Cephalochordate Hemocytes: First Demonstration for *Asymmetron lucayanum* (Bahamas Lancelet) Plus Augmented Description for *Branchiostoma floridae* (Florida Amphioxus)

Nicholas D. Holland* **and Linda Z. Holland**

Marine Biology Research Division, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093-0202

Abstract

*Within phylum Chordata, the subphylum Cephalochordata (amphioxus and lancelets) has figured large in considerations of the evolutionary origin of the vertebrates. To date, these discussions have been predominantly based on knowledge of a single cephalochordate genus (*Branchiostoma*), almost to the exclusion of the other two genera (*Asymmetron *and* Epigonichthys*). This uneven pattern is illustrated by cephalochordate hematology, until now known entirely from work done on* Branchiostoma. *The main part of the present study is to describe hemocytes in the dorsal aorta of a species of* Asymmetron *by serial block-face scanning electron microscopy. This technique, which demonstrates three-dimensional fine structure, showed that the hemocytes have a relatively uniform morphology characterized by an oval shape and scanty cytoplasm. Ancillary information is also included for* Branchiostoma *hemocytes, known from previous studies to have relatively abundant cytoplasm; our serial block-face scanning electron microscopy provides more comprehensive views of the highly variable shapes of these cells, which typically extend one or several pseudopodium-like protrusions. The marked difference in hemocyte morphology found between* Asymmetron *and* Branchiostoma *was unexpected and directs attention to investigating comparable cells in the genus* Epigonichthys. *A broader knowledge of the hemocytes in all three cephalochordate genera would provide more balanced insights into the evolution of vertebrate hematopoiesis.*

Introduction

Cephalochordate hemocytes: a possible early stage of circulatory system evolution in chordates

Within phylum Chordata, the subphylum Cephalochordata is sister group to the tunicates plus vertebrates (Delsuc *et al.*, 2008). Cephalochordates, in addition to occupying a key phylogenetic position, are evolving extremely slowly (Yue *et al.*, 2014). They are therefore the best available stand-ins for the proximate ancestor of the vertebrates, thus affording insights into the starting conditions for vertebrate evolution (Shimeld and Holland, 2005; Holland *et al.*, 2008; Holland and Holland, 2022). From this evolutionary point of view, we are examining the hemocytes of cephalochordates. Until now, everything known about this subject derived from studies of the relatively accessible genus *Branchiostoma*. For the other two genera (*Asymmetron* and *Epigonichthys*), the very existence of hemocytes had never been determined. Here we use serial block-face scanning electron microscopy (SBSEM) to describe the 3-D fine structure of these cells in *Asymmetron lucayanum*. The present contribution also includes new information

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* Corresponding author; email: nholland@ucsd.edu.
Abbreviations: l-rl, laminin-rich layer; SBSEM, serial block-face scanning electron microscopy.

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on *Branchiostoma* hemocytes; although these cells have often been studied before, SBSEM provided the first reliable overview of their strikingly varied shapes.

Cephalochordate hemocytes in the context of the vascular system as a whole

The literature on the cephalochordate circulatory system is extensive and unusually controversial (as is evident from Table 1). There have been diverse opinions about the number of hemocyte types (and even about whether they exist at all) as well as about the possible presence of an endothelium and a lymphatic system. Available knowledge favors a single kind of hemocyte (at least within a given genus); moreover, there is no solid evidence for an endothelium or a lymphatic system.

Müller (1844), the first to elucidate the overall course of cephalochordate hemal channels, realized that they are quite similar to the circulatory circuit of a fish. The main hemal channels in a cephalochordate are the veins and endostylar artery ventrally and the dorsal aortae dorsally

Table 1

Opinions about features of the circulatory system of cephalochordates

Feature	Absent	Only in some places	Present throughout
Endothelium	Hausmann, 1932 Altschul, 1954 Moller and Philpot, 1973b[b] Rähr, 1981a, 1981b Tomonaga, 1992 Ruppert, 1997 Schmitz *et al.*, 2000 Stach, 2000 Shigei *et al.*, 2001 Arid, 2006 Grimes and Kirby, 2009 Monahan-Earley *et al.*, 2013 Pascual-Anaya *et al.*, 2013	Zarnik, 1905[a] Joseph, 1914 Franz, 1927 Kampmeier, 1969 Casley-Smith, 1987	Legros, 1902 Schneider, 1902 Franz, 1925 Nakao, 1965 Azariah, 1966 Casley-Smith, 1971 Randall and Davie, 1980 Simoes-Costa *et al.*, 2005
Hemocyte(s)	Absent	One kind	More than one kind
	Quatrefages, 1845 Hatschek, 1888 Metchnikoff, 1892 Franz, 1933[i] Rowley *et al.*, 1984 Zhang *et al.*, 1992 Mao, 1997	Huxley, 1848[c] Marcusen, 1864[e] Krukenberg, 1881[g] Jordan, 1933 Andrew, 1965 Moller and Philpot, 1973a Welsch, 1975 Rähr, 1981a, 1981b[k] Holland and Holland, 1991 Ruppert, 1997 Kučera *et al.*, 2009 Monahan-Earley *et al.*, 2013 Pascual-Anaya *et al.*, 2013	Jones, 1846[d] Rohon, 1882[f] Shima, 1933[h] Hilton, 1943[j]
Lymph system	Absent	Not sure	Present
	Lankester, 1889 Franz, 1927 Casley-Smith, 1971, 1987	Kampmeier, 1969	Schneider, 1878 Dubowik, 1928 Tomonaga, 1992 Zhang *et al.*, 1992 Lin *et al.*, 2011

[a] Misinterpreted the acellular laminin-rich layer of the aortae as a cellular endothelium.
[b] Hemocytes said to be scattered endothelial cells; interpreted here as no endothelium.
[c] Pale green cells: presumably microalgae from gut lumen of cut amphioxus.
[d] Granule cells and agranular cells.
[e] Anucleate round blood cells.
[f] Red cells and white cells.
[g] Reddish cells: color possibly subliminally suggested by vertebrate erythrocytes.
[h] Granule cells (of several sizes and colors) and agranular cells plus rafts of about a dozen cohering epithelial cells, probably fragments of broken-up epithelia.
[i] No "real" hemocytes present (whatever that means).
[j] Granule cells (of several colors) and agranular cells reported in juice from cut-open amphioxus.
[k] Rähr referred to these as "several cell types," but that is interpreted here as a single cell type with varied shapes.

(Fig. 1A). The chief connections between these ventral and dorsal channels are passages, comparable to vertebrate aortic arches, running within the gill bar skeleton.

As seen at the histological level, the hemal channels are bounded, not by a cellular endothelium but by basal laminae of adjacent epithelia or mesothelia or by extracellular matrices of neighboring connective tissues (Fig. 1B, C). On the ventral side of the body, coeloms associated with the veins and subendostylar artery are lined with cells containing microfilaments (Storch and Welsch, 1974). Contractions of these microfilaments account for peristalsis along the ventral hemal channels in a predominantly anterior direction (Fig. 1B). In contrast, the aortic channels on the dorsal side of the body are not associated with myofilament-containing epithelial cells (Fig. 1C). Moreover, the hemal fluid in the aortae is surrounded by an acellular laminin-rich layer that is relatively thick and firm (Kučera *et al.*, 2009). Thus, posterior flow in the dorsal portion of the circulatory system is driven not by peristalsis but by pressure from behind.

The fluid within the hemal channels, as seen by conventional transmission electron microscopy (TEM) or by SBSEM, comprises a densely packed mass of about 20-nm particles rich in glycoprotein and lipid. It has been proposed that these components are combined into a single macromolecule of phospolipoglycoprotein, evidently synthesized by endodermal cells and exported into adjacent hemal channels to serve, among other things, as a vitellogenin (Sun and Zhang, 2001; Han *et al.*, 2006). The liquid nature of the hemal fluid was demonstrated by Rähr (1981a, 1981b) by ink injection, although the viscosity has yet to be measured. Moreover, because easily visualized particles (like cells) have never been seen suspended in the fluid, it has not been possible to visualize flow velocity *in situ*. Certainly, there is no justification for equating the flow velocity with the speed of the anteriorly progressing peristaltic contractions along the ventral vessels (*e.g.*, the 300 μm s^{-1} measured by Skramlik, 1938). In spite of these unknowns, cephalochordate circulation is often overconfidently qualified by such adjectives as "vigorous" (*e.g.*, by Furst, 2020). As an added complication, the anterior peristalsis of the ventral vessels can switch periodically to posterior peristalsis—infrequently (Skramlik, 1938), often (Azariah, 1965), or not at all (Ruppert, 1997), depending on the species.

The hemal fluid typically occupies the full volume of the channels in the ventral region of the body. By contrast, however, the hemal fluid in the dorsal aortae is limited to the center of the vessel and is surrounded by a cortical zone of coarser and more loosely packed material. This peripheral layer was first seen by Zarnik (1905), who mistakenly thought it was cellular and represented an endothelium. Moller and Philpot (1973a) later correctly identified the layer as a kind of acellular connective tissue. Subsequently, Rähr (1981a) injected the circulatory system of living cephalochordates with ink and, as already mentioned, showed that the marker was transported in the hemal fluid while the peripheral material in the aorta remained stationary.

The work of Rähr (1981a), although superb, did include a potential source of confusion by designating the peripheral connective tissue in the aorta as a "basement lamella"—a term not easily distinguished from "basal lamina," which, as mentioned, is a component of tissues bordering some

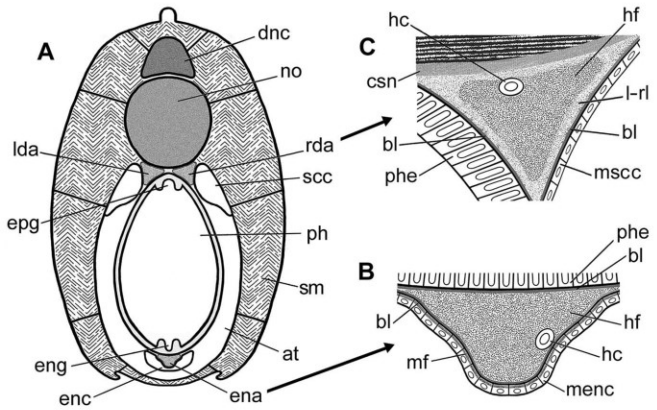

Figure 1. Schematic views of the histology of the major hemal channels of a cephalochordate. (A) The entire body cross-sectioned through the pharyngeal region to provide context for the more detailed diagrams of the endostylar artery (B) and the dorsal aorta (C). at, atrial cavity; bl, basal lamina; csn, collagenous sheath of notochord; dnc, dorsal nerve cord; ena, endostylar artery; enc, endostylar coelom; eng, endostylar groove; epg, epipharyngeal groove; hf, hemal fluid; l-rl, laminin-rich layer; lda, left dorsal aorta; menc, mesothelium of endostylar coelom; mf, myofilaments; mscc, mesothelium of subchordal coelom, no, notochord; ph, pharyngeal lumen; phe, pharyngeal endoderm; rda, right dorsal aorta; scc, subchordal coelom; sm, segmental musculature.

of the hemal channels and extraneous to them. Because the danger of confusion is considerable, the present paper will refer to the peripheral extracellular material as the "laminin-rich layer" (l-rl), a name justified by the results of Kučera *et al.* (2009), who studied postlarval cephalochordates and found strong immunostaining for laminin associated with the dorsal aortae, not only in the thin basal laminae of adjacent tissues but also in the thick zone peripheral to the hemal fluid.

Advantages and drawbacks of the serial block-face scanning electron microscopy technique

It is useful at the outset to outline the chief steps in the SBSEM procedure that was used in the present study. The first step is to scan the block face of the embedded specimen and record a backscattered image; then a microtome in the specimen chamber shaves away a thin layer from the block. The alternating scanning and shaving of the block face yields a *Z*-series of images (Kornfield and Denk, 2018; Schneider *et al.*, 2021) that can be converted to 3-D by a computer program. Each image superficially resembles conventional TEM, although at somewhat lower resolution. In the present study, this technique brought to light considerable differences in hemocyte size and shape between the two genera studied (*Asymmetron* and *Branchiostoma*). This diversity has major consequences for discussions of the evolution of circulatory systems in the phylum Chordata, as will be discussed.

Materials and Methods

Adults of the Bahamas lancelet, *Asymmetron lucayanum* Andrews, 1893, were sieved from the soft substratum at low tide in the lagoon (25.72297° N, 79.29288° W) on North Bimini Island, Bahamas. The animals were 1.8-cm long, the commonest size class of lancelets collected in Bimini at any time of year. For a study of the Florida amphioxus, *Branchiostoma floridae* Hubbs, 1922, unripe adults (2 cm long) were obtained from a laboratory culture maintained at Scripps Institution of Oceanography. For both species, the notochord with adjacent tissues was dissected from the rest of the animal about midway along the body length.

Primary fixation for SBSEM was at 4 °C for 2 weeks in 0.15 mol L^{-1} cacodylate buffer (pH 7.4) containing 2% formaldehyde, 1.5% glutaraldehyde, and 2 mmol L^{-1} $CaCl_2$ (Deerinck *et al.*, 2010). The sequence of postfixation steps was in reduced osmium tetroxide, thiocarbohydrazide, osmium tetroxide, uranyl acetate, and lead aspartate under conditions detailed in Wanner *et al.* (2016). After dehydration in ethanol, specimens were transferred through acetone and embedded in Durcupan resin. Each tissue block was oriented to show block faces oriented perpendicular to the long axis of the body. We gathered images for two specimens of each species to check for the degree of difference between individual animals in hemocyte morphology (no substantial differences were found).

For SBSEM, the tissues were observed in a 3View system (Gatan, Pleasanton, CA) in a Zeiss Merlin SEM (Oberkochen, Germany). Block faces were imaged every 0.25 μm. The series of 2-D images was converted to 3-D with the Reconstruct program (Fiala, 2005; Borrett and Hughes, 2016). The Reconstruct program permitted expression of the data as Boissonnat surfaces in 3-D and was also used to render some tissues and cells semitransparent, thus allowing visualization of internal structures.

Results

Hemocytes of Asymmetron lucayanum

Figure 2A is a side view of a segment of the right dorsal aorta rendered semitransparent to show the hemocytes within; those associated with the vessel wall toward the observer are marked with asterisks. These cells are relatively widely scattered and not at all associated with one another. Moreover, much of the cell volume is taken up by the nucleus, which is surrounded by a relatively scanty amount of cytoplasm. Figure 2B shows a view down the axis of the same aortal segment. In this view, the hemocyte shapes can be seen to range from squamous to cuboidal. Furthermore, the entire basal surface of most cells is closely associated with the l-rl, but with two exceptions. First, hemocyte 6 is associated with the l-rl only by two footlike projections, while the rest of the cell extends centrally, far into the hemal fluid, and, second, hemocyte 8 is located in a channel entirely within the substance of the l-rl.

Figure 2C is an individual SBSEM block-face image of hemocyte 2. The basal side of the cell is closely associated with the inner surface of the l-rl, while the apical side is bathed by the hemal fluid. In the scanty cytoplasm, a few mitochondria are present, but the other organelles visible (mostly small vesicles and granules) are not identifiable in our relatively low-resolution SBSEM. It could, however, be determined that the cytoplasm included neither obvious profiles of endoplasmic reticulum nor the microtubule-packed spheres characterizing *Branchiostoma* hemocytes and described below. In contrast to the more typical hemocytes, two of those shown in Figure 2A, as already mentioned, are conspicuously different. One of them, hemocyte 6, is shown as a block-face image in Figure 2D. It is only tenuously attached to the l-rl by two short cytoplasmic projections, while most of the cell body projects apically into the hemal fluid. The other exception is hemocyte 8, shown in Figure 2E, which is penetrating the l-rl and leaving behind a channel filled with hemal fluid. These two exceptional hemocytes will be considered further in the Discussion.

Hemocytes of Branchiostoma floridae

Figure 3A shows a cross section of the right dorsal aorta; when viewed from the arrowed direction, the bracketed region was relatively planar. This insured minimally distorted

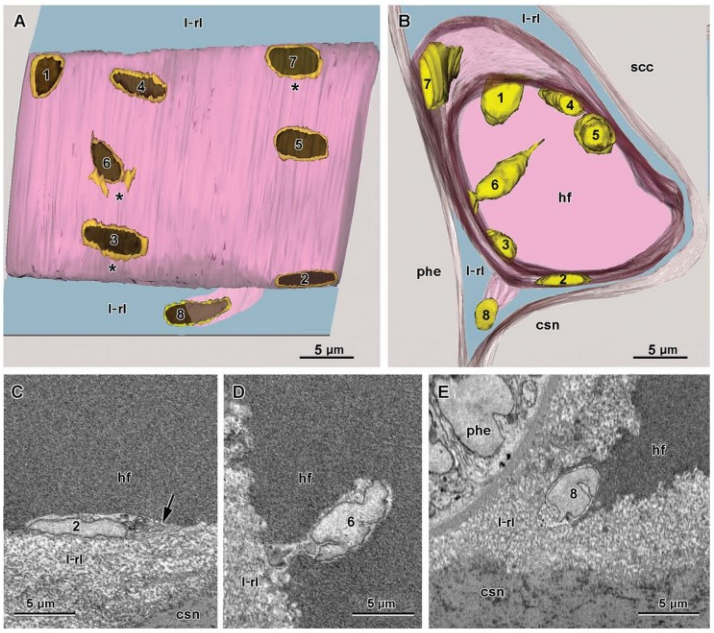

Figure 2. *Asymmetron lucayanum*. (A) Serial block-face scanning electron microscopy reconstruction of segment of right aorta in side view and depicted semitransparently to demonstrate the hemocytes within; the hemocyte cytoplasm is also semitransparent to reveal the nucleus within. Asterisks mark hemocytes on the aortic wall toward the observer. (B) Same segment of aorta in axial view showing hemocytes 1–7 associated the inner layer of the l-rl and bulging into the hf; in contrast, hemocyte 8 appears to be entirely embedded within the l-rl. (C) Serial block-face scanning electron microscopy image of block face showing hemocyte 2 closely associated with the inner surface of the l-rl; the arrow indicates a mitochondrion. (D) Serial block-face scanning electron microscopy image of hemocyte 6 attached to l-rl by two footlike processes and extending into the hf. (E) Serial block-face scanning electron microscopy image of block face showing hemocyte 8 occupying a burrow on the l-rl. csn, collagenous sheath of notochord bordering aorta, hf, hemal fluid; l-rl, laminin-rich layer; phe, pharyngeal endoderm bordering aorta; scc, subchordal coelom bordering aorta.

views of hemocyte shapes. In Figure 3B, the eight illustrated hemocytes of *Branchiostoma*, like those of *Asymmetron*, are widely scattered and not at all associated with one another. However, there the resemblance ends because the former have abundant cytoplasm and an overall shape that varies markedly from one cell to the next. The 3-D reconstructions showed that the cytoplasm typically extends away from the nuclear region in one or several long, relatively narrow cytoplasmic projections.

We also include SBSEM block-face images of *Branchiostoma* hemocytes here, confirming previous conventional TEM studies by others (*e.g.*, the high-quality work of Monahan-Earley *et al.*, 2013). Our Figure 3C and Figure 3D, respectively, show SBSEM block-face images of *Branchiostoma* hemocytes 8 and 4. The cytoplasmic contents of such cells (unlike those of *Asymmetron*) include conspicuous profiles of distended rough endoplasmic reticulum, a feature previously noted by Moller and Philpot (1973a), as well as dense spheres, about 0.5 μm in diameter, noted in almost all previous TEM studies of *Branchiostoma* hemocytes. Such spheres have been described as having a microtubular substructure in a few conventional TEM investigations (Moller and Philpot, 1973a; Zhang *et al.*, 1992; Monahan-Earley *et al.*, 2013). However, most such studies overlooked this detail, and, similarly, we could not demonstrate it in our SBSEM images. These distinctive spheres have not been found in any other cephalochordate cell type and will be considered further in the Discussion.

Discussion

Cephalochordate hemocytes: possible infrequent transport in hemal fluid and cell mobility

Most of the hemocytes in aortae of *Asymmetron* and *Branchiostoma* appear firmly associated with the inner surface of the l-rl. This agrees with the general assumption (*e.g.*, Monahan-Earley *et al.*, 2013) that cephalochordate hemocytes do not ordinarily circulate. However, there may be rare exceptions to this rule. For example, hemocyte 6 described here for *Asymmetron* extends far into the hemal

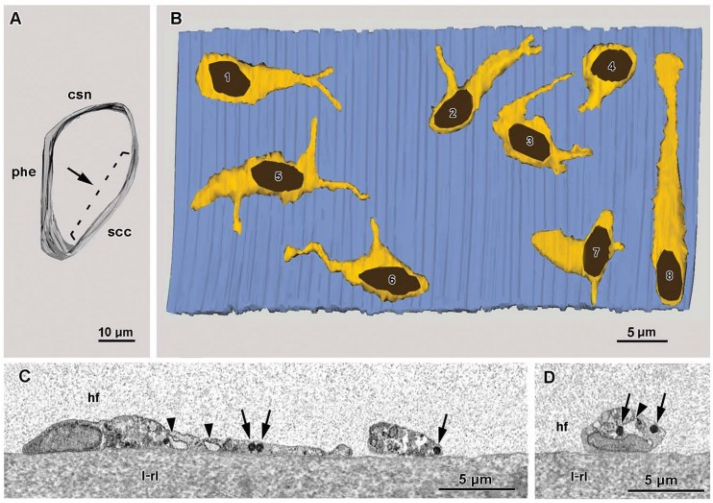

Figure 3. *Branchiostoma floridae*. (A) Axial view of segment of right aorta. The arrow and dashed bracket indicate, respectively, the direction of viewing the inner surface of the aorta and the extent of the view shown in (B). (B) Inner surface of aorta showing eight hemocytes closely associated with the l-rl (blue); semitransparent cytoplasm reveals the nucleus within. (C) Serial block-face scanning electron microscopy block-face image of hemocyte 8, in the long axis of its cytoplasmic protrusion. (D) Serial block-face scanning electron microscopy block-face image of hemocyte 4, the smallest seen. In (C) and (D), the arrowheads and arrows, respectively, indicate profiles of rough endoplasmic reticulum and microtubule-containing granules (content not visible here). Arrowheads indicate profiles of distended rough endoplasmic reticulum; arrows indicate dense spherules specific for *Branchiostoma* hemocytes. csn, collagenous sheath of notochord bordering aorta; hf, hemal fluid; l-rl, laminin-rich layer; phe, pharyngeal endoderm bordering aorta; scc, subchordal coelom bordering aorta.

fluid and is only tenuously anchored to the l-rl. This configuration suggests that this cell might be reattaching after a journey in the hemal fluid or, conversely, might be on the point of releasing itself to enter the circulation.

Although cephalochordate hemocytes, when observed in fixed material, appear tightly associated with the inside of the l-rl, it would not be surprising to find that, on an extended timescale, the living cells progress along that surface. In addition, slow changes in overall shape of the hemocytes probably also occur. *Asymmetron* hemocytes might alternate between squamous and cuboidal states, while *Branchiostoma* hemocytes might undergo striking shape changes like amebae. The fine structure of *Asymmetron* hemocytes is so unspecialized that one is at a loss to suggest specialized functions for them. However, the apparent excavation of the l-rl by hemocyte 8 (Fig. 2A, B, E) suggests that the hemocytes in general may help sculpt the inner contours of the vessels or initiate new circulatory channels, as considered below.

Possible functions of cephalochordate hemocytes

Very little is known about the functions of *Branchiostoma* hemocytes. Moller and Philpot (1973b) showed that the cells could take up ferritin and horseradish peroxidase *via* coated- and smooth-surfaced vesicles; those authors tentatively suggested that the cells roam about, vacuuming contaminating particles from the surface of the l-rl. Kučera *et al.* (2009) claimed that *Branchiostoma* hemocytes engage in phagocytosis and thus resemble vertebrate macrophages, but this is a mistake based on a misreading of Rhodes *et al.* (1982), who studied only the phagocytic celomocytes and not the hemocytes of *Branchiostoma*.

The cytoplasm of *Branchiostoma* hemocytes also includes profiles of distended rough endoplasmic reticulum, which suggests that they export proteinaceous material. It is unlikely that such export would be to augment the endodermally derived phospolipoglycoprotein component of the hemal fluid. It also seems unlikely that the exported proteins contribute to the l-rl (as suggested by Strillić *et al.*, 2010) because in *Asymmetron*, the hemocytes include no detectable rough endoplasmic reticulum. Thus, for cephalochordates in general, it is very likely that the l-rl is synthesized by epithelia neighboring the hemal channels.

A role for the distinctive microtubule-filled spheres from *Branchiostoma* hemocytes was suggested by Moller and Philpot (1973a), who proposed (not very convincingly) that the spheres released their microtubules into their cytoplasmic matrix to ensure a high level of motility by the pseudopodia-like extensions of the hemocytes. However, the same authors correctly discounted the possibility that the spheres are related to vertebrate Weibel-Palade bodies. In vertebrates, such organelles are packed with structures

that resemble microtubules but consist not of tubulin but of the glycoprotein von Willebrand factor (Fujimura and Holland, 2022), a vertebrate-specific molecule not encoded by the genomes of cephalochordates (our data) or tunicates (Grant *et al.*, 2017).

In sum, the available information on cephalochordate hemocytes is predominantly static—even for hemocytes of *Branchiostoma*, which have been described many times by electron microscopists over the last half century. At this juncture, there is much need for studies of hemocyte function. For instance, techniques of single-cell transcriptomics and cell-specific proteomics have recently provided new insights into vertebrate hematopoietic cell differentiation and detailed function (Zhang *et al.*, 2022). Moreover, with such techniques, one might discover how cephalochordate hemocytes might function in the wide spectrum of innate immune responses that have been reported for *Branchiostoma* (Lin *et al.*, 2011; Xu, 2015).

Cephalochordate hemocytes arise during development in the absence of an endothelium

In some ventral vessels of the *Branchiostoma* hemal system, hemocytes may be locally so concentrated in some places as to have been considered a localized region of endothelium (Table 1). However, as pointed out by Ruppert (1997), such cells are not united by any sort of specialized cell-to-cell association zones and certainly not by the tight junctions and adherens junctions characterizing vertebrate endothelial cells. The present discussion is written from the point of view that the cephalochordate vascular system lacks an endothelium and examines how this affects discussions of chordate circulatory system evolution.

A still unanswered question about cephalochordate hemocytes is their provenance during embryology. It is possible that they originate from cells of the ventral mesoderm (the equivalent of vertebrate lateral plate mesoderm), because the latter is the source of hematopoietic cells appearing during vertebrate embryology (Prummel *et al.*, 2020). From their first appearance at the early larval stage (Holland and Holland, 2010), all the cells within cephalochordate hemal vessels evidently comprise only hemocytes. This is supported by the genetic signature similarities between developing cephalochordate hemocytes and differentiating blood cells of vertebrates (Pascual-Anaya *et al.*, 2013). It should be mentioned in passing that the purported extravascular origin of lymphocytic hemocytes in cephalochordates (Huang *et al.*, 2007) is a mistake caused by cutting tangential instead of cross sections though an epithelial layer (for comparison, a true cross section of the same tissue is in Ruppert, 1997).

A discussion of the origins of endothelial cells and hematopoietic cells during chordate evolution is hampered by still-unsettled controversies about the development of these cell types in vertebrates. The traditional concept of a hemangioblast as a bipotent progenitor of both endothelial and hemopoietic cells is starting to be replaced by the notion of an early appearance of an endothelial cell population that subsequently gives rise to a hematopoietic population (Hou *et al.*, 2020; Chambers *et al.*, 2021). If one assumes that this sequence is correct, difficulties still remain for discussions of the evolution of hemal systems between cephalochordates (with hemocytes as the first and only representatives) and vertebrates (with endothelial cells early and hematogenic cells later). For instance, if one starts from the cephalochordate condition and proposes conversion of some hemocytes into endothelium later in chordate evolution, one would still need to assume an even later reversal of the cytodifferentiative series to bring about the endothelium-first situation in vertebrates.

The reversal difficulty mentioned above points to the desirability of broadening the discussion of chordate circulatory system evolution to include the chordate subphylum Tunicata, sister group of the vertebrates. The cells of tunicate hemal channels are hemocytes, and there is no clearly defined endothelium, in spite of the occasional designation of some regions of the tunicate epidermis as a "functional equivalent of endothelium" or a "vascular epithelium" (Burighel and Cloney, 1997). In fact, the cellular architecture of cephalochordate and tunicate hemal channels is essentially the same, except perhaps in one interesting respect. A study by Rinkevich *et al.* (2010) suggests that tunicates have "small cells" with stemlike properties widely scattered at the periphery of the hemal channels. In addition to including this possible early stage of endothelium formation, tunicates are also more vertebrate-like in having rapidly flowing blood plasma containing several hemocyte types.

Diverse modes of lumen formation in vertebrate and cephalochordate circulatory systems

Because cephalochordates lack the endothelium characteristic of vertebrates, the vessels of the circulatory system must be established and extended quite differently in the two groups. In the vertebrates, vasculogenesis is *de novo* blood vessel formation not arising from existing vasculature and considered to proceed from a single cell type. These cells were long considered hemangioblasts that later produce both endothelium and blood cells (Wu and Hirschi, 2021). However, as already mentioned, there is an emerging conception that endothelial cells appear first and subsequently give rise to hematogenic cells (Chambers *et al.*, 2021).

Vertebrate angiogenesis, by contrast to vasculogenesis, involves the prior existence of a solid cord of primordial endothelial cells that transforms into a hollow vessel by sequential production of tip and stalk endothelial cells followed by lumenization and only later by production of the hematogenic lineage (Eelen *et al.*, 2020). The important point is that vertebrate vasculogenesis and angiogenesis both evidently involve early participation of endothelial cells and not blood cells. Thus, neither vertebrate process

appears have anything in common with hemal vessel formation and extension in cephalochordates.

During cephalochordate development, it is likely that the main course of the circulatory system is laid out in the late neurula embryo, in which spaces derived from the blastocoel are being sandwiched between the neighboring epithelia of the developing organs and are beginning to accumulate small amounts of extracellular material (Mansfield *et al.*, 2015). Presumably, the next step would be colonization of these spaces by hemocytes derived from ventral mesoderm cells (as already mentioned). The next stage would be the filling of the dorsal hemal channels with laminin-rich extracellular matrix followed by its partial removal from the center of the channel by the action of the hemocytes as demonstrated by Kučera *et al.* (2009). As shown by Figure 2E, the hemocyte burrowing through the l-rl shows no obvious signs of endocytosis and presumably progresses by the release of lytic secretions, cell motility, or both.

Conclusion

The present study of cephalochordate hemocytes provides the first information on such cells for genus *Asymmetron* and adds new findings for genus *Branchiostoma*. Unexpectedly, hemocyte morphology differs strikingly between these two genera. It is not clear whether these differences reflect retrogressive evolution in *Asymmetron* or innovation in *Branchiostoma*. In any event, this wide discrepancy raises the question of which, if either, genus should be used to represent the plausible ancestral starting condition for discussions of circulatory system evolution within the chordates. It is possible that the situation could be improved by studying the hemocytes of the third cephalochordate genus, *Epigonichthys*. At the least, this would permit discussion of genus-level evolution of the character within the subphylum Cephalochordata, and it could lead to more realistic discussions of blood cell evolution within the phylum Chordata as a whole.

Acknowledgments

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