Published in final edited form as:
Circ Res. 2012 January 6; 110(1): 34–46. doi:10.1161/CIRCRESAHA.111.249847.

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

Stryder M. Meadows[1], **Peter J. Fletcher**[1], **Carlos Moran**[2], **Ke Xu**[1], **Gera Neufeld**[3], **Sophie Chauvet**[4], **Fanny Mann**[4], **Paul Krieg**[2], and **Ondine Cleaver**[1,*]

[1]Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas, USA 75390

[2]Department of Cell Biology and Anatomy, University of Arizona College of Medicine, 1656 E. Mabel Street - MRB311, Tucson, AZ, 85724

[3]Cancer Research and vascular Biology Center, The Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, P.O. Box 9679, 1 Efron St., Haifa, 31096, ISRAEL

[4]Developmental Biology Institute of Marseille Luminy, CNRS UMR 6216, University of Mediterranee, Case 907, Parc Scientifique de Luminy, 13288 Marseille cedex 09, France

Abstract

Rationale—Positive signals, such as vascular endothelial factor (VEGF), 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' (NGCs) 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 via the generation of stereotyped avascular zones by EC-repulsive cues. We demonstrate that Semaphorin3E (Sema3E) is a key factor that shapes the paired DA 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 NGCs. Mouse embryos that lack notochords fail to form cohesive aortic vessels due to loss of the avascular midline, yet maintain lateral avascular zones. We demonstrate that lateral avascular zones are directly generated by the lateral plate mesoderm (LPM), 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 LPM. Integration of multiple, and in some cases redundant, repulsive cues from various tissues is critical to patterning the first embryonic blood vessels.

[*]Corresponding author: Ondine Cleaver Department of Molecular Biology, University of Texas Southwestern Medical Center 5323 Harry Hines Blvd., NA8.300 Dallas, Texas 75390-9148, USA. Phone: (214) 648-1647 Fax: (214) 648-1196 ondine.cleaver@utsouthwestern.edu.

Disclosures
None

Keywords

Neuronal guidance cues; Sema3E; notochord; lateral plate mesoderm; endothelial

INTRODUCTION

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 two 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 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, BMP antagonists Chordin and Noggin are secreted from the notochord and inhibit EC migration, creating an avascular midline[18] 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.

Here, 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*. Eosin staining: sections dewaxed in xylenes for 10 min, followed by ethanol washes from 100% to 70%, then submerged in Eosin for 30 sec, ethanol washes to 100%, xylenes for 5 min, 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 1X PBS, transferred to 0.25% Trypsin (Hyclone) for 2 min, rinsed in 1X PBS, blocked in CAS-Block (Invitrogen) for 1 hour (hr) at RT and incubated overnight with PECAM antibody (BD Pharmingen; 1:300) in PBST at 4°C. The next day, embryos were washed with 1X PBST, 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

HUVEC 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. Co-culture experiments using HUVEC, HEK293T and HEK293T cells were carried out as previously described [21]. Briefly, HUVEC cells were seeded onto gelatinized 24 (2×10^4 cells/well) well plates. The next day, 5% of HEK293T and HEK293T cells, incubated with 5ug/ml of fluorescent vital DiI for 30 min, were seeded on top of the HUVECs. Cells were assessed after 24 hrs 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 18hr cultures.

Cell implant experiments in avian embryos

HEK293T and HEK293T-Sema3E cells were cultured using standard 'hanging-drop' method, and resulting aggregates were implanted into pre-vascular, 2-3 somite stage chicken and quail embryos at location of developing DA. New Cultures method used and incubated at 37°C in 95% oxygen until the 10-15 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

Dorsal aortae 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 mice [25] to pinpoint the positioning and timing of angioblast aggregation during vessel formation. At this stage, Flk1 expression is restricted to angioblasts. At the 1-2 somite (1-2S) stage of development, or embryonic day (E) 8.0, individual ECs were observed in two bilateral stripes. A wide avascular zone was present at the midline, separating the presumptive aortic vessels (**Figures 1A,A'**). In addition, two 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 (**Figures 1B,B'**). At 4S, all three avascular zones were distinct and cords began forming lumenized vessels in the anterior region of the embryo (**Figures 1C,C'**). Of note, lateral avascular regions became more defined in an anterior-posterior manner, similar to the forming DA (**Figures 1B-1C'**). By 5-6S, the DA consisted of tubes patent along their entire length, and all three avascular zones were well demarcated (**Figures 1D-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 (prior to 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 (**Figures 2I-2N'**), with high levels in the notochord (**Figures 2J,L,N**). *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 (**Figures 2E-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 (**Figures 2D,D'**), 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 three expressed in the DA throughout vasculogenesis (**Figure I**), confirming that aortic ECs are competent to respond to repulsive NGC signaling.

The notochord is required for patterning the murine dorsal aortae

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 three distinct phenotypes, based upon 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 (**Figures 3A,B** 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 disorganized vessel fragments and presence of ECs at the embryonic midline (**Figures 3C-3K**, **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 (**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 (**Figures 3F,H,L** and Supplementary **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 (**Figure III**). However, the DA resembled early EC cord structures and failed to undergo lumen formation in embryos in which *Foxa2* was depleted in the endoderm (**Figure III**). Similarly, deletion of *Foxa2* in ECs caused no obvious abnormalities in EC patterning (**Figures 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). 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 (**Figures 4A-H'**, **Figure**

IV), however expression was largely unaffected outside the midline (compare **Figures 4A,B** to **4C,D**, **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 **Figures 4E,F** to **4G,H**). 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 (**Figures 3C,D,I,J**, **Figure II**, **Figures 5A,B**) 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 **Figures 2M,N** to **Figures 4E,F,G,H** and **Figures 5CF**).

Of all the guidance cues surveyed, only *sema3E* exhibited strong LPM expression (**Figure 2**). We assessed all seven semaphorin3 genes (*sema3A-3H*, data not shown), and while *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,H and Figure V**). This nested location of the aortae between *sema3E*-expressing tissues suggested that ECs are corralled between avascular zones into aortic cords via negative cues on either side. Interestingly, *sema3E* expression in midline tissues and the LPM is down-regulated at the time of DA fusion (**Figure V**). We therefore proposed that midline Sema3E acts in an overlapping manner with other notochord repulsive cues, while in LPM Sema3E creates lateral avascular zones in a unique, non-redundant 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 two parallel and unbranched vascular tubes, extending from the head to the tail (**Figure 1**) [19, 32]. We found that while *sema3E*$^{+/-}$ aortae were indistinguishable from wild type (**Figure 6A,C**), *sema3E*$^{-/-}$ aortae were severely disrupted (**Figure 6B,D,G,H**), 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, due to loss of sema3E, or indirect, due to changes in endothelial promoting signals, we assessed the expression of growth factors known to influence developing blood vessels. We found that the VEGF, Shh, Bmp and Fgf signaling pathways were unaffected in *sema3E* mutants (**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** to **F**). EC differentiation also occurred normally (**Figure 6D, Figure VII**), as arterial *cx40* and *dll4* expression initiated in vessels of the *sema3E*$^{-/-}$ aortic plexus, suggesting that arteriovenous differentiation occurred normally (**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** to **F**). 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,F, Figure VII**). We had anticipated that since 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, 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 (**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 to 8.25 using a Flk1-LacZ allele, which labels initial angioblasts. We found that angioblasts emerged normally, in both the yolk sac and in two rows of intra-embryonic angioblasts presaging the aortae, but that the area encompassing the pre-aortic angioblasts appeared wider in the *sema3E* null embryos (**Figure VIII)**. In addition, at E7.5-7.75 extra-embryonic 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 human umbilical vein ECs (HUVEC) to assess EC behavior. Following 24 hours of incubation, HUVECs were found to be closely associated with control HEK293 cells, while Sema3E-expressing HEK293 cells efficiently repelled ECs, creating avascular zones (**Figures 6J,K**).

Since 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 (**Figures 6L-Q**). Results reflected decreases in EC migration, not proliferation, as doubling time of MS1 and bEnd.3 cells is over 24 hours [20].

To test the ability of Sema3E to repel ECs *in vivo*, we implanted control HEK293 and HEK293-Sema3E cell aggregates into pre-vascular regions of early quail embryos and assessed vascular development. Following 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,T,U**). In contrast, vessels did not contact Sema3E-expressing cells (**Figure 6S,T,U**). 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 (**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 (**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 (**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 multi-cue, 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 dorsal aortae formation in mouse. Our analyses of *FoxH1*$^{-/-}$and *Foxa2*$^{-/-}$ mice demonstrated the importance of the notochord in mammals, since 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, while 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 co-expressed by the notochord. Sema3E has been shown to inhibit EC migration through filipodia 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 Robo4 [43]). Studies of Netrin1 function suggest that it can act either as a positive or negative EC cue, dependent on receptors present (Neogenin 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 here, additional factors, such as thrombospondin-1 and -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 would likely 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. While 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 prior to 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 3S stage), but quickly clear as Sema3E expression initiates in the LPM (**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 two lateral wedges. Instead, angioblasts appear to emerge within a wide corridor resulting in formation of paired plexus rather than two 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 (**Figure VI**).

An interesting question arises as to the anatomical differences observed between the aortae of mammalian versus avian embryos. The lateral aspects of the avian paired aortae are less sharply demarcated [18] 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 likely species-specific cue usage may dictate formation of two 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], fibroblast growth factors (Fgf)[60], bone morphogenetic proteins (Bmp) [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 work [18] along with our findings, indicate that repulsive cues counterbalance abundant pro-angiogenic 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 comprised 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 anti-angiogenic 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 an effective and possibly inevitable approach to fighting tumor growth and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Chenghua Gu for the *sema3E* mice; Pamela Hoodless for the *Foxh1* mice; Klaus Kaestner for the *Foxa3-cre* and *Foxa2 m*ice; Tom Sato for the *Tie2-cre* mice; Eli Keshet for the *Flk1-LacZ* and Janet Rossant *Flk1-EGFP* mice; and Thomas Carroll for the *Sox2-cre* mice.

Sources of Funding

This work was supported by the Hartwell Foundation and NIH HL007360-32 (SM), AHA predoctoral fellowship 09PRE2070035 (KX), the Centre National de la Recherche Scientifique (CNRS) and University of Mediterranee (FM), Israel Science Foundation (GN), HL074184-02 (PAK), and NIH DK079862 and AHA Grant-in-Aid 0755054Y (OC).

NON-STANDARD ABBREVIATIONS AND ACRONYMS

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

REFERENCES

1. Mukouyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ. Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. Cell. 2002; 109(6):693–705. [PubMed: 12086669]
2. Adams RH, Eichmann A. Axon guidance molecules in vascular patterning. Cold Spring Harb Perspect Biol. 2010; 2(5):a001875. [PubMed: 20452960]
3. Hogan KA, Ambler CA, Chapman DL, Bautch VL. The neural tube patterns vessels developmentally using the VEGF signaling pathway. Development. 2004; 131(7):1503–1513. [PubMed: 14998923]
4. Stone J, Itin A, Alon T, Pe'er J, Gnessin H, Chan-Ling T, Keshet E. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J Neurosci. 1995; 15(7 Pt 1):4738–4747. [PubMed: 7623107]
5. Eichmann A, Makinen T, Alitalo K. Neural guidance molecules regulate vascular remodeling and vessel navigation. Genes Dev. 2005; 19(9):1013–1021. [PubMed: 15879551]
6. Torres-Vazquez J, Gitler AD, Fraser SD, Berk JD, Van NP, Fishman MC, Childs S, Epstein JA, Weinstein BM. Semaphorin-plexin signaling guides patterning of the developing vasculature. Developmental cell. 2004; 7(1):117–123. [PubMed: 15239959]
7. Autiero M, De Smet F, Claes F, Carmeliet P. Role of neural guidance signals in blood vessel navigation. Cardiovasc Res. 2005; 65(3):629–638. [PubMed: 15664389]
8. Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. Nature. 2005; 436(7048):193–200. [PubMed: 16015319]

9. Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. Science. 1996; 274(5290):1123–1133. [PubMed: 8895455]
10. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell. 1998; 93(5): 741–753. [PubMed: 9630219]
11. Helbling PM, Saulnier DM, Brandli AW. The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. Development. 2000; 127(2): 269–278. [PubMed: 10603345]
12. Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Breant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M, Eichmann A. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature. 2004; 432(7014):179–186. [PubMed: 15510105]
13. Bedell VM, Yeo SY, Park KW, Chung J, Seth P, Shivalingappa V, Zhao J, Obara T, Sukhatme VP, Drummond IA, Li DY, Ramchandran R. roundabout4 is essential for angiogenesis in vivo. Proc Natl Acad Sci U S A. 2005; 102(18):6373–6378. [PubMed: 15849270]
14. Shoji W, Isogai S, Sato-Maeda M, Obinata M, Kuwada JY. Semaphorin3a1 regulates angioblast migration and vascular development in zebrafish embryos. Development. 2003; 130(14):3227–3236. [PubMed: 12783793]
15. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD. Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. Science. 2005; 307(5707):265–268. [PubMed: 15550623]
16. Gitler AD, Lu MM, Epstein JA. PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. Developmental cell. 2004; 7(1):107–116. [PubMed: 15239958]
17. Fouquet B, Weinstein BM, Serluca FC, Fishman MC. Vessel patterning in the embryo of the zebrafish: guidance by notochord. Dev Biol. 1997; 183(1):37–48. [PubMed: 9119113]
18. Reese DE, Hall CE, Mikawa T. Negative regulation of midline vascular development by the notochord. Developmental cell. 2004; 6(5):699–708. [PubMed: 15130494]
19. Xu K, Sacharidou A, Fu S, Chong DC, Skaug B, Chen ZF, Davis GE, Cleaver O. Blood vessel tubulogenesis requires Rasip1 regulation of GTPase signaling. Developmental Cell. 2011:20. [PubMed: 21763602]
20. Xu K, Chong DC, Rankin SA, Zorn AM, Cleaver O. Rasip1 is required for endothelial cell motility, angiogenesis and vessel formation. Dev Biol. 2009
21. Guttmann-Raviv N, Shraga-Heled N, Varshavsky A, Guimaraes-Sternberg C, Kessler O, Neufeld G. Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. J Biol Chem. 2007; 282(36):26294–26305. [PubMed: 17569671]
22. Vokes SA, Yatskievych TA, Heimark RL, McMahon J, McMahon AP, Antin PB, Krieg PA. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. Development. 2004; 131(17):4371–4380. [PubMed: 15294868]
23. Drake CJ, Fleming PA. Vasculogenesis in the day 6.5 to 9.5 mouse embryo. Blood. 2000; 95(5): 1671–1679. [PubMed: 10688823]
24. Coffin JD, Harrison J, Schwartz S, Heimark R. Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. Dev Biol. 1991; 148(1):51–62. [PubMed: 1936575]
25. Ema M, Takahashi S, Rossant J. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. Blood. 2006; 107(1):111–117. [PubMed: 16166582]
26. Yamamoto M, Meno C, Sakai Y, Shiratori H, Mochida K, Ikawa Y, Saijoh Y, Hamada H. The transcription factor FoxH1 (FAST) mediates Nodal signaling during anterior-posterior patterning and node formation in the mouse. Genes Dev. 2001; 15(10):1242–1256. [PubMed: 11358868]
27. Hoodless PA, Pye M, Chazaud C, Labbe E, Attisano L, Rossant J, Wrana JL. FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse. Genes Dev. 2001; 15(10):1257–1271. [PubMed: 11358869]

28. Ang SL, Rossant J. HNF-3 beta is essential for node and notochord formation in mouse development. Cell. 1994; 78(4):561–574. [PubMed: 8069909]
29. Ding Q, Motoyama J, Gasca S, Mo R, Sasaki H, Rossant J, Hui CC. Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development. 1998; 125(14): 2533–2543. [PubMed: 9636069]
30. Lee CS, Sund NJ, Behr R, Herrera PL, Kaestner KH. Foxa2 is required for the differentiation of pancreatic alpha-cells. Dev Biol. 2005; 278(2):484–495. [PubMed: 15680365]
31. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol. 2001; 230(2): 230–242. [PubMed: 11161575]
32. Strilic B, Kucera T, Eglinger J, Hughes MR, McNagny KM, Tsukita S, Dejana E, Ferrara N, Lammert E. The molecular basis of vascular lumen formation in the developing mouse aorta. Dev Cell. 2009; 17(4):505–515. [PubMed: 19853564]
33. Eichmann A, Le Noble F, Autiero M, Carmeliet P. Guidance of vascular and neural network formation. Current opinion in neurobiology. 2005; 15(1):108–115. [PubMed: 15721752]
34. Poole TJ, Coffin JD. Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. J Exp Zool. 1989; 251(2):224–231. [PubMed: 2671254]
35. Kigel B, Varshavsky A, Kessler O, Neufeld G. Successful inhibition of tumor development by specific class-3 semaphorins is associated with expression of appropriate semaphorin receptors by tumor cells. PLoS One. 2008; 3(9):e3287. [PubMed: 18818766]
36. Cleaver O, Krieg PA. VEGF mediates angioblast migration during development of the dorsal aorta in Xenopus. Development. 1998; 125(19):3905–3914. [PubMed: 9729498]
37. Bressan M, Davis P, Timmer J, Herzlinger D, Mikawa T. Notochord-derived BMP antagonists inhibit endothelial cell generation and network formation. Dev Biol. 2009; 326(1):101–111. [PubMed: 19041859]
38. Sakurai A, Gavard J, Annas-Linhares Y, Basile JR, Amornphimoltham P, Palmby TR, Yagi H, Zhang F, Randazzo PA, Li X, Weigert R, Gutkind JS. Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. Mol Cell Biol. 2010; 30(12):3086–3098. [PubMed: 20385769]
39. Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Chien CB, Wu JY, Urness LD, Li DY. Robo4 is a vascular-specific receptor that inhibits endothelial migration. Dev Biol. 2003; 261(1): 251–267. [PubMed: 12941633]
40. Jones CA, London NR, Chen H, Park KW, Sauvaget D, Stockton RA, Wythe JD, Suh W, Larrieu-Lahargue F, Mukouyama YS, Lindblom P, Seth P, Frias A, Nishiya N, Ginsberg MH, Gerhardt H, Zhang K, Li DY. Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. Nat Med. 2008; 14(4):448–453. [PubMed: 18345009]
41. London NR, Zhu W, Bozza FA, Smith MC, Greif DM, Sorensen LK, Chen L, Kaminoh Y, Chan AC, Passi SF, Day CW, Barnard DL, Zimmerman GA, Krasnow MA, Li DY. Targeting Robo4-dependent Slit signaling to survive the cytokine storm in sepsis and influenza. Sci Transl Med. 2010; 2(23):23ra19.
42. Marlow R, Binnewies M, Sorensen LK, Monica SD, Strickland P, Forsberg EC, Li DY, Hinck L. Vascular Robo4 restricts proangiogenic VEGF signaling in breast. Proc Natl Acad Sci U S A. 2010; 107(23):10520–10525. [PubMed: 20498081]
43. Koch AW, Mathivet T, Larrivee B, Tong RK, Kowalski J, Pibouin-Fragner L, Bouvree K, Stawicki S, Nicholes K, Rathore N, Scales SJ, Luis E, del Toro R, Freitas C, Breant C, Michaud A, Corvol P, Thomas JL, Wu Y, Peale F, Watts RJ, Tessier-Lavigne M, Bagri A, Eichmann A. Robo4 maintains vessel integrity and inhibits angiogenesis by interacting with UNC5B. Developmental cell. 2011; 20(1):33–46. [PubMed: 21238923]
44. Park KW, Crouse D, Lee M, Karnik SK, Sorensen LK, Murphy KJ, Kuo CJ, Li DY. The axonal attractant Netrin-1 is an angiogenic factor. Proc Natl Acad Sci U S A. 2004; 101(46):16210–16215. [PubMed: 15520390]
45. Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally

indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci U S A. 1990; 87(17): 6624–6628. [PubMed: 1697685]

46. Iruela-Arispe ML, Bornstein P, Sage H. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. Proc Natl Acad Sci U S A. 1991; 88(11):5026–5030. [PubMed: 1711216]
47. Noh YH, Matsuda K, Hong YK, Kunstfeld R, Riccardi L, Koch M, Oura H, Dadras SS, Streit M, Detmar M. An N-terminal 80 kDa recombinant fragment of human thrombospondin-2 inhibits vascular endothelial growth factor induced endothelial cell migration in vitro and tumor growth and angiogenesis in vivo. J Invest Dermatol. 2003; 121(6):1536–1543. [PubMed: 14675207]
48. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 1997; 277(5322):55–60. [PubMed: 9204896]
49. Kane R, Godson C, O'Brien C. Chordin-like 1, a bone morphogenetic protein-4 antagonist, is upregulated by hypoxia in human retinal pericytes and plays a role in regulating angiogenesis. Mol Vis. 2008; 14:1138–1148. [PubMed: 18587495]
50. Maeshima K, Maeshima A, Hayashi Y, Kishi S, Kojima I. Crucial role of activin a in tubulogenesis of endothelial cells induced by vascular endothelial growth factor. Endocrinology. 2004; 145(8):3739–3745. [PubMed: 15117880]
51. Toyofuku T, Yabuki M, Kamei J, Kamei M, Makino N, Kumanogoh A, Hori M. Semaphorin-4A, an activator for T-cell-mediated immunity, suppresses angiogenesis via Plexin-D1. EMBO J. 2007; 26(5):1373–1384. [PubMed: 17318185]
52. Garriock RJ, Czeisler C, Ishii Y, Navetta AM, Mikawa T. An anteroposterior wave of vascular inhibitor downregulation signals aortae fusion along the embryonic midline axis. Development. 2010; 137(21):3697–3706. [PubMed: 20940228]
53. Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell. 1996; 87(6):1001–1014. [PubMed: 8978605]
54. McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. 1998; 12(10):1438–1452. [PubMed: 9585504]
55. Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J, De Robertis EM. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature. 2000; 403(6770):658–661. [PubMed: 10688202]
56. Salminen M, Meyer BI, Bober E, Gruss P. Netrin 1 is required for semicircular canal formation in the mouse inner ear. Development. 2000; 127(1):13–22. [PubMed: 10654596]
57. Plump AS, Erskine L, Sabatier C, Brose K, Epstein CJ, Goodman CS, Mason CA, Tessier-Lavigne M. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron. 2002; 33(2):219–232. [PubMed: 11804570]
58. Nguyen-Ba-Charvet KT, Plump AS, Tessier-Lavigne M, Chedotal A. Slit1 and slit2 proteins control the development of the lateral olfactory tract. J Neurosci. 2002; 22(13):5473–5480. [PubMed: 12097499]
59. Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn. 1995; 203(1):80–92. [PubMed: 7647376]
60. Murakami M, Simons M. Fibroblast growth factor regulation of neovascularization. Curr Opin Hematol. 2008; 15(3):215–220. [PubMed: 18391788]
61. Danesh SM, Villasenor A, Chong D, Soukup C, Cleaver O. BMP and BMP receptor expression during murine organogenesis. Gene Expr Patterns. 2009; 9(5):255–265. [PubMed: 19393343]
62. Patan S. TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. Microvasc Res. 1998; 56(1):1–21. [PubMed: 9683559]

63. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell. 1996; 87(7):1161–1169. [PubMed: 8980223]

64. Kalin RE, Kretz MP, Meyer AM, Kispert A, Heppner FL, Brandli AW. Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis. Dev Biol. 2007; 305(2):599–614. [PubMed: 17412318]

65. Cox CM, D'Agostino SL, Miller MK, Heimark RL, Krieg PA. Apelin, the ligand for the endothelial G-protein-coupled receptor, APJ, is a potent angiogenic factor required for normal vascular development of the frog embryo. Dev Biol. 2006; 296(1):177–189. [PubMed: 16750822]

66. Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005; 438(7070):932–936. [PubMed: 16355210]

67. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 2008; 8(8):592–603. [PubMed: 18650835]

68. Sund NJ, Ang SL, Sackett SD, Shen W, Daigle N, Magnuson MA, Kaestner KH. Hepatocyte nuclear factor 3beta (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. Mol Cell Biol. 2000; 20(14):5175–5183. [PubMed: 10866673]

69. Lee CS, Friedman JR, Fulmer JT, Kaestner KH. The initiation of liver development is dependent on Foxa transcription factors. Nature. 2005; 435(7044):944–947. [PubMed: 15959514]

70. Hayashi S, Lewis P, Pevny L, McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mech Dev. 2002; 119(Suppl 1):S97–S101. [PubMed: 14516668]

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 (BMP) 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, non-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 development. 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 pro- and anti-angiogenic therapies.

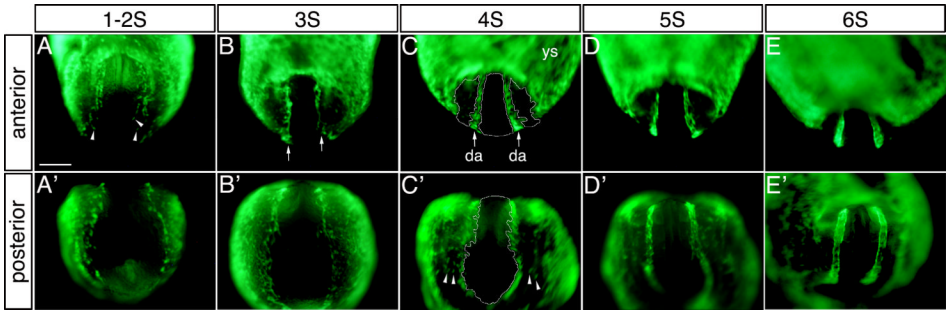

Figure 1. Formation of the dorsal aortae and avascular zones during mouse vasculogenesis

(**A**-**E**) *Flk1-EGFP* highlights ECs (green) in 1-6S stage embryos (anterior views). (**A**) Free angioblasts (white arrowheads) align in two 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'-E'**) Ventral views of 1-6S embryos. Lateral avascular zones are forming from 1-4S (**A'-C'**), with angioblasts (arrowheads) still present. By 5S(**D'**), lateral regions are virtually EC-free. Scale bar: 200 μm. da, dorsal aorta; S, somite; ys, yolk sac.

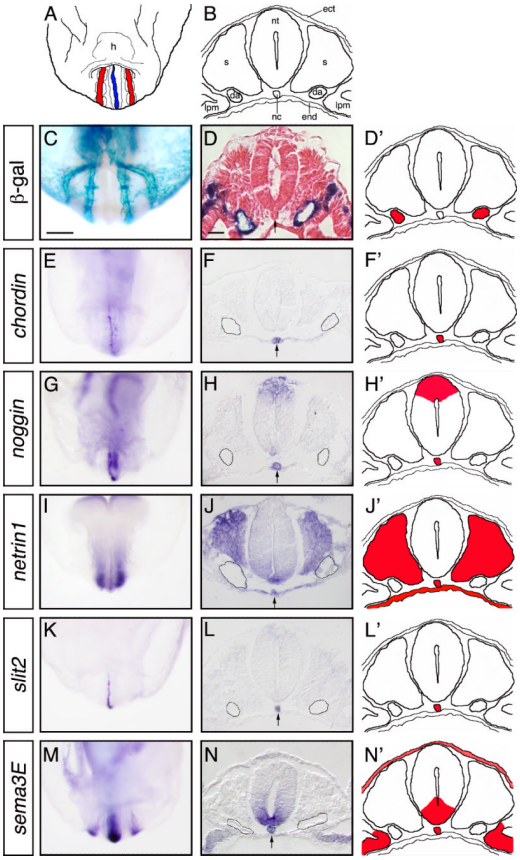

Figure 2. The notochord expresses multiple repulsive guidance cues during embryonic vasculogenesis

(**A,B**) Cartoon depiction of an E8.25 embryo; (**A**) anterior and (**B**) cross-section views showing DA (red), notochord (blue) and surrounding tissues. (**C,D**) E8-8.25 *Flk1-LacZ* embryos stained for β-galactosidase (light blue) and eosin (**D**, red); (**C**) anterior and (**D**) cross-section views. (**E-N**) In situ hybridization for *chordin*, *noggin*, *netrin1*, *slit2* and *sema3E* at E8-8.25. (**E,G,I,K, M**) Anterior and (**F,H,J,L,N**) cross-section views. Arrows mark the notochord and DA are outlined in black. Note expression of *chordin*, *noggin*, *netrin1*, *slit2* and *sema3E* in the notochord. The scale bars represent 200 μm (**C,E,G,I,K,M**) and 25 μm (**D,F,H,J,L,N**). (**D',F',H',J',L',N'**) Cartoon schematics of **D,F,H,J,L,N** respectively. Stained tissues shown in red. da, dorsal aorta; ect, ectoderm; end, endoderm; h, heart; lpm, lateral plate mesoderm; nc, notochord; nt, neural tube; s, somites;

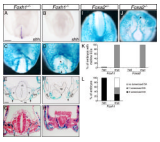

Figure 3. Dorsal aortae are severely disrupted in notochordless *Foxh1* and *Foxa2* null embryos

(**A, 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-J**) β-galactosidase staining (light blue) and eosin staining (**G,H,** red) of E8.25 *Foxh1*$^{+/-}$*;Flk1-LacZ*, *Foxh1*$^{-/-}$*;Flk1-LacZ*, *Foxa2*$^{+/-}$*;Flk1-LacZ* and *Foxa2*$^{-/-}$*;Flk1-LacZ* embryos; anterior (**C,D**), posterior (**I, J**) and cross-section (**E-H**) views. Note presence of midline ECs (arrowheads) in *Foxh1*$^{-/-}$ and *Foxa2*$^{-/-}$ embryos (**D,F,H,J**). Scale bars: 200 μm (**A-D,I,J**) and 50 μm (**E-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**) The percent of *Foxh1* embryos with lumenized DA (het, n = 7; mut, n = 7). Arrows, DA. lpm, lateral plate mesoderm; nc, notochord; np, neural plate; nt, neural tube; s, somite.

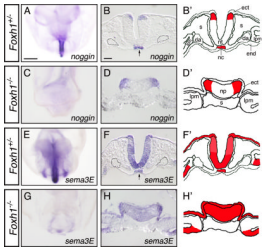

Figure 4. Midline repulsive guidance cues are lost in notochordless embryos

(**A-H**) *In situ* hybridization for *noggin* and *sema3E* transcripts in E8.0 *Foxh1*$^{+/-}$ and *Foxh1*$^{-/-}$ embryos: (**A,C,E,G**) anterior and (**B,D,F,H**) cross-section views. Arrows mark the notochord and DA are outlined in black. (Note F and H show posterior sections where Sema3E is expressed throughout the neural tube). Scale bars: 200 μm (**A,C,E,F**) and 25 μm (**B,D,F,H**). (**B',D',F',H'**) Cartoon depiction of stained tissues (red) in **B,D,F,H** respectively. da, dorsal aorta; ect, ectoderm; end, endoderm; lpm, lateral plate mesoderm; nc, notochord; np, neural plate; nt, neural tube: s, somite.

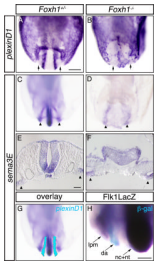

Figure 5. *Sema3E* expression corresponds to the lateral avascular zones in wild-type and *Foxh1*$^{-/-}$ embryos

(**A-H)** Expression of *plexinD1* and *sema3E* in E8.25 *Foxh1*$^{+/-}$, *Foxh1*$^{-/-}$ and *Flk1-LacZ* embryos; (**A,C,G,H**) anterior and (**E,F**) cross-section views. Lateral avascular zones (arrows), *sema3E* expression in the lpm (arrowhead) 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**) Cartoon of *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 μm (**A-D,G**), 100 μm (**H**) and 25 μm (**E,F**). da, dorsal aorta; lpm, lateral plate mesoderm; nc, notochord; nt, neural tube.

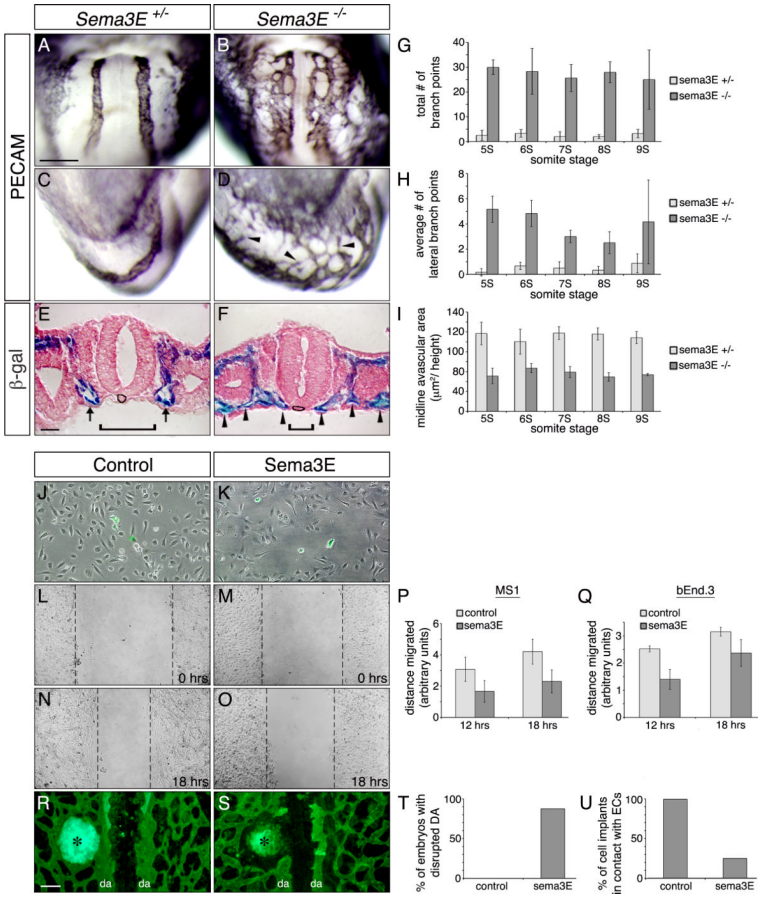

Figure 6. Sema3E is both sufficient for lateral avascular zones and required for dorsal aortae patterning

Sema3E$^{+/-}$*;Flk1-LacZ* and *sema3E*$^{-/-}$*;Flk1-LacZ* embryos stained for (**A-D**) PECAMor β-galactosidase (**E,F**). (**A,B**) Anterior view of 5S, (**C,D**) lateral view of 6S and (**E,F**) cross-section view of 8S embryos. **E** and **F** stained with eosin (red). (**B,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 (**G**) total aortic branch points and (**H**) ectopic branchpoints within lateral avascular regions, in *sema3E*$^{+/-}$ and *sema3E*$^{-/-}$ embryos. Branchpoints within 100 sq μm 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 μm/height) in *sema3E*$^{+/-}$ and *sema3E*$^{-/-}$ embryos, in anterior regions of 5-9S embryos (n = 3-4 embryos per somite stage). (**J-U**) Cultured ECs are repelled by HEK293-Sema3E cells (**K,** green), but not by control HEK293 cells (**J,** green). (**L-Q**) 'Wound-healing' assays with mouse MS1 (**L-P**) or bend.3 EC lines (**Q**). MS1 cells at 0 hours (hrs) (**L, M**) and after 18 hrs cultured (**N, O**) in media conditioned by control or HEK293-Sema3E cells. (**P, Q**) Quantification of MS1 and bend.3 cells migration (arbitrary units) at 12 and 18 hrs post 'scratch' (n=3). (**R-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 auto-fluorescing cell implants. Quantification of embryos with disrupted DA (**T**) and of cell implants contacting ECs (**U**) (control n=3, and Sema3E n=8). da, dorsal aorta. Scale bars: 200 Γm (**A-D**), 25 Γm (**E,F**) and (**R,S**) 100 μm.

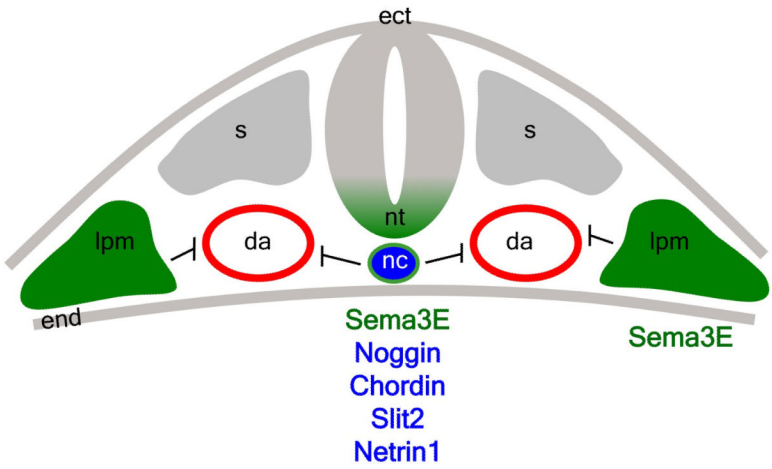

Figure 7. Model: Dorsal aortae formation is regulated by repulsive signals from the notochord and lateral plate mesoderm

Redundant EC-repulsive cues are expressed by the midline notochord (blue/green), while Sema3E is the primary cue in the lateral plate mesoderm (green). da, dorsal aortae; ect, ectoderm; end, endoderm; lpm, lateral plate mesoderm; nc, notochord; nt, notochord; s, somite.