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Integration of Repulsive Guidance Cues Generates Avascular Zones That Shape Mammalian Blood Vessels

Stryder M. Meadows, Peter J. Fletcher, Carlos Moran, Ke Xu, Gera Neufeld, Sophie Chauvet, Fanny Mann, Paul A. Krieg, Ondine Cleaver

Rationale: Positive signals, such as vascular endothelial growth factor, direct endothelial cells (ECs) to specific locations during blood vessel formation. Less is known about repulsive signal contribution to shaping vessels. Recently, “neuronal guidance cues” have been shown to influence EC behavior, particularly in directing sprouting angiogenesis by repelling ECs. However, their role during de novo blood vessel formation remains unexplored.

Objective: To identify signals that guide and pattern the first mammalian blood vessels.

Methods and Results: Using genetic mouse models, we show that blood vessels are sculpted through the generation of stereotyped avascular zones by EC-repulsive cues. We demonstrate that Semaphorin3E (Sema3E) is a key factor that shapes the paired dorsal aortae in mouse, as *sema3E*^{-/-} embryos develop an abnormally branched aortic plexus with a markedly narrowed avascular midline. In vitro cultures and avian grafting experiments show strong repulsion of ECs by Sema3E-expressing cells. We further identify the mouse notochord as a rich source of multiple redundant neuronal guidance cues. Mouse embryos that lack notochords fail to form cohesive aortic vessels because of loss of the avascular midline, yet maintain lateral avascular zones. We demonstrate that lateral avascular zones are directly generated by the lateral plate mesoderm, a critical source of Sema3E.

Conclusions: These findings demonstrate that Sema3E-generated avascular zones are critical regulators of mammalian cardiovascular patterning and are the first to identify a repulsive role for the lateral plate mesoderm. Integration of multiple, and in some cases redundant, repulsive cues from various tissues is critical to patterning the first embryonic blood vessels. (*Circ Res.* 2012;110:34-46.)

Key Words: neuronal guidance cues ■ Sema3E ■ notochord ■ lateral plate mesoderm ■ endothelial

During initial formation of the vasculature (vasculogenesis) in vertebrates, endothelial precursor cells (angioblasts) arise de novo within the mesoderm, migrate, coalesce, and differentiate into endothelial cells (ECs) as they form blood vessels. Blood vessels develop at specific locations in a highly stereotyped manner. In birds and mammals, the first blood vessels formed are the paired dorsal aortae (DA), which emerge in 2 bilateral stripes on either side of the embryonic midline. The precise nature of the patterning and positioning of the DA suggests that instructive, paracrine signals from surrounding tissues guide aortic ECs in a genetically determined manner during vasculogenesis.

Indeed, it is increasingly understood that a number of paracrine signals from neighboring tissues shape developing

blood vessels throughout the embryo.¹⁻⁴ Recently, the molecular cues that guide neuronal axon pathfinding have also been shown to guide ECs in the embryo.^{5,6} “Neuronal guidance cues” (NGCs) are secreted or membrane bound ligands that act as attractive or repulsive cues, depending on cognate EC receptors.⁷⁻⁹ Four major classes of NGCs include the Ephrins and Eph receptors, Slits and Robo receptors, Netrins and Unc5 receptors, and Semaphorins and Plexin receptors.

Loss-of-function studies have uncovered a role for NGCs and their receptors in directing the growth of intersomitic blood vessels (ISV), which sprout from the DA. Disruption of EphrinB2/EphB4 signaling in mice and *Xenopus* embryos leads to irregular growth of ISVs into adjacent tissues.^{10,11} In zebrafish, antisense morpholino

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knockdown of *Semaphorin 3a1*, *Semaphorin 3a2*, and *Netrin1a* NGCs and *PlexinD1*, *Unc5b*, and *Robo4* receptors commonly resulted in aberrant growth of ISVs into neighboring somitic tissues.^{6,12,13} Interestingly, loss of *Semaphorin 3a1* also results in disruption of the developing dorsal aorta.¹⁴ *Semaphorin 3e*, *PlexinD1*, and *Unc5b* gene ablation experiments in mice resemble those in the fish, as loss of ISV guidance is observed in mutant embryos.^{15,16} In general, these results indicate that NGCs act as repulsive signals and direct formation of vessels to locations where repulsive signals are absent. Although these data show that NGC signaling regulates angiogenic growth, the role of NGCs during assembly of the original vascular network in mammalian embryos has not been explored.

Studies in fish and avian embryos revealed crucial but opposite roles for the notochord in guiding the formation of the initial circulatory system. In zebrafish *no tail (ntl)* and *floating head (flh)* mutant embryos, which lack notochords, the single midline dorsal aorta fails to form.¹⁷ Wild-type notochord cells transplanted into *flh* mutants can direct host ECs to aggregate and form the aorta, suggesting that the notochord attracts aortic precursors. By contrast, in avian embryos, bone morphological protein (BMP) antagonists Chordin and Noggin are secreted from the notochord and inhibit EC migration, creating an avascular midline¹⁸ that repels ECs and shapes the paired aortae. The role of the notochord during mammalian vascular formation remains unexplored, and signaling centers for vascular patterning have yet to be identified.

We show that coordination of NGCs, both from the notochord and the lateral plate mesoderm (LPM), establishes avascular zones that are critical for formation of the first embryonic vessels in mammals. Expression analysis of NGCs during initial organization of angioblasts reveals that the notochord is a potent source of multiple repulsive NGCs as well as BMP antagonists. Notochordless embryos show that the murine notochord, similar to the avian notochord, is a repulsive signaling center required for generating an avascular midline and shaping the paired DA. We identify *Semaphorin3E* (*Sema3E*) as a key notochord NGC, as its absence leads to significant reduction of the midline avascular zone and ectopic aortic branching. *Sema3E* is also uniquely expressed by the LPM and, unlike the avian embryo, defines DA boundaries by creating lateral avascular zones. Together, our data demonstrate that multiple, largely redundant, repulsive guidance cues at the midline, and for the first time *Sema3E* from the LPM, are required for formation of avascular regions that coordinate to sculpt the mouse DA. These results underline the importance of paracrine signal coordination in directing formation of mammalian blood vessels.

Methods

Mouse Embryos and Histology

Embryos were processed for paraffin sectioning, in situ hybridization, and β -galactosidase staining as previously described.²⁰ For double stains, β -gal staining was followed by in situ hybridization for *sema3E*. For eosin staining, sections were

Non-standard Abbreviations and Acronyms

BMP	bone morphological protein
DA	dorsal aortae
E	embryonic day
EC	endothelial cell
FGF	fibroblast growth factors
HUVEC	human umbilical vein endothelial cell
ISV	intersomitic vessels
LPM	lateral plate mesoderm
NGC	neuronal guidance cues
Sema3E	semaphorin3E
VEGF	vascular endothelial growth factor

dewaxed in xylenes for 10 minutes, followed by ethanol washes from 100% to 70%, then submerged in eosin for 30 seconds, ethanol washes to 100%, xylenes for 5 minutes, then coverslipped with Permount (Fisher).

Whole-Mount In Situ Hybridization and RNA Probes

Whole-mount in situ hybridization in mouse and chicken embryos was carried out using digoxigenin-labeled probes and standard procedures previously described.²⁰ Full-length *Chordin*, *Dll4*, *Cx40*, *Netrin1*, *PlexinD1*, *Rasip1*, *Robo4*, *Sema3E*, *Slit2*, and *Unc5b* mouse clones were obtained from Open Biosystems to generate RNA probes. We recently demonstrated that *Rasip1* is required for vascular tubulogenesis and is an early vasculogenic marker.¹⁹ *PlexinD1* (pgm2n.pk010.p8) and *sema3E* (pgn1c.pk009.e13) chicken clones were obtained from the Delaware Biotechnology Institute.

PECAM Staining

Embryos fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS were washed in 1× PBS, transferred to 0.25% Trypsin (Hyclone) for 2 minutes, rinsed in 1× PBS, blocked in CAS-Block (Invitrogen) for 1 hour at room temperature, and incubated overnight with PECAM antibody (BD Pharmingen; 1:300) in PBST at 4°C. The next day, embryos were washed with 1× PBST and stained with DAB solution as per kit instructions (Vector labs). Staining was stopped by rinsing in water and fixation in 4% PFA.

Cell Culture and Endothelial Assays

Human umbilical vein ECs (HUVECs) were grown in M199 medium supplemented with 10% FCS; Mouse MS1 and bEnd.3 (ATCC) in DMEM (GIBCO) 5% FBS. HEK293T and HEK293T-*Sema3E* cells were maintained in DMEM 10% FBS. Coculture experiments using HUVEC, HEK293T, and HEK293T cells were carried out as previously described.²¹ Briefly, HUVEC cells were seeded onto gelatinized 24 (2×10⁴ cells/well) well plates. The next day, 5% of HEK293T and HEK293T cells, incubated with 5 μ g/mL of fluorescent vital DiI for 30 minutes, were seeded on top of the HUVECs. Cells were assessed after 24 hours in culture.

“Wound-healing” assays were performed in triplicate and as previously explained.²⁰ ECs were cultured in conditioned media from 60-mm dishes of control HEK293T and HEK293T-*Sema3E* cells for 18 hours. Images of the cell-free area were taken immediately after “scratching” and after 12- and 18-hour cultures.

Cell Implant Experiments in Avian Embryos

HEK293T and HEK293T-*Sema3E* cells were cultured using standard “hanging-drop” method, and resulting aggregates were

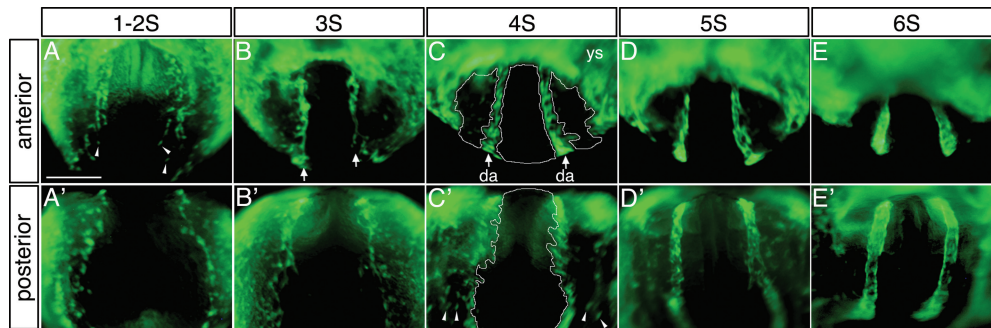


Figure 1. Formation of the dorsal aortae and avascular zones during mouse vasculogenesis. **A** through **E**, *Flk1-EGFP* highlights ECs (green) in 1–6S stage embryos (anterior views). **A**, Free angioblasts (**white arrowheads**) align in 2 bilateral rows, at 1–2S. Note absence of angioblasts at the midline and lateral regions. **B**, At 3S, angioblasts coalesce into cord-like structures (**white arrowhead**). Few angioblasts are located lateral to aortic cords. **C**, Avascular zones (outlined in white) surround the DA at 4S. By 5S (**D**), angioblasts differentiate into ECs and begin forming lumens. **E**, DA are lumenized by 6S, and avascular zones are clearly demarcated. **A'** through **E'**, Ventral views of 1–6S embryos. Lateral avascular zones are forming from 1S to 4S (**A'** through **C'**), with angioblasts (**arrowheads**) still present. By 5S (**D'**), lateral regions are virtually EC-free. Scale bar: 200 μ m. da indicates dorsal aorta; S, somite; and ys, yolk sac.

implanted into prevascular, 2 to 3 somite stage chicken and quail embryos at the location of developing DA. New Cultures method used and incubated at 37°C in 95% oxygen until the 10 to 15 somite stage. Chick embryos were fixed in 4% PFA/PBS and analyzed using in situ hybridization for *VE-cadherin*. Quail embryos were stained for QH1 (Developmental Studies Hybridoma Bank), using previously described procedures.²²

Results

DA Form Between Avascular Zones

Development of the DA has been previously described in mice^{23,24}; however, the strikingly avascular zones that flank the aortae have largely been ignored. To better understand the relationship between developing DA and surrounding EC-free regions, we examined *Flk1-EGFP* transgenic mice²⁵ to pinpoint the positioning and timing of angioblast aggregation during vessel formation. At this stage, *Flk1* expression is restricted to angioblasts. At the 1 to 2 somite (1–2S) stage of development, or embryonic day (E) 8.0, individual ECs were observed in 2 bilateral stripes. A wide avascular zone was present at the midline, separating the presumptive aortic vessels (Figure 1A and 1A'). In addition, 2 more additional regions largely devoid of ECs appeared lateral to each aortic vessel, separating embryonic from extraembryonic ECs.

At 3S, angioblasts aligned and coalesced into cohesive vascular cords, in an anterior-posterior fashion, while the midline avascular zone narrowed slightly and the lateral avascular zones became more prominent (Figure 1B and 1B'). At 4S, all 3 avascular zones were distinct and cords began forming lumenized vessels in the anterior region of the embryo (Figure 1C and 1C'). Of note, lateral avascular regions became more defined in an anterior-posterior manner, similar to the forming DA (Figure 1B through 1C'). By 5–6S, the DA consisted of tubes patent along their entire length, and all 3 avascular zones were well demarcated (Figure 1D through 1E'). These observations show that avascular zones in the mouse embryo are present from the onset of angioblast

emergence and vessel formation (1–5S) and are maintained as the DA form (before aortic fusion).

Multiple Repulsive Cues Are Expressed in the Notochord

The stereotyped location of avascular zones in early mouse embryos suggested that precisely controlled signals repel angioblasts to shape and pattern aortic vessels. In the chicken embryo, Chordin and Noggin secreted from the notochord are responsible for establishing the midline avascular zone.¹⁸ However, there are many other repulsive molecules known to repel ECs, such as those expressed from neural tissues.² We hypothesized that NGCs contributed to creating avascular regions and guiding murine DA formation.

Analysis of NGC expression during DA development (E8–8.25) revealed *slit2*, *sema3E*, and *netrin1* transcripts at the embryonic midline at 4S–8S (Figure 2I through 2N'), with high levels in the notochord (Figure 2J, 2L, and 2N). *Sema3E* was also detected in other tissues, such as the LPM, neural floor plate, and ectoderm, and *netrin1* was detected in the somites. In addition, we surveyed *chordin* and *noggin* expression and found that, similar to chickens, transcripts were present in the notochord at this stage (Figure 2E through 2H'). Multiple repulsive guidance cues, including NGCs, Chordin and Noggin, are thus expressed by the murine notochord during the formation of the paired DA. We note that the notochord lies at the heart of the midline avascular zone and that the *Sema3E* expressing LPM sits atop the lateral avascular zones (Figure 2D and 2D'), suggesting these tissues are candidate sources of repulsive EC signals that create avascular regions.

It stands to reason that for NGCs to repel aortic ECs from the notochord and LPM, the appropriate cognate receptors must be expressed by aortic ECs to receive, interpret, and integrate inhibitory signals. Indeed, we found that the endogenous *Slit2* receptor (*robo4*), *Sema3E* receptor (*plexinD1*), and *Netrin1* receptor (*unc5b*) were all expressed in the DA throughout vasculogenesis (Online Figure I; online figures available in the Data Supplement at <http://circres.ahajournals.org>), confirming that aortic ECs are competent to respond to repulsive NGC signaling.

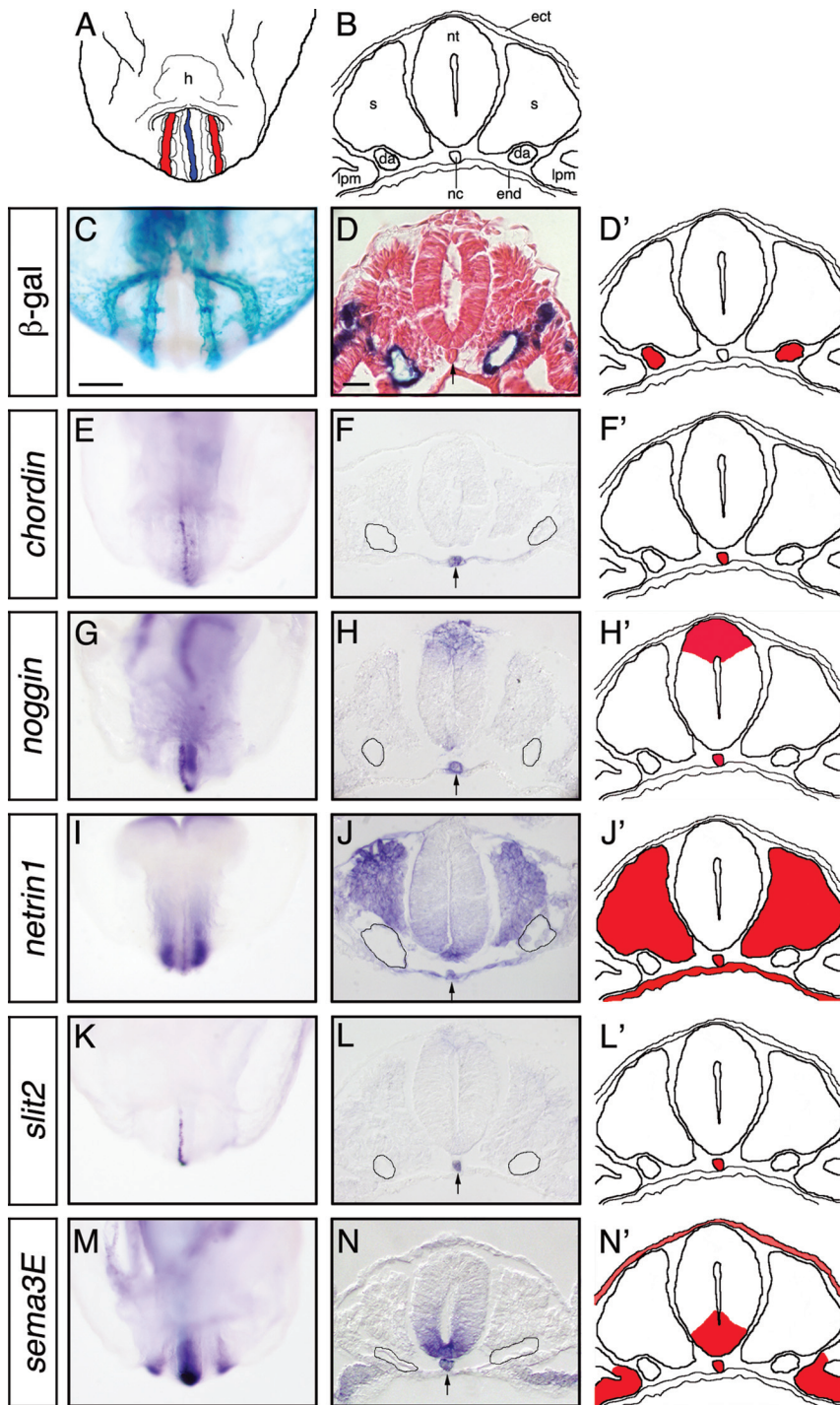


Figure 2. The notochord expresses multiple repulsive guidance cues during embryonic vasculogenesis. **A** and **B**, Depiction of an E8.25 embryo; **A**, anterior, and **B**, cross-sectional views showing DA (red), notochord (blue), and surrounding tissues. **C** and **D**, E8–8.25 *Flk1-LacZ* embryos stained for β -galactosidase (light blue) and eosin (**D**, red); anterior (**C**) and cross-sectional (**D**) views. **E** through **N**, In situ hybridization for *chordin*, *noggin*, *netrin1*, *slit2*, and *sema3E* at E8–8.25. **E**, **G**, **I**, **K**, and **M** are anterior views; **F**, **H**, **J**, **L**, and **N**, cross-sectional views. **Arrows** mark the notochord and DA are outlined in black. Note expression of *chordin*, *noggin*, *netrin1*, *slit2*, and *sema3E* in the notochord. Scale bars represent 200 μ m (**C**, **E**, **G**, **I**, **K**, and **M**) and 25 μ m (**D**, **F**, **H**, **J**, **L**, and **N**). **D'**, **F'**, **H'**, **J'**, **L'**, and **N'**, Schematics of **D**, **F**, **H**, **J**, **L**, and **N**, respectively. Stained tissues shown in red. da indicates dorsal aorta; ect, ectoderm; end, endoderm; h, heart; lpm, lateral plate mesoderm; nc, neural tube; and s, somites.

The Notochord Is Required for Patterning the Murine DA

The expression of multiple NGCs by the notochord suggested that repulsive redundancy at the midline might have evolved to ensure proper formation of the DA. It also suggested that loss of any single repulsive cue would have reduced impact on aortic patterning. Therefore, we investigated DA formation in *Foxh1* and *Foxa2* mutant embryos that fail to develop notochords.^{26–28} *Foxh1* null embryos present 3 distinct phenotypes, based on severity of abnormalities.²⁷ We analyzed the most morphologically normal

Foxh1^{-/-} and *Foxa2*^{-/-} embryos to assess aortic patterning.

Sonic hedgehog (*shh*) expression in *Foxh1* and *Foxa2* null was assessed to verify absence of notochord at E8–8.25 (Figure 3A and 3B and data not shown). To analyze the developing vasculature in notochordless mutants, the *Flk1-LacZ* allele was mated into the *Foxh1*[±] and *Foxa2*[±] background to create *Foxh1*[±];*Flk1-LacZ*, and *Foxa2*[±];*Flk1-LacZ* lines. This analysis revealed an essential requirement for the notochord during mouse DA formation. *Foxh1* and *Foxa2* null embryos displayed severely disrupted DA, showing

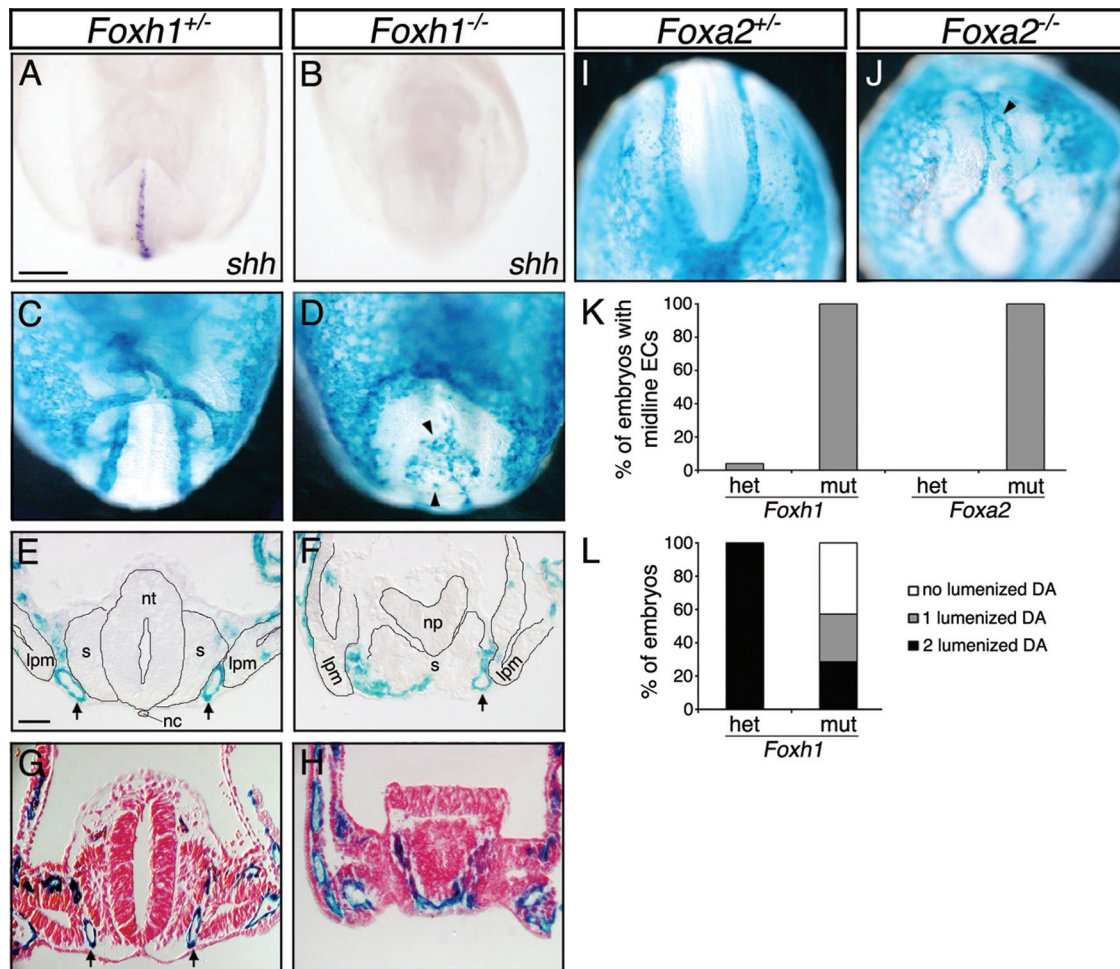


Figure 3. Dorsal aortae are severely disrupted in notochordless *Foxh1* and *Foxa2* null embryos. **A** and **B**, Expression of *shh* in *Foxh1*^{+/+} and *Foxh1*^{-/-} E8.25 embryos (anterior views). *Shh* marks the notochord (**A**) but is absent in *Foxh1*^{-/-} mutants (**B**). **C** through **J**, β -galactosidase staining (light blue) and eosin staining (**G** and **H**, red) of E8.25 *Foxh1*^{+/+};*Flk1-LacZ*, *Foxh1*^{-/-};*Flk1-LacZ*, *Foxa2*^{+/+};*Flk1-LacZ*, and *Foxa2*^{-/-};*Flk1-LacZ* embryos; anterior (**C** and **D**), posterior (**I** and **J**), and cross-sectional (**E** through **H**) views. Note presence of midline ECs (arrowheads) in *Foxh1*^{-/-} and *Foxa2*^{-/-} embryos (**D**, **F**, **H**, and **J**). Scale bars: 200 μ m (**A** through **D**; **I** and **J**) and 50 μ m (**E** through **H**). **K**, The percent of *Foxh1* (het, n=23; mut, n=14) and *Foxa2* (het, n=11; mut, n=8) embryos with ECs located at the midline. **L**, Percentage of *Foxh1* embryos with lumenized DA (het, n=7; mut, n=7). **Arrows**, DA. lpm indicates lateral plate mesoderm; nc, notochord; np, neural plate; nt, neural tube; and s, somite.

disorganized vessel fragments and presence of ECs at the embryonic midline (Figure 3C through 3K and Online Figure II). We note, however, that lateral avascular zones remained present in notochordless embryos. In addition, expression of early EC genes indicated that angioblast specification occurred normally, suggesting only vascular patterning was disrupted (Online Figure II). Interestingly, approximately 50% of notochordless mutants failed to form completely lumenized aortic vessels in the anterior region of the embryo where the foregut endoderm is absent and the somites are fused (Figure 3F, 3H, and 3L and Online Figure II).

As *Foxa2* is expressed in a number of tissues, including the notochord, floor plate, and endoderm,²⁹ we verified that expression of *Foxa2* was not required in tissues outside the notochord for proper vascular development. Conditional deletion of *Foxa2* in the embryonic endoderm and endothelium, using *Foxa3-Cre*³⁰ or *Tie2-Cre*³¹ driver lines, respectively, did not result in vascular patterning defects and avascular zones appeared normal in size (Online Figure III). How-

ever, the DA resembled early EC cord structures and failed to undergo lumen formation in embryos in which *Foxa2* was depleted in the endoderm (Online Figure III). Similarly, deletion of *Foxa2* in ECs caused no obvious abnormalities in EC patterning (Online Figure III). These results indicated that disrupted DA in *Foxa2* null embryos was due specifically to the lack of notochord and not to a cell autonomous requirement in ECs. Vascular analysis of embryos with tissue-specific deletion of *Foxh1* was not possible, as a *Foxh1* conditional allele is currently unavailable. Overall, our studies underline the importance of the notochord in the generation of an avascular midline to properly pattern the developing DA.

Loss of Midline Repulsive Cues in Notochordless Mutants

In the previous experiments, we reasoned that by genetically blocking notochord formation we could simultaneously eliminate all midline repulsive cues (both known and unknown).

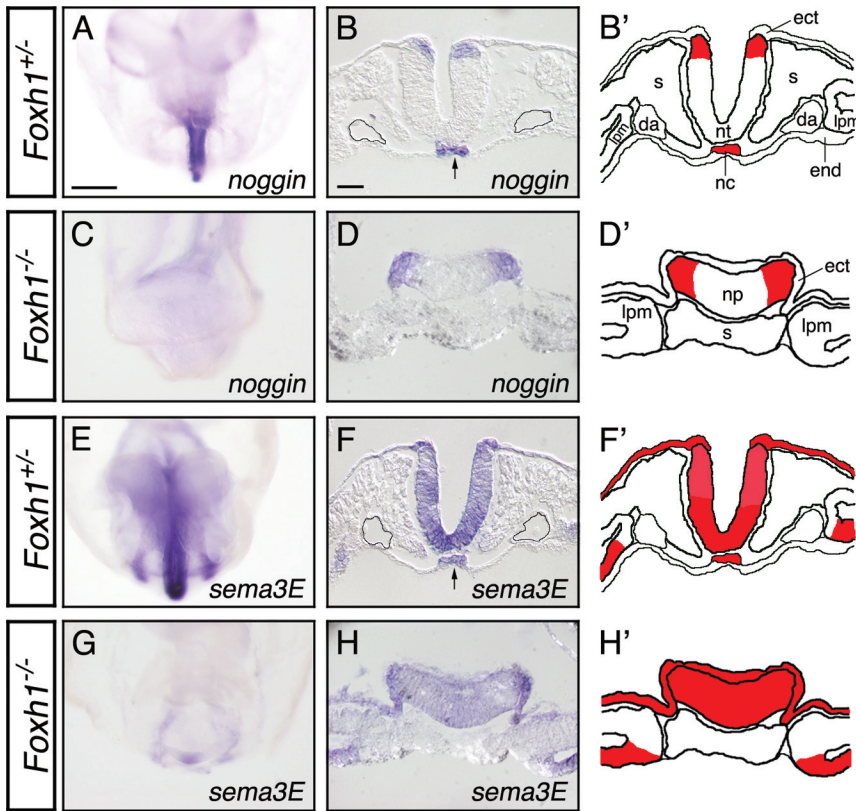


Figure 4. Midline repulsive guidance cues are lost in notochordless embryos. **A through H,** In situ hybridization for *noggin* and *sema3E* transcripts in E8.0 *Foxh1*[±] and *Foxh1*^{-/-} embryos: anterior views (**A, C, E,** and **G**) and cross-sectional views (**B, D, F,** and **H**). **Arrows** mark the notochord and DA are outlined in black. **F and H** show posterior sections where *Sema3E* is expressed throughout the neural tube. Scale bars: 200 μ m (**A, C, E,** and **F**) and 25 μ m (**B, D, F,** and **H**). **B', D', F',** and **H',** Depiction of stained tissues (red) in **B, D, F,** and **H** respectively. da indicates dorsal aorta; ect, ectoderm; end, endoderm; lpm, lateral plate mesoderm; nc, notochord; np, neural plate; nt, neural tube; and s, somite.

To verify absence of midline signals, we assessed NGCs, *chordin* and *noggin* expression in *Foxh1* null embryos. No trace of midline expression could be seen (Figure 4A through 4H' and Online Figure IV); however, expression was largely unaffected outside the midline (compare Figure 4A and 4B with Figure 4C and 4D; Online Figure IV). *Sema3E* transcripts remained in the LPM in both *Foxh1*[±] and *Foxh1*^{-/-} embryos but were absent from the anterior neural plate in *Foxh1*^{-/-} embryos (compare Figure 4E and 4F with Figure 4G and 4H). Similar to *Foxh1*^{-/-} embryos, *Foxa2* null embryos also exhibited absence of repulsive cues at the embryonic midline (n=2 per probe, data not shown). Together, these data confirmed that notochordless mutants lack midline repulsive cues as a result of loss of source tissues, such as the notochord.

LMP *Sema3E* Underlies Lateral Avascular Zones

The presence of lateral avascular zones in both heterozygous and homozygous null *Foxh1* and *Foxa2* embryos (Figure 3C, 3D, 3I, and 3J; Online Figure II; and Figure 5A and 5B) suggested that these regions did not depend on notochord signals. Furthermore, *sema3E* expression in the LPM correlated with lateral avascular regions, in both wild-type and notochordless embryos (compare Figure 2M and 2N with Figure 4E, 4F, 4G, and 4H and Figure 5C through 5F).

Of all the guidance cues surveyed, only *sema3E* exhibited strong LPM expression (Figure 2). We assessed all 7 semaphorin3 genes (*sema3A-3H*, data not shown), and, whereas *sema3A*, *3C*, and *3F* were expressed at low levels in the LPM, *sema3E* LPM expression was distinctly robust. Indeed, each

aortic vessel was closely flanked on one side by *sema3E* expression in the LPM (lateral) and on the other side by notochord/ventral neural tube (midline) (Figure 5G, 5H, and Online Figure V). This nested location of the aortae between *sema3E*-expressing tissues suggested that ECs are corralled between avascular zones into aortic cords through negative cues on either side. Interestingly, *sema3E* expression in midline tissues and the LPM is downregulated at the time of DA fusion (Online Figure V). We therefore proposed that midline *Sema3E* acts in an overlapping manner with other notochord repulsive cues, whereas in LPM *Sema3E* creates lateral avascular zones in a unique, nonredundant manner.

DA Disruption and Loss of Lateral Avascular Zones in *Sema3E*^{-/-} Embryos

To determine the role of *Sema3E* during vasculogenesis, we examined DA development in *sema3E*^{-/-} embryos. Although *sema3E*-deficient mice exhibit ISV (angiogenic) patterning defects at E10.5–11.5,¹⁵ DA formation and vasculogenic patterning had not been previously examined. In wild-type embryos, the DA form at E8.0–8.25 as 2 parallel and unbranched vascular tubes, extending from the head to the tail (Figure 1).^{19,32} We found that whereas *sema3E*[±] aortae were indistinguishable from wild-type (Figure 6A and 6C), *sema3E*^{-/-} aortae were severely disrupted (Figure 6B, 6D, 6G, and 6H), exhibiting a plexus-like appearance with numerous ectopic vessels that extended into lateral avascular regions, underlying the LPM. To distinguish whether the observed patterning defects were direct, caused by loss of *sema3E*, or indirect, due to changes in endothelial promoting signals, we assessed the expression of growth factors known

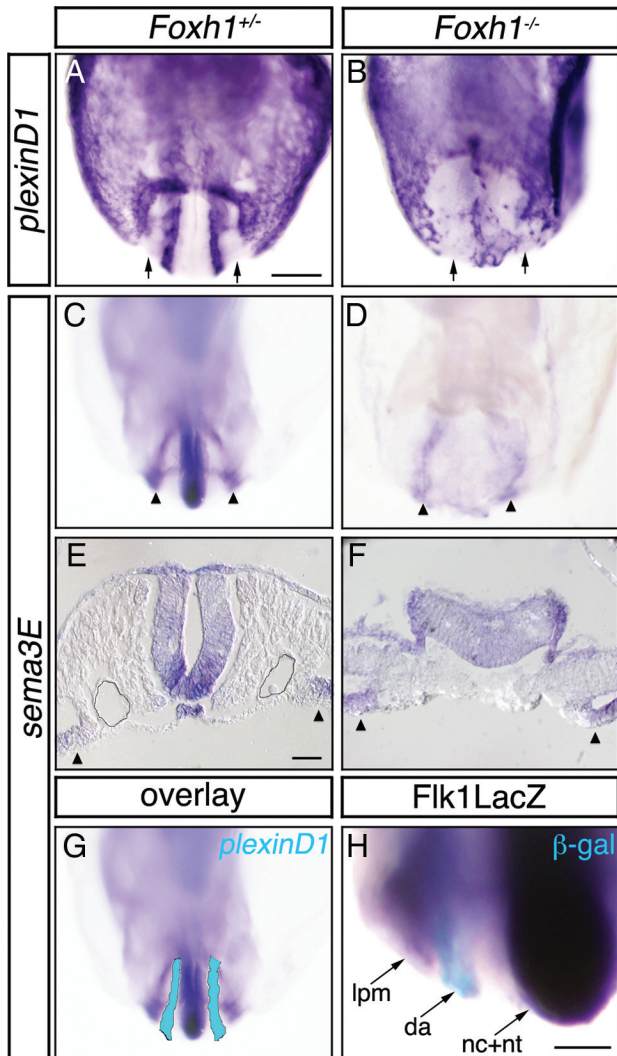


Figure 5. *Sema3E* expression corresponds to the lateral avascular zones in wild-type and *Foxh1*^{-/-} embryos. **A** through **H**, Expression of *plexinD1* and *sema3E* in E8.25 *Foxh1*^{+/+}, *Foxh1*^{-/-}, and *Flk1-LacZ* embryos: anterior views (**A**, **C**, **G**, and **H**) and cross-sectional views (**E** and **F**). Lateral avascular zones (**arrows**), *sema3E* expression in the lpm (**arrow-head**), and DA (outlined in black) are indicated. Anterior *sema3E* expression is lost at the midline (**D**) but maintained in the posterior neural plate (**F**) of *Foxh1* mutants. **G**, *plexinD1* expression (light blue) in the DA of embryo (**A**) superimposed onto (**C**). **H**, *Sema3E* expression (purple) and β -galactosidase staining (light blue) in a *Flk1-LacZ* embryo. Scale bars: 200 μ m (**A** through **D** and **G**), 100 μ m (**H**), and 25 μ m (**E** and **F**). da indicates dorsal aorta; lpm, lateral plate mesoderm; nc, notochord; and nt, neural tube.

to influence developing blood vessels. We found that the VEGF, Shh, BMP, and FGF signaling pathways were unaffected in *sema3E* mutants (Online Figure VI and data not shown), suggesting that *sema3E* affected angioblasts directly.

Defects of *sema3E*^{-/-} aortae appeared limited to vessel patterning rather than vessel integrity or differentiation. Lumens in *sema3E*^{-/-} aortae were largely present (compare Figure 6E with 6F). EC differentiation also occurred normally (Figure 6D and Online Figure VII), as arterial *cx40* and *dll4* expression initiated in vessels of the *sema3E*^{-/-} aortic plexus, suggesting that arteriovenous differentiation occurred

normally (Online Figure VII). In addition, defects were limited to the vasculature in *sema3E* null embryos, as the morphology of the LPM, neural tube, and notochord were indistinguishable from wild-type (compare Figure 6E with 6F). These results indicated that loss of *Sema3E* did not interfere with basic mechanisms required for vascular tube formation or surrounding tissues.

Unexpectedly, aortic ECs in *sema3E* mutants were much closer to the embryonic midline (Figure 6B and 6F and Online Figure VII). We had anticipated that because several repulsive cues are expressed by the notochord, loss of *Sema3E* alone would have little effect on the midline avascular zone. However, we observed ECs near and sometimes in direct contact with the notochord (Figure 6F and Online Figure VII) in *sema3E* mutants. Measurements of the avascular midline revealed an approximately 50% reduction in width in *sema3E*-deficient embryos (Figure 6I). However, despite reduction of the midline avascular zone, we never observed vascular branches crossing the notochord. These results show that *Sema3E* is a robust repulsive cue and that although other repulsive signals are still present in the notochord in *sema3E* mutants (Online Figure VII), they are not sufficient to maintain the normally broad avascular midline nor restrain aortic ECs into smooth aortic vessels. Therefore, *Sema3E* is a powerful repulsive cue during initial vasculogenesis, defining both lateral and midline avascular zones and guiding aortic ECs to precise locations during DA development.

To determine the events underlying the development of the highly branched *sema3E* null aortae, we examined the onset of their formation during vasculogenesis. Using tightly staged series, we sought to distinguish: whether angioblasts arose in a wider area due to lack of *Sema3E* restraint or whether aortae first formed normally but then developed excessive sprouting, or whether angioblasts migrated precociously from the yolk sac. We examined *sema3E*[±] and *sema3E*^{-/-} embryos from E7.75–8.25 using a *Flk1-LacZ* allele, which labels initial angioblasts. We found that angioblasts emerged normally in both the yolk sac and in 2 rows of intraembryonic angioblasts presaging the aortae but that the area encompassing the preaortic angioblasts appeared wider in the *sema3E* null embryos (Online Figure VIII). In addition, at E7.5–7.75 extraembryonic angioblasts did not precociously invade embryonic tissues of the *sema3E* null embryos. Although we cannot conclusively exclude the possibility that angioblasts migrate more quickly (either from their initial positions in the mesoderm or from the yolk sac), our observations tend to suggest that the abnormal *sema3E*^{-/-} aortae form initially as a plexus rather than from aberrant sprouting from initially normal aortae or from precocious or excessive yolk sac angioblast invasion.

Sema3E Robustly Repels Endothelial Cells

To determine whether murine notochord *Sema3E* can repulse ECs, we carried out both in vitro and in vivo repulsion experiments. First, we seeded HEK293 cells that constitutively secrete *Sema3E* (HEK293-*Sema3E*; Kigel et al, 2008) onto monolayers of HUVECs to assess EC behavior. After 24 hours of incubation, HUVECs were

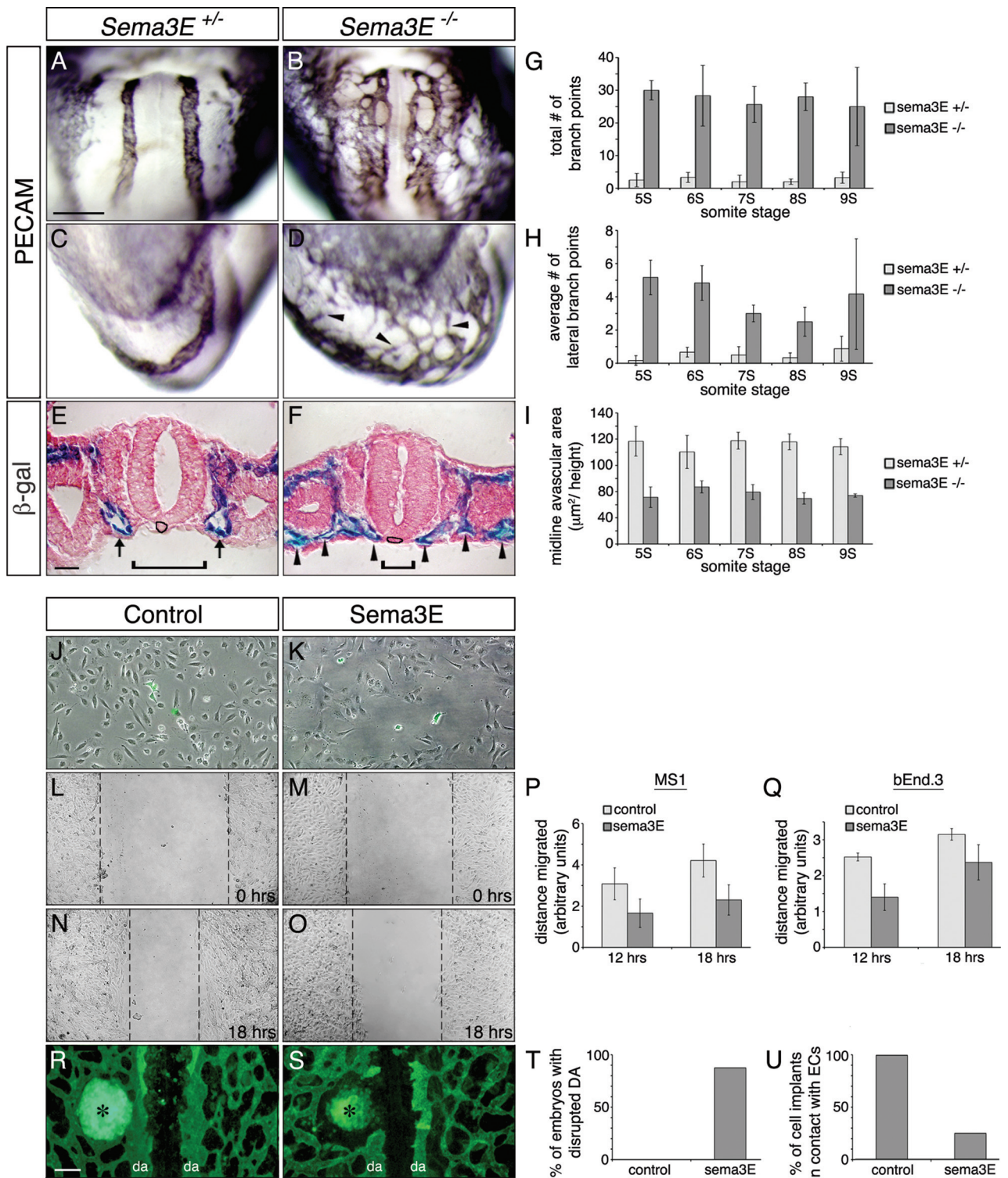


Figure 6. Sema3E is both sufficient for lateral avascular zones and required for dorsal aortae patterning. *Sema3E*[±]/*Flk1-LacZ* and *Sema3E*^{-/-}/*Flk1-LacZ* embryos stained for PECAM (A through D) or β -galactosidase (E and F). A and B, Anterior view of 5S; C and D, lateral view of 6S; and E and F, cross-sectional view of 8S embryos. E and F, Eosin stain (red). B and D, Blood vessels in *Sema3E* mutants form a plexus-like network across lateral avascular regions (arrowheads). Note vessels (arrowheads) in closer proximity to the notochord (outlined in black) in a *Sema3E* mutant (F) than in a heterozygote (arrows, DA) (E). Brackets indicate width of avascular zone around the notochord. Quantification of total aortic branch points (G) and ectopic branch points (H) within lateral avascular regions, in *Sema3E*[±] and *Sema3E*^{-/-} embryos. Branch points within 100 sq μ m areas, in both left and right lateral regions (anterior, representative fields of view), were counted in 5–9S embryos (n=3–4 embryos per somite stage). I, Quantification of midline avascular areas (sq μ m/height) in *Sema3E*[±] and *Sema3E*^{-/-} embryos, in anterior regions of 5–9S embryos (n=3–4 embryos per somite stage). J through U, Cultured ECs are repelled by HEK293-Sema3E cells (K, green) but not by control HEK293 cells (J, green). L through Q, “Wound-healing” assays with mouse MS1 (L through P) or bend.3 EC lines (Q). MS1 cells at 0 hours (L and M) and after 18 hours cultured (N and O) in media conditioned by control or HEK293-Sema3E cells. P and Q, Quantification of MS1 and bend.3

found to be closely associated with control HEK293 cells, whereas Sema3E-expressing HEK293 cells efficiently repelled ECs, creating avascular zones (Figure 6J and 6K).

Because NGCs can also inhibit cell migration,^{8,33} we tested the influence of Sema3E on EC migration using an in vitro “wound-healing” scratch assay. Monolayers of MS1 and bEnd.3 cells, which both express PlexinD1 (data not shown), were incubated in conditioned media from control HEK293 or HEK293-Sema3E cells. Migration distances across the cell-free wound area were measured at 12 and 18 hours to assess “healing rates.” In the presence of Sema3E, ECs healed at significantly slower rates (migrated a shorter distance) than those cultured in control HEK293 media (Figure 6L through 6Q). Results reflected decreases in EC migration, not proliferation, as doubling time of MS1 and bEnd.3 cells is >24 hours.²⁰

To test the ability of Sema3E to repel ECs in vivo, we implanted control HEK293 and HEK293-Sema3E cell aggregates into prevascular regions of early quail embryos and assessed vascular development. After overnight incubation, vessels were visualized with the quail-specific EC marker, QH1.³⁴ We found that ECs of the DA and lateral plexus made direct contact with HEK293 control cells, exhibiting normal vessel patterning (Figure 6R, 6T, and 6U). In contrast, vessels did not contact Sema3E-expressing cells (Figure 6S, 6T, and 6U). In fact, HEK293-Sema3E cells were extremely efficient in disrupting formation of the DA and creating avascular regions at locations where aortic vessels would normally develop. These results were also observed in chick embryos (Online Figure VII). Together, our in vitro and in vivo data confirm that Sema3E is a potent EC repulsive guidance cue that guides blood vessel patterning during vasculogenesis. These results support previous studies showing that Sema3E is repulsive to ECs.^{15,35} We propose, however, that Sema3E carries out its repulsion of ECs in the context of additional redundant cues (Figure 7).

Sema3E Expression Reflects Evolutionary Changes in DA Patterning

Formation and patterning of the DA in mammalian and avian embryos is remarkably similar.^{18,24} Paired aortic vessels, separated by an avascular midline, form at defined bilateral regions along the edge of the LPM. One interesting difference, however, is that unlike mice, avian embryos lack lateral EC-free zones (Online Figure VI). The paired DA of chicken and quail are closely associated with and connected to vessels of the adjacent yolk sac plexus. This suggested a possible evolutionary difference in Sema3E expression between mammals and birds, in that absence of lateral avascular regions in birds might be attributable to an absence of Sema3E expression in the LPM.

We therefore assessed *sema3E* expression in chicks during DA development. We found that although *sema3E* transcripts are present in the notochord, there is a complete

absence of expression in the LPM (Online Figure IX). This result suggests that *sema3E* expression in the notochord has been retained throughout evolution; however, mice may have adapted to express *sema3E* in the LPM to define the lateral boundaries of the developing DA. This observation also demonstrates that both mammals and birds utilize multiple repulsive cues from the notochord to ensure a strictly avascular midline for proper DA formation.

Discussion

In the present study, we identify Sema3E as a regionally expressed repulsive guidance cue, which creates stereotyped avascular regions that shape developing blood vessels. We identify Sema3E as an important notochord cue, as *sema3E*^{-/-} mouse embryos exhibit a markedly reduced midline avascular zone. A key finding of this work is that Sema3E expressed by the LPM creates lateral avascular zones that further shape the dorsal aortae. Together, these results constitute the first demonstration of notochord and LPM impact during vasculogenesis in mammalian embryos and underscore the powerful influence of Sema3E in shaping the first embryonic vessels.

The Notochord Is a Multicue, EC-Repulsive Signaling Center

Given our observations that multiple repulsive cues were expressed in the mammalian notochord, as well as prior work showing the notochord is required for dorsal aorta formation in fish, frogs and chick,^{17,18,36} we asked whether presence of the notochord was required for DA formation in mouse. Our analyses of *FoxH1*^{-/-} and *Foxa2*^{-/-} mice demonstrated the importance of the notochord in mammals, because in the absence of the notochord, aortic ECs cross the midline and fail to organize into distinct DA vessels. Our data supported recent findings that the avian notochord creates an avascular midline, thereby separating the forming DA. Interestingly, although observations in birds identify BMP antagonists as principal EC-repulsive midline cues,^{18,37} our studies point to multiple repulsive cues in the mammalian notochord, of which Sema3E is essential for normal formation of the paired aortae during vasculogenesis.

Indeed, expression analysis revealed that multiple repulsive cues are found in the mouse notochord. In addition to *chordin* and *noggin*, several NGCs, including *sema3E*, *slit2*, and *netrin1*, were coexpressed by the notochord. Sema3E has been shown to inhibit EC migration through filopodia retraction, collapse of lamellipodia, and disassembly of integrin-mediated adhesion, and *sema3E*^{-/-} mice exhibit ISV patterning defects.^{15,38} *Slit2* has also been shown to inhibit EC migration and restrain angiogenesis^{39–42} (although recent data suggests an alternative receptor than Robo4⁴³). Studies of *Netrin1* function suggest that it can act either as a positive or negative EC cue, dependent on receptors present (Neogenin

Figure 6 Continued. cells migration (arbitrary units) at 12 and 18 hours after “scratch” (n=3). **R** through **U**, In vivo response of ECs in control and Sema3E-HEK293 cells in quail embryos at 11S. ECs contact implanted control cells (**R**) but not HEK293-Sema3E cells (**S**). Asterisks denote autofluorescing cell implants. Quantification of embryos with disrupted DA (**T**) and of cell implants contacting ECs (**U**) (control n=3, and Sema3E n=8). da indicates dorsal aorta. Scale bars: 200 μm.

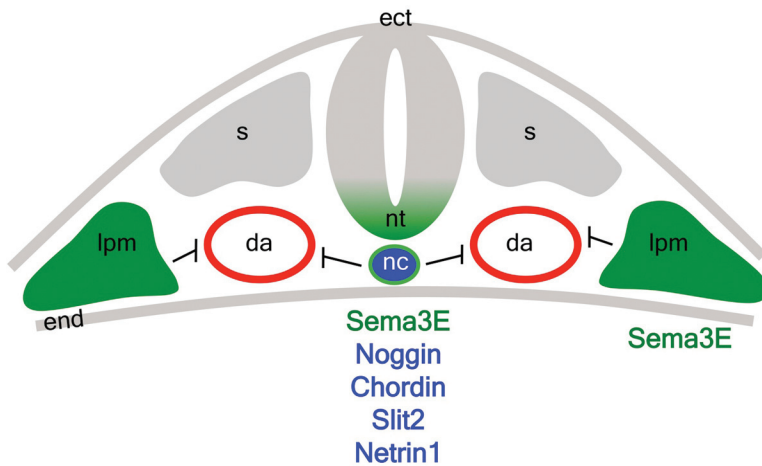


Figure 7. Model: Dorsal aortae formation is regulated by repulsive signals from the notochord and lateral plate mesoderm. Redundant EC-repulsive cues are expressed by the midline notochord (blue/green), whereas Sema3E is the primary cue in the lateral plate mesoderm (green). da indicates dorsal aortae; ect, ectoderm; end, endoderm; lpm, lateral plate mesoderm; nc, notochord; nt, notochord; and s, somite.

or *Unc5b*).^{12,44} *Netrin1* treatment *in vivo* caused filipodia retraction, and knockdown of *netrin1a* in zebrafish resulted in aberrant ISV growth,¹² suggesting a repulsive role. In addition, although we surveyed many candidate repulsive guidance cues, additional factors, such as thrombospondin-1 and thrombospondin-2,^{45–47} angiopoietin-2,⁴⁸ chordin-related 1,⁴⁹ follistatins,⁵⁰ and semaphorin-4A,⁵¹ can inhibit EC migration and angiogenesis and could influence DA formation. Expression analysis during vasculogenesis would help determine if any of these are expressed at the right time and place to repel aortic ECs.

Ablation of Sema3E Leads to Reduction of the Avascular Midline

Given that the notochord expresses a multitude of known EC-repulsive cues, we predicted that ablation of any single cue probably would have little effect on DA patterning. Surprisingly, however, *Sema3E*-deficient mice exhibited dramatic reduction of the avascular midline, with ECs often immediately adjacent to the notochord. In addition, Sema3E-expressing cells strongly inhibited EC migration, both *in vitro* and *in vivo*. Together, these results demonstrate that Sema3E is a robust, endogenous inhibitory cue required to actively maintain the avascular midline in the early embryo by restraining angioblast migration and thereby patterning the DA.

The presence of a residual midline avascular zone in *sema3E*^{-/-} embryos supports the idea that multiple notochord signals simultaneously repulse ECs. Despite their close proximity to the notochord, ECs in *sema3E* mutant mice never cross the midline. This observation suggests that remaining repulsive cues, presumably including Chordin, Noggin, Slit2, and Netrin1, are sufficient to locally repel ECs. Although we speculate that the repulsive effect of BMP antagonists, previously observed in chicks,^{18,52} is also at work in the mouse notochord, both the high level of *sema3E* midline expression and the reduced avascular zone in the *sema3E*^{-/-} mutants suggest *sema3E* plays a critical role in shaping the mammalian aortae.

Is a narrowed midline avascular space unique to deficiency in *sema3E* alone? Or does loss of any other individual NGCs, NGC receptors, or BMP antagonists

result in similar phenotypes? Assessment of paired DA formation (E8–E8.5) in *chordin*, *noggin*, *netrin1*, *slit2*, *unc5b*, *robo4*, and *plexinD1*^{-/-} mice has not yet been carried out.^{12,15,16,40,53–58} Analysis of DA development in these mutant backgrounds should reveal the relative repulsive strength and coordination of individual cues during embryonic vascular patterning.

Sema3E Establishes Lateral DA Boundaries in Mouse

To date, the lateral avascular zones that flank the paired DA have been largely ignored. In contrast to the midline avascular region, which is present before the genesis of the DA (1–2S) and is completely EC-free, angioblasts are initially present within lateral avascular zones as they form (from the 1S to the 3S stage) but quickly clear as Sema3E expression initiates in the LPM (Online Figure V). We propose that for a short time, angioblasts migrate throughout both lateral “wedges” of embryonic mesoderm but that aortic angioblasts are segregated from the extraembryonic angioblasts by the appearance of intervening LPM Sema3E.

In *sema3E*^{-/-} embryos, by contrast, angioblasts are not excluded from these 2 lateral wedges. Instead, angioblasts appear to emerge within a wide corridor resulting in formation of paired plexus rather than 2 large and parallel aortae. Furthermore, expression of *sema3E* in the LPM was unperturbed in notochordless embryos, correlating with residual lateral avascular zones observed in those mutant embryos. These experiments are the first to identify the LPM as an important source of vascular patterning cues and the paracrine LPM signal Sema3E as a locally non-redundant factor that shapes the lateral boundaries of the DA.

Interestingly, blood vessels in *sema3E*^{-/-} embryos appeared relatively normal in many respects. They retained their ability to form patent vessels and expressed arterial differentiation markers (*cx40* and *dll4*). This argues that Sema3E functions primarily to pattern developing blood vessels but does not affect EC specification or morphogenetic processes, such as their ability to adhere, coalesce, or form tubes. In addition, these results suggest that positive cues are present

and actively promoting blood vessel development, within the context of repulsive cues. Indeed, examination of *sema3E*^{-/-} mutants revealed normal expression of EC-promoting factors, including *vegf*, *shh*, *bmps*, and *fgfs* near the developing DA (Online Figure VI).

An interesting question arises as to the anatomic differences observed between the aortae of mammalian versus avian embryos. The lateral aspects of the avian paired aortae are less sharply demarcated¹⁸ than in mouse, as they connect directly to lateral plexus of vessels along the length of the embryonic axis. What is the molecular basis for this difference? We speculate that this patterning difference between mouse and chicks may reflect the differential expression of *Sema3E* in the LMP. In addition, it is probably species-specific cue usage that may dictate formation of 2 initial aortae in mouse and chicks, in contrast to the single midline aorta formed in frogs and fish. Future studies to address these questions will be of great interest.

The Busy EC Cue Environment

How can ECs interpret the numerous cues within the embryonic tissue microenvironment? Many EC-promoting factors, such as VEGF,^{3,59} FGF,⁶⁰ BMPs,^{18,61} angiopoietins,^{62,63} and apelin^{64,65} are expressed widely during vasculogenesis, yet the aortic vessels form at strikingly specific locations within the embryo. Previous work¹⁸ along with our findings indicate that repulsive cues counterbalance abundant proangiogenic signals by generating avascular zones that guide formation of the DA by “corralling” migrating angioblasts. In other words, aortic ECs receive and integrate both positive and negative signals from the surrounding environment to form vessels where positive factors are present and inhibitory signals are absent (Figure 7).

Similar to the early embryo, tumor environments are composed of numerous EC-promoting signals that are largely responsible for causing prolific angiogenic growth and tumor expansion.⁶⁶ It is therefore not surprising that therapeutic treatments targeting single positive cues have modest effects in regulating tumor blood vessel growth.⁶⁷ Our findings show that avascular tissues are not merely poor in angiogenic cues, as might be expected, but also rich in endogenous antiangiogenic factors, presenting a powerful blueprint for designing new therapeutic approaches. Recreating avascular environments with multiple EC-repulsive cues, in combination with current therapies, might prove to be an effective and possibly inevitable approach to fighting tumor growth and progression.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Mechanisms underlying the formation and patterning of the first mammalian blood vessels, the paired dorsal aortae (DA), are not well understood.
- In avian embryos, bone morphogenetic protein antagonists from the notochord separate and pattern the DA at the embryonic midline.
- Repulsive guidance cues influence endothelial cell (EC) behavior.

What New Information Does This Article Contribute?

- The notochord is required for formation of the DA during mammalian development and expresses multiple, redundant repulsive guidance cues.
- The lateral plate mesoderm (LPM) shapes the mammalian DA by creating avascular boundaries.
- Semaphorin 3E (Sema3E) is a critical EC-repulsive guidance cue that shapes and patterns the DA in mammals.

Elucidating the mechanisms involved in shaping the initial blood vessels is important to understanding basic cardiovascular devel-

opment. Our studies demonstrate that EC-repulsive signals, notably Sema3E, emanate from the notochord and LPM to guide aortic ECs to specific locations during formation of the first mammalian blood vessels, the paired DA. Similar to avian embryos that lack a notochord, notochordless mutant mice embryos display severe disruption of DA formation, with ECs present throughout the normally avascular midline that separates the paired vessels. Our results show that the mammalian notochord expresses multiple repulsive cues that coordinate during vasculogenesis to pattern the DA. In particular, Sema3E is strongly expressed in the notochord, and *sema3E*^{-/-} embryos exhibit a marked reduction of the avascular midline. Interestingly, in mammals, the DA are also constrained by lateral avascular zones, which have until now been largely ignored. Our studies are the first to demonstrate that LPM, specifically Sema3E from the LPM, generates the lateral avascular zones that define the DA boundaries. Without repulsive boundaries, as in *sema3E*^{-/-} embryos, the DA develop into highly branched plexus-like vessels. Understanding fundamental aspects of guidance cue regulation during blood vessel formation will impact the future development of proangiogenic and antiangiogenic therapies.