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Notch signaling in regulating angiogenesis in a 3D biomimetic environment

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Abstract

Angiogenesis is a complex cellular process involving highly orchestrated invasion and organization of endothelial cells (ECs) in a three-dimensional (3D) environment. Recent evidence indicates that Notch signaling is critically involved in regulating specialized functions and distinct fates of ECs in newly formed vasculatures during angiogenesis. Here we demonstrated, for the first time, the application of a microengineered biomimetic system to quantitatively investigate the role of Notch signaling in regulating early angiogenic sprouting and vasculature formation of ECs in 3D extracellular matrix. Morphological features of angiogenesis including invasion distance, invasion area, and tip cell number were quantified and compared under pharmacological perturbations of Notch signaling. In addition, influences of Notch signaling on EC proliferation in angiogenic vasculatures and directional invasion of tip cells were also investigated. Moreover, leveraging a novel nanobiosensor system, mRNA expression of Dll4, a Notch ligand, was monitored in invading tip cells using live cell imaging during dynamic angiogenic process. Our data showed that inhibition of Notch signaling resulted in hyper-sprouting endothelial structures, while activation of Notch signaling led to opposite effects. Our results also supported the role of Notch signaling in regulating EC proliferation and dynamic invasion of tip cells during angiogenesis.

Keywords

Angiogenesis; Biomimetic; Notch signaling; Nanobiosensor; Microenvironment

Introduction

Angiogenesis is the development of neo blood vessels from pre-existing vascular networks, which plays a prominent role in both normal physiological processes (*e.g.*, embryonic development and wound healing) and cancer metastasis [1–4]. Angiogenesis is a highly orchestrated process, in which endothelial cells (ECs) migrate collectively, make new connections, and remodel nascent vascular structures to form functional blood vessels [5]. According to recent studies, tip cells at the head of growing angiogenic sprouts, also called leader cells, are actively involved in probing and sensing surrounding environmental cues and providing guidance to stalk cells which follow tip cells and contribute to the establishment of cell-cell junctions and vessel lumens [6]. Angiogenesis is a dynamic process, with tip cells and stalk cells constantly rearranging their relative positions. The balance and dynamics of tip cells and stalk cells during angiogenic sprouting and vasculature formation are commonly believed to depend on Delta-Notch signaling [7,8].

Notch signaling is an evolutionarily conserved, intercellular, and contact-dependent signaling mechanism that plays a fundamental role in regulating cell fate during development and tissue homeostasis [5,9–12]. Multiple ligands are involved in Notch signaling, among which the most notable is Dll4 (Delta-like ligand 4) [9,11,13–15]. A high level of Dll4 in tip cells of angiogenic sprouts activates Notch signaling in stalk follower cells to inhibit their transformation into new tip cells [6,7,10,16,17]. Previous studies have shown that inhibition of Dll4 results in a broad spectrum antitumor activity [9,18,19]. For instance, deletion of a single allele of Dll4 inhibits tumor growth in a variety of tumor models [20–22]. However, there are also studies suggesting that Notch signaling functions as a negative regulator of tumor angiogenesis, as inhibition of Notch signaling results in hyper-angiogenic sprouting [9,18–22]. Nonetheless, the most commonly accepted theory for Notch signaling in tumor angiogenesis is that inhibition of Notch signaling through down-regulation of Dll4 leads to overgrowth of tumor vasculatures, even though such increased vascularity is non-productive [9,13,21,23–25]. Given its importance in tumor angiogenesis and progression, a better understanding of Notch signaling in a suitable angiogenesis model will provide new insights for targeting tumors that are resistant to anti-angiogenesis therapy [9,26,27].

Existing investigations of the regulatory role of Notch signaling in angiogenesis primarily rely on animal models, especially mouse retina [10,12,16,17,28,29]. In contrast to humans, mouse (and other rodent) pups have an immature retinal vasculature and persistent hyaloid vessels at birth, rendering it an ideal model for studying physiological angiogenic processes [30–32]. However, even though animal models can provide physiologically relevant environments, they are not ideal for modeling human diseases, due to critical genetic, molecular, immunologic, and cellular differences between species [33]. Moreover, uncontrolled laboratory environments, difficulty in isolating single parameters, and low

reproducibility of parametric studies are all intrinsic disadvantages associated with animal models. Finally, dynamic high-resolution, live cell imaging is also challenging with animal tissues.

Herein, we demonstrated, for the first time, the usage of a microengineered three-dimensional (3D) biomimetic angiogenesis device to investigate the role of Notch signaling in regulating angiogenic sprouting and vasculature formation of ECs in a 3D extracellular matrix. The unique compartmentalized design of the biomimetic angiogenesis device provided physiologically relevant biomimicry for recapitulating the dynamic angiogenic sprouting process. Morphological features of angiogenesis including endothelial invasion distance and area, tip cell number, and proliferation pattern of newly generated vasculatures were investigated and compared under different pharmacological perturbations of Notch signaling. Enabled by the 3D biomimetic angiogenesis device, the influence of Notch signaling on directional invasion of ECs during angiogenic sprouting was also examined. Importantly, a gold nanorod-locked nucleic acid (GNR-LNA) nanobiosensor was utilized in conjunction with the 3D biomimetic angiogenesis device to investigate Dll4 mRNA expression in invading tip cells at the head of angiogenic sprouts using live cell imaging. Together, our results confirmed the important role of Notch signaling in regulating morphogenesis, proliferation, and directional invasion of ECs during angiogenesis. Our data provide convincing evidence supporting the utility of the 3D biomimetic angiogenesis device for advancing understanding of molecular and cellular mechanisms underlying angiogenesis in a 3D biomimetic and physiologically relevant environment.

Results and Discussions

Design of biomimetic angiogenesis device and mechanism of GNR-LNA nanobiosensor

To examine the involvement of Notch signaling in angiogenesis, herein we adapted a compartmentalized microscale device, previously utilized by our group and others [34–38], to provide an efficient biomimetic means to promote and visualize early angiogenic processes. As shown in Fig. 1A, this biomimetic angiogenesis device consisted of two parallel side channels (100 μm in height and 1000 μm in width; designated as endothelial channel and fibroblast channel, respectively), separated by a central channel defined by trapezoid-shaped supporting posts designed for confining fibrin gel by surface tension prior to gelation. The fibrin gel in the central channel served as a scaffold to promote angiogenic sprouting of ECs, while allowing convenient microscopic imaging to examine morphological features of angiogenesis and Notch signaling. Human bone marrow fibroblasts (HS5) suspended in fibrin gel were carefully placed in the two reservoirs of the fibroblast channel without blocking the channel. The same fibrin gel was injected into the central gel channel. Upon fibrin gel polymerization, ECs were loaded into the endothelial channel and allowed to adhere to the gel interface in the endothelial channel by tilting the device by 90°. For pharmacological perturbations of Notch signaling, chemicals were added to culture medium and introduced into the angiogenesis device. The angiogenesis device was continuously imaged for 48 hr to examine morphological features of angiogenesis including endothelial invasion distance, invasion area, and tip cell number (SI Fig. 1).

A GNR-LNA nanobiosensor was utilized to examine Notch signaling during dynamic angiogenic process in the angiogenesis device. The GNR-LNA nanobiosensor is a complex of gold nanorod and locked nucleic acid (LNA) probe, a 20-base pair single strand alternative DNA/LNA oligonucleotide sequence labeled with a fluorophore (6-FAM (fluorescein)) (Fig. 1B). The LNA probe sequence is complimentary to target Dll4 mRNA sequence. Design and characterization of such GNR-LNA nanobiosensors have been reported previously [39,40]. Briefly, the LNA probe will bind to the GNR spontaneously to form the GNR-LNA complex. Due to their close physical proximity, fluorophore of the LNA probe is quenched by the GNR. After internalization by cells and in the presence of target mRNA molecules in the cytoplasm, the LNA probe dissociates from the GNR before binding target mRNA molecules while reacquiring fluorescence signal (Fig. 1B). Thus, fluorescence intensity of individual cells containing the GNR-LNA nanobiosensor can serve as a quantitative measure of the amount of target mRNA molecules inside individual single cells [41,42]. In this study, ECs were cultured with the GNR-LNA probe prior to cell seeding into the angiogenesis device. Internalized GNR-LNA probes in ECs through endocytosis was clearly evident in the dark-field image in Fig. 1C.

Involvement of Notch signaling in angiogenesis

Using the 3D biomimetic angiogenesis device, we first examined the involvement of Notch signaling in regulating angiogenic sprouting of ECs using two well characterized pharmacological drugs, DAPT and Jagged1 peptide (Fig. 1D) [11,14,42]. DAPT is a γ -secretase inhibitor that blocks Notch endoproteolysis and thus serves indirectly as a Notch inhibitor. Jagged1 peptide (hereafter referred to as Jagged1) can activate Notch signaling by inhibiting the function of endogenous Jagged1, a Notch ligand that has weak signaling capacity but competes with Dll4.

HS5 cells cultured in the fibroblast channel reservoirs could effectively induce angiogenesis of ECs into the fibrin gel by establishing growth factor gradients in the gel (SI Fig. 1 and Fig. 2A) [38]. 48 hr after initial cell seeding, we observed prominent directional migration and invasion of ECs from the endothelial channel into the fibrin gel toward the fibroblast channel forming neo blood vessel networks (SI Fig. 1, SI Fig. 2, and Fig. 2A). Pharmacological treatments with DAPT and Jagged1 effectively induced morphological changes of invading tip cells and blood vessel network. Specifically, both DAPT and Jagged1 treatment led to increased branching in the vascular network compared with untreated control. In addition, compared with untreated control, under DAPT treatment, tip cells located at the invasion front of angiogenic sprouting possessed more tiny sprouts, particularly for the furthest tip cells. For ECs treated with Jagged1, cell membrane of tip cells appeared much smoother, consistent with the theory that Notch signaling serves as a negative regulator to control the potency of EC sprouting from an existing vascular network [5,9–12].

We further quantified the effects of DAPT and Jagged1 treatments on invasion area, invasion distance, and tip cell number during angiogenic sprouting and blood vessel formation of ECs in the angiogenesis device. Both DAPT and Jagged1 treatments led to an overall increase of invasion area into the fibrin gel during angiogenesis (Fig. 2B). Inhibition of Notch signaling

by DAPT led to decreased invasion distance but potently increased tip cell number (including both isolated tip cells and tip cells with stalks) during angiogenic sprouting (Fig. 2C-D). These observations are in a good agreement with *in vivo* experiments reported in the literature [10,17,28,43], in which inhibition of Notch signaling has resulted in greater tip cell number and larger angiogenic invasion area. In contrast, up-regulation of Notch signaling by Jagged1 resulted in increased EC invasion but decreased tip cell numbers (including both isolated tip cells and tip cells with stalks; Fig. 2C-D), also in agreement with *in vivo* studies [5,9–12].

Even though both DAPT and Jagged1 treatments led to an overall increase of invasion area of angiogenic sprouting, spatial features of angiogenic sprouting were distinct between DAPT and Jagged1 treated samples. To quantify such spatial angiogenesis features, we divided the rectangular area between trapezoid-shaped supporting posts (where ECs were initially seeded) and the furthest leading tip cell into ten sub-regions of equal areas (labeled from 1–10; Fig. 2E). Invasion coverage percentage, defined as the ratio of projected area covered by ECs and the area of each sub-region, was calculated for each sub-region (SI Fig. 3). As shown in Fig. 2F, vascular network formation under Jagged1 treatment showed higher invasion coverage percentage, compared with untreated control, in sub-regions close to the invasion front, whereas DAPT treatment led to a higher invasion coverage in areas closer to the supporting posts where ECs were initially seeded. These morphogenetic distinctions could possibly be resulted from altered proliferation or invasion potency of ECs under different pharmacological perturbations of Notch signaling. These potentials were further investigated in the following sections. Together, our data supported that DAPT treatment led to EC hyper-sprouting, resulting in a “nonfunctional vasculature” exhibiting limited resemblance of normal vascular networks [6,8,15,24,27]. Even though Jagged1 treatment led to increased branching in the vessel network, such vascular architecture possessed a greater similarity to untreated control.

Notch signaling regulates EC proliferation during angiogenesis

Based on the distinct morphological features of neo blood vessel formation during angiogenesis observed in Fig. 2, we hypothesized that, in addition to invading tip cell behaviors, proliferation and remodeling of follower stalk cells might also be important for establishing such distinct morphological features of vascular networks under different conditions. To examine this possibility, Click-iT EdU assays were conducted to identify EdU-positive, proliferating ECs in the fibrin hydrogel (Fig. 3A; see Materials and Methods). As shown in Fig. 3B&C, the number of EdU positive nuclei in the fibrin gel was significantly greater under both DAPT and Jagged1 treatments compared with untreated control. However, the percentages of EdU positive nuclei relative to the total cell number (as labeled by DAPI) under DAPT and Jagged1 treatments were comparable with untreated control (Fig. 3B), suggesting that DAPT and Jagged1 treatments did not alter the proliferative behavior of ECs in the fibrin gel, but had caused more ECs to invade into the gel. This contention was further supported by standard cell proliferation and toxicity assays conducted using transwells (see Materials and Methods), revealing no significant differences in cell proliferation or toxicity between different monoculture or co-culture conditions treated without or with DAPT or Jagged1 (SI Fig. 4).

We further examined spatial patterns of EC proliferation in angiogenic vasculatures in the fibrin hydrogel, by dividing the rectangular area between supporting posts (where ECs were initially seeded) and the furthest leading tip cell into ten equal sub-regions. Under all experimental conditions, the highest percentages of EdU positive cell nuclei were in the middle portion of the vessel network, while both tip cells and ECs located close to supporting posts showed lowest percentages of EdU-positive nuclei (Fig. 3D). Compared with control, DAPT treatment resulted in lower percentages of proliferating EdU-positive ECs near the invasion front and greater cell proliferation for the rest vasculature regions in the fibrin hydrogel (Fig. 3D). In contrast, Jagged1 treatment led to significantly greater EC proliferation at the invasion front but less proliferation for the rest vasculature regions in the fibrin hydrogel compared with untreated control (Fig. 3D). These distinct spatial patterns of EC proliferation under different conditions could partially explain the greater invasion coverage in the middle portion of the vascular network under DAPT treatment and the higher invasion coverage at the invasion front under Jagged1 treatment when compared with untreated control. In conclusion, manipulation of Notch signaling using pharmacological drugs didn't affect the overall proliferation rate of ECs. Under DAPT treatment, a higher percentage of ECs were "trapped" in the vicinity around the supporting posts where the cells were initially seeded, likely due to compromised migratory potency of ECs, the evidence of which is presented in the following section.

Notch signaling in regulation of directional invasion of ECs

Our data so far suggested that the role of Notch signaling in regulating angiogenic morphogenesis was likely through its effect on mediating tip cell behaviors. Thus, we further performed live cell imaging to track migratory behaviors of individual tip cells during angiogenesis using ECs expressing red fluorescence protein (RFP) (Fig. 4A). Tip cells were continuously monitored for 48 hr after initial cell seeding (Fig. 4A; videos supplemented as SI GIF 1,2 and 3). In the angiogenesis device, tip cells preferentially migrated in the fibrin gel along growth factor gradients established toward the fibroblast channel with a relatively narrow range of cell migration orientation angle between $\pm 25^\circ$.

To further evaluate the directionality of tip cell invasion, distributions of migration orientation angle of tip cells were plotted in polar histograms (Fig. 4B). Perturbations of Notch signaling using DAPT and Jagged1 induced significant changes of tip cell migratory behaviors. Specifically, inhibition of Notch signaling by DAPT resulted in a decreased fraction of tip cells migrating along growth factor gradients toward the fibroblast channel, whereas activation of Notch by Jagged1 increased this fraction. This observation is also reflected in Fig. 4C where percentages of invading tip cells with migration orientation angles falling between $\pm 4^\circ$ were compared between different conditions (control: 34%; DAPT: 18%; Jagged1: 44%). Notably, we also observed decreased migration velocity for DAPT-treated ECs, but not for Jagged1 treated cells (Fig. 4D), suggesting that inhibition of Notch signaling negatively impacted both tip cell invasion directionality and speed.

Notch signaling in regulation of Dll4 mRNA expression

We further utilized the GNR-LNA nanobiosensor in conjunction with the 3D angiogenesis device to examine Dll4 mRNA expression in invading tip cells at the head of angiogenic

sprouts. The GNR-LNA probe was designed to target specifically Dll4 mRNA, and its specificity was validated using 2D culture assays before implementing it for 3D angiogenesis experiments (SI Fig. 5). A random probe was also included in this work as a negative control (SI Fig. 6). ECs were incubated with GNR-LNA probes for 4 hr to allow cellular uptake before seeding cells at the fibrin gel interface. Tip cells treated without (control) or with DAPT and Jagged1 were continuously monitored in the 3D angiogenesis device for 18 hr after initial cell seeding (Fig. 5A and SI Fig. 7). Live imaging of the 3D angiogenesis device to monitor single-cell Dll4 mRNA expression was limited to 18 hr due to photobleaching. As shown in Fig. 5B&C, compared with untreated control or cells treated with Jagged1, tip cells treated with DAPT consistently exhibited higher fluorescence intensity, suggesting greater Dll4 mRNA expression in DAPT-treated tip cells. In contrast, Jagged1 treatment led to significantly decreased Dll4 mRNA expression in tip cells. Differences in fluorescence intensity and thus Dll4 mRNA expression in tip cells between different conditions (DAPT vs. control and Jagged1 vs. control) became greater over time. These observations were further confirmed using EC networks formed on 2D Matrigel substrates (SI Fig. 5).

Conclusion

The Notch signaling pathway is an evolutionarily conserved intercellular pathway that is important in regulating cell fate patterning during normal development and tissue homeostasis [44]. There are four Notch receptors including Notch1, Notch2, Notch3 and Notch4, and five Notch transmembrane ligands including Jagged1, Jagged2, and Delta-like 1 (Dll1), Dll3 and Dll4 [5]. Notch signaling mediated through Dll4 has been particularly suggested to negatively regulate tip cell formation during angiogenesis and control leader cell formation during collective epithelial cell migration [28,45,46]. In this study we demonstrated, for the first time, the usage of a microengineered 3D biomimetic device to examine the role of Notch signaling in regulating angiogenic sprouting and vasculature formation. The administration of γ -secretase inhibitor DAPT, which inhibits Notch signaling through blocking Notch cleavage and signaling, to ECs in our biomimetic device resulted in an abnormal angiogenic network, including hyper-sprouting endothelial structures and increased tip cell number and invasion area, consistent with previous *in vivo* experiments [10,17,28,43]. In contrast, activation of Notch signaling through Jagged1 treatment showed the opposite effects, including increased invasion distance but decreased tip cell number, also in agreement with *in vivo* studies [5,9–12]. Moreover, enabled by the 3D biomimetic angiogenesis device, the influence of Notch signaling on EC proliferation in newly generated neo blood vessel networks and directional migration of invading tip cells were also investigated, with findings supporting the potent role of Notch signaling in regulating angiogenic dynamics of ECs. By using the GNR-LNA nanobiosensor for Dll4 mRNA detection, we further examined Dll4 mRNA expression in invading tip cells at the head of angiogenic sprouts. Our results suggested that DAPT and Jagged1 treatments perturbed Dll4 mRNA expression in tip cells during angiogenic sprouting, which could explain the different angiogenic sprouting phenotypes observed under DAPT and Jagged1 treatments. Together, the capability of the 3D biomimetic angiogenesis device to recapitulate *in vivo* 3D microenvironment, combined with the application of live-cell nanobiosensors, enabled us to

study in detail the role of Notch signaling in regulating angiogenesis. The 3D biomimetic angiogenesis device offers excellent assay repeatability and allows for live cell imaging at a high spatiotemporal resolution, which will provide an attractive alternative approach to existing animal models for studying angiogenesis.

Materials and Methods

Device fabrication

The 3D microengineered biomimetic angiogenesis device consisted of a polydimethylsiloxane (PDMS) microchannel layer and a bottom coverslip. The PDMS microchannel layer was fabricated using soft lithography. PDMS was mixed at a 1:10 curing agent to elastomer base ratio and casted onto a positive mold made of SU-8 patterned using standard lithography, then cured at 120°C for 20 min. Four loading reservoirs and two gel loading inlets were punched into the PDMS layer by 8 mm and 1.2 mm Harris Uni-Core punch tools (Ted Pella), respectively. The PDMS microchannel layer and the coverslip were cleaned with ethanol and treated with oxygen plasma for 80 sec before bonded together. The 3D angiogenesis device was baked at 80°C for 2 hr to restore surface hydrophobicity before further sterilized by exposing to UV light for 25 min.

Cell culture

Two cell lines were used in this work: human umbilical vein endothelial cells (HUVECs) and bone marrow stromal fibroblast (HS5). HUVECs and HS5 were acquired from Lonza and ATCC, respectively. HUVECs were maintained in endothelial cell growth medium-2 (EGM; Lonza) and harvested using 0.05% Trypsin-EDTA (Lonza) when cells reached 90% confluency. Passages 2–5 were used for HUVECs. HS5 cells were cultured in DMEM basal medium supplemented with 10% FBS and 0.5% penicillin-streptomycin. All cells were cultured under 5% CO₂ at 37°C, and culture medium was exchanged every 2 days.

Cells seeding

Fibrinogen (Sigma) was dissolved in PBS at a concentration of 2.5 mg/mL before supplemented with aprotinin (0.15 U/mL, Sigma). HS5 cells were then suspended in the fibrinogen solution at a concentration of 2×10^6 cells/mL before mixed with thrombin (0.5 U/mL) and loaded into the two reservoirs of the fibroblast channel without blocking the channel [35,47]. The same fibrin gel was prepared and injected into the middle gel channel of the 3D angiogenesis device. The 3D angiogenesis device was then incubated at 37°C for 15 min to allow complete gelation of the fibrin gel. EGM solution (300 μ L) were then added to the 4 loading reservoirs of the endothelial and fibroblast channels before filling the channels with EGM solution by gently applying vacuum. The 3D angiogenesis device was incubated for another 24 hr to eliminate bubbles trapped inside the device. 20 μ L HUVEC solution with a cell concentration of 5×10^6 cells/mL was introduced into the endothelial channel. The 3D angiogenesis device was then tilted by 90° and incubated for 15 min at 37°C to allow HUVECs to adhere to the fibrin gel interface.

To examine the involvement of Notch signaling in regulating angiogenic sprouts, HUVECs were cultured with EGM supplemented with 20 μ M γ -secretase inhibitor, DAPT (Sigma)

and 20 μ M Jagged1 peptide (188–204, AnaSpec). After cell seeding, the 3D angiogenesis device was incubated at 37°C with 5% CO₂ and monitored for 2 days, with culture medium exchanged daily.

Immunofluorescence staining

The vascular network in the 3D angiogenesis device was washed 3 times with PBS, 10 min each time. Cells were then fixed with 4% paraformaldehyde solution (PFA) for 10 min before permeabilized and blocked with the Perm/Block solution PBST (PBS + 0.5% Triton + 1% BSA) for 1 hr. After washing with PBS for 3 times to remove PBST, cells were incubated with primary Dll4 antibody diluted in PBST (1:200, R&D) at 4°C overnight. After incubation, cells were washed 3 times with PBST, 15 min each time. Cells were then incubated with Dll4 secondary antibody (1:500, Abcam), phalloidin (1:30), and DAPI (1:2000) for 2 hr at room temperature (all chemicals diluted in PBST). Cells were then washed 3 times with PBST, 15 min each time, before fluorescence imaging and analysis.

Imaging and analysis

Immunofluorescence images of vascular networks in the 3D angiogenesis device were recorded using an Olympus DSU-IX81 Spinning Disc Confocal Microscope equipped with an EMCCD camera (iXon X3, Andor). Z-stack images were acquired with a slice thickness of 1 μ m. Time-lapse images of angiogenesis were acquired during a time period of 48 hr with an interval of 30 min after initial cell seeding. Cells were kept at 37°C using a Tokai Hit incubator chamber placed on top of the microscope stage. Migration trajectories of individual invading tip cells were tracked manually using MTrackJ plugin of ImageJ. Orientation angle of tip cell invasion was calculated using coordinates of the starting and end points of an individual cell invasion trajectory. Fluorescence images of EC angiogenesis with internalized GNR-LNA probes were also acquired using confocal microscopy at 6, 12 and 18 hr after cell seeding and analyzed with ImageJ to determine fluorescence intensity of LNA probes.

Probe design

LNA probes for Dll4 mRNA have 20 base pair nucleotides (+AA+GG+GC+AG +TT+GG +AG+AG+GG+TT) designed for capturing Dll4 mRNA with a nucleotide sequence complementary to the probe. For mRNA detection, a fluorophore (6-FAM (fluorescein)) was attached to the 5' end of the LNA probe. Specificity of the Dll4 mRNA probe sequence was characterized using the NCBI Basic Local Alignment Search Tool (BLAST) database. A random probe (+AC+GC+GA+CA+AG+CG+CA+CC+GA+TA) was designed as negative control. All LNA probes were synthesized by Integrated DNA Technologies.

GNR-LNA probe preparation

GNRs with a diameter of 10 nm and a length of 67 nm were acquired from Nanopartz. GNRs were coated with 11-mercaptopoundecyltrimethylammonium bromide (MUTAB) for enhancing cellular uptake and its ability to bind LNA probes. LNA probes were diluted in Tris-EDTA buffer at a concentration of 10 mM before incubated with GNRs at 37°C for 30 min. GNR-LNA probes were then incubated with cells for endocytic uptake.

GNR-LNA probe assay

HUVECs were seeded in T25 cell culture flask with a seeding density of 5×10^4 cells/mL. When cells reached 80% confluency, GNR-LNA nanobiosensors were added into the flask and incubated with HUVECs for 4 hr for endocytic uptake. After intracellular delivery of GNR-LNA probes, cells were washed 3 times with PBS to remove extra probes, before harvested using 0.05% Trypsin-EDTA. For 2D Matrigel assays, glass bottom 24-well cell culture plates were treated with plasma for 1 min and coated with Matrigel (Corning). HUVECs were seeded into Matrigel coated wells at a seeding density of 200 cells/mm². Self-organized EC microvasculature networks were recorded every 3 hr for 12 hr using fluorescence microscopy. For microfluidic angiogenesis assays, HUVECs were seeded onto the fibrin gel interface within the endothelial channel before imaged every 6 hr for 18 hr using fluorescence microscopy.

The fluorescence intensity of GNR-LNA nanobiosensors is presented in arbitrary unit (a.u.). Cumulative probability (Fig. 5C) refers to the probability that the fluorescence intensity of tip cells is lower than or equal to the intensity value at the x -axis

Cell viability assay

To evaluate the cytotoxicity of GNRs, DAPT, and Jagged1, viability of ECs was characterized using a cell cytotoxicity assay (Cell Counting Kit-8 or CCK-8, Sigma) according to the manufacturer's instructions. Briefly, HUVECs were suspended in a 96-well plate with 5000 cells per well. HUVECs were incubated with GNRs, or treated with 20 μ M DMSO, 20 μ M DAPT, or 20 μ M Jagged1 for 24 hr or 48 hr. The CCK-8 solution was then added to each well for 2 hr. The absorbance of samples in each well was measured at 450 nm using a microplate reader (BioRad).

Transwell cell proliferation assay

The effect of stromal fibroblasts HS5 on EC proliferation was evaluated using a transwell 96-well plate (HTS transwell plate, Corning). HUVECs and HS5 were separated by a porous membrane with a 0.8 μ m pore size in the transwell chamber. HUVECs were seeded at 2000 cells per well into the lower compartment of the transwell. HS5 cells were mixed with the fibrin gel before seeded into the transwell insert chamber. Culture medium was exchanged after 24 hr incubation. After 2 days of co-culture, the upper transwell chamber was removed, and the CCK-8 solution was added to the lower compartment of transwell and incubated for additional 1 hr. The absorbance of samples was measured at 450 nm using a microplate reader every hour for 4 hr at 37°C.

In situ proliferation assay

For *in situ* proliferation measurements, Click-iT EdU Kit (Invitrogen) was used per manufacturer's protocol. HUVECs in the 3D angiogenesis device were cultured with EGM or with EGM supplemented with either 20 μ M DAPT or 20 μ M Jagged1 for 21 hr. Diluted EdU solution (10 μ M) was then introduced into all reservoirs of the device. Before fixation, cells were incubated with EdU solution for 3 hr to label newly synthesized DNA. The numbers of total nuclei (stained with Hoechst) and EdU labeled nuclei in confocal images were counted manually.

Quantification of invasion distance, invasion area, and tip cell number

The tip cell number was selected as a morphological indicator of vascular networks. Based on our observation, tip cell number can properly describe angiogenic morphogenic differences in the 3D biomimetic angiogenesis device, especially when isolated tip cells are prevailing. For each confocal image (Z-scanning images with a slice thickness of 1 μm), the number of tip cells (either trailed with a multicellular stalk or isolated) was assessed blindly by two independent observers. Vertical distance between leading tip cells and the fibrin gel interface within the endothelial channel was quantified as the invasion distance. A custom-designed MATLAB image processing program was used for quantification of invasion area and coverage percentage. Briefly, after adaptive filtering, binarization with adaptive threshold was applied to each image. Projected area in binarized images was measured as invasion area. A rectangular area was placed between trapezoid-shaped supporting posts and leading tip cells to define a region of interest (ROI). The ROI was segmented into 10 sub-regions with equal areas, designated as 1 – 10 starting from supporting posts toward leading tip cells. For each sub-region, the coverage percentage was calculated as the ratio between the number of pixels with a binary value of 1 and the total pixels of the sub-region.

Statistical analysis

Results were analyzed using independent, two-tailed Student *t*-test in Excel (Microsoft). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference

- [1]. Chung AS, Ferrara N, Developmental and Pathological Angiogenesis, *Annu. Rev. Cell Dev. Biol.*, 27 (2011) 563–584.
- [2]. Bergers G, Benjamin LE, Tumorigenesis and the angiogenic switch, *Nat. Rev. Cancer*, 3 (2003) 401–410. [PubMed: 12778130]
- [3]. Weis SM, Cheresh DA, Tumor angiogenesis: molecular pathways and therapeutic targets, *Nat. Med.*, 17 (2011) 1359–1370. [PubMed: 22064426]
- [4]. Vasudev NS, Reynolds AR, Anti-angiogenic therapy for cancer: Current progress, unresolved questions and future directions, *Angiogenesis*, 17 (2014) 471–494. [PubMed: 24482243]
- [5]. Blanco R, Gerhardt H, VEGF and Notch in tip and stalk cell selection, *Cold Spring Harb. Perspect. Med.*, 3 (2013) 1–19.
- [6]. Serra H, Chivite I, Angulo-Urarte A, Soler A, Sutherland JD, Arruabarrena-Aristorena A, Ragab A, Lim R, Malumbres M, Fruttiger M, Potente M, Serrano M, Fabra A, Viñals F, Casanovas O, Pandolfi PP, Bigas A, Carracedo A, Gerhardt H, Graupera M, PTEN mediates Notch-dependent stalk cell arrest in angiogenesis, *Nat. Commun.*, 6 (2015) 7935. [PubMed: 26228240]

- [7]. Bentley K, Franco CA, Philippides A, Blanco R, Dierkes M, Gebala V, Stanchi F, Jones M, Aspalter IM, Cagna G, Weström S, Claesson-Welsh L, Vestweber D, Gerhardt H, The role of differential VE-cadherin dynamics in cell rearrangement during angiogenesis, *Nat. Cell Biol*, 16 (2014) 309–321. [PubMed: 24658686]
- [8]. Bernier-Latmani J, Cisarovsky C, Demir CS, Bruand M, Jaquet M, Davanture S, Ragusa S, Siegert S, Dormond O, Benedito R, Radtke F, Luther S. a., Petrova TV, DLL4 promotes continuous adult intestinal lacteal regeneration and dietary fat transport, *J. Clin. Invest*, 125 (2015) 4572–4586. [PubMed: 26529256]
- [9]. Liu Z, Fan F, Wang A, Zheng S, Lu Y, Dll4-Notch signaling in regulation of tumor angiogenesis, *J. Cancer Res. Clin. Oncol*, 140 (2014) 525–536. [PubMed: 24114288]
- [10]. Benedito R, Roca C, Sörensen I, Adams S, Gossler A, Fruttiger M, Adams RH, The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis, *Cell*, 137 (2009) 1124–1135. [PubMed: 19524514]
- [11]. Patel NS, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL, Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function, *Cancer Res.*, 65 (2005) 8690–8697. [PubMed: 16204037]
- [12]. Liu Z-J, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M, Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis, *Mol. Cell. Biol*, 23 (2003) 14–25. [PubMed: 12482957]
- [13]. Phng LK, Gerhardt H, Angiogenesis: A Team Effort Coordinated by Notch, *Dev. Cell*, 16 (2009) 196–208. [PubMed: 19217422]
- [14]. Moya IM, Umans L, Maas E, Pereira PNG, Beets K, Francis A, Sents W, Robertson EJ, Mummery CL, Huylebroeck D, Zwijsen A, Stalk Cell Phenotype Depends on Integration of Notch and Smad1/5 Signaling Cascades, *Dev. Cell*, 22 (2012) 501–514. [PubMed: 22364862]
- [15]. Dufraigne J, Funahashi Y, Kitajewski J, Notch signaling regulates tumor angiogenesis by diverse mechanisms, *Oncogene*, 27 (2008) 5132–5137. [PubMed: 18758482]
- [16]. Stenzel D, Franco CA, Estrach S, Mettouchi A, Sauvaget D, Rosewell I, Schertel A, Armer H, Domogatskaya A, Rodin S, Tryggvason K, Collinson L, Sorokin L, Gerhardt H, Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo, *EMBO Rep.*, 12 (2011) 1135–1143. [PubMed: 21979816]
- [17]. Jakobsson L, a Franco C, Bentley K, Collins RT, Ponsioen B, Aspalter IM, Rosewell I, Busse M, Thurston G, Medvinsky A, Schulte-Merker S, Gerhardt H, Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting, *Nat. Cell Biol*, 12 (2010) 943–953. [PubMed: 20871601]
- [18]. Hoey T, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, Fitch-Bruhns M, Lazetic S, Park IK, Sato A, Satyal S, Wang X, Clarke MF, Lewicki J, Gurney A, DLL4 Blockade Inhibits Tumor Growth and Reduces Tumor-Initiating Cell Frequency, *Cell Stem Cell*, 5 (2009) 168–177. [PubMed: 19664991]
- [19]. Yen WC, Fischer MM, Hynes M, Wu J, Kim E, Beviglia L, Yeung VP, Song X, Kapoun AM, Lewicki J, Gurney A, Simeone DM, Hoey T, Anti-DLL4 has broad spectrum activity in pancreatic cancer dependent on targeting DLL4-notch signaling in both tumor and vasculature cells, *Clin. Cancer Res*, 18 (2012) 5374–5386. [PubMed: 22952347]
- [20]. Djokovic D, Trindade A, Gigante J, Badenes M, Silva L, Liu R, Li X, Gong M, Krasnoperov V, Gill PS, Duarte A, Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis, *BMC Cancer*, 10 (2010) 641. [PubMed: 21092311]
- [21]. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang W-C, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo J-AS, de Sauvage F, Plowman G, Yan M, Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis, *Nature*, 444 (2006) 1083–1087. [PubMed: 17183323]
- [22]. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G, Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis, *Nature*, 444 (2006) 1032–1037. [PubMed: 17183313]

- [23]. Roca C, Adams RH, Regulation of vascular morphogenesis by Notch signaling Regulation of vascular morphogenesis by Notch signaling, 21 (2007) 2511–2524.
- [24]. Kuhnert F, Kirshner JR, Thurston G, Dll4-Notch signaling as a therapeutic target in tumor angiogenesis, Vasc. Cell, 3 (2011) 20. [PubMed: 21923938]
- [25]. Ramasamy SK, Kusumbe a P., Wang L, Adams RH, Endothelial Notch activity promotes angiogenesis and osteogenesis in bone, Nature, 507 (2014) 376–380. [PubMed: 24647000]
- [26]. Li JL, Sainson RCA, Shi W, Leek R, Harrington LS, Preusser M, Biswas S, Turley H, Heikamp E, Hainfellner JA, Harris AL, Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo, Cancer Res., 67 (2007) 11244–11253. [PubMed: 18056450]
- [27]. Xu Z, Wang Z, Jia X, Wang L, Chen Z, Wang S, Wang M, Zhang J, Wu M, MMGZ01, an anti-DLL4 monoclonal antibody, promotes nonfunctional vessels and inhibits breast tumor growth, Cancer Lett., 372 (2016) 118–127. [PubMed: 26739060]
- [28]. Hellström M, Phng L-K, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson A-K, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalén M, Gerhardt H, Betsholtz C, Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis, Nature, 445 (2007) 776–780. [PubMed: 17259973]
- [29]. Claxton S, Fruttiger M, Periodic Delta-like 4 expression in developing retinal arteries, Gene Expr. Patterns, 5 (2004) 123–127. [PubMed: 15533827]
- [30]. Stahl A, Connor KM, Sapieha P, Chen J, Dennison RJ, Krah NM, Seaward MR, Willett KL, Aderman CM, Guerin KI, Hua J, Löfqvist C, Hellström A, Smith LEH, The mouse retina as an angiogenesis model, Invest. Ophthalmol. Vis. Sci, 51 (2010) 2813–2826. [PubMed: 20484600]
- [31]. Uemura A, Kusuha S, Katsuta H, Nishikawa S-I, Angiogenesis in the mouse retina: a model system for experimental manipulation, Exp. Cell Res, 312 (2006) 676–83. [PubMed: 16337189]
- [32]. Gariano RF, Gardner TW, Retinal angiogenesis in development and disease, Nature, 438 (2005) 960–966. [PubMed: 16355161]
- [33]. Mak IW, Evaniew N, Ghert M, Lost in translation: animal models and clinical trials in cancer treatment, Am. J. Transl. Res, 6 (2014) 114–8. [PubMed: 24489990]
- [34]. Huang CP, Lu J, Seon H, Lee AP, a Flanagan L, Kim H-Y, Putnam AJ, Jeon NL, Engineering microscale cellular niches for three-dimensional multicellular co-cultures, Lab Chip, 9 (2009) 1740–8. [PubMed: 19495458]
- [35]. Kim S, Chung M, Jeon NL, Three-dimensional biomimetic model to reconstitute sprouting lymphangiogenesis in vitro, Biomaterials, 78 (2015) 115–128. [PubMed: 26691234]
- [36]. Zervantonakis IK, Hughes-Alford SK, Charest JL, Condeelis JS, Gertler FB, Kamm RD, Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function, Proc. Natl. Acad. Sci. U. S. A, 109 (2012) 13515–13520. [PubMed: 22869695]
- [37]. Jeon JS, Bersini S, Gilardi M, Dubini G, Charest JL, Moretti M, Kamm RD, Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation, Proc. Natl. Acad. Sci. U. S. A, (2014) 2–7.
- [38]. Zheng Y, Sun Y, Yu X, Shao Y, Zhang P, Dai G, Fu J, Angiogenesis in Liquid Tumors: An In Vitro Assay for Leukemic-Cell-Induced Bone Marrow Angiogenesis, Adv. Healthc. Mater., 5 (2016) 1014–1024. [PubMed: 26924785]
- [39]. Tao S, Wang S, Moghaddam SJ, Ooi A, Chapman E, Wong PK, Zhang DD, Oncogenic KRAS confers chemoresistance by upregulating NRF2, Cancer Res., 74 (2014) 7430–7441. [PubMed: 25339352]
- [40]. Riahi R, Wang S, Long M, Li N, Chiou PY, Zhang DD, Wong PK, Mapping photothermally induced gene expression in living cells and tissues by nanorod-locked nucleic acid complexes, ACS Nano, 8 (2014) 3597–3605. [PubMed: 24645754]
- [41]. Wang S, Riahi R, Li N, Zhang DD, Wong PK, Single Cell Nanobiosensors for Dynamic Gene Expression Profiling in Native Tissue Microenvironments, Adv. Mater, 27 (2015) 6034–6038. [PubMed: 26314800]
- [42]. Wang S, Sun J, Zhang DD, Wong PK, A nanobiosensor for dynamic single cell analysis during microvascular self-organization, Nanoscale, (2016) 19–22. [PubMed: 27934987]

- [43]. Siekmann AF, Lawson ND, Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries, *Nature*, 445 (2007) 781–784. [PubMed: 17259972]
- [44]. Iso T, Hamamori Y, Kedes L, Notch signaling in vascular development, *Arterioscler. Thromb. Vasc. Biol*, 23 (2003) 543–553. [PubMed: 12615665]
- [45]. Riahi R, Sun J, Wang S, Long M, Zhang DD, Wong PK, Notch1-Dll4 signalling and mechanical force regulate leader cell formation during collective cell migration, *Nat. Commun.*, 6 (2015) 6556. [PubMed: 25766473]
- [46]. Tung JJ, Tattersall IW, Kitajewski J, Tips, stalks, tubes: Notch-mediated cell fate determination and mechanisms of tubulogenesis during angiogenesis, *Cold Spring Harb. Perspect. Med.*, 2 (2012).
- [47]. Kim S, Lee H, Chung M, Jeon NL, Engineering of functional, perfusable 3D microvascular networks on a chip, *Lab Chip*, 13 (2013) 1489–500. [PubMed: 23440068]

Figure 1.

A 3D microengineered biomimetic angiogenesis device and the gold nanorod-locked nucleic acid (GNR-LNA) nanobiosensor for investigation of Notch signaling in angiogenesis. (A) Schematic of the 3D angiogenesis device and cell seeding configuration. Two side channels (designated as endothelial channel and fibroblast channel) were separated by the central (gel) channel filled with fibrin gel. Endothelial cells (ECs) were loaded into the endothelial channel and allowed to adhere on the gel interface, whereas human bone marrow fibroblasts (HS5) suspended in fibrin gel were placed in the two reservoirs of the fibroblast channel. Paracrine interaction between HS5 and ECs led to invasion of ECs into the central gel region to form neo vessels. (B) Biosensing mechanism of the GNR-LNA nanobiosensors. Binding of LNA probe, a 20-base nucleic acid molecule labeled with a fluorophore (6-FAM (fluorescein)) at the 5' end, to GNR quenches the fluorophore. After internalization by ECs via endocytosis, LNA probes bind targeted mRNA in the cytoplasm and reacquire

fluorescence. Fluorescent intensity thus serves as an indicator of the expression level of target mRNA. (C) Dark-field image showing an EC with internalized GNR (bright spots in the image). DAPI staining (blue) labels the nucleus. (D) Dll4-Notch signaling between a tip cell and a stalk cell.

Figure 2.

Involvement of Notch signaling in regulating angiogenic morphogenesis. (A) Representative confocal images in z-projection showing angiogenic morphogenesis of ECs treated without (control) or with 20 μM DAPT or 20 μM Jagged1 as indicated. Images were acquired 48 hr after initial cell seeding. Samples were stained for Dll4 (green), F-actin (red; by phalloidin), and nucleus (blue; by DAPI), respectively. Dashed trapezoids highlight PDMS supporting structures at the fibrin gel interface within the endothelial channel. The white rectangles in the top images mark the areas recorded using 40 $\times$ objectives to show tip cell morphologies (middle). Single confocal image slices (bottom) show branching of neo blood vessel networks. (B-D) Bar plots of invasion area (B), invasion distance (C), and tip cell number (D). (E) Schematic of vessel branching. (F) Bar plot of invasion coverage percentage.

(isolated: crosshatch, with stalks: solid; D) under different conditions as indicated. (E) Representative confocal image slices showing 10 sub-regions with equal areas between supporting posts and leading tip cells. (F) Quantification of angiogenic invasion area coverage (%) in the 10 sub-regions between supporting posts and leading tip cells under different conditions as indicated. Error bars, S.E.M., with $n = 10 - 15$ fields of view from > 4 devices. p -values were calculated using two-sample t -test with respect to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Figure 3.

Involvement of Notch signaling in regulation of proliferation of ECs during angiogenesis. (A) Fluorescent images showing proliferation of invading ECs in the fibrin gel. Images were taken 21 hr after initial cell seeding. Cell nuclei with newly synthesized DNA within last 3 hr were labeled in red using the Click-iT EdU Imaging Kit (Invitrogen), while all cell nuclei were labeled in green. Bright-field images were superimposed to outline angiogenic sprouts. (B&C) Percentage (B) and number (C) of EdU labeled nuclei in the angiogenic network per field of view. (D) Bar plot showing percentage of EdU labeled nuclei in the 10 sub-regions between supporting posts and leading tip cells under different conditions as indicated. Error

bars, S.E.M., with $n = 10 - 15$ fields of view from > 4 devices. p -values were calculated using two-sample t -test with respect to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

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Figure 4.

Notch signaling regulates directional invasion of ECs during angiogenesis. (A) Live-cell confocal images showing dynamic directional EC invasion in the fibrin hydrogel under the control condition. Orientation angle α of tip cell invasion was calculated using coordinates of the starting and end points of an individual cell invasion trajectory 48 hr after initial cell seeding as shown in the last image. (B) Polar histograms showing distribution of orientation angle of tip cell invasion trajectory without (control) or with DAPT and Jagged1 treatments as indicated. Each bar in the histograms represents the percentage of orientation angle across the entire population. (C&D) Bar plots showing percentage of tip cells with the orientation angle α within $\pm 4^\circ$ (C) and migration velocity of tip cells (D) under different conditions as indicated. Error bars, S.E.M., with $n = 40 - 50$ cells from two devices. p -values were calculated using two-sample t -test with respect to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Figure 5.

Dll4 mRNA expression in tip cells during angiogenesis. (A) Confocal images showing fluorescence signals from GNR-LNA nanobiosensors for detection of Dll4 mRNA in invading tip cells under different conditions as indicated. Images were recorded 12 hr after initial cell seeding. (B) Mean fluorescence intensity of invading tip cells at different time points after initial cell seeding under different conditions as indicated. (C) Cumulative probability of tip cell fluorescence intensity (see Materials and Methods) at different time points after initial cell seeding under different conditions as indicated. Error bars, S.E.M., with $n = 130 - 150$ cells from 3 devices. p -values were calculated using two-sample t -test with respect to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.