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## Notch Decoys that Selectively Block DII/Notch or Jagged/Notch Disrupt Angiogenesis by Unique Mechanisms to Inhibit Tumor Growth

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### Abstract

A pro-angiogenic role for Jagged-dependent activation of Notch signaling in the endothelium has yet to be described. Using proteins that encoded different NOTCH1 EGF-like repeats, we identified unique regions of DLL-class and JAG-class ligand/receptor interactions, and developed Notch decoys that function as ligand-specific Notch inhibitors. N1<sub>10-24</sub> decoy blocked JAG1/JAG2-mediated NOTCH1 signaling, angiogenic sprouting *in vitro* and retinal angiogenesis, demonstrating JAG-dependent Notch signal activation promotes angiogenesis. In tumors, N1<sub>10-24</sub> decoy reduced angiogenic sprouting, vessel perfusion, pericyte coverage, and tumor growth. JAG/NOTCH signaling uniquely inhibited expression of anti-angiogenic sVEGFR-1/sFlt-1. N1<sub>1-13</sub> decoy interfered with DLL1/DLL4-mediated NOTCH1 signaling and caused endothelial hypersprouting *in vitro*, in retinal angiogenesis and in tumors. Thus, blockade of JAG- or DLL-mediated Notch signaling inhibits angiogenesis by distinct mechanisms. JAG/Notch signaling positively regulates angiogenesis by suppressing sVEGFR-1/sFlt-1 and promoting mural/endothelial cell interactions. Blockade of JAG-class ligands represents a novel, viable therapeutic approach to block tumor angiogenesis and growth.

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## Keywords

Soluble Notch; Jagged; Delta-like; tumor; angiogenesis

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## INTRODUCTION

Tumor angiogenesis is regulated by a variety of signaling pathways, some of which are validated targets of anti-angiogenic therapies. Molecular and genetic studies reveal that Notch signaling regulates cell fate, proliferation, differentiation, and apoptosis, depending on the cellular context. In the endothelium, Delta-like 4 (Dll4)/Notch signaling suppresses endothelial cell proliferation, migration, and sprouting (1-3). Agents that block gamma-secretase activity, required for Notch signal activation, or that block Dll4, disrupt tumor angiogenesis (4-6) but have toxicities that may limit their utility (5-7).

Notch proteins and their ligands interact on neighboring cells requiring direct cell-cell contact. The highly conserved mammalian Notch gene family encodes transmembrane receptors, Notch1, Notch2, Notch3, and Notch4. The ligands for Notch are transmembrane proteins of two classes: Jagged ligands (Jag), Jag1 and Jag2; and Delta-like ligands (Dll), Dll1, Dll3, and Dll4. Upon ligand activation, a cleaved Notch intracellular domain is released by a gamma-secretase-dependent proteolytic cleavage and transits to the nucleus, where it converts the CSL transcriptional repressor to an activator (8). The human NOTCH1 extracellular domain comprises 36 EGF-like repeats. Notch ligands share a conserved degenerate EGF-like repeat, the DSL domain, which is required for ligand binding to Notch (9,10), followed by an EGF-like repeat region that varies; Jaggeds have 16 EGF-like repeats, and Dlls contain 8 or fewer. Notch EGF-like repeats 11 and 12 and the DSL domain of ligands are necessary for Notch interaction with all ligands (11,12). It is unknown if there are distinct Notch EGF-like repeats that interact with Dll-class versus Jag-class ligands, and this gap in knowledge has limited our understanding of ligand-specific interactions that elicit unique Notch signaling outcomes.

Notch proteins and ligands are upregulated in several cancers and the roles of Notch signaling in tumor cells includes both tumor promoting and suppressing activities, depending on the tumor type (13,14). Previous studies have established a key role for DLL4/Notch signaling in tumor angiogenesis, reviewed in (15). DLL4 blockade inhibits tumor growth by dysregulating tumor angiogenesis, characterized by increased endothelial cell proliferation and a hypersprouted but non-functional tumor vasculature (5,6). DLL4/Notch function in angiogenesis involves an intricate cross-regulation of Notch and VEGF signaling pathways. VEGF induces expression of Notch1, Notch4 and Dll4 (16-18). Notch activation reduces expression of VEGFR-2 but increases expression of VEGFR-1 (19,20). In endothelial cells, VEGFR-3 can be either induced (20,21) or reduced (22) by Notch signaling. In murine retinal angiogenesis, Dll4 and Jag1 have unique activities in endothelium, as loss of function experiments result in distinct phenotypes. Dll4 heterozygotes display angiogenic hypersprouting, while endothelial-specific loss of Jag1 impairs retinal angiogenesis (23,24).

We have created soluble Notch inhibitors consisting of different EGF-like repeats of the NOTCH1 extracellular domain fused to human IgG Fc (NOTCH1 decoy). A human NOTCH1 decoy with all 36 EGF-like repeats functioned similarly to a rat Notch1 decoy that inhibits Jag1, Dll1, and Dll4 (25). We asked whether NOTCH1 decoys that incorporate distinct NOTCH1 EGF-like repeats would differentially antagonize Notch ligand classes. NOTCH1 decoy variants were identified that selectively inhibited DLL-class versus JAG-class Notch ligands, providing the first delineation of ligand-specific interaction domains in human NOTCH1. NOTCH1 decoy variants were evaluated for effects on *in vitro*, retinal, and tumor angiogenesis. A NOTCH1 decoy variant that interferes with JAG1 and JAG2 reduced NOTCH1 signaling, blocked angiogenic growth in retinas and tumors, and reduced tumor growth. Furthermore, JAG blockade specifically increased anti-angiogenic soluble VEGFR-1 (sVEGFR-1/sFlt-1) levels and disrupted pericyte coverage, providing a mechanism by which JAG blockade disrupts tumor growth. A NOTCH1 decoy variant that interfered with DLL1 and DLL4 caused a hypersprouting phenotype, promoted dysfunctional tumor angiogenesis, and inhibited tumor growth.

## RESULTS

### NOTCH1 decoy variants define unique JAG and DLL interaction domains

We developed a human NOTCH1 decoy (N1<sub>1-36</sub> decoy), encoding the 36 extracellular EGF-like repeats of human NOTCH1 fused to human IgG  $\gamma$  heavy chain (Fc) and variants consisting of EGF-like repeats 1-13 (N1<sub>1-13</sub>) or 1-24 (N1<sub>1-24</sub>) (Fig. 1A), and tested their ability to interfere with ligand-specific Notch activation. These N1 decoy variants were secreted from 293T cells into the media (Fig. 1B). The inhibitory activity against specific Notch ligands, DLL4 and JAG1, was assessed using a Notch/CSL reporter assay. HeLa cells expressing either DLL4 or JAG1, and individual N1 decoy variants or Fc, were co-cultured with HeLa cells expressing full-length rat Notch1 and a CSL-Luciferase reporter. Compound E, a gamma-secretase inhibitor (GSI), was used as a control for Notch1 signal inhibition. Expression of N1<sub>1-24</sub> and N1<sub>1-36</sub> decoys significantly blocked both DLL4- and JAG1-induced Notch1 signaling (Fig. 1C and 1D). N1<sub>1-13</sub> decoy inhibited DLL4-induced Notch1 signaling but not JAG1-mediated Notch1 signaling (Fig. 1C and 1D). Thus, N1<sub>1-24</sub> and N1<sub>1-36</sub> decoys acted as pan-ligand inhibitors, blocking both DLL4 and JAG1, whereas N1<sub>1-13</sub> decoy inhibited only DLL4 and was insufficient for blocking JAG1.

Based upon the activity of N1<sub>1-13</sub> decoy, we hypothesized that EGF-like repeats downstream of repeat 13 have a role in productive JAG1/NOTCH1 signaling, and thus created N1<sub>10-24</sub> and N1<sub>10-36</sub> decoy variants (Fig. 1E). It has been demonstrated that EGF-like repeats 11-12 are necessary for functional and physical interaction between Notch and its ligands (11,26). To further assess the importance of EGF-like repeats 11-12, we generated N1 decoys encoding EGF-like repeats 14-24 (N1<sub>14-24</sub>) and 14-36 (N1<sub>14-36</sub>). These N1 decoy variants were secreted from 293T cells (Fig. 1F). We probed for their inhibitory activity against DLL4/Notch1 and JAG1/Notch1 signaling. N1<sub>10-24</sub> and N1<sub>10-36</sub> decoys did not inhibit DLL4-mediated Notch1 signaling, indicating EGF-like repeats 1-9 are required for DLL4/Notch interactions (Fig. 1G). In contrast, N1<sub>10-24</sub> decoy significantly blocked JAG1-induced Notch1 signaling (Fig. 1H). N1<sub>10-36</sub> decoy did not inhibit JAG1, possibly due

to its poor secretion. N1<sub>14-24</sub> and N1<sub>14-36</sub> decoys did not block Notch1 signaling via either DLL4 or JAG1 (Fig. 1G and 1H), demonstrating EGF-like repeats 10-13 are critical for JAG1/Notch interaction. We broadened our analysis to include all known activating Notch ligands. We found that N1<sub>1-13</sub> decoy blocked DLL1/Notch1 signaling (Supplementary Fig. S1A) but not JAG2/Notch1 signaling (Supplementary Fig. S1B). N1<sub>10-24</sub> decoy did not block DLL1/Notch1 signaling (Supplementary Fig. S1C) and blocked JAG2/Notch1 signaling (Supplementary Fig. S1D). N1<sub>1-24</sub> decoy and N1<sub>1-36</sub> inhibited both DLL1 and JAG2 activation of Notch1 (Supplementary Fig. S1A and S1B). Thus, ligand specificity for N1<sub>1-13</sub> decoy and N1<sub>10-24</sub> decoy was also observed with DLL1 and JAG2.

A dose-response analysis of N1<sub>1-13</sub> decoy protein purified from conditioned media produced by Chinese hamster ovary (CHO) cell clones showed that N1<sub>1-13</sub> decoy preferentially inhibited DLL4 activation of Notch1, and only inhibited JAG1 activation of Notch1 at a high dose of 50µg/mL (Supplementary Fig. S2A and S2B). N1<sub>1-24</sub> decoy protein was able to inhibit both DLL4 and JAG1 activation of Notch1 at all concentrations (Supplementary Fig. S2C and S2D). Thus, at high doses, the N1<sub>1-13</sub> decoy was less discriminatory, which is consistent with the role of Notch1 EGF-like repeats 11 and 12 in interactions with Dll and Jagged ligands. We conclude N1<sub>1-13</sub> decoy functions as a dose-dependent, selective DLL-class ligand inhibitor, and N1<sub>10-24</sub> decoy functions as a JAG-class ligand inhibitor.

To confirm binding specificity, N1 decoys and full-length FLAG-tagged Notch ligands, DLL4 and JAG1 were co-expressed in 293T cells and co-immunoprecipitations performed (Fig. 2A). N1<sub>1-13</sub> decoy co-immunoprecipitated with DLL4, but not JAG1. N1<sub>10-24</sub> decoy coimmunoprecipitated with JAG1, but not DLL4. N1<sub>1-24</sub> decoy co-immunoprecipitated with both DLL4 and JAG1. Similar ligand specificity of the decoys was observed when binding assays were done with soluble ligands lacking transmembrane and cytoplasmic domains (Supplementary Fig. S3). Full-length Notch1 did not co-immunoprecipitate with N1 decoys (Fig. 2B), demonstrating that N1 decoys do not bind receptors. We conclude that N1 decoys function to block ligand-specific Notch signaling by competitively binding the extracellular domains of DLL-class or JAG-class ligands.

### NOTCH1 decoy variants have unique effects on angiogenesis in vitro

To determine the angiogenic effects of N1 decoys, we used an *in vitro* angiogenesis assay where HUVEC-coated collagen/dextran beads are embedded in fibrin (27). In response to angiogenic factors secreted by a fibroblast feeder layer, HUVEC sprout from the bead to form branched, lumenized sprouts. The sprouts formed by HUVEC expressing Fc or N1 decoys were evaluated on day 7. In the Fc control, endothelial cell sprouts merged to form multicellular, branched, and lumen-containing vascular networks (Fig. 3A). HUVEC expressing N1<sub>1-13</sub> decoy had a hypersprouting phenotype characterized by increased branch points, as seen by a 76% increase in the number of branch points over control (Fig. 3A and 3B). The N1<sub>1-13</sub> decoy phenotype is consistent with attenuation of DLL4/Notch signaling, as has been shown using an anti-DLL4 antibody (5). In contrast, HUVEC expressing N1<sub>10-24</sub> and N1<sub>1-24</sub> decoys showed reduced network formation compared to control (Fig. 3A and 3B). N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy HUVEC exhibited stunted sprouts and a 40% and 68% decrease in the number of branch points, respectively (Fig. 3B). Thus, JAG blockade

resulted in an anti-angiogenic response, and this effect dominated over DLL inhibition when using the pan-ligand inhibitor, N1<sub>1-24</sub> decoy.

### NOTCH1 decoy variants have unique effects on murine retinal angiogenesis

To determine how ligand-specific Notch inhibition affects developmental angiogenesis, we assessed N1 decoy treatment during murine retinal angiogenesis, where Dll4/Notch function is well understood (2,3). The effects of circulating N1 decoys on target tissues were assessed using injected adenoviruses that expressed N1 decoy proteins. To deliver N1 decoy to the bloodstream, adenovirus vectors expressing N1 decoys or Fc were injected into murine neonates, leading to hepatocyte infection and decoy secretion into circulation. All N1 decoys were detected in serum by western blot analysis at time of retina collection (Supplementary Fig. S4). N1<sub>1-13</sub> decoy significantly increased retinal vascular density (Fig. 3C and 3D), consistent with the increase in tip cells typical of DLL4 inhibition (Fig. 1C, 1D, and 3A). In contrast, N1<sub>10-24</sub> decoy reduced blood vessel density in the retina (Fig. 3C and 3D). N1<sub>1-24</sub> decoy increased retinal vasculature density (Fig. 3C and 3D), indicating that it predominantly functions as a Dll4 antagonist in murine retinal angiogenesis. This is in contrast to the predominant function of N1<sub>1-24</sub> decoy during *in vitro* sprouting, where it acts as JAG antagonist (Fig. 3A and 3B).

Jag1 plays a role in recruitment of vascular smooth muscle cells to arteries (23,24), a role that we evaluated in retinas of mice treated with N1 decoys. A decrease in  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) expressing vascular smooth muscle cell coverage was observed in neonate retinas on the arteries in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy-treated groups (Fig. 3E, quantified in Supplementary Fig. S5A), a phenotype also seen in endothelial-specific *Jag1* mutant mice (23,24). Vascular smooth muscle cell coverage of N1<sub>1-13</sub> decoy-treated group was similar to the Fc-treated group, indicating that, while the effect of N1<sub>1-24</sub> decoy on sprouting represents Dll signaling inhibition, its effect on smooth muscle cell coverage represents Jag signaling inhibition. No significant effects on smooth muscle cell coverage were observed when the N1 decoys were administered to adult mice (Fig. S5B), suggesting that the effect of decoy-mediated inhibition is limited to periods of active angiogenesis.

### Notch1 decoys inhibit tumor growth and angiogenesis by unique JAG- versus DLL-dependent mechanisms

Previous work has shown that Notch inhibition can disrupt tumor growth and angiogenesis (5,6,25,28,29). However, ligand class-specific blockade has yet to be assessed. We hypothesized that the diverse ligand-inhibitory activities of N1 decoy variants would have distinct anti-angiogenic and anti-oncogenic efficacies.

We tested the *in vitro* effects of N1 decoys (N1<sub>1-13</sub>, N1<sub>10-24</sub>, and N1<sub>1-24</sub> decoys) on colony formation, proliferation and apoptosis of four different tumor cell lines, Mm5MT-FGF4 (mouse mammary tumor (25)), KP1-VEGF (human pancreatic tumor (25)), LLC (mouse lung tumor), and B16-F10 (mouse melanoma) tumor cell lines. All N1 decoys significantly inhibited colony formation of Mm5MT-FGF4 cells in a soft agar assay, but not other tumor cell lines (Supplementary Fig. S6A and S6B). Thus, N1 decoys have the potential to inhibit both Mm5MT-FGF4 tumor cells and host cells. N1 decoys did not affect tumor cell

proliferation or apoptosis in any of the tumor lines grown in monolayer cultures (Supplementary Fig. S6C and S6D).

To evaluate N1 decoys in tumors *in vivo*, we performed xenograft studies using the four different tumor cell lines. Our goal was to evaluate N1 decoys as therapeutic proteins. The adenoviral expression system was used to allow N1 decoy proteins to be delivered to tumors, simulating the effects of protein delivery via circulation. Adenoviruses encoding N1 decoys were administered to nude mice 3 days after subcutaneous tumor implantation, and decoy proteins were detected in the serum from 2 days after injection until time of sacrifice at day 21 (Supplementary Fig. S7A). Using a human Fc ELISA, we found varying serum levels of N1 decoys, with the larger N1<sub>1-24</sub> and N1<sub>1-36</sub> decoys secreted into the serum at lower levels than the N1<sub>1-13</sub> and N1<sub>10-24</sub> decoys (Supplementary Fig. S7B). All N1 decoys tested significantly decreased growth of Mm5MT-FGF4, LLC, and B16-F10 tumors; while only N1<sub>10-24</sub> and N1<sub>1-24</sub> decoys inhibited the growth of KP1-VEGF tumors (Fig. 4A).

We sought to determine how blockade of Dll-class versus Jag-class Notch ligands affected tumor angiogenesis, and found that the different N1 decoys had distinct effects. N1<sub>1-13</sub> decoy significantly increased endothelial cell density in all tumor models (Fig. 4B and 4C), similar to that seen with DLL4 blockade (5). In contrast, tumors from the N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy groups had decreased endothelial cell density (Fig. 4B and 4C). In the Mm5MT-FGF4 model, vessel perfusion was determined by lectin perfusion followed by endomucin staining of tumor endothelium. The different N1 decoys all caused reduced tumor perfusion in Mm5MT-FGF4 tumors, with perfusion relative to control decreased 72% (N1<sub>1-13</sub>), 90% (N1<sub>10-24</sub>), and 84% (N1<sub>1-24</sub>) (Fig. 5A). Consistent with poor perfusion, N1 decoy-treated tumors had increased hypoxia (Fig. 5B). Tumor perfusion and hypoxia were quantified (Fig. 5C and 5D) and were found to be significantly different from control. To evaluate vessel regression, tumors were immunostained for endomucin and collagen IV, to detect areas where a collagen sleeve remains after endothelial degeneration. Collagen IV deposition was increased in N1<sub>1-13</sub> decoy treated tumors and reduced in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy tumors (Fig. 5E). When normalized to endomucin staining, however, there was no difference between Fc groups and N1 decoy-treated groups (Fig. 5F), indicating that the reduced tumor vasculature in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy tumors was not due to vessel regression. We conclude that blockade of Dll-class versus Jag-class Notch ligands had opposing effects on tumor vessel density, but uniformly reduced tumor perfusion and increased hypoxia. In tumor angiogenesis, N1<sub>1-24</sub> decoy behaved as a Jag-class inhibitor, consistent with the results when assessed *in vitro* (Fig. 3A and 3B).

As N1 decoys affected mural cell coverage during retinal angiogenesis (Fig. 3E), we evaluated mural cell coverage of N1 decoy-treated Mm5MT-FGF4 tumor vasculature, which is rich in NG2+ pericytes. Tumor sections were immunostained for endomucin and NG2 or  $\alpha$ SMA to visualize pericytes and vascular smooth muscle cells, respectively. Relative to the Fc group, N1<sub>1-13</sub> decoy did not alter pericyte-endothelial association and pericyte vascular coverage was unchanged (Fig. 5G and 5H). In Fc tumors, NG2-positive pericytes were closely associated with endothelial cells (Fig. 5H). In contrast, pericytes were disassociated from endothelial cells in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy-treated tumors (Fig. 5H). N1<sub>1-13</sub> decoy-treated tumors showed increased overall presence of NG2-positive cells, while N1<sub>10-24</sub> and



N1<sub>1-24</sub> decoy-treated tumors showed decreased overall NG2-positive cells (Supplementary Fig. S8A). When normalized to endothelial content, N1<sub>10-24</sub> decoy-treated tumors alone displayed reduced pericyte vascular coverage relative to control (Fig. 5G). Assessment of larger vessels in the tumors revealed reduced vascular smooth muscle  $\alpha$ SMA immunostaining in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy-treated tumors (Supplementary Fig. S8B and S8C). Thus, in tumor angiogenesis, Dll-class inhibition had no apparent effect on vascular mural cells, while blocking Jag-class ligands via N1<sub>10-24</sub> decoy resulted in defective pericyte and vascular smooth muscle cell coverage. N1<sub>1-24</sub> decoy resulted in defective mural cell coverage of tumor vessels, but did not reduce pericyte coverage, thus incompletely mimicking N1<sub>10-24</sub> decoy effects in tumor vasculature.

### JAG and DLL differentially regulate sVEGFR-1/sFlt-1

We explored the mechanisms by which DLL- and JAG-specific N1 decoy variants elicited their distinct effects in endothelial cells. HUVECs were infected with lentiviruses encoding Fc, N1<sub>1-13</sub>, N1<sub>10-24</sub>, or N1<sub>1-24</sub> decoys, and the effects on endothelial Notch downstream targets determined. Expression of N1<sub>1-13</sub>, N1<sub>10-24</sub>, and N1<sub>1-24</sub> decoys and J1KD suppressed the expression of HEY1, HEYL and HES1 (Fig. 6A), direct targets of Notch/CSL transactivation (30). Unlike N1 decoys that are able to block DLL-class ligands, neither N1<sub>10-24</sub> decoy nor J1KD reduced HEY2 transcripts (Fig. 6A).

Notch signaling regulates VEGF signaling in endothelial cells, largely through the regulation of VEGF receptor expression (15). Quantitative RT-PCR and FACs was performed to determine the effect of N1 decoys or J1KD on VEGF receptors. In HUVEC, N1<sub>1-13</sub>, N1<sub>10-24</sub>, N1<sub>1-24</sub> decoys and J1KD knockdown increased VEGFR-2 expression (Fig. 6B) and significantly decreased VEGFR-3 expression (Fig. 6C).

Inhibition of DLL-class or JAG-class Notch signaling differentially regulated VEGFR-1 expression. N1<sub>1-13</sub> and N1<sub>1-24</sub> decoys decreased VEGFR-1 transcripts. Conversely, N1<sub>10-24</sub> decoy or J1KD increased VEGFR-1 transcripts (Fig. 6D). However, VEGFR-1 surface expression was not increased in N1<sub>10-24</sub> decoy or J1KD HUVEC (Fig. 6D). VEGFR-1 exists as two splice variant that produce either a transmembrane receptor (VEGFR-1) or a soluble protein (sVEGFR-1/sFlt-1). Using PCR primers specific for sVEGFR-1/sFlt-1 transcripts, we found that N1<sub>10-24</sub> decoy or J1KD significantly increased sVEGFR-1/sFlt-1 transcripts (Fig. 6E). The pan-ligand inhibitor, N1<sub>1-24</sub> decoy, also increased sVEGFR-1/sFlt-1 expression in HUVEC, though to a lesser degree (Fig. 6E). The sVEGFR-1/sFlt-1 splice variant transcript levels was unaffected by DLL-specific N1<sub>1-13</sub> decoy (Fig. 6E). We validated these findings by ELISA using conditioned media from HUVECs expressing different N1 decoys, J1KD or a constitutively signaling Notch1 intracellular domain (N1IC). The level of sVEGFR-1/sFlt-1 in the media was significantly increased with N1<sub>10-24</sub> and N1<sub>1-24</sub> decoys or JAG1 knockdown, and unaffected by N1<sub>1-13</sub> decoy or N1IC (Fig. 6F).

This complex regulation of VEGFR-1/sFlt-1 levels by JAG-mediated signaling was also seen in N1 decoy-treated tumors. A significant increase in VEGFR-1/sFlt-1 was observed in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy-treated Mm5MT-FGF4 tumors (Fig. 6G and 6H). A diffuse and nonvascular staining pattern observed in N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy-treated tumors was indicative of increased soluble VEGFR-1/sFlt-1 (Fig. 6G). As sVEGFR-1/sFlt-1 functions as

a competitive antagonist of VEGF/VEGFR-2 signaling, the decrease in tumor angiogenesis we observed in the N1<sub>10-24</sub> and N1<sub>1-24</sub> decoy-treated tumors may arise due to decreased VEGFR-2 signaling.

We investigated the role of sVEGFR-1/sFlt-1 as a downstream effector of JAG-induced Notch signaling. HUVECs were generated with sVEGFR-1/sFlt-1 shRNA (sFlt-1 KD), or scrambled shRNA (Scr), and N1 decoys or Fc and the HUVEC bead sprouting angiogenesis assay performed. N1<sub>1-13</sub> decoy/Scr HUVEC had increased branching relative to control, while N1<sub>1-24</sub> or N1<sub>10-24</sub> decoy/Scr or Fc/J1KD HUVECs had significantly reduced endothelial branching (Fig. 7A and 7B). Co-expression of sFlt-1 shRNA with N1<sub>1-24</sub>, N1<sub>10-24</sub> or J1 shRNA rescued endothelial branching inhibition elicited by Jagged-inhibition (Fig. 7A and 7B). sFlt-1 knockdown consistently suppressed sFlt-1 levels in HUVEC with JAG-inhibitors to basal levels (Fig. 7C). These results demonstrate that the anti-angiogenic effects of JAG-blockade by either N1<sub>1-24</sub> decoy, N1<sub>10-24</sub> decoy or JAG1 shRNA likely result from an increase in sVEGFR-1/sFlt-1 secretion.

### **NOTCH1 decoys elicit limited gastrointestinal, hepatic, and renal toxicity compared to gamma-secretase inhibition**

Intestinal goblet cell hyperplasia occurs in mice treated with GSIs, or combined Notch1/Notch2 blockade (29,31) and represents a potential dose-limiting toxicity of GSI use in the clinic. Our animal studies with N1<sub>1-13</sub>, N1<sub>1-24</sub> or N1<sub>10-24</sub> decoys showed only a modest increase in goblet cell numbers, less than 2-fold, in the intestines of tumor-bearing mice, at the end of the 3-week experiment (Supplementary Fig. S9A and S9B). In contrast, GSI (Compound E) treatment for only 5 days resulted in a 5-fold increase in goblet cells (Supplementary Fig. S9A and S9B). Consistent with this mild intestinal phenotype, weight loss was not observed in N1 decoy variant-expressing, tumor-bearing mice (Supplementary Fig. S9C). These results suggest that N1 decoy expression levels capable of eliciting anti-oncogenic activity do not cause significant intestinal toxicity.

We observed no significant effects on levels of markers of hepatic damage (Supplementary Fig. S10). Histopathological analysis of the livers of mice infected with N1<sub>1-13</sub> decoy-expressing adenovirus demonstrated signs of minor sinusoidal dilation, while treatment with all other N1 decoys had no effect (Supplementary Fig. S11A). No changes in renal histomorphology were appreciable in any of the Fc- or N1 decoy-treated mice (Supplementary Fig. S11B). We conclude that Notch decoys represent a class of alternative Notch-targeting agents for anti-angiogenic therapy with minimal gastrointestinal toxicities.

## **DISCUSSION**

Notch proteins require EGF-like repeats 11 and 12 and calcium ions to interact productively with ligands (26,32). Since the discovery of mammalian Notch1 (33), decades of study have yet to uncover domains of Notch that can distinguish Jag-class versus Dll-class ligand interactions. By fusing different regions of the NOTCH1 extracellular domain to human Fc, we developed NOTCH1 decoy proteins that selectively interact with either JAG- versus DLL-class Notch ligands and thus inhibit productive Notch signaling. Using NOTCH1 decoys, we discovered unique downstream signaling events in endothelial cells dependent



on DLL- versus JAG-mediated Notch signaling. Specifically, DLL blockade reduced total VEGFR-1 levels, whereas JAG blockade selectively increased the soluble splice variant, sVEGFR-1/sFlt-1. The therapeutic potential of the NOTCH1 decoys was assessed in mouse tumor models, focusing on the effects on inhibiting DLL-class, JAG-class or both classes of ligands. We found that all classes of ligand-blockade inhibited tumor growth with minimal intestinal toxicity. Although both DLL-class and JAG-class Notch inhibitors disrupted tumor angiogenesis, they did so by distinct mechanisms. Dll/Notch blockade caused a hypersprouting phenotype resulting in dysfunctional, poorly perfused tumor vessels, whereas Jag/Notch blockade increased sVEGFR-1 levels, reducing tumor angiogenesis and perfusion. Jag/Notch blockade additionally disrupted pericyte association with the tumor endothelium. We conclude that blockade of JAG/NOTCH represents a distinct and novel therapeutic approach to inhibit tumor growth, resulting in reduced tumor angiogenesis and vessel functionality, while being tolerated by mice.

These studies shed new light on Notch signaling in endothelium. We demonstrated that JAG/NOTCH and DLL/NOTCH signaling have overlapping and unique molecular targets in endothelial cells. Pan-ligand N1<sub>1-24</sub> decoy, DLL-specific N1<sub>1-13</sub> decoy, and JAG-specific N1<sub>10-24</sub> decoy all reduced the mRNA levels of Notch targets HEY1, HEYL, HES1, and VEGFR-3, and increased VEGFR-2, suggesting these genes are targets of both DLL/NOTCH and JAG/NOTCH signaling. Analysis of Notch regulation of soluble VEGFR-1/sFlt-1, an anti-angiogenic agent that functions as a decoy receptor for VEGF (34), revealed ligand-specific responses. DLL-specific N1<sub>1-13</sub> decoy reduced expression of VEGFR-1, and this correlated with decreased expression of the Notch target and transcriptional repressor, HEY2. JAG-specific N1<sub>10-24</sub> decoy increased sVEGFR-1/sFlt-1 levels.

Unlike Dll4, the exact role of Jag1 in angiogenesis has been elusive. Endothelial Jag1 has been shown to reduce endothelial Dll4/Notch signaling when Notch is modified by Manic Fringe (24). In support of this model, endothelial-specific loss of Jag1 increased expression of Notch targets Hey1 and Hes1 in the retinal endothelium and reduced retinal vascular density (24). Although consistent with previous studies where loss of endothelial Jag1 reduced retinal angiogenic sprouting (23,24), the mechanism of action we uncovered demonstrates that JAG-class ligands can be positive effectors of endothelial Notch signaling and elicit a pro-angiogenic response. We found that JAG1 activates Notch signaling in endothelial cells to suppress sVEGFR-1/sFlt-1 levels thus promoting angiogenesis. In fact, expression of N1<sub>10-24</sub> decoy suppressed sprouting angiogenesis *in vitro* similar to that observed for JAG1 knockdown, which was reversed by using a sVEGFR-1/sFlt-1 shRNA. This mechanism is consistent with the significant elevation of sVEGFR-1/sFlt-1 levels and reduced vascular density observed in tumors treated with N1<sub>10-24</sub> decoy. Thus, the anti-angiogenic phenotype observed for JAG-specific NOTCH1 decoys in the *in vitro* sprouting assays and tumor xenografts is likely a result of increased levels of sVEGFR-1/sFlt-1.

Our studies do not exclude the possibility that Jag/Notch signaling may be both pro- and anti-angiogenic and this may depend on the modification state of Notch. When Jag1 is an activating ligand, endothelial cells would respond by reducing sVEGFR-1/sFlt-1, whereas if Notch is modified by manic fringe and less responsive to Jag1, increased DLL4/Notch signaling would restrict sprouting and branch point formation. Thus, the particular role of

endothelial Jag1 in angiogenesis is likely context dependent, differing based upon the levels and glycosylation state of endothelial Notch, or the cell type presenting Jag1 to endothelial Notch. Tumor cells that over-express JAG1 have been shown to promote tumor angiogenesis in mice (25,35). Thus, tumor-derived JAG1 could serve as an angiogenic stimulus and may contribute to resistance to VEGF blockade. Selective inhibition of JAG-mediated Notch signaling is thus an important therapeutic approach to prevent tumor- or endothelial derived JAG1 function.

Jag1-specific inhibition disrupted vascular mural cells associated with neonatal retinal and tumor vessels, revealing another role for Jag/Notch signaling in angiogenesis. N1<sub>10-24</sub> decoy reduced vascular smooth muscle cell coverage in arterioles of neonates and disrupted pericyte association with the tumor endothelium. Both observations are consistent with previous studies that showed that Jag1/Notch interactions are required for proper smooth muscle cell differentiation on arteries (23,24). Notch regulates a wide range of signaling molecules that promote endothelial-mural cell interactions (36). Endothelial Jag1 signals to Notch3 in mural cells to promote vascular smooth muscle cell differentiation (37). Pericytes and vascular smooth muscle cells can produce VEGF-A, which is known to promote endothelial cell survival (38). We propose that deregulation of pericyte-endothelial interactions by JAG-blockade contributes to vessel instability observed in tumors. The two major effects of N1<sub>10-24</sub> decoy on tumor vessels, disruption of tumor endothelial pericyte coverage and elevated sVEGFR-1/sFlt-1, has the combined effect of destabilizing tumor vessels and reducing VEGF/VEGFR-2 signaling in tumor endothelium. We propose that this double anti-angiogenic mechanism underlies the potent anti-tumor effect of JAG blockade.

The N1<sub>1-13</sub> decoy represents a therapeutic entity that functions similarly to DLL4 blocking antibodies. Blockade of Dll4/Notch leads to increased endothelial cell proliferation and increased tip cells, ultimately causing incomplete angiogenesis and poor vessel perfusion (5,6,28,39). The DLL-specific N1<sub>1-13</sub> decoy identified EGF-like repeats 1-13 as critical for DLL-class ligand association and proved to function as a tumor inhibitor that resulted in poor vessel perfusion. N1<sub>1-13</sub> decoy caused elevation of VEGFR-2 and a reduction of VEGFR-1, a change that is proposed to underlie the hypersprouting phenotype caused by Dll4 blockade (40). Thus, the angiogenic phenotype of N1<sub>1-13</sub> decoy matched the biochemical activity predicted of a DLL4 inhibitor.

By developing N1 decoys that block both DLL- and JAG-class ligands and N1 decoys selective for each class, we were able to compare the effects of combined DLL and JAG blockade with ligand-class selective blockade. Similar to N1<sub>10-24</sub> decoy, N1<sub>1-24</sub> decoy blocked endothelial network formation during *in vitro* angiogenesis and increased sVEGFR1/sFlt-1 levels in HUVEC, albeit not as strongly as N1<sub>10-24</sub> decoy. However, in retinal angiogenesis treatment with the N1<sub>1-24</sub> decoy displayed a mixed phenotype. N1<sub>1-24</sub> decoy caused hypersprouting consistent with Dll4 blockade, but reduced mural cell coverage consistent with Jag blockade. In tumors, N1<sub>1-24</sub> decoy phenocopied N1<sub>10-24</sub> decoy in four different tumor models, causing reduced tumor vasculature and elevating sVEGFR-1/sFlt-1 in the Mm5MT tumor model. From these studies we conclude that N1<sub>1-24</sub> decoy acts primarily as a JAG inhibitor in the microenvironment of the assayed tumors. Of course, the

phenotype caused by N1<sub>1-24</sub> decoy in any vascular bed, including those of tumors, will be dependent on the presence and activities of different Notch ligands and Fringe proteins.

A major adverse effect of Notch blockade using gamma-secretase inhibitors (31) or combined Notch1/Notch2 blocking antibodies (29) is compromised gastrointestinal function. We found that N1<sub>1-13</sub>, N1<sub>10-24</sub>, N1<sub>1-24</sub> decoys were effective against four different tumor types and induced only minimal goblet cell metaplasia relative to GSI treatment, and were well tolerated by mice for three weeks, in which time GSI treatment could already be seen to cause adverse severe gastrointestinal effects. DLL4 blockade has also been found to have potential adverse events due to the hypersprouting in normal tissue vasculature (7). Consistent with this, we found that mice treated with the Dll-class inhibitor (N1<sub>1-13</sub> decoy) showed signs of minor hepatic angiogenic dysfunction in the form of dilated hepatic sinusoids, though this effect was slight and did not result in significant serum elevation of marker enzymes associated with hepatic damage. Conversely, the Jag-class N1<sub>10-24</sub> decoy and pan-ligand N1<sub>1-24</sub> decoy did not exhibit this effect on hepatic sinusoids. Further, as the JAG class inhibitor blocked angiogenesis, one may anticipate that hypersprouting in normal tissues will likely not occur with N1<sub>10-24</sub> decoy even with longer treatments. By developing a novel set of therapeutic agents that block either DLL or JAG class Notch ligands that are efficacious against tumors and lack severe toxicity in mice, the stage is set to advance Notch decoys for assessment in the clinic.

## MATERIALS AND METHODS

### Endothelial and Cancer Cell Lines

HUVECs isolated from human umbilical veins (IRB-AAAE4646) were grown in EGM-2 Media (Lonza). HeLa, 293T, Mm5MT, LLC, CHO and B16-F10 were obtained in 2008 from American Type Culture Collection (ATCC), and KP1 from Health Science Research Resource Bank in 2005. Cell lines were passaged for less than 5 months following resuscitation and were not authenticated. Cancer cells were maintained in DMEM, 10% fetal bovine serum and 100 U/mL Penicillin-Streptomycin.

### Notch Reporter Assay

pBOS-Notch1, pGL3-11CSL-Luc and Renilla or either pCRIII-JAG1-FLAG, pCRIII-DLL4-FLAG or pCRIII-GFP-FLAG were introduced to HeLa cells with Effectene (Qiagen). 24 hours later, receptor and ligand expressing HeLa cells were co-cultured in a 24-well plate. Luciferase activity was measured 24 hours after co-culture, using the Dual-Luciferase Reporter Assay System (Promega). Assays were performed in triplicate.

### Co-Immunoprecipitation

N1 decoys and full-length DLL4-FLAG or JAG1-FLAG were co-transfected into 293T cells. 24 hours after transfection, 20nmol/ml DSG (Thermo Scientific) was added to the culture, incubated for 30 minutes, and quenched with 10mM Tris for 15 minutes. N1 decoy complexes were pulled down with Protein A/G Agarose and 50μmol/ml DTT was added to purified proteins and boiled for 5 minutes to reverse the crosslink.

### Sprouting Angiogenesis Assay

The fibrin gel sprouting angiogenesis assay has been previously described (27). HUVECs were adhered to Cytodex 3 dextran beads (GE Healthcare Bio-Sciences Corp.) at 400 cells per bead and embedded in a fibrin clot composed of 2mg/ml fibrinogen, 0.15U/ml aprotinin, and 0.0625U/ml thrombin (Sigma–Aldrich) in a 24-well plate. After one hour, Detroit 551 fibroblasts (ATCC) were seeded on top of the fibrin gel and cultures allowed to grow for 7 days. Experiments were performed in triplicate.

### Mouse Husbandry

C57BL/6 mice and NCr-nude mice were purchased from NCI-Frederick and maintained in the barrier facility at the Irving Cancer Research Center at Columbia University. All mice studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Columbia University (Protocols: AC-AAAB8961 and AC-AAAD3700).

### Retinal Analysis

C57BL/6 mice postnatal day 2 (P2) pups were subcutaneously injected with  $2.5 \times 10^8$  ffu adenoviruses (Ad) encoding N1 decoys or Fc. Eyes were isolated at P5 and fixed in 4%PFA. For adults,  $5 \times 10^8$  ffu N1 decoy adenovirus was administered via retro-orbital intravenous injection to 4-6 week old female NCr-nude mice and eyes were isolated 21 days later. Dissected Retinas were permeabilized with 1%BSA and 0.5% Triton X-100 in 1XPBS at room temperature for 2 hours and subsequently washed 3 times in PBLEC buffer (1% Triton X-100, 0.1mM MgCl<sub>2</sub>, 0.1mM CaCl<sub>2</sub>, 0.1mM MnCl<sub>2</sub> in 1XPBS pH6.8). For immunofluorescence, retinas were incubated with Biotin-conjugated isolectin B4 (Vector Laboratories) detected with streptavidinconjugated Alexa Fluor 488 (Invitrogen), and Cy3-conjugated  $\alpha$ SMA (Sigma Aldrich) washed with PBLEC, post-fixed with 4%PFA, and mounted. Images were acquired using laser scanning confocal Zeiss LSM 510 Meta microscope and LSM software.

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

RNA was collected RNeasy Mini Kit (Qiagen), treated with DNase I for 30 minutes and cDNA synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR reactions were done with Absolute Blue QPCR SYBR Green Mix (Thermo Scientific).

### Tumor Experiment

$1.0 \times 10^5$  Mm5MT-FGF4 cells,  $2.0 \times 10^6$  KP1-VEGF cells,  $5.0 \times 10^5$  LLC or  $5.0 \times 10^5$  B16-F10 cells were subcutaneously implanted into the upper flank of 4-6 week-old female NCr-nude mice. 3 days later,  $5 \times 10^8$  ffu N1 decoy adenovirus was administered via retro-orbital intravenous injection. Tumors were harvested and weighed at day 21. To measure hypoxia, 60mg/kg hypoxyprom-1 (Hypoxyprom, Inc) was injected intraperitoneally 30 minutes prior to sacrifice and sections immunostained with an anti-hypoxyprom antibody. To assess vessel perfusion, 100 $\mu$ g fluorescein *Lycopersicon esculentum* lectin (Vector

Laboratories) was intracardiac injected into the left ventricle, and mice were perfused with 1%PFA after 2 minutes. Lectin bound to the endomucin-positive endothelial cell surface was scored as a perfused vessel.

### Tissue analysis in mice expressing N1 decoys

For intestinal toxicity, duodena were harvested at time of tumor harvest. For renal and liver toxicity, adenovirus was retro-orbitally administered to 4-6 week old NCr-nude mice and serum and tissue were harvested after 21 days. Duodena and kidney sections were Periodic Acid-Schiff (PAS) stained. Liver sections were stained by hematoxylin and eosin. Serum was sent for liver panel analysis to ANTECH Diagnostics (New York, NY). Statistical analyses were performed using non-parametric T test or one-way ANOVA, as indicated.

### Immunofluorescent Staining

Fresh-frozen 7µm tumor sections were incubated with primary antibodies: endomucin (Santa Cruz Biotechnology), NG2 (Millipore), αSMA (Sigma Aldrich), Collagen IV (COSMO bio), VEGFR-1 (Abcam). Alexa Flour conjugated 588 or 594 secondary antibody (Invitrogen) were used and slides mounted with Vectashield with DAPI (Vector Laboratories).

### Enzyme-linked immunosorbent assay (ELISA)/Fluorescence-activated cell sorting (FACS)

sVEGFR-1 ELISA was performed with Human sVEGFR-1/Flt-1 Quantikine ELISA Kit (R&D Systems). VEGFR-1/2/3 FACS was performed with Monoclonal Anti-Human VEGF Receptor Phycoerythrin Sampler Pack (R&D systems).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### ACKNOWLEDGEMENTS

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**STATEMENT OF SIGNIFICANCE**

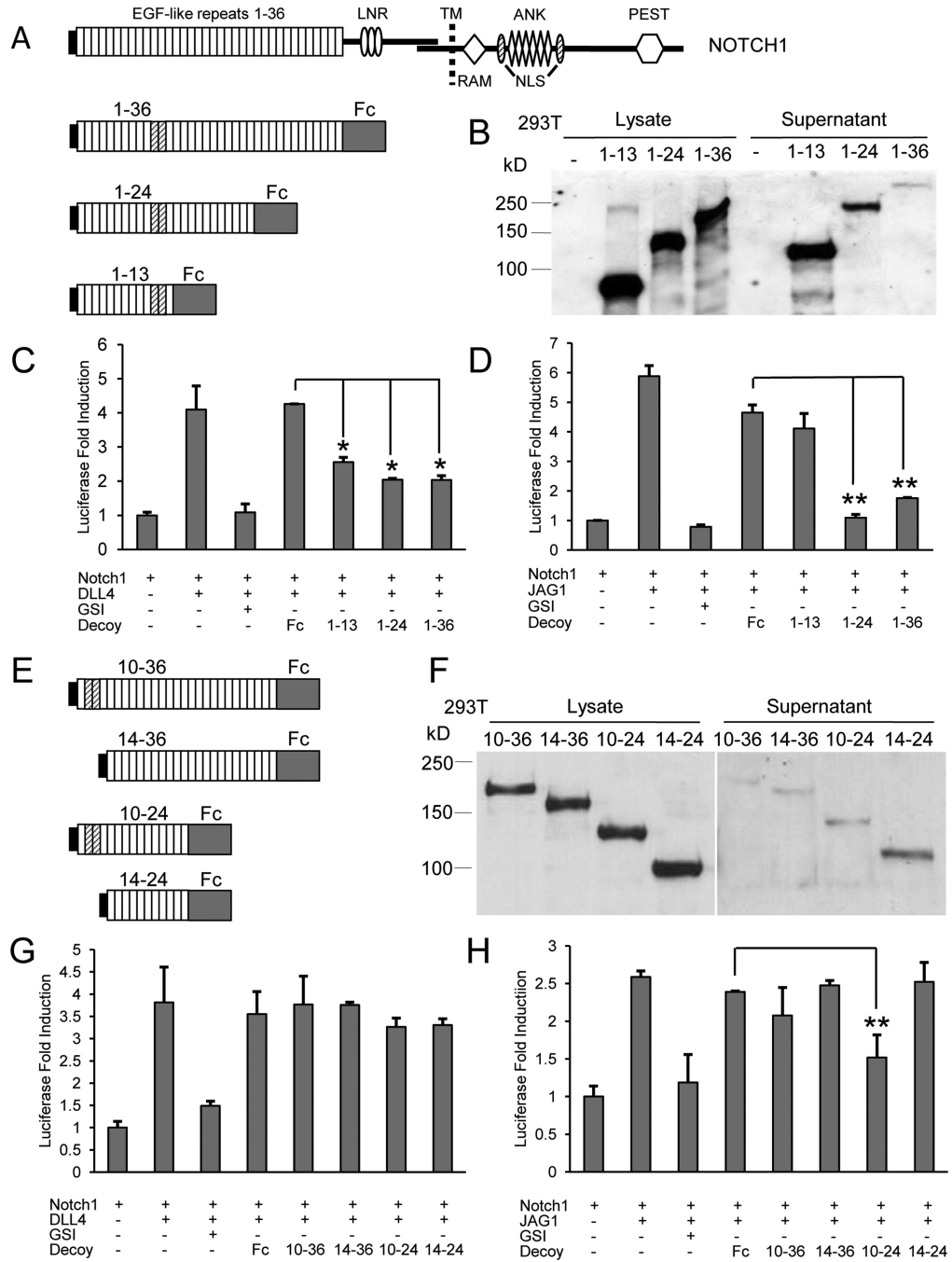
This is the first report identifying unique regions of the NOTCH1 extracellular domain that interact with JAG-class and DLL-class ligands. Using this knowledge, we developed therapeutic agents that block JAG-dependent Notch signaling and demonstrate for the first time that JAG blockade inhibits experimental tumor growth by targeting tumor angiogenesis.

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**Figure 1. N1 decoys differently inhibit ligand-induced Notch signaling**

(A and E) Schematic of NOTCH1 decoy variants. (A) NOTCH1 protein: EGF-like repeats 11-12 indicated with shading. EGF: epidermal growth factor, LNR: LIN-12/Notch repeats, TM: transmembrane domain, RAM: CSL interaction domain, ANK: ankyrin repeats, NLS: nuclear location signal, PEST: proline, glutamic acid, serine, threonine rich sequence. EGF-like repeats 1-36 of human NOTCH1 (N1<sub>1-36</sub>), repeats 1-24 (N1<sub>1-24</sub>), or repeats 1-13 (N1<sub>1-13</sub>) are fused in frame with human IgG Fc. (E) EGF-like repeats 10-36 of human NOTCH1 (N1<sub>10-36</sub>), repeats 14-36 (N1<sub>14-36</sub>), repeats 10-24 (N1<sub>10-24</sub>), or repeats 14-24

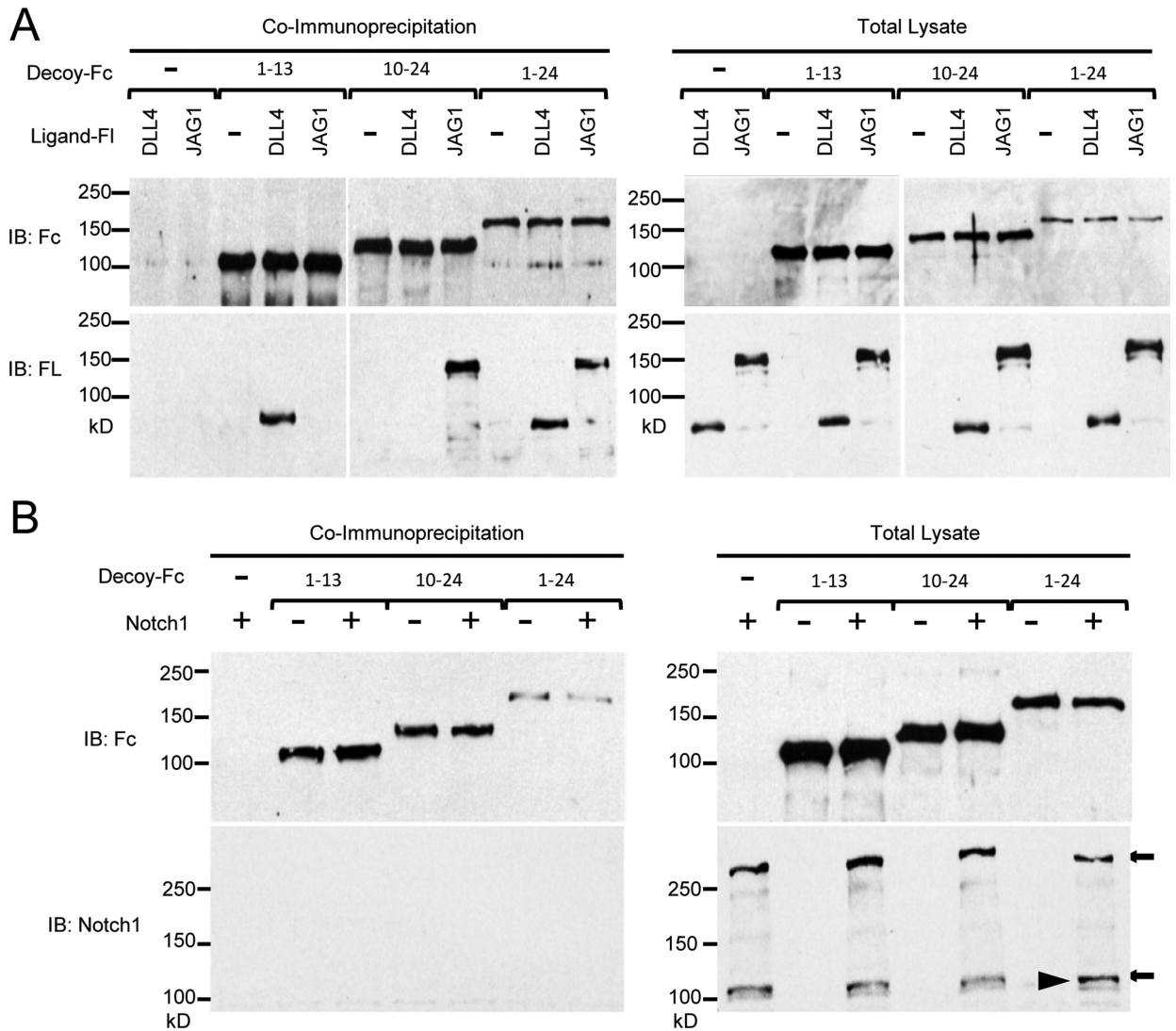
(N1<sub>14-24</sub>) are fused in frame with human IgG Fc. EGF-like repeats 11-12 indicated with shading. (**B** and **F**) N1 decoy variants expressed in 293T cells detected from total cell lysates and supernatant by immunoblotting with an anti-human Fc antibody. (**C**, **D**, **G**, and **H**) Notch signal activation measured in HeLa cells expressing full-length rat Notch1, N1 decoys and 11CSL-Luc co-cultured with HeLa cells expressing Notch ligands. Co-culture assays were performed in triplicate and repeated three times. Mean luciferase fold induction  $\pm$  S.D. \* P value < 0.002. \*\* P value < 0.005.

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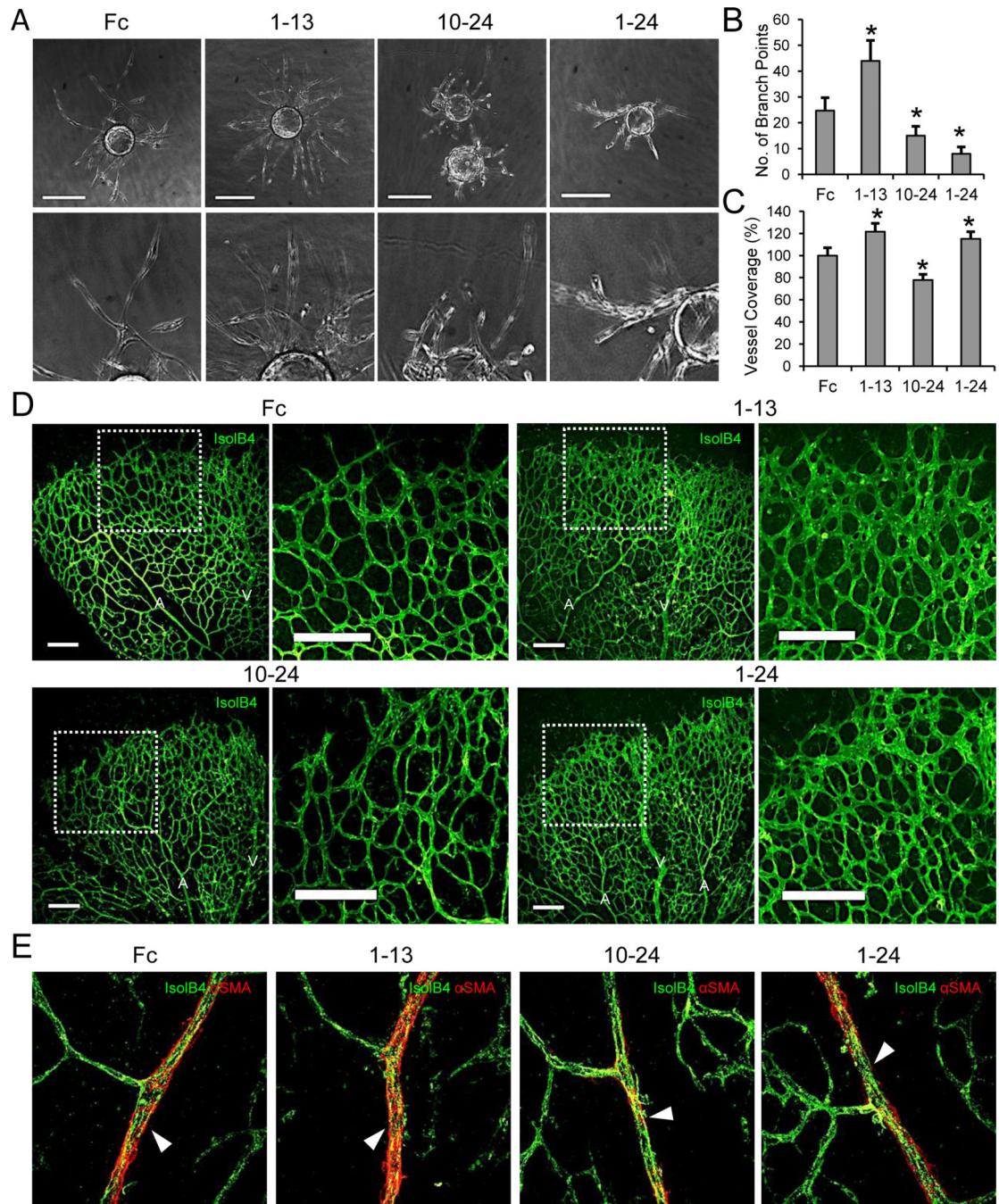
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**Figure 2. N1 decoy variants demonstrate ligand-specific binding**

(A) N1 decoys and Flag tagged DLL4 or JAG1 were co-transfected in 293T cell. Lysates immunoprecipitated with anti-Fc or anti-Flag antibodies or total cell lysates were immunoblotted with anti-Fc or anti-FLAG antibody. (B) N1 decoys and full-length rat *Notch1* were cotransfected in 293T cells. Lysates immunoprecipitated with anti-Fc or anti-NOTCH1 antibodies or total cell lysates were immunoblotted with anti-Fc or anti-NOTCH1 antibody. Anti-NOTCH1 antibody recognizes full-length rat Notch1 and furin-cleaved rat Notch1 (arrows) as well as endogenous furin-cleaved human NOTCH1 (arrowhead). These assays were repeated twice.

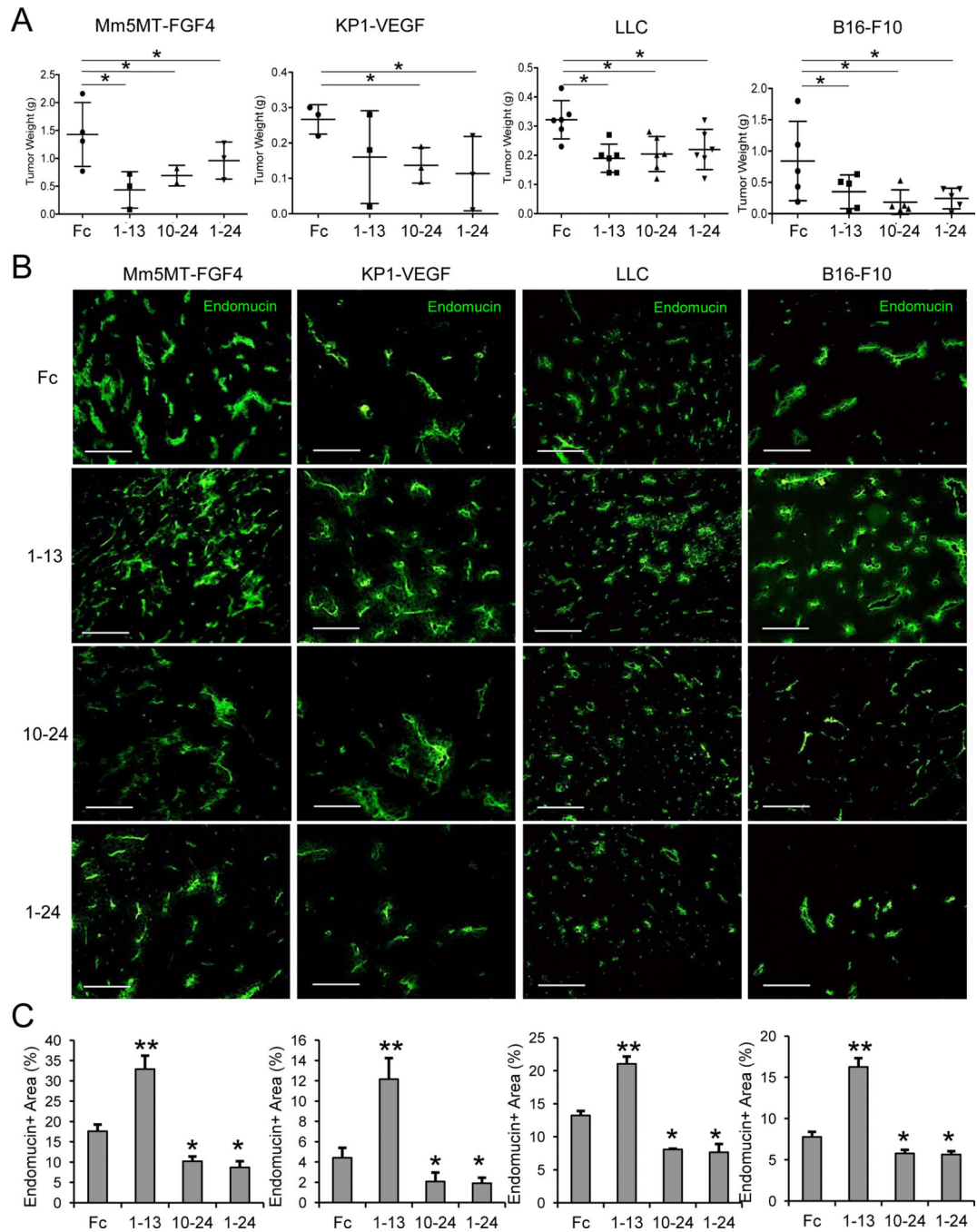




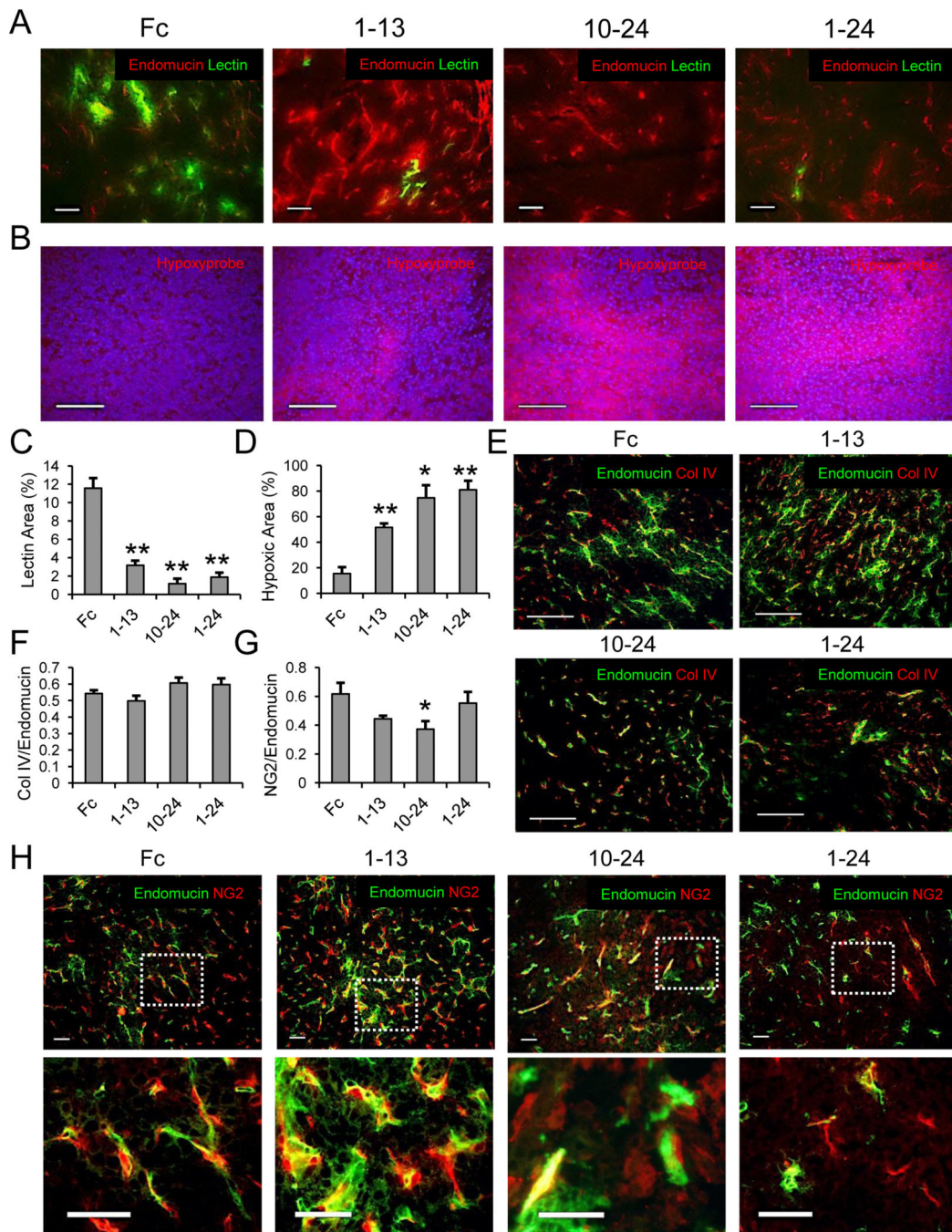
**Figure 3. N1 decoys variants function distinctly *in vitro* and in retinal angiogenesis**

(A) N1 decoy assessment in the HUVEC fibrin bead sprouting assay at day 7. Scale bars: 200  $\mu$ m. (B) Quantification of the mean number of branch points per bead  $\pm$  S.D. \* P value < 0.05. Fibrin bead sprouting assays were performed in triplicate and repeated twice. (C) Quantification of the mean percent vascular density of the P5 retinas  $\pm$  S.D. \* P value < 0.05. (D) Isolectin B4 (IsoIB4) staining of P5 retinas. A: artery, V: vein. (E) Isolectin B4 (IsoIB4) and  $\alpha$ SMA staining of P5 retinas. Vascular smooth muscle cell covered retinal arteries noted with arrowhead (n = 6).





**Figure 4. N1 decoys block xenograft tumor growth and disrupt tumor angiogenesis**  
**(A)** Mm5MT-FGF4, KP1-VEGF, LLC, and B16-F10 weigh significantly less in the N1 decoy-treated groups compared to Fc control group. Tumor weight measured at the time of harvest. Data presented as mean tumor weight  $\pm$  S.D. \* P value < 0.05 (n = 4-5). **(B)** Endomucin staining of N1 decoy treated Mm5MT-FGF4, KP1-VEGF, LLC, and B16-F10 tumors. Scale bars: 30  $\mu$ m. **(C)** Quantification of endomucin-positive vascular density. Data presented as mean percentage of endomucin-positive area  $\pm$  S.D. \* P value < 0.003 (n= 4-5).



**Figure 5. N1 decoys reduced perfusion and JAG-specific decoys disrupted pericyte association in Mm5MT-FGF4 tumors**

(A) Endomucin staining of FITC-lectin perfused tumors. (B) Antibody detection of tumors from hypoxyprobe injected mice and co-stained with DAPI. (C) Quantitation of vessel perfusion by mean percentage of lectin-positive area  $\pm$  S.D. \* P value < 0.006 (n = 4-5). (D) Quantitation of mean percentage of hypoxyprobe-positive area  $\pm$  S.D. \* P value < 0.002, \*\* P value < 0.05 (n = 4-5). (E) Collagen type IV (ColIV) and endomucin staining of Mm5MT-FGF4 tumor sections. (A, B, and E) Scale bars: 30  $\mu$ m. (F) Quantification of mean ColIV

area divided by mean endomucin area,  $\pm$  S.D. (n = 4-5). **(G)** Quantification of mean NG2 area divided by mean endomucin area,  $\pm$  S.D. \* P value < 0.02 (n = 4-5). **(H)** Endomucin and NG2 staining of Mm5MT-FGF4 tumor sections. Scale bars: 10  $\mu$ m.

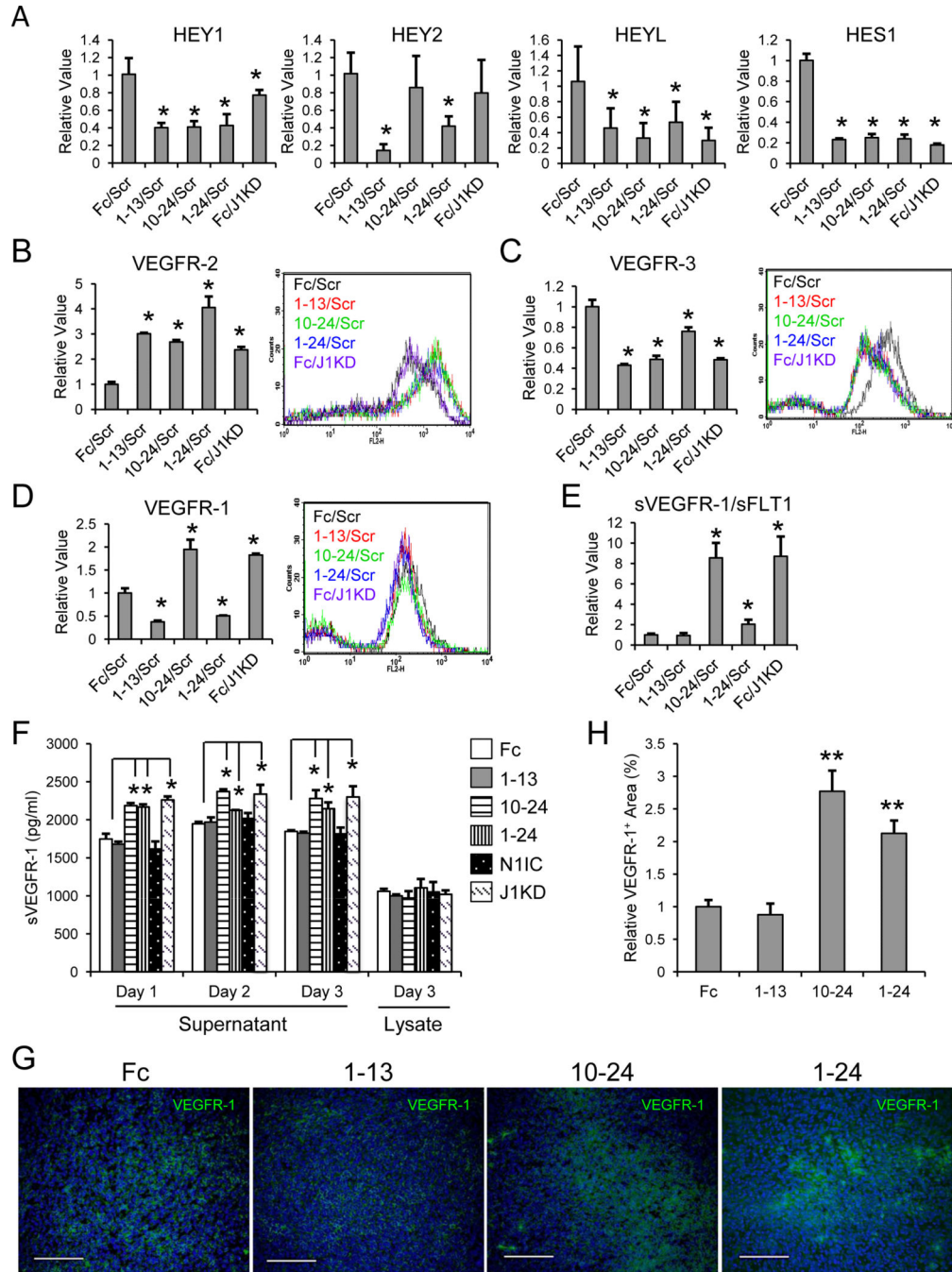
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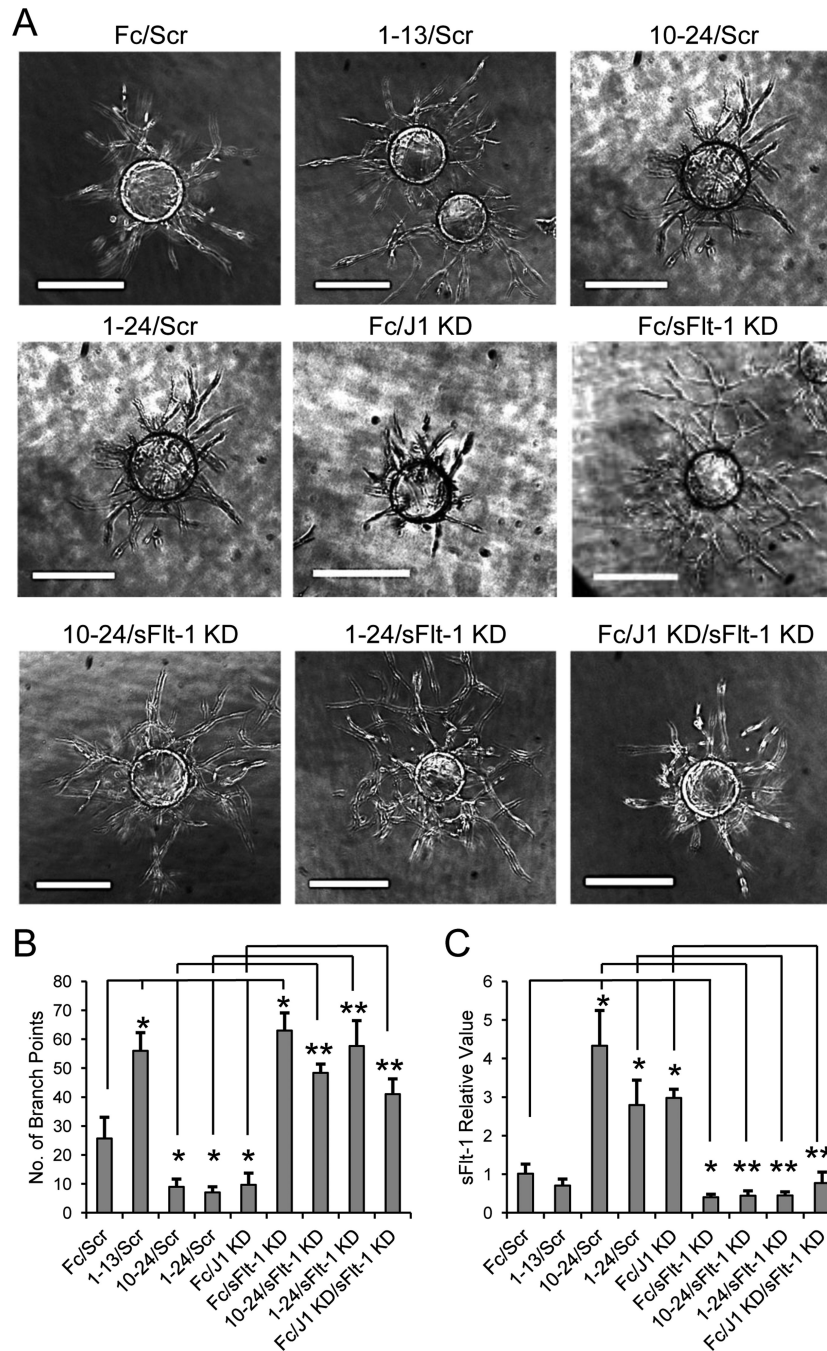
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**Figure 6. N1 decoys that block JAG elevate sVEGFR-1/sFlt-1**

(A) HEY1, HEY2, HEYL, HES1 qRT-PCR of N1 decoys or JAG1 knockdown (KD) HUVEC. (B-D) VEGF receptor qRT-PCR and flow cytometry of N1 decoys or JAG1 knockdown (KD) HUVEC. (E) Soluble VEGFR-1 (sVEGFR-1/sFLT1) qRT-PCR of N1 decoys or JAG1 knockdown (KD) HUVEC. (A-E) Data presented as  $\pm$  S.D. \* P value < 0.01. (F) Soluble VEGFR-1/sFlt-1 ELISA presented as  $\pm$  S.D. \* P value < 0.02. (G) VEGFR-1 staining of Mm5MT-FGF4 tumor sections. Scale bars: 30  $\mu$ m. (H) Quantitation of percent mean VEGFR-1-positive area. (n=3).



**Figure 7. sVEGFR-1/sFlt-1 knockdown rescues anti-angiogenic effects of N1 decoys that block JAG**

(A) Day 7 assessment in the HUVEC capillary sprouting assay, employing HUVECs transduced by different combinations of lentiviruses. Fc was used as control for N1 decoy overexpression, and scrambled shRNA (Scr) as control for sVEGFR-1/sFlt-1 or JAG1 knockdown (KD). Scale bar: 200  $\mu$ m. (B) Quantification of the mean number of branch points per bead  $\pm$  S.D. (C) sVEGFR-1/sFLT1 qRT-PCR  $\pm$  S.D. (B and C)\* P value < 0.05. \*\* P value < 0.005. Fibrin bead sprouting assays were performed in triplicate.