(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 November 2017 (30.11.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/205651 A1

(51) International Patent Classification:
A61K 38/00 (2006.01) *C07K 14/705* (2006.01)
A61K 38/17 (2006.01) *C07K 19/00* (2006.01)

(21) International Application Number:
PCT/US2017/034521

(22) International Filing Date:
25 May 2017 (25.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/341,331 25 May 2016 (25.05.2016) US

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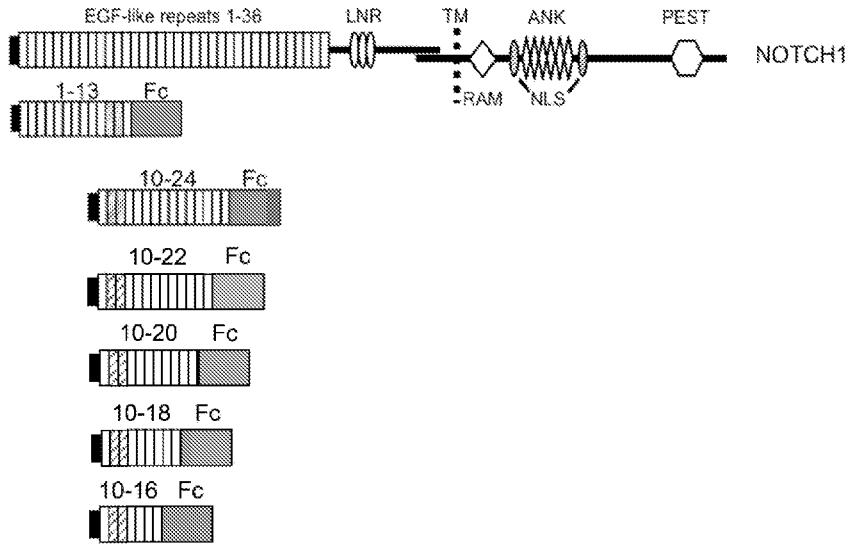
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH,

WO 2017/205651 A1

(54) Title: HUMAN NOTCH1 BASED FUSION PROTEINS AS DECOY INHIBITORS OF JAGGED-NOTCH SIGNALING AND DLL-NOTCH SIGNALING

Figure 3



(57) Abstract: This invention provides a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in: (a) an extracellular domain of a human Notch1 receptor protein, followed by (b) an Fc portion of an antibody, wherein the extracellular domain of the human Notch1 receptor protein (i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and (ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18 or 20. Also provided is a composition comprising the any of the fusion proteins of this invention and a pharmaceutically acceptable carrier, a method of treating a subject suffering from age-related macular degeneration (AMD), diabetic retinopathy, or cancer which comprises administering to the subject any of the compositions of this invention in an amount effective to treat the subject's cancer.

[Continued on next page]

GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

HUMAN NOTCH1 BASED FUSION PROTEINS AS DECOY INHIBITORS OF JAGGED-NOTCH SIGNALING AND DLL-NOTCH SIGNALING

This application claims priority of U.S. Provisional Patent Application No. 62/341,331 filed May 25, 2016, the entire contents of which are hereby incorporated herein by reference.

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

This invention was made with government support under grant number 1R01 HL112626 awarded by the National Institutes of Health.

This application incorporates-by-reference nucleotide and/or amino acid sequences which are present in the file named "170525_88010-A-PCT_SequenceListing_DH.txt" which is 32.6 kilobytes in size, and was created May 24, 2017 in the IBM-PCT machine format, having an operating system compatibility with MS-Windows, which is contained in the text file filed May 25, 2017 as part of this application.

BACKGROUND OF THE INVENTION

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

linked to both tumor angiogenesis and tumor lymphangiogenesis. The expression or function of several other potential Notch targets has also been linked to tumor angiogenesis; including ephrinB2, Id3, Angiopoietin 1, and PDGF-B. Insights on the role of these targets in Notch gene function will clearly facilitate future analysis of Notch in human pathologies.

SUMMARY OF THE INVENTION

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

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

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Also provided is a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

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

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

Also provided is a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

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

(b) a linker sequence, followed by

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Also provided is a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

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

(b) a linker sequence, followed by

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

Also provided is a fusion protein the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) a signal peptide, followed by

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

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Also provided is a fusion protein the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) a signal peptide, followed by

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

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

Also provided is a composition comprising the any of the fusion proteins of this invention and a pharmaceutically acceptable carrier.

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

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

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

BRIEF DESCRIPTION OF THE FIGURES

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

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

Figure 3: Schematic of design of Notch1 Decoys.

Figure 4: Assay demonstrating that Notch1 10-20, 10-18, and 10-16 decoys are secreted at higher levels than Notch1 1-13 and 10-24 decoys.

Figure 5: Results of assay demonstrating that Notch1 10-20 decoy blocks Jag1-Notch and Notch1 10-18 decoy blocks both Jag1 and Dll4-Notch.

Figure 6: Results of assay demonstrating that Notch1 10-20 decoy is secreted at higher levels than Notch1 10-24 decoy and maintains structural integrity after purification and storage.

Figure 7: Schematic depiction of assay and results of assay demonstrating that purified Notch1 10-20 decoy retains functional inhibition of Jag1-Notch activation.

Figure 8: Schematic depiction of assay and results of assay demonstrating that Notch1 10-20 decoy retains functional inhibition of sprouting angiogenesis similar to Notch1 10-24 Decoy.

Figure 9: Results of assay demonstrating that Notch1 1-13, 10-24, and 10-20 decoys inhibit migration of triple negative breast cancer cells.

Figure 10: Results of assay demonstrating that Notch1 10-20 decoy inhibits transendothelial migration of tumor cells across endothelial barriers.

DETAILED DESCRIPTION OF THE INVENTION

Terms

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

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

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

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

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

"Antibody" shall include, without limitation, (a) an immunoglobulin molecule comprising two heavy chains and two light chains and which recognizes an antigen; (b) a polyclonal or monoclonal immunoglobulin molecule; and (c) a monovalent or divalent fragment thereof. Immunoglobulin molecules may derive from any of the commonly known classes, including but not limited to IgA, secretory IgA, IgG, IgE and IgM. IgG subclasses are well known to those in the art and include,

but are not limited to, human IgG1, IgG2, IgG3 and IgG4. Antibodies can be both naturally occurring and non-naturally occurring. Furthermore, antibodies include chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. Antibodies may be human or nonhuman. Nonhuman antibodies may be humanized by recombinant methods to reduce their immunogenicity in humans. Antibody fragments include, without limitation, Fab and Fc fragments. The "Fc portion of an antibody", in one embodiment, is a crystallizable fragment obtained by papain digestion of immunoglobulin that consists of the C-terminal half of two heavy chains linked by disulfide bonds and known as the "effector region" of the immunoglobulin. In another embodiment, "Fc portion of an antibody" means all, or substantially all, of one C-terminal half of a heavy chain.

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

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

As used herein, "effective amount" refers to an amount which is capable of treating a subject having a tumor, a disease or a disorder. Accordingly, the effective amount will vary with the subject being

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

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

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

"Notch", "Notch protein", and "Notch receptor protein" are synonymous. In addition, the terms "Notch-based fusion protein" and "Notch decoy" are synonymous. The following Notch amino acid sequences are known and hereby incorporated by reference: Notch1 (Genbank accession no. S18188 (rat)); Notch2 (Genbank accession no. NP_077334 (rat)); Notch3 (Genbank accession no. Q61982 (mouse)); and Notch4 (Genbank accession no. T09059 (mouse)). The following Notch nucleic acid sequences are known and hereby incorporated by reference: Notch1 (Genbank accession no. XM_342392 (rat) and NM_017617 (human)); Notch2 (Genbank accession no. NM_024358 (rat), M99437 (human and AF308601 (human)); Notch3

(Genbank accession no. NM_008716 (mouse) and XM_009303 (human)); and Notch4 (Genbank accession no. NM_010929 (mouse) and NM_004557 (human)).

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

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

As used herein, "pharmaceutically acceptable carrier" means that the carrier is compatible with the other ingredients of the formulation and is not deleterious to the recipient thereof, and encompasses any of the standard pharmaceutically accepted carriers. Such carriers include, for example, 0.01-0.1 M and preferably 0.05 M phosphate

buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers can be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions and suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like.

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

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

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

There has been considerable evidence *in vivo*, including clinical observations, that abnormal angiogenesis is implicated in a number of disease conditions, which include rheumatoid arthritis, inflammation,

cancer, psoriasis, degenerative eye conditions and others.

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

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

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

The following abbreviations are used herein: ECD: extracellular domain; IC: intracellular domain; NECD/Fc: Notch-based fusion protein; N1: Notch1; N2: Notch2; N3: Notch3; N4: Notch4; Dll: Delta-like; DLL1: Delta-like 1; DLL4: Delta-like 4; JAG: JAGGED; JG: JAGGED; JAGGED-1: JAGGED 1; JG1: JAGGED 1; EC: endothelial cells; HUVEC: human umbilical vein endothelial cell; m.o.i.: multiplicity of infection; VEGF: vascular endothelial cell growth factor; VEGFR: vascular endothelial cell growth factor receptor; sp: signal peptide; PDGF: platelet

derived growth factor; PDGFR: platelet derived growth factor receptor; P1GF: placental growth factor.

Embodiments of the Invention

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

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

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Also provided is a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

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

(b) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

Also provided is a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

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

(b) a linker sequence, followed by

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Also provided is a fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

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

(b) a linker sequence, followed by

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

Also provided is a fusion protein the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) a signal peptide, followed by

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

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Also provided is a fusion protein the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:

(a) a signal peptide, followed by

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

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

(i) commences with the amino acid present at the N-terminus of EGF-

like repeat 10 and

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

In an embodiment of the fusion proteins of this invention, the signal peptide is the signal peptide of human Notch1 protein or the signal peptide of an IgG heavy chain.

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

Also provided is a composition comprising any of the fusion proteins of this invention and a pharmaceutically acceptable carrier.

In one embodiment of the composition of this invention, the fusion protein is present in an amount effective to inhibit Jagged-Notch signaling.

In one embodiment of the composition of this invention, the fusion protein is present in an amount effective to inhibit Delta-like-Notch signaling.

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

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

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

In one embodiment of any of the methods of this invention, the method further comprises administering an inhibitor of Vascular Endothelial Growth Factor (VEGF). In one embodiment, the inhibitor of VEGF is an

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

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

Also provided is a method of treating a subject suffering from cancer which comprises administering to the subject any of the compositions of this invention in an amount effective to treat the subject's cancer. In one embodiment the cancer is pancreatic cancer. In another embodiment the cancer is breast cancer.

Also provided is a method of inhibiting angiogenesis or metastasis in a tumor in a subject, the method comprising administering to the subject any of the compositions of this invention in an amount effective to reduce angiogenesis or metastasis in the tumor.

Also provided is a method of inhibiting Jag1-specific Notch activation, the method comprising administering a composition of this invention comprising, the composition comprising a fusion protein of this invention wherein the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 10 and extends to and includes the C-terminal amino acid of EGF-like repeat 20. In an embodiment the method does not inhibit Dll4-specific Notch activation. In an embodiment, the method inhibits angiogenic sprouting, cancer cell migration, and/or transendothelial migration of cancer cells across endothelial barriers.

Also provided is a method of inhibiting Jag1 and Dll4 Notch activation, the method comprising administering a composition of this invention comprising, the composition comprising a fusion protein of this invention wherein the extracellular domain of the human Notch1 receptor protein commences with the amino acid present at the N-terminus of EGF-like repeat 10 and extends to and includes the C-terminal amino acid of EGF-like repeat 18.

Each embodiment disclosed herein is contemplated as being applicable to each of the other disclosed embodiments. Thus, all combinations of the various elements described herein are within the scope of the invention.

EXAMPLES

Design of Notch1 Decoys

All decoys contain a designated number of EGF-like repeats from the extracellular domain of Notch1 fused at the C-terminus with human IgG1 Fc separated by a linker sequence. EGF-like repeats are designated in parentheses. Previous iterations of the decoys included Notch1 1-36, Notch1 1-13, and Notch1 10-24. Due to difficulty in producing relevant amounts of purified recombinant protein, inventors tested whether different variants of the decoys would be more easily secretable and examined ligand specificity with regards to inhibition of Notch activation. Using overlap extension PCR cloning, inventors were able to clone new variants including Notch1 10-22, Notch1 10-20, Notch1 10-18, and Notch1 10-16. See Figure 3.

Notch1 decoy secretory profile shows newer decoys are produced at higher levels than previous decoys.

Specific sequences coding for the cDNA of each decoy were cloned into a lentiviral expression construct, which was used to create chinese hamster ovary (CHO) cells stably expressing each decoy. CHO cells are the gold standard for recombinant biotherapeutic production. Conditioned media was collected from each CHO variant after 48 hours post-infection and equivalent volumes were evaluated by western blot analysis for visual, semi-quantitative determination of relative production. As expected, the Fc-only control is produced at the highest level. From our previous work, we observed that Notch1 1-13 is produced at higher amounts than Notch1 10-24. However, both are secreted at significantly lower amounts than Fc, and Notch1 10-24 is particularly difficult to produce. Notch1 10-24Fc(rl) represents a religated Notch1 10-24 as a control product of the cloning process. Notch1 10-22 appears to be secreted at a similar level as Notch1 10-24. However, Notch1 10-20 is secreted at even higher levels as Notch1 1-13 and Notch1 10-18 and Notch1 10-16 exhibit further enhanced secretion, providing three new potential decoys that are secreted at markedly higher levels than the previous decoys. See Figure 4.

Ligand Specificity of lentivirally-expressed Notch1 Decoys.

HeLa cells transfected with a Notch classical reporter construct to examine Notch activation were co-cultured with HeLa cells expressing

a vehicle control (GFP-expressing construct), JAG1 overexpression construct, or a DLL4 overexpression construct. In addition, ligand-expressing HeLa cells were also infected to express Notch1 decoys or Fc control. As illustrated in Figure 5, JAG1 and DLL4 both activate Notch. Notch1 10-24 and Notch1 1-13 inhibit JAG1 and DLL4-specific Notch activation, respectively, as previously observed. Notch1 10-22 does not inhibit JAG1-specific Notch activity, but does appear to reduce DLL4-specific Notch activity. Notch1 10-20 inhibits JAG1-specific Notch activity, but does not inhibit DLL4-specific Notch activity. Notch1 10-18 inhibits both JAG1 and DLL4-specific Notch activation, suggesting it may be a pan-Notch ligand inhibitor. Notch1 10-16 does not appear to inhibit either JAG1 or DLL4-specific Notch activation. These results show that of the newer decoys that are secreted at much higher levels than the previous decoys, Notch1 10-20 decoy retains Jagged specificity.

JAG1-specific Notch1 10-20 decoy is produced and secreted at significantly higher levels and maintains structural integrity after purification.

Decoy-expressing suspension CHO cells were cultured in shaking flask bioreactors in animal-component free media and media were collected and protein produced was purified by affinity chromatography using protein A containing chromatography columns. After purification, protein was stored in phosphate buffered saline, frozen at -80C, thawed and was analyzed by western blot analysis. Notch1 10-20 decoy is clearly produced at least two to three times more than Notch1 10-24 decoy without appreciable breakdown of the protein. Spectrophotometric analysis confirmed a 4-fold increase in production of Notch1 10-20 over Notch1 10-24, further confirming the potential of Notch1 10-20 as a Jag-specific decoy that can be produced at relevant levels and maintain structural integrity. See Figure 6.

Purified Notch1 10-20 decoy inhibits Jag1-specific activation of Notch in a Jag1-tethered plate assay.

Jag1 protein adhered to cell culture plates along with fibronectin to promote cell attachment stimulated Notch activation of a classical Notch reporter in a dual luciferase reporter assay as measured after 24hr of exposure of MDA-MB-231 triple negative breast cancer cells to

Jag1. This Jag1-specific activation of Notch in the breast cancer cells was inhibited by 5ug/mL of Notch1 10-20 purified decoy indicating that the purified decoy maintains inhibitory activity after purification and this can be easily evaluated in an in vitro test of activity using a tethered ligand approach. See Figure 7.

Production/Secretion/Purification of New Decoys

Previously, Notch1 1-13, 1-24, and 10-24 decoys were difficult to produce and purify.

Cloning of novel decoys, Notch1 10-20 and 10-18 decoys has provided decoys that are much more highly produced/secreted and are able to be purified while retaining functional inhibition of ligand-specific Notch. Notch1 10-20 decoy inhibits Jag1-specific Notch activation and Notch1 10-18 inhibits both Dll4 and Jag1-specific Notch.

Functional Use of New Decoys

Notch1 1-13 decoy promotes angiogenic hypersprouting but leads to dysfunctional vasculature and reduced perfusion in tumors, while Notch1 1-24 and 10-24 decoys inhibit angiogenic sprouting and tumor perfusion.

The following sections of this example illustrate the anti-angiogenic activity and potential anti-metastatic activity of Notch1 10-20 decoy, initially performed by overexpressing decoy in endothelial or tumor cells.

The following data will be repeated with purified protein.

Lentivirally expressed Notch1 10-20 decoy inhibits angiogenesis more robustly than Notch1 10-24 decoy.

As previously shown in PCT International Application Publication No. WO/2013/052607, Dll4-specific inhibition by Notch1 1-13 stimulates angiogenic hypersprouting in an in vitro fibrin bead angiogenesis assay, while inhibition of Jag1 by Notch1 10-24 leads to reduced sprouting angiogenesis. While Notch1 10-20 decoy is also Jag1 specific, it also inhibits angiogenesis, and in comparison to Notch1 10-24, Notch1 10-20 inhibited angiogenesis more robustly, most likely

due to its greater secretion. See Figure 8.

Notch1 decoys reduce migration of breast cancer cells in an assay that evaluates metastatic potential.

Notch inhibition by Notch1 1-13, Notch1 10-24, and Notch1 10-20 decoys delays lateral migration of MDA-MB-231 triple negative breast cancer cells in a wound closure assay. See Figure 9.

Lentivirally expressed Notch1 10-20 decoy inhibits migration of tumor cells across endothelial barriers indicating its potential as an anti-metastatic Jagged-specific Notch inhibitory therapeutic.

Non-specific Notch inhibition by the gamma secretase inhibitor DAPT and Dll4-specific inhibition by Notch1 1-13 decoy reduced transendothelial migration of MDA-MB-231 breast cancer cells across endothelial barriers, while Notch1 10-24 and Notch1 10-20 decoys robustly inhibited transendothelial migration. See Figure 10.

DISCUSSION

PCT International Application Publication No. WO/2013/052607, the contents of which are incorporated by reference, showed that Dll4-specific inhibition by Notch1 1-13 stimulates angiogenic hypersprouting in an in vitro fibrin bead angiogenesis assay, while inhibition of Jag1 by Notch1 10-24 leads to reduced sprouting angiogenesis. However, these previous decoys had the following limitations:

1. Decoys produced/secreted at low levels, suggesting manufacturing concerns for therapeutic production.
2. Due to difficulty in producing/secreting purified protein, functionality of purified decoys could not be thoroughly evaluated.

The objective of the new decoys was to achieve high production/secretion of decoys while maintaining ligand-specificity, and achieve functionality against angiogenesis, tumor growth, and other aspects of tumor progression such as metastasis.

What inventors have achieved with new decoys: Notch1 10-20 and Notch1

10-18 Decoys

1. Smaller decoys
2. Significantly greater production/secretion
3. Maintained ligand-specificity
4. Notch1 10-20 decoy is Jagged-specific, is produced/secreted at significantly higher levels than Notch1 10-24 decoy, inhibits in vitro angiogenic sprouting, inhibits cancer cell migration, and inhibits transendothelial migration of cancer cells across endothelial barriers.
5. Notch1 10-18 decoy appears to be a pan-ligand inhibitor. We must still evaluate its physiological function.

One major difference between the decoys of this invention and previous Notch inhibitors is that these decoys will specifically target Jagged-Notch or Dll-Notch signaling. Based on preliminary work targeting Jagged-Notch or Dll-Notch signaling, when used as therapeutics, these decoys will exhibit little to no toxic effects, which are seen with other Notch inhibitors.

A major improvement of the decoys of this invention is the fact that the general smaller size is designed to maximize potential to secrete and purify these decoys while still retaining the Notch ligand inhibitory properties. Thus they are a significant improvement on previously evaluated decoys, such as decoys comprising EGF-like repeats 10-24, 1-13 or 1-24.

WHAT IS CLAIMED IS:

1. A fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:
   (a) an extracellular domain of a human Notch1 receptor protein, followed by
   (b) an Fc portion of an antibody,
   wherein the extracellular domain of the human Notch1 receptor protein
   (i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and
   (ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

2. A fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:
   (a) an extracellular domain of a human Notch1 receptor protein, followed by
   (b) an Fc portion of an antibody,
   wherein the extracellular domain of the human Notch1 receptor protein
   (i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and
   (ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

3. A fusion protein, the sequence of which, commencing at the N-terminus of the fusion protein, is identical to the sequence of amino acids in:
   (a) an extracellular domain of a human Notch1 receptor protein, followed by
   (b) a linker sequence, followed by
   (c) an Fc portion of an antibody,
   wherein the extracellular domain of the human Notch1 receptor protein
   (i) commences with the amino acid present at the N-terminus of

EGF-like repeat 10 and

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

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

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

(b) a linker sequence, followed by

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

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

(a) a signal peptide, followed by

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

(c) an Fc portion of an antibody,

wherein the extracellular domain of the human Notch1 receptor protein

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

(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 18.

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

(a) a signal peptide, followed by
(b) an extracellular domain of a human Notch1 receptor protein, followed by
(c) an Fc portion of an antibody,
wherein the extracellular domain of the human Notch1 receptor protein
(i) commences with the amino acid present at the N-terminus of EGF-like repeat 10 and
(ii) extends to and includes the C-terminal amino acid of EGF-like repeat 20.

8. The fusion protein of claim 6 or 7, wherein the signal peptide is the signal peptide of human Notch1 protein or the signal peptide of an IgG heavy chain.

9. A composition comprising the fusion protein of any one of claims 1-5 and a pharmaceutically acceptable carrier.

10. The composition of claim 9, wherein the fusion protein is present in an amount effective to inhibit Jagged-Notch signaling.

11. The composition of claim 9, wherein the extracellular domain of the human Notch1 receptor protein of the fusion protein extends to and includes the C-terminal amino acid of EGF-like repeat 18 and wherein the fusion protein is present in an amount effective to inhibit Delta-like-Notch signaling.

12. A method of treating a subject suffering from age-related macular degeneration (AMD) which comprises administering to the subject the composition of any of claims 7-10 in an amount effective to treat the subject's AMD.

13. The method of claim 12, wherein the AMD is wet AMD.

14. The method of claim 12, wherein the AMD is dry AMD.

15. A method of treating a subject suffering from diabetic retinopathy which comprises administering to the subject the

composition of any of claims 9-11 in an amount effective to treat the subject's diabetic retinopathy.

16. The method of any one of claims 12-15, further comprising administering an inhibitor of Vascular Endothelial Growth Factor (VEGF).

17. The method of claim 16, wherein the inhibitor of VEGF is an inhibitor of VEGF-A, PGIF, VEGF-B, VEGF-C, or VEGF-D.

18. The method of any one of claims 12-15, further comprising administering a VEGF receptor inhibitor.

19. The method of claim 86, wherein the VEGF receptor inhibitor is a VEGFR-1 or a VEGFR-2 inhibitor.

20. A method of treating a subject suffering from cancer which comprises administering to the subject the composition of any of claims 9-11 in an amount effective to treat the subject's cancer.

21. The method of claim 20, wherein the cancer is pancreatic cancer.

22. The method of claim 20, wherein the cancer is breast cancer.

Figure 1:

Human NOTCH1 Protein Sequence (SEQ ID NO:1)

SP EGF-Like Repeat 1

1 MPPLLAPLLC LALLPALAAR GPRCSQPGET CLNGGKCEAA NGTEACVCGG AFVGPRCQDP

2 3

61 NPCLSTPCKN AGTCHVVDRR GVADYACSCA LGFSGPLCLT PLDNACLTNP CRNGGTCDLL

4

121 TLTEYKCRCP PGWSGKSCQQ ADPCASNPCA NGGQCLPFEA SYICHCPPSF HGPTCRQDVN

5 6

181 ECGQKPGLCR HGGTCHNEVG SYRCVCRATH TGPNCERPYV PCSPSPCQNG GTCRPTGDVT

7 8

241 HECACLPGFT GQNCEENIDD CPGNNCKNGG ACVDGVNTYN CRCPPEWTGQ YCTEDVDECQ

9

301 LMPNACQNGG TCHNTHGGYN CVCVNGWTGE DCSENIDDCA SAACFHGATC HDRVASFYCE

10 11

361 CPHGRTGLLC HLNDACISNP CNEGSNCDTN PVNGKAICTC PSGYTGPACS QDVDECSLGA

12

421 NPCEHAGKCI NTLGSFECQC LQGYTGPRCE IDVNECVSNP CQNDATCLDQ IGEFQCICMP

13 14

481 GYEGVHCEVN TDECASSPCL HNGRCLDKIN EFQCECPTGF TGHLCQYDVD ECASTPCKNG

15

541 AKCLDGPNTY TCVCTEGYTG THCEVDIDEC DPDPCHYGSC KDGVATFTCL CRPGYTGHHC

16 17

601 ETNINECSSQ PCRHGGTCQD RDNAYLCFCL KGTTGPNCEI NLDDCASSPC DSGTCLDKID

18 19

661 GYECACEPGY TGSMCNINID ECAGNPCHNG GTCEDGINGF TCRCPEGYHD PTCLSEVNEC

20

721 NSNPCVHGAC RDSLNGYKCD CDPGWSGTNC DINNNECESN PCVNGGTCKD MTSGYVCTCR

21 22

Figure 1 Continued

781 EGFSGPNCQT NINECASNPC LNQGTCIDDV AGYKCNCLLP YTGATCEVVL APCAPSPCRN

23

841 GGECRQSEDY ESFSCVCPTG WQGQTCEVDI NECVLSPCRH GASCQNTHGG YRCHCQAGYS

24 25

901 GRNCETDIDD CRPNPCHNGG SCTDGINTAF CDCLPGFRGT FCEEDINECA SDPCRNGANC

26

961 TDCVDSYTCT CPAGFSGIHC ENNTPDCTES SCFNGGTCVD GINSFTCLCP PGFTGSYCQH

27 28

1021 DVNECDSQPC LHGGTCQDGC GSYRCTCPQG YTGPNCQNLV HWCDSSPCKN GGKCWQTHTQ

29

1081 YRCECPSGWT GLYCDVPSVS CEVAAQRQGV DVARLCQHGG LCVDAGNTHH CRCQAGYTGS

30 31

1141 YCEDLVDECS PSPCQNGATC TDYLGGYSCK CVAGYHGVNC SEEIDECLSH PCQNGGTCLD

32

1201 LPNTYKCSCP RGTQGVHCEI NVDDCNPPVD PVSRSPKCFN NGTCVDQVGG YSCTCPPGFV

33 34

1261 GERCEGDVNE CLSNPCDARG TQNCVQRVND FHCECRAGHT GRRCESVING CKGKPCKNGG

35

1321 TCAVASNTAR GFICKCPAGF EGATCENDAR TCGSLRCLNG GTCISGPRSP TCLCLGPFTG

36 |

1381 PECQFPASSP CLGGNFCYNQ GTCEPTSESP FYRCLCPAKF NGLLCHILDY SFGGGAGRDI

LNR 1 2

1441 PPPLIEEACE LPECQEDAGN KVCSLQCNNH ACGWDGGDCS LNFNDPWKNC TQSLQCWKYF

3

1501 SDGHCDSQCN SAGCLFDGFD CQRAEGQCNP LYDQYCKDHF SDGHCDQGCN SAECEWDGLD

|

1561 CAEHVPERLA AGTLVVVVLM PPEQLRNSSF HFLRELSRVL HTNVVFKRDA HGQQMIFPYY

S1

Figure 1 Continued

1621 GREEELRKHP IKRAAEGWAA PDALLGQVKA SLLPGGSEGG RRRRELDPMD VRGSIVYLEI

S2 TM

1681 DNPQCVQASS QCFQSATDVA AFLGALASLG SLNIPYKIEA VQSETVEPPP PAQLHFMYVA

S3

1741 AAAFVLLFFV GCGVLLSRKR RRQHGQLWFP EGFKVSEASK KKRREPLGED SVGLKPLKNA

1801 SDGALMDDNQ NEWGDEDLET KKFRFEEPVV LPDLDDQTDH RQWTQQHLDA ADLRMSAMAP

1861 TPPQGEVDAD CMDVNVRGPD GFTPLMIASC SGGGLETGNS EEEEDAPAVI SDFIYQGASL

ANK Repeat 1

1921 HNQTDRTGET ALHLAARYSR SDAAKRLLEA SADANIQDNM GRTPLHAAVS ADAQGVFQIL

1981 IRNRATDLDA RMHDGTTPLI LAARLAVEGM LEDLINSHAD VNAVDDLGKS ALHWAAAVNN

2041 VDAAVVLLKN GANKDMQNNR EETPLFLAAR EGSYETAKVL LDHFANRDIT DHMDRLPRDI

2101 AQERMHHDIV RLLDEYNLVR SPQLHGAPLG GTPTLSPPLC SPNGYLGSLK PGVQGKKVRK

2161 PSSKGLACGS KEAKDLKARR KKSQDGKGCL LDSSGMLSPV DSLESPHGYL SDVASPPLLP

2221 SPFQQSPSVP LNHLPGMPDT HLGIGHLNVA AKPEMAALGG GGRLAFETGP PRLSHLPVAS

2281 GTSTVLGSSS GGALNFTVGG STSLNGQCEW LSRLQSGMVP NQYNPLRGSV APGPLSTQAP

2341 SLQHGMVGPL HSSLAASALS QMMSYQGLPS TRLATQPHLV QTQQVQPQNL QMQQQNLQPA

2401 NIQQQQSLQP PPPPPQPHLG VSSAASGHLG RSFLSGEPSQ ADVQPLGPSS LAVHTILPQE

PEST

Figure 1 Continued

2461 SPALPTSLPS SLVPPVTAAQ FLIPPSQHSY SSPVDNTPSH QLQVPEHPFL TPSPESPDQW

2521 SSSSPHSNVS DWSEGVSSPP TSMQSQIARI PEAFK

FIGURE 1

Figure 2:

Notch1 Decoy 10-24 (2502)

Human Notch1 signal peptide: 1-69

EGF-like repeats 10-24: 70-1788

Human Fc: 1789-2502

DNA Sequence (SEQ ID NO:2)

```
   1 ATGCCGCCGC TCCTGGCGCC CCTGCTCTGC CTGGCGCTGC TGCCCGCGCT CGCCGCACGA
  61 GGCCCGCGAT GCATCAGCAA CCCCTGTAAC GAGGGCTCCA ACTGCGACAC CAACCCTGTC
 121 AATGGCAAGG CCATCTGCAC CTGCCCCTCG GGGTACACGG GCCCGGCCTG CAGCCAGGAC
 181 GTGGATGAGT GCTCGCTGGG TGCCAACCCC TGCGAGCATG CGGGCAAGTG CATCAACACG
 241 CTGGGCTCCT TCGAGTGCCA GTGTCTGCAG GGCTACACGG GCCCCCGATG CGAGATCGAC
 301 GTCAACGAGT GCGTCTCGAA CCCGTGCCAG AACGACGCCA CCTGCCTGGA CCAGATTGGG
 361 GAGTTCCAGT GCATCTGCAT GCCCGGCTAC GAGGGTGTGC ACTGCGAGGT CAACACAGAC
 421 GAGTGTGCCA GCAGCCCCTG CCTGCACAAT GGCCGCTGCC TGGACAAGAT CAATGAGTTC
 481 CAGTGCGAGT GCCCCACGGG CTTCACTGGG CATCTGTGCC AGTACGATGT GGACGAGTGT
 541 GCCAGCACCC CCTGCAAGAA TGGTGCCAAG TGCCTGGACG GACCCAACAC TTACACCTGT
 601 GTGTGCACGG AAGGGTACAC GGGGACGCAC TGCGAGGTGG ACATCGATGA GTGCGACCCC
 661 GACCCCTGCC ACTACGGCTC CTGCAAGGAC GGCGTCGCCA CCTTCACCTG CCTCTGCCGC
 721 CCAGGCTACA CGGGCCACCA CTGCGAGACC AACATCAACG AGTGCTCCAG CCAGCCCTGC
 781 CGCCACGGGG GCACCTGCCA GGACCGCGAC AACGCCTACC TCTGCTTCTG CCTGAAGGGG
 841 ACCACAGGAC CCAACTGCGA GATCAACCTG GATGACTGTG CCAGCAGCCC CTGCGACTCG
 901 GGCACCTGTC TGGACAAGAT CGATGGCTAC GAGTGTGCCT GTGAGCCGGG CTACACAGGG
 961 AGCATGTGTA ACATCAACAT CGATGAGTGT GCGGGCAACC CCTGCCACAA CGGGGGCACC
1021 TGCGAGGACG GCATCAATGG CTTCACCTGC CGCTGCCCCG AGGGCTACCA CGACCCCACC
1081 TGCCTGTCTG AGGTCAATGA GTGCAACAGC AACCCCTGCG TCCACGGGGC CTGCCGGGAC
1141 AGCCTCAACG GGTACAAGTG CGACTGTGAC CCTGGGTGGA GTGGGACCAA CTGTGACATC
1201 AACAACAATG AGTGTGAATC CAACCCTTGT GTCAACGGCG GCACCTGCAA AGACATGACC
1261 AGTGGCTACG TGTGCACCTG CCGGGAGGGC TTCAGCGGTC CCAACTGCCA GACCAACATC
1321 AACGAGTGTG CGTCCAACCC ATGTCTGAAC CAGGGCACGT GTATTGACGA CGTTGCCGGG
1381 TACAAGTGCA ACTGCCTGCT GCCCTACACA GGTGCCACGT GTGAGGTGGT GCTGGCCCCG
```

Figure 2 Continued

```
1441 TGTGCCCCCA GCCCCTGCAG AAACGGCGGG GAGTGCAGGC AATCCGAGGA CTATGAGAGC
1501 TTCTCCTGTG TCTGCCCCAC GGGCTGGCAA GGGCAGACCT GTGAGGTCGA CATCAACGAG
1561 TGCGTTCTGA GCCCGTGCCG GCACGGCGCA TCCTGCCAGA ACACCCACGG CGGCTACCGC
1621 TGCCACTGCC AGGCCGGCTA CAGTGGGCGC AACTGCGAGA CCGACATCGA CGACTGCCGG
1681 CCCAACCCGT GTCACAACGG GGGCTCCTGC ACAGACGGCA TCAACACGGC CTTCTGCGAC
1741 TGCCTGCCCG GCTTCCGGGG CACTTTCTGT GAGGAGGACA TCAACGAGGA TCTGGGCCCG
1801 GGCGAGCCCA AATCTTGTGA CAAAACTCAC ACATGCCCAC CGTGCCCAGC ACCTGAACTC
1861 CTGGGGGGAC CGTCAGTCTT CCTCTTCCCC CCAAAACCCA AGGACACCCT CATGATCTCC
1921 CGGACCCCTG AGGTCACATG CGTGGTGGTG GACGTGAGCC ACGAAGACCC TGAGGTCAAG
1981 TTCAACTGGT ACGTGGACGG CGTGGAGGTG CATAATGCCA AGACAAAGCC GCGGGAGGAG
2041 CAGTACAACA GCACGTACCG TGTGGTCAGC GTCCTCACCG TCCTGCACCA GGACTGGCTG
2101 AATGGCAAGG AGTACAAGTG CAAGGTCTCC AACAAAGCCC TCCCAGCCCC CATCGAGAAA
2161 ACCATCTCCA AAGCCAAAGG GCAGCCCCGA GAACCACAGG TGTACACCCT GCCCCCATCC
2221 CGGGAGGAGA TGACCAAGAA CCAGGTCAGC CTGACCTGCC TGGTCAAAGG CTTCTATCCC
2281 AGCGACATCG CCGTGGAGTG GGAGAGCAAT GGGCAGCCGG AGAACAACTA CAAGACCACG
2341 CCTCCCGTGC TGGACTCCGA CGGCTCCTTC TTCCTCTACA GCAAGCTCAC CGTGGACAAG
2401 AGCAGGTGGC AGCAGGGGAA CGTCTTCTCA TGCTCCGTGA TGCATGAGGC TCTGCACAAC
2461 CACTACACGC AGAAGAGCCT CTCCCTGTCT CCGGGTAAAT GA
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Protein Sequence (SEQ ID NO:3)

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   1 MPPLLAPLLC LALLPALAAR GPRCISNPCN EGSNCDTNPV NGKAICTCPS GYTGPACSQD
  61 VDECSLGANP CEHAGKCINT LGSFECQCLQ GYTGPRCEID VNECVSNPCQ NDATCLDQIG
 121 EFQCICMPGY EGVHCEVNTD ECASSPCLHN GRCLDKINEF QCECPTGFTG HLCQYDVDEC
 181 ASTPCKNGAK CLDGPNTYTC VCTEGYTGTH CEVDIDECDP DPCHYGSCKD GVATFTCLCR
 241 PGYTGHHCET NINECSSQPC RHGGTCQDRD NAYLCFCLKG TTGPNCEINL DDCASSPCDS
 301 GTCLDKIDGY ECACEPGYTG SMCNINIDEC AGNPCHNGGT CEDGINGFTC RCPEGYHDPT
 361 CLSEVNECNS NPCVHGACRD SLNGYKCDCD PGWSGTNCDI NNNECESNPC VNGGTCKDMT
 421 SGYVCTCREG FSGPNCQTNI NECASNPCLN QGTCIDDVAG YKCNCLLPYT GATCEVVLAP
 481 CAPSPCRNGG ECRQSEDYES FSCVCPTGWQ GQTCEVDINE CVLSPCRHGA SCQNTHGGYR
```

Figure 2 Continued

541 CHCQAGYSGR NCETDIDDCR PNPCHNGGSC TDGINTAFCD CLPGFRGTFC EEDINECLGP

601 GEPKSCDKTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK

661 FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALPAPIEK

721 TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT

781 PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK*

FIGURE 2

Figure 3

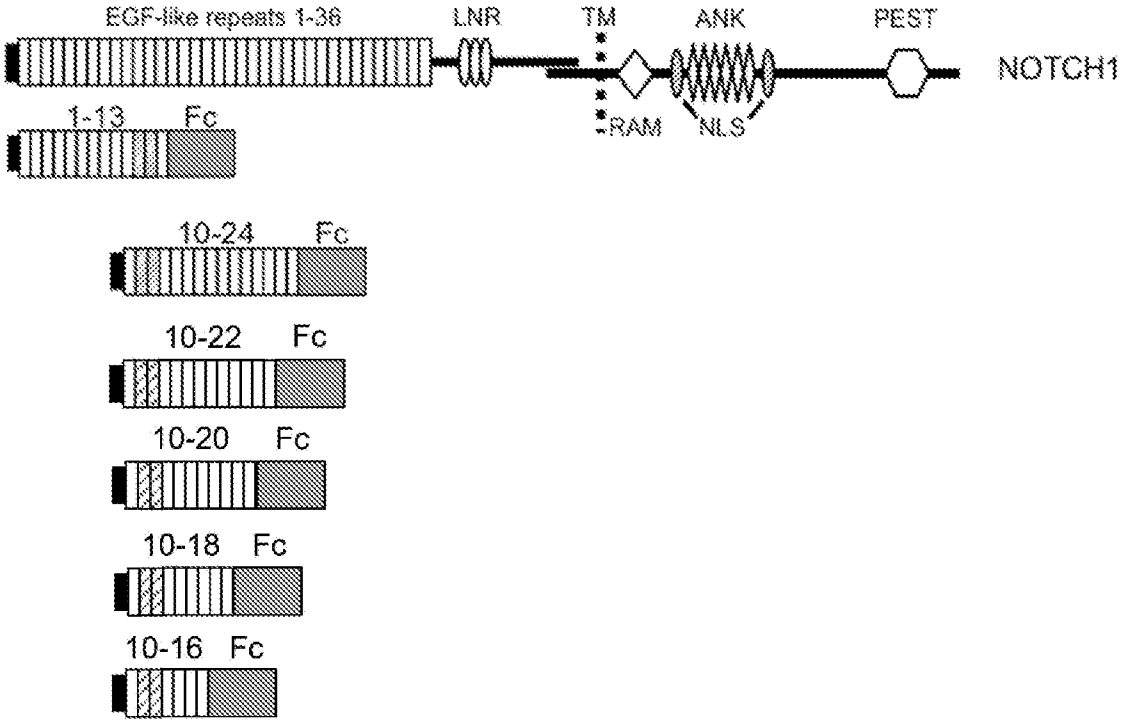


Figure 4

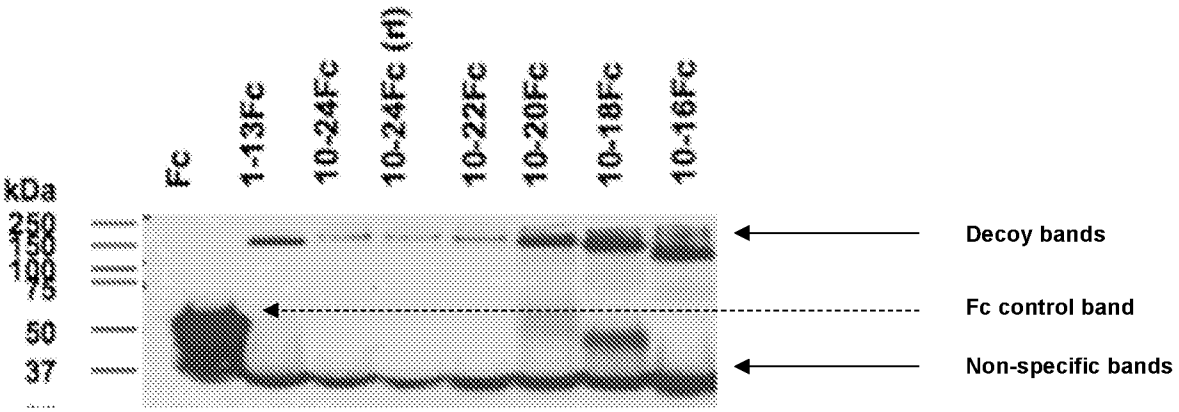


Figure 5

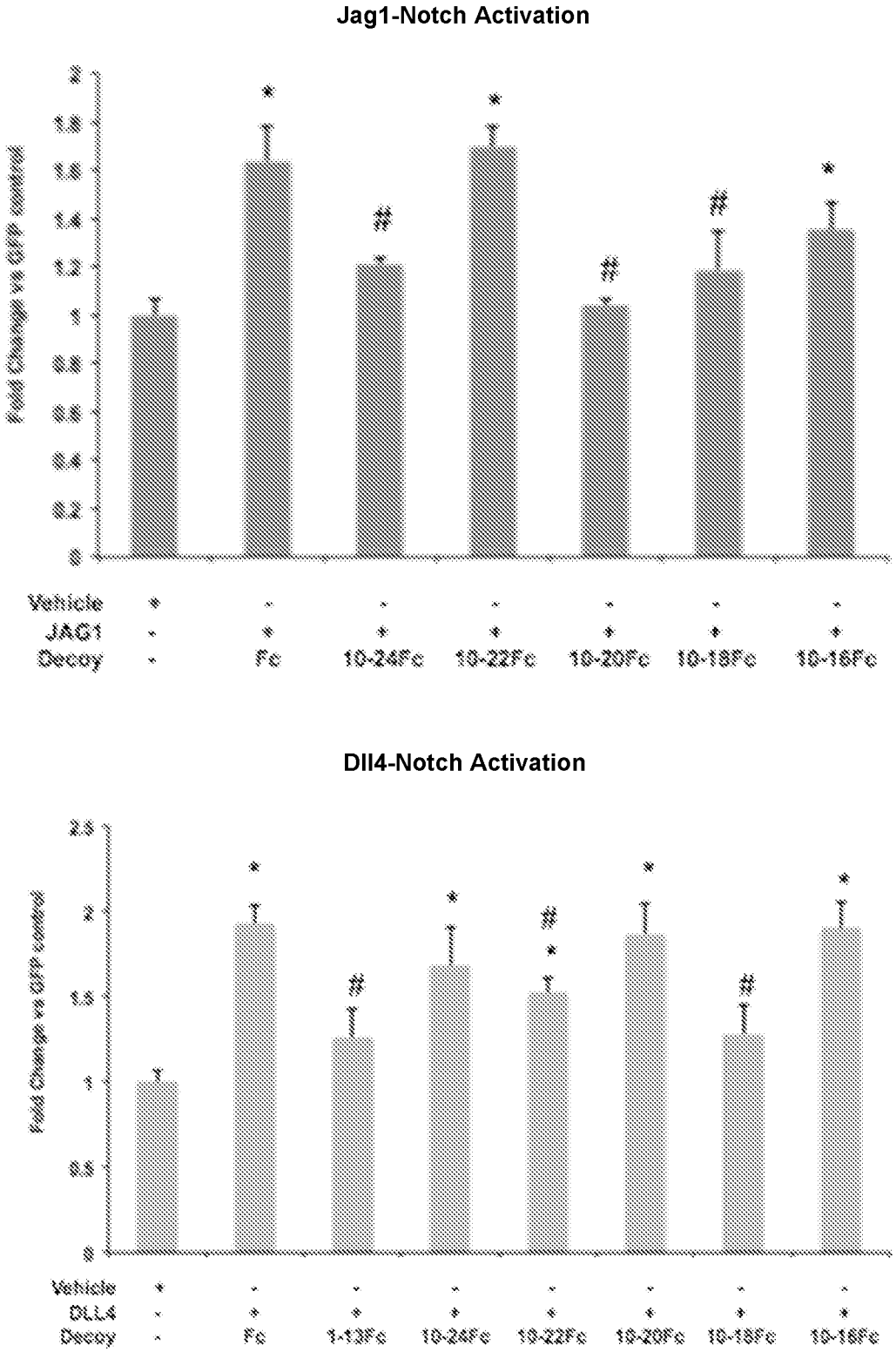

Jag1-Notch Activation
Fold Change vs GFP control
Vehicle
JAG1
Decoy
Fc
10-24Fc
10-22Fc
10-20Fc
10-18Fc
10-16Fc
Dll4-Notch Activation
Fold Change vs GFP control
Vehicle
DLL4
Decoy
Fc
1-13Fc
10-24Fc
10-22Fc
10-20Fc
10-18Fc
10-16Fc

Figure 6

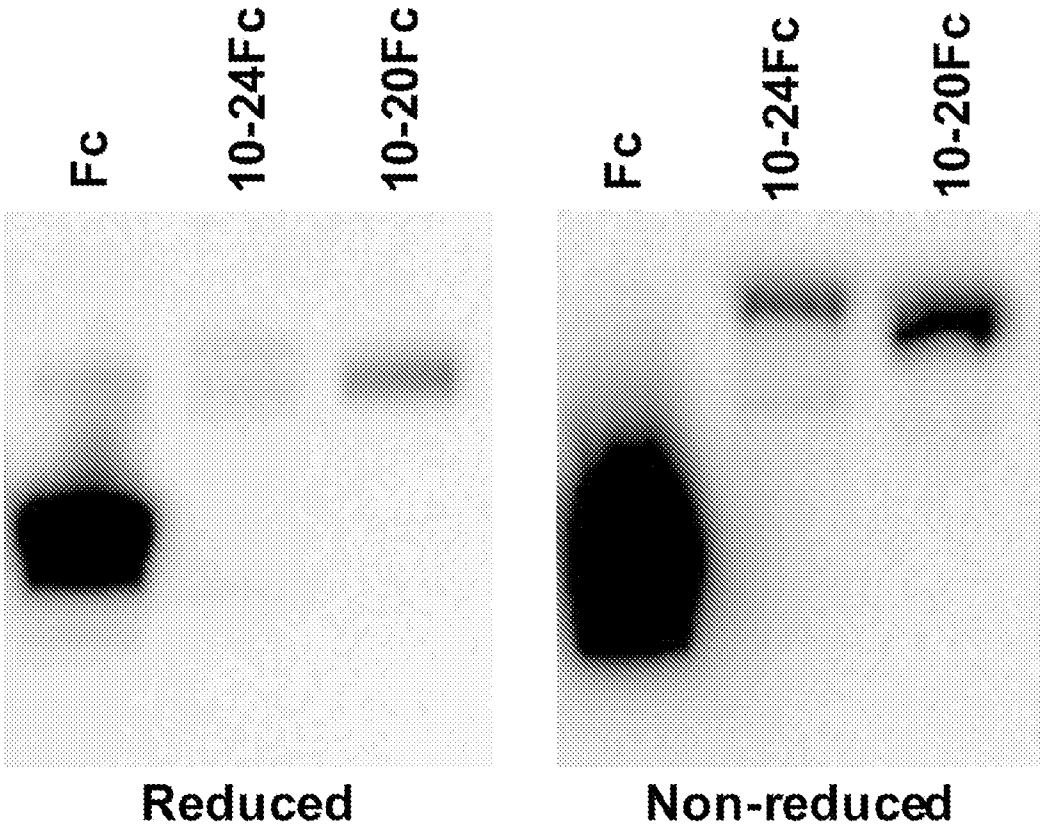
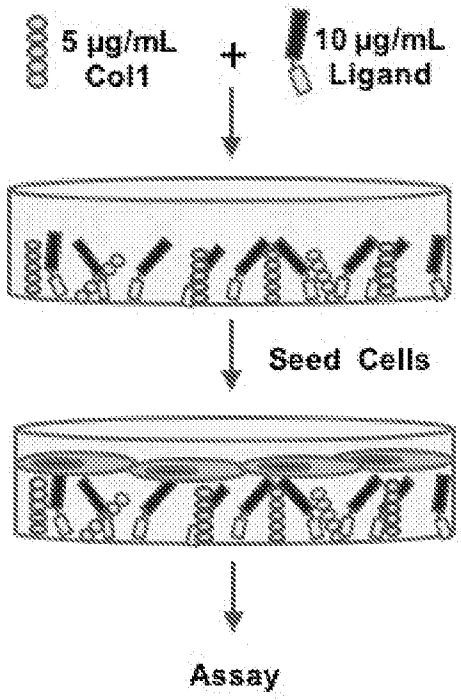


Figure 7


5 µg/mL
Col1
+
10 µg/mL
Ligand
Seed Cells
Assay

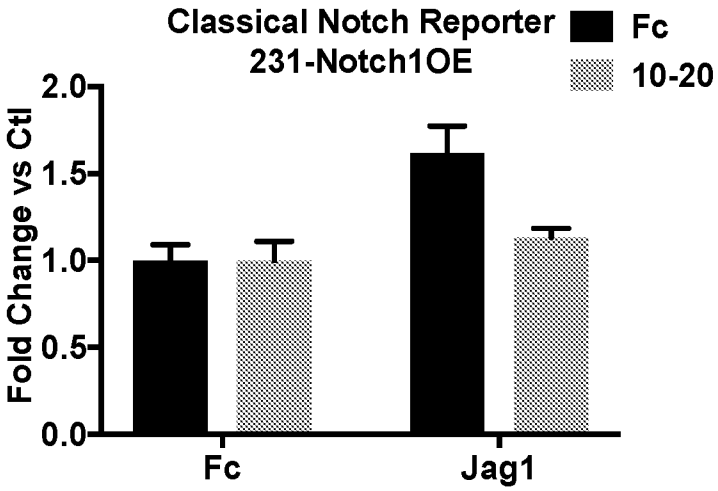

Classical Notch Reporter
231-Notch1OE
Fc
10-20
Fold Change vs Ctl
2.0
1.5
1.0
0.5
0.0
Fc
Jag1

Figure 8

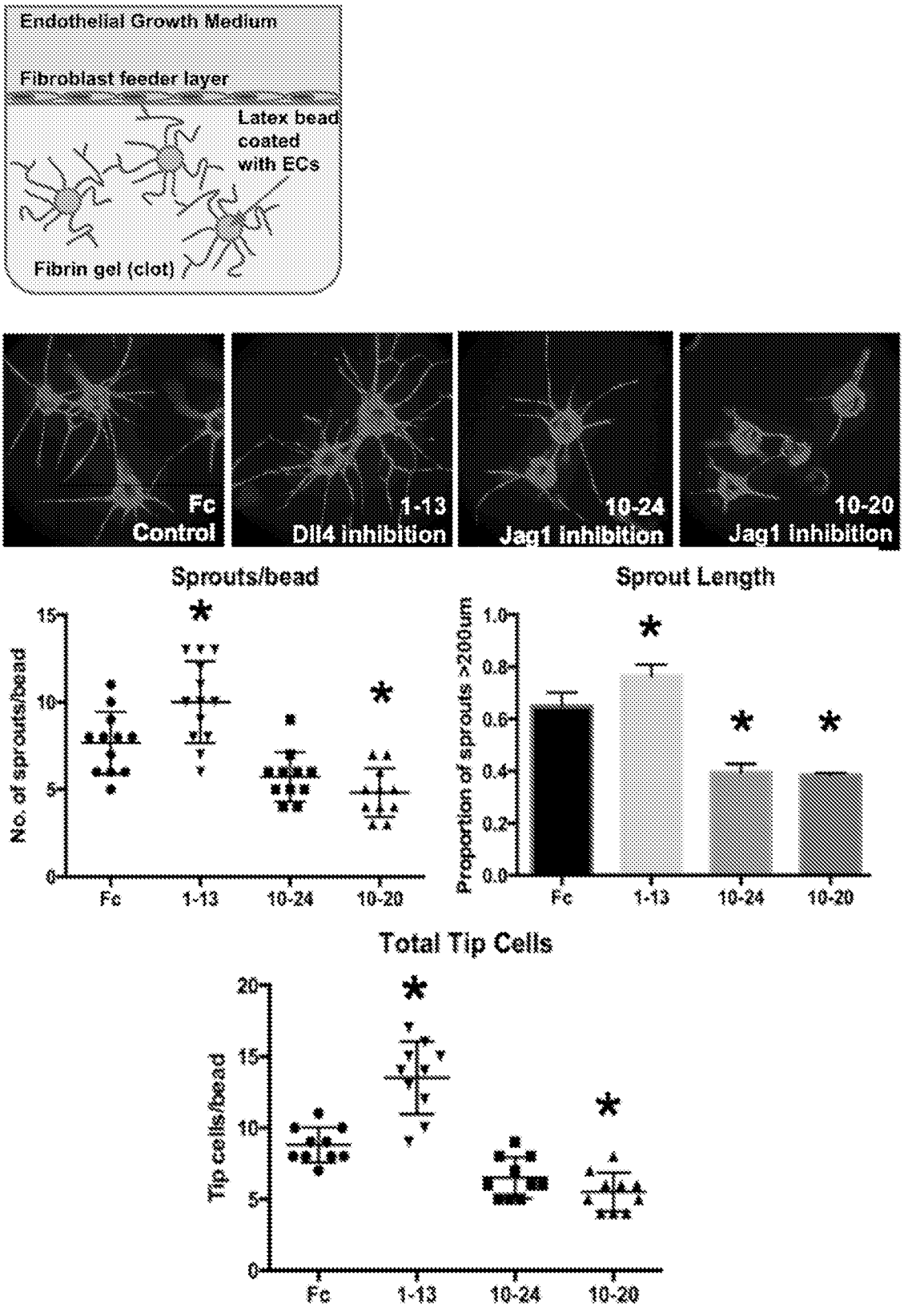
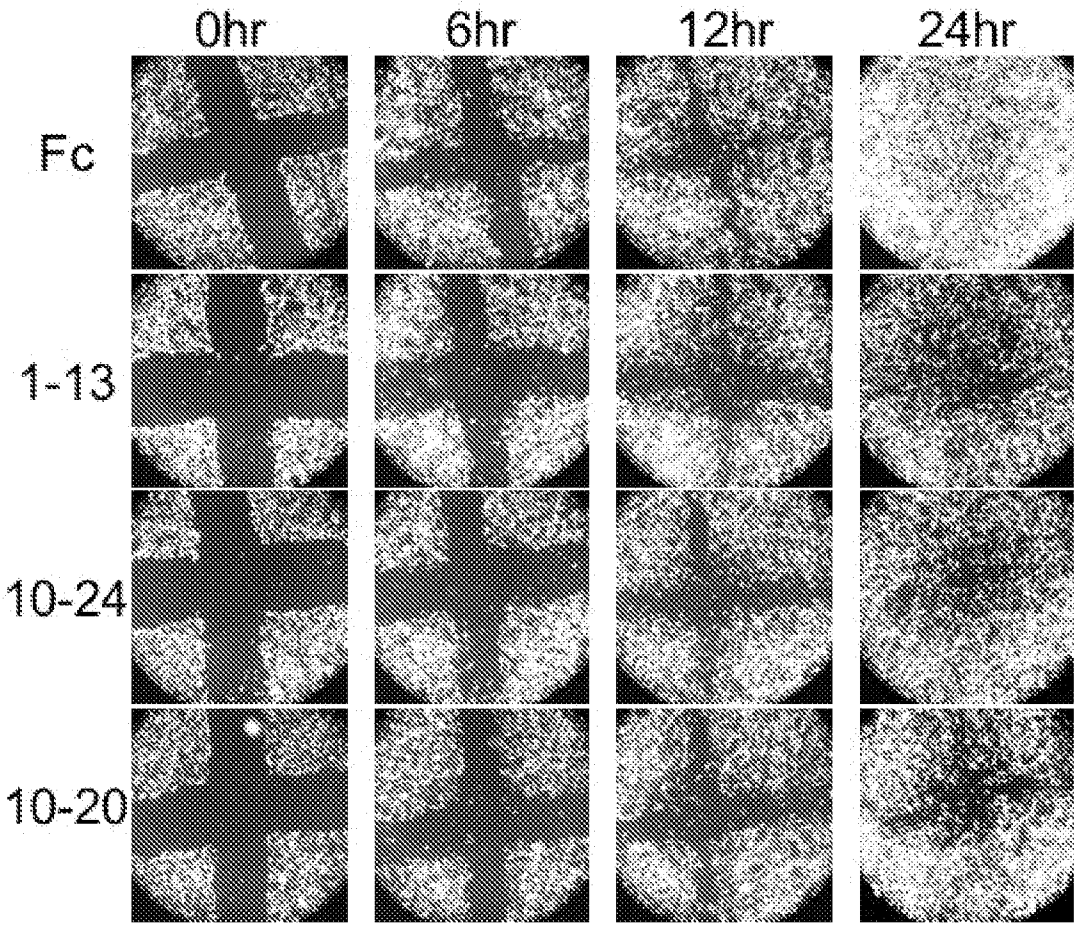

Endothelial Growth Medium
Fibroblast feeder layer
Latex bead coated with ECs
Fibrin gel (clot)
Fc Control
1-13 Dll4 inhibition
10-24 Jag1 inhibition
10-20 Jag1 inhibition
Sprouts/bead
No. of sprouts/bead
Fc
1-13
10-24
10-20
Sprout Length
Proportion of sprouts >200um
Total Tip Cells
Tip cells/bead

Figure 9



DLL4-specific: Notch1 1-13
JAG1-specific: Notch1 10-24
JAG1-specific: Notch1 10-20

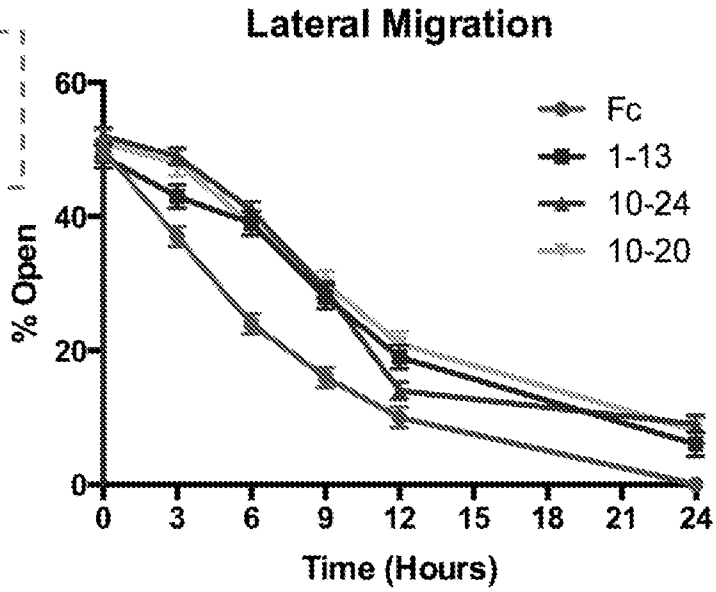
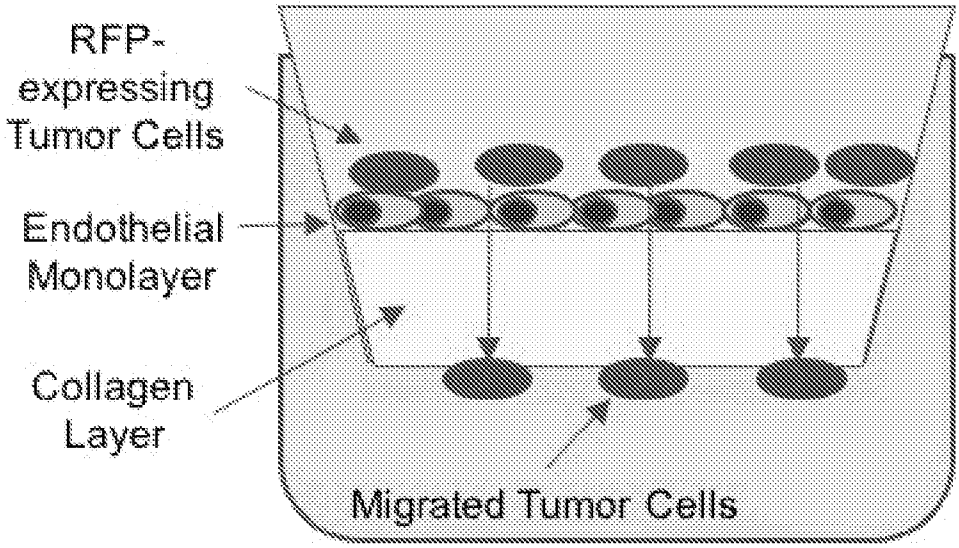
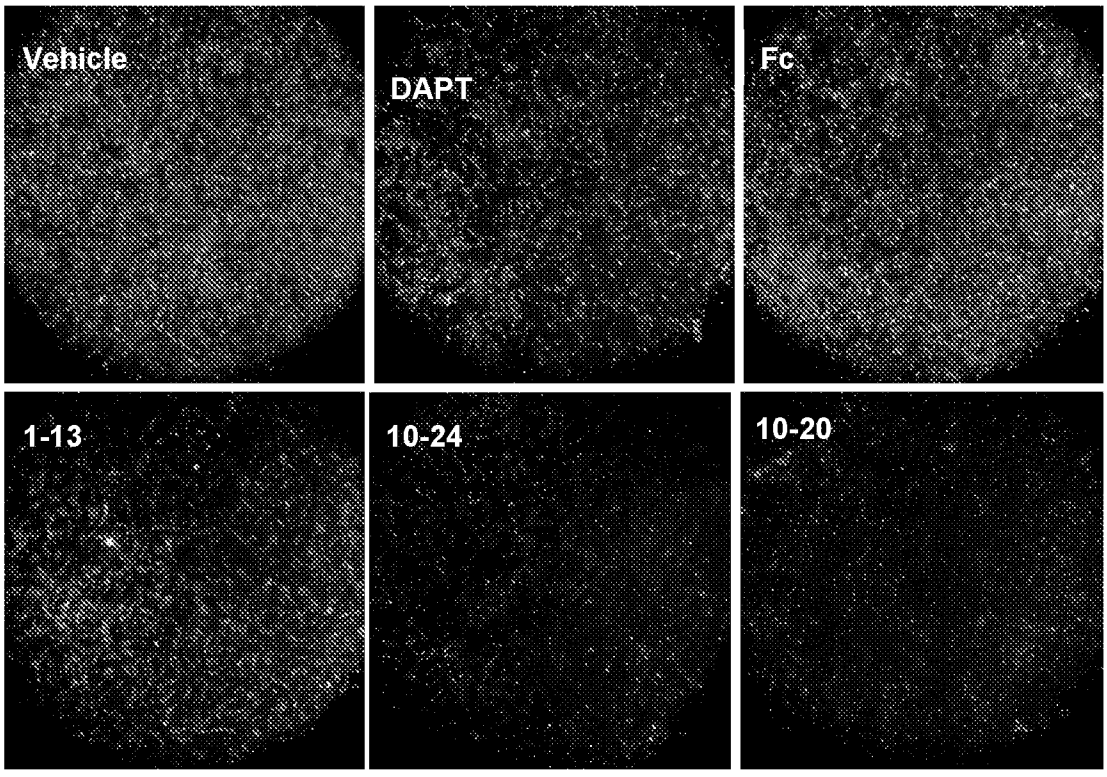


Figure 10





DAPT: Gamma secretase inhibitor
Fc: Decoy Control
DLL4-specific: Notch1 1-13
JAG1-specific: Notch1 10-24
JAG1-specific: Notch1 10-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/034521

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/00; A61K 38/17; C07K 14/705; C07K 19/00 (2017.01)
CPC - A61K 38/00; A61K 38/177; C07K 14/705; C07K 2319/30 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/134.1; 424/178.1; 435/69.1; 514/12; 530/387.3; 536/23.5 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0271643 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 18 September 2014 (18.09.2014) entire document	1, 2, 5-8
X	US 2016/0030513 A1 (PAJVANI et al) 04 February 2016 (04.02.2016) entire document	3, 4
A	US 2005/0261477 A1 (CHAMPION et al) 24 November 2005 (24.11.2005) entire document	1-8
A	US 2016/0115217 A1 (KITAJEWSKI et al) 28 April 2016 (28.04.2016) entire document	1-8
A	US 2015/0329615 A1 (KITAJEWSKI et al) 19 November 2015 (19.11.2015) entire document	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2017	11 AUG 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/034521

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/034521

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.