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Ultrastructure of Zebrafish Dorsal Aortic Cells

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ABSTRACT

Expression of vascular smooth muscle cell (VSMC) markers such as serum response factor (SRF) is complicated in zebrafish because of the ill-defined histology of the dorsal aorta and the presence of perivascular pigment. We report the ultrastructure of aortic cells in 7-day, 1-month, and 3 month-old zebrafish and provide clear evidence for the presence of perivascular melanocytes harboring an abundance of melanin. In 7-day-old larvae, endothelial cells (EC) and synthetic mural cells that display little evidence of VSMC differentiation comprise the dorsal aorta. The latter mural cells appear to fully differentiate into VSMC by 1 month of age. In 3-month-old adult zebrafish, EC exhibit greater differentiation as evidenced by the accumulation of electron-dense bodies having a diameter of ~200 nm. Adult zebrafish aortae also exhibit at least one clear layer of VSMC with the characteristic array of membrane-associated dense plaques, myofilament bundles, and a basement membrane. Subjacent to VSMC are collagen-producing adventitial fibroblasts and melanocytes. These studies indicate that fully differentiated VSMC occur only after day 7 in zebrafish and that such cells are arranged in at least one lamellar unit circumscribing the endothelium. These findings provide new data about the timing and accumulation of VSMC around the zebrafish aorta, which will be useful in phenotyping mutant zebrafish that exhibit defects in blood circulation.

INTRODUCTION

THE CARDIOVASCULAR SYSTEM is the first organ system to develop in vertebrates. Its role in both sustaining normal development and contributing to a variety of diseases has been appreciated for over a century. Only recently have genetic studies begun to assign functions of genes to specific processes related to the cardiovascular system. In mammals, evaluating the consequence of deleting cardiovascular genes on the development of this critical organ system is complicated due to the inherent difficulties in assessing cardiovascular form and function *in utero*.

Over the past decade, the zebrafish has emerged as a powerful model system for evaluating gene mutations and morphogenesis at

the earliest time points of cardiovascular development.^{1,2} Elegant microangiographic studies, for example, have shown a remarkable consistency in the general organization of blood vessels between zebrafish and mammals.³ The high degree of homology in basic patterning of the vasculature and the definition of common mutants altering vascular structure have made the zebrafish an attractive model system to complement and extend studies in mammals.⁴

Much of the genetic and anatomical work in the vasculature has focused on the endothelial cell (EC), principally because EC define the earliest blood vessels. For example, the zebrafish *cloche* mutant manifests as a vascular defect due to loss of differentiated EC.⁵ In addition to EC, vascular smooth muscle cells (VSMC) are recruited to the developing vascular wall where

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they function initially as supportive cells and later as highly specialized cells that control vessel wall caliber. The ability of VSMC to carry out such functions in the vessel wall relates to their unique transcriptome, which encompasses cell-restricted contractile, cytoskeletal, and transcription factor genes.^{6,7} To date, there has been only one report on the expression of a VSMC-restricted gene (*SM22*), but its expression in zebrafish has been rather difficult to interpret because of the extremely close juxtaposition of EC and VSMC in this animal.⁸ Further, the mutant *radar* exhibits circulatory arrest at 50 hours post fertilization (hpf), which the authors speculate could be due to defects in VSMC recruitment or function, though studies were not undertaken to address this hypothesis because of the lack of VSMC markers in zebrafish.⁹

To date, no study has reported the essential changes that must occur in zebrafish aortic ultrastructure from development to adulthood. We have been particularly interested in understanding when and to what extent VSMC invest the zebrafish aorta (e.g., how many layers of VSMC exist in the adult aorta?). Herein, we provide the first ultrastructural analysis of the larval versus adult zebrafish aorta. We have compared 7 day post fertilization (dpf) larvae with 1- and 3-month-old zebrafish and report striking differences in both EC and VSMC ultrastructure at these time points. At 3 months, but not 7 dpf or 1 month, EC are shown to harbor electron-dense organelles of unknown origin and function. While synthetic mural cells are observed as early as day 7 of development, they do not exhibit the ultrastructural features of a fully differentiated adult VSMC (i.e., little evidence of dense plaques and myofilaments). Finally, we show that the adult aorta has one clear layer of differentiated VSMC that lies between the EC layer and an adventitial layer composed of collagen-synthesizing fibroblasts and melanin-generating melanocytes. Collectively, these data provide new insight into the emergence of differentiated VSMC in zebrafish, thus providing a foundation for future studies on the gene expression profile and function of these cells in a variety of mutant phenotypes.

RESULTS

Our studies were motivated by the continuing difficulty in assessing unambiguously the expression of VSMC markers in adult zebrafish aorta. For example, expression of serum response factor (SRF), a known VSMC marker, in wild-type zebrafish aorta is difficult to interpret owing to the presence of pigment circumscribing the entire adult aortic wall (Fig. 1A, B). On the basis of positive Schmorl's histochemical staining, we have confirmed the identity of the pigment as melanin (data not shown).

In rare instances, SRF expression is seen in longitudinal sections of the dorsal aorta of wild-type zebrafish (Fig. 1C, D). *Golden* zebrafish mutants having less pigment exhibit clear and consistent SRF staining in cross-sections of the adult (3 month) aorta (Fig. 1E, F). Similar findings have been seen with the VSMC marker, smooth muscle α -actin (data not shown). The finding of SRF expression in VSMC was not reported in a previous publication of SRF staining in zebrafish.¹⁰

The presence of melanin also complicates a clear definition of the extent of VSMC investment around the dorsal aorta. To address this issue and to begin characterizing the onset of VSMC differentiation in zebrafish, we selected three time points (7 dpf, 1 month, and 3 months) to study the ultrastructure of the zebrafish aorta. High-powered light microscopic images at these three time points reveal striking differences in vessel wall caliber (diameters of $\sim 10 \mu\text{m}$, $25 \mu\text{m}$, and $50 \mu\text{m}$ at 7 dpf, 1 month, and 3 month, respectively) (Fig. 2). At the light microscopic magnification indicated, we could not ascertain with certainty the number of VSMC layers circumscribing the EC monolayer. We therefore prepared samples of dorsal aortae from each time point for transmission electron microscopic (TEM) examination.

Representative images in Figure 3 show that 7 dpf larvae exhibit an EC tube with closely juxtaposed mural cells rich in synthetic organelles. The mural cells show occasional myofibrils (data not shown) but are otherwise devoid of ultrastructural features consistent with differentiated VSMC (e.g., membrane converging actin cytoskeletal elements that create dense

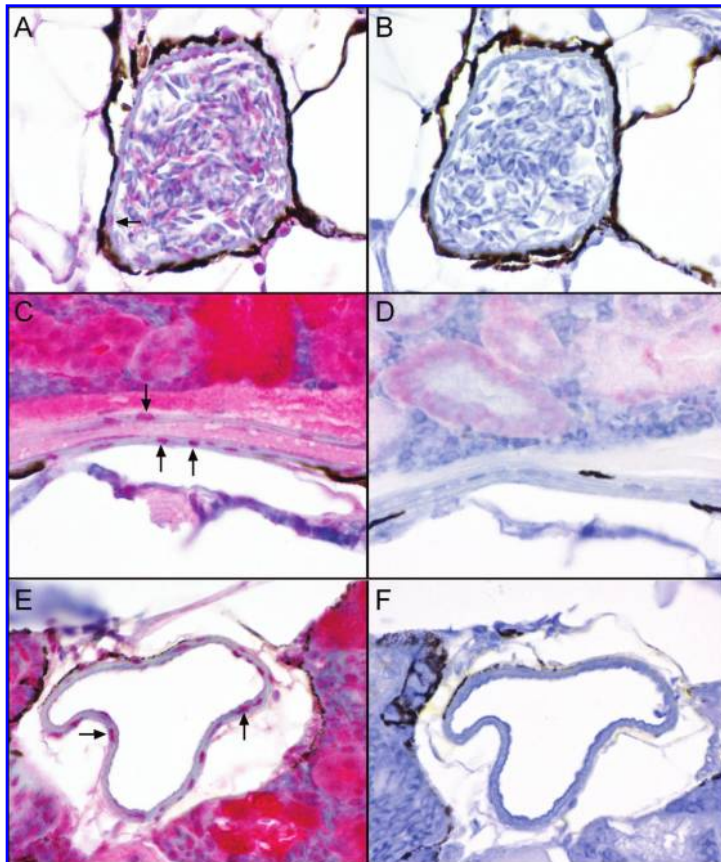


FIG. 1. Immunohistochemistry of SRF in adult zebrafish. (A) Cross-section of 3 month-old wild-type zebrafish aorta reveals positive staining for SRF in VSMC (arrow). Note extensive pigment circumscribing the dorsal aorta. (B) Adjacent section with the SRF antibody preabsorbed to purified peptide reveals loss in VSMC staining. Note extensive pigment circumscribing the dorsal aorta. (C) Longitudinal section of wild-type dorsal aorta showing positive SRF nuclear staining in VSMC (arrows). (D) The positive SRF nuclear staining is abolished upon preabsorption with SRF peptide; some pigment can be seen at both ends of the dorsal aorta. (E) Cross-section of 3-month-old *golden* zebrafish mutant aorta (with minimal pigment) stains positively for SRF in VSMC of the dorsal aorta (arrows indicate positive VSMC). (F) Preabsorption of antibody with purified SRF peptide abolishes SRF staining in VSMC. All images, 600 \times magnification.

plaques at the periphery of VSMC and a basement membrane). Moreover, no internal elastic lamina (IEL) is seen between EC and the underlying mural cells in 7 dpf larvae, though tight junctions between EC are readily evident (data not shown). Finally, very few melanin-

producing cells are observed at this time point. These data suggest that while both EC and mural cells (presumably fated for the VSMC lineage) are present in close association to one another in 7 dpf larvae, differentiation, particularly of VSMC, is incomplete.

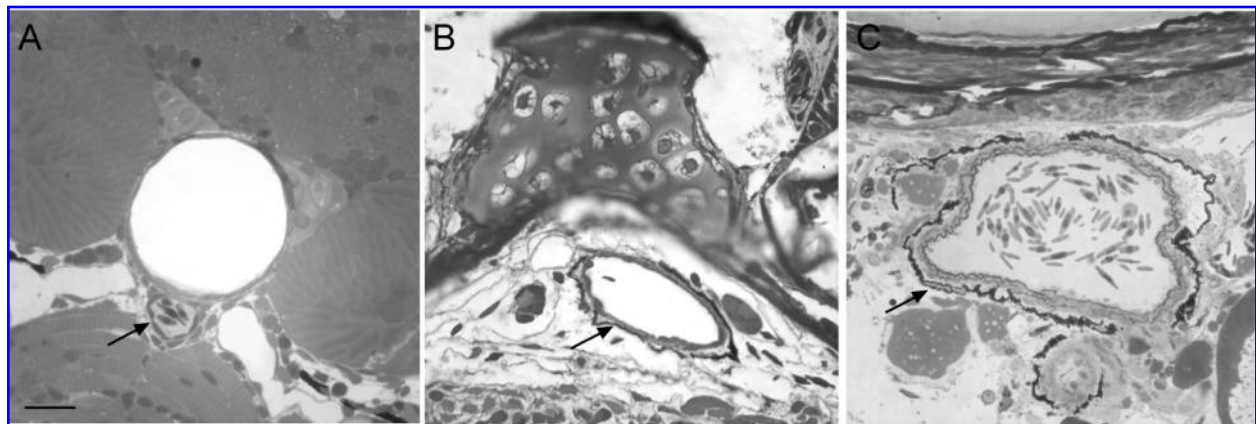


FIG. 2. Comparative light microscopy of developing dorsal aorta. One micron cross-sections of 7 dpf larval (A), 1 mpf juvenile (B), and 3 mpf adult (C) zebrafish aorta (denoted with arrows in each panel) stained with toluidine blue. Dorsal aspect of each panel is at top. Even at 600 \times magnification, it is difficult to ascertain the extent of VSMC investment. Scale bar at bottom of A = 10 μ m. Note progressive increase in caliber of dorsal aorta from 7 dpf to 3 mpf.

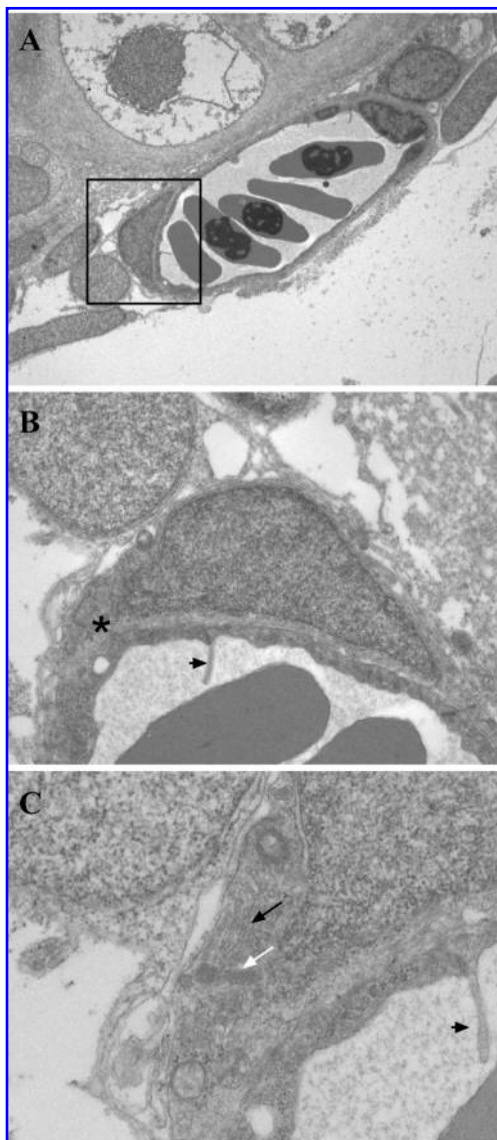


FIG. 3. Ultrastructure of 7 dpf larval aorta. (A) Transmission electron micrograph (TEM) of a mural cell (boxed) opposite a large EC nuclear profile at the 7 dpf stage. The lumen of this section contains 5 red blood cells. Magnification, 4000 \times . (B) Higher magnification of the mural cell boxed in A and rotated 90 degrees clockwise. Note the absence of any dense plaques at the cell's periphery (see Figs. 4B and 6B for comparison). Magnification, 15000 \times . (C) Higher magnification of region marked with asterisk in B showing rough endoplasmic reticulum (black arrow) and mitochondria (white arrow) with little evidence of packed myofilaments (see Fig. 6 for comparison). Arrowheads indicate extended EC process into lumen. Magnification, 30000 \times .

By 1 month of age, the zebrafish aorta shows clear evidence of melanin pigment (Fig. 4A). TEM identifies periaortic melanosomes within the melanocyte. Internal to the melanocytes are collagen-generating adventitial fibroblasts,

VSMC with peripheral dense plaques indicative of a mature actin cytoskeleton, and EC (Fig. 4B). We could readily observe the polymerization of collagen within adventitial fibroblasts

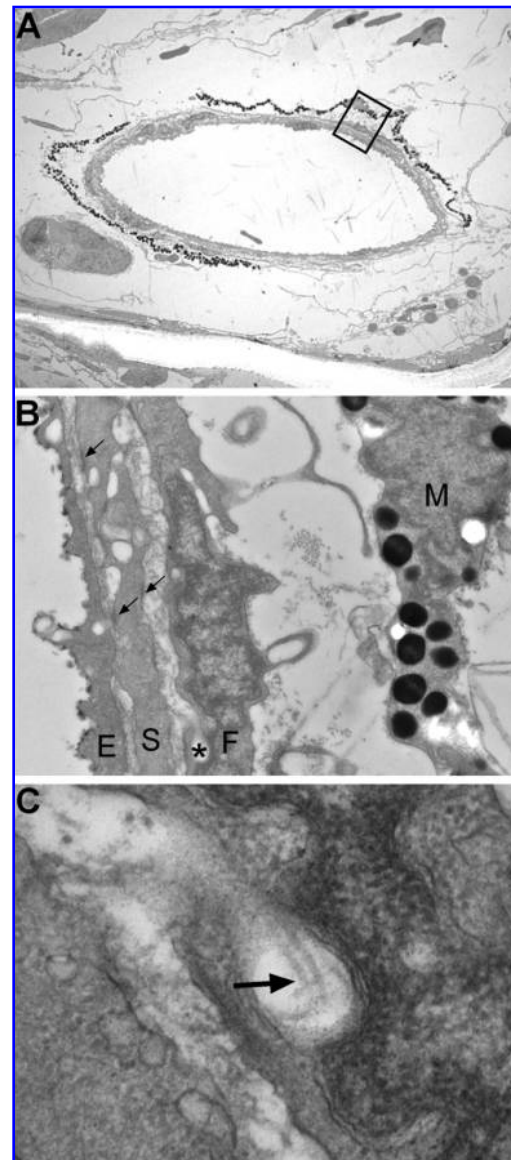


FIG. 4. Ultrastructure of 1 mpf aorta. (A) Low power TEM showing the dorsal aorta with melanin pigment surrounding much of the periphery. Magnification, 1500 \times . (B) Higher magnification of boxed region in A. Shown are 4 cell types, labeled E (EC), S (VSMC), F (adventitial fibroblast), and M (melanocyte) from lumen to outer wall. Arrows point to peripheral dense plaques in a mature VSMC. Note electron-dense melanosomes within the melanocyte external to the vessel wall. Magnification, 15000 \times . (C) High magnification image of region marked with asterisk in B. The arrow points to a region of the adventitial fibroblast in which mature collagen fibers are clearly evident. Magnification, 80000 \times .

(Fig. 4C). As in 7 dpf aortae, no IEL could be seen in the dorsal aorta of 1-month-old zebrafish (Fig. 4B and data not shown). These findings reveal that VSMC differentiation and the recruitment and differentiation of periaortic melanocytes occur between 1 and 4 weeks of development in wild-type zebrafish.

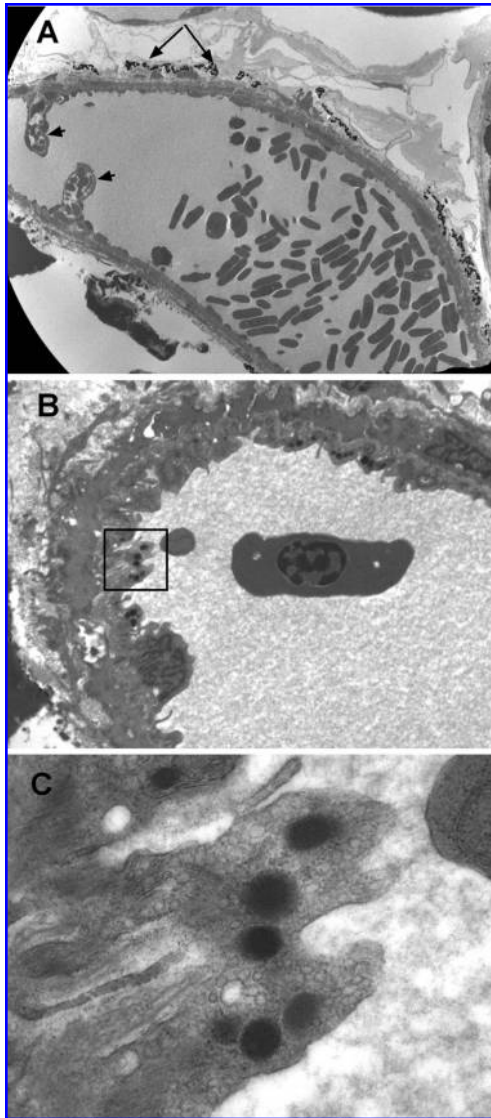


FIG. 5. Ultrastructure of 3 mpf aorta with focus on EC. (A) Low power TEM showing collections of electron dense periaortic melanosomes (arrows) and two valve leaflets (small arrowheads) extending into the lumen of the aorta. Magnification, 1000 \times . (B) Higher magnification of an independent adult aorta showing the EC layer containing electron dense bodies (boxed) and a differentiated VSMC with peripheral membrane-associated dense plaques. Magnification, 6000 \times . (C) High magnification of boxed EC in B. These organelles have a membrane and are typically, though not always, concentrated nearest the luminal edge of EC. Magnification, 40000 \times .

In 3-month-old adult zebrafish aorta, several features beyond the much greater diameter of the vessel seen in Fig. 2 are evident. Aortic valves are seen sporadically in these vessels, presumably to maintain forward flow of blood under low systolic pressure (Fig. 5A). EC in adult aorta show an accumulation of electron-dense structures that measure \sim 200 nm in diameter (Fig. 5B, C). These electron-dense EC structures are never seen in 7 dpf larvae and only rarely seen in 1-month-old fish. Because of their position in the EC, their smaller size, and weaker electron density, we do not believe the electron-dense EC structures represent melanosomes. A previous publication suggested these may be lipid inclusions;¹¹ however, staining of frozen sections with Oil-Red-O failed to support this suggestion (data not shown). High magnifications of these electron-dense EC-associated structures indicated the presence of a membrane, strongly suggestive of an identity related to an organelle (Fig. 5C and data not shown). The definitive identity and function of these organelles is presently unclear; we refer to them here as electron-dense EC organelles.

In contrast to 7 dpf larvae and 1-month-old zebrafish aorta, the adult zebrafish aorta exhibits a clear IEL that separates the EC of the tunica intima from the underlying VSMC comprising the tunica media (Fig. 6A, B). Immediately subjacent to the IEL are highly differentiated VSMC as evidenced by the presence of numerous dense plaques at the periphery of these cells (Fig. 6B), arrays of tightly packed myofilaments (Fig. 6C, D), and a basement membrane (Fig. 6E, F). At least one layer of VSMC is present in adult dorsal aorta and, in some regions, a potential second lamellar unit of VSMC is observed (Fig. 6B). Dense plaques are not seen in either EC or adventitial fibroblasts, the latter of which are frequently associated with a network of collagen fibers (Fig. 6A), likely of the type I isoform.¹²

Collectively, these results reveal a basic ultrastructural organization of the dorsal aorta in zebrafish that is similar to that in mammals with the notable exceptions of arterial valves, electron-dense EC organelles, reduced investment of VSMC, and an outer layer of melanin-generating melanocytes.

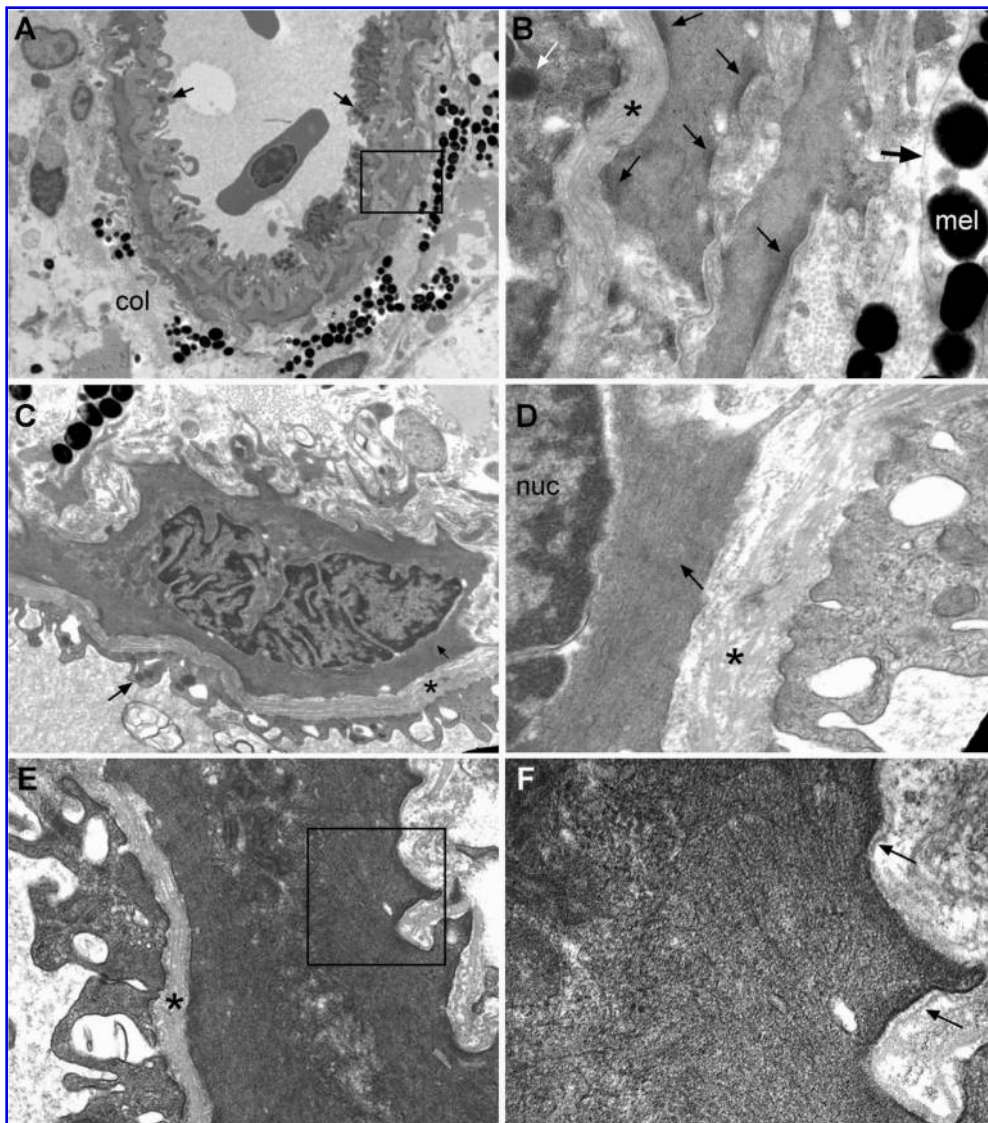


FIG. 6. Ultrastructure of 3 mpf aorta with focus on VSMC. (A) TEM of adult zebrafish aorta showing clear evidence of a wavy IEL separating EC from contractile VSMC having dense plaques at their periphery. Note the electron-dense melanosomes outside the vessel wall as well as the rich accumulation of collagen (col) fibers. Magnification, 5000 \times . (B) Higher magnification TEM of the boxed region in A. The asterisk denotes the IEL. Small black arrows point to peripheral dense plaques that define two apparent layers of differentiated VSMC. The melanosomes (mel) contained within a cell membrane (large black arrow) are bigger and more electron-dense than the EC organelles (white arrow). Magnification, 30000 \times . (C) Differentiated VSMC with large nucleus, myofilaments running throughout the cytosol, and peripheral dense plaques. The IEL is marked with an asterisk and separates the VSMC from the overlying EC (larger arrow points to a pair of electron-dense EC organelles). Magnification, 12000 \times . (D) Higher powered TEM of region marked with asterisk and small arrow in C. The nucleus (nuc) is labeled, as are a bundle of packed myofilaments (arrow) and the IEL (asterisk). Magnification, 40000 \times . (E) Another differentiated VSMC at right of IEL (asterisk). Magnification, 15000 \times . (F) High power TEM of boxed region in E. The arrows point to a classic basement membrane that encapsulates the differentiated VSMC. Magnification, 80000 \times .

DISCUSSION

We were prompted to examine the ultrastructure of the zebrafish aorta because of difficulties in interpreting our own and other reported data^{8,13,14} on the expression of VSMC

differentiation markers in VSMC of this animal. In this report, we have for the first time defined the temporal changes in ultrastructure between larval and adult zebrafish dorsal aorta. Incompletely differentiated EC and VSMC circumscribe a $\sim 10 \mu\text{m}$ diameter dorsal aorta in 7 dpf

larvae. In adult zebrafish dorsal aorta, the diameter increases ~5-fold and consists of differentiated EC overlying a well-developed IEL. Adult, but not larval or 1-month, aortic EC contain electron-dense organelles of unknown origin and function.

At least one and possibly two lamellar units of VSMC invest the adult lumen wall, and these cells have the signature ultrastructural characteristics (peripheral dense plaques, myofilaments, and basement membrane) of a differentiated phenotype that is not evident in 7 dpf larvae, but appears to be manifest by 1 month of age. Fibroblasts within the tunica adventitia appear to organize a rich collagen network similar to that seen in higher animal species.¹² Thus, the zebrafish aorta shares the same tissue organization as mammalian aortae, although the number of VSMC layers in zebrafish is only one or two.

Relatively little information exists regarding the ultrastructure of zebrafish blood vessels. Eriksson and Löfberg first commented on zebrafish aortic ultrastructure in the context of the hypochord, a transient structure immediately ventral to the notochord that is thought to position the dorsal aorta.¹⁵ Their study was confined to early aortic development (less than 24 hours pf, hpf) and showed an EC tube first emerging around the 17–20 somite-stage (~19 hpf) with no mention of mural VSMC. While we report the occasional presence of synthetic mural cells in the 7 dpf larval dorsal aorta, VSMC may in fact be recruited to the EC tube as early as 96 hpf.¹⁶ Very recently, however, EM analysis of the dorsal aorta did not reveal the presence of VSMC between 1 and 5 dpf of zebrafish development.¹⁷

Whether VSMC first emerge on 7 dpf or earlier, they are not fully differentiated, since the presence of membrane-associated dense plaques, basement membrane, and a rich array of myofilaments is not evident in such cells. We conclude therefore that fully differentiated VSMC arise only after 7 dpf. In this context, we suggest exercising caution in the interpretation of whole mount immunostaining of larval zebrafish aortae since it is impossible at the level of low-power light microscopy to discriminate EC versus VSMC expression of a gene. Further, histologic sectioning and colorimetric im-

munostaining of VSMC-restricted proteins (e.g., SRF) on even adult dorsal aorta are complicated by the presence of periaortic melanosomes, which we observe to confound accurate localization of signal to VSMC. Thus, until more VSMC-restricted genes are cloned from zebrafish and carefully analyzed, perhaps with the aid of an EM, it will be difficult to ascertain with precision the timing of VSMC differentiation.

In this report, we have defined an unusual organelle in EC that only emerges after 1 month of zebrafish development. Similar electron-dense bodies were previously reported in EC of the bulbous arteriosus and suggested to be of lipid origin.¹¹ If these were lipid droplets, they would stain readily for lipophilic stains such as Oil-Red-O and would not be membrane-bound. In fact, we could not demonstrate any positive staining for Oil-Red-O and, under high magnification, the electron-dense bodies clearly contain a membrane.

Based on these findings and a previous ultrastructural study of another teleost,¹⁸ it is possible the electron-dense EC organelles represent Weibel-Palade bodies which store clotting factor VIII. It is important to point out however that the electron-dense EC organelles reported here do not exhibit obvious tubulations that typify mammalian Weibel-Palade bodies¹⁹ and are not manifest in EC until after 1 month of development. The precise identity of these organelles awaits future investigative work.

In the mouse aorta, the presence of advanced differentiated VSMC (e.g., Myh11-staining) around the EC tube coincides with the establishment of functional circulation (e10–e10.5), rapid myocardial expansion, and elevations in blood pressure.^{20,21} In contrast, there appears to be a protracted time period between blood circulation in zebrafish (24 hpf) and the emergence of differentiated VSMC (later than 7 dpf). One reason for this may relate to the very low blood pressures that likely exist in the early developing zebrafish. Hu and colleagues measured blood pressure in the adult zebrafish and found peak systolic pressure gradients of 2.16 and 1.51 mm Hg in ventral and dorsal aorta, respectively.¹¹ Consistent with these low pressure gradients, the adult zebrafish aorta has

only one clear lamellar unit of VSMC circumscribing the endothelium.

Nevertheless, knockdown of *radar* (*Gdf6*) results in circulatory arrest at 50 hpf that is not associated with perturbations in vascular patterning, raising the possibility that a defect in recruitment of VSMC may underlie the phenotype.⁹ Given the myriad number of mouse knockouts exhibiting clear circulatory defects associated with impaired VSMC recruitment and/or differentiation,^{22–30} it will be of major interest to better explore the role of VSMC in many zebrafish mutant phenotypes. Such an analysis will be facilitated by the accurate determination of VSMC marker expression during development and the use of EM techniques.

MATERIALS AND METHODS

Microscopy

Adult zebrafish (~3 months pf), 1-month-old juveniles, and 7 dpf zebrafish larvae ($n = 4$ for each time point) were fixed overnight at 4°C in 0.1M phosphate buffered 2.5% glutaraldehyde, postfixed 60 min in phosphate buffered 1.0% osmium tetroxide, dehydrated in a graded series of ethanol to 100% (3 times), transitioned to propylene oxide, infiltrated with EPON/Araldite resin, embedded in fresh resin, and polymerized for two days at 70°C. One-micron sections were stained with a 1.0% toluidine blue stain and photographed with a digital camera at a magnification of 600×. Thin sections were cut at 70 nm and placed onto copper grids, stained with 2.0% aqueous uranyl acetate, and subsequently stained with lead citrate. The grids were examined using a Hitachi 7100 transmission electron microscope and digital images were captured using a Megaview III digital camera with AnalySIS software.

Immunostaining

Adult zebrafish were fixed in 4% buffered paraformaldehyde, paraffin embedded, and cut at 5-micron thickness. All slides were deparaffinized and rehydrated to PBS (pH, 7.4). Endogenous peroxidase activity was quenched using 3% aqueous hydrogen peroxide for 10 min

and antigen retrieval was performed utilizing heat-induced epitope retrieval in 0.05% citraconic anhydride.³¹ Smooth muscle α -actin (*Acta2*) slides were blocked in 5% normal horse serum (Vector Laboratories S-2000, Burlingame, CA) for 30 min and incubated in anti-Acta2 1:1000 (DAKO M 0851, Carpinteria, CA) for 90 min at room temperature. The secondary antibody, biotinylated horse anti-mouse 1:400 (Vector BA-2000), was applied for 30 min at room temperature. Alkaline phosphatase detection system (Vector AK-5000) was applied for 30 min followed by Vector Red chromagen (Vector SK-5100) in the dark for 30 min. SRF slides were blocked in 5% normal goat serum (Vector S-1000) for 30 min and incubated in SRF antibody 1:1200 (Santa Cruz sc-335, Santa Cruz, CA) overnight at 4°C. The secondary antibody, biotinylated goat anti-rabbit 1:400 (Vector BA-1000), was applied for 30 min at room temperature. Alkaline phosphatase detection system (Vector AK-5000) was applied for 30 min followed by Vector Red chromagen (Vector SK-5100) in the dark for 30 min. Melanin detection was done with Schmorl's histochemical reagent kit (Newcomer Supply, Middleton, WI) according to the manufacturer's instructions.

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