TOPICAL REVIEW

Origin and differentiation of vascular smooth muscle cells

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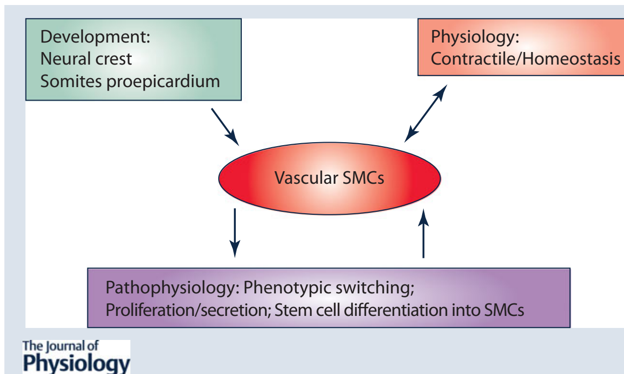

Abstract Vascular smooth muscle cells (SMCs), a major structural component of the vessel wall, not only play a key role in maintaining vascular structure but also perform various functions. During embryogenesis, SMC recruitment from their progenitors is an important step in the formation of the embryonic vascular system. SMCs in the arterial wall are mostly quiescent but can display a contractile phenotype in adults. Under pathophysiological conditions, i.e. vascular remodelling after endothelial dysfunction or damage, contractile SMCs found in the media switch to a secretory type, which will facilitate their ability to migrate to the intima and proliferate to contribute to neointimal lesions. However, recent evidence suggests that the mobilization and recruitment of abundant stem/progenitor cells present in the vessel wall are largely responsible for SMC accumulation in the intima during vascular remodelling such as neointimal hyperplasia and arteriosclerosis. Therefore, understanding the regulatory mechanisms that control SMC differentiation from vascular progenitors is essential for exploring therapeutic targets for potential clinical applications. In this article, we review the origin and differentiation of SMCs from stem/progenitor cells during cardiovascular development and in the adult, highlighting the environmental cues and signalling pathways that control phenotypic modulation within the vasculature.

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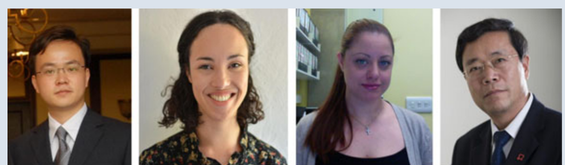
Abbreviations EndMT, endothelial-to-mesenchymal transition; HDAC, histone deacetylase; PDGF, platelet-derived growth factor; PDGFR-β, PDGF receptor-β; TGF-β, transforming growth factor-β; SmαA, α-actin; SMC, smooth muscle cell; SMMHC, smooth muscle myosin heavy chain; SRF, serum-response factor; VEGF, vascular endothelial growth factor.



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Introduction

Smooth muscle cells (SMCs) provide the main support for the structure of the vessel wall and regulate vascular tone in order to maintain intravascular pressure and tissue perfusion. It is a well-known fact that SMCs retain significantly more plasticity than other cell types in order to carry out different functions including contraction, proliferation and extracellular matrix synthesis (Alexander and Owens, 2012*a*). SMCs can undergo profound changes between two phenotypes: a quiescent one with differentiated SMCs, and a proliferating one with dedifferentiated SMCs (Salmon *et al.* 2012). The former type of SMC express a set of up-regulated smooth muscle markers, such as cytoskeleton and contractile proteins, which comprise smooth muscle α-actin (SMαA), smooth muscle myosin heavy chain (SMMHC), calponin and SM22α. All are necessary for the main function of SMCs, the contraction of the vessel wall. The expression of these markers is normally down-regulated in the latter type of SMCs, and is regarded as a phenotypic switch, originally described by Chamley *et al.* (1974), and amplified by Ross (1979) and Owens (1995*b*).

When repairing vascular injury, dedifferentiated SMCs participate in the formation of neointima by decreasing the expression of contractile proteins and increasing proliferation, migration and matrix protein synthesis (Yoshida *et al.* 2008). Similarly, during various disease states such as atherosclerosis, the recruited SMCs also acquire a synthetic phenotype in the course of lesion formation. In the embryo, following organization of endothelial cells into primary vascular plexus, SMCs are crucial for the maturation of the vessel (Jain, 2003). Vascular SMCs originate from various mesodermal lineages such as the splanchnic mesoderm, lateral plate mesoderm, somatic or paraxial mesoderm (Wasteson *et al.* 2008) and neural crest (Jiang *et al.* 2000). These migrating and differentiating SMCs play a key role in vasculogenesis and angiogenesis with continuing phenotypic switches.

Due to the complicated origins of SMCs during the early stages of embryogenesis, conflicting points of view exist about whether the SMCs in the vessel wall are heterogeneous or derived from multipotent vascular stem cells that differentiated into specific subpopulations with different functions (Frid *et al.* 1997; Nguyen *et al.* 2013; Tang *et al.* 2013). Bochaton-Piallat *et al.* (2001) seeded cultured arterial SMCs with distinct phenotypic features into the intima of denuded rat carotid artery and confirmed that SMC heterogeneity may be controlled genetically and not influenced by local stimuli. However, we believe that further investigation is essential to examine the specific roles of vascular progenitors and SMCs using different animal and embryo models. Therefore, the present article reviews the current state of research mainly on SMC differentiation and dedifferentiation during embryonic development and vascular remodelling, with a focus on (1) the different origins and development of SMCs during vasculogenesis, (2) the mechanisms of stem cell differentiation into SMCs, and (3) the role of stem cells and progenitors as a source of SMCs in the pathogenesis of arteriosclerosis or vascular remodelling.

SMC origins in embryonic development

Vasculogenesis and angiogenesis in the embryo. Both vasculogenesis and angiogenesis take place in the early developing embryo, enabling the establishment of the vascular system. The formation of a functional *de novo* vascular network from embryonic mesoderm via the process of vasculogenesis is critical for embryonic survival and later organogenesis (Amali *et al.* 2013). Vasculogenesis is driven by the invagination of epiblastic cells through the primitive streak and the formation of the mesoderm during gastrulation (Amali *et al.* 2013). Endothelial precursor cells are mainly derived from the splanchnic mesoderm by undergoing a transition from epithelium to mesenchyme due to the induction of the endoderm. Consequently, these precursor cells will form vesicles, which will accumulate along the future pathways of some of the earliest blood vessels such as the dorsal aorta, and may fuse either to each other or to existing vessels (Bellairs & Osmond, 2005).

Once the main vessels have been laid down, angiogenesis takes place to refine the pattern of the vessels and determine whether the vessel turns into an artery or a vein. The recruitment and differentiation of SMCs are the main events during this progress (Yao *et al.* 2014). Most SMCs contribute to multiple concentric layers of artery and vein, whereas pericytes, which may have a common ancestor with SMCs, exist in smaller vessels such as arterioles, capillaries and venules, and share their basal membrane with the endothelium (Stapor *et al.* 2014; Wang *et al.* 2014). Embryonic differentiating SMCs exhibit high rates of cell proliferation and migration. However, they also produce a large amount of extracellular matrix proteins and factors, which are different from the ones secreted by adult immature SMCs but necessarily required for angiogenesis (Shang *et al.* 2008). There are several important signalling pathways involved in smooth muscle development. For instance, platelet-derived growth factor (PDGF)-BB and transforming growth factor-β (TGF-β) serve as two important chemoattractants for migrating mural cells expressing PDGF receptor-β and perform multiple functions in proliferation or differentiation of both mural cells. It was also demonstrated by Yao *et al.* (2014) that sonic hedgehog could be expressed by SMCs of neovessels and promote PDGF-BB-induced migration via up-regulating extracellular signal-regulated kinase 1/2 and Akt phosphorylation. Most recently, it has been

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demonstrated that the T-box family transcription factor Tbx18 signalling pathway is involved in cell survival as Tbx18+ progenitors can differentiate into SMCs during development. However, tissue-specific transgenic mice are needed to confirm these findings and further investigation of the regulatory mechanisms is deemed essential (Xu *et al.* 2014).

Embryonic SMC development is complicated because of both complex regulatory signalling pathways and a mosaic pattern of differentiation, which has been recognized as evolutionarily conserved in many different vertebrate species (Hutson & Kirby, 2003). As shown in Fig. 1 the SMCs of coronary arteries, dorsal aortas and branchial arteries are derived from different cell groups during early embryonic development, a topic which will be discussed in the following sections. Additionally, nephrogenic stromal cells, a subpopulation of metanephric mesenchyme, can migrate within the developing kidney and differentiate into SMCs of renal vessels (Xu *et al.* 2014). Wilms tumour-1 positive proepicardium was previously reported to contribute to cardiomyocytes, endothelial cells and SMCs in the coronary artery system (Mikawa & Fischman, 1992; Zhou *et al.* 2008). Recent studies made further progress and showed that Wilms tumour-1+ pleural mesothelial cells could undergo mesothelial-to-mesenchymal transition and differentiate into SMCs in lung vessels (Que *et al.* 2008; Batra & Antony, 2014). Moreover, the embryonic endothelium is recognized as a source of haematopoietic stem cells, which can differentiate into several mesodermal lineages including SMCs (Bertrand *et al.* 2010). However, later research from Azzoni *et al.* (2014) found that not just embryonic endothelium but also early extra-embryonic endothelium was able to generate mesoangioblasts, which expressed haemangioblastic, haematopoietic, endothelial and SMC markers. Thus, different progenitors are recruited to differentiate into SMCs in different parts of the vascular system under elaborate control of different mechanisms (for reviews see De Val, 2011; Mack, 2011).

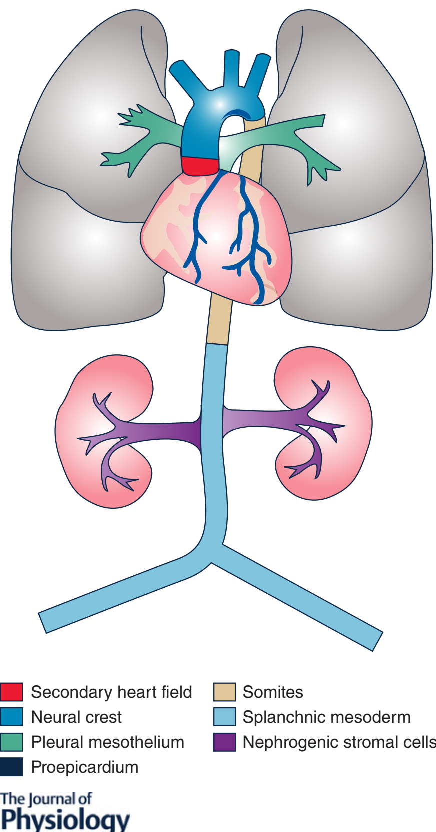

Figure 1. Developmental fate map for SMCs
The different colours represent the different embryonic origins for SMCs as indicated in the key.

Neural crest and branchial arch angiogenesis. The neural crest is present in the early embryo only as a strip of cells situated between the neural and the non-neural ectoderm (Mayor & Theveneau, 2013; Noisa & Raivio, 2014). As the neuroepithelium closes, neural crest cells become positioned on the dorsal neural tube and delaminate in a rostrocaudal wave. Neural crest cells migrate along specific routes and contribute to a wide range of mesenchymal structures such as melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons as well as glia. A few groups have reported the contribution of cardiac neural crest cells to SMCs of branchial arch arteries in the last two decades (Xie *et al.* 2013). Before transgenic techniques were broadly used, Bergwerff *et al.* (1998) applied a quail-chick chimera and infected pre-migratory neural crest cells with a retroviral vector including a *LacZ* reporter gene to study the chick embryonic neural crest differentiation. They found that neural crest cells are the only cell lineage that contributes to the smooth muscle of branchial arch arteries, although later on, *LacZ* positive cells also contributed to adventitial fibroblasts and non-muscular cells of the media and intima. More specifically, embryologists have demonstrated that neural crest cells residing at different levels of rhombomeres migrate into branchial arch arteries and differentiate towards SMCs at day 3 in chick embryonic development (Lumsden *et al.* 1991; Peterson *et al.* 1996; Kulesa & Fraser, 2000; Trainor & Krumlauf, 2000; Voiculescu *et al.* 2008). These findings were confirmed in two individual studies by using a *Wnt1-Cre/Rosa26* reporter mouse model (Jiang *et al.* 2000) and neural crest specific transgenic mouse (Nakamura *et al.* 2006). However, the molecular mechanisms controlling neural crest cell induction, migration and differentiation are still not fully understood.

Somite and dorsal aorta angiogenesis. Mesodermal structures between the endoderm and the ectoderm are

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created by gastrulation. Paraxial mesoderm is gradually separated into blocks of cells which become somites and are situated on both sides of the neural tube. Somites are transient structures that give rise to a series of tissues and organs, such as the cartilage of the vertebrae, ribs, muscles of the back, limbs, tongue and the dermis of the back skin (Sato, 2013). Since the dorsal aorta develops in very close proximity to the somites, the contribution of somite to vascular cells has long been speculated upon. In fact, when using chick-quail chimera experiments, somite-derived cells were found to migrate into the dorsal aorta and form endothelium as well as smooth muscle in the tunica media (Pouget *et al.* 2006). Furthermore, Pouget *et al.* (2008) reported that only the sclerotome gives rise to SMCs and pericytes, which expressed PDGF receptor-β (PDGFR-β) and myocardin, whereas the dermomyotome only gives rise to endothelium. Further studies on chicks revealed that the splanchnic mesoderm also temporarily contributed to the SMCs in the floor of the dorsal aorta, which were called primary SMCs. However, sclerotome-derived SMCs, also called secondary SMCs, have migrated ventrally and replaced the primary SMCs (Wiegreffe *et al.* 2009). These results resemble those of another study performed on a transgenic mouse line (Wasteson *et al.* 2008). Moreover, it has been reported that some SMCs in the dorsal aorta may arise from the myotome and share a common clonal origin with skeletal muscle (Esner *et al.* 2006).

Coronary artery angiogenesis in the developing heart. Previously, studies using lineage mapping and genetic mouse lines have shown that proepicardial cells give rise to the epicardium, SMCs of coronary artery, and other heart cells (Zhou *et al.* 2008; Olivey & Svensson, 2010). During the early embryonic development, proepicardial cells migrate to the myocardium and produce the epicardium. The epicardium of the neonatal heart is constituted of a continuous sheet of epithelial cells, some of which will undergo an epithelium-to-mesodermal transition to form a vascular plexus via vasculogenesis and angiogenesis (Wu *et al.* 2013; Diman *et al.* 2014). Postnatal coronary vessels were presumed to arise from these embryonic coronary vessels, but recent reports demonstrated that different sources of SMCs contribute to postnatal coronary artery growth (Tian *et al.* 2014). By using genetic lineage tracing, they found that a substantial portion of postnatal coronary vessels arise *de novo* in the neonatal mouse heart rather than expanding from the preexisting embryonic vasculature. This lineage conversion occurs within a brief period after birth and provides an efficient means of rapidly augmenting the coronary vasculature (Tian *et al.* 2014). This mechanism of postnatal coronary vascular growth provides venues for understanding and stimulating cardiovascular regeneration following injury and disease.

SMC origins in arteriosclerotic lesions

Mature SMC plasticity. Mature SMCs in the vessel wall can be defined as the contractile phenotype and are characteristic of normal physiological conditions. However, SMCs are sensitive to environmental stimuli, such as growth factors, mitogens, inflammatory mediators and mechanical influences, and are able to undergo rapid changes in their functional and morphological properties. During these changes, SMCs lose the ability to contract, but migrate, proliferate and accumulate in the intima (House *et al.* 2008). This process is named phenotypic modulation switching (Owens, 1995*a*). Conversely, SMC dedifferentiation to a synthetic phenotype is an early event in numerous cardiovascular pathologies, including atherosclerosis, restenosis and aortic aneurysm disease (Owens, 2007). The regulation of SMC phenotype is complex and has been thoroughly reviewed (Owens, 1995*a*). Most SMC marker genes are regulated by CArG box motifs within their promoters that are bound by serum-response factor (SRF), which induces transcription and differentiation. Principal co-activators of SRF are myocardin and myocardin-related factors that are crucially involved in SMC marker gene expression. Although much is known about the factors and mechanisms that control SMC plasticity in cell culture conditions, *in vivo* evidence, for example in native atherosclerosis in human or animal models, is still far from complete.

Evidence of mature SMC contribution to neointimal cells. Many reports from different groups have demonstrated the conversion of normal contractile vascular SMCs to a less differentiated, proliferative and migratory cell type in culture. There is indirect evidence indicating the contribution of mature SMCs to arteriosclerotic lesions, including neointima formation after endothelial injury, vein graft arteriosclerosis and native atherosclerosis (Alexander & Owens, 2012*b*). A more compelling lineage tracing study of vascular SMCs performed by Nemenoff *et al.* (2011) provided evidence that differentiated SMCs undergo phenotypic modulation in response to vascular injury using tamoxifen-inducible SMMHC-CreER mice. Nemenoff *et al.* (2011) and Herring *et al.* (2014) showed that β-Gal+ SMCs down-regulate *SMαA* and contribute to neointima formation at 7 days after femoral artery wire injury and that a fraction of β-Gal+ SMCs are BrdU+ within the intima and media 3 weeks after injury. These data are consistent with the prevailing dogma wherein mature SMCs undergo injury-induced SMC phenotypic switching with onset of cell proliferation. Very recently, Feil *et al.* (2014) provided *in vivo* evidence for smooth muscle-to-macrophage transdifferentiation and supported an important role of SMC plasticity in atherogenesis. However, many phenotypically modulated

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SMCs within atherosclerotic lesions have not been identified as being of SMC origin. In addition, multiple cell types other than SMCs can be found within lesions and can express SMC marker genes such as *SMαA*, a marker that has routinely been used to identify SMCs within lesions (Andreeva *et al.* 1997).

As mentioned above, little information is available on SMC lineage tracing during the development of atherosclerosis. A recent report using *SM22α* as a tracing marker for mature SMCs, which labelled about 11% of total medial SMCs, demonstrated that very few (<5% of total SMCs) labelled cells found in lesions were identified (Feil *et al.* 2014). These labelled cells displayed a macrophage-like cell phenotype, but were not derived from bone marrow cells. These data have several implications. Firstly, a very small proportion, if any, of SMCs in atherosclerotic lesions were derived from medial mature SMCs. Secondly, it cannot be excluded that cell fusion might occur during the formation of atherosclerotic lesions. It was discovered that the normal ploidy of a number of SMCs in the vessel is tetraploid (Barrett *et al.* 1983; Goldberg *et al.* 1984), which is related to induction of proliferation (Owens, 1989). Fusion itself may also be a naturally occurring mechanism in the physiological state (Terada *et al.* 2002; Wang *et al.* 2003), or atherogenesis. Finally, there is also evidence that macrophages can be induced to express multiple SMC markers including *SMα*A and *SM22α* (Martin *et al.* 2009; Stewart *et al.* 2009). As such, a subset of *SM22α* marker-positive cells in lesions may not be derived from mature SMCs. Thus, it would be essential to use rigorous lineage-tracing methods that permit identification of mature SMC origin in arteriosclerotic lesions.

Endothelial-to-mesenchymal transition (EndMT). Endothelial cells exhibit a wide range of phenotypic variability throughout the cardiovascular system (Chi *et al.* 2003). The most remarkable feature is their plasticity of endothelial-to-mesenchymal transition (EndMT), which is involved in the development of atherosclerosis (Chade *et al.* 2008). These cells lose cell–cell junctions due to decreased VE-cadherin, acquire invasive and migratory properties and lose other endothelial markers such as CD31. On the other hand, these cells gain mesenchymal markers, e.g. fibroblast-specific protein 1, N-cadherin and SMαA (Potts & Runyan, 1989; Nakajima *et al.* 2000; Armstrong & Bischoff, 2004; Arciniegas *et al.* 2007; Zeisberg *et al.* 2007). Pathological vascular remodelling of vein grafts occurs in response to altered biomechanical stress, in which Cooley *et al.* (2014) found that endothelial-derived cells contribute to neointimal formation through EndMT. TGF-β signalling activation is at the core of EndMT, which is a process where endothelial cells 'dedifferentiate' to acquire a mesenchymal and possible SMC-like phenotype. The authors (Cooley *et al.* 2014) demonstrated that early activation of the TGF-β/Smad2/3-Slug signalling pathway is crucial for EndMT, suggesting that some neointimal lesion SMCs could be derived from mature endothelial cells via EndMT. However, there may be a downside to the linear tracing system for endothelial cells used in this study, which is the Tie2/Cre reporter. It is now well known that Tie2 can be expressed in the progenitors of myeloid precursors and macrophages. Tie2-GFP+ cells detected in the neointima of vein grafts might be in fact myeloid cells, a case which will need to be further investigated in future studies.

Stem/progenitor cells contribute to SMC accumulation

Bone marrow stem cells. There is also evidence demonstrating that SMCs or SMC-like cells within arteriosclerotic lesions may be derived from a variety of sources, including vascular resident stem/progenitor cells, transdifferentiation of endothelial cells (DeRuiter *et al.* 1997) and adventitial fibroblasts (Scott *et al.* 1996; Li *et al.* 2000; Sartore *et al.* 2001), as well as bone marrow cells. Specifically, bone marrow and vessel wall-derived progenitors have been shown to have the ability to differentiate into SMCs, which can participate in angiogenesis and vascular remodelling (Abedin *et al.* 2004; Hirschi & Majesky, 2004; Urbich & Dimmeler, 2004; Aicher *et al.* 2005; Xu, 2007). In native atherosclerosis, Sata *et al.* (2002) demonstrated that SMCs in atherosclerotic plaques originate from bone marrow progenitors, implying that SMCs were derived from haematopoietic stem cells. One group has shown that the majority of neointimal SMCs within plaques of experimental atherosclerosis in sex-matched chimeric scenarios and transgenic bone marrow transplant settings are derived from the bone marrow (Shimizu *et al.* 2001). However, subsequent rigorous lineage-tracing and confocal studies by Bentzon *et al.* (Bentzon & Falk, 2010; Bentzon *et al.* 2007), Daniel *et al.* (Daniel *et al.* 2010) and the Nagai group (Iwata *et al.* 2010) showed that the majority of SMC-like cells within atherosclerotic lesions of ApoE$^{-/-}$ mice on a Western diet are not of haematopoietic origin. These results confirm early observations from our laboratory (Hu *et al.* 2002).

Stem cells in the adventitia. The vascular adventitia is defined as the outermost connective tissue of vessels. The adventitia is increasingly considered to be a highly active segment of vascular tissue that contributes to a variety of disease pathologies, including atherosclerosis and restenosis (Shi *et al.* 1996; Wilcox *et al.* 1996; Zalewski & Shi, 1997; Sartore *et al.* 2001; Rey & Pagano, 2002). Table 1 summarizes these data describing the characteristics and nature of stem/progenitor cells from different laboratories

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Table 1. Summary of published reports on progenitor cells found in the adventitia

Publication	Source/species	Cell marker expression	Summary
Hu *et al.* 2004	ApoE−/− mouse	Sca-1+/c-kit+/Lin-	Sca-1+ cells added to adventitia; migration to the intima observed.
Howson *et al.* 2005	Rat aorta	CD34/Tie-2, NG2, Nestin, PDGFR	Non-EC mesenchymal cells are pericyte precursors.
Invernici *et al.* 2007	Human fetal aorta	CD34+, CD133+, VEGFR2+, DES	Vascular progenitor cells formed by undifferentiated mesenchymal cells that co-express endothelial and myogenic markers. Under permissive culture conditions EC, mural cell or myocytes can be generated. *In vitro*, they form 3D-cord-like vascular structures. In a mouse model of limb ischaemia, they promote neovascularisation and muscular regeneration.
Zengin *et al.* 2006	Human arteries/veins	CD34+, CD31−, VEGFR2+, TIE-2	Arteries/veins from a range of organ cells were identified between media and adventitia. Capillary like outgrowths into the lumen were CD34+/CD31+ versus CD34+/CD31– in adventitial outgrowths.
Pasquinelli *et al.* 2010	Human thoracic aorta	CD34+ or c-kit+	Total vessel wall cell isolates showed expression of mesenchymal markers CD44+, CD90–, CD105+ and stem cell markers, i.e. OCT4, upon culture. Within tissue sections CD34+/c-kit+ cells were identified in the media–adventitia region.
Torsney *et al.* 2007	Human aorta and mammary arteries	CD34, c-kit, Sca-1	Progenitors were identified within neointimal lesions and the adventitia with variable expression of CD34, Sca-1, c-kit and VEGF receptor 2 markers, but no CD133 expression.
Passman *et al.* 2008	Mouse embryonic/adult arteries	Sca-1+	Cells in the media–adventitia have an Shh signalling domain; in Shh−/− mice adventitial Sca-1 cells reduced, Sca-1+ cells express SMC differentiation markers.
Campagnolo *et al.* 2010	Human saphenous vein	CD34, DES, VIM, NG2, PDGFRb, CD44, CD90, CD105, CD29, CD13, CD59, CD73, SOX2	Total vessel wall cell isolates contain CD34+/CD31– cells, which upon culture express pericyte/mesenchymal markers. Integrate into vascular networks *in vitro* and *in vivo*.
Klein *et al.* 2011	Adult human arterial	CD44+,CD90+,CD73+ CD34−,CD45−	Mesenchymal stem cells function as vasculogenic cells.
Tang *et al.* 2012	Mouse artery	Sox17, Sox10 and S100β	Multipotent vascular stem cells. Cloneable. Responsible for most, if not all, proliferating SMCs *in vitro* and neointimal SMCs *in vivo*.
Cho *et al.* 2013	Mouse aorta	Sca-1+/PDGFRα(–) Sca-1+/PDGFRα(+)	Bidirectional differentiation potential towards both osteoblastic and osteoclastic lineages.
Psaltis *et al.* 2014	Mouse aorta	Sca-1+/CD45+	Macrophage progeny particularly in the adventitia and to a lesser extent the atheroma.

EC, endothelial cell.

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(Torsney *et al.* 2007; Invernici *et al.* 2007; Passman *et al.* 2008; Campagnolo *et al.* 2010; Klein *et al.* 2011). In 2004, Hu *et al.* reported for the first time the existence of vascular progenitor cells in the adventitia that can differentiate into SMCs and participate in lesion formation in vein grafts (Hu *et al.* 2004). Cells expressing each of the progenitor markers Sca-1, c-kit, CD34 and Flk1, but not SSEA-1, were identified in the adventitia, particularly in the region of the aortic root. Zengin *et al.* (2006) identified vascular wall resident progenitor cells in the border zone of adventitia and media in human arteries and veins. The vessels were isolated from a range of organs including the liver, prostate, heart and kidney. The cells were characterized by expression of CD34+, VEGFR2+ and TIE2. Furthermore, it was found that the adventitia in aortic roots harboured large numbers of cells expressing stem cell markers in adult ApoE-deficient mice. When Sca-1+ cells carrying the *LacZ* gene were transferred to the adventitial side of vein grafts in ApoE-deficient mice, β-gal+ cells were found in atherosclerotic lesions of the intima and these cells enhanced the development of the lesions. Thus, in this model a large population of vascular progenitor cells existing in the adventitia could differentiate into SMCs that contributed to atherosclerosis (Hu *et al.* 2004).

Moreover, Sca-1+ stem cells in the adventitia can also differentiate into other types of cells participating in vascular remodelling. It was demonstrated that Sca-1+ progenitor cells exhibited greater osteoblastic differentiation potentials via activation of PPARγ triggered receptor activator for nuclear factor-κB expression, indicating the involvement of calcification for the vessel (Cho *et al.* 2013). In addition, single-cell disaggregates from the adventitial tissues of adult mice showed a unique predisposition for generating macrophage colony-forming units (Psaltis *et al.* 2012). These aortic macrophage colony-forming unit progenitors coexpressed Sca-1 and CD45, where they were the predominant source of proliferating cells in the aortic wall (Psaltis *et al.* 2014). As it has been observed that foam cells in atherosclerotic lesions can express both SMC and macrophage markers, it is possible that adventitia stem/progenitor cells might be responsible for the differentiation of macrophages, foam cells and SMCs during vascular remodelling, which display different phenotypes depending on the microenvironment.

Stem cells in the media. Sainz *et al.* (2006) reported the presence of stem cells in the media, which can be isolated from healthy murine thoracic and abdominal aortas. These side population cells were characterised by a Sca-1+, c-kit(–/low) Lin-CD34(–/low) expression profile. *In vitro* culture with vascular endothelial growth factor (VEGF) or PDGF-BB/TGF-β1 induced differentiation to endothelial cells and SMCs, respectively. Additionally, it was found that mesenchymal stromal cells exist within the wall of a range of vessel segments such as the aortic arch, and thoracic and femoral arteries (Pasquinelli *et al.* 2010). These cells were identified by expression of *Oct-4*, *Stro-1*, *Sca-1* and *Notch-1*, and lacked haematopoietic or endothelial markers. Resident multipotent mesenchymal stromal cells were recovered from fresh arterial segments by enzymatic digestion for *in vitro* analysis. Multipotent mesenchymal stromal cells cultured *in vitro* exhibited SMC, adipogenic and chondrogenic potential. Recently, Tang et al. (2012*c*) provided data from *in vitro* cell culture and lineage tracing experiments indicating differentiated vascular mature SMCs are incapable of proliferation either *in vivo* in response to injury or *in vitro* in cell culture. Instead, there exists a small population ($<$10%) of undifferentiated cells in the media that activate markers of mesenchymal stem cells, including Sox17, Sox10 and S100α, and proliferate to completely reconstitute medial cells in response to vascular injury. In addition, these media-derived multipotent vascular stem cells can proliferate and express several mesenchymal stem cell markers when placed in cell culture and can be induced to differentiate into neuronal, chondrogenic and SMC lineages with appropriate culture methods. These findings support the presence of medial stem/progenitor cells in the media that could be a source of SMCs within arteriosclerotic lesions.

Signal pathways involved in SMC differentiation

The mechanisms for stem cell differentiation into SMCs are still not completely defined. As mentioned previously, SMCs can arise from various multipotent progenitors and can then further mature into different SMC subtypes, which vary in their function, cardiovascular location and phenotype (Majesky, 2007). This process is dependent on several stimuli, including cytokines or growth factors, the extracellular matrix, microRNAs, chromosome structural modifiers and mechanical forces amongst others. The following section does not cover all recognized aspects of SMC differentiation, but will give a brief summary of a selection of key signalling pathways that need to be activated in order to render SMC differentiation possible.

TGF-β signalling. TGF-β is a potent multifunctional soluble cytokine that exists in at least three isoforms, TGF-β1, -2 and -3, and is involved in cell signalling regulation of events such as cell differentiation, proliferation, survival and apoptosis (Gadue *et al.* 2006). *In vivo*, early embryogenesis lethality, yolk sac vasculogenesis defect, reduced angiogenesis, haematopoiesis and cell adhesion as well as abnormal capillary tube formation and SMC hypoplasia could be observed in TGF-β1, TGF-β2 and TGF-β3 receptor null mice (Sinha *et al.* 2004; Ferreira *et al.* 2007; Churchman & Siow, 2009). *In vitro*,

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TGF-*β*1 signalling has been shown to be involved in the development of embryonic stem cell-derived SMCs and the maturation of mural cells by the positive regulation of SMMHC and SM*α*A through the Smad2 and Smad3 pathways or the Notch signalling pathway, which also involves Smad3 (Hirschi *et al.* 1998; Xie *et al.* 2011*b*; Cheung *et al.* 2014; Sinha *et al.* 2014). TGF-*β*1 signalling also promotes the contractile phenotype of adult SMCs by the increased expression of *SMMHC*, *SMαA* and calponin (Hao *et al.* 2003). It seems that TGF-*β*1 signalling is crucial for both SMC differentiation from embryonic stem cells and mature SMC phenotypic switching (Fig. 2).

PDGF signalling. PDGF exists in five isoforms (PDGF-A, PDGF-B, PDGF-C, PDGF-D and PDGF-AB homo- or hetero-dimmer including PDGF-AA, PDGF-BB and PDGF-AB), is mainly derived from platelets upon activation by various stimuli, e.g. low oxygen tension, and acts via its cell surface receptors tyrosine kinase receptors, PDGFR-*α* and PDGFR-*β*, as a potent mitogen for cells of mesenchymal origins such as SMCs (Heldin & Westermark, 1999). PDGF is also expressed by endothelial cells and can induce the maturation of mural cell precursors such as the multipotent mouse embryonic 10T1/2 cells into SMCs (Hirschi *et al.* 1998). It has been demonstrated that Sca1+ and Flk-1+ progenitor cells can differentiate into SMCs via PDGFR-*β*-mediated signalling (Hu *et al.* 2004) and culture on collagen IV (Sone *et al.* 2007; Xiao *et al.* 2007). This signalling pathway can be controlled by Wnt7b (Cohen *et al.* 2009) and can also be activated by cyclic strain. The latter triggers the SMC differentiation of Flk-1+ progenitors by the phosphorylation of PDGFR-*β*, resulting in increased expression of *SMMHC* and *SMαA* (Shimizu *et al.* 2008). Interestingly, PDGF-BB can also have a negative effect on mature SMCs through the PDGF-*β* receptor, notably

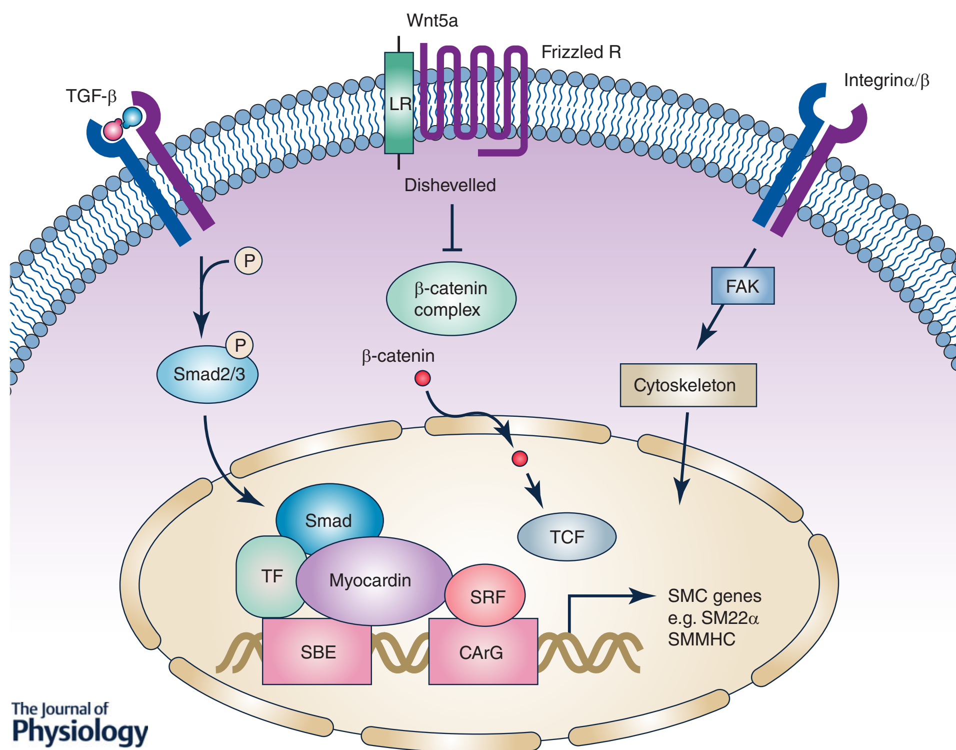

Figure 2. An overview of the involvement of TGF-*β*, Wnt and integrin signalling in the differentiation of stem cells towards the smooth muscle lineage
In TGF-*β* signalling, the binding of a TGF-*β* ligand to the TGF-*β* receptor catalyses the phosphorylation of the Smad2/3 molecule prior to its translocation to the nucleus. The Smads can then bind to a Smad binding element with various transcription factors. In canonical Wnt signalling, the Wnt ligand, Frizzled receptor protein and LRP form complexes to activate a cytosolic protein called Dishevelled. Activated Dishevelled inhibits the *β*-catenin destruction complex and thus increases the stabilization of *β*-catenin by escaping destruction via proteasomes and then accumulates in the cytosol and nucleus. In the nucleus, *β*-catenin forms a complex with T-cell factor (TCF) proteins. The complex activates the transcription of specific target genes, which drives mesoderm and SMC gene expression. This promotes the recruitment and the binding of the SRF–myocardin complex to the CArG elements found in the promoter region of most SMC-specific gene. Meanwhile, integrins bind to collagen that initiates signalling for cytoskeleton rearrangement, which is essential for SMC differentiation. SBE: Smad binding element; SRF: serum response factor; TF: transcription factor.

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by the repression of SMC markers such as *SMαA*, *SMMHC* and *SM22α*. This effect is cell density dependent and is mediated by the ETS1 increased expression (Dandre & Owens, 2004). Thus, PDGF-initiated signalling plays a different role in embryonic stem cell differentiation into SMCs and dedifferentiation of mature SMCs.

Wnt/Notch signalling. Wnt signalling is through a highly-conserved family of cysteine-rich glycosylated ligands, which act by extracellular signalling and are central to the regulation of cell fate, cell morphology and cell proliferation, the latter by inhibiting apoptosis (Dale, 1998; Blauwkamp *et al.* 2012). Absence of the Wnt receptor Frizzled-5 is embryonic lethal due to poor yolk sac angiogenesis (Ishikawa *et al.* 2001). Wnt signalling plays an important role in the onset of gastrulation and primitive-streak formation, as well as neuroectoderm, neuromesoderm and mesoendoderm lineage commitment depending on Wnt concentration (Blauwkamp *et al.* 2012; Tsakiridis *et al.* 2014; Turner *et al.* 2014). Indeed in human, embryonic stem cells expressing high concentrations of Wnt are pushed towards the mesoendoderm lineage, while cells expressing lower concentrations of Wnt further commit to the neuro-ectoderm lineage (Blauwkamp *et al.* 2012). Further differentiation into SMCs involves the canonical Wnt signalling pathway via the specific expression of Wnt3a and its downstream target, β-catenin, leading in turn to the increased expression of *SM22α* (Shafer & Towler, 2009). Our laboratory exploited this latter property by overexpressing *DKK3* in partially induced pluripotent stem cells (Karamariti *et al.* 2013). Indeed, unlike other members of the dickkopf family, which inhibit Wnt, DKK3 interacts with Kremen 1 to activate the canonical Wnt/β-catenin signalling pathway and downstream SMC differentiation (Karamariti *et al.* 2013).

As mentioned previously, Wnt concentration plays a role in lineage commitment. Its temporal expression is also important for cell differentiation. With regard to the development of the cardiovascular system, the canonical Wnt/β-catenin pathway first needs to be activated by TGF-β to signal initiation and subsequent mesoderm formation (Gadue *et al.* 2006; Bakre *et al.* 2007; Maretto *et al.* 2003), before being repressed at later stages for cardiac lineage specification (Ueno *et al.* 2007). This property has been exploited for efficient *in vitro* generation of embryonic stem cell-derived cardiomyocytes (Lian *et al.* 2012). Due to their EndMT properties, most multipotent mesoderm progenitors are common to SMCs and endothelial cells. However, the separation between SMC and endothelial cell commitment and the generation of SMC-specific *HAND+* progenitors is directed by Notch signalling and the mediation of Wnt and bone morphogenetic protein expression (Shin *et al.* 2009). Notch can also act as an antagonist of SMC differentiation from neural crest cells (High *et al.* 2007) and promote endothelial cell differentiation (Dejana, 2010). In adult SMCs, active Notch signalling inhibits SMC differentiation and their contractile phenotype (Havrda *et al.* 2006). Activation of Notch receptors in human SMCs using immobilized Jag-1 promotes up-regulation of contractile proteins (Boucher *et al.* 2011). Although the precise mechanism(s) of Jag-1/Notch-induced maturation is still poorly understood, a number of studies have systematically investigated the molecular pathways leading to the pro-differentiation and pro-proliferative effects of Notch signalling in SMCs. Thus, further defining of Notch receptor expression and function during stem cell differentiation and pathological settings will enhance our understanding of the signals required for maintaining vascular homeostasis.

HDACs and epigenetics. Histone deacetylases (HDACs) can be divided into three groups according to their phylogenetic class. Amongst these groups are class I (HDAC1, 2, 3 and 8) and class II (HDAC4, 5, 6, 7, 9 and 10) HDACs. They are essential regulators of gene expression by controlling chromatin structure and function. Accordingly, HDACs are also active in SMC fate (de Ruijter *et al.* 2003). For instance, class I HDAC inhibition prevents Notch signalling from up-regulating SMC markers essential for the SMC contractile phenotype, such as *SMMHC*, *SMαA*, calponin or *SM22α* (Tang *et al.* 2012*a*). Our laboratory has demonstrated that a particular class I HDAC, HDAC7, plays an essential role in embryonic stem cell-derived SMC generation (Margariti *et al.* 2009). As mentioned previously, PDGF-BB can up-regulate SMC differentiation. One mechanism for such an event is the up-regulation and subsequent splicing of HDAC7, followed by its preferential localization to the nucleus. There, HDAC7 can increase SRF by binding to myo-cardin, which in turn leads to the recruitment of the SRF–myocardin complex to the *SM22α* promoter and activation of SMC marker gene expression in order to induce stem cell differentiation towards an SMC lineage (Margariti *et al.* 2009). Other significant HDAC class I members include HDAC3, which is important for the derivation of neural crest-derived SMCs and the formation of the cardiac outflow tract (Singh *et al.* 2011); and HDAC8, which is exclusively expressed by cells showing SMC (visceral and vascular) differentiation (Waltregny *et al.* 2004).

miRNA. MicroRNAs (miRNA) are endogenous, single-stranded, short, non-coding 22-nucleotide RNAs. They are highly conserved and act as positive and negative regulators of gene expression by inhibiting mRNA trans-lation or inducing mRNA degradation. miRNAs can be expressed in a stage- and/or tissue-specific manner,

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giving them an important role in cell differentiation, proliferation and apoptosis (Baehrecke, 2003; Bushati & Cohen, 2007). miRNA also play an important role in the maintenance of stem cell pluripotency (Houbaviy *et al.* 2003; Suh *et al.* 2004) and the miR-302-367 cluster has even been successfully used to reprogramme fibroblasts into iPS cells (Anokye-Danso *et al.* 2011). When this is antagonized, mir-373, a downstream target of activin/nodal signalling, becomes overexpressed and leads to embryonic stem cell differentiation towards the mesendodermal lineage (Rosa *et al.* 2014). Similarly, miR-145 has been shown to directly repress the expression of pluripotent factors *OCT4*, *SOX2* and *KLF4* to promote human stem cell differentiation (Xu *et al.* 2009). More specifically, miR-145 has been shown to be the most abundant miRNA in differentiated SMCs (Cheng *et al.* 2009) and to be sufficient to generate neural-crest-derived SMCs (Cordes *et al.* 2009). Along with miR-143, miR-145 is positively regulated by SRF and myocardin to promote SMC differentiation by down-regulating factors such as *KLF4* and *ELK-1* that are normally expressed in less differentiated, more proliferative SMCs (Cordes *et al.* 2009). Recent studies also show that miR-1 regulates SMC differentiation by repressing *KLF4* (Xie *et al.* 2011*a*). As shown previously, retinoic acid treatment is beneficial for embryonic stem cell-derived SMC generation (Drab *et al.* 1997). It has since been demonstrated that stem cell treatment with retinoic acid increases the expression of miR-10a, which leads to increased SMC differentiation via the down-regulation of HDAC4, an SMC differentiation repressor (Huang *et al.* 2010). PDGF, another important regulator of SMC differentiation, can induce the expression of miR-221, which in turn represses c-Kit expression and causes the down-regulation of myocardin and downstream SMC marker genes, promoting SMCs to go from a contractile to a synthetic phenotype (Davis *et al.* 2009). Therefore, miRNAs are undoubtedly crucial players in modulating SMC differentiation (Kane *et al.* 2011). More targets of miRNAs await identification and how miRNAs themselves are regulated during lineage determination needs further elucidation. Since stem/progenitor cells in the vessel wall are involved in arteriosclerosis and vascular remodelling, therapeutic manipulation of miRNAs that regulate SMC differentiation and phenotypic modulation will present new options towards vascular disease treatment (Fig. 3) .

SRF–myocardin complex. As mentioned in the previous section, SMC differentiation can be marked by the expression of SMC-specific markers such as *SM22α*. The aforementioned can be triggered by the increased expression of transcription factors such as SRF and its cardiac and SMC-specific transcriptional cofactors myocardin and myocyte enhancer factor 2 (MEF2). Altogether, this complex can bind to a *cis*-acting DNA sequence, known as a CArG box ($CC(A/T)_6GG$), which is found in the regulatory regions of several immediate-early genes as well as SMC-specific genes (Shore & Sharrocks, 1995; Wang *et al.* 2001). Lack of SRF in stem cells leads to impaired SMC differentiation due to inactive *SMαA* and *SM22α* promoters. PKA-dependent phosphorylation of SRF can mimic the same effect by inhibiting the binding of SRF to the CArG box within SMC-specific promoters, subsequently inhibiting SMC-specific gene transcription (Blaker *et al.* 2009). It has been shown that myocardin is also a key element of this process and its loss-of-function mutation is lethal in mouse embryos due to vascular abnormalities such as lack of SMCs in the dorsal aortas of myocardin-deficient embryos (Li *et al.* 2003). Myocardin is exclusively expressed in SMCs and cardiomyocytes.

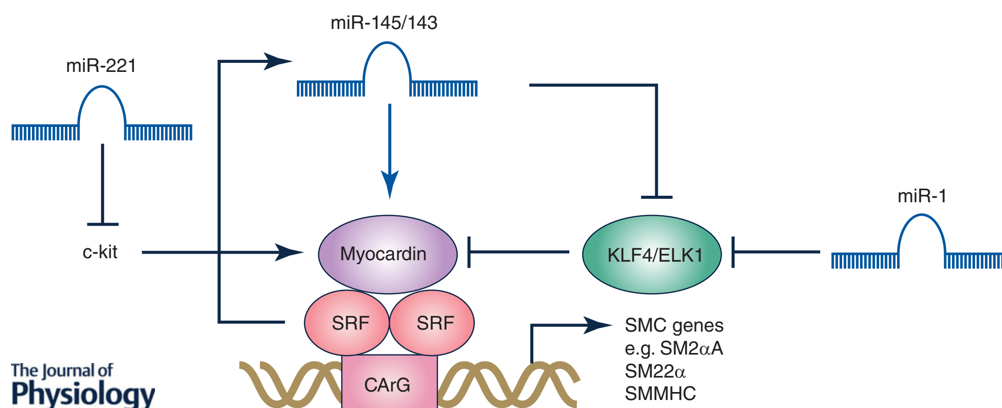

Figure 3. miRNA mediated stem cell differentiation into SMCs
miR-145 and miR-143 enhance the binding of myocardin and SRF to the CArG box, which in turn positively regulates their expression. Myocardin expression is also enhanced by miR-221 via the inhibition of c-kit expression. miR-145 and miR-143 expression, along with mIR-1 also inhibit myocardin repressors such as *KLF4* and *ELK-1*. This promotes the expression of SMC differentiation markers such as *SMαA*, *SM22α* and *SMMHC*.

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TGF-β1, which is a key player in SMC differentiation, can induce NADPH oxidase 4 (*Nox4*) and the production of the reactive oxygen species H_2O_2 that in turn activates the SRF–myocardin complex to enhance SMC differentiation (Clempus *et al.* 2007; Xiao *et al.* 2009). *Nrf3* expression is also important for SMC differentiation. Nrf3 can bind and also recruit the SRF–myocardin complex to the CArG box within the promoter region of SMC-specific genes, such as *SMαA* and *SM22α* (Pepe *et al.* 2010). Interestingly, Nrf3 also increases *Nox4* expression, which as mentioned above, is beneficial for SMC differentiation (Pepe *et al.* 2010). Using nuclear proteomics and bioinformatics, our lab has found that chromobox protein homolog 3 (Cbx3), a nuclear protein, can promote SMC differentiation via recruiting SMC transcription factor SRF and regulator Dia-1 to the promoter regions of SMC-specific genes (Fig. 4) (Xiao *et al.* 2011). Additionally, we found that an upstream regulator for Cbx3, heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) also plays an important role in smooth muscle development during chick embryonic development. Our results indicated that apart from direct activation of SMC gene transcription, hnRNPA2/B1 also regulates *Cbx3* to promote SMC differentiation (Wang *et al.* 2012). Therefore, a variety of signalling pathways leads to SRF–myocardin complex formation that binds to CArG box and results in SMC gene expression.

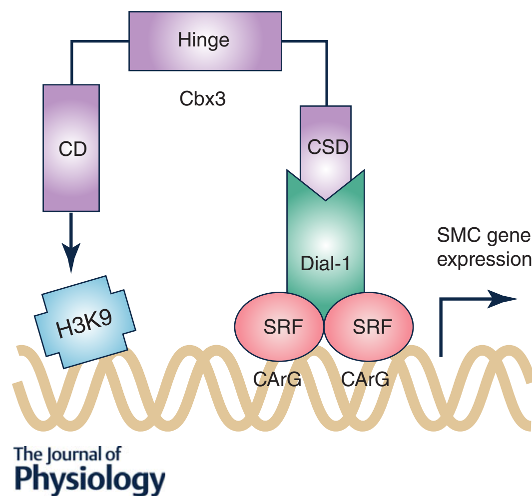

Figure 4. Proposed model for the role of Cbx3 in SMC differentiation
During the early phases of stem cell differentiation, histone modifications such as H3K9 occur within the promoter region of SMC differentiation genes. These regions can be recognized specifically by Cbx3 through the CD domain. After binding, Cbx3 functions as a bridge/anchor protein to recruit the SMC specific transcription factor SRF to the chromosome through interaction with Dia-1. This in turn facilitates SRF binding to the CArG elements within the promoter–enhancer region of SMC-specific genes, thereby regulating SMC differentiation from stem cells. (Adapted from Supplemental Figure VI of Xiao *et al.* (2011).)

Summary and perspectives

In embryonic development, there is obvious heterogeneity in the origins of vascular SMCs (Majesky, 2007). It has been demonstrated that even in neonatal hearts, SMCs in coronary arteries can be derived from different sources (Tian *et al.* 2014). The recent progress in understanding the molecular and cellular pathways that contribute to the origins and differentiation of SMCs in a variety of the vessels have made a significant contribution to our understanding of vascular SMC development. However, there are some questions that still need to be addressed in future studies. For example, new lineage-specific *in vitro* models of SMC development would be essential to test a long-standing question in developmental vascular biology – whether the heterogeneity of SMC origins contributes to the development and distribution of vascular SMCs. Other major challenges that may be amenable to suitable *in vitro* modelling include a detailed understanding of the SMC regulatory machinery during development. The rapid progress in this field could synergistically bring together the complementary fields of stem cell and vascular biology to make further major advances.

In adults, SMC accumulation in the intima is a key event in the development of arteriosclerosis (Ross, 1986) and as described above, the most accepted theory has been that the majority of intimal SMCs are derived from the media of the vessel (Ross & Glomset, 1973). This long-standing dogma is being revisited following the discovery that different sources of cells may be responsible for smooth muscle accumulation in atherosclerosis. In fact, earlier work by Holifield *et al.* (1996) indicated that mature SMCs in the media of canine carotid artery did not display the ability to proliferate and undergo phenotypic modification. Emerging evidence has demonstrated the existence of a population of vascular stem/progenitor cells that may be directly or indirectly involved in cardiovascular disease development (Xu, 2006; Anversa *et al.* 2007) and participate in atherosclerotic plaque development and neointima formation (Sata, 2003; Dimmeler & Zeiher, 2004; Hibbert *et al.* 2004; Hu *et al.* 2004; Wassmann *et al.* 2006; Foteinos *et al.* 2008). Tang *et al.* (2012) implied that mature SMCs in the media might not have the ability to dedifferentiate and contribute to lesional SMC accumulation. However, the lack of definitive mature SMC lineage tracing studies in the context of atherosclerosis and problems in pinpointing phenotypically modulated SMCs within lesions raise major questions regarding the contributions of mature SMC at all stages of atherogenesis.

Early work from Benditt & Benditt (1973) described their monoclonal theory of SMCs in atherosclerotic lesions in which SMCs displayed a monoclonal origin, or in other words were derived from a single cell. According to this theory, SMCs in arteriosclerosis could originate

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from one (stem/progenitor) cell that may be present in the arterial wall. It was eventually discovered that the arterial wall contains stem cells that can differentiate into SMCs (Margariti *et al.* 2006) in which the adventitia has been the focus as a potential source of SMC progenitors (Hu *et al.* 2004). Now it is generally accepted that vascular resident stem/progenitor cells can contribute to SMC accumulation in lesions depending on the differential degrees of vessel damage and the models used. The precise frequency and roles of progenitor cell-derived SMCs in arteriosclerosis remain uncertain. Yet, there is still uncertainty about the origin and niche of smooth muscle progenitors *in vivo* and given the innate heterogeneity of SMCs it is not surprising that there are conflicting data. Further study on vascular stem/progenitor cells could focus on the frequency of these cells contributing to lesional SMC accumulation in vascular disease.

Resident vascular stem/progenitor cells may play an important role in the pathogenesis of atherosclerosis, but regardless of the SMC source, the principle of local environmental cues impacting the pattern of gene expression and behaviour of these cells applies. Although significant progress in understanding the molecular mechanisms of signalling pathways and gene expression during stem cell differentiation into SMCs has been achieved, a key issue of molecular switching of SMCs has yet to be discovered. In addition, how do stem cells respond to the environmental stimuli and switch between SMC phenotypes? In other words, how do they know which signals to obey *in vivo*? At present there is no direct evidence that could give an answer to this question. Further investigation on this issue would enhance our understanding of the mechanisms of stem cell differentiation into SMCs. Furthermore, accumulating evidence indicates abundant stem/progenitor cells in a variety of vessels, including artery, vein and microvessels (Torsney and Xu, 2011). Can stem/progenitor cells resident in the vessel wall be released into circulating blood to form a stem cell pool that responds to different stimuli? Finally, it is unknown if pathophysiological processes could trigger stem cell mobilization and differentiation toward SMCs. Such challenges, just a few of the many we face, highlight the critical importance of continued and vigorous studies in this field.

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Additional information

Competing interests

None declared.

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