Sema3d controls collective endothelial cell migration by distinct mechanisms via Nrp1 and PlxnD1

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During cardiovascular development, tight spatiotemporal regulation of molecular cues is essential for controlling endothelial cell (EC) migration. Secreted class III Semaphorins play an important role in guidance of neuronal cell migration and were lately linked to regulating cardiovascular development. Recently, SEMA3D gene disruptions were associated with cardiovascular defects in patients; however, the mechanisms of action were not revealed. Here we show for the first time that Sema3d regulates collective EC migration in zebrafish through two separate mechanisms. Mesenchymal Sema3d guides outgrowth of the common cardinal vein via repulsion and signals through PlexinD1. Additionally, within the same ECs, we identified a novel function of autocrine Sema3d signaling in regulating Actin network organization and EC morphology. We show that this new function requires Sema3d signaling through Neuropilin1, which then regulates Actin network organization through RhoA upstream of Rock, stabilizing the EC sheet. Our findings are highly relevant for understanding EC migration and the mechanisms of collective migration in other contexts.

Introduction

Cardiovascular development requires coordinated specification and migration of endothelial cells (ECs) and depends on the tight spatiotemporal regulation of attractive and repulsive guidance cues. Misregulation of these cues can result in improper EC guidance and developmental defects and has implications for disease etiologies in the adult organism.

The Class III Semaphorins are a group of seven secreted proteins: Sema3a, b, c, d, e, f, and g. They have been shown to act as guidance cues and can have repulsive or attractive functions (Raper, 2000). Class III Semaphorins were first discovered as axon guidance molecules (Kolodkin et al., 1992; Luo et al., 1993) and later were shown to play an important role in cardiovascular morphogenesis (Gu and Giraudo, 2013). Loss of SEMA3C in mice results in aortic arch and outflow tract septation defects (Feiner et al., 2001), whereas SEMA3E is required for intersomitic artery patterning (Gu et al., 2005; Meadows et al., 2012). Recently, SEMA3D has been shown to be necessary for pulmonary vein development and pulmonary venous connections in mice (Degenhardt et al., 2013). In addition, disruptions in the SEMA3D gene in human patients resulted in congenital heart defects and anomalous pulmonary vein formations (Degenhardt et al., 2013; Sanchez-Castro et al., 2015). These analyses of SEMA3D deficiencies in mice and humans, however, were confined to phenotypic descriptions and failed to elucidate the mechanisms by which SEMA3D fulfills its diverse functions. Sema3d has been shown to inhibit (Aghajanian et al., 2014) or promote (Foley et al., 2015) cell migration. These contrary functions could be caused by distinct spatiotemporal gene expression regulation, but also by signaling through different receptors. Class III Semaphorins can signal through receptors of the Neuropilin (Nrp) and Plexin (Plxn) families in various combinations (Gu and Giraudo, 2013). However, it is unknown how differentially regulated Sema3d acts in vivo to elicit distinct endothelial responses.

The technical difficulties of addressing this question stem from the inaccessibility of early embryonic development and the limitations of time- and tissue-specific knockout strategies. Therefore, we used the translucent and externally developing model organism zebrafish for the analysis of vascular development by endothelial-specific transgenic fluorophore expression (Schuermann et al., 2014). The zebrafish vasculature has high structural homology with other vertebrates (Isogai et al., 2001), and most signaling pathways are highly conserved (Ellertsdtir et al., 2010). We use the developing common cardinal vein (CCV) as a model for collective EC migration during cardiovascular morphogenesis (Helker et al., 2013). The bilateral CCVs, which connect the venous system to the heart, develop as open-ended tubes from the cardinal veins and extend ventrally as sin-
Sema3d is required for vascular development

To assess the role of Sema3d in cardiovascular morphogenesis of zebrafish embryos, we generated Sema3d-deficient zebrafish embryos. We performed morpholino (MO)-mediated gene knockdown and also generated zebrafish sema3d mutants using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genome editing. We observed two phenotypes upon sema3d knockdown: first, a significantly shorter but wider CCV; and second, impaired collective and directional migration resulting in a disrupted EC sheet with impaired cell morphology. By using time- and tissue-specific knockdown as well as Sema3d receptor analysis, we showed that Sema3d elicits its differential functions by signaling through different receptors. Whereas mesenchymal Sema3d controls CCV outgrowth by signaling through PlxnD1 via repulsion, EC-specific Sema3d regulates Actin network organization and junction formation in the leading edge of collectively migrating ECs. There, Sema3d signals through Nrp1 via RhoA and Rock in an autocrine manner, facilitating consistent cell sheet organization.

Sema3d regulates collective EC migration by controlling CCV width and EC sheet organization

To investigate Sema3d function in collective EC migration, we analyzed CCV development in Tg(kdrl:EGFP) embryos. We showed that both sema3d morphants and mutants failed to form a proper CCV (Fig. 1, D–H; and Fig. S2 E). CCV length was significantly reduced by ~40% in morphants and mutants, whereas the CCV front width was nearly doubled at 30 hpf (Fig. 1, E, G, and H). We measured the CCV outgrowth angle and showed that Sema3d-deficient embryos exhibited a fanned-out CCV outgrowth. Whereas in control (Ctr) MO and wild-type (WT) embryos, the CCV ECs migrated out at a nearly orthogonal angle from the anterior and posterior cardinal vein, this angle was shifted by ~20° upon knockdown, resulting in a widened CCV (Fig. S2 D).

In addition to this phenotype of increased CCV width, we identified a second phenotype exhibiting lesions in the leading edge of the EC sheet. For a more detailed investigation of cell morphology and especially Actin polymerization in the leading edge, we generated the transgenic line Tg(fli1a:lifeactEGFP)z260. We used the fli1 promoter to drive the Lifeact marker (Riedl et al., 2008) for visualization of F-Actin in endothelial and blood cells. Ctr morphants showed an ordered cell sheet with intact cell–cell contacts, whereas sema3d morphants and mutants showed a disorganized cell sheet with lesions (Fig. 1 F).

This disorganized cell sheet could be caused by a reduced number of cells. Thus, we investigated the CCV cell number, which was not affected before 32 hpf in sema3d morphants and mutants (Fig. S2 B). Because the two described phenotypes already occurred before 30 hpf, the reduced cell number cannot be causative, but EC migration itself might be impaired. Because Sema3d deficiency affected not only the CCV but also Se outgrowth, we investigated whether the stalled Se phenotype might be caused by reduced cell number or reduced sprouting. We quantified EC numbers at 30 hpf and sprouting events at 22 hpf (Fig. S3, B and C). Neither sprouting nor cell number was impaired in sema3d morphants or mutants, indicating that the stalled Se phenotype is also caused by impaired EC migration.

Based on these findings, we further investigated the migrational parameters of single CCV ECs from 28 to 32 hpf (Fig. 1, I and J; and Video 1 and 2). We found that neither the track length nor the migration speed was altered by Sema3d deficiency (Fig. 1, I and J). However, we observed changes in the outgrowth orientation, which we measured by determining the angle of the migration tracks relative to the perpendicular. We showed that this angle was on average more than doubled upon loss of Semad3, which led to the fanned-out phenotype (Fig. 1 J). Moreover, in Ctr morphants, the ECs migrated ventrally in a highly directional manner. In contrast, upon sema3d knockdown, ECs changed direction and made detours. To quantify the altered directionality, we measured the migration track displacement length. In Ctr morphants, the EC track displacement amounted to 64 µm over 4 h, whereas in Semad3-deficient embryos it was reduced to 48 µm (Fig. 1 J).

In sum, we observed two phenotypes upon loss of Sema3d: first, fanned-out cell migration tracks, leading to a shorter but wider CCV; and second, impaired collective and directional migration combined with a disrupted EC sheet (Fig. 1 K).

Sema3d is localized in the mesenchyme next to the CCV and in the ECs of the CCV leading edge

To understand these two distinct phenotypes of CCV collective migration upon sema3d knockdown, we investigated Sema3d localization.
Figure 1. Sema3d regulates collective EC migration by controlling CCV width and EC sheet organization. (A) Vasculature of a 30-hpf-old zebrafish embryo visualized by GFP expression of Tg(kdrl:EGFP)s843. (B) Illustration of CCV development. (C) CCV ECs migrate as a collective cell sheet; F-Actin visualized by GFP expression of Tg(fli1a:lifeactEGFP)mu240 at 32 hpf. (D–F) CCV development is impaired in sema3d morphants and mutants. (D) Widened CCV outgrowth upon Sema3d loss at 24 hpf (white arrowheads); confocal projections of Tg(kdrl:EGFP)s843 embryos. (E) Shorter but wider CCVs of sema3d morphants and mutants at 30 hpf (white brackets). (F) The CCV cell sheet exhibits lesions (black arrowheads) in the leading edges of 30-hpf Sema3d-deficient Tg(fli1a:lifeactEGFP)mu240 embryos. Confocal projections were color-inverted. (G) Quantification of CCV length shows a shortening in sema3d morphants and mutants at 30 hpf (each n = 25). (H) Quantification of CCV front width shows an increase in sema3d morphants and mutants at 30 hpf (each n = 18). (I and J) Tracking of single CCV EC migration from 28 to 32 hpf. (I) Confocal projections at 32 hpf with migration tracks (turquoise lines) and displacement distance (white arrows). (J) Quantification of migration parameters (each condition 84 cells and 14 cells per embryo/movie). Track length and migration speed are not impaired in sema3d morphants. In contrast, track displacement length is reduced and the angles of tracks to the perpendicular are increased in sema3d morphants compared with Ctr morphants, leading to a fanned-out CCV. (K) Model of sema3d knockdown (k.d.) representing the two distinct phenotypes: first, fanned-out cell migration tracks leading to a shorter but wider CCV; second, impaired collective and directional migration and a disrupted EC sheet. DA, dorsal aorta; ACV, anterior cardinal vein; PCV, posterior cardinal vein. ***, P < 0.001; n.s., not significant. Error bars indicate SD.
localization. Antibody staining revealed that Sema3d is localized in the mesenchyme next to the CCV at 25 and 32 hpf (Fig. 2 A). At 32 hpf, Sema3d was additionally expressed in the ECs of the CCV leading edge. By confocal imaging, we clearly visualized the anti-Sema3d signal in vesicles inside the green kdrl:GFP-positive cells of the CCV leading edge cells (Fig. 2, A and B). sema3d morphants and mutants completely lacked Sema3d staining (Fig. S1 E). By in situ hybridization, we confirmed the sema3d expression from the mesenchyme and the leading edge ECs (Fig. S2 C). Likewise, Sema3d expression by ECs has been shown in human umbilical vein ECs (HUV ECs; Serini et al., 2003). Overall, we showed that there are two distinct sema3d expression domains (Fig. 2 C): sema3d is expressed in the mesenchyme on both sides of the CCV and in the ECs of the CCV leading edge.

Mesenchymal Sema3d acts as a repulsive cue for CCV outgrowth
Sema3d has been shown to act as a repulsive cue during axon guidance (Liu et al., 2004; Wolman et al., 2004). To figure out whether Sema3d mediates CCV repulsion, we performed global sema3d overexpression with sema3d mRNA injection (Fig. 3 A) or sema3d overexpression upon heat shock in the transgenic line Tg(hsp70:sema3dGFP) (Fig. 3 B). Overexpression did not abolish outgrowth completely, but CCV width was significantly reduced by one third (from 120 to 80 µm; Fig. 3 C). We hypothesized that mesenchymal Sema3d acts as a repulsive cue on CCV collective migration. To test this, we performed mosaic sema3d knockdown through injection of sema3d MO together with DiI for cell tracking into 1 of 16 cells (Fig. 3 D). Partial loss of sema3d in the mesenchyme next to the CCV induced ECs to migrate into the mesenchyme area. This indicates that the bilateral mesenchymal sema3d expression domains provide Sema3d as a repulsive cue to direct medial CCV outgrowth.

EC-specific Sema3d is required for EC sheet organization and collective migration
At 24 hpf, the CCV is an open-ended cylinder (Fig. 1 B), located between the two mesenchymal sema3d expression domains (Fig. 2 A). From the roof of the cylinder, the ECs migrate ventrally as a cell sheet underneath the epidermis. At 30 hpf, no other tissue is located in proximity of the extended cell sheet (Fig. S4 C). Based on EC-specific sema3d expression in the leading edge (Fig. 2 and Fig. S2 C), we hypothesized that the sema3d knockdown phenotype in the CCV leading edge depends on EC-specific Sema3d. Therefore we aimed to delete sema3d selectively in the CCV leading edge ECs by two different approaches: time-specific sema3d photo-MO activation (Fig. 4) and mosaic sema3d knockdown through injection of MO in only one of 16 blastomeres (Fig. S4).

We used light-inducible cleavage of an inhibiting sense (SE)–MO to specifically activate the sema3d antisense (AS)-MO at chosen time points. For this approach, the sema3d AS-MO was injected in combination with a pairing sema3d SE-photo-MO, resulting in an inactive, bound MO pair (called AS+SE-photo-MO). UV light–induced cleavage of the SE-MO destroys the pairing and thereby frees the sema3d AS-MO to bind to sema3d mRNA and inhibit protein translation. In short, the sema3d MO is active only after UV induction (Fig. 4 A). We showed that UV exposure itself had no effect on Ctr MO-injected embryos in terms of CCV width and cell sheet morphology (Fig. 4, B–C′). Likewise, SE-photo-MO–injected embryos that were not exposed to UV light showed a WT phenotype, similar to Ctr MO-injected embryos (Fig. 4, E and E′). To control whether UV light exposure of AS+SE-photo-MO indeed reproduces the phenotype obtained by sema3d MO injection, we exposed whole AS+SE-photo-MO–injected embryos to UV light at 9 hpf (Fig. 4, F and F′). Indeed, MO activation at 9 hpf completely reproduced the phenotypes observed in Sema3d-deficient embryos and induced wider CCVs and disrupted cell sheet morphology as in sema3d morphants and mutants.
(Fig. 4 B, compare with Fig. 1, E and F; and Fig. 4, compare F and F’ with D and D’).

Next, we chose to activate the sema3d MO and thereby stop Sema3d protein production at different time points of CCV development. At 24 hpf, the CCVs formed between the two mesenhexymal sema3d expression domains, but did not extend beyond them. UV exposure at 24 hpf resulted in a mean CCV width of 173 µm (Fig. 4, B and G) and therefore affected the CCV width much less severely than UV-mediated induction at 30 hpf. (C) Quantification of CCV front width (each n = 18). Error bars indicate SD. (D) Mosaic sema3d k.d. through sema3d MO injection into one of 16 cells in Tg(kdrl:EGFP) embryos (magenta, coinjection with DiI for cell tracking). Partial loss of sema3d in the mesenchyme (white asterisks) induced ECs (white arrowhead) to migrate into the mesenchyme (n = 3), indicating that sema3d acts as a repulsive cue in the mesenchyme next to the CCV. Ctr MO-injected mesenchymal cells (white asterisks in right panel) did not induce ectopic EC migration. ***, P < 0.001.

Sema3d regulates Actin network organization in leading edge ECs controlling cell shape and contacts

In addition to the described lesions in the CCV front in sema3d morphants and mutants, ECs also exhibit impaired cell morphology (Figs. 1 F and 5 A). To assess changes in morphology, we measured the cell shape factor, with a value of 1 indicating a perfectly round shape (Fig. 5 B). Whereas Ctr MO-injected cells had a cell shape factor of 0.7, Sema3d-deficient cells had a mean cell shape factor of 0.4, confirming that Sema3d regulates cell morphology directly or indirectly at the leading front of the CCV migrating cell sheet (Fig. 5 B).

We hypothesized that loss of Sema3d could alter the Actin cytoskeleton of leading edge ECs or act on EC junctions. Therefore, we first investigated the cellular Actin structure by imaging of Tg(fli1a:lifeactEGFP) embryos. In sema3d morphants and mutants the cohesive cell sheet was disrupted and cells lacked cell–cell contacts (Fig. 5 A). Live imaging illustrated the inability of sema3d morphant ECs to form a consistent cell sheet (Ctr MO in Video 3 vs. sema3d morphant in Video 4). Moreover, in Ctr MO and WT siblings Actin cables were arranged in parallel to the leading front. These organized Actin cables were not present in Sema3d-deficient cells (Fig. 5, A and E). To analyze whether partial impairment of Actin polymerization is sufficient to cause the observed phenotype, we inhibited Actin/Mysin polymerization by applying blebbistatin or latrunculin A from 22 to 30 hpf. Interestingly, inhibition of Actin/Mysin polymerization completely phenocopied the disrupted CCV sheet morphology that we observed in sema3d mutants, but did not lead to a wider CCV (Fig. 5 C). Analysis of this experiment with Tg(fli1a:lifeactEGFP) embryos was not possible, as inhibition of Actin polymerization prevents incorporation of the fluorescent F-Actin fragments of the Lifeact reporter.

To elucidate the mechanism by which Sema3d regulates Actin network organization, we investigated potential Sema3d...
downstream effectors. Class III Semaphorins and their receptors have been shown to interact with multiple GTPases (Püschel, 2007). The GTPases RhoA, Rac1, and Cdc42 have been linked to regulation of Actin cytoskeleton organization (Sit and Manser, 2011). We used pharmacological inhibitors against each of these GTPases from 22 to 30 hpf and analyzed Actin cable formation and cell–cell contacts in Tg(fli1a:lifeactEGFP) mu240 embryos.

We showed that Rac1 and Cdc42 are not involved in the regulation of CCV collective cell migration (Fig. 5, D and F). However, inhibition of either RhoA or its downstream effector Rock induced the same leading edge phenotype as Sema3d depletion, characterized by lesions in the cell sheet as well as a loss of intracellular parallel Actin cables (Fig. 5 F). To quantify the CCV cell–cell contact phenotype, we determined the distance of cell–cell contacts per cell in relation to the perimeter (Fig. 5 D). Free cell edges such as the leading front, which cannot make any cell contact, were added to the cell–cell contact length. Because of the variable size of leading-front lengths, the minimum percentage of cell–cell contacts would therefore be roughly 20%. Ctr cells exhibited ~100% of perimeter with cell–cell contacts, whereas this number was reduced to 65–69% in Sema3d morphants and mutants as well as in RhoA or Rock inhibitor–treated embryos (Fig. 5 D). Accordingly, the percentage of CCV front cells with aligned Actin cables was also reduced by at least 75% upon knockdown of Sema3d and inhibition of RhoA or Rock (Fig. 5, E and F).

Additionally, it has been shown that FAK can control Actin assembly (Serrels et al., 2007). We therefore also investigated the contribution of FAK to the phenotype. Inhibition of FAK resulted in loss of Actin cables but not of cell–cell contacts (Fig. 5, D–F). We conclude that Actin cable assembly can depend on FAK; however, formation of the cohesive cell sheet and CCV migration seemed to be independent of FAK. Taken together, these experiments indicate that Sema3d regulates cell–cell contact formation and Actin assembly in ECs through the effector kinases RhoA and Rock.

Interestingly, we observed a comparable function of Sema3d in mediating cell–cell contacts in Ses. As noted...
Figure 5. **Sema3d acts on Actin network organization in leading edge ECs controlling Actin cable formation, cell shape, and contacts.** (A) The cohesive cell sheet in the CCV leading edge is disrupted and exhibits lesions (pink arrowheads) in sema3d morphants and mutants at 30 hpf. In Ctr embryos, Actin cables are arranged parallel to the leading front; they are absent in Sema3d-deficient embryos (open arrowheads). High-resolution confocal projections of Tg(fli1a:lifeactEGFP) embryos from Fig. 1F were color-inverted. (B) Quantification of the circularity of CCV leading edge cells illustrates that loss of **sema3d** significantly alters cell shape (each n = 24). (C) Inhibition of Myosin/Actin phenocopies **sema3d** phenotype of disrupted cell–cell contacts (pink arrowheads) but does not lead to a wider CCV. Confocal projections of Tg(kdrl:EGFP) embryos at 30 hpf. (D) The length of cell perimeter with cell–cell contacts of CCV leading edge cells is reduced in **sema3d** morphants and mutants and after Rock or RhoA inhibition. Inhibition of Rac1, Cdc42, or FAK had no effect on cell–cell contacts (n = 30 for morphants and mutant cells and n = 18 for inhibitor experiments). (E) Quantification of front cells with aligned Actin cables.
Earlier, Ses were stalled at the horizontal myoseptum because of migrational defects in Sema3d-deficient embryos (Fig. S3, A–C). To elucidate the effect of EC-specific sema3d knockdown, we transplanted sema3d MO-injected Tg(kdrl:EGFP) injected donor cells into WT Tg(kdrl:HRAS-mCherry) hosts and analyzed the vascular defects (Fig. S3 D). In the case of global sema3d knockdown or transplanted Sema3d-deficient muscle tissue, Ses were stalled at the horizontal myoseptum (Fig. S3 E). In the case of EC-specific knockdown, Se cells lost their connections to neighboring cells (Fig. S3 F). We conclude that EC-specific Sema3d is required for cell–cell contact formation in Ses and for Se cell migration (Fig. S3 G).

Next, we analyzed the contribution of cell–cell junctions to the regulation of EC migration. We hypothesized that loss of cell–cell contacts could be caused by a misregulation of junctional proteins. Previously, we showed that loss of the adherens junction molecule Cdh5 impairs the directional migration of CCV cells (Helker et al., 2013). Interestingly, we observed a similar effect on directional EC migration in sema3d mutants and morphants (Fig. 1, I and J). To determine whether misregulation of Cdh5 downstream of Sema3d impairs directionality, we analyzed cdh5 expression. cdh5 mRNA expression was not altered in sema3d mutants and morphants (Fig. S2, E and F). Additionally, we analyzed Cdh5 protein distribution together with the tight junction molecule zonula occludens 1 (ZO-1; Fig. 6 A). sema3d morphant ECs exhibited altered distribution of junctional proteins: although ZO-1 staining was equally distributed at the cell edges, Cdh5 staining was aberrantly distributed in a patchy pattern and lost in several locations. We quantified the total length of Cdh5-positive cell perimeter in relation to the whole perimeter and found it to be reduced by more than 20% in Sema3d-deficient cells (Fig. 6 B). However, when analyzing cell–cell contact length using Tg(fli1a:lifeactEGFP) embryos, we found no changes in cdh5 mutants (Fig. 6, C and E). Interestingly, Cdh5-deficient CCV ECs exhibited tiny holes within the ECs themselves (Fig. 6 C, pink arrowheads). We speculate that these holes within the cells might contribute to a loss of cell sheet tension, much as the reduction in cell–cell contacts upon Sema3d deficiency might lead to the described phenotype of impaired directional migration. To exclude direct regulation of cell–cell contacts by blood flow, we inhibited flow with nifedipine and tricaine from 22 to 30 hpf. We observed no change in cell–cell contact length under these conditions (Fig. 6, D and E).

Taken together, our experiments reveal that sema3d knockdown alters the distribution of junctional proteins and affects the cytoskeleton, resulting in an impaired EC shape as well as a loss of cell–cell contacts and Actin cables. We conclude that EC-specific Sema3d regulates Actin polymerization via RhoA and Rock in leading edge ECs independently of Cdh5 and flow.

The differential functions of Sema3d are transduced by different receptors

We have shown that on the one hand Sema3d acts as a repulsive cue and on the other hand as a regulator of Actin network organization facilitating a consistent cell sheet organization. Therefore, we asked how the same molecule could act on the same EC population but elicit very different phenotypic responses. One hypothesis is that the different responses are mediated by signaling through different Sema3d receptors. Class III Semaphorin proteins are secreted ligands and have been reported to signal through class A and D Plexins and Neuropilin 1 and 2 (Gu and Giraudo, 2013). Therefore, we used in situ hybridization to investigate the expression pattern of all potential Sema3d receptor candidates: plxnA1b, plxnA2, plxnA3, plxnD1, nrp1a, nrp1b, nrp2a, and nrp2b. Of these only plxnD1, nrp1a, and nrp1b were expressed in the CCV (Fig. 7 A). cdh5 was used as a control, as it labels all CCV ECs. In comparison to cdh5, plxnD1 was specifically expressed in the most dorsal CCV ECs. In contrast, nrp1a was expressed within the whole CCV, including the leading edge.

To analyze whether the differential roles of Sema3d are mediated by differential receptor signaling, we analyzed PlxnD1- or Nrp1-deficient embryos. MO-mediated loss of plxnD1 led to an increased CCV width (Fig. 7 B). Ctr MO-injected embryos exhibited a CCV width of ∼120 µm, whereas plxnD1 MO-injected embryos had CCV widths of ∼200 µm (Fig. 7 D). Indeed, loss of plxnD1 phenocopied the increased CCV width phenotype of sema3d morphants and mutants (Fig. 1, compare E and H). However, analysis of the CCV leading edge cell sheet did not show any disruption of the cohesive EC sheet (Fig. 7 B). Measurement of the percentage of cell perimeter with cell–cell contacts showed no significant difference between plxnD1 morphants and Ctr morphants (Fig. 7 G).

In contrast, nrp1ahu1002 mutant embryos and nrp1a/b morphants did not show an increased CCV width (Fig. 7, C, D, and F). Nevertheless, the CCV leading edge exhibited a disorganized cell sheet with an increased number of lesions in Tg(kdrl:EGFP) embryos, whereas plxnD1 MO-injected embryos had CCV widths of ∼200 µm (Fig. 7 D). Indeed, loss of plxnD1 phenocopied the increased CCV width phenotype of sema3d morphants and mutants (Fig. 1, compare E and H). However, analysis of the CCV leading edge cell sheet did not show any disruption of the cohesive EC sheet (Fig. 7 B). Measurement of the percentage of cell perimeter with cell–cell contacts showed no significant difference between plxnD1 morphants and Ctr morphants (Fig. 7 G).

In summary, loss of Sema3d results in a wide CCV with lesions, plxnD1 knockdown in a wide CCV only, and Nrp1 knockdown in a disrupted cell sheet with lesions (Fig. 7 H). Nrp1 is localized in the whole CCV and especially in the leading edge ECs, whereas PxnD1 is localized only in the upper CCV (Fig. 7). Pxn receptor activation always occurs downstream of Sema binding (Janssen et al., 2010), but Nrp is a multifunctional coreceptor that can be activated by different ligands and act in complex with different receptors (Pellet-Many et al., 2008). We specifically addressed the involvement of Vegf and Vegf receptor (VegfR) in the regulation of CCV collective migration (Fig. S5). Although our data did not support involvement of Vegf ligands, we could not resolve whether VegfR acts as a Nrp1 coreceptor in the context of EC migration (Fig. S5, A–D). However, we could clearly show that Sema binding to Nrp1 is required for regulating Actin cable and cell–cell contact formation, as Nrp1

Actin cables [n = 10 for morphants or mutants and n = 7 for inhibitor experiments]. [F] Lateral confocal projections of Tg(fli1a:lifeactEGFP) embryos at 30 hpf. Leading edge high magnifications were color-inverted. Inhibition of Rock and RhoA induced lesions in the leading edge [pink arrowheads]. Parallel-aranged Actin cables [open arrowheads] are missing after inhibition of Rock, RhoA, or FAK. *** P < 0.001. Error bars indicate SD.
protein variants lacking the Sema3d binding domain (Nrp1Δa) could not rescue Nrp1 deficiency (Fig. S5, E–H). To summarize, we propose that mesenchymal Sema3d signals through PlxnD1 via repulsion inducing a straight CCV outgrowth, whereas EC-specific Sema3d signals through Nrp1 via RhoA and Rock and facilitates coherent cell sheet organization.

Sema3d-Nrp1 signaling regulates cell–cell contacts and Actin cable formation in the CCV leading edge by activating RhoA

To investigate whether RhoA- and Rock-mediated Actin network organization in the CCV leading edge acts downstream of Sema3d-Nrp1 signaling, we performed rescue experiments using pharmacological activation of RhoA (Fig. 8). Untreated Tg(fli1a:lifeactEGFP)mu240 Sema3d-deficient embryos as well as nrp1a/b morphants exhibited lesions in the leading edge and had few cells with Actin cables (Fig. 8 A). RhoA activation in these morphants and mutants from 22 to 30 hpf rescued cell–cell contact formation in the CCV cell sheet and restored the formation of Actin cables (Fig. 8, B and C). RhoA activation increased cell–cell contact length in Sema3d-deficient and nrp1a/b morphant embryos by 19% and 17%, respectively (Fig. 8 C). Similarly, RhoA activation restored the percentage of CCV front cells with aligned Actin cables from 15% to 87% in sema3d morphants, from 19% to 80% in sema3d mutants, and from 22% to 87% in nrp1a/b morphants (Fig. 8 D). Interestingly, the increase of CCV width seen in sema3d morphants and mutants was not normalized by RhoA activation. Taken together, our data show that Sema3d-Nrp1 signaling regulates cell–cell contacts and Actin cable formation in the CCV leading edge though RhoA activation.

Sema3d directly regulates collective cell migration

Based on Sema3d localization in the mesenchyme next to the CCV as well as in the leading edge ECs, we propose two mechanisms of Sema3d regulating collective migration in vascular development. On the one hand, mesenchymal Sema3d signals in a paracrine fashion through PlxnD1 via repulsion and induces straight CCV outgrowth; on the other hand, EC-specific Sema3d signals in an autocrine fashion through Nrp1 via RhoA and Rock and facilitates consistent cell sheet organization and migration through affecting cell–cell contact and Actin cable formation (Fig. 9).

Hereby we demonstrate that Sema3d is a novel regulator of collective EC migration. Class III Semaphorins have so far

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Figure 6. Loss of cell–cell contacts in the CCV leading edge did not depend on flow or Cdh5. (A) Antibody staining for ZO-1 and Cdh5 showed aberrant junction distribution and impaired cell shape upon sema3d loss. Arrowheads indicate gaps in Cdh5 protein distribution. (B) The length of Cdh5-positive cell–cell connections is reduced in sema3d morphants (n = 4). (C) Cell–cell connections in the CCV leading edge of cdh5as mutant embryos are present, but the ECs exhibit holes inside the cells (pink arrowheads). (D) Flow inhibition with nifedipine and tricaine did not lead to lesions or impaired Actin cable alignment in the CCV leading edge. Parallel-arranged Actin cables are indicated by open arrowheads. (E) Quantification of cell–cell-contact length of CCV leading edge cells (n = 16). ***, P < 0.001; n.s., not significant. Error bars indicate SD.
Figure 7. Differential Sema3d functions are transduced by signaling through the different receptors Nrp1 or PlxnD1. (A) In situ hybridization (ISH) of 30-hpf embryos showing cdh5 expression in all CCV cells, plxnD1 expression in the dorsal-most CCV cells (open arrowheads), and nrp1 expression in all CCV cells (open arrowheads). (B–G) Analysis of PlxnD1 or Nrp1 deficiency in Tg(kdrl:EGFP)s843 or Tg(fli1a:lifeactEGFP)mu240 embryos at 30 hpf. (B) Loss of PlxnD1 led to a wider CCV (white arrowheads), but cell–cell contacts were not impaired, and parallel-arranged Actin cables were present (open arrowheads). (C) nrp1ahu10012 mutant embryos had reduced cell–cell contacts (pink arrowheads) but did not exhibit a wider CCV. (D) Quantification of CCV front width (each n = 11; nrp1ahu10012 n = 4). (E) Number of lesions in nrp1ahu10012 mutant embryos is increased compared with WT (n = 4). (F) nrp1a/b and nrp1a morphant CCVs exhibit a normal width, but the leading edges showed lesions (pink arrowheads) and lacked Actin cables (compare with open arrowhead in Ctr). (G) Quantification of cell–cell contact length (each n = 13). (H) Schematic overview of phenotypes comparing Sema3d deficiency with plxnD1 and nrp1 knockdown. (I) Schematic illustration of ligand-receptor localization indicating autocrine Sema3d-Nrp1 signaling and paracrine Sema3d-PlxnD1 signaling. Magenta, sema3d expression; green, CCV ECs; blue, plxnD1 expression; and yellow, nrp1 expression. ***, P < 0.001; **, P < 0.01; n.s., not significant. Error bars indicate SD.
been reported only for guiding neural crest cell streams via a repulsive mechanism (Eickholt et al., 1999; Yu and Moens, 2005; Gammill et al., 2007). Additionally, Sato et al. (2006) reported that in the cardiac neural crest, knockdown of sema3d results in impairment of migration, but the mechanism of action was not elucidated. To analyze whether Sema3d might regulate collective cell migration of other tissues, we investigated collective cell migration of the zebrafish lateral line primordium (LLP).

Figure 8. Sema3d-Nrp1 signaling regulates cell–cell contacts and Actin cable formation in the CCV leading edge by activating RhoA. (A and B) Lateral confocal projections of Tg(fli1a:lifeactEGFP)m240 embryos at 30 hpf. Magnifications of the leading edges were color-inverted. (A) sema3d morphants and mutants as well as nrp1a/b morphants exhibited lesions in the leading edge (pink arrowheads) and lacked parallel-arranged Actin cables (open arrowhead in Ctr MO). (B) Activation of RhoA from 24 to 30 hpf reduced lesions (pink arrowheads) and rescued Actin cable formation (open arrowheads) in sema3d morphants and mutants as well as in nrp1a/b morphants. (C and D) Quantification of cell–cell contact length (C, n = 16) and cells with Actin cables (D, each n = 8; sema3d<sup>m268</sup> + RhoA activator, n = 4). ***, P < 0.001; **, P < 0.01. Error bars indicate SD.
We showed by antibody staining and in situ hybridization that Sema3d is expressed in the LLP and its deposited neuromasts (Fig. 10 A). Upon sema3d knockdown, LLP migration distance was reduced and rosette organization was impaired (Fig. 10, B–D). The observed phenotype of reduced migration and impaired cellular organization resembles the phenotype observed in CCV ECs, illustrating the importance of Sema3d in regulating collective cell migration in other developmental contexts.

Discussion

Our work reveals two separate roles of Sema3d for the regulation of collective EC migration. Spatiotemporally regulated Sema3d expression in combination with signal transduction through different receptors induces the differential functions of Sema3d-mediated repulsion of ECs on the one hand and migration of these ECs on the other hand (Fig. 9). This is the first study to elucidate the mechanisms by which Sema3d fulfills these contrary functions in vivo.

We show that mesenchymal Sema3d controls straight and directional migration of ECs, most likely by repulsion through the EC-specific receptor PlxnD1, in a paracrine manner (Fig. 9 B). Thereby Sema3d guides the migration route of the ECs and supports collective migration. PLXND1 was previously shown to act as a receptor for SEAMA3D and to coimmunoprecipitate with SEAMA3D (Foley et al., 2015). In mice, Sama3C has recently been shown to mediate repulsion of ECs through PLXND1 (Yang et al., 2015). Additionally, Sema3a-PlxnD1 signaling has been shown to guide Se patterning via a repulsive mechanism, leading to ectopic sprouting in either Sema3a- or PlxnD1-deficient embryos (Torres-Vázquez et al., 2004). In contrast to ectopic sprouting induced by Sema3a deficiency, we show here that loss of Sema3d impairs cell migration and cell morphology of Se ECs, which is in line with Nrp1-mediated signaling in the Ses (Fantin et al., 2015).

Many diverse roles of Class III Semaphorins have been published, but difficulties existed in dissecting the various functions mediated by different tissues and receptors in space and time. Sema3d was reported to act as a repellent or an attractant in axon guidance in zebrafish (Wolman et al., 2004; Taku et al., 2016). Accordingly, Moret et al. (2007) studied how intrinsic and extrinsic Sema3a repels and promotes axon migration at the same time. However, our analysis enables us for the first time to dissect these contradictory roles. We show that Sema3d is not only mediating repulsion of ECs but also promoting collective migration of leading edge ECs (Fig. 9 C). Our work reveals a novel mechanism by which EC-specific Sema3d regulates Actin network organization, mediating a cohesive cell sheet. (A) Schematic overview of ligand-receptor localization in the CCV. Sema3d (magenta) is localized in the mesenchyme next to the CCV and in the CCV leading edge. PlxnD1 (blue) is localized in the dorsal part of the CCV. Nrp1 (yellow) is localized in the whole CCV. Seam3d regulates collective EC migration via repulsion (B) and regulation of Actin network organization controlling cell sheet organization (C). (B) Sema3d is secreted from the mesenchyme and mediates repulsion on ECs via the EC-specific receptor PlxnD1 in a paracrine manner. Thus, Sema3d mediates straight outgrowth of the CCVs. (C) In contrast, EC-specific Sema3d in the leading edge regulates cell migration by autocrine signaling through Nrp1. Sema3d-Nrp1 signaling mediates Actin network organization via RhoA (red) and Rock (orange) regulating cell shape, cell–cell contacts, and Actin cable formation.
al., 2015). We show in vivo that Sema3d-Nrp1 signaling controls Actin dynamics in the leading edge cells of collectively migrating CCV ECs, independently of PlxnD1. In accordance with our observations, Sema3d has been shown to affect cytoskeleton reorganization in HUVECs independently of PlxnD1 (Aghajanian et al., 2014). Additionally, we were able to show for the first time that this autocrine EC Sema3d-Nrp1 signaling acts via RhoA and Rock on the Actin network. A previous study (Takamatsu et al., 2010) showed that inhibition of Myosin II or Rock blocked Sema3a-induced dendritic cell migration; however, these components were not placed in a direct signaling pathway. Nevertheless, how Nrp1-Sema3d activity is linked to RhoA-Rock will need further investigations. Recently, Yoshida et al. (2015) showed that Syx, a novel RhoA guanine exchange factor, activates RhoA downstream of Nrp1 signaling. However, because Syx-deficient zebrafish embryos die during gastrulation (Goh and Manser, 2010), we could not test this hypothesis.

We identified Sema3d as a novel regulator of collective migration in different contexts. Collective cell migration is a highly important process during embryogenesis and organogenesis (Scarpa and Mayor, 2016); it also drives processes during tissue repair, immune responses, tissue vascularization, and cancer metastasis (Friedl and Gilmour, 2009). Patients with congenital heart defects and anomalous pulmonary veins have been shown to have disruptions in the Sema3d gene (Siversides et al., 2012; Degenhardt et al., 2013; Sanchez-Castro et al., 2015). Recently, Sema3d autocrine signaling has been shown to promote tumor cell migration (Foley et al., 2015). As we have unraveled the complexity of Sema3d-mediated signaling for regulating collective migration for the first time in vivo, it will be interesting to apply our results to elucidate disease etiology in the future.

Materials and methods

Zebrafish husbandry and strains

Zebrafish (Danio rerio) embryos were maintained under standard husbandry conditions at 28.5°C (Westerfield, 2000). Zebrafish strains used were Tg(kdrl:EGFP)$^{het}$ (Jin et al., 2005), Tg(kdrl:HRAS-mCherry)$^{glh}$ (Chi et al., 2008), Tg(8.0cldnb:lynEGFP)$^{glh}$ (Haas and Gilmour, 2006), and Tg(hsp70:sema3dGFP) (obtained from M.C. Halloran, University of Wisconsin, Madison, WI; Liu et al., 2004). The following zebrafish mutants were used: cdx5$^{mut}$ (Lenard et al., 2013), npt1d$^{mut}$ (de Bruin et al., 2016), and kdrh$^{mut}$ (Bussmann et al., 2010).
Generation of sema3d mutants by CRISPR/Cas9-mediated mutagenesis

Annealed CRISPR target site primers (CRIsama3d SE, 5'-TAGGAG ACAGTGCAAGCGGCA-3', and AS, 5'-AAATCTCCGGCTGGA CCGTGTCT-3') were cloned into the guide RNA (gRNA) expression vector pDR274 as previously described (Hwang et al., 2013). After Dral digestion (New England Biolabs, Inc.), gRNA was transcribed using MEGASHortscript T7 kit (Ambion). Nls-zCas9-nls mRNA was synthesized as previously described (Jao et al., 2013). 2 nl of sema3d gRNA (12.5 ng/µl) and nls-zCas9-nls-mRNA (300 ng/µl) were injected into one-cell-stage zebrafish embryos. sema3d mutant genotypes were analyzed by PCR using primers sema3d-genos SE, 5'-CTGGCAAGACCTTC TTCA-3' and AS, 5'-ATATGGCGACATCCTCCCAT-3', followed by restriction digestion with Hpy188III (New England Biolabs, Inc.).

Generation of Tg(fli1a:lifeactEGFP)ma240

The fli1a:lifeactEGFP plasmid was generated via gateway cloning using Lifeact-EGFP (Riedl et al., 2008) and pTolfli1epDest (Villefranc et al., 2008). Probes were amplified from 24-hpf cDNA with the following T7 promoter site primers: sema3d SE, 5'-TGACAGGTT GCAAAATTTAT-3', and AS, 5'-GAATCGACTCTATAGAGGA TTGTGCAAGGCTGGATT-3'; pxnDI SE, 5'-ACATCGAGAAGA CGACACC-3', and AS, 5'-GAATACGACTCTATAGAGGA AAGAAGGCGGAGTT-3'; and ntp1a SE, 5'-ATGTGGAGACAT ACCTTTTT-3', and AS, 5'-GAATACGACTCTATAGGTT TTCTTTGAAAGGGCATT-3'. The cdh5 probe was synthesized as described previously (Larson et al., 2004).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Blum et al., 2008) using rabbit anti–zebrafish Cdh5 (Blum et al., 2008), mouse anti–human ZO-1 (Invitrogen), rabbit anti–human Sema3d (Sigma-Aldrich), goat anti–mouse Alexa Fluor 546 (Invitrogen), goat anti–rabbit Alexa Fluor 546 (Invitrogen), and goat anti–rabbit Alexa Fluor 647 (Invitrogen).

Transplantations

Donor Tg(kdrl:HRAS-mCherry)s896 embryos were injected with sema3d MO and 2.5% Alexa Fluor 647 Dextran (Thermo Fisher Scientific). Cells were transplanted into Tg(kdrl:HRAS-mCherry)ma240 hosts as described previously (Helker et al., 2015).

Pharmacological experiments

Inhibitor treatments were performed on dechorionated embryos from 22 hpf to fixation at 30 hpf using 400 µM ROCK inhibitor H-1152 (Enzo Life Sciences), 100 µM (−)-blebbistatin (Sigma-Aldrich), 24 nM latrunculin A (Sigma-Aldrich), 100 µM Cdc42 inhibitor ML141 (Sigma-Aldrich), 1 mM Rac1 inhibitor (Tocris Bioscience), 50 µM FAK inhibitor 14 (Sigma-Aldrich), 12.5 µM nifedipine (Sigma-Aldrich), 3 µg/ml Rhôl inhibitor (Cytoskeleton, Inc.), 5 µg/ml Rhôl activator II (Cytoskeleton, Inc.), and 1 µM VegfR inhibitor SU5416 (Sigma-Aldrich).

Microscopy, in vivo time-lapse analysis, and statistical analysis

Either 4% PFA-fixed or living embryos were mounted in 0.3% agarose (containing 19.2 mg/l triacine and 30 mg/l phenylthiourea for living embryos) for imaging. Bright-field and epifluorescence images were acquired with a stereomicroscope M165 FC or M205 C (Leica Microsystems) equipped with a C10600 camera (Hamamatsu Photonics) or a MicroPublisher 5.0 RTV camera (Qimaging). For live time-lapse imaging, embryos were kept in a 28.5°C heated chamber. Fluorescent confocal images were acquired using an LSM 780 (ZEISS); objective lenses: 20x Plan Apo NA 0.80, 40x LD C Apochromat NA 1.0, or 63x Plan-APo NA 1.40) or an Sp5 inverted confocal microscope (Leica Biosystems; objective lenses: 20x HCX PL S-APo NA 0.50). Imaris 7/8 (Bitplane) was used to assemble confocal stacks and movies. Imaris 7/8 and Fiji 1.45b (ImageJ) software were used for tracking, length, and angle measurements. Determination of cell migration parameters was performed as described previously (Helker et al., 2013). For all quantifications, SDs and p-values with two-tailed t test were calculated using Excel 2011 (Microsoft) and Prism 6 (GraphPad). All error bars indicate SD.

Online supplemental material

Fig. S1 shows genetic and phenotypic details for the sema3d mutants and morphants. Fig. S2 shows impaired vascular development in Sema3d-deficient embryos. Fig. S3 shows that Sema3d additionally regulates intersegmental vessel development. Fig. S4 shows that EC-specific Sema3d ensures cohesive EC sheet migration. Fig. S5 shows that Nrp1 signaling in the CCV leading edge requires Sema3 binding, but might not depend on VegfA. It also addresses the involvement of VegfR as a Nrp1 coreceptor. Videos 1 and 2 show normal versus...
impaired CCV migration in Ctr morphants (Video 1) versus sema3d morphants (Video 2) using Tg(kdr:EGFP)ryt1. Videos 3 and 4 show detailed analysis of Actin cable formation during CCV migration using the novel Tg(fltl:lifeactEGFP)m280. Ctr morphants exhibit a cohesive EC sheet with prominent Actin cables (Video 3), whereas sema3d morphants exhibit a severely disrupted EC sheet (Video 4). Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201603100.dv.

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