COMPOSITIONS OF HUMANIZED NOTCH FUSION PROTEINS AND METHODS OF TREATMENT

This application claims the benefit of U.S. Provisional Application No. 60/966,052, filed August 23, 2007, the contents of which is hereby incorporated by reference.

The invention disclosed herein was made with United States government support under grant number R01 HL62454 from the National Institutes of Health and grant number DAMRDCW81XWH-04-1-054 and grant number DAMD17-03-1-0218 from the Department of Defense. Accordingly, the United States government has certain rights in this invention.

Throughout this application, various publications are referenced by arabic numbers within parentheses or by author and publication date within parentheses. Full citations for these publications may be found at the end of the specification. 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

Vascular Development

During mammalian embryogenesis, formation of the vascular system is an early and essential process. In the embryo, vascular development initiates with the pluripotent hemangioblast derived from the paraxial and lateral plate mesoderm. The hemangioblast has the potential to differentiate into either a hematopoietic progenitor or an endothelial cell progenitor, known as the angioblast.

Vascular development begins with a process known as vasculogenesis whereby angioblasts differentiate into endothelial cells and migrate together to form the primitive vascular plexus. This initial vascular network consists of vessels that are homogenous in size and made up wholly of endothelial cells. The vascular plexus is then remodeled via angiogenesis.

Angiogenesis involves the sprouting of new vessels, the migration of these vessels into avascular regions, and the recruitment of accessory cells, pericytes and smooth muscle cells (Gale and Yancopoulos, 1999). The smooth muscle cells that differentiate and form the contractile vessel walls originate from multiple progenitors including neural crest cells, mesenchymal cells and even endothelial cells (Owens, 1995). In adults, angiogenesis is involved in follicular development, wound healing, and pathological processes such as tumor angiogenesis and heart disease.

The Notch Family and Notch Ligands

Studies of *Drosophila*, *C. Elegans*, zebrafish and mammals have demonstrated that the Notch pathway is an evolutionarily conserved signaling mechanism that functions to modulate numerous cell-fate decisions. Notch signaling is required for the proper patterning of cells originating from all three germ layers. Depending on the cellular context, Notch signaling may both inhibit and induce differentiation, induce proliferation, and promote cell survival (Artavanis-Tsakonas et al., 1995; Lewis,

1998; Weinmaster, 1997). In *Drosophila*, a single Notch protein is activated by two ligands, Serrate and Delta. In mammals these families have been expanded to four Notch genes (Notch1, Notch2, Notch3 and Notch4) and five ligands, 2 Serrate-like (Jagged1-2) and 3 Delta (Dl1, 3, 4) (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Gallahan and Callahan, 1997; Lardelli et al., 1994; Lindsell et al., 1995; Shawber et al., 1996a; Shutter et al., 2000a; Uyttendaele et al., 1996; Weinmaster et al., 1992; Weinmaster et al., 1991). During embryogenesis, Notch receptors and ligands are expressed in dynamic spatial and temporal patterns. However, it is not known if all ligands activate all receptors.

Notch Signaling and Function

Notch signaling influences many different types of cell-fate decisions by providing inhibitory, inductive or proliferative signals depending on the environmental context (reviewed in Artavanis-Tsakonas et al., 1995; Greenwald, 1998; Robey, 1997; Vervoort et al., 1997). This pleiotropic function suggests that Notch modulates multiple signaling pathways in a spatio-temporal manner.

Consistent with Notch regulating cell-fate decisions, both the receptors and ligands are cell surface proteins with single transmembrane domains (Figure 1). The regulatory extracellular domain of Notch proteins consists largely of tandemly arranged EGF-like repeats that are required for ligand binding (Artavanis-Tsakonas et al., 1995; Weinmaster, 1998). C-terminal to the EGF-like repeats are an additional three cysteine-rich

repeats, designated the LIN12/Notch repeats (LNR) (Greenwald, 1994). Downstream of the LNR lies the proteolytic cleavage sequence (RXRR) that is recognized by a furin-like convertase. For Notch1, cleavage at this site yields a 180 kilodalton extracellular peptide and a 120 kilodalton intracellular peptide that are held together to generate a heterodimeric receptor at the cell surface (Blaumueller et al., 1997; Kopan et al., 1996; Logeat et al., 1998).

The intracellular domain of Notch (NotchICD, Figure 1) rescues loss-of-function Notch phenotypes indicating that this form of Notch signals constitutively (Fortini and Artavanis-Tsakonas, 1993; Lyman and Young, 1993; Rebay et al., 1993; Struhl et al., 1993).

The cytoplasmic domain of Notch contains three identifiable domains: the RAM domain, the ankyrin repeat domain and the C-terminal PEST domain (Figure 1). Upon ligand-activation Notch undergoes two additional proteolytic cleavages which results in the release of the cytoplasmic domain (Weinmaster, 1998). This Notch peptide translocates to the nucleus and interacts with transcriptional repressors known as CSL (CBF, Su (H), Lag-2) and converts it to transcriptional activator. The CSL/Notch interaction is dependent on the presence of the RAM domain of Notch; while, transcriptional activity also requires the presence of the ankyrin repeats (Hsieh et al., 1996; Hsieh et al., 1997; Roehl et al., 1996; Tamura et al., 1995; Wettstein et al., 1997). Both *in vivo* and *in vitro* studies indicate that the HES and Hey genes are the direct targets of Notch/CSL-dependent signaling (Bailey and Posakony, 1995; Eastman et al., 1997;

Henderson et al., 2001; Jarriault et al., 1995; Nakagawa et al., 2000; Wettstein et al., 1997). The HES and Hey genes are bHLH transcriptional repressor that bind DNA at N-boxes (Nakagawa et al., 2000; Sasai et al., 1992; Tietze et al., 1992). Notch has also been proposed to signal by a CSL-independent pathway. In fact, expression of just the ankyrin repeat domain is necessary and sufficient for some forms of Notch signaling (Lieber et al., 1993; Matsuno et al., 1997; Shawber et al., 1996b).

Finally, the PEST domain has been implicated in protein turnover by a SEL-10/ubiquitin-dependent pathway (Greenwald, 1994; Oberg et al., 2001; Rogers et al., 1986; Wu et al., 1998; Wu et al., 2001). Similar to the receptors, the extracellular domain of the Notch ligands also consist mostly of tandemly arranged EGF-like repeats (Figure 1). Upstream of these repeats is a divergent EGF-like repeat known as the DSL (Delta, Serrate, Lag-2) that is required for ligand binding and activation of the receptors (Artavanis-Tsakonas et al., 1995).

Notch Signaling and Vascular Development

Although many of the genes that function to induce vasculogenesis and angiogenesis have been identified, little is known about how cell-fate decisions are specified during vascular development. A number of observations suggest that the Notch signaling pathway may play a role in cell fate determination and patterning of the vascular system.

Notch1, Notch4, Jagged1 and Dll4 are all expressed in the developing vasculature, while Notch3 is expressed in the accessory smooth muscle cells (Krebs et al., 2000; Shutter et al., 2000b; Uyttendaele et al., 1996; Villa et al., 2001; Xue et al., 1999). Mice lacking Jagged1 are embryonic lethal and have severe vascular defects (Xue et al., 1999). Mice nullizygous for Notch1 are embryonic lethal and die of severe neuronal defects, but also have defects in angiogenesis (Krebs et al., 2000; Swiatek et al., 1994). Mice lacking Notch4 are born and appear to be normal, but embryos that have lost both Notch1 and Notch4 die at E9.5 of severe hemorrhaging and vascular patterning defects indicating Notch1 and Notch4 may be functionally redundant during vascular development (Krebs et al., 2000). Exogenous expression of an activated form of Notch4 in endothelium also resulted in vascular defects similar to those seen for the double Notch1/Notch4 nullizygous mice, suggesting that appropriate levels of Notch signaling is critical for proper development of the embryonic vasculature (Uyttendaele et al., 2001).

Taken together, the data from mice mutant for Notch/Notch signaling components uncover several processes dependent on Notch including vascular remodeling, arterial venous specification, vascular smooth muscle cell recruitment and heart/heart outflow vessel development.

Recent experiments have implicated Notch signaling in arterial/venous endothelial cell specification. *In situ* analysis of E13.5 embryos found that Notch1, Notch3, Notch4, Dl4, Jagged1 and Jagged2 expression was restricted to the arteries and absent in the veins (Villa

et al., 2001). Consistent with expression data, disruption of Notch signaling in Zebrafish was associated with loss of the arterial marker ephrinB2; while, ectopic expression of an activated form of Notch lead to a loss in the venous cell marker EphB4 within the dorsal aorta (Lawson et al., 2001). These data suggest that Notch signaling may help to specify arterial and venous cell fates during angiogenesis.

Taken together, the data from mice mutant for Notch/Notch signaling components uncover several processes dependent on Notch including vascular remodeling, arterial venous specification, vascular smooth muscle cell recruitment and heart/heart outflow vessel development.

Notch signaling has also been suggested to function in the adult vascular system. In humans, missense mutations in the extracellular domain of Notch3 correlate with the development of the degenerative vascular disease, CADASIL (Caronti et al., 1998; Desmond et al., 1998; Joutel et al., 2000; Joutel et al., 1996). In a wound healing model, an increase in Jagged1 expression was observed at the regenerating endothelial wound edge, suggesting Notch signaling may function during processes of adult angiogenesis (Lindner et al., 2001). Taken together these data support Notch signaling functions at a number of critical steps during vascular development: vasculogenesis, vascular patterning/angiogenesis, and arterial/venous specification. However, the molecular mechanism(s) by which the Notch signaling pathways influence these different steps has yet to be elucidated.

Significance

Shimizu et al. (*J. Biol. Chem.* 274(46): 32961-32969 (1999)) describe the use of Notch1ECD/Fc, Notch2ECD/Fc and Notch3ECD/Fc in binding studies. However, Shimizu et al. do not mention the use of such proteins for inhibiting angiogenesis.

U.S. Patent No. 6,379,925 issued April 30, 2002 to Kitajewski et al. describes murine Notch4. However, it does not describe Notch-based fusion proteins as set forth in the subject application.

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.

Summary of the Invention

This invention provides a fusion protein comprising a signal peptide, an extracellular domain of human Notch receptor protein and an Fc portion of an antibody bound thereto.

This invention provides a method for treating a subject having a tumor comprising administering to the subject an amount of the above fusion protein effective to treat the subject, thereby treating the subject having a tumor.

This invention provides a method for inhibiting angiogenesis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit angiogenesis in the subject, thereby inhibiting angiogenesis in the subject.

This invention provides a method for treating a subject having ovarian cancer comprising administering to the subject an amount of the above fusion protein effective to treat the subject, thereby treating the subject having ovarian cancer.

This invention provides a method for treating a subject having a metabolic disorder comprising administering to the subject an amount of the above fusion protein effective to treat the subject, thereby treating the

subject having a metabolic disorder.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for the treatment of a subject having a tumor.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for inhibiting angiogenesis in a subject.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for treating a subject having ovarian cancer.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for for treating a subject having a metabolic disorder.

This invention provides a method for inhibiting physiological lymphangiogenesis or pathological lymphangionesis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit physiological lymphangiogenesis or pathological lymphangionesis in the subject.

This invention provides a method of inhibiting tumor metastasis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit tumor metastasis in the subject.

This invention provides a method of inhibiting growth of a secondary tumor in a subject comprising administering to the subject an amount of the above fusion protein of

effective to inhibit growth of the secondary tumor in the subject.

This invention provides a method of inhibiting blood vessel cooption by a tumor in subject comprising administering to the subject an amount of the above fusion protein effective to inhibit blood vessel cooption by a tumor in the subject.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of Vascular Endothelial Growth Factor (VEGF), VEGF-A, P1GF, VEGF-B, VEGF-C, orVEGF-D, each in an amount effective to treat the cancer in the subject.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and a VEGF receptor antagonist, a VEGFR-1 antagonist, a VEGFR-2 antagonist or a VEGFR-3 antagonist, each in an amount effective to treat the cancer in the subject.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of Platelet Derived Growth Factor (PDGF), PDGF-A or PDGF-B, each in an amount effective to treat the cancer in the subject.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and a PDGF receptor antagonist, each in an amount effective to treat the cancer in the subject.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of HER2/neu, each in an amount effective to treat the cancer in the subject.

This invention provides a method of treating vascular proliferative retinopathy comprising administering to the subject the above fusion protein in an emount effective to treat the vascular proliferative retinopathy.

Brief Description of the Figures

Figure 1

This Figure shows the schematic structure of Notch and Notch ligands: Notch1, Notch2, Notch3, Notch4, Jagged-1, Jagged-2, Delta-like 1, Delta-like 3, Delta-like 4.

Figure 2

This Figure shows the schematic design of Notch-based fusion proteins (NotchECD/Fc). The extracellular domain of Notch1, Notch2, Notch3, or Notch4 containing the EGF-repeats is fused to the Fc portion of an antibody.

Figure 3

This Figure shows a co-culture assay for testing the activity of Notch-based fusion proteins. Notch and Notch responsive transcriptional reporters are expressed in a "Notch-responsive" cell, HeLa. Notch ligands, Jagged-1, Delta-like 1, or Delta-like 4 are expressed in a "ligand-presenting" cell, 293. Expression is mediated by transfection of individual cell populations, cells are co-cultured, and then assayed for Notch-dependent reporter activity.

Figure 4

This Figure shows the inhibitory activity of Notch-based fusion protein against activation of Notch signaling by interaction between Notch and Notch ligand. Induction of Notch signaling was detected by co-cultivating both Notch1- and 3 types of Notch ligand-expressing cells and these inductions were inhibited by co-transfection of Notch-based fusion protein-expressing vector into Notch1-

expressing cells. Therefore, Notch-based fusion proteins can be used as Notch inhibitor based on inhibition of interaction between Notch and Notch ligand.

Figure 5

This Figure shows the expression of Notch1-based fusion protein (Notch1ECD/Fc) in 293. *Panel A*: expression in cell lystates (lys) or secreted into media (sup). *Panel B*: expression in 293 lysates of NECD/Fcs, as listed.

Figure 6

This Figure shows activation of Notch signaling in HUVEC infected with adenoviral-encoding VEGF-165. Activation of Notch signaling can be detected by using CBF1 promoter activity. Transcriptional activity of CBF1 promoter is activated by binding of Notch-IC to CBF1. We measured CBF1 promoter activity in HUVEC which was infected with adenovirus-encoding VEGF-165 at different MOI. Induction of CBF1 promoter was clearly detected in Ad-VEGF-infected HUVEC, compared to Ad-LacZ-infected cells in the MOI dependent manner. This data showed overexpression of VEGF could activate Notch signaling in HUVEC.

Figure 7

This Figure shows the effect of Notch-based fusion proteins on VEGF-induced activation of Notch signaling. Co-infection of Ad-Notch-based fusion protein with Ad-VEGF clearly reduced activation of CBF1 promoter activity induced by Ad-VEGF infection alone. In the case of infection at 40 MOI for each adenovirus in panel A, 60% inhibition at 24 hour and 90% inhibition at 48 hour after reporter gene transfection was detected. This inhibitory

activity of Notch trap was dependent on MOI of Ad-Notch-based fusion protein.

Figure 8

This Figure shows an experiment in which we evaluated the effect of Notch-based fusion proteins on induction of budding by overexpressed VEGF-165 in HUVEC. When Ad-VEGF-infected HUVEC were cultured on type collagen gel for 8 days, budding was induced into collagen gel. This induction of budding by overexpressed VEGF was clearly inhibited by coinfection of adenoviral-encoding Notch-based fusion proteins. Ad-Notch-based fusion protein itself had less effect on morphology.

Figure 9

This Figure shows the result of counting buds per field under microscope. Ad-VEGF-infection into HUVEC increased the number of buds depending on used MOI. Even though a half MOI of Notch-based fusion protein was used, compared to Ad-VEGF, Ad-VEGF-induced budding was clearly inhibited. These data suggested that VEGF induced budding of HUVEC through activation of Notch signaling and Notch-based fusion protein could inhibit VEGF-induced budding.

Figure 10

This Figure shows the amino acid sequence of the extracellular domain of the rat Notch1 protein (SEQ ID NO:1) and a linker sequence (SEQ ID NO:2).

Figure 11

This Figure shows the amino acid sequence of the extracellular domain of the rat Notch2 protein (SEQ ID NO:3) and a linker sequence (SEQ ID NO:2).

Figure 12

This Figure shows the amino acid sequence of the extracellular domain of the mouse Notch3 protein (SEQ ID NO:4).

Figure 13

This Figure shows the amino acid sequence of the extracellular domain of the mouse Notch4 protein (SEQ ID NO:5) and a linker sequence (SEQ ID NO:2).

Figures 14A and 14B

This Figure shows the nucleic acid sequence of the extracellular domain of the rat Notch1 gene (SEQ ID NO:6).

Figures 15A and 15B

This Figure shows the nucleic acid sequence of the extracellular domain of the rat Notch2 gene (SEQ ID NO:7).

Figures 16A and 16B

This Figure shows the nucleic acid sequence of the extracellular domain of the mouse Notch3 gene (SEQ ID NO:8).

Figures 17A and 17B

This Figure shows the nucleic acid sequence of the extracellular domain of the mouse Notch4 gene (SEQ ID NO:9) and the nucleic acid sequence (SEQ ID NO:10) and the amino acid sequence (SEQ ID NO:2) of a linker sequence.

Figures 18A and 18B

This Figure shows the nucleic acid sequence of the extracellular domain of the human Notch1 gene (SEQ ID NO:11).

Figures 19A and 19B

This Figure shows the nucleic acid sequence of the extracellular domain of the human Notch2 gene (SEQ ID NO:12).

Figures 20A and 20B

This Figure shows the nucleic acid sequence of the extracellular domain of the human Notch3 gene (SEQ ID NO:13).

Figures 21A and 21B

This Figure shows the nucleic acid sequence of the extracellular domain of the human Notch4 gene (SEQ ID NO:14).

Figures 22A - 22I

These Figures show that VEGF activates Notch signaling to induce HUVEC budding. HUVEC were transduced with Ad-VEGF at 40 MOI (Figs. 22A, 22H, 22I) or 20 MOI (Figs. 22C, 22G). Ad-LacZ was co-transduced to HUVEC to make the same total amount of adenovirus 60 MOI (Fig. 22G), 80 MOI (Fig. 22A) and 100 MOI (Figs. 22H, 22I). Figure 22A shows RT-PCR analysis of Notch and Notch ligand expression. Numbers show PCR cycles. Figure 22B shows the effect of transduced VEGF on CSL reporter activity. Figure 22C shows the effect of SU5416 on CSL reporter activity transactivated by Ad-VEGF. Figure 22D shows the construct of Notch decoy (N1ECDFc). Figure 22E shows secretion of

N1ECDFc from HUVEC trasduced with Ad-N1ECDFc. Figure 22F shows the effect of N1ECDFc against ligand-induced CSL reporter activity in a co-culture assay (□: (-); ■: 0.33 ng pHyTC-N1ECDFc; ■: 0.67 ng pHyTC-N1ECDFc). Figures 22G-I show the effect of N1ECDFc against Ad-VEGF-transduced HUVEC. Notch signaling was activated with transduction of Ad-VEGF in HUVEC in the absence or presence of co-transduction of Ad-N1ECDFc at indicated dosage. Figure 22G shows the effect of N1ECDFc on CSL reporter activity transactivated by Ad-VEGF. Figure 22H shows inhibition of budding of Ad-VEGF-transduced HUVEC with co-transduction of Ad-N1ECDFc at 40 MOI. Figure 22I shows quantification of the effect of N1ECDFc on budding of Ad-VEGF-transduced HUVEC (□: bud; ■: cell number).

Figures 23A - 23J

These Figures show that Notch signaling up-regulates Flt1 expression to induce HUVEC budding. HUVEC were transduced with either Ad-LacZ or Ad-N1IC at 40 MOI. Figures 23A-23C show the effect of inhibitors for receptor tyrosine kinases on Notch-induced HUVEC budding. Figure 23A is a photograph of budding of Ad-N1IC-transduced HUVEC treated with PD166866, ZD1893 at 1 μM and SU5416 at 0.5 μM. Figure 23B shows quantification of the effect of inhibitors at 1 μM (□: bud; ■: cell number). Figure 23C shows dose-dependency of the effect of SU5416 (□: bud; ■: cell number). Figures 23D-E show induction of Flt-1 expression in Ad-N1IC-transduced HUVEC. Figure 23D shows RT-PCR analysis of Flt-1 mRNA expression. Figure 23E shows W.B. analysis of Flt-1 protein expression. Figures 23F-G show promotion of Notch-induced HUVEC budding with PlGF stimulation. Ad-N1IC-transduced HUVEC were cultured on

collagen gel with SFM, instead of complete medium, in the absence or presence of 50 ng/ml PlGF. Figure 23F shows PlGF-induced budding of Ad-N1IC-transducec HUVEC (arrow head: buds with single filopodia; arrow: buds with multiple filopodia). Figure 23G shows the quantification of the effect of PlGF on budding of Ad-N1IC-transduced HUVEC (□: multi; ■: total). Figures 23H-I show the effect of Flt-1 siRNA transfection on Flt1 expression. Ad-N1IC-transduced HUVEC were transfected with 200 pmol of either control (CT) or Flt-1 siRNA. Figure 23H shows the reduction of Flt-1 mRNA expression. Figure 23I shows the reduction of Flt-1 protein expression. Figure 23J shows the effect of Flt-1 siRNA transfection on Notch-induced HUVEC budding. Ad-N1IC-transduced HUVEC were transfected with either 100 or 200 pmol of siRNA and cultured on collagen gel for 2 days.

Figures 24A - 24E

These Figures show that VEGF regulates gelatinase activity via Notch signaling by up-regulation of both MMP-9 and MT1-MMP. Figures 24A-B show gelatin zymography analysis of MMP-9 and MMP-2 activity stimulated by VEGF in HUVEC. Figure 24A shows the effect of N1ECDFc on MMP-9 activity. Transduced HUVEC were cultured on fibrin gel on the indicated day (i.e. D2, D4, D6, D8). Similar results were also obtained by using collagen gel, although induction of MMP-9 was stronger on fibrin gel than collagen gel (data not shown). Figure 24B shows the effect of N1ECDFc on MMP-2 activity. HUVEC were transduced with Ad-N1ECDFc at the indicated doses and condition medium was collected from HUVEC cultured on collagen gel at day 4. Figures 24C-D show up-regulation of MMP-9 and MT1-MMP with Notch signaling. HUVEC were

transduced with either Ad-LacZ or Ad-N1IC at 40 MOI. Numbers show PCR cycles. Figure 24C shows RT-PCR analysis of the effect of Notch signaling on expression of MMP-9 and MMP-2. Figure 24D shows the induction of MT1-MMP expression of both transcript and protein with Notch signaling. Figure 24E shows RT-PCR analysis of MMP-9 and MT1-MMP expression in Ad-VEGF-HUVEC with co-transduction of Ad-N1ECDFc. HUVEC were transduced with Ad-VEGF in the absence or presence of co-transduction of Ad-N1ECDFc at 40 MOI each. Ad-LacZ was co-transduced to make the same total amount of adenovirus at 80 MOI.

Figures 25A - 25D

These Figures show the role of Notch signaling in VEGF-dependent *in vivo* angiogenesis. Figures 25A-25D show inhibition of VEGF-induced angiogenesis with N1ECDFc in mouse DAS assay. Representative photographs are shown. Figure 25A show subcutaneous induced angiogenesis with 293/VEGF transfectant versus 293/VEGF also expressing Notch decoy (Notch-based fusion protein) N1ECDFc. Figure 25B shows the quantitation of degree of vascularization induced by 293/VEGF in control versus 293 expressing Notch decoy (Notch-based fusion protein)- N1ECDFc. Figure 25C shows subcutaneous induced angiogenesis with Ad-LacZ infected MDA-MB-231 cells versus Ad-N1ECDFc (Notch-based fusion protein) infected MDA-MB-231 cells. MDA-MB-231 breast cancer cells produce VEGF (data not shown). Figure 25D shows quantitation of degree of vascularization induced by Ad-LacZ infected MDA-MB-231 cells versus Ad-N1ECDFc (Notch-based fusion protein) infected MDA-MB-231 cells.

Figures 26A and 26B

These Figures show proliferation of Ad-VEGF165-transduced HUVEC. HUVEC were transduced with Ad-VEGF165 at the indicated dosages. Ad-LacZ was also co-infected to make the same total amount of adenovirus at a MOI of 40 pfu/cell. HUVEC were suspended in SFM supplemented with 1% FBS and then plated at 1 x 10^4 cells/well in 24-well multi-wll plates with 0.4 ml of medium. After 4 days, cell numbers were determined using the CCK-8 kit and the results are indicated as the ratio of cell numbers determined to the number of control cells, which were transduced with Ad-GFP at a MOI of 40 pfu/cell. Figure 26A shows the effect of transduced VEGF on proliferation. Figure 26B shows the inhibitory effect of SU5416. Ad-VEGF-transduced HUVEC were treated with SU5416 at the indicated dosages.

Figures 27A and 27B

These Figures show the induction of HUVEC buds on type I collagen gel. HUVEC were transduced with either Ad-VEGF165 or AD-N1IC at the indicated dosages. Ad-LacZ was also co-infected to make the same total amount of adenovirus at a MOI of 40 pfu/cell. Transduced HUVEC were cultured on collagen gel with complete medium. The amount of budding was evaluated under microscopy at day 7.

Figures 28A and 28B

These Figures show the effect of alteration of Notch signaling on cell proliferation. The cells were transduced with the indicated adenoviruses. Ad-GFP was also co-infected to make the same total amount of adenovirus at a MOI of 60 pfu/cell. After 4 days, cell numbers were determined using the CCK-8 kit and results are indicated as the ratio of cell numbers determined to

the number of control cells, which were transduced with AD-GFP at MOI of 60 pfu/cell. Figure 28A shows the effect of transduced N1IC and Notch fusion protein on the proliferation of HUVEC. Transduced HUVEC were suspended in complete medium and then plated at 1 x 10^4 cells/well in 24-well multiwell plates with 0.4 ml of indicated medium (□: Ad-N1IC; ■: Ad-N1ECDFc). Figure 28B shows the effect of Notch fusion protein on proliferation of KP1/VEGF transfectants. Transduced KP1/VEGF transfectants were suspended in RPMI1640 medium and then plated at 2x 10^4 cells/well in 24-well multiwell plates with 0.5 ml of medium.

Figure 29

This Figure shows the RT-PCR analysis of induction of PIGF expression in Ad-N1IC-transduced HUVEC. HUVEC were infected with either Ad-LacZ or Ad-N1IC at a MOI of 40 pfu/cell. Total RNA was isolated from transduced HUVEC cultured on collagen gel for 5 days with complete medium.

Figures 30A - 30C

These Figures show inhibition of budding of either Ad-N1IC- or Ad-VEGF-transduced HUVEC with Flk-1 siRNA transfection. Figure 30A shows reduction of Flk-1 mRNA and protein expression in Ad-VEGF-HUVEC with transfection of 200 pmol Flk-1 siRNA. Ad-VEGF-HUVEC at a MOI of 40 pfu/cell were transfected with 200 pmol of either control (CT) or Flk-1 siRNA. Total RNA was isolated 48 hours after transfection. Total cell lysate was collected from serum starved cells with SFM for 48 hours after transfection. Figures 30B and 30C show the inhibitory effect of Flk-1 siRNA transfection on either VEGF or Notch-induced HUVEC buds. Either Ad-N1IC- or Ad-VEGF-

HUVEC at a MOI of 40 pfu/cell were transfected with 200 pmol of siRNA as indicated and cultured on collagen gel for 5 days. Figure 30B shows the effect of Flk-1 siRNA transfection on HUVEC buds (□: Ad-VEGF; ■: Ad-N1IC). Figure 30C shows quantification of the inhibitory effect of Flk-1 siRNA transfection.

Figures 31A and 31B

These Figures show inhibition of budding of Ad-N1IC-transduced HUVEC with treatment of matrix metalloproteinase inhibitor GM6001. Either Ad-LacZ or Ad-N1IC-HUVEC at a MOI of 40 pfu/cell were cultured on collagen gel for 5 days in the absence or presence of GM6001 at 50 μm. Figure 31A shows the effect of GM6001 on Notch-induced HUVEC buds. Figure 31B shows quantification of the inhibitory effect of GM6001.

Figures 32A - 32D

These Figures show that Notch1 decoy inhibits activation of Notch signaling stimulated by Notch ligands. Figure 32A shows a schematic of Notch1 decoy (N1ECDFc) and Western blotting to detect secreted Notch1 decoy in conditioned medium. HUVECs transduced with adenovirus coding Notch1 decoy (Ad-N1ECDFc) at indicated m.o.i. Figure 32B shows that Notch1 decoy inhibits ligand-induced CSL reporter activity in co-culture signaling assay. Activation of Notch signaling was measured in HeLa cells expressing Notch1 co-cultured with 293 cells expressing Notch ligands. Data is shown as mean ± SD. Figure 32C shows ectopic expression of Notch4 induces the morphogenesis of HUVECs cultured on fibrin gel. HUVECs were transduced with adenovirus encoding Notch4 (Ad-Notch4) at 30 m.o.i. and Ad-GFP at 10 m.o.i, to mark

infected cells. Two days later, HUVECs were co-cultured with stable HUVEC transfectants on fibrin gel and morphological changes were documented using fluorescence microscopy. Notch 4 induces cell extensions (upper left, white arrows) and treatment with 200 nM compound E blocks Notch4-induced extensions (upper right). Notch1 decoy expression blocks Notch4-induced cell extensions. Adenovirus-transduced HUVECs were co-cultured on fibrin gels with stable HUVEC transfectants expressing either Fc (lower left) or Notch1 decoy (lower right) and photographed two days later. Bar = 200 μm. Figure 32D shows quantification of effect of Notch signal inhibition on Notch4-induced extensions. Reduction in sprouting was statistically significant after treatment with compound E and transduction of N1ECDFc ($p<0.0001$, both; data is shown as mean $\pm$ SD).

Figures 33A - 33D

These Figures show that FGF4 induces the expression of Notch ligands in murine mammary carcinoma Mm5MT cells. Stable Mm5MT transfectants generated by retroviral gene transfer. Figure 33A shows quantitative RT-PCR analysis of the expression of Notch ligands showing induction of Jagged1 and Dll1 in Mm5MT transfectants expressing FGF4 (Mm5MT-FGF4), compared to mock transfectants (Mm5MT-X). Figure 33B shows Jagged1 protein is elevated in Mm5MT-FGF4 versus Mm5MT-X, as determined by western blotting. Figure 33C shows reduction of Notch ligand expression in Mm5MT-FGF4 cells with PD166866, an inhibitor of FGF receptor kinase. Figure 33DB shows immunohistochemical analysis of Jagged1 staining in Mm5MT transfectants. Bar = 50 μm.

Figures 34A - 34C

These Figures show that Notch1 decoy inhibits angiogenesis and subcutaneous tumor growth of Mm5MT-FGF4 tumors in mice. Figure 34A shows tumor volumes of Mm5MT-FGF4-X and Mm5MT-FGF4-Fc differ significantly from Mm5MT-FGF4-N1ECDFc transfectants in mice (day 21, P=0.037 and P=0.008, Mm5MT-FGF4-X and Mm5MT-FGF4-Fc versus Mm5MT-FGF4-N1ECDFc respectively; data is shown as mean $\pm$ SD). Figure 34B shows immunohistochemical analysis of neovessels with CD31 staining within tumors of Mm5MT-FGF4 transfectants. Upper panels, Bar = 100 μm, Lower panels, Bar = 50 μm. Figure 34C shows quantitative analysis demonstrated a reduction in CD31(+) neovessels in Mm5MT-FGF4-N1ECDFc transfectants as compared to Fc or mock-transfected tumors ($P<0.001$ for both Mm5MT-FGF4-X and Mm5MT-FGF4-Fc versus Mm5MT-FGF4-N1ECDFc; data is shown as mean $\pm$ SD). Xenografts were harvested 22 days after inoculation and stained with anti-CD31 antibody.

Figures 35A - 35D

These Figures show Notch1 decoy expression disrupts angiogenesis and impairs tumor viability in human NGP xenografts. We have previously reported that these human neuroblastoma xenografts in mice have a mature, hierarchical vasculature that is relatively resistant to VEGF blockade (16). To determine whether Notch receptor activation contributed to NGP angiogenesis, we transfected NGP cells with the Notch1 decoy construct, which did not affect their ability to grow in culture (data not shown). There was, however, a marked decrease in tumor viability *in vivo* (Figure 35A) TUNEL=red

fluorescence, erythrocytes=green fluorescence, Bar = 100 μm), with (Figure 35B) significantly increased tumor cell apoptosis (P=0.0002, TUNEL-positive cells in NGP-N1ECDFc vs. NGP-LacZ tumors), and (Figure 35C) increased intratumoral hemorrhage ($p<0.0001$, quantitation of parenchymal erythrocyte signal). In addition, the tumor vessel networks in NGP-N1ECDFc xenografts appeared to have been physically disrupted as compared to NGP-LacZ controls, with (Figure 35D) immunostaining for ECs and VMCs (using anti-CD31 and αSMA antibodies, respectively) demonstrating lack of continuity of these vascular cell layers, (Bar = 50 μm). Individual vascular cells appeared detached from one another. Taken together, these results suggest that Notch1 decoy expression disrupted the ability of ECs and VMCs to form stable vascular conduits, causing vessel breakdown, hemorrhage, and ischemia of tumor tissues.

Figure 36

This Figure shows that SKOV3 tumor cells programmed expressing the Notch1 decoy block the growth of ovarian cancer xenografts.

Figure 37

This Figure shows regulation of myoblast differentiation by Foxo and Notch. C2C12 cells were immunostained with anti-Myosin antibody (green) and DAPI (blue). See text for panel description. Each experiment was repeated ≥ six times.

Figures 38A - 38C

These Figures show the quantitative analysis of C2C12

differentiation. Figure 38A shows Western blotting analysis of myosin expression in C2C12 cells. Figure 38B shows morphometric analysis of Myosin-positive cells. Results from differentiation experiments were analyzed by scoring the number of Myosin-immunostained cells as percentage of all DAPI-positive cells. Figure 38C shows DBD-Foxo1ADA reporter gene assays. We carried out reporter gene assays using the canonical Foxo1-responsive *Igfbp1* promoter (left panel) and the *Hes1* promoter (right panel) in cells co-transfected with Foxo1-ADA or DBD-Foxo1ADA. Western blot (inset) demonstrates that expression levels of the two proteins are similar. An asterisk indicates $P<0.01$ by ANOVA.

Figure 39A - 39E

These Figures show Foxo1 co-immunoprecipitates with Csl. **a**, Co-immunoprecipitation of endogenous Foxo1 and Csl in C2C12 cells co-cultured with LacZ- (denoted by the "-" sign) or Jagged1-expressing HEK293 cells (denoted by the "+" sign). **b-c**, Co-immunoprecipitation experiments in C2C12 cells co-transfected with FLAG-Csl and HA-Foxo1. **d-e**, Co-immunoprecipitation experiments in C2C12 cells co-transfected with FLAG-Csl and the truncated mutant Myc- or HA-tagged Δ256 Foxo1.

Figures 40A - 40D

These Figures show that Foxo1 binds directly to Csl. **a**, GST pull-down assays of GST-Foxo1 fusion protein with Csl immunoprecipitated from HEK293 cells. **b-c**, Binding of GST-Foxo1 and GST-FLAG-Csl in a cell-free system and mapping of the Csl interaction domain. Full-length and truncated fragments of GST-Foxo1 and GST-FLAG/Csl were

purified from bacteria and co-incubated. Thereafter, Csl was isolated using anti-FLAG antibody, and the immunoprecipitate was analyzed by immunoblotting with anti-Foxo1 or anti-FLAG antibodies. **d**, *Hes1* promoter ChIP spanning the Csl binding site in C2C12 cells to detect endogenous Foxo1, Csl and Notch1 (Endog) or following transduction with Foxo1-ADA (Foxo1-ADA) during myoblast differentiation. Input represents DNA extracted from chromatin prior to immunoprecipitation. *Hes1* (semiquantitative RT-PCR) and Myosin (Western blot) expression corresponding to each time point are shown. Day 0 is defined as the time when cells are serum-deprived to induce myoblast fusion. Abbreviations: IP: immunoprecipitation; IB: immunoblotting; TCL: total cellular lysate.

Figures 41A - 41B

These Figures show that Foxo1 regulates Notch-induced Hes1, Hes5 and Hey1 expression. **a**, *Hes1*, *Hes5* and *Hey1* expression measured by semiquantitative RT-PCR in C2C12 cells transduced with Foxo1-ADA or Notch1-IC following transfection of Gfp, Foxo1 or Csl siRNA as indicated. **b**, *Hes1* reporter gene assays in HEK293 cells transduced with Foxo1-ADA, Notch1-IC, Foxo1 siRNA, GFP siRNA or control plasmid. We measured luciferase activity and normalized it by β-galactosidase activity. The data represent arbitrary units relative to control empty vector.

Figures 42A - 42F

These Figures show that Foxo1 is required for Notch binding to the Hes1 promoter and activation of Hes1 target genes.

a, ChIP assays of endogenous Foxo1 and Notch1 in C2C12 cells co-cultured with LacZ- (denoted by a "-" sign) or or Jagged1-expressing HEK293 cells (denoted by a "+" sign) in the absence (lanes 1-2) and presence (lanes 3-4) of Csl siRNA. **b**, ChIP assays of endogenous Notch1 in co-culture system in the absence (lanes 1-2) and presence (lanes 3-4) of Foxo1 siRNA. **c**, Hes1 promoter assays following co-culture in the absence and presence of Foxo1 or Gfp siRNA. **d**, ChIP assays of Ncor and Smrt and Maml1 binding to *Hes1* in the co-culture system in the absence (lanes 1-2) and presence (lanes 3-4) of Foxo1 siRNA. **e**, Expression of *MyoD*, *Myf5* and *β-actin* in C2C12 cells by semiquantitative RT-PCR. **f**, Model of Foxo1 and Notch regulation of Hes1 promoter.

Figures 43A - 43D

These Figures show conditional ablation of Foxo1 in skeletal muscle.

a, Western blot analysis of Foxo1 and Foxo4 expression levels in various muscle types. **b,** Metachromatic and immunohistochemical analysis of soleus and plantaris muscle from Myog-Foxo1 mice and control (lox/lox) littermates. **c,** Gene expression analysis of Myog-Foxo1 (sold bars) and control mice (empty bars); TropC: troponin-C; TropT: troponin-T; Mlc: myosin light chain; Myog: Myogenin; Mck: muscle-type creatine kinase. Data are means ± SEM of three independent measurements (n= 6 for each genotype). An asterisk indicates $P<0.05$ by ANOVA. **d,** Treadmill performance test in 8 week-old Myog-*Foxo1* mice and *lox/lox* littermates (n=6 for each genotype). An asterisk indicates $P<0.05$ by ANOVA.

Figure 44

This Figure shows the efficiency of adenoviral transduction in C2C12 cells. We transduced cells with HA-Foxo1-ADA or HA-Notch1-IC adenovirus and performed immunohistochemistry with anti-HA antibody (red) and DAPI (blue).

Figure 45

This Figure shows inhibition of transfected Foxo1 expression. We tested the ability of Foxo1 siRNA to inhibit expression of endogenous (left panel) and transfected (right panel) Foxo1 following adenoviral transduction.

Figure 46

This Figure shows the specificity of Foxo1 siRNA. Western blot analysis of Foxo1, Foxo3 and Foxo4 expression in C2C12 cells transfected with Foxo1 siRNA.

Figure 47

This Figure shows that Foxo1-ADA and Notch1-IC do not affect cell proliferation. We transduced C2C12 cells with LacZ, Foxo1-ADA or Notch1-IC adenovirus, performed immunohistochemistry with anti-Ki67 antibody and DAPI and quantitated the Ki67 labeling index as percentage of Ki67-positive cells by counting at least 1,000 cells.

Figure 48

This Figure shows siRNA-resistant Foxo1-ADA. Western blot of Foxo1-ADA and siRNA-resistant Foxo1-ADA in cells transfected with Foxo1 siRNA.

Figure 49

This Figure shows the specificity of Foxo1-Csl co-immunoprecipitation. Following co-transfection with Foxo3 or Foxo4 expression vectors, we performed co-immunoprecipitation experiments with Csl.

Figure 50

This Figure shows Hes1 promoter assays. We used a synthetic *Hes1* reporter gene containing four tandem repeats of the Csl binding site in promoter assays with Foxo1 and Notch1-IC in C2C12 cells.

Figure 51

This Figure shows inhibition of Csl expression by siRNA. We measured Csl levels by western blot following transfection of C2C12 cells with Csl siRNA at different concentrations.

Figure 52

This Figure shows a schematization of eleven formulations of human Notch1 decoys.

Figure 53

This Figure shows signal sequence analysis of Notch 1 to determine where Notch1 signal peptide ends. The prediction results of analysis utilizing the SignalI 3.0 Server provided online by the Technical Univeristy of Denmark are shown. The results predict a major site of cleavage located between alanine 18 (A19) and A19 and a minor site of cleavage between A19 and Arginine 20 (R20). These two cleavage sites are indicated by the "/" in amino acid sequence 1-20 of human Notch 1: MPPLLAPLLCLALLPALA/A/R (SEQ ID NO:15) The nucleotide

sequence encoding amino acids 1-23 of human Notch1 is atgccgccgc tcctggcgcc cctgctctgc ctggcgctgc tgcccgcgct cgccgcacga ggcccgcga (SEQ ID NO:16).

Figure 54

This Figure shows signal sequence analysis of human Hc to determine where human Hc signal peptide ends. MWGWKCLLFWAVLVTATLCTA/R (SEQ ID NO: 17). The prediction results of analysis utilizing the SignalIP 3.0 Server provided online by the Technical University of Denmark are shown above. These results predict a major site of cleavage located between alanine 21 (A21) and arginine 22 (R22). This cleavage site is indicated by the "/" in amino acid sequence 1-22 of human Hc (SEQ ID NO:17) provided above. The nucleotide sequence encoding amino acids 1-22 of human Hc is atgtggggct ggaagtgcct cctcttctgg gctgtgctgg tcacagccac tctctgcact gccagg (SEQ ID NO:18).

Figure 55

This Figure shows the human Notch1/Fc fusion sequence for all constructs that end after EGF Repeat 36 of human Notch1.

Figure 56

This Figure shows the human Notch1/Fc fusion sequence for all constructs that end after EGF Repeat 13 of human Notch1.

Figure 57

This Figure shows the human Notch1/Fc fusion sequence for all constructs that end after EGF Repeat 23 of human

Notch1.

Figure 58

This Figure shows the human Notch1/Fc fusion sequence for all constructs that end after EGF Repeat 24 of human Notch1.

Figure 59A and 59B

This Figure shows the full-length amino acid (aa) sequence of human Notch1, consisting of aa residue 1 (M = methionine) to aa residue 2555 (K = lysine) (SEQ ID NO: 52). The signal peptide and first 36 EGF-like repeat domains are present in aa 1-1433 of this sequence. Amino acids 1-1433, or a subset of these aa, were utilized for the design of the human Notch1 decoy proteins, described in the ensuing sections. The amino acids encompassing EGF-repeats 1-36 are underlined.

Figure 60

This Figure shows the human Fc amino acid sequence utilized to generate the Fc tag on Notch1 decoy proteins (SEQ ID NO: 53). The 237 amino acids of human Fc were fused at the C-terminus of all Notch1 decoy constructs, just downstream of Notch1 EGF-like repeats. This region of human Fc allows for detection and purification of the Notch decoys and serves to stabilize the secreted human Notch1-human Fc fusion proteins.

Figure 61

This Figure shows the amino acid sequence of h-Notch1$^{(1-36)}$ decoy protein (SEQ ID NO: 54). h-Notch1$^{(1-36)}$ decoy protein consists of the following three components: (1)

human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 1-36 of human Notch1 consisting of amino acids 24-1433 followed by (3) amino acids 1434-1670 that contain the human HC tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1670 amino acids.

Figure 62

This Figure shows the amino acid sequence of h-Notch1$^{(1-13)}$ decoy protein (SEQ ID NO: 55). h-Notch1$^{(1-13)}$ decoy protein consists of the following three components:(1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 1-13 of human Notch1 consisting of amino acids 24-531 followed by (3) amino acids 532-768 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 768 amino acids.

Figure 63

This Figure shows the amino acid sequence of h-Notch1$^{(1-24)}$ decoy protein (SEQ ID NO: 56). h-Notch1$^{(1-24)}$ decoy protein consists of the following three components:(1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 1-24 of human Notch1 consisting of amino acids 24-948 followed by (3) amino acids 949-1185 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1185 amino

acids.

Figure 64

This Figure shows the amino acid sequence of h-sp^{N}Notch1$^{(9-23)}$ decoy protein (SEQ ID NO: 57). h-sp^{N}Notch1$^{(9-23)}$ decoy protein consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch1 consisting of amino acids 24-594 followed by (3) amino acids 595-831 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 829 amino acids.

Figure 65

This Figure shows the amino acid sequence of h-sp^{HC}Notch1$^{(9-23)}$ decoy protein (SEQ ID NO: 58). h-sp^{HC}Notch1$^{(9-23)}$ decoy protein consists of the following three components: (1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch1 consisting of amino acids 23-593 followed by (3) amino acids 594-830 that contain the human HC tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 829 amino acids.

Figure 66

This Figure shows the amino acid sequence of h-sp^{N}Notch1$^{(9-36)}$ decoy protein (SEQ ID NO: 59). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation. h-sp^{N}Notch1$^{(9-36)}$ decoy protein consists

of the following three components:(1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 9-36 of human Notch1 consisting of amino acids 24-1118 followed by (3) amino acids 1119-1355 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1355 amino acids.

Figure 67

This Figure shows the amino acid sequence of h-$sp^{HC}Notch1^{(9-36)}$ decoy protein (SEQ ID NO: 60). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-$sp^{HC}Notch1^{(9-36)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 9-36 of human Notch1 consisting of amino acids 23-1117 followed by (3) amino acids 1118-1354 that contain the human HC tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1354 amino acids.

Figure 68

This Figure shows the amino acid sequence of h-$sp^{N}Notch1^{(13-24)}$ decoy protein (SEQ ID NO: 61). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation. h-$sp^{N}Notch1^{(13-24)}$ decoy protein consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 13-24 of human Notch1

consisting of amino acids 24-478 followed by (3) amino acids 479-715 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 715 amino acids.

Figure 69

This Figure shows the amino acid sequence of h-$sp^{HC}Notch1^{(13-24)}$ decoy protein (SEQ ID NO: 62). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-$sp^{HC}Notch1^{(13-24)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 13-24 of human Notch1 consisting of amino acids 23-477 followed by (3) amino acids 478-714 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 714 amino acids.

Figure 70

This Figure shows the amino acid sequence of h-$sp^{N}Notch1^{(25-36)}$ decoy protein (SEQ ID NO: 63). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation. h-$sp^{N}Notch1^{(25-36)}$ decoy protein consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 25-36 of human Notch1 consisting of amino acids 24-508 followed by (3) amino acids 509-745 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the

human Fc tag is underlined and italicized. This formulation contains 745 amino acids.

Figure 71

This Figure shows the amino acid sequence of h-sp^{HC}Notch1$^{(25-36)}$ decoy protein (SEQ ID NO: 64). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-sp^{HC}Notch1$^{(25-36)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 25-36 of human Notch1 consisting of amino acids 23-507 followed by (3) amino acids 508-744 that contain the human HC tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 744 amino acids.

Figure 72A and 72B

This Figure shows the nucleic acid sequence which encodes h-Notch1$^{(1-36)}$ decoy protein set forth in Figure 61 (SEQ ID NO: 65).

Figure 73

This Figure shows the nucleic acid sequence which encodes h-Notch1$^{(1-13)}$ decoy protein set forth in Figure 62 (SEQ ID NO: 66).

Figure 74A and 74B

This Figure shows the nucleic acid sequence which encodes h-Notch1$^{(1-24)}$ decoy protein set forth in Figure 63 (SEQ ID NO: 67).

Figure 75

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch1$^{(9-23)}$ decoy protein set forth in Figure 64 (SEQ ID NO: 68).

Figure 76

This Figure shows the nucleic acid sequence which encodes h-sp^{HC}Notch1$^{(9-23)}$ decoy protein set forth in Figure 65 (SEQ ID NO: 69).

Figure 77A and 77B

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch1$^{(9-36)}$ decoy protein set forth in Figure 66 (SEQ ID NO: 70).

Figure 78A and 78B

This Figure shows the nucleic acid sequence which encodes h-sp^{Hc}Notch1$^{(9-36)}$ decoy protein set forth in Figure 67 (SEQ ID NO: 71).

Figure 79

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch1$^{(13-24)}$ decoy protein set forth in Figure 68 (SEQ ID NO: 72).

Figure 80

This Figure shows the nucleic acid sequence which encodes h-sp^{HC}Notch1$^{(13-24)}$ decoy protein set forth in Figure 69 (SEQ ID NO: 73).

Figure 81

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch1$^{(25-36)}$ decoy protein set forth in Figure 70 (SEQ ID NO: 74).

Figure 82

This Figure shows the nucleic acid sequence which encodes h-sp^{Hc}Notch1$^{(25-36)}$ decoy protein set forth in Figure 71 (SEQ ID NO: 75).

Figure 83

This Figure shows the full-length amino acid (aa) sequence of human Notch4, consisting of aa 1 (M = methionine) to aa 2003 (K = lysine) (SEQ ID NO: 76). The signal peptide and first 29 EGF-like repeat domains are present in aa 1-1174 of this sequence. Amino acids 1-1174, or a subset of these aa, were utilized for the design of the human Notch4 decoy proteins, described in the ensuing sections. The amino acids encompassing EGF-repeats 1-29 are underlined.

Figure 84

This Figure shows the Human Fc sequence utilized to generate the Fc tag on Notch4 decoy proteins (SEQ ID NO: 77). The 237 amino acids of human Fc, shown here, were fused at the C-terminus of all Notch4 decoy constructs, just downstream of Notch4 EGF-like repeats. This region of human Fc allows for detection and purification of the Notch decoys and serves to stabilize the secreted human Notch4-human Fc fusion proteins.

Figure 85

This Figure shows the amino acid sequence of h-Notch4$^{(1-29)}$

decoy protein (SEQ ID NO: 78). h-Notch4$^{(1-29)}$ decoy protein consists of the following three components:(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 1-29 of human Notch4 consisting of amino acids 28-1173 followed by (3) amino acids 1174-1410 that contain the human HC tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1410 amino acids.

Figure 86

This Figure shows the amino acid sequence of h-Notch4$^{(1-13)}$ decoy protein (SEQ ID NO: 79). h-Notch4$^{(1-13)}$ decoy protein consists of the following three components:(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 1-13 of human Notch4 consisting of amino acids 28-554 followed by (3) amino acids 555-791 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 791 amino acids.

Figure 87

This Figure shows the amino acid sequence of h-Notch4$^{(1-23)}$ decoy protein (SEQ ID NO: 80). h-Notch4$^{(1-23)}$ decoy protein consists of the following three components:(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 1-23 of human Notch4 consisting of amino acids 28-933 followed by (3) amino acids 934-1170 that contain the human Fc tag. The predicted signal peptide

sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1170 amino acids.

Figure 88

This Figure shows the amino acid sequence of h-sp^{N}Notch4$^{(9-23)}$ decoy protein (SEQ ID NO: 81). The abbreviation sp^{N} denotes that the human Notch4 signal peptide is used in this formulation. h-sp^{N}Notch4$^{(9-23)}$ decoy protein consists of the following three components:(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch4 consisting of amino acids 28-602 followed by (3) amino acids 603-839 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 839 amino acids.

Figure 89

This Figure shows the amino acid sequence of h-sp^{HC}Notch4$^{(9-23)}$ decoy protein (SEQ ID NO: 82). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-sp^{HC}Notch4$^{(9-23)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch4 consisting of amino acids 23-597 followed by (3) amino acids 598-834 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 834 amino acids.

Figure 90

This Figure shows the amino acid sequence of h-sp^{N}Notch4$^{(9-29)}$ decoy protein (SEQ ID NO: 83). The abbreviation sp^{N} denotes that the human Notch4 signal peptide is used in this formulation. h-sp^{N}Notch4$^{(9-29)}$ decoy protein consists of the following three components:(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 9-29 of human Notch4 consisting of amino acids 28-843 followed by (3) amino acids 844-1080 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1080 amino acids.

Figure 91

This Figure shows the amino acid sequence of h-sp^{HC}Notch4$^{(9-29)}$ decoy protein (SEQ ID NO: 84). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-sp^{HC}Notch4$^{(9-29)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 9-29 of human Notch4consisting of amino acids 23-838 followed by (3) amino acids 839-1075 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1075 amino acids.

Figure 92

This Figure shows the amino acid sequence of h-sp^{N}Notch4$^{(13-23)}$ decoy protein (SEQ ID NO: 85). The

abbreviation sp^N denotes that the human Notch4 signal peptide is used in this formulation. h-sp^NNotch4$^{(13-23)}$ decoy protein consists of the following three components: (1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 13-23 of human Notch4 consisting of amino acids 28-444 followed by (3) amino acids 445-681 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 681 amino acids.

Figure 93

This Figure shows the amino acid sequence of h-sp^{HC}Notch4$^{(13-23)}$ decoy protein (SEQ ID NO: 86). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-sp^{HC}Notch4$^{(13-23)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 13-23 of human Notch4 consisting of amino acids 23-439 followed by (3) amino acids 440-676 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 676 amino acids.

Figure 94

This Figure shows the amino acid sequence of h-sp^NNotch4$^{(21-29)}$ decoy protein (SEQ ID NO: 87). The abbreviation sp^N denotes that the human Notch4 signal peptide is used in this formulation. h-sp^NNotch4$^{(21-29)}$

decoy protein consists of the following three components: (1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 21-29 of human Notch4 consisting of amino acids 28-392 followed by (3) amino acids 393-629 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 629 amino acids.

Figure 95

This Figure shows the amino acid sequence of h-$sp^{HC}Notch4^{(21-29)}$ decoy protein (SEQ ID NO: 88). The abbreviation sp^{HC} denotes that the human HC signal peptide is used in this formulation. h-$sp^{HC}Notch4^{(21-29)}$ decoy protein consists of the following three components:(1) human HC signal sequence consisting of amino acids 1-22 of human HC, followed by (2) amino acids encoding the EGF-like repeats 21-29 of human Notch4 consisting of amino acids 23-387 followed by (3) amino acids 388-624 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 624 amino acids.

Figure 96A and 96B

This Figure shows the nucleic acid sequence of human Notch4 (SEQ ID NO: 89).

Figure 97

This Figure shows human Notch4 signal peptide sequence (SEQ ID NO: 90). The underlined sequence encodes Signal Peptide.

Figure 98

This Figure shows the nucleic acid sequence of human HC signal peptide (nt 1-66) (SEQ ID NO: 91).

Figure 99

This Figure shows the nucleic acid sequence of the human FC Tag (SEQ ID NO: 92).

Figure 100

This Figure shows the nucleic acid sequence which encodes h-Notch4$^{(1-29)}$ decoy protein (SEQ ID NO: 93). Human Notch4 decoy (EGF like repeats 1-29) [nt 1-3522].

Figure 101

This Figure shows the nucleic acid sequence which encodes h-Notch4$^{(1-13)}$ decoy protein (SEQ ID NO: 94). Human Notch4 decoy (EGF like repeats 1-13) [nt 1-1662].

Figure 102

This Figure shows the nucleic acid sequence which encodes h-Notch4$^{(1-23)}$ decoy protein (SEQ ID NO: 95). Human Notch4 decoy (EGF like repeats 1-23) [nt1-2799].

Figure 103

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch4$^{(9-29)}$ decoy protein (SEQ ID NO: 96). Human Notch4 decoy (EGF like repeats 9-29) [nt 1-81, 1075-3522].

Figure 104

This Figure shows the nucleic acid sequence which encodes

h-sp^{HC}Notch4$^{(9-29)}$ decoy protein (SEQ ID NO: 97). Human Notch4 decoy (EGF like repeats 9-29) [nt 1075-3522] & HC Signal Peptide [nt 1-66].

Figure 105

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch4$^{(9-23)}$ decoy protein (SEQ ID NO: 98). Human Notch4 decoy (EGF like repeats 9-23) [nt 1-81, 1075-2799].

Figure 106

This Figure shows the nucleic acid sequence which encodes h-sp^{HC}Notch4$^{(9-23)}$ decoy protein (SEQ ID NO: 99). Human Notch4 decoy (EGF like repeats 9-23) [nt 1075-2799] & HC Signal Peptide [nt 1-66].

Figure 107

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch4$^{(13-23)}$ decoy protein (SEQ ID NO: 100). Human Notch4 decoy (EGF like repeats 13-23) [nt 1-81, 1549-2799].

Figure 108

This Figure shows the nucleic acid sequence which encodes h-sp^{HC}Notch4$^{(13-23)}$ decoy protein (SEQ ID NO: 101). Human Notch4 decoy (EGF like repeats 13-23) [nt 1549-2799] & HC Signal Peptide [nt 1-66].

Figure 109

This Figure shows the nucleic acid sequence which encodes h-sp^{N}Notch4$^{(21-29)}$ decoy protein (SEQ ID NO: 102). Human Notch4 decoy (EGF like repeats 21-29) [nt 1-81, 2428-3522].

Figure 110

This Figure shows the nucleic acid sequence which encodes h-sp^{HC}Notch4$^{(21-29)}$ decoy protein (SEQ ID NO: 103). Human Notch4 decoy (EGF like repeats 21-29) [nt 2428-3522] & HC Signal Peptide [nt 1-66].

Figure 111

This Figure shows a schematization of eleven formulations of human Notch4 decoys.

Figure 112

This Figure shows signal sequence analysis of Notch 4 to determine where Notch4 signal peptide ends.

Figure 113

This Figure shows the human Notch4/Fc fusion sequence for all constructs that end after EGF Repeat 29 of human Notch4.

Figure 114

This Figure shows the human Notch4/Fc fusion sequence for all constructs that end after EGF Repeat 13 of human Notch4.

Figure 115

This Figure shows the human Notch4/Fc fusion sequence for all constructs that end after EGF Repeat 23 of human Notch4.

Figure 116

This Figure shows Notch4 insufficiency reduces circulating glucose and insulin levels. We are currently

analyzing the blood glucose and insulin levels of *Notch4* (*N4*) mutant mice. At weaning (P21), multiple litters generated from N4+/- matings have either been fed a normal diet or a high fat diet consisting of 45% of calories from fat. At 5 months of age, blood was draw and glucose and insulin levels determined from random fed mice or mice after 6 hours of fasting. In females, reduction in *N4* alleles correlated with a significant decrease in circulating glucose levels independent of the diet. In contrast, *N4* insufficiency was associated with a decrease in glucose levels in only males fed a normal diet. Insulin levels of all females were unaffected (data not shown). This may due to female mice being genetically protected against insulin resistance, and thus the metabolic abnormalities are exquisitely mild. On the normal diet, there were no differences in the insulin levels of the male mice. However, abalation of *N4* correlated with a significant decrease in the random fed insulin levels of male mice fed a high fat diet. A similar trend was observed for N4 knockout males that had been fasted 6 hours. Thus, loss of *N4* correlated with a significant decrease in blood glucose levels in both males and females fed a normal diet. In females, this reduction of glucose levels, but not insulin levels was observed in *N4* mutant females fed a high fat diet. In contrast, glucose levels were unchanged in *N4* knockout males, whereas insulin levels were reduced. These results are consistent with *Notch4* insufficiency protecting against genetic and environmental forms of hyperglycemia due to disrupted insulin signaling

Figure 117

This Figure shows loss of Notch4 expression suppressed weight gain in mice fed a high fat diet.

Figure 118

This Figure shows Rat Notch1 decoy is present in murine serum. The stability of the rat Notch1 decoy formulation in the mammalian blood stream was tested. Western blot analysis demonstrates that the full-length protein can be expressed in mice and is present at detectable levels with little evidence of degradation.

Figure 119

This Figure shows Human Notch 1 decoy (n-Notch(1-36)decoy) and rat Notch1 decoy block mouse mammary tumor growth. The growth curve presented here demonstrates that either Rat Notch1 decoy or human Notch1 decoy reduced the growth of tumor xenografts in nude mice.

Figure 120

This Figure shows rat Notch1 decoy inhibits SKNEP1 metastasis to lung tissue. SKNEP1 Ewings Sarcoma cells were programmed to express control Fc protein or rat Notch1 decoy s1 (sort 2) or rat Notch1 decoy s4 (sort 4). These SKNEP1 cell lines were orthotopically implanted into kidney of nude mice. After 6 weeks of tumor growth, metastasis to lung was assessed histologically. SKNEP1 cells expressing Rat Notch1 decoy showed fewer lungs that were positive for metastasis. We conclude that expression of the rat Notch1 decoy in nude mice diminishes the capacity of SKNEP1 cells to metastasize to lung.

Figure 121:

This figure shoes Notch1 and Notch4 are co-expressed with VEGFR-3 and LYVE-1 in lymphatics of mouse skin. The expression of Notch1 and Notch4 was analyzed in the vasculature of mouse P4 dorsal skin. At this time point, the dermal lymphatics are actively remodeling into the lymphatic capillaries near the surface and collecting ducts in the lower dermal layers. 5 μm cross-sections of skin were co-stained with antibodies against Notch1 or Notch4 (red), and PECAM, VEGFR-3 or LYVE-1 (green). Notch1 and Notch4 share an overlapping pattern of expression with the blood and lymphatic endothelial cell marker, PECAM (upper panels). Notch1 and Notch4 were co-expressed with both VEGFR-3 (middle panels) and LYVE-1 in the dermal vasculature (lower panels). This expression pattern demonstrates that Notch1 and Notch4 are expressed and may function in the lymphatic vessels of the neonatal dermis.

Figure 122

This Figure shows dermal lymphatic capillaries are altered in *Notch4* homozygous knockout mice. We examined the dermal lymphatics of P4 mice. Sections of wildtype and *Notch4* nullizygous were immunostained with antibodies against PECAM and LYVE-1 (green). Analysis of PECAM staining appeared similar between mutant and wildtype skin (upper panels). In contrast, LYVE-1-positive vessels in the dermis of *Notch4* mutants had a different morphology than that of wildtype (middle panels). *Notch4* mutant LYVE-1 vessels were often dilated and LYVE-1 staining was discontinuous (lower panels). These results suggest that Notch4 signaling may be involved in remodeling of the lymphatic vascular plexus.

Figure 123

This Figure shows loss of Notch4 correlates with reduced LYVE1 expression in murine dermal lymphatics. *Notch4* heterozygous (*N4+/-*) mice were mated and the dorsal skin of the resulting pups removed and embedded 14 days postnatally. Cros-sections of skin were immunostained for the endothelial cell marker, PECAM (data not shown), or the lymphatic endothelial cell marker, LYVE1 (A). Five areas for each were captured by microscopy and PECAM and LYVE1 staining quantitated using imaging software (B, C). PECAM expression was reduced approximately 25% in the *N4-/-* dermis compared to wild-type (WT) dermis (B). LYVE-1 staining was more affected than the PECAM with LYVE1 staining decreased nearly 50% in *N4-/-* relative to WT mice (C). There was also a reduction in the intensity of the LYVE1 staining in the *N4-/-* lymphatics relative to the WT (A).

Figure 124

This Figure shows Notch1 and Notch4 are expressed in human breast cancer lymphatic vessels. We performed double immunohistochemistry with antibodies against VEGFR-3 or LYVE-1 (green) and Notch1 or Notch4 (red) of human breast cancers. Notch1 and Notch4 were expressed in the extratumoral blood and lymphatic endothelium of human micropapillary breast carcinomas. To determine if Notch1 signaling was activated within the tumoral lymphatic endothelium, we double stained with an antibody against podoplanin (green) and N1Val (red; Cell Signaling), an antibody that specifically detects the activated Notch1 peptide. Expression of the activated Notch1 peptide was

observed in most (white arrows) but not all (yellow arrows) of the lymphatic endothelial nuclei(lower panel). These results demonstrate that Notch1 was actively signaling in the pathological lymphatic vessels.

5

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.

"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 F_c 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 beteen 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. "Extracellular domain", "ECD" and "Ectodomain" are synonymous.

"Half-life-increasing moiety" means a moiety which, when operably affixed to a second moiety, increases the in vivo half-life of the second moiety. Half-life-increasing moieties include, for example, Fc portions of antibodies, glycosylation tags (i.e. glycosylated polypeptides), polyethylene glycol (PEG), polypeptides having PEG affixed thereto, and lipid-modified polypeptides.

"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.

"Operably affixed" means, with respect to a first moiety affixed to a second moiety, affixed in a manner permitting the first moiety to function (e.g. binding properties) as it would were it not so affixed.

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 the preferred embodiment, the subject is a human being.

"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, Macular degeneration, Retinopathy of Prematurity, Pre-eclampsia, Diabetic retinopathy, Ischemia, Stroke, Cardiovascular Disease, Psoriasis, lymphedema, tumorigenesis and tumor lymphangiogenesis.

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 glucocse 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; EC: endothelial cells; FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; HUVEC: human umbilical vein endothelial cell; m.o.i.: multiplicity of infection; VMC: vascular mural cells; 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

This invention provides a fusion protein comprising a signal peptide, an extracellular domain of human Notch receptor protein and an Fc portion of an antibody bound thereto.

In a first embodiment of the fusion protein, the Notch receptor protein is Notch1 receptor protein. In one embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-36. In another embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-13. In another embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-24. In another embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 9-23. In another

embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 9-36. In another embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 13-24. In a further embodiment, the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 25-36.

In a second embodiment of the fusion protein, the Notch receptor protein is Notch2 receptor protein.

In a third embodiment of the fusion protein, the Notch receptor protein is Notch3 receptor protein.

In a fourth embodiment of the fusion protein, the Notch receptor protein is Notch4 receptor protein. In one embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 1-29. In another embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 1-13. In another embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 1-23. In another embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 9-23. In another embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 9-29. In another embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 13-23. In a further embodiment, the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 21-29.

In one embodiment of the fusion protein, the Fc portion of the antibody is the Fc portion of a human antibody.

In one embodiment of the fusion protein, the signal peptide is the signal peptide of Notch1, Notch2, Notch3, Notch4, or the Hc (HC; Heavy Chain) portion of an antibody.

In one embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 54. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 55. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 56. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 57. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 58. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 59. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 60. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 61. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 62. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 63. In a further embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 64.

In one embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 65. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 66. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 67. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 68. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 69. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 70. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 71. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 72. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 73. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 74. In a further embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 75.

In one embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 78. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence

of which is set forth in SEQ ID NO: 79. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 80. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 81. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 82. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 83. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 84. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 85. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 86. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 87. In another embodiment, the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 88.

In one embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 89. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 90. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 91. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the

sequence of which is set forth in SEQ ID NO: 92. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 93. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 94. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 95. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 96. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 97. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 98. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 99. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 100. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 101. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 102. In another embodiment, the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 103.

This invention provides a method for treating a subject having a tumor comprising administering to the subject an amount of the above fusion protein effective to treat the

subject, thereby treating the subject having a tumor.

This invention provides a method for inhibiting angiogenesis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit angiogenesis in the subject, thereby inhibiting angiogenesis in the subject.

This invention provides a method for treating a subject having ovarian cancer comprising administering to the subject an amount of the above fusion protein effective to treat the subject, thereby treating the subject having ovarian cancer.

This invention provides a method for treating a subject having a metabolic disorder comprising administering to the subject an amount of the above fusion protein effective to treat the subject, thereby treating the subject having a metabolic disorder. In one embodiment, the metabolic disorder is diabetes, obesity, atherosclerosis, ischemia, stroke, or cardiovascular disease.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for the treatment of a subject having a tumor.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for inhibiting angiogenesis in a subject.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for

treating a subject having ovarian cancer.

This invention provides use of the above fusion protein for the preparation of a pharmaceutical composition for for treating a subject having a metabolic disorder. In one embodiment, the metabolic disorder is diabetes, obesity, atherosclerosis, ischemia, stroke, or cardiovascular disease.

This invention provides a method for inhibiting physiological lymphangiogenesis or pathological lymphangionesis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit physiological lymphangiogenesis or pathological lymphangionesis in the subject. In one embodiment the pathological lymphangiogenesis is tumor lymphangiogenesis or lymph node metastasis that may be dependent on tumor lymphangiogenesis.

This invention provides method of inhibiting tumor metastasis in a subject comprising administering to the subject an amount of the above fusion effective to inhibit tumor metastasis in the subject. In on embodiment, the metastasis occurs via a blood vessel, the lymphatic vasculature or a lymph node. Tumor metastasis is the spread of cancer from one organ to another non-adjacent organ.

This invention provides a method of inhibiting growth of a secondary tumor in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit growth of the secondary tumor in the subject. Inhibition may also be of the tumor

angiogenesis associated with the secondary or metastatic tumor. In one embodiment the secondary tumor growth is inhibited by inhibition of angiogenesis associated with the secondary tumor.

This invention provides a method of inhibiting blood vessel cooption by a tumor in subject comprising administering to the subject an amount of the above fusion protein effective to inhibit blood vessel cooption by a tumor in the subject. The process of vessel coopion is a process whereby tumor cells associate with pre-existing vessels and growth with assistance of coopted vessels. This growth of tumors on coopted vessels may be in the absence of, precede, or be in conjunction with tumor angiogenesis.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of Vascular Endothelial Growth Factor (VEGF), each in an amount effective to treat the cancer in the subject. In one embodiment the inhibitor of VEGF is an inhibitor of VEGF-A, an inhibitor of P1GF, an inhibitor of VEGF-B, an inhibitor of VEGF-C, or an inhibitor of VEGF-D. Examples of VEGF-inhibitors include, but are not limited to, bevacizumab, PTK787, Bay43-9006, SU11248, AG013676, ZD6474, VEGF-trap and Anti-VEGFR2. Examples of such inhibitors are more fully described in Ferrara et al., (2004) *Nature Reviews Drug Discovery*, Vol.3:391-400 and Ellis et al. (2008) *Nature Reviews Cancer* Vol8:579-591, the contents of each of which are hereby incorporated by reference.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of VEGFR-1, VEGFR-2 or VEGFR-3, each in an amount effective to treat the cancer in the subject. In one embodiment, the inhibitor targets one or more of the VEGFR.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of Platelet Derived Growth Factor (PDGF), each in an amount effective to treat the cancer in the subject. In on embodiment the inhibitor of Platelet Derived Growth Factors is an inhibitor of PDGF-A or an inhibitor of PDGF-B

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and a PDGF receptor antagonist, each in an amount effective to treat the cancer in the subject. In one embodiment the PDGF receptor antagonist is a PDGF Receptor-B antagonist.

This invention provides a method of treating cancer in a subject comprising administering to the subject the above fusion protein and an inhibitor of HER2/neu, each in an amount effective to treat the cancer in the subject.

This invention provides a method of treating vascular proliferative retinopathy comprising administering to the subject the above fusion protein in an emount effective to treat the vascular proliferative retinopathy.

101. The method of claim 100, wherein the vascular

proliferative retinopathy is diabetic retinopathy, macular defernation or retinopathy of prematurity

This invention also provides a first method for treating a subject having a tumor comprising administering to the subject an effective amount of a composition of matter comprising the extracellular domain of a Notch receptor protein operably affixed to a half-life-increasing moiety, so as to thereby treat the subject.

This invention also provides a second method for inhibiting angiogenesis in a subject comprising administering to the subject an effective amount of a composition of matter comprising the extracellular domain of a Notch receptor protein operably affixed to a half-life-increasing moiety, so as to thereby inhibit angiogenesis in the subject.

In a first embodiment of the above methods, the Notch receptor protein is Notch1 receptor protein. In one embodiment, the Notch1 receptor protein is human Notch1 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the half-life-increasing moiety are within the same polypeptide chain.

In a second embodiment of the above methods, the Notch receptor protein is Notch2 receptor protein. In one embodiment, the Notch2 receptor protein is human Notch2 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In

another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the half-life-increasing moiety are within the same polypeptide chain.

In a third embodiment of the above methods, the Notch receptor protein is Notch3 receptor protein. In one embodiment, the Notch3 receptor protein is human Notch3 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the half-life-increasing moiety are within the same polypeptide chain.

In a fourth embodiment of the above methods, the Notch receptor protein is Notch4 receptor protein. In one embodiment, the Notch4 receptor protein is human Notch4 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the half-life-increasing moiety are within the same polypeptide chain.

In a fifth embodiment of the above methods, the subject is a mammal. In one embodiment, the mammal is a human.

In a sixth embodiment of the above methods, the angiogenesis is tumor angiogenesis.

In a further embodiment of the second method, the subject has a tumor. In another embodiment, the subject is

afflicted with a pathologic vascular hyperplasia. In one embodiment, the pathologic vascular hyperplasia is a benign hemagioma. In a further embodiment, the subject is afflicted with a lymphatic vascular proliferative disease.

This invention provides a first composition of matter comprising the extracellular domain of Notch4 receptor protein operably affixed to a half-life-increasing moiety. In one embodiment, the extracellular domain is covalently bound to the half-life-increasing moiety. In another embodiment, the extracellular domain and the half-life-increasing moiety are within the same polypeptide chain.

This invention also provides a second composition of matter comprising the extracellular domain of Notch4 receptor protein operably affixed to a half-life-increasing moiety and a pharmaceutically acceptable carrier.

This invention further provides an article of manufacture comprising (i) a packaging material having therein a composition of matter comprising the extracellular domain of a Notch receptor protein operably affixed to a half-life-increasing moiety and (ii) a label indicating that the composition is intended for use in treating a subject having a tumor or other disorder treatable by inhibiting angiogenesis in the subject.

In a first embodiment of the above article, the Notch receptor protein is Notch1 receptor protein. In one embodiment, the Notch1 receptor protein is human Notch1

receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the Half-life-increasing moiety are within the same polypeptide chain.

In a second embodiment of the above article, the Notch receptor protein is Notch2 receptor protein. In one embodiment, the Notch2 receptor protein is human Notch2 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the Half-life-increasing moiety are within the same polypeptide chain.

In a third embodiment of the above article, the Notch receptor protein is Notch3 receptor protein. In one embodiment, the Notch3 receptor protein is human Notch3 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the Fc portion of a human antibody. In a further embodiment, the extracellular domain and the Half-life-increasing moiety are within the same polypeptide chain.

In a fourth embodiment of the above article, the Notch receptor protein is Notch4 receptor protein. In one embodiment, the Notch4 receptor protein is human Notch4 receptor protein. In another embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the Fc portion of the antibody is the

Fc portion of a human antibody. In a further embodiment, the extracellular domain and the Half-life-increasing moiety are within the same polypeptide chain.

In another embodiment of the above article, the composition is admixed with a pharmaceutical carrier. In a final embodiment, the subject is a human.

This invention provides a replicable vector which encodes a polypeptide comprising the extracellular domain of a Notch4 receptor protein operably affixed to a half-life-increasing moiety. In one embodiment, the half-life-increasing moiety is an Fc portion of an antibody. In another embodiment, the vector includes, without limitation, a plasmid, a cosmid, a retrovirus, an adenovirus, a lambda phage or a YAC.

This invention also provides a host vector system which comprises a replicable vector which encodes a polypeptide comprising the extracellular domain of a Notch receptor protein operably affixed to a half-life-increasing moiety and a suitable host cell. In one embodiment, the host cell is a eukaryotic cell. In another embodiment, the eukaryotic cell is a CHO cell. In a another embodiment, the eukaryotic cell is a HeLa cell. In a further embodiment, the host cell is a bacterial cell.

Finally, this invention provides a third method of producing a polypeptide which comprises growing a host vector system which comprises a replicable vector which encodes a polypeptide comprising the extracellular domain of a Notch receptor protein operably affixed to a half-life-increasing moiety and a suitable host cell under

conditions permitting production of the polypeptide, and recovering the polypeptide so produced.

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

Human Notch1 Fusion Proteins (Notch Decoys)

The Notch1 decoys are assembled using sequences encoding a signal peptide, a portion of the Notch1 extracellular domain encompassing all or a subset of the EGF-like repeat domains, and a portion of the human Fc protein (amino acids 1-237). The complete full-length sequence of human Notch1 is provided in Figure 59.

The signal peptides utilized are either the native Notch1 signal peptide or the human Hc signal peptide, each fused to a region of Notch1. The signal peptide allows for secretion of the Notch decoy proteins.

The Notch1 extracellular domains used are designed to bind to Notch ligands and consist of all or a subset of the 36 EGF-like repeat domains of the human Notch1 protein.

The Fc tag is fused to the C-terminus of a given EGF-like repeat of human Notch1 and serves to allow for purification, detection, and stabilization of the Notch1 decoy proteins.

The overall design of the human Notch1 decoys, eleven formulations, is to encode for; (1) a signal peptide to allow for secretion of Notch1 decoy proteins into the extracellular media of eukaryotic cells that are used to

produce the proteins, (2) a portion of the extracellular domain of all or a portion of the EGF-like repeats of human Notch1 to allow for association with Notch ligands, and (3) a portion of the human Fc protein to allow for detection.

The following eleven formulations of human Notch1 decoys will be described and are schematized in Figure 52.

1) h-Notch1$^{(1-36)}$ decoy (N1-1 of Figure 52)
2) h-Notch1$^{(1-13)}$ decoy (N1-2 of Figure 52)
3) h-Notch1$^{(1-24)}$ decoy (N1-3 of Figure 52)
4) h-sp^{N}Notch1$^{(9-23)}$ decoy (N1-4 of Figure 52)
5) h-sp^{HC}Notch1$^{(9-23)}$ decoy (N1-5 of Figure 52)
6) h-sp^{N}Notch1$^{(9-36)}$ decoy (N1-6 of Figure 52)
7) h-sp^{HC}Notch1$^{(9-36)}$ decoy (N1-7 of Figure 52)
8) h-sp^{N}Notch1$^{(13-24)}$ decoy (N1-8 of Figure 52)
9) h-sp^{HC}Notch1$^{(13-24)}$ decoy (N1-9 of Figure 52)
10) h-sp^{N}Notch1$^{(25-36)}$ decoy (N1-10 of Figure 52)
11) h-sp^{HC}Notch1$^{(25-36)}$ decoy (N1-11 of Figure 52)

Human Notch1 sequence

The full-length amino acid (aa) sequence of human Notch1, consisting of aa residue 1 (M = methionine) to aa residue 2555 (K = lysine) is set forth in Figure 59. The signal peptide and first 36 EGF-like repeat domains are present in aa 1-1433 of this sequence. Amino acids 1-1433, or a subset of these aa, were utilized for the design of the human Notch1 decoy proteins, described in the ensuing sections. The amino acids encompassing EGF-repeats 1-36 are underlined.

Human Fc sequence utilized to generate the Fc tag on Notch1 decoy proteins

The 237 amino acids of human Fc, shown in Figure 60, were fused at the C-terminus of all Notch1 decoy constructs, just downstream of Notch1 EGF-like repeats. This region of human Fc allows for detection and purification of the Notch decoys and serves to stabilize the secreted human Notch1-human Fc fusion proteins.

Signal Peptides utilized in Notch1 decoy proteins

Two distinct signal peptide sequences were incorporated into the design of the human Notch1 decoy proteins. The first is the human Notch1 signal peptide that is predicted to encompass amino acids 1-20 of human Notch1. This determination was made using the Signal IP 3.0 Server program provided by the Technical University of Denmark. The second is the human Hc signal peptide that is predicted to encompass amino acids 1-22 of human IgG heavy chain (HC) signal peptide.

1. Human Notch1 signal peptide (aa 1-20)

MPPLLAPLLCLALLPALA/A/R (SEQ ID NO:16)

Amino acid sequence of the predicted human Notch1 signal peptide is schematized in Figure 53. The prediction results of analysis utilizing the SignalIP 3.0 Server provided online by the Technical University of Denmark are shown in Figure 53. These results predict a major site of cleavage located between alanine 18 (A18) and alanine 19 (A19) and a minor site of cleavage between A19 and Arginine 20 (R20). These two cleavage sites are

indicated by the "/" in amino acid sequence 1-20 of human Notch1, provided above.

2. Human Notch1 signal peptide fusion peptide (aa 1-23) utilized in Notch1 decoys that utilize this signal sequence.

In order to make sure that the Notch1 signal peptide is utilized efficiently three additional amino acids beyond the predicted minor site of cleavage are provided in the human Notch1 decoys. Thus the amino acid sequence utilized in the human Notch1 decoy formulation, that incorporate a Notch1 signal peptide, contains glycine-proline-arginine (GPR- bold/underlined) between the sites of predicted signal peptide cleavage and the Notch1 EGF-like repeats as shown below.

MPPLLAPLLCLALLPALAAR**GPR** (SEQ ID NO:130)

3. Human HC signal peptide (aa 1-22)

The amino acid sequence of the predicted human Hc signal peptide is

MWGWKCLLFWAVLVTATLCTA/R (SEQ ID NO:18)

The prediction results of analysis utilizing the SignalIP 3.0 Server provided online by the Technical University of Denmark are shown above. These results predict a major site of cleavage located between alanine 21 (A21) and arginine 22 (22). This cleavage site is indicated by the "/" in amino acid sequence 1-22 of human Hc provided

above.

h-Notch1$^{(1-36)}$ decoy

h-Notch1$^{(1-36)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 1-36 of Notch1 (N1-1 of Figure 52).

h-Notch1$^{(1-36)}$ decoy protein which is set forth in Figure 61 consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 1-36 of human Notch1 consisting of amino acids 24-1433 followed by (3) amino acids 1434-1670 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1670 amino acids.

h-Notch1$^{(1-13)}$ decoy

h-Notch1$^{(1-13)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 1-13 of Notch1 (N1-2 of Figure 52).

h-Notch1$^{(1-13)}$ decoy protein which is set forth in Figure 62 consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 1-13 of human Notch1 consisting of amino acids 24-531 followed by (3) amino acids 532-768 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 768 amino acids.

h-Notch1$^{(1-24)}$ decoy

h-Notch1$^{(1-24)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 1-24 of Notch1 (N1-3 of Figure 52).

h-Notch1$^{(1-24)}$ decoy protein which is set forth in Figure 63 consists of the following three components:

(1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 1-24 of human Notch1 consisting of amino acids 24-948 followed by (3) amino acids 949-1185 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1185 amino acids.

h-sp^{N}Notch1$^{(9-23)}$ decoy

h-sp^{N}Notch1$^{(9-23)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 9-23 of Notch1 (N1-4 of Figure 52). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation.

h-sp^{N}Notch1$^{(9-23)}$ decoy protein which is set forth in Figure 64 consists of the following three components:

(1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch1 consisting of amino acids 24-593 followed by (3) amino acids 594-830 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 830 amino acids.

h-sp^{HC}Notch1$^{(9-23)}$ decoy

h-sp^{HC}Notch1$^{(9-23)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 9-23 of Notch1 (N1-5 of Figure 52). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch1$^{(9-23)}$ decoy protein which is set forth in Figure 65 consists of the following three components: (1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch1 consisting of amino acids 23-592 followed by (3) amino acids 593-829 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 829 amino acids.

h-sp^{N}Notch1$^{(9-36)}$ decoy

h-sp^{N}Notch1$^{(9-36)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 9-36 of Notch1 (N1-6 of Figure 52). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation.

h-sp^{N}Notch1$^{(9-36)}$ decoy protein which is set forth in Figure 66 consists of the following three components:
(1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 9-36 of human Notch1 consisting of amino acids 24-1118 followed by (3) amino acids 1119-1355 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This

formulation contains 1355 amino acids.

h-sp^{HC}Notch1$^{(9-36)}$ decoy

h-sp^{HC}Notch1$^{(9-36)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 9-36 of Notch1 (N1-7 of Figure 52). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch1$^{(9-36)}$ decoy protein which is set forth in Figure 67 consists of the following three components: (1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 9-36 of human Notch1 consisting of amino acids 23-1117 followed by (3) amino acids 1118-1354 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1354 amino acids.

h-sp^{N}Notch1$^{(13-24)}$ decoy

h-sp^{N}Notch1$^{(13-24)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 13-24 of Notch1 (N1-8 of Figure 52). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation.

h-sp^{N}Notch1$^{(13-24)}$ decoy protein which is set forth in Figure 68 consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 13-24 of human Notch1 consisting of amino acids 24-478 followed by (3) amino acids 479-715 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the

human Fc tag is underlined and italicized. This formulation contains 715 amino acids.

h-sp^{HC}Notch1$^{(13-24)}$ decoy

h-sp^{HC}Notch1$^{(13-24)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 13-24 of Notch1 (N1-9 of Figure 52). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch1$^{(13-24)}$ decoy protein which is set forth in Figure 69 consists of the following three components: (1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 13-24 of human Notch1 consisting of amino acids 23-477 followed by (3) amino acids 478-714 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 714 amino acids.

h-sp^{N}Notch1$^{(25-36)}$ decoy

h-sp^{N}Notch1$^{(25-36)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 25-36 of Notch1 (N1-10 of Figure 52). The abbreviation sp^{N} denotes that the human Notch1 signal peptide is used in this formulation.

h-sp^{N}Notch1$^{(25-36)}$ decoy protein which is set forth in Figure 70 consists of the following three components: (1) human Notch1 signal sequence consisting of amino acids 1-23 of human Notch1, followed by (2) amino acids encoding the EGF-like repeats 25-36 of human Notch1 consisting of amino acids 24-508 followed by (3) amino

acids 509-745 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 745 amino acids.

h-sp^{HC}Notch1$^{(25-36)}$ decoy

h-sp^{HC}Notch1$^{(25-36)}$ decoy denotes the human Notch1 decoy that encompass EGF-like repeats 25-36 of Notch1 (N1-11 of Figure 52). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch1$^{(25-36)}$ decoy protein which is set forth in Figure 71 consists of the following three components: (1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 25-36 of human Notch1 consisting of amino acids 23-507 followed by (3) amino acids 508-744 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 744 amino acids.

Methods

Construction of human Notch1 decoys

Total RNA from human umbilical venous endothelial cells (HUVEC) was used to generate the human Notch1 decoy variants. Total RNA was reverse transcribed with M-MLV reverse transcriptase and either random hexamer primers or a Notch1 decoy specific primer. The synthesized cDNA was then amplified with Notch1 decoy specific upstream (sense) and downstream (antisense) primers.

The downstream primer encodes either BamHI or BglII restriction site at the 5′ end that will ligate with the BglII site in the Fc sequence to generate an in frame human Notch1/Fc chimera.

In the case of Notch1 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 36, a BglII site will be generated to create the fusion site and this fusion sequence is provided (Notch1, Figure 4). This applies to formulations; h-Notch1$^{(1-36)}$ decoy, h-sp^{N}Notch1$^{(9-36)}$ decoy, h-sp^{HC}Notch1$^{(9-36)}$ decoy, h-sp^{N}Notch1$^{(25-36)}$ decoy, h-sp^{HC}Notch1$^{(25-36)}$ decoy.

In the case of Notch1 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 13, a BamHI site will be generated to create the fusion site and this fusion sequence is provided (Notch1, Figure 5). This applies to formulation h-Notch1$^{(1-13)}$ decoy.

In the case of Notch1 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 23, a BglII site will be generated to create the fusion site and this fusion sequence is provided (Notch1, Figure 6). This applies to formulations h-sp^{N}Notch1$^{(9-23)}$ decoy, h-sp^{HC}Notch1$^{(9-23)}$ decoy.

In the case of Notch1 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 24, a BglII site will be generated to create the fusion site and this fusion sequence is provided (Notch1, Figure 7). This applies to formulations h-Notch1$^{(1-24)}$ decoy, h-sp^{N}Notch1$^{(13-24)}$ decoy, h-sp^{HC}Notch1$^{(13-24)}$ decoy.

The amplified PCR product is sublconed into pBluescript SK II Fc to generate the different human Notch1/Fc chimeras. The human Notch1/Fc decoy sequences are then shuttled into mammalian expression vectors (pAd-lox, pCCL, pcDNA3) for expression and purification of human Notch1 decoy proteins.

Human Notch4 Fusion Proteins (Notch Decoys)

The Notch4 decoys are assembled using sequences encoding a signal peptide, a portion of the Notch4 extracellular domain encompassing all or a subset of the EGF-like repeat domains, and a portion of the human Fc protein (amino acids 1-237). The complete full-length sequence of human Notch4 is provided in Figure 83.

The signal peptides utilized are either the native Notch4 signal peptide or the human Hc signal peptide, each fused to a region of Notch4. The signal peptide allows for secretion of the Notch decoy proteins.

The Notch4 extracellular domains used are designed to bind to Notch ligands and consist of all or a subset of the 29 EGF-like repeat domains of the human Notch4 protein

The Fc tag is fused to the C-terminus of a given EGF-like repeat of human Notch4 and serves to allow for purification, detection, and stabilization of the Notch4 decoy proteins.

The overall design of the human Notch4 decoys, eleven formulations, is to encode for; (1) a signal peptide to allow for secretion of Notch4 decoy proteins into the extracellular media of eukaryotic cells that are used to produce the proteins, (2) a portion of the extracellular domain of all or a portion of the EGF-like repeats of human Notch4 to allow for association with Notch ligands, and (3) a portion of the human Fc protein to allow for detection.

The following eleven formulations of human Notch4 decoys will be described and are schematized in Figure 111.

1) h-Notch4$^{(1-29)}$ decoy (N4-1 of Figure 111)
2) h-Notch4$^{(1-13)}$ decoy (N4-2 of Figure 111)
3) h-Notch4$^{(1-23)}$ decoy (N4-3 of Figure 111)
4) h-sp^{N}Notch4$^{(9-23)}$ decoy (N4-4 of Figure 111)
5) h-sp^{HC}Notch4$^{(9-23)}$ decoy (N4-5 of Figure 111)
6) h-sp^{N}Notch4$^{(9-29)}$ decoy (N4-6 of Figure 111)
7) h-sp^{HC}Notch4$^{(9-29)}$ decoy (N4-7 of Figure 111)
8) h-sp^{N}Notch4$^{(13-23)}$ decoy (N4-8 of Figure 111)
9) h-sp^{HC}Notch4$^{(13-23)}$ decoy (N4-9 of Figure 111)
10) h-sp^{N}Notch4$^{(21-29)}$ decoy (N4-10 of Figure 111)
11) h-sp^{HC}Notch4$^{(21-29)}$ decoy (N4-11 of Figure 111)

Human Notch4 sequence

The full-length amino acid (aa) sequence of human Notch4, consisting of aa residue 1 (M = methionine) to aa residue 2003 (K = lysine) is set forth in Figure 83. The signal peptide and first 29 EGF-like repeat domains are present in aa 1-1174 of this sequence. Amino acids 1-1174, or a subset of these aa, were utilized for the design of the human Notch4 decoy proteins, described in the ensuing

sections. The amino acids encompassing EGF-repeats 1-29 are underlined.

Human Fc sequence utilized to generate the Fc tag on Notch4 decoy proteins

The 237 amino acids of human Fc, shown in Figure 84, were fused at the C-terminus of all Notch4 decoy constructs, just downstream of Notch4 EGF-like repeats. This region of human Fc allows for detection and purification of the Notch decoys and serves to stabilize the secreted human Notch4-human Fc fusion proteins.

Signal Peptides utilized in Notch4 decoy proteins

Two distinct signal peptide sequences were incorporated into the design of the human Notch4 decoy proteins. The first is the human Notch4 signal peptide that is predicted to encompass amino acids 1-24 of human Notch4. This determination was made using the Signal IP 3.0 Server program provided by the Technical University of Denmark. The second is the human Hc signal peptide that is predicted to encompass amino acids 1-22 of human Hc.

1. Human Notch4 signal peptide (aa 1-24)

MQPPSLLLLLLLLLLLCVSVVRP/R (SEQ ID NO:104)

Amino acid sequence of the predicted human Notch4 signal peptide is schematized in Figure 112. The prediction results of analysis utilizing the SignalIP 3.0 Server provided online by the Technical University of Denmark are shown in Figure 112. These results predict a site of cleavage located between proline 23 (A23) and and

Arginine 24 (R24). The cleavage site is indicated by the "/" in amino acid sequence 1-24 of human Notch4, provided above.

2. Human Notch4 signal peptide fusion peptide (aa 1-27) utilized in Notch4 decoys that utilize this signal sequence

In order to make sure that the Notch4 signal peptide is utilized efficiently three additional amino acids beyond the predicted minor site of cleavage are provided in the human Notch4 decoys. Thus the amino acid sequence utilized in the human Notch4 decoy formulation, that incorporate a Notch4 signal peptide, contains glycine-proline-arginine (GLL- bold/underlined) between the sites of predicted signal peptide cleavage and the Notch4 EGF-like repeats.

MQPPSLLLLLLLLLLLCVSVVRPR**GLL** (SEQ ID NO: 131)

3. Human HC signal peptide (aa 1-22)

The amino acid sequence of the predicted human Hc signal peptide is

MWGWKCLLFWAVLVTATLCTA/R (SEQ ID NO: 17)

The prediction results of analysis utilizing the SignalIP 3.0 Server provided online by the Technical University of Denmark are shown above. These resutls predict a major site of cleavage located between alanine 21 (A21) and arginine 22 (22). This cleavage sites is indicated by the

"/" in amino acid sequence 1-22 of human Hc provided above.

h-Notch4$^{(1-29)}$ decoy

h-Notch4$^{(1-29)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 1-29 of Notch4 (N4-1 of Figure 111).

h-Notch4$^{(1-29)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 1-29 of human Notch4 consisting of amino acids 28-1173 followed by (3) amino acids 1174-1410 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1410 amino acids.

h-Notch4$^{(1-13)}$ decoy

h-Notch4$^{(1-13)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 1-13 of Notch4 (N4-2 of Figure 111).

h-Notch4$^{(1-13)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 1-13 of human Notch4 consisting of amino acids 28-554 followed by (3) amino acids 555-791 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This

formulation contains 791 amino acids.

h-Notch4$^{(1-23)}$ decoy

h-Notch4$^{(1-23)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 1-23 of Notch4 (N4-3 of Figure 111).

h-Notch4$^{(1-23)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 1-23 of human Notch4 consisting of amino acids 28-933 followed by (3) amino acids 934-1170 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1170 amino acids.

h-sp^{N}Notch4$^{(9-23)}$ decoy

h-sp^{N}Notch4$^{(9-23)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 9-23 of Notch4 (N4-4 of Figure 111). The abbreviation sp^{N} denotes that the human Notch4 signal peptide is used in this formulation.

h-sp^{N}Notch4$^{(9-23)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch4 consisting of amino acids 28-602 followed by (3) amino acids 603-839 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This

formulation contains 839 amino acids.

h-sp^{HC}Notch4$^{(9-23)}$ decoy

h-sp^{HC}Notch4$^{(9-23)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 9-23 of Notch4 (N4-5 of Figure 111). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch4$^{(9-23)}$ decoy protein consists of the following three components:

(1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 9-23 of human Notch4 consisting of amino acids 23-597 followed by (3) amino acids 598-834 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 834 amino acids.

h-sp^{N}Notch4$^{(9-29)}$ decoy

h-sp^{N}Notch4$^{(9-29)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 9-29 of Notch4 (N4-6 of Figure 111). The abbreviation sp^{N} denotes that the human Notch4 signal peptide is used in this formulation.

h-sp^{N}Notch4$^{(9-29)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 9-29 of human Notch4 consisting of amino acids 28-843 followed by (3) amino acids 844-1080 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the

human Fc tag is underlined and italicized. This formulation contains 1080 amino acids.

h-sp^{HC}Notch4$^{(9-29)}$ decoy

h-sp^{HC}Notch4$^{(9-29)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 9-29 of Notch4 (N4-7 of Figure 111). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch4$^{(9-29)}$ decoy protein consists of the following three components:

(1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 9-29 of human Notch4consisting of amino acids 23-838 followed by (3) amino acids 839-1075 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 1075 amino acids.

h-sp^{N}Notch4$^{(13-23)}$ decoy

h-sp^{N}Notch4$^{(13-23)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 13-23 of Notch4 (N4-8 of Figure 111). The abbreviation sp^{N} denotes that the human Notch4 signal peptide is used in this formulation.

h-sp^{N}Notch4$^{(13-23)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 13-23 of human Notch4 consisting of amino acids 28-444 followed by (3) amino acids 445-681 that contain the human Fc tag. The

predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 681 amino acids.

h-sp^{HC}Notch4$^{(13-23)}$ decoy

h-sp^{HC}Notch4$^{(13-23)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 13-23 of Notch4 (N4-9 of Figure 111). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch4$^{(13-23)}$ decoy protein consists of the following three components:

(1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 13-23 of human Notch4 consisting of amino acids 23-439 followed by (3) amino acids 440-676 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 676 amino acids.

h-sp^{N}Notch4$^{(21-29)}$ decoy

h-sp^{N}Notch4$^{(21-29)}$ decoy denotes the human Notch4 decoy that encompasses EGF-like repeats 21-29 of Notch4 (N4-10 of Figure 111). The abbreviation sp^{N} denotes that the human Notch4 signal peptide is used in this formulation.

h-sp^{N}Notch4$^{(21-29)}$ decoy protein consists of the following three components:

(1) human Notch4 signal sequence consisting of amino acids 1-27 of human Notch4, followed by (2) amino acids encoding the EGF-like repeats 21-29 of human Notch4

consisting of amino acids 28-392 followed by (3) amino acids 393-629 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 629 amino acids.

h-sp^{HC}Notch4$^{(21-29)}$ decoy

h-sp^{HC}Notch4$^{(21-29)}$ decoy denotes the human Notch4 decoy that encompass EGF-like repeats 21-29 of Notch4 (N4-11 of Figure 111). The abbreviation sp^{HC} denotes that the human Hc signal peptide is used in this formulation.

h-sp^{HC}Notch4$^{(21-29)}$ decoy protein consists of the following three components:

(1) human Hc signal sequence consisting of amino acids 1-22 of human Hc, followed by (2) amino acids encoding the EGF-like repeats 21-29 of human Notch4 consisting of amino acids 23-387 followed by (3) amino acids 388-624 that contain the human Fc tag. The predicted signal peptide sequence is underlined and the human Fc tag is underlined and italicized. This formulation contains 624 amino acids.

Methods

Construction of human Notch4 decoys

Total RNA from human umbilical venous endothelial cells (HUVEC) was used to generate the human Notch4 decoy variants. Total RNA was reverse transcribed with M-MLV reverse transcriptase and either random hexamer primers or a Notch4 decoy specific primer. The synthesized cDNA was then amplified with Notch4 decoy specific upstream

(sense) and downstream (antisense) primers.

The downstream primer encodes either BamHI or BglII restriction site at the 5′ end that will ligate with the BglII site in the Fc sequence to generate an in frame human Notch4/Fc chimera.

In the case of Notch4 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 29, a BglII site will be generated to create the fusion site and this fusion sequence is provided (Notch4, Figure 113). This applies to formulations; h-Notch4$^{(1-29)}$ decoy, h-sp^{N}Notch4$^{(9-29)}$ decoy, h-sp^{HC}Notch4$^{(9-29)}$ decoy, h-sp^{N}Notch4$^{(21-29)}$ decoy, h-sp^{HC}Notch4$^{(21-29)}$ decoy.

In the case of Notch4 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 13, a BamHI site will be generated to create the fusion site and this fusion sequence is provided (Notch4, Figure 114). This applies to formulation h-Notch4$^{(1-13)}$ decoy.

In the case of Notch4 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 23, a BglII site will be generated to create the fusion site and this fusion sequence is provided (Notch4, Figure 115). This applies to formulations h-Notch4$^{(1-23)}$ decoy, h-sp^{N}Notch4$^{(9-23)}$ decoy, h-sp^{HC}Notch4$^{(9-23)}$ decoy, h-sp^{N}Notch4$^{(13-23)}$ decoy, h-sp^{HC}Notch4$^{(13-23)}$ decoy.

The amplified PCR product is sublconed into pBluescript SK II Fc to generate the different human Notch4/Fc chimeras. The human Notch4/Fc decoy sequences are then shuttled into mammalian expression vectors (pAd-lox,

pCCL) for expression and purification of human Notch4 decoy proteins.

5

Second Series of Experiments

Materials & Methods

Plasmid Constructs

Adenovirus constructs encoding LacZ, full-length Notch4, or the activated form of Notch4/int3 have been previously described (Shawber et al., 2003). An activated form of Notch1 cDNA fused in frame with 6 myc tags (Kopan et al., 1994) was cloned into the adenovirus expression vector, pAd-lox. Both VEGF165 and N1ECDFc was also cloned into the pAd-lox. Adenoviral stocks were generated and titered as previously described (Hardy et al., 1997). The retroviral expression vector pHyTc encoding either LacZ, the activated form of Notch4/int3, J1, Dll1 and Dll4 have been previously described (Uyttendaele et al., 2000, Shawber et al., 2003, Das et al., 2004 in print). Plasmids encoding the intracellular domain of Notch1 (bp 5479-7833, Genbank accession# X57405) and the extracellular domain of Dll4 (bp 1-1545, Genbank accession# AF253468, provided by Chiron) fused in frame with a myc/His tag, were engineered into pHyTC.

Notch1ECD, Notch2ECD, Notch3ECD and Notch4ECD are engineered using the Fc sequences contained in the plasmid pCMX-sFR1-IgG using the methods set forth in *Clin. Exp. Immunol.* (1992) 87(1):105-110 to create the Notch-based fusion proteins, i.e. Notch1ECD/Fc, Notch2ECD/Fc, Notch3ECD/Fc and Notch4ECD/Fc.

Adenoviral Gene Transfer

7.5×10^5 cells of HUVEC at passage 3 were seeded into type

I collagen-coated 6 well plates on the day before adenoviral infection. Adenoviral infection with Ad-lacZ, Ad-VEGF165 or Ad-N1ECDFc was performed at indicated m.o.i., and incubated at 37ºC for 1 hr with occasional swirling of plates.

Luciferase reporter assays

To determine ligand-induced Notch signaling, co-culture assays were performed using HeLa and 293-derived Bosc cells. Transient transfections were performed by calcium phosphate precipitation. Hela cells plated 1-day prior in 10-cm plates at $1.5x10^6$ were transfected with 333 ng of pBOS Notch1, 333 ng pGA981-6, and 83 ng pLNC lacZ with either 666 ng pCMV-Fc or pHyTC-N1ECDFc (333 ng for x1, 666 ng for x2). Bosc cells plated 1-day prior in 10-cm plates at $4x10^6$ were transfected with either 680 ng pHyTc-Jagged1, pHyTc-Dll1, pHyTc-Dll4, or pHyTc-x (empty vector). One day after transfection, the cells were co-cultured in triplicate (HeLa:Bosc, 1:2) on 12-well plates for 24 hours. Cells were harvested and luciferase activity was determined 2-days post-transfection using the Enhanced Luciferase assay kit (BD PharMingen), and β-galactosidase activity was determined using the Galacto-Light Plus kit (PE Biosystems). All assays were performed in a Berthold dual-injection luminometer.

To determine VEGF-induced Notch signaling, HUVEC which were infected with adenovirus were used. HUVEC plated 1-day prior in 6 well plates at $8.0x10^5$ were infected with either Ad-LacZ as control or Ad-VEGF at indicated m.o.i. in the presence or absence of Ad-N1ECD/Fc. Two days after infection, infected HUVEC were re-seeded into 24-well plate at 1.5×10^5 cell in triplicate and cultured for 24

hours, and then transfected with 12.5 ng pRL-SV40 (Promega) and 137.5 ng pGA981-6 using Effectene transfection reagent (Qiagen). Cells were harvested either 1 or 2 days post-transfection and luciferase activity was determined by using the Dual-Luciferase® Reporter Assay System (Promega).

Sprouting assay

For making collagen gels, an ice-cold solution of porcine type I collagen (Nitta gelatin, Tokyo, Japan) was mixed with 10xRPMI1640 medium and neutralization buffer at the ratio of 8:1:1. 400 µl aliquots of collagen gel were then added to 24-well plates and allowed to gel for at least 1 hour at 37°C. Following adenoviral infection (above), HUVEC was harvested and plated at $1.3x10^5$ cells per well onto the top of the collagen gel in 24-well plates in 0.8 ml of EGM2 medium. HUVEC became nearly confluent 48 hours after plating. After seeding, medium was changed every 2 days for 1 week. Sprouting was observed and photographs taken after 8 days with an Olympus digital camera mounted to a microscope. For quantification of the number of sprouts, 5 fields per each well were randomly selected and sprouting was counted under microscopy in a blind manner by two investigators.

Results and Discussion

NOTCHECD/Fc Fusion Proteins Function As Antagonists Of Notch

Notch Antagonists-NotchECD/Fc Fusion Proteins

We have made several Notch antagonists (Figure 2). Our

strategy was to fuse the coding sequence of Notch EGF repeats in the Extracellular Domain (ECD) to the human or mouse Fc domain. This design makes a secreted protein without signaling function but which retains the ligand-binding domain and thus should bind to and inhibit ligand function. We refer to these proteins as "NotchECD/Fc" and all four Notch1-4ECD/Fcs have been made. The Fc domain facilitates affinity purification and protein detection by immunoblotting or immunohistochemistry.

Testing Notch Antagonists

An *in vitro* co-culture system (Figure 3) with ligands expressed on one cell and Notch receptor activation scored in another cell was used to measure transcriptional activation of the Notch pathway. We used this co-culture assay to show that Notch1ECD/Fc functions to block ligand-dependent Notch signaling (Figure 4). The N1ECD/Fc expression vector was co-transfected at different ratios with full-length Notch1 and the CSL-luciferase reporter in HeLa cells, followed by co-culture with ligand expressing 293 cells. We observed that activation of Notch1 signaling by Notch ligands was reduced by N1ECD/Fc expression. This effect displayed concentration-dependency; a 2:1 ratio of N1ECD/Fc to Notch1 was more effective in inhibiting signaling than a 1:1 ratio. Notch1ECD/Fc could block signaling mediated by Jagged1, Delta-like 1 or Delta-like 4.

Expressing and Purifying Notch Antagonists

We have made CHO and HeLa cell lines expressing NotchECD/FCs using retroviral vectors for the purpose of protein purification. N1ECD/Fc proteins are secreted

(Figure 5); as shown in conditioned media collected from HeLa-NotchECD/Fc lines and purified with Protein-A(pA) agarose. The pA purified sample (Sup) and whole cell lysates (Lys) were immunoblotted with α-Fc antibody (Figure 5, panel A) demonstrating that N1ECD/Fc is secreted into the media. Adenovirus vectors for NotchECD/Fc were used to infect HeLa cells and lysates from these cells were immunoblotted with α-Fc antibodies demonstrating that they express NotchECD/Fc(1, 2, 3, 4) proteins (Figure 5, panel B). We are currently purifying N1ECD/Fc from CHO cell conditioned media using pA-affinity chromatography.

Defining Angiogenic Inhibition
Using Notch Fusion Proteins

Activation of Notch signaling can be detected by using CBF1 promoter activity

One can measure Notch signaling function by measuring transcriptional activity of CBF1 promoter, which is activated by binding of Notch-IC to CBF1. We measured CBF1 promoter activity in HUVEC which was infected with adenovirus encoding VEGF-165 at different MOI (Figure 6). Induction of CBF1 promoter was clearly detected in Ad-VEGF-infected HUVEC, compared to Ad-LacZ-infected cells in the MOI dependent manner. This data showed over-expression of VEGF could activate Notch signaling in HUVEC. Thus VEGF induced Notch signaling activity.

We asked whether Notch fusion proteins could block VEGF-induced activation of Notch signaling. Co-infection of Ad-Notch fusion protein with Ad-VEGF clearly reduced

activation of CBF1 promoter activity induced by Ad-VEGF infection alone (Figure 7). In the case of infection at 40 MOI for each adenovirus in Figure 7 (panel A), 60% inhibition at 24 hr and 90% inhibition at 48 hr after reporter gene transfection were detected also the inhibitory activity of Notch decoy was dependent on MOI of Ad-Notch fusion protein.

Notch fusion proteins block initiation of angiogenic sprouting induced by VEGF

In this experiment, we evaluated the effect of Notch decoy on induction of budding (initiation of sprouting) by over-expressed VEGF-165 in HUVEC. When Ad-VEGF-infected HUVEC were cultured on type collagen gel for 8 days, budding was induced into collagen gel. This induction of budding by overexpressed VEGF was clearly inhibited by coinfection of adenoviral encoding Notch fusion protein (Figure 8). Ad-Notch fusion protein itself had less effect on morphology.

In Figure 9 we counted buds per field using the microscope. Ad-VEGF-infection into HUVEC increased the number of buds depending on the MOI used. Ad-VEGF-induced budding was clearly inhibited. These data suggest that VEGF induced budding of HUVEC through activation of Notch signaling and that the Notch fusion protein could inhibit VEGF-induced budding.

References cited in Second Series of Experiments

1. Artavanis-Tsakonas, S., K. Matsuno, and M.E. Fortini. 1995. Notch signaling. *Science* 268:225-232.

2. Bailey, A.M., and J.W. Posakony. 1995. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes & Development* 9:2609-22.

3. Bettenhausen, B., M. Hrabe de Angelis, D. Simon, J.L. Guenet, and A. Gossler. 1995. Transient and restricted expression during mouse embryogenesis of *Dll*, a murine gene closely related to *Drosophila Delta*. *Development* 121:2407-18.

4. Blaumueller, C.M., H. Qi, P. Zagouras, and S. Artavanis-Tsakonas. 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* 90:281-91.

5. Caronti, B., L. Calandriello, A. Francia, L. Scorretti, M. Manfredi, T. Sansolini, E.M. Pennisi, C. Calderaro, and G. Palladini. 1998. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL). Neuropathological and in vitro studies of abnormal elastogenesis. *Acta Neurol Scand*. 98:259-67.

6. Desmond, D.W., J.T. Moroney, T. Lynch, S. Chan, S.S. Chin, D.C. Shungu, A.B. Naini, and J.P. Mohr. 1998. CADASIL in a North American family: clinical,

pathologic, and radiologic findings [see comments]. *Neurology* 51:844-9.

7. Dunwoodie, S.L., D. Henrique, S.M. Harrison, and R.S. Beddington. 1997. Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* 124:3065-76.

8. Eastman, D.S., R. Slee, E. Skoufos, L. Bangalore, S. Bray, and C. Delidakis. 1997. Synergy between suppressor of Hairless and Notch in regulation of Enhancer of split m gamma and m delta expression. *Mol Cell Biol*. 17:5620-5634.

9. Fortini, M.E., and S. Artavanis-Tsakonas. 1993. Notch: neurogenesis is only part of the picture. *Cell* 75:1245-7.

10. Gale, N.W., and G.D. Yancopoulos. 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, Angiopoietins, and ephrins in vascular development. *Genes and Development* 13:1055-1066.

11. Gallahan, D., and R. Callahan. 1997. The mouse mammary tumor associated gene INT3 is a unique member of the NOTCH gene family (NOTCH4). *Oncogene* 14:1883-90.

12. Greenwald, I. 1994. Structure/function studies of

lin-12/Notch proteins. *Current Opinion in Genetics & Development* 4:556-62.

13. Greenwald, I. 1998. LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev.* 12:1751-62.

14. Henderson, A.M., S.J. Wang, A.C. Taylor, M. Aitkenhead, and C.C.W. Hughes. 2001. The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation. *J Biol Chem.* 276:6169-6176.

15. Hicks, C., S.H. Johnston, G. diSibio, A. Collazo, T.F. Vogt, and G. Weinmaster. 2000. Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nature Cell Biology* 2:515-520.

16. Hsieh, J.J., T. Henkel, P. Salmon, E. Robey, M.G. Peterson, and S.D. Hayward. 1996. Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Molecular & Cellular Biology* 16:952-9.

17. Hsieh, J.J., D.E. Nofziger, G. Weinmaster, and S.D. Hayward. 1997. Epstein-Barr virus immortalization: Notch2 interacts with CBF1 and blocks differentiation. *J Virol.* 71:1938-45.

18. Jarriault, S., C. Brou, F. Logeat, E.H. Schroeter, R. Kopan, and A. Israel. 1995. Signaling downstream of activated mammalian Notch. *Nature* 377:355-358.

19. Joutel, A., F. Andreux, S. Gaulis, V. Domenga, M. Cecillon, N. Battail, N. Piga, F. Chapon, C. Godfrain, and E. Tournier-Lasserve. 2000. The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients [see comments]. *J Clin Invest*. 105:597-605.

20. Joutel, A., C. Corpechot, A. Ducros, K. Vahedi, H. Chabriat, P. Mouton, S. Alamowitch, V. Domenga, M. Cecillion, E. Marechal, J. Maciazek, C. Vayssiere, C. Cruaud, E.A. Cabanis, M.M. Ruchoux, J. Weissenbach, J.F. Bach, M.G. Bousser, and E. Tournier-Lasserve. 1996. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* 383:707-10.

21. Kopan, R., E.H. Schroeter, H. Weintraub, and J.S. Nye. 1996. Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc Natl Acad Sci U S A* 93:1683-8.

22. Krebs, L.T., Y. Xue, C.R. Norton, J.R. Shutter, M. Maguire, J.P. Sundberg, D. Gallahan, V. Closson, J. Kitajewski, R. Callahan, G.H. Smith, K.L. Stark, and T. Gridley. 2000. Notch signaling is essential for vascular morphogenesis in mice. *Genes and Development* 14:1343-1352.

23. Lardelli, M., J. Dahlstrand, and U. Lendahl. 1994. The novel Notch homologue mouse Notch3 lacks

specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mechanism of Development* 46:123-136.

24. Lawson, N.D., N. Scheer, V.N. Pham, C. Kim, A.B. Chitnis, J.A. Campos-Ortega, and B.M. Weinstein. 2001. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* 128:3675-3683.

25. Lewis, J. 1998. Notch signaling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol*. 9:583-589.

26. Lieber, T., S. Kidd, E. Alcomo, V. Corbin, and M.W. Young. 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev.* 7:1949-1965.

27. Lindner, V., C. Booth, I. Prudovsky, D. Small, T. Maciag, and L. Liaw. 2001. Members of the Jagged/Notch gene familites are expressed in injured arteries and regulate cell phenotype via alteration in cell matrix and cell-cell interations. *Pathology* 159:875-883.

28. Lindsell, C.E., C.J. Shawber, J. Boulter, and G. Weinmaster. 1995. Jagged: A mammalian ligand that activates Notch1. *Cell* 80:909-917.

29. Logeat, F., C. Bessia, C. Brou, O. LeBail, S.

Jarriault, N.G. Seidah, and A. Israel. 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci USA* 95:8108-12.

30. Lyman, D., and M.W. Young. 1993. Further evidence for function of the Drosophila Notch protein as a transmembrane receptor. *Proc Natl Acad Sci USA* 90:10395-10399.

31. Matsuno, K., M.J. Go, X. Sun, D.S. Eastman, and S. Artavanis-Tsakonas. 1997. Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements. *Development* 124:4265-4273.

32. Nakagawa, O., D.G. McFadden, M. Nakagawa, H. Yanagisawa, T. Hu, D. Srivastava, and E.N. Olson. 2000. Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. *Proc Natl Acad Sci USA* 97:13655-13660.

33. Oberg, C., J. Li, A. Pauley, E. Wolf, M. Gurney, and U. Lendahl. 2001. The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J Biol Chem.* 276:35847-35853.

34. Owens, G.K. 1995. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev.* 75:487-527.

35. Rebay, I., R.G. Fehon, and S. Artavanis-Tsakonas. 1993. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. *Cell* 74:319-29.

36. Robey, E. 1997. Notch in vertebrates. *Curr Opin Genet Dev.* 7:551-7.

37. Roehl, H., M. Bosenberg, R. Blelloch, and J. Kimble. 1996. Roles of the RAM and ANK domains in signaling by the C. elegans GLP-1 receptor. *Embo J.* 15:7002-7012.

38. Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degrade proteins: The PEST hypothesis. *Science* 234:364-368.

39. Sasai, Y., R. Kageyama, Y. Tagawa, R. Shigemoto, and S. Nakanishi. 1992. Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes & Dev.* 6:2620-2634.

40. Shawber, C., J. Boulter, C.E. Lindsell, and G. Weinmaster. 1996a. Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev Biol.* 180:370-6.

41. Shawber, C., D. Nofziger, J.J. Hsieh, C. Lindsell, O. Bogler, D. Hayward, and G. Weinmaster. 1996b. Notch signaling inhibits muscle cell differentiation through a CBF1- independent pathway. *Development* 122:3765-73.

42. Shimizu, K., S. Chiba, T. Saito, T. Takahashi, K. Kumano, H. Hamada, and H. Hirai. 2002. Integrity of intracellular domain of Notch ligand is indespensable for cleavage required for the release of the Notch2 intracellular domain. *Embo J.* 21:294-302.

43. Shutter, J.R., S. Scully, W. Fan, W.G. Richards, J. Kitajewski, G.A. Deblandre, C.R. Kintner, and K.L. Stark. 2000a. Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.* 14:1313-1318.

44. Shutter, J.R., S. Scully, W. Fan, W.G. Richards, J. Kitajewski, G.A. Deblandre, C.R. Kitner, and K.L. Stark. 2000b. Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes and Development* 14:1313-1318.

45. Struhl, G., K. Fitzgerald, and I. Greenwald. 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* 74:331-45.

46. Swiatek, P.J., C.E. Lindsell, F. Franco del Amo, G. Weinmaster, and T. Gridley. 1994. *Notch 1* is essential for postimplantation development in mice. *Genes & Development* 8:707-719.

47. Tamura, K., Y. Taniguchi, S. Minoguchi, T. Sakai, T. Tun, T. Furukawa, and T. Honjo. 1995. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J

kappa/Su(H). *Curr Biol.* 5:1416-1423.

48. Tietze, K., N. Oellers, and E. Knust. 1992. Enhancer of splitD, a dominant mutation of Drosophila, and its use in the study of functional domains of a helix-loop-helix protein. *Proc Natl Acad Sci USA* 89:6152-6156.

49. Uyttendaele, H., J. Ho, J. Rossant, and J. Kitajewski. 2001. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *PNAS.* 98:5643-5648.

50. Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski. 1996. Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* 122:2251-9.

51. Vervoort, M., C. Dambly-Chaudiere, and A. Ghysen. 1997. Cell fate determination in Drosophila. *Curr Opin Neurobiol.* 7:21-28.

52. Villa, N., L. Walker, C.E. Lindsell, J. Gasson, M.L. Iruela-Arispe, and G. Weinmaster. 2001. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mechanisms of Development* 108:161-164.

53. Weinmaster, G. 1997. The Ins and Outs of Notch Signaling. *Mol Cel Neurosci.* 9:91-102.

54. Weinmaster, G. 1998. Notch signaling: direct or what? *Curr Opin Genet Dev.* 8:436-42.

55. Weinmaster, G., V.J. Roberts, and G. Lemke. 1992. *Notch* 2: a second mammalian *Notch* gene. *Development* 116:931-941.

56. Weinmaster, G., V.J. Roberts, and G.A. Lemke. 1991. A homolog of Drosophila Notch expressed during mammalian development. *Development* 113:199-205.

57. Wettstein, D.A., D.L. Turner, and C. Kintner. 1997. The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* 124:693-702.

58. Wu, G., E.J. Hubbard, J.K. Kitajewski, and I. Greenwald. 1998. Evidence for functional and physical association between Caenorhabditis elegans SEL-10, a Cdc4p-related protein, and SEL-12 presenilin. *Proc Natl Acad Sci USA* 95:15787-91.

59. Wu, G., S.A. Lyapina, I. Das, J. Li, M. Gurney, A. Pauley, I. Chui, R.J. Deshaies, and J. Kitajewski. 2001. SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol Cell Biol.* 21:7403-7015.

60. Xue, Y., X. Gao, C.E. Lindsell, C.R. Norton, B. Chang, C. Hicks, M. Gendron-Maguire, E.B. Rand, G. Weinmaster, and T. Gridley. 1999. Embryonic lethality and vascular defects in mice lacking the

Notch ligand Jagged1. *Hum Mol Genet*. 8:723-30.

Third Series of Experiments

VEGF initiates angiogenesis via an activation of Notch signaling

Both the VEGF and Notch signaling pathways are critical for vascular development. Here we show that VEGF activates Notch signaling to initiate angiogenesis. VEGF increased the expression of Delta4 and Notch4 causing Notch signal activation and inducing filopodia in cultured primary endothelial cells. Studies using VEGF Receptor inhibitors show that Notch signal activation in turn enhances VEGF action by inducing VEGFR-1 (Flt-1) expression. Other elements of VEGF action, including the induction of MMP-9 and MT1-MMP, are mediated by Notch. Using *in vivo* assays to model VEGF-induced skin neovascularization, we found that a secreted Notch inhibitor (Notch-based fusion protein) blocks VEGF-induced neo-vascularization and induction of VEGFR-1 expression. Thus, Notch signaling is requisite for angiogenesis regulated by VEGF, likely at the level of initiation.

VEGF is a key regulator of angiogenesis progression consisting of multiple processes, such as degradation of ECM, budding (filopodia formation), proliferation, survival, and migration of endothelial cells. Although most of the steps might be co-operated with downstream molecules of VEGF signaling, it is not known how these steps are coordinately regulated to result in more complex morphogenetic events, such as angiogenic sprouting. Notch signaling is an evolutionarily conserved

signaling mechanism that functions to regulate cell fate decisions (1). Upon binding by a ligand, such as Jagged and Delta-like, the cytoplasmic domain of Notch (NotchIC) is released by presenilin/γ-secretase, translocates to the nucleus, interacts with the transcriptional repressor CSL (CBF1/Su(H)/lag2), and converts it to a transcriptional activator (1). Roles of Notch signaling in vascular development were suggested by studies of mice with targeted mutation (2). Since Notch activation within the endothelium also disrupts vascular remodeling, proper Notch signaling is essential for vascular development (3). Although relevance of Notch to VEGF signaling is suggested (4-6), it is still unclear how Notch signaling has a role in VEGF-regulated angiogenesis and whether Notch signaling participates in physiological and pathological angiogenesis in the adult vasculature.

HUVEC (Human Umbilical Vein Endothelial cells) growth are dependent on VEGF (Figs. 26A and 26B) and differentiation-related biological responses, such as sprouting, and can be evaluated at an early stage (7). At first, we examined whether adenovirally transduced VEGF induced both Notch and Notch ligand expression in HUVEC cultured with complete medium containing bFGF (Fig. 22A), as reported (5). RT-PCR analysis showed that both Dl4 and Notch4 mRNA was up-regulated in adenovirally-transduced VEGF HUVEC (Ad-VEGF-HUVEC), compared to adenovirally-transduced LacZ HUVEC (Ad-LacZ-HUVEC) (Fig. 22A). Transduced VEGF did not appear to induce Jagged1 and Notch1 expression. Transduced-VEGF also activated Notch signaling in a dose-dependent manner by measuring CSL-luciferase reporter activity (Fig. 22B), which was

transactivated with Notch signaling (8). Notch signaling was activated at a higher dosage of Ad-VEGF, compared to proliferation (Fig. 26A). Since SU5416, which is an inhibitor of VEGFR kinases, decreased VEGF-induced CSL-luciferase reporter activity (Fig. 22C), VEGF induced Notch signaling through activation of receptor kinase. Since Notch mutants lacking both transmembrane and cytoplasmic domains functioned as dominant negative inhibitors against Notch signaling (9), we made a Notch-based fusion protein or decoy (N1ECDFc) to inhibit Notch signaling (Fig. 22D). Western blotting analysis of conditioned medium of Ad-N1ECDFc-transduced HUVEC (Ad-N1ECDFc-HUVEC) demonstrated that N1ECDFc was expressed and secreted well (Fig. 22E). By using a co-culture assay, in which Bosc cells expressing Notch ligands (either J1, Dl1 or Dl4) activated Notch signaling in HeLa cells expressing Notch1 compared to control Bosc cells, we determined inhibition of Notch signaling with transfection of a N1ECDFc-expression plasmid (Fig. 22F). Then, we examined whether N1ECDFc inhibited activation of Notch signaling by transduced VEGF in HUVEC (Fig. 22G). Co-transduction of Ad-N1ECDFc with Ad-VEGF into HUVEC clearly decreased CSL luciferase activity induced by VEGF. Gerhardt et al. reported that VEGF controlled angiogenesis in the early postnatal retina by guiding filopodia extension at the tips of the vascular sprouts (10). During angiogenic sprouting, the formation of a specialized endothelial cell making filopodia projections among quiescent endothelial cells, might be one of the early events. Here we mean formation of a single endothelial cell making filopodia protrusions as budding. Budding of the primary endothelial cells is induced by cultivating them 3-dimensionally on either fibrin or

collagen gel (11). In the case where Ad-VEGF-HUVEC were cultured on collagen gel with complete medium, transduced-HUVEC made filopodia extensions into the collagen gel for 5 days (Fig. 22H) and the number of buds was increased in a dose-dependent manner (Fig. 27A). Activation of Notch signaling by adenovirus encoding the activated form of Notch4 (Ad-Notch4/int3) induced HUVEC budding (12) and that of Notch1 (Ad-N1IC) also induced HUVEC budding (Fig. 23A & 27B). Since both VEGF and Notch signaling induce HUVEC budding, we examined whether N1ECDFc inhibited VEGF-induced HUVEC budding (Fig. 22H-I). Budding of Ad-VEGF-HUVEC was clearly inhibited by co-transduction of Ad-N1ECDFc. Neither Ad-LacZ or Ad-N1ECDFc-transduced HUVEC formed buds (Fig. 22H). N1ECDFc inhibited VEGF-induced HUVEC budding without affecting cell number (Fig. 22I). Transduced-N1ECDFc did not clearly alter proliferation of HUVEC, while that of Ad-N1IC-transduced HUVEC was inhibited in a dose-dependent manner (Fig. 28A), consistent with the inhibitory efficacy of Notch signaling against endothelial proliferation (13).

To test whether Notch signaling is down-stream of VEGF, we evaluated three distinct inhibitors for receptor tyrosine kinases, including VEGFR on N1IC-induced HUVEC budding, because three growth factors existed in complete medium (Fig. 23A-C). At a concentration of 1 μM, each compound showed selective inhibition against each kinase (data not shown). Neither PD166866 or ZD1893 affected budding of Ad-N1IC-HUVEC, while SU5416 clearly inhibited it (Fig. 23A-B). SU5416 selectively inhibited budding of Ad-N1IC-HUVEC with less reduction of viability at lower

concentrations (Fig. 23C). Since Taylor et al. reported that Notch down-regulated Flk1/KDR/VEGFR2 expression (14), it was unlikely that Notch co-operated with Flk1 to promote budding. Thus, we examined whether activation of Notch signaling affected Flt1/VEGFR1 expression in HUVEC, because SU5416 inhibits both Flt1 and Flk1 kinase activity (15). RT-PCR analysis demonstrated that expression of Flt1 mRNA was up-regulated in Ad-N1IC-HUVEC, while expression of endothelial cell maker, CD31 mRNA, was not compared to that in Ad-LacZ-HUVEC (Fig. 23D). Western blotting analysis also showed that expression of Flt1 protein was up-regulated in Ad-N1IC-HUVEC (Fig. 23E). Thus, we examined whether PlGF, which is a selective ligand for Flt1, promoted budding of HUVEC in which Flt1 was up-regulated via activation of Notch signaling (Fig. 23F-G). PlGF increased the number of Ad-N1IC-HUVEC buds by 150%, compared to the absence of PlGF (Fig. 23F). Moreover, PlGF increased HUVEC buds containing multiple filopodia by 250% (Fig. 23G). While reduction of Flt1 expression using small interfering RNA (siRNA) for Flt1 inhibited budding of Ad-N1IC-HUVEC (Fig. 23J), transfection of which selectively decreased expression of Flt1 mRNA (Fig. 23H) and that of Flt1 protein (Fig. 23I). Although reduction of Flk1 expression with Flk1 siRNA also inhibited budding of Ad-N1IC-HUVEC (Fig. 30B), the inhibitory efficacy of Flk1 siRNA was less than that of Flt1 siRNA (Fig. 23J). Effects of Flk1 siRNA were more effective on budding of Ad-VEGF-HUVEC than that of Ad-N1IC-HUVEC (Fig. 30B-C). Transfection with Flt1 siRNA inhibited budding of both Ad-N1IC- and Ad-VEGF-HUVEC to a similar extent (data not shown).

Several studies demonstrated that VEGF regulated

gelatinase activities in endothelial cells and the significance of gelatinase activity like MMP-2 and MMP-9 has been firmly established to induce angiogenic sprouting (16). We examined whether VEGF regulated gelatinase acitivity via Notch signaling in HUVEC.

In Gelatin zymography, conditioned medium of Ad-VEGF-HUVEC showed both induction and activation of MMP9, which started to be detected at day 6 (Fig. 24A) and activation of MMP2, which was detected at day 4 (Fig. 24B), compared to those of Ad-LacZ-HUVEC. Co-transduction of Ad-N1ECDFc with Ad-VEGF showed inhibition of both induction and activation of MMP9 (Fig. 24A) and an activation of MMP2 (Fig. 24B). RT-PCR analysis demonstrated that expression of MMP9 mRNA was up-regulated in Ad-N1IC-HUVEC, but expression of MMP2 mRNA was decreased in Ad-N1IC-HUVEC (Fig. 24C). Since induction of MMP2 activity was not detected in gelatin zymography (Fig. 24B), this result was a likely consequence. While expression of MT1-MMP, which is able to activate MMP2 at the cell surface (17), was up-regulated at both the transcript and protein levels in Ad-N1IC-HUVEC (Fig. 24D). As VEGF can regulate both gelatinase and MT1-MMP expression (16), RT-PCR analysis demonstrated that both MMP9 and MT1-MMP were up-regulated in Ad-VEGF-HUVEC, compared to Ad-LacZ-HUVEC and this induction was inhibited with co-transduction of Ad-N1ECDFc (Fig. 24E). Ad-N1ECDFc infection alone did not affect expression of either MMP9 or MT1-MMP in Ad-LacZ infected HUVEC (data not shown). Requisition of MMPs for angiogenic sprouting has been established by synthetic MMP inhibitors (16). GM6001 is one broad inhibitor against MMPs including MMP2, MMP9 and MT1-MMP (18). GM6001 clearly decreased budding of Ad-N1IC-HUVEC on both

collagen (Fig. 31A-B) and fibrin gel (data not shown).

In the mouse Dorsa Air Sac (DAS) assay (19), stable transfectant of 293 cells over-expressing VEGF121 (293/VEGF) significantly induced *in vivo* angiogenesis (Fig. 25A, left panel). This VEGF-induced angiogenesis was clearly inhibited by coexpression of N1ECDFc, compared to 293/VEGF alone (Fig. 25A). Vessel density was measured and an index of angiogenesis given in Fig. 25B, demonstrating the 293/VEGF induced angiogenesis is inhibited by co-expression of 293/N1ECDFc (Fig. 25B).

Also, in the mouse Dorsa Air Sac (DAS) assay (19), the human breast cancer cell line, MDA-MB-231 significantly induced *in vivo* angiogenesis, presumably via the secretion of VEGF (Fig. 25C, left panel). This VEGF-induced angiogenesis was clearly inhibited by adenovirus mediated expression of N1ECDFc, compared to adenovirus expressing LacZ. (Fig. 25C). Vessel density was measured and an index of angiogenesis given in Fig. 25D, demonstrating the MDA-MB-231 induced angiogenesis is inhibited by expression of N1ECDFc.

Flk1 is a major positive signal transducer for angiogenesis through its strong tyrosine kinase activity in the embryo, while Flt1 is thought to be a negative signal transducer for angiogenesis. However, a positive role for Flt-1 was demonstrated in adult mice, as *in vivo* growth of LLC over-expressing PlGF2 was severely compromised in mice lacking the cytoplasmic Flt-1 kinase domain (20). Notch might function to alter VEGF signaling by inducing Flt-1 signaling and moderate Flk-1

signaling either to induce filopodia extension or potentiate angiogenic sprouting, since PlGF/Flt-1 signaling altered the phospholyration site of Flk-1 and potentiated ischemic myocardial angiogenesis (21). Interestingly, Notch signaling also up-regulated PlGF expression (Fig. 29). However, continuous activation of Notch signaling inhibits formation of multi-cellular lumen-containing angiogenic sprouts, as previously reported (22). Notch signaling should be turned off after budding/filopodia formation and transient activation of the Notch pathway might be required. In a transgenic mouse model of pancreatic beta-cell carcinogenesis (Rip1Tag2 mice) in which tumor angiogenesis is VEGF dependent, the level of VEGF expression is not increased, but mobilization of extracellular VEGF stored in the matrix to VEGF receptors occurs. MMP-9 is responsible for this mobilization and tumor progression was inhibited in Rip1Tag23MMP-9-null double-transgenic mice (23). Notch up-regulated MMP-9 expression and might increase local VEGF level at the site for angiogenic sprouting. While Notch also up-regulates MT1-MMP expression, extracellular MMP-2 might be targeted to the cell membrane of Notch-activated endothelial cells. Notch might determine the site for angiogenic sprouting by regulating gelatinase activity and VEGF concentration. Since endothelial MMP-9 was regulated by Flt-1 in lung specific metastasis (20), Flt-1 might participate in induction of MMP-9 indirectly.

References cited in Third Series of Experiments

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch Signaling: Cell Fate Control and Signal Integration in Development. Science 1999;284(5415):770-776.

2. Shawber CJ, J. K. Notch function in the vasculature: insights from zebrafish, mouse and man. Bioessays. 2004;26(3):225-34.

3. Uyttendaele H, Ho J, Rossant J, J. K. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proc Natl Acad Sci U S A. 2001;98(10):5643-8.

4. Lawson ND, Vogel AM, BM. W. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell 2002;3(1):127-36.

5. Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, et al. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol. 2003;23(1):14-25.

6. Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, et al. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A. 2004;101(45):5949-54.

7. Montesano R, L. O. Phorbol esters induce angiogenesis in vitro from large-vessel endothelial cells. J Cell Physiol. 1987;130(2):284-91.

8. Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, A. I. Signalling downstream of activated mammalian Notch. Nature. 1995;377(6547):355-8.

9. Small D, Kovalenko D, Kacer D, Liaw L, Landriscina M, Di Serio C, et al. Soluble Jagged 1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. J Biol Chem 2001;276(34):32022-30.

10. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 2003;161(6):1163-77.

11. Koolwijk P, van Erck MG, de Vree WJ, Vermeer MA, Weich HA, Hanemaaijer R, et al. Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J Cell Biol 1996;132(6):1177-88.

12. Das I, Craig C, Funahashi Y, Jung KM, Kim TW, Byers R, et al. Notch oncoproteins depend on gamma-secretase/presenilin activity for processing and function. J Biol Chem 2004;279(29):30771-80.

13. Noseda M, Chang L, McLean G, Grim JE, Clurman BE, Smith LL, et al. Notch activation induces

endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. Mol Cell Biol 2004;24(20):8813-22.

14. Taylor KL, Henderson AM, CC. H. Notch activation during endothelial cell network formation in vitro targets the basic HLH transcription factor HESR-1 and downregulates VEGFR-2/KDR expression. Microvasc Res 2002;64(3):372-83.

15. Itokawa T, Nokihara H, Nishioka Y, Sone S, Iwamoto Y, Yamada Y, et al. Antiangiogenic effect by SU5416 is partly attributable to inhibition of Flt-1 receptor signaling. Mol Cancer Ther 2002;1(5):295-302.

16. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 2001;21(7):1104-17.

17. Seiki M, Koshikawa N, I. Y. Role of pericellular proteolysis by membrane-type 1 matrix metalloproteinase in cancer invasion and angiogenesis. Cancer Metastasis Rev 2003;22(2-3):129-43.

18. Yamamoto M, Tsujishita H, Hori N, Ohishi Y, Inoue S, Ikeda S, et al. Inhibition of membrane-type 1 matrix metalloproteinase by hydroxamate inhibitors: an examination of the subsite pocket. J Med Chem 1998;41(8):1209-17.

19. Funahashi Y, Wakabayashi T, Semba T, Sonoda J, Kitoh

K, K. Y. Establishment of a quantitative mouse dorsal air sac model and its application to evaluate a new angiogenesis inhibitor. Oncol Res. 1999;11(7):319-29.

20. Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, et al. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2002;2(4):289-300.

21. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, et al. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 2003;9(7):936-43.

22. Leong KG, Hu X LL, Noseda M, Larrivee B, Hull C, Hood L, et al. Activated Notch4 inhibits angiogenesis: role of beta 1-integrin activation. Mol Cell Biol 2002;22(8):2830-41.

23. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2(10):737-44.

Fourth Series of Experiments

A Novel Construct Derived From The Notch1 Ectodomain Inhibits Notch Signaling, Endothelial Morphogenesis, And Tumor Angiogenesis

Notch signaling is required for vascular development, but also functions in tumor angiogenesis. Inhibition of vascular endothelial growth factor (VEGF) is a validated anti-angiogenic therapy, and VEGF can induce both Notch and Notch ligand Delta-like 4 (Dll4) expression in endothelial cells (EC). Although Dll4 inhibition can restrict tumor growth and disrupt neovasculature, the effect of inhibiting Notch receptor function on tumor angiogenesis has yet to be defined. In this study, we generated a soluble fusion protein of the Notch1 receptor (N1ECDFc, or Notch1 decoy) to block this pathway, and assessed its effect on angiogenesis *in vitro* and *in vivo*. Notch1 decoy expression reduced signaling stimulated by the binding of three distinct Notch ligands to Notch1, and also inhibited morphogenesis of EC overexpressing Notch4. We tested the effects of Notch1 decoy expression on tumor angiogenesis using two models: mouse mammary tumor Mm5MT cells overexpressing fibroblast growth factor 4 (Mm5MT-FGF4), and NGP human neuroblastoma cells. Exogenously expressed FGF4 induced the expression of Notch ligands Jagged1 and Delta-like 1 (Dll1) in Mm5MT-FGF4 cells, and Jagged1 was expressed in Mm5MT-FGF4 xenografts. Overexpression of Notch1 decoy did not affect tumorigenicity of Mm5MT-FGF4 cells *in vitro*, but restricted Mm5MT-FGF4 xenograft growth in mice, while markedly impairing neoangiogenesis. Similarly, Notch1

decoy expression did not affect NGP cells *in vitro* but disrupted vessels and decreased tumor viability in NGP xenografts. These results strongly suggest that Notch receptor signaling is required for tumor neoangiogenesis, and provides a new target for tumor therapy.

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

The highly conserved Notch gene family encodes transmembrane receptors (Notch1, -2, -3, -4) and ligands (Jagged1, -2; Delta-like or Dll1, -3, -4), also transmembrane proteins. Upon ligand binding, the Notch cytoplasmic domain (NotchIC) is released by presenilin/γ-secretase (3). Notch signaling defects produce severe vascular defects in embryos (4), with haploinsufficiency of Dll4 causing lethality. The potential role of Notch signaling in tumor angiogenesis has thus excited much

recent interest. Mice transgenic for a Dll4-reporter construct demonstrate expression in tumor endothelial cells (EC) (5), and increased Dll4 expression has been detected in human cancers (6, 7). Two recent reports confirm that this role is critical by demonstrating that Dll4 blockade suppresses growth and perfusion in experimental tumors (8, 9). Intriguingly, in these studies Dll4 inhibition disorganized tumor vasculature rather than simply preventing vessel proliferation, suggesting that Dll4 is required for functional vessel assembly.

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

In these studies, we evaluated the role of Notch receptor activation in angiogenesis using a novel soluble construct based on the extracellular domain of Notch1 (N1ECDFc, or Notch1 decoy). *In vitro*, Notch1 decoy inhibited both ligand-induced activation of Notch signaling and morphogenesis of EC adenovirally over-expressing Notch4. *In vivo*, Notch1 decoy expression delayed growth of murine Mm5MT xenografts in which Jagged1 expression was up-regulated by transduction of FGF4, and disrupted vasculature and tumor viability in NGP neuroblastoma tumors. These data support a

requirement for Notch receptor function during tumor neoangiogenesis, and suggest that inhibition of this pathway may provide an effective new anti-tumor strategy.

Materials and Methods

Reagents and Expression Vectors

Compound E was purchased from Calbiochem (San Diego, CA), and PD166866 from Eisai (Tokyo, Japan). Notch1 decoy (N1ECDFc) encodes the rat Notch1 ectodomain (bp 241-4229, Genbank accession #X57405) fused in frame to human IgG Fc. Retroviral pHyTC-Jagged1, -Dll1, -Dll4, and pBos-Notch1 have been described (11). Notch1 decoy and Fc were engineered into retroviral vector pHyTCX, and mouse FGF4 engineered into pQNCX. Adenoviral constructs encoding LacZ and mouse Notch4 and pAdlox-GFP have been described (12)(13).

HUVECs, Adenoviral, and Retroviral Infections

HUVECs were isolated as described (14) and mouse mammary carcinoma Mm5MT obtained (ATCC, Manassas, VA). We used adenovirus at indicated multiplicity of infection (m.o.i.) and retroviral supernatants from GP2-293 cells (BD Biosciences, Bedford, MA) for infection. HUVECs were selected using 300μg/ml hygromycinB (Invitrogen, Carlsbad, CA) and Mm5MT-FGF4 selected in 1mg/ml G418 (Gibco-Invitrogen, Grand Island, NY), with double transfectants in 300μg/ml hygromycinB.

Western Blotting

Ad-N1ECDFc-transduced HUVEC were cultured in endothelium serum-free medium (GIBCO-Invitrogen) 48h, and Mm5MT-FGF4

transfectants in DMEM. Western blots were performed using anti-human Fc (Pierce, Rockford, IL).

Quantitative RT-PCR

Mm5MT transfectants were cultured 7 days with vehicle or 1μM PD166866 (inhibitor of FGF receptor-kinase), total RNA isolated (RNeasy mini-kit, Qiagen, Valencia, CA), and first-strand cDNA synthesized (SuperScript™ First-Strand Synthesis System, Invitrogen). Quantitative RT-PCR for β-actin, FGF4, Jagged1, Dll1, and Dll4 (SYBER Green PCR Master Mix, 7300 Real Time PCR; Applied Biosystems, Foster City, CA) was performed in triplicate and values normalized for β-actin. Values are shown for fold induction compared to controls (primer sequences available on request).

Co-culture Signaling Assay

Notch1 decoy inhibition of ligand-induced signaling was performed as described (11). HeLa cells were transfected with 333ng pBOS-Notch1, 333ng pGA981-6, and 83ng pLNC-LacZ with either 666ng pCMV-Fc or pHyTC-N1ECDFc (333ng for x1, 666ng for x2). 293 cells were transfected with 680ng pHyTc-Jagged1, pHyTc-Dll1, pHyTc-Dll4, or pHyTc-X (empty vector). Cells were harvested, luciferase activity determined 48h post-transfection (Enhanced Luciferase assay kit, BD PharMingen, San Diego, CA), and β-galactosidase activity determined (Galacto-Light Plus kit, Applied Biosystems). Assays were performed in triplicate.

Endothelial Co-Culture Morphogenesis Assay

HUVEC morphogenesis was assessed as described (11),

modified by adding co-culturing of Ad-Notch4 transduced HUVEC with Notch1 decoy- or Fc-HUVEC transfectants. Ad-GFP at 10m.o.i. was co-transduced in HUVECs with Ad-LacZ or Ad-Notch4 at 30m.o.i and 48h later seeded on fibrin gels (24-well plates, $1.5x10^4$ cells/well). Stable HUVEC-mock (HUVEC-X), HUVEC-Fc, or HUVEC-N1ECDFc transfectants were seeded at $1.35x10^5$ cells/well, and vehicle or 200nM compound E added 3h later. Seven days later, HUVEC morphogenesis was calculated as the number of GFP-positive cells with processes compared to total GFP-positive cells/field.

Mm5MT Tumor Model

6-8 week-old female C3H mice (Taconic, Hudson, NY) underwent subcutaneous implantation of 10^6 Mm5MT transfectants (N=10 each). Tumor diameters were measured with calipers, and volume calculated (length (mm) x width $(mm)^2$ x ½). Tumors were harvested at day 22 and analyzed. Experiments were performed thrice.

Immunohistochemistry

5μm fresh-frozen Mm5MT tissue sections were immunostained (15) (see supplemental data for antibody list). CD31 quantitation was performed using an Eclipse E800 microscope and ImagePro Plus v.4.01 (Silver Spring, MD). 20 different fields/slide were measured, and density ratios calculated as (area of specific staining)/(total area, each field). Data is shown as the ratio of the mean of average density ratios of each Mm5MT transfectant to Mm5MT mock-transfectant.

NGP Tumor Model

The NGP tumor model has previously been described in detail (16). NGP cells were transfected with LacZ or N1ECDFc, as above, and 10^6 NGP-LacZ or NGP-N1ECDFc cells implanted intrarenally in 4-6 week old NCR nude mice (Taconic, Germantown, NY; NGP-LacZ n=11, NGP-N1ECDFc n=13). At 6 weeks, tumors were harvested for analysis. 5uM paraffin-embedded sections were immunostained for CD-31/PECAM and α-smooth muscle actin (αSMA). To detect apoptosis (TUNEL assay) we used the Apoptag Red *in situ* Kit (Chemicon). Signal was quantified by photographing 20-23 randomly selected fields of each tissue, excluding areas of normal kidney. Each frame was photographed in both red (TUNEL signal) and green channels. Using Adobe Photoshop, green channel signals were subtracted, in order to eliminate erythrocyte autofluorescence. A uniform red-channel threshold was arbitrarily selected, and total signal area measured in 4 NGP-N1ECDFc and 3 NGP-LacZ tumors. Erythrocyte quantification was performed similarly.

Statistical analysis

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

Results

Notch1 decoy inhibits ligand-induced Notch signaling in cells expressing Notch1

Notch1 decoy is based on the ectodomain of Notch1 fused to human IgG Fc and is secreted, as determined by

blotting of media conditioned by adenovirus Notch1 decoy (Ad-N1ECDFc)-infected HUVEC (Fig. 32A). We assessed Notch1 decoy activity using co-culture signaling assays (11). 293 cells expressing Notch ligands (Jagged1, Dll1, Dll4) activated Notch signaling when cultured with HeLa cells expressing Notch1, measured by CSL-luciferase reporter activity (Fig. 32B). Expression of Notch1 decoy in either HeLa (Fig. 32B) or 293 cells (data not shown) blocked Notch1 signaling in co-culture assays, indicating that Notch1 decoy prevented activation by Jagged1, Dll1, or Dll4.

Notch1 decoy blocks morphogenesis of HUVEC induced by Notch4

Notch4 expression induced cellular extensions from HUVECs cultured on fibrin gels (Fig. 32C), resembling morphologic changes induced by VEGF and FGF2 (17, 18). Fibrin can induce Jagged1 in EC (19). We tested the hypothesis that such extensions reflect endogenous Notch ligand activation of Notch4 transduced in HUVECs using either Compound E (CE), a (-secretase inhibitor (GSI), or Notch1 decoy. Compared to vehicle, treatment with 200nM CE clearly inhibited extensions in Notch4-HUVECs, (Fig. 32C upper panels, 32D). Reduction in sprouting was significant (Fig 32D, $p<0.0001$ for both compound E treatment and N1ECDFc transduction; data shown as mean $\pm$ SD). Notch4 + Notch1 decoy co-expression in HUVECs similarly blocked endothelial extensions relative to Notch4 + Fc control (Fig. 32C, lower panels; 32D). Collectively, these data indicate that Notch receptor activation is both necessary and sufficient to induce HUVEC extensions in this assay, and that the Notch1 decoy

functions similarly to GSI, further validating its activity as a Notch receptor inhibitor.

FGF4 induced the expression of Notch ligands, Jagged1 and Dll1, in mouse mammary tumor Mm5MT cells

Overexpression of FGF4 in Mm5MT cells promoted tumorigenicity in clonogenic and xenograft assays (data not shown). Since HGF/MAPK signaling induced Jagged1 expression in HNSCC (10), we asked whether FGF4 would stimulate expression of Notch ligands in Mm5MT cells. We detected up-regulation of Jagged1 and Dll1 in Mm5MT-FGF4 transfectants using quantitative PCR (Dll4 expression was unaltered) (Fig. 33A). The FGFR-kinase inhibitor PD166866 suppressed induction of both Jagged1 and Dll1 in Mm5MT-FGF4 transfectants (Fig. 33C) indicating that FGF4-induced Jagged1 and Dll1 expression requires FGFR signaling. Immunoblotting confirmed up-regulation of Jagged1 protein in Mm5MT-FGF4 cells (Fig. 33B), and immunostaining demonstrated strikingly increased Jagged1 in Mm5MT-FGF4 tumors (Fig. 33D). In addition, Notch4 was detected in Mm5MT-FGF4 tumor endothelium (not shown).

Notch1 decoy expression inhibited angiogenesis and growth of Mm5MT-FGF4 tumors in mice

We hypothesized that Mm5MT-FGF4 tumors expressing Jagged1 promote angiogenesis by signaling via endothelial Notch receptors. Thus, we evaluated the effect of Notch1 decoy expression on Mm5MT-FGF4 xenograft growth in mice. Tumorigenicity of Mm5MT-FGF4 stably overexpressing either Fc or Notch1 decoy was unaltered by clonogenic assay (data not shown). However, Mm5MT-FGF4-N1ECDFc xenograft growth was significantly delayed as compared to both

Mm5MT-FGF4 mock- and Fc-transfectants, suggesting that Notch inhibition had impaired a critical element in tumorigenesis (Fig. 34A). Immunostaining for the endothelial marker CD31/PECAM demonstrated marked inhibition of angiogenesis in Mm5MT-FGF4-N1ECDFc tumors (Fig. 34B). Consistent with a requirement for Notch in vessel assembly, EC appeared as detached solitary cells or small clusters, with few organized vessels detected. Quantitative analysis of anti-CD31 staining demonstrated a 58% decrease in microvessel density in Notch1 decoy-expressing tumors ($P<0.001$ for both Mm5MT-FGF4-X and Mm5MT-FGF4-Fc versus Mm5MT-FGF4-N1ECDFc; data shown as mean $\pm$ SD; Fig. 34C).

Notch1 decoy expression disrupted angiogenesis in human NGP neuroblastoma xenografts

NGP xenografts in mice form a mature hierarchical vasculature that is comparatively resistant to VEGF blockade (16). To determine whether Notch receptor activation contributed to NGP angiogenesis, we transfected NGP cells with N1ECDFc, as above. Similar to results observed with Mm5MT-FGF4-N1ECDFc cells, growth of NGP-N1ECDFc cells in culture was unaffected by transfection (not shown). However, xenograft viability was strikingly impaired (Fig. 35A), with significantly increased tumor cell apoptosis ($P=0.0002$, TUNEL-positive cells in NGP-N1ECDFc vs. NGP-LacZ tumors, Fig. 35B). Intratumoral hemorrhage was significantly increased in NGP-N1ECDFc tumors, suggesting that vessels were physically disrupted ($P<0.0001$, Fig. 36C). Immunostaining for the vascular basement membrane component collagen IV indicated an overall decrease in vasculature, with

diminished branching, although remaining collagen sleeves appeared smooth and intact (not shown). However, immunostaining for EC and vascular mural cells (VMC) (using anti-CD31 and anti-αSMA antibodies, respectively) demonstrated disorder of these normally contiguous cell layers. Individual vascular cells appeared irregular, and were erratically detached from one another, with loss of vessel continuity (Fig. 35D). Taken together, these results suggest that Notch1 decoy expression disrupted endothelial and VMC interactions in tumor vasculature, leading to instability, hemorrhage, and defective perfusion of tumor tissues.

Discussion

Recent reports confirm the critical role of the Notch ligand Dll4 in angiogenesis, and demonstrate that Dll4 blockade can effectively repress tumor growth by disrupting vasculature (8, 9). In this study, we show that blockade of Notch receptor function using a novel construct derived from the Notch1 ectodomain also effectively reduces tumor perfusion, although its effects on vasculature are distinct. The Notch1 decoy inhibited signaling induced by the binding of ligands Jagged1, Dll1 and Dll4 to Notch1. Consistent with a role for Notch receptor activation in neoangiogenesis, overexpression of Notch4 induced endothelial cell extensions, which could be prevented by blocking Notch signaling with either Notch1 decoy or GSI. Although Notch1 decoy did not inhibit tumor cell growth *in vitro*, expression of Notch1 decoy inhibited growth and angiogenesis of Mm5MT-FGF4 xenografts, in which Jagged1 expression is up-regulated. Similarly, Notch1 decoy expression had no effect on NGP

tumor cell proliferation *in vitro*, but disrupted tumor vessels and viability *in vivo*.

Notch4 overexpression in HUVECs was sufficient to induce endothelial extensions on fibrin gel without exogenous expression of Notch ligands. Since fibrin is known to induce Jagged1 expression in EC, and thus may have functioned to promote HUVEC expression of Jagged1 in this assay, we speculate that this caused activation of Notch4. In HeLa co-culture signaling assays, the Notch1 decoy inhibited signaling via ligand-Notch1 receptor interaction. We were unable to similarly evaluate Notch4 activity as Notch4 was poorly processed and presented in HeLa cells (not shown). However, processed Notch4 is found on HUVECs after adenoviral Notch4 transduction (not shown), indicating that Notch1 decoy can block ligand-induced Notch4 activation.

The multiple roles recently demonstrated for Notch signaling in tumorigenesis increase the attractiveness of this pathway as a potential target for cancer therapy. While Notch activation is likely to function directly in malignant transformation in human cancers (20, 21), it may also be required for angiogenesis (8, 9). Interestingly, Notch ligand induction can be regulated by growth factor signals. For example, Jagged1 is induced in tumor cells by HGF (10), and Dll4 induced in EC by VEGF (22). Here we show that FGF4 can similarly stimulate Jagged1 and Dll1 expression in murine Mm5MT cells. Notch1 decoy reduced Mm5MT-FGF4 tumor growth and angiogenesis *in vivo* but did not affect tumorigenicity *in vitro*. Thus, these results suggest that Notch receptor activation in

Mm5MT vasculature rather than tumor cells is required for neoplastic growth in this system.

While both Mm5MT-FGF4 and NGP xenografts displayed striking disorder of tumor vasculature after Notch1 decoy expression, the differences in vascular phenotype observed in these models suggest that tumor-specific patterns of Notch function may fine-tune vessel assembly. Mm5MT-FGF4 tumors proliferate rapidly, and develop dense, erratic endothelial networks relatively devoid of recruited VMC. These immature vascular beds express Dll4 (data not shown) and are extensively ablated by Notch1 decoy expression, leaving small clusters or individual EC isolated in tumor parenchyma. Consistent with this initial failure to form vessel networks, decoy-expressing tumor remnants are not necrotic and are significantly smaller than controls. In contrast, NGP tumors develop a mature vascular plexus, with near-uniform coverage of endothelium by VMC. NGP vessels express Notch1 and relatively little Dll4 (not shown). Notch1 decoy expression in NGP tumors causes intratumoral hemorrhage and necrosis, with loss of vessel continuity, suggesting that perfused vessels become unstable after some degree of tumor growth has occurred.

Collectively, these data provide support for a model in which Notch signaling controls multiple aspects of tumor angiogenesis. While Notch activation is broadly required for neoangiogenesis, individual Notch proteins may differentially regulate vascular remodeling. Our results confirm the importance of Notch ligand-receptor interactions in tumor vasculature, and suggest that perturbing Notch receptor function may provide a novel

and effective means of disrupting tumor angiogenesis.

References For Fourth Series of Experiments

1. Ferrara, N. Vascular Endothelial Growth Factor: Basic Science and Clinical Progress. Endocr. Rev., *25:* 581-611, 2004.
2. Jain, R., Duda, D., Clark, J., and Loeffler, J. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol. 2006 Jan;3(1):24-40., *3:* 24-40, 2006.
3. Kopan, R. Notch: a membrane-bound transcription factor. J Cell Sci., *115:* 1095-1097, 2002.
4. Shawber, C. and Kitajewski, J. Notch function in the vasculature: insights from zebrafish, mouse and man. Bioessays., *26:* 225-234, 2004.
5. Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., Murphy, A. J., Adams, N. C., Lin, H. C., Holash, J., Thurston, G., and Yancopoulos, G. D. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci *101:* 15949-15954, 2004.
6. Patel, N. S., Li, J.-L., Generali, D., Poulsom, R., Cranston, D. W., and Harris, A. L. Up-regulation of Delta-like 4 Ligand in Human Tumor Vasculature and the Role of Basal Expression in Endothelial Cell Function. Cancer Res., *65:* 8690-8697, 2005.
7. Patel, N. S., Dobbie, M. S., Rochester, M., Steers, G., Poulsom, R., Le Monnier, K., Cranston, D. W., Li, J.-L., and Harris, A. L. Up-Regulation of Endothelial Delta-like 4 Expression Correlates with

Vessel Maturation in Bladder Cancer. Clin Cancer Res, *12:* 4836-4844, 2006.

8. Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chanthery, Y., Kowalski, J., Watts, R. J., Callahan, C., Kasman, I., Singh, M., Chien, M., Tan, C., Hongo, J. A., de Sauvage, F., Plowman, G., and Yan, M. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature, *444:* 1083-1087, 2006.

9. Noguera-Troise, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., Lin, H. C., Yancopoulos, G. D., and Thurston, G. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature, *444:* 1032-1037, 2006.

10. Zeng, Q., Li, S., Chepeha, D. B., Giordano, T. J., Li, J., Zhang, H., Polverini, P. J., Nor, J., Kitajewski, J., and Wang, C.-Y. Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell, *8:* 13-23, 2005.

11. Das, I., Craig, C., Funahashi, Y., Jung, K.-M., Kim, T.-W., Byers, R., Weng, A. P., Kutok, J. L., Aster, J. C., and Kitajewski, J. Notch Oncoproteins Depend on {gamma}-Secretase/Presenilin Activity for Processing and Function. J Biol Chem., *279:* 30771-30780, 2004.

12. Shawber, C. J., Das, I., Francisco, E., and Kitajewski, J. A. N. Notch Signaling in Primary Endothelial Cells. Ann NY Acad Sci, *995:* 162-170, 2003.

13. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai,

Y., and Phipps, M. L. Construction of adenovirus vectors through Cre-lox recombination. J Virol., *71:* 1842-1849, 1997.

14. Jaffe, E., Nachman, R., Becker, C., and Minick, C. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest., *52:* 2745-2756, 1973.

15. Vorontchikhina, M. A., Zimmermann, R. C., Shawber, C. J., Tang, H., and Kitajewski, J. Unique patterns of Notch1, Notch4 and Jagged1 expression in ovarian vessels during folliculogenesis and corpus luteum formation. Gene Expression Patterns, *5:* 701-709, 2005.

16. Kim, E. S., Serur, A., Huang, J., Manley, C. A., McCrudden, K. W., Frischer, J. S., Soffer, S. Z., Ring, L., New, T., Zabski, S., Rudge, J. S., Holash, J., Yancopoulos, G. D., Kandel, J. J., and Yamashiro, D. J. Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci U S A, *99:* 11399-11404, 2002.

17. Montesano R and L., O. Phorbol esters induce angiogenesis in vitro from large-vessel endothelial cells. J Cell Physiol., *130:* 284-291., 1987.

18. Koolwijk P, van Erck MG, de Vree WJ, Vermeer MA, Weich HA, Hanemaaijer R, and VW., v. H. Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. J Cell Biol, *132:* 1177-1188, 1996.

19. Zimrin, A. B., Pepper, M. S., McMahon, G. A., Nguyen, F., Montesano, R., and Maciag, T. An Antisense Oligonucleotide to the Notch Ligand Jagged Enhances Fibroblast Growth Factor-induced Angiogenesis in Vitro. J Biol Chem, *271:* 32499-32502, 1996.

20. Nickoloff, B., Osborne, B., and L., M. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene, *22:* 6598-6608, 2003.

21. Radtke, F. and Raj, K. THE ROLE OF NOTCH IN TUMORIGENESIS: ONCOGENE OR TUMOUR SUPPRESSOR? Nature Reviews Cancer, *3:* 756-767, 2003.

22. Liu, Z.-J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G. P., Fairman, R. M., Velazquez, O. C., and Herlyn, M. Regulation of Notch1 and Dll4 by Vascular Endothelial Growth Factor in Arterial Endothelial Cells: Implications for Modulating Arteriogenesis and Angiogenesis. Mol Cell Biol *23:* 14-25, 2003.

Fifth Series of Experiments

Notch as a Therapeutic Target in Ovarian Cancer

Epithelial ovarian carcinoma is the leading cause of death from gynecological cancer in the U.S. Patients suffer as a result of our inability to diagnose the disease at an early stage, and from the lack of novel therapeutics directed against this tumor. Vascular endothelial growth factor (VEGF) is produced by malignant epithelial ovarian cancer cells, which, in turn, promotes tumor angiogenesis that nourishes the tumor [1]. Recent clinical trials demonstrate that targeting VEGF for therapeutic intervention can improve outcome, in some cases [2]. Thus, anti-angiogenic therapeutics have been validated as an approach to treat ovarian cancer, but more work needs to be done to build on these gains. We have identified the Notch signaling pathway as a novel angiogenic pathway in both physiological and pathological angiogenesis [3, 4]. Notch is a cell surface receptor (Figure 1- Notch1) that promotes cell fate determination, cell survival and proliferation. Our lab has developed a Notch inhibitor, called the Notch decoy (schematized in Figure 1), which is a fusion protein comprising the **N**otch**1** **e**xtra-**c**ellular **d**omain fused to an **Fc** tag (N1ECDFc) which can inhibit ligand-dependent Notch signaling (data not shown). We have demonstrated that the Notch decoy can block VEGF-induced angiogenesis and tumor angiogenesis in a Wilms Tumor mouse model (data not shown). We generated human ovarian cancer cells SKOV3 that over-express the Notch decoy, SKOV3-N1ECDFc. We found that expression of Notch decoy in SKOV3 cell

significantly inhibited the growth of tumor when xenografted into mice (Figure 36). Thus, the Notch decoy blocks ovarian cancer growth. We hypothesize that this inhibition is targeting tumor angiogenesis. Further, we have surveyed human ovarian cancer samples and have found that several Notch ligands and Notch receptors are highly expressed in tumor vessels associated with ovarian cancers, thus supporting the hypothesis that one can target Notch in human ovarian tumor vessels and ultimately block their growth.

This study will test the hypothesis that Notch signaling promotes tumor angiogenesis in ovarian cancer. In addition, we hypothesize that inhibition of Notch, utilizing a "Notch decoy," will block tumor growth. We will determine if Notch decoy action is against tumor vessels or growth of tumor cells or both. The overall goal is to establish Notch decoy as a therapeutic for treatment of ovarian cancer.

Specific Aims:

Specific Aim 1: Determine if Notch decoy expression blocks tumor xenograft growth of SKOV3 or OVCAR3 cells and define whether Notch decoy is targeting tumor angiogenesis.

Specific Aim 2: Modify the Notch decoy design to make versions with increased efficacy / stability.

Specific Aim 3: Use purified Notch decoys to block ovarian cancer tumor xenografts in mice, also in combination with standard chemotherapeutic agents.

Research Strategy:

Specific Aim 1: *Determine if Notch decoy blocks tumor xenograft growth of SKOV3 or OVCAR3 cells and define whether Notch decoy is targeting tumor angiogenesis.*

Our preliminary results demonstrate that SKOV3 tumor cells programmed expressing the Notch1 decoy block the growth of ovarian cancer xenografts (Fig. 36). This finding will be replicated in another ovarian cancer cell line, OVCAR3. The Notch decoy may inhibit growth of tumor cells, tumor vessels or both. To determine if Notch decoy targets tumor cells, we will determine if ovarian cancer lines expressing Notch decoy grow more poorly in soft-agar assays. To determine if the decoy is targeting tumor angiogenesis, we will evaluate xenografted tumors for evidence of such inhibition. Measures of tumor angiogenesis for control xenografts will include microvessel density assessment, proliferative index of tumor endothelial cells, and imaging tumor vasculature via lectin perfusion. Measures of inhibition of tumor angiogenesis in Notch decoy expressing xenografts will include assessment of apoptotic endothelial cells, reduction of tumor microvasculature and reduced ascites accumulation.

Specific Aim 2: *Modify the Notch decoy design to make versions with increased efficacy / stability.*

The current Notch1 decoy, although effective as a Notch blocking agent, may not be readily amenable to purification, as it is a relatively large protein. We propose to develop variants of Notch1 decoy that encompass smaller parts of the extracellular domain. These variants will be screened as inhibitors of Notch

signaling in cell culture-based assays. Inhibitory variants will also be screened for: their efficiency of secretion from producing cells, their stability once secreted, their solubility, and the ease of purification via the Fc affinity tag. Purified variants will be injected into mice to evaluate potential toxicity.

Specific Aim 3: *Use purified Notch decoys to block ovarian cancer tumor xenografts in mice, also in combination with standard chemotherapeutic agents.* The purified decoys, from *Specific Aim 2,* will be employed, either as single agents, or in combination with chemotherapy, to evaluate their efficacy against ovarian cancer xenografts (SKOV3/OVCAR3). Using the purified Notch decoy as a single agent, we will attempt to replicate the block to tumor xenograft growth seen in experiments of *Specific Aim 1* and determine the optimal concentration for inhibition. To accomplish this end, we will determine the optimal dose, dosing schedule, and duration of treatment needed to block SKOV3/OVCAR3 xenograft growth in mice. Next, we will use the Notch decoy in combination with paclitaxol. An optimal dose of Notch decoy will be used with or without paclitaxol to determine efficacy in blocking xenograft growth.

Impact: This study is aimed at directly advancing the treatment of ovarian cancer. Clinical studies have recently established the success of treating ovarian cancers with VEGF blocking agents. Few new therapeutic agents have been developed to supplement chemotherapeutic treatment. Despite this success, targeting of VEGF to block tumor growth may only limit tumor growth to an extent. It may also lead to new tumor vessels growing

that are resistant to VEGF blockade. We propose to target an alternative tumor angiogenic pathway implicated in ovarian cancer, the Notch signaling pathway. This study will advance treatment by developing a novel therapeutic entity, the Notch decoy. Our preliminary studies already establish that Notch decoy can block ovarian cancer growth in a mouse model. Thus, the impact of development of the Notch decoy as a therapeutic entity may be highly significant.

Innovation: Our recent development of the Notch decoy presents a novel and previously untested paradigm to treat ovarian cancer. No other published study has yet implicated this pathway in the treatment of ovarian cancer, yet our preliminary evidence defines this as a key area for exploration. In addition, despite the recent success of VEGF-blockade as a new therapeutic approach for treatment of ovarian cancer, we feel that this approach may need to be supplemented by inhibition of other angiogenic pathways, such as Notch.

References For Fifth Series of Experiments

1. Abu-Jawdeh, G.M., et al., *Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms.* Lab Invest, 1996. **74**(6): p. 1105-15.
2. Monk, B.J., et al., *Activity of bevacizumab (rhuMAB VEGF) in advanced refractory epithelial ovarian cancer.* Gynecol Oncol, 2005. **96**(3): p. 902-5.
3. Shawber, C., J.J. Kandel, and J. Kitajewski, *Notch: cell fate determination from vascular development to*

human vasculopathy. Drug Discovery Today: Disease Models, 2004. **1**(3): p. 351-8.

4. Zeng, Q., et al., *Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling*. Cancer Cell, 2005. **8**(1): p. 13-23.

Sixth Series of Experiments

A Foxo/Notch pathway controls myogenic differentiation and fiber type specification

Foxo transcription factors govern metabolism and cellular differentiation. Unlike Foxo-dependent metabolic pathways and target genes, the mechanisms by which these proteins regulate differentiation have not been explored. Activation of Notch signaling mimics the effects of Foxo gain-of-function on cellular differentiation. Using muscle differentiation as a model system, we show that Foxo physically and functionally interacts with Notch by promoting co-repressor clearance from the Notch effector Csl, leading to activation of Notch target genes. Inhibition of myoblast differentiation by constitutively active Foxo1 is partly rescued by inhibition of Notch signaling, while Foxo1 loss-of-function precludes Notch inhibition of myogenesis and increases MyoD expression. Accordingly, conditional Foxo1 ablation in skeletal muscle results in increased formation of MyoD-containing (fast-twitch) muscle fibers and altered fiber type distribution at the expense of Myogenin-containing (slow-twitch) fibers. Notch/Foxo1 cooperation may integrate environmental cues through Notch with metabolic cues through Foxo1 to regulate progenitor cell maintenance and differentiation.

A central question in regenerative medicine is to understand how highly specialized cell types arise from undifferentiated stem or progenitor cells (1). Germane to

this issue is how biochemical signals engendered by microenvironmental and endocrine/nutritional cues are transcriptionally integrated to activate cellular differentiation processes.

The O subfamily of forkhead (Fox) proteins regulates hormonal, nutrient and stress responses to promote cell survival and metabolism. The ability to fine-tune Foxo transcription is essential to control these cellular functions, and is largely dependent on post-transcriptional modifications, including phosphorylation and acetylation (2). In addition to their role in terminally differentiated cells, Foxo proteins have also been implicated in myoblast (3), pre-adipocyte (4) and endothelial cell differentiation (5). Moreover, Foxo4 regulates vascular smooth muscle cells differentiation through interactions with Myocardin (6). *Foxo3* knockout mice display premature ovarian failure, consistent with a role for this gene in ovarian follicle maturation (7). The mechanisms by which Foxo proteins control cellular differentiation remain unclear, and recent conditional ablation studies are consistent with a significant degree of functional overlap amongst the three Foxo isoforms in the hematopoietic lineage (8, 9).

The Notch pathway plays an important role in neural, vascular, muscular and endocrine differentiation during embryogenesis (10). Upon ligand-induced cleavage, the intracellular domain of the Notch receptor translocates to the nucleus, where it interacts with the DNA binding protein Csl, changing its transcriptional properties from a suppressor to an activator of transcription (11). Csl targets include the *Hairy* and *Enhancer of Split* (Hes,

Hey) genes. Hes1 controls gut endoderm (12), pre-adipocyte (13) and neurogenic differentiation (14). Active Notch signaling, or gain of Notch1 receptor function, inhibits differentiation of C2C12 and 10T/2 myoblasts by suppressing *MyoD* transcription (15-21).

It is noteworthy that Foxo1 gain-of-function (3-5) phenocopies Notch1 activation (13, 17, 22, 23) in every cellular differentiation context. Moreover, Foxo1 ablation (24) phenocopies Notch1 ablation (25) in mice. Despite these intriguing similarities, Foxo and Notch signal through two seemingly distinct mechanisms, the phosphatidylinositol-3-kinase pathway (Foxo), and the Hes/Hey pathway (Notch). In this study, we show that Foxo physically and functionally interacts with Notch by promoting co-repressor clearance from Csl, thus controlling the myogenic program.

Myogenic precursors arise from mesodermal stem cells (26) and are converted into myotubes by a multi-step process culminating in the expression of myogenic transcription factors of the MRF family (MyoD, Myogenin, MRF4 and Myf5) (27). Myogenic transcription factors heterodimerize with E proteins and promote expression of muscle-specific genes, acting in close coordination with myocyte-specific MEF2 enhancer factors (28).

Adult muscle is a heterogeneous tissue, primarily defined by its myofiber content (29). Different myosin heavy chain (MyHC) sub-types characterize different myofibers. Type I fibers express primarily slow-twitch MyHC, whereas type II fibers express fast-twitch MyHC (29). The process of fiber-type specification is controlled at multiple

steps. First, there appears to be heterogeneity among myogenic precursor cells, and evidence from avian embryo cross-transplantation experiments indicates that early precursors contribute primarily to slow muscle fibers, and later precursors to fast fibers (29). Post-natally, fiber type specification is also affected by cell autonomous factors, including innervation and endocrine/nutritional cues (28). The Foxo co-activator Pgc1α plays a critical role in promoting the formation of slow-twitch fibers (30), and recent data have also implicated the Foxo deacetylase Sirt1 in this process (31). Using conditional mutagenesis in mice, we show that Foxo1's role in suppressing MyoD-dependent myogenesis in C2C12 cells is mirrored by an increase of MyoD-containing myofibers in Foxo1-deficient skeletal muscle, consistent with a key function in myoblast lineage specification.

Results

Interaction of Foxo1 and Notch signaling in C2C12 differentiation

To understand whether Notch and Foxo interact to control muscle development, we used a cellular differentiation model. C2C12 cells undergo myogenic conversion and myotube fusion upon growth factor withdrawal, a process associated with Foxo1 nuclear translocation (3). Accordingly, transduction of adenovirus encoding a constitutively active Foxo1 mutant (Foxo1-ADA)(4) blocked the effect of serum withdrawal to induce C2C12 differentiation, as reflected by inhibition of myoblast fusion (Fig. 37a-c). Conversely, Foxo1 inhibition by siRNA did not affect these processes (Fig. 37d).

Similarly, constitutively active Notch (Notch1-IC) phenocopied Foxo1-ADA in blocking myoblast differentiation (Fig. 37e). Virtually all cells became transduced with the adenoviruses (Fig. 44). Foxo1 siRNA effectively suppressed expression of both endogenous Foxo1 and transfected FLAG-Foxo1 (Fig. 45) in a dose-dependent manner, without affecting control proteins or other Foxo isoforms (Fig. 46). Neither Foxo1-ADA nor Notch1-IC affected C2C12 proliferation (Fig. 47).

We asked whether we could preempt the effect of Foxo1-ADA by inhibition of endogenous Notch signaling. To this end, we used a truncated Notch1 receptor lacking the transmembrane anchor and intracellular domain, which acts as a decoy receptor by binding Notch ligands (32, 33) (Y.F. and J.K., unpublished observation). The decoy did not affect C2C12's ability to undergo differentiation in response to growth factor withdrawal (Fig. 37f), but partly rescued Foxo1-ADA inhibition of myoblast differentiation (Fig. 37g). As an alternative probe to block Notch signaling, the presenilin inhibitor (PSI) Compound E (34) also rescued Foxo1-ADA inhibition of myoblast differentiation (Fig. 37h).

To examine the effect of Foxo1 on Notch signaling, we co-transfected Foxo1 siRNA and Notch-IC. Foxo1 siRNA rescued inhibition of myoblast differentiation and Myosin expression by Notch1-IC (Fig. 37i), while control siRNA had no effect (data not shown). To rule out non-specific effects of Foxo1 siRNA on myoblast differentiation, we generated a siRNA-resistant Foxo1-ADA (Fig. 48). Foxo1 siRNA reversed the effects of Foxo1-ADA (Fig. 37j), but failed to rescue inhibition of C2C12 differentiation

caused by siRNA-resistant Foxo1-ADA (Fig. 37k). We present a quantitative analysis of these data in Fig. 38a, showing that Foxo1 and Notch1-IC decreased myosin levels by >80%, while Notch decoy and Foxo1 siRNA restored them to ~70% of fully differentiated cells. We obtained similar data by performing a morphometric analysis of Myosin-positive cells (Fig. 38b). These data indicate that Foxo1 is required for the effect of Notch on myoblast differentiation.

We next tested whether Foxo1 affects differentiation via its transcriptional function. To this end, we generated a DNA Binding Deficient mutant in the backbone of the ADA mutant, by replacement of N208A and H212R (DBD-Foxo1ADA) (6, 35). We confirmed that this mutant is unable to bind DNA by measuring *Igfbp1* promoter activity, a canonical Foxo1 target. Foxo1-ADA increased *Igfbp1* promoter activity by 10-fold, whereas DBD-Foxo1ADA was unable to do so (Fig. 38c, left panel). Surprisingly, this mutant was as effective as the DNA binding-competent Foxo1-ADA at inhibiting differentiation (Fig. 37l). These data indicate that Foxo1 controls differentiation independently of its ability to bind DNA in a sequence-specific manner.

Foxo1 binds to Csl and is recruited to the Hes1 promoter

Notch1-IC binds to and co-activates Csl to promote *Hes* and *Hey* expression (11). Based on the results with the DBD-Foxo1ADA mutant, we tested whether Foxo1 interacts with Csl in a Notch-dependent manner using co-culture of C2C12 cells expressing Notch1 receptor with HEK293 cells expressing the Notch ligand Jagged1 (denoted by the "+"

sign), or LacZ as a negative control (denoted by the "-" sign). We provide several lines of evidence that Foxo1 and Csl interact in cultured cells. We detected endogenous Foxo1 in endogenous Csl immunoprecipitates, and the co-immunoprecipitation was significantly enhanced by activation of Notch signaling (Fig. 39a). To confirm the specificity of the interaction, we expressed HA-tagged Foxo1 and FLAG-tagged Csl in C2C12 cells. Following immunoprecipitation with anti-HA (Foxo1) antiserum, we detected FLAG-Csl in immunoblots (Fig. 39b). Conversely, following immunoprecipitation with anti-FLAG (Csl) antiserum, we detected HA-Foxo1 in immunoblots (Fig. 39c). The ability to co-immunoprecipitate with Csl appears to be specific to Foxo1, as we failed to detect other Foxo isoforms in Csl immunoprecipitates (Fig. 42). A truncated Foxo1 mutant (Δ256, encoding amino acids 1-256)(36) retained the ability to interact with Csl. We detected FLAG-Csl in Myc-Δ256 immunoprecipitates (Fig. 39d), and HA- Δ256 in FLAG-Csl immunoprecipitates (Fig. 39e), indicating that Csl interacts with the Foxo1 N-terminal domain.

To determine whether this is a direct protein-protein interaction and map the interaction domain(s), we first carried out pull-down assays with affinity-purified GST-Foxo1 produced in bacteria and FLAG-Csl expressed in HEK293 cells. We detected Csl association with full-length and N-terminal Foxo1 (amino acids 1-300), but not with C-terminal Foxo1 (aa. 290-655) or GST (Fig. 40a). We next mapped the Csl domain that interacts with Foxo1 using a cell-free system with GST-Foxo1 and GST-Flag/Csl purified from bacterial cultures. Again, we recovered full-length (1-655) and N-terminal (1-300), but not C-

terminal (290-655) Foxo1 in Csl immunoprecipitates. Conversely, N-terminal Foxo1 interacts with N-terminal Csl (Fig. 40b).

We used Csl deletion mutants to map the Foxo1-binding domain in Csl. These studies indicate that Foxo1 binds to a domain encompassing amino acids 172-279 (Fig. 40c), which is contained within the Csl NTD domain (37) (Fig. 40c, diagram). Interestingly, this domain is required for DNA and corepressor binding, but does not contribute to Notch binding (38, 39).

Csl binds to a consensus sequence in the *Hes1* promoter (40), which thus provides a useful readout assay of the Foxo/Csl interaction. If the latter were required to regulate C2C12 differentiation, three predictions should be met: (a) Foxo1 should be detected in chromatin immunoprecipitation assays (ChIP) spanning the Csl element in the *Hes1* promoter, (b) the interaction should be differentiation-dependent and (c) inhibition of differentiation by Foxo1-ADA should be accompanied by constitutive binding to the Csl element in the *Hes1* promoter. Figure 40d demonstrates that all predictions are fulfilled. First, we performed ChIPs using primers spanning the Csl binding site of *Hes1* in differentiating C2C12 cells. We detected endogenous Foxo1, Notch1 and Csl in immunoprecipitates from undifferentiated cells (Fig. 40d Endog lanes, Day 0). As the PCR-amplified sequence contains no forkhead binding sites, we conclude that Foxo1 binds to this DNA fragment via Csl. Moreover, binding of both Foxo1 and Notch1 decreased as cells became differentiated (day 1 and 2). When we transduced

cells with constitutively nuclear Foxo1-ADA, differentiation was inhibited (Fig. 37c) and the mutant Foxo1 was persistently bound to the *Hes1* promoter, as were Csl and Notch1 (Fig. 40d, Foxo1-ADA lanes).

We next analyzed *Hes1* expression. The prediction was that *Hes1* levels should correlate with occupancy of the *Hes1* promoter by Foxo1 and Notch1. Indeed, *Hes1* mRNA expression declined as Foxo1 and Notch1 binding to Csl decreased, while Myosin protein levels increased (Fig. 40d). To rule out a direct effect of Foxo1 on *Csl* transcription, we carried out reporter gene assays with the *Csl* promoter. Foxo1 failed to activate expression of a *Csl* reporter gene, despite the presence of ten repeats of a forkhead binding site in the *Csl* promoter (41) (Data not shown). Moreover, Csl expression was unaffected in C2C12 cells expressing Foxo1-ADA (not shown). These data indicate that Foxo1 regulates Notch-dependent differentiation via protein/protein interactions with Csl.

Foxo1 is required for Notch induction of Hes and Hey genes via Csl

We examined the ability of Foxo1-ADA to promote expression of endogenous *Hes1, Hes5* and *Hey1* in C2C12 cells. Both Foxo1-ADA and Notch1-IC increased the expression of the three genes, while Foxo1 siRNA inhibited *Hes1, Hes5* and *Hey1* expression induced by Notch1-IC (Fig. 41a). Foxo1 siRNA had no effect on *Hes1, Hes5* and *Hey1* expression in growth factor-deprived cells (Fig. 41a).

We focused the next set of experiments on *Hes1*, as a prototypical Notch target gene. We tested Foxo1's ability to regulate *Hes1* transcription using reporter assays with the *Hes1* promoter, as well as measurements of *Hes1* expression. Foxo1-ADA and Notch1-IC induced Hes1 promoter activity by 1.8- and 2.5- fold, respectively. Co-transfection of Foxo1-ADA with Notch1-IC caused a 2.5-fold increase (Fig. 41b). Co-transfection of Foxo1 siRNA suppressed Notch-induced *Hes1* activity in a dose-dependent manner, while control siRNA had no effect (Fig. 41b). We obtained similar results with a synthetic *Hes1* reporter containing four tandem repeats of the Csl binding motif (Fig. 50). Moreover, the DBD-Foxo1ADA was able to induce *Hes1* reporter gene activity to an even greater extent than Foxo1-ADA, confirming that direct DNA binding is not required for Foxo1 activation of Hes1 (Fig. 38c, right panel).

The failure of Notch1-IC to induce *Hes1* expression in cells expressing Foxo1 siRNA suggests that Foxo1 is required for Csl/Notch interaction. Thus, we investigated the binding of Foxo1 and Notch1 to the *Hes1* promoter in a co-culture system. We co-cultured C2C12 cells expressing Notch1 with HEK293 cells expressing the Notch ligand Jagged1 to induce activation of endogenous Notch signaling. Co-culture in the presence of Jagged1-expressing cells increased endogenous Foxo1 (Fig. 42a, lanes 1-2) and Notch1 binding to the *Hes1* promoter in ChIP assays (Fig. 42a, b, lanes 1-2) (42). These data are consistent with the observation that Foxo1 co-immunoprecipitation with Csl increased upon co-culture (Fig. 39a). To test whether Foxo1 binding to the *Hes1*

promoter is Csl-dependent, we inhibited Csl expression with siRNA (Fig. 51). Transfection of Csl siRNA inhibited both Foxo1 and Notch1 binding to *Hes1* promoter (Fig. 42a, lanes 3-4), indicating that they are Csl-dependent. Moreover, Foxo1-ADA failed to induce *Hes1* expression in the presence of Csl siRNA (Fig. 41a, lane 5). The results of ChIP experiments were corroborated by *Hes1* promoter assays. Expression of Jagged1 or Notch1 alone had no effect on *Hes1* activity, but co-culturing yielded a 3.7-fold increase in *Hes1* reporter gene activity (Fig. 42c). Foxo1 siRNA abolished Notch binding to the *Hes1* promoter in ChIP assays (Fig. 42b, lanes 3-4) and induction of *Hes1* promoter activity (Fig. 42c). These results suggest that Foxo1 is required for binding of Notch1 to the *Hes1* promoter, and provide a mechanism whereby inhibition of Foxo1 expression restores differentiation of myoblasts expressing Notch1-IC. The ability of Foxo1 siRNA to inhibit Notch induction of *Hes1* in a co-culture system rules out the possibility that the effects observed in differentiation experiments with Notch1-IC are due to non-physiologic activation of Notch signaling by the truncated intracellular Notch1 mutant (15).

Foxo1 promotes corepressor clearance and Maml1 binding to Csl

To clarify the molecular mechanism of Foxo1-dependent activation of *Hes1* expression, we investigated corepressor/coactivator exchange at the *Hes1* promoter. Activation of Notch cleared the corepressors Ncor and Smrt (43) and recruited the coactivator Maml1 (42) to the *Hes1* promoter. Foxo1 siRNA prevented Notch-induced corepressor exchange (Fig. 42d). These data are

consistent with the observation that Foxo1 binds to the region 172-279 of Csl (Fig. 40c), which has been shown to contain the Ncor/Smrt binding sites (38, 39).

To demonstrate that the observed changes in the transcriptional complex result in changes in Hes1 activity, we investigated expression of Hes1 target genes involved in myogenesis. Hes1 has been proposed to suppress myoblast differentiation by inhibiting the bHLH transcription factor MyoD, without affecting Myf5 (16, 17). Expression analyses revealed that Notch1-IC or Foxo1-ADA suppressed *MyoD*, while *Myf5* was unaffected. Notch decoy or Foxo1 siRNA partly restored *MyoD* expression (Fig. 42e).

Altered fiber type composition in skeletal muscle lacking Foxo1

Based on the cellular data, we undertook to probe Foxo1 function in muscle differentiation *in vivo* using conditional gene inactivation. The predicted outcome of this experiment is accelerated differentiation of MyoD-containing, but not Myf5-containing myoblasts. Because MyoD is the predominant myogenic factor in fast fibers, while Myogenin is predominant factor in slow fibers (44), the removal of Foxo/Notch inhibition on MyoD expression should result in increased formation of fast fibers, potentially at the expense of slow fibers.

There are three Foxo isoforms in mice: Foxo1, 3, and 4 (8, 9). The latter is predominant in most muscle types (45) except soleus, where Foxo1 is the most abundant (Fig. 43a). Coincidentally, soleus is also

physiologically enriched in slow-twitch fibers, and thus allowed us to readily test our hypothesis. We inactivated Foxo1 expression in skeletal muscle by crossing mice homozygous for a floxed *Foxo1* allele with Myogenin-cre transgenics. mRNA analysis indicated that the knockout occurred as planned (data not shown). Histological analyses revealed a reduction of type I (slow-twitch) fibers in soleus of Myog-Foxo1 mice, while type II fiber-enriched muscles were unaffected (Figure 43b). Consistent with the histological findings, expression of type I fiber markers decreased, while type II fiber markers increased in Myog-Foxo1 mice (Figure 43c). We then analyzed expression of the myogenic transcription factors MyoD, Myf5 and Myogenin. MyoD is the predominant factor in fast fibers, and Myogenin in slow fibers (44). Consistent with the histopathology, we found a twofold increase in MyoD expression and ~80% decrease in Myogenin, while Myf5 expression was unchanged (Fig. 43c). Moreover, expression the Foxo1 coactivator Pgc1α, which regulates type I fiber determination (30) was unchanged, indicating that the phenotype of Myog-Foxo1 mice cannot be accounted for by decreased Foxo1-dependent Pgc1α transcription (Fig. 43c) (46). As a functional correlate of the observed fiber type switch, we examined running performance on a treadmill. Indeed, Myog-Foxo1 mice displayed reduced running capacity, as predicted from the reduction in type I (endurance) fibers (Fig. 43d).

Finally, to determine whether these changes reflected developmental alterations in fiber-type specification, as opposed to adaptive or cell-nonautonomous factors, we determined MyoD expression in Foxo1 (24) and Notch1 knockout (25) embryos at E9.5. In *Foxo1*$^{-/-}$ embryos, MyoD

levels increased 3.1±1.1 fold, and in *Notch1*$^{-/-}$ embryos 7.3±2.9 fold compared to controls (P<0.05 in both mutants vs. wildtype, n=4). The increase in MyoD expression observed *in vivo* is consistent with the physical and functional interactions between Foxo1 and Notch at this key signaling nexus in myoblast differentiation. Thus, we propose that the fiber-type switch in Myog-Foxo1 mice is the result of accelerated differentiation of MyoD-containing myoblasts during embryonic development.

Discussion

This study provides biochemical, cellular and genetic evidence that Foxo and Notch pathways cooperate in the regulation of muscle differentiation. The data reveal a novel mode of Foxo1 action to promote corepressor exchange at the *Hes1* promoter via direct binding to the Csl NTD region (Fig. 42f). We propose that Foxo1 binding to this domain stabilizes the Notch/Csl complex and promotes corepressor clearance and Maml1 recruitment, consistent with the proposed role of NTD from structural studies (37). The findings also provide a mechanism by which two major biochemical pathways, the phosphoinositol-3-kinase/Akt pathway and the Notch/Hes pathway, converge in a synergistic manner to control cellular differentiation *in vivo*.

The proposed role for Foxo1 is independent of its transcriptional function, and involves a direct interaction with Csl. While our studies have focused on Hes-1 as a prototypical effector of Notch1 signaling, our data should not be construed to indicate that Hes-1 is

the sole mediator of the Notch/Foxo interaction. For example, we have observed a similar Foxo/Notch epistasis in the differentiation of pre-adipocytes, PC-12, and HUVECs, suggesting that Foxo interacts with Notch in multiple cell contexts (data not shown). We propose that Notch/Foxo cooperation integrates environmental cues through Notch with metabolic cues through Foxo1 to regulate progenitor cell maintenance and differentiation. This two-tiered mechanism allows committed progenitor cells in various tissues to avoid differentiation in response to developmental cues (Notch) when Foxo1 is active, i.e., in the absence of growth factors. These cells would then persist in a dormant state in adult tissues, where they can terminally differentiate in response to a combination of Notch ligand and hormonal/nutritional cues leading to Foxo1 inhibition. This interpretation is consistent with the fiber-type switch observed in Foxo1-deficient muscle, an observation that appears to position Foxo1 as a fate decider within the myogenic lineage, as opposed to an inducer of the myogenic program. It remains to be seen whether other Foxo and Notch isoforms also interact and how they contribute to this process.

The demonstration that Foxo1 is a coregulator of gene expression provides a potential explanation for the protean functions of this transcription factor. An interesting question emerging from our studies is how the switch from one function to the other is effected, and how the complex post-translational modifications of Foxo1 in response to growth factors, hormones and nutrients impinge on this process. The findings have broad

implications for the pathophysiology of disease processes that involve Foxo1 signaling. A potential implication of our observation is the ability to explore the use of agents that inhibit Notch signaling (47) as a treatment of metabolic disorders characterized by excessive Foxo function (48).

Materials and Methods

Animal generation and analysis

Myogenin-cre (49) and *Foxo1*^{flox} mice have been described (9). The wild-type, null and *Foxo1*^{flox} alleles were detected using PCR with primers 5'-GCT TAG AGC AGA GAT GTT CTC ACA TT-3', 5'-CCA GAG TCT TTG TAT CAG GCA AAT AA-3' and 5'-CAA GTC CAT TAA TTC AGC ACA TTG A-3'. Prior to the treadmill performance test, mice were trained for 2 days (Columbus Instruments). The test was performed at 15 m/min for the first 30 min, followed by 1m/min increases at 10 min intervals until exhaustion. Skeletal muscle samples were quickly frozen in OCT matrix, and 7 μm serial sections were obtained. Muscle fibers were typed using metachromatic ATPase (50) or immunostaining with anti-skeletal slow myosin (Sigma). For embryonic studies, we set up timed matings of heterozygous Foxo1 (24) or Notch1 (25) mice and recovered embryos at E9.5. mRNA was isolated from whole embryos and real-time RT-PCR was performed as described below.

Viral expression studies

C2C12 cells were differentiated as described (3, 4). Foxo1-ADA, Notch1-IC, Jagged1, Csl and Notch decoy adenoviral and mammalian expression vectors have been

described (36, 51). We generated retroviruses expressing Foxo1-ADA and Notch1-IC using the pQCXIH vector. To generate Notch decoy (pAdlox Notch1ECD-Fc), the extracellular domain of Notch1 (bp 241-4229, GenBank accession# X57405) was fused in frame with human IgG Fc tag and cloned into pAdlox. Retroviral supernatant was produced from cells transiently co-transfected with pVSV-G vector and designated pQCXIH vector into GP2-293 cells (BD Bioscience). To generate the DNA binding-deficient Foxo1, we replaced N208 and H212 with alanine and arginine, respectively, using QuikChange Mutagenesis Kit (Stratagene). The mutations were then cloned in the backbone of the Foxo1-ADA mutant.

Luciferase assay and co-culture assay

We transfected HEK293 cells with Hes1-luciferase (-194 to 160 from transcription start site) (Hes1/pGL2 basic), Synthetic Hes1- luciferase (containing a 4x Csl binding site, 4 x Csl/pGL2 basic) or Csl-luciferase (-1536 to 22, Csl/pGL2 basic) reporter genes along with pCMV5, pCMV5-Foxo1-ADA, pQNC-Notch1-IC, pHyTc Notch decoy or Foxo1 siRNA. We used plasmid pRSV-β-galactosidase as a control of transfection efficiency (51). For co-culture assay, we expressed Notch1 in C2C12 cells and Jagged1 or LacZ in HEK293 cells by transfection. We then harvested HEK293 cells and seeded them on C2C12 cells. After 1hr incubation, we used the co-cultured cells for experiments.

Western blotting and immunoprecipitation

We performed these assays according to standard techniques using anti-Myosin (MF-20), anti-HA (12CA5, Boehringer Mannheim), anti-FLAG (M2, Sigma), anti-Foxo1

(H128 and N20, Santa Cruz), anti-Notch1 (C-20, Santa-Cruz), anti-Csl (Chemicon and Santa-Cruz), anti-NcoR (Santa-Cruz), anti-SMRT (Santa-Cruz) or anti-MAML1 (Chemicon) antibodies. For Foxo/Csl co-immunoprecipitation, we used purified nuclear fractions (52). Because Csl migrates close to IgG heavy chain on SDS-PAGE, we used dimethylpyrimilidate (DMP from Pierce) to cross-link antibodies to Protein A beads and avoid IgG contamination of eluted protein complexes (52).

Chromatin immunoprecipitation assays

We performed ChIP assay in C2C12 cells as described previously (4) and in co-cultured cells as described by Fryer (42). The primer pairs employed to amplify the Csl binding site of the Hes1 promoter are: 5'-GCAAAGCCCAGAGGAAAGAGTTAG-3' and 5'-AGGAGAGAGGTAGACAGGGGATTC-3'.

siRNA transfection and siRNA-resistant Foxo1

The Foxo1-specific siRNA sequence is 5'-ACGGAGGATTGAACCAGTATA-3'. The Csl specific siRNA sequence is 5'-TAGGGAAGCTATGCGAAATTA-3'. siRNA was transfected using lipofectamine-plus reagent (Invitrogen). We generated siRNA-resistant Foxo1 by replacing three residues (underlined) in the sequence 5'-ACGGCGGTCTGAACCAGTATA-3'. Primer sequences employed for real-time RT-PCR studies are available on request.

Recombinant proteins and interaction assays

We generated GST-FLAG-Csl encompassing amino acids 1-527, 1-279, 1-172 and 279-527 fragments by cloning into pGEX6P-1. GST-Foxo1 constructs have been described (53). Following bacterial culture and IPTG induction, we

purified GST fusion proteins and incubated them together. Thereafter, we isolated GST-FLAG/Csl by immunoprecipitation with anti-FLAG antibody, washed the immune pellets extensively and performed immunoblot with anti-Foxo1 antiserum.

References For Sixth Series of Experiments

1. Singec, I., Jandial, R., Crain, A., Nikkhah, G., and Snyder, E.Y. 2007. The leading edge of stem cell therapeutics. *Annu Rev Med* 58:313-328.
2. Accili, D., and Arden, K.C. 2004. FoxOs at the Crossroads of Cellular Metabolism, Differentiation, and Transformation. *Cell* 117:421-426.
3. Hribal, M.L., Nakae, J., Kitamura, T., Shutter, J.R., and Accili, D. 2003. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* 162:535-541.
4. Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W.H., Arden, K.C., and Accili, D. 2003. The forkhead transcription factor foxo1 regulates adipocyte differentiation. *Dev Cell* 4:119-129.
5. Potente, M., Urbich, C., Sasaki, K.I., Hofmann, W.K., Heeschen, C., Aicher, A., Kollipara, R., Depinho, R.A., Zeiher, A.M., and Dimmeler, S. 2005. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest* 115:2382-2392.
6. Liu, Z.P., Wang, Z., Yanagisawa, H., and Olson, E.N. 2005. Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. *Dev Cell*

9:261-270.

7. Castrillon, D.H., Miao, L., Kollipara, R., Horner, J.W., and DePinho, R.A. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 301:215-218.

8. Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. 2007. FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. *Cell* 128:325-339.

9. Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. 2007. FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. *Cell* 128:309-323.

10. Shawber, C.J., and Kitajewski, J. 2004. Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays* 26:225-234.

11. Lai, E.C. 2002. Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. *EMBO Rep* 3:840-845.

12. Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O.D. 2000. Control of endodermal endocrine development by Hes-1. *Nat Genet* 24:36-44.

13. Ross, D.A., Rao, P.K., and Kadesch, T. 2004. Dual roles for the Notch target gene Hes-1 in the differentiation of 3T3-L1 preadipocytes. *Mol Cell Biol* 24:3505-3513.

14. Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi,

S., Guillemot, F., and Kageyama, R. 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *Embo J* 18:2196-2207.

15. Shawber, C., Nofziger, D., Hsieh, J.J., Lindsell, C., Bogler, O., Hayward, D., and Weinmaster, G. 1996. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development* 122:3765-3773.

16. Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. 1992. Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev* 6:2620-2634.

17. Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. 1999. Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J Biol Chem* 274:7238-7244.

18. Nofziger, D., Miyamoto, A., Lyons, K.M., and Weinmaster, G. 1999. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* 126:1689-1702.

19. Wilson-Rawls, J., Molkentin, J.D., Black, B.L., and Olson, E.N. 1999. Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. *Mol Cell Biol* 19:2853-2862.

20. Hirsinger, E., Malapert, P., Dubrulle, J., Delfini, M.C., Duprez, D., Henrique, D., Ish-Horowicz, D., and Pourquie, O. 2001. Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation. *Development* 128:107-116.

21. Conboy, I.M., and Rando, T.A. 2002. The regulation of Notch signaling controls satellite cell

activation and cell fate determination in postnatal myogenesis. *Dev Cell* 3:397-409.

22. Shawber, C.J., Das, I., Francisco, E., and Kitajewski, J. 2003. Notch signaling in primary endothelial cells. *Ann N Y Acad Sci* 995:162-170.

23. Limbourg, F.P., Takeshita, K., Radtke, F., Bronson, R.T., Chin, M.T., and Liao, J.K. 2005. Essential role of endothelial Notch1 in angiogenesis. *Circulation* 111:1826-1832.

24. Hosaka, T., Biggs, W.H., 3rd, Tieu, D., Boyer, A.D., Varki, N.M., Cavenee, W.K., and Arden, K.C. 2004. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci U S A* 101:2975-2980.

25. Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., et al. 2000. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 14:1343-1352.

26. McKinsey, T.A., Zhang, C.L., and Olson, E.N. 2001. Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev* 11:497-504.

27. Rudnicki, M.A., and Jaenisch, R. 1995. The MyoD family of transcription factors and skeletal myogenesis. *Bioessays* 17:203-209.

28. Bassel-Duby, R., and Olson, E.N. 2006. Signaling pathways in skeletal muscle remodeling. *Annu Rev Biochem* 75:19-37.

29. Schiaffino, S., and Reggiani, C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 76:371-423.

30. Lin, J., Wu, H., Tarr, P.T., Zhang, C.Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., et al. 2002. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418:797-801.

31. Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., et al. 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 127:1109-1122.

32. Nickoloff, B.J., Osborne, B.A., and Miele, L. 2003. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* 22:6598-6608.

33. Nickoloff, B.J., Qin, J.Z., Chaturvedi, V., Denning, M.F., Bonish, B., and Miele, L. 2002. Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. *Cell Death Differ* 9:842-855.

34. Pan, Y., Lin, M.H., Tian, X., Cheng, H.T., Gridley, T., Shen, J., and Kopan, R. 2004. gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. *Dev Cell* 7:731-743.

35. Dowell, P., Otto, T.C., Adi, S., and Lane, M.D. 2003. Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. *J Biol Chem* 278:45485-45491.

36. Nakae, J., Kitamura, T., Silver, D.L., and Accili, D. 2001. The forkhead transcription factor Foxo1

(Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* 108:1359-1367.

37. Kovall, R.A., and Hendrickson, W.A. 2004. Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. *Embo J* 23:3441-3451.

38. Hsieh, J.J., and Hayward, S.D. 1995. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* 268:560-563.

39. Kao, H.Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C.R., Evans, R.M., and Kadesch, T. 1998. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* 12:2269-2277.

40. Tun, T., Hamaguchi, Y., Matsunami, N., Furukawa, T., Honjo, T., and Kawaichi, M. 1994. Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. *Nucleic Acids Res* 22:965-971.

41. Kawaichi, M., Oka, C., Shibayama, S., Koromilas, A.E., Matsunami, N., Hamaguchi, Y., and Honjo, T. 1992. Genomic organization of mouse J kappa recombination signal binding protein (RBP-J kappa) gene. *J Biol Chem* 267:4016-4022.

42. Fryer, C.J., White, J.B., and Jones, K.A. 2004. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell* 16:509-520.

43. Liang, Y., Chang, J., Lynch, S.J., Lukac, D.M., and Ganem, D. 2002. The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jkappa (CSL), the target of the Notch signaling pathway. *Genes Dev* 16:1977-1989.

44. Hughes, S.M., Taylor, J.M., Tapscott, S.J., Gurley,

C.M., Carter, W.J., and Peterson, C.A. 1993. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* 118:1137-1147.

45. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W.H., 3rd, Wright, C.V., White, M.F., Arden, K.C., and Accili, D. 2002. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest* 110:1839-1847.

46. Daitoku, H., Yamagata, K., Matsuzaki, H., Hatta, M., and Fukamizu, A. 2003. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* 52:642-649.

47. Miele, L., Miao, H., and Nickoloff, B.J. 2006. NOTCH signaling as a novel cancer therapeutic target. *Curr Cancer Drug Targets* 6:313-323.

48. Accili, D. 2004. Lilly lecture 2003: the struggle for mastery in insulin action: from triumvirate to republic. *Diabetes* 53:1633-1642.

49. Knapp, J.R., Davie, J.K., Myer, A., Meadows, E., Olson, E.N., and Klein, W.H. 2006. Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. *Development* 133:601-610.

50. Ogilvie, R.W., and Feeback, D.L. 1990. A metachromatic dye-ATPase method for the simultaneous identification of skeletal muscle fiber types I, IIA, IIB and IIC. *Stain Technol* 65:231-241.

51. Das, I., Craig, C., Funahashi, Y., Jung, K.M., Kim, T.W., Byers, R., Weng, A.P., Kutok, J.L., Aster, J.C., and Kitajewski, J. 2004. Notch oncoproteins depend on gamma-secretase/presenilin activity for

processing and function. *J Biol Chem* 279:30771-30780.

52. Chi, T., Yan, Z., Xue, Y., and Wang, W. 2004. Purification and functional analysis of the mammalian SWI/SNF-family of chromatin-remodeling complexes. *Methods Enzymol* 377:299-316.

53. Puigserver, P., Rhee, J., Donovan, J., Walkey, C.J., Yoon, J.C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D., et al. 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* 423:550-555.

Seventh Series of Experiments

Diabetic patients often develop obesity and vascular pathologies. The molecular mechanisms that contribute to diabetic complications remain to be elucidated. In the past two years, I have been evaluating Notch4 knockout mice for postnatal defects. These analyses revealed that Notch4 mutant mice develop hallmarks of diabetes: 1) early onset obesity as seen by a dramatic increase in subcutaneous fat, and 2) reduced pericyte content in retinal vasculature reminiscent of diabetic retinopathy. We have found that Notch and Foxo1, a transcriptional regulator of insulin signaling, cooperate to regulate adipogenesis and angiogenesis. Mice deficient for Notch1, Notch1/Notch4, or Foxo1 die *in utero* with angiogenic defects. These data lead us to hypothesize that dysregulated Notch signaling contributes to diabetic obesity and vasculopathologies. The proposal objective is to examine this hypothesis and define the roles of Notch and insulin signaling interactions in adipogenesis and angiogenesis. Mouse models will be used to alter Notch, Foxo1, and insulin receptor activity via genetic manipulation. Adipogenesis and metabolic dysfunction will be evaluated in Notch4 and insulin receptor knockout mice and embryonic fibroblasts derived from these mice. Embryonic and retinal angiogenesis will be evaluated in mice haploinsufficient for Notch1, Notch4 and/or Foxo1. Finally, the function of Notch and Foxo1 signaling in proliferative retinopathy will be evaluated in a hypoxia-driven retinal angiogenesis mouse model. My career goals are to become an independent scientific investigator in the field of diabetic research.

Evaluation of Notch function in metabolism.

We have found that Notch and Foxo1, a transcriptional regulator of Insulin signaling, form a transcriptional complex with CSL to regulate cell fate decision. Loss of one allele of Foxo1 rescues hyperglycemia and hyperinsulinemia in insulin receptor haploinsufficient mice. Similarly, loss of Notch4 in mice correlated with lower blood glucose levels as compared to wildtype littermates. Foxo1 and Notch4 share an overlapping expression pattern in the β-cells of the adult murine pancreas, while Notch1 is expressed in both the α- and β-cells. In this aim, we will further characterize the metabolism of Notch mutant mice. We will also determine if defects are present in the pancreases of Notch and Notch/Foxo1 mutant mice.

Evaluation of Notch4 function in adipogenesis.

We have found that *Notch4* knockout mice have larger adipose tissue depots. In the skin, Notch4 is expressed in both the adipocytes and vessels. Notch4 may regulate adipogenesis by either a cell autonomous or cell non-autonomous mechanism. In the cell autonomous model, Notch functions in the adipocyte to regulate differentiation from committed preadipocytes in the stromavascular fraction. Alternatively, Notch may regulate angiogenesis within adipose tissue, which then affects adipogenesis. We have established that Notch and Foxo1, cooperate to inhibit hormone-induced adipogenesis of cultured fibroblasts. In insulin receptor mutant mice, adipogenesis was perturbed and differentiation was partially rescued by inhibiting Foxo1. However, it is

unknown whether abnormalities of Notch function can affect insulin-dependent adipocyte differentiation and function *in vivo*. To begin to address this question, we will further characterize the adipose phenotype in Notch mutant mice with a focus on the subcutaneous and visceral adipose depots. We will then deterimine if Notch4 insufficiency rescues the subcutaneous adipose phenotype in Insr mutant mice. Finally, we will evaluate Notch function in adipocyte differentiation of embryonic fibroblasts derived from *Notch4* and *Insr* deficient mice. Our goal is to determine the role of Notch and Insulin signaling interactions in adipocyte differentiation.

Diabetes and obesity

Obesity is a primary risk factor for insulin resistance, hyperglycemia and the development of type 2 diabetes {Eckel, 2005 #769}. It is also associated with cardiovascular dysfunction. Adipose tissue has an important metabolic function in storing triacylglycerol in times of energy excess and releasing free fatty acids and glycerol in times of energy deprivation. In addition, adipocytes regulate metabolic homeostasis by producing a number of bioactive substances, termed adipokines. Adipokines consist of hormone cytokines, growth factors, and other bioactive compounds. Theses include leptin, tumor necrosis factor alpha, angiotensin II, interleukin-6, interleukin-1, adiponectin, resistin, and prostaglandins. These secreted factors play a major role in regulating both metabolic and vascular biology and as such have been proposed to be the connection between insulin resistance and cardiovascular disease.

The development of obesity appears to be regulated by insulin signaling and dependent on angiogenesis. In adipocytes, insulin signaling induces Vascular Endothelial Growth Factor (VEGF) {Mick, 2002 #767}. VEGF is a potent inducer of angiogenesis that can promote endothelial cell proliferation, migration, and differentiation as well as vessel wall leakiness {Yancopoulos, 2000 #65}. In mouse models of obesity, antagonists of VEGF disrupt not only angiogenesis, but prevent adipogenesis {Rupnick, 2002 #766;Fukumura, 2003 #768}. Thus in adipose tissue, there is a reciprocal paracrine regulation of adipocyte differentiation and angiogenesis.

Notch Function in Adipogenesis

A role for Notch in adipocyte differentiation is just beginning to be elucidated. Using *in vitro* assays, Notch signaling has been shown to both promote and inhibit hormone-induced adipogenesis of fibroblasts. In stromal cell lines, Notch perturbed osteoblast differentiation that then led to an increase in adipocyte differentiation {Sciaudone, 2003 #772}. In contrast, both ligand-mediated Notch signaling, or ectopic expression of a constitutively active Notch1 inhibited adipogenesis of 3T3-L1 fibroblasts {Garces, 1997 #177;Ross, 2004 #773}. Similarly, overexpression of the Notch target gene, HES1 inhibited adipocyte differentiation of fibroblasts {Ross, 2004 #773}. Disrupting HES1 expression with siRNA also blocked fibroblast adipocyte differentiation. Thus, both inhibition and activation of Notch perturbs adipogenesis suggesting adipogenesis is dosage sensitive to Notch signaling.

Notch-mediated inhibition of fibroblast adipogenesis correlated with loss of the adipocyte-specific genes C/EBPα and PPARγ. Adipocyte differentiation of fibroblasts in which Notch signaling was activated was rescued by the expression either C/EBPα or PPARγ□, suggesting that Notch inhibits adipogenesis by suppressing the expression of these two genes. Consistent with the fibroblast data, retinoic acid induced adipogenesis was slightly enhanced in Notch1 knockout embryoid bodies {Nichols, 2004 #770}. Finally, Notch1 expression was found to be reduced in adipose tissues isolated from insulin-resistant patients relative to insulin-sensitive subjects suggesting a role for Notch in diabetes related adipocyte differentiation {Yang, 2003 #777}.

Vascular Complications in Diabetes

Diabetic patients display multiple vascular complications, including arterial hypertension, strokes, ischemia, retinopathy, atherosclerosis and heart attacks. However, little is known as to the molecular mechanisms contributing to diabetic vascular complications. Blindness is one of such complications that has a vascular origin but is poorly understood. Within 20 years of diagnosis, a quarter of diabetics develop proliferative retinopathy leading to blindness. Diabetic retinopathy initiates with an increase in vascular permeability, thickening of the basement membrane, and loss of pericytes in the retinal microvasculature followed by a proliferative phase of neovascularization {Cukiernik, 2004 #749}. Vascular growth factor (VEGF) has been implicated in the development of diabetic

proliferative retinopathy. VEGF signaling can promote endothelial cell proliferation, migration, differentiation and vessel wall leakiness {Yancopoulos, 2000 #65}. In mouse models of type I and type II diabetes, the hypoxia sensing transcription factor, HIF-1α, and VEGF are found to be increased within the eyes {Kondo, 2004 #744} suggesting that hypoxia is an initiating event in the development of diabetic retinopathy. The induction of VEGF is likely mediated by HIF-1α, as *VEGF* is a direct transcriptional target of HIF-1α {Yancopoulos, 2000 #65}. Supporting the role for VEGF in diabetic retinopathy, ectopic expression of VEGF in primate eyes leads to rapid development of proliferative retinopathy and macular edema {Lebherz, 2005 #748}. In a diabetic rat model, subcutaneous injection of the VEGF receptor signaling inhibitor SU5416 suppresses VEGF induced retinal microvascular permeability and vasoconstriction {Cukiernik, 2004 #749}. Finally, an intravitreal injection of an inhibitor of all three VEGFRs, PTK/ZK, reduces retinal neovascularization in a hypoxic mouse model {Maier, 2005 #750}. Thus, dysregulation of VEGF signaling plays a critical role in the development of proliferative retinopathy and may also contribute to other diabetic vascular complications.

EVALUATION OF NOTCH FUNCTION IN METABOLISM.

We have found that Notch and Foxo1, a transcriptional regulator of Insulin signaling, form a transcriptional complex with CSL to regulate cell fate decisions. Loss of one allele of Foxo1 rescues hyperglycemia and hyperinsulinemia in insulin signaling deficient mice (Nakae 2002 ng). In mice, loss of *N4* correlated with a

significant decrease in blood glucose levels compare to wildtype littermates. Like Foxo1, Notch4 may oppose insulin signaling in regulating the metabolism. Circulating glucose and insulin levels are regulated by both the glucose producing tissues, such as liver and the insulin producing islet β-cells of the pancreas. Increased Foxo1 signaling in the β-cells of *Foxo1* transgenic leads to □-cell failure and the development of diabetes (Nakea 2002 ng). Foxo1 and Notch4 share an overlapping expression pattern in the β-cells of the adult murine pancreata, while Notch1 is expressed in both the α- and β-cells. Thus, Notch4 and/or Notch1 may have a function in the endocrine cells of the pancreatic islets. In this aim, we will further characterize the metabolism and pancreata of Notch mutant mice. Notch mutant mice will be crossed with L2 *Ttr-Insr*$^{-/-}$ mice (Fig. 21), and used to determine if *N4* and/or *N1* insufficiency suppresses the diabetic and pancreatic defects observed in this *Insr* deficient background.

EVALUATION OF NOTCH FUNCTION IN ADIPOGENESIS.

We have found that *N4* knockout mice have dermal adipose hypertrophy. This adipose tissue phenotype in *N4* mutant mice may arise from a cell autonomous defect in the adipocytes or from a non-cell autonomous angiogenic defect. In contrast to *N4* nullizygous mice, *Insr* deficient mice display adipose tissue hypotrophy {Cinti, 1998 #774;Kitamura, 2004 #745} (Okamoto JCI 2004). Ectopic expression of dominant negative Foxo1 restores adipogenesis of *Insr*$^{-/-}$ embryonic fibroblasts {Nakae, 2003 #765}. We found that Notch and Foxo1 cooperate to inhibit hormone-induced adipogenesis of fibroblasts.

Since Foxo1 functions in epistasis with insulin signaling (Fig. 3), Notch may also have an opposing function to insulin signaling in adipogenesis. Consistent with adipose cell autonomous function for Notch4, Notch4 is expressed within the subcutaneous adipocytes. Thus, we will evaluate Notch function adipogenesis of embryonic fibroblasts derived from *Notch* and *Notch;Insr* deficient mice. We will further characterize the subcutaneous fat defect and evaluate the visceral fat depot in Notch mutant mice. Finally, we will deterimine if Notch4 insufficiency rescues the dermal adipose tissue defect in *Insr* mutant mice.

In mouse models of obesity, antagonists of VEGF block both angiogenesis and adipogenesis {Rupnick, 2002 #766;Fukumura, 2003 #768}, indicating that there is reciprocal regulation of adipogenesis and angiogenesis. Since *N4* nullizygous mice also display defects in retinal angiogenesis, the observed increase in subcutaneous adipose tissue may arise from endothelial cell dysfunction. Therefore, we will also determine if there are differences in the adipose tissue vasculature of *Notch* mutant mice.

Eighth Series of Experiments

Rat Notch1 decoy present in murine serum.

The stability of rat Notch1 decoy formulation in the mammalian blood stream was tested. As shown in Figure 118, Notch decoys are stable in the mammalian circulatory system.

Nude mice were injected with control adenovirus or adenovirus expressing rat Notch1 decoy (rN1 decoy). 2 weeks after injection, serum was collected and 4 microliters were evaluated by Western blot analysis. This analysis demonstrates that the full-length rat Notch1 decoy protein (see arrow in Figure 118) can be expressed in mice and is present at detectable levels with little evidence of degradation.

Ninth Series of Experiments

Human Notch1 decoy (h-Notch (1-36) decoy) and rat Notch 1 decoy block mouse mammary tumor growth

The activity of the human Notch1 decoy and the Rat Notch 1 decoy was compared against the growth of the mammary tumor cell line, Mm5MT-FGF4. As shown in Figure 119, both hNotch1 decoy and rNaotch1 decoy reduce the growth rate of Mm5Mt-FGF4.

We developed a tumor model which utilized Mm5MT-FGF4 cells grown in nude mice. In this experiment, $2X10^5$ Mm5MT-FGF4 cells were implanted into nude mice and four days later adenovirus encoding Fc control, rat Notch1 decoy or human Notch1 decoy was injected into ocular vein. Notch decoys are produced by adenovirus infected liver of mice and secreted into the bloodstream (example in Figure 118). The growth curve presented in Figure 119 demonstrates that either Rat Notch1 decoy or human Notch1 decoy reduced the growth of tumor xenografts in nude mice.

Rat Notch1 decoy inhibits SKNEP1 metastasis to lung tissue

Notch1 decoys can block metastasis in mouse model. We have tested the activity of the rat Notch1 decoy against tumor growth and metastasis of Ewing's Sarcoma cell line, SKNEP. In this tumor model, SKNEP tumor cells are orthotopically implantedinto the kidney where the tumor grows and then metastasized to the lung. Expression of

rat Notch1 decoy in SKNEP tumor cells reduced tumor growth and metastasis to lung, as shown in Figure 120.

SKNEP1 Ewings Sarcoma cells were programmed to express control Fc protein or rat Notch1 decoy s1 (sort 2) or rat Notch1 decoy s4 (sort 4). These SKNEP1 cell lines were orthotopically implanted into kidney of nude mice. After 6 weeks of tumor growth, metastasis to lung was assessed histologically. SKNEP1 cells expressing Rat Notch1 decoy showed fewer lungs that were positive for metastasis. We conclude that expression of the rat Notch1 decoy in nude mice diminishes the capacity of SKNEP1 cells to metastasize to lung.

Tenth Series Of Experiments

Notch1 and Notch4 are co-expressed with VEGFR-3 and LYVE-1 in lumphatics of mouse skin

Notch1 and Notch4 are expressed in lymphatics of mouse skin

We analyzed the expression of Notch1 and Notch4 in the vasculature of mouse P4 dorsal skin. At this time point, the dermal lymphatics are actively remodeling into the lymphatic capillaries near the surface and collecting ducts in the lower dermal layers. 5 µm cross-sections of skin were co-stained with antibodies against Notch1 or Notch4 (red), and PECAM, VEGFR-3 or LYVE-1 (green). Notch1 and Notch4 share an overlapping pattern of expression with the blood and lymphatic endothelial cell marker, PECAM (upper panels, figure 121). Notch1 and Notch4 were co-expressed with both VEGFR-3 (middle panels, Figure 121) and LYVE-1 in the dermal vasculature (lower panels, Figure 121). This expression pattern demonstrates that Notch1 and Notch4 are expressed and may function in the lymphatic vessels of the neonatal dermis.

Dermal lymphatic capillaries are altered in Notch4 mutant mice

We examined the dermal lymphatics of P4 mice. Sections of wildtype and *Notch4* nullizygous were immunostained with antibodies against PECAM and LYVE-1 (green). Analysis of PECAM staining appeared similar between

mutant and wildtype skin (upper panels, Figure 122). In contrast, LYVE-1-positive vessels in the dermis of *Notch4* mutants had a different morphology than that of wildtype (middle panels, Figure 122). *Notch4* mutant LYVE-1 vessels were often dilated and LYVE-1 staining was discontinuous (lower panels, Figure 122). These results suggest that Notch4 signaling may be involved in remodeling of the lymphatic vascular plexus.

Loss of Notch4 results in reduced LYVE-1 positive vessels

Notch4 heterozygous ($N4^{+/-}$) mice were mated and the dorsal skin of the resulting pups removed and embedded 14 days postnatally. The results are set forth in Figure 123. Cros-sections of skin were immunostained for the endothelial cell marker, PECAM (data not shown), or the lymphatic endothelial cell marker, LYVE1 (A). Five areas for each were captured by microscopy and PECAM and LYVE1 staining quantitated using imaging software (B, C). PECAM expression was reduced approximately 25% in the $N4^{-/-}$ dermis compared to wild-type (WT) dermis (B). LYVE-1 staining was more affected than the PECAM with LYVE1 staining decreased nearly 50% in $N4^{-/-}$ relative to WT mice (C). There was also a reduction in the intensity of the LYVE1 staining in the $N4^{-/-}$ lymphatics relative to the WT (A).

Loss of Notch4 function in mice disrupts development of dermal lymphatic's suggesting a role in lymphangiogenesis.

Notch1 and Notch4 Expressed in human breast cancer

We performed double immunohistochemistry with antibodies against VEGFR-3 or LYVE-1 (green) and Notch1 or Notch4 (red) of human breast cancers. The results are set forth in Figure 124. Notch1 and Notch4 were expressed in the extratumoral blood and lymphatic endothelium of human micropapillary breast carcinomas. To determine if Notch1 signaling was activated within the tumoral lymphatic endothelium, we double stained with an antibody against podoplanin (green) and N1Val (red; Cell Signaling), an antibody that specifically detects the activated Notch1 peptide. Expression of the activated Notch1 peptide was observed in most (white arrows) but not all (yellow arrows) of the lymphatic endothelial nuclei(lower panel). These results demonstrate that Notch1 was actively signaling in the pathological lymphatic vessels. These results also demonstrate that Notch1 and Notch4 may function in tumor lymphangiogenesis.

What is claimed is:

1. A fusion protein comprising a signal peptide, an extracellular domain of human Notch receptor protein and an Fc portion of an antibody bound thereto.

2. The fusion protein of claim 1, wherein the Notch receptor protein is Notch1 receptor protein.

3. The fusion protein of claim 1, wherein the Notch receptor protein is Notch2 receptor protein.

4. The fusion protein of claim 1, wherein the Notch receptor protein is Notch3 receptor protein.

5. The fusion protein of claim 1, wherein the Notch receptor protein is Notch4 receptor protein.

6. The fusion protein of claim 1, wherein the Fc portion of the antibody is the Fc portion of a human antibody.

7. The fusion protein of claim 2, wherein the Fc portion of the antibody is the Fc portion of a human antibody.

8. The fusion protein of claim 3, wherein the Fc portion of the antibody is the Fc portion of a human antibody.

9. The fusion protein of claim 4, wherein the Fc portion of the antibody is the Fc portion of a human antibody.

10. The fusion protein of claim 5, wherein the Fc portion of the antibody is the Fc portion of a human

antibody.

11. The fusion protein of claim 1, wherein the signal peptide is the signal peptide of Notch1, Notch2, Notch3, Notch4, or IgG Heavy Chain.

12. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-36.

13. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-13.

14. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-24.

15. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 9-23.

16. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 9-36.

17. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 13-24.

18. The fusion protein of claim 2, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 25-36.

19. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 1-29.

20. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 1-13.

21. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 1-23.

22. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 9-23.

23. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 9-29.

24. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 13-23.

25. The fusion protein of claim 5, wherein the extracellular domain of Notch4 receptor protein comprises EGF-like repeats 21-29.

26. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 54.

27. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 55.

28. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 56.

29. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 57.

30. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 58.

31. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 59.

32. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 60.

33. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 61.

34. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 62.

35. The fusion protein of claim 2, wherein the fusion

protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 63.

36. The fusion protein of claim 2, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 64.

37. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 65.

38. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 66.

39. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 67.

40. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 68.

41. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 69.

42. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 70.

43. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the

sequence of which is set forth in SEQ ID NO: 71.

44. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 72.

45. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 73.

46. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 74.

47. The fusion protein of claim 2, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 75.

48. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 78.

49. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 79.

50. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 80.

51. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 81.

52. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 82.

53. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 83.

54. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 84.

55. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 85.

56. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 86.

57. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 87.

58. The fusion protein of claim 5, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO: 88.

59. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 89.

60. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 90.

61. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 91.

62. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 92.

63. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 93.

64. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 94.

65. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 95.

66. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 96.

67. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 97.

68. The fusion protein of claim 5, wherein the fusion

protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 98.

69. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 99.

70. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 100.

71. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 101.

72. The fusion protein of claim 5, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 102.

73. The fusion protein of claim 5 wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO: 103.

74. A method for treating a subject having a tumor comprising administering to the subject an amount of the fusion protein of claims 1-73 effective to treat the subject, thereby treating the subject having a tumor.

75. A method for inhibiting angiogenesis in a subject comprising administering to the subject an amount of the fusion protein of claims 1-73 effective to inhibit angiogenesis in the subject, thereby

inhibiting angiogenesis in the subject.

76. A method for treating a subject having ovarian cancer comprising administering to the subject an amount of the fusion protein of claims 1-73 effective to treat the subject, thereby treating the subject having ovarian cancer.

77. A method for treating a subject having a metabolic disorder comprising administering to the subject an amount of the fusion protein of claims 1-73 effective to treat the subject, thereby treating the subject having a metabolic disorder.

78. The method of claim 77, wherein the metabolic disorder is diabetes, obesity, atherosclerosis, ischemia, stroke, or cardiovascular disease.

79. Use of the fusion protein of claims 1-73 for the preparation of a pharmaceutical composition for the treatment of a subject having a tumor.

80. Use of the fusion protein of claims 1-73 for the preparation of a pharmaceutical composition for inhibiting angiogenesis in a subject.

81. Use of the fusion protein of claims 1-73 for the preparation of a pharmaceutical composition for treating a subject having ovarian cancer.

82. Use of the fusion protein of claims 1-73 for the preparation of a pharmaceutical composition for treating a subject having a metabolic disorder.

83. The use of claim 82, wherein the metabolic disorder is diabetes, obesity, atherosclerosis, ischemia, stroke, or cardiovascular disease.

84. A method for inhibiting physiological lymphangiogenesis or pathological lymphangionesis in a subject comprising administering to the subject an amount of the fusion protein of any of claims 1-73 effective to inhibit physiological lymphangiogenesis or pathological lymphangionesis in the subject.

85. The method of claim 84, wherein the pathological lymphangiogenesis is tumor lymphangiogenesis or lymph node metastasis.

86. A method of inhibiting tumor metastasis in a subject comprising administering to the subject an amount of the fusion protein of any of claims 1-73 effective to inhibit tumor metastasis in the subject.

87. The method of claim 86, wherein the metastasis occurs via a blood vessel, the lymphatic vasculature or a lymph node.

88. A method of inhibiting growth of a secondary tumor in a subject comprising administering to the subject an amount of the fusion protein of any of claims 1-73 effective to inhibit growth of the secondary tumor in the subject.

89. The method of claim 88, wherein the secondary tumor growth is inhibited by inhibition of angiogenesis

associated with the secondary tumor.

90. A method of inhibiting blood vessel cooption by a tumor in subject comprising administering to the subject an amount of the fusion protein of any of claims 1-73 effective to inhibit blood vessel cooption by a tumor in the subject.

91. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-73 and an inhibitor of Vascular Endothelial Growth Factor (VEGF), each in an amount effective to treat the cancer in the subject.

92. The method of claim 91, wherein the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

93. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-73 and a VEGF receptor inhibitor, each in an amount effective to treat the cancer in the subject.

94. The method of claim 93, wherein the VEGF receptor inhibitor is a VEGFR-1 inhibitor, VEGFR-2 inhibitor, or VEGFR-3 inhibitor.

95. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-73 and an inhibitor of Platelet Derived Growth Factor (PDGF), each in an amount effective to treat the cancer in the subject.

96. The method of claim 95, wherein the inhibitor of Platelet Derived Growth Factors is an inhibitor of PDGF-A or an inhibitor of PDGF-B

97. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-73 and a PDGF receptor antagonist, each in an amount effective to treat the cancer in the subject.

98. The method of claim 97, wherein the PDGF receptor antagonist is a PDGF Receptor-B antagonist.

99. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-73 and an inhibitor of HER2/neu, each in an amount effective to treat the cancer in the subject.

100. A method of treating vascular proliferative retinopathy comprising administering to the subject the fusion protein of any of claims 1-73 in an emount effective to treat the vascular proliferative retinopathy.

101. The method of claim 100, wherein the vascular proliferative retinopathy is diabetic retinopathy, macular defernation or retinopathy of prematurity.