The evolutionary origins of chordate hematopoiesis and vertebrate endothelia.

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ABSTRACT

The vertebrate circulatory system is the most complex vascular system among those of metazoans, with key innovations including a multi-chambered heart and highly specialized blood cells. Invertebrate vessels, on the other hand, consist of hemal spaces between the basal laminae of epithelia. How the evolutionary transition from an invertebrate-type system to the complex vertebrate one occurred is, however, poorly understood. We investigate here the development of the cardiovascular system of the cephalochordate amphioxus *Branchiostoma lanceolatum* in order to gain insight into the origin of the vertebrate cardiovascular system. The cardiac markers *Hand*, *Csx* (*Nkx2-5*) and *Tbx4/5* reveal a broad cardiac-like domain in amphioxus; such a decentralized organization during development parallels that seen in the adult anatomy. Our data therefore support the hypothesis that amphioxus never possesses a proper heart, even transiently during development. We also define a putative hematopoietic domain, supported by the expression of the hematopoietic markers *Scl* and *Pdvegfr*. We show that this area is closed to the dorsal aorta anlages, partially linked to excretory tissues, and that its development is regulated by retinoic acid, thus recalling the aorta-gonads-mesonephros (AGM) area of vertebrates. This region probably produces *Pdvegfr*+ hemal cells, with an important role in amphioxus vessel formation, since treatments with an inhibitor of PDGFR/VEGFR lead to a decrease of Laminin in the basal laminae of developing vessels. Our results point to a chordate origin of hematopoiesis in an AGM-like area from where hemal *Pdvegfr*+ cells are produced. These *Pdvegfr*+ cells probably resemble the ancestral chordate blood cells from which the vertebrate endothelium later originated.

Keywords: amphioxus, hematopoiesis, evo-devo, endothelium, VEGFR-2/Flk-1,

Scl/Tal-1

INTRODUCTION

The vertebrate circulatory system, despite its high level of specialization and physiological relevance, nevertheless remains poorly understood, both in terms of its origin and its evolutionary transition from invertebrate hemal systems. The invertebrate hemal systems are usually composed of a network of cavities located between the basal laminae of epithelia (Ruppert and Carle, 1983). Frequently, these epithelia contain myofilaments and are contractile, contributing to the circulation of the hemal fluid. In these animals, the pumping organ is a specialized peristaltic vessel composed of myoepithelial cells. However, in vertebrates the endothelial cells delimit the vascular lumen and the heart is a multilayered and multi-chambered muscular organ. Although there exist important differences between the cellular elements involved in cardiovascular development of vertebrate and invertebrate phyla, a common basic gene network has been identified (Davidson and Erwin, 2006), suggesting that the extant circulatory systems and pumping organs of very diverged animals share a common evolutionary origin (Xavier-Neto et al., 2007). However, the evolutionary steps leading to the acquisition of complex vertebrate cardiovascular systems remain to be elucidated (Muñoz-Chápuli and Pérez-Pomares, 2010; Pérez-Pomares et al., 2009; Simões-Costa et al., 2005).

Hematopoiesis, the process that gives rise to the different blood cell lineages from hematopoietic stem cells (HSCs), generally takes place concomitantly with cardiovascular development. HSCs are responsible for maintenance and self-renewal of all blood cells in vertebrates (reviewed by Orkin and Zon, 2008). During vertebrate ontogeny, hematopoiesis occurs first in the so-called blood islands (Galloway and Zon,

2003), situated in the extraembryonic tissues surrounding the yolk sac (or equivalent regions depending on the animal group), whereas in the embryo proper it occurs first in the aorta-gonads-mesonephros (AGM) region (Godin and Cumano, 2002; Robin et al., 2003). Members of the PDGFR/VEGFR subfamily and other tyrosine kinase receptors (RTKs) (especially *VEGFR-2/Flk-1*) (Kattman et al., 2006), and the transcription factors SCL/TAL-1 and GATA1-3 (Gering et al., 1998; Pimanda et al., 2007) have a crucial function in hematopoiesis (reviewed by Cumano and Godin, 2007). They are important elements of a gene regulatory network playing a key role in the determination of mouse HSCs in the yolk sac, in the AGM and in the fetal liver. Later in development, the endothelial lineage is marked by *VEGFR-2/Flk-1*, in contrast to the hematopoietic lineage. It is believed that both lineages originate from the same cellular progenitors, the hemangioblasts (Ema et al., 2003). Although the molecular mechanisms underlying hematopoiesis have been widely studied in vertebrate embryos and in embryonic stem cells, little is known about its evolutionary origin.

From an evolutionary point of view, three key issues are (i) the transition from the invertebrate to the vertebrate cardiovascular system, (ii) the evolutionary relationship between vertebrate and invertebrate hematopoiesis and (iii) the origin of vertebrate endothelium from invertebrate-type hemal cells. The cephalochordate amphioxus is placed in a key phylogenetic position to understand the origin of chordates (Bertrand and Escriva, 2011), as it represents the sister group of the tunicate-vertebrate clade (Delsuc et al., 2006). Amphioxus possesses a closed hemal system; the anatomical distribution of main vessels and the direction of flow of hemal fluid (backwards dorsally and forwards ventrally) are reminiscent of those in the vertebrate embryo (Rähr, 1979). However, as has been widely described in the literature, adult amphioxus do not have a proper heart from a morphological point of view (Fig. 1), and the hemal fluid

circulates by the contraction of several main vessels (depicted in Fig. 1) (Franz, 1927; Moller and Philpott, 1973; Rähr, 1981; Randall and Davie, 1980; Ruppert, 1997). However, if amphioxus develops a heart during development that is secondarily lost in the adult still remains to be investigated. As in other invertebrates, the contractile capacities of these vessels are due to myofilaments arranged basally in the coelomic epithelia (Moller and Philpott, 1973). Free hemal cells have been described within and lining the lumen of amphioxus vessels in some regions (Kučera et al., 2009; Rhodes et al., 1982). Kučera et al. (2009) described a possible role of these cells in the degradation of the extracellular matrix to open the vessel lumen, where Laminin is one of the main components. However, as in other invertebrates, a true endothelium is absent.

In order to better understand the transition from an invertebrate-type to a vertebrate hematopoietic and vascular system, we have analyzed a number of hematopoietic and cardiac markers in embryos of the European amphioxus *Branchiostoma lanceolatum*. Only two cardiac markers have been previously studied, *BMP2/4* (Panopoulou et al., 1998) and *Csx* (*Nkx2.5/tinman*) (Holland et al., 2003), with contradictory conclusions. While Panopoulou and colleagues proposed the endostylar artery as a vertebrate heart homologue, Holland *et al*. proposed so for the subintestinal vessel. Furthermore, Onimaru *et al*. have suggested a separation of the amphioxus ventral mesoderm into an anterior pharyngeal domain and a posterior cardiac domain. Here, we study the expression of the cardiac markers *Csx* (*Nkx2-5/tinman*), *Tbx4/5* and *Hand*, which define a broader cardiac area than previously reported including both pharyngeal and ventral trunk mesoderm. This suggests that all developing vessels in the pharynx (e.g., endostylar artery) and the trunk (e.g, subintestinal vessel), which are indeed contractile in the adult, represent the “cardiac” domain. On the other hand, the expression of three important hematopoietic markers (*Pdvegfr*, *Scl* and *Gata1/2/3*)

suggests that during development, amphioxus embryos possess a hematopoietic domain in the anterior part of the body close to the two dorsal aortas, associated with the developing excretory system and regulated by retinoic acid (RA). This hitherto undescribed domain strongly resembles the vertebrate aorta-gonads-mesonephros area. Finally, using results from experiments in which we inhibit PDVEGFR, we discuss the putative function of free *Pdvegfr*+ hemal cells in vessel formation, and its implications for the evolutionary origin of the vertebrate endothelium.

MATERIAL AND METHODS

Gene annotation, cloning and phylogenetic analysis

We looked for putative *Scl/Tal-1* orthologous sequences in the genome of *B. floridae* JGI v1.0 by means of tBLASTN and using aminoacidic sequences of vertebrate counterparts SCL/TAL-1 and TAL2 as queries. The corresponding genomic sequences were retrieved and a model was predicted by GeneWise2 and GeneScan, as previously described (D'Aniello et al., 2008). Only one candidate was predicted. Alignment of the sequences with vertebrate *Scl/Tal-1* orthologues was done with MAFFT multiple sequence aligner (Katoh et al., 2002). To confirm that our protein was the true Scl orthologue, we carried out a phylogenetic analysis: a phylogenetic tree was inferred with MrBayes 3.2 (Ronquist et al., 2012) using two independent runs (each with four chains). Model selection was performed using ProtTest (Abascal et al., 2005; Drummond and Strimmer, 2001; Guindon and Gascuel, 2003). The tree was considered to have converged when the standard deviation was <0.01, and 25% of the trees were burned to generate the consensus tree.

The sequences of *B. floridae GATA1/2/3* and *GATA4/5/6* genes were kindly provided by William Q. Gillis (Gillis et al., 2009). Primers based on *B. floridae* sequences were used to amplify a fragment of each gene from a liquid cDNA library of *B. lanceolatum* in pDNR222 (CloneMinerII kit, Invitrogen). The primers used for cloning and PCR conditions are described in Suppl. Table 1. The sequences of the clones used in this work have been submitted to the NCBI GenBank database under the accession numbers JQ942471-7, except for probes based in exons 2a and 2b of *GATA4/5/6*, which were submitted to the NCBI Probe database under the accession numbers 12859234 and 12859235, respectively.

Whole mount in situ *hybridizations and sectioning.*

Ripe adult amphioxus (*B. lanceolatum*) were sampled in Argelès-sur-mer, France, during the spawning season of 2009. Spawning was induced as reported in Fuentes et al. (2007) in Barcelona, Spain. After *in vitro* fertilization, embryos were cultured at 17°C and fixed at different stages with 4% PFA in MOPS buffer overnight at 4°C. Wholemount *in situ* hybridizations were performed as previously described (Irimia et al., 2010). Following wholemount *in situ* hybridization, embryos were embedded in Spurr's resin and sectioned with an ultramicrotome at 3 μm, as previously described (Candiani et al., 2007).

SU5416 and retinoic acid treatments

The embryos were maintained in 0.22 μm-filtered fresh seawater and were treated with different concentrations of SU5416 (Calbiochem), a permeable, ATP-competitive and

selective inhibitor of tyrosine kinase receptors of the VEGFR and PDGFR family. SU5416 was dissolved in DMSO and tested at three concentrations: 0.1 μM, 1μM and 20 μM from 8hpf (hours post-fertilisation), using as a negative control the same concentration of DMSO. The drug-containing seawater was changed every 24 hours. The embryos were fixed for wholemount *in situ* hybridization as described above at different stages from late gastrulae until 3 day-old larvae. Retinoic acid and BMS009 treatments were performed as described in Escriva et al. (2002), but using only a concentration of 10^{-6} M of all-trans retinoic acid (Sigma-Aldrich) or BMS009.

Immunohistochemistry

For immunolocalisation experiments, 5-10 animals from each of the control and treatment conditions were used in 3 different experiments, following previously reported procedures (Somorjai et al., 2012). Primary antibodies included α-acetylated Tubulin (1:500, Sigma), α-Laminin (1:25, rabbit anti-laminin-111, Sigma; (Kučera et al., 2009)) and Alexa Fluor 568 Phalloidin (1:400, Invitrogen) for F-Actin staining. DAPI was used to label nuclei (1:5000 of 5mg/ml stock, Invitrogen). Samples were mounted in Prolong Gold antifade reagent (Invitrogen), and images were acquired on a Leica SPII confocal microscope.

Quantification

Quantification of Laminin and F-Actin levels on confocal images was performed with ImageJ software (n=5 each DMSO and treated larvae). The RGB line profiler was used to simultaneously collect pixel intensities from all three channels. For each individual,

3-5 "lines" were profiled in equivalent posterior tail regions of control and treated larvae, when possible from different confocal sections, and the median values considered representative. In order to be able to compare across animals, Laminin values were normalized with respect to the highest value collected from the basal lamina below the epidermis (e.g., "de" or "pm" in figure 5E, value of 1). For F-Actin, only dorsal and ventral notochord membranes were considered, and normalization was with respect to the highest of the two. Means were compared using Welch´s *t* test statistic for unequal variances at a global $P \leq 0.5$. Comparisons were considered significant when they passed the Bonferroni correction for multiple tests at $P \leq 0.008$.

RESULTS

Expression of cardiovascular markers in amphioxus

To better understand the development of the amphioxus vascular system, we have re-evaluated several cardiac markers in the European amphioxus *B. lanceolatum* for which expression had been reported in the Floridian amphioxus. *B. lanceolatum Csx* (correct naming for *Nkx2.5/tinman* after Holland et al., 2007) expression is generally comparable to that of its *B. floridae* orthologue (Holland et al., 2003). *B. lanceolatum Csx* expression is first detected in the right side of the pharynx and the ventral part of the first six somites (Fig. 2A-B). At the pre-mouth larval stage, it is expressed in the anlage of the subintestinal vessel in addition to the pharynx, although it is weaker in the most caudal region as compared to the expression in the Floridian amphioxus (Fig. 2C; Holland et al., 2003). As in *B. floridae*, the European amphioxus *Csx* is no longer detected in the subintestinal vessel from the second day of development onwards (Fig.

2D).

Vertebrate *Hand1* and *Hand2* are bHLH family genes with important functions in cardiac development, especially *Hand2* (McFadden et al., 2005). Amphioxus possesses only one orthologous gene (*Hand*) for both vertebrate *Hand1* and *Hand2* (Onimaru et al., 2011). Since Onimaru *et al*. (Onimaru et al., 2011) reported the Floridian amphioxus *Hand* expression pattern in a restricted window of development, here we investigated its complete expression profile in *B. lanceolatum* (supplementary Fig. S1). Interestingly, *B. lanceolatum Hand* shows clear asymmetrical expression: more anteriorly, it occurs in the right coelomic diverticulum (Fig. 2E-F, I), and then in the ventral part of the somites, showing stronger expression in the somites on the right side until neurula stages (Fig. 2E-F, J and supplementary Fig. S1). Regarding its possible cardiac function, we detected expression in both ventral and posterior parts of ectoderm and mesoderm (Fig. 2K), in a domain surrounding the coelomic space where the anlage of the subintestinal vessel will later open. This represents an early expression domain not detected in the previous report for *B. floridae* (Onimaru et al., 2011). Subsequently, as in *B. floridae*, European amphioxus *Hand* is expressed in the ventral mesoderm, not only in the anlage of the subintestinal vessel, but also in the pharyngeal mesoderm of pre-mouth and 2 day-old larvae (Fig. 2G, L, H, M-N), as can be clearly observed in sections of the pharyngeal region (Fig. 2M). *Hand* is expressed until 84 hours post-fertilization (hpf) in *B. lanceolatum*, and its expression is detected in the very posterior part of the subintestinal vessel, the posterior part of the hindgut, and in a few cells in the pharynx and pre-oral pit (Fig. 2O).

The vertebrate T-box containing gene *Tbx5* is crucial for heart development in vertebrates (Naiche et al., 2005). The amphioxus orthologue *Tbx4/5* has also been related to cardiac development by its expression in the most posterior part of the

subintestinal vessel, although only in very late larval stages (Horton et al., 2008; Minguillon et al., 2009). We investigated the expression pattern of this gene in earlier stages of *B. lanceolatum* and detected a previously unreported expression pattern in the pharyngeal and ventral mesoderm of pre-mouth larvae (Fig. 2P-S), similarly to the previously discussed cardiac markers *Csx* and *Hand* (Fig. 2C and G). *Tbx4/5* expression subsequently weakens through development, and in 2 day-old larvae, is restricted to the most caudal portion of the subintestinal vessel, as well as to a few scattered cells around the pharynx (Fig. 2Q). At 84 hours of development, only the weak expression in the posterior subintestinal vessel persists, expression that coincides with that previously reported (Horton et al., 2008; Minguillon et al., 2009). Again, as for Hand, Tbx4/5 is clearly expressed in the pharyngeal mesoderm (Fig. 2R). Therefore, contrary to what was reported by Onimaru et al. (2011), the pharyngeal mesoderm likely develops into cardiac elements, as does the rest of the ventral mesoderm, which was defined as the only cardiac domain by Onimaru et al. (2011).

We further investigated other important orthologous amphioxus counterparts of vertebrate cardiac genes, including *B. lanceolatum Islet* and *GATA4/5/6.* As for *Islet*, our results in *B. lanceolatum* confirm previous reports in *B. floridae*; namely, its lack of expression in the cardiac domain (supplementary Fig. S2 and see Jackman et al., 2000). We also investigated the expression of two isoforms of amphioxus *GATA4/5/6* (Gillis et al., 2009) and, interestingly, neither of them were detected in the ventral mesoderm (see supplementary Fig. S3 for the complete expression pattern).

Expression of hematopoietic markers during amphioxus development

In vertebrates, the genes *Scl/Tal-1*, encoding a bHLH transcription factor, and *VEGFR-*

2 (*Flk-1*), encoding a member of the PDGFR/VEGFR family of tyrosine kinase receptors, are necessary players for the generation of HSCs (Mead et al., 2001; Shalaby et al., 1995). In a previous study in amphioxus, we identified only one member of the PDGFR/VEGFR subfamily: *Pdvegfr* (D'Aniello et al., 2008). Herein, we have also identified a single orthologue in the amphioxus genome corresponding to *Scl/Tal-1*, *Tal-2* and *lyl-1* vertebrate paralogues, which we name *Scl*. Amino acidic multiple sequence alignment and phylogenetic analysis clearly show that the amphioxus protein we have identified is a clear orthologue of the vertebrate *Tal-1/Tal-2/Lyl-1* family (supplementary Figs. S4 and S5). The expression patterns of amphioxus *Pdvegfr* and *Scl* genes are very similar during the ontogeny of amphioxus, starting to be expressed in two anterior, bilateral and slightly asymmetrical groups of mesodermal cells, with the left signal located more anteriorly (Fig. 3A and M). Strikingly, this bilateral mesodermal expression is very similar to that found in vertebrates, such as zebrafish embryos (Gering et al., 1998). Sections of embryos at this stage show that both genes are co-expressed in the same cells (Fig. 3D-E, O-P). The topographical position of the left signal corresponds to the developing Hatschek's nephridium (HN), which is a mesodermal tissue localized between somites 1 and 2 (identified as 2 and 3 in Goodrich, 1934, since Goodrich interpreted the anterior most coelomic diverticula as somites). To confirm that this left signal corresponds properly to the HN, we used the amphioxus orthologue of *Pax2/5/8*, a marker of the HN (Kozmik et al., 1999; Somorjai et al., 2008). Double single-stained *in situ* hybridization using both *Pax2/5/8* and *Pdvegfr* shows that the left *Pdvegfr* signal coincides with that of *Pax2/5/8* in the HN (Fig. 3T and U). Thus, this result indicates that *Pdvegfr* and *Scl* are expressed in one of the developing excretory organs of amphioxus.

Later in development, at the pre-mouth larval stage, single cells expressing both

Pdvegfr and *Scl* can be progressively detected in most posterior parts throughout the right side (Fig. 3B, N and AA and AB for a ventral view). In zebrafish, the early expression of *Scl* is also posteriorly extended during development (Gering et al., 1998). As in the neurula stage, the most anterior expression of both *Pdvegfr* and *Scl* corresponds to the left side, and seems to have been enlarged medially following the outline between the gut and the notochord, in a region where the dorsal aorta (split in two branches in the anterior region) (Ruppert, 1997) will develop (Fig. 3F and Q). Similarly, the right signal is also located close to the presumptive dorsal aorta, and it expands ventro-laterally into the mesoderm of the pharynx (Fig. 3G and R). The most posterior cells seem to have migrated caudally through the pharyngeal mesoderm (Fig. 3H and S), which is strongly displaced to the right, probably due to the specific morphological features associated with larval amphioxus´ feeding behavior (van Wijhe, 1919). Amphioxus *Scl* and *Pdvegfr* are most likely co-expressed from neurula to pre-mouth larval stages (Fig. 3A-B, M-N). However, while *Scl* expression is no longer detected, *Pdvegfr* is continuously expressed in 2 day-old larvae, in the anlage of the two dorsal aorta branches, in cells laterally located between notochord and gut as well as under the somites (Fig. 3C, I-L). At this stage, *Pdvegfr* expression is also detected in the club-shaped gland (Fig. 3I) and in isolated, scattered cells distributed along the main body axis in both dorsal aorta and subintestinal vessel anlages (Fig. 3K, L). The narrow, elongated morphology of these cells suggest that they might be migrating, and probably originate in more anterior regions (inset in Fig. 3C).

During vertebrate hematopoiesis, *GATA1-3* genes are also essential players in hematopoiesis (Cumano and Godin, 2007). In vertebrates, *GATA1-3* genes are not only specifically expressed by the HSCs, but also by the surrounding mesenchyme (Cumano and Godin, 2007). In amphioxus, the single orthologue *GATA1/2/3* (Gillis et al., 2009)

is broadly expressed in the anterior part of the embryo, except in the ectoderm and neural plate (Fig. 3V and W); in the posterior half of the embryo, *GATA1/2/3* is only expressed in the ventral part of the somites. In pre-mouth larvae, the expression is slightly more restricted, with expression in both the right and left anterior coelomic diverticula, in the club-shaped gland, endostyle and pharynx, and in the surrounding mesoderm (Fig. 3X). However, from the mid- to posterior pharynx, the expression becomes restricted to the right side (Fig. 3Y). Comparison of ventral views of the *GATA1/2/3* and *Pdvegfr*/*Scl* patterns suggests that the latter is enclosed in a wide domain encompassing the former (Fig. 3Y, AA and AB). Eventually, *GATA1/2/3* expression becomes highly reduced in this presumptive hematopoietic area, coinciding with the lack of expression of *Scl*. It is strongly expressed in the anterior right coelomic cavity, preoral pit and pharynx, and faintly where *Pdvegfr* is detected (compare Fig. 3C and Z, white arrowheads).

Retinoic acid treatment severely inhibits hematopoiesis in amphioxus embryos

Retinoic acid (RA) signaling plays a crucial role in the determination of the HSCs in vertebrates. In zebrafish, RA treatment inhibits early hematopoiesis (de Jong et al., 2010), and the same effect is seen in mouse embryonic stem cells (Szatmari et al., 2010). Thus, we investigated if RA treatment (see Material and Methods) had a similar effect on the *Pdvegfr*+/*Scl*+ cells, strong candidates for HS-like cells in amphioxus. In RA-treated embryos the expression of both *Pdvegfr* and *Scl* is detected neither in neurula nor in pre-mouth stages when compared with the DMSO-treated control embryos (Fig. 4A-D and G-J). In 2 day-old larvae, the small population of *Pdvegfr*+ cells in the reduced pharynx is detected as in the control, but putative migrating cells of the dorsal

aorta and subintestinal vessel anlage are drastically reduced (supplementary Fig. S6A). This expression even disappears in some larvae (supplementary Fig. S6B). Thus, these results suggest a function of RA in the determination of the *Pdvegfr*+/*Scl*+ cell population in amphioxus. In contrast, the RA-antagonist BMS009 had no effect in the determination and development of this putative haematopoietic tissue (Fig. 4E, F, K and L).

Inhibition of PDVEGFR leads to posterior defects in the amphioxus larva

In order to assess if these *Pdvegf*+ cells have a function in vessel development, as *VEGFR-2*+ cells (endothelial cells) do in vertebrate angiogenesis and vasculogenesis, we treated amphioxus embryos with SU5416, a specific inhibitor of PDGFR and VEGFR, (see Material and Methods). This drug specifically acts by blocking the cross-phosphorylation of tyrosine residues of these receptors. Continuous treatment from early stages, when *Pdvegfr* starts to be expressed, causes the posterior part of the embryo to hook from pre-mouth larval stages onwards, an effect that becomes stronger in later stages (supplementary Fig. S7).

The curly-tail phenotype obtained upon inhibition of PDGFR/VEGFR may result from a variety of defects in morphogenesis. For instance, it could indicate a problem with elongation of the notochord, maturation of muscle fibres, or subintestinal vessel formation posteriorly. In order to begin to distinguish among these possibilities, we performed immunohistochemistry using a variety of antibodies. Comparing SU5416-treated larvae at 55hrs with age-matched controls revealed no major morphogenetic or cytological defects at multiple levels. First, acetylated Tubulin expression shows that both epidermal and intestinal cilia appear grossly normal.

Moreover, the axons of the neural tube extend posteriorly similarly in control and SU5416-treated embryos (Fig. 5A, A′ and supplementary Fig. S8). Second, our results using Phalloidin staining of F-actin suggest that blocking PDVEGFR causes no overt defects in notochord formation or muscle differentiation (Fig. 5B-C′). The superficial longitudinal muscle fibers extend to their attachment sites at the edges of the myomeres equally well in treated and control larvae (Fig. 5B, B′). We also found no significant difference in the number of somites formed (n=5, mean 14.33 vs 14.8, 2-tailed t-test, *P*=0.4558). Finally, we also saw no apparent differences in Laminin expression at the somite level between DMSO and SU5416-treated larvae (Fig. 5B, B′). Although we cannot exclude fine structural differences in treated and untreated larvae, taken together our data suggest no major disruption in morphogenesis.

While we saw no gross structural defects upon blocking of PDVEGFR, careful examination of Laminin staining revealed a strong reduction in the posterior tail, specifically in the hooked region (Fig. 5C, C′). Quantification of Laminin levels showed a highly statistically significant reduction in the basal lamina of SU4516-treated larvae in only three regions (Fig. 5D, E): dorsal to the intestine (t=5.00, df=7, *P*=0.0016), ventral to the intestine (t=6.71, df=7, *P*=0.0003) and in the visceral ceolomic epithelia (t=6.07, df=5, *P*=0.0018). The former is part of the dorsal aorta, while the two latter define the subintestinal vessel. However, no differences were found in F-Actin in equivalent locations dorsal and ventral to the notochord. This suggests a specific defect in dorsal aorta and subintestinal vessel formation, and perhaps of basal lamina deposition processes, after blocking PDVEGFR signalling.

DISCUSSION

The core of a gene regulatory network controlling the early development of the vertebrate heart and of the pumping organs of invertebrates appears to be deeply conserved, since orthologous genes, namely *Hand*, *Nkx2-5* and *Tbx* family genes, play crucial roles in their formation (Davidson and Erwin, 2006; Olson, 2006). Despite this 'deep homology' (Shubin et al., 2009), the specific functions of these genes in vertebrates and invertebrates are distinct, and both clades have likely undergone independent, parallel modifications of the gene regulatory network, leading to particular innovations in their respective circulatory systems (Medioni et al., 2009; Xavier-Neto et al., 2007). Therefore, understanding the formation of the cardiovascular and blood systems in the closest invertebrate relatives to vertebrates, such as cephalochordates, may shed light on the evolutionary changes that led to the origin of the complex vertebrate circulatory system.

The amphioxus decentralized cardiac domain is a derived feature

It is likely that the ancestral condition of the pumping organs resembled a simple contractile tube, with hemal spaces opening between the endodermal and visceral coelomic epithelia (Xavier-Neto et al., 2007). The pumping function of this primitive heart was probably a co-option of the function of the visceral coelomic myoepithelium for intestinal peristalsis (Pérez-Pomares et al., 2009).

Among the contractile vessels of amphioxus, either the subintestinal or the endostylar vessels have been claimed to be homologous to the vertebrate heart, based on only one vertebrate cardiac marker, *Csx* (*Nkx2-5*) (Holland et al., 2003), or on the expression of the growth factor *BMP2/4* (Panopoulou et al., 1998), respectively. However, a distinct morphological heart in adult amphioxus does not exist. This lack

of a heart in the adult could originate during development in two ways: either the heart is formed at some developmental stage, and is secondarily lost; or, alternatively, it never develops, and cardiac ontogeny would then also be decentralized (i.e. not restricted to a specific area). Although similarity of gene expression does not necessarily imply homology, the co-localization of *Hand* and *Csx* in the coelomic epithelium under the gut and, importantly, also in the pharyngeal mesoderm (Fig. 2C and G; see Onimaru et al. (2011) for *B. floridae*) indicates that the amphioxus cardiac domain is not restricted but decentralized. The new expression pattern of *Tbx4/5* reported here (Fig. 2P-S) in these tissues strongly supports this hypothesis. Thus, at the pre-mouth larval stage, a cardiac domain appears to be characterized by some of the molecular players involved in vertebrate cardiogenesis, namely *Tbx4/5*, *Hand* and *Csx* (*Nkx2.5*). Other members of the T-box containing family have also been associated with a cardiovascular function, such as *Tbx20*, whose expression in amphioxus resembles that of the markers studied here (Belgacem et al., 2011). It is therefore remarkable that adult pharyngeal vessels and those more linked to the gut are all contractile, and derived from the embryonic pharyngeal and ventral trunk mesoderm. Interestingly, the expression of other important orthologous genes of vertebrate cardiac markers, such as *Islet* and *GATA4/5/6* (supplementary Fig. S2 and S3), do not co-localize with *Csx*, *Hand* or *Tbx4/5*. However, in *Ciona intestinalis,* one GATA factor has been implicated in cardiovascular determination, *GATAa* (Ragkousi et al., 2011), and *Islet* is expressed in a population resembling a secondary heart field (Stolfi et al., 2010). Whether the absence of cardiac expression of *GATA4/5/6* and *Islet* in amphioxus is associated with its cardiac 'decentralization' remains to be investigated. Taken together, our results suggest that the cardiac domain of amphioxus, unlike that found in hemichordates (where it is centralized in the heart-kidney complex, Fig. 1) and other chordates, is not restricted to

a unique contractile vessel as previously suggested (Holland et al., 2003; Panopoulou et al., 1998). Taken into consideration that hemichordates are an outgroup of chordates, and given that they possess a main pumping organ in the prosome, the lack of a central pumping organ in amphioxus is likely a derived morphological characteristic, resulting from a derived ontogeny of the cardiac domain.

Hematopoiesis in amphioxus is carried out in an AGM-like area

Although blood cells have been identified in a wide range of invertebrates (Hartenstein, 2006), how these cells are determined and whether this process is similar or not to vertebrate hematopoiesis are still obscure. For instance, it has been reported that hematopoiesis in the lymph gland of *Drosophila* is similar to that occurring in the AGM of vertebrates (Mandal et al., 2004). However, although some of the genetic elements are the same, probably belonging to an ancient gene regulatory network (Davidson and Erwin, 2006), the anatomy of the process in *Drosophila* is essentially different from that of vertebrates, and key factors like the *Drosophila GATA1/2/3* orhologue *grain* (Gillis et al., 2008) or *Scl* are not expressed in the cardiogenic mesoderm. Thus, the similarities between the cardiogenic mesoderms of *Drosophila* and vertebrates are rather superficial and are likely not homologous (Medioni et al., 2009).

Studies in closer relatives of vertebrates may shed light on the hitherto obscure origin and evolution of vertebrate hematopoiesis. We have found here only one amphioxus orthologue for vertebrate paralogues *Scl/Tal-1*, *Tal-2* and *lyl-1*. These genes have roles in both hematopoietic development (Ema et al., 2003; Giroux et al., 2007) and in the neural tube (Ferran et al., 2009; van Eekelen et al., 2003). In contrast, in amphioxus the expression of *Scl* is present only in mesodermal derivatives, at least in

the window of development studied here. The function of *Scl/Tal-1* and *Tal-2* in the central nervous system was acquired in the vertebrate lineage, probably due to generation of new enhancers after the two rounds of whole genome duplication that took place at the origin of vertebrates (Jiménez-Delgado et al., 2009). Thus, amphioxus *Scl* is a good hematopoietic marker. Also, in a previous study (D'Aniello et al., 2008) we identified only one member of the PDGFR/VEGFR tyrosine kinase receptor family. Given that all vertebrate VEGFR members have important roles in the development of the vascular system and hematopoiesis (Otrock et al., 2007), we believe that amphioxus *Pdvegfr* is also a good marker for hematopoiesis and vessel development.

We have shown in amphioxus that early expression of these two important hematopoietic markers, *Pdvegfr* and *Scl,* occurs in two bilateral, slightly asymmetrical domains. It is probably in the neurula stage that determination of the hematopoietic domain occurs (Fig. 6A). The co-expression with the *Pax2/5/8* orthologue indicated that the left domain corresponds to the HN, which is tightly associated with the left dorsal aorta (Stach, 1998). The right domain is likely the anlage of the glomus, a highly vascularized area in the adult, formed at the rostral side of the right dorsal aorta (Franz, 1927). This common expression of hematopoietic genes in areas where excretory and vascular domains converge strongly recalls the vertebrate AGM. Moreover, it more generally highlights the close relationship between hematopoiesis and nephrogenesis in more basal vertebrates (Ma et al., 2011). Later, this hematopoietic domain slightly broadens in the pre-mouth larva, where some cells appear to have been displaced posteriorly, probably through migration, entering the cardiac domain (Fig. 6B). Meanwhile, amphioxus *GATA1/2/3* is expressed in the same area as the aforementioned factors, especially on the right side, from where the *Pdvegfr*+/*Scl*+ cells seem to start migrating caudally (Fig. 3X, AA and AB). Importantly, GATA-2 forms a complex with

SCL/TAL-1 in vertebrates, thereby regulating hematopoiesis (Mead et al., 2001; Pimanda et al., 2007). Therefore, we suggest that a hematopoietic process occurring in an AGM-like area was present in the last common ancestor of chordates.

A variety of studies suggest that RA is involved in HSC development in vertebrates. For instance, treatment with RA blocks primitive hematopoiesis in zebrafish and mouse, upstream of SCL (de Jong et al., 2010; Szatmari et al., 2010). Interestingly, in RA-treated amphioxus embryos the development of *Pdvegfr/Scl*-expressing anterior domains is highly impaired (Fig. 4 and supplementary Fig. S6), and *Pdvegfr*+ cells are strongly reduced in the dorsal aorta and the subintestinal vessel, indicating that they are not very well produced or specified. Although this could be due to a loss of the hematopoietic tissues, such as the HN, the expression of *Pax2/5/8* in the HN of RA-treated embryos indicates that this is not the case (Schubert et al., 2006). Thus, not only are some important hematopoietic factors expressed in these tissues, but it is also likely that their regulation is controlled by the same players as in vertebrates. This strongly supports our hypothesis that a hematopoietic function is carried out by these tissues, and that it is homologous to that carried out in the AGM area of vertebrates.

Vertebrate endothelial cells might have derived from ancestral free hemal Pdvegfr+ *cells*

The last step of our model concerns the specification of blood cells (Fig. 6C). The final piece of evidence that supports our hypothesis of a hematopoietic AGM-like area is the generation of *Pdvegfr*+ cells scattered along the dorsal aorta and the subintestinal vessel in later stages. In vertebrates, while VEGFR-2 is an important marker of multipotent cells with hemato-cardiovascular specification (Kattman et al., 2006), in late

development it is expressed in endothelial cells, but not in HSCs (Ishitobi et al., 2011; Yamaguchi et al., 1993). As discussed above, the early function of amphioxus *Pdvegfr* in hematopoiesis is revealed by its co-expression with *Scl*. However, in 2 day-old larvae, *Scl* is not detected anymore, giving to the aforementioned hematopoietic domain a transitional nature, and *Pdvegfr* is expressed in isolated cells within the amphioxus vessels. These cells may well correspond to blood cells, or amoebocytes (Muñoz-Chápuli et al., 2005), that have been specified later, as occurs in vertebrate endothelial cells. In vertebrates, VEGFR-2 has an important role in vasculogenesis and angiogenesis. Accordingly, the inhibition of PDVEGFR by SU5416 in amphioxus embryos leads to what appears to be a vascular malformation, possibly due to defective deposition of Laminin in the vessels. Laminin is usually present in the basal lamina of the epithelia that constitute invertebrate vascular systems, including amphioxus (Kučera et al., 2009). Thus, *Pdvegfr*+ amoebocytes likely have a function in amphioxus vessel development.

In conclusion, although cephalochordates lack endothelial cells, as do other invertebrates, these amoebocytes, originating in an AGM-like area, may be similar to the evolutionary progenitors of the vertebrate endothelium (Muñoz-Chápuli, 2011; Muñoz-Chápuli and Pérez-Pomares, 2010). Thus, the close ontogenetic relationship between endothelium and blood cells in vertebrates would be accounted for by an evolutionary relationship, i.e., the endothelial cells of vertebrates probably originated as a specialization of free blood cells, akin to the amoebocytes of amphioxus (Muñoz-Chápuli et al., 2005; Muñoz-Chápuli and Pérez-Pomares, 2010).

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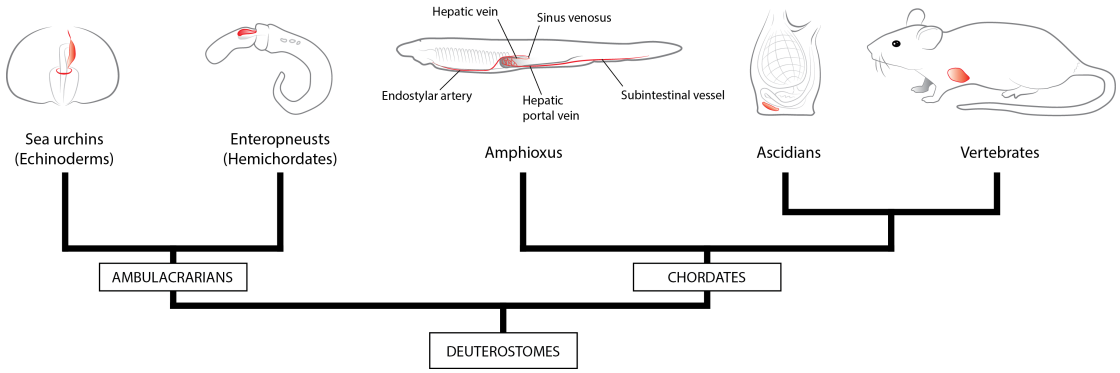

Figure 1. Phylogenetic tree of deuterostomes depicting the heart and pumping organs. The hemichordate enteropneusts have a heart-kidney complex on the rostral tip of the stomochord, in the prosome. Adult amphioxus are widely described as not possessing a proper centralized pumping organ or heart. Instead, several main vessels are contractile (labeled in the amphioxus scheme). Adult ascidians have a localized pumping vessel surrounded by a pericardium. Vertebrates possess complex chambered hearts, which represent an innovation of this group. The different pumping organs are

colored in red.

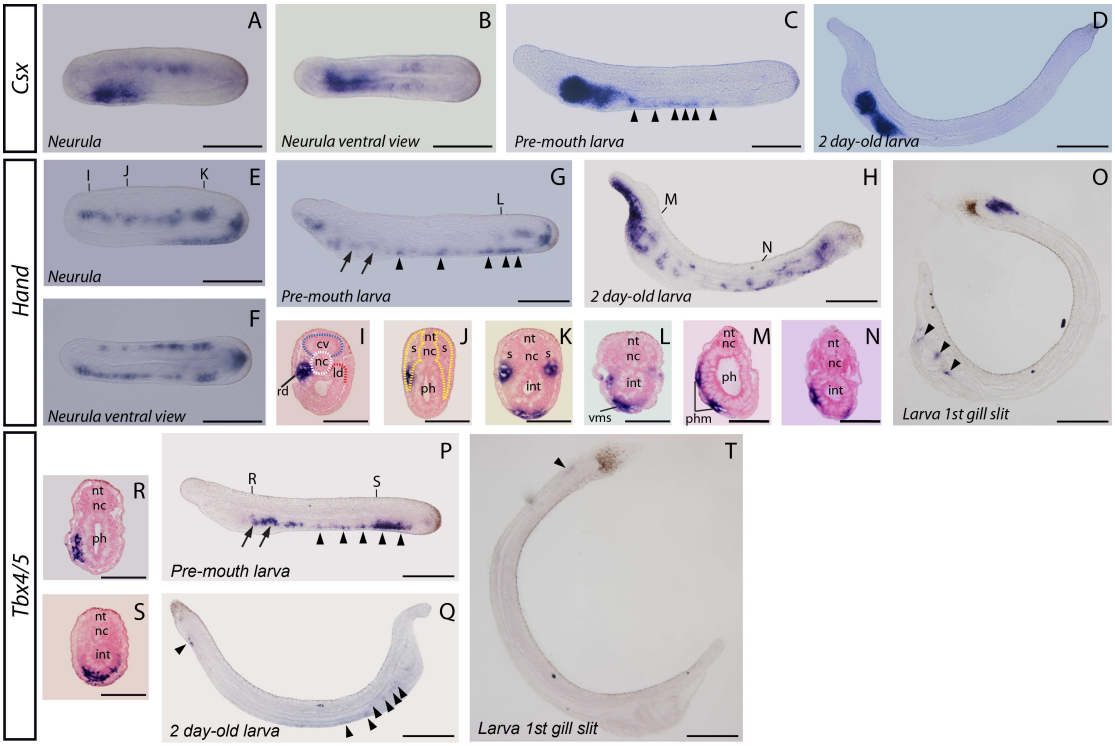

Figure 2. Cardiac marker gene expression during *B. lanceolatum* development. Expression patterns of *Csx* (*Nkx2.5*) (A-D), *Hand* (E-O) and *Tbx4/5* (P-T). For whole mounts, dorsal is towards the top, except for ventral views (indicated), and anterior is towards the left. In transverse sections, the view is from the anterior part of the embryo, with dorsal towards the top. Scale bars, 100 µm in wholemounts and 50 µm in sections. *Csx* is first expressed in the pharyngeal endoderm and somites (A, B), mainly on the right side (B). Later, *Csx* is expressed by mesothelial cells in the subintestinal vessel anlage (C, arrowheads), although it is no longer expressed there at later stages (D). At the neurula stage, *Hand* is asymmetrically expressed in the ventral half of the somites (E, F, J, K) and in the right diverticulum (F, I). It is also expressed in posterior-ventral ectoderm and posterior mesothelial cells of the subintestinal vessel (E, K). In pre-mouth larvae, it is expressed in the pharyngeal mesoderm (arrows) and more clearly in the ventral mesoderm (vms, arrowheads) in the presumptive subintestinal vessel (G, L). Somitic expression is restricted to the posterior part at later stages (G, H). In larvae (H), expression in the pharyngeal mesoderm (phm) and mesothelial cells of the subintestinal

vessel is still detected (H, M, N), unlike *Hand* (D). However, eventually it is restricted to some cells in the pharyngeal mesoderm (arrowheads) and the posterior tip of the subintestinal vessel and endoderm (O). *Tbx4/5* is first detected in pre-mouth larvae (P) in both ventral pharyngeal mesoderm (arrows) and subintestinal mesothelial cells (arrowheads), similarly to *Csx* and *Hand*. This expression is clear in sections (R, S). *Tbx4/5* expression decreases progressively, although it is still detectable in the pharynx and subintestinal vessel in 2 day-old larvae (Q) and finally is reduced to the very posterior tip of the subintestinal vessel (T, arrowhead).

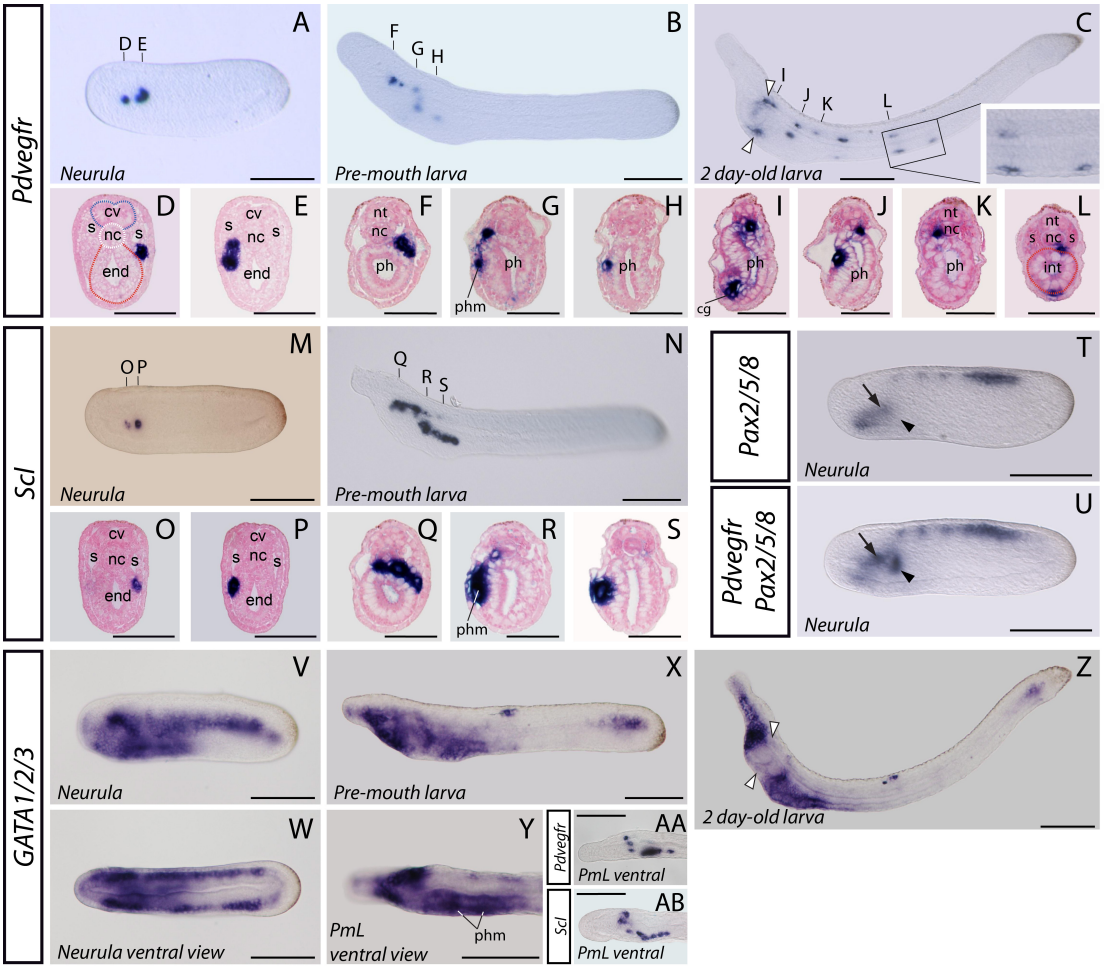

Figure 3. Hematopoietic marker gene expression in amphioxus. Expression patterns of *Pdvegfr* (A-L and AA), *Scl* (M-S and AB) and *Gata1/2/3* (V-Z). For whole mounts, dorsal is towards the top, except for ventral views (indicated), and anterior is towards the left. In transverse sections, the view is from the anterior part of the embryo. Scale bars, 100 µm in wholemounts and 50 µm in sections. *Pdvegfr* and *Scl* are expressed in the same tissues at early stages (A-E and M-P, respectively). On the left side, both *Pdvegfr* and *Scl* are expressed in Hatschek's nephridium (D and O, respectively), and on the right side, in a region of mesodermal origin between the somites (s) and the endoderm (end), likely the glomus anlage (E and P). At later stages, they are detected between the notochord (nc), pharynx (ph) and somites (F and Q), and are expanded posterior-ventrally along the pharyngeal mesoderm (phm; G, H, R and S). In 2 day-old larvae (C), *Scl* is no longer detected, and *Pdvegfr* is expressed in the club-shaped gland

(cg; I); *Pdvegfr*+ cells are detected in both anterior branches of the dorsal aorta (I-L) and the subintestinal vessel (L). These scattered cells present an elongated shape typical of migrating cells (C, inset). Comparison of *Pax2/5/8* expression (T) with that of *Pdvegfr* in a double *in situ* hybridization (U) shows that the left signal of the latter coincides with the expression of the former in Hatschek's nephridium (arrow), while the right signal is more posterior (arrowhead). *GATA1/2/3* is expressed in both right and left coelomic diverticula, the club-shaped gland, the endostyle and the pharynx, and in the surrounding mesoderm (V-Z). At early stages, it is expressed in the ventral half of all somites (V), and at later stages this domain is restricted posteriorly (X, Z). The expression in pharyngeal mesoderm is restricted to the right side (Y) engulfing *Pdvegfr*+/*Scl*+ cells (compare ventral views in V, AA and AB). In 2 day-old larvae, expression is faintly detected where anterior *Pdvegfr*+ cells are located (white arrowheads in Z; compare with C). cv, cerebral vesicle; cg, club-shaped gland; end, endoderm; int, intestine; nc, notochord; nt, neural tube; ph, pharynx; PmL, Pre-mouth larva; s, somites.

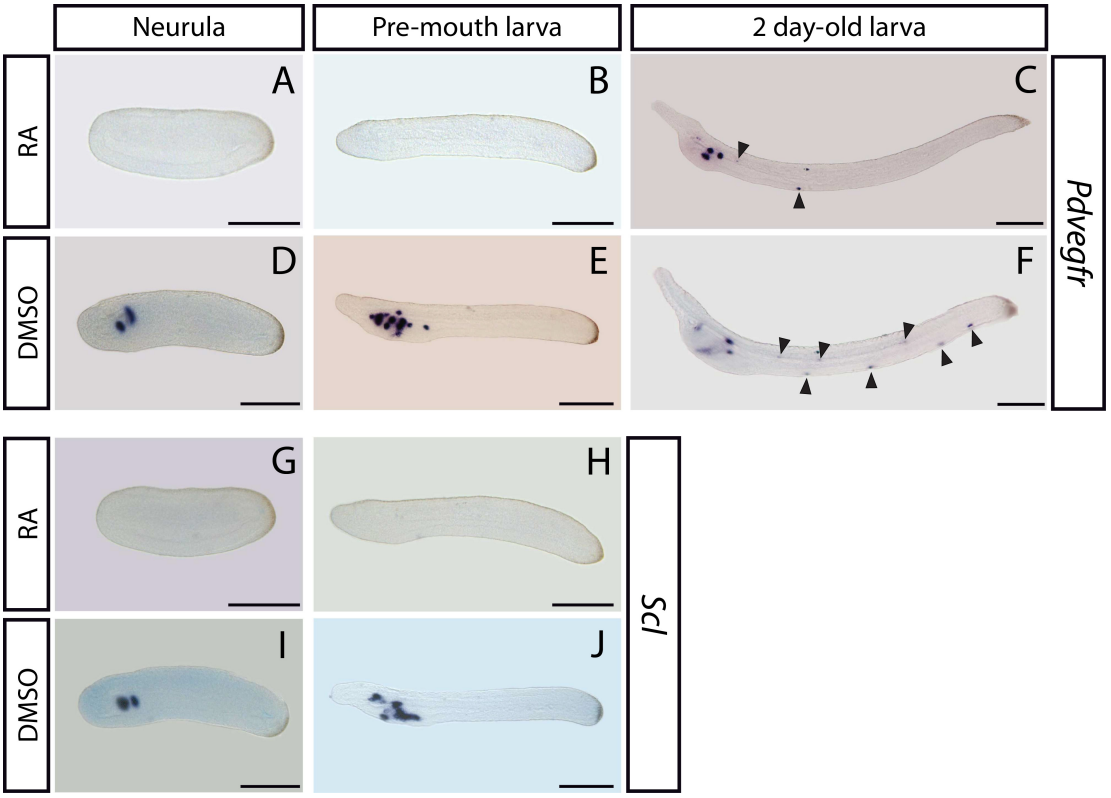

Figure 4. Effects of RA and BMS009 treatment on hematopoietic tissues during amphioxus development. Excess RA blocks the expression of amphioxus *Pdvegfr* (A-C) and *Scl* (G, H), in comparison to control DMSO-treated embryos (D-F and I-J, respectively). The expression of *Pdvegfr* is detected neither at neurula (A) nor at pre-mouth larva (B) stages. It is only detected at the 2 day-old larval stage (C), and almost no positive cells (arrowheads) are found in the subintestinal vessel or dorsal aorta. Scl^{+} cells are not detected at any stage in RA-treated embryos (G-H). Scale bars, 100 μm.

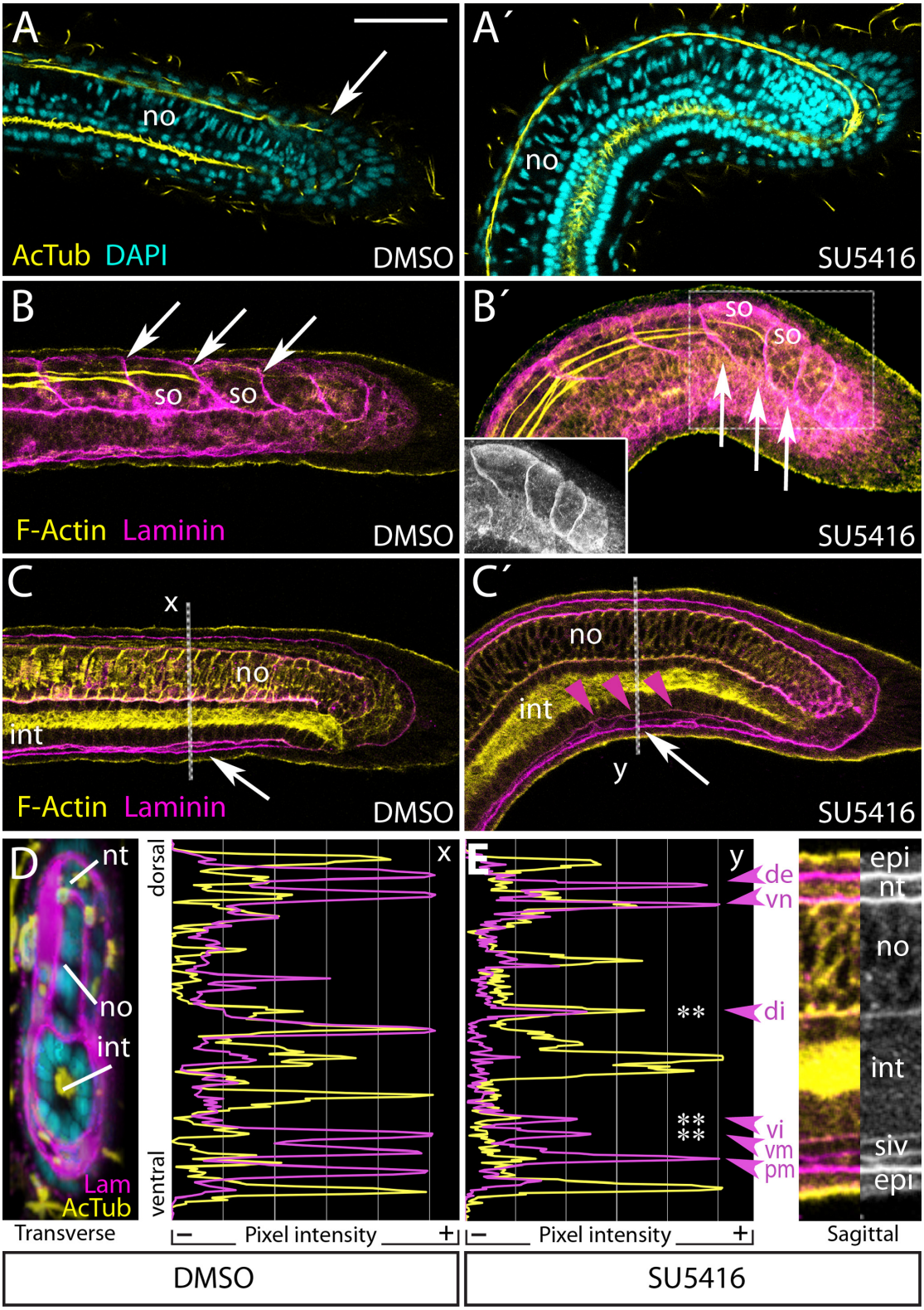

Figure 5. PDGFR/VEGFR inhibitor SU5416 reduces Laminin expression posteriorly. No overt defects are evident in the axons of the neural tube as revealed by acetylated Tubulin staining (arrows in A, A´). At the level of the somites (so), Laminin appears

normal in both DMSO (B) and SU5416-treated (B´ and inset) larvae, as do the maturing muscle fibres (yellow, F-Actin). More medially, in spite of the curled tail, the notochord (no) and intestine (int) are also largely unaffected in treated (C´) *vs* control (C) animals, as evidenced by Phallodin and Laminin staining. However, postero-ventrally, at the level of the subintestinal vessel (white arrows), SU4516-treated larvae show reduced Laminin levels (magenta arrowheads) that are not apparent in DMSO-treated controls. Quantification of levels of expression of Laminin in control (D, right, level x in C) compared to treated (E, left, level y in C´) larvae reveals a specific reduction in the basal lamina of the dorsal intestine epithelium (di), where the dorsal aorta is located, and in both ventral intestine epithelium (vi) and visceral mesothelium (vm), which together delimit the subintestinal vessel (siv). White asterisks, $P \leq 0.0018$ with Welch´s t-test. No differences were observed in the dorsal basal lamina (de) between the epidermis (epi) and the neural tube (nt), in that ventral to the neural tube (vn), or in the parietal mesothelium (pm) located between the subintestinal vessel and the epidermis (detail, E right). Yellow and magenta represent F-Actin and Laminin, respectively, unless otherwise noted. Scale bar, 50μm.

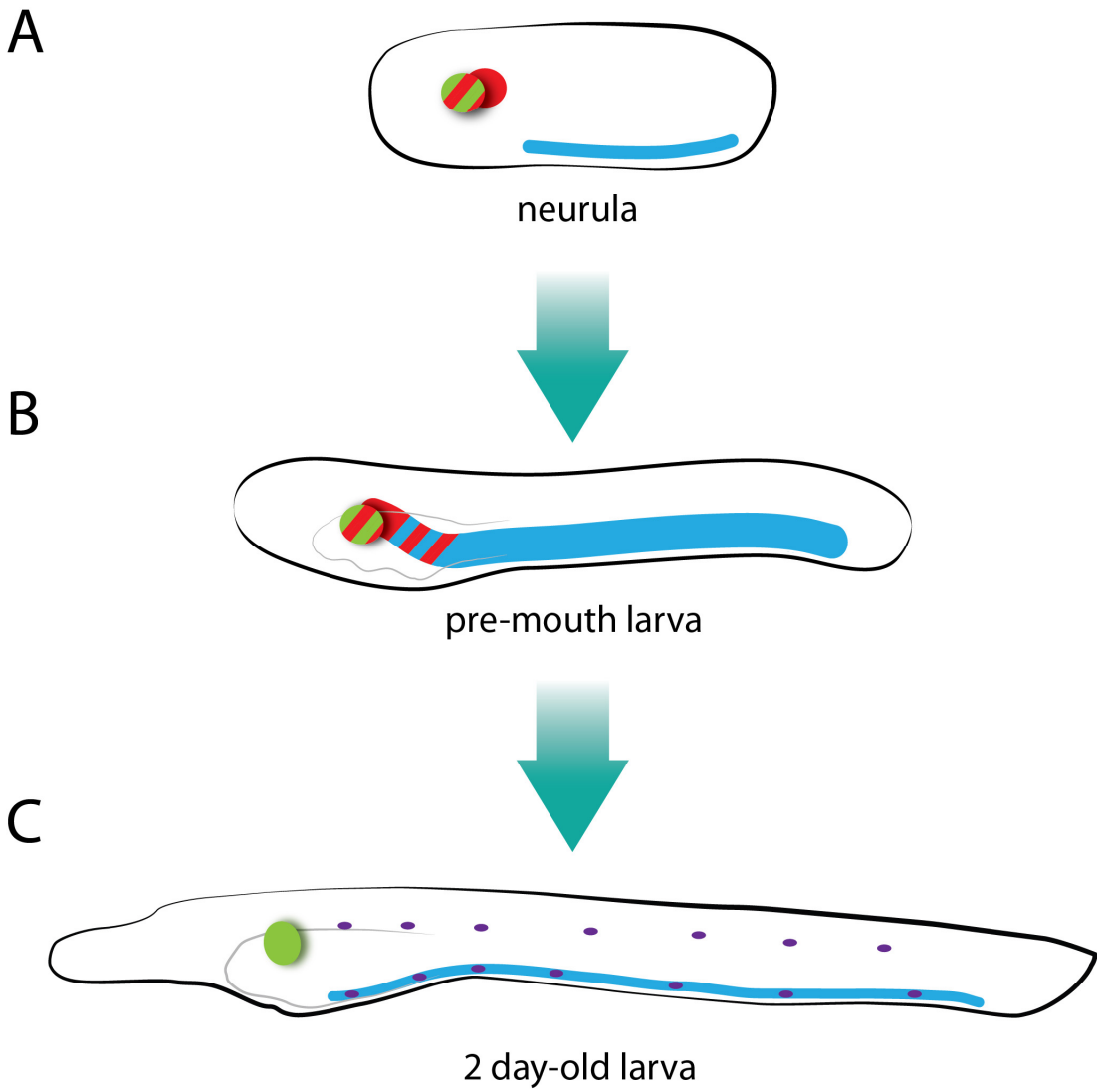

Figure 6. Schematic representation of the development of the cardiac and hematopoietic domains in amphioxus. (A) At the neurula stage, the neprhogenic (green), hematopoietic (red) and cardiogenic (blue) domains are determined. The nephrogenic and hematopoietic domains are associated in the Hatschek's nephridium, on the left side. The cardiac domain consists of ventral mesoderm, corresponding to the subintestinal vessel anlages. (B) At the pre-mouth larval stage, the cardiogenic mesodermic domain broadens from pharynx to tail. The hematopoietic domain expands from lateral spots to medial and more posterior cells, the latter entering contact with the cardiac domain. (C) Finally, *Scl* expression is no longer detected, indicating that early hematopoiesis has finished, and specified *Pdvegfr*+ hemal cells (purple) are detected in

1 both the dorsal aorta and subintestinal vessel. These *Pdvegfr*+ cells have an important
2 role in the development of such vessels, and are probably similar to the invertebrate-
3 type hemal cells from which the vertebrate endothelium originated. The cardiac vessels
4 have already been specified, consisting of pharyngeal and subintestinal vessels. The
5 Hatschek's nephridium is formed in the dorsal, left side of the pharynx.