New models to study vascular mural cell embryonic origin: implications in vascular diseases

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Abstract

A key question in vascular biology is how the diversity of origin of vascular mural cells, namely smooth muscle cells and pericytes influences vessel properties, in particular the regional propensity to vascular diseases. This review therefore first describes the role and regulation of mural cells during vascular formation, with a focus on embryonic origin. We then consider the evidence that connects heterogeneities in smooth muscle cell and pericyte origins with disease. Since this idea has major implications for understanding and modelling human disease, then there is a pressing need for new model systems to investigate mural cell development and the consequences of heterogeneity. Recent advances arising from in vitro strategies for deriving mural cells from human pluripotent stem cells as well as from the zebrafish model will be discussed and the medical relevance of these discoveries will be highlighted.
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**Abstract**

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**1. Introduction**

Cardiovascular diseases such as myocardial infarction and stroke are the main causes of death in Europe. The vast majority of these cases are secondary to atherosclerotic disease of the arterial wall. Other diseases of the vessel wall such as aortic aneurysms are less common but can have devastating consequences, often in young people with no warning. The high prevalence and severity of arterial diseases has resulted in an urgent need to better understand the underlying biology of the vessel wall in both health and disease.

An increasing body of work suggests that developmental factors continue to influence the response of adult blood vessels under physiological and pathological conditions. The vasculature is heterogeneous in terms of structural composition and function. Moreover there are striking regional heterogeneities in disease development that are thought to be governed by hemodynamic factors as well as vessel structure. Although structure and anatomy are developmentally determined, the prevailing hemodynamic view does not take into account the multiple different embryonic origins of vascular mural cells, namely smooth muscle cells (SMC) and pericytes, and the consequences of this heterogeneity on disease.
This review will therefore initially discuss the development and regulation of SMCs and pericytes during blood vessel formation, with an emphasis on their embryonic origins. We will accordingly focus on lineage specific variations in the signalling pathways that regulate mural cell development and impact on their distinct functional properties. These functional differences may lead to distinct differences in pathological responses and we will next emphasise the evidence connecting heterogeneities in SMC origins with disease and will examine both clinical and experimental studies. Since the idea of a developmental basis for the vascular disease response has important connotations for our understanding of human disease and for vascular regenerative medicine, then new experimental model systems that would allow us to examine vascular cell development and the consequences of mural cell heterogeneity, are urgently required. In recent years, we and others have progressed human pluripotent stem cells and the zebrafish model for investigating mural cells of distinct embryonic origins and their role in vascular development and disease. These model systems will be discussed in detail and we will highlight the translational potential of these exciting new tools.

2. Vascular mural cell embryonic development

Vascular mural cells are an essential component of the vasculature and are required for the normal development and homeostasis of mature blood vessels. The initial events in vascular development are differentiation of a primary endothelial vascular plexus from angioblasts which then undergoes angiogenic remodelling\(^6,7\). Vascular mural cells are recruited by developing blood vessels in a process called vascular myogenesis\(^8\). During this process, mesenchymal precursors are recruited by endothelial cells and ensheathe established blood vessels\(^9\)–\(^12\).

Vascular mural cells have been traditionally classified into vascular SMC and pericytes depending on their density, morphology, location, and expression of specific markers\(^13\)–\(^16\). Vascular SMCs are found mainly in medium and large blood vessels where a continuous single or multilamellar SMC layer surrounds the endothelial cell lining and provides contractility to modulate blood flow and stability. SMCs are separated from the endothelium by a basement membrane. SMCs secrete most of the extracellular matrix in the media, consisting mainly of elastin and fibrillar collagens I and III, which together with fibroblast-derived adventitial collagens largely determine the mechanical properties of the vessel wall\(^2\). Other common matrix components include laminin, collagen IV, nidogen, perlecan, and fibulins\(^17\)–\(^19\). Secretion of extracellular matrix from SMCs is essential for normal function and development while matrix abnormalities are commonly seen in vascular disease\(^20,21\). Modulation of the mural cell phenotype by extracellular matrix components is dependent on their binding to specific integrin and non-integrin receptors\(^22,23\).

Pericytes are mural cells found in microvessels such as terminal arterioles and capillaries, with high densities particularly in the brain, eye and kidney\(^24\)–\(^27\). Pericytes are isolated cells and unlike SMCs do not form a continuous layer. Their key feature is that pericytes are embedded within the basement membrane and make direct contact with the underlying endothelial cells. Functionally, their predominant roles likely include regulating capillary permeability, endothelial stabilisation and microvascular contractility\(^28\).

It is important to appreciate that SMC and pericytes are found as a continuum of mural cells, with a gradual change in morphology and features as the vasculature branches into smaller vessels. Indeed there are no specific molecular markers that unequivocally distinguish pericytes from SMCs and
validated markers for pericytes such as RG5S, CD146, NG2 (CSPG4), PDGFR-β and desmin may all be expressed in SMCs under a variety of conditions\textsuperscript{29}. For these and other reasons, pericytes and SMCs are often thought of as related cell types with developmental links\textsuperscript{10}. For the purposes of this review, we will use the general term ‘mural cell’ when referring to both SMCs and pericytes or when the cell type has not been clearly defined.

Vascular SMCs represent a mosaic tissue produced from multiple unique and non-overlapping developmental origins\textsuperscript{31} (figure 1). Pioneering studies conducted in avian embryos showed the first indication that vascular smooth muscle origins might be diverse. SMCs in the great vessels were traced back to a distant source of progenitors at the dorsal surface of the cranial neural tube\textsuperscript{32,33}. We now know that SMCs of the aorta and many of its proximal branches have multiple different developmental origins. The ascending aorta and arch, the pulmonary trunk, the brachiocephalic, subclavian and carotids are all derived from the neural crest. The secondary heart field contributes to a domain overlapping neural crest derived SMCs at the base of the aortic root\textsuperscript{34} while the descending aorta is derived from somites\textsuperscript{35}.

Although most of the work on ontology has been carried out on SMC, in cases where studies have been carried out on the embryonic origin of pericytes, they have been found to have a concordant origin with the SMC in the larger vessels supplying that territory. The majority of the SMC and pericytes in the head region, including those that vascularise the central nervous system, are neural crest derived, as demonstrated in chick-quail chimeras\textsuperscript{24,36} and indicated by marker expression in mice\textsuperscript{37}. Recent studies on thymus development demonstrated that also the mural cells in the thymus are derived from neural crest. The origins of vascular SMC in the gut\textsuperscript{38} and liver\textsuperscript{39} have been mapped to the mesothelium, the single-layer squamous epithelium that lines the coelomic cavities and its organs and originates from the lateral plate mesoderm. Coronary vessel mural cells in the heart appear to have a similar development. Here, the epicardial mesothelium is thought to give rise to cardiac mesenchymal cells, including coronary SMCs and pericytes. The case of pulmonary mural cells is interesting, as despite the proximal large pulmonary vessels originating from the neural crest, the more distal pulmonary artery SMCs and pericytes, consistent with vessels in other coelomic organs appear to be derived from pulmonary mesothelium\textsuperscript{40}. Further studies are still required to determine to what extent there is concordance between SMC and pericyte ontology and whether there are any vascular beds, in normal development or in response to disease processes that result in discordant origins for these mural cells.

3. Signaling pathways implicated in the developmental of mural cells

Mural cells are significant contributors to vascular development and vessel remodeling as well as disease. Although a unique list of markers defining mural cells is a desiderata for many biologists and clinicians such effort remains a puzzling task (reviewed in detail by Shen and McCloskey\textsuperscript{30}). Some of these markers vary between pericytes and vascular SMCs and change due to the heterogeneity and plasticity of these cells during time and space. The anatomical relationship between mural and endothelial cells suggests close interactions involving paracrine or juxtacrine signaling but also less studied factors such as mechanical forces, cell-matrix contacts and lipid signaling. Here we summarize the major endothelial-mural cross-talk signaling described so far. Importantly, the role of many of these pathways varies depending on the embryonic lineage of the mural cells.

3.1 PDGF-BB/PDGFR-β
PDGF receptor (PDGFR)-β expression is considered as one of the first indicators of mural cell lineage. PDGF-BB is released from angiogenic endothelial cells and binds to PDGFR-β expressed on the surface of emerging mural cells. As a result, PDGFR-β-positive mural cells (or their mesenchymal progenitors) are recruited with the angiogenic sprouts and microvessel wall. PDGFR-β is expressed broadly by pericytes and developing vascular SMCs, and PDGF-BB probably plays a role also in the proliferation and differentiation of aortic and venous vSMCs. Knockout of the pdgfb or pdgfrb genes in mice results in virtually identical phenotypes and perinatal lethality resulting from vascular dysfunction caused by mural cell deficiency. The degree of this deficiency varies extensively between different organs, suggesting that other signaling pathways may play a similar role to that of PDGF-BB/PDGFR-β in mural cell recruitment. Regional variations in other signaling pathways have been shown to depend on embryonic origin (see TGF-β and Notch sections) suggesting a possible developmental explanation for the regional variation in PDGF-BB signaling.

3.2 TGF-β signaling

Cytokines of the transforming growth factor-β (TGF-β) family, including TGF-βs, bone morphogenic proteins (BMPs), activins, and nodal, play crucial roles in embryonic development and adult tissue homeostasis by regulating cell proliferation, survival, and differentiation, as well as stem-cell self-renewal and lineage-specific differentiation. In the cardiovascular system, TGF-β signaling has been shown to play crucial roles in vasculogenesis, angiogenesis, and lymphangiogenesis including regulation of mural cell differentiation, proliferation and migration. Recently, pericytes have been suggested to differentiate from tissue myeloid progenitors in the skin vasculature through TGF-β signaling. TGF-β pathways also interact with other key vascular signaling pathways. For example, in cerebral endothelial cells the TGF-β/SMAD4 pathway has been shown to cooperate with Notch/RBP-Jk pathway to regulate N-cadherin expression and stabilize contacts between endothelial cells and pericytes.

Importantly, studies have shown a specific requirement for Smad2 and Mkl2 in development by the neural crest-derived aortic arch SMCs. Meanwhile in adult SMCs, TGF-β elicits key differences in the response of SMC of different embryonic origins with proliferation of neural crest-derived aortic arch SMCs and suppression of proliferation in mesoderm derived SMCs. Additionally, angiotensin II signaling which enhances TGF-β signaling also has a lineage dependent effect on SMC. Owens et al demonstrated a divergent effect of angiotensin II infusion with SMC hyperplasia in the ascending aorta and hypertrophy in the descending aorta. These findings highlight that the complex and pleiotropic actions of various growth factor signaling cascades on SMC development and function are further modified by their embryonic origins.

3.3 Notch signaling

In the development and differentiation of vascular SMCs, the roles of the Notch pathway can be categorized into two different types: constructing a mature blood vessel wall and artery-vein differentiation. Once recruited to endothelial tubes, Notch ligands in endothelial cells can induce Notch activation in mural cells, which promotes integrin adhesion to endothelial basement membrane and initiates maturation and differentiation. Besides, the Notch activation in vascular SMCs can further lead to the increased expression of Notch ligand, Jagged1 which allows for lateral induction of Notch signaling by homotypic vascular SMC-SMC interactions through multiple layers of smooth muscle to promote SMC differentiation. Furthermore, Notch can also be closely tied to the
many possible phenotypic endpoints of vascular SMCs. For example, Notch signaling in vascular SMCs can promote contractile differentiation and also there are contrasting data supporting that Notch can promote or inhibit vascular SMC proliferation in vitro and in vivo. A key factor that may explain opposing responses could be the embryonic lineage of the SMC. Recent studies have shown that SMC of different embryonic origins respond differently to notch3 depending on developmental stage. In the zebrafish model Notch3 enhances brain vascular integrity by regulating pericyte numbers.

Several reports support a role of Notch signaling for vascular SMC and/or pericyte survival, proliferation, differentiation and even migration. Further evidence proposes the role of Notch-PDGFBB interactions in pericyte differentiation with Dll4 and PDGF-BB that can convert skeletal myoblasts to pericytes. These data indicate that Notch is required for mural cell recruitment to the blood vessel wall in response to PDGF-BB signal.

Overall, both endothelial cells and mural cells express several Notch receptors and ligands indicating that this signaling pathway is crucial in both angiogenesis and vascular maturation. Hemodynamic and environmental conditions have been shown to modulate this pathway as well, altering the response of endothelial cell and SMC physiology. Mutant analyses of Notch receptors and its ligands in human, mice and zebrafish also support different functions of this pathway among species. Overall, our understanding on how Notch pathway regulates the association of mural cells and endothelial cells is still very limited and far from being completely decoded.

4. Developmental heterogeneity and disease

The presence of different embryonic origins for distinct mural cell populations or vascular beds raises a crucially important question: does embryonic origin impact regional susceptibility or development of disease? In this section we review clinical studies that suggest that embryonic origin may have a role in regulating disease development and then examine experimental data from animal models that supports this hypothesis.

Studies by deBakey in cardiac patients and the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study suggest a regional heterogeneity in disease development. deBakey and Glaeser reviewed 5,568 patients who initially had documented occlusive atherosclerotic disease in a single region and required surgery and had repeat angiography to measure progression. These patients were divided into 4 arterial disease categories: coronary, aortic branches, abdominal viscera and terminal abdominal aorta and branches. After 25 years follow up, different arterial beds showed different responses to common risk factors. The patients in this study were selected as already having established occlusive disease. The PDAY study on the other hand examined unselected young people who had died of noncardiac reasons and compared atherosclerotic disease in different regions. The investigators found that systemic risk factors had differential effects on disease development in different vascular regions. For example males had more fatty streaks in the right coronary artery than women but women showed increased early disease in the aorta. Smoking predisposed to increased fatty streaks only in the abdominal aorta while glycated haemoglobin levels predicted early disease in the right coronary artery only. Together the findings from the
deBakey and PDAY studies suggest that different vascular regions have different susceptibilities to common systemic risk factors.

It should be noted that these findings do not inform us as to the cause of the differing susceptibilities to disease. One possible mechanism, as posited in this review article, is the distinct embryonic origins of the SMC leading to differences in responses to disease mediators between different vascular regions. Other causes could include differences in vessel wall structure. For example, Sims found an increased number of discontinuities in the internal elastic laminae of coronary arteries versus internal mammary arteries. The greater elastic lamina discontinuities were associated with increased cellular content in the coronary intima, which may predispose the coronary artery to develop atherosclerosis compared to the internal mammary. It is of course possible that these differences in elastic tissue structure are also developmentally mediated.

Another vascular disease that has characteristic distribution is aortic aneurysm and dissection. These are categorised typically as abdominal or thoracic aortic aneurysms (Figure 2). Abdominal aneurysms are associated with many of the same risk factors as for atherosclerosis, while thoracic aneurysms have an association with genetic syndromes (e.g. Marfan syndrome) and frequently present in the aortic root and ascending aorta. In particular, there is increasing evidence for a possible role of different SMC lineages in the development of thoracic aortic aneurysms.

Since the majority of elastin is deposited in fetal and early neonatal life, it seems differences in vessel wall mechanical properties are developmentally programmed and again raise the possibility of embryonic lineage having a role. The aortic media is composed of extracellular matrix and SMC. Elastic fibres and collagen make up at least 50% of dry weight. Elastic fibres give rise to aortic elasticity while collagens provide overall stiffness and strength. These mechanical parameters vary along the course of the vascular tree (examples of local variability). Of note the aorta provides 65% of all arterial compliance and the majority of this is in the ascending aorta and arch while the ascending aorta has the highest elastin content. Moreover, while these variable local mechanical properties may be advantageous for pump function and blood distribution, they may predispose to aortic disease especially at transition zones between different SMC lineages.

A key requirement for development of aortic aneurysm and dissection is medial weakness and predisposing factors include hypertension, genetic connective tissue disorders, pregnancy, atherosclerosis and arterial inflammation. If embryonic origins predispose different regions of the aortic wall to produce differing amounts of structural proteins or distinct cellular behaviour, then the outcome may be regional differences in strength and disease development. In this context, the entry intimal tear in dissecting thoracic aneurysms is usually found in the ascending aorta (29%) or at the sino-tubular junction (29%) in type A dissections, an area where SMC of different embryonic origins are juxtaposed.

The interaction between SMC populations of different embryonic origins has been proposed to be detrimental in some genetic aortopathies. Increased TGF-β signalling has been invoked as a driver of aortic aneurysms in Marfan syndrome. It is then paradoxical that in Loeys-Dietz syndrome, a related aortopathy syndrome, that the mutations in TGFbRI or TGFbRII are loss of function mutations. Although in vitro studies show reduced signalling through these mutant receptors, there is evidence of increased Smad2 phosphorylation in late stage aneurysms and the observations have been reconciled by proposing compensatory increases in TGF-β release/activation by one SMC.
lineage that leads to excess signalling in adjacent SMC from a different lineage\(^79,80\). While the precise nature of TGF-\(\beta\) in aneurysm development is now subject to considerable debate\(^81-84\), the concept of adjacent populations of SMC from different lineages leading to the development of a maladaptive extracellular milieu for one or both of the populations which predisposes to disease is an interesting idea that needs further experimental validation. Certainly distinct regions of SMC are juxtaposed in the aortic root and at the aortic isthmus, both regions that are highly susceptible to an intimal tear\(^77\).

In addition to clinical observations, a number of animal studies have suggested that regional embryonic heterogeneity may predispose to disease. Aortic arch SMC derived from the neural crest, were shown to have a greater propensity to calcify compared to the mesodermal derived descending aorta, using either aortic explants under high phosphate conditions or in vivo in a MGP null mouse model\(^85\). The authors concluded that the aorta was developmentally regionalised and this heterogeneity had an effect on pathological calcification. Similarly, Pruett et al\(^86\) identified that Hox genes identified positional identities in the vasculature and that transgenic overexpression of Hoxc11 resulted in region-specific remodelling of the arterial tree\(^87\). Meanwhile Andres and colleagues identified regional differences in Hox family member gene expression and NFkB activity that anti-correlated in the aortas of atherosclerosis susceptible mice\(^88\). They further demonstrated a reciprocal inhibitory action between NFkB and HoxA9, thus identifying a possible regulatory mechanism that defined an atherosclerosis susceptible aortic arch with high NFkB activity and low HoxA9 expression, while conversely the atherosclerosis-resistant descending thoracic aorta had low NFkB activity and high expression of HoxA9. The most intriguing finding in this study however was that similar differences in Hox family member gene expression patterns including HoxA9 were detected between SMC generated in vitro from neuroectoderm (analogous to the aortic arch) versus paraxial mesoderm (analogous to the descending aorta). This finding suggests that at least some of the differences in gene expression that define regional vascular susceptibility to disease are programmed during development, since there were no confounding factors such as flow or vessel structure in the in vitro studies.

Despite our focus on embryonic origin, it should be remembered that a major determinant of the site specific nature of vascular disease development is regional differences in hemodynamic forces. In particular for the development of atherosclerosis, low shear stress\(^89\) and turbulent flow are thought to be important drivers of disease location. Accordingly mouse models of atherosclerosis show increased disease at branch points and along the lesser curvature of the aorta. Indeed there is considerable evidence of differential hemodynamic profiles predisposing different endothelial populations to display distinct responses to systemic risk factors\(^3\). Thus we propose that the effect of embryonic origins is to provide a basal regional susceptibility to disease that then responds to highly variable local hemodynamic cues.

5. New models to study SMC and pericyte developmental heterogeneity

5.1 Human pluripotent stem cells

Generation of mural cells from embryonic stem cells and induced pluripotent stem cells, together termed pluripotent stem cells (PSC), offers the opportunity to recapitulate many of the developmental steps that take place in the embryo. In particular, the availability of human PSCs
enables the study of SMC and pericyte development and disease in a human context. A number of groups have developed protocols to generate mural cells from PSCs and these have been reviewed recently by Dash et al\textsuperscript{90} and Sinha et al\textsuperscript{91}. These approaches initially depended on spontaneous differentiation in embryoid bodies, which results in a heterogenous mixture of different types of SMCs and/or pericytes. Further developments in in vitro methods included the use of stromal feeder cells, or specific matrix coatings such as collagen IV, or a combination of growth factors along with serum to induce SMCs. Although these systems were efficient in producing mural cells, the limitations included the lack of chemically defined systems and difficulties in relating the in vitro stages to known developmental events in the embryo. In addition the phenotypic characterisation of the resultant smooth muscle-like cells and their precise developmental stage and functionality were not always fully defined.

Indeed, given that the primary distinction between SMC and pericytes is related to endothelial cell contact and sharing of the endothelial basement membrane, it can be difficult to distinguish between these cells in conditions where morphological criteria are lacking such as in vitro. Thus the majority of in vitro protocols use molecular markers to identify the cells, although as most of these are shared between SMC and pericytes, then the precise identity of in vitro generated mural cells can be indeterminate\textsuperscript{92}. In many protocols, cells were shown to express mature SMC specific markers such as MYH11 and SMTN\textsuperscript{93,94}, suggesting the generation of bona fide SMCs although such marker expression can be limited and variable or poorly characterised\textsuperscript{95}, and may reflect a degree of plasticity of PSC-derived mural cells in vitro. Alternatively, mural cells that did not express MYH11 or SMTN have been labelled either as general mural cells\textsuperscript{92} or as pericytes\textsuperscript{96}, although given the overlapping nature of pericyte markers the latter may also represent immature SMCs.

One of the biggest limitations was that the embryonic origin of mural cells in these in vitro systems was relatively neglected. To address this limitation, we described the use of a chemically defined system that used human PSCs to first generate neuroectoderm, lateral plate mesoderm and paraxial mesoderm, the 3 embryonic tissues that give rise to the majority of vascular SMCs\textsuperscript{97}. We then generated lineage specific SMCs from these intermediate embryonic tissues that displayed all the markers and functionality of contractile SMCs but differed in behaviour in the same way as already described for SMCs from different aortic or vascular regions\textsuperscript{98}. For example, during development MKL2 is required specifically by neural crest derived SMCs, while these same SMC in the adult proliferate in response to TGF-\beta while mesoderm-derived SMCs do not. The lineage specific hPSC-derived SMC platform was able to reproduce these differences in the need for MKL2 during development or in the response to TGF-\beta in neuroectoderm-derived SMCs compared to those from mesoderm. This system was further refined by the generation of epicardium, the precursor for coronary SMCs, from the lateral plate mesoderm\textsuperscript{99}. Epicardial cells expressed multiple epicardial markers and when injected \textit{in ovo}, were able to localise to their subepicardial niche in a chick model. Taken together, these findings supported the validity of an in vitro lineage specific system.

In addition to validating known differences between different SMC populations, a key requirement for such an in vitro model is to make novel predictions that can be tested in vivo or in primary SMC cultures. Our in vitro model was firstly able to predict the differential proteolytic responses of different regions of SMC from different regions of the rat aorta when treated with IL-1\beta\textsuperscript{97}. Indeed we hypothesise that this differential degradation of underlying extracellular matrix in different aortic regions may predispose to increased wall stress and intimal tear at sites where there is a
juxtaposition of two different SMC lineages. Secondly, further in vitro studies revealed a key role for Notch3 in SMC development specifically from the neuroectoderm lineage. While this finding predicts abnormalities in neural crest derived SMC in vivo, the Notch3 null mouse had not been shown to have any vascular abnormalities during fetal development. However, a more detailed analysis unearthed a mild phenotype with reduced maturation of carotid SMC in Notch3 null embryos at E13.5 compared to wild type controls. Finally, gene expression studies from this in vitro model also predict a diversity of functions between SMCs of distinct embryonic origins. For example, the increase in genes related to calcification in neuroectoderm-derived SMC is consistent with the high frequency of calcification in the ascending aorta and aortic arch. Further differences in gene expression and corresponding functional differences in vitro between the different lineages, such as SMC migration or inflammatory activation, also warrant investigation as to their in vivo relevance. Together these findings reinforce the validity of this lineage specific approach and highlight the need to consider embryonic origins when considering SMC biology.

One of the most exciting uses of human PSC-derived SMC systems is in modelling human vascular diseases in vitro using patient-derived induced iPS cells. Since it is generally difficult to obtain human vascular tissues, and tissues obtained at the time of surgery usually represent end-stage disease, then an in vitro system enables an unprecedented analysis of early mechanisms that may be driving the disease and be amenable to therapy. Initial studies on SMCs examined genetic diseases such as Hutchinson-Gilford Progeria syndrome and Williams-Beuren syndrome. These resulted in key insights into the cellular and molecular pathologies that develop in these conditions and the in vitro platform then offers a system to screen for and test new therapies. However, limitations such as the precise type of SMC generated, an inability to account for the localised nature of some of these conditions and a lack of rigorous isogenic control lines remained.

We recently addressed some of these limitations using the lineage specific system of SMC generation to model the aortic abnormalities in Marfan syndrome. SMCs derived from iPSCs generated from two patients with severe aortic disease recapitulated multiple phenotypical abnormalities seen in the Marfan aorta including increased matrix metalloproteinase activity, disruption and loss of extracellular microfibrils, reduction in SMC contraction and proliferation and increased SMC death. We identified increased TGF-β signalling through both canonical Smad pathway and the non-canonical ERK1/2 and p38. These structural, biochemical and functional abnormalities were reversed on CRISPR/Cas9-mediated correction, verifying that the disease phenotypes seen were indeed caused by the single nucleotide mutation. Importantly, treatment with losartan – an angiotensin receptor blocker that was highly promising in mouse studies, but disappointing in recent clinical trials – was only able to rescue the extracellular matrix and proliferative phenotypes, while cell death was regulated independently by p38 and KLF4. Cyclic stretch exacerbated the disease phenotype through a β1-integrin mediated pathway, thus validating a mechanosensory component of the disease mechanism. Intriguingly, the phenotypic abnormalities were most severe in neural crest-derived SMCs, suggesting a developmental explanation for the location of the aortic aneurysms in Marfan syndrome in the aortic root, ascending aorta and arch. In fact the earliest onset of the aneurysm in this condition is in the aortic root, a region with extensive overlap between secondary heart field and neural crest-derived SMCs. These observations support the hypothesis that juxtaposition of SMCs of different embryonic origins may potentially be detrimental to vascular health, and reinforce the importance of using lineage specific SMC systems for disease models.
These iPSC-based strategies could also be used with other genetically mediated aortic syndromes, such as Loeys-Dietz or vascular Ehlers-Danlos syndromes. Given the principal location of Loeys-Dietz aneurysms in the aortic root and ascending aorta, it is likely that as for Marfan syndrome, there will be differences in disease susceptibility in SMC of different embryonic origins. Indeed, the ‘Loeys-Dietz paradox’ of loss of TGF-β function mutations leading to increased TGF-β signalling is postulated to involve maladaptive compensatory responses in juxtaposed SMC populations. This hypothesis could be tested in vitro using lineage specific SMC from patient-derived iPSCs. Conversely, the aneurysmal distribution in vascular Ehlers-Danlos is more widespread and so it would be predicted that all SMC lineages would display a disease phenotype equally. A lineage based disease modelling approach also has value beyond single gene disorders. In the aortopathy associated with bicuspid aortic valve, there were differences in contractility and maturation of SMCs from neural crest vs paraxial mesoderm, including reduced canonical TGF-β signalling and increased mTOR activity in neural crest-derived SMC. These findings shed light on the underlying abnormalities in this relatively common condition and again suggested a developmental explanation for the distribution of the disease.

Although many diseases have been studied using mouse or other animal models, these are not always representative of human physiology or disease. In particular, animal models cannot replicate the genetic context of patient derived cells that may be essential for disease development. For example, genetic variants that affect noncoding regions of the genome frequently have no corresponding mouse sequences. This is clearly illustrated by the findings of the recent genome wide association studies. These have so far identified at least 58 variants that are associated with cardiovascular disease. However, the majority of these variants are located in noncoding regions, which are poorly homologous in mice, and the genes involved in the pathology are unknown. Using hiPSC with and without the risk haplotypes may enable these variants to be investigated in a human vascular context.

Recently the ability to efficiently carry out site specific single nucleotide changes using CRISPR/Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) methods offers a powerful tool to validate causal variants. This approach is based on a bacterial defence system adapted for use in mammalian cells. Bacteria respond to infecting viruses by incorporating foreign DNA into the bacterial genome as clusters of short repeats which can be used to provide adaptive immunity. On reinfection, these clusters are transcribed and act as guides for Cas proteins that will target and interfere with the identified invading DNA. Using the same approach, mutations may also be introduced into wild type PSCs to create disease models obviating the need for patient samples. It should be noted however that one advantage of patient derived iPSCs is that the extent of clinical disease is usually clear; with ‘patient-free’ in vitro engineered disease models it would not be clear in advance whether the genetic background would be permissive for disease development.

Other translational fields where SMC lineage may be relevant include vascular regeneration and new drug testing. hPSC-derived SMC are being used to regenerate or tissue-engineer both large vessels and the microvasculature. To date no studies have compared the use of different lineage specific SMC, although given their distinct biological properties it is likely that there will be distinct functional responses depending on the mural cell origin resulting in different vessel properties. As a first attempt at this question, Bargehr and colleagues found that SMC derived from hPSCs supported network formation by endothelial cells in vitro and that the extent of support varied according to the
embryonic origin of the SMC\textsuperscript{116}. Differential expression of a range of angiogenic factors such as midkine, mediated the differential supportive properties of distinct SMC lineages. While the developmental significance of these findings is unclear, the results have implications for regenerative medicine strategies where vascular regeneration is planned. In terms of drug testing, lineage specific neuroectoderm-derived SMC have recently been used in an endothelial cell co-culture system that includes physiological or pathological shear stress when testing compounds\textsuperscript{117}. While this study was the first to compare hiPSC-derived SMC and endothelial cells with primary cells and validated the use of these in vitro generated cells for drug testing, further studies comparing the responses of multiple different lineages would be revealing.

Although PSC based strategies for generating lineage specific SMCs offer great promise for understanding regional variations in disease development, the underlying mechanisms and testing new drugs, there are a number of limitations that need to be borne in mind when using this approach. The most important consideration is to what extent the in vitro findings reflect in vivo events. It is inevitably difficult to replicate the multiplicity of other non-SMC that may interact with SMC in the vessel wall, although endothelial cell co-cultures are a first step in this direction\textsuperscript{117}. Furthermore effects of vessel wall structure, blood flow and cyclic stretch should be borne in mind and can be modelled in vitro to some extent\textsuperscript{106,117,118}. Indeed tissue engineered vessels offer the opportunity to study several of these factors in an integrated manner, although they suffer from usually being low throughput and time-intensive\textsuperscript{112}. Another consideration is the extent to which embryonic differences are maintained in the adult vasculature. Pfalzgraf and colleagues have described a loss of the transcriptional and phenotypic differences seen in embryonic aortas when re-examined in adult mice\textsuperscript{119}. These findings are somewhat controversial due to possible cellular heterogeneity in the adult tissues used for transcriptional analyses and the use of immortalised SMC lines, and need further validation. Nevertheless, they raise the interesting idea of phenotypic convergence in adult vessels, although since embryonic genes are frequently re-expressed in disease, then differences mediated by embryonic origin may still have a role in regulating adult disease.

5.2 Zebrafish as model system to study mural cell origin in vertebrates

The zebrafish (\textit{Danio Rerio}) has emerged in recent years as an excellent vertebrate model organism to study a wide variety of biological processes. Some of the advantages of the zebrafish model system include high fecundity, external fertilization, rapid development and optical clarity. Last, but not least, zebrafish has low-cost maintenance compared to other vertebrate model systems. The importance of zebrafish for cardiovascular research relies on some unique characteristics such as the early development of a functional cardiovascular system (beating heart, aorta, cardinal vein, and blood) that is already formed by 24 hours post fertilization (hpf). Since the zebrafish embryo is relatively small and aquatic, oxygen can diffuse passively through tissues and thus embryos can live up to three days without a functional cardiovascular system\textsuperscript{120,121}. This allows genetic manipulation of cardiovascular development for longer than would be possible in mammals, where the absence of a functional cardiovascular system is fatal during its early development.

A major advantage of the zebrafish system is the combination of optical clarity and simple transgenics, which has resulted in a powerful model system for analysing the molecular basis of
During cardiovascular development mural cells appear shortly after the onset of circulation of blood flow. As consequence, mural cell coverage and mechanical signalling promote accumulation of extracellular matrix and vessel’s wall maturation. Recently, the role of flow-dependent forces and development. Until 5 days post fertilization (dpf), zebrafish embryos are nearly transparent, allowing in vivo visualization (even at single cell resolution) of the heart, blood vessels, as well as other tissues, without instrumentation or manipulation other than the use of a stereomicroscope. Furthermore, the generation of tissue-specific transgenic lines is relatively easy. Such a method usually uses a native tissue-specific promoter to drive expression of a fluorescent reporter protein, such as green fluorescent protein or mCherry. Coupled with impressive optical clarity, these transgenic lines allow observation of in vivo cellular behaviour in a way impossible in other models. Transparent zebrafish embryos are also well suited for in vivo time-lapse imaging. The fast acquisition speed of a spinning disk and 2-photon confocal microscopy reduces the recording time significantly when millimeter-sized embryos need to be imaged at high resolution and at short time intervals. Light Sheet Fluorescence Microscopy could also be very useful in zebrafish. These attributes, including the possibility of carrying out gain- and loss-of-function studies, have led to the emergence of the zebrafish as an excellent embryological model that provides a unique opportunity to uncover novel insights into the molecular genetics of endothelial and mural cell development. It should be noted that in early zebrafish development, due to the lack of specific reagents for characterization of vascular basement membrane as well as the lack of selective markers for mesenchymal cells associated with blood vessels, it is difficult to label cells definitively as pericytes or SMCs so we use the more general term: mural cell.

In the beginning we showed that zebrafish embryos develop mural cells that share many of the morphological, molecular and functional characteristics of mammalian mural cells. This Initial investigation has been essential to other investigations using the zebrafish model to study the biology of vascular mural cells. Early zebrafish mural cells expressed characteristic SMC/pericyte markers such as PDGFR-β, ACTA2 and Transgelin. Other well-known mammalian pericyte markers such as NG2, RGSS and CD146 has never been tested in zebrafish due to the lack of species-specific reagents. Dorsal aorta mural cells appeared to originate from the lateral plate mesoderm on the ventral side of the dorsal aorta, suggesting evolutionary conservation of embryonic origin and function. Subsequently, specific transgenic fish were generated and used to trace the live development of mural cells in embryonic and larval stages. Acta2, pdgfr and tagl-based transgenic animals are currently available to follow vascular SMC and pericyte recruitment from early stages of development to adulthood.

Recently, by using live imaging of mural cells and by lineage tracing in vivo Ando and colleagues illustrate the developmental origins and biology by which mural cells develop and cover endothelial cells in zebrafish embryos. They show that in cranial vessels and in the aortic arches mural cells were derived from neural crest cells, while vessels in the hind brain were of mesoderm origin. In the trunk vasculature, mural cells derived from mesoderm covered the ventral side of the dorsal aorta (DA), but not the posterior cardinal vein. Furthermore, live imaging clarified that mesoderm-derived mural cells are induced and recruited at the ventral portion of the dorsal aorta and then migrate from the dorsal aorta around intersegmental vessels (ISVs) preferentially covering arterial rather than venous ISVs. These findings suggest that arterial endothelial cells promote greater mural cell recruitment and maturation compared to their venous counterparts.

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primary cilia in vascular mural cell coverage of developing vessels in zebrafish embryos has also been investigated\textsuperscript{128}. By using advanced genetic tools it has been shown that mural cells are recruited by arterial-fated vessels and that this process is strictly dependent on selective hemodynamic-dependent arterial Notch signalling. This flow-dependent Notch activation and the associated endothelial genetic program are driven by endothelial primary cilia as determined by tissue-specific CRISPR gene targeting. Ultimately, zebrafish foxc1b was identified as a cilia-dependent Notch specific target that is required in endothelial cells to drive mural cell coverage in a flow-dependent manner\textsuperscript{128}. These data offer a novel hemodynamic-dependent mechanism driving vascular myogenesis in developing vasculature that may provide a new and better understanding of endothelial and mural cell interaction in health and in disease.

Most studies in zebrafish that have examined vascular disease have focused on endothelial patterning and blood components\textsuperscript{130}. However, the SMC developmental homology between zebrafish and mammals and the potential advantages of high-resolution imaging and tissue-specific genetic approaches raise the prospect of using the zebrafish to study the genetics of vascular SMC-related human diseases. It has been shown that in zebrafish mural cells surrounding brain central arteries are Notch3 dependent for proliferation and integrity\textsuperscript{64}. Zebrafish Notch3 mutants display both cerebral hemorrhages and pericyte loss supporting a mechanism whereby in zebrafish Notch3 promotes mural cell integrity and models CADASIL human disease.

Challenges to such an approach in zebrafish may include the relative sparsity of mural cells in the zebrafish vasculature, the low pressure arterial environment and the lack of optical clarity in adult fish. Nevertheless, the zebrafish model has been used to provide insights into thoracic aortic aneurysm development. Rare variants in MAT2A and FOXE3 have been identified that were associated with familial thoracic aneurysms\textsuperscript{131,132}. Knockdown of the orthologous gene in zebrafish led to abnormalities in aortic arch development, which were rescued by the wild type human MAT2A or FOXE3 to a greater extent than by the mutant versions. It wasn’t clear in these studies whether the defect was solely in endothelial cell patterning or whether there were primary defects in the developing arch mural cells. However, FOXE3 was investigated further in a mouse model which showed reduced medial SMC numbers, density and SMC marker expression in the ascending aorta and arch along with an increased propensity to SMC death and rupture in response to transverse aortic constriction. More detailed studies of the role of mural cells in zebrafish models of aortic aneurysms would clarify the utility of the zebrafish system for SMC disease modelling.

There are some limitations with the zebrafish system as mentioned above with a relative sparsity of mural cell density and loss of optical clarity in adults. In addition, mammalian antibodies frequently do not cross react with fish antigens and require either the development of fish-specific antibodies or the use of in situ hybridisation or transgenic reporter techniques. Finally, genome duplication events after evolutionary divergence of zebrafish and land-based invertebrates results in either a lack or duplication of orthologous human genes\textsuperscript{133}. Accordingly, the absence of conditional knock-out and knock-in technology still represents a weakness of this model system. Despite these issues, the many advantages of the zebrafish system render it a powerful and exciting new model for studying the role of mural cell lineage on vascular development and disease.

6. Summary and Conclusions
A key question in vascular biology is how the diversity of origin of mural cells influences vessel properties, in particular the regional propensity to vascular diseases. There is now increasing evidence that the mechanisms regulating development and function of distinct vascular regions depends on their embryological origins. Moreover, pathological responses appear to be influenced by the origin of the SMC or pericytes involved. The challenge to the field is to identify in detail the effects of mural cell lineage as distinct from other undeniably important factors such as prevailing haemodynamics and vessel wall structure. To facilitate these studies, we have highlighted 2 new models. First, human PSCs enable the generation of lineage specific SMC in vitro, providing opportunities for comprehensive in vitro analyses of development and disease on a human genetic background. Secondly, the zebrafish model combines the power of an in vivo system with unmatched access to live imaging of the developing vasculature. These and other model systems offer the prospect of new understanding of disease mechanisms and new therapies for a range of vascular diseases.

Conflict of Interest

The authors declare no conflict of interest.

Figure legends

Figure: 1. Schematic representation of vascular mural cell origin during development.

Fate mapping studies identify a highly mosaic distribution of mural cells with different developmental origins for vascular smooth muscle and pericytes. Neural crest cells contribute to mural cells of the aortic root, ascending aorta, arch and branches as well as pulmonary trunk (not shown). Lateral plate mesoderm (LPM) gives rise to secondary heart field and the base of the aortic root, as well as to the proepicardium. The proepicardium is a developmentally complex structure that also has origins from the septum transversum (ST), a rostral mesodermal structure that folds to lie between the developing heart and liver. During early cardiac development, the proepicardium forms the epicardium, a mesothelial structure around the developing heart, which contributes to the coronary arteries and to coronary pericytes. Paraxial mesoderm (PM) gives rise to the somites, which contribute to the descending aorta and its distal branches.

Figure 2: Thoracic and abdominal aortic aneurysms.

Aortic aneurysms, caused by weakness and dilatation of the vessel wall, are commonly seen in the aortic root and ascending aorta or in the abdominal aorta. Thoracic aneurysms are prone to dissection; classified as type A for those that involve the ascending aorta and arch and type B when only the descending aorta is involved. The sites of dissection (arrows) occur frequently at the sinotubular junction or at the aortic isthmus, regions where there is juxtaposition of SMC of different embryonic origins.
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