HUMAN NOTCH1 DECOYS

This application claims priority of U.S. Provisional Application No. 61/543,186, filed October 4, 2011, the contents of which are hereby incorporated by reference.

Throughout this application, various publications are referenced by author and publication date within parentheses. Full citations for these publications may be found at the end of the specification or at the end of each experimental section. The disclosures of these publications are hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Background of the Invention

Notch proteins play key roles in developmental decisions involving the vasculature, the hematopoietic system, and the nervous system. As such, an understanding of their function is key to understanding how cell-fate decisions and commitment are controlled during development and in adult tissues. To date, several reports on Notch or Notch ligand gene disruptions have described vascular phenotypes providing emphasis that this pathway is a fundamental part of the machinery that guides vascular development. Aberrant Notch activity has been linked to human pathologies; including both cancer and vascular disorders (CADASIL). The analysis of Notch in tumor angiogenesis has only recently begun; however, our discovery of potential downstream targets of Notch suggests a role in pathological processes associated with angiogenesis. For instance, VEGFR-3 has been linked to both tumor angiogenesis and tumor lymphangiogenesis. The expression or function of several other potential Notch targets has also been linked to tumor angiogenesis; including ephrinB2, Id3, Angiopoietin 1, and PDGF-B. Insights on the role of these targets in Notch gene function will clearly facilitate future analysis of Notch in human pathologies.

Additional background to this invention can be found in U.S. Patent Application Publication No. US 2011-0008342 A1, the entire contents of

which are hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Summary of the Invention

This invention provides a fusion protein which comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of the amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 23.

In one embodiment, a fusion protein of this invention comprises an amino acid sequence identical to the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extending up to the C-terminal amino acid of EGF-like repeat 24. In another embodiment, the fusion protein comprises an amino acid sequence identical to the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extending up to the C-terminal amino acid of EGF-like repeat 36.

In one presently preferred embodiment the fusion protein of this invention comprises Notch1 EGF-like repeats 10-24 (also designated herein as Notch1 decoy 10-24.) This fusion protein binds to JAGGED-1 without binding to Dll4 enabling the protein to be free from unpleasant side effects caused by inhibition of the Dll4 pathway such as liver toxicity, vascular neoplasm or necrosis in the heart and lung.

Due to its ligand specificity for JAGGED-1, it is also contemplated and expected that the Notch1 decoy 10-24 fusion protein will show superior anti-tumor activity against JAGGED associated tumor malignancies such as breast cancer, head and neck squamous cell carcinoma (HNSCC) and the related cancers in which the JAGGED ligand is reported to be highly

expressed or induced by growth factors.

Additionally, it is contemplated that this fusion protein will have a superior secretion profile relative to the secretion profile from transfected cells of previously described Notch1 fusion proteins. This in turn will provide improved efficiency for protein purification

This invention further provides compositions comprising such fusion proteins and a carrier.

This invention also provides a method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject a fusion protein of this invention in an amount effective to treat the subject's AMD.

In addition, this invention provides a method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject a fusion protein of this invention in an amount effective to treat the subject's diabetic retinopathy.

This invention still further provides a method of treating a subject suffering from cancer which comprises administering to the subject a fusion protein of this invention in an amount effective to treat the subject's cancer.

This invention also provides a method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject a fusion protein in an amount effective to treat the subject's AMD, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1, the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

This invention also provides a method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject a fusion protein in an amount effective to treat the subject's diabetic retinopathy, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is an N-terminal amino acid of EGF-like repeat 1, the C-terminal amino acid is not a C-terminal amino acid of EGF-like repeat 36.

This invention further provides a method of treating a subject suffering from a cancer which comprises administering to the subject a fusion protein in an amount effective to treat the subject's cancer, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids present in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1, the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

Brief Description of the Figures

Figures 1a – 1b: Schematic of truncated Notch1 decoy variants. Fig 1a. is a schematic of Notch1 decoy 1-36 and Notch1 decoy 1-24 which each interact with both Dll4 and JAGGED-1 and act as pan-Notch inhibitors. Fig 1b. is a schematic of four truncated Notch1 decoy variants 10-36, 14-36, 10-24 and 14-24.

Figures 2a – 2b: Expression and secretion of truncated Notch1 decoy variants in 293T cells. Figure 2a shows western blot of total cell lystates and supernatants and the molecular weights of Notch1 decoys 1-13, 1-24 and 1-36. Figure 2b shows western blot of total cell lystates and supernatants and the molecular weights of Notch1 decoys 10-36, 14-36, 10-24 and 14-24.

Figures 3a – 3c: Notch Reporter Assay for Decoys 1-13, 1-24 and 1-36. Figure 3a Schematic of Notch reporter construct containing multiple CSL-binding sites linked to the luciferase gene. Figure 3b shows the results for HELA cells expressing DLL4. Figure 3c shows the results for HELA cells expressing JAGGED-1.

Figures 4a – 4b: Notch Reporter Assay for Decoys 10-26, 14-36, 10-24 and 14-24. Figure 4a shows the results for HELA cells expressing DLL4. Figure 4b shows the results for HELA cells expressing JAGGED-1.

Figures 5a – 5b: Immunoblot using anti-Fc or anti-FLAG antibody of 293T cell lysates which were co transfected with Notch1 decoys and soluble (Figure 5a) or full length (Figure 5b) Notch1 ligands.

Figure 6: Co-Immunoprecitipation of Notch1 decoys and Notch1.

Figures 7a – 7c: Isolectin B4 staining of P5 mouse retinas after injection of adenoviruses expressing different Notch1 decoys at day P2.

Fig. 7a is control decoy, Notch1 decoy 1-13, Notch 10-24 decoy and DAPT. Fig 7b is Fc, Notch1 decoy 1-13, Notch1 decoy 10-24 and Notch1 decoy 1-24. The vascular areas of the retina are quantified in Figure 7c. Mean vessel coverage areas ± S.D. * P value < 0.05.

Figures 8a – 8d: Gene Expression profiling of HUVECs expressing Notch1 decoys. Quantitative RT-PCR for mRNA transcripts of the Notch receptors (Notch1, Notch2, Notch3, and Notch4) are set forth in Figure 8a, 8b, 8c and 8d, respectively.

Figures 9a – 9b: Gene Expression profiling of HUVECs expressing Notch1 decoys. Quantitative RT-PCR for mRNA transcripts of HEY1 is in Figure 9a. Quantitative RT-PCT for mRNA transcripts of HEY2 is in Figure 9b.

Figures 10a – 10b: Gene Expression profiling of HUVECs expressing Notch1 decoys. Quantitative RT-PCR for mRNA transcripts of HEYL is in Figure 10a. Quantitative RT-PCT for mRNA transcripts of HES1 is in Figure 10b.

Figures 11a – 11e: Gene Expression Profiling of HUVECs expressing Notch1 decoys. Quantitative RT-PCR for mRNA transcripts of DLL4 is in Figure 11a; for JAGGED-1 is in Figure 11b; for VEGFR-1 in Figure 11c; For VEGFR-2 in Figure 11d; and for VEGFR-3 in Figure 11e.

Figures 12a – 12c: Flow cytometry for VEGF receptors. Histograms of HUVECs expressing Notch decoys for VEGFR-1 (Figure 12a); VEGFR-2 (Figure 12b) and VEGFR-3 (Figure 12c).

Figures 13a and 13b: Gene Expression Profiling of HUVECs expressing Notch1 decoys. Quantitative RT-PCR for mRNA transcripts of full-length VEGFR-1 and soluble VEGFR-1 in both Figure 13a and 13b.

Figure 14: Gene Expression profiling of Notch receptors and ligands expressed in mouse mammary tumor cells (Mm5MT), human pancreatic cancer cells (KP1), mouse Lewis lung carcinoma cells (LLC), and mouse melanoma cells (B16-F10).

Figures 15a - 15d: Effect of Notch decoys 1-13, 10-24 and 1-24 on cell proliferation and apoptosis of Mm5MT and KP1 tumor cells. Figure 15a is results of tumor proliferation studies in Mm5MT-FGFT cells. Figure 15b is results of proliferation studies in KP1-VEGF. The percentage of apoptotic cells is indicated in the upper right quadrant. Average apoptotic cell percentage ± S.D. Figure 15c is the results of apoptosis studies in Mm5MT-FGF4. Figure 15d is the results of the apoptosis studies in KP1-VEGF.

Figures 16a - 16c: Figure 16a is Western blot analysis of Fc, Notch1 decoys 1-13, 10-24 and 1-24 in serum of mice in which Adenoviruses expressing Notch1 decoys 1-13, 10-24 and 1-24, or Fc as control, were injected intravenously. Figure 16b and 16c are tumor sections immunostained by an anti-human IgG Fc antibody and counterstained with DAPI. Scale bars: 30 micrometers.

Figures 17a - 17c: Figure 17a is imaging of mice expressing Notch1 decoys 1-13, 10-24, 1-24 or Fc as control. Notch1 decoys reduced growth of Mm5MT tumors. Tumor growth was monitored by assessing the total radiance from luminescence signals using the Xenogen IVIS Imaging system and the results are shown in Figure 17b. Tumor weight was measured on last day immediately before tumor harvesting and the results are shown in Figure 17c.

Figures 18a - 18c: Figure 18a is imaging of mice expressing Notch1 decoys 1-13, 10-24, 1-24 or Fc as control. Notch1 decoys reduced growth of KP1 tumors. Tumor growth was monitored by assessing the total radiance from luminescence signals using the Xenogen IVIS Imaging system and the results are shown in Figure 18b. Tumor weight was measured on last day immediately before tumor harvesting and the results are shown in Figure 18c.

Figures 19a - 19b: Effects of Notch1 decoys on tumor vasculature. Tumor sections were immunostained for Endomucin (green) and Dll4 (red) and the results are shown in Figure 19a. Quantification of tumor vasculature was based on Endomucin-positive areas in tumor sections and the results are in Figure 19b.

Figures 20a – 20b: Immunofluorescence analysis of tumor endothelia content. KP1 tumors were immunostained for Endomucin and Dll4 and the results are shown in Figure 20a. Quantification of tumor vasculature was based on Endomucin-positive areas in KP1 tumor sections and the results are in Figure 20b.

Figures 21a – 21b: Effects of Notch1 decoys on tumor vasculature. Flurescein-conjugated lectin (100µg) was injected into mice 2 minutes before tumor harvesting. Tumor sections were immunostained for Endomucin (red) and perfused lectin (green) was associated with tumor vessels. The results of the immunostaining are in Figure 21a. The amount of vessel-associated lectin reflected function tumor vasculature and results are shown in Figure 21b.

Figures 22a – 22b: Tumor sections were coimmunostained for endomucin (green) and NG2 (red) and the results are set forth in Figure 22a. The percentage of NG2-positive areas was measured as a parameter of pericyte recruitment in tumors and the results are shown in Figure 22b.

Figures 23a – 23c: Day 12 photographs of LLC tumor-bearing mice with luminescence signals from different Notch decoy groups is in Figure 23a. Tumor growth was monitored and quantified based on the total radiance and the results are set forth in Figure 23b. Tumor weight at day 12 was measured before tumor harvesting and the results are shown in Figure 23c.

Figures 24a – 24c: Day 12 photographs of B16-F10 tumor-bearing mice with luminescence signals from different Notch decoy groups is in Figure 24a. Tumor growth was monitored and quantified based on the total radiance and the results are set forth in Figure 24b. Tumor weight at day 12 was measured before tumor harvesting and the results are shown in Figure 24c.

Figure 25a – 25b: Lungs and livers from LLC tumor-bearing mice were harvested at day 12, incubated in 30 mg/ml D-Luciferin and analyzed by the Xenogen IVIS Imaging System. Imaging results are set forth in

Figure 25a. Total radiance results are set forth in Figure 25b.

Figures 26a – 26c: Lungs and livers from mice B16-F10 tumor-bearing mice were harvested at day 12, incubated in 30mg/ml D-Luciferin and analyzed using the Xenogen IVIS Imaging System. Imaging from the Fc group for the lungs is set forth in Figures 26a and 26b and the liver in 26c.

Figures 27a – 27c: Notch1 decoys induce mild goblet cell hyperplasia. Similar to pan-Notch inhibitors, Notch1 decoys 1-24 and 1-36, ligand-specific decoys slightly increased the number of goblet cells. Normal architecture of the small intestine was preserved and compared to the control. Results are shown in Figure 27a. The average goblet cell number per filed was calculated and the results are shown in Figure 27b. Figure 27c shows that Notch1 decoys are well tolerated by tumor-bearing mice. No significant difference in weight change between the treatment groups and the control is observed. Mean weight change ± S.D. (n = 5).

Figure 28: The amino acids sequence of the human NOTCH1 protein (SEQ ID NO:1).

Figure 29: The nucleic acid sequence of human Notch1 decoy 10-24 is set forth in SEQ ID NO:2. Human Notch 1 signal peptides corresponds to nucleotides 1-69 of SEQ ID NO:2, EGF-like repeats 10-24 correspond to nucleotides 70-1788 of SEQ ID NO:2 and Human Fc corresponds to nucleotides 1789-2502 of SEQ ID NO:2. The amino acid sequence of human Notch1 decoy 10-24 is set forth in SEQ ID NO:3.

Figure 30: The nucleic acid sequence of human Notch1 decoy 10-36 is set forth in SEQ ID NO:4. Human Notch 1 signal peptide corresponds to nucleotides 1-69 of SEQ ID NO:4, EGF-like repeats 10-36 correspond to nucleotides 70-3243 of SEQ ID NO:4 and Human Fc corresponds to nucleotides 3244-3957 of SEQ ID NO:4. The amino acid sequence of human Notch1 decoy 10-36 is set forth in SEQ ID NO:5.

Figure 31: The nucleic acid sequence of human Notch1 decoy 14-24 is

set forth in SEQ ID NO:6. Human Notch 1 signal peptide corresponds to nucleotides 1-69 of SEQ ID NO:6, EGF-like repeats 14-24 correspond to nucleotides 70-1320 of SEQ ID NO:6 and Human Fc corresponds to nucleotides 1321-2034 of SEQ ID NO:6. The amino acid sequence of human Notch1 decoy 14-24 is set forth in SEQ ID NO:7.

Figure 32: The nucleic acid sequence of human Notch1 decoy 14-36 is set forth in SEQ ID NO:8. Human Notch 1 signal peptide corresponds to nucleotides 1-69 of SEQ ID NO:8, EGF-like repeats 14-36 correspond to nucleotides 70-2775 of SEQ ID NO:8 and Human Fc corresponds to nucleotides 2776-3489 of SEQ ID NO:8. The amino acid sequence of human Notch1 decoy 14-36 is set forth in SEQ ID NO:9.

Figures 33a - 33c: Notch1 decoys variants display unique effects on in vitro and retinal angiogenesis. Figure 33a shows Notch1 decoy assessment using HUVEC fibrin bead sprouting assay. After 7 days, endothelial cells form tube-like structures. HUVECs expressing $N1^{1-13}$ decoy show significantly increased sprouting. In contrast, HUVECs expressing $N1^{10-24}$ or $N1^{1-24}$ decoys form shorter, thinner sprouts, as opposed to the Fc control. The number of lumen-containing sprouts is quantified in Figure 33b. Mean number of sprouts ± S.D. * P value < 0.05. Retinas were immunostained with αSMA to identify vascular smooth muscle cells. $N1^{10-24}$ and $N1^{1-24}$ decoys significantly decreased vascular smooth muscle cell coverage along retinal arteries. Results are shown in Figure 33c.

Figures 34a - 34c: Notch1 decoys increase endothelial cell migration. Lentivirally transduced HUVECs were seeded at 1.0×10^5 cells per well in a 24-well plate and allowed to become confluent overnight. Then, scratches were made in each well, and HUVEC migration into the wounded area was photographed (Figure 34a) and quantified using the TScratch program. Average migration rate ± S.D. * P value < 0.03 (Figure 34b). Figure 34 c shows that Notch1 decoys increase endothelial cell proliferation. HUVECs lentivirally transducer with Fc, Notch1 decoys 1-13, 1-24, 1-36, or N1IC, were seeded at 1.0×10^4 cells per well in a 24-well plate. Cell numbers were quantified on day 1 and day 4. Average cell number ± S.D. * P value < 0.005.

Figures 35a - 35b: HUVECs expressing Notch1 decoys show increased endothelial network and branching. Figure 35a. HUVECs were seeded between collagen layers at 1.0 x 10^5 cells per well in a 24-well plate and cultured for 4 days. Figure 34b. HUVEC ability to form network was quantified by counting the number of branch points. HUVEC-N1IC and HUVEC-Notch4/int3 were not included due to its lack of proper network. Average number of branch points ± S.D. * P value < 0.005.

Figure 36: Quantitative real-time PCR for Notch ligands, JAGGED-1 and DLL4. Compared to those on a normal culture plate or collagen gel, HUVECs cultured on fibrin gel significantly upregulated JAGGED-1 expression and at the same time downregulated DLL4 expression. Average relative value of mRNA transcripts ± S.D. * P value < 0.002.

Figures 37a - 37b: Notch1 decoys 1-13, 10-24, and 1-24 reduce colony formation in Mm5MTFGF4. Figure 37a. Mm5MT-FGF4, KP1-VEGF, LLC, and B16-F10 tumor cells were seeded in semi-solid agar medium at 3 x 10^3 cells/well in a 24-well plate and cultured for 3 weeks. 3 mg/ml of MTT was added to culture for colony visualization. Figure 37b. Quantification of the colony area was performed. Average percentage of colony area ± S.D. * P value < 0.001.

Figure 38: Notch1 decoys block xenografted tumor growth. Using Mm5MT-FGF4, KP1-VEGF, LLC, or B16-F10 subcutaneously injected into nude mice, tumor growth was assessed. Ad encoding different Notch1 decoy variants are intravenously injected 3 days after tumor implantation. Tumors are significantly smaller in the Notch1 decoy-treated groups. Tumor weight is measured at the time of harvest. Mean tumor weight ± S.D. * P value < 0.05 (n = 4-5).

Figures 39a - 39c: Notch1 decoys 1-24 and 1-36 similarly reduce growth of Mm5MT-FGF4 (Figure 39a) and KP1-VEGF tumors (Figure 39b). Tumor volume (V) was calculated from length (L) and width (W) (V = 0.5 x L x W), which were measured on a weekly basis. Average tumor volume ± S.D. * P value < 0.05. Notch1 decoys 1-24 and 1-36 significantly reduced tumor vasculature. CD31 immunofluorescence showed decreased vascular content and disrupted structure in tumors from the Notch1

decoy groups. Scale bars: 30 micrometers. Data shown in Figure 39c.

Figures 40a – 40b: Notch1 decoys block xenografted tumor growth. Figure 40a shows that Notch1$^{1-13}$ decoy blocks Dll4/Notch activity and results in increased tumor vasculature whereas Notch1$^{10-24}$ or Notch1$^{1-24}$ decoys reduce tumor vessels in all tumor models. Tumor vasculature is analyzed by endomucin immunofluorescence (green). Figure 40b shows data for the images of Figure 40a quantified for endomucin-positive areas from multiple tumor sections. Mean percentage of endomucin-positive area ± S.D. * P value < 0.003 (n= 4-5). Scale bars: 30 micrometers.

Figures 41a – 41c: Notch1 decoys disrupt tumor angiogenesis, reduce perfusion and induce hypoxia. Tumor sections from mice injected with fluorescein-conjugated lectin are immunostained for endomucin (red), and perfused lectin (green). The amount of endomucin-associated lectin reflects functional tumor vasculature and normal perfusion. Tumor sections from mice intraperitoneally injected with hypoxyprobe are immunostained with an APC-conjugated anti-hypoxyprobe antibody (red) and DAPI (blue), and quantified for tumor hypoxia. Data shown in Figure 41a. Mean percentage of lectin-positive area ± S.D. * P value < 0.006 (n = 4-5) is shown in Figure 41b. Mean percentage of hypoxyprobe-positive area ± S.D. * P value < 0.002, ** P value < 0.05 (n = 4-5) is shown in Figure 41c. Tumors from the decoy-treated mice showed a significantly increased hypoxia and necrosis. Scale bars: 30 micrometers.

Figures 42a – 42c: Notch1 decoys disrupt tumor angiogenesis. Collagen type IV (red) and endomucin (green) immunofluorescence of Mm5MT-FGF4 tumor sections. The presence of collagen type IV correlates with tumor vasculature, suggesting that Notch1 decoys inhibit tumor angiogenesis without causing vessel regression. Images are shown in Figure 42a. Mean Col IV-positive areas and mean endomucin-positive areas ± S.D. are shown in Figures 42b and 42c respectively * P value < 0.002.

Figures 43a – 43d: Notch1 decoys that target JAGGED-1 disrupt mural-

endothelial cell interactions in tumors. Tumor sections are co-immunostained for endomucin (green) and NG2 (red). Images are shown in Figure 43a. Scale bars: 10 micrometers. The percentage of NG2-positive areas is measured as a parameter of pericyte coverage in tumors. Mean percentage of endomucin or NG2-positive areas ± S.D. are shown in Figured 43a and 43b respectively. Figure 43d shows NG2-positive / endomucin-positive area * P value < 0.02 (n = 4-5).

Figure 44: Notch1 decoys that target JAGGED-1 disrupt mural-endothelial cell interactions in tumors. Tumor sections are co-immunostained for endomucin (green) and αSMA (red). Large vessels with vascular smooth muscle cell coverage are normally located at the tumor periphery. Scale bars: 10 micrometers.

Figure 45: Quantitative RT-PCR on HUVECs expressing N1 decoys or JAGGED-1 shRNA for Notch downstream targets: HEY1, HEY2, HEYL, HES1.

Figure 46: Quantitative RT-PCR and flow cytometry on HUVECs expressing Notch1 decoys or JAGGED-1 shRNA for VEGF receptors.

Figures 47a - 47b: Figure 47a shows quantitative RT-PCR on HUVECs expressing Notch1 decoys or for VEGF receptors. Figure 47b shows results of an enzyme-linked immunosorbent assay for soluble VEGFR-1.

Figures 48a - 48b: VEGFR-1 immunofluorescence on tumor sections confirmed that $N1_{10-24}$ decoy and 1-24 increase soluble VEGFR-1 expression. Immunofluorescence images are shown in Figure 48A. Mean VEGFR-1-positive areas ± S.D. are shown in Figure 48b * P value < 0.02. Scale bars: 30 micrometers.

Figure 49: Ligand Specificity of Notch1 decoys for other Dll and Jag family members. Notch signal activation is measured in HeLa cells expressing full length rat Notch1 and 11CSL-Luc co-cultured with HeLa cells expressing Notch ligands. Only $N1_{1-13}$, $N1_{1-24}$, and $N1_{1-36}$ decoys inhibit DLL1-induced Notch signaling, suggesting that EGF-like repeats 1-9 are indispensable for inhibiting DLL1-induced Notch signaling. However, $N1_{1-13}$ decoy does not inhibit JAG2. Only $N1_{10-24}$ decoy is able to

block JAG2, implicating that EGF-like repeats 10-24 of Notch1 confer JAGGED specificity. Mean luciferase fold induction ± S.D. * P value < 0.005.

Detailed Description of the Invention

Terms

As used in this application, except as otherwise expressly provided herein, each of the following terms shall have the meaning set forth below.

"Administering" may be effected or performed using any of the methods known to one skilled in the art. The methods comprise, for example, intralesional, intramuscular, subcutaneous, intravenous, intraperitoneal, liposome-mediated, transmucosal, intestinal, topical, nasal, oral, anal, ocular or otic means of delivery.

"Affixed" shall mean attached by any means. In one embodiment, affixed means attached by a covalent bond. In another embodiment, affixed means attached non-covalently.

"Amino acid," "amino acid residue" and "residue" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide or peptide. The amino acid can be, for example, a naturally occurring amino acid or an analog of a natural amino acid that can function in a manner similar to that of the naturally occurring amino acid.

"C-terminal" and "N-terminal" amino acid, as used herein, refers to an amino acids at or in close proximity to the carboxy or amino terminal ends, respectively, of a given protein, protein domain or amino acid sequence motif such that no amino acid residue essential to the structure, function, or characterization of the protein, protein domain or amino acid sequence motif lie beyond said C-terminal amino acid or N-terminal amino acid.

"Antibody" shall include, without limitation, (a) an immunoglobulin

molecule comprising two heavy chains and two light chains and which recognizes an antigen; (b) a polyclonal or monoclonal immunoglobulin molecule; and (c) a monovalent or divalent fragment thereof. Immunoglobulin molecules may derive from any of the commonly known classes, including but not limited to IgA, secretory IgA, IgG, IgE and IgM. IgG subclasses are well known to those in the art and include, but are not limited to, human IgG1, IgG2, IgG3 and IgG4. Antibodies can be both naturally occurring and non-naturally occurring. Furthermore, antibodies include chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. Antibodies may be human or nonhuman. Nonhuman antibodies may be humanized by recombinant methods to reduce their immunogenicity in humans. Antibody fragments include, without limitation, Fab and Fc fragments. The "Fc portion of an antibody", in one embodiment, is a crystallizable fragment obtained by papain digestion of immunoglobulin that consists of the C-terminal half of two heavy chains linked by disulfide bonds and known as the "effector region" of the immunoglobulin. In another embodiment, "Fc portion of an antibody" means all, or substantially all, of one C-terminal half of a heavy chain.

"Humanized", with respect to an antibody, means an antibody wherein some, most or all of the amino acids outside the CDR region are replaced with corresponding amino acids derived from a human immunoglobulin molecule. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they do not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules include, without limitation, IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. Various publications describe how to make humanized antibodies, e.g., United States Patent Nos. 4,816,567, 5,225,539, 5,585,089 and 5,693,761, and PCT International Publication No. WO 90/07861.

As used herein, the term "composition", as in pharmaceutical composition, is intended to encompass a product comprising the active ingredient(s) and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly from combination, complexation, or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or

from other types of reactions or interactions of one or more of the ingredients.

As used herein, "effective amount" refers to an amount which is capable of treating a subject having a tumor, a disease or a disorder. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated. A person of ordinary skill in the art can perform routine titration experiments to determine such sufficient amount. The effective amount of a compound will vary depending on the subject and upon the particular route of administration used. Based upon the compound, the amount can be delivered continuously, such as by continuous pump, or at periodic intervals (for example, on one or more separate occasions). Desired time intervals of multiple amounts of a particular compound can be determined without undue experimentation by one skilled in the art. In one embodiment, the effective amount is between about 1µg/kg - 10 mg/kg. In another embodiment, the effective amount is between about 10µg/kg - 1 mg/kg. In a further embodiment, the effective amount is 100µg/kg.

"Extracellular domain" as used in connection with Notch receptor protein means all or a portion of Notch which (i) exists extracellularly (i.e. exists neither as a transmembrane portion or an intracellular portion) and (ii) binds to extracellular ligands to which intact Notch receptor protein binds. The extracellular domain of Notch may optionally include a signal peptide ("sp"). "Extracellular domain", "ECD" and "Ectodomain" are synonymous.

"Inhibiting" the onset of a disorder or undesirable biological process shall mean either lessening the likelihood of the disorder's or process' onset, or preventing the onset of the disorder or process entirely. In the preferred embodiment, inhibiting the onset of a disorder or process means preventing its onset entirely.

"Notch", "Notch protein", and "Notch receptor protein" are synonymous. In addition, the terms "Notch-based fusion protein" and "Notch decoy" are synonymous. The following Notch amino acid sequences are known and hereby incorporated by reference: Notch1 (Genbank accession no. S18188

(rat)); Notch2 (Genbank accession no. NP_077334 (rat)); Notch3 (Genbank accession no. Q61982 (mouse)); and Notch4 (Genbank accession no. T09059 (mouse)). The following Notch nucleic acid sequences are known and hereby incorporated by reference: Notch1 (Genbank accession no. XM_342392 (rat) and NM_017617 (human)); Notch2 (Genbank accession no. NM_024358 (rat), M99437 (human and AF308601 (human)); Notch3 (Genbank accession no. NM_008716 (mouse) and XM_009303 (human)); and Notch4 (Genbank accession no. NM_010929 (mouse) and NM_004557 (human)).

The terms "nucleic acid", "polynucleotide" and "nucleic acid sequence" are used interchangeably herein, and each refers to a polymer of deoxyribonucleotides and/or ribonucleotides. The deoxyribonucleotides and ribonucleotides can be naturally occurring or synthetic analogues thereof. "Nucleic acid" shall mean any nucleic acid, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form nucleic acid molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA). Nucleic acids include, without limitation, anti-sense molecules and catalytic nucleic acid molecules such as ribozymes and DNAzymes. Nucleic acids also include nucleic acids coding for peptide analogs, fragments or derivatives which differ from the naturally-occurring forms in terms of the identity of one or more amino acid residues (deletion analogs containing less than all of the specified residues; substitution analogs wherein one or more residues are replaced by one or more residues; and addition analogs, wherein one or more resides are added to a terminal or medial portion of the peptide) which share some or all of the properties of the naturally-occurring forms.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein, and each means a polymer of amino acid residues. The amino acid residues can be naturally occurring or chemical analogues thereof. Polypeptides, peptides and proteins can also include modifications such as glycosylation, lipid attachment, sulfation, hydroxylation, and ADP-ribosylation.

As used herein, "pharmaceutically acceptable carrier" means that the carrier is compatible with the other ingredients of the formulation and is not deleterious to the recipient thereof, and encompasses any of the standard pharmaceutically accepted carriers. Such carriers include, for example, 0.01-0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers can be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions and suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like.

"Subject" shall mean any organism including, without limitation, a mammal such as a mouse, a rat, a dog, a guinea pig, a ferret, a rabbit and a primate. In one embodiment, the subject is a human.

"Treating" means either slowing, stopping or reversing the progression of a disease or disorder. As used herein, "treating" also means the amelioration of symptoms associated with the disease or disorder. Diseases include, but are not limited to, Tumor Angiogenesis, Atherosclerosis, Wound Healing, Retinopathy of Prematurity, Pre-eclampsia, Diabetic retinopathy, Ischemia, Stroke, Cardiovascular Disease, Psoriasis, lymphedema, tumorigenesis and tumor lymphangiogenesis, age-related macular degeneration (AMD), wet AMD, pancreatic cancer and breast cancer.

Angiogenesis is encountered during wound healing processes, the female menstrual cycle and endometrial remodeling, as well as during embryonic development and organ growth. In the pathological setting, angiogenesis plays an important role in different diseases like rheumatoid arthritis, psoriasis, macular degeneration, diabetic retinopathy, and

tumor growth.

There has been considerable evidence *in vivo*, including clinical observations, that abnormal angiogenesis is implicated in a number of disease conditions, which include rheumatoid arthritis, inflammation, cancer, psoriasis, degenerative eye conditions and others.

Other diseases for use of Notch fusion proteins are metabolic disorders such as, but not limited to, Diabetes, Obesity, Prediabetic state, Atherosclerosis, Ischemia, Stroke, Cardiovascular Disease, Regulating expression of Insulin, and Regulating the function of Insulin.

The use of Notch fusion proteins is also indicated for Metabolic Syndrome refers to a combination of medical disorders that increases the risk to a person for cardiovascular disease and diabetes. Other known names referring to such syndrome is syndrome X, insulin resistance syndrome, Reaven's syndrome. Several features of the syndromes include: fasting hyperglycemia, high blood pressure, central obesity (also known as visceral obesity), decreased High Density Lipoprotein (LDL), elevated triglycerides, elevated uric acid levels. Fasting hyperglycemia, listed above, includes diabetes mellitus type 2 or impaired fasting glucose and impaired glucose tolerance or insulin resistance. In addition to metabolic syndrome, the Notch decoy may have indications for pre-diabetic states.

Units, prefixes and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acid sequences are written left to right in 5'to 3'orientation and amino acid sequences are written left to right in amino- to carboxy-terminal orientation. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

The following abbreviations are used herein: ECD: extracellular domain; IC: intracellular domain; NECD/Fc: Notch-based fusion protein; N1: Notch1; N2: Notch2; N3: Notch3; N4: Notch4; Dll: Delta-like; DLL1: Delta-like 1; DLL4: Delta-like 4; JAG: JAGGED; JG: JAGGED; JAGGED-1:

JAGGED 1; JG1: JAGGED 1; EC: endothelial cells; HUVEC: human umbilical vein endothelial cell; m.o.i.: multiplicity of infection; VEGF: vascular endothelial cell growth factor; VEGFR: vascular endothelial cell growth factor receptor; sp: signal peptide; PDGF: platelet derived growth factor; PDGFR: platelet derived growth factor receptor; P1GF: placental growth factor.

Embodiments of the Invention

In one embodiment, the fusion protein is a fusion protein which comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 23.

In another embodiment of the fusion protein of this invention comprises an amino acid sequence identical to the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extending up to the C-terminal amino acid of EGF-like repeat 24.

In another embodiment the sequence of the consecutive amino acids comprises the sequence set forth in SEQ ID NO: 3, commencing with cystine at position 24 and ending with lysine at position 833.

In another embodiment of the fusion protein of this invention comprises an amino acid sequence identical to the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extending up to the C-terminal amino acid of EGF-like repeat 36.

In another embodiment the sequence of the consecutive amino acids comprises the sequence set forth in SEQ ID NO: 5, commencing with

cystine at position 24 and ending with lysine at position 1318.

In another embodiment of any of the fusion proteins of this invention, the Fc portion of the antibody is the Fc portion of a human antibody.

In another embodiment of the fusion proteins of this invention, (a) the extracellular domain of the human Notch1 receptor protein is preceded by a signal peptide. In a further embodiment, the signal peptide is the signal peptide of human Notch 1 protein or the signal peptide of an IgG heavy chain.

In another embodiment of any of the fusion proteins of this invention, the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extends up to the C-terminal amino acid of EGF-like repeat 24. In a further embodiment the fusion protein comprises the sequence of consecutive amino acids set forth in SEQ ID NO: 3.

In another embodiment of any of the fusion proteins of this invention, the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extends up to the C-terminal amino acid of EGF-like repeat 36. In a further embodiment the fusion protein comprises the sequence of consecutive amino acids set forth in SEQ ID NO: 5.

Also provided is a composition comprising any of the fusion protein of this invention and a carrier.

In one embodiment the fusion protein is present in an amount effective to inhibit the activity of JAGGED-1 in a pharmaceutically acceptable carrier.

Also provided is a method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject any of the fusion proteins of this invention in an amount effective to treat the subject's AMD.

In one embodiment the AMD is wet AMD. In another embodiment the AMD is dry AMD.

Also provided is a method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject any of the fusion proteins of this invention in an amount effective to treat the subject's diabetic retinopathy.

In one embodiment of any of the methods of this invention the method further comprises administering an inhibitor of Vascular Endothelial Growth Factor (VEGF). In another embodiment, the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

In one embodiment of any of the methods of this invention the method further comprises administering a VEGF receptor inhibitor. In another embodiment, the VEGF receptor inhibitor is a VEGFR-1 or a VEGFR-2 inhibitor.

Also provided is a method of treating a subject suffering from cancer which comprises administering to the subject any of the fusion proteins of this invention in an amount effective to treat the subject's cancer.

In one embodiment the cancer is pancreatic cancer. In another embodiment the cancer is breast cancer.

Also provided is a method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject a fusion protein in an amount effective to treat the subject's AMD, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1 the C-terminal amino acid is not the C-terminal

amino acid of EGF-like repeat 36.

In one embodiment the AMD is wet AMD. In another embodiment the AMD is dry AMD.

Also provided is a method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject a fusion protein in an amount effective to treat the subject's diabetic retinopathy, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1 the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

In one embodiment of any of the methods of this invention the method further comprises administering an inhibitor of Vascular Endothelial Growth Factor (VEGF). In a further embodiment, the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

In one embodiment of any of the methods of this invention the method further comprises administering a VEGF receptor inhibitor. In a further embodiment, the VEGF receptor inhibitor is a VEGFR-1 or a VEGFR-2 inhibitor.

Also provided is a method of treating a subject suffering from cancer which comprises administering to the subject a fusion protein in an amount effective to treat the subject's cancer, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the

sequence of amino acids present in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1 the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

In one embodiment the cancer is pancreatic cancer. In another embodiment the cancer is breast cancer.

In one embodiment of any of the methods of this invention the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 9 and extends up to the C-terminal amino acid of EGF-like repeat 23.

In another embodiment of any of the methods of this invention the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 9 and extends to the C-terminal amino acid of EGF-like repeat 36.

In another embodiment of any of the methods of this invention the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 1 and extends to the C-terminal amino acid of EGF-like repeat 13.

In another embodiment of any of the methods of this invention the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 1 and extends to the C-terminal amino acid of EGF-like

repeat 24.

In one embodiment of any the fusion proteins of this invention, the amino acid sequence of the extracellular domain of a human Notch1 receptor protein consists of EGF-like repeats 10-24. In a further embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 3.

In one embodiment of any of the fusion proteins of this invention, the amino acid sequence of the extracellular domain of the human Notch1 receptor protein consists of EGF-like repeats 10-36. In a further embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 5.

In one embodiment of any of the fusion proteins of this invention, the amino acid sequence of the extracellular domain of the human Notch1 receptor protein consists of EGF-like repeats 14-24. In a further embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO:7.

In one embodiment of any of the fusion proteins of this invention, the amino acid sequence of the extracellular domain of the human Notch1 receptor protein consists of EGF-like repeats 14-36. In a further embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO:9.

In one embodiment of any of the fusion proteins of this invention, the Fc portion of the antibody is the Fc portion of a human antibody. In another embodiment of any of the fusion proteins of this invention the signal peptide is the signal peptide of human Notch 1 protein or the signal peptide of an IgG heavy chain.

Also provided is a pharmaceutical composition comprising any one of the fusion proteins described herein and a pharmaceutically acceptable carrier.

This invention provides a method of treating age-related macular degeneration (AMD) in a subject comprising administering to the subject

the fusion protein of any one the fusion proteins of this invention in an amount effective to treat the subject, thereby treating AMD in the subject.

In one embodiment, the fusion protein is any one of the fusion proteins of this invention for use in treating age-related macular degeneration in a subject.

This invention also provides a method of treating age-related macular degeneration (AMD) in a subject comprising administering to the subject a JAGGED-1 inhibitor in an amount effective to reduce angiogenesis thereby treating the AMD in the subject. In one embodiment the AMD is wet AMD. In another embodiment the JAGGED inhibitor is a Notch1 fusion protein.

Additional Notch1 fusion proteins are described, for example, in PCT International Application NO. PCT/US2008/010045, filed August 22, 2008, on behalf of The Trustees of Columbia University in the City of New York, the entire contents of which are hereby incorporated by reference into the subject application. In another embodiment, the Notch1 fusion protein is any of the fusion proteins described herein.

This invention also provides for a JAGGED-1 inhibitor for use in treating age-related macular degeneration in a subject. In one embodiment the AMD is wet AMD. In another embodiment the JAGGED inhibitor is a Notch1 fusion protein. In another embodiment, the Notch1 fusion protein is any of the fusion proteins described herein.

In one embodiment of this invention, the method further comprises administering an inhibitor of Vascular Endothelial Growth Factor (VEGF). In a further embodiment, the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

In one embodiment of this invention, the method further comprises administering a VEGF receptor inhibitor. In a further embodiment the VEGF receptor inhibitor is a VEGFR-1 or a VEGFR-2 inhibitor.

This invention provides a method of treating pancreatic cancer in a

subject comprising administering to the subject any one of the fusion proteins described herein in an amount effective to treat the subject, thereby treating the subject having pancreatic cancer.

In one embodiment, the fusion protein is any one of the fusion proteins of this invention for use in treating pancreatic cancer in a subject.

This invention provides a method of delaying or inhibiting tumor growth, wherein the tumor comprises pancreatic cancer cells, which method comprises contacting the tumor with an amount of any one of the fusion proteins described herein effective to delay or inhibit the growth of the tumor.

In one embodiment, the fusion protein is any one of the fusion proteins of this invention for use in inhibiting tumor growth, wherein the tumor comprises pancreatic cancer.

This invention provides a method of treating breast cancer in a subject comprising administering to the subject any one of the fusion proteins of this invention in an amount effective treat the subject, thereby treating the breast cancer in the subject.

In one embodiment, the fusion protein is any one of the fusion proteins of this invention for use in treating breast cancer in a subject.

This invention provides a method of delaying or inhibiting tumor growth, wherein the tumor comprises breast cancer cells, which method comprises contacting the tumor with an amount of any one of the fusion proteins of this invention in an amount effective to delay or inhibit the growth of the tumor.

In one embodiment, the fusion protein is any one of the fusion proteins of this invention for use in inhibiting tumor growth, wherein the tumor comprises breast cancer cells.

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit

in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series of Experiments

Introduction

Notch signaling requires cell-cell contact that allows Notch receptors and ligands to interact. The main part of the Notch extracellular domain comprises up to 36 EGF-like repeats which contain a Ca^{2+}-binding consensus sequence. Notch ligands also contain EGF-like repeats in their extracellular regions, but they can be distinguished by the presence or absence of the cysteine-rich domain. The JAGGED ligand family contains 16 EGF-like repeats and the cysteine-rich domain whereas the Delta-like ligand family contains 8 or fewer EGF-like repeats. Several lines of evidence showed that the DSL region conferred specificity to Notch binding and the C-terminal EGF-like repeats helped facilitate binding (Shimizu et al.; Glittenberg et al.; Henderson et al.). Here, it is shown that EGF-like repeats 11-13 are necessary for Notch/Dll1 and Notch/JAGGED-1 interactions (Cordle et al.; Hambleton et al.).

A novel soluble construct was created based on the 36-EGF-like repeats of the rat Notch1 extracellular domain fused with the human IgG Fc (Notch1 decoy) as a Notch inhibitor (Funahashi et al.). It has been shown that Notch1 decoy inhibited Notch activity stimulated by Notch ligands JAGGED-1, Dll1, and Dll4. This finding implicates that EGF-like repeats of Notch are sufficient to effectively interact with Notch ligands and, therefore, to prevent the Notch receptor from being activated by its ligands. It was investigated whether Notch-ligand specificity was determined by the extracellular EGF-like repeats. In addition, it has been well established that Notch is one of the targets of anti-angiogenic therapies, so we created the new Notch1 decoy construct based on human NOTCH1 for therapeutic purpose. Rat Notch1 and human NOTCH1 protein sequences are 96% homologous (92.6% identical), and it has been shown that the rat and human Notch1 decoys

have undistinguishable activity in Notch signaling and functional assays. Here, the human Notch1 decoy is used, which will simply be referred to as Notch1 decoy, is used in *in vitro* studies and *in vivo* and tumor experiments in the next chapter.

Despite extensive genetic and cellular studies of the Notch pathway, molecular characterization of Notch-ligand interactions still remains elusive. In order to increase the understanding of the molecular basis of Notch-ligand recognition, truncated fragments of Notch1 decoy including EGF-like repeats 1-13 and 1-24 was also created. Because EGF-like repeats 11-13 are implicated in ligand binding, it was hypothesized that 11-13 would be the shortest form of Notch that still retained ligand-binding activity. The molecular weight of Notch1 decoy 1-36 is approximately 180 kD or over 250 kD in its glycosylated, secreted form, so it was explored whether it could be modified or shortened so that it was produced and secreted at a higher level for therapeutic purpose. These new decoy variants were utilized to assess their inhibitory effects as well as their ligand specificity. The results set forth herein demonstrate that Notch1 decoy 1-13 acts as a Dll4-specific inhibitor, and Notch1 decoy 1-24 as a pan-Notch inhibitor.

A JAGGED-1-specific inhibitor also was successfully constructed. A second generation of the Notch1 decoys, including 10-24, 10-36, 14-24, and 14-36 was constructed and tested. Only Notch1 decoy 10-24 has proven to be JAGGED-1-specific and exhibit a distinct activity in endothelial cell-based assays, retinal angiogenesis, and gene profiling analysis. Based on their diverse activities, the tumor studies were performed with the decoys 1-13, 10-24, and 1-24 as possible anti-tumor and anti-angiogenic agents.

Materials and Methods

Generation of Notch1 decoy variants

A schematic of full length Notch 1 decoy 1-36 and Notch decoy 1-24 are shown in Figure 1(a). Four truncated Notch 1 decoy variants derived from PCR mutagenesis of the Notch1 decoy 1-36 and 1-24 constructs were

generated. These were Notch 10-36 decoy, Notch 14-36 decoy, Notch 10-24 decoy and Notch 14-24 decoy. Schematic representations of these decoys are set forth in Figure 1b. The amino acid sequence of human Notch 1 is set forth in SEQ ID NO:1. The nucleic acid sequence of human Notch1 decoy 10-24 is set forth in SEQ ID NO:2 and the amino acid sequence is set forth in SEQ ID NO:3. The nucleic acid sequence of human Notch1 decoy 10-36 is set forth in SEQ ID NO:4 and the amino acid sequence is set forth in SEQ ID NO:5. The nucleic acid sequence of human Notch1 decoy 14-24 is set forth in SEQ ID NO:6 and the amino acid sequence is set forth in SEQ ID NO:7. The nucleic acid sequence of the human Notch1 decoy 14-26 is set forth in SEQ ID NO:8 and the amino acid sequence is set forth in SEQ ID NO:9.

Expression and Secretion of Truncated Notch1 decoy variants in 293T cells.

293T cells were transfected with pCCL-based Notch1 decoy plasmids by calcium phosphate transfection. Total cell lysates were collected 2 days after transfections, and Western blotting was performed using the rabbit anti-human Fc antibody. The molecular weight of Notch1 decoys 1-13, 1-24 and 1-36 decoys are 83 kD, 127 kD, and 178 kD respectively. See Figure 2a. The molecular weights of Notch1 decoys 10-36, 14-36, 10-24 and 14-24 are 140Kd, 128 kD, 91kD and 73 kD, respectively. See Figure 2b.

Notch Reporter Assay

To assess effects of the Notch decoys on Notch signaling a Notch reporter construct containing multiple CSL-binding sites linked to the luciferase gene (11CSL-Luc) was utilized. Activation of Notch signaling was measured in HeLa cells expressing Notch1 and 11CSL-Luc co-cultured with HeLa cells expressing Notch ligands. All Notch decoys inhibited DLL4-induced Notch signaling. However, Notch 1-13 did not inhibit JAGGED-1. Average luciferase fold induction ± S.D. *P value <0.002. See Figure 3. EGF-like repeats 1-9 were shown to be indispensable for inhibiting DLL4-induced Notch signaling (See Figure 4a). Only Notch 1 decoy 10-24 was able to block JAGGED-1, implicating that EGF-like repeats 10-24 may harbor JAGGED-1 specificity. See

Figure 4b.

Co-Transfection of Notch1 decoys with Soluble Ligands

Notch1 decoys were co-transfected with soluble ligands (see Figure 5a) or full length ligands (see Figure 5b) in 293T cells. 293T cells were co-transfected with pcDNA3.1-decoy and either pCRIII-DLL4-FLAG or pCRIII-JAGGED-1-FLAG or the empty vector. DSG, a crosslinking agent, was also added to stabilize the interaction of the decoy and the ligand as a protein complex. Then, cell lysates were collected and pulled down by Protein A/G agarose. The pulldown complex was then immunoblotted by an anti-FLAG antibody. Notch1 decoy 1-13 interacts with DLL4 and Notch1 decoy 10-24 interacts with JAGGED-1.

Co-Immunoprecipitation Assays

Co-immunoprecipitation was performed with Notch1 decoys and full-length Notch1 receptor. Cell lysates were pulled down by Protein A/G agarose and blotted with an anti-Fc or anti-Notch1 antibody. Notch1 decoys do not interact with Notch1 receptor. See Figure 6.

Retinal Angiogenesis

50µl of 5.0 x 10^9 ffu/ml Adenoviruses expressing different decoys (1-13, 10-24, 1-24, and 1-36) or 50 µl of 2mg/ml DAPT were subcutaneously injected into P2 neonatal pupils. Retinas were collected at P5 and fixed and immunostained with isolectin B4 for the retinal vasculature. Expression and secretion of the decoys were confirmed by human Fc western blotting of the blood serum. Results are shown in Figure 7a-7d. Notch1 decoys 1-13 and 10-24 displayed opposite effects on retinal angiogenesis.

Gene Expression Profiling

i. Primary Cells and Cancer Cell Lines

Cell cultures were maintained at 37°C in 5% CO2 and 95% humidified air. HUVECs were grown in EGM-2 Media (Lonza Group, Walkersville, MD).

Mm5MT, LLC, and B16-F10 were from the American Type Culture Collection (ATCC, Manassas, VA). Cancer cell lines were maintained in 1X High Glucose DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and Pen-Strep.

ii. HUVECs expressing Notch1 decoys

RNA was harvested from lentivirally transduced HUVECs for reverse transcription and quantitative RT-PCR.

Quantitative RT-PCR for mRNA transcripts of the Notch receptors, Notch 1-Notch4, was performed. Results are shown in Figures 8a-8d. Average relative value ± S.D. *P value <0.03.

Quantitative RT-PCR for mRNA transcripts of HEY1 and HEY2 was performed. All Notch1 decoys downregulated HEY1 but only 1-13 and 1-24 downregulated HEY2. Results are shown in Figures 9a and 9b. Average relative value ± S.D. *P value <0.03.

Quantitative RT-PCT for mRNA transcripts of HEYL and HES1 was performed. Downstream targets of Notch signaling, HEYL and HES1, were also downregulated by expression of Notch1 decoys in HUVECs. Results are shown in Figure 10a and 10b. Average relative value ± S.D. *P value <0.03.

Quantitative RT-PCR for mRNA transcripts of DLL4, JAGGED-1, VEGFR-1, VEGFR-2 and VEGFR-3 was performed. Notch1 decoys 1-13 and 10-24 had different effects of VEGFR-1 transcripts. Results are shown in Figures 11a – 11e. Average relative value ± S.D. *P value <0.03.

iii. Gene Expression Profiling of Cancer Cell Lines

Four different cell lines: mouse mammary tumor cells (Mm5MT), human pancreatic cancer cells (KP1), mouse Lewis lung carcinoma cells (LLC), and mouse melanoma cells (B16-F10) were utilized. RNA was isolated from cultured tumor cells and reversely transcribed. PCR was done to explore expressions of all Notch receptors and ligands in these cell lines. The PCR results are shown in Figure 14.

Cell Proliferation and Apoptosis Cell Assays

Tumor cells were lentivirally transduced with different Notch1 decoy variants and assessed for cell proliferation and apoptosis. Apoptosis assay was performed using FITC-conjugated Annexin V antibody. The percentage of apoptotic cells is indicated in the upper right quadrant of Figures 15c and 15d.

Notch1 detection by Western Blotting and Immunostaining

Adenoviruses expressing Notch1 decoys 1-13, 10-24 and 1-24, or Fc as control, were injected intravenously into adult mice. Western blot analysis was performed, see Figure 16a. Tumors were harvested and the decoy levels in tumor sections were assessed by immunofluorescence see Figure 16b. The results are set forth in Figures 16a and 16b.

Tumor Growth Experiments

Mm5MT-FGF4 and KP1-VEGF tumor cells were first lentivirally transduced to express Luciferase to monitor tumor growth with luciferase activity or luminescence signals. Experiments were performed in 2 ways: first, by introducing different Notch1 decoys or Fc directly into tumor cells by lentiviral transduction; second, by using the adenoviruses. Hypoxyprobe™, a marker for hypoxia in tissues, and FITC-conjugated lectin were injected into mice before tumor harvesting in order to analyze tumor hypoxia and vessel perfusion. Tumor growth was monitored by assessing the total radiance from luminescence signals using the Xenogen IVIS Imaging System. Average total flux ± S.D. *P value <0.05 (n = 4-5). Results are set forth in Figures 17a-17c and 18a-18c.

Tumor Vasculature Characterization

Tumor sections were immunostained for Endomucin-positive areas (green) and Dll4 (red). Quantification of tumor vasculature was based on Endomucin-positive areas in tumor sections. Average Endomucin-positive area ± S.D. *P value <0.003 (n=4-5). Scale bars: 30 micrometers. Results are set forth in Figures 19a and 19b and Figures 20a and 20b.

Fluorescein-conjugated lectin (100µg) was injected into mice 2 minutes before tumor harvesting. Tumor sections were immunostained for Endomucin (red) and perfused lectin (green) was associated with tumor vessels. The amount of vessel-associated lectin reflected function tumor vasculature. Average lectin-positive area ± S.D. *P value <0.006 (n=4-5). Scale bars: 30 micrometers. Results are set forth in Figures 21a and 21b.

NG2 and Endomucin immunofluorescence on Mm5MT and KP1 tumor sections.

Tumor sections were co-immunostained for Endomucin (green) and NG2 (red). The percentage of NG2-positive areas was measured as a parameter of pericyte recruitment in tumors. Average NG2-positive area ± S.D. *P value <0.02 (n=4-5). Scale bars: 10 micrometers. Results set forth in Figures 22a and 22b.

Tumor invasion and metastasis

Subcutaneous LLC and B16-F10 tumors expressing Luciferase were used to assess the Notch1 decoy activities in tumor invasion and metastasis. Photographs were taken at day 12 of tumor-bearing mice with luminescence signals from different decoy groups (1-13, 10-24 and 1-24). Tumor growth was monitored and quantified based on the total radiance. Average total flux ± S.D. (n=5). Tumor weight was measured at day 12 before tumor harvesting. Average tumor weight ± S.D. *P value <0.05 (n=5). Results are set forth in Figures 23a-23c and 24a-24c.

Lungs and livers from tumor-bearing mice were harvested at day 12, incubated in 30 mg/ml D-Luciferin, and analyzed by the Xenogen IVIS Imaging System. Mice from each group began to develop lung metastasis at day 12 for the LLC model and tumor burden was not significantly different between groups. There was no liver metastasis found in either group. Average total flux ± S.D. (n=5). The results are shown in Figures 25a and 25b.

Lungs and livers from tumor-bearing mice were harvested at day 12,

incubated in 30 mg/ml D-Luciferin, and analyzed by the Xenogen IVIS Imaging System. Imaging of the organs displayed metastatic foci from the Fc group in the lungs (Figures 26a and 26b) and the liver (Figure 26c). There was no metastasis found in decoy treated groups. B16-F10 tumor-bearing mice showed a delay in developing lung and liver metastasis in decoy treated groups.

Results

Construction of JAGGED-1-specific Notch1 decoy

As shown by several *in vitro* functional assays, Notch1 decoy variants exhibited differential inhibitory activities, depending on Notch ligands. Notch1 decoy 1-13 was shown to inhibit only DLL4-induced Notch signaling, and its inhibitory effects in functional assays were very similar to those of other DLL4-neutralizing agents, including soluble DLL4-Fc and anti-DLL4 antibodies. Based on the original constructs, EGF-like repeats 1-24 were required for both DLL4- and JAGGED-1-induced Notch activation. And, EGF-like repeats 1-13 blocked only DLL4 but not JAGGED-1. Therefore, it was hypothesized that EGF-like repeats 14-24 and 14-36 may exhibit JAGGED-1 specificity. Nevertheless, several lines of evidence suggest that EGF-like repeats 11-13 are necessary for NOTCH interaction with JAGGED-1. Thus, Notch1 decoy variants 10-24 and 10-36 were created as possible JAGGED-1-specific agents. PCR mutagenesis was performed on either Notch1 decoy 1-24 or 1-36 plasmids to delete the first 9 or 13 EGF-like repeats. The new constructs were confirmed by expression and secretion in 293T cells as shown in Figure 2. Their expression levels were similar to those of the previous decoy variants in that larger variants were expressed and secreted at a lower level than smaller ones.

Notch1 decoy variants lacking EGF-like repeats 1-9 or 1-13 were unable to block DLL4, but Notch1 decoy 10-24 significantly inhibited JAGGED-1-induced Notch signaling

Notch1 decoy 1-13 functions as a DLL4-specific inhibitor. After the construction of the new Notch1 decoys had been accomplished, co-culture signaling assay were utilized to test the inhibitory effects and ligand

specificity of the Notch decoys. All of the 2nd generation Notch1 decoys did not inhibit DLL4-induced Notch signaling (Figures 3a-c). This suggested that EGF-like repeats 1-9 were required for receptor interaction with DLL4. However, Notch1 decoy 10-24 significantly blocked JAGGED-1-induced Notch signaling while the other decoys did not have any effect (Figures 4a-b). Notch1 decoy 10-36 also exhibited minor JAGGED-1 inhibition, but its inhibitory effect was not consistent or significant. This finding is of particular interest because EGF-like repeats 11-13 have been shown to interact with both Delta-like and JAGGED ligands, but the cell-based signaling assay set forth herein demonstrated that the absence of EGF-like repeats 1-9 seemed to shift Notch receptor affinity toward JAGGED-1.

Notch1 decoys bind to Notch ligands with specificity

Notch1 decoys have been proved to be Notch inhibitors, and it was hypothesized that based on the nature of the receptor decoy itself, that they blocked Notch signaling by competitively interacting with Notch ligands and thereby preventing Notch receptors from being activated. To further explore the mechanism of inhibition, co-immunoprecipitation of the Notch1 decoys and full-length Notch ligands, DLL4 and JAGGED-1, was performed. As expected, Notch1 decoy 1-13 was shown to interact with only DLL4 but not JAGGED-1 while Notch1 decoy 10-24 interacted with JAGGED-1 only (Figures 5a and 5b). Notch1 decoy 1-24 interacted with both DLL4 and JAGGED-1, which supported the previous functional assays.

Although oligomerization of Notch ectodomains is presently unknown, it was investigated whether Notch1 decoys can interact with Notch receptors and block Notch activity. Co-immunoprecipitation of Notch decoys and full-length rat Notch1 was performed in 293T cells and the results are shown in Figure 6. No Notch1 was detected from the Fc pulldown, suggesting that Notch1 decoys were likely to inhibit Notch signaling by competing with the ligands and not interacting with the receptors.

Notch1 decoys are anti-angiogenic in retinal angiogenesis

The early postnatal mouse retina has been an extensively studied angiogenesis model. It develops a vascular pattern in a well-defined series of events including vascular sprouting at the periphery and pruning and remodeling at the center. Therefore, retinal angiogenesis was utilized as a model to further explore the effects of our Notch1 decoys. It has been demonstrated that DLL4 blockade or DLL4 deletion increased angiogenic sprouting (Lobov et al.) while endothelial cell-specific JAGGED-1 deficiency resulted in reduced angiogenic sprouting (Benedito et al.). One hallmark of retinal angiogenesis is the emergence of filopodia-extending endothelial tip cells at the vascular front. Notch decoy 1-13 phenocopied DLL4 deficiency in that the retinal vasculature showed a significant increase in the number of tip cells and angiogenic sprouting. However, Notch decoy 10-24 resulted in reduced angiogenesis similar to the loss of JAGGED-1 in endothelial cells. The difference in the retinal vasculature between the two decoys was strikingly dramatic and clearly indicative of differential inhibition of Notch ligands. Notch1 decoys 1-24 and 1-36 and the GSI, DAPT, caused increased but severely disrupted sprouting angiogenesis (Figure 7a).

Gene profiling of HUVECs expressing Notch1 decoy variants demonstrates they block Notch signaling

Next, the effects of Notch1 decoys on Notch signaling and its downstream targets were explored (See Figure 8-11). The transcript level of NOTCH1 was significantly reduced with expressions of the decoys (99%, 97%, and 97% respectively), indicating that NOTCH1 was autoregulated by the level of Notch activity. Interestingly, other NOTCH transcript levels were not affected by these decoys. NOTCH2 and NOTCH3 are not normally expressed in endothelial cells although they can sometimes be detected in cultured HUVECs. Expressions of Notch ligands, JAGGED-1 and DLL4, did not alter with Notch1 decoy expressions. In addition, the direct downstream targets of Notch signaling were also explored to validate the results from the *in vitro* assays. Most targets, including HEY1, HEYL, and HES1, were significantly decreased with expression of all decoys, indicating that

Notch1 decoys effectively blocked Notch activity. Unlike other decoys, Notch1 decoy 10-24, however, did not reduce the expression level of HEY2. This result may suggest differential regulation of Notch activity through different HEYs and HESs by Notch ligands. The mechanisms of JAGGED-1 and DLL4 (or JAG and DLL ligands in general) regulation of Notch signaling have not been completely understood, and these expression profiling data might give us clues to what downstream targets are important in ligand-specific Notch signaling. The Notch pathway has been well established to regulate VEGF signaling. Thus, it was further explored whether Notch1 decoys had any effect on expressions of VEGF receptors. All Notch1 decoy variants increased VEGFR-2 expression in HUVECs as opposed to N1IC. This finding supported the observation that inhibition of Notch activity with Notch1 decoys increased HUVEC proliferation and migration, which likely resulted from the increase in VEGFR-2 expression and activity. Furthermore, inhibition of Notch seemed to significantly decrease VEGFR-3 expression. Notch1 decoys 1-13 and 1-24 decreased VEGFR-1 expression; however, Notch1 decoy 10-24 increased its expression, similar to that in HUVECs expressing N1IC. This result was unexpected because Notch1 decoy 10-24 showed significant Notch inhibitory activity. Gene expression profiling of VEGF receptors was validated by flow cytometry. Cell surface expression of VEGFR-2 was increased in HUVECs expressing the decoys, and VEGFR-3 was reduced as shown in the histograms in Figure 12. However, VEGFR-1 surface expression was not dramatically shifted as the qRT-PCR data suggested. VEGFR-1 is known to exist in multiple isoforms: transmembrane receptor and soluble proteins. These isoforms are derived from different mRNA splice variants. The data from qRT-PCR and flow cytometry suggested that increased VEGFR-1 expression was attributable to the soluble isoform transcript, but not the surface receptor. Then, the expression analysis was repeated, utilizing the PCR primers specific to the splice variant of the soluble isoform. As shown in Figure 13, the soluble VEGFR-1 transcript was 14-fold increased by Notch1 decoy 10-24 but not significantly affected by Notch1 decoys 1-13 and 1-24. This finding implicated that JAGGED-1 inhibition led to an upregulation of soluble VEGFR-1 but not full-length receptor, and that DLL4 inhibition decreased full-length VEGFR-1 and had no effect on soluble VEGFR-1.

Gene expression profiling of cancer cell lines to define Notch/Notch ligands expressed

To explore the effects of Notch1 decoys on tumor angiogenesis, we utilized 4 different cell lines: mouse mammary tumor cells (Mm5MT), human pancreatic cancer cells (KP1), mouse Lewis lung carcinoma cells (LLC), and mouse melanoma cells (B16-F10). Mm5MT and KP1 cell lines do not metastasize with subcutaneous implantation, but LLC and B16-F10 metastasize to the lungs and the liver. Therefore, these tumor models enabled us to investigate not only the decoy effects on tumor growth and tumor angiogenesis but also tumor cell invasion and metastasis. The results are shown in Figure 14. Mm5MT and LLC similarly expressed high levels of Notch1, Notch2, and Dll1 and low levels of Notch3, Notch4, and JAGGED-1. KP1 cells expressed NOTCH1, NOTCH2, NOTCH3, JAGGED-1, and DLL4. And, B16-F10 cells expressed Notch2, Notch3, Notch4, and only one ligand JAGGED-1.

Notch1 decoys did not have any effect on tumor cell proliferation and apoptosis

Prior to utilizing these tumor cells for tumor experiments in mice, we tested whether our Notch1 decoys would have any effect on tumor cell growth in culture. First, all Mm5MT and KP1 tumor cells were lentivirally transduced with different Notch1 decoy variants, 1-13, 10-24, and 1-24, and performed proliferation and apoptosis assays. Cell proliferation was observed over a 4-day period, and there was no significant difference in the number of cells at the end of the experiment (Figure 15). For apoptosis, cultured cells were resuspended, incubated with the FITC-conjugated AnnexinV antibody, and analyzed by flow cytometry. No significant difference in the number of AnnexinV positive cells was observed.

Notch decoys were secreted into the blood circulation and detected in tumors when expressed via an adenovirus vectors

To mimic systemic delivery of the decoys, an adenovirus delivery approach was utilized. Adenoviruses expressing Notch1 decoys or Fc as control, were injected intravenously into adult mice. The injected

adenoviruses infected hepatocytes and produced high serum levels of the encoded Notch1 decoys which could be detected by Western blots (See Figures 16a and b). After the tumors were harvested, the decoy levels in tumor sections were also assessed by immunofluorescence which showed that all decoy variants reached the tumors.

All Notch1 decoys 1-13, 10-24, and 1-24 significantly reduced tumor growth and showed differential effects on tumor angiogenesis

Since Notch1 decoys 1-24 and 1-36 inhibited tumor growth and tumor angiogenesis in a similar fashion, subsequent tumor experiments with only the 1-24 variant alongside the ligand-specific decoys was performed. DLL4 blockade has been extensively shown to reduce tumor growth by increasing non-functional tumor vasculature (Noguera-Troise et al.; Ridgway et al.; Hoey et al.). Therefore, it was expected that the Dll4-specific decoy 1-13 behaved the same way. Mm5MT-FGF4 and KP1-VEGF tumor cells were first lentivirally transduced to express Luciferase so that tumor growth can be monitored for luciferase activity or luminescence signals. The tumor experiments were performed in 2 ways: first, by introducing different Notch1 decoys or Fc directly into tumor cells by lentiviral transduction; second, by using the adenoviruses. In addition, Hypoxyprobe™, a marker for hypoxia in tissues, and FITC-conjugated lectin were injected into mice before tumor harvesting in order to analyze tumor hypoxia and vessel perfusion. The presence of different decoys resulted in a significant decrease in tumor growth. The 1-13, 10-24, and 1-36 Mm5MT tumors were smaller in weight than the control by 69%, 52%, and 39% (Figure 17a-17c), and the KP1 tumors were smaller by 40%, 49%, and 57% respectively (Figure 18a-18c). The effects of the decoys were seen after the tumor began to grow rapidly which usually took about one week after implantation. Tumor growth from the decoy groups appeared to be delayed or even reversed, as seen in the KP1 tumors. Since the tumor data were collected from luminescence signals which represented luciferase activity in live cells, we predicted that a decrease in tumor size toward the end of the experiment may have resulted from tumor cell death and necrosis.

Notch1 decoy 1-13 led to an increase in non-functional tumor vasculature

Tumor vasculature was analyzed by Endomucin immunofluorescence and lectin perfusion. As shown in Figures 19a & 19b and 20a & 20b, tumors with Notch1 decoy 1-13 showed a marked increase in vessel density in both Mm5MT and KP1 models. The vasculature in 1-13 tumors was significantly more highly branched and had more extensive endothelial networks than the control tumors. Dll4 immunofluorescence also showed an increase in Dll4-positive endothelial cells which supported hypersprouting networks of Endomucin-positive cells. In contrast, the tumors from the 10-24 and 1-24-treated mice distinctly showed a decrease in vascular content by Endomucin immunostaining. These morphologic changes were also reflected by quantification of vascular areas from Endomucin immunofluorescence. Additionally, histologic assessment showed more extensive tumor hypoxia across all the experimental groups which suggested that increased tumor vasculature in the decoy 1-13 group may not be properly functional. The distribution of vessel perfusion was compared by intravascular lectin, which was quantified for lectin-positive area, and endothelial Endomucin immunofluorescence. As shown in Figures 21a and 21b, the vasculature from all decoy-treated groups showed poor vessel perfusion, decreased by 72%, 90%, and 84% respectively. Some large tumor vessels were normally perfused, but most small branching vessels did not contain fluorescent lectin. Therefore, the tumor hypoxia and lectin perfusion data suggested that Notch1 decoy 1-13 inhibited Notch/Dll4 signaling pathway and led to increased non-functional vascular network.

Tumor vessel dilation and disrupted morphology indicated distinct activities of Notch1 decoy 10-24

Unlike increased vascular network in the 1-13 tumors, tumor vasculature in the 10-24 tumors showed a significant decrease in endothelial cell content and disrupted vessel structure. Most tumor vessels appeared large and dilated. Notch1 decoy 10-24 has been shown in vitro to act as a JAGGED-1-specific inhibitor, thus it was predicted that these morphological changes in tumor vasculature may result from JAGGED-1 inhibition. Notch3 is the predominant Notch receptor for arterial

identity and vascular smooth muscle cell maturation, and its expression and activity require endothelial-expressed JAGGED-1 (Domenga et al.; Liu, Kennard, and Lilly). Inhibition of JAGGED-1-mediated Notch signaling, therefore, may result in disrupted mural cell coverage and abnormal tumor vessel maturation.

JAGGED-1 blockade by Notch decoys 10-24 and 1-24 resulted in dysregulated endothelial-pericyte interactions

Because the endothelial cell content in tumors was significantly affected in the decoy-treated group, it was further explored whether perivascular components were also changed, especially pericytes and macrophages. Figure 22 shows NG2 and Endomucin immunofluorescence on Mm5MT and KP1 tumor sections. There was a significant increase in NG2-positive pericyte content in sections from the 1-13 group. Pericytes were found around tumor vessels, and their interactions with tumor endothelium appeared normal and similar to the control tumor sections. NG2-positive pericytes were rarely seen as free components without Endomucin-positive vessels. However, NG2 immunofluorescence on the 10-24 and 1-24 tumor sections showed a significant increase in the number of pericytes not associated with tumor vessels. NG2 and Endomucin signals appeared to be diminished and physically disrupted. Individual pericytes were erratically detached from tumor vessels, implicating loss of normal vascular structure. These results suggest that JAGGED-1-mediated Notch activation was required for regulation and maintenance of endothelial-pericyte interactions and functions, and that deregulation of these interactions led to vessel instability and defective vessel perfusion.

Notch1 decoys did not affect LLC tumor metastasis but delayed formation of B16-F10 micrometastases in the lungs and the liver

Subcutaneous LLC and B16-F10 tumors metastasize to the lungs and the liver in mice. Therefore, we utilized these two additional tumor models to assess the decoy activities in tumor invasion and metastasis, using the tumor lines expressing Luciferase. Figure 23 and 24 show that all Notch1 decoys significantly inhibited growth of both LLC and B16-F10 tumors by 40%, 37%, 32% and 58%, 78%, 71% in weight (LLC and

B16-F10; 1-13, 10-24, and 1-24 respectively). The lungs and livers from the tumor-bearing mice were harvested and analyzed (Table 1 and 2). Analysis of luminescence signals from the lungs and livers reliably detected metastatic foci (Figure 25 and 26). Total metastatic burden was quantified from the total photon radiance of the entire organ whereas the number and size of metastatic foci were also assessed from individual signals. For LLC, the total metastasis burden was not statistically different between the decoy groups, and neither was the number of micrometastases. There was no liver metastasis at this time point. Interestingly, the B16-F10 tumor data showed that some of the mice from the control group had lung and liver metastases, while the decoy groups had no metastasis. Although the tumor metastasis experiments may require a different experimental design to extend the length of tumor growth, these data suggest that Notch1 decoys might delay tumor metastasis.

Table 1. The number of lung metastases in LLC tumor-bearing mice

Group		Number of Lung Metastases
Fc	1	1
	2	5
	3	2
	4	0
	5	3
1-13	1	3
	2	3
	3	1
	4	3
	5	1
10-24	1	0
	2	0
	3	5
	4	0
	5	4
1-24	1	1
	2	2
	3	1
	4	0
	5	0

Table 2. The number of lung and liver metastases in B16-F10 tumor-bearing mice

Group		Number of Lung Metastases	Number of Liver Metastases
Fc	1	3	3
	2	5	0
	3	0	0
	4	0	0
	5	0	0
1-13	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
10-24	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
1-24	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0

Notch1 decoys induced mild goblet cell hyperplasia

An obstacle to the therapeutic application of pan-Notch inhibitors has been gut toxicity by goblet cell hyperplasia. It has been demonstrated that such toxicity required inhibition of both Notch1 and Notch2 receptors as seen following GSI treatment (Wu et al.). In Figure 27, it was found that Notch1 decoys slightly induced gut toxicity in nude mice. However, this effect was drastically mild relative to GSI treatment. The structure of intestinal crypts remained unchanged, and there was no significant change in the weight of the mice with decoy treatment. Therefore, individual Notch1 decoy proved to be effective in blocking Notch signaling and significantly reducing goblet cell hyperplasia in mice.

Discussion

The primary findings of the *in vivo* study are (1) Notch1 decoys do not affect tumor cell proliferation and apoptosis. (2) Notch1 decoys significantly inhibit tumor growth by disrupting tumor angiogenesis and perivascular components. (3) Tumor cell invasion and metastasis appear to be delayed by Notch1 decoy treatment. (4) Unlike GSIs, all Notch1 decoys cause mild gut toxicity in mice. This study is the first evidence to show that Notch1 decoy variants, as ligand-specific (1-13 and 10-24) or pan-Notch (1-24) inhibitors, have differential effects on tumor angiogenesis but all block tumor growth and tumor metastasis.

Differences between Notch1 decoy 1-36 and the small decoy variants

Previous findings showed that Notch1 decoy 1-36 inhibited Notch1 signaling induced by ligands JAGGED-1, Dll1, and Dll4 (Funahashi et al.). Mm5MT and human neuroblastoma tumor (NGP) studies proved that Notch1 decoy 1-36 disrupted tumor vessels and viability. These angiogenic effects are distinct from those previously reported for Dll4 blockade in tumors, particularly the lack of endothelial hypersprouting. Therefore, it is clear that Notch1 decoy 1-36 activity is unique and likely reflecting inhibition of multiple Notch-ligand interactions as opposed to those observed in Dll4 blockade. These data also suggest that a vascular network is regulated by different Notch

receptors and ligands which may play distinct roles in different angiogenic processes or in different cell types.

Based on the construct of the original Notch1 decoy, the EGF-like repeats in the extracellular domain of Notch1 proved to be sufficient in inhibit Notch activation by binding to the ligands. It has been shown that EGF-like repeats 11 and 12 mediate interactions with Delta and, to a lesser degree, Serrate (Rebay et al.). Notch1 decoy constructs were modified to be Dll4-specific or JAGGED-1-specific. Since the role of Notch/Dll4 signaling has been well established in both developmental and tumor angiogenesis, Dll4 blockade effectively served as a control for us to assess the effects of Notch1 decoy variants. In addition, it has been shown that different Notch ligands were upregulated in different types of tumors. For example, JAGGED-1 and Dll1 were induced by FGF4 in Mm5MT cells whereas only JAGGED-1 was expressed in B16-F10 mouse melanoma cells. Notch activity in the endothelium in these tumors is, therefore, likely to be induced by different sets of Notch ligands. It is conceivable that ligand-specific Notch1 decoys may show different effects in different types of tumors. Ultimately, ligand-specific decoys would allow us to understand different roles of Notch ligands in the angiogenesis process in tumors in order to better design therapeutic agents for cancer treatment.

Bioavailability of the Notch1 decoys

The smaller decoy variants, 1-13, 10-24, and 1-24, were shown to be produced and secreted at a higher level than 1-36. Immunohistochemistry on tumor sections also suggested that Notch1 decoy 1-36 was restricted to the tumor vasculature as opposed to the smaller decoys which highly permeated into tumor cells. Differential effects of the decoys in inhibiting tumor angiogenesis may partly be attributable to their bioavailability. Since tumor vessel regression and overproduction often comes with non-functional vessels with poor perfusion, the smaller decoys may have some advantages over the larger variants in that they can easily diffuse and better access the tumor even when tumor vasculature has been compromised.

Anti-angiogenic and anti-tumor activity of the Notch1 decoys

Both JAGGED-1-specific and Dll4-specific decoys similarly reduced tumor growth and disrupted tumor vasculature in all of our tumor models. These data suggest that perturbation of Notch signaling can introduce a significant effect on tumor endothelium and maybe tumor cells themselves. However, it is likely that these effects result from different mechanisms. It is well established that blocking Dll4/Notch signaling leads to an increase in non-functional angiogenesis and poor vessel perfusion. Notch1 decoy 1-13 possesses similar activity and gives similar effects to Dll4 blockade. However, JAGGED-1 inhibition reduced tumor angiogenesis. It has been shown that Notch regulates a wide range of signaling molecules that promote endothelial-pericyte interactions (Armulik, Abramsson, and Betsholtz). Therefore, one possible mechanism is that JAGGED-1 blockade through decoys 10-24 and 1-24 disrupted pericyte coverage of the blood vessels, therefore suppressing tumor angiogenesis.

Anti-metastatic activity of the Notch1 decoys

Two metastasis models were utilized to investigate the role of Notch1 decoys in tumor cell invasion and metastasis. An important finding of this study is that Notch1 decoys 1-13, 10-24, and 1-24 all appeared to delay pulmonary metastasis in B16-F10 model but not LLC. Since these tumors grew considerably fast in nude mice, the experiment was terminated after 12 days which only allowed analysis of normal-sized tumors but rather early-stage metastatic process. These tumors were derived from subcutaneous implantation of the tumor cells, thus lung and liver metastases must have come from tumor cell invasion from the primary site. Therefore, the effects of the decoys on metastasis can be focused on: tumor cell intravasation, survival and transport in the circulatory system, promotion of metastatic niche, and homing and colonization. Some evidence showed that genetic disruption of pericyte coverage elicited increased metastasis in Rip1/Tag2 pancreatic tumor model (Xian et al.). Since the decoys reduced tumor vascular integrity and decreased pericyte coverage, it is likely that tumor cell dissemination and metastasis were inhibited beyond the primary tumors.

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SECOND SERIES OF EXPERIMENTS

Introduction:

Notch is a transmembrane receptor that interacts with ligands expressed on the cell surface. In mammals, four Notch genes (1-4) and five

ligands, referred to as JAGGED (JAGGED1 and JAGGED2) or Delta-likes (1, 3, and 4). Delta-like 4 (Dll4) acting through Notch1 has been established to function in angiogenic sprout restriction during physiological and pathological retinal angiogenesis (Thurston, G and Kitajewski J, 2008). In contrast, JAGGED-1 has been implicated as a pro-angiogenic factor, however, the mechanism of action for JAGGED-1 in this capacity is not well understood (Benedito R, et al. 2009).

Age related macular degeneration (AMD) is a common cause of blindness and has a significant negative impact on the health of aging individuals. In wet (exudative) form of AMD, blood vessels grow up from the choroid behind the retina. These abnormal blood vessels are leaky and can cause the retina to become detached. Frontline treatment of wet AMD has been via anti-angiogenic agents that reduce the abnormal growth of blood vessels. VEGF inhibiting agents are currently used in such treatment. Although these agents are effective means of wet AMD treatment it is possible that other anti-angiogenic agents may be equally effective, may be useful when combined with anti-VEGF agents, or may be effective when VEGF-blockade does not lead to long term restoration of vision. Here the anti-angiogenic agents that target Notch, a critical signaling pathway in vascular growth and differentiation are studied (Dufraine, J. et al. 2008). Specifically, protein-based, receptor antagonists of the Notch pathway, Notch1 decoys were developed (Funahashi Y, 2008). Notch1 decoys have been developed that target the JAGGED-1/Notch1 pathway and these have proven to be anti-angiogenic in mouse models of retinal angiogenesis and tumor angiogenesis. Here the effect of therapeutic inhibition of JAGGED-1-Notch1 in retinal angiogenesis using mouse models of pathological angiogenesis is determined.

Preliminary Studies:

Notch1$^{1-36}$ decoy, schematized in Fig. 1, binds and inactivates several Notch ligands, including Dll4 and JAGGED-1 (Funahashi Y, et al. 2008). Inhibition of Dll4 alone, by inhibitory antibodies, has been reported to promote non-functional angiogenesis, resulting in hyper-sprouted but poorly perfused vessels (Yan, M. et al. Nature 463 and Ridgway J, et al. Nature 2006). In contrast, the Notch1$^{1-36}$ decoy blocks angiogenesis

with no evidence of increased sprouting (Funahashi Y, et al. 2008). Thus, the Notch1$^{1-36}$ decoy works via a mechanism that is distinct from Dll4 blockade and does not elicit effects on vessels that lead to vascular neoplasia in normal tissue (Yan, M. et al., Clin. Cancer Res. 207 and Yan, M. et al., Nature 2010). Ligand-specific Notch1 decoy variants have been generated that are derived from the original Notch1$^{1-36}$ decoy. Using *in vitro* Notch signaling assays, Notch1$^{1-24}$ and Notch1$^{1-36}$ decoys inhibit both Dll4-and JAGGED-1-mediated Notch signaling. A Notch1$^{1-13}$ decoy inhibited Dll4-mediated Notch signaling but not JAGGED-1. Since the Notch1$^{1-13}$ decoy is specific for Dll4 and Notch1$^{1-24}$ blocks both Dll4 and JAGGED-1, it was hypothesized that EGF-repeats 14-24 may encompass a region of JAGGED-1 specificity. A Notch1$^{10-24}$ decoy was made and shown to block JAGGED-1 but not Dll4 mediated Notch1 signaling. Thus, there are Notch1 decoys that block both JAGGED-1 and Dll4 (Notch1$^{1-24}$ decoy), a variant that blocks Dll4 but not JAGGED-1 (Notch1$^{1-13}$ decoy), and one that blocks JAGGED-1 but not Dll4 (Notch1$^{10-24}$ decoy). These variants were tested using *in vitro* angiogenesis assays and demonstrated that Notch1$^{1-13}$ decoy caused excess sprouting of endothelial cells, as predicted of a Dll4 inhibitor, whereas Notch1$^{10-24}$ decoy reduced *in vitro* angiogenic sprouting (data not shown). Notch1 decoy variants were tested using retinal angiogenesis mouse models by expressing Notch1 decoys in neonates. Wholemount isolectin staining, used to visualize the newly grown vasculature, demonstrated that expression of Notch1$^{1-13}$ decoy caused overgrowth of retinal vasculature, similar to that seen when a chemical Notch signaling gamma-secretase inhibitor, DAPT, is administered to neonates. In contrast, Notch1$^{10-24}$ decoy caused reduced vascular branching and growth in neonatal retina (Fig. 2C). The effects of inhibition of Dll4, JAGGED-1, or combined Dll4/JAGGED-1 blockade in mouse models of pathological retinal angiogenesis is described herein.

Experimental Procedures:

We determine the effects of Notch1 decoys on physiological and pathological retinal angiogenesis. JAGGED-1 or pan-Notch ligand inhibition via Notch1 receptor antagonists can block hypoxia driven retinal angiogenesis via their anti-angiogenic effects.

Specific inhibition of JAGGED-1/Notch1 is a highly novel approach at anti-angiogenesis applied to the treatment of wet AMD and deserves to be explored to facilitate improved treatments for blindness due to abnormal angiogenesis.

Determining the consequences of Notch inhibition for physiological retinal angiogenesis in mice.

Using adenovirus vectors to infect neonates at birth, Notch1 decoys that inhibit all Notch ligand ($Notch1^{1-24}$), Dll4 ($Notch1^{1-13}$), or JAGGED-1 ($Notch1^{10-24}$) are expressed in neonate mice and retinal angiogenesis is studied. The retina is an excellent model to study sprouting angiogenesis(Connolly, SE. et al., 1988 and Gerhardt, H. et al. 2003). Retinas from Notch1 decoy expressing neonates are isolated at postnatal day 4 (P4), P8 and P21. At P4, the retina is undergoing sprouting angiogenesis. At P8, the primary plexus has reached the retinal edge, by P21 retinal angiogenesis has completed. Whole-mount immunohistochemistry (IHC) with isolectin is used to identify endothelial cells, and CD11b or F4/80 to identify retinal myeloid cells, which actively participate in retinal angiogenesis. Endothelial tip cells will be determined by IHC for tip cell markers, high VEGFR-2 and Dll4. Standard and confocal microscopy will evaluate vascular density, vessel diameter, endothelial cell content, number of intercapillary junctions, and quantity/location of filopodia. The distance of the primary plexus has grown from the optic nerve will be determined. Double staining for endomucin+ or VE-cadherin+ cells and phospho-Histone-3 or ApoTag® antibodies is done to visualize endothelial cell proliferation or apoptosis, respectively. Albumin staining of retinal sections is done to evaluate capillary permeability, scored as extravascular retinal albumin staining.

Determining the consequences of Notch inhibition for hypoxia driven retinal angiogenesis in mice.

In premature babies, improper oxygen exposure leads to retinopathy of prematurity (ROP), a proliferative retinopathy driven by hypoxia with increased vascular permeability, thickening of basement membrane and

uncontrolled growth of vessels. A mouse model of oxygen-induced ischemic retinopathy (OIR) (Smith, LE et al. 1994) is used as a means of assessing Notch1 decoy efficacy in blocking pathological retinal angiogenesis. After expression of Notch1 decoys in neonates, as above, an OIR model is conducted, where P7 mice are exposed to 75% oxygen for 5 days. Retinas exhibit central retinal capillary obliteration at P8, becoming extensive by P12. Returning to room air at P12 causes the inner retina to become hypoxic, VEGF is up-regulated, and uncontrolled retinal neovascularization occurs from P12 to P17. The OIR model has been used to show that macrophages are recruited from the bone marrow during active neovascularization (P17)(Kataoka, K. et al. 2011) and facilitate normalization of the vasculature(Ritter, MR. et al. 2006) is assessed for macrophage content. For OIR studies, experimental mice will be evaluated by wholemount and section IHC of retinas at P8, P12 and P17. P8 and P12 retinas are evaluated for extent of central retina vasoobliteration, and macrophage density by double staining for isolectin and CD11b or F4/80. P17 retinas are evaluated for extent of dysregulated neovascularization and macrophage density.

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THIRD SERIES OF EXPERIMENTS

Introduction:

Tumor angiogenesis is regulated by a variety of signaling pathways, some of which are validated targets of anti-angiogenic therapies. The

first approved anti-angiogenic drug, bevacizumab (Avastin), is used in several types of cancers and has been proved somewhat successful. However, anti-angiogenic agents targeting the VEGF pathway do not exhibit durable tumor responses, eventually inducing drug resistance or influencing tumor metastasis (Bergers and Hanahan, 2008; Ebos et al., 2009; Pàez-Ribes et al., 2009). Disruption of tumor vasculature prevents tumor perfusion and results in hypoxia. This, in turn, can induce a wide range of factors and chemoattractants that promote tumor angiogenesis and tumor growth, despite VEGF inhibition. The Notch signaling pathway represents a target for anti-angiogenic therapy, and several Notch inhibitors have been developed. Agents that block gamma-secretase activity, required for Notch signal activation, or block Delta-like 4 (DLL4), disrupt tumor angiogenesis (Noguera-Troise et al., 2006; Ridgway et al., 2006; Kalén et al., 2011). Molecular and genetic studies reveal that Notch signaling regulates cell fate, cell proliferation, differentiation, and apoptosis, depending on the cellular context. In the endothelium, Notch signaling regulates proliferation, migration, and sprouting (Hellström et al., 2007).

Notch signaling requires cell-cell contact, allowing Notch proteins and their ligands to interact on neighboring cells. The highly conserved Notch gene family encodes transmembrane receptors, Notch1, Notch2, Notch3, and Notch4. The ligands for Notch are transmembrane proteins of two classes: the Jagged ligands (Jag), JAGGED-1 and Jag2; and the Delta-like ligands (Dll), Dll1, Dll3, and Dll4. Upon ligand activation, an intracellular Notch peptide is released by a gamma-secretase-dependent proteolytic cleavage and transits to the nucleus converting the CSL transcriptional repressor to an activator (Kopan and Ilagan, 2009). The Notch1 ligand binding domain comprises 36 EGF-like repeats. Notch ligands share a conserved degenerate EGF-like repeat, the DSL domain, which confers specificity to Notch binding (Henderson et al., 1997; Shimizu et al., 1999; Glittenberg et al., 2006) followed by an EGF-like repeat region that varies; JAGGEDs have 16 EGF-like repeats, and Dlls contain 8 or fewer. Notch EGF-like repeats 11 and 12, and the DSL domain are necessary for Notch interaction with either Dll1 or JAGGED-1 (Hambleton et al., 2004; Cordle et al., 2008). EGF-like repeats 24-29, or the Abruptex region, oppose Notch activation by competing with Dll ligands for the ligand-binding site (Pei and Baker,

2008). It is unknown if there are distinct Notch EGF-like repeats that interact with Dll versus Jag ligands. This gap in knowledge has limited our understanding of ligand-specific interactions with Notch and signaling outcome.

Notch receptors and ligands have been shown to be upregulated in several cancers. The roles of Notch signaling in tumor cells include both tumor promoting and suppressing activities depending on the tumor type (Takebe et al., 2010; Ranganathan et al., 2011). Inhibition of endothelial Notch function disrupts tumor angiogenesis. DLL4 blockade inhibits tumor growth by dysregulating tumor angiogenesis, characterized by increased endothelial cell proliferation and tip cell numbers resulting a non-functional vasculature (Noguera-Troise et al., 2006; Ridgway et al., 2006).

Notch and VEGF signaling pathways are intricately linked. VEGF induces expression of Notch receptors and Dll4 (Liu et al., 2003; Funahashi et al., 2011), and Notch activation reduces expression of VEGFR-2 but increases expression of VEGFR-1/sFlt-1 (Taylor et al., 2002; Shawber et al., 2007). In endothelial cells, VEGFR-3 can be either induced by Notch (Shawber et al., 2007; Geudens et al., 2010) or reduced by Notch signaling (Tammela et al., 2011). In retinal angiogenesis, Dll4 and JAGGED-1 have been demonstrated to have unique activities in endothelium, as endothelial loss of function experiments result in distinct phenotypes (High et al., 2008; Benedito et al., 2009).

We have created soluble, extracellular domain NOTCH1 constructs encoding different EGF-like repeats fused with human IgG Fc (NOTCH1 decoy). The NOTCH1 decoys function as Notch inhibitors. A human NOTCH1 decoy with all 36 EGF-like repeats functioned similarly to a rat Notch1 decoy that inhibits JAGGED-1, Dll1, and Dll4 (Funahashi et al., 2008). We asked whether NOTCH1 decoys that incorporate different NOTCH1 EGF-like repeats would antagonize selective Notch ligands. NOTCH1 decoy variants were identified that selectively inhibited DLL4 or JAGGED-1, providing the first delineation of ligand-specific interaction domains in human NOTCH1. NOTCH1 decoy variants were evaluated for effects on in vitro, retinal, and tumor angiogenesis. A NOTCH1 decoy variant that specifically interfered with DLL4, caused a hypersprouting phenotype, promoted dysfunctional tumor angiogenesis, and inhibited tumor growth.

A NOTCH1 decoy variant that blocks JAGGED-1 caused reduced NOTCH1 signaling, blocked angiogenic growth in retinas and tumors, and reduced tumor growth. JAGGED-1 blockade specifically increased anti-angiogenic soluble VEGFR-1 (sVEGFR-1/sFlt-1) levels and disrupted pericyte coverage, providing a mechanism by which JAGGED-1 blockade disrupts tumor growth.

Materials and Methods

Primary Cells and Cancer Cell Lines

Cell cultures were maintained at 37°C in 5% CO_2 and 95% humidified air. HUVECs were grown in EGM-2 Media (Lonza Group, Walkersville, MD). Mm5MT, LLC, and B16-F10 were from the American Type Culture Collection (ATCC, Manassas, VA). KP1 was obtained from Health Science Research Resource Bank (Osaka, Japan). Cancer cell lines were maintained in 1X High Glucose DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and Pen-Strep.

Notch Reporter Assay

HeLa cells were transfected with pBOS-Notch1, pGL3-11CSL-Luc and Renilla with Effectene Transfection Reagent (Qiagen, Germantown, MD), or with either pCRIII-JAGGED-1-FLAG or pCRIII-DLL4-FLAG or pCRIII-GFP-FLAG as control. 24 hours after transfection, receptor and ligand cells were co-cultured in a 24-well plate. Cells were harvested, and luciferase activity measured 24 hours after co-culture, using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI). Assays were performed in triplicates.

Co-Immunoprecipitation

Notch1 decoys and full-length DLL4-FLAG or JAGGED-1-FLAG were co-transfected into 293T cells by calcium phosphate transfection. DSG (Thermo Scientific, Waltham, MA), was added to the culture 24 hours after transfection at a final concentration of 20 nmol/ml, incubated for 30 minutes, and quenched with 10 mM Tris for 15 minutes. The lysate

was pulled down by 20 µl of Protein A/G Agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To reverse the crosslink, the immunocomplex was treated with 50 µmol/ml DTT and boiled for 5 minutes before electrophoresis.

Sprouting Angiogenesis Assay

A sprouting assay (Nakatsu and Hughes, 2008) used HUVECs adhered to Cytodex 3 dextran beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) at 400 cells per bead. Beads were embedded at 250 beads per well in a 24-well plate in a fibrin clot composed of 2 mg/ml fibrinogen (Sigma-Aldrich, St. Louis, MO), 0.15 U/ml aprotinin (Sigma-Aldrich, St. Louis, MO), and 0.0625 U/ml thrombin (Sigma-Aldrich, St. Louis, MO). After one hour, Detroit 551 fibroblasts (ATCC, Manassas, VA) were seeded on top of the fibrin gel at 1.0×10^5 cells per well. Experiments were performed in triplicates.

Retinal Analysis

P2 pups were subcutaneously injected with adenoviruses (Ad) encoding different Notch1 decoys or Fc. Ad was prepared in 1X PBS at 5.0×10^9 ffu/ml, and each pup received a single dose of 50 µl. Eyeballs collected at P5 were fixed in 4% PFA. Retinas were dissected, permeabilized in 1X PBS with 1% BSA and 0.5% Triton X-100 for 2 hours at room temperature and subsequently washed 3 times in PBLEC buffer (1% Triton X-100, 0.1 mM MgCl2, 0.1 mM $CaCl_2$, 0.1 mM MnCl2 in 1X PBS pH 6.8). For immunofluorescence, retinas were incubated overnight with FITC-conjugated isolectin B4 (Vector Laboratories, Inc., Burlingame, CA), washed with PBLEC, post-fixed with 4% PFA, and mounted.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was collected from cultured cells with RNeasy Mini Kit (Qiagen, Germantown, MD). Isolated RNA was treated with DNase I for 30 minutes and used in reverse-transcription PCR using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). For qRT-PCR, reactions were done with ABsolute Blue QPCR SYBR Green Mix (Thermo

Scientific, Waltham, MA).

Tumor Experiment

4-6 week-old female NCr-nude mice (NCI-Frederick, Frederick, MD) underwent subcutaneous implantation of 1.0 x 10^5 Mm5MT-FGF4 cells, or 2.0 x 10^6 KP1-VEGF cells, or 5.0 x 10^5 LLC or B16-F10 cells in the upper flank. 2 days later, Ad encoding Notch1 decoys were administered via retro-orbital intravenous injection. Tumors were harvested at day 21 and analyzed. To measure tumor hypoxia, mice were injected intraperitoneally 30 minutes before sacrifice with hypoxyprobe-1 at 60 mg/kg (Hypoxyprobe, Inc., Burlington, MA). Tumors were immunostained with an anti-hypoxyprobe antibody. To assess vessel perfusion, mice received an intracardiac injection at the left ventricle with 100 µg of fluorescein Lycopersicon esculentum lectin (Vector Laboratories, Inc., Burlingame, CA). After 2 minutes, the mice were perfused with 1% PFA. Tumors were analyzed for lectin bound to the endothelial cell surface by fluorescence microscopy. For gut toxicity analysis, the duodena were harvested from Ad-infected, tumor-bearing mice. The tissue was fixed in 4% PFA overnight and dehydrated in 30% sucrose solution before paraffin embedding. PAS staining was performed to analyze goblet cells.

Immunofluorescent Staining

Fresh-frozen tumor tissue sections of 7-µm thickness were post-fixed in cold acetone for 3 minutes and washed in 1X PBS for 5 minutes twice and then blocked for 1 hour at room temperature in the blocking solution containing 3% BSA and 2% serum. Then, primary antibody solution was added to the blocking solution, added to slides, and incubated at 4°C overnight. Slides were washed for 5 minutes twice in 1X PBS. The fluorescently conjugated secondary antibody was added to the sections and incubated for 30 minutes. The slides were washed twice in 1X PBS and mounted.

Results

Notch1$^{1-13}$ Decoy Increases Capillary Sprouting, Whereas Notch1$^{10-24}$ Decoy Reduces Sprouting and Vascular Smooth Muscle Cell Coverage

To determine the effects of Notch1 decoys on angiogenesis and lumen-containing sprout formation by endothelial cells, we expressed Notch1 decoys in HUVECs and performed in vitro sprouting assays. HUVEC-coated dextran beads were embedded in fibrin and sprout formation assessed on day 7. In the Fc control, endothelial cell sprouts anastomosed to form multicellular, branched, and lumen-containing networks (Figure 33A). HUVECs expressing Notch1$^{1-13}$ decoy showed a hypersprouting phenotype characterized by increased tip cells, as seen by a 76% increase in the number of capillary sprouts (Figures 33A and 33B). The Notch1$^{1-13}$ decoy phenotype is consistent with the attenuation of DLL4/Notch signaling, as it has been shown that an anti-DLL4 antibody enhanced endothelial cell proliferation and sprouting (Ridgway et al., 2006). In contrast, HUVECs expressing Notch1$^{10-24}$ and Notch1$^{1-24}$ decoys exhibited stunted sprouts and a decrease in the number of sprouts by 40% and 68% respectively (Figure 33B). Anastomosis, observed in the control group, was absent in HUVECs expressing Notch1$^{10-24}$ and Notch1$^{1-24}$ decoys. These results suggest that inhibition of JAGGED-1 is anti-angiogenic and that the effect dominates over DLL4 inhibition.

All Notch1 decoys tested (Notch1$^{1-13}$, Notch1$^{10-24}$, Notch1$^{1-24}$, Notch1$^{1-36}$) increased HUVEC migration and proliferation when grown in monolayers (Figures 34A-34C), the opposite of Notch signal activation by Notch1IC. In a capillary-like network formation assay, with HUVECs embedded between collagen gels, Notch11-13 decoy caused HUVECs to form a more complex vascular network with an increase in branch points, whereas Notch1$^{10-24}$ failed to form a complete network (Figures 35A and 35B). The ability of Notch1$^{1-13}$ and Notch1$^{10-24}$ to elicit different angiogenic responses in 3-dimensional (3D) in vitro assays (Figure 33A) was not seen in monolayer assays. A possible explanation for the differences is that Notch ligand expression is influenced by extracellular matrix (ECM). JAGGED-1 expression was significantly increased and DLL4 decreased when HUVECs were grown on fibrin as compared to collagen (Figure 36). Thus, ligand-specific responses elicited by Notch1 decoy variants in HUVECs are influenced by ECM and are manifested when evaluation of capillary-like sprouting is modeled in vitro in 3D.

We asked what effects the Notch1 decoys would have on murine retinal

angiogenesis, where Notch signaling restricts sprout formation (Lobov et al., 2007; Suchting et al., 2007). Adenovirus vectors expressing the decoys or Fc were injected into neonates, decoy expression in the circulation verified by western blotting, and effects of the circulating decoys on retinal angiogenesis assessed. Adenovirus infection led to detectable serum levels of the Notch1 decoys (data not shown). Notch1$^{1-13}$ decoy significantly increased endothelial sprouting and the number of sprouting tip cells (Figure 33B), consistent with its ability to selectively block DLL4 (Figure 33A). Notch1$^{10-24}$ decoy reduced blood vessel density in the retina (Figure 7B). Notch1$^{1-24}$ decoy increased retinal vasculature density (Figure 7B). Thus, Notch1$^{1-24}$ decoy behaved as a Dll4 antagonist in murine retinal angiogenesis and a JAGGED-1 antagonist during in vitro sprouting.

JAGGED-1 has been shown to be important for the recruitment of vascular smooth muscle cells (High et al., 2008; Benedito et al., 2009). Analysis of α-smooth muscle actin (αSMA) immunofluorescence revealed decreased vascular smooth muscle cell coverage along the arteries in Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy-treated groups (Figure 33C), a phenotype also seen in endothelial-specific JAGGED-1 mutant mice (High et al., 2008; Benedito et al., 2009). NG2 immunofluorescence showed no significant difference in retinal pericyte coverage (data not shown). When evaluated for effects on sprouting angiogenesis in vitro and in vivo, Notch1$^{1-13}$ decoy functioned as a DLL4 inhibitor and Notch1$^{10-24}$ decoy as a JAGGED-1 inhibitor.

<u>Notch1$^{1-13}$, Notch1$^{10-24}$, and Notch1$^{1-24}$ Decoys Significantly Reduce Tumor Growth and Dysregulate Tumor Angiogenesis</u>

We tested the ability of Notch1 decoys to directly affect tumor cells; assessing if Notch1$^{1-13}$, Notch1$^{10-24}$, and Notch1$^{1-24}$ decoys would affect in vitro colony formation, proliferation and apoptosis of Mm5MT-FGF4 (mouse mammary tumor), KP1-VEGF (human pancreatic tumor), LLC (mouse lung tumor), and B16-F10 (mouse melanoma) tumor cell lines. All Notch1 decoys significantly inhibited colony formation of Mm5MT-FGF4 cells, but not other tumor cell lines (Figure 37). Thus, in Mm5MT-FGF4 tumors, Notch1 decoys have the potential to inhibit both tumor cells and host cells, such as endothelial and mural cells. Notch1 decoys did not

affect tumor cell proliferation or apoptosis in any of the tumor lines grown in monolayer cultures (Figure 15).

To evaluate the action of Notch1 decoy variants in tumors, we performed xenograft studies using the 4 different tumor cell lines. Adenoviruses encoding different Notch1 decoys were injected intravenously into mice 3 days after subcutaneous tumor implantation. High levels of proteins were detected in the serum by Western blots as early as 2 days after injection and in tumors by immunofluorescence (Figure 16). All Notch1 decoys tested significantly decreased growth of Mm5MT-FGF4, LLC, and B16-F10 tumors; while only Notch1$^{10-24}$ and Notch1$^{1-24}$ decoys inhibited the growth of KP1-VEGF tumors (Figures 38A-38D). The ability of Notch1$^{1-24}$ decoy to perturb Mm5MT-FGF4 and KP1-VEGF tumor growth was similar to that observed for the full-length Notch1 decoy (Notch1$^{1-36}$) (Figure 39). The effects of Notch1 decoys were seen after the tumor began to grow rapidly which took about one week after implantation (data not shown).

Notch1$^{1-13}$ decoy, Notch1$^{10-24}$ and Notch1$^{1-24}$ decoys had distinct effects on tumor angiogenesis. Notch1$^{1-13}$ decoy significantly increased endothelial cell density in all tumor models (Figures 40A-40B), similar to that seen with DLL4 blockade (Ridgway et al., 2006). In contrast, tumors from the Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy groups showed a decrease in endothelial cell content (Figures 40A-40B). In the Mm5MT-FGF4 model, vessel perfusion was determined by lectin perfusion followed by endomucin staining of tumor endothelium. Compared to Fc tumors, the vasculature from all Notch1 decoy-treated groups showed poor vessel perfusion, decreased 72% (Notch1$^{1-13}$), 90% (Notch1$^{10-24}$), and 84% (Notch1$^{1-24}$) (Figures 41A and 41B). Consistent with poor perfusion, Notch1 decoy-treated tumors had increased hypoxia and tumor necrosis (Figure 41A and 41C). To determine vessel regression, tumors were immunostained for endomucin and collagen IV. Collagen IV deposition was increased in Notch1$^{1-13}$ decoy treated tumors and reduced in Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy tumors (Figures 42A-42C). When normalized to endomucin staining, there was no difference between Fc groups and Notch1 decoy-treated groups (Figure 42C), indicating that the reduced tumor vasculature was not due to vessel regression.

In conclusion, DLL4 and JAGGED-1 inhibition resulted in distinct

angiogenic phenotypes in murine tumor xenografts. Notch1$^{1-13}$ decoy, a DLL4 inhibitor, increased endothelial cell content, reduced vessel perfusion and increased tumor hypoxia and necrosis. Notch1$^{10-24}$ decoy, a JAGGED-1 inhibitor, reduced tumor angiogenesis and tumor vessels were poorly perfused leading to increased hypoxia.

Notch1 Decoys Display Distinct Abilities to Perturb Mural Cell Coverage of Tumor Vessels

Based upon the effect of Notch1 decoys on retinal mural cell coverage, we evaluated mural cells in Notch1 decoy-treated Mm5MT-FGF4 tumors. Tumor sections were immunostained for endomucin and NG2 or αSMA to visualize pericytes and vascular smooth muscle cells, respectively. In the Notch1$^{1-13}$ decoy tumors, pericytes were closely associated with endothelial cells (Figure 43A). Relative to the Fc group, Notch1$^{1-13}$ decoy caused an increase in pericyte content coincident with increased endothelial cell content (Figures 43B-43D), indicating that pericyte density was unchanged. Pericytes were disassociated from endothelial cells in Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy-treated tumors (Figure 43A). The number pericytes relative to endothelial cells was significantly reduced in Notch1$^{10-24}$ decoy tumors (Figure 43D); that is, overall pericyte coverage of vessels was decreased. αSMA immunostaining revealed reduced vascular smooth muscle cell coverage of large arterial vessels for Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy-treated tumors (Figure 44). Similar effects of Notch1$^{10-24}$ and Notch1$^{1-24}$ decoys were observed on the mural cells of B16-F10 tumors (data not shown). KP1-VEGF and LLC control tumors have poor mural cell coverage of vessels (data not shown). Thus in tumor angiogenesis, Dll4 inhibition had no apparent effect on vascular mural cells, while blocking JAGGED-1 via Notch1$^{1-24}$ and Notch1$^{10-24}$ decoys resulted in defective pericyte and vascular smooth muscle cell coverage.

The JAGGED-specific Notch1$^{10-24}$ Decoy Increases Soluble VEGFR-1/sFlt-1

We explored the mechanisms by which DLL4- and JAGGED-1-specific Notch1 decoy variants elicited distinct effects in endothelial cells by evaluating Notch target gene expression. HUVECs were infected with

lentiviruses encoding Fc, Notch1$^{1-13}$, Notch1$^{10-24}$, or Notch1$^{1-24}$ decoys and the effects on endothelial Notch downstream targets determined. JAGGED-1 was knocked down in HUVECs using an shRNA containing lentivirus (J1KD). Expression of Notch1$^{1-13}$, Notch1$^{10-24}$, and Notch1$^{1-24}$ decoys and J1KD suppressed the expression of HEY1, HEYL and HES1 (Figure 45), direct targets of Notch/CSL transactivation (Nakagawa et al., 2000). Unlike other Notch1 decoys, Notch1$^{10-24}$ decoy or J1KD did not reduce HEY2 transcripts (Figure 45). Thus, DLL4 and JAGGED-1 activation of Notch differentially regulates the expression of HEY2 in endothelial cells.

Notch signaling regulates VEGF signaling in endothelial cells, largely through the regulation of VEGF receptors (Thurston and Kitajewski, 2008). We used quantitative RT-PCR and FACs to determine the effect of Notch1 decoy variants or J1KD on the expression of VEGF receptors. Notch1$^{1-13}$, Notch1$^{10-24}$, Notch1$^{1-24}$ decoy variants and J1KD knockdown increased VEGFR-2 expression in HUVECs (Figure 46), as opposed to the Notch1 intracellular domain (Notch1IC), which reduced VEGFR-2 (data not shown). The increase of VEGFR-2 by Notch1 decoys likely contributes to the increase in HUVEC proliferation and migration (Figure 34). All Notch1 decoy variants and J1KD significantly decreased VEGFR-3 expression (Figure 45).

Inhibition of DLL4- or JAGGED-1-mediated Notch signaling by Notch1 decoys differentially regulated VEGFR-1 expression. Notch1$^{1-13}$ and Notch1$^{1-24}$ decoys decreased VEGFR-1 transcripts; while, Notch1$^{10-24}$ decoy or J1KD increased VEGFR-1 (Figure 46). However, VEGFR-1 surface expression was not increased in Notch1$^{10-24}$ decoy HUVEC (Figure 46).

VEGFR-1 exists as two splice variant that produce either a transmembrane receptor (VEGFR-1) or a soluble protein (sVEGFR-1/sFlt-1). Using PCR primers specific for sVEGFR-1/sFlt-1 transcripts, we found that Notch1$^{10-24}$ decoy or J1KD significantly increased sVEGFR-1/sFlt-1 transcripts (Figure 47A). Notch1 Notch1$^{1-24}$1-24 decoy, that inhibits both Dll4 and JAGGED-1, also increased sVEGFR-1/sFlt-1 expression in HUVEC. The sVEGFR-1/sFlt-1 splice variant was not affected by Dll4-specific Notch1$^{1-13}$ decoy in HUVEC (Figure 47A). We validated the finding that JAGGED-1/NOTCH signaling regulates sVEGFR-

1/sFlt-1 using ELISA on conditioned media from HUVECs expressing different Notch1 decoys, J1KD or Notch1IC. The levels of sVEGFR-1/sFlt-1 were significantly increased with Notch1$^{10-24}$ and Notch1$^{1-24}$ decoys or JAGGED-1 knockdown, and unaffected by Notch1$^{1-13}$ decoy or Notch1IC expression (Figure 47B).

VEGFR-1/sFlt-1 immunofluorescence of Mm5MT-FGF4 tumors showed a significant increase in VEGFR-1/sFlt-1 in Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy groups (Figures 48A and 48B). The diffuse and non-vascular staining pattern in Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy-treated tumors is indicative of increased soluble VEGFR-1/sFlt-1. Thus, we found that inhibiting JAGGED-1/Notch signaling with either Notch1$^{1-24}$ or Notch1$^{10-24}$ decoy specifically increased sVEGFR-1/sFlt-1 levels in HUVEC and murine tumor xenografts. As sVEGFR-1/sFlt-1 functions as a competitive antagonist of VEGF/VEGFR-2 signaling, the decrease in tumor angiogenesis we observed in the Notch1$^{10-24}$ and Notch1$^{1-24}$ decoy-treated tumors may arise due to decreased VEGFR-2 signaling.

Notch1 Decoys Are Not Toxic to Tumor-Bearing Mice

Previous publications reported intestinal goblet cell hyperplasia in mice treated with GSIs, or combined Notch1/Notch2 blockade (van Es et al., 2005; Wu et al., 2010). Expression of Notch1$^{1-13}$, Notch1$^{1-24}$ or Notch1$^{10-24}$ decoys modestly increased goblet cell numbers, less than 2-fold, in the intestines of tumor-bearing mice, at the end of the 3-week experiment (Figure 27A and 27B). In contrast, GSI (Compound E) treated mice had a 5-fold increase in goblet cells. Consistent with the mild gut phenotype, weight loss was not observed in Notch1 decoy variant tumor-bearing mice (Figure 27C). These results suggest that Notch1 decoys lack significant gut toxicity and represent alternative Notch-targeting agents for anti-angiogenic therapy.

Discussion

To interact productively with Notch ligands, Notch receptors require EGF-like repeats 11 and 12 and calcium ions (Rebay et al., 1991; Rand et al., 2000); however, little is known about ligand-specific interaction domains on Notch. We utilized biochemical and functional

assays to define domains for ligand-specific interactions with NOTCH1, focusing on DLL4 and JAGGED-1. Using this knowledge we uncovered unique downstream signaling events for DLL4- or JAGGED-1-mediated Notch signaling. Specifically, Notch1$^{1-24}$ decoy functions as a pan-ligand inhibitor, interacting with and blocking signaling induced by DLL4 or JAGGED-1. Notch1$^{1-13}$ decoy functions as DLL4-specific antagonist, defining the first 13 EGF-like repeats as capable of interfering with DLL4, but not JAGGED-1. Conversely, Notch1$^{10-24}$ decoy, containing EGF-like repeats 10-24 of NOTCH1, inhibited JAGGED-1 but not DLL4. Using these ligand-selective Notch1 decoys, we found opposite regulation of sVEGFR-1/sFlt-1 levels elicited by DLL4 or JAGGED-1 inhibition, demonstrating that DLL4 and JAGGED-1 have distinct signaling effects downstream of NOTCH1. Finally, we demonstrate that tumor inhibition can be accomplished using Notch1 decoys that either selectively inhibit DLL4 or JAGGED-1, or inhibit both. However, inhibition of DLL4 or JAGGED-1 resulted in distinct angiogenic phenotypes in the retina and tumor xenografts.

JAGGED-1 versus DLL4 Ligand-Specific Notch Signaling in Endothelial Cells

We previously described a rat Notch1$^{1-36}$ decoy that blocked Notch1 signaling by JAGGED-1, Dll1, or Dll4 (Funahashi et al., 2008). We generated human Notch1$^{1-36}$ and Notch1$^{1-24}$ decoys (Figure 36) and showed they blocked both JAGGED-1 and DLL4. Notch1$^{1-36}$ and Notch1$^{1-24}$ decoys functioned as anti-angiogenic agents, despite the fact that they interact with DLL4, whose inhibition should elicit a hypersprouting response. We hypothesized that the anti-angiogenic activity of Notch1$^{1-36}$ and Notch1$^{1-24}$ decoys reflects a phenotype elicited by blocking JAGGED-1 or both DLL4 and JAGGED-1.

All Notch1 decoy variants that were active antagonists contained EGF-like repeats 11-13 of NOTCH1. We discovered that EGF-like repeats of NOTCH1 upstream of 10-13 conferred inhibitory activities against DLL4 (Figures 3B and 3C) and specific binding to DLL4 (Figure 5B). Conversely, the EGF-like repeats downstream of 10-13 conferred inhibitory activities against JAGGED-1 (Figures 4A and 4B) and binding to JAGGED-1 (Figure 5B), but not DLL4. This is the first description of

ligand-selective association domains in the NOTCH1 protein.

Our studies demonstrate that JAGGED-1/Notch and DLL4/Notch signaling have overlapping and unique molecular targets. Pan-ligand Notch1$^{1-24}$ decoy, DLL4-specific Notch1$^{1-13}$ decoy, and JAGGED-1 specific Notch1$^{10-24}$ decoys all caused a reduction of the levels of Notch targets HEY1, HEYL, HES1, VEGFR-3 and an increase in VEGFR-2, demonstrating these genes are targets of both DLL4/NOTCH and JAGGED-1/NOTCH signaling. A difference in Notch1 decoy variant activities was discovered when we analyzed the ligand-specific regulation of soluble VEGFR-1/sFlt-1, an anti-angiogenic agent that functions as a decoy receptor for VEGF and antagonizes VEGFR-2 signaling (Shibuya, 2006). DLL4-specific Notch1$^{1-13}$ decoy reduced sVEGFR-1/sFlt-1 splice variant and protein levels, whereas JAGGED-1 specific Notch1$^{10-24}$ decoy resulted in increased sVEGFR-1/sFlt-1. Thus, the anti-angiogenic phenotype observed for JAGGED-1-specfic Notch decoys in in vitro sprouting assays and tumor xenografts may arise from the increase in sVEGFR-1/sFlt-1.

Anti-Angiogenic and Anti-Tumor Activity of Notch1 Decoys

JAGGED-1-specific and DLL4-specific Notch1 decoys both reduced tumor growth and induced hypoxia in tumors, indicating that the Notch1 decoys effectively block blood flow to tumors. Our analysis demonstrated that the tumor-inhibitory effects of Notch1 decoy variants result from different angiogenic mechanisms when DLL4 and/or JAGGED-1 are targets.

Blockade of Dll4/Notch leads to increased endothelial cell proliferation and increased tip cells, ultimately resulting in non-functional angiogenesis and poor vessel perfusion (Noguera-Troise et al., 2006; Ridgway et al., 2006; Li et al., 2007; Hoey et al., 2009). Consistent with these studies, the DLL4-specific Notch1$^{1-13}$ decoy caused hypersprouting in in vitro fibrin bead assay (Figures 33A and 33B), during retinal angiogenesis (Figures 7B and 7C) and in four different tumor xenografts (Figure 38). Notch1$^{1-13}$ decoy caused elevation of VEGFR-2 and a reduction of VEGFR-1, a change that is proposed to underlie the hypersprouting phenotype caused by Dll4 blockade (Potente et al., 2011). Thus, the angiogenic phenotype of Notch1$^{1-13}$ decoy matched the biochemical activity as a DLL4 inhibitor.

The JAGGED-1 inhibitor, Notch1$^{10-24}$ decoy, caused reduced sprouting in vitro (Figure 33A), during retinal angiogenesis (Figures 7B and 7C) and in multiple tumor xenografts (Figure 38). This is consistent with previous studies where loss of endothelial JAGGED-1 reduces retinal angiogenic sprouting (High et al., 2008; Benedito et al., 2009). JAGGED-1 inhibition by Notch1$^{10-24}$ decoy was associated with reduced Notch signaling as seen by a decreased JAGGED-1/NOTCH1 induced CSL-reporter expression and HEY1, HEYL, and HES1 expression. However, inhibition of JAGGED-1 specifically increased sVEGFR-1/sFlt-1 production in endothelial cells. Thus, the effect of JAGGED-1 blockade by Notch1$^{10-24}$ decoy was to elevate the levels of an anti-angiogenic agent produced by endothelial cells. In fact, significant elevation of sVEGFR-1/sFlt-1 was seen in tumors treated with Notch1$^{10-24}$ decoy (Figure 48); thus reduced tumor angiogenesis correlated with high sVEGFR-1/sFlt-1 levels.

Notch1$^{10-24}$ decoy expression reduced and disrupted vascular mural cells associated with both retinal and tumor vessels. In retinas, JAGGED-1-specific inhibition reduced vascular smooth muscle cell coverage of arterioles (Figure 33D). Pericytes also failed to associate with the tumor endothelium in Notch1$^{10-24}$ decoy treated tumors. The disruption of mural cell coverage observed with Notch1$^{10-24}$ decoy is also consistent with previous studies that showed that JAGGED-1/Notch interactions are required for proper smooth muscle cell association on arteries (High et al., 2008; Benedito et al., 2009).

We found that JAGGED-1-mediated Notch activation is required for regulation and maintenance of endothelial-pericyte interactions, and posit that deregulation of these interactions contributes to vessel instability. Thus, in addition to elevating sVEGFR-1/sFlt-1, we propose an additional mechanism by which Notch1$^{10-24}$ decoy blocks tumor angiogenesis. Notch1$^{10-24}$ decoy, through inhibition of JAGGED-1, destabilizes tumor vessels by disrupting endothelial pericyte interactions. Notch regulates a wide range of signaling molecules that promote endothelial-mural cell interactions (Armulik et al., 2005) and Notch in smooth muscle cells responds to endothelial JAGGED-1 by promoting differentiation (Domenga et al., 2004). Pericytes produce

VEGF-A and are known to promote endothelial cell survival (Franco et al., 2011). As Notch1$^{10-24}$ decoy severely disrupted pericyte coverage of tumor blood vessels and elevated sVEGFR-1/sFlt-1, this tumor endothelium would be particularly sensitive to the lack of pro-survival signals provided by VEGF-A. The Notch1$^{10-24}$ decoy thus represents a potent anti-angiogenic agent in tumors that acts to disrupt pericytes and elevate sVEGFR-1/sFlt-1.

JAGGED-1: Pro-Angiogenic Notch Ligand

The regulation of Notch signaling in blood vessels is attributed to endothelial Notch ligands, JAGGED-1 and Dll4. Unlike Dll4, the role of JAGGED-1 remained somewhat elusive until it was demonstrated that endothelial JAGGED-1 has reduced capacity to activate Notch signaling if Notch is glycosylated by Manic Fringe (Benedito et al., 2009). This data suggests a model where endothelial JAGGED-1 interferes with Dll4/Notch signaling, either by preventing Dll4/Notch interaction or by promoting lower Notch signaling than that mediated by Dll4 (Benedito et al., 2009). In support of this model, endothelial-specific loss of JAGGED-1 led to increased Notch targets Hey1 and Hes1 in retinal vessels (Benedito et al., 2009).

We propose that endothelial JAGGED-1 can act via Notch signal activation to promote angiogenesis by downregulating sVEGFR-1/sFlt1, and possibly other JAGGED-1-specific Notch targets yet to be identified. In cultured endothelial cells, the ability of JAGGED-1 to activate Notch signaling was largely similar to DLL4 (Figures 3B-3C and 4A-4b). Blocking JAGGED-1 activity through Notch1$^{10-24}$ decoy or J1KD down-regulated most Notch downstream targets, including HEY1, HEYL, HES1, VEGFR-3 and up-regulated VEGFR-2 (Figure 45). However, inhibition of JAGGED-1/NOTCH signaling by either Notch1$^{10-24}$ decoy or J1KD did not repress HEY2 and elevated sVEGFR-1/sFlt-1, unlike DLL4 blockade. Thus, loss-of-function experiments using either Notch1$^{10-24}$ decoy or J1KD demonstrates that endothelial JAGGED-1 can promote angiogenesis by activating Notch signaling which results in down-regulation of sVEGFR-1/sFlt-1. When JAGGED-1 is an activating ligand, endothelial cells would respond by reducing sVEGFR-1/sFlt-1, whereas if JAGGED-1 is manic fringe-modified and less active as a ligand, increased DLL4 signaling

would restrict sprout formation. Thus, the particular role of JAGGED-1 in angiogenesis is context dependent, differing based upon the levels and glycosylation state of NOTCH, or the cell type presenting JAGGED-1 to endothelial Notch. All evidence from our study is consistent with the conclusion that JAGGED-1 activity is critical for productive angiogenesis.

Function of Notch1 Decoys that Block Both DLL4 and JAGGED-1

By developing Notch1 decoys that block both DLL4 and JAGGED-1 and Notch1 decoys selective for each, we had the opportunity to compare the effects of combined DLL4 and JAGGED-1 blockade with ligand selective blockade. Similar to $Notch1^{10-24}$ decoy, $Notch1^{1-24}$ decoy blocked endothelial sprouting using in vitro fibrin bead sprouting assays (Figure 33A) and increased the protein levels of sVEGFR1/sFlt-1 (Figure 47B), albeit not as strongly as $Notch1^{10-24}$ decoy. However, $Notch1^{1-24}$ decoy also functioned similar to the DLL4-specific $Notch1^{1-13}$ decoy, as seen by increased HUVEC proliferation, migration and network formation. In retinas, $Notch1^{1-24}$ decoy displayed mixed phenotypes, causing hyper-sprouting (Figure 7B), but also reducing mural cell coverage (Figure 33D). Thus, $Notch1^{1-24}$ decoy can perturb both Dll4 and JAGGED-1 function in retinal vessels. In contrast, $Notch1^{1-24}$ decoy phenocopied $Notch1^{10-24}$ decoy in four different tumor models, causing reduced tumor vasculature and elevating sVEGFR-1/sFlt-1 in the Mm5MT tumor model. $Notch1^{1-24}$ decoy acted primarily as a JAGGED-1 inhibitor in the tumor microenvironment and it's utility in tumors will clearly be dependent on the presence and activities of different Notch ligands.

Therapeutic Potential of Notch1 Decoys

Differential effects of Notch1 decoys in blocking tumor angiogenesis will be influenced by their bioavailability. $Notch1^{1-13}$ and $Notch1^{1-24}$ decoys were expressed and secreted at higher levels than $Notch1^{1-36}$ decoy, and thus may be easier to produce and potentially more effective. Analysis of tumor sections demonstrated that $Notch1^{1-36}$ decoy was restricted to the tumor vasculature as opposed to the smaller

Notch1 decoy variants that were detected around the tumor vessels and diffused over the tumor cells (Figure 39). Being more diffusible, Notch1$^{1-13}$ and Notch1$^{10-24}$ decoys have the potential to affect tumor angiogenesis, tumor cells, cancer stem cells, and other cells in the tumor microenvironment. Tumor cells over-expressing JAGGED-1 promote tumor angiogenesis in mice (Zeng et al., 2005; Funahashi et al., 2008), suggesting tumor-derived JAGGED-1 could serve as an alternative angiogenic pathway in cases of VEGF blockade. Selective inhibition of JAGGED-1-mediated Notch signaling thus is important for targeting pro-tumor activities of JAGGED-1 derived from many cell types.

The potential advantage of the vascular localization of Notch1$^{1-36}$ decoy could be to minimize off-target side effects. A major adverse affect of Notch blockade using gamma-secretase inhibitors (van Es et al., 2005) or combined Notch1/Notch2 blocking antibodies (Wu et al., 2010) is compromised gastrointestinal function. We found Notch1$^{1-13}$, Notch1$^{10-24}$, Notch1$^{1-24}$ decoys induced only minimal goblet cell metaplasia relative to GSI treatment, and were tolerated by mice expressing the Notch1 decoys for up to eight weeks (data not shown).

Despite differences in activities and targets, Notch1$^{1-13}$, Notch1$^{10-24}$, Notch1$^{1-24}$ decoys were all effective at limiting tumor growth in four different tumor models, with minimal toxicity. The complexity of the Notch pathway and the variety of processes that Notch functions in has provided us with opportunities to investigate and develop a wide range of therapeutic agents that can modulate the signaling pathway differently and offer new alternatives for cancer therapy.

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What is claimed is:

1. A fusion protein which comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 23.

2. The fusion protein of claim 1, wherein the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extends to a C-terminal amino acid of EGF-like repeat 24.

3. The fusion protein of claim 1, wherein the amino acid sequence of the extracellular domain of the human Notch1 receptor protein extends to a C-terminal amino acid of EGF-like repeat 36.

4. The fusion protein of any one of claims 1-3, wherein the Fc portion of the antibody is the Fc portion of a human antibody.

5. The fusion protein of claim 2, wherein the sequence of the consecutive amino acids comprises the sequence set forth in SEQ ID NO: 3, commencing with cystine at position 24 and ending with lysine at position 833.

6. The fusion protein of claim 3, wherein the sequence of the consecutive amino acids comprises the sequence set forth in SEQ ID NO: 5, commencing with cystine at position 24 and ending with lysine at position 1318.

7. The fusion protein of claim 1, wherein (a) the extracellular domain of the human Notch1 receptor protein is preceded by a

signal peptide.

8. The fusion protein of claim 7, wherein the signal peptide is the signal peptide of human Notch1 protein or the signal peptide of an IgG heavy chain.

9. The fusion protein of claim 8, wherein the sequence of consecutive amino acids is set forth in SEQ ID NO: 3.

10. The fusion protein of claim 8, wherein the sequence of consecutive amino acids is set forth in SEQ ID NO: 5.

11. A composition comprising the fusion protein of any one of claims 1-10 and a carrier.

12. A composition of claim 11, wherein the fusion protein is present in an amount effective to inhibit the activity of JAGGED-1 in a pharmaceutically acceptable carrier.

13. A method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject the fusion protein of any one of claims 1-10 in an amount effective to treat the subject's AMD.

14. The method of claim 13, wherein the AMD is wet AMD.

15. The method of claim 13, wherein the AMD is dry AMD.

16. A method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject the fusion protein of any one of claims 1-10 in an amount effective to treat the subject's diabetic retinopathy.

17. The method of any one of claims 13-16, further comprising administering an inhibitor of Vascular Endothelial Growth Factor (VEGF).

18. The method of claim 17, wherein the inhibitor of VEGF is an

inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

19. The method of any one of claims 13-16, further comprising administering a VEGF receptor inhibitor.

20. The method of claim 19, wherein the VEGF receptor inhibitor is a VEGFR-1 or a VEGFR-2 inhibitor.

21. A method of treating a subject suffering from cancer which comprises administering to the subject the fusion protein of any one of claims 1-10 in an amount effective to treat the subject's cancer.

22. The method of claim 21, wherein the cancer is pancreatic cancer.

23. The method of claim 21, wherein the cancer is breast cancer.

24. A method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject a fusion protein in an amount effective to treat the subject's AMD, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids present in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises yhe amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1 the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

25. The method of claim 24, wherein the AMD is wet AMD.

26. The method of claim 24, wherein the AMD is dry AMD.

27. A method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject a fusion protein in an amount effective to treat the subject's diabetic retinopathy, wherein the fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids present in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1 the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

28. The method of any one of claims 24-27, further comprising administering an inhibitor of Vascular Endothelial Growth Factor (VEGF).

29. The method of claim 28, wherein the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

30. The method of any one of claims 24-27, further comprising administering a VEGF receptor inhibitor.

31. The method of claim 30, wherein the VEGF receptor inhibitor is a VEGFR-1 or a VEGFR-2 inhibitor.

32. A method of treating a subject suffering from cancer which comprises administering to the subject a fusion protein in an amount effective to treat the subject's cancer, wherein the

fusion protein comprises consecutive amino acids the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids present in:

(a) an extracellular domain of a human Notch1 receptor protein, followed by

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) comprises the amino acid present at the N-terminus of EGF-like repeat 9 and

(ii) extends at least through the C-terminal amino acid of EGF-like repeat 13,

provided that if the N-terminal amino acid is the N-terminal amino acid of EGF-like repeat 1 the C-terminal amino acid is not the C-terminal amino acid of EGF-like repeat 36.

33. The method of claim 32, wherein the cancer is pancreatic cancer.

34. The method of claim 32, wherein the cancer is breast cancer.

35. The method of any one of claims 24-34, wherein the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 9 and extends to the C-terminal amino acid of EGF-like repeat 23.

36. The method of any one of claims 24-34, wherein the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 9 and extends to the C-terminal amino acid of EGF-like repeat 36.

37. The method of any one of claims 24-34, wherein the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 1 and extends to the C-terminal amino acid of EGF-like repeat 13.

38. The method of any one of claims 24-34, wherein the amino acid sequence of the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 1 and extends to the C-terminal amino acid of EGF-like repeat 24.