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Review

Adult Stem Cells in Vascular Remodeling

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Abstract

Understanding the contribution of vascular cells to blood vessel remodeling is critical for the development of new therapeutic approaches to cure cardiovascular diseases (CVDs) and regenerate blood vessels. Recent findings suggest that neointimal formation and atherosclerotic lesions involve not only inflammatory cells, endothelial cells, and smooth muscle cells, but also several types of stem cells or progenitors in arterial walls and the circulation. Some of these stem cells also participate in the remodeling of vascular grafts, microvessel regeneration, and formation of fibrotic tissue around biomaterial implants. Here we review the recent findings on how adult stem cells participate in CVD development and regeneration as well as the current state of clinical trials in the field, which may lead to new approaches for cardiovascular therapies and tissue engineering.

Key words: Cardiovascular disease; Stem cell; atherosclerosis; vascular grafts; vascular smooth muscle cell.

Introduction

Cardiovascular diseases (CVDs) such as ischemic heart disease, stroke, and peripheral artery disease are the leading cause of mortality and morbidity around the world: about 30% of global deaths and 10% of global disease burden a year are due to CVDs [1, 2]. In the past three decades, these diseases have been increasing in underdeveloped and developing countries. Although deaths from CVDs have declined in some developed countries with better healthcare interventions and systems and primary prevention, population growth and aging will drive up global CVDs in coming decades [1, 2].

Atherosclerosis is a chronic inflammatory disease resulting in clogged arteries or unstable plaque rupture [3, 4]. Currently, treatment of atherosclerosis includes reducing risk factors such as treatment of hypercholesterolemia and hypertension [1, 2] and, for advanced disease, surgery such as stent implantation and bypass surgery using autologous vessels or tissue-engineered vascular grafts [5]. However, thrombosis and secondary atherosclerosis are common complications following stent and graft implantation, particularly in small-diameter arteries and grafts [6]. New therapies are thus urgently needed for better prevention and treatment of atherosclerosis.

It is widely accepted that endothelial cell (EC) dysfunction, inflammatory cell recruitment, and vascular smooth muscle cell (SMC) de-differentiation contribute to atherogenesis [3, 4, 7]. In the past two decades, several types of vascular stem cells (VSCs), in addition to circulating progenitors, have been identified and characterized, with evidence that they are not only involved, but also play pivotal roles in blood vessel remodeling and disease development. VSCs or similar stem cells in mesenchymal tissues, for instance, also participate in the regeneration of blood vessels following the implantation of vascular grafts.

Elucidating the regulatory mechanisms of these VSCs is fundamental to understanding vascular remodeling and may pave the way to developing novel, successful therapies for atherosclerosis. In this review, we analyze vascular remodeling through the lens of stem cells, and discuss the challenges we face in developing improved therapies for vascular diseases and regeneration.

Overview of atherosclerotic vascular remodeling

Large and medium size blood vessels have three distinct layers: 1) the tunica intima, an inner lining of ECs, which may contain a small number of endothelial progenitor cells (EPCs) [8, 9]; 2) the tunica media, a thick middle layer composed of smooth muscle cells (SMCs) and a small number of stem cells; and 3) the tunica adventitia, an outer layer of connective tissue containing a heterogeneous population of cells, including fibroblasts, resident inflammatory cells (including macrophages, dendritic cells, T cells and B cells), microvascular (vasa vasorum) ECs around which pericytes reside, adrenergic nerves, and also stem cells (including multipotent mesenchymal stem cells, or MSCs) and progenitor cells (including those with macrophage, endothelial, smooth muscle, and hematopoietic potential) [10-18]. All these cells contribute, to varying extents, to the pathogenesis of atherosclerosis and vascular remodeling.

Atherosclerosis is thought to be initiated by dysfunctional or activated ECs [3, 7]. Various risk factors include genetic defects and environmental risks, behaviors like cigarette smoking and harmful use of alcohol, as well as disturbed blood flow, hypertension, hypercholesterolemia, infections, and other chronic conditions such as diabetes, obesity, autoimmune diseases, and aging [1, 2]. The injured endothelial area may be repaired by adjacent EC proliferation or EPCs from bone marrow or resident endothelium [19]. Disease begins when such endothelial repair does not occur properly.

Malfunctioning ECs secrete cytokines and upregulate expression of surface adhesive molecules to recruit circulating platelets, monocytes, T cells, neutrophils, dendritic cells, and mast cells to adhere to the site of endothelial injury and infiltrate into the subendothelial space. Within this space, monocytes differentiate into macrophages and scavenge lipid deposited from the circulation, becoming foam cells in the process [3, 20-22]. Notably, most of these foam cells are initially derived from preexisting intimal-resident myeloid progenitors rather than recently recruited blood monocytes [23]. In addition, the inflammatory cells activate medial SMCs and stem cells, prompting adventitial stem cells to proliferate and migrate into the intima, where they may differentiate and also obtain some properties of myofibroblasts and macrophages [3, 20-22, 24, 25]. Disease proceeds as the abnormal vascular wall processes prompt macrophages, together with leukocytes, activated ECs, and SMCs, to secrete increasing amounts of inflammatory cytokines to recruit more inflammatory cells from the circulation and resident adventitial tissues. This forms a cycle of inflammatory responses in local atherosclerotic lesions [3, 4, 26-28]. All these events lead to the development of fatty streaks, formation of neointima, and thickening of arterial walls seen at the early stages of atherosclerosis [3, 26]. The extracellular matrix, too, may play a role in lipid retention [29]. As these atherosclerotic lesions continue to grow and narrow the lumen, arteries may attempt to compensate by gradual dilation; however, this compensation reaches its limit beyond a certain size of atherosclerotic lesion.

Advanced atherosclerotic plaques have developed a fibrous cap that sequesters the underlying inflammatory mixture, which includes foam cells and extracellular lipid droplets, infiltrated T cells, macrophages, and mast cells, and necrotic tissue [3, 26]. The cap itself is mainly comprised of SMCs and collagen matrix, which can be degraded and ruptured by metalloproteases released by macrophages and mast cells. Stability of plaques is thus defined by thickness of the fibrous cap. Severe thrombosis may occur upon fibrous cap rupture, leading to acute coronary artery disease (myocardial infarction) and stroke [3, 26].

Several groups provide direct evidence that smooth muscle myosin heavy chain $(SM\text{-}MHC)^+$ SMCs are a major contributor to neointimal thickening and atherosclerotic lesions, using transgenic mice with tamoxifen-regulated CreER under the control of a SM-MHC promoter (SM-MHC-CreER) [22, 30-33]. Interestingly, some studies suggest that SMCs in human atherosclerotic lesions are monoclonal [34, 35], implying heterogeneity of the SMC population. By using multi-colored lineage tracing in $ApoE^{-/-}$/SM-MHC-CreER/Rosa26-Confetti transgenic mice, a recent study demonstrates that only a small number of SMCs proliferate and contribute to atherosclerotic plaques [36]. This is consistent with our single-cell analysis of SMCs showing that only a small subpopulation of SMCs is capable of proliferation and differentiation (unpublished data). However it is worth noting that, in addition to medial SMCs, other non-SMCs such as stem cells and ECs also contribute to the SMCs of neointima and atherosclerotic lesions [22, 33, 37, 38],

while lesional macrophage-like cells can also be derived from SMCs [39], suggesting alternative mechanisms may also account for vascular disease development.

Endothelial to mesenchymal transition (EndoMT) is one possible mechanism. Some studies utilized Tie2-Cre mice for lineage tracing ECs and found that ECs contribute to pulmonary artery neointimal formation by differentiating into cells positive for smooth muscle α-actin (α-SMA) [40, 41]. However, other researchers found a very low frequency, in contrast, of EndoMT in the neointima [38]. Similarly, using Tie2-Cre mice to trace ECs in carotid artery neointimal formation, we found that although ECs contributed to neointimal formation, they still maintained endothelial identities and expressed CD31 but no or low α-SMA expression [37]. This discrepancy requires further investigation with different animal models and tissue locations, and still leaves open the possibility of additional mechanisms for neointimal pathogenesis.

Stem cells in vascular remodeling

In addition to vascular SMCs and ECs, vascular stem and progenitor cells have been isolated from the circulation and from different layers of the artery wall, and have been implicated in vascular disease development. Key examples found in or around the vasculature are summarized in Table 1. The list is organized based on differentiation potential and tissue(s) of origin, and is discussed in detail below.

Table 1. Vascular stem cells and progenitors

Location	Markers	Species/Tissue	Cell	Differentiation	*In vivo* function	Year
Adventitia	Stro1$^+$, CD105$^+$, CD73$^+$, CD44$^+$, CD90$^+$, CD29$^+$, Oct4$^+$, Sox2$^+$ [92, 93]	Human internal thoracic artery	Vascular wall-resident multipotent stem cells	Adipocyte, chondrocyte, osteocyte, SMC	Neovascularization, (Putative) neointima formation and tumor vascularization	2011, 2013
Adventitia	CD34$^+$, vWF$^-$, CD31$^-$, Sox2$^+$ [94]	Human saphenous vein	Saphenous vein-derived progenitor	Myocyte , osteoblast, adipocytes, neuron-like cell	Neovascularization	2011
Adventitia	Sca-1$^+$ [99, 100, 193]	Mouse aortic root	Adventitia progenitors	SMC, EC	Atherosclerotic lesion	2004, 2008, 2012
Adventitia	Sca-1$^+$, CD45$^+$ [28]	Mouse aorta	Macrophage progenitors	Macrophages	Inflammatory response	2014
Adventitia	Sca-1$^+$, CD34$^+$ [33]	Mouse aortic root, carotid arteries, descending aorta, femoral arteries	Vascular, myeloid progenitors	Mature SMCs, resident Macrophages, Endothelial-like cells	(Putative) Maintenance of resident vascular progenitor cell population	2016
Adventitia	Gli1$^+$, Sca1$^+$, CD34$^+$, PDGFRβ$^+$[118]	Mouse arteries	Adventitial progenitors	SMCs, osteoblast-like cells	Neointima, calcification	2016
Adventitia and media	Sox10$^+$, Sox17$^+$, CD29$^+$, CD44$^+$, S100β$^+$, NFM$^+$ [25, 194]	Human, rat and mouse arteries and veins; normal and diseased vessels	MVSC	SMC, osteoblast, chondrocyte, adipocyte, neural lineages	Neointima, proliferative SMC, osteochondrogenic	2012
Media	CD29$^+$, CD44$^+$ 3G5$^+$, SMA$^+$ [83, 84]	Bovine and human thoracic aorta	CVC/MSC	SMC, osteoblast, chondrocyte	Not reported (N/R)	2002, 2010
Media	Sca-1$^+$, c-kit$^{-/low}$ Lin$^-$, CD34$^{-/low}$ [85]	Mouse thoracic and abdominal arteries	Side population	SMC, EC	N/R	2006
Media	CD44$^+$, CD56$^+$, CD90$^+$, CD105$^+$, CD34$^-$ and CD45$^-$ [87]	Porcine aorta	Pericyte-like, MSC-like vascular progenitors	Adipocyte, Osteocyte, Chondrocyte	N/R	2014
Intima	CD13$^+$, CD29$^+$, CD44$^+$,CD54$^+$, CD90$^+$ [98]	Human saphenous vein-internal surface	Vein MSC	Osteoblasts, chondrocytes, adipocytes	N/R	2005
Around microvessel	N/R [195, 196]	Bovine retina	Pericyte	Osteoblast, chondrocyte, adipocyte	Chondrogenic and adipogenic in diffusion chambers	1990, 2004
Around microvessel	NG2$^+$, alkaline phosphatase$^+$ or CD146$^+$, NG2$^+$, and PDGFR-β$^+$ [197, 198]	Human skeletal muscle, pancreas, adipose tissue, and placenta	Pericyte/ peri-vascular MSCs	Skeletal muscle, Osteoblast, chondrocyte, adipocyte	Muscle regeneration, ectopic bone formation	2007, 2008
N/R	CD34$^+$, Tie-2$^+$, NG2$^+$, nestin$^+$, PDGFR-α$^+$, PDGFR-β$^+$ [112]	Rat aorta	Pericyte progenitor	Pericyte	N/R	2005
N/R	Oct-4$^+$, Stro-1$^+$, Sca-1$^+$, Notch-1$^+$, CD44$^+$, CD90$^+$, CD105$^+$, CD73$^+$, CD29$^+$, CD166$^+$ [14]	Human aortic arches, thoracic and femoral arteries	MSC	SMC, chondrocyte, adipocyte	N/R	2010

Bone marrow-derived progenitor or stem cells

Bone marrow cells were reported to differentiate into SMCs in neointima and atherosclerotic lesions in the early 2000s [42-45]. These findings, however, remain controversial, as later studies in vascular transplant and injury models countered by arguing that bone marrow-derived cells did not in fact differentiate into neointimal SMCs, although they did participate in the inflammatory response [46-48]. A mouse wire injury model, for instance, found that some bone marrow cells were recruited to the neointima and expressed α-SMA, but never became positive for mature SMC marker SM-MHC. Rather, these bone marrow cells expressed markers of monocytes and macrophages [48].

Other bone marrow-derived cells – specifically, certain EPCs – have also been identified as important for endothelial regeneration. It should be noted that the term "endothelial progenitor cell" has been applied to many different cell types, and defining what precisely it means to be an EPC is a source of controversy. Classification traditionally is divided into two methods: antigen classification, and culture-based classification. Both have been used to identify vascular-relevant EPCs.

Using the first method, cell-surface antigens are examined typically with flow cytometry to quantify relevant populations. Putative EPCs were first isolated by Asahara *et al.* (1997) from human peripheral blood by flow cytometry using surface markers CD34 and vascular endothelial growth factor receptor 2 (VEGFR-2, also known as kinase insert domain receptor, KDR, or fetal liver kinase 1, Flk1), both of which are characteristically expressed by ECs [49]. These circulating cells could contribute to neoangiogenesis postnatally by homing to angiogenic sites and acquiring characteristics of endothelium. Thereafter, other groups reported that EPCs contribute to endothelial regeneration in rodent models after various arterial injuries including vein graft atherosclerosis and mechanical injury [50-52], as well as in human diabetic wound healing [53].

Studies further showed that EPCs are in fact a heterogeneous population comprised of different subpopulations with different cell surface markers. In addition to CD34 and VEGFR-2, in an attempt to distinguish between immature and mature endothelial cells, investigators also commonly use markers like CD133 (also known as AC133), which is lost during endothelial maturation [54]. For example, Peichev et al. (2000) identified a unique subpopulation of EPCs ($CD34^+/VEGFR\text{-}2^+/AC133^+$) in human fetal liver and peripheral blood [55]; another subpopulation of $Flk1^+/AC133^+/CD34^-/VE\text{-}cadherin^-$ cells were also identified as EPCs in human bone marrow [56]. Despite the advantages of having specific markers for lineage tracing and drawing ties between disparate populations, one can see here too how antigen-based definitions may still be somewhat nonspecific in phenotype. The more antigen markers utilized, the more specific the definition, but also the fewer the cells identified – particularly considering the inherently probabilistic nature of antigen carriage for given cell types.

In the second method of classification, cells are isolated based on *in vitro* culture. Given the difficulties of finding specific surface markers for EPCs, some research groups isolated EPCs by single-cell colony-formation assay (SCCFA) based on the high self-renewal and proliferation potential of stem cells. Some studies subdivided EPCs based on their time of appearance in culture into populations which, interestingly, have different differentiation potential: early EPCs cannot differentiate into ECs, but only differentiate into macrophages and contribute to angiogenesis through paracrine factors, and thus were named as myeloid angiogenic cells (MACs); and late EPCs can differentiate into ECs and contribute to *de novo* blood vessel formation, and were dubbed endothelial colony forming cells (ECFCs) [57-61].

In addition to circulation-derived EPCs, EPCs with similar properties have been derived based on colony-formation assay from the vascular endothelium of large human blood vessels, placenta, and adipose tissue [62-64]. Mouse ECFCs have also been isolated from endothelial culture by surface markers $lin^-CD31^+CD105^+Sca1^+c\text{-}Kit^+$, with c-Kit expression found to be critical for the clonal expansion of these ECFCs [65].

Beyond the nature of EPC classification, their functions, too, remain controversial. The concept of bone marrow-derived EPCs playing a fundamental role in the mechanism of vascular repair and regeneration has acquired many proponents as we described, though it remains hotly debated [66]. Pre-clinical animal studies showed that transplanted human EPCs formed microvessels and promoted vascular regeneration *in vivo* [49, 55, 56, 67, 68]. In mouse models of vascular graft transplantation, for instance, bone marrow cells contributed to the regenerated ECs of the grafts [50, 69, 70]. Nevertheless, another study countered that bone marrow-derived EPCs do not contribute to vascular endothelium in mouse models of bone marrow transplantation, tumor formation, and a parabiotic system [71].

A role for bone marrow-derived EPCs in atherogenesis similarly has been inferred, but accumulation of solid evidence in this role is mixed

and still work in progress [52]. In an ApoE$^{-/-}$ mouse model, bone marrow-derived Sca-1^{+}/CD34^{+}/Flk-1^{+}/CD133^{+} EPCs were found in the lesion-prone area of endothelium, possibly for repairing the injured endothelium [72]. However, other studies have said that, although there may exist a population of bone marrow-derived EPCs, ECs derived from the vascular bed are instead responsible for the EC replacement and regeneration seen in transplant arteriosclerosis [73].

In the clinical context, the role of EPCs remains unclear. Large-scale clinical studies suggested that high levels of EPCs were associated with reduced risk of cardiovascular diseases [74, 75] and improved outcomes after acute ischemic stroke [76-78] (versus poorer stroke outcomes if blood EPCs failed to increase [79]), and that vascular trauma, acute coronary diseases, and stroke induced elevated level of EPCs [76, 80, 81], presumably for purposes of vascular repair and maintenance. However, some also found no clear correlation between EPC level and endothelial function [82].

To date, much ambiguity and controversy remains in regards to the existence of true EPCs that can differentiate into ECs, their marker expression, location, and contribution to endothelial regeneration. It is possible that EPCs are a rare but dynamic population that respond to specific stimuli such as severe endothelial injury of large arteries or vascular transplantation [50, 69, 70], but not to tumor growth, which involves microvessels [71].

Medial stem cells

Stem and progenitor cells resident to vasculature have been identified across the different vessel wall layers. Similar to the bone marrow-derived progenitor cells, isolation has relied on antigen selection or culture-based characterization. Although those derived from the adventitia are better characterized and supported – evidence which will be elaborated momentarily – a few groups of stem cells have also been characterized in the media.

A population of calcifying vascular cells (CVCs) was first isolated from human atherosclerotic lesions in the arterial medial layer by Boström *et al*. (1993) and Tintut *et al*. (2003) and found to differentiate into SMC, osteogenic, and chondrogenic lineages [83, 84]. CVCs were harvested by tissue explant culture and were identified as expressing CD29 and CD44, two non-specific mesenchymal cell markers (adhesion receptors). However, no specific transcriptional markers were identified.

Later, in 2006, Sainz *et al*. isolated a small population of Sca-1^{+}, c-kit $^{(-/low)}$, Lin^{-}, CD34$^{-/low}$ cells from the media layer (around 6±0.8% prevalence in tunica media) of healthy murine thoracic and abdominal aortas [85]. They used a Hoechst DNA binding dye method to identify non-tissue-specific stem/progenitor cells based on their ability to expel the dye via the transmembrane transporter ATP-binding cassette transporter subfamily G member 2 (ABCG2). These cells gave rise to ECs (as determined by VE-cadherin, CD31, and von Willebrand factor expression) and SMCs (determined by α-SMA, calponin, and SM-MHC expression) when cultured with vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGF-β1)/platelet-derived growth factor BB (PDGF-BB) respectively, similar to Flk-1^{+} mesoangioblasts found in the embryonic dorsal aorta, and also produced (VE-cadherin^{+} and α-SMA^{+}) vascular-like branching structures of cells [85, 86].

Another population of vascular progenitors were isolated by Zaniboni, *et al.* from the media by internal digestion of porcine aortas with collagenase [87]. These cells were described as similar to both MSCs and pericytes. Like MSCs, they had elongated, spindle-shaped, fibroblast-like morphology, and met minimum MSC criteria [88] for CD90 and CD105 positivity while lacking expression of CD34 and CD45. They also expressed additional MSC markers CD44 and CD56 and displayed classic MSC differentiation potential into adipocytes, chondrocytes, and osteocytes. At the same time, in behavior considered distinctive of pericytes, in coculture with human umbilical vein endothelial cells they were able to form network-like structures [87].

MSCs themselves have also been implicated in atherosclerosis [89]. MSCs expressing Oct-4, Stro-1, Sca-1, and Notch-1, for instance, were identified in the wall of a range of vessel segments such as the aortic arch, and thoracic and femoral arteries. These multipotent cells exhibited adipogenic, chondrogenic, and leiomyogenic potential [14, 15].

Our group, too, has identified a population of multipotent vascular stem cells (MVSCs) in the arterial medial and adventitial layers that could significantly contribute to the population of traditionally defined "proliferative and synthetic SMCs" in SMC culture and in neointima [25, 37]. Upon vascular injury (e.g., denudation injury), Sox10^{+} MVSCs are activated, become proliferative, and migrated from both medial and adventitial layers to contribute to neointima formation [25, 37]. In addition, some Sox10^{-} cells became Sox10^{+}, suggesting Sox10 may be a marker of activated cells (Fig. 1). In wound healing and scar formation, MVSC-like Sox10^{+} cells (which are also found in soft tissues around blood vessels and throughout the body) can differentiate into both myofibroblasts and SMCs [24].

Following the implantation of polymer vascular grafts for instance, these cells, rather than SMCs, are recruited to the outer surface of the grafts and gradually differentiate into SMCs [70], recapitulating some aspect of vascular development.

Of special note is that vessel-derived stem/progenitor cells as well as MSCs isolated from ApoE$^{-/-}$ mice respond to the inflammatory environment and undergo calcification in the form of significantly greater osteogenesis and chondrogenesis [90]. MVSCs can also differentiate mesenchymally into osteogenic, chondrogenic, and adipogenic cells *in vitro* [25] and *in vivo* (unpublished observation), suggesting a possible role for them in vascular fat accumulation and calcification. As CVCs, in contrast, can differentiate into osteogenic and chondrogenic cells but not adipogenic cells *in vitro*, it is possible that CVCs are derived from MVSCs that have partially differentiated. Because almost all VSCs share some characteristics of MSCs, it is also possible that MSCs are derived from one or multiple subpopulations of VSCs.

Adventitial stem cells

The adventitia is the outermost layer of a blood vessel and is composed of a collagen-rich extracellular matrix embedded with a mixture of cells. The complexity of cellular composition reflects the pivotal role of the adventitia in vascular remodeling. Indeed, of the three blood vessel layers, evidence for vascular stem/progenitor cell enrichment in the adventitia, specifically along its border with the media, is the most abundant and robust. Its significance makes physiological and anatomical sense. Proximity to the vasa vasorum, which connect to the peripheral circulation, enable vessel wall communication with otherwise removed stem cell niches including the aforementioned bone marrow [14, 15], and the pivotal role of vasa vasorum density, structural integrity, and expansion in atheroma development and complications is well documented [91].

In human arteries, in addition to the Sox10^{+} MVSCs we described in the previous section [25], a population of vascular wall-resident multipotent stem cells (VW-MPSCs) were isolated from the adventitia by Klein, *et al.* [92]. They expressed certain MSC surface markers (including Stro1, CD105, CD73, CD44, CD90 and CD29) and positivity for stem cell-associated transcription factors Oct4 and Sox2, and demonstrated lack of contaminating mature EC or EPCs and hematopoietic stem cells (HPCs) by negativity for CD31, CD34, CD45, CD68, CD11b, and CD19. These VW-MPSCs also demonstrated adipocyte, chondrocyte, and osteocyte differentiation in culture conditions. *In vivo* transplantation with human umbilical vein endothelial cells (HUVECs) into immunodeficient mice via Matrigel resulted in new vessel formation covered with VW-MPSC-derived pericyte- and smooth muscle-like cells, an effect enhanced by VEGF, FGF-2, and TGFβ1 stimulation [92]. These authors more recently identified that HOX genes may epigenetically regulate VW-MPSC differentiation into SMCs, potentially contributing to neointimal formation and tumor vascularization [93].

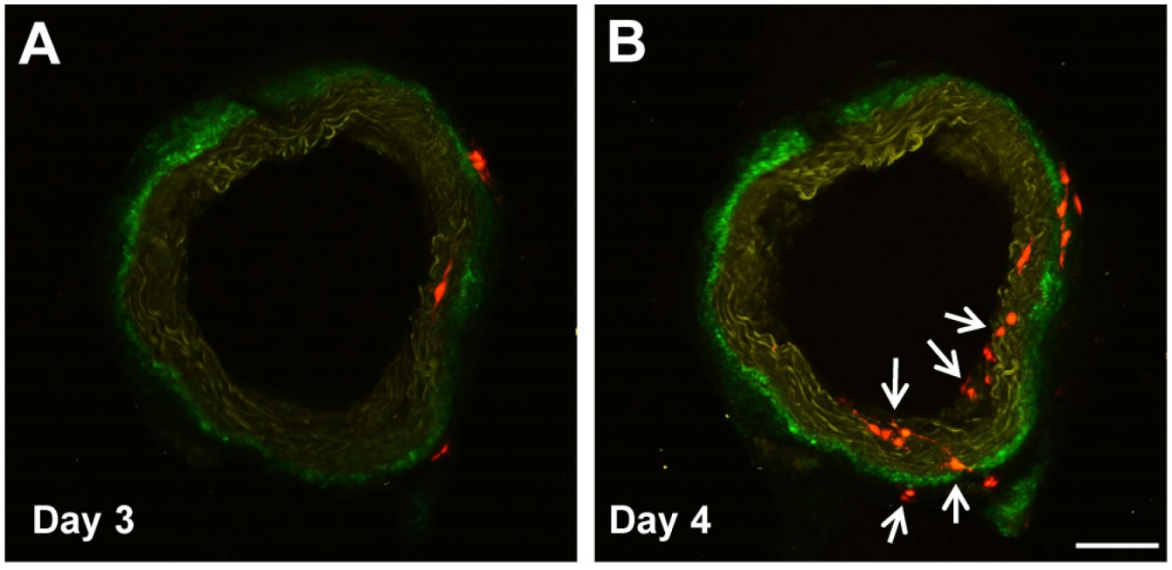

Figure 1. Sox10^{+} MVSCs in aorta ring *ex vivo* culture. Aorta rings of Sox10-Cre/Rosa-RFP mice were cultured *ex vivo*, and imaged by two-photon microscopy. Arrows indicate the emerging Sox10^{+} cells. Scale bar, 100 μm.

Progenitors have also been derived from human veins, dubbed "saphenous vein-derived progenitor cells" (SVPs) for their specific location of origin. Assessing endothelial markers CD34, CD31, and von Willebrand factor (vWF) in these cells showed $CD34^+$, $CD31^-$, vWF^-. These highly proliferative cells were found to be localized around adventitial *vasa vasorum*, and expressed pericyte/mesenchymal antigens as well as stem cell marker Sox2. In an ischemic hindlimb model in immunodeficient mice, intramuscular injection of SVPs improved neovascularization and blood flow recovery, and the cells established N-cadherin-mediated physical contact with the capillary endothelium by day 14 post-transplantation [94]. These therapeutic benefits of vein-derived adventitial stem cells have been replicated in other studies using mouse models of ischemia, with one beginning to look towards manufacturing these cells for human angina therapy [95-97]. Spindle shaped MSCs ($CD13^+$, $CD29^+$, $CD44^+$, $CD54^+$) have also been isolated from human varicose saphenous vein intima. Displaying a similar gene expression profile to bone marrow-derived MSCs, these could differentiate into osteoblasts, chondrocytes, and adipocytes [98].

In rodents, another important progenitor population, Sca-1^+ stem cells, has been described in the adventitia along the medial border. This population also expresses other stem cell markers including c-kit, CD34, and Flk1 and was first identified by Hu *et al.* in the aortic roots of $ApoE^{-/-}$ mice [99]. They had demonstrated capacity to differentiate into SMCs *in vivo*, with LacZ-labeled Sca-1^+ cells found in vein graft atherosclerotic lesions after transplantation in the adventitial space, implying the migration of Sca-1^+ cells from the adventitia to the neointima [99]. Years later, the same group illustrated the multipotency of the cells by demonstrating in a decellularized vessel graft mouse model the cells' *in vitro* differentiation into SMCs (with PDGF) and ECs (with VEGF) [100]. Implications to reduce neointimal thickness by applying VEGF to the adventitial layer, promoting stem cell differentiation into ECs rather than SMCs, were made clear as well [100].

Other studies have since further implicated Sca-1^+ stem cells in atherosclerosis and adventitial remodeling [28, 101, 102]. The later stages of atherosclerosis, for instance, mainly involve resident proliferating macrophages rather than those differentiated from bone marrow monocytes [27]. These local resident proliferating macrophages were found to be derived from a subpopulation of Sca-1^+ stem cells, resident macrophage progenitors, that also expressed CD45 [28]. In aging, $Sca1^+$ adventitial cells enriched for monocyte/macrophage markers and CD45 were shown to be depleted by 3-fold in mature versus young mice, raising the question of whether age-related vascular degeneration may be due to such effects on progenitors in the vascular wall [103].

Recently, Majesky *et al.* used two *in vivo* SMC lineage-tracing approaches and showed that some $Sca1^+$ vascular adventitial progenitors ($CD34^+$) are derived from differentiated SMCs, potentially thereby contributing to maintenance of the resident vascular progenitor cell population [33]. In an earlier study, Shankman *et al.* had suggested that SMCs could de-differentiate into progenitor-like cells capable of differentiating into MSC- and macrophage-like cells [32]. Interestingly, in both cases, KLF4 was identified as a key modulator of cell phenotypic changes. This intriguing relationship between SMCs and VSCs (or VSC-like cells) warrants further investigation.

Overall, although a human ortholog of Sca-1 has yet to be identified, study of pathways and mechanisms surrounding these cells have been of great value, and results suggest that locally manipulating microenvironment is a possible angle for treating atherosclerotic disease [104].

Pericytes

Pericytes play important roles in regulating microvascular stability and dynamics [105]. They were first described over a century ago, and defined as another type of vascular mural cell that surround microvessels, forming an incomplete envelope around ECs and found within the microvascular basement membrane [106]. Pericyte-like cells have also been reported in the inner intima (mostly subendothelium) in human arteries of all sizes [107]. Several markers have been used to identify pericytes, including NG2 [108], CD146 [109, 110], PDGFRβ, and α-SMA [111].

In recent years, accumulating studies have discovered important roles for pericytes in development and diseases. Pericyte-like cells were identified in atherosclerotic lesions and thought to be one of the sources of atherosclerotic cells [83, 112], which may come from the vasa vasorum, a specialized microvessel inside large vessel walls [91]. Cells histologically characterized as "true pericytes" were also found to comprise a second net-like subendothelial tissue layer, which combines with the endothelium to form the intimal barrier in healthy human and bovine microvasculature. In contrast with the endothelium, these pericytes were highly prothrombotic when exposed to serum and display overshooting growth behavior in endothelium-denuded vascular areas, making them potential key players in atherosclerosis, thrombosis, and thrombotic side-effects of venous coronary bypass grafting [92].

In the porcine aortic media, novel vascular progenitor cells with pericyte- and MSC-like properties were also found capable of differentiating into osteocytes, chondrocytes, and adipocytes [87]. Pericytes around microvessels in skeletal muscle are another type of myogenic progenitor cell distinct from satellite cells [113, 114].

Pericytes in multiple organs have been reported to have properties of MSCs [111]. Moreover, pericytes can differentiate into myofibroblasts and are another important cellular source of organ fibrosis [115-117]. It is likely that pericytes include subpopulations of stem cells or progenitors. In our recent work, we found $Sox10^+$ stem cells in the stroma of subcutaneous connective tissues which had the same properties as MVSCs in large vessels [24, 25]. These $Sox10^+$ stem cells are precursors of pericytes and fibroblasts, as described in the previous section, and contribute to both fibrosis and microvessel formation during tissue repair and regeneration [24]. $Gli1^+$ stem cells had similarly wide distribution as the $Sox10^+$ stem cells and were found in the perivascular space and also adventitial layer of large arteries. They could differentiate into myofibroblasts contributing to organ fibrosis, and neointimal SMCs contributing to atherosclerotic lesions and arterial calcification [115, 118].

Therapeutically, two separate studies examined the benefit of pericyte transplantation in mouse models of myocardial infarction. They found that pericytes from both saphenous vein [119] and skeletal muscle [120] attenuated left ventricular dilation, improved cardiac contractility and ejection fraction, reduced myocardial fibrosis and scarring, and improved neovascularization and angiogenesis. Saphenous vein-derived pericytes also reduced cardiomyocyte apoptosis, attenuated vascular permeability, and improved myocardial blood flow [119], while the skeletal muscle-derived pericytes significantly diminished host inflammatory cell infiltration at the infarct site as well [120]. Both studies attributed benefits to cellular interactions and paracrine effects [119, 120].

Dellavalle, *et al.* demonstrated the skeletal muscle-regenerating properties of both normal human pericytes and dystrophin-reprogrammed human Duchenne patient pericytes when transplanted into mouse models of muscular dystrophy [113]. In small-diameter tissue-engineered vascular grafts (TEVGs), exogenously seeded pericytes improved maintenance of patency after TEVG implantation into the aorta of rats (100% at 8 weeks, versus 38% unseeded controls) [121]. An endogenous approach has met with similar success, where promoting the differentiation of $Sca\text{-}1^+$ stem/progenitor cells into the endothelial lineage has reduced neointimal thickness by up to 80% [100]. Altogether, these findings highlight stem cells as important players and potentially significant therapeutic targets in vascular remodeling, and underscore the multifactorial complexity of vascular disease pathogenesis.

Microenvironment of vascular cells

The microenvironment plays important roles in regulating vascular cell function and the stem cell renewal and fate decision, and includes both biochemical factors (e.g., growth factors, cytokines) and biophysical factors (e.g., extracellular matrix, stiffness, flow shear stress and mechanical stretch).

Inflammatory cytokines, in addition to adhesion molecules, govern recruitment of relevant immune cells to the arterial wall in atherosclerosis. Beyond these traditional roles in regulating cell function and homeostasis, though, and notably for our discussion here, in recent years cytokines have also been found to regulate stem cell recruitment and activation during vascular remodeling [122, 123]. Cytokines like stromal cell-derived factor 1α (SDF-1α), for example, has been shown to recruit bone marrow EPCs to form microvessels in hindlimb ischemic angiogenesis [124, 125] and to promote adventitial $Sca1^+$ stem cells to migrate through vein graft walls and differentiate into neointimal SMCs [126]. In advanced atherosclerotic plaques, it is also believed that SDF-1α recruits SMC progenitor cells from bone marrow to the fibrotic cap [127]. Another cytokine, tumor necrosis factor-α (TNF-α), induces adventitial $Sca1^+$ stem cells to differentiate into ECs, while suppressing SMC gene activation [128]. Growth factors like VEGF and PDGF-BB/TGF-β1 can stimulate adventitial and medial stem cells to differentiate into ECs and SMCs, respectively [85, 100].

Among the biophysical factors found important for vascular cells, local disturbed flow is a major factor that induces EC dysfunction in the branches and curvatures of the arterial tree [129]. Disturbed flow shear stress can induce a series of intracellular signaling pathways in ECs and activate proliferative and inflammatory gene expression, initiating neointimal formation and atherosclerosis even in newborns [129, 130].

The extracellular matrix (ECM) is also important in regulating vascular dynamics. Subendothelial matrix proteoglycans are thought to contribute to lipid retention in the early stages of atherosclerosis [29]. ECM stiffness and embedded growth factors are critical in regulating cell functions. Our previous work has showed that stiff surfaces, together with TGFβ, promoted MSC differentiation into SMCs *in*

vitro [131]. Collagen IV, too, has been reported to be critical in promoting embryonic stem cell differentiation into Sca-1$^+$ stem cells, and to act together with aforementioned cytokines and growth factors to promote differentiation [132, 133]. Mechanical stretch and microtopography can regulate SMC differentiation and function as well [134, 135].

To date, the niche of VSCs has not been well defined. Although we know connection to the peripheral circulation via the *vasa vasorum* enables vessel niche communication with other stem cell niches like the bone marrow, how VSCs are activated by such communication, inflammatory signals, and local microenvironmental changes remains to be investigated.

Clinical implications

As our understanding of the importance and mechanism of stem and progenitor cell involvement in human vascular remodeling has evolved, two therapeutic angles have arisen: 1) influencing endogenous VSC behavior to prevent initiation and progression of disease, and 2) exogenous stem cell delivery to promote disease reversal and healing of tissue injury. The application of more immature stem cells with greater differentiation potential such as embryonic and induced pluripotent stem cells to cardiovascular disease (including myocardial infarction, vascular regeneration in coronary and peripheral artery disease) has been reviewed elsewhere [136-138]. Adult stem cells such as those we have discussed pose multiple advantages in their accessibility (e.g., the stromal vascular fraction of adipose aspirates contain human blood vessel fragments; coronary bypass surgery makes pieces of aorta or segments of internal thoracic artery, radial artery, and saphenous vein readily available), decreased risk of uncontrolled differentiation (e.g., teratomas), and immune-privileged nature (in the case of MSCs and pericytes) that enables allogeneic use as well [139].

That said, clinical trials and therapies utilizing such VSCs are still sadly lacking. No human clinical trials to date have examined application of pericytes or resident VSCs for vascular disease. MSCs and EPCs, perhaps because of the broadness of their definition, have accumulated a more substantial body of clinically relevant evidence. The majority of clinical trials for atherosclerosis and diseases for which it is the primary cause – such as angina, myocardial ischemia, and ischemic stroke, all diseases primarily of the macrovasculature – utilize MSCs and EPCs instead. These trials focus, too, more on stem cell/progenitors for disease treatment rather than disease prevention. Limited evidence for underlying mechanisms suggests stem cell angiogenic roles play a large part in measurable therapeutic benefit; evidence for a therapeutic role in neointimal regression, in contrast, is lacking [140, 141]. It should be noted that MSCs and EPCs have also been utilized therapeutically to promote angiogenesis in diseases of the *micro*vasculature such as diabetic ischemia-induced chronic wounds [53, 142] and peripheral occlusive disease [140, 141, 143], but we focus on *macro*vascular plaque-related diseases here instead.

Bone marrow-derived EPCs

In 2013, a phase III trial for refractory angina locally transplanted (G-CSF-stimulated) autologous blood cells positive for the EPC marker CD34 via percutaneous intramyocardial injection. The trial showed preliminary results consistent with those of earlier phase studies [144], although with higher placebo effects than previously detected, and animal studies lead us to believe benefit is derived from cell contribution to myocardial neoangiogenesis, and possible differentiation into cardiomyocytes and ECs [145-147]. If completed, it would have provided the requisite information for regulatory approval of the first cellular therapeutic for a cardiovascular indication [148]. Results may merit an expanded examination of therapeutic EPC transplantation, perhaps in combination with other vasculogenic mediators and scaffolds to improve EPC survival and function.

Other clinical trials have also attempted direct exogenous transplantation of adult bone-marrow-derived stem cells, but for myocardial ischemia (MI) and ischemic stroke patients. Several have found such intracoronary transplantation improves regional systolic function recovery and infarct size reduction in MI patients [149, 150], and a number of recent meta-analyses have confirmed improvements in not only left ventricular contractility after therapy [151-153] but also decreased mortality, acute MI recurrence, and readmission for heart failure [150, 152]. Still, effects of transplantation on infarct volume and remodeling are contradictory and inconclusive [150, 152-156]. BM cells, rather than incorporating, may prompt ischemic tissues to secrete paracrine signals (e.g., angiogenic factors); these signals in conjunction with transdifferentiation potential may underlie functional recovery [149, 156-158].

In stroke, promising results in experimental models [159] prompted clinical trials of intra-arterial or intravenous transplantation of autologous bone marrow mononuclear cells (including CD34$^+$ progenitors). A phase I/II clinical trial in middle

cerebral artery stroke patients transplanted 5-9 days after stroke found that changes in serum levels of GM-CSF, PDGF-BB, and MMP-2 associated with better functional outcomes were induced; however, varied impact on functional outcomes themselves was not measured [160]. Another phase II randomized control trial (RCT) found that cell therapy was safe, but had no beneficial effect on stroke outcome [161]. The first trial to explore dose-dependent efficacy of intra-arterial transplantation of bone marrow mononuclear cells in moderate-to-severe acute ischemic stroke patients is currently ongoing (IBIS trial, prospective phase II RCT) [162]. Despite promising animal studies, which suggest BM cell-based treatments can benefit endogenous neurorestoration by promoting contralesional pyramidal axon sprouting and preservation, increasing neurotrophic factor secretion, and possible synergistic effects between microvascular angiogenesis and neurogenesis, demonstrable long-term clinical therapeutic benefit of cell therapies for stroke is still being determined [141].

Secondary stimulation of endogenous progenitors has also been attempted. Granulocyte colony-stimulating factor (G-CSF) is one agent that can stimulate the bone marrow to release EPCs, in addition to release of granulocytes and hematopoietic stem cells [18]. Multiple clinical trials, encouraged by prior positive results in various animals [163], sought to assess its utility in upregulating endogenous EPC release in patients with ischemic heart disease. Results, however, have been mixed: although one study found an improvement of severe ischemia in severe MI patients [164] and a meta-analysis of seven RCTs including 364 acute MI patients found improvement of left ventricular ejection fraction (LVEF) [165], others (including an RCT and a meta-analysis of ten clinical trials including 445 patients) concluded no impact on infarct size, LV function, or coronary restenosis [166-168]. Interestingly, physical exercise, strongly established by many large-scale epidemiological studies as being robustly associated with decreased cardiovascular mortality and potent primary and secondary CVD prevention [169-173], has been found to mobilize EPCs from the bone marrow and is thought to exert its benefits mechanistically via the maintenance of an intact endothelial layer [174].

Using G-CSF in stroke patients has been less studied. A phase IIb RCT concluded in 2012 that G-CSF successfully and safely increased $CD34^+$ cells by 9.5-fold relative to placebo, with a trend of reducing ischemic lesion volume [175]. Further study, though, is necessary.

Bone marrow-derived MSCs

The majority of completed clinical trials (as reported on clinicaltrials.gov) involving MSC transplantation for vascular disease focuses on treatment of myocardial ischemia, finding that treatment is tolerable and safe with improvements seen in metrics such as LVEF [176-178] and global EF [179], LV end-systolic [176, 178, 179] and diastolic volumes [178], and functional walk and cardiac tests [176] and global symptom scores [177]. A phase I/II clinical trial for patients with severe stable coronary artery disease and refractory angina transplanted autologous bone marrow-derived MSCs into their viable myocardium, and found similarly promising results. The trial showed sustained safety three years post-transplantation, significant clinical improvements in symptomatic and functional metrics, as well as reduced hospital admissions for CV disease [180].

Delivery route of MSCs, furthermore, was found by meta-analysis of six clinical trials involving 334 MI patients to shape efficacy of treatment. Greatest improvement in LVEF was seen if transendocardial injection and intravenous infusion, rather than intracoronary infusion, were used to deliver MSCs [181].

In 2015 an observational clinical study for coronary atherosclerosis examined outcomes of plasmonic resonance therapy using silica-gold nanoparticles that had been incubated with allogeneic mesenchymal $CD73^+$ $CD105^+$ stem-progenitor cells. Results showed highly safe, significant plaque regression relative to stenting controls (reduction of total atheroma volume up to $60mm^3$, or 37.8% of plaque burden, relative to current maximal success of conventional drugs of $6\text{-}14mm^3$) and late lumen enlargement without arterial remodeling [182].

Overall, although animal and preliminary clinical studies have revealed much promise, there remains much to be done in understanding the mechanism of VSC therapeutic benefits in order to appropriately target them for effective therapy.

Future directions and perspectives

Strong evidence has accumulated to demonstrate the involvement of various stem and progenitor cells in vascular regeneration and disease, including atherosclerotic neointimal formation. These stem cells display a nonuniform distribution both across the vessel wall as well as across different vascular territories, a distribution perhaps contributing to explanations of why different vascular segments may have variable susceptibility to vascular disease despite similar hemodynamics and environment [183]. Different populations of vascular cells,

including SMCs, ECs, inflammatory cells (including macrophage and dendritic cell progenitors), and stem cells, may interact with and be subject to regulation by each other and by the local microenvironment during neointimal thickening. Recent studies show exosomes, nanometer lipid bilayer signaling particles secreted by cells with important roles in many physiological and pathological processes [184-187], have a hand in this regulation by mediating vascular calcification as found in atherosclerosis [188], atheroprotective communication between ECs and SMCs [189], and anti-inflammatory effects of MSCs [187]. Exosomes could thus be therapeutic targets of interest as well [190].

Identifying proper cellular targets (e.g., using screening methods such as RNA-sequencing and epigenetic profiling to characterize VSCs, along with other techniques such as laser microdissection and immunofluorescence to identify key VSC markers) and understanding the underlying regulatory mechanisms will facilitate the development of successful therapies for vascular disease. Given their differentiation potential into SMCs and ECs, these stem cells could also be good cellular sources for fabricating vascular grafts or otherwise promoting vascular regeneration.

Far as the field has come, several critical questions remain to be addressed. First, given the diversity of stem cells discovered by different research groups, confirming whether these cells are distinct populations and determining their relationship with proliferative/synthetic SMCs will be necessary. It will be helpful to obtain consensus on specific panels of markers to define different stem cell populations. Examining to what degree the difference in their marker expression profiles may be a result of different culture conditions *in vitro*, too, will be of importance.

Second, the niche of VSCs needs to be further characterized to define the macro and microenvironmental factors that maintain VSCs in a quiescent versus activated state, and how such factors promote healthy survival.

Third, stem cell fate needs to be determined in long-term *in vivo* experiments. However, stem cells may become activated and differentiated quickly at the early phase of neointimal thickening *in vivo*, which makes capture of the phenotype by immunohistology difficult. Genetic lineage tracing techniques would address this problem, if obstacles of selection of good markers and of availability of transgenic animal models can be surmounted. Such techniques could also address the relative contributions of different cell types, and multi-color reporter mice could be used to investigate heterogeneity within the same population.

Fourth, the behavior of VSCs under various pathological conditions should be elucidated. Stem cell activation and differentiation are regulated by various microenvironmental factors. Changes in biochemical and biophysical factors in a disease state and the effects of these factors, individually or in combination, may have profound effects on stem cell functions. Conversely, taking creative inspiration from current successful therapies for atherosclerosis and brainstorming approaches for cellular therapies to target their same mechanisms could yield therapies with fewer side-effects and more targeted results. For instance, any conversation on atherosclerosis would be incomplete without mention of statins, the current mainstay of treatment [191, 192]. Research has shown that, independent of cholesterol reduction, statins may exert their beneficial effects via EPC mobilization. This may be a promising direction for future therapies [52]. Similarly, piggybacking on the putative plaque-stabilizing mechanism of statins by use of the chemokine SDF-1 to recruit bone marrow-derived SM progenitor cells to the fibrous cap has yielded increases in cap thickness without altering artery diameter in mice [127]. This finding may prove useful for unstable atherosclerosis if further studies in large animals and humans continue to yield promising results.

Fifth, especially with sourcing of vascular wall MSCs becoming increasingly feasible [17], there is great promise in cell therapies if details on differences in identity and manufacturing based on specific vascular and cell source can be fleshed out. Despite their mechanistic significance, EPCs and other progenitors without immune-privilege, in contrast with MSCs and pericytes which do, may pose a challenge in clinical application if the goal is exogenous transplantation [139]. Endogenous recruitment and processes may be more feasible for these other progenitors. Although stem cell transplantation has been proven to be safe and benefit tissue regeneration, the mechanisms of benefit, too, are unclear at present. Overall, clinical trials certainly remain of value – as phenomena in humans are ultimately distinct from those in animals – but it is clear that such applications are yet in the early stages. The mixed results clearly indicate that an improved understanding of underlying mechanisms is necessary not only for effective design of therapeutic translation and study, but also for interpretation of results. Ongoing risk and safety assessment will continue to be necessary in parallel.

Finally, besides delivery of exogenous stem cells for therapies, the potential of endogenous recruitment or of using stem cells as novel targets of therapies needs to be further investigated *in vitro* and *in vivo*. *In*

vitro isolated VSCs can be used for drug screening. A well-defined culture model, such as co-culture with SMCs, mechanical loading, and 3D culture that mimics the *in vivo* microenvironment, would be valuable. Blood vessel tissue *ex vivo* culture is better than cell culture as it mimics the niche of cell-cell interactions and native extracellular matrix, which may be useful when combined with tissue clarity techniques and transgenic animal models. All these new tools and technologies will continue to facilitate further discoveries in vascular stem cell biology, enabling development of diagnostic and therapeutic strategies with unprecedented efficacy and capability to combat vascular disease and promote regeneration.

Abbreviations

CVD: cardiovascular disease; EC: endothelial cell; EPC: endothelial progenitor cell; SMC: smooth muscle cell; VSC: vascular stem cell; MSC: mesenchymal stem cell; MVSC: multipotent vascular stem cell; CVC: calcifying vascular cells; α-SMA: smooth muscle α-actin; SM-MHC: smooth muscle myosin heavy chain.

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Competing Interests

The authors have declared that no competing interest exists.

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