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PRINCIPAL INVESTIGATOR:Jan K. Kitajewski, PhD

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New York, NY 10032

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Jan K. Kitajewski
Email: jkk9@columbia.edu

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14. ABSTRACT

We hypothesize that Notch and Jagged-1 expression on breast cancer cells, as well as Delta like-4 on breast tumor vessels, present a diagnostic and therapeutic target for detection and treatment of this devastating disease. Our combined objective is to utilize mouse models to establish whether 1) human breast cancer cells that express Jagged-1 and tumor vessels that express Delta-4, respectively can be detected using a novel diagnostic platform, 2) tumor derived proteins that shed upon Notch activation can be detected in serum of tumor bearing animals, and 3) blockade of Notch pathway activation, using neutralizing antibodies and a decoy receptor, will mitigate the growth of breast tumor in animals. The ultimate goal of these studies is to utilize the expertise of Drs. Diacovo and Kitajewski to validate Notch as a diagnostic and therapeutic target in breast cancer in order to develop paradigms that can be used in the clinic to detect and treat breast cancer.

15. SUBJECT TERMS
Notch, Jagged1, Breast Cancer, Angiogenesis

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INTRODUCTION

This proposal focuses on Notch signaling in breast cancer and asks whether Notch and Notch ligands represent diagnostic markers of and therapeutic targets for breast cancer.

The Notch family and Ligands. In the context of cancer biology, Notch signaling has been proposed to promote tumor-induced angiogenesis, and tumorigenesis by blocking cell differentiation. Notch is a transmembrane receptor that interacts with ligands expressed on the surface of cells. In mammals, there are four Notch genes (1-4) and five ligands, referred to as Jagged (Jagged-1 and Jagged-2) or Deltas (1-3) (also called Delta-like). Many of the Notch proteins are widely expressed with the exception of Delta-4, which appears to be highly expressed on tumor endothelium as described below. Moreover, both Notch1 and Jagged-1 are preferentially expressed on breast cancer cells [1]. Based on these observations, this proposal will focus on the receptor, *Notch1*, and the ligands, *Delta-4* and *Jagged-1,* in developing new diagnostic strategies and therapies in the war against breast cancer. Of key importance to the diagnostic part of this proposal is an understanding that during Notch signal activation there is release of the extracellular domains of Notch1, Delta-4 and Jagged-1. The shed products are schematized in Figure 1B, consisting of cleavage products, soluble Jagged-1 (sJagged-1), soluble Delta-4 (sDelta-4), and Notch1 ectodomain (Notch1 ECD).

Delta-4 as a Tumor Endothelial Marker.

The growth and metastasis of breast cancer relies on the formation of new blood vessels by the process of angiogenesis. Angiogenesis involves the sprouting of new vessels from pre-existing normal vasculature, with the migration of these vessels into the tumor [2]. Recent clinical trials with the anti-angiogenic agent Avastin (a function blocking antibody to VEGF) validate this concept in women with breast cancer where survival is enhanced [3-5]. Interestingly, evidence is mounting that blockade of VEGF activity may be overcome through Notch signal activation in tumor endothelium (our unpublished observations), suggesting that inhibition of both pathways may be advantageous in the treatment of breast cancer [6]. In addition, Delta-4 (a Notch ligand) has a provocative expression pattern in breast cancer as it is highly expressed on human breast tumor vessels [7]. Thus, Delta-4 is a true tumor endothelial cell marker that may be exploited for diagnosis and targeted therapies as outlined in this proposal.

Notch1 and Jagged-1 are over-expressed in human breast cancers. Notch signaling may be activated in greater than 50% of human breast cancers, implicating its role in tumor development [8, 9]. Moreover, expression of Notch1 and its ligand Jagged-1 on breast tumor cells has been shown to correlate with poor prognosis in terms of survival [1].

Specific Aims

1. Diagnositc Imaging of Jagged-1/Delta-4 Expressing Breast Tumors.

Utilize a nanoparticle based imaging platform (Diacovo) to detect for the elevated expression of the Notch ligands (Dll4) in animals bearing murine or xenografted human breast tumors (Kitajewski).

2. Detection of Jagged-1 ectodomain shedding from Breast Tumors.

Determine if the extracellular domain of Delta-4 and Jagged-1 are shed into serum in tumor bearing mice (Kitajewski). Generate antibodies to detect human forms of these proteins (Diacovo) for development of an ELISA based detection system (Kitajewski).

3. Inhibition of Breast Tumors with Notch Neutralizing Agents and Notch decoys.

Explore the consequences of therapeutic intervention of Notch signaling in breast tumor models with either Notch decoy (Kitajewski) or neutralizing antibodies (Diacovo).

Body

Specific Aims 1: **Diagnostic Imaging of Jagged-1/Delta-4 Expressing Breast Tumors.**

> *Goal: Utilize a nanoparticle based imaging platform (Diacovo) to detect for the elevated expression of the Notch ligands (JAGGED1 and DLL4) in whole animals bearing murine or xenografted human breast tumors (Kitajewski).*

We have made progress and report here on **Task2**: *Development of Hybridoma lines producing monoclonal antibodies against Jagged-1 (Year 1) and Delta-4 (Year 2)*

We have completed the production of several hybridoma's producing monoclonal antibodies against the extracellular domaon of JAGGED1. We first briefly summarize our accomplishments from Year 1 and then provide data for accomplishments of Year 2 (current report).

Brief synopsis of work on JAGGED1 antibody generation achieved in Year 1:
In Year 1 we were able to generate an expression construct that produces the extracellular domain of human JAGGED1. This JAGGED1 cDNA was fused to the coding region of the FLAG tag to encode a hJAGGED1-ECD-Flag (hJAG1-ECD-Flag) expression construct which was introduced into CHO cells. CHO cell clones that produced and secreted JAG1-ECD-FLAG were identified and used to generate conditioned media for purification. JAG1-ECD-Flag was purified in milligram quantities. At the end of Year 1 we had purchased the mice that would be used to generate hybridomas.
Please see below for continuation of update on JAGGED1 antibody generation for Year 2 (page 7).

Brief synopsis of work on DLL4 antibody generation done in Year 1:
The full-length human DELTA-LIKE 4 (DLL4) cDNA was cloned and used to generate several expression vectors. We generated a construct fusing the extracellular part of human DLL4 with the FLAG tag, generating a cDNA that would encode a secreted form containing the extracellular domain of DLL4. The soluble DLL4-ECD-Flag was expressed in Chinese hampster ovary K1 (CHO-K1) cells and then CHO clones were screened for their ability to express high levels of secreted DLL4-ECD-Flag.

Year 1 culminated with the successful production of CHO lines producing the extracellular domains of human Jagged1 and Dll4 and purification of hJAG-ECD-Flag for use as an immunogen.

In Figure 1, we demonstrate the utility of the CHO lines in producing either JAG1-ECD-Flag or DLL4-ECD-Flag in conditioned media. Western blots were conducted on cell lysates (top panel) or conditioned media (bottom panel) from the CHO cell lines listed at the top of the gel. Westerns were probed with anti-Flag antibodies. Robust expression of DLL4-ECD-Flag in conditioned media is seen in lines DLL4-ECD-F6-8 and -F6-10. Robust expression of hJAG1-ECD is seen in conditioned media from lines JAG1-ECD-1-14, -1-19, and -1-24.

Figure 2 provides a brief outline of the purification strategy to obtain hJAG1-ECD-Flag and analysis of purified preparations by SDS-PAGE electrophoresis and coomassie blue staining of gels to allow for determination of purity of hJAG1-ECD-Flag and to roughly quantify amount purified. We determined that the preparation is highly purified and the yield was approximately 3 mg of hJAG1-ECD-Flag; an abundant amount to be used as an antigen for antibody production.

Figure 1. Western blot analysis of hJAG1-ECD and hDLL4-ECD expression in CHO

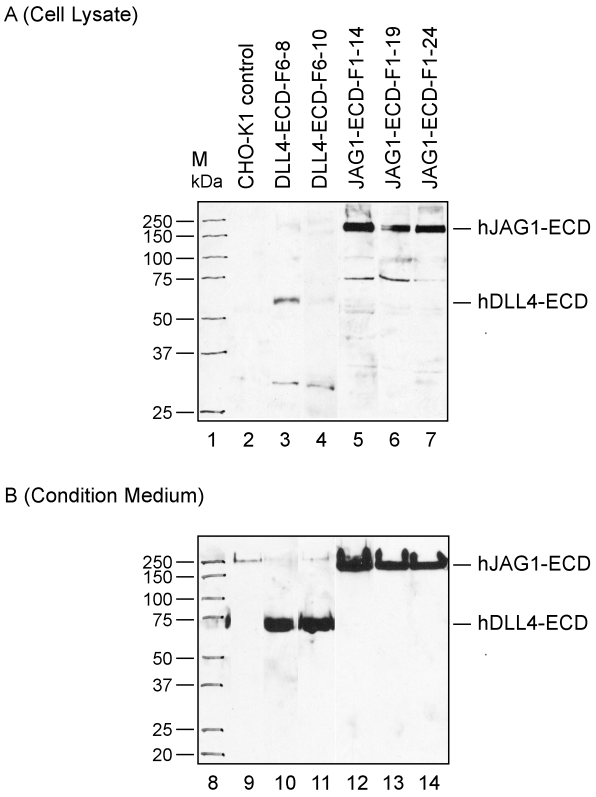


Figure 2. Purification of hJAG1-ECD expressed in CHO

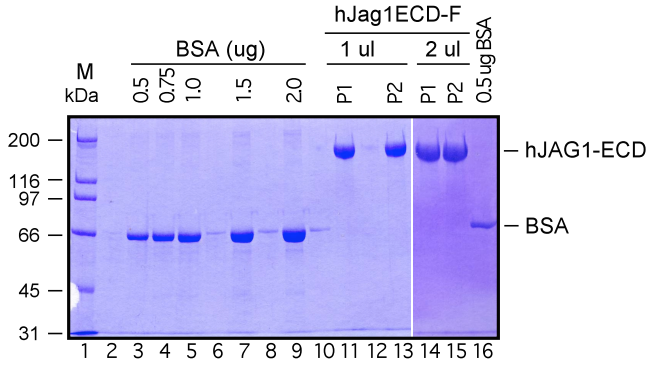
Purification of hJAG1-ECD-Flag

Source: condition medium from CHO cell stably expressing hJAG1-ECD-F

Methods: a) Q-sepharose Fast Flow Ion Exchange column
b) M2 agarose affinity column

Post process: All positive fractions are pooled and divided into P1 and P2.
P1 and P2 was concentrated by Amicon Ultra-4 (10k) column.

Storage: Total volume with P1 and P2 together is about 1100 ul.
Final protein is stored in 3 tubes with 250ul each and therest in the 4th tube.



Update on JAGGED1 antibody generation for Year 2 progress report

I. Producing hybridomas against extracellular domain of human JAG1:
Using the purified protein preparation that was analyzed in Figure 2, a total of 1.5 mg purified extracellular domain of human JAG1 was injected into 3 mice for antibody production. The mice sera was collected regularly and screened by using the sera in western blot analysis to establish that a positive response was elicited; that is, mice were generating antibodies against the extracellular domain of human Jagged1. Two of the mice were used to generate hybridoma cell lines. The spleen cells from the mice were collected and cells from the spleen dispersed into culture dishes and then these cells were fused with NS1 myeloma cells to form hybridomas.

II. Screening hybridomas against extracellular domain of human JAG1:
The hybridomas were grown in multi-well plates and the media was collected from a total of 960 hybridomas-containing wells and used to screen for positive reaction. The initial screen consisted of use of the hybridoma media to test for staining of surface Jagged1 expressed by CHO cells and this was followed up by FACS analysis of cells expressing human Jagged1, or by western blotting. **Figure 3** displays an example of such screening using CHO cells that express Jagged1. Cells were fixed and then immuno-stained with hybridoma media. DAPI staining (Figure 3, top left) allows for identification of cells on a slide, anti-Flag staining (Figure 3, bottom left) is a positive control, as the expressed Jagged1 is flag-tagged. A hybridoma supernatant producing anti-Jagged1 antibodies is detected by their ability of this supernatant to stain cells (Figure 3, top right).

Figure 3. Staining of hJAG1-Flag expressed in CHO with anti-serum from immunized mice (M2 is monoclonal antibody against Flag tag)

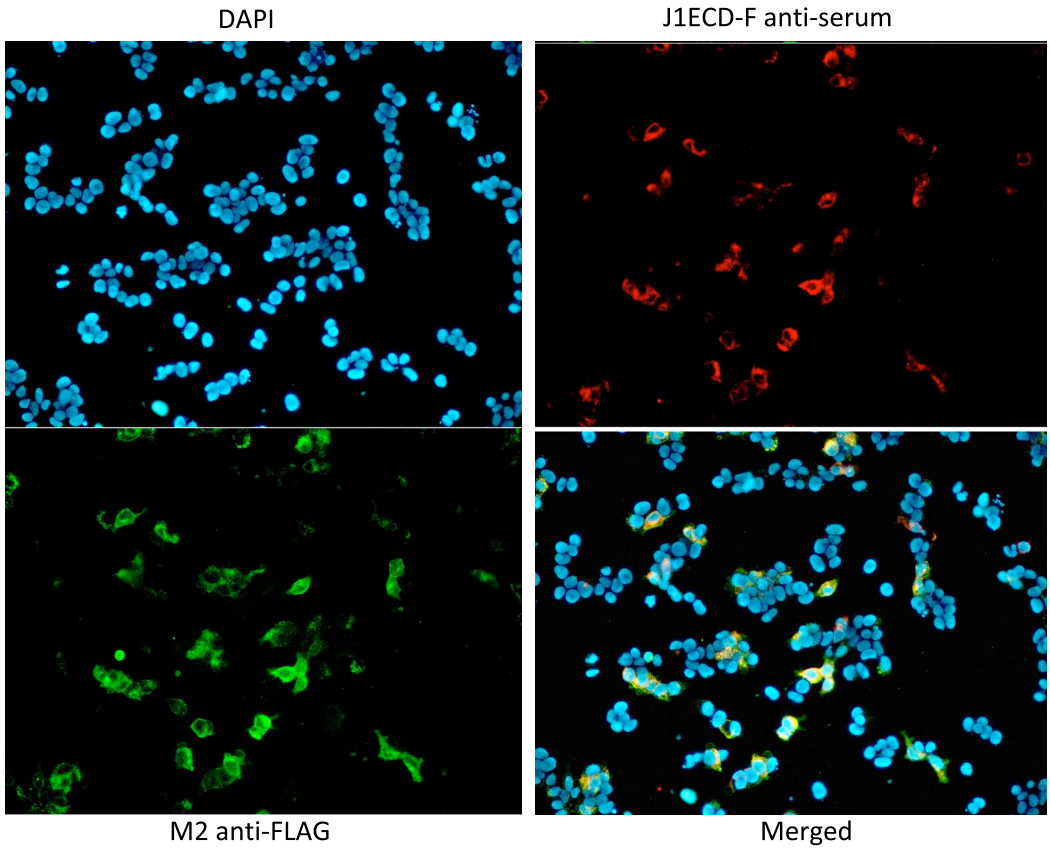
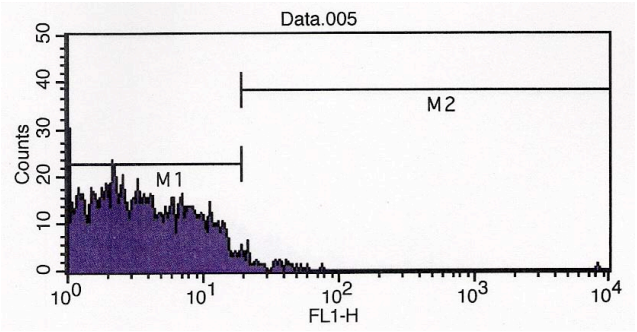
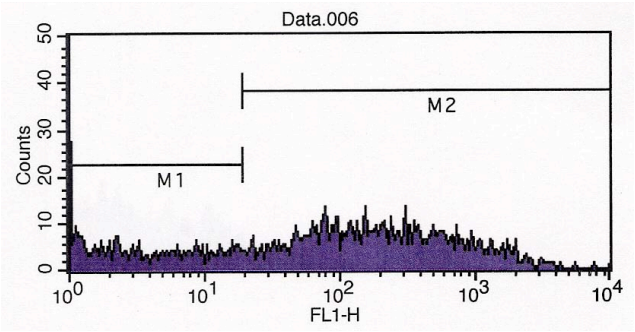


Figure 4 displays a typical FACS analysis experiments where CHO cells expressing full length human Jagged1Flag are analyzed using select supernatants containing anti-Jagged1 antibodies (4B4), which show increased intensity of surface expression of human Jagged1, whereas other supernatants (3D12) do not show activity on FACS analysis.

Figure 4. FACS screening of positive clones with CHO cell expressing hJAG1-Flag
(3D12 is a typical negative, 4B4 is a typical positive)



3D12

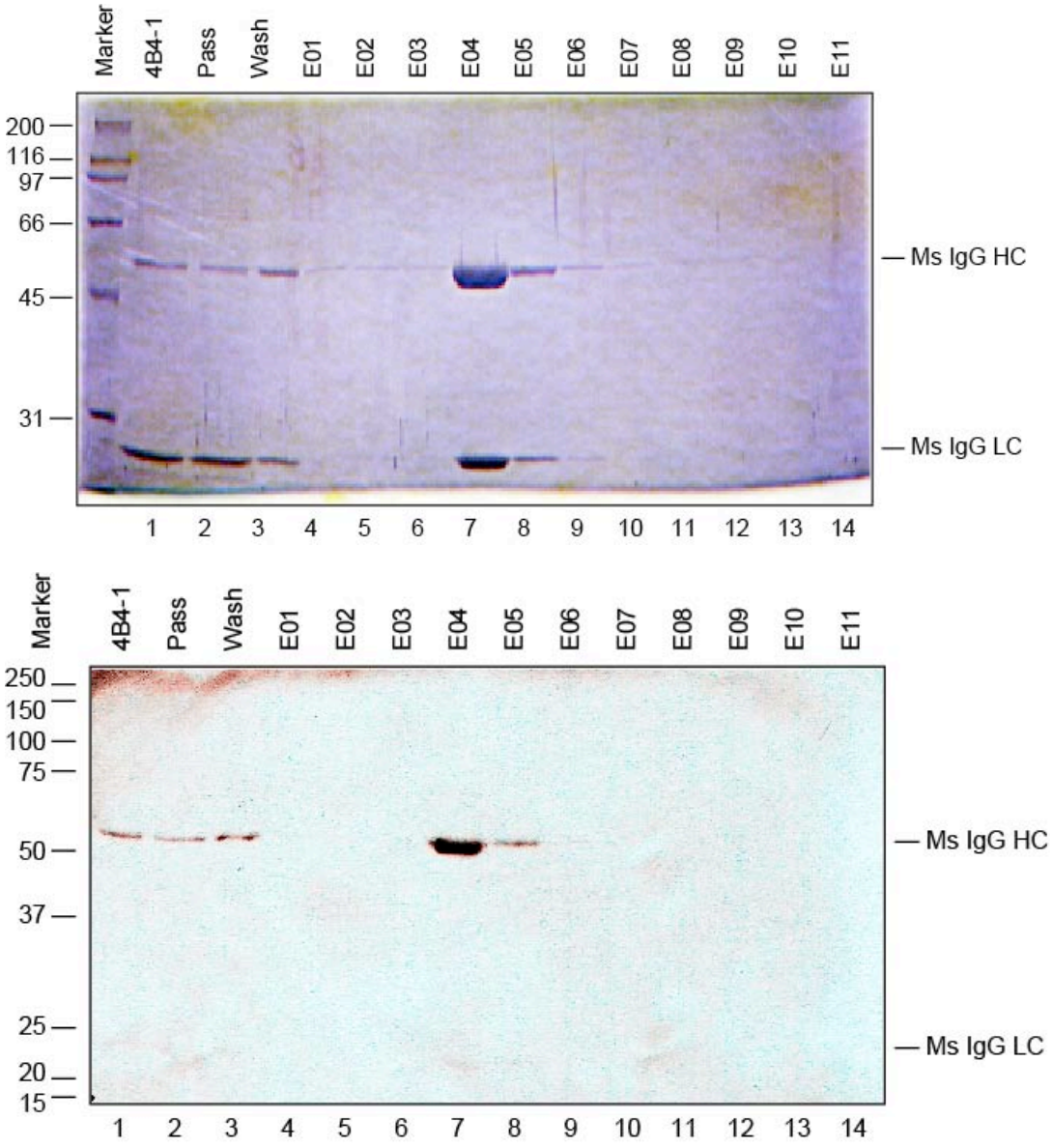


4B4

III. Selection of hybridoma clones and purificatin of *monoclonal antibody against hJAG1-ECD:*

Using the screens described in section II, above, 11 hybridoma clones were selected from 960 potential hybridomas screened. The 11 positive hybridoma clonse were expanded, frozen away, and then grown in sufficient quantities to condition media (serum-free) in preparation of purification of the antibodies. The purification of antibodies was done with Protein G-sepharose 4B column chromatgraphy. After column loading by passing over conditioned media, and washing, bound proteins are eluted with 0.1M Glycine (pH 3.5, with neutralizing buffer in the collection tube). Samples from fractions were subjected for SDS-PAGE gel analysis with Coomassie-blue staining (Figure 5, top panel) or were analyzed by western blot using anti-mouse Fc antibodies (Figure 5, bottom panel). The purified antibody was dialyzed against phosphate buffered saline (PBS) and concentrated using a Centricon 30K. The final concentration was determined by BCA Protein Assay (Pierce) and the yield was approximately 5 mg, an abundant amount to be used in further functional assays.

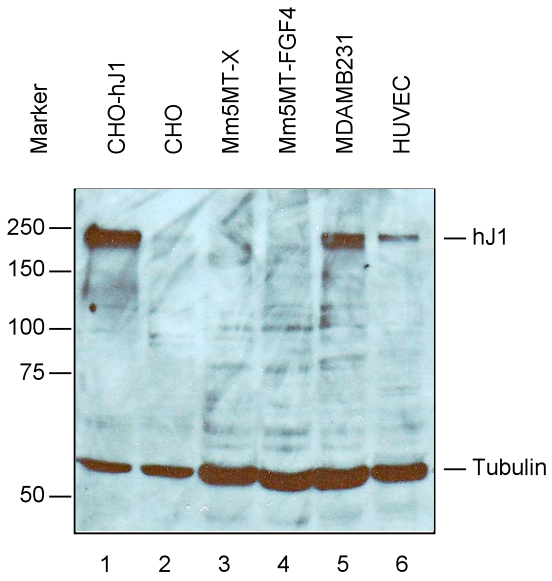
Figure 5. Purification of monoclonal antibody against hJAG1-ECD (clone: 4B4-2)



IV. Use of purified monoclonal antibodies to detect endogenous human Jagged1:

In order to further validate the monoclonal antibodies that were generated we sought to use them to detect endogenous human Jagged1 protein expressed by primary human endothelial cells and other cell types that were known to express human Jagged1 protein. HUVEC (human umbilical venous endothelial cells) were used as primary endothelial cells for analysis. MDAMB231 is human breast cancer cell line with elevated Jagged1 transcript expression, based upon previous quantitative RT-PCR analysis (data not shown). Mm5MT-FGF4 is mouse breast cancer cell line that is expressing murine Jagged1. In addition, we used CHO cells transfected with a cDNA expression vector expressing full-length human Jagged1 as positive control. As shown in **Figure 6**, human Jagged1 was detected in a western blot conducted with monoclonal 4B4-2 using extracts from the various cell lines described above. Western blotting with 4B4-2 successfully detected human Jagged1 expressed in CHO cells (lane 1), human Jagged1 expressed in breast cancer line MDA-MB-231 (lane 5) and primary endothelial cells HUVEC (lane 6). Monoclonal 4B4-2 did not strongly detect the murine Jagged1 expressed by Mm5MT-FGF4 cells (lane 4) or the Jagged1 that might be expressed by chinese hamster ovary (CHO) cells (lane 2). Thus, the 4B4-2 monoclonal antibody is capable of detecting human Jagged1 expressed by a variety of cells whereas it is not able to detect murine or hamster Jagged1.

Figure 6. Western blot analysis of endogenous JAG1 expression in various cell lines with the monoclonal antibody



Our efforts in Years 1 and 2 were put fully into identifying anti-human Jagged1 antibodies and characterizing their utility, thus, we were not able to utilize year 2 to begin the process of generating anti-Dll4 antibodies. We noted that several anti-Dll4 antibodies have been generated by the pharmaceutical sector and we are in the process of obtaining these for the diagnostic studies proposed and thus have focused our efforts into characterizing the unique anti-Jagged1 antibodies.

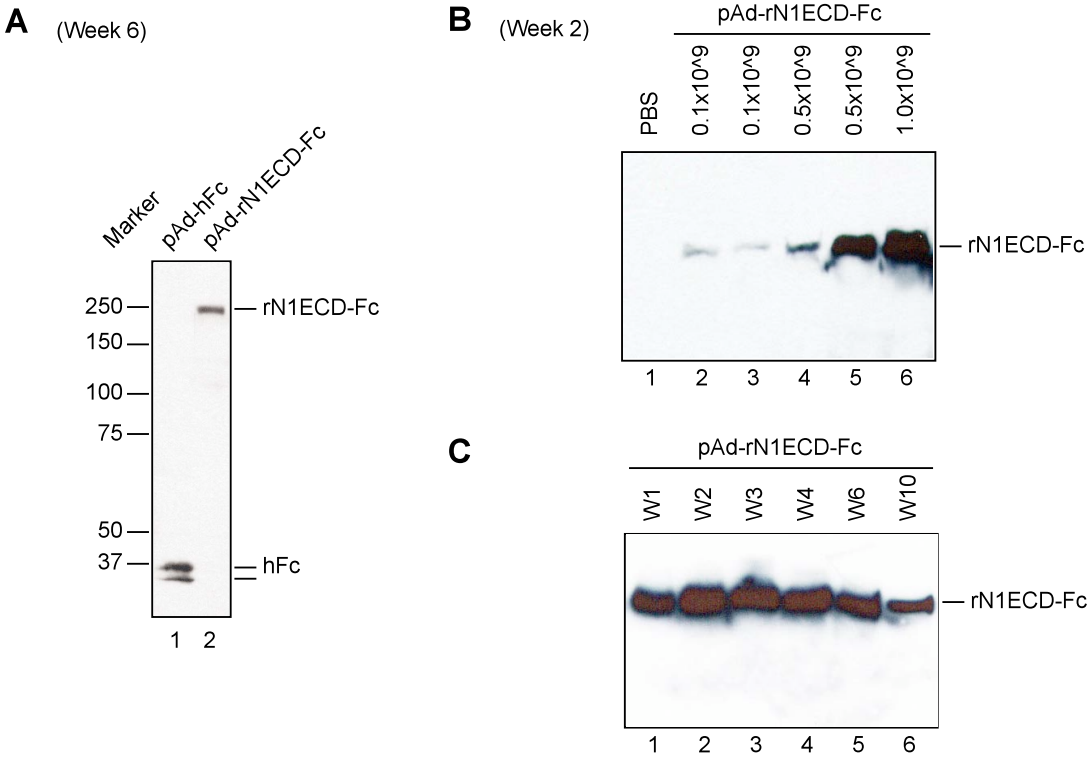
Specific Aims 2: **Detection of Jagged-1 ectodomain shedding from Breast Tumors.**

Determine if the extracellular domain of Delta-4 and Jagged-1 are shed into serum in tumor bearing mice (Kitajewski). Generate antibodies to detect human forms of these proteins (Diacovo) for development of an ELISA based detection system (Kitajewski).

We have made progress and report here on **Task5**. Screen tumor xenografts for shed Jagged-1 or Delta-4 in serum of tumor xenografts. (Months 8-24)

We have completed a pilot study to determine how readily we can detect secreted Notch antagonists in murine serum. Injection of an adenovirus into the eye vein was done with Ad encoding either Fc or N1ECDFc into immunocompromised mice. Eight immunocompromised mice for each were injected with 2.5 x10^9 Ad-N1ECDFc and six with Ad-Fc. We reported in our Year 1 report, that serum collected from the mice was used in Western blot analysis and we were able to detect N1ECDFc within the serum by immunoblotting. In Year 2, we continued this analysis and optimized several aspects of the procedure. In **Figure 7** we demonstrate that Notch decoy, rat Notch1 extracellular domain-Fc fusion (rN1ECD-Fc) and Fc control can be detected six weeks after injection of adenovirus (Figure 7A). We next determined the minimal dose that would allow for robust expression of rN1ECD-Fc, which was found to be 0.5X10e9 virus per mouse (Figure 7B). At the optimal dose (0.5X10e9 virus per mouse) we demonstrated that one single injection was sufficient to allow for production of rN1ECD-Fc and presence in murine serum of up to ten weeks (Figure 7C). We have already generated an adenovirus expressing the JAG1-ECD-Flag protein, described above, and will used the procedure at optimal conditions as a positive control for the detection of shed JAGGED1.

Figure 7. Detection of N1ECDFc in serum of mice injected with adenovirus with various dose and duration.



Specific Aims 3:

Inhibition of Breast Tumors with Notch Neutralizing Agents and Notch decoys.

Explore the consequences of therapeutic intervention of Notch signaling in breast tumor models with either Notch decoy (Kitajewski) or neutralizing antibodies (Diacovo).

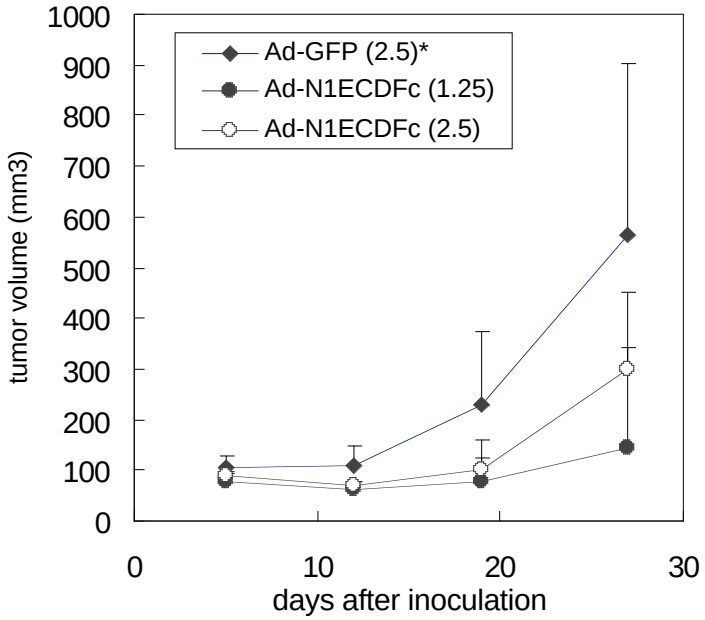
We have made progress and report here on **Task 4:** Use Neutralizing Antibodies and Notch1 antagonist to block Jagged-1 driven mammary tumors.

Introduction to Notch decoy: Notch1ECDFc. We have generated Notch antagonists that are composed of the signal peptide and EGF-like repeats of Notch1 fused in frame with Fc fragment of human IgG. Using an *in vitro* co-culture assay, we have found that the Notch antagonists N1ECDFc 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. The research on Notch1ECDFc has led to a reportable outcome in Year 1; a published paper on the effect of Notch1ECDFc on mouse mammary tumor growth and angiogenesis. Please see Appendix paper; Funahashi *etal.,* "A Notch1 ectodomain construct inhibits endothelial Notch signaling, tumor growth, and angiogenesis" ***Cancer Research*** 68:4727-35.

The work most relevant to the annual report is briefly described here. In **Year 1** we reported that N1ECDFc suppresses angiogenesis in a mouse model of breast cancer. 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- We evaluated our Notch antagonist, N1ECDFc, in a mouse model of breast cancer to determine its effects on tumor growth and angiogenesis. In this tumor model, FGF4-expressing Mm5MT (FGF4-Mm5MT) are tumorigenic when subcutaneously implanted in syngeneic mice (data not show). The expression of rat Notch1 decoy (rN1ECDFc) was able to block the growth of Mm5MT-FGF4 xenografts (10).

*In **Year 2** we further explored a novel model for expressing N1ECDFc in liver via adenovirus vector which then produces inhibitory protein in serum of mice.* We established an alternate approach to express Notch antagonists in mice, described in Update for *Specific Aim 2.* By injecting an adenovirus encoding either GFP or N1ECDFc into either tail vein or eye vein in immunocompromised mice we could elicit high levels of such inhibitory proteins in serum of mice (Figure 7). Immuno-compromised mice for each were injected with 2.5 $x10^9$ Ad-GFP or Ad-rN1ECDFc, or 1.25 x 10^9 Ad-rN1ECDFc. 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 8). 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 collected serum from the mice were able to detect N1ECDFc within the serum by immunoblotting (data not shown). Thus, we conclude that circulating rat N1ECDFc suppressed mouse mammary tumor growth. This represents a model we will use to compare and contrast Notch1ECDFc activity against Jagged1 neutralizing agents (antibody or decoys).

Figure 8. Vein injection of Ad-rN1ECDFc suppresses growth of xenografted mammary tumors (Mm5MT).

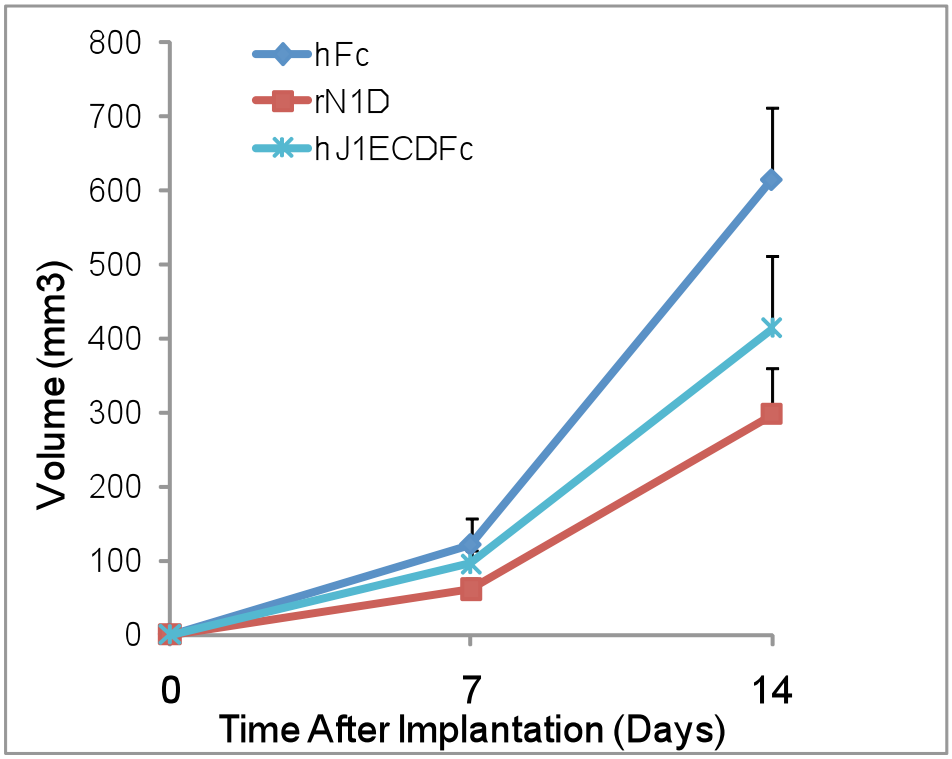


* x10^9 pfu per mouse

Development of Jagged1 decoy to evaluate role of Jagged1 in mammary tumor growth.

During the period of time while anti-Jagged1 antibodies were under development, we opted to develop an alternate strategy for Jagged1 inhibition that could further validate the importance of the Jagged1/Notch axis in growth of breast cancer. Using a design that proved to be successful for developing the rat Notch1 decoy, we used our cloned human Jagged1 cDNA to generate a human Jagged1 decoy protein. The complete coding region of extracellular domain (ECD) of human Jagged1 was fused to the human Fc protein generating a cDNA encoding human JaggedECDFc protein. The cDNA was used to generate an adenovirus expression vector (Ad-J1ECDFc). We tested the activity of J1ECDFc in the Mm5MT-FGF4 tumor model. For this analysis we chose retro-orbital injection into eye vein of an adenovirus that expresses hFc, rat N1ECDFc, or the hJagECDFc into immunocompromised mice. Five immunocompromised mice for each were injected with 1.0 x10^9 Ad-hFc or Ad-rN1ECDFc, or Ad-J1ECDFc at 2 days after subcutaneous implantation of 2 x 10^5 tumor cells (Mm5MT-FGF4). After 14 days, a suppression of tumor growth was observed in the mice that were injected with Ad-rN1ECDFc or Ad-J1ECDFc (Figure 9). This inhibition was also evident when animals were sacrificed at 20 days. We also collected serum from the mice were able to detect rN1ECDFc and hJagECDFc within the serum by immunoblotting (data not shown). Thus, we demonstrate that circulating rN1ECDFc or hJagECDFc was able to suppressed tumor growth. This represents a model we will use to compare and contrast Notch1ECDFc activity against Jagged1 neutralizing agents (J1ECDFc or antibody).

Figure 9. Eye vein injection of Ad-Fc, Ad-rN1ECDFc (rN1D) and Ad-hJ1ECDFc suppressed growth of xenografted mammary tumors (Mm5MT-FGF4)



KEY RESEARCH ACCOMPLISHMENTS

- Cloned the human JAGGED1 and DLL4 cDNAs, sequenced constructs and generated expression clones to be used to purify antigen.
- Generated CHO cells producing secreted Jagged1 ECD and purified 3 milligrams of Jagged1 Extracellular domain (ECD) to be used as antigen for hybridoma production.
- Generated B-cell line expressing either GFP tagged Jagged1 or DLL4.
- Elicited immune response in several mice against human Jagged1 extracellular domain.
- Developed ELISA, Immunofluorescence, and FACS assays for screening hybridoma supernatants.
- Generated eleven hybridoma clones producing monoclonal antibodies that bind human Jagged1.
- Purified monoclonal anti-hJAG1ECD from condition medium of positive hybridoma clones.
- Valided the monoclonal antibodies against human Jagged1 in various assays.
- Found that the expression of Notch antagonists altered the growth of murine mammary tumor xenografts.
- Developed a system to generate circulating Notch antagonists in mice using tail vein or eye vein injections of adenovirus.
- Demonstrated that circulating Notch antagonist inhibited tumor growth of subcutaneous xenografts in immuno-compromised mice.
- Developed a soluble Jagged1 decoy, human Jagged1 ECDFc and expression constructs.
- Demonstrated that circulating Notch and Jagged1 antagonists inhibited tumor growth of subcutaneous xenografts of mouse mammary tumor cell sin immuno-compromised mice.

REPORTABLE OUTCOMES (PUBLICATIONS/ABSTRACTS)

Publications (Year 1):

Funahashi Y, Hernandez S, Das I, Ahn A, Huang J, Vorontchinkhina M, Sharma A, Kanamaru E, Borisenko V, DeSilva DM, Suzuki A, Wang X, Shawber CJ, Kandel JJ, Yamashiro DJ, **Kitajewski J.** (2008) A Notch1 ectodomain construct inhibits endothelial Notch signaling, tumor growth and angiogenesis. *Cancer Research*, **68:** 4727-35*.

Dufraine J, Funahashi Y, **Kitajewski J**. (2008). Notch signaling regulates tumor angiogenesis by diverse mechanisms. *Oncogene*, **27**: 5132-7.

CONCLUSIONS

The proposal ***objective*** is to explore the importance of Jagged1 and Dll4 in mammary tumor growth and to determine the diagnostic potential of assessing Jagged1 or Dll4 levels in tumors or as shed proteins in serum. Much of our efforts have been toward developing hybridoma antibodies against human JAGGED1 and DLL4 extracellular domains. To this end we have generated full length cDNA clones of JAGGED1 and DLL4, sequenced these cDNAs and then generated a variety of expression constructs to be used in hybridoma production. We have generated CHO cells secreting a FLAG tagged extracellular domain of JAGGED1 and DLL4 and purified substantial amounts of the human Jagged1ECDFlag protein to be used as antigen. We have successfully elicitied immune responses in mice injected with purified human Jagged1ECD and used spleen to make a series of hybridoma clones. The clones were screened for utility in recognizing human Jagged1 in ELISA, Immunofluorescence, FACS, and western blotting and eleven clones were identified. Milligram amounts of several anti-Jagged1 monoclonal antibodies have been purified for further analysis.
We have also demonstrated that we can detect Notch proteins in the serum of mice expressing these via adenovirus vectors and we propose to use this system as a positive control for the development of ELISA assays to detect shed JAGGED1 or DLL4 proteins. In addition, we have developed a system that uses injection of Notch antagonist expressing adenoviruses into immunocompromised mice that allow for studies assessing the effect of Notch antagonists in nude mice with tumor xenografts. This leads to expression of the antagonist in the circulation and inhibition of mouse mammary tumor xenografts. We found that Notch decoy blocked tumor growth, presumably by blocking tumor angiogenesis. Finally, we have pursued an alternate approach to Jagged1 blockade by generating a human Jagged1 extracellular domain (ECD) Fc clone (hJag1ECDFc) and have developed an adenovirus vectors to express this protein. Use of the Ad-hJag1ECDFc in a mouse mammary tumor model display that the hJag1ECDFc has activity against mammary tumor growth that is comparable to the inhibition seen using rat Notch1 decoy.

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A Notch1 Ectodomain Construct Inhibits Endothelial Notch Signaling, Tumor Growth, and Angiogenesis

Yasuhiro Funahashi,[1] Sonia L. Hernandez,[3,4] Indranil Das,[1] Audrey Ahn,[1] Jianzhong Huang,[4,5] Marina Vorontchikhina,[1] Anshula Sharma,[1] Emi Kanamaru,[1] Valeriya Borisenko,[1] Dinuka M. DeSilva,[1] Akihiko Suzuki,[7] Xing Wang,[1] Carrie J. Shawber,[1] Jessica J. Kandel,[4,5,6] Darrell J. Yamashiro,[2,4,6] and Jan Kitajewski[1,2,6]

Departments of [1]Obstetrics and Gynaecology, [2]Pathology, [3]Nutrition, [4]Pediatrics, and [5]Surgery, [6]Institute of Cancer Genetics, Columbia University Medical Center, New York, New York; and [7]Tohoku University Medical Center, Sendai, Japan

Abstract

Notch signaling is required for vascular development and tumor angiogenesis. Although inhibition of the Notch ligand Delta-like 4 can restrict tumor growth and disrupt neovasculature, the effect of inhibiting Notch receptor function on angiogenesis has yet to be defined. In this study, we generated a soluble form of the Notch1 receptor (Notch1 decoy) and assessed its effect on angiogenesis *in vitro* and *in vivo*. Notch1 decoy expression reduced signaling stimulated by the binding of three distinct Notch ligands to Notch1 and inhibited morphogenesis of endothelial cells overexpressing Notch4. Thus, Notch1 decoy functioned as an antagonist of ligand-dependent Notch signaling. In mice, Notch1 decoy also inhibited vascular endothelial growth factor–induced angiogenesis in skin, establishing a role for Notch receptor function in this process. We tested the effects of Notch1 decoy on tumor angiogenesis using two models: mouse mammary Mm5MT cells overexpressing fibroblast growth factor 4 (Mm5MT-FGF4) and NGP human neuroblastoma cells. Exogenously expressed FGF4 induced Notch ligand expression in Mm5MT cells and xenografts. Notch1 decoy expression did not affect tumorigenicity of Mm5MT-FGF4 cells *in vitro* but restricted Mm5MT-FGF4 xenograft growth in mice while markedly impairing neoangiogenesis. Similarly, Notch1 decoy expression did not affect NGP cells *in vitro* but disrupted vessels and decreased tumor viability *in vivo*. These results strongly suggest that Notch receptor signaling is required for tumor neoangiogenesis and provides a new target for tumor therapy. [Cancer Res 2008;68(12):4727–35]

Introduction

Angiogenesis is exquisitely regulated by multiple signal pathways, including vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), and hepatocyte growth factor (HGF). Among these, VEGF critically influences almost all steps of angiogenesis, including endothelial proliferation, survival, and tube formation (1). Consistent with this protean role, VEGF inhibitors reduce angiogenesis in preclinical models and have been clinically validated as cancer therapy (2). Despite this established efficacy, different tumor types exhibit widely varying susceptibility to VEGF blockade (2). The underlying reasons for this variability are not clear. One possibility is that alternative signals rescue tumor vasculature, allowing for perfusion despite VEGF inhibition. Identification of such pathways is therefore of clear therapeutic importance.

The highly conserved Notch gene family encodes transmembrane receptors (Notch1, Notch2, Notch3, Notch4) and ligands [Jagged1, Jagged2, Delta-like 1 (Dll1), Dll3, Dll4], also transmembrane proteins. Upon ligand binding, the Notch cytoplasmic domain (NotchIC) is released by presenilin/γ-secretase (3). Notch signaling defects produce severe vascular defects in embryos (4), with haploinsufficiency of Dll4 causing lethality. The potential role of Notch signaling in tumor angiogenesis has thus excited much recent interest. Mice transgenic for a Dll4 reporter construct show expression in tumor endothelial cells (EC; ref. 5) and increased Dll4 expression has been detected in human cancers (6, 7). Two recent reports confirm that Dll4 plays a critical role in neoplastic endothelium, as Dll4 blockade suppresses growth and perfusion in experimental tumors (8, 9). Intriguingly, in these studies Dll4 inhibition disorganized tumor vasculature rather than simply preventing vessel proliferation, suggesting that Dll4 is required for the assembly of functional vessels.

Recent data indicate that Notch receptors also play a role in tumor angiogenesis. For example, in head and neck squamous cell carcinoma (HNSCC), HGF was shown to up-regulate expression of Jagged1 on tumor cells, but not on endothelium. Increased Jagged1 expression activated Notch signaling in neighboring ECs, stimulating tumor angiogenesis and growth in mice (10). Thus, these data suggest that there are at least two distinct mechanisms for activating Notch signaling in tumor endothelium.

Accumulating evidence shows the intricate linkage of Notch activation and VEGF signaling. VEGF can induce the expression of Notch receptors and Dll4 (11), with Dll4 reducing expression of VEGF receptor 2 (VEGFR2) in ECs, contributing to feedback regulation of VEGF (12). More recently, Notch receptors have been shown to regulate the expression of endothelial VEGFRs (13). These studies suggest that VEGF-mediated and Notch-mediated signal pathways cross-regulate one another by mechanisms yet to be fully understood.

In these experiments, we evaluated the role of Notch receptor activation in angiogenesis using a novel soluble construct based on the extracellular domain of Notch1 (Notch1 decoy). *In vitro*, Notch1 decoy inhibited both ligand-induced activation of Notch signaling and morphogenesis of ECs adenovirally overexpressing Notch4. *In vivo*, Notch1 decoy reduced VEGF-stimulated angiogenesis in murine skin. Notch1 decoy expression delayed growth of murine Mm5MT xenografts in which Jagged1 expression was up-regulated

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J.J. Kandel and D.J. Yamashiro contributed equally to this manuscript.

Requests for reprints: Jan Kitajewski, 1130 St. Nicholas Avenue, Irving Cancer Research Center, New York, NY 10032. Phone: 212-851-4688; Fax: 212-851-4690; E-mail: jkk9@columbia.edu.

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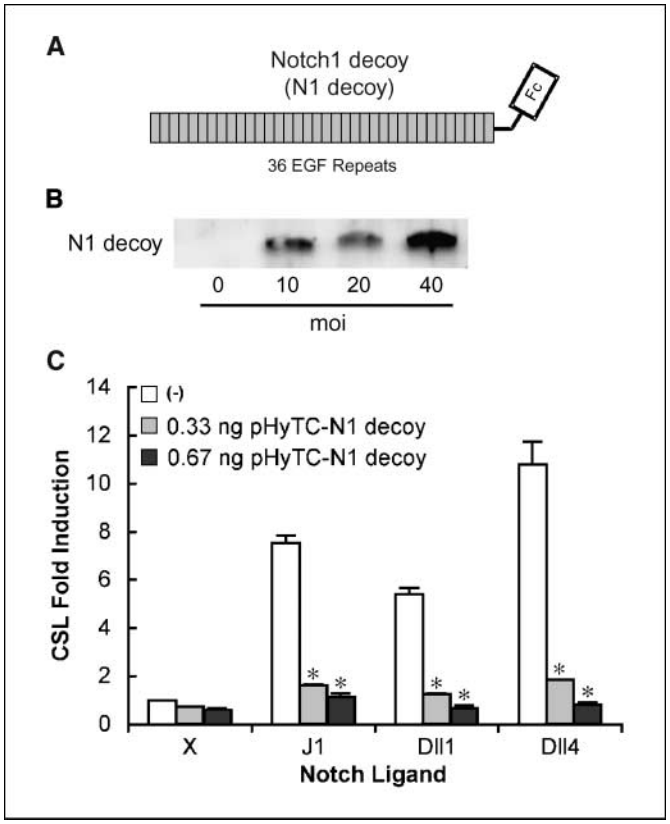

Figure 1. Notch1 decoy inhibits activation of Notch signaling stimulated by Notch ligands. *A,* schematic of Notch1 decoy containing the 36 endothelial growth factor repeats of rat Notch1 fused to human Fc. *B,* Western blotting to detect secreted Notch1 decoy in conditioned medium from HUVECs transduced with Ad-Notch1 decoy at indicated m.o.i. *Bar,* 100 μm. *C,* Notch1 decoy inhibits ligand-induced CSL reporter activity in coculture signaling assay. Activation of Notch signaling was measured in HeLa cells expressing Notch1 cocultured with 293 cells expressing Notch ligands. *Columns,* mean; *bars,* SD. *, $P < 0.05$.

by ectopic expression of FGF4 and disrupted vasculature and tumor viability in NGP neuroblastoma tumors. Taken together, these data support a requirement for Notch receptor function during neoangiogenesis, including VEGF-induced angiogenesis, and suggest that inhibition of this pathway may provide an effective new antitumor strategy.

Materials and Methods

Reagents and expression vectors. Compound E was purchased from Calbiochem, and PD166866 (14) was from Eisai Co., Ltd. Notch1 decoy encodes the rat Notch1 ectodomain (bp 241-4229, accession no. X57405) fused in frame to human IgG Fc. Retroviral pHyTC-Jagged1, pHyTC-Dll1, pHyTC-Dll4, and pBos-Notch1 have been described (15). Notch1 decoy and Fc were engineered into retroviral vector pHyTCX, and mouse FGF4 engineered into pQNCX. Adenoviral constructs encoding LacZ and mouse Notch4 and pAdlox-GFP have been described (16, 17).

Human umbilical vein ECs, adenoviral, and retroviral infections. Human umbilical vein ECs (HUVEC) were isolated as described (18), and mouse mammary carcinoma Mm5MT was obtained (American Type Culture Collection). We used adenovirus (Ad) at indicated multiplicity of infection (m.o.i.) and retroviral supernatants from GP2-293 cells (BD Biosciences) for infection. HUVECs were selected using 300 μg/mL hygromycinB (Invitrogen), and Mm5MT transfectants expressing FGF4 (Mm5MT-FGF4) was selected in 1 mg/mL G418 (Life Technologies-Invitrogen) with double transfectants in 300 μg/mL hygromycinB. Both Mm5MT-FGF4-Notch1 decoy and NGP-Notch1 decoy lines expressed Notch1 decoy, with protein detected in both cell lysates and in conditioned media (data not shown). Thus, a portion of Notch1 decoy is soluble in the extracellular environment.

Western blotting. Ad encoding Notch1 (Ad-Notch1) decoy-transduced HUVEC were cultured in endothelium serum-free medium (Life Technologies-Invitrogen) at 48 h and Mm5MT-FGF4 transfectants in DMEM. Western blots were performed using antihuman Fc (Pierce).

Quantitative reverse transcription–PCR. Mm5MT transfectants were cultured 7 d with vehicle or 1 μmol/L PD166866 [inhibitor of FGF receptor (FGFR) kinase], total RNA isolated (RNeasy mini-kit, Qiagen), and first-strand cDNA synthesized (SuperScript First-Strand Synthesis System, Invitrogen). Quantitative reverse transcription–PCR (RT-PCR) for β-actin, FGF4, Hey1, VE-Cadherin, Jagged1, Dll1, and Dll4 (SYBER Green PCR Master Mix, 7300 Real-Time PCR; Applied Biosystems) was performed in triplicate, and values were normalized for β-actin. Values are shown for fold induction compared with controls (primer sequences available on request).

Coculture signaling assay. Notch1 decoy inhibition of ligand-induced signaling was performed as described (15). HeLa cells were transfected with 333 ng pBOS-Notch1, 333 ng pGA981-6 (19), and 83 ng pLNC-LacZ with either 666 ng pCMV-Fc or pHyTC-Notch1 decoy (333 ng for 1×, 666 ng for 2×). 293 cells were transfected with 680 ng pHyTc-Jagged1, pHyTc-Dll1, pHyTc-Dll4, or pHyTc-X (empty vector). Cells were harvested, luciferase activity was determined 48 h posttransfection (enhanced luciferase assay kit, BD PharMingen), and β-galactosidase activity was determined (Galacto-Light Plus kit, Applied Biosystems). Assays were performed in triplicate.

Endothelial coculture morphogenesis assay. HUVEC morphogenesis was assessed as described (15), modified by adding coculturing of Ad-Notch4–transduced HUVEC with Notch1 decoy-HUVEC or Fc-HUVEC transfectants. Ad-GFP at 10 m.o.i. was cotransduced in HUVECs with Ad-LacZ or Ad-Notch4 at 30 m.o.i. and, 48 h later, seeded on fibrin gels (24-well plates, 1.5×10^4 cells per well). Stable HUVEC-mock (HUVEC-X), HUVEC-Fc, or HUVEC-Notch1 decoy transfectants were seeded at 1.35×10^5 cells per well, and vehicle or 200 nmol/L compound E added 3 h later. Seven days later, HUVEC morphogenesis was calculated as the number of GFP-positive cells with processes compared with total GFP-positive cells per field.

Mouse dorsal air sac assay. The dorsal air sac (DAS) angiogenesis assay was performed as described (20) with minor changes. Millipore chambers were packed with 5.0×10^6 KP1/VEGF cells transduced (60 m.o.i.) with either Ad-GFP or Ad-Notch1 decoy and transplanted into the DAS of C57BL/6 mice (n = 3–5 each, with experiments performed in triplicate). Photographs were taken 4 d after implantation. To control for the effects of Notch1 decoy on growth of KP1 cells, we established that decoy expression did not affect growth of KP1 cells in culture (data not shown).

Colony formation (Clonogenic) assay. 0.66% agar (250 μL; Agra noble, Difco) in DMEM (agar solution) was added into 24-well plates. After agar became solid, 250 μL of 1:1 mixture of agar solution and cell suspension were overlaid at 1.5×10^3 cells per well and kept at 4°C for 30 min and then 250 μL of agar solution was added again. DMEM (750 μL) was aliquoted into well and changed twice a week for 2 wk. Cell numbers were measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies). Data was shown as percentage of control compared with mock transfectants (Mm5MT-FGF4-X).

Mm5MT tumor model. Female C3H mice (6–8 wk old; Taconic) underwent s.c. implantation of 10^6 Mm5MT transfectants (n = 10 each). Tumor diameters were measured with calipers, and volume was calculated [length (mm) × width $(mm)^2 \times {}^1/_2$]. Tumors were harvested at day 22 and analyzed. Experiments were performed thrice.

Immunohistochemistry. Fresh-frozen Mm5MT tissue sections (5 μm) were immunostained (see supplementary data for antibody list; ref. 21). CD31 quantitation was performed using an Eclipse E800 microscope and ImagePro Plus v.4.01. Twenty different fields per slide were measured, and density ratios were calculated as (area of specific staining)/(total area, each field). Data are shown as the ratio of the mean of average density ratios of each Mm5MT transfectant to Mm5MT mock-transfectant.

NGP tumor model. The NGP tumor model has previously been described in detail (22). NGP cells were transfected with LacZ or Notch1

decoy, as above, and 10^6 NGP-LacZ or NGP-Notch1 decoy cells implanted intrarenally in 4-wk-old to 6-wk-old NCR nude mice (Taconic; NGP-LacZ n = 11, NGP-Notch1 decoy n = 13). At 6 wk, tumors were harvested for analysis. Paraffin-embedded sections (5 μmol/L) were immunostained for CD-31/PECAM and α-smooth muscle actin (αSMA). To detect apoptosis [terminal transferase deoxyuridine nick end labeling (TUNEL) assay], we used the Apoptag Red *in situ* kit (Chemicon). Signal was quantified by photographing 20 to 23 randomly selected fields of each tissue, excluding areas of normal kidney. Each frame was photographed in both red (TUNEL signal) and green channels. Using Adobe Photoshop, green channel signals were subtracted to eliminate erythrocyte autofluorescence. A uniform red-channel threshold was arbitrarily selected, and total signal area was measured in four NGP-Notch1 decoy and three NGP-LacZ tumors. Erythrocyte quantification was performed similarly.

Statistical analysis. Significance in quantitative studies was assessed using Tukey-Kramer tests (CD31 quantitation) and Kruskal-Wallis analysis (all others).

Results

Notch1 decoy inhibits ligand-induced Notch signaling in cells expressing Notch1. Notch1 decoy is based on the ectodomain of rat Notch1 fused to human IgG Fc (Fig. 1*A*) and is secreted, as determined by blotting of conditioned media from Ad-Notch1 decoy–infected HUVEC (Fig. 1*B*). We assessed Notch1 decoy activity using coculture signaling assays (15). 293 cells expressing Notch ligands, Jagged1, Dll1, or Dll4 activated Notch signaling when cultured with HeLa cells expressing Notch1, as measured by CSL-luciferase reporter activity (Fig. 1*C*). Expression of Notch1 decoy in either HeLa (Fig. 1*C*) or 293 cells (data not shown) blocked Notch1 signaling in coculture assays, indicating that this construct prevented Notch1 activation by Jagged1, Dll1, or Dll4 and, thus, is likely to function as a pan-ligand inhibitor of Notch1 signaling.

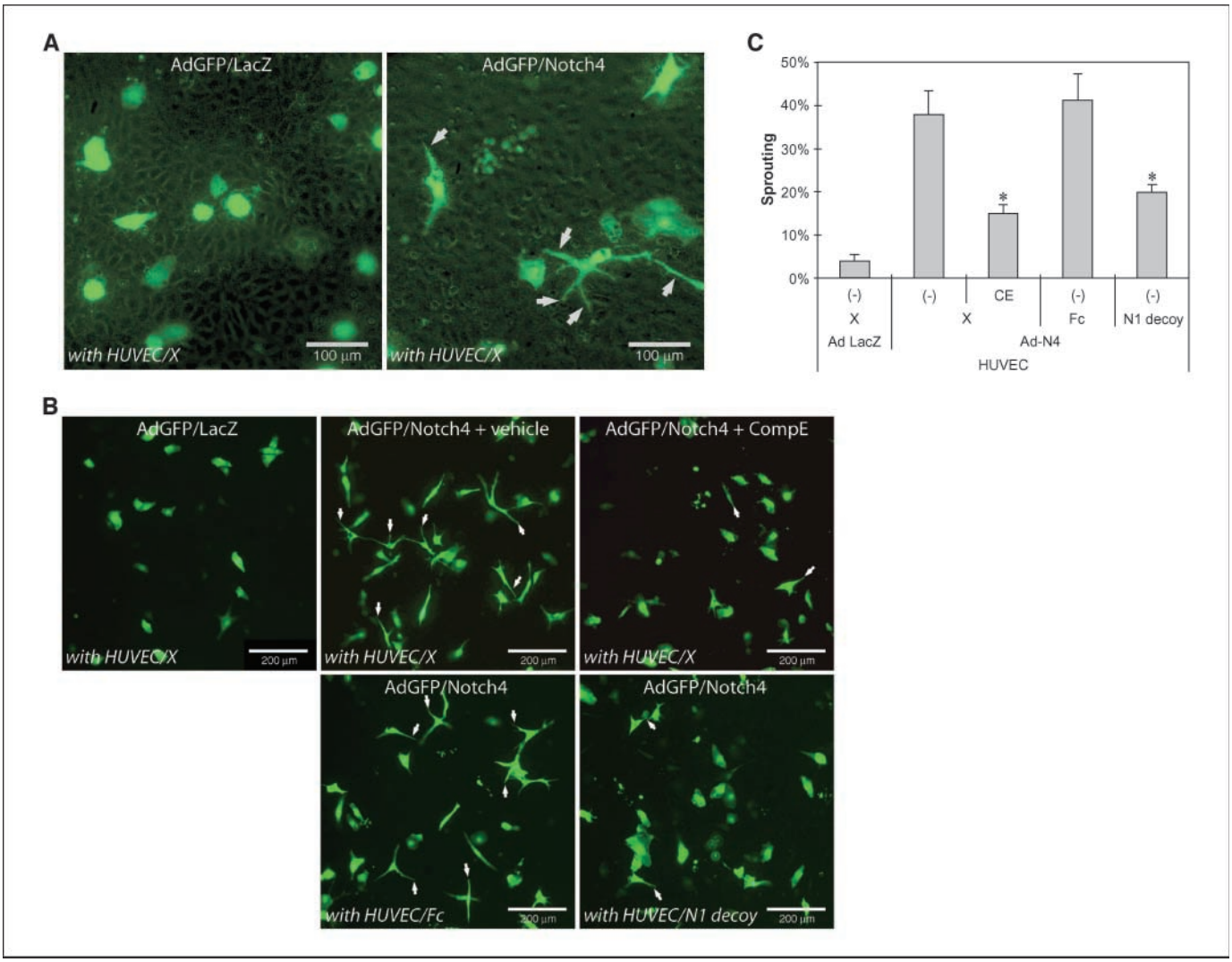

Figure 2. Notch1 decoy or compound E blocks Notch4-mediated HUVEC extensions. *A,* ectopic expression of Notch4 induces morphogenetic changes by HUVECs cultured on fibrin gel. HUVECs were transduced with Ad-Notch4 at 30 m.o.i. and Ad-GFP at 10 m.o.i. to mark-infected cells. Two days later, HUVEC transfectants were cocultured with transduced HUVECs on fibrin gel and morphologic changes were documented using fluorescence microscopy. Notch4-induced cell extensions (*right, white arrows*). *B,* Notch inhibition blocks Notch4-mediated HUVEC extensions. Notch4 expression induced cell extensions (*top center*) compared with control LacZ expressing HUVEC (*top left*), whereas treatment with 200 nmol/L compound E blocked Notch4-induced extensions (*top right*). Notch1 decoy expression blocks Notch4-induced cell extensions. Adenovirus-transduced HUVECs were cocultured on fibrin gels with stable HUVEC transfectants expressing either Fc (*bottom left*) or Notch1 decoy (*bottom right*) and photographed 2 d later. *Bar,* 200 μm. *C,* quantification of effect of Notch signal inhibition on Notch4-induced extensions. Reduction in extensions was statistically significant after treatment with compound E and expression of Notch1 decoy ($P < 0.0001$, both). *Columns,* mean; *bars,* SD.

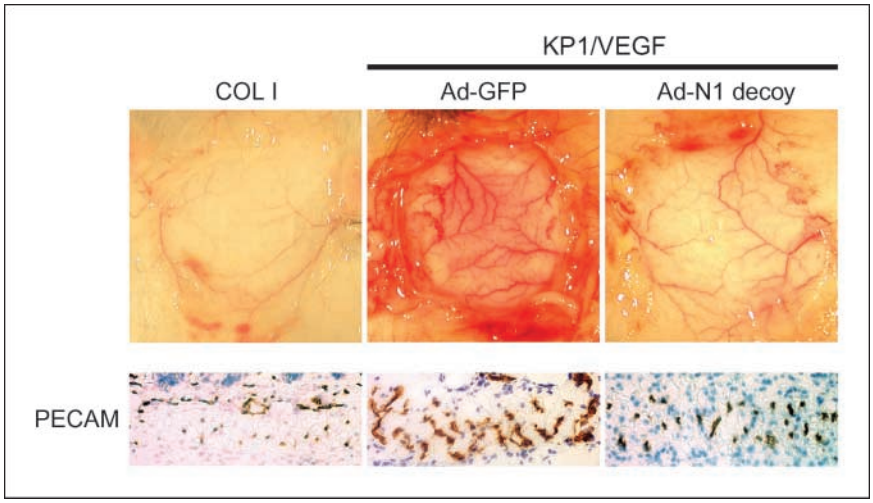

Figure 3. Role of Notch signaling in VEGF-dependent *in vivo* angiogenesis. Inhibition of KP1/VEGF-induced angiogenesis with Notch1 decoy in mouse DAS assay. Representative photographs. *Top,* subcutaneous VEGF-induced angiogenesis with control COL1 (*left*) and KP1/VEGF cells transduced with GFP (*middle*) or with Notch1 decoy (*right*). *Bottom,* immunohistochemical analysis with CD31/PECAM antibody in muscle layer of skin (20×).

Notch1 decoy blocked morphogenesis of HUVEC induced by Notch4. HUVECs transduced with Notch4 formed cellular extensions when cocultured with control HUVECs on fibrin gels (Fig. 2*A*), resembling morphologic changes induced by VEGF and FGF2 (23, 24). Using a CSL-Notch reporter introduced into HUVEC, we found that ectopic Notch4 expression in HUVEC or ectopic Notch4/Dll4 coexpression can increase Notch signaling over basal signaling (Supplementary Fig. S1). Previous reports have shown that growth of ECs on fibrin induced Jagged1 expression, therefore, it is highly likely that Jagged1 was expressed in HUVECs grown on fibrin (25). In our assay, endogenous Notch ligands expressed by HUVECs may activate the exogenous Notch4 to promote formation of cellular extensions. We examined this possibility using either compound E, a γ-secretase inhibitor (GSI), or Notch1 decoy. Compared with vehicle, treatment with 200 nmol/L compound E clearly inhibited extensions in Notch4-HUVECs (Fig. 2*B*, *top* and Fig. 2*C*). Coculturing of Ad-Notch4 transduced HUVECs with Notch1 decoy–HUVEC transfectants similarly blocked endothelial extensions relative to Fc-HUVEC transfectants (Fig. 2*B*, *bottom* and Fig. 2*C*). Reduction in formation of cellular extensions was significant (Fig. 1*D*; $P < 0.0001$ for both compound E treatment and Notch1 decoy transduction; data shown as mean ± SD).

Collectively, these data indicate that Notch receptor activation seems to be involved and in part required to induce HUVEC extensions in this assay and that the Notch1 decoy functions similarly to GSI, further validating its activity as an inhibitor of multiple Notch ligand-receptor interactions.

The Notch1 decoy inhibits VEGF-induced angiogenesis in murine dermis. The role of Notch in physiologic angiogenesis was evaluated using a DAS assay (26), in which a chamber containing VEGF-A^{121}–expressing pancreatic KP1 tumor cells KP1 (KP1/$VEGF^{121}$) is implanted under the dorsal skin of a mouse. Angiogenesis was induced in the dermal smooth muscle layer overlying the KP1/$VEGF^{121}$ chamber (Fig. 3) but was significantly inhibited when KP1/$VEGF^{121}$ cells also expressed Notch1 decoy (Fig. 3, *top*), as evidenced by immunostaining for the endothelial marker CD31/PECAM (Fig. 3, *bottom*). KP1/$VEGF^{121}$ and Ad-GFP–infected KP1/$VEGF^{121}$ cells elicit a similar angiogenic response (data not shown). These data suggest that dermal angiogenesis induced by VEGF requires Notch receptor activation. In this model, the Notch1 decoy is secreted from the cells in the implanted chamber and acts on vessels in adjacent tissue by diffusing out of the chamber as a soluble agent. To detect secreted Notch1 decoy, we immunostained for human Fc and found this deposited perivascularly and diffusely in tissues external to the implanted chamber (Supplementary Fig. S2).

FGF4 induced the expression of Notch ligands, Jagged1, and Dll1, in mouse mammary tumor Mm5MT cells. Overexpression of FGF4 in Mm5MT cells promoted tumorigenicity in clonogenic and xenograft assays (data not shown). Because the tyrosine kinase pathway involving HGF/mitogen-activated protein kinase signaling induced Jagged1 expression in HNSCC (10), we asked whether FGF4, via stimulation of tyrosine kinase signaling, would stimulate analogous expression of Notch ligands in Mm5MT cells. We detected up-regulation of Jagged1 and Dll1 in Mm5MT-FGF4 transfectants using quantitative PCR (Dll4 expression was unaltered; Fig. 4*A*). Immunoblotting confirmed up-regulation of Jagged1 protein in Mm5MT-FGF4 cells compared with control Mm5MT cells (Fig. 4*B*). The FGFR kinase inhibitor PD166866 (14) suppressed induction of both Jagged1 and Dll1 in Mm5MT-FGF4 transfectants (Fig. 4*C*), indicating that FGF4-induced Jagged1 and Dll1 expression requires FGFR signaling. We also evaluated the expression of several known angiogenic factors by quantitative RT-PCR, comparing Mm5MT-FGF4 cells to Mm5MT-FGF4 cells expressing Notch1 decoy. No major difference in the expression of VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor, FGF1, FGF2, FGF4, platelet-derived growth factor-B, or angiopoietin 4 were detected when Mm5MT-FGF4 or Mm5MT-FGF4-Fc cells were compared with Mm5MT-FGF4 cells expressing Notch1 decoy (Supplementary Fig. S3*A*).

Notch1 decoy expression inhibited angiogenesis and growth of Mm5MT-FGF4 tumors in mice. Tumorigenicity of Mm5MT-FGF4 expressing Notch1 decoy was unaltered compared with Mm5MT-FGF4 cells or Mm5MT-FGF4 cells stably overexpressing Fc, as evaluated by a clonogenic assay *in vitro* (Fig. 5*A*). We hypothesized that Mm5MT-FGF4 tumors expressing Jagged1 would promote angiogenesis by signaling via endothelial Notch receptors. Thus, we evaluated the effect of Notch1 decoy expression on Mm5MT-FGF4 xenograft growth after s.c. implantation in mice. Immunostaining confirmed strikingly increased Jagged1 in Mm5MT-FGF4 tumors (Fig. 4*D*). In addition, Notch4 was detected in Mm5MT-FGF4 tumor endothelium (data not shown). Mm5MT-FGF4-Notch1 decoy xenograft growth was significantly delayed compared with both Mm5MT-FGF4 mock

and Fc transfectants, suggesting that Notch inhibition had impaired a critical element in tumorigenesis (Fig. 5*B*). Immunostaining for CD31/PECAM showed marked inhibition of angiogenesis in Mm5MT-FGF4-Notch1 decoy tumors (Fig. 5*C*). Consistent with a requirement for Notch in vessel assembly, ECs appeared as detached solitary cells or small clusters, with few organized vessels detected. Quantitative analysis of anti-CD31 staining showed a 58% decrease in microvessel density in Notch1 decoy–expressing tumors ($P < 0.001$ for both Mm5MT-FGF4-X and Mm5MT-FGF4-Fc versus Mm5MT-FGF4-Notch1 decoy; data shown as mean ± SD; Fig. 5*D*). Consistent with the immunohistochemical data, quantitative RT-PCR analyses of pooled tumor RNA revealed a 60% reduction in VE-Cadherin expression in the Mm5MT-FGF4-Notch1 decoy tumors compared with the Fc control tumors (Supplementary Fig. S3*B*). Expression of Hey1, a direct target of Notch/CSL signaling in ECs, was decreased 2.7-fold in the Notch1 decoy–expressing tumors compared with controls (Supplementary Fig. S3*C*). Taken together, these data indicate that Notch1 decoy expression inhibited Notch signaling in tumors concurrent with a quantitative decrease in vasculature.

The effects of Notch1 decoy on Mm5MT-FGF4 tumor angiogenesis we observed differed from previous reports of inhibition of the Notch ligand Dll4 in tumor xenografts, in which tumor growth was restricted concurrent with overgrowth of a dysfunctional tumor vessel network (8, 9). Therefore, to determine whether an overgrowth phase occurred in our system, we evaluated tumor vasculature at an earlier time point during Mm5MT-FGF4 tumor growth. The first evidence of reduction in Mm5MT-FGF4-Notch1 decoy tumor growth (compared with Mm5MT-FGF4-X or Mm5MT-FGF4-Fc controls) is detectable at day 12. We quantitated tumor endothelium at this time point (day 12) but found no significant difference in PECAM-positive endothelium and no significant difference in the appearance of Dll4-positive tumor vessels when comparing control to Notch1 decoy–expressing tumors (Supplementary Fig. S4). We examined apoptosis in Mm5MT-FGF4-Notch1 decoy tumors compared with Mm5MT-FGF4-X and Mm5MT-FGF4-Fc controls at both time points by TUNEL assay but no significant difference was identified (data not shown).

Notch1 decoy expression disrupted angiogenesis in human NGP neuroblastoma xenografts. NGP xenografts in mice form a mature hierarchical vasculature that is comparatively resistant to VEGF blockade (22). To determine whether Notch receptor activation contributed to NGP angiogenesis, we transfected NGP cells with Notch1 decoy, as above. To confirm the presence of Notch1 decoy, we immunostained tumor sections using antihuman Fc antibodies and detected signal both around the tumor cells and perivascularly (data not shown). Thus, Notch1 decoy may interact with multiple different cell types in tumor xenografts. Similar to results observed with Mm5MT-FGF4-Notch1 decoy cells, neither NGP cell proliferation in culture nor colony formation in soft agar was affected by expression of the Notch1 decoy (data not shown). However, xenograft viability was strikingly impaired (Fig. 6*A*), with significantly increased tumor cell apoptosis ($P = 0.0002$; TUNEL-positive cells in NGP-Notch1 decoy versus NGP-LacZ tumors; Fig. 6*B*). Intratumoral hemorrhage was significantly increased in NGP-Notch1 decoy tumors, suggesting that vessels were physically disrupted ($P < 0.0001$; Fig. 6*C*). Immunostaining for the vascular basement membrane component, collagen IV indicated an overall decrease in vasculature, with diminished branching, although remaining collagen sleeves seemed smooth and intact (not shown). However, immunostaining for ECs and vascular mural cells (VMC; using anti-CD31 and anti-αSMA antibodies, respectively) showed disorder of these normally contiguous cell layers. Individual vascular cells seemed irregular and were erratically detached from one another, with loss of vessel continuity (Fig. 6*D*). NGP tumor cells did not express Jagged-1, although immunostaining showed

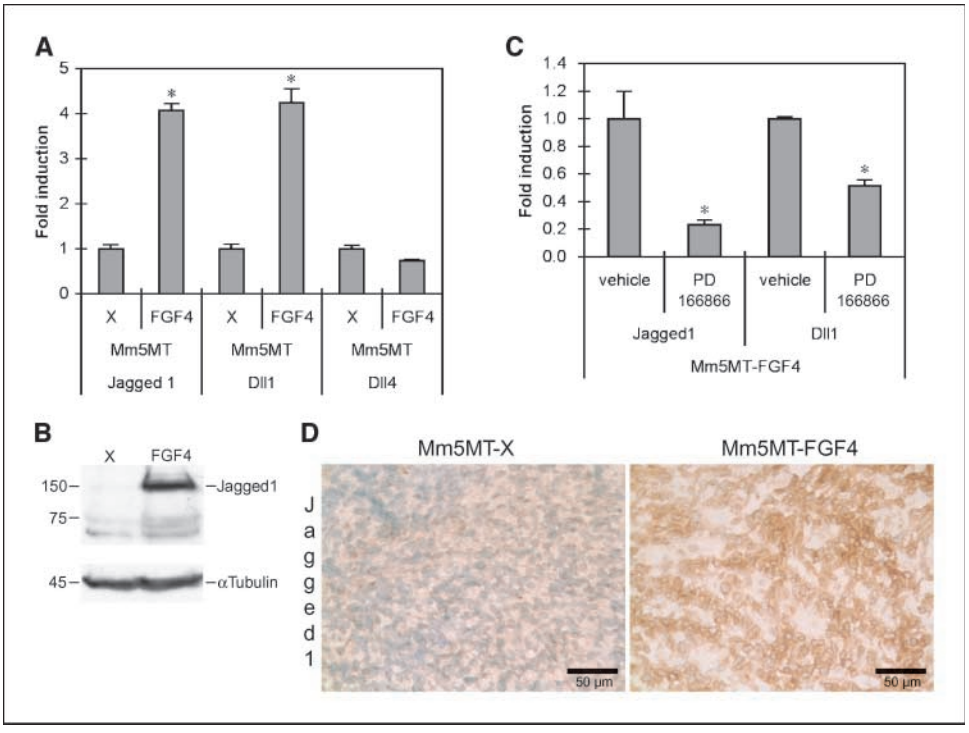

Figure 4. FGF4 induces the expression of Notch ligands in murine mammary carcinoma Mm5MT cells. Stable Mm5MT transfectants generated by retroviral gene transfer. *A,* quantitative RT-PCR analysis of the expression of Notch ligands showing induction of Jagged1 and Dll1 in Mm5MT-FGF4 compared with mock transfectants (Mm5MT-X). *, $P < 0.05$. *B,* Jagged1 protein is elevated in Mm5MT-FGF4 versus Mm5MT-X, as determined by Western blotting. *C,* reduction of Notch ligand expression in Mm5MT-FGF4 cells with PD166866, an inhibitor of FGFR kinase. *, $P < 0.05$. *D,* immunohistochemical analysis of Jagged1 staining in Mm5MT transfectants. *Bar,* 50 μm.

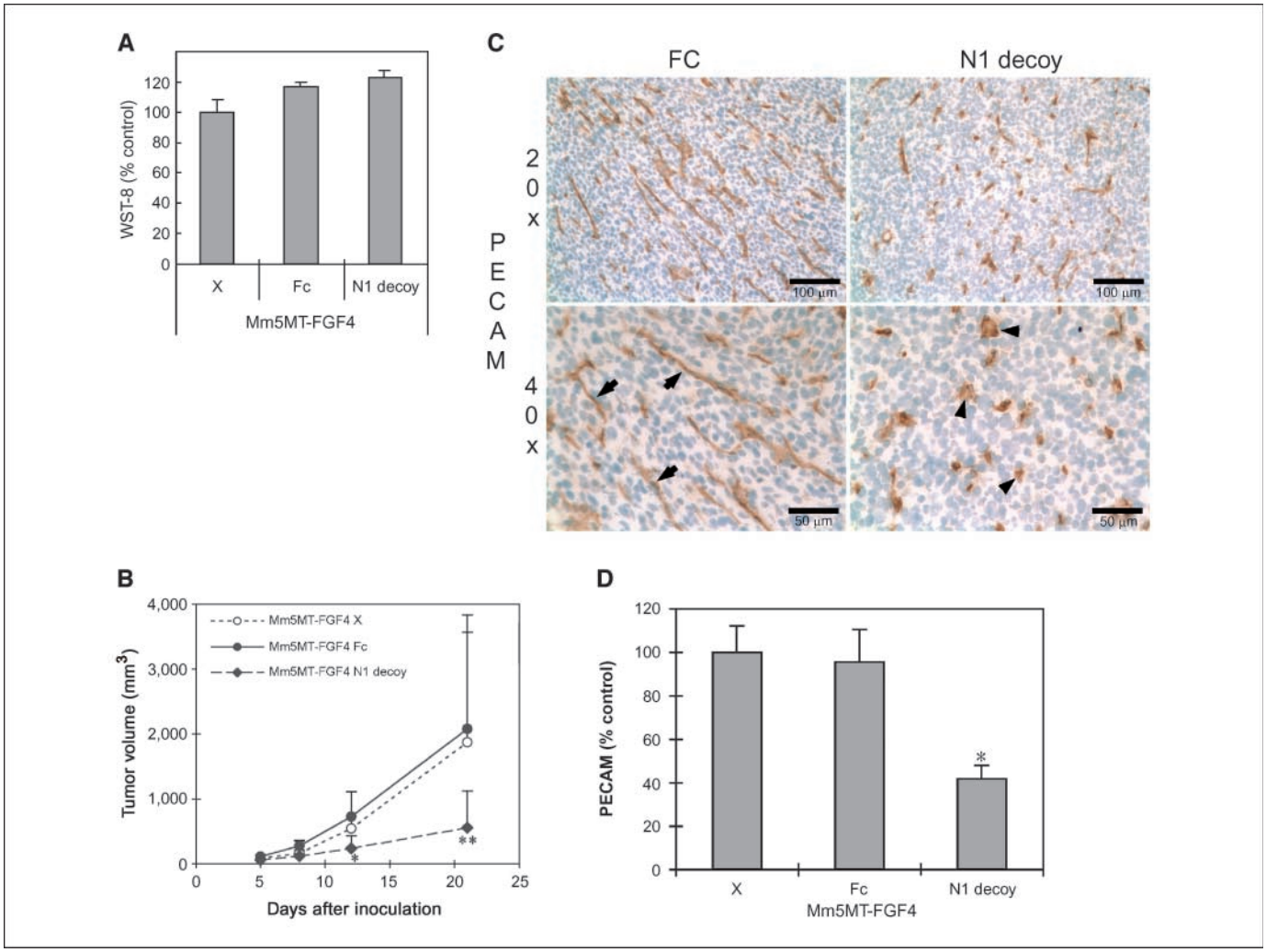

Figure 5. Notch1 decoy inhibits angiogenesis and s.c. tumor growth of Mm5MT-FGF4 tumors in mice. *A,* Mm5MT-FGF4 tumor growth in soft agar is little affected by expression of Fc or N1 decoy. *B,* tumor volumes of Mm5MT-FGF4-X and Mm5MT-FGF4-Fc differ significantly from Mm5MT-FGF4-Notch1 decoy transfectants in mice (**, day 21, $P = 0.037$ and $P = 0.008$, Mm5MT-FGF4-X and Mm5MT-FGF4-Fc versus Mm5MT-FGF4-Notch1 decoy, respectively). *Points,* mean; *bars,* SD. *C,* immunohistochemical analysis of neovessels with CD31/PECAM staining within day 21 tumors derived from Mm5MT-FGF4 transfectants. *Top, bar,* 100 μm; *bottom, bar,* 50 μm. *D,* quantitative analysis showed a reduction in CD31(+) neovessels in Mm5MT-FGF4-Notch1 decoy transfectants compared with Fc or mock-transfected tumors (*, $P < 0.001$ for both Mm5MT-FGF4-X and Mm5MT-FGF4-Fc versus Mm5MT-FGF4-Notch1 decoy). *Columns,* mean; *bars,* SD.

expression in vasculature (data not shown). Taken together, these results suggest that Notch1 decoy expression disrupted Notch-mediated endothelial and VMC interactions in tumor vasculature, leading to instability, hemorrhage, and defective perfusion of tumor tissues.

Discussion

Recent reports confirm the critical role of the Notch ligand Dll4 in angiogenesis and show that Dll4 blockade can effectively repress tumor growth by deregulating vascular development (8, 9). In this study, we show that blockade of Notch receptor function using a novel secreted construct derived from the Notch1 ectodomain effectively inhibits angiogenesis. The Notch1 decoy inhibited ligand-dependent Notch1 signal activation induced by ligands Jagged1, Dll1, and Dll4. Thus, Notch1 decoy likely acts to block multiple distinct ligand receptor combinations that participate in physiologic or pathologic angiogenesis. Consistent with a role for Notch receptor activation in neoangiogenesis, overexpression of Notch4-induced EC extensions, which could be prevented by blocking Notch signaling with either Notch1 decoy or GSI. Similarly, diffusion of Notch1 decoy from an implanted chamber reduced VEGF-stimulated angiogenesis in the dermis. Although Notch1 decoy did not inhibit tumor cell growth *in vitro*, expression of Notch1 decoy inhibited growth and angiogenesis of Mm5MT-FGF4 xenografts, in which Jagged1 expression is up-regulated. Similarly, Notch1 decoy expression had no effect on NGP tumor cell proliferation *in vitro* but disrupted tumor vessels and viability *in vivo*.

Overall, the effects of Notch1 decoy on dermal and tumor angiogenesis in these studies are distinct from those previously reported for Dll4 blockade in tumors. Most notable among these differences is the lack of observable overgrowth of endothelium in response to Notch1 decoy expression observed in all three of the *in vivo* angiogenesis models we used. In the Mm5MT-FGF4 model, evaluation of both early (day 12) and later (day 21) time points for tumor evaluation showed either equivalent (day 12) or reduced (day 21) EC content. Similarly, in the NGP tumor model, Notch1 decoy expression resulted in vascular disruption, without evidence of overgrowth. It is thus clear that response to Notch1

decoy expression is unique, likely reflecting the interruption of multiple Notch ligand–receptor interactions acting on endothelium as opposed to the effects observed after the selective inhibition of Dll4.

Notch4 overexpression in HUVECs was sufficient to induce endothelial extensions on fibrin gel without exogenous expression of Notch ligands (Fig. 2). Because fibrin is known to induce Jagged1 expression in EC and, thus, may have functioned to promote HUVEC expression of Jagged1 in this assay, we speculate that this caused activation of Notch4; in reporter assays, increased Notch activity was found when HUVECs engineered to express Dll4 and Notch4 were cocultured, suggesting that paracrine signaling is responsible for enhanced Notch signaling. In HeLa coculture signaling assays, the Notch1 decoy inhibited signaling via ligand-Notch1 receptor interaction. We were unable to similarly evaluate Notch4 activity, as Notch4 was poorly processed and presented on the surface of HeLa cells (data not shown). However, processed Notch4 is found on HUVECs after adenoviral Notch4 transduction (not shown), indicating that Notch1 decoy can block ligand-induced Notch4 activation (27).

Expression of the Notch1 decoy also blocked new vessel growth stimulated by $VEGF^{121}$ in the dermis, consistent with previous work demonstrating that Notch receptor activation is required for VEGF-induced up-regulation of target genes (28). One of these endothelial target genes is Dll4, which acts to reduce VEGFR2 and neuropilin-1 expression in a negative feedback loop (12). As above, inhibition of Dll4 disrupts this loop and results in deregulated

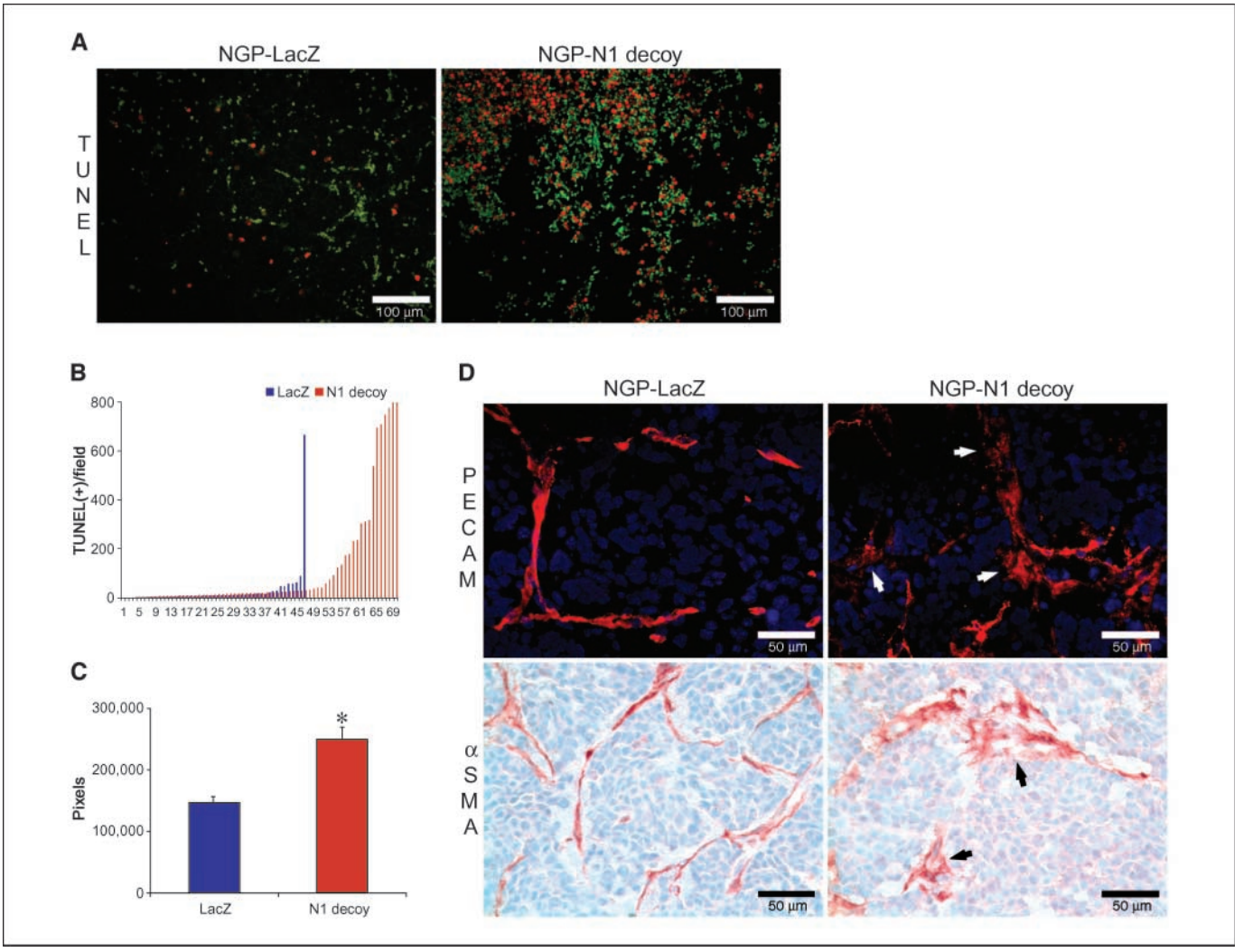

Figure 6. Notch1 decoy expression disrupts angiogenesis and impairs tumor viability in human NGP xenografts. We have previously reported that human neuroblastoma xenografts in mice have a mature, hierarchical vasculature that is relatively resistant to VEGF blockade (22). To determine whether Notch receptor activation contributed to NGP angiogenesis, we transfected NGP cells with the Notch1 decoy construct, which did not affect their ability to grow in culture (data not shown). There was, however, a marked decrease in tumor viability *in vivo* (*A*; *red fluorescence,* TUNEL; *green fluorescence,* erythrocytes; *bar,* 100 μm), with significantly increased fields that had high levels of tumor cell apoptosis [*B*; $P = 0.0002$; TUNEL-positive cells in NGP-Notch1 decoy (■) versus NGP-LacZ (■) tumors], and increased intratumoral hemorrhage [*C*; *, $P < 0.0001$, quantitation of parenchymal erythrocyte signal, NGP-Notch1 decoy (■) versus NGP-LacZ (■) tumors]. In addition, the tumor vessel networks in NGP-Notch1 decoy xenografts seemed to have been physically disrupted compared with NGP-LacZ controls, with immunostaining for ECs and VMCs (using anti-CD31/PECAM and αSMA antibodies, respectively) demonstrating lack of continuity of these vascular cell layers (*D*; *bar,* 50 μm). Individual vascular cells seemed detached from one another. Taken together, these results suggest that Notch1 decoy expression disrupted the ability of ECs and VMCs to form stable vascular conduits, causing vessel breakdown, hemorrhage, and ischemia of tumor tissues.

sprouting, forming a nonfunctional vasculature (8, 9). However, Notch1 decoy expression exerts a different effect, repressing neoangiogenesis, potentially reflecting disruption of other Notch ligand: receptor interactions (e.g., Jagged1/Notch1, as discussed below). Taken together, these data suggest a model in which construction of a vessel network is exquisitely regulated by cross-talk between Notch and VEGF pathways at multiple points, with different Notch receptor and ligand pairs playing distinct roles in this process.

The multiple roles recently shown for Notch signaling in tumorigenesis increase the attractiveness of this pathway as a potential target for cancer therapy. Whereas Notch activation is likely to function directly in malignant transformation in human cancers (29, 30), it seems to be required for angiogenesis in a number of tumor systems (8, 9) and in our models presented here. Interestingly, Notch ligand induction can be regulated by growth factor signals. For example, Jagged1 is induced in tumor cells by HGF (10) and Dll4 induced in ECs by VEGF (11). Here, we show that FGF4 can similarly stimulate Jagged1 and Dll1 expression in murine Mm5MT cells. Notch1 decoy reduced Mm5MT-FGF4 tumor growth and angiogenesis *in vivo* but did not affect tumorigenicity *in vitro*. Similarly, expression of Notch1 decoy did not affect NGP tumor growth *in vitro* while strikingly disrupting NGP vasculature *in vivo*. Thus, these results suggest that Notch receptor activation in Mm5MT and NGP vessels rather than tumor cells is required for neoplastic growth in these neoplasms.

While both Mm5MT-FGF4 and NGP xenografts displayed striking disorder of tumor vasculature after Notch1 decoy expression, the differences in vascular phenotype observed in these models suggest that tumor-specific patterns of Notch ligand/receptor interaction may fine-tune vessel assembly. Mm5MT-FGF4 tumors strongly express Jagged-1, proliferate rapidly, and develop dense, erratic endothelial networks relatively devoid of recruited VMCs. Consistent with previous data indicating that tumor cell expression of Jagged-1 can stimulate Notch-dependent angiogenesis (10), Notch1 decoy expression caused profound ablation of Mm5MT-FGF4 vasculature by day 21, leaving small clusters or individual ECs isolated in tumor parenchyma. In contrast, NGP tumors developed a mature vascular plexus, with near-uniform coverage of endothelium by VMCs. Also in contrast to the Mm5MT-FGF4 model, NGP vessels strongly expressed Jagged-1, whereas NGP tumor cells did not. Notch1 decoy expression in NGP tumors causes intratumoral hemorrhage and necrosis, with loss of vessel continuity. We speculate that the strikingly different patterns of Jagged-1 expression in the Mm5MT-FGF4 and NGP models (tumor cell versus EC) may contribute to the distinct effects of the Notch1 decoy in each. For example, engagement of Jagged-1 expressed by Mm5MT-FGF4 tumor cells by endothelial Notch receptors may be required for new tumor vessels to sprout. Notch1 decoy expression disrupted this interaction and limited new tumor growth. In contrast, NGP vessels may be stabilized by Notch-mediated signaling between adjacent vascular cells, so that Notch1 decoy caused discontinuity in already-formed vessels with loss of tumor perfusion.

Collectively, these data provide support for a model in which Notch signaling controls interactions between the multiple cell types responsible for tumor angiogenesis. Whereas Notch activation is broadly required for new vessel formation, tumor and vascular cell expression of individual Notch proteins may differentially regulate vascular sprouting and remodeling. Our results confirm the importance of Notch ligand receptor interactions in tumor vasculature and suggest that perturbing Notch receptor function may provide a novel and effective means of disrupting tumor angiogenesis.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interest in this work.

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We dedicate this work to the memory of Dr. Judah Folkman.

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