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REVIEW

Smooth muscle cell differentiation: Mechanisms and models for vascular diseases

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BACKGROUND: Vascular smooth muscle cells (VSMCs) are mature cells that play critical roles in both normal and aberrant cardiovascular conditions. In response to various environmental cues, VSMCs can dedifferentiate from a contractile state to a highly proliferative synthetic state through the so-called 'phenotypic switching' process. Changes in VSMC phenotype contribute to numerous vascular-related diseases, including atherosclerosis, calcification, and restenosis following angioplasty. Adventitial VSMC progenitor cells also contribute to formation of the neointima.
METHODS/RESULTS: Herein, we review both, the roles of VSMC differentiation in vascular diseases, and the in vitro models used to investigate the molecular mechanisms involved in the regulation of VSMC differentiation and phenotype modulation.
CONCLUSION: A comprehensive understanding of VSMC behavior in vascular diseases is essential to identify new therapeutic targets for the prevention and treatment of cardiovascular diseases.

Keywords vascular smooth muscle cells, progenitor, differentiation, transcription factor, cardiovascular disease

Introduction

Vascular smooth muscle cells (VSMCs) are highly specialized cells that play crucial roles in the cardiovascular system under normal and numerous pathologic conditions. In arteries, the main function of VSMCs is to provide structural support to the vasculature and regulate blood vessel tone and diameter, blood pressure, and blood flow distribution, under normal conditions. VSMCs are quiescent and typically express a unique range of contractile proteins, including smooth muscle cell actin (ACTA2), smoothelin, SM22α, and smooth muscle cell myosin heavy chain (MYH11) (Owens, 1995).

The origin of VSMCs is far more diverse than originally thought. They develop from a wide range of embryonic tissues. Lineage mapping studies have shown that the vascular smooth muscle in developing vertebrate embryos is a mosaic tissue. Different vessels, or even different segments of the same vessel, are composed of subtypes of VSMCs arising from different embryonic tissues (Majesky, 2007). For example, neural crest-derived VSMCs constitute the ascending aorta and the aortic arch, while VSMCs in the descending thoracic aorta, the abdominal aorta, and distal portions of the internal carotid arteries are mesoderm-derived (Majesky, 2007). The origin of coronary vascular smooth muscle is thought to be the pro-epicardium (Mikawa and Gourdie, 1996). Thus, it is essential to emphasize that the underlying mechanisms controlling the differentiation of VSMCs from different progenitors are likely to be different (Shi and Chen, 2016). VSMCs contribute significantly to formation of the neointima in vascular diseases; in this classical view intimal cells are derived from medial VSMCs (Baumgartner and Studer, 1963; Stemerman and Ross, 1972; Schwartz et al., 1975; Clowes et al., 1983; Regan et al., 2000). However, recent studies have suggested that adventitial VSMC progenitor cells also contribute to formation of the neointima. Several cellular models have been established to address VSMC origin in development and pathogenesis (see below). In this review, we provide a brief summary of the current understanding of the regulatory mechanisms in

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VSMC phenotypic switching and their role in vascular diseases.

VSMCs in vascular diseases

The blood vessel wall is composed of three layers: a single layer of the endothelium which constitutes the intima, the media which is composed of VSMCs, and the ECM synthesized by these VSMCs, and the adventitia which is a complex layer consisting of fibroblasts, collagen fibers, immune cells, and nerves. Blood vessels continuously generate mechanical signals and biochemical factors, and in response to these, VSMCs are involved in regulating physiologic functions as well as pathological changes taking place in the vascular wall (Alexander and Owens, 2012). Unlike skeletal and cardiac muscle cells which are terminally differentiated, VSMCs maintain a high degree of plasticity both *in vivo* and *in vitro*. Following an injury such as angioplasty, the insertion of a stent, or in vascular diseases, VSMCs dedifferentiate, reduce the expression levels of MYH11, ACTA2, and cause a series of other conventional 'VSMC markers' in normal blood vessels, as has been previously described (Owens,1995; Wang and Olson, 2004). During this so-called 'phenotypic switching' process, VSMCs dedifferentiate from a 'contractile' state to a highly proliferative 'synthetic' state. VSMCs that undergo phenotypic switching show increased rates of proliferation, migration, and synthesis of extracellular matrix (ECM) components, while at the same time also acquiring macrophage markers and properties (Rzucidlo et al., 2007; Shi and Chen, 2016). This dedifferentiated phenotype plays an important role in a large number of major diseases in humans, including atherosclerosis, restenosis, calcification, and tumor development. Hence, unraveling the molecular regulatory pathway involved in VSMC phenotypic switching should provide useful insights into the pathogenesis of cardiovascular diseases.

Atherosclerosis

Atherosclerosis is a chronic progressive inflammatory disease characterized by the formation of plaque in the intima of medium-sized arteries. It is the leading cause of morbidity and mortality worldwide (Virmani et al., 2000; Libby et al., 2011). Atherosclerotic lesions most often go through a partial resolution process characterized by the formation of a fibrous cap. This fibrous cap serves as a 'protective' barrier to separate platelets in the blood stream from pro-thrombotic materials in the plaque. The thickness of the fibrous cap, and the extent of cap inflammation determine the stability of the atherosclerotic plaque. Acute rupture of unstable or 'vulnerable plaques' frequently results in acute thrombotic vascular diseases, like myocardial infarction and stroke (Legein et al., 2013; Vilahur and Badimon, 2013). The historical view of the role played VSMCs in atherosclerosis is that VSMCs in advanced plaques are completely beneficial in preventing rupture of the fibrous cap. However, recent studies have shown that the role of VSMCs within the atherosclerotic plaque is determined by the balance between cell proliferation and migration and between cell senescence and cell death (Lacolley et al., 2012). It has been suggested that VSMC proliferation may be predominantly protective, not just in advanced lesions but throughout the entire process of atherosclerosis.

The molecular regulation of VSMC phenotypic switching in atherosclerosis has been discussed extensively, and there has been significant progress in illuminating the underlying mechanisms. One of the most classic discoveries was of serum response factor (SRF), which is a muscle-specific transcription factor known to drive VSMC-specific gene expression (Owens et al., 2004). In addition, myocardin (MYOCD), a powerful myogenic coactivator that associates firmly with SRF, and stabilizes the binding of SRF at the degenerate CC(A/T-rich)6GG(CArG) cis-elements of all known CArG-dependent VSMC marker genes, is also involved (Wang et al., 2003b). The MYOCD-SRF regulatory module is a central component of the regulatory mechanism that facilitates combinatorial interactions between activating and repressing signals/co-factors that act on most VSMC contractile genes (Bennett et al., 2016). Studies have shown that $ApoE^{-/-}$ mice having a MYOCD-specific conditional knockout exhibit increased atherosclerosis associated with VSMC phenotypic switching, compared to $ApoE^{-/-}$ mice lacking the MYOCD-specific conditional knockout. Loss of myocardin therefore represents a crucial permissive step in the process of phenotypic transition and inflammatory activation, including increased macrophage recruitment, at the onset of vascular disease (Ackers-Johnson et al., 2015).

In addition, Krüppel-like factors (KLFs), a group of transcription factors principally involved in regulating cell growth, differentiation, proliferation, and apoptosis, also play important roles in the progression of atherosclerosis (McConnell and Yang, 2010). Investigators have found that loss of KLF4 in VSMCs *in vivo* results in a transient delay in phenotypic switching following vascular injury (Yoshida et al., 2008). Recent studies using $ApoE^{-/-}$ mice have shown that a VSMC specific conditional knockout of KLF4 results in significant reductions in plaque size with increased fibrous cap thickness, and increases multiple indices of plaque stability. In addition, the plaques in the these knockout mice contained reduced numbers of VSMC-derived mesenchymal stem cell-like cells, as well as macrophage-like cells, suggesting that KLF4 promotes the transition to a 'macrophage' phenotype (Shankman et al., 2015). Similarly, studies have found that another KLF family member, KLF5, induces the phenotypic conversion of VSMCs and facilitates loss of contractile function in smooth muscle (Kim et al., 2015). Dramatically, recent studies have shown that the transcriptional activities of myocardin, KLF4, KLF8, and KLF5 orchestrate the mechanism of the VSMC phenotype switch

(see below) (Ha et al., 2017). More extensive studies have shown that KLF4 also has a critical role in the generation of vascular progenitor cells in the adventitia. A group of vascular progenitor cells in the adventitia of ApoE$^{-/-}$ mice and human vessels that express the progenitor markers Sca1 and CD34 (AdvSca1 progenitors) can differentiate into VSMCs *in vitro* (Hu et al., 2004; Zengin et al., 2006; Campagnolo et al., 2010). Interestingly, further studies by Majesky's group have established that AdvSca1 cells are generated from VSMCs and that generation of AdvSca1 cells is KLF4-dependent both *in vivo* and *in vitro* (Majesky et al., 2017). VSMC-derived AdvSca1 cells can differentiate into macrophage-like cells and endothelial-like cells *in vivo* and play important roles in arterial homeostasis and disease. In summary, these studies all indicate that KLF4 plays a key role in atherosclerosis and other vascular-related diseases.

Besides the aforementioned transcription factors, several other stimulating cues that modulate VSMC plasticity in atherosclerosis have been reported, including growth factors and inflammatory mediators (Beamish et al., 2010). For example, platelet-derived growth factor (PDGF), tumor necrosis factor α (TNFα), and interleukin-1β (IL1β) stimulate the phenotypic switching from the contractile type to the synthetic type (Ha et al., 2015). Conversely, the synthetic VSMC phenotype is significantly converted into the contractile VSMC phenotype by stimulation of the insulin and insulin-like growth factor-1 (IGF-1) signaling pathways (Wang et al., 2003a; Hayashi et al., 2004). Therefore, the inflammatory response is closely associated with these VSMC phenotypic conversions in disease states. Previously, investigators have reported that that TNFα plays a critical role in vascular remodeling. For example, a deficiency in TNFα reduces the thickness of vascular walls and the size of atherosclerotic lesions in TNFα/ApoE double knockout mice (Ohta et al., 2005). However, the mechanism underlying the TNFα-induced phenotypic conversion of VSMCs is still unclear. Recent data from a study conducted by Ha et al. (2017) suggest that KLF8 stimulates the differentiation of VSMCs by enhancing myocardin, KLF4, and NFκB, while suppressing KLF5; In the presence of TNFα, the expression of KLF8 is downregulated, thereby relieving KLF5 repression, inducing VSMC dedifferentiation and promoting vascular disease (Fig. 1).

Although the majority of studies have focused on the intracellular signals within VSMCs that regulate phenotypic switching, VSMCs synthesize ECM that separates VSMCs from each other. The conventional view is that the ECM maintains VSMCs in the 'contractile' state and suppresses

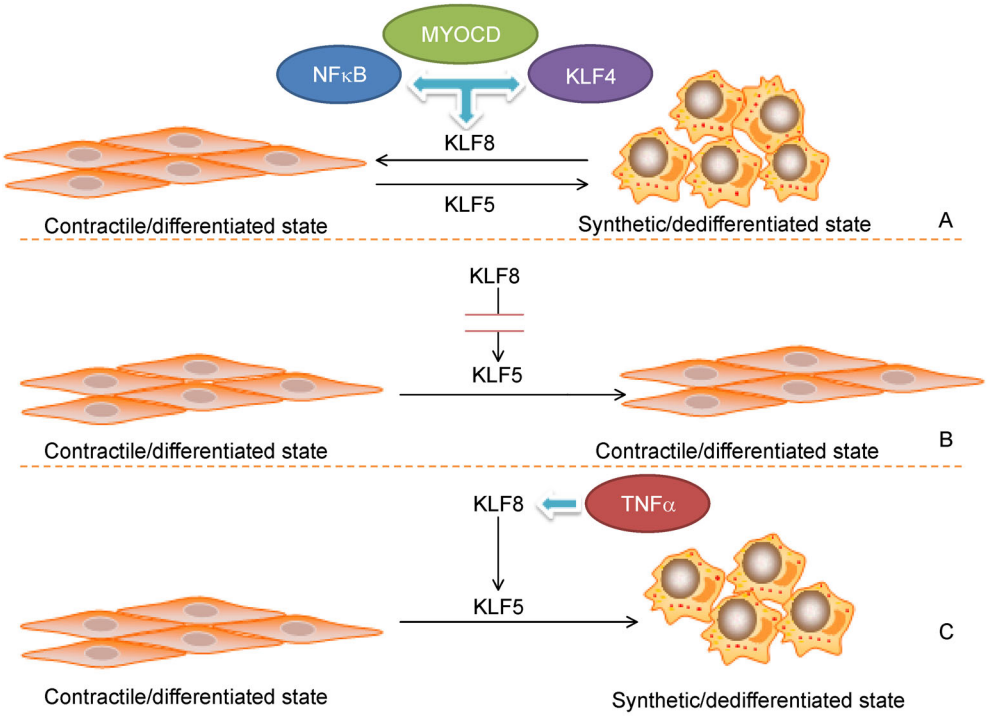

Figure 1 Schematic summarizing the regulation of VSMC phenotype by cross-regulation of myocardin, KLF4, KLF8, and KLF5, and the role of KLFs during the TNFα-induced phenotypic conversion of VSMCs. (A) KLF5 induces phenotypic conversion (dedifferentiation) of VSMCs facilitating the loss of the contractile function of smooth muscle; In contrast, KLF8 stimulates the differentiation of VSMCs. Myocardin, KLF4, and NFκB signaling pathways mediate the expression of KLF8, and thus stimulate the differentiation of VSMCs. (B) KLF8 blocks the expression of KLF5 which plays an essential role in the dedifferentiation of contractile VSMCs. (C) In the presence of TNFα, the expression of KLF8 is downregulated, thereby relieving its repressive effect on KLF5 expression, leading to VSMC dedifferentiation.

phenotypic switching. However, ECM, collagen, and elastin can be broken down by matrix metalloproteinases (MMPs) released from macrophages and VSMCs. This process will boost phenotypic switching and promote cell proliferation and migration (Koyama et al., 1996; Li et al., 1998; Fukumoto et al., 2004). However, the mechanisms by which the ECM affects VSMCs may be more complex than previously thought. For example, fibronectin (FN) is one of the earliest ECM proteins deposited at atherosclerosis-prone sites, and it is thought to promote atherosclerotic lesion formation. A study has demonstrated that while FN worsens the process of atherosclerosis by increasing the atherogenic plaque area, it also facilitates the formation of the protective fibrous cap (Rohwedder et al., 2012). Moreover, although VSMCs produce and respond to collagens in vitro, there is no direct evidence *in vivo* that VSMCs are a crucial source of collagens and impact lesion development or fibrous cap formation. A recent study has demonstrated that a VSMC-derived collagen, collagen type XV (COL15A1), is critical for atherosclerotic lesion development. Interestingly, previous studies have also reported that VSMC *Col15a1* knockout results in a dramatic attenuation rather than an exacerbation of atherosclerotic lesion formation (Durgin et al., 2017). This study is the first to examine how knocking out a given ECM gene exclusively in VSMCs impacts lesion pathogenesis *in vivo*.

There are multiple cell types involved in the process of atherosclerosis, including macrophages, lymphocytes, endothelial cells (ECs), and VSMCs. In particular, macrophages and VSMCs are key contributors (Tabas et al., 2015). The conventional viewpoint is that plaques are composed primarily of macrophages, and that these macrophage-derived foam cells are more vulnerable to rupture compared to VSMCs (Virmani et al., 2000; Libby et al., 2011). In other words VSMCs in advanced plaque lesions are usually considered to be athero-protective, whereas macrophages are considered to be athero-promoting with respect to plaque stabilization. However, due to limitations, this viewpoint may be incorrect. In advanced plaques, there is an over-dependence on markers that are either not specific or are upregulated in other cell types. For example, a study has shown that approximately 50% of foam cells in advanced human coronary artery lesions express the VSMC-specific marker, SM α-actin. Nevertheless, most of these cells also express the macrophage marker CD68, and thus, it is uncertain whether these cells represent VSMC-derived cells expressing macrophage markers or are macrophages expressing VSMC markers (Allahverdian et al., 2014). Lineage tracing studies in advanced plaques in $ApoE^{-/-}$ mouse have also indicated that medial VSMCs can convert to macrophage-like cells and constitute a major component in advanced atherosclerotic lesions. These macrophage-like cells express various macrophage markers including Mac-2/LGLS3 and CD68, and simultaneously they reduce the expression of classic VSMC markers, such as ACTA2 (Feil et al., 2014). Of note, an editorial on this latter paper emphasized that the labeling efficiency of VSMCs in these studies was too low to illustrate what proportion of macrophage-like cells were derived from VSMCs, and how these cells contribute to lesion pathogenesis in advanced lesions (Swirski and Nahrendorf, 2014). Similar studies have found that cholesterol loading of VSMCs converts them to a macrophage-like state by downregulating the miR-143/145-myocardin axis, which positively regulates the master VSMC differentiation transcription factor myocardin. Although these cells would be classified as macrophages by immunohistochemistry analysis, their transcriptome and functional properties imply that their contribution to atherogenesis is not that of classical macrophages (Vengrenyuk et al., 2015). In summary, the results from all these studies support the conclusion that most previous studies have misidentified VSMCs and macrophages in atherosclerotic lesions and that VSMCs exhibit an even greater degree of plasticity than previously recognized. Nevertheless, VSMCs and macrophages are inextricably linked in atherosclerotic lesions. Consistent with this view, recent experiments using a transwell chamber model have shown that VSMC-macrophage communication can induce changes in the protein composition of the ECM by significantly decreasing the expression of ECM proteins (collagen I, III, elastin) in VSMCs, while increasing the expression and activity of metalloprotease MMP-9 and expression of the collagenase MMP-1 in both macrophages and VSMCs. As discussed above, these changes result in the progression of the atheroma toward a 'vulnerable plaque' (Butoi et al., 2016), suggesting that targeting the smooth muscle-to-macrophage transdifferentiation or cellular cross-talk in the atherosclerotic plaque may be a novel therapeutic strategy to slow-down or retard plaque progression.

Calcification

Vascular calcification (VC) is an active and complicated process regulated by multiple factors that involves several cytokines and their associated signaling pathways. VC is widely seen in numerous vascular-related diseases, including atherosclerosis, chronic kidney disease (CKD), hypertension, and diabetes (Bessueille and Magne, 2015). The conventional viewpoint suggests that vascular calcification mainly occurs in the arterial intima and media. However, *in vitro* studies have observed calcified lesions in the aortic adventitia in humans, suggesting that adventitial calcification may arise from fibroblasts which had been transformed into myofibroblasts or VSMCs (Li et al., 2015). There are numerous vascular cells, including VSMCs, myofibroblasts, vascular mesenchymal progenitors, and ECs that have been shown to be involved in vascular calcification (Steitz et al., 2001; Bostrom et al., 2011). VSMCs are major participants in

vascular calcification and undergo an osteogenic phenotype switch characterized by the loss of VSMC-specific markers and acquisition of osteogenic markers, including Runx1/Cbfa1, osteopontin, osteocalcin, and alkaline phosphatase (Steitz et al., 2001; Speer et al., 2009). Apoptosis of VSMCs also promotes this deposition. Osteoblast-like cells promote calcification of the collagen in the ECM, ultimately leading to increased stiffness and decreased pliability of the arterial wall (Shanahan et al., 2011).

It is generally accepted that serum phosphate and calcium levels, magnesium, bone morphogenetic proteins (BMPs), leptin, and inflammatory cytokines drive this phenotypic transition by inducing oxidative stress in VSMCs. In addition, other vascular tissue factors such as adrenomedullin, C-type natriuretic peptide, angiotensin, and aldosterone are also involved in regulating vascular calcification (McCarty and DiNicolantonio, 2014). It is worth mentioning that expression of the osteogenic transcription factor Runx2 plays a critical role in VSMC calcification *in vitro* (Chen et al., 2006; Byon et al., 2008). Runx2 belongs to the runt-related transcription factor family, and its expression is significantly increased in calcified vascular tissue compared with normal vascular tissue, suggesting that Runx2 may be important in vascular calcification (Steitz et al., 2001; Tyson et al., 2003). Runx2 either directly or indirectly promotes the expression of many proteins found in osteoblasts that regulate the extracellular deposition of hydroxyapatite (Liu and Lee, 2013). Studies using small interfering RNA, as well as VSMC-specific Runx2 deficient mouse models, have demonstrated that Runx2 is essential for oxidative stress-induced VSMC calcification, and is sufficient to induce VSMC calcification by itself, both *in vitro* and *in vivo* (Byon et al., 2008; Sun et al., 2012). In addition, elevated serum phosphate and calcium, two other vital mediators of mediators of VC, have been independently correlated with inflammation. Using incubation of human aortic smooth muscle cells (HASMCs) in a high phosphate medium, a recent study has demonstrated that elevated calcium and phosphate levels have direct effects on vascular calcification, accompanied by the activation of NF-κB signaling, increased expression of the pro-inflammatory mediators, and increased production of reactive oxygen/nitrogen species (ROS/RNS). Therefore, these studies provide evidences for novel mechanisms whereby high phosphate can directly trigger vascular calcification (Martinez-Moreno et al., 2017).

Restenosis

Restenosis refers to an abnormal ($> 50\%$) narrowing of the vessel diameter compared with the normal vessel, and often occurs following percutaneous angioplasty procedures. VSMC accumulation in the arterial intima is an important event in the pathogenesis of post-angioplasty restenosis (Glass and Witztum, 2001; Marx et al., 2011). Restenosis is mainly caused by formation of the neointima. Injury to the arterial wall induces the dedifferentiation, migration, and proliferation of medial-derived VSMCs, and initiates an inflammatory response, all of which contribute to restenosis (Mitra and Agrawal, 2006). Thus, the molecular mechanisms underlying the pathological restenosis response and the formation of the neointima have been widely investigated, and consequently have remarkably improved the treatment of patients with coronary artery disease.

During the development of restenosis, several processes similar to those occurring during atherosclerosis promote a dedifferentiated VSMC phenotype (Newby and Zaltsman, 2000; Schober, 2008. Both are characterized by activation of VSMCs, resulting in an inflammatory environment, neointimal hyperplasia, and vessel occlusion (Libby et al., 2011; Alexander and Owens, 2012). Phosphatase and tensin homolog (PTEN), which is a dual-specificity protein and lipid phosphatase, has been implicated as a negative regulator of VSMC proliferation and injury-induced vascular remodeling. PTEN functions as a cytoplasmic lipid phosphatase to regulate both basal and growth factor-stimulated PI3-kinase/Akt-mediated signaling (Dahia, 2000; Vazquez et al., 2000; Tamguney and Stokoe, 2007). Thus, changes in VSMC PTEN/Akt signaling following vascular injury are related to increased VSMC proliferation and neointima formation. Furgeson et al. have shown that carotid ligation in mice having a VSMC-specific heterozygous PTEN deletion resulted in enhanced neointima formation, increased VSMC hyperplasia, reduced expression of VSMC markers (α-SMA and calponin), increased PI3-kinase/Akt/mTOR signaling, and NF-κB activity compared with wild-type mice (Furgeson et al., 2010). These data indicate that inactivation of PTEN exclusively in VSMCs promotes a pro-inflammatory phenotype and enhances neointima formation. Consistent with this viewpoint, a further study, using both mouse genetic models and in vitro approaches, demonstrated that PTEN is an essential regulator of the transcriptional activity of SRF which plays an important role in the mechanism that dynamically regulates the expression of VSMC contractile genes (Horita et al., 2016). Thus, loss of the PTEN-SRF axis promotes reprogramming of VSMCs into a proliferative, and inflammatory phenotype. Therefore, targeting PTEN-SRF nuclear interactions has the potential to produce novel therapeutics that preserve the mature differentiated VSMC phenotype in order to inhibit in-stent restenosis. Collectively, these data support the conclusion that an alteration in VSMC PTEN signaling acts as a critical initiating determinant driving pathological vascular remodeling.

Abdominal aortic aneurysms (AAAs)

AAA is a progressive degenerative disease with no available pharmacological treatment which may result in significant morbidity and mortality, occurring mainly in older adults

(Baxter et al., 2008). AAA is defined as an acquired focal dilation (aneurysm) greater than 1.5 times the normal size of abdominal aorta.

Extensive studies have suggested that inflammation in the aortic wall causes the production of local inflammatory mediators, with macrophages and VSMCs releasing proteases such as MMPs in response to cytokine production. These events lead to the breakdown of the ECM proteins, collagen and elastin, which is followed by VSMC apoptosis in the later stages of the aneurysm (Ailawadi et al., 2009). Thus, loss of VSMCs and the resulting degradation of collagen and elastin are now viewed as critical steps in aneurysm development and progression (Ailawadi et al., 2003; Owens, 2007). However, as an important contributor to aneurismal change, less attention has been paid to the role of VSMCs compared to the role of inflammation. There is growing evidence that aortic VSMCs have the potential to directly participate in the degenerative process. As an example, recent studies have demonstrated that AAA-SMCs have a unique gene expression profile and a so called pro-elastolytic phenotype, which is able to degrade significantly more insoluble elastin than cells derived from normal aorta, that directly contributes to the pathogenesis of the aneurysm (Airhart et al., 2014).

Other cell signaling mechanisms involved in the formation of these aortic aneurysms remain incompletely understood. TGF-β signaling is known to regulate VSMC growth and apoptosis, MMP-dependent proteolysis, and vascular inflammation. TGF-β signaling is widely recognized as being an important signaling pathway in the initiation of aneurysms and their progression, although, interestingly, the role of TGF-β signaling is still controversial (Wang et al., 2013). Early studies have shown that increased TGF-β signaling leads to aneurismal dilatation in AAAs (Fukui et al., 2003). In contrast, Wang et al. have shown that TGF-β acts as a protector against inflammatory AAA progression and complications in an angiotensin II-infusion mouse model (Wang et al., 2010). Yet, similar studies using a smooth muscle-specific disruption of the TGF-β type II receptor (Tgfbr2) animal model has suggested that TGF-β signaling in VSMCs contributes to the pathogenesis of elastase-induced AAA, and that disruption can prevent its formation (Gao et al., 2014). How can we explain these contradictory findings? A variety of molecules that may play opposing functions exist in the TGF-β pathway. According to the locations of the different aneurysms (e.g. thoracic vs. abdominal) and the major types of cells (SMCs vs. inflammatory cells) that are regulated by TGF-β signaling, TGF-β signaling can act as a master upstream modulator that regulates both pro- and anti-inflammatory pathways (Li et al., 2014). This may explain why TGF-β1 has been reported to promote thoracic aortic aneurysm development, while at the same time playing a protective role in AAA development (Wang et al., 2013). This protective role of TGF-β1 in AAA development was addressed in a recent study that showed that the expression of TGF-β1 is markedly elevated in aneurysmal tissue group compared to nonaneurysmal tissue (Doyle et al., 2015).

Resident vascular progenitor cells and VSMCs

It is widely accepted that VSMCs contribute significantly to neointimal formation following vascular injury. The origin of these neointimal VSMCs, however, is still under debate. The fact that most intimal cells express VSMC marker genes, including α-SMA and SM22α, after vascular injury led to the hypothesis that intimal cells are derived from medial VSMCs (Baumgartner and Studer, 1963; Stemerman and Ross, 1972; Schwartz et al., 1975; Clowes et al., 1983; Regan et al., 2000). However, this hypothesis was challenged by the finding that adventitial cells initially respond to injury by increasing cell proliferation and then later migrating into the neointima (Holifield et al., 1996; Scott et al., 1996; Shi et al., 1996; Mason et al., 1999; Oparil et al., 1999). An early report showed that cells from the adventitial side of uninjured canine carotid arteries proliferate *in vitro* and express α-SMA rather than SM-MHC. In contrast, VSMCs from adult carotid media did not proliferate *in vitro,* and maintained expression of VSMC marker proteins (Holifield et al., 1996). Direct evidence for the ability of adventitial cells to migrate through the media into the neointima was obtained by seeding these adventitial cells onto the adventitial side of an artery and detecting the movement of these cells following arterial endothelial injury (Mason et al., 1999; Li et al., 2000; Hu et al., 2004; Rodriguezmenocal et al., 2009). In this experiment, β-galactosidase was used to label adventitial cells prior to endothelial injury of rat common carotid arteries. As a result, β-galactosidase-positive cells were observed within the medial layer after 3 days, and in the neointima 7 and 14 days after endothelial injury (Holifield et al., 1996).

In recent years, numerous reports have demonstrated the existence of resident cardiovascular progenitor cells (Kovacic and Boehm, 2009; Campagnolo et al., 2010; Orlandi and Bennett, 2010; Hu and Xu, 2011; Majesky et al., 2011a, 2011b; Psaltis et al., 2011; Torsney and Xu, 2011; Plass et al., 2012; Li and Izpisua Belmonte, 2016). Emerging data has suggested that several distinct progenitor populations have the capacity to differentiate into VSMCs. The recruitment of stem/progenitor cells present into the vessel wall are largely responsible for VSMC accumulation in the intima during vascular remodeling that occurs following such events as neointimal hyperplasia and arteriosclerosis (Scott et al., 1996; Li, 2000; Sartore et al., 2001; Abedin et al., 2004; Hirschi and Majesky, 2004; Aicher et al., 2005; Xu, 2007; Du et al., 2012). Among all the resident vascular progenitor cells, adventitial $Sca1^+$ progenitor cells are among the most important (Hu, 2004; Passman et al., 2008). Hu et al. first identified a $Sca1^+$ cell residing in the adventitia as being a VSMC progenitor cell (Hu et al., 2004). When $Sca\text{-}1^+$ cells

expressing the *LacZ* gene were transferred to the adventitial side of vein grafts in ApoE-deficient mice, *β*-gal$^+$ cells were found in the adventitial media at 2 weeks, and in the adventitial intima at 4 weeks, where they no longer expressed the Sca1 antigen and became immunopositive for VSMC marker proteins. *In vitro*, adventitial Sca1$^+$ progenitor cells can differentiate into smooth muscle cells expressing the mature VSMC markers α-smooth muscle actin (ACTA2), calponin (CNN1), and smooth muscle myosin heavy chain (MYH11). Passman et al. have also reported that Sca1$^+$, CD34$^+$, and PDGFRβ$^+$ cells residing in an adventitial niche, characterized by sonic hedgehog (Shh) signaling, could be differentiated into smooth muscle-like cells *in vitro* (Passman et al., 2008). The differentiation of adventitial Sca1$^+$ progenitors into VSMC can be regulated by PDGF-BB. MMP-811 and stromal-cell derived factor-12 also seem to regulate adventitial Sca1$^+$ progenitor cell recruitment during atherosclerosis or neointimal formation after arterial endothelial injury (Shikatani et al., 2016).

However, the origin of the adventitial Sca1$^+$ progenitor cells remains unknown. Little attention has been paid to the possibility that VSMCs may also move in the opposite direction, that is, into the adventitia. Recent reports have demonstrated that a high percentage of VSMCs in atherosclerotic lesions lack detectable expression of conventional VSMC markers, but exhibit a macrophage-like phenotype (Feil et al., 2014; Vengrenyuk et al., 2015). These findings suggest that VSMCs exhibit an even greater degree of plasticity than that previously recognized. Using fate-mapping and lineage-tracing approaches, Majesky et al. have demonstrated that a distinct subpopulation of adventitial Sca1$^+$ progenitor cells derived from differentiated VSMCs can undergo a reprogramming-like process in situ to generate multipotent progenitor cells (Majesky et al., 2017).

Other than Sca1$^+$ cells, Rafael Kramann have demonstrated that Gli1$^+$ progenitor cells are also an important adventitial cell source for VSMCs and contribute to neointimal formation after acute arterial injury. Gli1$^+$ cells located in the arterial adventitia are progenitors of VSMCs and contribute to neointima formation and repair after acute injury to the femoral artery. Genetic fate tracing has indicated that adventitial Gli1$^+$ MSC-like cells migrate into the media and neointima during arteriosclerosis in ApoE$^{-/-}$ mice with chronic kidney disease (Kramann et al., 2016).

Furthermore, Tang et al. isolated cells from the medial layer of the blood vessel wall and identified a new type of multipotent vascular stem cell (MVSC) expressing markers which included Sox17, Sox10, S100β, and neural filament-medium polypeptide (NFM) (Tang et al., 2012). MVSCs can differentiate into MSC-like cells and subsequently VSMCs. Importantly, lineage-tracing experiments have shown that MVSCs, as well as proliferative or synthetic VSMCs, are not derived from mature VSMCs. Following vascular injury, MVSCs become proliferative and contributed significantly to vascular remodeling and neointima formation. These findings define a novel MVSC-VSMC differentiation pathway, and support a new viewpoint that the differentiation of MVSCs contributes to vascular remodeling and diseases instead of the de-differentiation of VSMCs

In summary, resident vascular progenitor cells significantly contribute to neointimal formation and vascular diseases.

In vitro VSMC differentiation models

Given that VSMC differentiation plays a critical role in the process of vascular disease, we will next summarize the in vitro models that have been established to define the molecular mechanisms involved in the regulation of VSMC differentiation and phenotypic modulation.

Mesoderm-derived models

C3H/10T1/2 Cells

The 10T1/2 cell line was generated from primary cultures of 14- to 17-day whole C3H mouse embryos (Reznikoff et al., 1973). It has been reported that these multipotent 10T1/2 cells can differentiate into VSMCs by coculture with ECs or treatment with transforming growth factor-β1 (TGF-β1, 1 ng/mL) for 24 to 48 h, as evidenced by a phenotypic change from a polygonal to a spindled-shaped phenotype, and the expression of VSMC-specific markers including α-SMA, SM22α, and calponin (Hirschi et al., 1998). This cell model is most commonly used for studying VSMC differentiation because of the ease of acquisition of these cells from the American Type Culture Collection, undemanding culture conditions (DMEM + 10% FBS), and rapid induction of differentiation by TGF-β1 (within 48 h).

However, it has been suggested that 10T1/2 cells cannot fully differentiate into VSMCs upon TGF-β1 treatment, but rather differentiate into myofibroblasts that do not express MYOCD and the mature VSMC marker MHY11 (Yoshida and Owens, 2005; Shi et al., 2012). These conflicting reports maybe due to the differences in culturing methods and manipulation of these cells in different laboratories. Regardless, the 10T1/2 cells differentiation system has been a useful model for studying the molecular mechanisms underlying TGF-β1-dependent VSMC differentiation.

Neural crest stem cell–derived models

Monc-1 cells

Monc-1 is a neural crest cell line that was immortalized by retroviral transfection of the *v-myc* gene in primary cultures of mouse neural crest cells (Rao and Anderson, 1997). Monc-1 cells can differentiate into neurons, gliacytes, chondrocytes, melanocytes, and VSMCs (Rao and Anderson, 1997; Jain et al., 1998). It has been shown that Monc-1 cells can differentiate to a VSMC lineage following stimulation with TGF-β1, resulting in the induction of VSMC markers. In

contrast, bone morphogenic protein 2 (BMP-2), another member of the TGF-β superfamily, is unable to induce the expression of VSMC marker genes in Monc-1 cells (Chen and Lechleider, 2004). This inconsistency is probably due to the antagonistic effect of the *v-myc* gene on BMP-2 signaling. In addition to TGF-β1, fetal bovine serum (FBS) can also induce VSMC differentiation from Monc-1 cells (Jain et al., 1998). FBS-induced Monc-1 cells exhibit a differentiation state similar to the proliferative synthetic phenotype of VSMCs, rather than the contractile phenotype, because of lack of a spindle-shaped morphology and a lack of contraction in response to carbachol (Jain et al., 1998; Chen and Lechleider, 2004). The limitation of this model is the requirement of a complicated medium for growth and maintenance of the undifferentiated state. On the other hand, Monc-1 cells expressing the *v-myc* proto-oncogene in a constitutive manner may generate undefined effects such as abnormal proliferation during the differentiation process.

JoMa1 cells

JoMa1 is an immortalized multipotent neural crest cell line derived from neural crest primary cultures from a transgenic mouse line expressing a conditional tamoxifen-inducible *c-Myc* oncogene (Maurer et al., 2007). TGF-β1 treatment, combined with tamoxifen removal from the culture medium, results in a loss of *c-Myc* and induces the differentiation of JoMa1 cells into VSMCs as evidenced by a change in cell morphology, a strong expression of α-SMA (over 90% of cells) and SM γ-actin, and a weak expression of calponin. A clonally derived subline of JoMa1 cells, termed JoMa1.3, showed a purer VSMC population expressing higher levels of CNN1 than its parental line following TGF-β1 treatment.

Compare to Monc-1 cells, JoMa1 and JoMa1.3 cells do not express *c-Myc* during differentiation due to the absence of tamoxifen, which avoids the potential interference of this oncogene in the differentiation program. In the presence of tamoxifen, TGF-β1 induction would lead to incompatibility between the proliferation and differentiation signals in these cells because the proliferative stimulus via *Myc,* and the differentiation stimuli via TGF-β1, delivered in parallel induces cell death (Maurer et al., 2007). Although JoMa1 is an attractive in vitro model for studying VSMC differentiation in a neural crest linage, it has limitations similar to the Monc-l line with respect to requiring complex culture conditions to maintain its undifferentiated state.

Stem/progenitor cell-derived models

P19 and A404 cells

The P19 cell line is derived from a teratocarcinoma that was formed following transplantation of a 7.5-day mouse C3H/He embryo into the testis (McBurney and Rogers,1982; McBurney, 1993). It appears to use mechanisms similar to those of normal embryonic stem cells (ESCs) to differentiate (McBurney, 1993). Studies have shown that P19 cells differentiate into fibroblast-like cells upon treatment with retinoic acid (RA; 10^{-6} mol/L for 48 h) or dimethyl sulfoxide and 7.5% fetal bovine serum (after 5 to 7 days), following which they express smooth muscle α-actin (ACTA2), exhibit calcium influx patterns, and acquire the ability to respond to phenylephrine, angiotensin II, and endothelin (Rudnicki et al., 1990; Blank et al., 1995). Although the P19 cell line can differentiate into functional VSMC-like cells, it still requires additional enrichment methods to increase the yield of VSMCs.

The A404 cell line is a P19-derived clonal cell line containing an ACTA2 promoter/intron-driven puromycin resistance gene (Manabe and Owens, 2001). All-*trans* RA, combined with puromycin treatment, stimulates a rapid differentiation of multipotent A404 cells into VSMCs that express myocardin and multiple VSMC markers including α-SMA, calponin, and SM-MHC, with a differentiation efficiency of more than 90% (Manabe and Owens, 2001; Spin et al., 2004). Although the A404 cell line is a highly efficient model system for VSMC differentiation, it is not a natural VSMC progenitor that exists in vivo because it is established by introducing the α-SMA promoter into the cells.

ESC differentiation system

Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass of blastocysts. ESCs can differentiate into VSMCs in an adherent monolayer culture system (Huang et al., 2006; Xie et al., 2009). When treated with all-*trans* RA, monolayers of hESC cultures differentiate toward a VSMC lineage, and after up to 30 days in culture, 40%–90% of the cells stably express α-SMA and SM-MHC, demonstrate morphological changes consistent with a contractile VSMC phenotype (Huang et al., 2006; Xie et al., 2009), and exhibit a smooth muscle-like contraction frequency (Huang et al., 2006). Moreover, these differentiated cells display functional calcium responses following treatment with the vasoconstrictor caffeine and the depolarizing agent KCl (Xie et al., 2009). To improve the efficiency of VSMC differentiation from ESCs, the ESC surface marker Sca-1 can be used to isolate VSMC progenitor subpopulations (Xiao et al., 2006, 2007). Treatment of ESC-derived Sca-1^+ cells with PDGF has been reported to induce VSMC differentiation at an efficiency of more than 95% after long-term culture on a surface containing collagen IV (Xiao et al., 2007).

Human embryonic stem cell-derived mesenchymal cells (hES-MCs), derived from H9 human embryonic stem cells, are natural mesoderm-derived VSMC progenitors (Boyd et al., 2009). hES-MCs have the capacity to produce three mesenchymal lineages including osteocytes, chondrocytes, and VSMCs (Boyd et al., 2009). It has been reported that hES-MCs can robustly differentiate into the VSMC lineage upon TGF-β1 induction, accompanied by a high level of

VSMC marker expression including α-SMA, SM22α, SM-MHC, and calponin, as well as contraction in response to the muscarinic agonist carbachol or KCl, suggesting a functional VSMC phenotype (Guo et al., 2013).

hES-MCs appear to be a more attractive *in vitro* model for VSMC differentiation than 10T1/2 cells. Similar to 10T1/2 cells, hES-MCs exhibit such advantages as simple culture methods (αMEM + 10% MSC-qualified FBS) and rapid differentiation (within 72 h of TGF-β1 induction). Remarkably, unlike 10T1/2 cells, hES-MCs can stably express SM-MHC and myocardin upon TGF-β1 treatment, signifying a full differentiation toward a mature and functional VSMC lineage rather than immature VSMCs or myofibroblasts (Guo et al., 2013). Most importantly, hES-MCs may be used to study mechanisms underlying VSMC differentiation in humans because of their human origin.

iPSC differentiation system

Recently, several studies have established methods for generating VSMCs from induced pluripotent stem cells (iPSCs), such as lateral plate mesoderm-derived, coronary-like, VSMCs from pluripotent stem cells, neural crest-derived VSMCs from skin-derived precursors, and contractile or synthetic VSMCs from human iPSCs (Steinbach and Husain, 2016; Yang et al., 2016). These methods may be very useful for personalized medicine and regenerative medicine approaches. They may also be used to establish models for hereditary familial syndromes that affect VSMCs such as Marfan's or Loeys-Dietz Syndrome, or even systemic conditions such as progeria (Liu et al., 2011). Compared to ESCs, iPSCs are mainly used for SM tissue engineering and regeneration to avoid both ethical problems and the recipient's immune response that are encountered with ESCs. In spite of the potential strengths of iPSC-derived VSMCs in the treatment of vascular disease, there are still issues that need to be considered. It has been reported by Xie et al. (2009) that there are different VSMC marker expression patterns in iPSCs generated by different methods. This may be due in part to the random insertion of the viral vectors encoding the four transcription factors used in iPS generation.

In summary, a variety of *in vitro* VSMC differentiation models are available. Careful consideration should be given to their relative advantages and their intrinsic limitations to select the model that is suitable for the experimental purpose.

Perspectives

Our understanding of the role of VSMCs in cardiovascular diseases has evolved remarkably over the last few years. Recent studies have clarified that the development and progression of numerous cardiovascular diseases are strongly associated with different VSMC phenotypes. Thus, further investigation of the mechanism behind VSMC phenotype regulation is essential to illuminate the pathophysiology of VSMC-related cardiovascular diseases to develop therapeutics to treat these diseases. However, many questions remain to be addressed: (1) How is VSMC differentiation from progenitors of different embryonic origin controlled? In other words, in the in vitro differentiation models, what are the mechanisms underlying progenitor-specific VSMC differentiation? (2) Many transcription factors and inflammatory mediators are responsible for stimulating the VSMC phenotype switch. While some evidence indicates that interactions exist between them, what is the mechanism underlying the regulation of transcription factors by inflammatory cytokines during VSMC phenotypic switching? (3) As many of the factors that play important roles in the control of VSMC differentiation are also involved in regulating many other cellular processes, Is there a cross talk between the diverse cellular processes among phenotypic switching? (4) What is the relation between VSMCs and other cell types, including ECs, macrophages, and circulating progenitor cells under pathological conditions, and how can they be distinguished? (5) Finally, and most importantly, as direct interventional studies to prevent phenotypic switching have been lacking, is it possible to identify the factors and mechanisms that promote beneficial changes in VSMC phenotype, and processes that can replace more conventional therapies in the near future?

Abbreviations

AAA: Abdominal aortic aneurysms; ACTA2: Smooth muscle alpha actin; CNN1: calponin; KLFs: Krüppel-like factors; EC: Endothelial cells; ECM: Extracellular matrix; MVSC: Multipotent vascular stem cell; MYH11: Smooth muscle-myosin heavy chain; SRF: Serum response factor; MYOCD: Myocardin; VC: Vascular calcification; VSMC: Vascular smooth muscle cell.

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Compliance with ethics guidelines

Yujie Deng, Caixia Lin, Huanjiao Jenny Zhou, and Wang Min declares that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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