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Characterization of vascular mural cells during zebrafish development

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ABSTRACT

Development and maturation of the nascent cardiovascular system requires the recruitment of mural cells (MCs) around the vascular tree in a process called vascular myogenesis. Understanding the origin and development of vascular MCs has been hampered by difficulties in observing these cells *in vivo* and performing defined genetic and experimental manipulations in available model organisms. Here, we investigate the origin of vascular MCs using molecular and genetic tools in zebrafish. We show that vascular MCs are present around the lateral dorsal aortae (LDA) and anterior mesenteric arteries (AMA) of developing animals, and that they also contribute to the outflow tract of the developing heart and ventral aorta (VA). Genetic data indicate that the vascular MCs of the LDA and AMA do not arise from blood or endothelial progenitors but from other derivatives of the lateral plate mesoderm. We further show that zebrafish vascular MCs share many of the morphological, molecular and functional characteristics of vascular smooth muscle cells and pericytes found in higher vertebrates. These data establish the zebrafish as a useful cellular and genetic model to study vascular myogenesis as well as tumor angiogenesis and other MC-associated diseases.

1. Introduction

In vertebrates, the development and maturation of the cardiovascular system require the recruitment of smooth muscle cells in a process called vascular myogenesis (Carmeliet, 2000; Jain, 2003). During this process, smooth muscle cell precursors, called mural cells (MCs), differentiate and surround vascular endothelial vessels promoting the maturation and specialization of the cardiovascular system (Carmeliet, 2003). During development, these vascular MCs undergo proliferation and/or differentiation depending on the size of the vessels. On large vessels, they become multi-layered and referred to as vascular smooth muscle cells (SMCs). On smaller vessels, mural cells remain as single sparse cells and usually referred to as pericytes (PCs). Large arterial vessels have strong, elastic vessel walls with dense populations of concentrically-arranged SMCs to withstand the higher blood pressures. SMCs in these large vessels support and regulate blood flow. Veins are generally irregularly covered by SMCs and have valves to prevent backflow of blood (Scultetus et al., 2001). Small blood vessels, which are composed of endothelial cells (ECs) surrounded by a basal lamina, are loosely covered by single PCs, which serve in both scaffolding and signaling functions, and can also regulate vasoconstriction and vasodilation within the vascular bed. It is not known whether PCs and SMCs have distinct progenitors or whether

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they represent phenotypic variants of the same lineage (Bergers and Song, 2005; Majesky, 2007).

In mammals SMCs are characterized by a set of contractile proteins that are distinct from those expressed in skeletal and cardiac muscles (Owens, 1995). Several dynamic molecular markers present in mammalian vascular SMCs are used for their detection, including smooth muscle-myosin heavy chain (MYH11) (Babij et al., 1991; Frid et al., 1993; Madsen et al., 1998; Miano et al., 1994), smooth muscle alpha-actin (ACTA2) (Gabbiani et al., 1981; Hungerford et al., 1996; Mack and Owens, 1999), Sm22alpha (aka Transgelin) (Li et al., 1996; Kim et al., 1997), Smoothelin (van der Loop et al., 1996; Kramer et al., 1999), Desmin and Calponin (Duband et al., 1993; Owens et al., 2004). The expression patterns of these markers can vary depending on the developmental stage of the vascular system. In particular, Sm22alpha and smooth muscle alpha-actin are the first markers expressed in SMCs and for this reason are considered embryonic SMC markers in mammals (Majesky, 2007).

Intriguingly, unlike skeletal or cardiac muscle cells which permanently exit the cell cycle and are terminally (and essentially irreversibly) differentiated, SMCs retain remarkable plasticity and can undergo rather profound and reversible phenotypic changes in response to changes in local environmental cues. The high degree of mammalian smooth muscle plasticity, especially during postnatal development, predisposes it to phenotypic switching, and thus may play a major role in human disease (Halayko and Solway, 2001). Unregulated growth, differentiation and plasticity of mammalian vascular SMCs is a key feature of vascular diseases such as atherosclerosis, restenosis and hypertension, as well as monogenic diseases such as telangiectasia and retinopathies (Ross, 1999; Conway et al., 2001; Sata et al., 2002; Hao et al., 2003). Several genes identified to play a key role in vascular myogenesis are also associated with human disease (Carmeliet, 2003; Rensen et al., 2007). Furthermore, in response to specific vascular injuries, vascular SMCs dramatically increase their rate of proliferation, migration and synthetic capacity and play a critical role in vascular repair and tumor angiogenesis (Gerhardt and Semb, 2008).

A limitation in studying mammalian vascular myogenesis is the lack of an easily manipulatable *in vivo* system to analyze smooth muscle development and differentiation. The zebrafish has already been established as a powerful model system to study cardiovascular development (Stainier, 2001; Lawson and Weinstein, 2002; Beis et al., 2005; Santoro et al., 2007). The zebrafish is also a genetically accessible vertebrate with an optically clear embryo permitting high-resolution *in vivo* cell imaging. Surprisingly, the existence of vascular SMCs or PCs has not yet been fully reported in this model organism. We carried out a series of morphological, molecular and functional studies to visualize and characterize vascular myogenesis in the developing zebrafish.

Since we were able to detect only a few cells surrounding early embryonic vessels, we decided to name these perivascular cells, mural cells (MCs). Our studies show that zebrafish embryos develop vascular MCs that share many of the characteristic features of embryonic SMCs found in higher vertebrates, and we provide evidence to support a functional role for zebrafish MCs during cardiovascular development. These studies identify the zebrafish as an embryological and genetic model system to further study the development, differentiation and plasticity of vascular MCs *in vivo*.

2. Results

2.1. *Vascular smooth muscle cells are present in zebrafish larvae*

Understanding the molecular mechanisms regulating vascular myogenesis remains a major issue, in part because of the challenges of studying this process in amniotes (Majesky, 2007). We therefore decided to investigate the origin of vascular MCs in zebrafish, particularly focusing on their contribution to the trunk vasculature (mainly dorsal aorta and cardinal vein) during development. We started by comparing the ultrastructure of the dorsal aorta (DA) and posterior cardinal vein (PCV) at various stages. Light microscopy images of 3 months old zebrafish revealed striking differences in vessel wall caliber between the DA and PCV, with the former clearly characterized by thick layers of vascular SMCs around the ECs (Fig. 1) (Miano et al., 2006). Interestingly, the PCV did not show the same extent of vascular SMC coverage as in mammals. To better characterize these vascular SMCs we analyzed samples of DA and PCV by transmission electron microscopy (TEM) at earlier time points. Analyses of 20-day-old DA revealed that at least two to three layers of synthetic vascular SMCs are present in the DA, while single and dispersed SMCs were found in the PCV (Supplementary Fig. 1). Interestingly, a dense layer of extracellular matrix, the internal elastic lamina (IEL), lies between the EC and the SMC layers, constituting the basement membrane. The synthetic SMCs in the DA at 20 dpf did not yet exhibit the ultrastructural features of fully differentiated adult vascular SMCs. Differentiated, contractile vascular SMCs are elongated, whereas undifferentiated, synthetic SMCs have a cobblestone morphology and contain a high number of organelles involved in protein synthesis. Moreover, synthetic SMCs show higher growth rates and higher migratory activity than contractile SMCs (Hao et al., 2003). Collectively, these results reveal a basic ultrastructural organization of the DA in zebrafish that is similar to that in mammals with the notable exception of a delay in zebrafish SMC maturation.

2.2. *Markers of embryonic smooth muscle cells identify specific perivascular tissue during zebrafish development*

The transcriptional program controlling smooth muscle differentiation is poorly understood. Unlike skeletal and cardiac muscle, SMCs do not terminally differentiate but retain remarkable plasticity and exhibit a diverse range of phenotypes under various physiological and pathological conditions (Halayko and Solway, 2001). Due to their heterogeneous origin and transcriptional profiles, no single marker can be used to identify all SMCs. However, when these markers are assayed in combination, and are considered in the morphological context of developing organs, they can be used to identify cells of the SMC lineage. A variety of genes and gene products have been identified in mammals and other vertebrates as useful markers of the relative state of induction and differentiation

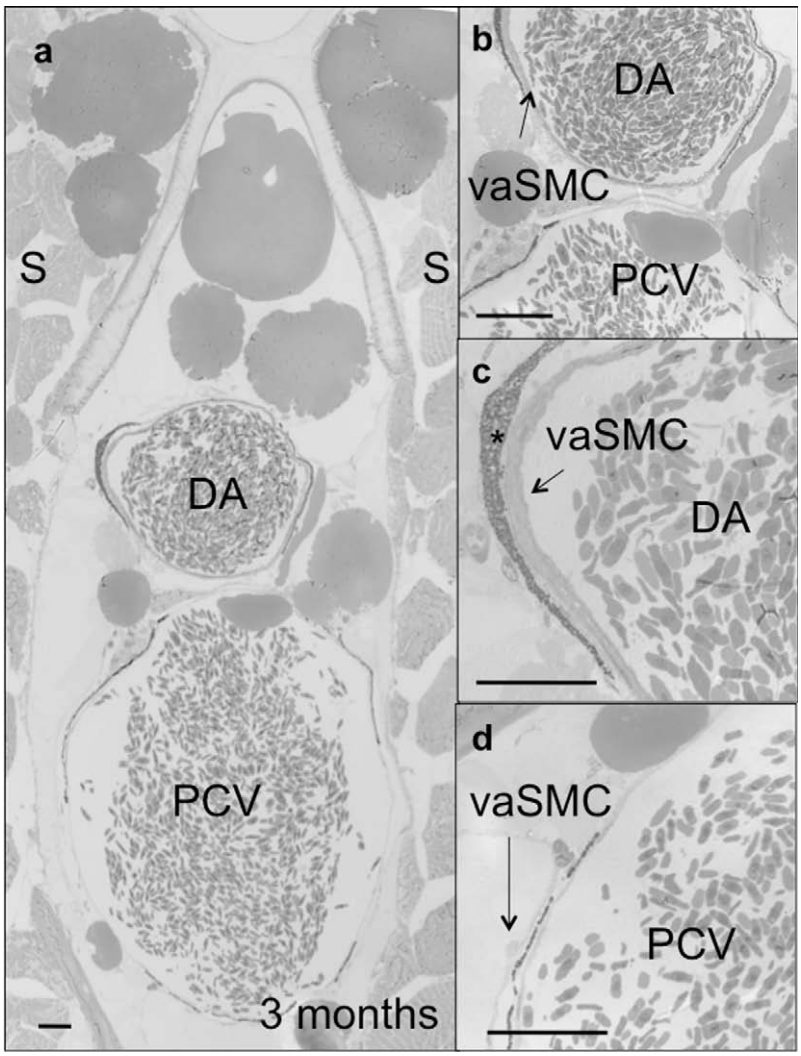

Fig. 1 – Histological analyses of the dorsal aorta and posterior cardinal vein of 3 months old zebrafish. (a–d) Toluidine blue-stained transverse sections of the trunk vasculature showing the dorsal aorta (DA) and posterior cardinal vein (PCV). A thick layer (arrows in (b and c)) of vascular SMCs (vaSMCs) is present around the DA but not the PCV, where single vaSMCs (arrow in (d)) are interspersed along the circumference of the PCV. Scale bars, 100 μm. *Peri-aortic melanocytes. Sections are at the level of the 10th somite.

of SMCs (Owens, 1995; Owens et al., 2004). These markers include mainly contractile, cytoskeletal and cytoskeleton-associated proteins such as smooth muscle alpha-actin, Sm22alpha, smooth muscle-myosin heavy chain, and Smoothelin. Similarly, in zebrafish, the heterogeneity of MCs precludes their identification by single markers (Georgijevic et al., 2007; Davis et al., 2008). Therefore, we identified and cloned a panel of zebrafish MC markers including the genes encoding Transgelin (Sm22alpha-b), Transgelin2 (Sm22alpha-a), Acta2, Myh11, Smoothelin, Calponin and Desmin. Among those we found that *acta2* and *transgelin* were expressed specifically in the perivascular region of zebrafish larvae (Fig. 2). Starting at 72 hpf these genes were not only expressed in visceral SMCs (Georgijevic et al., 2007) and the fin bud, but also in a specific region of the vasculature corresponding to the anterior portion of the DA, the lateral DA (LDA) as well as the primitive internal carotid arteries (PICA). Cross-section analyses revealed specific staining around the dorsal aorta and in the mesenchyme surrounding the gut epithelium. At this time, expression was relatively weak and discontinuous throughout the length of the dorsal aorta. However, this expression became more evident at 96 hpf especially around the DA and LDA. At this later stage, *acta2* and *transgelin* expression also became evident in the heart, specifically the outflow tract and ventral aorta (VA) suggesting the "induction" of MC-like tissue in this region (Fig. 2g–j).

Altogether these data support the existence of zebrafish vascular MCs in specific regions of the developing vasculature starting at 72 hpf. Progressively, these MCs became more evident in these anatomical regions and are possibly involved in cardiovascular maturation.

2.3. *Vascular mural cells are present around the hyaloid, dorsal and mesenteric arteries throughout zebrafish development*

To better investigate zebrafish vascular MC development at cellular resolution we decided to use antibody staining

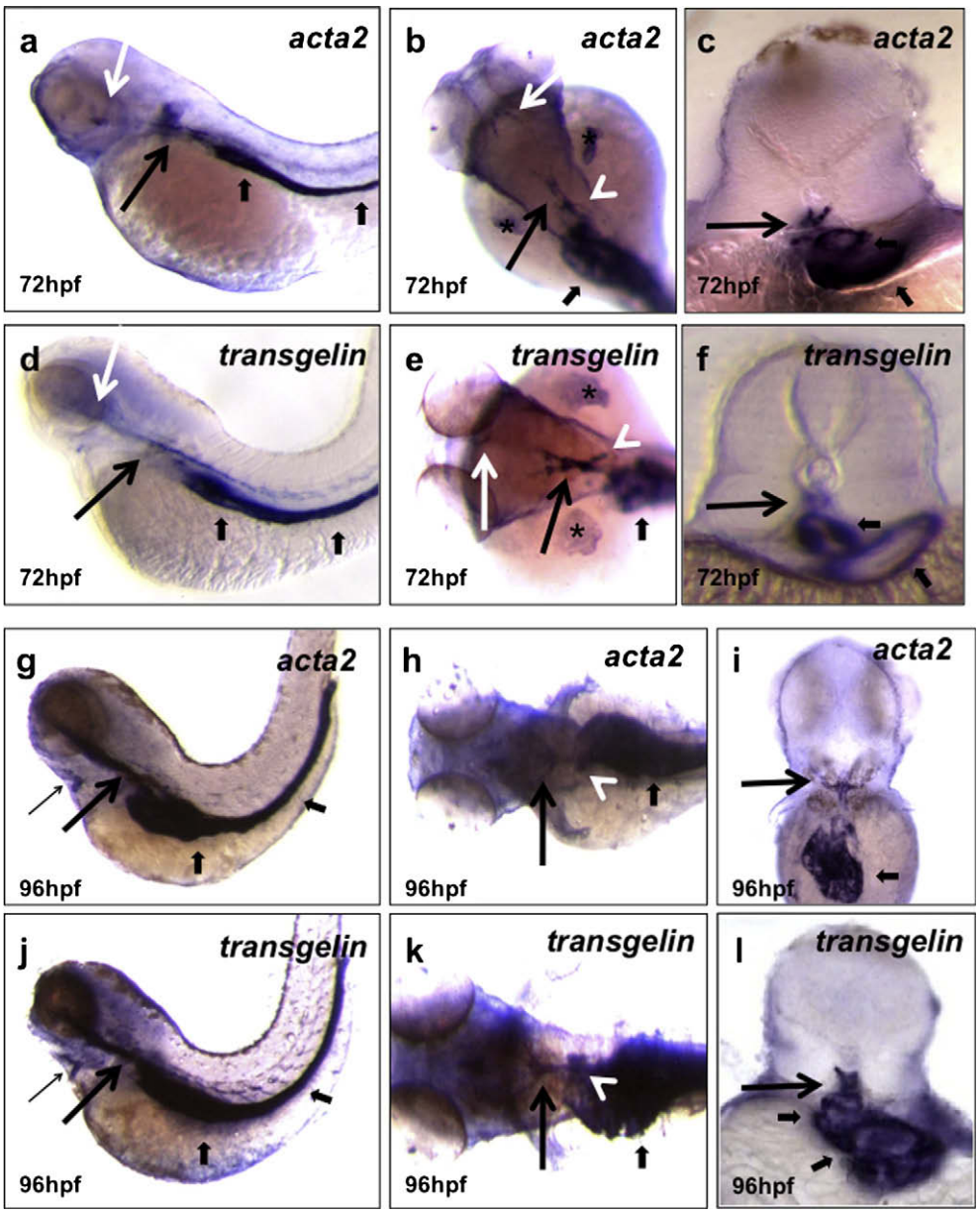

Fig. 2 – Mural cell markers are observed in specific perivascular districts. Wild-type larvae analyzed for *acta2* (a–c; g-i) and *transgelin* (d–f; j–l) expression at 72 (a–f) and 96 (g–l) hpf. At 72 hpf, *acta2* and *transgelin* are strongly expressed in the anterior lateral dorsal aortae (LDA) (long black arrows), internal carotid arteries (ICA) (long white arrows) and anterior mesenteric artery (AMA) (white arrowheads). At 96 hpf, a specific and diffuse staining appears at the Y junction of the bilateral dorsal aortae (long black arrows), AMA (white arrowheads) and in the heart region at the level of the bulbus arteriosus (BA) and ventral aorta (VA) (thin arrows). Visceral smooth muscle around the gut and swim bladder (short black arrows) are also evident at both stages. Cross-section analyses reveal specific staining around the dorsal aorta (vascular MCs) (long black arrows) and the gut epithelium (visceral smooth muscle cells) (short black arrows). Images (a, d, g and j) are lateral views, anterior to the left; (b, e, h and k) are dorsal views, anterior to the left; (c, f, i and l) are cross-sections at the level of the 2nd somite. At 72 hpf, both genes are also expressed in the fin buds (asterisks).

followed by confocal imaging. As previously reported by Georgijevic et al. (2007) the epitope for the commonly used mammalian ACTA2 antibody is not conserved in zebrafish and thus the mammalian antibody is not useful to distinguish between the different muscle types. Therefore, we developed a specific antibody that recognizes zebrafish Transgelin (aka Sm22alpha-b), one of the earliest mammalian vascular SMC markers (Zhang et al., 2001). We investigated the localization of MCs throughout the length of the animal at 80 hpf (Fig. 3a–c). Cross-sections at the level of the 2nd somite showed Transgelin expression around the dorsal aorta (DA) and the gut (G). At more posterior positions (10th somite), the expression of Transgelin was lost around the vasculature (DA and PCV) and maintained only around the visceral organs (G).

No expression was detected at the level of the 18th somite. Higher magnification imaging of DA sections at the level of the 2nd somite showed that Transgelin-positive cells are individual cells located in close proximity to the ventral and lateral sides of the DA (Fig. 3d). Transgelin-positive MCs lined the outside of the vascular tube and are clearly different from endothelial cells (ECs) (Fig. 3e). Interestingly, the PCV was not yet covered by MCs at this stage of development. We also investigated the morphology of the Transgelin-positive MCs using ultra-thin cross-sections and TEM (Fig. 4). Representative images of the DA at 80 and 120 hpf at the level of the 2nd somite show a vascular lumen (L) and the lining ECs surrounded by tightly apposed MCs. At this stage, no internal elastic lamina (IEL) was observed between the ECs and the adjacent MCs, though tight junctions between ECs were readily evident.

To further characterize the anteroposterior coverage of these MCs we performed whole-mount staining on 80 hpf larvae with the Transgelin antibody. We found that the MCs appear to form a bulge of cells in the ventral portion of the DA that appears to migrate towards the anterior portion of the DA as well as posteriorly towards the mesenteric artery (Fig. 5a–e and Supplementary Movies). Cross-sections of the bulge region revealed a number of MCs in the ventral and lateral portion of the DA (Fig. 5d–e). These data correlate well with the *acta2* and *transgelin in situ hybridization* data indicating that zebrafish vascular MCs may start to differentiate on the ventral side of the DA at the 2nd somite level around 72 hpf.

It has been previously shown that the adult zebrafish retina vasculature features PCs that are located between the vascular endothelium and the basal lamina of the retinal vessels (Alvarez et al., 2007). In situ hybridization analyses with *acta2* and *transgelin* as well as immunostaining with the Transgelin antibody staining revealed the presence of putative MCs in the eyes of developing larvae as early as 72 hpf (Supplementary Fig. 2). These cells appeared to originate from a specific region of the retinal vasculature called the inner optic circle (IOC) in close association with the optic vein (OV). As the eye vasculature further developed, these MCs continued to cover the IOC (data not shown).

2.4. *Developmental origin of vascular mural cells in zebrafish embryos*

In zebrafish, as in other vertebrates, the dorsal aorta develops in close association with the splanchnic mesoderm, which also contributes to the primitive vascular

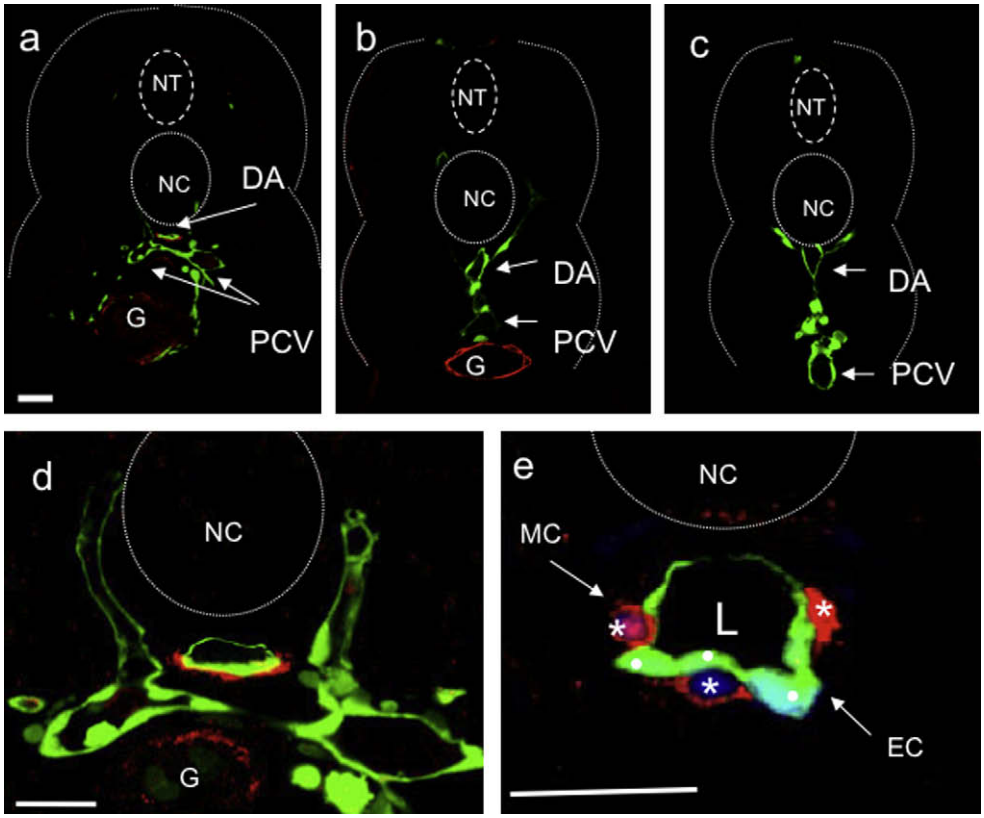

Fig. 3 – Vascular mural cells are present around the dorsal aorta in larvae. Confocal images of transverse sections of 80 hpf *Tg(flk1:gfp)*^{s843} wild-type larvae stained for Transgelin (red; (a–e)) and DNA (blue; (e)). Vascular MCs are Transgelin-positive cells surrounding endothelial cells (ECs) (green) of the dorsal aorta (DA). Sections shown are at the level of the 2nd (a, d and e), 10th (b) and 18th (c) somite. Vascular Transgelin-positive MCs are absent from the PCV in all sections (a–d). High-magnification images show that Transgelin-positive MCs (white asterisks) are single cells clearly distinguishable from endothelial cells (ECs: white dots). Scale bars, 20 μm. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein; G, gut; L, vascular lumen.

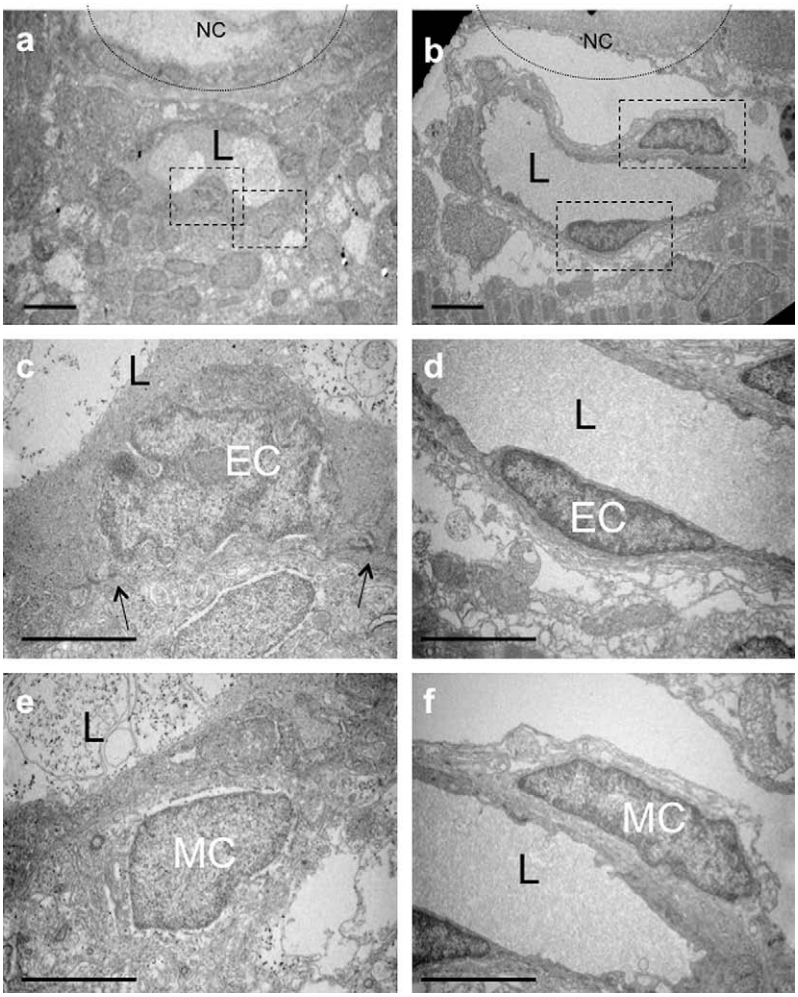

Fig. 4 – TEM of single mural cells surrounding endothelial vessels in larvae. Transmission electron microscopy (TEM) images of transverse sections of 80 (a, c and e) and 120 (b, d and f) hpf larvae. (c and e) Higher magnification of MCs and ECs boxed in (a). MCs and ECs exhibit different morphologies, and tight junctions are evident between ECs (black arrows). (d–f) Higher magnification images of MCs and ECs boxed in (b). Both ECs and MCs exhibit a more elongated morphology at 120 than 80 hpf. L, lumen. Scale bars, 5 μm.

tube (Lawson and Weinstein, 2002). This observation suggests that the early MCs in the zebrafish aortic floor also arise from the splanchnic mesoderm. Alternatively, these MCs might derive from the ECs of the aortic floor through a process called endothelial transdifferentiation (DeRuiter et al., 1997). To address this point, we took advantage of two zebrafish mutants, *cloche* (*clo*^{s5}) and *hands off* (*han*^{s6}). *cloche* mutant embryos lack all endothelial precursors in the head and trunk regions and do not form functional blood vessels (Stainier et al., 1995). The *hands off* gene encodes the bHLH transcription factor Hand2 and is expressed in the lateral plate mesoderm during gastrulation and somitogenesis stages, and *han*^{s6} mutants have defects in lateral plate mesoderm-derived tissues (Yelon et al., 2000). We tested for the presence of vascular MCs in these mutants using the Transgelin antibody (Fig. 6). Interestingly, while *clo*^{s5} mutants showed Transgelin-positive cells in the region corresponding to the aortic floor, *han*^{s6} mutants completely lacked these cells. These experiments suggest that in zebrafish, MCs derive from the lateral plate mesoderm and not from the transdifferentiation of ECs.

2.5. *Vascular mural cells are present in the developing outflow tract in zebrafish*

The zebrafish is a widely accepted model for the study of early cardiogenesis (Stainier, 2001; Beis et al., 2005). Much of this research has been concentrated on the development of the atrium and ventricle, at stages long before the outflow tract is formed. As a result, this structure has been poorly characterized. Several reports have shown that the adult zebrafish outflow tract is invested with a considerable number of smooth muscle layers (Hu et al., 2000; Grimes et al., 2006). We therefore decided to investigate the onset of zebrafish MC induction in the bulbus arteriosus (BA) and outflow tract. Whole mount in situ hybridization analyses showed that *acta2* and *transgelin* are expressed in the heart region at 96 hpf (Fig. 2). To gain a better resolution of these MC-positive cardiac regions, we analyzed thin cross-sections of whole mount

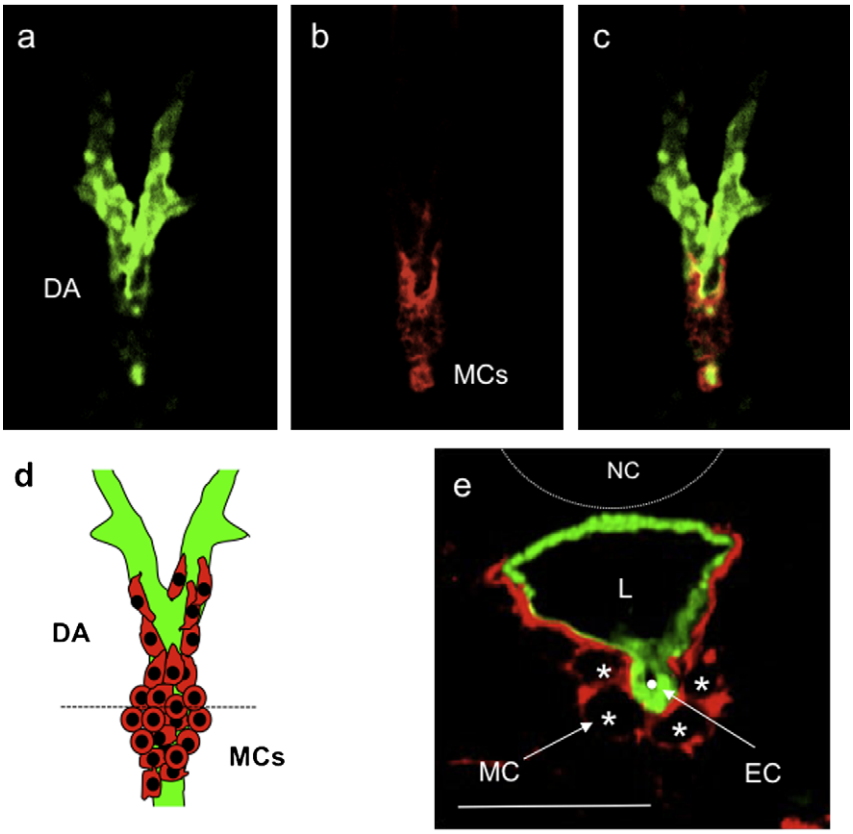

Fig. 5 – Vascular mural cells first appear in the anterior region of the dorsal aorta. (a–c) Confocal images of horizontal sections of a 80 hpf *Tg(flk1:GFP)*^{s843} wild-type larva stained for Transgelin (red) show that MCs develop as a "bulge" of cells at the Y junction between the lateral dorsal aortae (LDA) and the dorsal aorta (DA). Images (a–d) are ventral views, anterior to the top. (d) Schematic representation of vascular MCs (red) and ECs (green) forming the anterior trunk vasculature at 80 hpf. (e) Confocal image of a transverse section of a 80 hpf *Tg(flk1:GFP)*^{s843} wild-type larva stained for Transgelin (red). Vascular MCs are present and appear to originate at the ventral region of the DA. Scale bars, 20 μm. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein; G, gut; L, vascular lumen.

stained larvae at 96 hpf (Fig. 7a and b). These analyses confirmed that the region involved is the BA and the proximal part of the VA. We confirmed the presence of this MC coverage in the outflow tract by Transgelin staining (Fig. 7c–e). Immunofluorescence on whole mount and cross-sections with Transgelin antibody clearly showed a single layer of vascular MCs around the VA at 96 hpf. Interestingly, at 72 hpf, while the BA was already formed and several layers of cuboidal cells were evident, the outflow tract did not yet express these MC markers (data not shown). These data are in agreement with a previous characterization of the zebrafish outflow tract where a cellular structure committed to become the BA appears after 48 hpf (Grimes et al., 2006), a stage when the atrium and ventricle are already formed and pumping blood through a rudimentary circulatory system. We speculate that between 72 and 96 hpf these cells are induced to differentiate into vascular MCs and contribute to the future outflow tract.

3. Discussion

Mammalian vascular SMCs are a highly heterogeneous cell population with diverse embryonic origins, properties and gene expression profiles. A major difficulty in the smooth muscle field has been the lack of an easily manipulatable model system for the study of SMC biology *in vivo*. Surprisingly, the development of vascular SMCs, in particular vaSMCs and PCs, has not yet been characterized in zebrafish, even though the transparency and easy manipulation of embryos has established it as an excellent model system to study the development of a variety of organ systems. Here, we analyzed the development and distribution of zebrafish vascular MCs and investigated the temporal and spatial expression patterns of several MC genes during cardiovascular development. Additionally, we examined the morphology of zebrafish vascular MCs with different microscopy techniques and identified these cells in the larval and adult vasculature. The goal of this study is to begin to provide an understanding of the ontogeny of zebrafish vascular SMCs and PCs and the morphological events that lead to their induction, differentiation and maturation. This understanding will help investigate the cellular and molecular mechanisms that regulate their development and to evaluate the importance of SMC biology as a susceptibility factor for vascular diseases like atherosclerosis, retinopathies and hereditary haemorrhagic telangiectasia

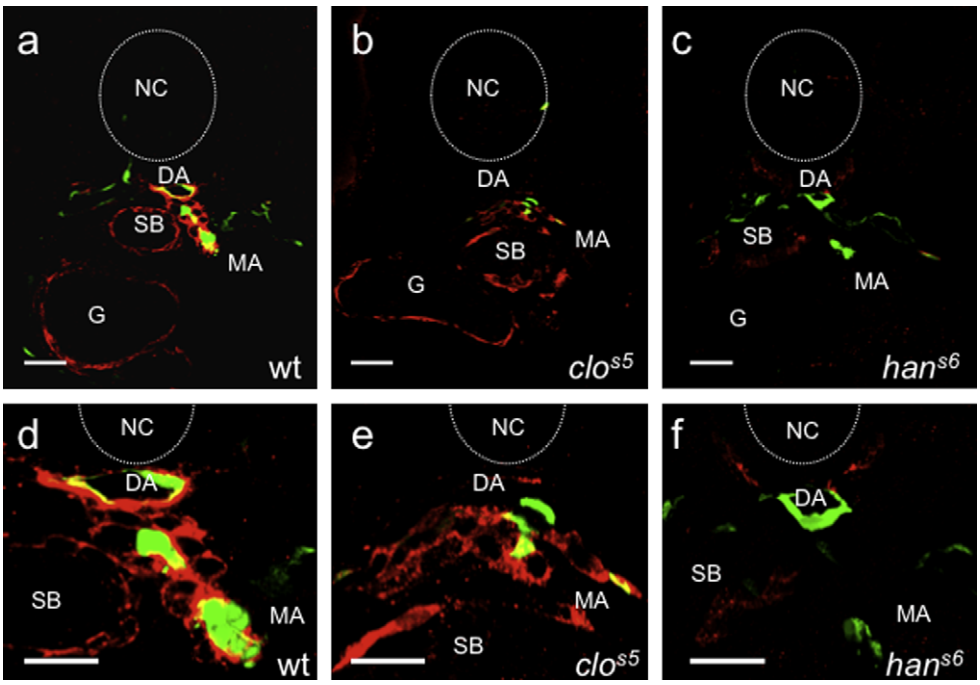

Fig. 6 – Vascular mural cells derive from the lateral plate mesoderm but not from the blood or endothelial lineages. Confocal images of transverse sections, at different magnifications, of 80 hpf *Tg(flk1:GFP)*^{s843} wild-type (a and d) or *cloche* (*clo*^{s5}) (b and e) or *hands-off* (*han*^{s6}) (c and f) mutant larvae analyzed for Transgelin expression (red). While wild-type (wt) and *clo*^{s5} mutant larvae show Transgelin-positive cells around both the dorsal aorta and visceral organs, *han*^{s6} mutant larvae completely lack both vascular and visceral MCs. Sections are at the level of the1st-2nd somite. Scale bars, 20 μm. NT, neural tube; DA, dorsal aorta; SB, swim bladder; G, gut; MA, mesenteric artery.

(HHT). The importance of developing a model system to study SMCs resides on the extensive contribution of these cells to various pathological conditions. The zebrafish represents an interesting new approach to connect developmental biology and associated diseases (Dodd et al., 2000; Lieschke and Currie, 2007; Chico et al., 2008). Our data suggest an evolutionarily conserved developmental origin and function of SMCs indicating that the zebrafish can be used to study the genetics of vascular SMC-associated human diseases. Generation of transgenic zebrafish lines with fluorescently labeled vascular MCs, as well as the identification and characterization of mutant lines showing defects in zebrafish vascular MC development will be the next steps. In fact, some zebrafish mutant phenotypes identified in genetic screens for developmental regulators resemble human disease states and these mutations, once isolated molecularly, will provide candidate genes for evaluation in human disorders (Lieschke and Currie, 2007). The identification of mutant phenotypes resembling human vascular diseases will be very useful to (i) dissect new genetic pathways involved in SMC-associated diseases, (ii) study the genetic origin of pathological conditions associated with MCs and (iii) facilitate the set-up of new therapeutic applications for these pathologies.

The origin of vertebrate vascular SMCs is complex with contributions from several independent cell lineages (Gittenberger-de Groot et al., 1998; Majesky, 2007). Neural crest cells (NCs) give rise to vascular SMCs in the cephalic region and in the cardiac outflow tract region (Wilm et al., 2005) and are required for patterning the great vessels (Hutson et al., 2003). The pro-epicardial organ contributes to SMCs in the coronary arteries and ventrally emigrating neural tube cells may contribute to SMCs in the great vessels and the coronary vessels in chicken (Mikawa and Gourdie, 1996; Gittenberger-de Groot et al., 1998). The mesothelium also gives rise to SMCs in mesenteric vessels in mouse (Wilm et al., 2005). In posterior parts of the body, vascular MCs are assumed to derive primarily from the splanchnic lateral plate mesoderm (Gittenberger-de Groot et al., 1999), but the precise source of these cells has not been rigorously determined. Observations of the early pattern of SMC contractile protein expression in mouse and quail aorta suggest that vascular MC differentiation is induced in mesenchymal cells that line the endothelium (Hungerford et al., 1996; Takahashi et al., 1996). Vascular SMC contractile proteins are first observed on the ventral side of the vessel, and it has been hypothesized that MCs are only induced in the ventral area of the aorta and later migrate to populate the lateral and dorsal areas. Our findings are in agreement with these findings and suggest that vascular MCs in the trunk originate from the lateral plate mesoderm as in other vertebrate model organisms (Wiegreffe et al., 2007; Wasteson et al., 2008). However, cell lineage experiments need to be carried out to determine the precise origin of MCs in different anatomical regions. Establishment of transgenic lines for zebrafish MCs should also be a useful tool to study the perivascular origin of mesenchymal stem cells and their potency in myogenesis, adipogenesis, chondrogenesis and tissue regeneration (Cossu and Bianco, 2003; Crisan et al., 2008).

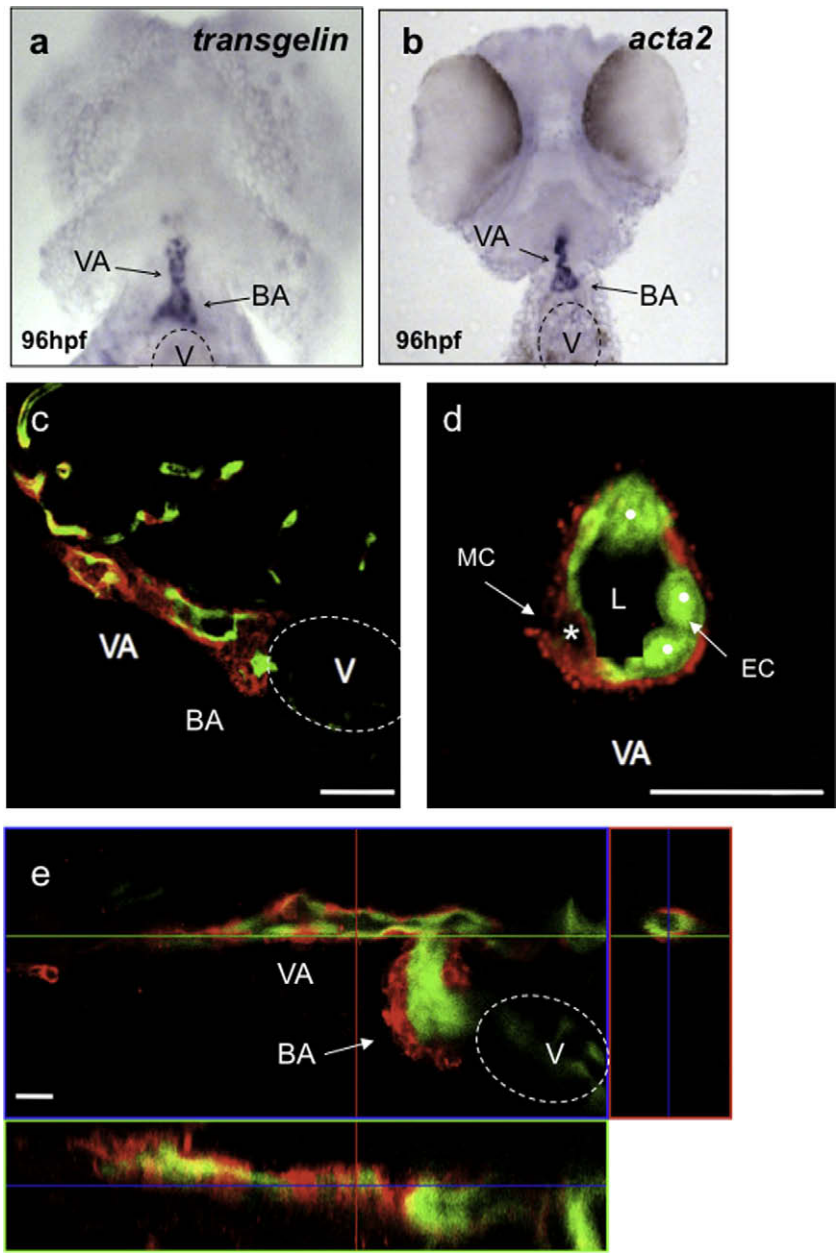

Fig. 7 – Vascular mural cells contribute to the ventral aorta and the bulbus arteriosus. Ninety-six hpf wild-type larvae analyzed for *transgelin* (a) and *acta2* (b) expression. *transgelin* and *acta2* are expressed in the bulbus arteriosus (BA) and ventral aorta (VA). (c) Confocal image of a sagittal section of a 96 hpf *Tg(flk1:EGFP)*^{s843} wild-type larva stained for Transgelin (red) show that vascular MCs surround the VA and contribute to the BA. (d) Confocal image of a transverse section of the VA shows single MCs around the ECs (white dots). (e) Confocal projection of the heart region of a 120 hpf *Tg(flk1:EGFP)*^{s843} wild-type larva stained for Transgelin (red). At this stage of development, vascular MCs are present in the BA as well as VA. Scale bar, 20 μm. V, ventricle; L, vascular lumen.

We also noticed that from early developmental stages until adulthood, zebrafish arteries and veins are differently covered by SMCs. While the aorta is covered with vascular MCs from an early time point, the cardinal vein is not. Lack of MCs in the posterior cardinal vein of zebrafish larvae is not surprising given that only a few scattered SMCs were found in the walls of the caudal vein and the posterior cardinal vein of 20 dpf animals (Fig. 1 and Supplementary Fig. 1). This situation resembles that found in the carp (*Cyprinus carpio*) (Satchell, 1992). Gravity exerts significant effect on venous return in terrestrial animals, affecting venous pressure and ventricular filling pressure. Hence, venous compliance must be continuously adjusted in terrestrial animals by MC contractility to ensure proper cardiac filling. In contrast, gravitational stress in water is much less than in a terrestrial environment. The specific gravity of water is similar to that of blood, resulting in constant transmural pressure along the length of the posterior cardinal vein (Scultetus et al., 2001; Satchell, 1992).

Therefore, modulation of venous blood pressure by smooth muscle contractility may not be crucial in juvenile zebrafish. On the other hand, we found an early vascular MC coverage in the anterior mesenteric artery of zebrafish larvae suggesting that this is a crucial vascular region to control blood flow towards the gastro-intestinal tract region at early time points.

While the use of atrioventricular-specific molecular markers has allowed extensive characterization of the development of the atrium and ventricle, the lack of any bulbus arteriosus (BA)-specific markers has meant that this region of the zebrafish heart is poorly characterized and quite possibly misunderstood (Hu et al., 2000; Grimes et al., 2006). In zebrafish, the BA forms by 48 hpf as an independent structure, at a stage when the atrium and ventricle are already formed and pumping blood through a rudimentary circulatory system. Because it forms so late, it is usually excluded from studies of the true cardiac chambers, and detailed analyses of its development need to be carried out. The function of the BA in fish is to reduce the high ventricular pressure during systole, and to maintain constant blood flow into the ventral aorta and gills during diastole. The elastic walls of the BA maintain a strong and even pressure on the blood flowing to the gills, maintaining a steady flow. The ability of the bulbus wall to expand and hold much of the stroke volume is critical to this process (Bushnell et al., 1992). Our finding of extensive smooth muscle coverage in the outflow tract and closely associated vessels is consistent with a role for the BA in maintaining an even blood pressure. It will be important to identify the progenitors of these outflow tract MCs as their biology gets further investigated (Sato and Yost, 2003).

Our results provide the first analysis of zebrafish vascular myogenesis. Collectively, these data provide new insights into the emergence of differentiated vascular MCs and PCs in zebrafish, thus providing a foundation for future studies on the gene expression profile and function of these cells in wild-type and mutant animals.

4. Materials and methods

4.1. *Zebrafish husbandry*

Zebrafish, *Danio rerio*, were maintained and staged based on developmental time and morphological criteria as previously described (Santoro et al., 2007). Fish were kept under a 14-h light and 10-h dark photoperiod at approximately 28 °C. Following fertilization, eggs were collected and embryos – wild-type or mutant – were raised at 28 °C under standard laboratory conditions in the presence of 0.003% 1-phenyl-2-thiourea to prevent formation of melanin pigments, greatly facilitating visualization of the vascular system.

4.2. *Whole mount in situ hybridization*

Wild-type and mutant larvae were collected at 72 or 96 hpf, fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4 °C, then transferred into 100% methanol (MeOH) for at least 2 h at −20 °C. Whole-mount in situ hybridization was performed as previously described (Santoro et al., 2007), using Digoxigenin-labeled antisense RNA probes of *acta2* and *transgelin*. For *acta2*, a 3′-UTR-specific probe was generated using the following primers: 5′-gtcccgtaattctgctattg-3′ and 5′-aattattgctgagctttatt-3′. For *transgelin2*, a specific probe was generated using the following primers: 5′-atggcaaataaaggtccgtc-3′ and 5′-gtcgtccgtaaccggtcatt-3′. For *transgelin*, a specific probe was generated using the following primers: 5′-actatggcaaacaaggggcc-3′ and 5′-acgcccctcttttggagccc-3′. To perform the in situ hybridization, the larvae were rehydrated using successive dilutions of methanol (75%, 50%, 25%) in PBS. Then the larvae were permeabilized by digestion with proteinase K (10 μg/ml) at room temperature for 30 min to allow the RNA probe to penetrate. Proteinase K digestion was stopped by incubation for 30 min in 4% (w/v) paraformaldehyde (PFA) in PBS. Hybridization took place overnight at 65 °C in the presence of approximately 150 ng antisense DIG-labeled RNA probes. After washing, the hybridized larvae were incubated overnight in a 1:10,000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche) at 4 °C. Color reactions (using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates) were used to detect the hybridized mRNA transcripts. The larvae were then embedded in 17% Gelatin–PBS–Azide, fixed in 4% paraformaldehyde for at least 24 h at room temperature and cut with a VT1000S vibratome (Leica) into 50 μm sections. Processed samples were mounted in Vectashield (Vector Laboratories) and images were acquired using a Zeiss Axiocam.

5. Histology and transmission electron microscopy (TEM)

Wild-type animals were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature or overnight at 4 °C. Semi-thin sections were cut at 0.5 or 1.0 μm and a metal loop was used to collect thick sections, and transfer them to a drop of distilled water on a glass slide. Sections were dried down on a glass slide by placing the slide on a slide warmer lamp. After the sections were completely dry, they were covered with a few drops of staining solution (1% toluidine blue and 2% borate in distilled water) for 1–2 min depending on the darkness of staining desired. Excess stain was gently rinsed off with distilled water. Slides were air dried and mounted. For TEM analysis, wild-type animals were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature or overnight at 4 °C. Samples were dissected in cold PBS, and fixed in 2% formaldehyde (Polysciences) and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. Embryos were post-fixed with 1% osmium tetraoxide, followed by staining in 1% uranyl acetate in 70% ethanol, dehydrated through a graded series of ethanol washes, followed by propylene oxide and embedded in resin (EMBED 812 KIT, Electron Microscopy Sciences) for 72 h at 60 °C in the vacuum oven. 80 nm sections were stained with uranyl acetate, followed by lead citrate and viewed on a Hitachi 7000 electron microscope.

6. Antibody production and embryo immunostaining

A polyclonal anti-Transgelin (aka Sm22alpha-b) antibody was generated using the TGYGRPRQIMNP peptide sequence. Antigen-affinity purification was performed according to Covance protocols. The larvae were washed at room temperature with PT buffer (0.3% Triton X-100 in phosphate-buffered saline) for 5 min, rinsed three times with phosphate-buffered saline (PBS), and then fixed in 3% paraformaldehyde (PFA) overnight at 4 °C. The fixed larvae were permeabilized with 1% BSA, 1% DMSO and 0.5% Triton X-100 in PBS for 1 h at room temperature and then incubated with primary antibody overnight at 4 °C. After washing in PBT-S (0.1% Triton X-100, 1% BSA in PBS) for 2 h and incubated with secondary antibodies and TOPRO (Molecular Probes) overnight at 4 °C, the larvae were washed in PBT-S and then in PBS, and finally embedded in 1.5% low melting agarose. Images were acquired using a Zeiss LSM5 Pascal confocal microscope and Zeiss confocal projection software.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2009.06.1080.

REFERENCES

Alvarez, Y., Cederlund, M.L., Cottell, D.C., Bill, B.R., Ekker, S.C., Torres-Vazquez, J., Weinstein, B.M., Hyde, D.R., Vihtelic, T.S., Kennedy, B.N., 2007. Genetic determinants of hyaloid and retinal vasculature in zebrafish. BMC Dev. Biol. 7, 114.

Babij, P., Kelly, C., Periasamy, M., 1991. Characterization of a mammalian smooth muscle myosin heavy-chain gene: complete nucleotide and protein coding sequence and analysis of the 5′ end of the gene. Proc. Natl. Acad. Sci. USA 88, 10676–10680.

Beis, D., Bartman, T., Jin, S.W., Scott, I.C., D'Amico, L.A., Ober, E.A., Verkade, H., Frantsve, J., Field, H.A., Wehman, A., Baier, H., Tallafuss, A., Bally-Cuif, L., Chen, J.N., Stainier, D.Y., Jungblut, B., 2005. Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. Development 132, 4193–4204.

Bergers, G., Song, S., 2005. The role of pericytes in blood-vessel formation and maintenance. Neuro-Oncol. 7, 452–464.

Bushnell, P.G., Jones, D.R., Farrell, A.P., 1992. The arterial system. In: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds.), The Cardiovascular System, XII, Part A. Academic Press, Inc., San Diego, CA, pp. 89–139.

Carmeliet, P., 2000. Mechanisms of angiogenesis and arteriogenesis. Nat. Med. 6, 389–395.

Carmeliet, P., 2003. Angiogenesis in health and disease. Nat. Med. 9, 653–660.

Chico, T.J., Ingham, P.W., Crossman, D.C., 2008. Modeling cardiovascular disease in the zebrafish. Trends Cardiovasc. Med. 18, 150–155.

Conway, E.M., Collen, D., Carmeliet, P., 2001. Molecular mechanisms of blood vessel growth. Cardiovasc. Res. 49, 507–521.

Cossu, G., Bianco, P., 2003. Mesoangioblasts – vascular progenitors for extravascular mesodermal tissues. Curr. Opin. Genet. Dev. 13, 537–542.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P.N., Traas, J., Schugar, R., Deasy, B.M., Badylak, S., Buhring, H.J., Giacobino, J.P., Lazzari, L., Huard, J., Peault, B., 2008. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3, 301–313.

Davis, J.L., Long, X., Georger, M.A., Scott, I.C., Rich, A., Miano, J.M., 2008. Expression and comparative genomics of two serum response factor genes in zebrafish. Int. J. Dev. Biol. 52, 389–396.

DeRuiter, M.C., Poelmann, R.E., VanMunsteren, J.C., Mironov, V., Markwald, R.R., Gittenberger-de Groot, A.C., 1997. Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. Circ. Res. 80, 444–451.

Dodd, A., Curtis, P.M., Williams, L.C., Love, D.R., 2000. Zebrafish: bridging the gap between development and disease. Hum. Mol. Genet. 9, 2443–2449.

Duband, J.L., Gimona, M., Scatena, M., Sartore, S., Small, J.V., 1993. Calponin and SM 22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. Differentiation 55, 1–11.

Frid, M.G., Printesva, O.Y., Chiavegato, A., Faggin, E., Scatena, M., Koteliansky, V.E., Pauletto, P., Glukhova, M.A., Sartore, S., 1993. Myosin heavy-chain isoform composition and distribution in developing and adult human aortic smooth muscle. J. Vasc. Res. 30, 279–292.

Gabbiani, G., Schmid, E., Winter, S., Chaponnier, C., de Ckhastonay, C., Vandekerckhove, J., Weber, K., Franke, W.W., 1981. Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a specific alpha-type actin. Proc. Natl. Acad. Sci. USA 78, 298–302.

Georgijevic, S., Subramanian, Y., Rollins, E.L., Starovic-Subota, O., Tang, A.C., Childs, S.J., 2007. Spatiotemporal expression of smooth muscle markers in developing zebrafish gut. Dev. Dyn. 236, 1623–1632.

Gerhardt, H., Semb, H., 2008. Pericytes: gatekeepers in tumour cell metastasis? J. Mol. Med. 86, 135–144.

Gittenberger-de Groot, A.C., DeRuiter, M.C., Bergwerff, M., Poelmann, R.E., 1999. Smooth muscle cell origin and its relation to heterogeneity in development and disease. Arterioscler. Thromb. Vasc. Biol. 19, 1589–1594.

Gittenberger-de Groot, A.C., Vrancken Peeters, M.P., Mentink, M.M., Gourdie, R.G., Poelmann, R.E., 1998. Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. Circ. Res. 82, 1043–1052.

Grimes, A.C., Stadt, H.A., Shepherd, I.T., Kirby, M.L., 2006. Solving an enigma: arterial pole development in the zebrafish heart. Dev. Biol. 290, 265–276.

Halayko, A.J., Solway, J., 2001. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. J. Appl. Physiol. 90, 358–368.

Hao, H., Gabbiani, G., Bochaton-Piallat, M.L., 2003. Arterial smooth muscle cell heterogeneity: implications for atherosclerosis and restenosis development. Arterioscler. Thromb. Vasc. Biol. 23, 1510–1520.

Hu, N., Sedmera, D., Yost, H.J., Clark, E.B., 2000. Structure and function of the developing zebrafish heart. Anat. Rec. 260, 148–157.

Hungerford, J.E., Owens, G.K., Argraves, W.S., Little, C.D., 1996. Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers. Dev. Biol. 178, 375–392.

Hutson, L.D., Jurynec, M.J., Yeo, S.Y., Okamoto, H., Chien, C.B., 2003. Two divergent slit1 genes in zebrafish. Dev. Dyn. 228, 358–369.

Jain, R.K., 2003. Molecular regulation of vessel maturation. Nat. Med. 9, 685–693.

Kim, S., Ip, H.S., Lu, M.M., Clendenin, C., Parmacek, M.S., 1997. A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. Mol. Cell. Biol. 17, 2266–2278.

Kramer, J., Aguirre-Arteta, A.M., Thiel, C., Gross, C.M., Dietz, R., Cardoso, M.C., Leonhardt, H., 1999. A novel isoform of the smooth muscle cell differentiation marker smoothelin. J. Mol. Med. 77, 294–298.

Lawson, N.D., Weinstein, B.M., 2002. In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev. Biol. 248, 307–318.

Li, L., Miano, J.M., Cserjesi, P., Olson, E.N., 1996. SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. Circ. Res. 78, 188–195.

Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. Nat. Rev. Genet. 8, 353–367.

Mack, C.P., Owens, G.K., 1999. Regulation of smooth muscle alpha-actin expression in vivo is dependent on CArG elements within the 5′ and first intron promoter regions. Circ. Res. 84, 852–861.

Madsen, C.S., Regan, C.P., Hungerford, J.E., White, S.L., Manabe, I., Owens, G.K., 1998. Smooth muscle-specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5′-flanking and first intronic DNA sequence. Circ. Res. 82, 908–917.

Majesky, M.W., 2007. Developmental basis of vascular smooth muscle diversity. Arterioscler. Thromb. Vasc. Biol. 27, 1248–1258.

Miano, J.M., Cserjesi, P., Ligon, K.L., Periasamy, M., Olson, E.N., 1994. Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. Circ. Res. 75, 803–812.

Miano, J.M., Georger, M.A., Rich, A., De Mesy Bentley, 2006. Ultrastructure of zebrafish dorsal aortic cells. Zebrafish 3, 455–463.

Mikawa, T., Gourdie, R.G., 1996. Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. Dev. Biol. 174, 221–232.

Owens, G.K., 1995. Regulation of differentiation of vascular smooth muscle cells. Physiol. Rev. 75, 487–517.

Owens, G.K., Kumar, M.S., Wamhoff, B.R., 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol. Rev. 84, 767–801.

Rensen, S.S., Doevendans, P.A., van Eys, G.J., 2007. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Neth. Heart J. 15, 100–108.

Ross, R., 1999. Atherosclerosis – an inflammatory disease. New Engl. J. Med. 340, 115–126.

Santoro, M.M., Samuel, T., Mitchell, T., Reed, J.C., Stainier, D.Y., 2007. Birc2 (cIap1) regulates endothelial cell integrity and blood vessel homeostasis. Nat. Genet. 39, 1397–1402.

Sata, M., Saiura, A., Kunisato, A., Tojo, A., Okada, S., Tokuhisa, T., Hirai, H., Makuuchi, M., Hirata, Y., Nagai, R., 2002. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat. Med. 8, 403–409.

Satchell, G.H., 1992. The venous system. In: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds.), The Cardiovascular System, vol. XII, Part A. Academic Press, Inc., San Diego, CA, pp. 141–183.

Sato, T., Yost, H.J., 2003. Cardiac neural crest contributes to cardiomyogenesis in zebrafish. Dev. Biol. 257, 127–139.

Scultetus, A.H., Villavicencio, J.L., Rich, N.M., 2001. Facts and fiction surrounding the discovery of the venous valves. J. Vasc. Surg. 33, 435–441.

Stainier, D.Y., Weinstein, B.M., Detrich 3rd, H.W., Zon, L.I., Fishman, M.C., 1995. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. Development 121, 3141–3150.

Stainier, D.Y., 2001. Zebrafish genetics and vertebrate heart formation. Nat. Rev. Genet. 2, 39–48.

Takahashi, K., Tazunoki, T., Okada, T., Ohgami, K., Miwa, T., Miki, A., Shibata, N., 1996. The 5′-flanking region of the human smooth muscle cell calponin gene contains a *cis*-acting domain for interaction with a methylated DNA-binding transcription repressor. J. Biochem. 120, 18–21.

van der Loop, F.T., Schaart, G., Timmer, E.D., Ramaekers, F.C., van Eys, G.J., 1996. Smoothelin, a novel cytoskeletal protein specific for smooth muscle cells. J. Cell Biol. 134, 401–411.

Wasteson, P., Johansson, B.R., Jukkola, T., Breuer, S., Akyurek, L.M., Partanen, J., Lindahl, P., 2008. Developmental origin of smooth muscle cells in the descending aorta in mice. Development 135, 1823–1832.

Wiegreffe, C., Christ, B., Huang, R., Scaal, M., 2007. Sclerotomal origin of smooth muscle cells in the wall of the avian dorsal aorta. Dev. Dyn. 236, 2578–2585.

Wilm, B., Ipenberg, A., Hastie, N.D., Burch, J.B., Bader, D.M., 2005. The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. Development 132, 5317–5328.

Yelon, D., Ticho, B., Halpern, M.E., Ruvinsky, I., Ho, R.K., Silver, L.M., Stainier, D.Y., 2000. The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development 127, 2573–2582.

Zhang, J.C., Kim, S., Helmke, B.P., Yu, W.W., Du, K.L., Lu, M.M., Strobeck, M., Yu, Q., Parmacek, M.S., 2001. Analysis of SM22alpha-deficient mice reveals unanticipated insights into smooth muscle cell differentiation and function. Mol. Cell. Biol. 21, 1336–1344.