong in lymphocytes	Weak in malignant epithelial cells Strong in endothelial cells Strong in lymphocytes.	Weak in non-invasive malignant epithelial cells Strong in invasive malignant epithelial cells Moderate in endothelial cells	Strong in invasive malignant epithelial cells, Weak and patchy in endothelial cells.

Evaluation of Notch and VEGFR-3 expression in human breast cancer. In breast tumors an activator of Notch, Dll4 and VEGFR-3 have both been reported to be up-regulated in the vasculature suggesting that Notch and VEGFR-3 signaling may function in pathological vessel development [1, 2]. To evaluate the relationship between Notch and VEGFR-3 in human breast cancer, we have analyzed their expression in histologically normal breast tissue from reduction mammoplasty, ductal carcinoma in situ (DCIS), invasive ductal carcinoma, lobular carcinoma and micropapillary carcinoma (6 samples of each). Five micron sections from paraffin embedded tissues were acquired through the Columbia University's Institute of Cancer Genetics Tumor Procurement Core Facility. For each tissue sample, hematoxylin and eosin staining was performed and histological diagnosis confirmed by a collaborating pathologist, Nikki Feirt. Sections were immunostained for Notch1, Notch4, Dll4, and VEGFR-3. The results are summarized in Table 2.

Notch1 expression was observed in the ductal epithelial cells of all breast tissues examined (Figure 10). In both the DCIS and invasive carcinoma, Notch1 expression was

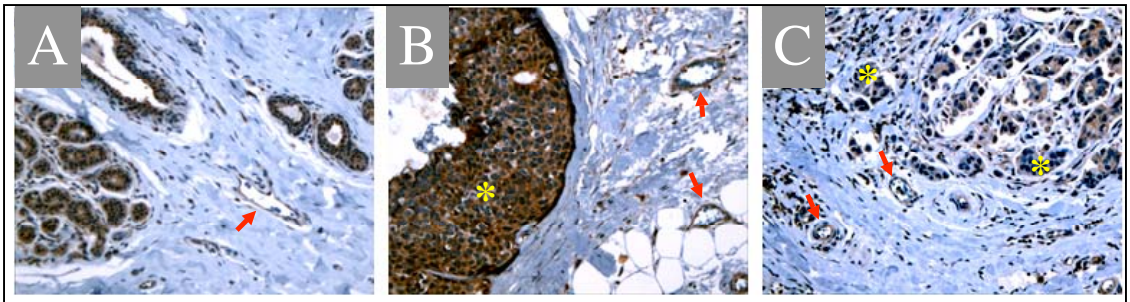

Figure 10. Notch1 immunostaining of A) normal tissue, B) DCIS and C) invasive ductal carcinoma. The red arrows indicate Notch1 expression within the vascular endothelium, and the yellow asterisk the malignant ductal epithelial cells.

detected in a subset of nuclei in the malignant epithelial cells. This is consistent with Notch1 actively signaling within these cells. As the tumor progresses, Notch1 expression became predominantly nuclear in the malignant epithelial cells. Notch1 was also expressed in vascular endothelial cells. Endothelial cell expression of Notch1 was weak in the normal and invasive vessels, but strong in the DCIS. The strong endothelial expression of Notch1 in DCIS correlates with an angiogenic phase of tumorigenesis prior to tumor invasion.

Similar to Notch1, Notch4 was also expressed in both the endothelial and epithelial cells in normal and malignant breast tissue (Figure 11). However, the level of Notch4 expression in the different tissues differed from the Notch1 expression pattern. Notch4 expression was strongest in the normal ductal epithelial cells. In the DCIS, only a subset of malignant epithelial cells at the periphery of the tumor expressed Notch4. Consistent with Notch4 actively signaling, Notch4 protein was localized to the nucleus of these malignant epithelial cells. All the invasive malignant epithelial cells expressed Notch4 and a subset of these expressed Notch4 in the nucleus. This pattern suggests that the Notch4 expressing epithelial cells of the DCIS, may be the cells that progress to an invasive phenotype. Notch4 was also expressed weakly in the endothelium of normal breast tissue and DCIS. An increase in Notch4 expression was observed in the vessels of the invasive carcinoma.

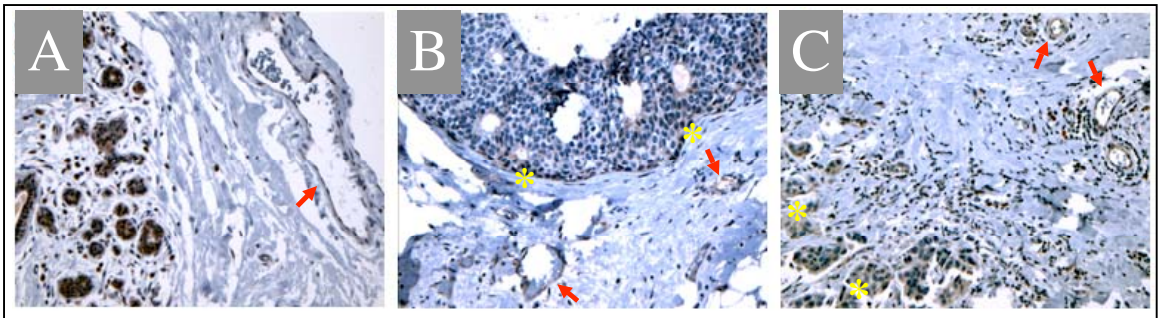

Figure 11. Notch4 immunostaining of A) normal tissue, B) DCIS and C) invasive ductal carcinoma. The red arrows indicate Notch4 expression within the vascular endothelium, and the yellow asterisk Notch4 expression in a subset of malignant ductal epithelial cells.

In the normal breast tissue, Dll4 was moderately expressed in the aciner ductal epithelial cells, but absent from the terminal ductal epthelium (Figure 12). In the mammary carcinomas, Dll4 expression was weak to moderate in the non-invasive malignant epithelial cells, and strong in the invasive malignant epithelial cells. In the endothelium, Dll4 expression was moderate in the vessels of the normal and DCIS tissues

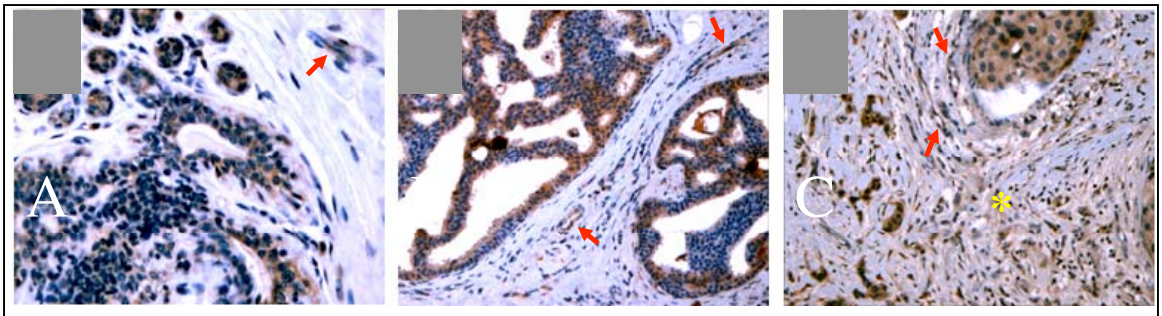

Figure 12. Dll4 immunostaining of A) normal tissue, B) DCIS and C) invasive ductal carcinoma. The red arrows indicate Dll4 expression within the vascular endothelium, and the yellow asterisk the malignant ductal epithelial cells.

and stronger in the vessels of the invasive carcinoma.

VEGFR-3 was strongly expressed in the ductal epithelial cells of all tissues examined (Figure 13). In contrast, VEGFR-3 expression was weak or absent in the endothelial cells of normal tissue and invasive carcinoma. In the angiogenic vessels of the DCIS, VEGFR-3 was moderate expressed within the endothelial cells. Taken together, the expression patterns of Notch1, Notch4, Dll4 and VEGFR-3 in human mammary carcinomas suggest that Notch and VEGFR-3 signaling may participate in both ductal tumorigenesis and pathological tumor angiogenesis.

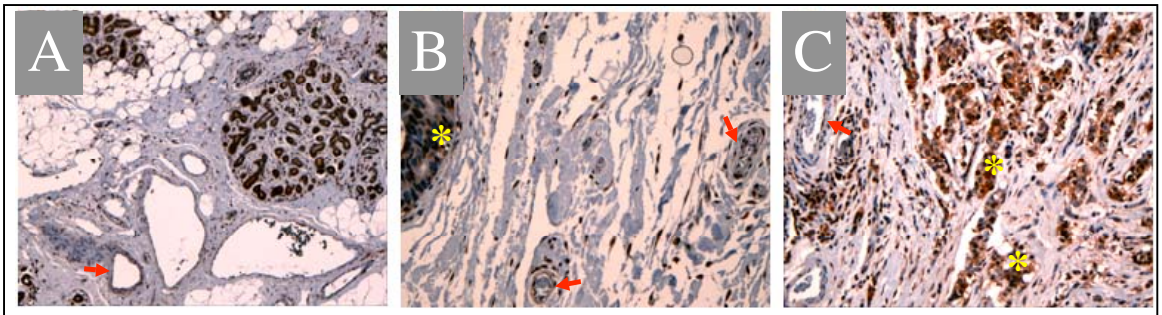

Figure 13. VEGFR-3 immunostaining of A) normal tissue, B) DCIS and C) invasive ductal carcinoma. The red arrows indicate VEGFR-3 expression within the vascular endothelium, and the yellow asterisk the malignant ductal epithelial cells.

Notch1 and Notch4 are co-expressed in the breast cancer lymphatic endothelium. We observed that Notch1, Notch4, Dll4 and VEGFR-3 were all expressed in the vasculature of human breast carcinomas (Figs 10-13). We also observed that Jagged1 is expressed with the vasculature of normal breast tissue (Fig. 8). Whether these proteins are expressed in both the blood and lymphatic endothelium remains to be determined. To discriminate between the blood and lymphatic vessels, we performed immunohistochemistry on human micropapillary breast carcinomas using antibodies against CD34, as a marker for blood endothelium and LYVE-1 and podoplanin as markers for lymphatic endothelium (Fig. 14).

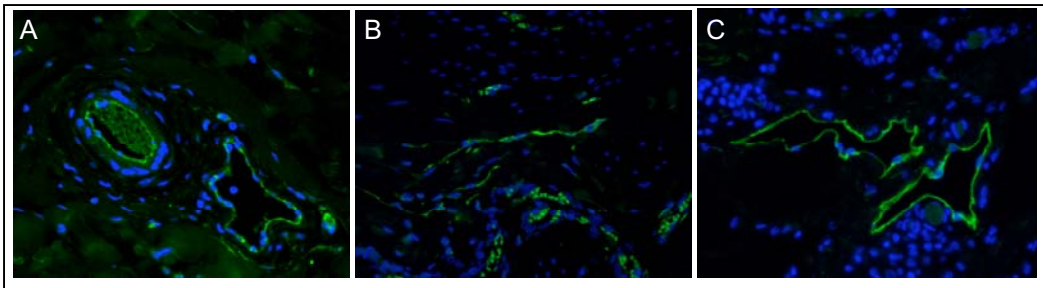

Figure 14. Expression of blood and lymphatic endothelial cell markers in micropapillary breast carcinomas. A) CD34 expression in the blood endothelium. Expression of the lymphatic markers **B)** LYVE-1 and **C)** podoplanin.

We then performed double immunohistochemistry with antibodies against LYVE-1 and Notch1 or Notch4 (Fig. 15). We found that both Notch1 and Notch4 are expressed in the lymphatic endothelium of human micropapillary breast carcinomas. Notch1 and Notch4 were co-expressed with LYVE-1 in the lymphatic endothelial cell bodies. Interestingly, Notch1 and Notch4 proteins were also detected in the nuclei of the lymphatic endothelial cells suggesting that Notch signaling was activated in these cells (Fig. 15 G & H). To further validate this observation, we co-immunostained the

micropapillary breast carcinoma tissue with Notch1Val, an antibody that only recognizes the activated Notch1 peptide and the lymphatic marker, podoplanin (Fig. 16). Activated Notch1 peptide was detected in a subset of the tumor lymphatic endothelium.

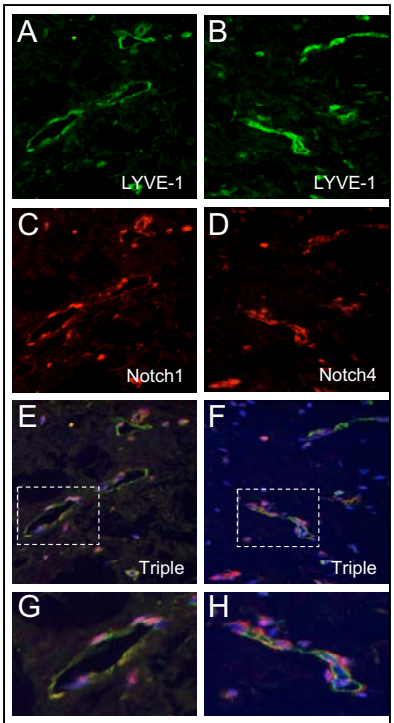

Figure 15. Notch1 and Notch4 are expressed in the lymphatics of micropapillary breast carcinomas. **A, B)** LYVE-1, **C**) Notch1, **D)** Notch4 double immunohistochemistry of breast tumor tissue. **E)** Notch1 and LYVE-1 co-expression in the lymphatic vessel. **F)** Notch4 and LYVE-1 co-expression in the lymphatic vessel. **G, H)** Magnified view of lymphatic vessels from panels **E** and **F**, respectively.

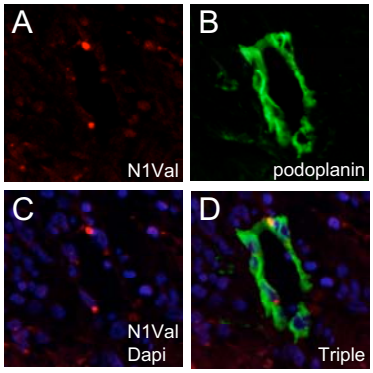

Figure 16. Notch1 is activated in a lymphatic endothelium of micropapillary breast carcinomas. **A)** Notch1Val, and **B)** Podoplanin double immunohistochemistry of breast tumor tissue. **C)** DAPI and Notch1Val image demonstrating perinuclear and nuclear expression of activated Notch1. **D)** Triple stain, Notch1Val, podoplanin and DAPI.

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 Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis [1]. Moreover, ectopic expression of the VEGFR-3 ligand, VEGF-C, in mammary xenografts induced lymphangiogenesis and nodal spread of the breast cancer cells [8]. VEGF-C-mediated lymphangiogenesis promoted tumor metastasis and VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors [9]. 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 mice.

Our expression studies of human mammary tissue demonstrated that Notch1, Notch4 and Dll4 were expressed in the normal and malignant ductal epithelial cells. This suggested to us 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 and FGF expressing mouse Mm5MT mammary tumor cell line. 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

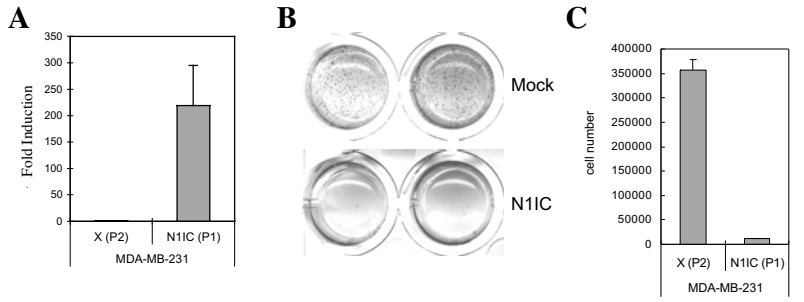

Figure 17. Notch signal activation inhibits adhesion independent growth of human MDA-MB-231 breast cancer cell line. A) N1IC transactivate a CSL luciferase reporter in retroviral MDA-MB-231 cell lines. **B)** Soft agar growth of Mock and N1IC MDA-MB-231 cell lines. **C)** Quantification of cell viability of Mock (X) and N1IC MDA-MB-231 in soft agar.

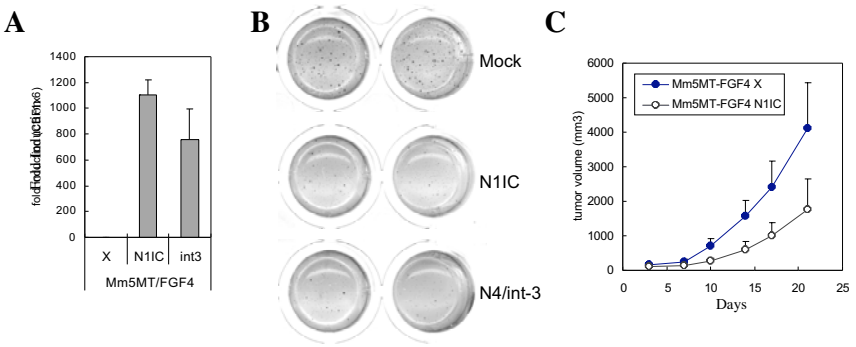

Figure 18. Notch signal activation inhibits adhesion independent growth and tumor xenografts of FGF4 expressing Mm5MT cells. A) N1IC and N4/int-3 transactivate a CSL luciferase reporter in retroviral Mm5MT lines. **B)** Notch1 and Notch4 signaling inhibits FGF4 expressing Mm5MT growth in soft agar. **C)** Notch1 inhibits subcutaneous FGF4 expressing Mm5MT tumor xenografts in mice.

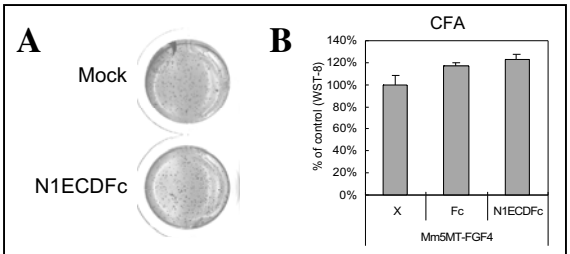

Figure 19. Expression of Notch decoys N1ECDFc in FGF4 expressing Mm5MT cells has a nominal affect on non-adherent tumor cell growth. A) Soft agar growth of Mock and N1ECDFc Mm5MT/FGF4 cells. **B)** Quantification of cell viability of Mock (X) and N1ECDFc Mm5MT/FGF4 cells in soft agar.

selected in hygromycin. 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 (Figs. 17A & 18A). Next, we determined the ability of the mock and Notch expressing mammary tumor populations to form colonies in soft agar (Figs. 17B & 18B). 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 17C). The inhibition of cell g rowth 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 18C). FGF expressing Mm5MT cells grew to a tumor volume of 4 cm2 within 20 days. In contrast, the growth of the N1IC expressing mouse mammary tumor cells was suppressed 60% relative to the mock controls.

Next, we determined the affect of expressing the Notch antagonist, N1ECDFc on the tumorigenicity of mammary tumor cells. FGF-expressing Mm5MT cells were retrovirally infected with empty virus, Fc (control) or N1ECDFc expressing viruses and

cell populations generated by hygromycin selection. In soft agar assays, expression of the Notch antagonists had a nominal affect on soft agar growth as determined by WST-8 assay (Fig. 19). 1 x 10^6 mock, Fc, or N1ECDFc expressing cells were injected subcutaneously into 10 C3H syngeneic mice each. Three independent trials were monitored for tumor growth for 3 weeks (Fig. 20). In the three trials, expression of N1ECDFc significantly inhibited tumor growth. This affect of the Notch1 antagonist may be on either the tumor epithelial cells, tumor angiogenesis or both. To discriminate between these possibilities, immunohistochemistry will be performed to analyze tumor angiogenesis, lymphangiogenesis, epithelial apoptosis and epithelial proliferation.

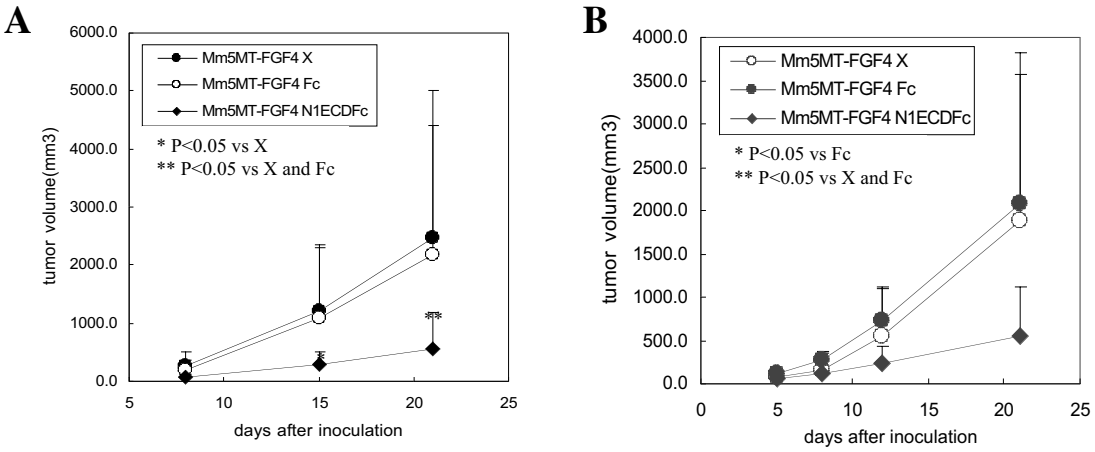

Figure 20. Notch decoy inhibits tumor growth of Mm5MT-FGF4 in syngeneic C3H mice. Tumor volume over 21 days post-inoculation of Mock, Fc and N1ECDFc expressing mammary tumor cells. **A)** Average of two independent experiments. **B)** Third independent experiment.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed Notch1 and Notch4 up-regulates VEGFR-3 at the cell surface of primary endothelial cells (HUVEC, HUAEC, HMVEC) consistent with our RT-PCR data.
- Demonstrated that ligand activation of Notch signaling also up-regulates VEGFR-3 in primary endothelial cells.
- Using FACS, demonstrated that Notch up-regulates VEGFR-3 in blood endothelial cells (BEC) isolated from HMVEC.
- Using embryos in which Notch signaling is specifically induced within the vascular endothelium, demonstrated that Notch4 induced precocious expression of VEGFR-3 within the vasculature.
- Demonstrated that Notch1, Notch4, Dll4 are expressed in the normal ductal epithelium and that Notch is actively signaling in a subset of epithelial cells.
- Demonstrated that Notch1, Notch4, Dll4 and VEGFR-3 are expressed in the endothelium and ductal epithelium of histologically normal breast, ductal carcinoma in situ, invasive ductal carcinoma, lobular carcinoma and micropapillary carcinoma tissue.
- Demonstrated that Notch1 and Notch4 are expressed and most likely active in the lymphatic endothelium of micropapillary breast carcinomas.
- Found that the expression of activated Notch constructs inhibited the growth of murine and human mammary tumor cell lines in soft agar.
- Demonstrated that the Notch1 antagonist inhibited tumor growth of subcutaneous xenografts of mouse mammary tumor cells.

REPORTABLE OUTCOMES

Publications

Lee, A., Frischer, J., Serur, A., Huang, J, Bae, J-O., Kornfield, Z.N., Eljuga, L., **Shawber, C.J.**, Feirt, N., Mansukhani, M., Stempak, D., Baruchel, S., Bender, J.G., Kandel, J.J., and Yamashiro, D.J. Inhibition of COX-2 disrupts tumor vascular mural cell recruitment and survival signaling. *Cancer Research*. 8: 4378-4384.

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Book Chapter

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Shawber, C.J., Francisco, E., Feirt, N., Roe, T., Podgrabinska, S., Kitamura, Y., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and Kitajewski, J. *Role of Notch/VEGFR-3 in breast tumor angiogenesis and lymphangiogenesis*. (2005) Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Philadephia, PA.

Shawber, C.J., Funahashi, Y., Franscisco, E., Feirt, N., Roe, T., Podgrabinska, S., Kitamura, Y., Vorontchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Accili, D., Skobe, M., and Kitajewski, J. *Notch and VEGFR-3 interactions in endothelial cells and breast cancer vasculature*. (2005) Era of Hope. Department of Defense Breast Cancer Research Program Meeting. Philadephia, PA.

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AWARDS AND HONORS

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CONCLUSIONS

The proposal objective is to define the interaction between Notch and VEGFR-3 in breast cancer. We investigated this relationship in four different settings: primary endothelial cell cultures, mouse embryos, human breast tissue and mouse tumor xenograft studies. We demonstrated that both activated forms of Notch1 and Notch4 as well as ligand activated Notch signaling up-regulates VEGFR-3 transcripts and proteins in primary human endothelial cell cultures. Using FACS analysis of BEC purified from HMVEC, we found that Notch increased the percentage of VEGFR-3 expressing blood endothelial cells.

In normal breast tissue, we found that Notch1, Notch4 and Dll4 are expressed in both the epithelial and endothelial cells. Jagged1 expression was restricted to the large vessels in the normal breast tissue. We also found that antibodies against an activated Notch1 peptide and the Notch-target gene Hey1 were expressed in a subset of normal ductal epithelial cells, indicating that Notch signal activation occurs in wildtype breast epithelium. We expanded our analysis to analyze Notch and VEGFR-3 signaling proteins in malignant breast tissue. Notch1, Notch4, Dll4, and VEGFR-3 were expressed in both epithelial and endothelial cells of malignant mammary tissues examined. We observed some differences between the tissue types such as a coincident increase in VEGFR-3 and Notch1 expression in the endothelium of DCIS. In contrast, Notch4 expression within the endothelium was strongest in invasive carcinomas suggesting that Notch1 and Notch4 signaling may have different roles in pathological angiogenesis. We also began to examine the expression of Notch proteins in the lymphatic and blood endothelium of micropapillary breast carcinomas. In a novel observation, we found that Notch1 and Notch4 were expressed in tumor lymphatics and that Notch1 was actively signaling in a subset of the tumor lymphatic endothelial cells.

We have also studied the use of a Notch1 antagonist of in blocking breast tumor growth in murine mammary tumor xenograft models. We found that our Notch1 antagonist significantly suppressed FGF-expressing Mm5MT mammary tumor cell growth. We are currently examining the mechanism by which the Notch1 antagonist suppressed tumor growth. This may occur through inhibition of tumor angiogenesis, lymphangiogenesis or tumor epithelial cell proliferation.

Complication from metastatic disease is the leading cause of breast cancer-related deaths. In breast cancer, the spread of tumor cells throughout the body is in part dependent on their access to blood and lymphatic vessels. Thus, there is a crucial need to identify the genes involved in regulating angiogenesis and lymphangiogenesis. Previous studies suggest that the angiogenic/lymphatic factor VEGFR-3, its ligand VEGF-C and the Notch ligand Dl4 may regulate these processes in human breast cancer [1, 2]. By examining the relationship between Notch and VEGFR-3 in both physiological and pathological vascular development, we will increase our understanding of these processes and may define new targets for potential cancer therapeutics.

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