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Dll4-containing exosomes induce capillary sprout retraction in a 3D microenvironment

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Delta-like 4 (Dll4), a membrane-bound Notch ligand, plays a fundamental role in vascular development and angiogenesis. Dll4 is highly expressed in capillary endothelial tip cells and is involved in suppressing neighboring stalk cells to become tip cells during angiogenesis. Dll4-Notch signaling is mediated either by direct cell-cell contact or by Dll4-containing exosomes from a distance. However, whether Dll4-containing exosomes influence tip cells of existing capillaries is unknown. Using a 3D microfluidic device and time-lapse confocal microscopy, we show here for the first time that Dll4-containing exosomes causes tip cells to lose their filopodia and trigger capillary sprout retraction in collagen matrix. We demonstrate that Dll4 exosomes can freely travel through 3D collagen matrix and transfer Dll4 protein to distant tip cells. Upon reaching endothelial sprout, it causes filopodia and tip cell retraction. Continuous application of Dll4 exosomes from a distance lead to significant reduction of sprout formation. This effect correlates with Notch signaling activation upon Dll4-containing exosome interaction with recipient endothelial cells. Furthermore, we show that Dll4-containing exosomes increase endothelial cell motility while suppressing their proliferation. These data revealed novel functions of Dll4 in angiogenesis through exosomes.

S prouting angiogenesis is a complex and highly dynamic process involving interactions between endothelial cells and their environment[1]. In response to VEGF stimulation, a migratory endothelial cell at the vessel's tip, called the tip cell, extends filopodia and increases motility whereas the following endothelial cells, called stalk cells, proliferate and form the trunk of the new vessel[2]. The main features of tip cells are their location at the forefront of vessel branches, highly polarized nature, and numerous filopodia probing the environment, while migrating towards angiogenic cues[2,3]. Studies in mouse and zebrafish development have illustrated the importance of the VEGF and Notch signaling pathways for the specification of the endothelial cells into tip cells and stalk cells during angiogenic sprouting process in physiological and pathological conditions[4].

One of the key factors in tip cell selection is Notch signaling. The Notch pathway is an evolutionary conserved intercellular signaling pathway involved in a number of biological processes including cell fate determination, cellular differentiation, proliferation, survival, and apoptosis[5–7]. In mammalian cells, the Notch family of proteins is composed of four single pass transmembrane receptors (Notch1-4) and five membrane bound ligands [Jagged (Jag)-1, -2, delta-like (Dll)-1, -3, and -4]. Notch signaling is unique as both ligand and receptor are transmembrane proteins. Hence for signal transduction to occur, the signal-sending cell and the signal-receiving cell have to be adjacent to one another. This unique form of juxtacrine signaling is of particular importance in development as lateral inhibition due to Notch signaling allows for tissue specification, whereby cells adopting a particular cell fate inhibits the neighbouring from doing likewise[4,8].

Mutations of Notch receptors and ligands in mice and human lead to abnormalities in the vascular system[9]. Endothelial cells express Notch1, 3, and 4 and Dll-1, Dll-4, Jag-1, and Jag-2. Upon ligand binding, Notch is cleaved intracellularly to generate the Notch intracellular domain (NICD) and act as a transcriptional regulator[4]. Studies have shown that a VEGF gradient is important in the process of selection and induction of endothelial tip cells[2]. Upon binding to VEGFR2, VEGF initiates a signaling cascade and enable one endothelial cell to take the lead and preventing other neighboring cell from becoming tip cells through juxtacrine Dll4/Notch signaling[10–12]. Dll4 induction by VEGF activates the Notch pathway in adjacent endothelial cells, reducing the expression of VEGFR2 and VEGFR3 in these cells, and consequently suppresses the tip cell phenotype and makes them stalk cells[13].

Further studies on the implication of VEGF pathway in tip and stalk cell selection using mosaic analysis *in silico* demonstrated that differential levels of VEGFR1 and VEGFR2 in endothelial cells affect their potential to become tip cell during sprouting angiogenesis[14]. Endothelial cells with lower VEGFR1 expression has a higher probability in adopting the tip cell position than those with lower VEGFR2 and this selection only occurs in the presence of a functional Notch system where it modulates the expression of Dll4[14]. More recently, Sheldon *et al* demonstrated a new form of signaling for Dll4, whereby it is incorporated into exosomes and can be transferred from one cell type to another beyond cell-cell contact and become incorporated into the recipient cells in vitro and in vivo. In this model they showed that Dll4-containing exosomes can inhibit Notch signaling of the recipient cells in vitro and appear to switch their phenotype towards tip cells by increasing Dll4 to Notch-receptor ratio[15].

Exosomes are small extracellular membrane vesicles from the endosomal origin with a mean size of 40–100 nm that are released from a variety of cell types either spontaneously or by induction. The main function of exosomes is cell-to-cell communication and influencing cell signaling pathway[16]. Exosomes come from the intraluminal vesicles of multivesicular bodies that are formed through the inward budding of late endosome membrane. Multivesicular bodies can either fuse with lysosomes to degrade their cargo or fuse with plasma membrane or release their internal vesicles into extracellular space[17]. Exosomes contain various molecular constituents of their cell of origin including proteins and nucleic acids and can be internalized by recipient cells through various mechanisms such as endocytosis, phagocytosis, micropinocytosis or even direct membrane lipid fusion[18–21]. Recent studies on exosome uptake mechanism using human umbilical vein endothelial cells (HUVECs) have suggested that exosomes are internalized by endocytosis rather than by membrane fusion at the plasma membrane and this uptake highly depends on ERK1/2-heat shock protein 27 signaling[22].

Nevertheless, it is not known whether Dll4-containing exosomes would influence an already formed capillary by transferring Dll4 into the sprouting endothelial cells. Answers to this question have more significance for in vivo situations where angiogenesis rather than vasculogenesis is the dominant mode of neovascularization.

In this study we used a unique microfluidic in vitro angiogenesis system in which a VEGF gradient created over the gel matrix, stimulates endothelial sprout formation in a 3D microenvironment. This 3D microenvironment more closely resembles in vivo tissues than the 2D vasculogenesis assays. We show that Dll4 exosomes can dose-dependently induce retraction of the tip cell in an existing capillary sprout after travelling through the 3D collagen matrix. We show that Dll4 exosomes can activate Notch signaling in recipient microvascular endothelial cells and propose that Dll4 exosomes can suppress angiogenic sprouting by activating Notch signaling.

Results

Dll4 exosomes and their characterization. It has been shown previously that Dll4 is incorporated into endothelial exosomes[15]. HMVECs contain low levels of Dll4 under normal conditions. They can however, be stimulated to produce more Dll4 by plating the cells onto Dll4 coated flasks. This leads to activation of Notch signaling, transcription and production of Dll4[26]. However, to increase the exosome yield, we transduced U87 human glioma cell line, which does not express endogenous Dll4, with recombinant lentivirus harboring an Dll4 expression cassette to overexpress human Dll4 (U87-Dll4). This allowed us to produce a larger amount of exosomes with and without Dll4.

Exosomes from both HMVECs and U87 were examined under TEM for their intact structure and sizes (Figure 1A). The size and homogeneity of the exosomes were also examined by Dynamic Light Scattering (DLS). Exosomes from HMVECs are homogeneous with an average hydrodynamic radius of 135 nm $\pm$ 8 nm whereas exosomes from U87 cells are heterogeneous with an average hydrodynamic radius of 191 nm $\pm$ 36 nm. Dll4 can be detected in exosomes isolated from both cell types where they appear to have the same molecular weight. Exosome markers Alix or CD63 are present in these Dll4-containing exosomes (Figure 1B and C). We analyzed the expression of four additional exosomes markers in our U87 derived exosomes to show that the overall composition of exosomes are similar (Figure 1B). Accordingly, Dll4 protein is only expressed in U87-Dll4 cells, but not in parental U87 cells (Figure 1D). For subsequent experiments, we used exosomes from U87 cell line unless specified and refer to them as Dll4 exosomes and/or Control exosomes.
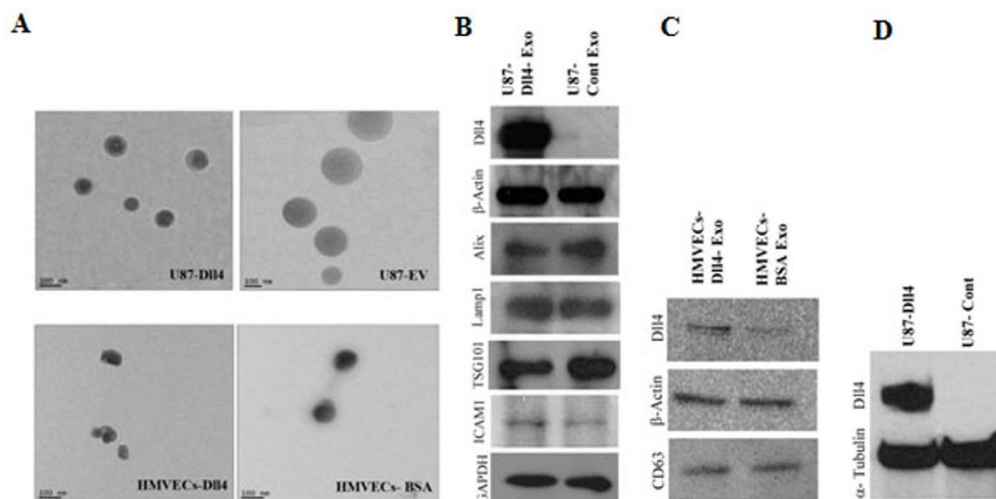

Figure 1 | Characterization of exosomes. Exosomes were isolated from U87 cells and HMVECs using ultracentrifugation protocol. They were re-suspended in PBS and fixed prior to imaging with TEM, showing that exosomes are intact and determine their sizes. A) Exosomes from Dll4-expressing retrovirus transduced U87 cells and from HMVECs cultured on recombinant human Dll4 coated flasks and BSA coated flasks (scale bar 100 nm). U87 exosomes are very heterogeneous whereas HMVECs exosomes are homogeneous. The biochemical assays on exosomes, using equal amount of total protein lysate, showing that the expression of Dll4 and exosome specific proteins in B) U87-Dll4 and U87-cont and C) HMVECs-Dll4 and HMVECs-BSA exosomes lysate. D) Cell lysate from U87 overexpressing Dll4 showing high expression level of Dll4 that is normally absent in the native U87 cell line. The blot images were cropped from the original full length images (Supplemental Figures S6–S8).

Dll4 exosomes cause sprout retraction after traveling through a 3D collagen matrix. To assess the effect of Dll4 exosomes on HMVECs capillary sprout, we seeded HMVECs into the microfluidic devices (MFDs) and conditioned them for three days with a VEGF gradient which allows the cells to form capillary sprouts. On the third day we applied 50 μgmL^{-1} of exosomes, as was reported by Sheldon *et al.* 2010, to the gradient media channel and monitored the effects on the sprouts with time-lapse confocal microscopy for nine hours. Application of Dll4 exosomes to the pre-formed sprouts in a gradient channel caused the retraction of the tip cell filopodia and the existing sprout itself. In contrast, control exosomes, isolated from U87 cells, did not affect the sprouts (Figure 2A) (Supplemental Figure S2, video A). Our data shows that as early as two hours after application of Dll4 exosomes, the sprouting tip cells and tip cell filopodia were retracted. The retractions are Dll4 exosome dose dependent with no significant retraction at 1 μgmL^{-1} of Dll4 exosomes (Supplemental Figure S2, video B). Significant retractions were observed from 5 μgmL^{-1} of Dll4 exosomes (Figure 2A). Furthermore, the application of exosomes either to the cell monolayer or to both cell monolayer and gradient channel, had no significant effect on the sprouts (data not shown).

Dll4 exosomes reduce the number of capillary sprouts in a dose dependent manner after its application to the gradient channel of MFD. In order to explore whether the immediate sprout retraction would influence the net sprout formation over the longer term, we extended the duration of the above experiment to three days. HMVECs in MFDs were conditioned for three days. On day three, images from all 37 slots of MFDs were taken using 10× objective. This is to set a base line of sprout formation before application of exosomes to the gradient channel. The sprout assays were incubated with four different concentrations of exosomes, 50, 20, 5, and 1 μgmL^{-1}, in MFD for 3 days before they were fixed and stained for actin cytoskeleton with rhodamine phalloidin. The number of sprouts in all 37 slots were recorded and analyzed. The results clearly indicated that the Dll4 exosomes causes a significant reduction in sprouts number at all concentrations except for 1 μgmL^{-1} concentration (Figure 2B). In comparison, exosomes applied directly to endothelial cells channel did not cause any significant changes in sprout numbers (Supplemental Figures S3). These data provide further evidence that Dll4 exosomes causes retraction of existing sprouts in a dose dependent maner, resulting in a net loss of capillary sprouts.

Dll4 exosomes can travel through collagen matrix and incorporate into endothelial cells. To confirm that the effect of sprout retraction is due to Dll4 exosomes that have travelled through the collagen matrix and interacted with the endothelial cells in the sprout upon reaching them, we labeled the exosomes with fluorescent membrane dye PKH26 prior to its application into the gradient channel in MFD. HMVECs were conditioned with VEGF for three days. Upon initiation of sprout formation on day three, labeled exosomes were applied to the gradient channel at 50 μgmL^{-1} and time-lapse microscopy was used to monitor the impact for 9 hours. As exosomes are too small to be visualized under light microscope, we fixed MFDs at the end point and carried out immunocytochemistry with Dll4 antibody and nuclear stain to assess the position of the exosomes as well as Dll4 expression using confocal microscopy. Endothelial cells in a distance clearly received and have taken up the labeled exosomes (red color) (Figure 3B). The collagen gel is porous and allows exosomes to travel through and reach the cell monolayer. Moreover, we show that Dll4 exosomes are expressing Dll4 as expected and appeared to have been internalized by endothelial cells (Figure 3C). The time required for exosomes to travel across the collagen gel in MFD (a distance of 1.3 mm) and reach the monolayer cells is estimated to be about 45–60 minutes. To
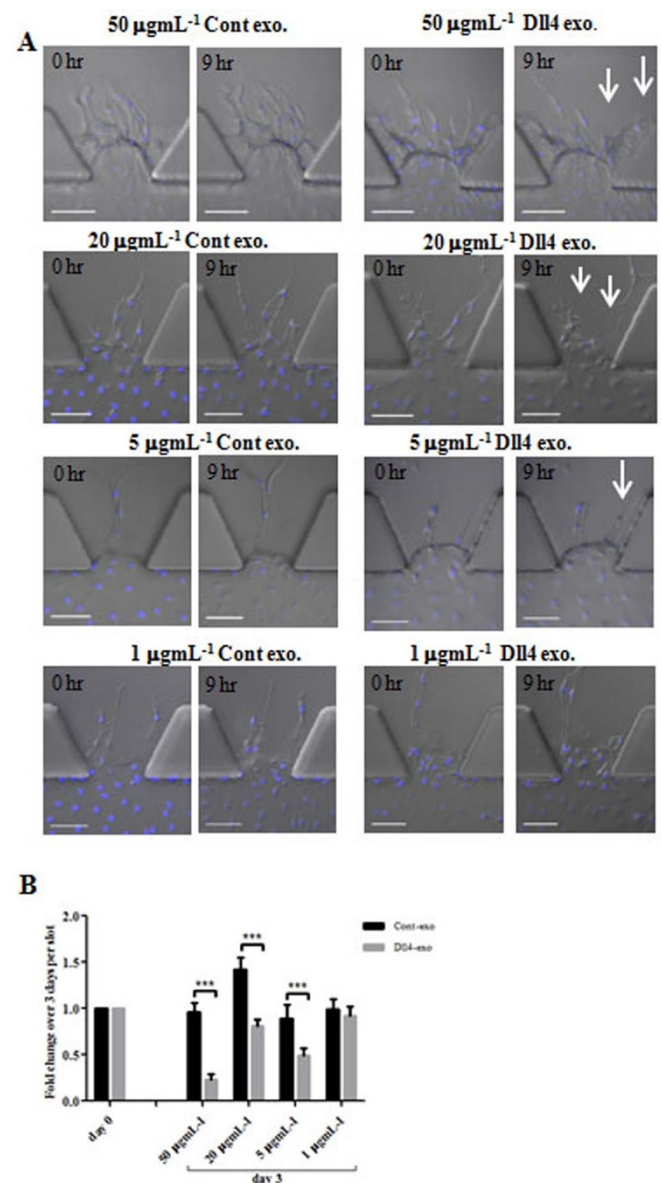

Figure 2 | Dll4 exosomes cause retraction of tip cells and their filopodia at the leading edge of sprouts upon its gradient application. (A) HMVECs were seeded into MFDs and upon formation of the monolayer, they were conditioned for 3 days with VEGF prior to exosomes application in the gradient i.e. into the media channel that contain 40 ng/ml VEGF. The experiment was carried out individually for four different concentrations of exosomes. They were immediately imaged using time-lapse confocal microscopy for 9 hours and then fixed. Arrows indicate the retraction of the sprouts after application of Dll4 exosomes. Scale bars 100 mm. (B) Dll4 exosomes reduces the number of sprouts after their continues application to the gradient (media channel) in a dose dependent manner. HMVECs were seeded and after formation of the monolayer they were conditioned with VEGF for three days to allow formation of the sprouts. At day three images were taken from all 37 slots by the light microscopy before addition of exosomes in the gradient at different concentration. This is labeled as day 0 on the bars. Fresh medium with exosomes were added daily for the next two days and images were taken again on day 3. The number of sprouts in each slots were counted from the images and plotted using Prism software. The experiment was performed at four concentrations of exosomes and three repeats. *** P < 0.01 significant.

further confirm that Dll4 exosomes can move across the collagen matrix, we injected 4 × 10^{12} of 100 nm and 200 nm fluoresbrite fluorescent microspheres separately into the cell seeding port of
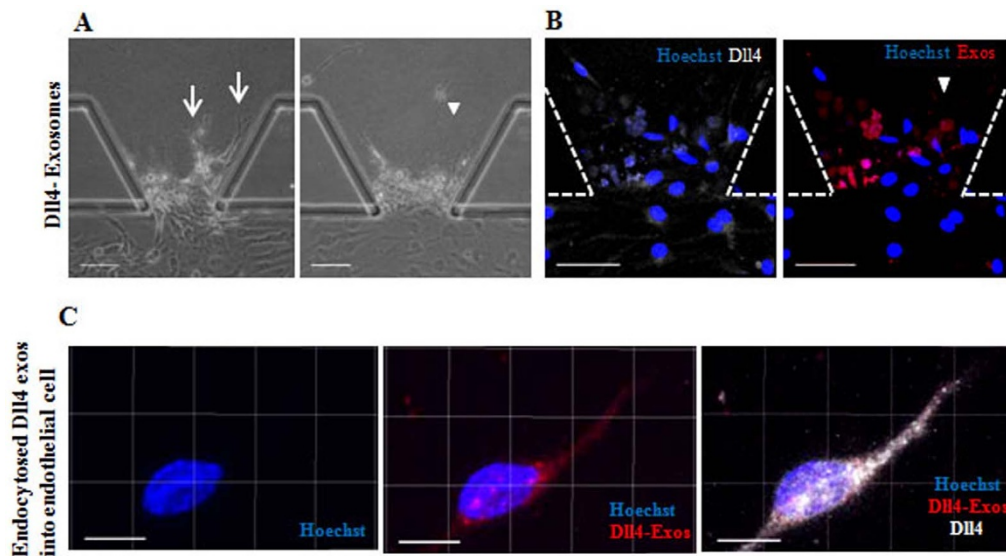
Figure 3 | Exosomes travel through the collagen matrix and are internalized by endothelial cells. Dll4 exosomes were labeled with red fluorescent dye PKH26 and applied in the gradient channel in MFD that has already been conditioned for 3 days with VEGF. Fresh medium with labeled exosomes were added daily for the next two days They were fixed and immunocytochemistry was performed. (A) Phase images of endothelial sprouts at Day 0 without Dll4 exosomes where white arrows indicating the sprouts, and on Day 3 after addition of exosomes every 24 hour for three days where the sprouts are retracted (white arrow head) Scale bar: 50 μm. (B) Confocal images of magnified regions of fixed cells from day 3 indicating Dll4 expression and co-localization of Dll4 with exosomes. Scale bar: 50 μm. (C) Examples of internalized exosomes and co-localization with Dll4 at higher magnification. Scale bar:10 μm.

MFDs and ran the time-lapse for nine hours as we did for exosomes. The result confirmed that microspheres of exosomes sizes can readily travel through the collagen matrix in MFD to reach the distant destination (Supplemental Figures S4).

Dll4 exosomes activates Notch signaling. We assessed the effect of Dll4 exosomes on Notch signaling by analyzing the downstream Notch target genes using qRT-PCR. HMVECs were cultured in a six well plate at a density of 2.5×10^5 cell per well and incubated for 36–48 hours to allow cells to reach 80–85% confluency. They were starved, and conditioned for 8 hours with 20 ngmL^{-1} VEGF and three concentrations of exosomes, i.e. 1 μgmL^{-1}, 5 μgmL^{-1} and 20 μgmL^{-1}. The RNA from the conditioned cells was extracted and analyzed for Notch target genes Hey1 and Hes1 by qRT-PCR. As shown in Figure 4, both were activated by Dll4 exosomes, with more apparent effects at higher concentration.

Dll4 exosomes increase cell motility. One other key behavioral changes that we observed in our time-lapse data was a significant increase in cell motility in the presence of Dll4 exosomes. In order to quantify the cell's motility, we tracked all the cell nuclei in time-lapse images using a custom MATLAB code. We analyzed all the time-lapse data from four concentrations of 50, 20, 5, and 1 μgmL^{-1} exosomes that were added with the gradient in each MFDs. Both cells in the monolayer and the ones already migrating into the 3D collagen gel were analyzed. Our data showed that there is a significant increase in cell motility in cells that were treated with Dll4 exosomes in comparison to that of control exosomes (Figure 5). This experiment further confirms the propagation of exosomes across the matrix.

It has been reported that the Snail family member *Slug* induces a motile phenotype binding and represses the VE-cadherin promoter[27]. It has also been proposed that as Notch signaling downregulates VEGFR2, VEGFR3 and neuropilin-1 (Nrp1) level, it could potentially reduces endothelial cell motility. Hence we analyzed the RNA expression level of slug and Nrp1 after exposing HMVECs to 1 μgmL^{-1} and 20 μgmL^{-1} exosomes. Interestingly, the results showed that the expression of Slug1 was significantly increased after

exposing the cells to Dll4 exosomes for 8 hr (Figure 5C) but there was no change in the expression level of Nrp1 (Supplemental Figure S5).

Dll4 exosomes reduce cell proliferation. It has been shown that Notch activation in human umbilical vein endothelial cells (HUVECs) reduces cell proliferation[8,28,29]. To investigate whether Dll4 exosomes can also affect endothelial cell proliferation via Notch activation, we examined the rate of proliferation in HMVECs under Dll4 exosome treatment in the MFD environment. We seeded HMVECs into MFDs and allow them to form a confluent monolayer. They were then conditioned with VEGF for three days before application of exosomes. After 24 hours of exosomes treatment, cells were fixed and stained with Ki67 antibody. The total number of Ki67 positive cells in all 37 slots of MFDs were counted. As there were fewer sprouts in those that were treated with higher concentration of Dll4 exosomes, the counting was performed on entire endothelial cell population. Interestingly, the proliferation rate was significantly lower in cells treated with 20 and 5 μgmL^{-1} Dll4 exosomes but not with 1 μgmL^{-1} Dll4 exosomes (Figure 6).

Discussion

In this work, we used a unique microfluidic device (MFD) capable of generating a chemically controlled 3D microenvironment with a concentration gradient of desired biological factors. This device allowed us to acquire the time-lapse data of sprout growth towards a VEGF gradient and the sprout response to Dll4 exosomes in the 3D microenvironment. This 3D MFD environment mimics the in vivo tissue environment more closely than cells cultured on 2D surfaces without any extracellular matrix and growth factor gradient. The biological importance of exosomes released from cells in a tissue either constitutively or upon environmental stimulation has become increasingly appreciated in various physiology and pathophysiology processes[30]. The discovery that the transmembrane Dll4 ligand is also incorporated into exosomes and can influence angiogenesis at a distance through a signaling mechanism distinct from juxtacrine cell-cell signal transduction is most intriguing[15]. However, how the Dll4 exosomes influence angiogenesis is not well understood.
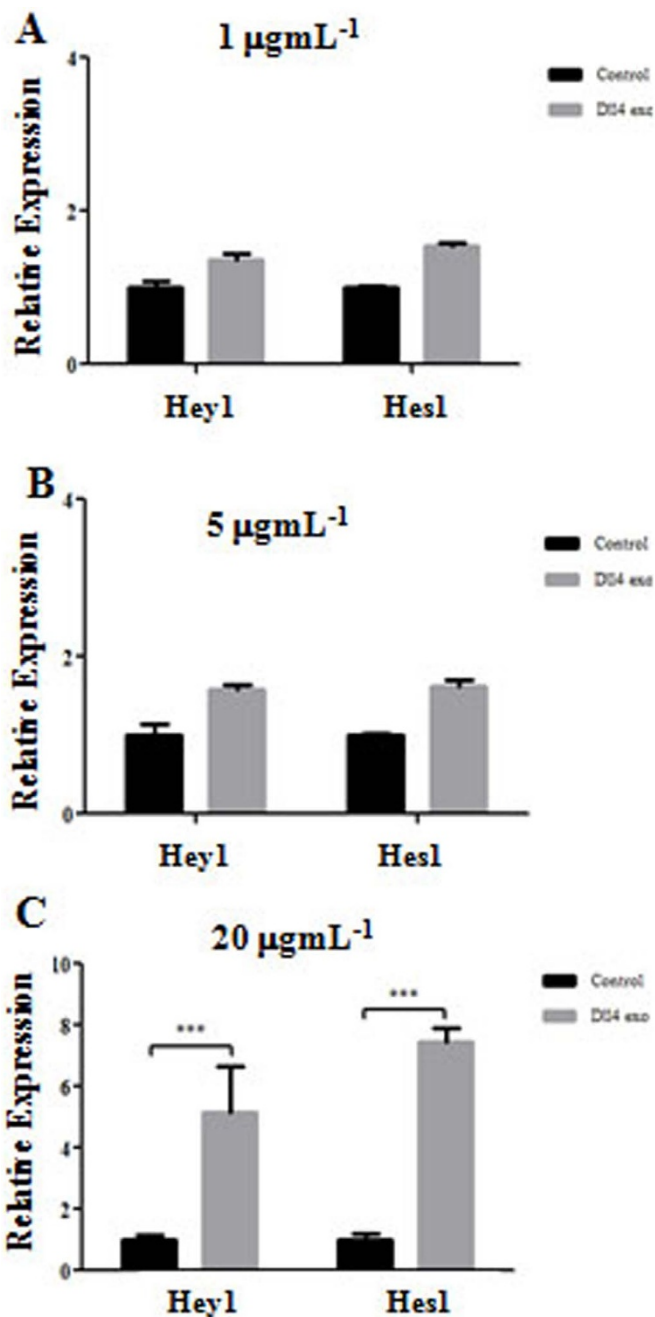
Figure 4 | Dll4 exosomes stimulate Notch target genes. HMVECs were plated on gelatin coated 6 well plate at a density of 2.5×10^5 per well. HMVECs were grown to 80–90% confluency before being starved for 2 hour. They were conditioned with three concentrations of control exosomes and Dll4 exosomes for 8 hours prior to cell extraction and RNA isolation. qPCR was performed to examine the Notch target genes, Hey1 and Hes1. Histograms indicating the relative expression of the genes at three different exosomes concentrations (A) 1 μgmL^{-1}, (B) 5 μgmL^{-1}, and (C) 20 μgmL^{-1}, *** $P < 0.01$ significant.

Figure 5 | Dll4-containing exosomes increase cell motility when applied in the gradient. HMVECs were seeded into MFDs and after formation of the monolayer, they were conditioned for 3 days with VEGF prior to exosomes application in the gradient. They were immediately imaged using time-lapse confocal miscoscopy for 9 hours. The cell motility in both monolayer channel and inside the 3D collagen gel was determined using the time lapse data and in house tracking algorithm. (A) a representative image of cell motility analysis from one of the slot regions in MFDs that are treated control and Dll4 exosomes. Each line shows the cell trajectory during the run. (B) The experiment was performed with four different concentrations of exosomes. Dll4 exosomes significantly increase the cell motility in all concentrations. (C) HMVECs increase the expression level of Slug1 mRNA in response to 20 mgmL^{-1} Dll4 exosomes. HMVECs were seeded in 6 well plates at a density of 2.5×10^5 cells per well. Once they reached 80–90% confluency, 20 μgmL^{-1} exosomes were applied in each well and incubated for 8 hr. RNA extraxtion was followed and used in qPCR. *** $P < 0.01$ significant.

Our results show for the first time that Dll4 exosomes cause retraction of the existing capillary sprouts when they traveled from a distance through an extracellular collagen matrix. We showed that Dll4 exosomes trigger the retraction of tip cell filopodia followed by the tip cell itself. We propose that upon binding of Dll4 exosomes to the tip cells, Dll4 exosomes act as a Nano cell in activating Notch receptor in the recipient cell. We have shown that Notch signaling is activated in the recipient cells upon interaction with Dll4 exosomes,
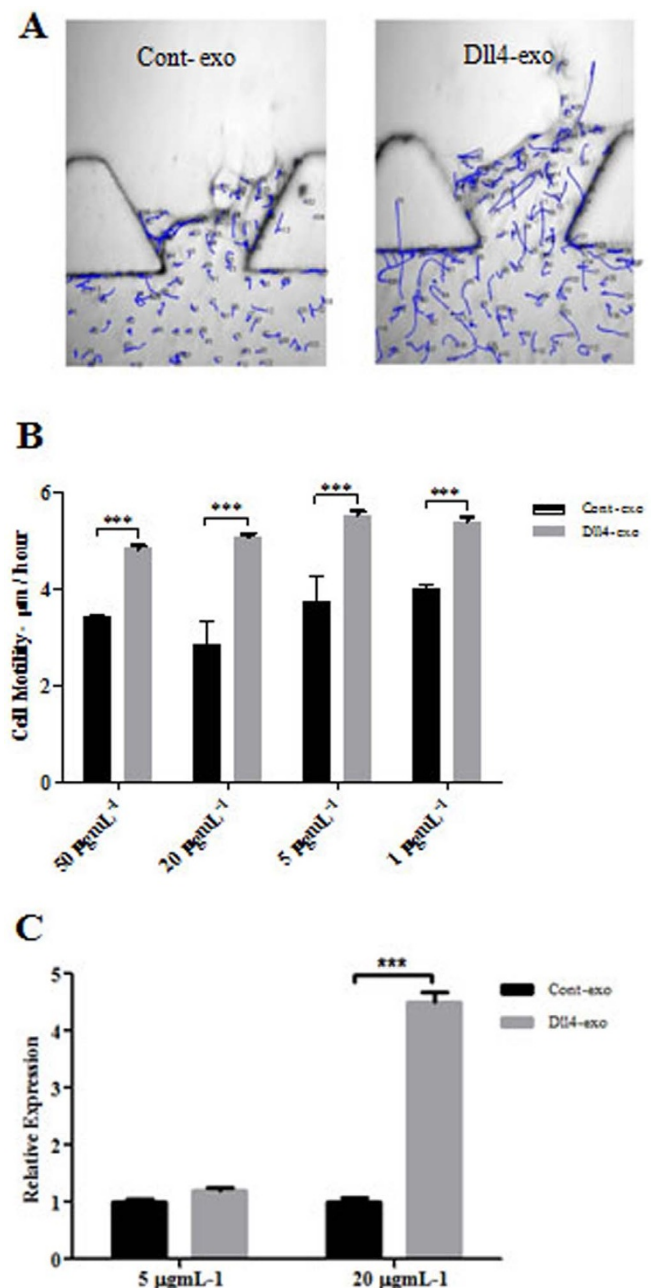
demonstrated by the transcription activation of the Notch target genes Hey1 and Hes1 (Figure 4). It has been previously shown that activation of Notch signaling leads to downregulation of VEGFR2 and VEGFR3, a phenomenon that occurs predominantly in stalk cells[13,31,32]. However, we did not observe any significant changes in VEGFR2 or VEGFR3 levels (data not shown). Hence we propose that contact with Dll4 exosomes immediately triggers Notch activation in the tip cell and cause a phenotype change from the tip to stalk cells, in a process which is likely to be independent of VEGFR2 and VEGFR3. This leads to a loss of the tip cell's leading role, resulting in retraction of filopodia and subsequently retraction of the sprout. Notch is known to be required cell-autonomously for stalk cell specification by actively repressing tip cell phenotypes[12]. Thus, our results suggest that Dll4 in exosomes function similarly to Dll4 on the cell membrane in activating Notch signaling in recipient cells and promoting a stalk cell phenotype in the recipient cells.

It is known that Notch1 and Notch4 are expressed by endothelial cells. Notch1 is the primary endothelial Notch receptor during developmental angiogenesis[33]. In addition, Dll4 has also been reported to bind Notch2 on endothelial cells where Notch2 may be responsible in regulating Hey2 expression[29,32]. It is perceivable that different Notch receptors can activate distinct downstream genes and generate varied impacts on endothelial cell behaviors. Further investigation is required to explore the role of Dll4 exosomes and their interaction with different Notch receptors and their involvement in regulating Notch target gene expression.

Our results showed different responses of ECs to Dll4 exosomes than Sheldon et al 2010. Sheldon et al also used Dll4-containing exosomes secreted by Dll4-overexpressing U87 glioma cells. However, the pro-angiogenic/pro-tip cell effects on HUVECs in a 2D uniform angiogenic environment differ from the sprout retracting effect/anti-tip cell effect in 3D VEGF gradient environment we observed using HMVECs. Different endothelial cell subtypes and the different microenvironment in experimental settings may be the reason for the different results observed. Thus, further investigations are required to clarify if local tissue microenvironment could influence the outcome of the Dll4-containing exosomes on angiogenesis.

The sizes of exosomes in our preparation appear to be larger on average than the published data in U87 exosomes which is reported to be 50–100 nm[15]. Different methods used to estimate the exosomes size may account for the differences. We have used both DLS and TEM to characterize the exosomes. Exosomes from HMVECs are homogeneous with average hydrodynamic radius of 135 nm ± 8 nm whereas exosomes from U87s are very heterogeneous with an average hydrodynamic radius of 191 nm ± 36 nm. Overall, the size distribution, ultrastructural morphology and protein composition strongly suggest that our derived microvesicles are largely exosomes. We have also analyzed mRNA using randomly selected primers, that were reported to be expressed in U87 exosomes[34], and showed that the overall composition of the exosomes were not compromised after Dll4 transduction of U87 MG cells (data not shown).

Stimulation of HMVEC migration correlated with the activation of Slug, a Notch downstream target gene, known to promote cell movement[27]. It was shown that Slug is able to bind to, and repress the VE-cadherin promoter in endothelial cells. Hence, it could be possible that the observed increase in cell motility could be to the downregulation of the cell-cell adherens junction protein, VE-cadherin[27,35]. Whether this is the cause of increased cell motility and Slug expression in our model after Dll4 exosomes application needs to be investigated. Moreover, it has been shown that VEGF co-receptor Nrp1 is involved in regulating cell motility via PI3K but not cell proliferation[36]. Although it has been reported that Nrp1 downregulates Notch signaling and its C-terminal domain stimulate endothelial migration[26,29,37], We did not observe any significant change in expression level of Nrp1 in our experiment. It has been shown previously that overexpression of Jag1 in endothelial cells induces cell-cycle arrest
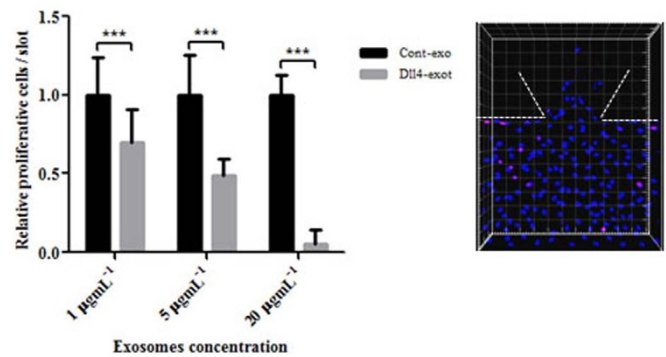

Figure 6 | Dll4 exosomes reduced cell proliferation when applied in the gradient. HMVECs were seeded into MFDs after formation of the monolayer they were starved and conditioned with VEGF for three days for sprouts to form. On day three, exosomes were applied in the gradient and incubated for 24 hours. They were fixed and stained on the following day with Ki67 antibody and imaged with the confocal microscope. The total and Ki67 positive cells both in the monolayer and the gel region were counted using Imaris software. The statistical analysis were carried out using Prism software. The experiment was performed with three concentrations of exosomes and three independent repeats. Dll4 exosomes significantly reduced cell proliferation at higher concentrations *** P < 0.01 but had no significant effect with 5 mgmL^{-1} p = 0.0537 and 1 mgmL^{-1} p = 0.35 exosomes. The panel is a representative of a single region of the MFD to indicate entire cell population (Hoechst nuclear stain) and Ki67 positive cells (pink stain).

similar to the effect of overexpressing active Notch1 and Notch4[28]. Whether Dll4 exosomes represent the similar model needs to be studied. Although we did not detect any changes in expression of Jag1 when we performed in vitro assay with Dll4 exosomes (Supplemental Figure S4), a better approach would be to test Jag1 expression in the retracted tip cell directly in the microfluidic device which is technically difficult at the present time.

Our data elaborate the complexity of Notch signaling in endothelial cells. It also demonstrated, for the first time, that Dll4 exosomes causes retraction of existing sprouts in a 3D VEGF gradient microenvironment. This study provided a platform in understanding the function of Notch signaling closer to the in vivo microenvironment. It also demonstrated that exosomes are more than biomarkers and can be developed as a tool for drug delivery.

Methods

Cell culture and reagents. Human microvascular endothelial cells (HMVEC, Lonza) were cultured in EGM-2 media (Lonza) and were used at passage 7 for all the experiments. U-87 primary glioblastoma cell line, U-87 MG, (including Dll4-overexpressing cells) were cultured in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum. We transduced U-87 tumor cell line with human Dll4 in pLVX-IRES-ZsGreen lentiviral vector to overexpress Dll4. Recombinant human Dll4 and VEGF-165 were purchased from R&D Systems.

Microfluidic device design. Microfluidic devices (MFDs) were fabricated from polydimethylsiloxane (PDMS – Dow Corning Sylgard 184) using standard soft lithography and plasma bonded to glass cover slip (Supplemental Figure S1A). The design details have been described previously[23]. The central gel region was filled with 2.5 mgmL^{-1} type I collagen at pH 7.4 (BD Biosciences) (Supplemental Figure S1A). A 2.5 × 10^6 cells mL^{-1} cell suspension was seeded into the cell seeding channel. Approximately 10 µL more total fluid volume was filled into the ports in channels during seeding to provide a minor interstitial flow to seed cells directly onto the vertical collagen wall (Supplemental Figure S1B). Cells were allowed to grow for 24 hours before the start of the experiment which found to be sufficient for a confluent monolayer to be formed on the glass substrate and the collagen-channel interface (Supplemental Figure S1B–C). By varying the composition of the cellular growth media in the channels, growth factor concentrations and gradient across the gel region can be established to stimulate cellular responses. Observation is attained via three-dimensional confocal imaging of the gel region (Supplemental Figure S1B and 1D). For all experiments using MFDs, HMVECs were starved for two hours prior

to conditioning with VEGF at 20 ngmL^{-1} in the cell monolayer and 40 ngmL^{-1} in the gradient media channel.

Exosome isolation and labeling. U-87 cells were incubated in Opti-MEM (Invitrogen) for 24 hours to obtain exosomes as described previously[15]. HMVECs were platted on Dll4 coated plates to induce the production of Dll4 and subsequently produce exosomes containing Dll4. To eliminate other exosomes contaminants in the media, EGM-2 had previously been centrifuged at 110000 g for 16 hours. Exosomes were harvested from cell media using ultracentrifugation as described previously[24]. The resulting exosomes were resuspended in PBS and subsequently used in experiments. The exosomes were labeled with the fluorescent membrane dye PKH26 (Sigma-Aldrich) according to manufacturer's instructions.

Transmission Electron Microscope and Dynamic light scattering of exosomes. Exosomes were re-suspended in PBS and fixed in 2% paraformaldehyde (PF) and 2.5% glutaldehyde and imaged using Transmission Electron Microscope (TEM, Jeol 2010). In addition to TEM, Dynamic Light Scattering (DLS), Dynapro (Protein Solutions) was used to analyze exosome's diameter in PBS suspension. The hydrodynamic radii were determined from the mean of at least 20 readings.

Time-lapse study. HMVECs in MFDs were allowed to form a confluent monolayer prior to conditioning. They were conditioned with VEGF for three days and on day 3, cells in MFD stained with Hoechst briefly before addition of exosomes in the gradient channel along with VEGF at 40 ngmL^{-1}. They were immediately imaged using Olympus FV1200 for nine hours. MFDs were fixed at the end point and immunostained. The video clips produced using Imaris 7.2 (Bitplane).

Western blotting. Proteins were extracted from cells and exosomes using standard methods and quantified using Bradford's protein assay. Proteins were separated using SDS-PAGE. 10–20 μg of proteins per run was used unless specified. Primary antibodies used were Alix and Dll4 (Cell Signaling), CD63, β- actin (Santa Cruz), α-tubulin (Sigma), GAPDH, TSG101, ICAM1 (abcam), and Lamp1 (R&D Systems).

Quantification of sprout number. HMVECs in MFDS were conditioned with 20 ngmL^{-1} in the cell monolayer channel and 40 ngmL^{-1} in the opposite gradient channel. The above conditioning was maintained throughout the experiment and the medium was changed every 24 hours. On the third day, just before addition of exosomes, images were taken from all 37 regions of each device using Olympus inverted microscope, CKX41. This would be day 0 in the experiments. They were medium changed as before, every 24 hours and images were taken at day two and day three before being fixed with 4% paraformaldehyde.

RNA preparation and quantitative Real- Time PCR. Total RNA was extracted using RNeasy mini-kit according to manufacture instructions (Qiagen). Quantitative Real-Time PCR (qRT-PCR) was set up as one step reaction using 50 ng RNA per reaction, 10 μl SYBR Green PCR master mix, and SuperScript® III reverse transcriptase (Invitrogen). The reactions performed in triplicate and in an ABI StepOne Real-Time-PCR Systems (Applied Biosystems). Primers used are: GAPDH forward (5′ATGGAAATCCCATCACCATCTT3′) and reverse (5′ CGCCCCACTT-GATTTTGG3′), Hey1 forward (5′GCGCACGCCCTTGCT3′) and reverse (5′GCC-AGGCATTCCCGAAAT3′), Hes1 forward (5′TCCGGAGCTGGTGCTGAT3′) and reverse (5′TCCAGGACCAAGGAGAGAGGTA3′), Slug1 forward (5′CAGCTACC-CAATGGCCTCTCT3′) and reverse (5′GGACTCACTCGCCCCAAAG3′). The differences between experimental groups were analyzed using student's t test.

Immunocytochemistry (ICC). Endpoint staining for F-actin and nuclei (rhodamine and Hoechst, Invitrogen), as well as Dll4 (R&D Systems) was performed after fixing cells in MFDs with 4% paraformaldehyde. A standard immunofluorescent staining protocol was followed in MFDs.

Automated image analysis to measure cell motility. Quantitative measurement of cell motility was performed for two hours of time-lapse data after conditioning and applying exosomes to MFDs. Automated image processing methods were developed to track multiple cell nuclei from time-lapse microscopy images using custom scripts written in MATLAB (Mathworks, USA) software. Manual cell tracking of a large amount of time-lapse data is not only time-consuming, but would also be subjected to human-to-human variance. The confocal time-lapse images are complex due to low signal-to-noise ratio and slow sampling rate as well as complex cell topologies, proliferation and death, and diverse cell density between monolayer and gel region. Time-sequence images were analyzed stochastically based on Kalman filter combined with Multiple Hypothesis testing (MHT), to provide the optimal estimate of cell trajectories, given statistic properties of the image[25] (Supplemental Method S1).

Cell proliferation assay. HMVECs were conditioned for three days and on the third day, exosomes were applied together with 40 ngmL^{-1} in the gradient channel at different concentrations and incubated at 37°C for 24 hours. On the following day, they were fixed and stained with Ki67 antibody (abcam) and Hoechst nuclei stain. They were imaged using Olympus FV1200 and cell counts were performed using Imaris 7.2.

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Author contributions

S.S.N. and E.T. performed experiments; S.S.N. designed the experiments and analyzed the data, made the figures and wrote the paper, L.L.S.O. designed automated image processing method; R.G. conceived the project, advised on research and wrote the paper; and H.H.A. secured research funding and advised on research.

Additional information

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