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Semaphorin3a1 regulates angioblast migration and vascular development in zebrafish embryos

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SUMMARY

Semaphorins are a large family of secreted and cell surface molecules that guide neural growth cones to their targets during development. Some semaphorins are expressed in cells and tissues beyond the nervous system suggesting the possibility that they function in the development of non-neural tissues as well. In the trunk of zebrafish embryos endothelial precursors (angioblasts) are located ventral and lateral to the somites. The angioblasts migrate medially and dorsally along the medial surface of the somites to form the dorsal aorta just ventral to the notochord. Here we show that in zebrafish Sema3a1 is involved in angioblast migration in vivo. Expression of *sema3a1* in somites and *neuropilin 1*, which encodes for a component of the Sema3a receptor, in angioblasts suggested that Sema3a1 regulates the pathway of the dorsally migrating angioblasts. Antisense knockdown of Sema3a1 inhibited the formation of the dorsal aorta. Induced ubiquitous expression of *sema3a1* in *hsp70:^{gfp}sema3a1^{myc}* transgenic embryos inhibited migration of angioblasts ventral and lateral to the somites and retarded development of the dorsal aorta, resulting in severely reduced blood circulation. Furthermore, analysis of cells that express angioblast markers following induced expression of *sema3a1* or in a mutant that changes the expression of *sema3a1* in the somites confirmed these results. These data implicate Sema3a1, a guidance factor for neural growth cones, in the development of the vascular system.

Key words: Cell migration, Semaphorin, Zebrafish, Angioblast, Neuropilin

INTRODUCTION

Organogenesis requires specific signaling between cells during embryogenesis. These signals include extrinsic signals that regulate locomotive behaviors, such as cell migration and neural growth cone extension. One family of molecules believed to act as guidance cues for both cell migration and growth cone extension is the semaphorin family. The semaphorins are a large and diverse gene family that is conserved from invertebrates to humans. These proteins are secreted, GPI-linked or transmembrane; may have an Ig or thrombospondin type 1 domain; and all share a large, conserved sema domain (reviewed by Tessier-Lavigne and Goodman, 1996; Kolodkin, 1998; Raper, 2000). The first member of this family that was identified to be repulsive for growth cones was chick Collapsin 1 (now called Sema3a), a secreted protein that causes the collapse of specific growth cones (Luo et al., 1993).

The receptor for Sema3a consists of a complex of neuropilin and plexin molecules (Tamagnone et al., 1999; Takahashi et al., 1999). Neuropilins are a small family of conserved proteins whereas plexins are a larger family of conserved proteins. Interestingly, neuropilins are also part of the receptors for a spliced isoform of vascular endothelial growth factor (Vegf), $Vegf_{165}$. $Vegf_{165}$ binds to a complex of neuropilin and Vegfr2 (KDR/flk1) on endothelial and tumor cells and elicits mitogenic and chemotactic responses (Soker et al., 1998). The fact that both Vegf and semaphorins use neuropilins as part of their receptors suggests the possibility of dynamic interactions between the formation of blood vessels and development of the nervous system. Indeed, Semas can inhibit the action of Vegf in vitro and vice versa via competition for binding to neuropilin. Sema3a can inhibit $Vegf_{165}$-mediated aortic endothelial cell migration and capillary angiogenesis, and $Vegf_{165}$ can inhibit Sema3a-induced collapse of dorsal root ganglion growth cones and apoptosis of neural progenitor cells (Miao et al., 1999; Bagnard et al., 2001). However, it is unknown whether interactions between semaphorins and Vegf occur in vivo.

In zebrafish, vascular endothelial precursors (angioblasts) along with hematopoietic progenitors arise initially within the lateral mesoderm at gastrula stages (Gering et al., 1998; Detrich et al., 1995; Brown et al., 2000). Subsequently, these cells converge to the midline where they give rise to axial blood vessels and blood cells (Al-Adhami and Kunz, 1977; Zon 1995; Childs et al., 2002; Zhong et al., 2001). At these stages,

Vegf is expressed by the ventromedial region of each somite that the angioblasts migrate on (Liang et al., 1998; Liang et al., 2001) and is required for early vasculature formation (Nasevicius et al., 2000).

Zebrafish contain two copies of the *sema3a* gene, *sema3a1* and *sema3a2* (Shoji et al., 1998; Roos et al., 1999; Yee et al., 1999). The expression of *sema3a1* by the somites guides the growth cones of spinal motor and posterior lateral line neurons (Shoji et al., 1998; Yee et al., 1999; Halloran et al., 2000). *sema3a1* is normally expressed by the dorsal and ventral regions of the somites but not the horizontal myoseptal region found in-between these regions. The horizontal myoseptal region is adjacent to the notochord and the dorsal aorta, suggesting that Sema3a1 may act to restrict migrating angioblasts to the vicinity of the notochord. Furthermore, some mutations that affect the notochord or notochord-derived factors lead to both expression of *sema3a1* in the entire somite including the horizontal myoseptal region (Shoji et al., 1998) and selectively delete the dorsal aorta (Fouquet et al., 1997; Brown et al., 2000). These correlations suggest that Sema3a1 is involved in dorsal aorta formation in addition to growth cone guidance.

Here we demonstrate that Sema3a1 can regulate vascular development in vivo. A subset of mesodermal cells that are probably angioblasts express *neuropilin 1*, and these cells migrate dorsally toward the notochord. Antisense knockdown of Sema3a1 inhibits the formation of the dorsal aorta. Furthermore, induced ubiquitous expression of *sema3a1* interferes with dorsal migration by angioblasts and adversely affects dorsal aorta formation.

MATERIALS AND METHODS

Fish colony

Zebrafish (*D. rerio*) were maintained in a laboratory breeding colony at 28.5°C on a 14/10-hour light/dark cycle. Embryos collected from breeding fish were allowed to develop at 28.5°C and were staged as described by Kimmel et al. (Kimmel et al., 1995). Embryo age was defined as hours post-fertilization (hpf).

Transgenic fish

Zebrafish *sema3a1* cDNA (Yee et al., 1999) was tagged with Egfp (Clontech) and 6 Myc epitopes (Roth et al., 1991). Egfp was inserted between the 25th and 26th amino acids, between the putative signal peptide and the sema domain, and the Myc epitope was added at the C-terminus. Following transfection of HEK 293 cells with the tagged *sema3a1* expression construct, the media conditioned by the transfected cells was compared with untagged Sema3a1 for activity using the chick dorsal root ganglion growth cone collapse assay (Luo et al., 1993). Both untagged and tagged Sema3a1 were effective in inducing collapse of DRG growth cones and there was no difference in the potency of the two recombinant proteins. The 1.5 kb zebrafish *hsp70* promoter (Halloran et al., 2000) was then linked to the tagged *sema3a1* and injected into one blastomere of embryos at 1-4 cell stage. The injected embryos were raised to sexual maturity, pair-wise mated with wild-type fish, and their F1 progeny screened with PCR for the transgene using primers for the transgene (tcaagtccgccatgcccgaa/cgtccaygccgagagtgatc) to identify founder fish. F1 embryos were also examined for expression by GFP fluorescence and western blotting (see below) following heat induction. We established two independent lines in which *sema3a1* is expressed ubiquitously after heat treatment. Embryos were heat-induced by raising the water temperature from 28.5°C to 38°C over a period of 15 minutes using a programmable water bath (BU150P, Yamato) and then holding the temperature at 38°C for another 30 minutes. Full-length fusion protein was detected by western blotting as early as 15 minutes after heat treatment. In all experiments, heat treatment was started at 15 hpf.

In situ hybridization

Digoxygenin-labeled riboprobes for *sema3a1*, *sema3a2*, *neuropilin 1*, *fli1* and *gata1* were synthesized by in vitro transcription and hydrolyzed to an average length of 200-500 base pairs by limited alkaline hydrolysis (Cox et al., 1984). Hybridization on wholemounted embryos was performed according to the protocol of Schulte-Merker et al. (Schulte-Merker et al., 1992). For double in situ hybridization, *fli1* and *neuropilin 1* riboprobes were labeled with FITC and digoxygenin, respectively. First the red color was developed with AP-conjugated anti-FITC and FAST Red (Sigma), then the green color was developed with HRP-conjugated anti-digoxygenin and the TSA system (Perkin Elmer Life Sciences). Sections were made with cryostat (Cryocut 1800, Leica) or microslicer (DTK-3000W, Dosaka EM) after wholemount hybridization. For cryostat sectioning, embryos were equilibrated in 30% sucrose, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and cut into 20 μm. For microslicer sectioning, embryos were embedded in 30% albumin, 0.5% gelatin, 0.8% glutaraldehyde in PBS and cut into 40 μm.

Western blotting

Whole zebrafish protein (10 μg) was separated with SDS-PAGE gel electrophoresis. The protein was transferred onto PVDF membrane (Millipore) and incubated with 1/100 dilution of anti-Myc (9E10, Roche) in 5% skim milk/PBS followed by an HRP conjugated anti-mouse IgG and ECL immunostain kit (Amersham).

Cell tracing

Embryos were anesthetized in 0.01% tricaine (3-aminobenzonic acid ethylester, Sigma) and mounted in 1% agar on a microslide (Shoji et al., 1998). A 0.2% solution of diI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes) dissolved in dimethylformamide was passed iontophoretically from a micropipette onto approximately 10 mesodermal cells lateral and ventral to somites 10-12 at 18 hpf. Nomarski images and epifluorescence images were captured by 3CCD Video Camera System (DEI-750 Optronics), and combined using Adobe Photoshop. The position of the dorsal-most diI-labeled cells were measured from the dorsal boundary of the yolk tube at 18 hpf and 22 hpf following heat induction at 15 hpf.

Labeling of the vascular system

Anesthetized embryos were embedded and their vascular system labeled with ink as previously described (Isogai et al., 2001). The sinus venosus was incised for drainage, and then 0.75% Berlin Blue solution was pressure-injected into the dorsal aorta. After perfusion, the embryos were fixed in 4% paraformaldehyde. In *yot* mutants, dye was injected into the heart cavity because the narrower dorsal aorta was difficult to visualize.

Morpholino oligonucleotide injection

Morpholino oligonucleotides (MOs) were obtained from Gene Tools, LLC. The antisense *sema3a1* morpholino sequence (25 mer) was complimentary to a sequence of the 5′UTR (–59 to –34). The control morpholino sequence had 4 bases mismatched compared with the *sema3a1* antisense morpholino sequence. Sequences were as follows: *sema3a1* antisense MO, 5′-CTTGTAGCCCACAGTGCCCAGAG-CA-3′; *sema3a1* control MO, 5′-CTTCTAGCCGACAGAGC-CCAGTGCA3′. Morpholino oligonucleotides were solubilized in 1x Danieau Solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6

mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) and injected into 1 cell-stage embryos. For the knockdown experiments, 0.3 pmol of these MOs were injected into *hsp70:^{gfp}sema3a1^{myc}* embryos.

RESULTS

Generation of transgenic fish

In order to study the role of Sema3a1 for vascular development, we generated transgenic zebrafish by injecting recently fertilized embryos with plasmid DNA encoding the zebrafish *sema3a1* gene (Yee et al., 1999) driven by the zebrafish *hsp70* promoter (Shoji et al., 1998; Halloran et al., 2000). The *sema3a1* cDNA was tagged with *egfp* and 6 *myc* epitopes. Egfp was inserted between the putative signal peptide and conserved sema domain near the amino terminus, and the Myc epitopes were fused to the carboxyl terminus. This fusion protein was expressed by HEK293 cells and found to be secreted and to collapse chick dorsal root ganglion growth cones in vitro (not shown).

Recently fertilized embryos were injected with the *hsp70:^{gfp}sema3a1^{myc}* construct. The injected embryos were raised to maturity and founder fish were identified by pairwise crosses with wild-type zebrafish and PCR of DNA isolated from the F1 embryos using primers for *gfp*. Of 96 injected fish, 4 transmitted the transgene to F1 offspring. Of the F1 progeny from different founders, 2.8-24.8% expressed the transgene as assayed by GFP fluorescence and Myc immunohistochemistry, indicating that the germlines of the founders were mosaic. As expected, each transgenic F1 produced F2 offspring that were 50% transgenic when crossed with wild-type fish. We also generated lines of fish homozygous for the *sema3a1* transgene, by incrossing pairs of F1 or F2 hemizygous fish. Mature homozygous offspring were identified based on their ability to generate 100% transgenic embryos when crossed with a wild-type fish. Homozygous transgenic embryos but not wild-type embryos showed strong, widespread induction of the *sema3a1* transgene as assayed by GFP fluorescence following exposure to increased temperatures, whereas transgenic embryos were not induced to express the transgene without elevated temperatures (Fig. 1A). Western blots against Myc-tag revealed an approximately 150 kDa protein corresponding to the predicted size of the fusion protein. Expression peaked 1-7 hours following heat induction and began to decrease after 15 hours (Fig. 1B).

Expression of *sema3a1*, *sema3a2* and *neuropilin 1* correlate with putative angioblast migration

There are two copies of the *sema3a* gene in zebrafish, *sema3a1* and *sema3a2*, that are equally homologous with *sema3a* in other species (Roos et al., 1999; Yee et al., 1999). The expression patterns of Sema3a1 and Sema3a2 and their receptor, neuropilin 1, were analyzed by wholemount in situ hybridization during early vasculogenesis in the trunk. *sema3a2* is expressed transiently in the posterior half of each somite during early stages of somite maturation (Fig. 2A) (Roos et al., 1999). Shortly after the onset of *sema3a2* expression, *sema3a1* is expressed by the posterior somites (Fig. 2B). Interestingly, the putative angioblasts that give rise to the dorsal aorta are located posterior to the border of each somite (see below), i.e. adjacent to the region of the somites not expressing the semaphorins. Subsequently, *sema3a1* expression changes so that it is expressed by dorsal and ventral regions of each somite but not in the horizontal myoseptal region that is in-between the expressing regions and adjacent to the site where the dorsal aorta forms (Fig. 2C,G) (Shoji et al., 1998; Yee et al., 1999). The change in expression of *sema3a1* correlates with dorsal migration of vascular endothelial precursors (angioblasts) along the anterior region of each segment (see below) to the eventual site just ventral to the notochord. Furthermore, it suggests that the semaphorins act to restrict the route taken by the angioblasts.

If the hypothesis that Sema3a1 and Sema3a2 regulate migration of angioblasts is correct, then angioblasts should express neuropilin 1, the putative receptor for Sema3a1 and Sema3a2. In fact, *neuropilin 1* is expressed by cells that are probably angioblasts (Lee et al., 2002). We confirmed that *neuropilin 1* is expressed transiently from 18 to 22 hpf by cells ventral to the somites and by cells that appear to be extending dorsally along the anterior border of each somite (Fig. 2D,E). The apparent dorsal migration by *neuropilin 1*-positive cells is most apparent in transverse sections (Fig. 2I,J,L). At any one stage of development the *neuropilin 1*-positive cells are generally found in a more dorsal position in anterior segments compared with those in posterior segments (Fig. 2E). Because the somites form sequentially from anterior to posterior, this suggests that *neuropilin 1*-positive cells are migrating dorsally. The pattern of *neuropilin 1*-positive cells is similar to that of migrating angioblasts (Fouquet et al., 1997; Brown et al., 2000), and *neuropilin 1*-positive cells co-express the endothelial marker *fli1* (Fig. 2L-N). These observations suggest that angioblasts express *neuropilin 1*.

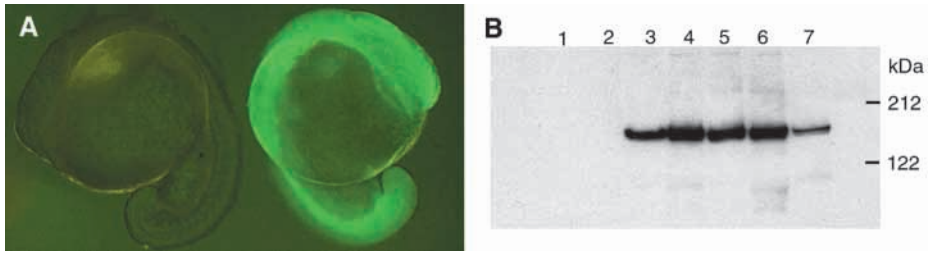

Fig. 1. The *hsp70:^{gfp}sema3a1^{myc}* transgene is heat inducible in transgenic embryos. (A) On the right, transgenic embryos (18 hpf) after heat induction at 15 hpf exhibit ubiquitous GFP fluorescence, indicating that the transgene was induced. On the left, transgenic embryos not heat induced exhibit no GFP fluorescence. (B) After heat induction of transgenic embryos, the ^{GFP}Sema3a1^{myc} fusion protein was detected by western blotting using an anti-Myc antibody. No band corresponding to the fusion protein is detectable in wild-type embryos (lane 1), nor non-heat induced transgenic embryos (lane 2). An approximately 150 kDa band corresponding to the fusion protein is labeled immediately after the heat treatment (lane 3), 1 hour (lane 4), 3 hours (lane 5), 7 hours (lane 6) and 15 hours (lane 7) after heat treatment of transgenic embryos. In lanes 3-7, heat treatment consisted of raising the water temperature from 28.5°C to 38°C over a period of 15 minutes, then holding at 38°C for 30 minutes and then decreasing to 28.5°C over the next 15 minutes in a programmable water bath.

Interestingly, by 21.5-22.5 hpf the dorsal-most *neuropilin 1*-positive cells have reached a site just ventral to the notochord in segments 10-15, whereas CaP axons labeled with monoclonal antibody Znp-1 are still located at the muscle pioneers (not shown), a site that is dorsal to the notochord. This suggests that the *neuropilin 1*-positive cells are not following the CaP axons from the ventrolateral somite to the notochord. Furthermore, following ubiquitous induction of *sema3a1* in *hsp70:*^{gfp}*sema3a1*^{myc} transgenic embryos at 15 hpf, there were many fewer *neuropilin 1*-positive cells along the somites between the notochord and the yolk tube (Fig. 2F,K), suggesting angioblast migration was retarded.

Putative angioblasts failed to migrate dorsally following induced ubiquitous expression of *sema3a1*

The distribution of *neuropilin 1*-positive cells along the anterior-posterior axis suggested that the cells expressing *neuropilin 1* migrate dorsally. To test this hypothesis, putative *neuropilin 1*-positive cells were labeled with the fluorescent dye, diI, to see if they migrate. DiI was injected into cells lateral and ventral to the somites. When diI was injected in 18 hpf wild-type embryos (n=5), some of the labeled cells were found in a more dorsal position several hours later, whereas others had not changed their positions suggesting that a subset of cells from ventrolateral mesoderm migrated dorsally (Table 1; Fig. 3A-C).

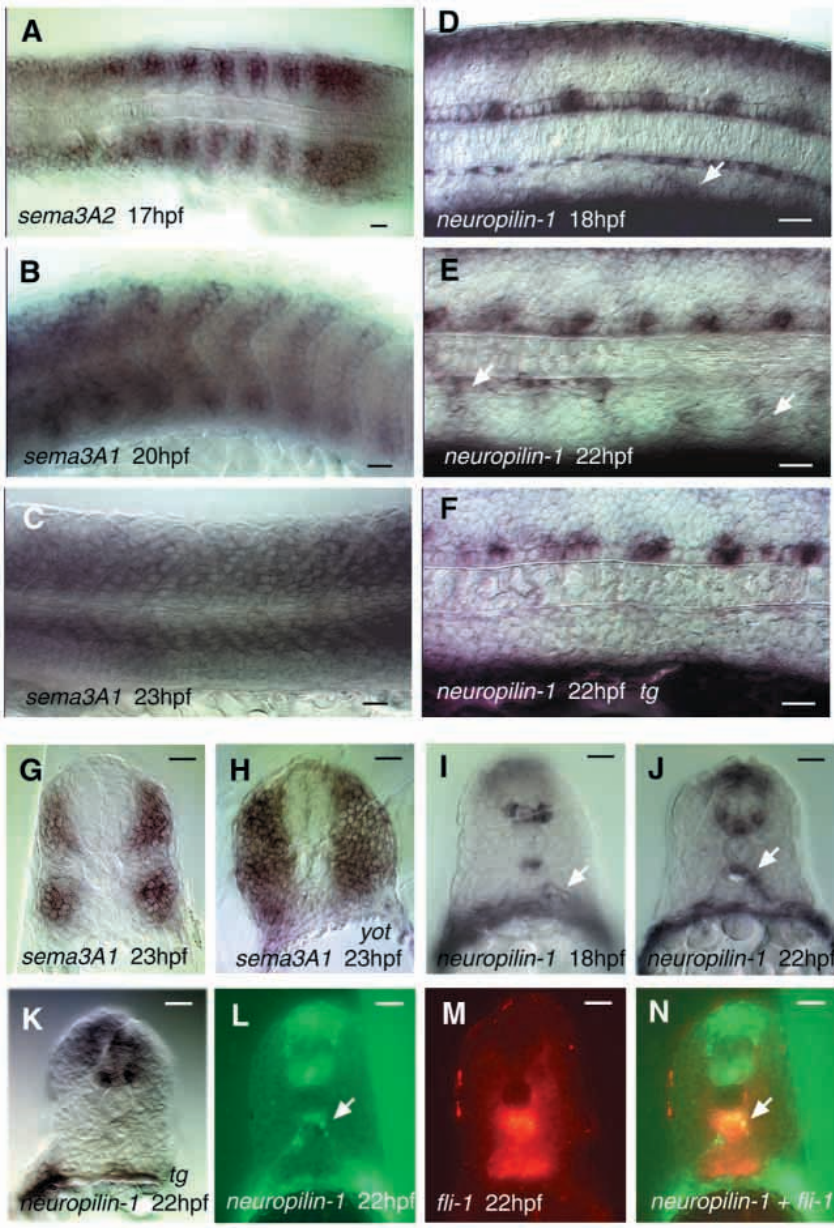

Fig. 2. The expression patterns of *sema3a1*/*sema3a2* and *neuropilin 1* in the trunk region correlate with the migration pathway of angioblasts. Expression of *sema3a1* (B,C,G,H), *sema3a2* (A), their receptor subunit *neuropilin 1* (D-F,I-K), and co-expression of *neuropilin 1* and *fli1* (L-N) were assayed by in situ hybridization. Unless otherwise noted, embryos are oriented with rostral towards the left and dorsal upwards. (A) At 17 hpf, *sema3a2* is expressed by the posterior part of newly segmented somites as seen in a dorsal view. (B) *sema3a1* is also initially expressed by the posterior regions of each somite, as seen in a lateral view of a 20 hpf embryo. (C) Subsequently, *sema3a1* expression changes so that it is expressed by the dorsal and ventral region of each somite, but not the horizontal myoseptal region as seen at 23 hpf. (D) At 18 hpf *neuropilin 1* is expressed by gut endoderm ventral to the somites and by a strip of cells that appear to be extending dorsally (arrow). These strips of cells were adjacent to the anterior regions of the somites as judged by comparison with the borders of the somites that are out of the plane of focus. *neuropilin 1* is also expressed by subsets of cells within the spinal cord dorsal to the notochord. (E) By 22 hpf many more strips of *neuropilin 1*-expressing cells were evident, with the anterior strips generally extending more dorsally compared with more posterior strips (arrows). (F) The *neuropilin 1*-expressing strips are missing in 22 hpf *hsp70:*^{gfp}*sema3a1*^{myc} transgenic embryos following ubiquitous induction of *sema3a1* at 15 hpf, whereas expression in the ventral spinal cord is unperturbed. (G) Transverse section of the trunk of a 23 hpf wild-type embryo showing expression of *sema3a1* in the dorsal and ventral but not horizontal myoseptal region of the somites. (H) In the U-type mutant, *you-too* (*yot*), *sema3a1* is expressed throughout the entire somite as seen in a transverse section. (I) Transverse section of the trunk of an 18 hpf wild-type embryo showing expression of *neuropilin 1* in the strip of cells that appears to be extending along the medial surface of the somites (arrow). *neuropilin 1* is also expressed by hypochord cells just ventral to the notochord. (J) At 22 hpf, cells expressing *neuropilin 1* (arrow) are seen extending to the dorsal aortic region beneath the hypochord. (K) Transverse section of a 22 hpf *hsp70:*^{gfp}*sema3a1*^{myc} transgenic embryo heat induced at 15 hpf showing that the *neuropilin 1*-expressing cells normally found between the notochord and the yolk tube are missing. (L-N) Transverse sections of a 22 hpf wild-type embryo double-labeled for *neuropilin 1* (green, L) and the endothelial marker *fli1* (red, M) showing that many of the *neuropilin 1*-positive cells (arrow) located between the notochord and yolk tube express *fli1* (red and green merged, N). Scale bars: 20 μm.

Table 1. Dorsal migration by ventrolateral mesoderm cells is inhibited by induced ubiquitous expression of *sema3A1*

Experiment number	Wild type	*hsp70:gfpsema3A1myc*
1	26.5	8.0
2	26.0	2.0
3	25.0	1.5
4	21.0	1.0
5	17.5	–0.5
Mean	23.2±3.9	2.4±3.3

Cells ventrolateral to the somites were labeled with diI at 18 hpf in wild-type embryos and *hsp70:gfpsema3A1myc* embryos that had been heat induced at 15 hpf. The position of the labeled cells were examined at 18 and 22 hpf (see Materials and Methods). Shown are the distances (μm) between the dorsalmost labeled cell at 18 hpf and the dorsalmost labeled cell in the same embryo at 22 hpf.

Following ubiquitous induction of *sema3a1*, many fewer *neuropilin 1*-positive cells appeared to migrate towards the notochord (Fig. 2F,K). To verify this, putative angioblasts were labeled with diI and examined following heat-induction of *sema3a1*. Following heat-induction of *sema3a1* in *hsp70:gfpsema3a1myc* embryos at 15 hpf, none of the labeled cells migrated dorsally to any significant amount (*n*=5; Table 1; Fig. 3D-F). The dorsal-most labeled cells were 2.4 μm more dorsal at 22 hpf compared with at 18 hpf in transgenics that were heat induced at 15 hpf. In comparison, the dorsal-most labeled cells were 23.2 μm more dorsal at 22 hpf compared with 18 hpf in wild-type embryos. Furthermore, heat treatment of wild-type embryos did not affect dorsal migration of labeled cells (*n*=3; not shown). These results demonstrate that some but not all cells from the ventro-lateral mesoderm migrate dorsally, and that Sema3a1 can regulate this migration in vivo.

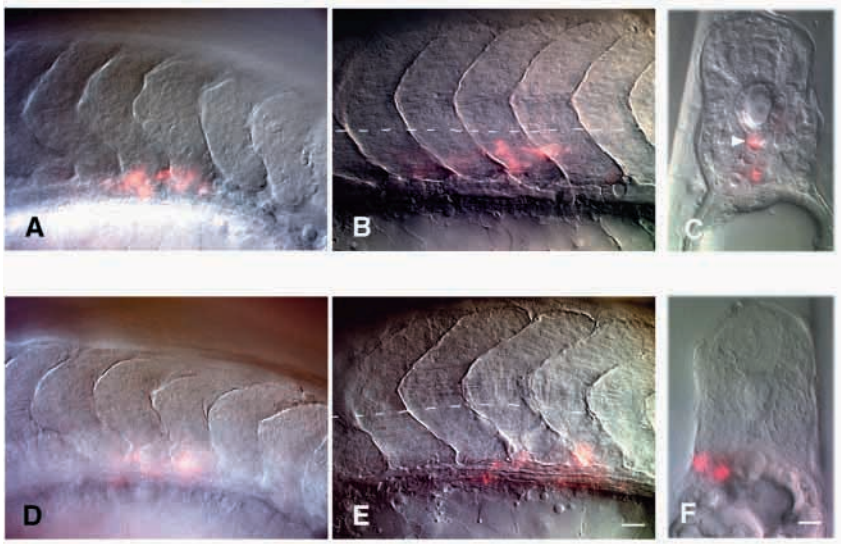

Fig. 3. Dorsal migration by putative angioblasts is interrupted by overexpression of *sema3a1*. In all panels, Nomarski and fluorescence images are overlaid. (A) DiI-labeled cells ventrolateral to the somites immediately following diI injection in an 18 hpf wild-type embryo. (B) Some of the diI-labeled cells have migrated dorsally by 22 hpf in the same embryo shown in A. Broken line designates the ventral boundary of the notochord. (C) Transverse section at 24 hpf of another embryo showing that some of the diI-labeled cells (arrowhead) labeled at 18 hpf had migrated to a position just ventral to the notochord. (D) DiI-labeled cells ventrolateral to the somites immediately following diI injection in an 18 hpf *hsp70:gfpsema3a1myc* transgenic embryo following heat induction at 15 hpf. (E) The diI-labeled cells failed to migrate dorsally and remained in ventrolateral positions at 22 hpf in the same embryo shown in D. Broken line designates the ventral boundary of the notochord. (F) Transverse section of a *hsp70:gfpsema3a1myc* transgenic embryo at 24 hpf showing that diI-labeled cells have not migrated dorsally and remain very ventral. Scale bars: 20 μm.

Sema3a1 regulates migration by vascular angioblasts but not hematopoietic progenitor cells

Development of endothelial and hematopoietic lineages are closely related in early embryogenesis (Stainer et al., 1996; Thompson et al., 1998). They express common early markers, emerge simultaneously, and mutations in mice and zebrafish can cause the loss of both lineages (Shalaby et al., 1995; Stainer et al., 1996). Furthermore, in zebrafish both lineages migrate in a similar manner from the lateral mesoderm (Detrich et al., 1995; Gering et al., 1998; Zhong et al., 2001). In order to see which population is regulated by Sema3a1, we examined *fli1*, which is expressed by differentiating endothelial cells as well as by immature endothelial and hematopoietic progenitors (Brown et al., 2000), and *gata1*, which is selectively expressed by hematopoietic progenitors following misexpression of Sema3a1 (Detrich et al., 1995).

Fli1 is an ETS domain transcription factor and is expressed by early vascular and hematopoietic precursor cells (Brown et al., 2000). At 20 hpf, *fli1* is expressed diffusely by many cells found between the notochord and the yolk tube (*n*=4; Fig. 4A), but at 25 hpf expression is clearly seen in presumptive dorsal aorta cells just ventral to the notochord (*n*=5; Fig. 4B) (Brown et al., 2000). In the notochord mutant *you-too* (*yot*) that fails to develop the dorsal aorta (Fouquet et al., 1997; Chen et al., 1996), and that expresses *sema3a1* in the entire somite (Fig. 2H) (Shoji et al., 1998), *fli1* expression is restricted abnormally in patches rather than in cells just ventral to the notochord at 25 hpf (*n*=5; Fig. 4C). Thus aberrant expression of *sema3a1* throughout the entire somite correlates with the abnormal distribution of *fli1* cells. Finally, following induction of *sema3a1* in *hsp70:gfpsema3a1myc* embryos at 15 hpf, the distribution of *fli1*-positive cells was abnormal with cells diffusely distributed between the notochord and yolk tube at 25 hpf (*n*=5; Fig. 4D), suggesting that ubiquitous misexpression of Sema3a1 partially inhibits dorsal migration of angioblasts. This result is consistent with our finding that misexpression of Sema3a1 interferes with dorsal migration by ventro-lateral mesodermal cells, and suggests that these cells are *fli1*-positive angioblasts that give rise to the dorsal aorta.

GATA1 is a transcription factor that regulates hematopoietic development (Pevny et al., 1991; Orkin, 1992), and is restricted to blood progenitors (Detrich et al., 1995). In contrast to *fli1*, the expression pattern of *gata1* was not perturbed following misexpression of *sema3a1* in transgenic embryos (*n*=5), nor in *yot* mutants (*n*=4; Fig. 4F-H). Thus, Sema3a1 appears to

regulate migration by *fli1*-positive angioblasts but not migration by *gata1*-positive hematopoietic progenitor cells.

Ubiquitous induction of *sema3a1* reduces blood circulation and interferes with the development of the dorsal aorta

The previous sections showed that induced ubiquitous expression of *sema3a1* interfered with dorsal migration of putative angioblasts from the ventrolateral mesoderm. This finding predicts that overexpression of *sema3a1* should lead to defects in the dorsal aorta and thus blood circulation. Indeed, blood circulation was abnormal at 30 hpf in transgenic embryos (n=20) following induction of *sema3a1* at 15 hpf (Fig. 5). Initially, the heart was seen to be beating in many transgenic embryos, but by 27 hpf the blood cells were restricted to the heart and yolk sac and tube and did not circulate into other parts of the embryo. Subsequently the heart cavity became swollen and eventually stopped beating (29-30 hpf). The dorsal aorta was present in *hsp70:*^{gfp}*sema3a1*^{myc} embryos (30 hpf) following induced ubiquitous expression, but the lumen of the dorsal aorta contained no blood cells and was constricted when visualized by dye injection (Fig. 5D,E). The axial vein and other cranial vessels at this stage appear relatively unaffected. Thus, it appears that constriction of the dorsal aorta is responsible for the lack of circulation in these embryos. Corroborating these findings, in *yot* mutants high pressures that actually ruptured the axial vein were required to fill the dorsal aorta, and the lumen of the dorsal aorta was constricted at various points (Fig. 5F). These results suggest that Sema3a1 in the somites acts to regulate angioblast migration and, thereby, the formation of the dorsal aorta.

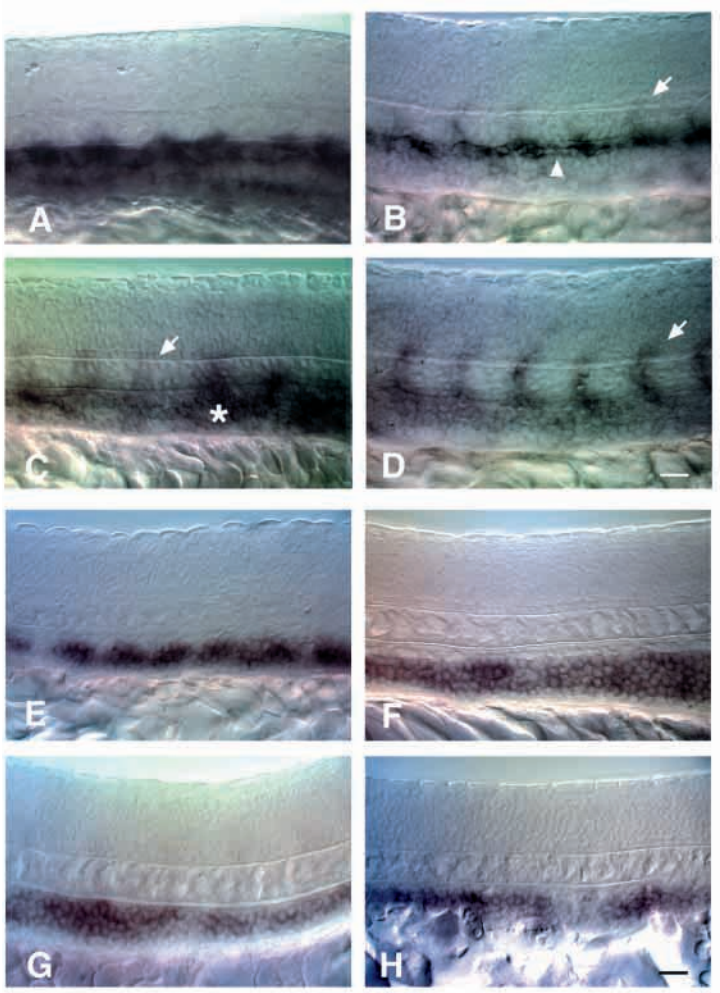

Fig. 4. Induced ubiquitous expression of *sema3a1* adversely affects the distribution of cells expressing the angioblast marker *fli1*, but not the hematopoietic marker, *gata1*. All panels show the trunk of whole-mount embryos following in situ hybridization for *fli1* (A-D) or *gata1* (E-H) in wild-type embryos (A,B,E,F), *yot* embryos that express *sema3a1* through the entire somites (C,G), and *hsp70:*^{gfp}*sema3a1*^{myc} transgenic embryos (D,H) following heat induction at 15 hpf. In wild-type embryos, *fli1* is expressed diffusely between the notochord and yolk tube at 20 hpf (A), but by 25 hpf *fli1* is expressed most strongly by presumptive dorsal aorta cells immediately ventral to the notochord (B, arrowhead). At 25 hpf the pattern of *fli1* is disrupted with aberrant patches of cells (C, asterisk) between the notochord and yolk tube in *yot* embryos, and with diffused pattern in transgenic embryos following heat induction (D). *fli1* expression can also be seen in the intersegmental arteries (arrows in B-D). *gata1* is expressed by many cells between the notochord and yolk tube in wild-type embryos at 20 (E) and 25 hpf (F). *gata1* expression is normal in *yot* embryos (G) and in *hsp70:*^{gfp}*sema3a1*^{myc} transgenic embryos at 25 hpf following heat induction at 15 hpf (H). Scale bar: 20 μm.

Antisense knockdown of Sema3a1 results in severe defects in dorsal aorta development

To see whether Sema3a1 is required for normal dorsal aorta formation, we injected antisense MOs (Nasevicius et al., 2000) against *sema3a1* into recently fertilized embryos. The efficacy of the *sema3a1* antisense MO to effectively knockdown translation of Sema3a1 was determined by injecting the antisense or control MO into recently fertilized *hsp70:*^{gfp}*sema3a1*^{myc} embryos, heat inducing them at 18 hpf, and assaying for induction of ^{GFP}Sema3a1^{Myc} by fluorescence at 25 hpf. GFP fluorescence was significantly reduced in the *sema3a1* antisense MO-injected transgenic embryos (n=22), whereas *sema3a1* control MO-injected transgenic embryos (n=19) showed GFP fluorescence comparable to uninjected transgenic embryos (Fig. 6A).

Knocking down Sema3a1 appeared to interfere with normal migration of angioblasts and disrupted formation of the dorsal aorta and normal circulation (Fig. 6B-E). In control MO-injected wild-type embryos, *neuropilin 1*-positive cells were seen to be extending dorsally along the anterior border of the somites from the yolk tube to the notochord at 22 hpf as in uninjected wild-type embryos (see Fig. 2E) and circulation was normal at 27-28 hfp (not shown). In contrast, in antisense MO-injected wild-type embryos there were fewer *neuropilin 1*-positive cells in the region between the yolk tube and the notochord (n=20; Fig. 6B, compare with Fig. 2E; Fig. 6C, compare with Fig. 2J), suggesting that their migration was inhibited. Furthermore, high pressures were required to inject Berlin blue ink into the vasculature at 30 hpf. In these embryos, the cardinal vein but not the dorsal aorta (Fig. 6D) or the cardinal vein and a constricted dorsal aorta (Fig. 6E) were labeled. Furthermore, the heart was swollen and no circulation was observable (not shown). These results indicate that Sema3a1 expressed by the somites is necessary for normal development of the dorsal aorta.

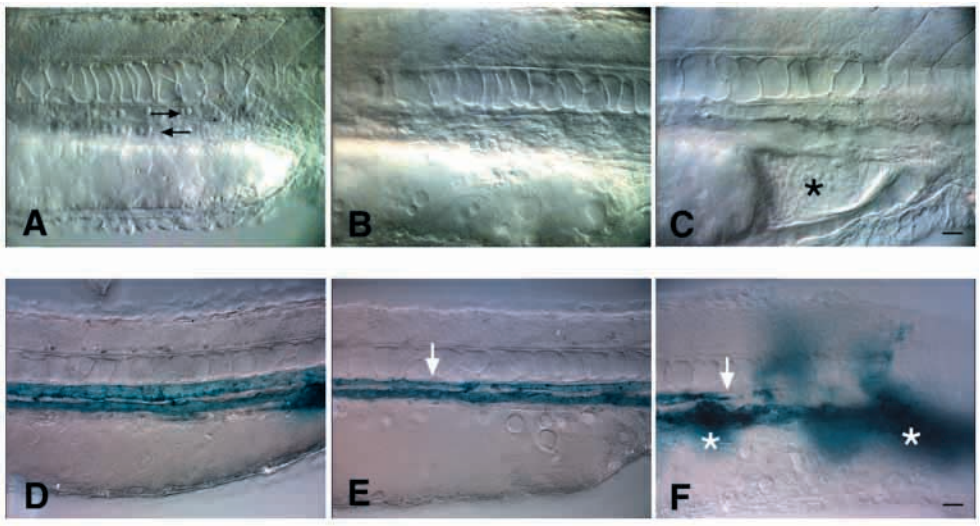

Fig. 5. Induced ubiquitous expression of *sema3a1* induces constriction of the dorsal aorta and blocks flow of blood cells in the trunk vascular system. In all panels, embryos are 30 hpf. Blood cells flow through the dorsal aorta (right-pointing arrow) and axial vein (left-pointing arrow) in wild-type embryos (A) but not in *hsp70:*^{gfp}*sema3a1*^{myc} embryos following heat induction at 15 hpf (B,C). Asterisk in C denotes abnormal cavity filled with putative blood cells. When labeled with Berlin Blue ink, the dorsal aorta can be seen to be constricted in *hsp70:*^{gfp}*sema3a1*^{myc} embryos following heat induction at 15 hpf (arrow in E) compared with wild-type embryos (D). In *yot* embryos, the lumen of the dorsal aorta was tapered (arrow in F). High pressures that caused the cardinal vein to rupture (asterisks in F) were required to fill the dorsal aorta with ink. Scale bar: 20 μm.

DISCUSSION

Sema3a1 regulates dorsal migration of ventrolateral mesoderm cells

Based upon previous work, we hypothesized that Sema3a regulates migration by angioblasts that give rise to the dorsal aorta. To test this hypothesis, we first showed that cells that are probably migrating angioblasts express neuropilin 1, a receptor for Sema3a. Second, the *neuropilin 1*-positive cells appear to migrate to regions that do not express Sema3a1 nor Sema3a2, suggesting that they avoid the Sema3a-expressing regions. Third, both misexpression and knockdown of Sema3a1 interfered with migration by putative angioblasts, and adversely affected development of the dorsal aorta and blood circulation. Both ubiquitous misexpression and knockdown of Sema3a1 would be expected to disrupt the normal dorsal/ventral gradient of Sema3a1, with overexpression masking the gradient and knockdown eliminating or dramatically decreasing the gradient. These results suggest that a gradient of Sema3a1 expressed by the somites does regulate migration by angioblasts that give rise to the dorsal aorta.

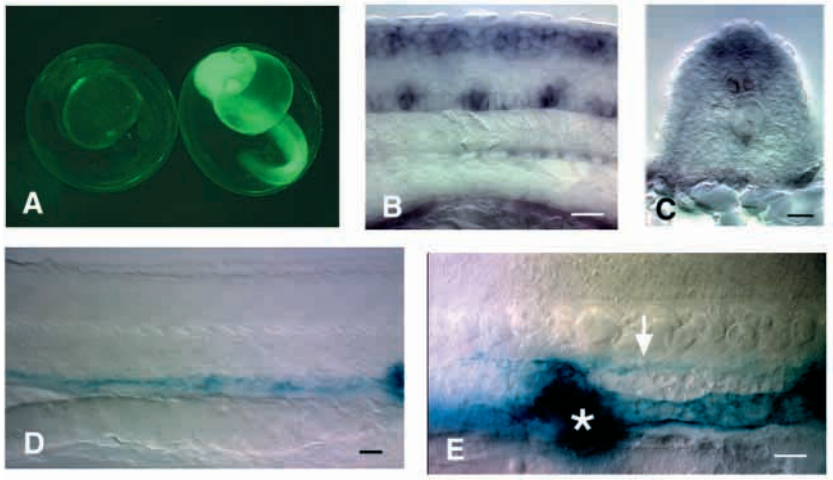

Fig. 6. Knockdown of Sema3a1 by antisense MO disrupts the migration of putative *neuropilin* 1-positive angioblasts and the formation of the dorsal aorta. (A) Heat induction of ^{GFP}Sema3a1^{myc} was significantly reduced in a *hsp70:*^{gfp}*sema3a1*^{myc} embryo at 25 hpf by prior injection of antisense *sema3a1* antisense MO (left), but not by injection of *sema3a1* control MO (right). (B,C) There are fewer *neuropilin 1*-expressing cells than normal between the notochord and yolk tube in a 22 hpf antisense *sema3a1* MO-injected wild-type embryo seen in a lateral view (B, compare with Fig. 2E) and a transverse section (C, compare with Fig. 2J). Some *neuropilin 1*-positive cells can be seen just ventral to the notochord but the strips of cells extending dorsally from the yolk tube are missing. (D) Normal pressures that would label the dorsal aorta and the cardinal vein in the trunk of wild-type embryos with Berlin Blue ink only label the cardinal vein in 30 hpf embryos injected with *sema3a1* antisense MO. (E) Higher pressures that lead to rupture of the cardinal vein (asterisk) sometimes labeled the dorsal aorta, but the aorta was severely constricted (arrow) in 30 hpf embryos injected with *sema3a1* antisense MO. Scale bar: 20 μm.

Recently, MO knockdown of neuropilin 1 was shown to lead to defective circulation in intersegmental vessels but not axial vessels, and MO knockdown of Vegf to defective circulation in both intersegmental and axial vessels (Lee et al., 2002). Because neuropilin 1 serves as a co-receptor for both Sema3a and Vegf, one would have expected a knockdown of neuropilin 1 to interfere with dorsal aorta formation. It is possible that defects of the dorsal aorta require higher levels of neuropilin 1 knockdown compared to defects of the intersegmental vessels. In fact, neuropilin 1 does appear to contribute to formation of the dorsal aorta because injection of neuropilin 1 antisense MO with a non-effective level of Vegf antisense MO did interfere with both intersegmental and axial circulation (Lee et al., 2002).

Mechanism of Sema3a1 action during dorsal aorta formation

How does Sema3a1 regulate angioblast

migration? One possibility is that migration by angioblasts is directed by CaP motor axons whose pathway partially coincides with that of the angioblasts. Consistent with this idea is that CaP growth cones are repulsed by Sema3a1 (Halloran et al., 2000). Thus, it is possible that the deleterious effects on angioblast migration following manipulation of Sema3a1 are secondary effects mediated via erroneous outgrowth by the CaP motor axons. However, our evidence suggests that angioblasts do not appear to follow the CaP axons. Angioblasts start ventrolateral to the somites and first migrate medially and then dorsally to the notochord. The CaP motor axon starts out in the spinal cord and extends ventrally then laterally to the ventrolateral edge of the somite (Myers et al., 1986). In midtrunk segments angioblasts are arriving at their destination just ventral to the notochord whereas the CaP motor axons are still at the muscle pioneers, a site that is dorsal to the destination of the angioblasts. Thus, many angioblasts have completed their migration prior to extension of the CaP motor axon beyond the notochord, suggesting that angioblasts need not follow CaP axons to complete their migration.

A second possibility is that Sema3a1 may directly repulse angioblasts. First, class 3 semaphorins are secreted molecules that repulse specific growth cones (Raper, 2000). Second, class 3 semaphorins regulate migration by neural crest cells (Eickholt et al., 1999) and neurons (Marin et al., 2001). Third, Dev cells, a human medulloblastoma cell line, avoid migrating on a Sema3a substrate in vitro (Bagnard et al., 2001).

A third possibility is that because class 3 semaphorins and Vegf both use neuropilins as a component of their functional receptor (Takahashi et al., 1999; Soker et al., 1998), Sema3a1 may regulate migration of angioblasts by interfering with the chemoattractant activity of Vegf for these cells. Because semaphorins can inhibit the action of Vegf in vitro and vice versa via competition for binding to neuropilin (Miao et al., 1999; Bagnard et al., 2001), the effects on migration of putative vascular endothelial cells following misexpression of Sema3a1 could be accounted for by this mechanism. In fact, migration of angioblasts that form the dorsal aorta in *Xenopus* is guided by Vegf expressed by the hypochord cells ventral to the notochord (Cleaver and Krieg, 1998). In zebrafish, Vegf is expressed by the ventromedial region of each somite (Liang et al., 1998), neuropilin 1 and Flk1/Vegfr2 is expressed by putative angioblasts (this study) (Fouquet et al., 1997), and knocking down Vegf leads to defective vascular development including that of the dorsal aorta (Nasevicius et al., 2000). Interestingly, the expression domains of Vegf and Sema3a1 within the somites appear to partially overlap (Fig. 7). Thus it is possible that Sema3a1 secreted by the ventral third of the somite may create a functional gradient of secreted Vegf activity such that the level of Vegf is high dorsal and low ventral in the ventral half of the somite. Such a functional Vegf gradient would be consistent with the dorsal migration of the angioblasts. Furthermore, it is possible that competitive interactions between Vegf and Sema3a1 may also generate a functional gradient of Sema3a1 within the ventral third of the somites. Here the repulsive activity of Sema3a1 would be high ventral and low dorsal in the ventral half of the somites. Thus, there exists the intriguing possibility that complementary gradients of attractive and repulsive molecules may regulate dorsal migration by angioblasts.

The manipulations of Sema3a1 expression are consistent with the possibility that Sema3a1 may repulse angioblasts and/or regulate attraction by Vegf. In the case of repulsion, overexpression of Sema3a1 would be expected to mask directed repulsion by a gradient of endogenous Sema3a1 and thereby interfere with angioblast migration. Similarly, knocking down Sema3a1 would significantly decrease or eliminate the Sema3a1 gradient and disrupt directed angioblast migration. In fact, a gradient of Sema3a is hypothesized to direct cortical axons via a repulsive activity and both ubiquitous application of exogenous Sema3a and elimination of Sema3a lead to similar and aberrant outgrowth by cortical axons (Polleux et al., 1998). This demonstrated that cortical axons were not inhibited from extending despite encountering a uniform distribution of a repulsive molecule. Similarly, temporal retinal axons will avoid posterior tectal membranes that they find repulsive, but nevertheless are able to extend on posterior tectal membranes in the absence of a better substrate (Walter et al., 1990). Disruption of angioblast migration would also be expected if Sema3a1 acted as a competitive inhibitor for Vegf. Ubiquitous misexpression of Sema3a1 would inhibit Vegf action by binding to neuropilin 1 and thus interfere with attraction by dorsal sources of Vegf. Knockdown of Sema3a1 might lead to a shallower functional gradient of Vegf and thus impede angioblast migration.

Whether migration of angioblasts is regulated by complementary gradients of Vegf and Sema3a1 is an open question. What is clear is that both Vegf and Sema3a1 can regulate the formation of the dorsal aorta. That they do so in

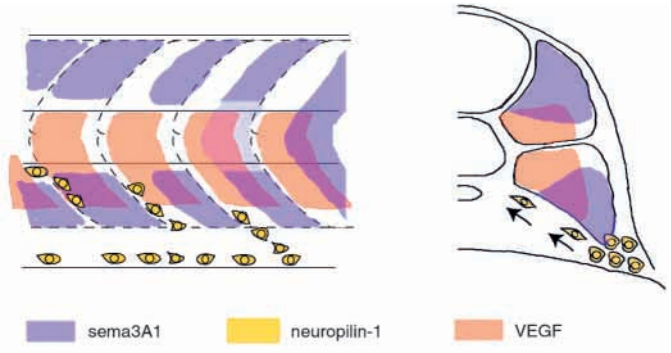

Fig. 7. The regulation of angioblast migration in the trunk by *sema3a1*, *vegf* and *neuropilin 1*. (Left) A side view of the trunk showing the expression of *sema3a1*, *vegf* and *neuropilin 1* with the posterior-most somite showing the earliest pattern of somitic *sema3a1* expression, and each successive anterior somite showing a slightly more mature pattern. (Right) Transverse section showing the expression of *sema3a1*, *vegf* and *neuropilin 1* in a segment corresponding to the middle segments shown in the side view. Initially expression of *vegf* by medial somitic cells attracts *neuropilin 1*-positive angioblasts to migrate dorsally (arrows), whereas expression of *sema3a1* in the posterior somitic cells channels these angioblasts to the anterior regions of each somite. As the angioblasts migrate dorsally to their position just ventral to the notochord, *sema3a1* is changing so that it is no longer expressed by the horizontal myoseptal cells at the level of the notochord but is expressed by the ventral third of the somite. The new pattern of Sema3a1 is proposed to restrict the angioblasts to their final destination. Overlapping expression of Vegf and Sema3a1 may generate functional gradients of attraction by Vegf and repulsion by Sema3a1

concert in vivo is suggested by the correlation of dorsal aorta defects and concomitant changes in expression of Vegf and Sema3a1 in the zebrafish *floating head* (*flh*) mutant. Vegf expression is missing in somites and Sema3a1 is expressed throughout the entire somite in *flh* embryos (Shoji et al., 1998; Liang et al., 2001). The defects of the dorsal aorta are more severe in *flh* (Brown et al., 2000; Liang et al., 2001) compared to that seen following antisense knockdown of Vegf (Nascevicius et al., 2000), knockdown of Sema3a1 or misexpression of Sema3a1 throughout the entire somite (this study). Furthermore, the fact that the notochord is missing in *flh* suggests that notochord signaling is critical for guiding angioblasts that form the dorsal aorta (Fouquet et al., 1997; Weinstein et al., 1999). Sonic hedgehog from the notochord appears to play a major role in this concerted process, because it downregulates Sema3a1 (Shoji et al., 1998) and upregulates Vegf (Lawson et al., 2002) on the somites. A similar dorsal aorta phenotype is found in other midline mutants, which appear to lack hedgehog signaling (Chen et al., 1996; Brown et al., 2000) (W.S., unpublished).

Sema3a regulates vascular formation in other vertebrates

Our evidence demonstrates that in zebrafish Sema3a1 can affect migration by putative angioblasts and the formation of the dorsal aorta. Intriguingly, Sema3a is expressed in early somites of mammals in a pattern similar to that seen in zebrafish (Giger et al., 1996; Taniguchi et al., 1997). Endothelial precursors in avian embryos migrate from the somitic mesoderm and splanchnopleural mesoderm to form the dorsal aorta (Pardanaud et al., 1996). Furthermore, Sema3a knockout mice and neuropilin 1 knockout mice exhibit cardiovascular defects (Behar et al., 1996; Kawasaki et al., 1999). This suggests that Sema3a may also regulate migration of angioblasts in other vertebrates as well.

Other class 3 semaphorins also regulate cardiovascular development. *sema3C* is expressed along the path followed by migrating cardiac neural crest cells to the truncus arteriosus and the aortic arch (Feiner et al., 2001). In Sema3C knockout mice these vascular elements are defective suggesting that Sema3C is an attractive cue for neural crest migration (Feiner et al., 2001). Thus, Sema3a and Sema3C appear to regulate different processes in cardiovascular development.

Ephrins and their Eph receptors are also involved in patterning of the vascular system. Ephrin B2 (Efnb2) is expressed by arterial but not venous endothelial cells, whereas Ephb4 the proposed Efnb2 receptor is expressed at higher levels by venous endothelial cells (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999). Furthermore, mice lacking Efnb2 or Efnb4 exhibit aberrant vascular patterning (Wang et al., 1998; Gerety et al., 1999). Because ephrins and their receptors are well-known regulators of growth cone guidance and cell migration, it is possible that they may also regulate migration by angioblasts during vascular development. In the zebrafish *efnb2* is expressed by somites (Durbin et al., 1998) in a pattern that is similar to that of *sema3a1*. Early the posterior half of each somite expresses *efnb2*, but later expression changes so that the dorsal and ventrolateral somite expresses *efnb2*. Thus the expression pattern of *efnb2* is appropriate for the guidance of angioblast migration as well. *efnb2* is also expressed by dorsal arterial angioblasts in zebrafish, but only after migration (Lawson et al., 2001). Similarly, Ephb4 is expressed by the venous angioblasts in the trunk after cell migration but is only diffusely expressed prior to vessel formation (Zhong et al., 2001). This later expression suggests that Efnb2/Ephb4 may be involved in the patterning and separation of dorsal arterial and posterior cardinal venous angioblasts in their final positions ventral to the notochord.

In conclusion, our experiments demonstrate that Sema3a1, a factor that guides growth cone extension, can also regulate migration of putative angioblasts. Interestingly, Vegf, which is an important regulator of migration by angioblasts, also affects growth cones (W.S., unpublished). Thus, factors that regulate migration/guidance in the nervous system also regulate migration in the vascular system and vice versa.

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