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Semaphorin 3d and Semaphorin 3e Direct Endothelial Motility through Distinct Molecular Signaling Pathways*

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Background: Class 3 semaphorins are guidance molecules for endothelial cells.
Results: In multiple endothelial cell assays, semaphorin 3d requires neuropilin 1 or PI3K/Akt but not plexin D1, whereas semaphorin 3e requires plexin D1 but not neuropilin 1 or PI3K/Akt.
Conclusion: Semaphorin 3d and 3e utilize different pathways to mediate similar effects in endothelial cells.
Significance: Related guidance molecules utilize distinct mechanisms to repel endothelial cells.

Class 3 semaphorins were initially described as axonal growth cone guidance molecules that signal through plexin and neuropilin coreceptors and since then have been established to be regulators of vascular development. Semaphorin 3e (Sema3e) has been shown previously to repel endothelial cells and is the only class 3 semaphorin known to be capable of signaling via a plexin receptor without a neuropilin coreceptor. Sema3e signals through plexin D1 (Plxnd1) to regulate vascular patterning by modulating the cytoskeleton and focal adhesion structures. We showed recently that semaphorin 3d (Sema3d) mediates endothelial cell repulsion and pulmonary vein patterning during embryogenesis. Here we show that Sema3d and Sema3e affect human umbilical vein endothelial cells similarly but through distinct molecular signaling pathways. Time-lapse imaging studies show that both Sema3d and Sema3e can inhibit cell motility and migration, and tube formation assays indicate that both can impede tubulogenesis. Endothelial cells incubated with either Sema3d or Sema3e demonstrate a loss of actin stress fibers and focal adhesions. However, the addition of neuropilin 1 (Nrp1)-blocking antibody or siRNA knockdown of Nrp1 inhibits Sema3d-mediated, but not Sema3e-mediated, cytoskeletal reorganization, and siRNA knockdown of Nrp1 abrogates Sema3d-mediated, but not Sema3e-mediated, inhibition of tubulogenesis. On the other hand, endothelial cells deficient in Plxnd1 are resistant to endothelial repulsion mediated by Sema3e but not Sema3d. Unlike Sema3e, Sema3d incubation results in phosphorylation of Akt in human umbilical vein endothelial cells, and inhibition of the PI3K/Akt pathway blocks the endothelial guidance and cytoskeletal reorganization functions of Sema3d but not Sema3e.

Patterning of the vascular system requires coordinated temporal and spatial direction to the developing endothelium together with the ability of the endothelium to receive guidance signals. This is accomplished by a combination of secreted attractive and repulsive cues as well as cell-to-cell communications (1). Disruption of these signaling pathways can result in improper endothelial cell guidance, developmental pathologies, and disease (2).

Semaphorins are a family of secreted and transmembrane signaling molecules that have been implicated in numerous and diverse biological processes (3). Originally discovered as axon guidance molecules (4), semaphorins have since been implicated in vascular patterning, tumor progression, and immune cell regulation (5–7). The class of secreted vertebrate semaphorins, class 3 (Sema3 proteins), has been shown to be particularly important in proper cardiovascular development (8). Loss of Sema3c in mice results in aortic arch malformations as well as defects of outflow tract septation (9). Sema3e is required for intersomitic vessel and aortic patterning (10, 11), and, most recently, we have demonstrated that Sema3d is necessary for proper pulmonary vein development and connection (12).

Several class 3 semaphorins can affect the migration and motility of endothelial cells (13), and this function is what is believed to be responsible for Sema3-mediated cardiovascular development. Canonical Sema3 protein signaling involves neuropilin/plexin heterodimeric receptors (14). With the exception of Sema3e, which can signal through Plxnd1 alone (10), all other Sema3 proteins are thought to require either neuropilin 1 (Nrp1) or neuropilin 2 (Nrp2) coreceptors. Although Sema3 proteins are capable of exerting both an attractive and repellent effect on neurons (14), in general, their effect on endothelial cells appears to be of a repellent nature. The mechanism of endothelial repulsion in the case of Sema3e is a result of loss of focal adhesions and dismantling of the actin cytoskeleton (15). Other Sema3 protein-mediated endothelial repulsion mechanisms remain poorly elucidated.

In this study, we show that Sema3d, like Sema3e, is capable of inhibiting endothelial cell motility, migration, and tube formation. Both of these Sema3 proteins accomplish these tasks by affecting cytoskeletal dynamics and cell adhesion. Interestingly, the mechanisms by which these two Sema3 ligands exert such

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similar effects on endothelial cells diverge at the level of receptor signaling. Unlike Sema3e, Sema3d does not require Plxnd1 for endothelial repulsion. And although Sema3d-mediated inhibition of endothelial tube formation and cytoskeletal rearrangements require Nrp1, Sema3e mediates these effects independently of Nrp1. We also observe distinct signaling pathways downstream of the receptors for these two Sema3 proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293T cells were cultured in DMEM (Sigma) with 10% FBS. Primary human umbilical vascular endothelial cells (HUVECs)[3] (lot no.1023) were cultured in human endothelial cell culture medium, VascuLife® EnGS (catalog no. LL-0002, Lifeline Cell Technology) (basal EnGS medium, 0.2% EnGS, 5 ng/ml recombinant human EGF, 50 μg/ml ascorbic acid, 10 mM L-glutamine, 1.0 μg/ml hydrocortisone hemisuccinate, 0.75 units/ml heparin sulfate, and 2% FBS).

Reagents and Antibodies—Human recombinant Sema3e (catalog no. 3239-S3-025), human recombinant Sema3d, and anti-neuropilin 1 antibody (catalog no. AF566) were obtained from R&D Systems. Anti-phospho-Akt (catalog no. 4060), anti-Akt (catalog no. 9272), anti-β-actin (catalog no. 4967), and wortmannin (catalog no. 9951) were purchased from Cell Signaling Technology. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent for HEK293T cells, and phalloidin Rhodamine (catalog no. PHDR1) was purchased from Cytoskeleton. Anti-vinculin antibody (catalog no. V9131) and Cytochalasin D (catalog no. C2618) were obtained from Sigma-Aldrich. Prolong Gold antifade reagent with DAPI (catalog no. P36935) and Alexa Fluor 488 goat anti-mouse (catalog no. A11001) were obtained from Invitrogen. AP assay reagent A (catalog no. Q501) was obtained from GenHunter. Coverslips and tissue culture plates were coated with type I rat tail collagen (catalog no. CB40236) from BD Biosciences. Tubulogenesis assays were performed using Matrigel® growth factor reduced (catalog no. 354230, lot no. 2222781, endotoxin 3.4) from BD Biosciences.

Production and Quantification of Conditioned Medium—HEK293T cells were transfected with the Sema3d-pAPtag5, Sema3e-AP-pA6, or pAPtag4 plasmid in antibiotic-free DMEM with 10% FBS. After 24 h, the medium was changed to DMEM with 1% FBS and penicillin-streptomycin. After 48 h, the conditioned medium was collected. The YFP-PCDMA3.2 plasmid transfected into HEK293T cells was used as a transfection control. A 50-μl sample of the conditioned medium was incubated at 65 °C for 15 min to inactivate endogenous AP activity. 50 μl of AP assay reagent A (2 M diethanolamine, 1 mM MgCl, 1 mg/ml BSA, and 24 mM *p*-nitrophenylphosphate (pH 6.8)) was added to the conditioned medium and incubated at 37 °C for 10 min. 100 μl of 0.5N NaOH was added to stop the reaction. Absorbance was measured at 405 nm.

Microscopy and Image Analysis—For time-lapse experiments, HUVECs were cultured in a Nikon BioStation IM live cell recorder for 3 h in the presence of 10 nM recombinant Sema3d, 10 nM recombinant Sema3e, or vehicle (PBS). Images were recorded with BioStation IM software. For immunofluorescence experiments, HUVECs were cultured on collagen-coated coverslips. They were treated with 10 nM recombinant Sema3d, 10 nM recombinant Sema3e, or vehicle (PBS). Alternatively, they were incubated with conditioned medium containing equal quantities of Sema3d-AP, Sema3e-AP, or AP at 37 °C, where indicated, as measured by alkaline phosphatase activity. The HUVECs were subsequently fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton for 5 min. Cells were stained with phalloidin Rhodamine for 30 min, blocked with 2% w/v nonfat milk in Dulbecco's phosphate-buffered saline for 60 min, and incubated with anti-vinculin antibody for 60 min and then Alexa Fluor 488 anti-mouse IgG for 60 min. Coverslips were mounted with medium containing DAPI stain, and the cells were visualized on a Nikon Eclipse 80i microscope using NIS Elements software. Segmentation and quantification of focal adhesions were achieved using ImageJ software (16). A threshold was applied to vinculin-stained images on the basis of measurements of the mean gray value to exclude non-fluorescent background pixels. The "Analyze Particles" function was then used to count focal adhesions. Particles smaller than 1 μm^2 or with a circularity greater than 0.75 were excluded on the basis of the sizes and shapes of vinculin-positive focal adhesions that have been published previously (17).

Matrigel Tubulogenesis Assay—Reduced growth factor Matrigel (289 μl/well) was plated in the wells of a 24-well plate. The plate was incubated at 37 °C for 30 min. HUVECs (6×10^4 cells/well) were plated, and recombinant Sema3d (2 μg/ml), Sema3e (2 μg/ml), IgG (3.6 μg/ml), Cytochalasin D (50 ng/ml) or dimethyl sulfoxide (2 μl) was added to the appropriate well. The cells were incubated for 8 h and visualized on an inverted Nikon Eclipse TE200 microscope using Capture Advanced software.

RNA Interference—HUVECs were transfected with Lipofectamine RNAiMAX using Nrp1 predesigned siRNA (Ambion, catalog no. 4914) or Silencer negative control siRNA 1 (Ambion, catalog no. AM4611) in antibiotic-free medium. The medium was changed to complete HUVEC medium after 24 h, and the cells were used at 48 h.

Endothelial Isolation—A timed pregnant female from a *Plxnd1* heterozygous mouse cross was sacrificed at embryonic day 16.5. The embryos were assessed for the presence of persistent truncus arteriosus to identify nulls and subsequently genotyped for verification. The embryos (without the head, heart, lungs, and liver) were minced and incubated with collagenase A (Sigma, catalog no. C-0130). Single-cell suspension was achieved by passing the cells through small gauge syringes and a 40-μm nylon cell strainer. Cells were incubated with a platelet/endothelial cell adhesion molecule antibody (BD Biosciences, catalog no. 557355) for 30 min at 4 °C, washed, incubated with protein G Dynabeads (Invitrogen, catalog no. 10003D), and washed again. Dynabeads were plated onto fibronectin (Roche, catalog no. 11051407001) in endothelial cell medium.

Transwell Migration—Transwell inserts (BD Biosciences, catalog no. 353097) in triplicate were coated on the underside with 10 μg/ml fibronectin (Roche, catalog no. 11051407001) and placed in individual wells of a 24-well plate containing

[3] The abbreviations used are: HUVEC, human umbilical vein endothelial cell; EnGS, endothelial cell growth supplement; AP, alkaline phosphatase; ANOVA, analysis of variance.

either 10 nM recombinant Sema3d, 10 nM recombinant Sema3e, or vehicle (PBS) in DMEM. Endothelial cells were trypsinized and resuspended in DMEM containing 0.2% BSA (Gemini, catalog no. 700-101P), and then 10^5 cells were seeded in each insert and allowed to migrate for 5 h. For inhibitor experiments, the cells were resuspended in medium containing either wortmannin (1 μM) or a dimethyl sulfoxide vehicle control when seeded in the inserts. The migrated cells were fixed in 4% paraformaldehyde for 2 min, permeabilized in methanol for 20 min, and stained with Giemsa (Sigma, catalog no. GS-500) for 25 min. Cells that did not migrate were scraped from the inside of the insert with a cotton swab. Three high-power fields of each insert were imaged using an Olympus MVX10 microscope and quantified using ImageJ.

Cell Adhesion Assay—Collagen I-coated cell adhesion plates (Cell Biolabs, catalog no. CBA-052) were allowed to warm to room temperature for 10 min. HUVECs were resuspended in DMEM containing 0.2% BSA and either 10 nM Sema3d, 10 nM Sema3e, or a vehicle control. 3×10^5 cells from each condition were transferred to individual wells and incubated for 30 min. Non-adherent cells were washed away, the remaining cells were stained and extracted, and the optical density was measured at 560 nm.

Western Blotting—Blots were probed with anti-phospho-Akt (1:2000), anti-Akt (1:1000), or anti-β-actin (1:1000) according to the instructions of the manufacturer. Visualization was achieved using ECL Prime (GE Life Sciences). Quantification of individual band intensities was performed using ImageJ.

Statistical Analysis—One-way analysis of variance (ANOVA) was used to assess statistical differences between groups. Significant ANOVA results were further analyzed by Tukey's multiple comparisons test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant). All data are represented as the mean ± S.E.

RESULTS

Sema3d and Sema3e Inhibit Endothelial Cell Motility and Tubulogenesis—To examine the effects of Sema3d and Sema3e on endothelial cell motility and migration, HUVECs were incubated with equal concentrations (10 nM) of Sema3d or Sema3e and imaged live for 3 h. A compilation of the tracks corresponding to individual endothelial cell migration paths showed that both Sema3d and Sema3e significantly decreased the total distance and the maximum displacement traveled by the cells compared with controls (Fig. 1, *a* and *b*). Moreover, HUVECs appeared to lose directional motility in the presence of both Sema3d and Sema3e because they failed to migrate away from the point of origin, as measured by maximum displacement normalized to total distance for each track (data not shown). These data suggest that Sema3d and Sema3e mediate endothelial cell repulsion through inhibition of general and directed cell motility.

We assessed how Sema3d and Sema3e affect the ability of HUVECs to form capillary-like tubes on a basement membrane matrix. This assay allows us to investigate the effects of these Sema3 proteins on endothelial cell migration and rearrangements necessary in angiogenesis. Both Sema3d and Sema3e significantly inhibited tube formation by HUVECs after 8 h when compared with controls (Fig. 1, *c* and *d*). These results demonstrate that both Sema3d and Sema3e similarly inhibit functional abilities of endothelial cells that are necessary for proper vascular development.

Sema3d-mediated Endothelial Repulsion Does Not Require Plxnd1—To compare the chemotactic properties of Sema3d and Sema3e on endothelial cells, we employed a transwell migration assay. It has been shown previously that endothelial cells are inhibited from migrating through a membrane toward a Sema3e gradient (18). To assess the necessity of Plxnd1 in this assay, endothelial cells were isolated from embryonic day 16.5 *Plxnd1*$^{+/-}$ and *Plxnd1*$^{-/-}$ mice. Both Sema3d and Sema3e were capable of inhibiting the migration of *Plxnd1*$^{+/-}$ endothelial cells through a transwell membrane compared with controls (Fig. 2*a*). As expected, Sema3e was unable to inhibit the migration of *Plxnd1*$^{-/-}$ endothelial cells (Fig. 2, *b* and *c*). In contrast, Sema3d was able to inhibit endothelial migration even in the absence of Plxnd1 expression (Fig. 2, *b* and *c*). These results provide evidence of divergent signaling pathways for Sema3d *versus* Sema3e despite similar functional activities.

Sema3d and Sema3e Cause Loss of Actin Stress Fibers and Focal Adhesions in Endothelial Cells—We next sought to investigate the cellular mechanisms by which these Sema3 proteins affect migration and motility. We compared the effects of Sema3d and Sema3e on actin stress fiber and focal adhesions in HUVECs. HUVECs were incubated with AP-tagged Sema3d, AP-Sema3e, or AP alone in equal amounts, as quantified by alkaline phosphatase activity. Both Sema3 proteins caused endothelial cells to lose their cytoskeletal stress fibers (Fig. 3*a*). The percentage of cells with a loss of stress fibers was significantly more in both the Sema3d and Sema3d groups in as little as 15 min compared with the control (Fig. 3*b*). Loss of actin stress fibers occurred at a similar rate in response to Sema3d *versus* Sema3e, and more than 80% of the exposed cells had lost stress fibers by 60 min (Fig. 3, *a* and *b*).

Concurrent with loss of actin stress fibers, focal adhesions, which adhere the cytoskeleton to the extracellular matrix, also decreased after exposure to Sema3d or Sema3e, as visualized by the focal adhesion marker vinculin (Fig. 3*a*). Quantification of the number of focal adhesions per cell revealed a statistically significant decrease at 15 min with Sema3d ($p < 0.01$) or Sema3e ($p < 0.01$) and a continued decline thereafter when compared with controls (Fig. 3*c*). A cell adhesion assay revealed that Sema3d and Sema3e were able to significantly inhibit HUVEC adhesion to a collagen matrix after 30 min of incubation (Fig. 3*d*). These results suggest that both Sema3d and Sema3e signal to modify the organization of the endothelial cell cytoskeleton and adhesion in a grossly similar manner.

Sema3d-mediated Endothelial Cell Cytoskeletal Reorganization and Tubulogenesis Is Dependent on Nrp1—We examined the role of Nrp1 in Sema3d- and Sema3e-mediated cytoskeletal rearrangement in HUVECs by incubating cells with a blocking anti-Nrp1 antibody and either Sema3d or Sema3e. Visualization of actin and vinculin showed that blocking Nrp1 inhibited Sema3d-mediated, but not Sema3e-mediated, cytoskeletal changes (Fig. 4, *a* and *b*). Knockdown of Nrp1 in HUVECs using Nrp1 siRNA also showed that Sema3d, but not Sema3e, requires Nrp1 to dismantle the cytoskeleton (Fig. 4*c*). The ability of Sema3d to inhibit tubulogenesis was also dependent on

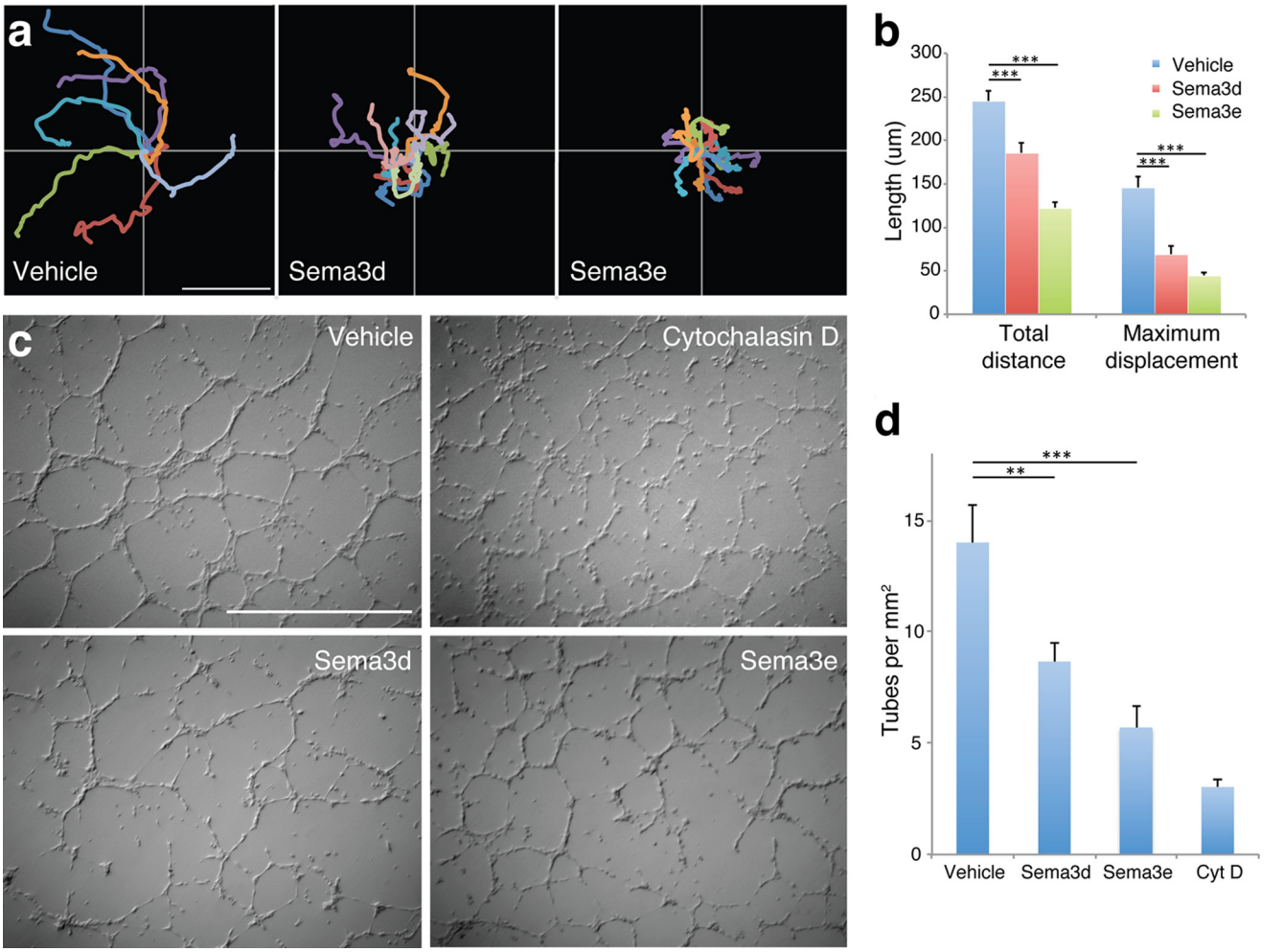


FIGURE 1. **Sema3d and Sema3e inhibit endothelial cell motility and tubulogenesis.** *a*, tracks representing the migration paths of individual HUVECs incubated with Sema3d, Sema3e, or a vehicle control for 3 h. *b*, quantification of total distance (in micrometers) and maximum displacement (in micrometers) of HUVECs in each condition. ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test). *c*, photomicrographs of HUVECs seeded in Matrigel and incubated with Sema3d, Sema3e, Cytocholasin D, or a vehicle control for 8 h. Cytochalasin D is used as a positive control for the inhibition of tubulogenesis. *d*, quantification of the number of tubules formed per high-power field. *Cyt D*, Cytochalasin D. **, $p < 0.01$; ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 9$). *Scale bars* = 50 μm (*a*) and 1 mm (*c*).

Nrp1, whereas the activity of Sema3e in this assay was unaffected by Nrp1 knockdown (Fig. 4, *d* and *e*). Therefore, Sema3d and Sema3e engage endothelial cells via different receptor complexes to mediate similar effects on migration, tubulogenesis, and cytoskeletal reorganization.

Sema3d Signals through the PI3K/Akt Pathway—During a screen of intermediate signaling molecules involved in cytoskeletal dynamics, we found that Sema3d increased the phosphorylation of Akt in HUVECs (not shown). We confirmed this result and also determined that this effect was not seen in response to Sema3e (Fig. 5, *a* and *b*). Further examination revealed that Sema3d incubation increased Akt phosphorylation in HUVECs in a dose-dependent manner (Fig. 5*c*). To test whether the PI3K/Akt pathway was necessary for functional Sema3d signaling, we employed a transwell endothelial cell migration assay. Similar to the repulsion seen using *Plexind1*$^{+/-}$ endothelial cells (Fig. 2*a*), both Sema3d and Sema3e were able to inhibit HUVECs from migrating through a transwell insert compared with vehicle control (Fig. 5*d*). However, incubation with the PI3K inhibitor wortmannin abrogated the ability of Sema3d to inhibit endothelial migration but did not have an effect on Sema3e-mediated inhibition (Fig. 5*d*).

To investigate whether the PI3K-dependent repulsive action of Sema3d on endothelial cells was due to actin cytoskeletal regulation, we visualized actin stress fibers in Sema3d- or Sema3e-incubated HUVECs in the presence or absence of the potent PI3K inhibitor wortmannin (Fig. 5*e*). As shown previously, Sema3d caused HUVECs to lose actin stress fibers, but this response was attenuated greatly in the presence of wortmannin (Fig. 5*f*). In contrast, wortmannin had no effect on Sema3e-mediated actin reorganization.

DISCUSSION

Our results indicate that Sema3d and Sema3e are capable of exerting similar effects on endothelial cells but that these effects are mediated by distinct molecular signaling pathways. We show that Sema3d signals through Nrp1 and PI3K independently of Plxnd1 and that Sema3e signals through Plxnd1 independently of Nrp1 although achieving similar functional endpoints. It will be of interest to determine the details of the

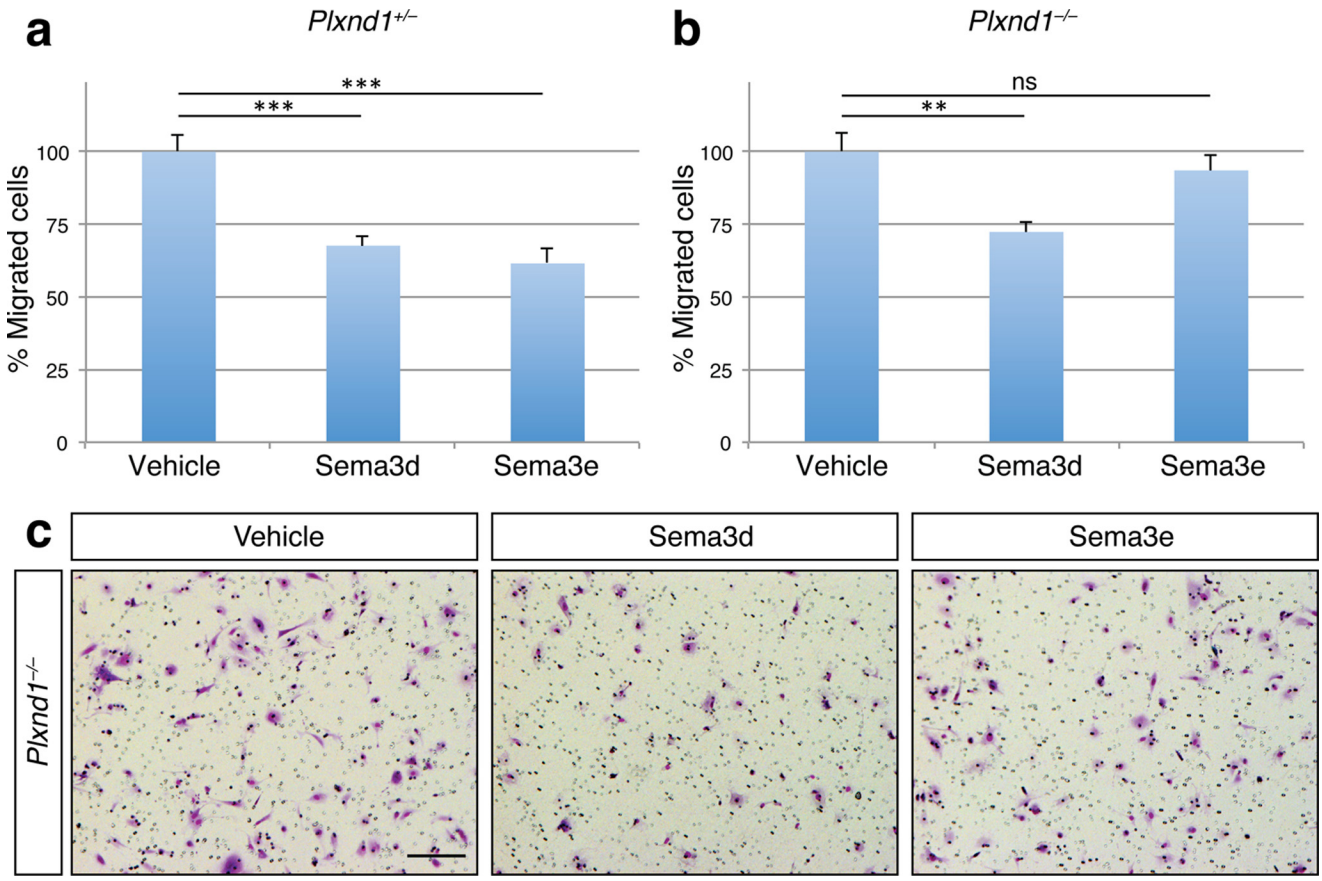
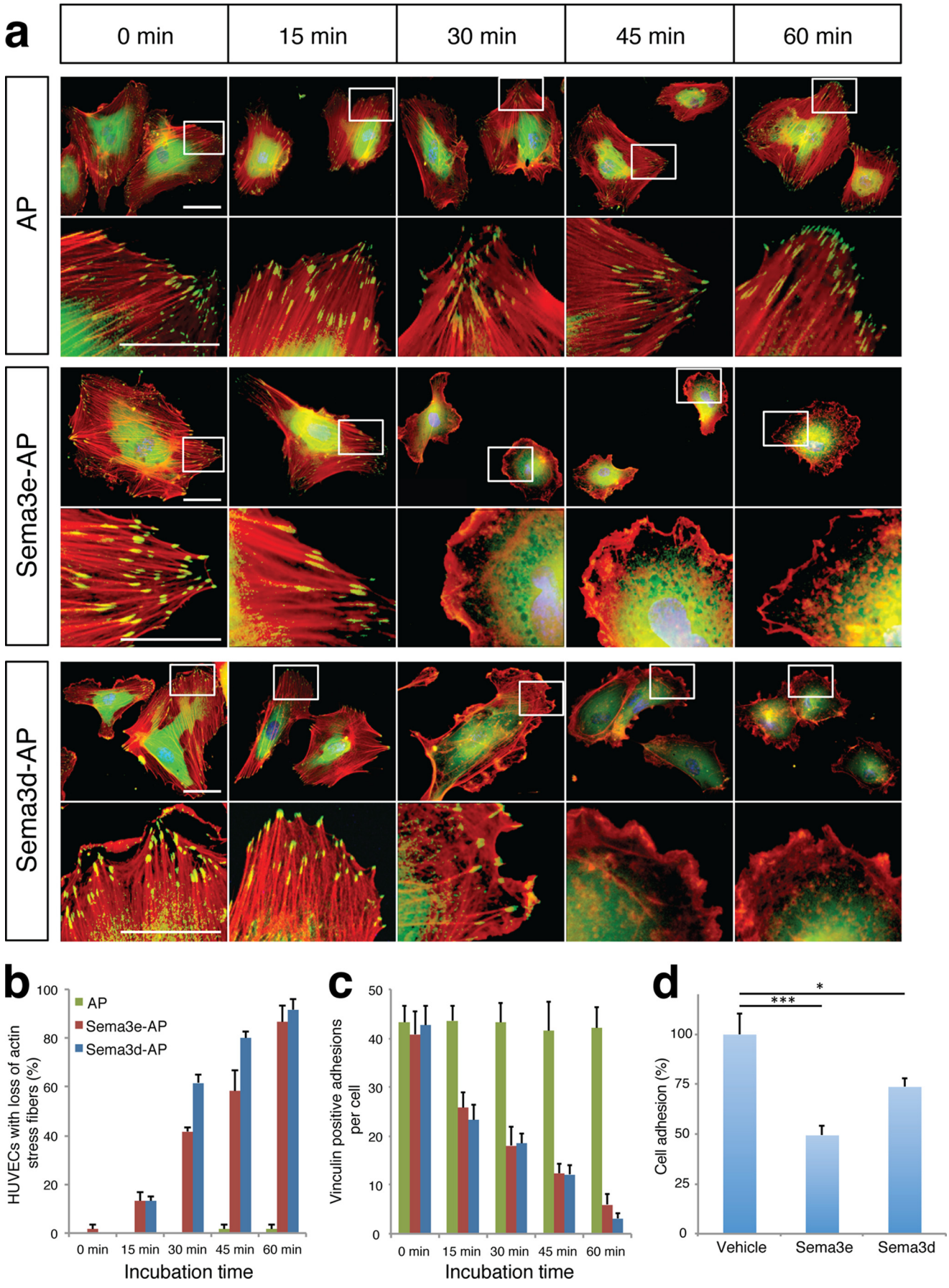


FIGURE 2. **Sema3d inhibits endothelial migration independently of Plxnd1.** *a*, graph representing the percentage of *Plxnd1*$^{+/-}$ endothelial cells that migrated through a porous transwell insert in the presence of Sema3d or Sema3e compared with a vehicle control. ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 3$). *b*, graph representing the percentage of *Plxnd1*$^{-/-}$ endothelial cells that migrated through a porous transwell insert in the presence of Sema3d or Sema3e compared with a vehicle control. **, $p < 0.01$; *ns*, not significant (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 3$). *c*, photomicrographs of stained endothelial cells that migrated through a transwell membrane in the presence of vehicle (*left panel*), Sema3d (*center panel*), or Sema3e (*right panel*). *Scale bar* = 100 μm.

intracellular signaling pathways activated in response to various class 3 semaphorins and at which points signaling via Sema3d and Sema3e converge. In this regard, the details of how plexin and neuropilin receptors signal are only partially understood, and the complexity of Sema3 ligand-receptor interactions continues to emerge in the literature. Although Sema3e can signal via Plxnd1 alone, the full description of the functional Sema3d receptor(s) are unknown and remain elusive, although our work indicates that Nrp1 is a necessary component of the Sema3d receptor in endothelial cells. We were unable to detect binding of Sema3d to any of the plexins we examined, including plexins A1, A2, A3, B2, and D1 (data not shown). In addition to plexin receptors, we did not detected binding of Sema3d to other candidate coreceptors, including Pdgfrα, Pdgfrβ, Vegfr1, Vegfr2, and Vegfr3 (data not shown).

We demonstrate that Sema3d, but not Sema3e, induces Akt phosphorylation in HUVECs and requires PI3K signaling for endothelial repulsion. This is in contrast to the several class 3 semaphorins that negatively regulate Akt signaling. Sema3a, Sema3b, and Sema3f have all been shown to inhibit or decrease Akt phosphorylation in various cell types (19–23). Sema3e can inhibit VEGF-mediated Akt phosphorylation in endothelial cells (24) but can also increase Akt phosphorylation in subicular neurons (25). Our results provide an example of semaphorin-mediated endothelial guidance requiring the PI3K/Akt signaling pathway.

Although our results suggest that Sema3d and Sema3e produce similar effects on endothelial cells, it remains possible that unique functions exist for Sema3d or Sema3e that were not detected by our assays. Sema3e signaling, for example, can induce apoptosis in tumor endothelial cells (18). Sema3c promotes proliferation and inhibits apoptosis of mouse glomerular endothelial cells and affects migration (26). Sema3a inhibits cancer cell proliferation and causes cellular contraction (27). Although Sema3b can induce a similar morphologic contraction in these cells, it does not have the same effect on cell proliferation. The effects of Sema3d and Sema3e may also depend on the specific types of endothelial cells that are utilized. Although our studies focused on HUVECs, it is becoming increasingly clear that an important heterogeneity exists between endothelial populations (28). For example, arterial, venous, and lymphatic endothelial cells differentially express neuropilin, VEGF, and Notch receptors. Perhaps guidance queues mediated by Sema3 proteins are differentially interpreted by the arterial, venous, and lymphatic endothelium, in part on the basis of receptor availability. It is worth noting that the primary defect resulting from Sema3d inactivation in the mouse is mispatterning of the pulmonary veins (12), whereas


a
0 min
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HUVECs with loss of actin stress fibers (%)
0
20
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80
100
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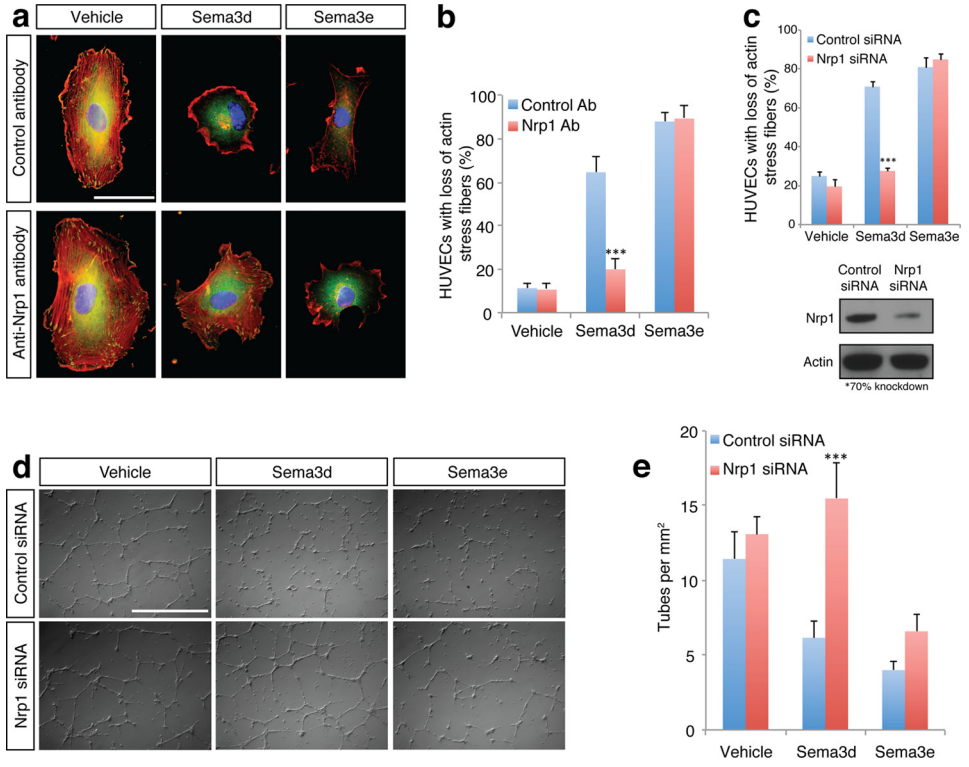

FIGURE 4. **Sema3d-mediated, but not Sema3e-mediated, cytoskeletal reorganization and inhibition of tubulogenesis is dependent on Nrp1.** *a*, HUVECs were exposed to Sema3d, Sema3e, or a vehicle control with or without an anti-NRP-1 blocking antibody for 60 min and subsequently stained for F-actin (*red*) and vinculin (*green*). *b*, quantification of the percentage of HUVECs displaying an absence of actin stress fibers under each condition. ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 60$ cells/condition). *Ab*, antibody. *c*, quantification of the percentage of HUVECs with loss of actin stress fibers after Nrp1 or control siRNA transfection incubated with Sema3d, Sema3e, or vehicle control. ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 60$ cells/condition). Also shown is a Western blot analysis of Nrp1 protein expression in HUVECs after siRNA-mediated knockdown. *d*, photomicrographs of HUVECs after Nrp1 or control siRNA-mediated knockdown, seeding in Matrigel, and incubation with Sema3d, Sema3e, or a vehicle control for 8 h. *e*, quantification of the number of tubules formed per high power field. ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 9$). *Scale bars* = 5 μm (*a*) and 1 mm (*d*).

inactivation of Sema3e produces abnormalities of intersomitic arteries (10).

In neurons, the cellular response to a given Sema3 protein may be dependent on the receptor complexes that are expressed, and different receptors can produce opposing responses to the same ligand. During mouse neural development, for example, neurons that express both Plxnd1 and Nrp1 are attracted to Sema3e and migrate toward the signal (29). In contrast, neurons expressing only Plxnd1 are repelled by Sema3e. Similarly, in zebrafish, sema3d can act as a repellent signal to axons expressing nrp1A and as an attractant to axons expressing both nrp1A and nrp2A (30). Sema3e can also signal through a heterotrimeric receptor complex composed of Plxnd1, Nrp1, and vascular endothelial growth factor 2 (Vegfr2) to promote axonal growth (25). In contrast, Sema3e inhibits the growth of axons expressing Plxnd1 only. The effects of Sema3 proteins are also modulated by the variable expression of intracellular signaling components. In the central nervous system, for example, the ability of Sema3f to induce stereotyped axonal pruning of neurons originating in the dentate gyrus is dependent upon the presence of an intracellular Rac GTPase-activating protein, called β2-chimaerin, that binds to the intracellular domain of Nrp2 and is activated by Sema3f binding (31). The ability of Sema3f to induce axon repulsion, however, does not require β2-chimaerin.

Nrp1, which, as we showed, is necessary for Sema3d repulsion of HUVECs, has also been demonstrated to serve as a subunit of a receptor for VEGF. Sema3 competition with VEGF for neuropilin binding can inhibit the angiogenic effects of VEGF on the endothelium (32). Furthermore, different members of the Sema3 family compete with VEGF with varying potency. We and others have recently shown that semaphorin-Plxnd1 activity can modulate VEGF signaling by inducing secretion of a soluble VEGF receptor (sFlt1) (33) and, conversely, VEGF can also act upstream of Plxnd1 to regulate its expression (34). The result of VEGF and semaphorin integration modulates Notch signaling in tip and stalk cells of angiogenic sprouts, and the combined actions of these signals contribute to the determination of tip and stalk cell identity and the angiogenic response. It seems likely that

FIGURE 3. **Sema3d and Sema3e induce loss of actin stress fibers and down-regulate focal adhesion complexes.** *a*, HUVECs were incubated with alkaline phosphatase-tagged Sema3d, Sema3e, or alkaline phosphatase alone (*AP*) as a control for 0, 15, 30, 45, and 60 min and stained for F-actin (*red*) and vinculin (*green*). *b*, quantification of the percentage of HUVECs displaying loss of actin stress fibers at each time point ($n = 60$ cells/condition). *c*, quantification of the number of vinculin units per HUVEC at each time point ($n = 10$ cells/condition). *d*, percentage of HUVEC adhesion to collagen I after a 30-min incubation with Sema3e, Sema3d, or a vehicle control as quantified by a colorimetric assay. *, $p < 0.05$; ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 5$). *Scale bars* = 50 μm (*smaller bar*) and 5 μm (*larger bar*).

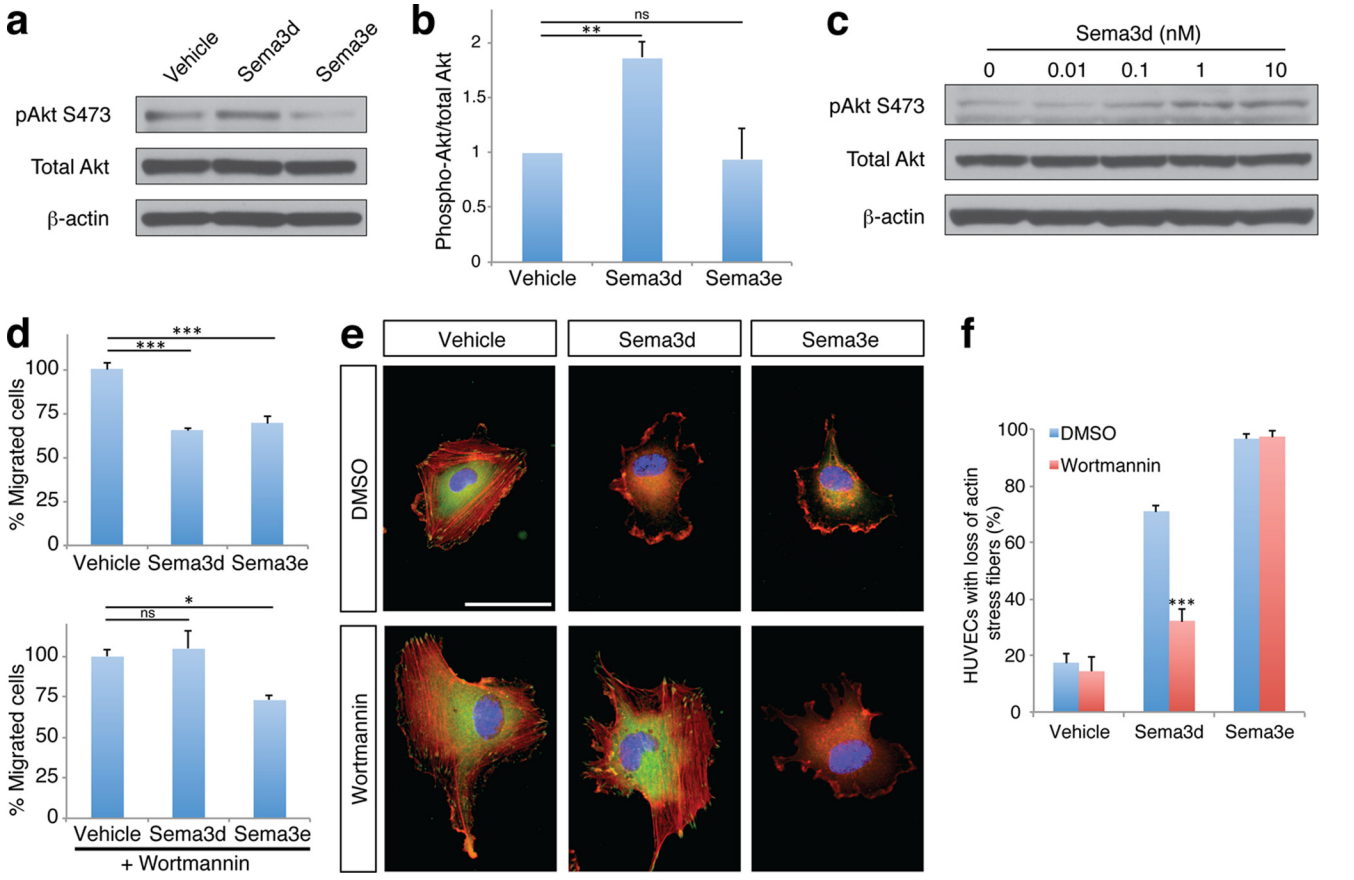

FIGURE 5. **Sema3d signals through PI3K/Akt to repel endothelial cells via actin cytoskeletal reorganization.** *a*, Western blot analysis for phospho-Akt (Ser-473) of HUVECs treated with Sema3d, Sema3e, or vehicle for 5 min. *b*, quantification of Akt phosphorylation normalized to total Akt of HUVECs incubated with 10 nM of Sema3d, Sema3e, or a vehicle control for 5 min. **, $p < 0.01$; *ns*, not significant (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 3$). *c*, Western blot for phospho-Akt (Ser-473) of HUVECs treated with increasing doses of Sema3d for 30 min. *d*, *top panel*, graph representing the percentage of HUVECs that migrated through a porous transwell insert in the presence of Sema3d or Sema3e compared with a vehicle control. ***, $p < 0.001$ (one-way ANOVA between groups, post-hoc multiple comparisons, Tukey's test, $n = 3$). *Bottom panel*, graph representing the percentage of HUVECs that migrated through a porous transwell insert toward medium containing wortmannin and either Sema3d or Sema3e compared with a vehicle control. *, $p < 0.05$; *ns*, not significant (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 3$). *e*, HUVECs were incubated with Sema3d, Sema3e, or a vehicle control with or without wortmannin (1 μM) for 30 min and subsequently stained for F-actin (*red*) and vinculin (*green*). *DMSO*, dimethyl sulfoxide. *f*, quantification of the percentage of HUVECs displaying an absence of actin stress fibers under each condition. ***, $p < 0.001$ (one-way ANOVA between groups, post hoc multiple comparisons, Tukey's test, $n = 60$ cells/condition). *Scale bar* = 5 μm.

Sema3d and Sema3e will modulate variably and intersect with other important angiogenic and antiangiogenic pathways, including VEGF.

The range and diversity of cellular effects in response to Sema3 proteins in different cell types speaks to the complexity of Sema3 protein-receptor signaling. We demonstrate that Sema3d and Sema3e can similarly affect endothelial motility and migration through distinct cellular mechanisms. Sema3e-Plxnd1 signaling in endothelial cells relies on the GTPase-activating protein (GAP) activity of Plxnd1 to inactivate R-Ras and activate Arf6 to modulate the cytoskeleton and cellular adhesion (15). It will be of interest to investigate these pathways in Sema3d signaling in endothelial cells and whether Sema3d requires a plexin coreceptor or an alternate signaling partner in association with Nrp1.

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