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Article

Polyethylenimine Triggers Dll4 Degradation to Regulate Angiogenesis In Vitro

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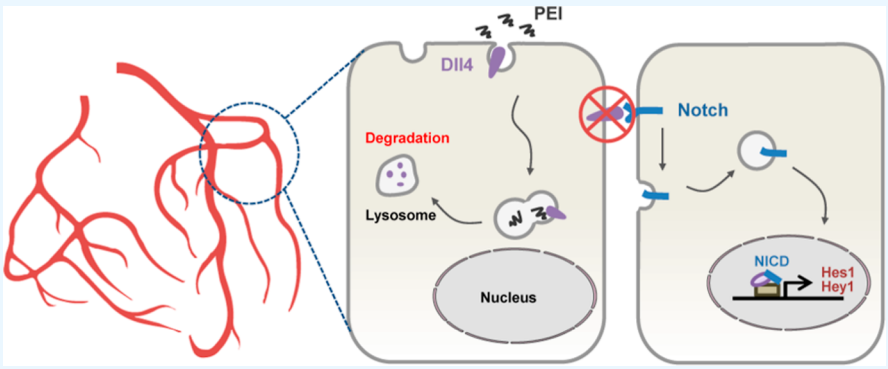

ABSTRACT: The Dll4-Notch signaling pathway plays a crucial role in the regulation of angiogenesis and is a promising therapeutic target for diseases associated with abnormal angiogenesis, such as cancer and ophthalmic diseases. Here, we find that polyethylenimine (PEI), a cationic polymer widely used as nucleic acid transfection reagents, can target the Notch ligand Dll4. By immunostaining and immunoblotting, we demonstrate that PEI significantly induces the clearance of cell−surface Dll4 and facilitates its degradation through the lysosomal pathway. As a result, the activation of Notch signaling in endothelial cells is effectively inhibited by PEI, as evidenced by the observed decrease in the generation of the activated form of Notch and expression of Notch target genes Hes1 and Hey1. Furthermore, through blocking Dll4-mediated Notch signaling, PEI treatment enhances angiogenesis in vitro. Together, our study reveals a novel biological effect of PEI and establishes a foundation for the development of a Dll4-targeted biomaterial for the treatment of angiogenesis-related disease.

INTRODUCTION

Angiogenesis is an important process of the formation of new blood vessels from preexisting vasculature, and it occurs in various physiological and pathological conditions throughout the lifespan.[1,2] The therapeutic potential of controlling angiogenesis is widely recognized. Inducing angiogenesis has been found to be beneficial in the treatment of wound healing, ischemic heart disease, and peripheral arterial disease. Conversely, reducing or inhibiting angiogenesis has shown therapeutic benefits in the management of cancer, ophthalmic conditions, rheumatoid arthritis, and various other diseases.[3−5] Over the past two decades, numerous drugs targeting angiogenesis have been approved for the treatment of associated diseases, with the majority of these drugs being specifically designed to target the VEGF/VEGFR pathway.[6] However, due to limited therapeutic efficacy and drug resistance,[4,7] there is a need to elucidate mechanisms underlying clinical benefits and develop new drugs based on other important molecular targets.

The Dll4-Notch signaling pathway plays a key role in angiogenesis.[8] Under normal physiological conditions, VEGF upregulates the expression of the Notch ligand Dll4 on the cellular surface. This ligand subsequently binds to the Notch receptor, leading to the activation of Notch signaling in endothelial cells. The activation of Notch signaling by Dll4 can influences multiple aspects of angiogenesis, particularly angiogenic sprouting.[9−13] In vivo studies with tumor models have shown that blocking Dll4 can stimulate blood vessel sprouting and increase tumor angiogenesis, but these new blood vessels have poor morphology and function, which renders the tumor more hypoxic and inhibits tumor growth.[14,15] In addition, the clinical data showed that the survival time of cancer patients with high Dll4 expression was significantly shorter than that of

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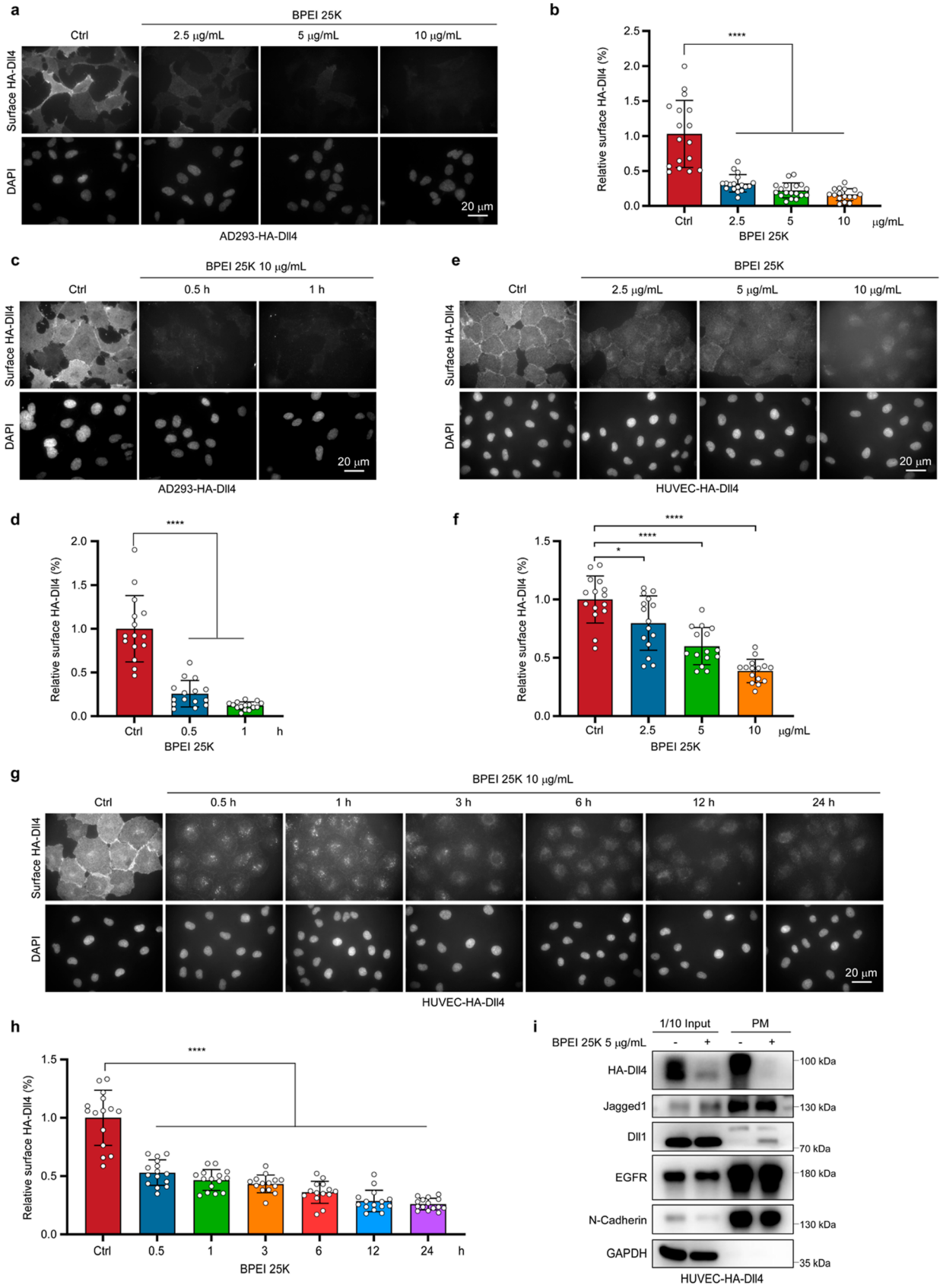

Figure 1. BPEI 25K treatment decreases cell−surface expression of Dll4. (a) Immunostaining of the surface Dll4 in HA-Dll4 stably expressing AD293 cells upon treatment with different concentrations of BPEI 25K (2.5, 5, and 10 μg/mL) for 12 h. (b) Quantification of surface HA-Dll4 immunofluorescence intensity in (a). Relevant statistical comparisons are indicated within the graph, and each dot represents the percentage of fluorescence intensity compared to the control (n = 16−18 cells for each condition, means ± S.D.). (c) Representative images of the surface Dll4 in AD293-HA-Dll4 cells after treatment with 10 μg/mL BPEI 25K for indicated times (0.5 and 1 h). (d) Quantification of surface HA-Dll4

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Figure 1. continued

immunofluorescence intensity in (c) (n = 15 cells for each condition, means ± S.D.). (e) HUVECs stably expressing HA-Dll4 were treated with the indicated concentration of BPEI 25K for 12 h, fixed, and stained for surface HA-Dll4. (f) Quantification of surface HA-Dll4 immunofluorescence intensity in (e) (n = 15 cells for each concentration, means ± S.D.). (g) Representative images of the surface Dll4 in HUVEC-HA-Dll4 cells upon treatment with 10 μg/mL BPEI 25K for the indicated times. (h) Quantification of surface HA-Dll4 immunofluorescence intensity at each time point after treatment with 10 μg/mL BPEI 25K in (g) (n = 15 cells for each condition, means ± S.D.). (i) HUVEC-HA-Dll4 cells were treated with 5 μg/mL BPEI 25K for 12 h and then biotinylated for 1 h at 4 °C. Biotinylated membrane proteins were precipitated and analyzed by immunoblotting as indicated. PM, plasma membrane. DNA was stained with DAPI. Scale bar: 20 μm.

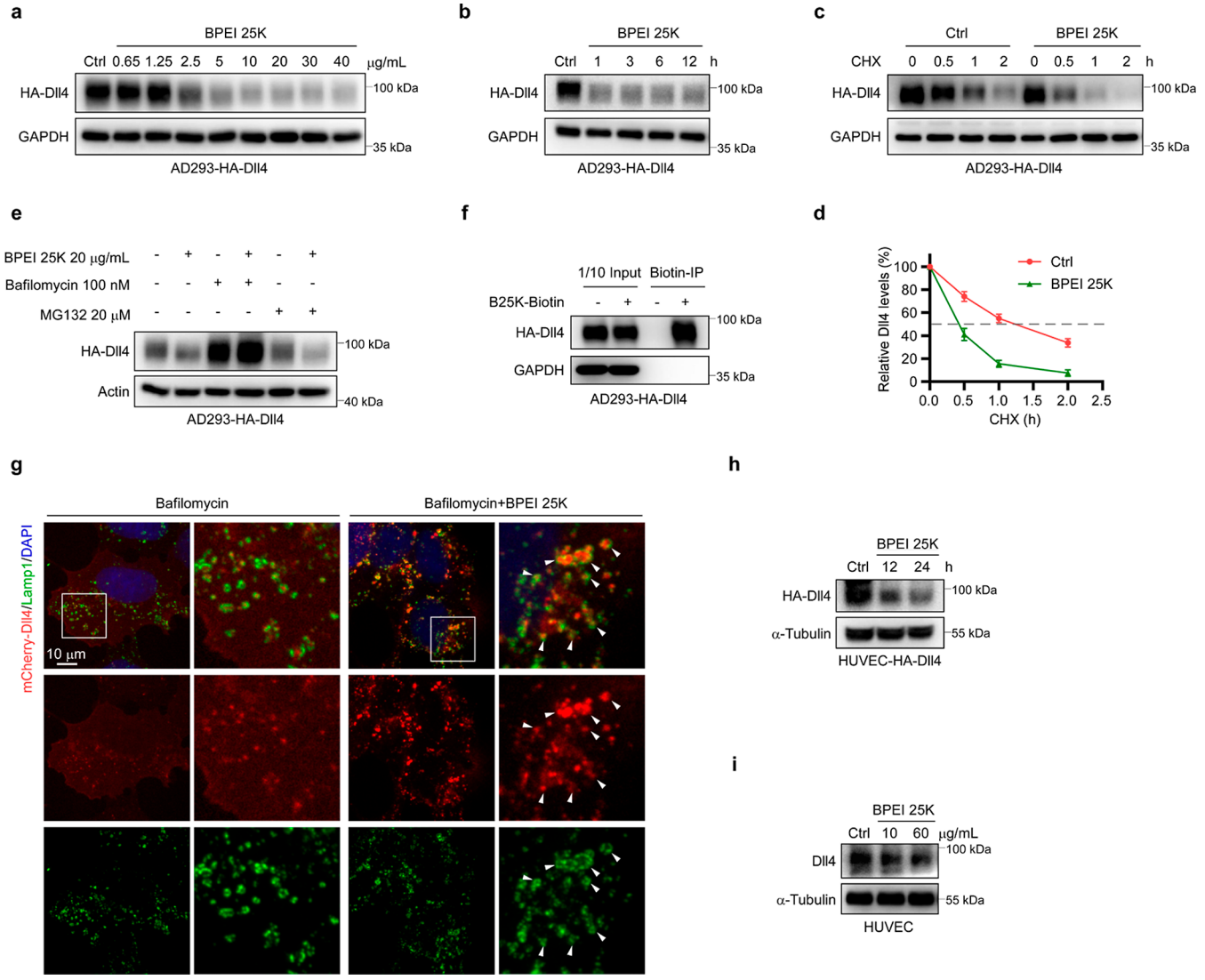

Figure 2. BPEI 25K treatment induces Dll4 degradation through the lysosomal pathway. (a) Western blot analysis of the Dll4 levels in AD293-HA-Dll4 cells treated with different doses of BPEI 25K for 24 h. (b) Western blot of the Dll4 in AD293-HA-Dll4 cells treated with 5 μg/mL BPEI 25K for the indicated times. (c,d) AD293-HA-Dll4 cells were cotreated with BPEI 25K and CHX (100 μg/mL) for 0, 0.5, 1, and 2 h, collected and subjected to immunoblotting, as indicated. HA-Dll4 protein levels were quantified, and the values are expressed relative to time 0 h. Abundance was normalized to GAPDH. (e) Dll4 levels were analyzed by Western blotting in AD293-HA-Dll4 cells treated with BPEI 25K for 12 h in the presence or absence of MG132 or bafilomycin A1. (f) Detect the interaction between BPEI 25K and Dll4 by Biotin-BPEI 25K immunoprecipitation and Western blot. (g) Confocal images of the colocalization of mCherry-Dll4 and Lamp1 in mCherry-Dll4 overexpressing AD293 cells after treatment with 5 μg/mL BPEI 25K in the presence of bafilomycin A1 for 1 h. (h) Western blot analysis of HA-Dll4 levels in HUVEC-HA-Dll4 cells upon treatment with 10 μg/mL BPEI 25K for the indicated times. (i) Western blot of endogenous Dll4 in primary HUVECs after treatment with indicated concentrations of BPEI 25K for 24 h. DNA was stained with DAPI. Scale bar: 10 μm.

patients with low Dll4 expression.[16,17] Therefore, Dll4 has emerged as a promising therapeutic target.

Polyethylenimine (PEI) is a cationic polymer, which is widely used as a gene transfection agent.[18−20] As PEI can bind to anionic residues on the plasma membrane and deliver cargos into cells through endocytosis,[20] we utilize it to construct protein degraders to target membrane proteins such as Dll4 in our unpublished work. Surprisingly, we found that PEI itself could eliminate cell−surface Dll4. In this study, we conducted a more comprehensive examination of the intrinsic bioactivity of

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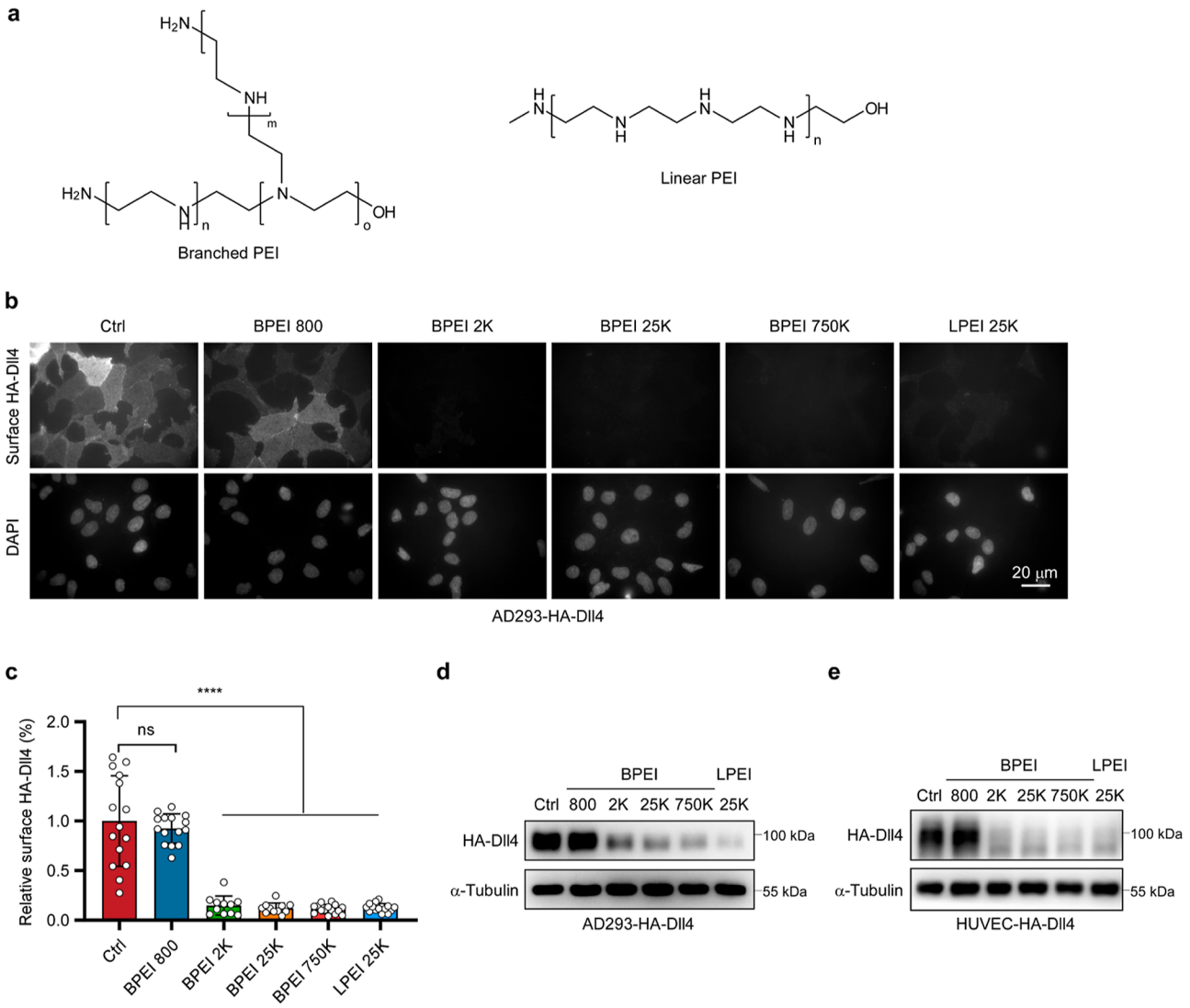

Figure 3. Effect of PEI on Dll4 degradation is dependent on its molecular weight. (a) Structure illustration of branched and linear PEI. (b) Immunostaining images showing the surface HA-Dll4 in AD293-HA-Dll4 cells after treatment with branched and linear PEI with different molecular weights (30 μg/mL B800, 10 μg/mL B2K, 10 μg/mL B25K, 2 μg/mL B750K, and 10 μg/mL L25K) for 12 h. (c) Corresponding quantification of surface HA-Dll4 immunofluorescence intensity in (b) (n = 15 cells for each concentration, means ± S.D.). (d,e) Immunoblotting detecting the HA-Dll4 abundance in AD293-HA-Dll4 cells (d) and HUVEC-HA-Dll4 cells (e) after treatment with 5 μg/mL different types of PEI (B800, B2K, B25K, B750K, and L25K) for 12 h. DNA was stained with DAPI. Scale bar, 20 μm.

PEI on Dll4 and Dll4-mediated Notch signaling and angiogenesis. Our results showed that PEI could induce Dll4 degradation via the lysosome pathway and consequently inhibit the activation of Notch signaling and promote angiogenesis in vitro. Our study reveals a novel biological effect of PEI, which implies promising potential for future biomedical applications in the field of angiogenesis-related therapy.

RESULTS

BPEI 25K Treatment Induces Cell−Surface Clearance of Dll4. To unambiguously explore the bioeffect of PEI on the Notch ligand Dll4, and to circumvent the problem of the unavailability of an appropriate antibody for detecting endogenous Dll4 at the cell surface, we employed previously established AD293 and HUVEC lines that stably express a HA-tagged Dll4 for our investigation.[21] We first treated HA-Dll4 expressing AD293 cells with 25 kDa branched PEI (BPEI 25K), a form of PEI widely used for gene delivery, at concentrations commonly employed in transfection experiments and examined the cell−surface localization of Dll4 by immunofluorescence staining. As shown in Figure 1a and b, Dll4 on the cell surface exhibited a partial reduction when cells were treated with BPEI 25K at a concentration of 2.5 μg/mL and almost disappeared when the concentration was increased to 5 μg/mL. Moreover, the reduction of cell−surface Dll4 occurred rapidly, and it nearly vanished within 30 min following treatment with 10 μg/mL of BPEI 25K (Figure 1c, d). We also observed BPEI 25K-induced cell−surface clearance of Dll4 in a concentration- and time-dependent manner in HA-Dll4 expressing HUVECs (Figure 1e−h). The results from the plasma membrane protein isolation assay further confirmed that treatment with BPEI 25K led to a significant reduction in Dll4 localization on the cell surface (Figure 1i). To be noted that this treatment did not exhibit any impact on other membrane proteins, such as EGFR and N-Cadherin (Figure 1i). Moreover, the typical Notch ligands Dll1 and Jag1 did not decrease after BPEI 25K treatment, and surface

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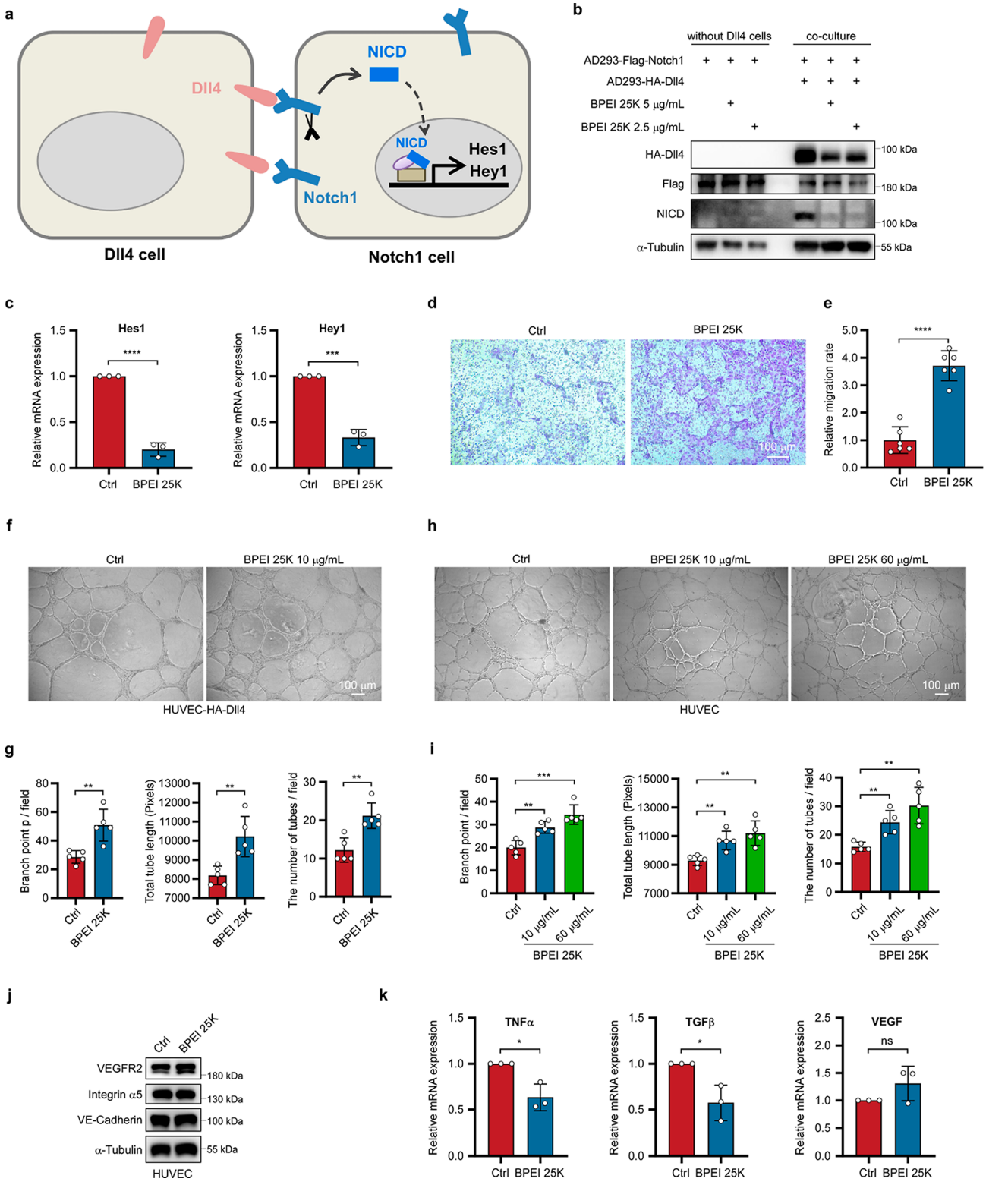

Figure 4. BPEI 25K treatment inhibits Notch signaling and promotes HUVEC tube formation. (a) Schematic of coculture assay for assessment of Notch activation. (b) Western blot analysis of the generation of NICD after treatment with 2.5 and 5 μg/mL BPEI 25 K by coculture assay, as described in (a). (c) Quantitative RT-PCR analysis of the mRNA level of Notch target genes Hes1 and Hey1 in HA-Dll4 expression HUVECs treated with 5 μg/mL BPEI 25K for 24 h (n = 3 biological replicates, means ± S.D.). (d,e) Transwell migration assay in HUVEC-HA-Dll4 cells upon treatment with 5 μg/mL BPEI 25 K for 18 h. Relative migration rate was quantified by ImageJ (n = 6 fields, means ± S.D.). (f–i) Representative images and quantification of tube formation of HUVEC-HA-Dll4 cells (f,g) and primary HUVEC cells (h,i) upon treatment with 10 μg/mL BPEI 25K for 24 and 8 h, respectively (n = 5 fields, means ± S.D.). (j) Western blot of proteins associated with vascular regulation in primary HUVECs after treatment with 10 μg/mL BPEI 25K for 24 h. (k) Quantitative RT-PCR analysis of the mRNA level of angiogenesis-related cytokines, TNFα, TGFβ, and VEGF in primary HUVECs after being treated with 5 μg/mL BPEI 25K for 24 h (n = 3 biological replicates, means ± S.D.).

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Dll1 even slightly increased, which may be attributed to the compensation in response to the loss of Dll4 (Figure 1i). In addition, we further examined the effect of BPEI 25K on the Notch receptor in Flag-Notch1 expressing AD293 cells. Immunofluorescence results showed that cell surface expression of Notch1 did not remarkably decrease like Dll4 after treatment with BPEI 25K, but the distribution of Notch1 was changed and it accumulated at cell−cell contacts (Supporting Information, Figure 1). Together, these results suggested that BPEI 25K could specifically cause cell−surface Dll4 clearance. In order to exclude the above bioeffect due to the potential toxicity of PEI, we performed a cell viability analysis. Our data showed that BPEI 25K had no deleterious effect on the viability of AD293 and HUVECs at concentrations known to induce Dll4 degradation (Supporting Information, Figure 2).

BPEI 25K Treatment Promotes Dll4 Degradation via the Lysosomal Pathway. We next sought to determine whether the decrease in cell−surface Dll4 caused by BPEI 25K was due to enhancing the endocytic degradation of Dll4. We first examined the global Dll4 protein levels in AD293 cells stably expressing HA-Dll4 by immunoblotting. The results showed that Dll4 was indeed degraded upon BPEI 25K treatment, and the degradation of Dll4 was concentration-dependent (Figure 2a). Temporal analysis revealed that Dll4 degradation was detected within 1 h of treatment with 5 μg/mL BPEI 25K (Figure 2b). These data were consistent with the previous results of cell−surface clearance of DLL4 (Figure 1a,b). We then used cycloheximide (CHX) to inhibit protein synthesis and estimated the half-life of Dll4 after treatment with BPEI 25K. As expected, the half-life of Dll4 in BPEI 25K-treated cells was obviously shorter than that in control cells, suggesting BPEI 25K significantly accelerated the degradation rate of Dll4 (Figure 2c,d). It is well-known that two degradation pathways exist in cells, the ubiquitin-dependent proteasome pathway and the lysosomal pathway.[22] To further determine the pathway by which PEI-induced degradation of Dll4 occurs, we treated cells with MG132 or bafilomycin A1, which inhibits proteasomal and lysosomal functions, respectively. The administration of MG132 did not obviously affect the protein level of Dll4 in both the control and BPEI 25K-treated cells. Conversely, treatment with bafilomycin A1 completely prevented the degradation of Dll4 induced by BPEI 25K, suggesting that PEI facilitates Dll4 degradation via the lysosomal pathway (Figure 2e). Given that PEI can deliver genes into lysosomes via endocytosis, we propose that PEI may bind to Dll4 and deliver it into the lysosome for degradation. To confirm this hypothesis, we conducted a Biotin-BPEI 25K co-IP to detect whether PEI can bind to Dll4, and our data showed that PEI can strongly bind to Dll4 (Figure 2f). Meanwhile, the confocal images also displayed that Dll4 was significantly delivered into the lysosome from the plasma membrane after treatment with BPEI 25K (Figure 2g). Furthermore, we have also substantiated that BPEI 25K has the capability to degrade exogenous Dll4 in HUVEC-HA-Dll4 cells and endogenous Dll4 in primary HUVECs (Figure 2h,i), thereby providing evidence that the degradation of Dll4 induced by PEI is not contingent upon the specific cell type.

As mentioned above, PEI was widely used as a transfection reagent; therefore, we were curious whether PEI could degrade Dll4 when mixed with plasmids. The surprising result is that BPEI 25K can also promote Dll4 degradation when it is used to transfect plasmids into cells (Supporting Information, Figure 3). This data suggests that delivery genes using PEI may have additional effects, which need to be carefully considered.

Degradation of Dll4 Induced by PEI Is Dependent on Its Molecular Weight. PEIs can be classified into two types, linear and branched, based on their chemical structure, and are available in a broad range of molecular weights[23] (Figure 3a). We then asked whether various PEIs possess similar effects. To this end, four additional PEIs, including branched (800 Da, 2 kDa, and 750 kDa) and linear (25 kDa) forms, were selected for examination. The immunofluorescence results displayed that all types of PEI, except low-molecular weight BPEI 800, could significantly reduce cell−surface Dll4 (Figure 3b,c). Consistent with the immunofluorescence data, these PEIs except BPEI 800 all have the ability to decrease total Dll4 protein levels in AD293 and HUVEC stable cell lines (Figure 3d,e). As PEI is a cationic polymer, we then wondered whether the cationic property of PEI is responsible for this effect of Dll4 degradation. To test this hypothesis, we examined another commonly used polycation, PolyLysine. Our data showed that PolyLysine cannot induce the degradation of Dll4 (Supporting Information, Figure 4). Based on these results, we speculate that the induction of Dll4 degradation is the universal bioactivity of PEIs and may be contingent upon the molecular weight and structure of PEI itself.

BPEI 25K Inhibits Dll4-Notch Signaling and Affects Angiogenesis In Vitro. As an important ligand of the Notch receptor, Dll4 is known to activate the Notch1 signaling pathway by binding to the Notch1 receptor. A typical feature of the activation of Notch1 signaling pathways is that the Dll4-triggered cleaved intracellular domain of Notch1 (NICD) can be transferred into the nucleus, and then it binds to CSL protein and activates the transcription of downstream target genes such as transcription factor HES-1 (Hes1) and hairy/enhancer-of-split related with YRPW motif protein 1 (Hey1).[8] Based on the previous results that PEI was able to induce Dll4 degradation and at the same time cause changes in the localization of Notch1, we proposed that PEI-induced Dll4 clearance would further inhibit the activation of Notch1 signaling. To test this hypothesis, we first conducted a coculture assay with one cell line overexpressing Dll4 and the other one overexpressing Notch1 to assess the NICD production (Figure 4a). Our results showed that PEI treatment significantly impaired NICD generation (Figure 4b). Next, we performed a qPCR assay to detect the mRNA levels of Notch1 downstream target genes Hes1 and Hey1 and found that, compared with the control HUVECs, the mRNA levels of these two genes in the PEI-treated HUVECs were significantly reduced to 25 and 35%, respectively (Figure 4c). Together, these data support that the Notch1 signaling pathway is inhibited by PEI.

The Dll4-Notch1 signaling pathway plays an important role in angiogenesis. Therefore, we further performed a well-established transwell migration assay and tube formation assay to verify the effect of PEI on the angiogenesis. With the migration assay, we found that 5 μg/mL BPEI 25K treatment dramatically promoted the migration of HUVECs stably expressing HA-Dll4 (Figure 4d,e). Consistent with the cell migration data, upon treatment with BPEI 25K, HUVECs stably expressing HA-Dll4 formed the vascular network structure with more branch points, length, and number of tubes than the untreated group (Figure 4f,g), indicating PEI treatment resulted in a pro-angiogenic effect. Similarly, as BPEI 25K was demonstrated to induce endogenous Dll4 degradation in primary HUVECs, the same effect of PEI on primary HUVEC tube formation was observed in a concentration-dependent manner (Figure 4h,i). To further validate the pro-angiogenic effect of PEI, we analyzed the expression levels of some

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angiogenic modifiers. Our results showed that the levels of VEGFR2, but not integrin α5 or VE-cadherin, were increased after treatment with BPEI 25K (Figure 4j), and the expressions of TNFα and TGFβ were decreased following the BPEI 25K treatment (Figure 4k). As VEGFR2, TNFα, and TGFβ can be regulated by Notch signaling,[8,24,25] the alterations observed in the expression of these genes may be attributed to the inhibition of the Notch signaling by PEI. Collectively, these data are consistent with the reports that blocking Dll4-Notch signaling could increase deficient angiogenesis,[14,15] implying that PEI may be used as a regulator of angiogenesis for the treatment of diseases.

DISCUSSION

Dll4, a transmembrane ligand for the Notch receptor, is a key regulator of vascular morphogenesis and has emerged as a promising therapeutic target for tumor angiogenesis. Here, we demonstrated that the commonly used polymeric material PEI can target Dll4 for lysosomal degradation, leading to the inhibition of Notch signaling. More importantly, this biological effect of PEI on Dll4-Notch signaling can further influence angiogenesis, like other documented small molecules and antibodies that specifically target this pathway.[9,14] In addition, we examined the generality of such an effect across different types of PEI and discovered that the molecular weight of PEI emerges as a significant determinant. PEI is a polycationic polymer due to the presence of abundant amino groups, and therefore it can bind anionic molecules such as cell−surface heparin sulfate proteoglycans and enter cells via endocytosis.[26] We speculate that the positive charge effect of PEI is responsible for its promotion of Dll4 endocytic degradation, but the molecular mechanism is still unclear and needs further study.

In conclusion, our data provide a basis for the development of Dll4-targeted biomaterials for angiogenesis-related disease therapy. During the past two decades, nanomedicines have demonstrated benefits in cancer treatment and management through favorable pharmacokinetics, precise targeting, reduced side effects, and drug resistance.[27] Given that PEI is often used to construct the backbone or coating material of nanomedicine,[28] we can use the properties of that PEI could specifically degrade Dll4 to synthesize new DLL4-targeted antitumor angiogenesis drugs by grafting PEI onto nanomaterials.

METHODS

Reagents. Branched PEI Mw 800 (Cat. No. 408719), Mw 2K (Cat. No. 408700), Mw 25K (Cat. No. 408727), and PolyLysine (Mw: 15−30K, Cat. No. P1399) were purchased from Sigma, and Mw 750K (Cat. No. 25449) was from Polysciences. Linear PEI Mw 25K (Cat. No. 23966) was obtained from Polysciences. Bafilomycin A1 (Cat. No. HY-100558), MG132 (Cat. No. HY-13259), and CHX (Cat. No. HY-12320) were purchased from MedChemExpress.

Biotin-BPEI 25K Synthesis. Branched PEI (Mw = 25,000, 20.0 mg, 1.0 equiv) and Biotin-NHS (0.55 mg, 2.0 equiv) were dissolved with PBS (1.0 mL, pH 9.0), and the reaction was incubated for 24 h at room temperature. After dialysis and lyophilization, Biotin-B25K was obtained as a white powder (14.67 mg, 72%).

Cell Lines and Cell Culture. HUVEC and AD293 cells stably expressing HA-Dll4 and Flag-Notch1 were established by the same protocols, as previously described.[21] The stable cell lines were cultured at 37 °C under 5% CO_2 in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin−streptomycin (Gibco). Primary human umbilical vein endothelial cells were obtained from Procell and cultured by an additional 10 ng/mL bFGF and 100 μg/mL heparin.

Immunoblotting. Cells were lysed with SDS lysis buffer containing a 1% protease inhibitor (MedChemExpress) on ice for 10 min, and then the lysates were centrifuged at 14,000*g* for 15 min at 4 °C to remove cellular debris. Protein concentration in cell lysates was quantified using a BCA assay (Beyotime Biotechnology). Equal amounts of each sample were resolved by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore). The membranes were blocked with PBST containing 5% skim milk for 30 min and incubated with primary antibodies against DLL4 (Cell Signaling, 2589), DLL1 (ProteinTech, 28544−1-AP), Jagged1 (Santa Cruz, sc-8303), HA (Covance, MMS-101P), EGFR (Cell Signaling, 4267), Notch1 (Cell Signaling, 3608), Cleaved Notch1 (Cell Signaling, 4147), N-Cadherin (BD Biosciences, 610921), VE-Cadherin (Cell Signaling, 2158), VEGFR2 (Cell Signaling, 2479), Integrin α5 (Abcam, ab150361), GAPDH (ProteinTech, 60004−1-lg), Flag (PROSPEC, ANT-146), α-Tubulin (Sigma, T9026), and β-Actin (Santa Cruz, sc-47778) by the dilution according to the official instructions overnight at 4 °C. Following three washes in PBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The chemiluminescence substrate was applied (Millipore), and blots were visualized using the Amersham Imager 600 system. ImageJ software was used for the relative quantification of bands by normalizing them to the loading control in each lane.

Immunofluorescence Staining. Cells were seeded on coverslips in 24-well plates and placed in the cell incubator overnight for adhering. After treatment, the cells were fixed with 4% paraformaldehyde for 15 min. For cell surface membrane protein staining, fixed cells were blocked directly in a blocking buffer (PBS, 3% BSA) for 30 min and followed by labeling with primary antibodies and Alexa Fluor 488 or Alexa Fluor 555-conjugated secondary antibodies, respectively. DNA was stained by DAPI at the same time as secondary antibody staining.

Membrane Protein Isolation. Proteins at the plasma membrane were isolated using the Cell Surface Protein Isolation Kit (Pierce, 89881). The principle is to label cell membrane proteins with biotin. Briefly, cells were first incubated in PBS containing 0.5 mg/mL Sulfo-NHS-Biotin for 1 h at 4 °C to label membrane proteins, and then lysed with western IP buffer (Beyotime Biotechnology) supplemented with protease inhibitors. Biotinylated membrane proteins were precipitated with a NeutrAvidin-agarose resin and then subjected to immunoblotting analysis.

BPEI 25K Immunoprecipitation. For BPEI 25K immunoprecipitation, cells were lysed with western IP buffer (Beyotime Biotechnology) plus a protease inhibitor cocktail, and equal amounts of total protein were incubated with 5 μg Biotin-BPEI 25K and biotin beads (Thermo) followed by 4 h of rotated incubation at 4 °C. The beads were washed and resuspended in 1 × SDS loading buffer, then heated for 10 min at 95 °C. Protein samples were analyzed by SDS-PAGE and immunoblotting.

Matrigel-Based Tube Formation Assay. The 48-well plate was coated with 150 μL of precooled matrigel (BD Sciences, 354234) on ice and then placed at 37 °C for 30 min to allow the gelling of matrigel. Then collect HA-Dll4 expression HUVECs or primary HUVECs and seed them at a density of 10 × 10^4 cells/well and 5 × 10^4 cells/well in cell culture media with

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the required concentration of BPEI 25K, respectively. After incubation at 37 °C, 5% CO_2 for 6 to 24 h, the tube formation of cells on matrigel was imaged with an Olympus IX81 live cell imaging system equipped with a 10× objective. The five random fields of tubule-like structures in images were quantified by the angiogenesis plugin of ImageJ software.

Cell Migration Assay. For the transwell migration assay, using the 8 μm PET millicell hanging cell culture 24 well inserts. HUVEC-HA-Dll4 cells were resuspended in the cell culture medium containing 1% FBS and 5 μg/mL BPEI 25K, 200 μL of cell suspension (2×10^4 cells) was added to the upper chambers, and the DMEM was supplemented with 20% FBS, and 10 ng/mL bFGF was added to the lower chamber (Millipore, PIEP12R48). After 18 h of incubation, the cells that migrated to the lower face of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. After being washed three times with PBS, the image was captured with an Olympus CKX53 microscope equipped with a 10× objective. The migration rate was measured by counting the migrating cells using ImageJ software.

Cell Vability Assay. AD293-HA-Dll4 and HUVEC-HA-Dll4 cells were plated in 96-well plates at 5×10^3 cells/well and incubated overnight for adhesion, then treated with 1.25, 2.5, 5, and 10 μg/mL BPEI 25K for 12 and 24 h, respectively. The cell proliferation was analyzed using a TransDetect Cell Counting Kit (TransGen Biotech, FC101−01), according to the manufacturer's instructions.

Coculture Assay. The coculture assay was performed, as previously described.[21] AD293-HA-Dll4 cells were resuspended in a culture medium (10% FBS, 1% penicillin−streptomycin) supplied with 2.5 or 5 μg/mL BPEI 25K. Then the cell suspension was seeded in 60 mm plates at a density of 5×10^6 cells/well and cultured overnight for cell adherence. AD293-Flag-Notch1 cells were seeded into these plates at a density of 5×10^5 cells/well the next day. The cell was cocultured for 4 h and collected for immunoblotting as described above.

qPCR. After 24 h of treatment with BPEI 25K, HA-Dll4 expression cells were harvested and washed three times with PBS. RNA was extracted using the TransZol RNA Extraction kit (TransGen Biotech, ET101−01-V2), according to the manufacturer's protocol. RNA concentration was quantified using NanoDrop spectrophotometry (Thermo Scientific) at optical density 260 and 280 nm. Two μg of total RNA was transcribed reversely into cDNA using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, AT311). qPCR of Hes1 and Hey1 was performed using the TransStart Top Green qPCR SuperMix (TransGen Biotech, AQ141) protocol. The primers used for the amplification of Hes1 and Hey1 are as follows: Hes1-Forward 5′- TCAACACGACACCGGATAAAC-3′, Reverse 5′-GCCGCGAGCTATCTTTCTTCA-3′; Hey1- Forward 5′-GTTCGGCTCTAGGTTCCATGT-3′, Reverse 5′-CGTCGGCGCTTCTCAATTATTC-3′; TNFα-Forward 5′-CCTCTCTCTAATCAGCCCTCTG-3′, Reverse 5′-GAGGACCTGGGAGTAGATGAG-3′; TGFβ -Forward 5′-TACCTGAACCCGTGTTGCTCTC-3′, Reverse 5′-GTTGCTGAGGTATCGCCAGGAA-3′; VEGF-Forward 5′-AGGGCAGAATCATCACGAAGT-3′, Reverse 5′-AGGGTCTCGATTGGATGGCA-3′. Results were calculated according to the $2^{-\Delta\Delta Ct}$ relative quantification method.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 9.0 software. All data are presented as the mean ± S.D. Each experiment was performed at least three times, unless otherwise specified. Statistical significance was determined by an unpaired Student's t test. Differences were considered statistically significant when P values were assigned as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06050.

BPEI 25K can modulate cell−surface Notch1 distribution; cell viability was not affected after treatment of BPEI 25K; complexes of PEI and nucleic acids also induce Dll4 degradation; and polylysine has no effect on Dll4 degradation (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Folkman, J. Opinion - Angiogenesis: an organizing principle for drug discovery? *Nat. Rev. Drug Discov.* **2007**, *6*, 273−286.
(2) Carmeliet, P. Angiogenesis in health and disease. *Nat. Med.* **2003**, *9*, 653−660.
(3) Timar, J.; Dome, B.; Fazekas, K.; Janovics, A.; Paku, S. Angiogenesis-dependent diseases and angiogenesis therapy. *Pathol. Oncol. Res.* **2001**, *7*, 85−94.
(4) Cao, Y.; Langer, R.; Ferrara, N. Targeting angiogenesis in oncology, ophthalmology and beyond. *Nat. Rev. Drug Discov.* **2023**, *22*, 476−495.
(5) Fallah, A.; Sadeghinia, A.; Kahroba, H.; Samadi, A.; Heidari, H. R.; Bradaran, B.; Zeinali, S.; Molavi, O. Therapeutic targeting of angiogenesis molecular pathways in angiogenesis-dependent diseases. *Biomed. Pharmacother.* **2019**, *110*, 775−785.
(6) Liu, Z. L.; Chen, H. H.; Zheng, L. L.; Sun, L. P.; Shi, L. Angiogenic signaling pathways and anti-angiogenic therapy for cancer. *Signal Transduct. Targeted Ther.* **2023**, *8*, 198.
(7) Carmeliet, P.; Jain, R. K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **2011**, *473*, 298−307.
(8) Phng, L. K.; Gerhardt, H. Angiogenesis: A Team Effort Coordinated by Notch. *Dev. Cell* **2009**, *16*, 196−208.
(9) Hellstrom, M.; Phng, L. K.; Hofmann, J. J.; Wallgard, E.; Coultas, L.; Lindblom, P.; Alva, J.; Nilsson, A. K.; Karlsson, L.; Gaiano, N.; et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **2007**, *445*, 776−780.
(10) Suchting, S.; Freitas, C.; le Noble, F.; Benedito, R.; Bréant, C.; Duarte, A.; Eichmann, A. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3225−3230.
(11) Lobov, I. B.; Renard, R. A.; Papadopoulos, N.; Gale, N. W.; Thurston, G.; Yancopoulos, G. D.; Wiegand, S. J. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3219−3224.
(12) Pitulescu, M. E.; Schmidt, I.; Giaimo, B. D.; Antoine, T.; Berkenfeld, F.; Ferrante, F.; Park, H.; Ehling, M.; Biljes, D.; Rocha, S. F.; et al. Dll4 and Notch signalling couples sprouting angiogenesis and artery formation. *Nat. Cell Biol.* **2017**, *19*, 915−927.
(13) Trindade, A.; Ram Kumar, S.; Scehnet, J. S.; Lopes-da-Costa, L.; Becker, J.; Jiang, W.; Liu, R.; Gill, P. S.; Duarte, A. Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. *Blood* **2008**, *112*, 1720−1729.
(14) Ridgway, J.; Zhang, G.; Wu, Y.; Stawicki, S.; Liang, W. C.; Chanthery, Y.; Kowalski, J.; Watts, R. J.; Callahan, C.; Kasman, I.; et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* **2006**, *444*, 1083−1087.
(15) Noguera-Troise, I.; Daly, C.; Papadopoulos, N. J.; Coetzee, S.; Boland, P.; Gale, N. W.; Chieh Lin, H.; Yancopoulos, G. D.; Thurston, G. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* **2006**, *444*, 1032−1037.
(16) Donnem, T.; Andersen, S.; Al-Shibli, K.; Al-Saad, S.; Busund, L.; Bremnes, R. M. Prognostic Impact of Notch Ligands and Receptors in Nonsmall Cell Lung Cancer Coexpression of Notch-1 and Vascular Endothelial Growth Factor-A Predicts Poor Survival. *Cancer* **2010**, *116*, 5676−5685.
(17) Jubb, A. M.; Soilleux, E. J.; Turley, H.; Steers, G.; Parker, A.; Low, I.; Blades, J.; Li, J. L.; Allen, P.; Leek, R.; et al. Expression of Vascular Notch Ligand Delta-Like 4 and Inflammatory Markers in Breast Cancer. *Am. J. Pathol.* **2010**, *176*, 2019−2028.
(18) Hsu, C. Y. M.; Uludag, H. A simple and rapid nonviral approach to efficiently transfect primary tissue-derived cells using polyethylenimine. *Nat. Protoc.* **2012**, *7*, 935−945.
(19) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in-Vivo - Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297−7301.
(20) Pandey, A. P.; Sawant, K. K. Polyethylenimine: A versatile, multifunctional non-viral vector for nucleic acid delivery. *Mater. Sci. Eng., C* **2016**, *68*, 904−918.
(21) Shao, X.; Ding, Z.; Zhao, M.; Liu, K.; Sun, H.; Chen, J.; Liu, X.; Zhang, Y.; Hong, Y.; Li, H.; et al. Mammalian Numb protein antagonizes Notch by controlling postendocytic trafficking of the Notch ligand Delta-like 4. *J. Biol. Chem.* **2017**, *292*, 20628−20643.
(22) Zhao, L.; Zhao, J.; Zhong, K. H.; Tong, A. P.; Jia, D. Targeted protein degradation: mechanisms, strategies and application. *Signal Transduct. Targeted Ther.* **2022**, *7*, 113.
(23) Chen, Z. H.; Lv, Z. Y.; Sun, Y. F.; Chi, Z. G.; Qing, G. Y. Recent advancements in polyethyleneimine-based materials and their biomedical, biotechnology, and biomaterial applications. *J. Mater. Chem. B* **2020**, *8*, 2951−2973.
(24) Zhou, J. S.; Jain, S.; Azad, A. K.; Xu, X.; Yu, H. C.; Xu, Z.; Godbout, R.; Fu, Y. Notch and TGFβ form a positive regulatory loop and regulate EMT in epithelial ovarian cancer cells. *Cell. Signal.* **2016**, *28*, 838−849.
(25) Wang, Y. D.; Wu, B.; Farrar, E.; Lui, W.; Lu, P.; Zhang, D.; Alfieri, C. M.; Mao, K.; Chu, M.; Yang, D.; et al. Notch-Tnf signalling is required for development and homeostasis of arterial valves. *Eur. Heart J.* **2015**, *38*, ehv520−686.
(26) Lungwitz, U.; Breunig, M.; Blunk, T.; Gopferich, A. Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm.* **2005**, *60*, 247−266.
(27) Shi, J. J.; Kantoff, P. W.; Wooster, R.; Farokhzad, O. C. Cancer nanomedicine: progress, challenges and opportunities. *Nat. Rev. Cancer* **2017**, *17*, 20−37.
(28) Li, J. C.; Yu, X. R.; Shi, X. Y.; Shen, M. W. Cancer nanomedicine based on polyethylenimine-mediated multifunctional nanosystems. *Prog. Mater. Sci.* **2022**, *124*, 100871.