HUMAN NOTCH3 BASED FUSION PROTEINS AS DECOY INHIBITORS OF NOTCH3 SIGNALING

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 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, EGF repeats 1-X of the extracellular domain of human Notch3 receptor protein wherein X is any integer from 12 to 34, and an Fc portion of an antibody bound thereto.

This invention provides a fusion protein comprising a signal peptide, EGF repeats 1-X of the extracellular domain of human Notch3 receptor protein wherein X is any integer from 1 to 10, and an Fc portion of an antibody bound thereto.

This invention provides a fusion protein comprising a signal peptide, at least 12 EGF repeats of the extracellular domain of human Notch3 receptor, and an Fc portion of an antibody bound thereto.

This invention provides a fusion protein comprising a signal peptide, EGF repeats of the extracellular domain of human Notch3 receptor protein, wherein at least 12 EGF repeats are present, 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 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 lymphangiogenesis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit physiological lymphangiogenesis or pathological lymphangiogenesis 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), 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 inhibitor, 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), 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 breast cancer in a subject comprising administering to the subject an amount of the above fusion protein effective to treat the breast cancer in the subject.

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

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 metallo-proteinase 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.

Figure 32A, 32B and 32C:

This Figure shows the full-length nucleotide sequence of human Notch3 (SEQ ID NO:15), consisting of the initiating ATG (nt 1) to the stop (TGA; nt 6964). The signal peptide and first 34 EGF-like repeat domains are present in nt 1-4158 of this sequence. Nucleotides 1-4158 are utilized for the design of the human Notch3 decoy proteins, described herein. The nucleotides encompassing EGF-repeats 1-34 are underlined.

Figure 33:

This Figure shows the full-length amino acid (aa) sequence of human Notch3 (SEQ ID NO:16), consisting of aa 1(M=methionine) to aa 2555 (K=lysine). The signal peptide and first 34 EGF-like repeat domains are present in aa 1-1386 of this sequence. Amino acids 1-1386 are

utilized for the design of the human Notch3 decoy proteins, described in the ensuing sections. The amino acids encompassing EGF-repeats 1-34 are underlined.

Figure 34

This figure shows the schematization of two human Notch3 decoy proteins, h-Notch3$^{(1-34)}$decoy and h-sp^{HC}-Notch3$^{(1-34)}$ decoy.

Figure 35

This figure shows the human Fc nucleotide sequence utilized to generate the Fc tag on Notch3 decoy proteins (SEQ ID NO:17). The 713 nucleotides of human Fc are fused at the 3'-end of the Notch3 decoy construct, just downstream of Notch3 EGF-like repeats. This region of human Fc allows for the detection and purification of the Notch decoys and serves to stabilize the secreted human Notch3-human Fc fusion proteins.

Figure 36

This figure shows the human Fc amino acid sequence utilized to generate the Fc tage on Notch3 decoy proteins (SEQ ID NO:18). The 237 amino acids of human FC were fused at the C-terminus of all Notch3 decoy constructs, just downstream of the Notch3 EGF-like repeats. This region of human Fc allows for the detection and purification of the Notch decoys and serves to stabilize the secreted human Notch3-human Fc fusion proteins.

Figure 37:

This figure shows the human Notch3/Fc fusion sequence for all constructs that end after EGF repeat 34 of human Notch 3.

Figure 38

This Figure shows the signal sequence analysis of human Notch3 signal peptide that is predicted to encompass amino acids 1-40 of human Notch3. This determination was made using the signal IP 3.0 Server program provided by the Technical Unviersity of Denmark. These results predict a major site of cleavage located between alanine 39 (A39) and alanine 40 (A40). The cleavage site is indivated by the "/" in amino acid sequence 1-40 of human Notch3 as depicted in this figure.

Figure 39:

This Figure shows the signal sequence analysis of human HC signal peptide that is predicted to encompass amino acids 1-22 of human HC. This determination was made using the signal IP 3.0 Server program provided by the Technical University of Denmark. These results predict a major site of cleavage located between alanine 21 (A21) and aringine 22 (A22). This cleavage site is indivated by the "/" in amino acid sequence 1-22 of human HC provided above.

Figure 40A and 40B:

This Figure shows the nucleotide sequence of h-Notch3$^{(1-34)}$ decoy protein (SEQ ID NO:31). The predicted human Notch3 signal peptide is underlined (nt 1-120). Notch3 EGF repeats 1-34 are encoded from nt 121-4158. The fusion junction, BglII site, is located at nt 4158-4163. The Fc tag sequence is underlined and italicized.

Figure 41

This Figure shows the amino acid sequence of h-Notch3$^{(1-34)}$ decoy protein (SEQ ID NO:32). The predicted human Notch3 signal peptide is underlined (AA 1-40). Notch3 EGF repeats 1-34 are encoded from aa 41-1386. The FC tag sequence is underlined and italicized.

Figure 42

This Figure shows the amino acid sequence of h-sp^{HC}Notch3$^{(1-34)}$ decoy protein (SEQ ID NO:33). The predicted human Notch3 signal peptide is underlined (AA 1-22). Notch3 EGF repeats 1-34 are encoded from aa 22-1386. The FC tag sequence is underlined and italicized.

Figure 43A and 43B

This Figure shows the nucleotide sequence of h-sp^{HC}Notch3$^{(1-34)}$ decoy protein (SEQ ID NO:34). The predicted human HC signal peptide is underlined (nt 1-66). Notch3 EGF-repeats are encoded from nt 67-4104. The fusion junction, BglII site, is from nt 5004 to 5009. The Fc tag sequence is underlined and italicized.

Figure 44

This Figure shows expression of Notch proteins and ligands in blood and lymphatic endothelial cells. RT-PCT was performed for Notch1-4, Dll4, Dll4 and Jagged1 on RNA isolated from blood endothelial cells (BEC) and lymphatic endothelial cells (LEC) purified from HMVEC. Notch1, Notch2, Notch4, Dll4 and Jagged1 were expressed in both BEC and LEC at a similar level. Expression of Notch 3 appears to be restricted to the LEC suggestive of Notch3 signaling functions in the lymphatic endothelium.

Figure 45

This Figure shows Notch3 is co-expressed with the lymphatic endothelial cell marker LYVE-1 and Prox1 in e13.5 embryos. 10 micron serial sections of embryonic day 13.5 mouse embryos were immunostained for either LYVE-1, Prox1 and Notch3. Notch3 was expressed in the cells that also expressed the lymphatic endothelial cell markers, LYVE-1 and Prox1.

Figure 46

This Figure shows Prox1 induced Notch3 expression in blood endothelial cells. **(A)** It was examined if extopic expression of Prox1 would alter the expression of Notch proteins or ligands. Twenty-four hours post adenoviral infection with either Ad-Prox1 or Ad-LacZ, HUVEC total RNA was isolated and quantitative RT-PCR for Notch1-4, Dll4 and Jagged1 performed. Prox-1 robustly upregulated the expression of Notch3. Notch1, Notch2, Notch4, Dll4 and Jagged1 expression was not significantly affected. **(B)** Compound E (cE), Presenlin inhibitor that inhibits Notch signaling, was incubated for 24 hours on either Ad-LacZ or Ad-Prox1 infected HUVEC. Total RNA was isolated and quantitative PCR performed to determine Notch3 expression. Prox1 induced Notch3 expression and this induction was inhibited by the addition of compound E. This suggests that the Prox1 induction of Notch3 is dependent on Notch signal activation.

Figure 47

This figure shows that Prox1 induces Notch-target genes in blood endothelial cells. HUVEC were infected with adenoviruses encoding, LacZ, Prox1, N1IC or N4/int-3 and total RNA isolated 24 hours post-infection. Quantitative

RT-PCR was performed for the endothelial Notch-target genes, VEGFR-3, EphrinB2, Hey1 and Hey2. Similar to Notch1 and Notch4 signal activation, Prox1 induced all four genes **(A and B)**. Expression of Hey1 and Hey2 in the lymphatic endothelium is unknown.

Figure 48

This Figure shows that Prox1 induces Notch-target genes is dependent on Notch signaling in blood endothelial cells. HUVEC were infected with adenoviruses encoding LacZ, Prox1, N1Ic or N4/int-3. Compound E(cE), Presenlin inhibitor that inhibits Notch signaling, was incubated for 24 hours on either Ad-LacZ or Ad-Prox1 infected HUVEC and total RNA isolated. Quantitative RT-PCR was performed for the endothelial Notch-target genes, VEGFR-3, EphrinB2, and Hey2. The Prox-1 mediated induction of the Notch target genes, ephrinB2, VEGFR-3 and Hey2 was inhibited by the addition of the Notch signaling inhibitor Compound E. Thus, Prox1 regulates the expression of ephrinB2, VEGFR-3 and Hey2 via Notch.

Figure 49

This figure shows a Schematic of N1IC knock in. An activated form of Notch1 was inserted into the EF1alpha locus flanked by two LoxP sites. Upon expression of Cre-recombinase, the neo/tpA cassette is lost and N1IC is expressed under the control of the ubiquitous EF1 alpha promoter.

Figure 50:

This Figure shows Notch activation in SM22 expressing vascular smooth muscle cells results in embryonic

lethality before E10.5. No viable SM22Cre/+; EF1αN1IC/ mice were observed at postnatal day 21 (P21) with a p value less than 0.001. At embryonic day E9.5, an predicted number of SM22Cre/+; EF1αN1IC/+ embryos were observed, but they were severly growth retarded compared with their control litter mates (Lower panel).

Figure 51:

This Figure shows Notch activation in SM22 expressing vascular smooth muscle cells alters alpha smooth muscle cell actin expression. E9.5 embryos were wholemount immunostained for alpha smooth muscle cell actin. Expression of alpha smooth muscle cell actin was altered in the SM22Cre/+; EF1αN1IC/+ embryos compared to the WT controls. Thus, Notch signal activation in vascular smooth muscle cells disrupts cardiovascular development.

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, and Psoriasis.

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.

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.; HC or Hc: Heavy Chain IgG; PDGF: Platelet derived growth factor; PlGF: placental growth factor;

Embodiments of the Invention

This invention provides a fusion protein comprising a signal peptide, EGF repeats 1-X of the extracellular domain of human Notch3 receptor protein wherein X is any integer from 12 to 34, and an Fc portion of an antibody bound thereto.

This invention provides a fusion protein comprising a signal peptide, EGF repeats 1-X of the extracellular domain of human Notch3 receptor protein wherein X is any integer from 1 to 10, and an Fc portion of an antibody bound thereto.

This invention provides a fusion protein comprising a signal peptide, at least 12 EGF repeats of the extracellular domain of human Notch3 receptor, and an Fc portion of an antibody bound thereto.

This invention provides a fusion protein comprising a signal peptide, EGF repeats of the extracellular domain of human Notch3 receptor protein, wherein at least 12 EGF repeats are present, and an Fc portion of an antibody bound thereto.

In one embodiment of the fusion protein, the extracellular domain of Notch3 receptor protein comprises EGF-like repeats 1-34.

In one embodiment of the dusion 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 Notch3 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:32. In another embodiment, the fusion protein comprises consecutive amino acids, the seuqnece

of which is set forth in SEQ ID NO:33.

In one embodiment, the fusion protein is encoded by consecutive nucleotide, the sequence of which is set forth in SEQ ID NO:31. In another embodiment, the fusion protein is encoded by consecutive nucleotide, the sequence of which is set forth in SEQ ID NO:34

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 use of the above fusion protein for the preparation of a pharmaceutical composition for treating a subject having cardiovascular disease. In one embodiment the cardiovascular disease is atherosclerosis, ischemia or stroke.

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 a method for inhibiting physiological lymphangiogenesis or pathological lymphangiogenesis in a subject comprising administering to the subject an amount of the above fusion protein effective to inhibit physiological lymphangiogenesis or pathological lymphangiogenesis 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 PGIF, 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 a VEGF receptor inhibitor, each in an amount effective to treat the cancer in the subject. In one embodiment, the VEGF receptor inhibitor is a VEGFR-1 inhibitor, a VEGFR-2 inhibitor, a VEGFR-3 inhibitor or a an inhibitor of any combination of VEGFRs.

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 breast cancer in a subject comprising administering to the subject an amount of the above-fusion protein effective

to treat the breast cancer in the subject.

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

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 Notch3 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 Notch3 Fusion Proteins (Notch Decoys)

The Notch3 decoys are assembled using sequences encoding a signal peptide, a portion of the Notch3 extracellular domain encompassing all the EGF-like repeat domains, and a portion of the human Fc protein (amino acids 1-237). The complete full-length nucleotide sequence of human Notch3 is provided in Figure 32. The complete full length amino acid sequence of human Notch3 is provided in Figure 33.

The signal peptides utilized are either the native Notch3 signal peptide or the human Hc signal peptide, each fused to a region of Notch3. The signal peptide allows for secretion of the Notch decoy proteins.

The Notch3 extracellular domains used are designed to bind to Notch ligands and consist of all or a subset of the 34 EGF-like repeat domains of the human Notch3 protein.

The Fc tag is fused to the C-terminus of a given EGF-like repeat of human Notch3 and serves to allow for purification, detection, and stabilization of the Notch3 decoy proteins.

The overall design of the human Notch3 decoys, two formulations, is to encode for; (1) a signal peptide to

allow for secretion of Notch3 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 the EGF-like repeats of human Notch3 to allow for association with Notch ligands, and (3) a portion of the human Fc protein to allow for detection.

The following two formulations of human Notch3 decoys will be described and are schematized in Figure 34.

1) h-Notch3$^{(1-34)}$ decoy
4) h-sp^{Hc}Notch3$^{(1-34)}$ decoy

Human Notch3 sequence

The full-length nucleotide (nt) sequence of human Notch3, consisting of the initiating ATG (nt 1) to the stop (TGA; nt 6964) is set forth in Figure 32. The signal peptide and first 34 EGF-like repeat domains are present in nt 1-4158 of this sequence. Nucleotides 1-4158 are utlizied for the design of the human Notch3 decoy proteins, described in the ensuing sections. The nucletodies encompassing EGF-repeats 1-34 are underlined.

The full-length amino acid (aa) sequence of human Notch3, consisting of aa 1(M= methionine) to aa 2555 (K=lysine) is set forth in Figure 33. The signal peptide and first 34 EGF-like repeat domains are present in aa 1-1386 of this sequence. Amino acids 1-1386 are utilized for the design of the human Notch3 decoy proteins, described in the ensuing sections. The amino acids encompassing EGF repeats 1-34 are underlined.

Human Fc sequence utilized to generate the Fc tag on Notch3 decoy proteins

The 713 nucleotides of human Fc, which are set forth in Figure 35, are fused at the 3' -end of the Notch3 decoy construct, just downstream of Notch3 EGF-like repeats. This region of human fc allows for detection and purification of the Notch decoys and serves to stabilize the secreted human Notch3-human Fc fusion proteins.

The 237 amino acids of human Fc, shown in Figure 36, were fused at the C-terminus of all Notch3 decoy constructs, just downstream of Notch3 EGF-like repeats. This region of human Fc allows for detection and purification of the Notch decoys and serves to stabilize the secreted human Notch3-human Fc fusion proteins.

Signal Peptides utilized in Notch3 decoy proteins

Two distinct signal peptide sequences were incorporated into the design of the human Notch1 decoy proteins. The first is the human Notch3 signal peptide that is predicted to encompass amino acids 1-40 of human Notch3. 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 Notch3 signal peptide (nt 1-20)

MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAA/A (SEQ ID NO:27)

Amino acid sequence of the predicted human Notch3 signal peptide is schematized in Figure 37. The prediction results of analysis utilizing the SignalIP 3.0 Server provided online by the Technical University of Denmark are shown in Figure 37. These results predict a major site of cleavage located between alanine 39 (A39) and alanine 40 (A40) These cleavage site is indicated by the "/" in amino acid sequence 1-40 of human Notch3, provided above.

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

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

MWGWKCLLFWAVLVTATLCTA/R (SEQ ID NO: 29)

The nucleotide sequence of the predicted human Hc signal peptide is:

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-Notch3$^{(1-34)}$ decoy

h-Notch1$^{(1-34)}$ decoy denotes the human Notch3 decoy that encompass EGF-like repeats 1-34 of Notch3.

The amino acid sequence of h-Notch3$^{(1-34)}$ decoy protein which is set forth in Figure 41. The predicted human Notch3 signal peptide is underlined (AA 1-40). Notch3 EGF-repeats 1-34 are encoded from aa 41-1386. The Fc tage sequence is underlined and italicized.

The nucleotide sequence of h-Notch3$^{(1-34)}$ decoy protein which is set forth in Figure 40. The predicted human Notch3 signal peptide is underlined (nt 1-120). Notch3 EGF repeats 1-34 are encoded from nt 121-4158. The fusion junction, BglII site is nt 4158 to 4163. The Fc tage sequence is underlined and italicized.

h-sp^{Hc}Notch1$^{(1-34)}$ decoy

h-sp^{Hc}Notch1$^{(1-34)}$ decoy denotes the human Notch3 decoy that encompass EGF-like repeats 1-34. The abbreviation sp^{Hc} denotes that the human Hc signal peptide is used in this formulation.

The amino acid sequence of h-Notch3$^{(1-34)}$ decoy protein which is set forth in Figure 42. The predicted human Hc signal peptide is underlined (AA 1-22). Notch3 EGF-repeats 1-34 are encoded from aa 22-1386. The Fc tag sequence is underlined and italicized.

The nucleotide sequence of h-Notch3$^{(1-34)}$ decoy protein which is set forth in Figure 43. The predicted human Hc signal peptide is underlined (nt 1-66). Notch3 EGF repeats 1-34 are encoded from nt 67-4104. The fusion junction, BglII site is nt 4104 to 4109. The Fc tag

sequence is underlined and italicized.

Methods

Construction of human Notch3 decoys

Total RNA from either human aortic smooth muscle cells (AoSMC) or human umbilical venous endothelial cells (HUVEC) that overexpress Prox1 were used to generate human Notch3 decoy variants. Total RNA was reverse transcribed with M-MLV reverse transcriptase and either random hexamer primers or Notch3 decoy specific primers. The synthesized cDNA was then amplified with Notch3 decoy specific upstream (sense) and downstream (antisense) primers. The Notch3 decoy was constructed from 4 individual amplicons. The3-prime amplicon was amplified with a downstream primer encoding a Bgl II restriction site at the 5-prime end for ligation into the BglII site in the Fc sequence to generate an in fram human Notch3/Fc chimera.

In the case of Notch3 decoys that generate the fusion after nucleotide sequence encoding EGF-like repeat 34, a BglIII site will be generated to create the fusion site and this fusion sequence is provided (Notch3, Figure 37). This applies to formulations h-Notch3(1-34)decoy and h-sp^{HC}Notch3(1-34)decoy.

The amplified PCR productes were subcloned into e pBluescript SK II Fc to generate the different human Notch3/Fc chimeras. The human Notch3/Fc decoy sequences are then shuttled into mammalian expression vectors (pAd-lox, pCCL or pcDNA3) for expression and purification of

human Notch3 decoy proteins.

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 into the Fc containing 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

$7.5x10^5$ 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 x 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.

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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.

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Fourth Series Of Experiments

Expression of Notch proteins and ligands in blood and lymphatic endothelial cells.

RT-PCR was performed for Notch1-4, Dll1, Dll4 and Jagged1 on RNA isolated from blood endothelial cells (BEC) and lymphatic endothelial cells (LEC) purified from HMVEC. As shown in in Figure 44, Notch1, Notch2, Notch4, Dll4 and Jagged1 were expressed in both BEC and LEC at a similar level. Expression of Notch 3 appears to be restricted to the LEC suggestive of Notch3 signaling functions in the lymphatic endothelium.

Notch3 is co-expressed with the lymphatic endothelial cell marker LYVE-1 and Prox1 in e13.5 embryos.

10 micron serial sections of embryonic day 13.5 mouse embryos were immunostained for either LYVE-1, Prox1 and Notch3. As shown in Figure 45, Notch3 was expressed in the cells that also expressed the lymphatic endothelial cell markers, LYVE-1 and Prox1.

Prox1 induced Notch3 expression in blood endothelial cells.

It was examined if ectopic expression of Prox1 would alter the expression of Notch proteins or ligands. As shown in Figure 46, section A, twenty-four hours post adenoviral infection with either Ad-Prox1 or Ad-LacZ, HUVEC total RNA was isolated and quantitative RT-PCR for Notch1-4, Dll4 and Jagged1 performed. Prox-1 robustly upregulated the expression of Notch3. Notch1, Notch2, Notch4, Dll4 and Jagged1 expression was not significantly affected. As shown in Figure 46, section B, Compound E

(cE), Presenlin inhibitor that inhibits Notch signaling, was incubated for 24 hours on either Ad-LacZ or Ad-Prox1 infected HUVEC. Total RNA was isolated and quantitative RT-PCR performed to determine Notch3 expression. Prox1 induced Notch3 expression and this induction was inhibited by the addition of compound E.

This suggests that the Prox1 induction of Notch3 is dependent on Notch signal activation.

Prox1 induces Notch-target genes in blood endothelial cells.

HUVEC were infected with adenoviruses encoding, LacZ, Prox1, N1IC or N4/int-3 and total RNA isolated 24 hours post-infection. Quantitative RT-PCR was performed for the endothelial Notch-target genes, VEGFR-3, EphrinB2, Hey1 and Hey2. Similar to Notch1 and Notch4 signal activation, Prox1 induced all four genes (Figure 47, sections A and B). Expression of Hey1 and Hey2 in the lymphatic endothelium is unknown.

Prox1 induces Notch-target genes is dependent on Notch signaling in blood endothelial cells.

HUVEC were infected with adenoviruses encoding LacZ, Prox1, N1Ic or N4/int-3. Compound E(cE), Presenlin inhibitor that inhibits Notch signaling, was incubated for 24 hours on either Ad-LacZ or Ad-Prox1 infected HUVEC and total RNA isolated. Quantitative RT-PCR was performed for the endothelial Notch-target genes, VEGFR-3, EphrinB2, and Hey2. The Prox-1 mediated induction of

the Notch target genes, ephrinB2, VEGFR-3 and Hey2 was inhibited by the addition of the Notch signaling inhibitor Compound E, as shown in Figure 48. Thus, Prox1 regulates the expression of ephrinB2, VEGFR-3 and Hey2 via Notch.

Fifth Series of Experiments

Background

Insights into a function for Notch in vascular homeostasis can be drawn from the human neurovascular disorder, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). In a majority of patients, CADASIL has been found to correlate with missense mutation in Notch3. CADASIL is a late-onset (average age of 45) autosomal dominant disorder characterized by migraines with aura and recurrent strokes that lead to psychiatric symptoms, progressive cognitive decline, dementia, and death [81]. These neuropathological symptoms arise secondary to a slow developing arteriopathy, associated with the disorganization and destruction of the vascular smooth muscle cells surrounding the cerebral arteries and arterioles. Regression of vascular smooth muscle cells is associated with a decrease in vessel wall thickness, a loss of extracellular matrix, and vessel wall weakness [82]. Within the vascular smooth muscle cells, there is an accumulation of the extracellular domain of Notch3 and in the extracellular matrix, an abnormal deposition of particles referred to as granular osmophilic materials (GOM) [83]. In this disorder, arterial lesions are not restricted to the brain and are found in arteries of the skin and retina [83-85].

The CADASIL phenotype correlates with the expression of Notch3 in vascular smooth muscle cells [70,81]. The hypothesis being that Notch3 functions to maintain cell-cell interactions or communication between vascular smooth muscle cells and arterial endothelial cells. A recent study has recreated the CADASIL vessel pathology in transgenic mice that express a Notch3 transgene encoding the CADASIL R90C mutation specifically in vascular smooth muscle cells [86]. The vasculature of these mice showed classic CADASIL arteriopathy, including GOM deposits and Notch3 accumulation. However, these hallmarks were preceded by the disruption of anchorage and adhesion of vascular smooth muscle cells to neighboring cells followed by degeneration of the vascular smooth muscle cells. Thus, CADASIL results from reduced vascular smooth muscle cell contact and viability and the GOM deposition and accumulation of the extracellular domain of Notch3 are secondary consequences of this cellular deterioration. Consistent with a role for Notch3 in cell survival, expression of a constitutively active form of Notch3 in rat aortic smooth muscle cells resulted in the induction of cFlip, an antagonist of Fas-dependent apoptosis [87]. In addition, ectopic expression of Hey1 in cultured vascular smooth muscle cells promoted cell survival via Akt and thus inhibited apoptosis in response to serum starvation and Fas ligand [88]. Taken together, this data indicate that Notch3 maintains arterial vessel homeostasis by promoting vascular smooth muscle cell survival. The resulting arterial vessel wall leakiness could arise from vascular smooth muscle cell death or a failure of vascular smooth muscle cells to communicate to their neighboring

endothelial cells. Disruption of Notch3 activity in mice may help define the nature of this defect.

The specific activity of CADASIL mutant Notch3 proteins is still poorly understood. One complication in interpreting mutant Notch3 function arises from conflicting *in vitro* studies that have shown that truncated cytoplasmic Notch3 can either inhibit or activate the CSL transcription factor [89,90].

Activation of Notch signaling in vascular smooth muscle cells results in embryonic lethality.

Notch3 is expressed and active in cells that surround blood vessels, the smooth muscle cells and pericytes. Smooth muscles cells are important for cardiovascular function and they must be healthy to prevent stroke. Pericytes can contribute to tumor vessel growth. Notch1 and Notch4 are not though to function in these cells types.

Therefore the Notch3 fusion proteins described herein may be useful to prevent stroke by preventing abnormal Notch3 activity. In addition, the Notch3 fusion proteins may be useful to maintain vascular smooth muscle cells, to restrain tumor pericyte growth or function, or to affect retinal angigogenesis by modulating pericyte function.

We have constructed a transgenic mouse that expresses an activated form of Notch1 (N1IC) under the control of the elongation factor 1-alpha promoter (EF1α) in tissues that express Cre-recombinase, referred to as *EF1α$^{N1IC/+}$*

(Fig.49). *EF1*$\alpha^{N1IC/+}$ is viable and fertile (Fig. 50). We have expressed N1IC in vascular smooth muscle cells by crossing *EF1*$\alpha^{N1IC/+}$ with an SM22-Cre mouse line (*SM22*$^{Cre/+}$). The resulting *SM22*$^{Cre/+}$;*EF1*$\alpha^{N1IC/+}$ double transgenic die at E9.5 (Fig. 50). *SM22*$^{Cre/+}$;*EF1*$\alpha^{N1IC/+}$ embryos display myocardial defects that we believe are responsible for embryonic lethality. Consistent with these myocardial smooth muscle cells defects , we observed an alteration in the expression of the vascular smooth muscle cell marker, alpha smooth muscle cell actin in E9.5 *SM22*$^{Cre/+}$;*EF1*$\alpha^{N1IC/+}$ transgenic animals (Fig 51). These results demonstrating that increased Notch signaling in vascular smooth muscle cells disrupts embryonic cardiovascular development.

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What is claimed is:

1. A fusion protein comprising a signal peptide, EGF repeats 1-X of the extracellular domain of human Notch3 receptor protein wherein X is any integer from 12 to 34, and an Fc portion of an antibody bound thereto.

2. A fusion protein comprising a signal peptide, EGF repeats 1-X of the extracellular domain of human Notch3 receptor protein wherein X is any integer from 1 to 10, and an Fc portion of an antibody bound thereto.

3. A fusion protein comprising a signal peptide, at least 12 EGF repeats of the extracellular domain of human Notch3 receptor, and an Fc portion of an antibody bound thereto.

4. A fusion protein comprising a signal peptide, EGF repeats of the extracellular domain of human Notch3 receptor protein, wherein at least 12 EGF repeats are present, and an Fc portion of an antibody bound thereto.

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

6. The fusion protein of any of claims 1-4, wherein the signal peptide is the signal peptide of Notch3 or IgG Heavy Chain.

7. The fusion protein of any of claims 1, 3 or 4, wherein the extracellular domain of Notch1 receptor protein comprises EGF-like repeats 1-34.

8. The fusion protein of any of claims 1, 3, or 4 wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO:32.

9. The fusion protein of any of claims 1, 3, or 4, wherein the fusion protein comprises consecutive amino acids, the sequence of which is set forth in SEQ ID NO:33.

10. The fusion protein of any of claims 1, 3, or 4, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO:31.

11. The fusion protein of any of claims 1, 3, or 4, wherein the fusion protein is encoded by consecutive nucleotides, the sequence of which is set forth in SEQ ID NO:34.

12. A method for treating a subject having a tumor comprising administering to the subject an amount of the fusion protein of claims 1-11 effective to treat the subject, thereby treating the subject having a tumor.

13. A method for inhibiting angiogenesis in a subject comprising administering to the subject an amount of the fusion protein of claims 1-11 effective to inhibit angiogenesis in the subject, thereby inhibiting angiogenesis in the subject.

14. A method for treating a subject having ovarian cancer comprising administering to the subject an amount of the fusion protein of claims 1-11 effective to treat the subject, thereby treating the subject having ovarian cancer.

15. A method for treating a subject having a metabolic disorder comprising administering to the subject an amount of the fusion protein of claims 1-11 effective to treat the subject, thereby treating the subject having a metabolic disorder.

16. The method of claim 15, wherein the metabolic disorder is diabetes, obesity, atherosclerosis, ischemia, stroke, or cardiovascular disease.

17. Use of the fusion protein of claims 1-11 for the preparation of a pharmaceutical composition for the treatment of a subject having a tumor.

18. Use of the fusion protein of claims 1-11 for the preparation of a pharmaceutical composition for inhibiting angiogenesis in a subject.

19. A method for inhibiting physiological lymphangiogenesis or pathological lymphangiogenesis in a subject comprising administering to the subject an amount of the fusion protein of any of claims 1-11 effective to inhibit physiological lymphangiogenesis or pathological lymphangiogenesis in the subject.

20. The method of claim 19, wherein the pathological

lymphangiogenesis is tumor lymphangiogenesis or lymph node metastasis.

21. A method of inhibiting tumor metastasis in a subject comprising administering to the subject an amount of the fusion protein of any of claims 1-11 effective to inhibit tumor metastasis in the subject.

22. The method of claim 21, wherein the metastasis occurs via a blood vessel, the lymphatic vasculature or a lymph node.

23. 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 1-11 effective to inhibit growth of the secondary tumor in the subject.

24. The method of claim 23, wherein the secondary tumor growth is inhibited by inhibition of angiogenesis associated with the secondary tumor.

25. 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 1-11 effective to inhibit blood vessel cooption by a tumor in the subject.

26. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-11 and an inhibitor of Vascular Endothelial Growth Factor (VEGF), each in an amount effective to treat the cancer in the subject.

27. The method of claim 26, wherein the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

28. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-11 and a VEGF receptor inhibitor, each in an amount effective to treat the cancer in the subject.

29. The method of claim 29, wherein the VEGF receptor inhibitor is a VEGFR-1 inhibitor, a VEGFR-2 inhibitor or a VEGFR-2 inhibitor.

30. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-11 and an inhibitor of Platelet Derived Growth Factor (PDGF), each in an amount effective to treat the cancer in the subject.

31. The method of claim 30, wherein the inhibitor of Platelet Derived Growth Factors is an inhibitor of PDGF-A or an inhibitor of PDGF-B

32. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-11 and a PDGF receptor antagonist, each in an amount effective to treat the cancer in the subject.

33. The method of claim 32, wherein the PDGF receptor antagonist is a PDGF Receptor-B antagonist.

34. A method of treating cancer in a subject comprising administering to the subject the fusion protein of any of claims 1-11 and an inhibitor of HER2/neu, each in an amount effective to treat the cancer in the subject.

35. A method of treating breast cancer in a subject comprising administering to the subject an amount of the fusion protein of any of claims 1-11 effective to treat the breast cancer in the subject.

36. The use of a fusion protein of any of claims 1-11 for the preparation of a pharmaceutical composition for treating a subject having breast cancer.

SEQUENCE LISTING

<110> The Trustees Of Columbia University In The City Of New York
Kitajewski, Jan
Shawber, Carrie

<120> Human Notch3 Based Fusion Proteins As Decoy Inhibitors Of Notch3 Signaling

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Gln His Gly Gly Thr Cys Tyr Pro Gln Arg Gln Pro Pro Tyr Tyr
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Cys Ala Cys Pro Pro Gly Tyr Gln Gly Gln Ser Cys Gln Ser Asp Ile
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His Leu Cys Leu Cys Pro Pro Gly Phe Thr Gly Leu Asp Cys Glu Met
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Leu Asp Gly Leu Asp Thr Tyr Thr Cys Leu Cys Pro Lys Thr Trp Lys
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Gly Trp Asp Cys Ser Glu Asp Ile Asp Glu Cys Glu Ala Arg Gly Pro
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Pro Arg Cys Arg Asn Gly Gly Thr Cys Gln Asn Thr Ala Gly Ser Phe
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His Cys Val Cys Val Ser Gly Trp Gly Gly Ala Gly Cys Glu Glu Asn
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Asn Ala Gln Cys Ser Thr Asn Pro Leu Thr Gly Ser Thr Leu Cys Ile
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Cys Gln Pro Gly Tyr Ser Gly Ser Thr Cys His Gln Asp Leu Asp Glu
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Cys Gln Met Ala Gln Gln Gly Pro Ser Pro Cys Glu His Gly Gly Ser
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Cys Ile Asn Thr Pro Gly Ser Phe Asn Cys Leu Cys Leu Pro Gly Tyr
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Thr Gly Ser Arg Cys Glu Ala Asp His Asn Glu Cys Leu Ser Gln Pro
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Cys His Pro Gly Ser Thr Cys Leu Asp Leu Leu Ala Thr Phe His Cys
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Leu Cys Pro Pro Gly Leu Glu Gly Arg Leu Cys Glu Val Glu Val Asn
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Glu Cys Thr Ser Asn Pro Cys Leu Asn Gln Ala Ala Cys His Asp Leu
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Asp Pro Cys Pro Val Gly Ala Ser Cys Leu Asp Leu Pro Gly Ala Phe
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Phe Cys Leu Cys Arg Pro Gly Phe Thr Gly Gln Leu Cys Glu Val Pro
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Glu His Arg Ala Pro Cys Leu Cys Pro Asp Gly Ser Pro Gly Cys Val
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Ser Cys Gln Lys Ala Ala Met Ser Gln Gly Ile Glu Ile Ser Gly Leu
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<213> Homo sapiens

<400> 16

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Leu Ala Gly Pro Gly Ala Ala Ala Pro Pro Cys Leu Asp Gly Ser Pro
35 40 45

Cys Ala Asn Gly Gly Arg Cys Thr Gln Leu Pro Ser Arg Glu Ala Ala
50 55 60

Cys Leu Cys Pro Pro Gly Trp Val Gly Glu Arg Cys Gln Leu Glu Asp
65 70 75 80

Pro Cys His Ser Gly Pro Cys Ala Gly Arg Gly Val Cys Gln Ser Ser
85 90 95

Val Val Ala Gly Thr Ala Arg Phe Ser Cys Arg Cys Pro Arg Gly Phe
100 105 110

Arg Gly Pro Asp Cys Ser Leu Pro Asp Pro Cys Leu Ser Ser Pro Cys
115 120 125

Ala His Gly Ala Arg Cys Ser Val Gly Pro Asp Gly Arg Phe Leu Cys
130 135 140

Ser Cys Pro Pro Gly Tyr Gln Gly Arg Ser Cys Arg Ser Asp Val Asp
145 150 155 160

Glu Cys Arg Val Gly Glu Pro Cys Arg His Gly Gly Thr Cys Leu Asn
165 170 175

Thr Pro Gly Ser Phe Arg Cys Gln Cys Pro Ala Gly Tyr Thr Gly Pro
180 185 190

Leu Cys Glu Asn Pro Ala Val Pro Cys Ala Pro Ser Pro Cys Arg Asn
195 200 205

Gly Gly Thr Cys Arg Gln Ser Gly Asp Leu Thr Tyr Asp Cys Ala Cys
210 215 220

Leu Pro Gly Phe Glu Gly Gln Asn Cys Glu Val Asn Val Asp Asp Cys
225 230 235 240

Pro Gly His Arg Cys Leu Asn Gly Gly Thr Cys Val Asp Gly Val Asn
245 250 255

Thr Tyr Asn Cys Gln Cys Pro Pro Glu Trp Thr Gly Gln Phe Cys Thr
260 265 270

Glu Asp Val Asp Glu Cys Gln Leu Gln Pro Asn Ala Cys His Asn Gly
275 280 285

Gly Thr Cys Phe Asn Thr Leu Gly Gly His Ser Cys Val Cys Val Asn
290 295 300

Gly Trp Thr Gly Glu Ser Cys Ser Gln Asn Ile Asp Asp Cys Ala Thr
305 310 315 320

Ala Val Cys Phe His Gly Ala Thr Cys His Asp Arg Val Ala Ser Phe
325 330 335

Tyr Cys Ala Cys Pro Met Gly Lys Thr Gly Leu Leu Cys His Leu Asp
340 345 350

Asp Ala Cys Val Ser Asn Pro Cys His Glu Asp Ala Ile Cys Asp Thr
355 360 365

Asn Pro Val Asn Gly Arg Ala Ile Cys Thr Cys Pro Pro Gly Phe Thr
370 375 380

Gly Gly Ala Cys Asp Gln Asp Val Asp Glu Cys Ser Ile Gly Ala Asn
385 390 395 400

Pro Cys Glu His Leu Gly Arg Cys Val Asn Thr Gln Gly Ser Phe Leu
405 410 415

Cys Gln Cys Gly Arg Gly Tyr Thr Gly Pro Arg Cys Glu Thr Asp Val
420 425 430

Asn Glu Cys Leu Ser Gly Pro Cys Arg Asn Gln Ala Thr Cys Leu Asp
435 440 445

Arg Ile Gly Gln Phe Thr Cys Ile Cys Met Ala Gly Phe Thr Gly Thr
450 455 460

Tyr Cys Glu Val Asp Ile Asp Glu Cys Gln Ser Ser Pro Cys Val Asn
465 470 475 480

Gly Gly Val Cys Lys Asp Arg Val Asn Gly Phe Ser Cys Thr Cys Pro

485 490 495

Ser Gly Phe Ser Gly Ser Thr Cys Gln Leu Asp Val Asp Glu Cys Ala
500 505 510

Ser Thr Pro Cys Arg Asn Gly Ala Lys Cys Val Asp Gln Pro Asp Gly
515 520 525

Tyr Glu Cys Arg Cys Ala Glu Gly Phe Glu Gly Thr Leu Cys Asp Arg
530 535 540

Asn Val Asp Asp Cys Ser Pro Asp Pro Cys His His Gly Arg Cys Val
545 550 555 560

Asp Gly Ile Ala Ser Phe Ser Cys Ala Cys Ala Pro Gly Tyr Thr Gly
565 570 575

Thr Arg Cys Glu Ser Gln Val Asp Glu Cys Arg Ser Gln Pro Cys Arg
580 585 590

His Gly Gly Lys Cys Leu Asp Leu Val Asp Lys Tyr Leu Cys Arg Cys
595 600 605

Pro Ser Gly Thr Thr Gly Val Asn Cys Glu Val Asn Ile Asp Asp Cys
610 615 620

Ala Ser Asn Pro Cys Thr Phe Gly Val Cys Arg Asp Gly Ile Asn Arg
625 630 635 640

Tyr Asp Cys Val Cys Gln Pro Gly Phe Thr Gly Pro Leu Cys Asn Val
645 650 655

Glu Ile Asn Glu Cys Ala Ser Ser Pro Cys Gly Glu Gly Gly Ser Cys
660 665 670

Val Asp Gly Glu Asn Gly Phe Arg Cys Leu Cys Pro Pro Gly Ser Leu
675 680 685

Pro Pro Leu Cys Leu Pro Pro Ser His Pro Cys Ala His Glu Pro Cys
690 695 700

Ser His Gly Ile Cys Tyr Asp Ala Pro Gly Gly Phe Arg Cys Val Cys
705 710 715 720

Glu Pro Gly Trp Ser Gly Pro Arg Cys Ser Gln Ser Leu Ala Arg Asp
725 730 735

Ala Cys Glu Ser Gln Pro Cys Arg Ala Gly Gly Thr Cys Ser Ser Asp
740 745 750

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Gly Met Gly Phe His Cys Thr Cys Pro Pro Gly Val Gln Gly Arg Gln
        755                 760                 765

Cys Glu Leu Leu Ser Pro Cys Thr Pro Asn Pro Cys Glu His Gly Gly
    770                 775                 780

Arg Cys Glu Ser Ala Pro Gly Gln Leu Pro Val Cys Ser Cys Pro Gln
785                 790                 795                 800

Gly Trp Gln Gly Pro Arg Cys Gln Gln Asp Val Asp Glu Cys Ala Gly
                805                 810                 815

Pro Ala Pro Cys Gly Pro His Gly Ile Cys Thr Asn Leu Ala Gly Ser
            820                 825                 830

Phe Ser Cys Thr Cys His Gly Gly Tyr Thr Gly Pro Ser Cys Asp Gln
        835                 840                 845

Asp Ile Asn Asp Cys Asp Pro Asn Pro Cys Leu Asn Gly Gly Ser Cys
    850                 855                 860

Gln Asp Gly Val Gly Ser Phe Ser Cys Ser Cys Leu Pro Gly Phe Ala
865                 870                 875                 880

Gly Pro Arg Cys Ala Arg Asp Val Asp Glu Cys Leu Ser Asn Pro Cys
                885                 890                 895

Gly Pro Gly Thr Cys Thr Asp His Val Ala Ser Phe Thr Cys Thr Cys
            900                 905                 910

Pro Pro Gly Tyr Gly Gly Phe His Cys Glu Gln Asp Leu Pro Asp Cys
        915                 920                 925

Ser Pro Ser Ser Cys Phe Asn Gly Gly Thr Cys Val Asp Gly Val Asn
    930                 935                 940

Ser Phe Ser Cys Leu Cys Arg Pro Gly Tyr Thr Gly Ala His Cys Gln
945                 950                 955                 960

His Glu Ala Asp Pro Cys Leu Ser Arg Pro Cys Leu His Gly Gly Val
                965                 970                 975

Cys Ser Ala Ala His Pro Gly Phe Arg Cys Thr Cys Leu Glu Ser Phe
            980                 985                 990

Thr Gly Pro Gln Cys Gln Thr Leu  Val Asp Trp Cys Ser  Arg Gln Pro
        995                 1000                1005

Cys Gln  Asn Gly Gly Arg Cys  Val Gln Thr Gly Ala  Tyr Cys Leu
    1010                1015                1020
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Cys Pro Pro Gly Trp Ser Gly Arg Leu Cys Asp Ile Arg Ser Leu
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Pro Cys Arg Glu Ala Ala Ala Gln Ile Gly Val Arg Leu Glu Gln
1040 1045 1050

Leu Cys Gln Ala Gly Gly Gln Cys Val Asp Glu Asp Ser Ser His
1055 1060 1065

Tyr Cys Val Cys Pro Glu Gly Arg Thr Gly Ser His Cys Glu Gln
1070 1075 1080

Glu Val Asp Pro Cys Leu Ala Gln Pro Cys Gln His Gly Gly Thr
1085 1090 1095

Cys Arg Gly Tyr Met Gly Gly Tyr Met Cys Glu Cys Leu Pro Gly
1100 1105 1110

Tyr Asn Gly Asp Asn Cys Glu Asp Asp Val Asp Glu Cys Ala Ser
1115 1120 1125

Gln Pro Cys Gln His Gly Gly Ser Cys Ile Asp Leu Val Ala Arg
1130 1135 1140

Tyr Leu Cys Ser Cys Pro Pro Gly Thr Leu Gly Val Leu Cys Glu
1145 1150 1155

Ile Asn Glu Asp Asp Cys Gly Pro Gly Pro Pro Leu Asp Ser Gly
1160 1165 1170

Pro Arg Cys Leu His Asn Gly Thr Cys Val Asp Leu Val Gly Gly
1175 1180 1185

Phe Arg Cys Thr Cys Pro Pro Gly Tyr Thr Gly Leu Arg Cys Glu
1190 1195 1200

Ala Asp Ile Asn Glu Cys Arg Ser Gly Ala Cys His Ala Ala His
1205 1210 1215

Thr Arg Asp Cys Leu Gln Asp Pro Gly Gly Gly Phe Arg Cys Leu
1220 1225 1230

Cys His Ala Gly Phe Ser Gly Pro Arg Cys Gln Thr Val Leu Ser
1235 1240 1245

Pro Cys Glu Ser Gln Pro Cys Gln His Gly Gly Gln Cys Arg Pro
1250 1255 1260

Ser Pro Gly Pro Gly Gly Gly Leu Thr Phe Thr Cys His Cys Ala
1265 1270 1275

Gln Pro Phe Trp Gly Pro Arg Cys Glu Arg Val Ala Arg Ser Cys
1280 1285 1290

Arg Glu Leu Gln Cys Pro Val Gly Val Pro Cys Gln Gln Thr Pro
1295 1300 1305

Arg Gly Pro Arg Cys Ala Cys Pro Pro Gly Leu Ser Gly Pro Ser
1310 1315 1320

Cys Arg Ser Phe Pro Gly Ser Pro Pro Gly Ala Ser Asn Ala Ser
1325 1330 1335

Cys Ala Ala Ala Pro Cys Leu His Gly Gly Ser Cys Arg Pro Ala
1340 1345 1350

Pro Leu Ala Pro Phe Phe Arg Cys Ala Cys Ala Gln Gly Trp Thr
1355 1360 1365

Gly Pro Arg Cys Glu Ala Pro Ala Ala Ala Pro Glu Val Ser Glu
1370 1375 1380

Glu Pro Arg Cys Pro Arg Ala Ala Cys Gln Ala Lys Arg Gly Asp
1385 1390 1395

Gln Arg Cys Asp Arg Glu Cys Asn Ser Pro Gly Cys Gly Trp Asp
1400 1405 1410

Gly Gly Asp Cys Ser Leu Ser Val Gly Asp Pro Trp Arg Gln Cys
1415 1420 1425

Glu Ala Leu Gln Cys Trp Arg Leu Phe Asn Asn Ser Arg Cys Asp
1430 1435 1440

Pro Ala Cys Ser Ser Pro Ala Cys Leu Tyr Asp Asn Phe Asp Cys
1445 1450 1455

His Ala Gly Gly Arg Glu Arg Thr Cys Asn Pro Val Tyr Glu Lys
1460 1465 1470

Tyr Cys Ala Asp His Phe Ala Asp Gly Arg Cys Asp Gln Gly Cys
1475 1480 1485

Asn Thr Glu Glu Cys Gly Trp Asp Gly Leu Asp Cys Ala Ser Glu
1490 1495 1500

Val Pro Ala Leu Leu Ala Arg Gly Val Leu Val Leu Thr Val Leu
1505 1510 1515

Leu Pro Pro Glu Glu Leu Leu Arg Ser Ser Ala Asp Phe Leu Gln

1520 1525 1530

Arg Leu Ser Ala Ile Leu Arg Thr Ser Leu Arg Phe Arg Leu Asp
1535 1540 1545

Ala His Gly Gln Ala Met Val Phe Pro Tyr His Arg Pro Ser Pro
1550 1555 1560

Gly Ser Glu Pro Arg Ala Arg Arg Glu Leu Ala Pro Glu Val Ile
1565 1570 1575

Gly Ser Val Val Met Leu Glu Ile Asp Asn Arg Leu Cys Leu Gln
1580 1585 1590

Ser Pro Glu Asn Asp His Cys Phe Pro Asp Ala Gln Ser Ala Ala
1595 1600 1605

Asp Tyr Leu Gly Ala Leu Ser Ala Val Glu Arg Leu Asp Phe Pro
1610 1615 1620

Tyr Pro Leu Arg Asp Val Arg Gly Glu Pro Leu Glu Pro Pro Glu
1625 1630 1635

Pro Ser Val Pro Leu Leu Pro Leu Leu Val Ala Gly Ala Val Leu
1640 1645 1650

Leu Leu Val Ile Leu Val Leu Gly Val Met Val Ala Arg Arg Lys
1655 1660 1665

Arg Glu His Ser Thr Leu Trp Phe Pro Glu Gly Phe Ser Leu His
1670 1675 1680

Lys Asp Val Ala Ser Gly His Lys Gly Arg Arg Glu Pro Val Gly
1685 1690 1695

Gln Asp Ala Leu Gly Met Lys Asn Met Ala Lys Gly Glu Ser Leu
1700 1705 1710

Met Gly Glu Val Ala Thr Asp Trp Met Asp Thr Glu Cys Pro Glu
1715 1720 1725

Ala Lys Arg Leu Lys Val Glu Glu Pro Gly Met Gly Ala Glu Glu
1730 1735 1740

Ala Val Asp Cys Arg Gln Trp Thr Gln His His Leu Val Ala Ala
1745 1750 1755

Asp Ile Arg Val Ala Pro Ala Met Ala Leu Thr Pro Pro Gln Gly
1760 1765 1770

Asp Ala Asp Ala Asp Gly Met Asp Val Asn Val Arg Gly Pro Asp
1775 1780 1785

Gly Phe Thr Pro Leu Met Leu Ala Ser Phe Cys Gly Gly Ala Leu
1790 1795 1800

Glu Pro Met Pro Thr Glu Glu Asp Glu Ala Asp Asp Thr Ser Ala
1805 1810 1815

Ser Ile Ile Ser Asp Leu Ile Cys Gln Gly Ala Gln Leu Gly Ala
1820 1825 1830

Arg Thr Asp Arg Thr Gly Glu Thr Ala Leu His Leu Ala Ala Arg
1835 1840 1845

Tyr Ala Arg Ala Asp Ala Ala Lys Arg Leu Leu Asp Ala Gly Ala
1850 1855 1860

Asp Thr Asn Ala Gln Asp His Ser Gly Arg Thr Pro Leu His Thr
1865 1870 1875

Ala Val Thr Ala Asp Ala Gln Gly Val Phe Gln Ile Leu Ile Arg
1880 1885 1890

Asn Arg Ser Thr Asp Leu Asp Ala Arg Met Ala Asp Gly Ser Thr
1895 1900 1905

Ala Leu Ile Leu Ala Ala Arg Leu Ala Val Glu Gly Met Val Glu
1910 1915 1920

Glu Leu Ile Ala Ser His Ala Asp Val Asn Ala Val Asp Glu Leu
1925 1930 1935

Gly Lys Ser Ala Leu His Trp Ala Ala Ala Val Asn Asn Val Glu
1940 1945 1950

Ala Thr Leu Ala Leu Leu Lys Asn Gly Ala Asn Lys Asp Met Gln
1955 1960 1965

Asp Ser Lys Glu Glu Thr Pro Leu Phe Leu Ala Ala Arg Glu Gly
1970 1975 1980

Ser Tyr Glu Ala Ala Lys Leu Leu Leu Asp His Phe Ala Asn Arg
1985 1990 1995

Glu Ile Thr Asp His Leu Asp Arg Leu Pro Arg Asp Val Ala Gln
2000 2005 2010

Glu Arg Leu His Gln Asp Ile Val Arg Leu Leu Asp Gln Pro Ser
2015 2020 2025

Gly Pro Arg Ser Pro Pro Gly Pro His Gly Leu Gly Pro Leu Leu
2030 2035 2040

Cys Pro Pro Gly Ala Phe Leu Pro Gly Leu Lys Ala Ala Gln Ser
2045 2050 2055

Gly Ser Lys Lys Ser Arg Arg Pro Pro Gly Lys Ala Gly Leu Gly
2060 2065 2070

Pro Gln Gly Pro Arg Gly Arg Gly Lys Lys Leu Thr Leu Ala Cys
2075 2080 2085

Pro Gly Pro Leu Ala Asp Ser Ser Val Thr Leu Ser Pro Val Asp
2090 2095 2100

Ser Leu Asp Ser Pro Arg Pro Phe Gly Gly Pro Pro Ala Ser Pro
2105 2110 2115

Gly Gly Phe Pro Leu Glu Gly Pro Tyr Ala Ala Ala Thr Ala Thr
2120 2125 2130

Ala Val Ser Leu Ala Gln Leu Gly Gly Pro Gly Arg Ala Gly Leu
2135 2140 2145

Gly Arg Gln Pro Pro Gly Gly Cys Val Leu Ser Leu Gly Leu Leu
2150 2155 2160

Asn Pro Val Ala Val Pro Leu Asp Trp Ala Arg Leu Pro Pro Pro
2165 2170 2175

Ala Pro Pro Gly Pro Ser Phe Leu Leu Pro Leu Ala Pro Gly Pro
2180 2185 2190

Gln Leu Leu Asn Pro Gly Thr Pro Val Ser Pro Gln Glu Arg Pro
2195 2200 2205

Pro Pro Tyr Leu Ala Val Pro Gly His Gly Glu Glu Tyr Pro Ala
2210 2215 2220

Ala Gly Ala His Ser Ser Pro Pro Lys Ala Arg Phe Leu Arg Val
2225 2230 2235

Pro Ser Glu His Pro Tyr Leu Thr Pro Ser Pro Glu Ser Pro Glu
2240 2245 2250

His Trp Ala Ser Pro Ser Pro Pro Ser Leu Ser Asp Trp Ser Glu
2255 2260 2265

Ser Thr Pro Ser Pro Ala Thr Ala Thr Gly Ala Met Ala Thr Thr
2270 2275 2280

Thr Gly Ala Leu Pro Ala Gln Pro Leu Pro Leu Ser Val Pro Ser
2285 2290 2295

Ser Leu Ala Gln Ala Gln Thr Gln Leu Gly Pro Gln Pro Glu Val
2300 2305 2310

Thr Pro Lys Arg Gln Val Leu Ala
2315 2320

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ctcatgatct cccggacccc tgaggtcaca tgcgtggtgg tggacgtgag ccacgaagac 180

cctgaggtca agttcaactg gtacgtggac ggcgtggagg tgcataatgc caagacaaag 240

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caggactggc tgaatggcaa ggagtacaag tgcaaggtct ccaacaaagc cctcccagcc 360

cccatcgaga aaaccatctc caaagccaaa gggcagcccc gagaaccaca ggtgtacacc 420

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accgtggaca agagcaggtg gcagcagggg aacgtcttct catgctccgt gatgcatgag 660

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<400> 18

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20 25 30

Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
35 40 45

Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
50 55 60

Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
65 70 75 80

Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
85 90 95

Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
100 105 110

Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
115 120 125

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
130 135 140

Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
145 150 155 160

Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
165 170 175

Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
180 185 190

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
195 200 205

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
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His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
225 230 235

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Leu Ala Gly Pro Gly Ala Ala Ala
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<212> PRT
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Thr Leu Cys Thr Ala Arg
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<212> DNA
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<212> DNA
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cccccttgcc tggacggaag cccgtgtgca aatggaggtc gttgcaccca gctgccctcc 180

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<400> 32

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35 40 45

Cys Ala Asn Gly Gly Arg Cys Thr Gln Leu Pro Ser Arg Glu Ala Ala
50 55 60

Cys Leu Cys Pro Pro Gly Trp Val Gly Glu Arg Cys Gln Leu Glu Asp
65 70 75 80

Pro Cys His Ser Gly Pro Cys Ala Gly Arg Gly Val Cys Gln Ser Ser
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Val Val Ala Gly Thr Ala Arg Phe Ser Cys Arg Cys Pro Arg Gly Phe
100 105 110

Arg Gly Pro Asp Cys Ser Leu Pro Asp Pro Cys Leu Ser Ser Pro Cys
115 120 125

Ala His Gly Ala Arg Cys Ser Val Gly Pro Asp Gly Arg Phe Leu Cys
130 135 140

Ser Cys Pro Pro Gly Tyr Gln Gly Arg Ser Cys Arg Ser Asp Val Asp
145 150 155 160

Glu Cys Arg Val Gly Glu Pro Cys Arg His Gly Gly Thr Cys Leu Asn
165 170 175

Thr Pro Gly Ser Phe Arg Cys Gln Cys Pro Ala Gly Tyr Thr Gly Pro
180 185 190

Leu Cys Glu Asn Pro Ala Val Pro Cys Ala Pro Ser Pro Cys Arg Asn
195 200 205

Gly Gly Thr Cys Arg Gln Ser Gly Asp Leu Thr Tyr Asp Cys Ala Cys
210 215 220

Leu Pro Gly Phe Glu Gly Gln Asn Cys Glu Val Asn Val Asp Asp Cys
225 230 235 240

Pro Gly His Arg Cys Leu Asn Gly Gly Thr Cys Val Asp Gly Val Asn
245 250 255

Thr Tyr Asn Cys Gln Cys Pro Pro Glu Trp Thr Gly Gln Phe Cys Thr
260 265 270

Glu Asp Val Asp Glu Cys Gln Leu Gln Pro Asn Ala Cys His Asn Gly
275 280 285

Gly Thr Cys Phe Asn Thr Leu Gly Gly His Ser Cys Val Cys Val Asn
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Gly Trp Thr Gly Glu Ser Cys Ser Gln Asn Ile Asp Asp Cys Ala Thr
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Ala Val Cys Phe His Gly Ala Thr Cys His Asp Arg Val Ala Ser Phe
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Tyr Cys Ala Cys Pro Met Gly Lys Thr Gly Leu Leu Cys His Leu Asp
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Asp Ala Cys Val Ser Asn Pro Cys His Glu Asp Ala Ile Cys Asp Thr
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Asn Pro Val Asn Gly Arg Ala Ile Cys Thr Cys Pro Pro Gly Phe Thr
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Gly Gly Ala Cys Asp Gln Asp Val Asp Glu Cys Ser Ile Gly Ala Asn
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Pro Cys Glu His Leu Gly Arg Cys Val Asn Thr Gln Gly Ser Phe Leu
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Cys Gln Cys Gly Arg Gly Tyr Thr Gly Pro Arg Cys Glu Thr Asp Val
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Asn Glu Cys Leu Ser Gly Pro Cys Arg Asn Gln Ala Thr Cys Leu Asp
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Arg Ile Gly Gln Phe Thr Cys Ile Cys Met Ala Gly Phe Thr Gly Thr
450 455 460

Tyr Cys Glu Val Asp Ile Asp Glu Cys Gln Ser Ser Pro Cys Val Asn
465 470 475 480

Gly Gly Val Cys Lys Asp Arg Val Asn Gly Phe Ser Cys Thr Cys Pro
485 490 495

Ser Gly Phe Ser Gly Ser Thr Cys Gln Leu Asp Val Asp Glu Cys Ala
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Ser Thr Pro Cys Arg Asn Gly Ala Lys Cys Val Asp Gln Pro Asp Gly
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Tyr Glu Cys Arg Cys Ala Glu Gly Phe Glu Gly Thr Leu Cys Asp Arg
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Asn Val Asp Asp Cys Ser Pro Asp Pro Cys His His Gly Arg Cys Val
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Asp Gly Ile Ala Ser Phe Ser Cys Ala Cys Ala Pro Gly Tyr Thr Gly
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Thr Arg Cys Glu Ser Gln Val Asp Glu Cys Arg Ser Gln Pro Cys Arg
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His Gly Gly Lys Cys Leu Asp Leu Val Asp Lys Tyr Leu Cys Arg Cys
595 600 605

Pro Ser Gly Thr Thr Gly Val Asn Cys Glu Val Asn Ile Asp Asp Cys
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Ala Ser Asn Pro Cys Thr Phe Gly Val Cys Arg Asp Gly Ile Asn Arg
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Tyr Asp Cys Val Cys Gln Pro Gly Phe Thr Gly Pro Leu Cys Asn Val
645 650 655

Glu Ile Asn Glu Cys Ala Ser Ser Pro Cys Gly Glu Gly Gly Ser Cys
660 665 670

Val Asp Gly Glu Asn Gly Phe Arg Cys Leu Cys Pro Pro Gly Ser Leu
675 680 685

Pro Pro Leu Cys Leu Pro Pro Ser His Pro Cys Ala His Glu Pro Cys
690 695 700

Ser His Gly Ile Cys Tyr Asp Ala Pro Gly Gly Phe Arg Cys Val Cys
705 710 715 720

Glu Pro Gly Trp Ser Gly Pro Arg Cys Ser Gln Ser Leu Ala Arg Asp

725 730 735

Ala Cys Glu Ser Gln Pro Cys Arg Ala Gly Gly Thr Cys Ser Ser Asp
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Gly Met Gly Phe His Cys Thr Cys Pro Pro Gly Val Gln Gly Arg Gln
755 760 765

Cys Glu Leu Leu Ser Pro Cys Thr Pro Asn Pro Cys Glu His Gly Gly
770 775 780

Arg Cys Glu Ser Ala Pro Gly Gln Leu Pro Val Cys Ser Cys Pro Gln
785 790 795 800

Gly Trp Gln Gly Pro Arg Cys Gln Gln Asp Val Asp Glu Cys Ala Gly
805 810 815

Pro Ala Pro Cys Gly Pro His Gly Ile Cys Thr Asn Leu Ala Gly Ser
820 825 830

Phe Ser Cys Thr Cys His Gly Gly Tyr Thr Gly Pro Ser Cys Asp Gln
835 840 845

Asp Ile Asn Asp Cys Asp Pro Asn Pro Cys Leu Asn Gly Gly Ser Cys
850 855 860

Gln Asp Gly Val Gly Ser Phe Ser Cys Ser Cys Leu Pro Gly Phe Ala
865 870 875 880

Gly Pro Arg Cys Ala Arg Asp Val Asp Glu Cys Leu Ser Asn Pro Cys
885 890 895

Gly Pro Gly Thr Cys Thr Asp His Val Ala Ser Phe Thr Cys Thr Cys
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Pro Pro Gly Tyr Gly Gly Phe His Cys Glu Gln Asp Leu Pro Asp Cys
915 920 925

Ser Pro Ser Ser Cys Phe Asn Gly Gly Thr Cys Val Asp Gly Val Asn
930 935 940

Ser Phe Ser Cys Leu Cys Arg Pro Gly Tyr Thr Gly Ala His Cys Gln
945 950 955 960

His Glu Ala Asp Pro Cys Leu Ser Arg Pro Cys Leu His Gly Gly Val
965 970 975

Cys Ser Ala Ala His Pro Gly Phe Arg Cys Thr Cys Leu Glu Ser Phe
980 985 990

Thr Gly Pro Gln Cys Gln Thr Leu Val Asp Trp Cys Ser Arg Gln Pro
995 1000 1005

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Pro Cys Arg Glu Ala Ala Ala Gln Ile Gly Val Arg Leu Glu Gln
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Tyr Cys Val Cys Pro Glu Gly Arg Thr Gly Ser His Cys Glu Gln
1070 1075 1080

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Cys Arg Gly Tyr Met Gly Gly Tyr Met Cys Glu Cys Leu Pro Gly
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Gln Pro Cys Gln His Gly Gly Ser Cys Ile Asp Leu Val Ala Arg
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Tyr Leu Cys Ser Cys Pro Pro Gly Thr Leu Gly Val Leu Cys Glu
1145 1150 1155

Ile Asn Glu Asp Asp Cys Gly Pro Gly Pro Pro Leu Asp Ser Gly
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Pro Arg Cys Leu His Asn Gly Thr Cys Val Asp Leu Val Gly Gly
1175 1180 1185

Phe Arg Cys Thr Cys Pro Pro Gly Tyr Thr Gly Leu Arg Cys Glu
1190 1195 1200

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Thr Arg Asp Cys Leu Gln Asp Pro Gly Gly Gly Phe Arg Cys Leu
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Cys His Ala Gly Phe Ser Gly Pro Arg Cys Gln Thr Val Leu Ser
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Pro Cys Glu Ser Gln Pro Cys Gln His Gly Gly Gln Cys Arg Pro
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Ser Pro Gly Pro Gly Gly Gly Leu Thr Phe Thr Cys His Cys Ala
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Gln Pro Phe Trp Gly Pro Arg Cys Glu Arg Val Ala Arg Ser Cys
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Arg Glu Leu Gln Cys Pro Val Gly Val Pro Cys Gln Gln Thr Pro
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Arg Gly Pro Arg Cys Ala Cys Pro Pro Gly Leu Ser Gly Pro Ser
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Cys Arg Ser Phe Pro Gly Ser Pro Pro Gly Ala Ser Asn Ala Ser
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Cys Ala Ala Ala Pro Cys Leu His Gly Gly Ser Cys Arg Pro Ala
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Pro Leu Ala Pro Phe Phe Arg Cys Ala Cys Ala Gln Gly Trp Thr
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Gly Pro Arg Cys Glu Ala Pro Ala Ala Ala Pro Glu Val Ser Glu
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Glu Pro Arg Asp Leu Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys
1385 1390 1395

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
1400 1405 1410

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
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Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
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His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
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Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
1460 1465 1470

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
1475 1480 1485

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
1490 1495 1500

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Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
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Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
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Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
    1550                1555                1560

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
    1565                1570                1575

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
    1580                1585                1590

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
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<213> Homo sapiens

<400> 33

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Asn Gly Gly Arg Cys Thr Gln Leu Pro Ser Arg Glu Ala Ala Cys Leu
        35                  40                  45

Cys Pro Pro Gly Trp Val Gly Glu Arg Cys Gln Leu Glu Asp Pro Cys
    50                  55                  60

His Ser Gly Pro Cys Ala Gly Arg Gly Val Cys Gln Ser Ser Val Val
65                  70                  75                  80

Ala Gly Thr Ala Arg Phe Ser Cys Arg Cys Pro Arg Gly Phe Arg Gly
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Pro Asp Cys Ser Leu Pro Asp Pro Cys Leu Ser Ser Pro Cys Ala His
            100                 105                 110
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Pro Pro Gly Tyr Gln Gly Arg Ser Cys Arg Ser Asp Val Asp Glu Cys
    130                 135                 140

Arg Val Gly Glu Pro Cys Arg His Gly Gly Thr Cys Leu Asn Thr Pro
145                 150                 155                 160

Gly Ser Phe Arg Cys Gln Cys Pro Ala Gly Tyr Thr Gly Pro Leu Cys
                165                 170                 175

Glu Asn Pro Ala Val Pro Cys Ala Pro Ser Pro Cys Arg Asn Gly Gly
            180                 185                 190

Thr Cys Arg Gln Ser Gly Asp Leu Thr Tyr Asp Cys Ala Cys Leu Pro
        195                 200                 205

Gly Phe Glu Gly Gln Asn Cys Glu Val Asn Val Asp Asp Cys Pro Gly
    210                 215                 220

His Arg Cys Leu Asn Gly Gly Thr Cys Val Asp Gly Val Asn Thr Tyr
225                 230                 235                 240

Asn Cys Gln Cys Pro Pro Glu Trp Thr Gly Gln Phe Cys Thr Glu Asp
                245                 250                 255

Val Asp Glu Cys Gln Leu Gln Pro Asn Ala Cys His Asn Gly Gly Thr
            260                 265                 270

Cys Phe Asn Thr Leu Gly Gly His Ser Cys Val Cys Val Asn Gly Trp
        275                 280                 285

Thr Gly Glu Ser Cys Ser Gln Asn Ile Asp Asp Cys Ala Thr Ala Val
    290                 295                 300

Cys Phe His Gly Ala Thr Cys His Asp Arg Val Ala Ser Phe Tyr Cys
305                 310                 315                 320

Ala Cys Pro Met Gly Lys Thr Gly Leu Leu Cys His Leu Asp Asp Ala
                325                 330                 335

Cys Val Ser Asn Pro Cys His Glu Asp Ala Ile Cys Asp Thr Asn Pro
            340                 345                 350

Val Asn Gly Arg Ala Ile Cys Thr Cys Pro Pro Gly Phe Thr Gly Gly
        355                 360                 365

Ala Cys Asp Gln Asp Val Asp Glu Cys Ser Ile Gly Ala Asn Pro Cys
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370 375 380

Glu His Leu Gly Arg Cys Val Asn Thr Gln Gly Ser Phe Leu Cys Gln
385 390 395 400

Cys Gly Arg Gly Tyr Thr Gly Pro Arg Cys Glu Thr Asp Val Asn Glu
405 410 415

Cys Leu Ser Gly Pro Cys Arg Asn Gln Ala Thr Cys Leu Asp Arg Ile
420 425 430

Gly Gln Phe Thr Cys Ile Cys Met Ala Gly Phe Thr Gly Thr Tyr Cys
435 440 445

Glu Val Asp Ile Asp Glu Cys Gln Ser Ser Pro Cys Val Asn Gly Gly
450 455 460

Val Cys Lys Asp Arg Val Asn Gly Phe Ser Cys Thr Cys Pro Ser Gly
465 470 475 480

Phe Ser Gly Ser Thr Cys Gln Leu Asp Val Asp Glu Cys Ala Ser Thr
485 490 495

Pro Cys Arg Asn Gly Ala Lys Cys Val Asp Gln Pro Asp Gly Tyr Glu
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Cys Arg Cys Ala Glu Gly Phe Glu Gly Thr Leu Cys Asp Arg Asn Val
515 520 525

Asp Asp Cys Ser Pro Asp Pro Cys His His Gly Arg Cys Val Asp Gly
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Ile Ala Ser Phe Ser Cys Ala Cys Ala Pro Gly Tyr Thr Gly Thr Arg
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Cys Glu Ser Gln Val Asp Glu Cys Arg Ser Gln Pro Cys Arg His Gly
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Gly Lys Cys Leu Asp Leu Val Asp Lys Tyr Leu Cys Arg Cys Pro Ser
580 585 590

Gly Thr Thr Gly Val Asn Cys Glu Val Asn Ile Asp Asp Cys Ala Ser
595 600 605

Asn Pro Cys Thr Phe Gly Val Cys Arg Asp Gly Ile Asn Arg Tyr Asp
610 615 620

Cys Val Cys Gln Pro Gly Phe Thr Gly Pro Leu Cys Asn Val Glu Ile
625 630 635 640

Asn Glu Cys Ala Ser Ser Pro Cys Gly Glu Gly Gly Ser Cys Val Asp
645 650 655

Gly Glu Asn Gly Phe Arg Cys Leu Cys Pro Pro Gly Ser Leu Pro Pro
660 665 670

Leu Cys Leu Pro Pro Ser His Pro Cys Ala His Glu Pro Cys Ser His
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Gly Ile Cys Tyr Asp Ala Pro Gly Gly Phe Arg Cys Val Cys Glu Pro
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Gly Trp Ser Gly Pro Arg Cys Ser Gln Ser Leu Ala Arg Asp Ala Cys
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Glu Ser Gln Pro Cys Arg Ala Gly Gly Thr Cys Ser Ser Asp Gly Met
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Gly Phe His Cys Thr Cys Pro Pro Gly Val Gln Gly Arg Gln Cys Glu
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Leu Leu Ser Pro Cys Thr Pro Asn Pro Cys Glu His Gly Gly Arg Cys
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Glu Ser Ala Pro Gly Gln Leu Pro Val Cys Ser Cys Pro Gln Gly Trp
770 775 780

Gln Gly Pro Arg Cys Gln Gln Asp Val Asp Glu Cys Ala Gly Pro Ala
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Pro Cys Gly Pro His Gly Ile Cys Thr Asn Leu Ala Gly Ser Phe Ser
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Cys Thr Cys His Gly Gly Tyr Thr Gly Pro Ser Cys Asp Gln Asp Ile
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Asn Asp Cys Asp Pro Asn Pro Cys Leu Asn Gly Gly Ser Cys Gln Asp
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Gly Val Gly Ser Phe Ser Cys Ser Cys Leu Pro Gly Phe Ala Gly Pro
850 855 860

Arg Cys Ala Arg Asp Val Asp Glu Cys Leu Ser Asn Pro Cys Gly Pro
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Gly Thr Cys Thr Asp His Val Ala Ser Phe Thr Cys Thr Cys Pro Pro
885 890 895

Gly Tyr Gly Gly Phe His Cys Glu Gln Asp Leu Pro Asp Cys Ser Pro
900 905 910

Ser Ser Cys Phe Asn Gly Gly Thr Cys Val Asp Gly Val Asn Ser Phe
915 920 925

Ser Cys Leu Cys Arg Pro Gly Tyr Thr Gly Ala His Cys Gln His Glu
930 935 940

Ala Asp Pro Cys Leu Ser Arg Pro Cys Leu His Gly Gly Val Cys Ser
945 950 955 960

Ala Ala His Pro Gly Phe Arg Cys Thr Cys Leu Glu Ser Phe Thr Gly
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Pro Gln Cys Gln Thr Leu Val Asp Trp Cys Ser Arg Gln Pro Cys Gln
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Asn Gly Gly Arg Cys Val Gln Thr Gly Ala Tyr Cys Leu Cys Pro Pro
995 1000 1005

Gly Trp Ser Gly Arg Leu Cys Asp Ile Arg Ser Leu Pro Cys Arg
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Cys Pro Glu Gly Arg Thr Gly Ser His Cys Glu Gln Glu Val Asp
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Pro Cys Leu Ala Gln Pro Cys Gln His Gly Gly Thr Cys Arg Gly
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Ser Cys Pro Pro Gly Thr Leu Gly Val Leu Cys Glu Ile Asn Glu
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Asp Asp Cys Gly Pro Gly Pro Pro Leu Asp Ser Gly Pro Arg Cys
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1160 1165 1170

Thr Cys Pro Pro Gly Tyr Thr Gly Leu Arg Cys Glu Ala Asp Ile
1175 1180 1185

Asn Glu Cys Arg Ser Gly Ala Cys His Ala Ala His Thr Arg Asp
1190 1195 1200

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Gly Phe Ser Gly Pro Arg Cys Gln Thr Val Leu Ser Pro Cys Glu
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Ser Gln Pro Cys Gln His Gly Gly Gln Cys Arg Pro Ser Pro Gly
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Pro Gly Gly Gly Leu Thr Phe Thr Cys His Cys Ala Gln Pro Phe
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Trp Gly Pro Arg Cys Glu Arg Val Ala Arg Ser Cys Arg Glu Leu
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Pro Phe Phe Arg Cys Ala Cys Ala Gln Gly Trp Thr Gly Pro Arg
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Cys Glu Ala Pro Ala Ala Ala Pro Glu Val Ser Glu Glu Pro Arg
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Asp Leu Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr
1370 1375 1380

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
1385 1390 1395

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Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp

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Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
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Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
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Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
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Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
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Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu
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Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
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Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
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Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
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Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
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