

# Atherosclerosis Compendium

## *Circulation Research* Compendium on Atherosclerosis

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*Peter Libby, Karin E. Bornfeldt, and Alan R. Tall, Editors*

## Vascular Smooth Muscle Cells in Atherosclerosis

Martin R. Bennett, Sanjay Sinha, Gary K. Owens

**Abstract:** The historical view of vascular smooth muscle cells (VSMCs) in atherosclerosis is that aberrant proliferation of VSMCs promotes plaque formation, but that VSMCs in advanced plaques are entirely beneficial, for example preventing rupture of the fibrous cap. However, this view has been based on ideas that there is a homogenous population of VSMCs within the plaque, that can be identified separate from other plaque cells (particularly macrophages) using standard VSMC and macrophage immunohistochemical markers. More recent genetic lineage tracing studies have shown that VSMC phenotypic switching results in less-differentiated forms that lack VSMC markers including macrophage-like cells, and this switching directly promotes atherosclerosis. In addition, VSMC proliferation may be beneficial throughout atherogenesis, and not just in advanced lesions, whereas VSMC apoptosis, cell senescence, and VSMC-derived macrophage-like cells may promote inflammation. We review the effect of embryological origin on VSMC behavior in atherosclerosis, the role, regulation and consequences of phenotypic switching, the evidence for different origins of VSMCs, and the role of individual processes that VSMCs undergo in atherosclerosis in regard to plaque formation and the structure of advanced lesions. We think there is now compelling evidence that a full understanding of VSMC behavior in atherosclerosis is critical to identify therapeutic targets to both prevent and treat atherosclerosis. (*Circ Res.* 2016;118:692-702. DOI: 10.1161/CIRCRESAHA.115.306361.)

**Key Words:** atherosclerosis ■ extracellular matrix ■ interleukin ■ platelet-derived growth factor ■ smooth muscle

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From the Division of Cardiovascular Medicine, Addenbrooke's Centre for Clinical Investigation, Addenbrooke's Hospital, Cambridge CB2 0QQ, United Kingdom (M.R.B., S.S.); and University of Virginia School of Medicine, Charlottesville (G.K.O.).

Correspondence to Martin R. Bennett, MD, PhD, Division of Cardiovascular Medicine, Addenbrooke's Centre for Clinical Investigation, Addenbrooke's Hospital, PO Box 110, Cambridge CB2 0QQ, United Kingdom. E-mail mrb@mole.bio.cam.ac.uk

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### Nonstandard Abbreviations and Acronyms

|              |                                       |
|--------------|---------------------------------------|
| <b>ACTA2</b> | smooth muscle $\alpha$ -actin         |
| <b>ApoE</b>  | apolipoprotein E                      |
| <b>ECM</b>   | extracellular matrix                  |
| <b>KLF4</b>  | Kruppel-like factor 4                 |
| <b>IL-1</b>  | interleukin-1                         |
| <b>MYH11</b> | smooth muscle cell myosin heavy chain |
| <b>Sca1</b>  | stem cell antigen 1                   |
| <b>VSMC</b>  | vascular smooth muscle cell           |

Atherosclerosis is a chronic progressive inflammatory disease and the leading cause of death worldwide<sup>1-3</sup> [<http://www.who.int/mediacentre/factsheets/fs310/en/>]. The major clinical consequences of atherosclerosis such as myocardial infarction or stroke are not a function of gradual narrowing of the lumen, but rather due to thrombotic events associated with acute rupture or erosion of an unstable plaque. Postmortem and clinical imaging studies have identified several features of plaque instability leading to rupture, including: (1) a thin or fragmented fibrous cap comprising smooth muscle  $\alpha$ -actin (ACTA2)-positive cells presumed to be derived from vascular smooth muscle cells (VSMCs), (2) large numbers of cells positive for markers such as CD68 or LGALS3 presumed to be macrophages, and (3) the presence of a large necrotic core containing cells filled with lipid (foam cells), presumed to be macrophages.

These observations underlie the general dogma that atherosclerotic plaques with a preponderance of macrophages and macrophage-derived foam cells relative to VSMCs, particularly within the fibrous cap and shoulder regions, are less stable and more prone to rupture.<sup>1,2</sup> That is, VSMCs in advanced lesions are generally regarded as having atheroprotective plaque-stabilizing properties, whereas macrophages are viewed as being athero-promoting and detrimental for plaque stabilization. However, this model may be overly simplistic and probably incorrect because of 3 major limitations. First, most studies have not unambiguously identified which cells within advanced lesions are derived from VSMCs or macrophages. This is because of reliance on markers that are not specific, and/or are downregulated, and/or are activated by another cell type in advanced plaques. Second, the role of individual cell types requires identification of the factors and mechanisms that regulate phenotypic transition of VSMCs and monocyte/macrophages, and defining how the functional properties of these phenotypically modulated cells affect disease pathogenesis. Third, VSMCs undergo multiple processes, often simultaneously, at different stages and in different regions of the plaque. The role of VSMCs is, therefore, the additive effect of these processes, and may vary throughout atherogenesis. This review will analyze the effect of embryological origin on VSMC behavior, the evidence for different origins of VSMCs in atherosclerosis, and the roles of specific processes implicated in atherosclerosis, including phenotypic switching, cell proliferation, migration, cell death, and cell senescence.

## Origin and Plasticity of VSMCs Within Atherosclerotic Lesions

### Embryological Origin of VSMCs and Susceptibility to Atherosclerosis

Lineage tracing studies have established that VSMCs originate from multipotent precursors from several developmental origins.<sup>4</sup> For example, VSMCs in the ascending aorta, arch, and pulmonary trunk as well as head and neck vessels are derived from neural crest, whereas Islet-1<sup>+</sup> progenitors in the secondary heart field contribute to the proximal aortic root. Coronary VSMCs are generated from the epicardium, whereas the descending aorta is predominantly derived from somitic precursors. Although these lineage-specific VSMC populations share considerable phenotypic similarities, there are key differences between them both in their requirement for developmental regulators such as myocardin-related transcription factor B,<sup>5,6</sup> and the responses of adult cells to key mediators and factors that may be relevant in disease development, such as transforming growth factor- $\beta$ .<sup>7,8</sup>

There is now evidence that these differences between distinct VSMC lineages influence the development of vascular diseases, including atherosclerosis. For example, pioneering clinical studies by DeBaakey and Glaeser<sup>9</sup> suggested that the progression of atherosclerotic lesions in response to systemic risk factors differed in 4 distinct vascular regions, including coronary arteries, the branches of the aorta, the abdominal visceral arteries, and the terminal abdominal aorta. Further studies of early atherogenesis in unselected young populations who died of noncardiac causes confirmed that disease development in distinct vascular regions responds differently to common risk factors, such as smoking or sex.<sup>10</sup> Thus, it is possible that there may be basal differences in VSMC susceptibility to systemic risk factors based on embryonic origins, although it seems likely that local vascular hemodynamic and structural factors still have a major role in defining precise patterns of plaque development.

Although one must be cautious in extrapolating results obtained in cultured cells, including VSMCs, to in vivo settings, the confounding effects of flow and local vessel characteristics may be overcome by study of cultured cells from different regions. Indeed, the latter has been aided recently by the generation of lineage-specific VSMCs in vitro from pluripotent stem cells.<sup>11</sup> For example, the atherosclerosis-resistant thoracic aorta of fat fed apolipoprotein E (ApoE)<sup>-/-</sup> mice has higher expression of a range of Homeobox (Hox) genes than the more atherosclerosis-prone aortic arch, with reciprocal inhibition between HoxA9 and nuclear factor  $\kappa$ B.<sup>12</sup> The resultant high nuclear factor  $\kappa$ B activity in the arch and low activity in the thoracic aorta defines a possible regulatory mechanism for this critical inflammatory regulator in atherosclerosis. Differences in Hox gene expression were also seen in an in vitro human embryonic stem cell-derived model, with high *HoxA9* expression in paraxial mesoderm-SMCs that corresponded to thoracic aorta and low expression in neuroectoderm-SMCs corresponding to the arch. Thus, the atherosclerosis-susceptibility or resistance seems to be related, in part, to developmental programming. The challenges now are to further characterize the identity of different VSMC

regions by both transcriptional and epigenetic mechanisms, to determine which developmental signatures are preserved in the adult vasculature, and how these mechanisms which define positional identity may regulate the development of atherosclerosis.

### Phenotypic Switching of VSMCs in Atherosclerosis

VSMCs in the normal arterial media express a range of SMC markers, conventionally including smooth muscle cell myosin heavy chain (MYH11), 22-kDa SMC lineage-restricted protein (SM22 $\alpha$ /tagln), ACTA2, smoothelin, and others. VSMCs in culture and in atherosclerosis reduce expression of these markers, and, at least in vitro, acquire increased capacity for cell proliferation, migration, and secretion of various extracellular matrix (ECM) proteins and cytokines.<sup>13</sup> VSMCs undergoing phenotypic switching can also acquire macrophage markers and properties. This phenotypic switching has long been considered of fundamental importance to atherosclerosis, generating a VSMC phenotype that is proatherogenic; however, direct interventional studies to prevent phenotypic switching have been lacking.

Regulation of VSMC phenotypic switching has been reviewed extensively elsewhere,<sup>14</sup> but recent studies have defined the role that phenotypic switching actually plays in atherosclerosis and plaque stability, and established that inhibiting VSMC phenotypic switching may be beneficial in advanced atherosclerosis. For example, the myocardin–serum response factor regulatory module is a central component of phenotypic regulation that facilitates combinatorial interactions of activating and repressing signals and cofactors that act on most VSMC contractile genes. Myocardin<sup>+/−</sup> mice on an ApoE<sup>−/−</sup> background exhibit increased atherosclerosis with increased accumulation of macrophage or macrophage-like cells compared with myocardin<sup>+/+</sup> littermates.<sup>15</sup> Although this was not a VSMC-specific loss of function study, the only cells in the vasculature that express myocardin are VSMCs. Loss of myocardin upregulated a variety of inflammatory pathways to increase macrophage recruitment, or switched VSMCs to a macrophage-like phenotype (see below). Conversely, gain of myocardin function inhibited inflammatory pathways and limited neointimal macrophage accumulation in vivo.<sup>15</sup>

Similarly, the stem cell and induced pluripotent stem cell factor Kruppel-like factor 4 (KLF4) has been shown previously to be required for phenotypic transition of cultured VSMCs in response to platelet-derived growth factor BB,<sup>16,17</sup> oxidized phospholipids,<sup>18,19</sup> or interleukin (IL)-1 $\beta$ <sup>20</sup> and silences SMC marker genes to inhibit myocardin-dependent gene activation.<sup>16,21</sup> Loss of KLF4 in VSMCs in vivo is also associated with a transient delay in phenotypic switching after ligation injury.<sup>22</sup> More recent studies have shown that VSMC-specific conditional knockout of KLF4 does not prevent VSMC phenotypic switching, but markedly reduces plaque size with increased fibrous cap area, an index of increased plaque stability.<sup>23</sup> Interestingly, KLF4 knockout did not alter overall VSMC numbers, but reduced the number of VSMC-derived macrophage-like and mesenchymal stem cell-like cells, indicating that KLF4 regulates the transition toward a macrophage phenotype. Indeed, results of KLF4 ChIP-seq analyses on brachiocephalic lesions of SMC-selective KLF4 knockout

versus wild-type mice identified a large number of putative SMC KLF4 target genes including many associated with pro-inflammatory processes.<sup>23</sup>

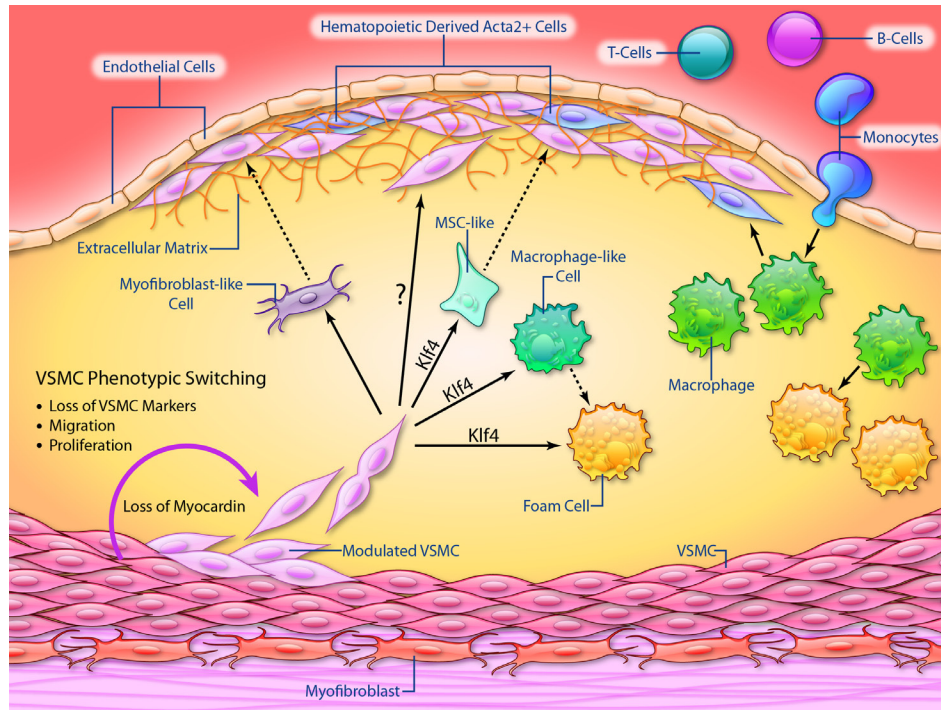
The switching of VSMCs to macrophage-like cells may be driven by lipid accumulation in the plaque because cholesterol loading of cultured VSMCs activated multiple proinflammatory genes, suppressed expression of VSMC marker genes, activated macrophage markers, and induced phagocytic activity, all of which were KLF4 dependent<sup>23</sup> (Figure 1). However, gene expression of these VSMC-derived macrophage-like cells is distinctly different from classical monocytes, macrophages, and dendritic cells,<sup>24</sup> and these cells have reduced phagocytic capacity compared with activated peritoneal macrophages. Reduced phagocytosis, for example of apoptotic cells, is evident in advanced atherosclerosis<sup>25</sup> and directly promotes formation of the necrotic core of the lesion. These studies indicate that SMC-derived macrophage-like cells may promote atherosclerosis by having reduced ability to clear lipids, dying cells, and necrotic debris, and by exacerbating inflammation. Although it has long been postulated that VSMCs within lesions play a beneficial role,<sup>1,26,27</sup> for example by protecting the fibrous cap from rupture and promoting plaque repair, recent studies show that this is an oversimplification, and VSMC function can vary dramatically depending on the nature of the phenotypic transitions.

Although we have focused on the signals within VSMCs that regulate phenotypic switching, VSMCs synthesize and are embedded in an ECM that separates them from each other, except at defined cell–cell contacts. The conventional view is that ECM suppresses phenotypic switching, keeping VSMCs in a contractile state that is less responsive to mitogens. Conversely, breakdown of ECM, collagen, or elastin, for example by matrix metalloproteinases released from macrophages and VSMCs, would promote phenotypic switching and facilitate both cell proliferation and migration.<sup>28–30</sup> However, the real effects of ECM on VSMCs may be more complex. For example, recent studies have shown that fibronectin deposition at sites of early plaque formation promotes atherosclerosis, but also promotes the formation of the protective fibrous cap.<sup>31</sup> Similarly, although it is widely believed that phenotypically modulated VSMCs within the fibrous cap produce ECM molecules critical for plaque stabilization, there are no studies that have examined how knockout of a given ECM gene exclusively in VSMCs affects lesion pathogenesis. Indeed, this is a critical area in need of further studies.

### Derivation of VSMCs From Within the Vessel Wall or Bone Marrow

Although it has long been assumed that differentiated (mature contractile state) VSMCs undergo phenotypic switching during atherogenesis,<sup>1</sup> direct evidence that VSMCs exhibit phenotypic switching in vivo during atherogenesis has only been proven recently based on rigorous SMC-specific conditional lineage tracing studies.<sup>23,32,33</sup> These studies showed that >80% of VSMC-derived cells within advanced ApoE<sup>−/−</sup> mouse plaques lacked detectable expression of commonly used SMC markers such as ACTA2, and that >30% of VSMC-derived cells expressed multiple markers of macrophages, including LGALS3/Mac2, CD11b, F4/80, and CD68. Similar studies





**Figure 1. Schematic summarization of the current knowledge of the identity and origins of vascular smooth muscle cells (VSMCs), macrophages, and putative derivatives of these cells within advanced atherosclerotic lesions.** The solid lines illustrate known pathways that give rise to lesion cells, whereas dotted lines with a “?” indicate putative pathways not yet directly validated in animal models or humans. Klf4 indicates Kruppel-like factor 4 (Illustration Credit: Ben Smith).

using a single-cell epigenetic assay<sup>32</sup> and Y-chromosome lineage tracing in humans who had a cross-sex heart transplant showed that nearly 20% of CD68+ cells in advanced coronary plaques are also of SMC not myeloid origin, demonstrating that transition of VSMCs to macrophage-like cells also occurs at a relatively high frequency in human lesions. These studies indicate that the conventional view of the macrophage-rich necrotic core may also be incorrect, and that VSMCs and dead VSMCs comprise a substantial component of the core. Interestingly, VSMC-derived cells that lacked detectable expression of ACTA2 expressed markers of mesenchymal stem cells (eg, stem cell antigen 1 [Sca1]<sup>+</sup> CD105<sup>+</sup>), as well as myofibroblasts (ACTA2<sup>+/−</sup> platelet-derived growth factor  $\beta$  receptor<sup>+</sup>), raising the concept of a progenitor population of SMCs within the vessel wall that selectively proliferate and accumulate in atherosclerosis<sup>23</sup> (see below).

These studies clearly indicate that a major fraction of VSMC-derived cells in advanced lesions have previously gone either unidentified or been incorrectly identified as being another cell type. However the converse is also true, that a subset of cells within lesions that express at least some SMC markers are not SMC derived. For example, several studies from 2002 onwards have shown that some SMC marker-positive cells within lesions were of myeloid origin both in mice<sup>34</sup> and in humans.<sup>35</sup> Later studies showed that hematopoietic (myeloid)-derived cells can activate early but not late stage markers of SMCs within ApoE-null mouse lesions including ACTA2 and SM22 $\alpha$  but not MYH11,<sup>36</sup> although all these markers can be expressed by bone marrow-derived cells in culture.<sup>37</sup> Similarly, studies of cross-sex bone marrow transplant human subjects showed that at least 10% to 15% of ACTA2<sup>+</sup> cells

within advanced human coronary artery lesions are of myeloid and not VSMC origin.<sup>36</sup> In contrast, cross-gender bone marrow transplant lineage tracing and arterial transplantation studies concluded that 100% of ACTA2<sup>+</sup>-positive cells within lesions of ApoE<sup>−/−</sup> mice are of local arterial wall origin and not derived from the bone marrow,<sup>38,39</sup> and lineage tracing using the SM22 $\alpha$  promoter indicated that <1% of cells expressing SM22 $\alpha$  were of myeloid origin.<sup>37</sup>

The discrepancy between these studies even in the same species may be because of the assumption that immunohistochemical SMC markers are specific for VSMCs and macrophage markers are specific for bone marrow-derived cells. However, recent studies showed that 40% of foam cells within advanced human coronary artery lesions express both the SMC marker ACTA2 and the macrophage marker CD68, although it is unclear if these represent VSMC-derived cells that have activated macrophage markers, are macrophages that have activated SMC markers, or neither.<sup>40</sup> Again, lineage tracing studies using epigenetic markers showed that 38% of cells within advanced human coronary artery lesions that were dual positive for CD68 and ACTA2 exhibited the SMC-specific MYH11 H3K4diMe epigenetic signature indicating they were of VSMC and not myeloid origin.<sup>23</sup> Similarly, Y-chromosome lineage tracing studies in cross-sex heart transplant coronary artery lesions showed that myeloid cells do not acquire the SMC-specific MYH11 H3K4diMe epigenetic signature and that nonmyeloid cells are CD68+. These studies indicate that myeloid cells can acquire some, but not all, SMC markers in advanced plaques, suggesting that they do not behave like vessel wall-derived VSMCs. Indeed, ablation studies indicated that in contrast to vessel wall-derived VSMCs,

myeloid-derived SMC marker—positive cells are likely to promote atherosclerosis.<sup>37</sup>

### VSMCs Derived From Stem and Progenitor Populations Within the Vessel Wall

Several studies have suggested several alternative sources of VSMC-like cells within atherosclerotic lesions. For example, Tang et al<sup>41</sup> found that MYH11-expressing medial VSMCs are terminally differentiated and incapable of phenotypic transition during vascular injury and disease, and the existence of a MYH11<sup>−</sup> medial stem cell population that gives rise to VSMC-like cells within lesions. Both of these findings have been refuted based on technical limitations that have been summarized recently.<sup>42</sup> These include (1) the failure to perform high resolution Z-stack confocal analyses to ensure the lineage tracing gene and other marker genes are expressed within the same cell and do not represent signals from overlapping cells, an essential requirement for lineage tracing<sup>43</sup>; (2) inappropriate reliance on assessing a negative population because it is impossible to ascertain if this represents failed cre-mediated recombination, silencing of the lineage tracing gene, or technical loss of the reporter marker; or whether the cell truly did not express the MYH11 SMC marker gene, that is, reliable lineage tracing should focus only on assessing a positively labeled cell population; (3) failure to provide rigorous validation that only the cell population of interest (in this case MYH11 expressing mature VSMCs) was labeled at time zero and not other cell types; and (4) methodological concerns including inappropriate fixation and cell permeabilization methods that might result in artificial loss of eGFP, for example validation of their lineage tracing model and image resolution to determine colocalization of markers<sup>42</sup> and functional studies using rigorous lineage tracing methodologies. Indeed, subsequent rigorous lineage tracing studies by several groups have shown that mature MYH11-expressing medial VSMCs are not terminally differentiated, and are capable of phenotypic transition in culture,<sup>23,32</sup> in atherosclerosis,<sup>33</sup> and after vascular injury.<sup>44</sup>

Several groups have postulated that adventitial cells including Sca1<sup>+</sup> stem cells,<sup>45</sup> adventitial pericytes,<sup>46</sup> and adventitial fibroblasts<sup>47,48</sup> may contribute to formation of neointimal lesions after vascular injury or within atherosclerotic plaques. Indeed, there is compelling evidence for the existence of a population of Sca1<sup>+</sup> adventitial cells that can be induced to activate multiple SMC marker genes in vitro.<sup>49,50</sup> Moreover, it is well established that pericytes and activated myofibroblasts express multiple SMC markers and thus may give rise to VSMC-like cells within lesions. Unfortunately, studies to date have relied on the use of single markers or panels of markers that do not clearly define the origins of cells nor exclude VSMCs as the source of the cells in question, and as yet there have been no rigorous high-resolution lineage tracing studies of any of these cell populations. Indeed, to our knowledge, no one has identified appropriate cell-specific conditional lineage tracing genes to specifically label these interesting cell populations. Adventitial Sca1<sup>+</sup> c-Kit<sup>+</sup> cells tagged with  $\beta$ -galactosidase gene product (LacZ) in vitro and then transplanted to the adventitial surface of a vein graft within ApoE<sup>−/−</sup> mice have been shown to contribute to vein graft neointima formation.<sup>45</sup>

However, this model results in huge decellularization of the media after transplant, and as yet there are no rigorous lineage tracing studies showing that endogenous adventitial cells normally contribute to primary atherosclerotic plaque formation, although this is an area deserving further study.

### Clonal Nature of the Atherosclerotic Plaque

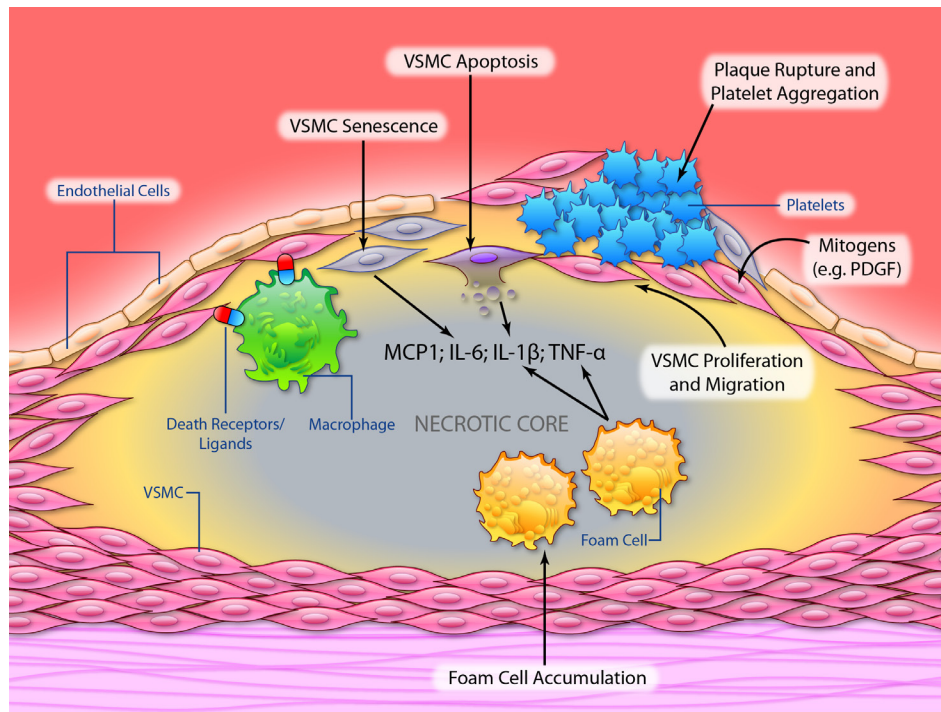
Although the most robust lineage tracing studies support a prominent role for phenotypic switching of MYH11<sup>+</sup> medial VSMCs in generating the atherosclerotic lesion, a key consideration is whether all medial MYH11<sup>+</sup> cells contribute to the intimal VSMCs. In particular, findings of monoclonality of human plaques both in historical and recent studies suggested that a subpopulation of medial VSMCs selectively proliferates to cause VSMC accumulation in atherosclerosis, and in particular in the fibrous cap. For example, >40 years ago Benditt and Benditt<sup>51</sup> found that fibrous caps of atherosclerotic plaques of females heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase were monoclonal in nature, whereas the medial VSMCs were a mixture. Although subsequent observations showed that normal vessels also comprised a mosaic of monoclonal patches because of the expansion of progenitor cells during normal vessel development,<sup>52–54</sup> recent lineage tracing studies showed preliminary evidence of clonal expansion of MYH11<sup>+</sup> cells during plaque development.<sup>33</sup> Although the latter study did not address the extent to which this occurs nor the cell types generated in the plaque, they do raise critical questions as to the nature of the MYH11<sup>+</sup> cells that contribute to atherosclerosis. For example, is there a specific subpopulation of MYH11<sup>+</sup> progenitors and if so, how could these be identified and what are their molecular characteristics? Alternatively, dynamic fluctuations within MYH11<sup>+</sup> medial VSMCs could lead to stochastic development of plaque progenitor cells. Clearly, further studies are needed to define precisely the extent to which atherosclerotic plaques are derived from a single progenitor and the molecular mechanisms underlying such an event.

### VSMC Processes in Atherosclerosis—Evidence and Consequences

The stability of the atherosclerotic plaque depends on the thickness of the fibrous cap and the degree of cap inflammation. Plaque rupture is increased by cap thinning promoted by death of VSMCs and breakdown of collagen and ECM, which may subsequently lead to myocardial infarction or stroke. However, plaque rupture is frequently subclinical because VSMCs repair the rupture and reorganize the associated thrombus. Indeed, complicated plaques frequently show evidence of multiple ruptures and repair, ultimately resulting in luminal narrowing. Successful plaque repair requires VSMCs to proliferate and synthesize matrix, both properties that are altered by death and cellular senescence. Indeed, the balance of cell proliferation and migration versus cell death and cell senescence determines the population of VSMCs within the atherosclerotic plaque (Figure 2). The role and regulation of these processes is crucial to both atherogenesis and plaque stability.

### Cell Proliferation

VSMCs in the normal vessel wall have a low turnover, with barely measurable proliferation indices. Increased cell



**Figure 2. Schematic illustration of several processes that vascular smooth muscle cells (VSMCs) undergo in advanced atherosclerotic plaques.** IL indicates interleukin; MCP, monocyte chemoattractant protein, PDGF, platelet-derived growth factor; and TNF, tumor necrosis factor (Illustration Credit: Ben Smith).

proliferation is observed during early atherogenesis and on vascular injury,<sup>55</sup> and aged VSMCs from rodents also show increased proliferation<sup>56–58</sup> compared with cells from younger animals. In contrast, human VSMCs derived from both aged vessels and advanced atherosclerotic plaques undergo reduced proliferation and prolonged population doubling times.<sup>59,60</sup> This observation corresponds to *in vitro* findings where plaque VSMCs in culture show decreased percentages in S-phase and increased percentages in G<sub>1</sub>, consistent with a G<sub>1</sub> growth arrest.<sup>60</sup> Although some of the arrest is associated with reduction in responses to mitogens, such as insulin-like growth factor 1,<sup>61</sup> the arrest is mediated by major changes in the expression of various cell cycle regulators, especially those involved in G<sub>1</sub>–S transition. Thus, increased expression of the cyclin-dependent kinase inhibitors p16<sup>INK4a</sup> and p21,<sup>62</sup> decreased cyclin D and cyclin E,<sup>63</sup> and hypophosphorylation of the retinoblastoma protein<sup>62–64</sup> are observed in both normal human VSMCs undergoing replicative senescence and human plaque VSMCs. Plaque VSMCs also show reduced expression of the transcription factors E2F1–3 and increased sequestration of E2F1 to retinoblastoma protein.<sup>62</sup> Importantly, these cell cycle regulators become potential markers of vascular cell senescence. Collectively, these observations suggest that in advanced lesions enhancement and not inhibition of VSMC proliferation may be beneficial for plaque stability and progression. Similarly, if VSMC proliferation is predominantly reparative in atherogenesis, enhancement of the ability of VSMCs to proliferate may also be beneficial early in disease.

### Cell Migration

The presence of a large number of intimal VSMCs, for example forming a fibrous cap, has been taken as evidence that

VSMC migration from the media plays an important role in atherogenesis. However, VSMC migration is a difficult process to quantify in human atherosclerosis, in part because there are no specific markers available, and also because human arteries also contain intimal VSMCs. In rodents, where there are no VSMCs in the intima in the normal vessel, intimal VSMCs must have arisen by migration from the intima or transdifferentiation of invading myeloid cells from the lumen. Indeed, seminal studies from the 1970s demonstrated VSMC migration both directly (via appearance in the intima) and indirectly (via proliferation labeling studies with demonstration of intimal VSMCs that had not proliferated). In contrast, such direct or indirect quantification of migration in human vessels is not possible, and we are left with evidence that human VSMCs can migrate to a variety of stimuli in culture, but the contribution of VSMC migration to the mature atherosclerotic plaque is unclear. Similarly, in humans it is not clear whether migration occurs independently or is dependent on cell proliferation.

### Cell Death

The presence of apoptosis in atherosclerotic plaques has been confirmed by several studies.<sup>55,60,65</sup> Apoptotic indices are low in early lesions (Stary grades I–III), but seen with increasing frequency as lesions develop, in both the necrotic core and fibrous cap. Apoptosis is predominantly restricted to macrophages and VSMCs, although all cell types within the vessel wall can undergo apoptosis. However, the same caveats apply to studies on apoptosis and other studies, that interpretation is now limited by their use of markers that are not lineage specific. Plaque rupture occurs most commonly in the shoulder area of the plaque, a region characterized by reduced VSMCs



and increased macrophages. This suggests that VSMC apoptosis, perhaps induced by macrophages through death ligand/death receptor interactions,<sup>66</sup> may be a central event in plaque rupture and its subsequent sequelae.<sup>67</sup> Indeed, symptomatic plaques exhibit increased levels of VSMC apoptosis<sup>68</sup> compared with stable lesions.

Although apoptosis is seen in vascular disease, these frequencies cannot be transposed into absolute rates of cell death, as we do not know how long the death process lasts in vivo in diseased vessels, and how much of the death process is associated with positive markers.<sup>69,70</sup> For example, delay of phagocytosis may result in increased apoptosis being detected,<sup>25</sup> and positive live cells will be marked if apoptotic bodies retain terminal UTP nick end-labeling positivity after engulfment. Although we cannot get accurate rates of apoptosis, VSMC apoptosis in atherosclerosis has profound consequences, promoting multiple features of vulnerable plaques<sup>71</sup> such as a thin fibrous cap, enlarged necrotic core, and macrophage infiltration into the cap. Chronic VSMC apoptosis accelerates both atherogenesis and progression of established lesions, promotes calcification, and also induces features of medial degeneration, including medial atrophy, VSMC loss, elastin fragmentation, increased glycosaminoglycans, and speckled calcification.<sup>72</sup> These features are seen in cystic medial degeneration, for example in Erdheim disease, in Marfan syndrome, and to a lesser extent in normal aging.<sup>73,74</sup> Importantly, loss of VSMCs is sufficient alone to trigger all these secondary features, suggesting that VSMC apoptosis is a primary and early event in these diseases.

VSMC apoptosis in atherosclerosis is also associated with inflammation, whereas in vascular aging, medial degeneration, and remodeling, there is remarkably little inflammation. The recent explanation for this phenomenon may rest on the efficiency of clearance of apoptotic cells and the cytokines released from dead and surrounding live VSMCs. Dying VSMCs release IL-1, apoptosis releases IL-1 $\beta$  and necrosis releases IL-1 $\alpha$ . Secondary necrosis (after apoptosis) releases both IL-1 $\alpha$  and IL-1 $\beta$ .<sup>75</sup> Apoptotic VSMCs are normally cleared from the vessel wall in  $\approx$ 48 hours because VSMCs themselves are efficient at clearing apoptotic VSMCs.<sup>76</sup> However, phagocytosis is delayed in the presence of hyperlipidemia, possibly because of the defective phagocytosis induced when VSMCs undergo phenotypic switching to macrophage-like cells, with the resultant subsequent inflammation that is dependent on IL-1.<sup>75</sup> Furthermore, a recent study has linked the human 9p21 gene locus, which has been shown to be highly correlated with enhanced cardiovascular disease, with reduced expression of cyclin-dependent kinase inhibitor 2B and calreticulin, a ligand required for activation of engulfment receptors on phagocytic cells.<sup>77</sup> Cyclin-dependent kinase inhibitor 2B-deficient apoptotic bodies were resistant to efferocytosis and not efficiently cleared by neighboring macrophages.<sup>77</sup> These data suggest that loss of cyclin-dependent kinase inhibitor 2B promotes atherosclerosis by increasing the size and complexity of the lipid-laden necrotic core through impaired efferocytosis.

As described above, recent studies have suggested that bone marrow-derived cells may migrate to the atherosclerotic plaque or neointima after injury and express SMC markers.

Indeed, VSMC apoptosis releases stromal cell-derived factor 1 $\alpha$  after injury, which may recruit SMC progenitors to sites of arterial injury.<sup>78</sup> However, bone marrow-derived smooth muscle-like cells are infrequent in primary atherosclerotic plaques, and unlike vessel wall-derived VSMCs, their apoptosis reduces atherosclerosis and reduces plaque inflammation.<sup>37</sup> In this case, their proatherogenic action is also dependent on cytokines released, including chemokine (C-X-C motif) ligand 16 (CXCL16), IL-6, and monocyte chemoattractant protein-1,<sup>37</sup> but apoptosis of these cells reduces plaque inflammation. Clearly, whether apoptosis induces inflammation depends on their origin; vessel wall-derived cells promote inflammation when they undergo apoptosis in atherosclerosis; bone marrow-derived SMC-like cells already have a proinflammatory phenotype and their apoptosis reduces inflammation.

### Cell Senescence

Cell senescence is defined as the irreversible loss of the ability of cells to divide. There are 2 general types of cell senescence, replicative senescence, and stress-induced premature senescence. Replicative senescence occurs with exhaustion of proliferative lifespan over time, a characteristic of aging, and is associated with critically shortened telomeres at chromosomal ends, which then induce a DNA damage response. In contrast, stress-induced premature senescence is triggered by external stimuli, including oxidizing agents and radiation, which activate the intracellular senescence cascade prematurely. Although stress-induced premature senescence shares many morphological and molecular characteristics to replicative senescence, stress-induced premature senescence is not usually characterized by telomere shortening.

In addition to the altered expression of cell cycle regulators, senescent cells are characterized by specific markers, including senescence-associated  $\beta$  galactosidase, a lysosomal enzyme seen in senescence of multiple human cell types.<sup>79</sup> Increased numbers of senescence-associated  $\beta$  galactosidase-positive cells expressing markers associated with VSMCs, endothelial cells (ECs), and monocyte/macrophages are observed in aged vessels and atherosclerotic lesions when compared with their respective young and normal counterparts,<sup>62,80</sup> reinforcing the idea that atherosclerosis is associated with premature cellular senescence. However, a word of caution is required when interpreting senescence-associated  $\beta$  galactosidase staining. In particular, cells with a high lysosomal content, such as macrophage foam cells, show senescence-associated  $\beta$  galactosidase reactivity that may not reflect senescence.

Shortened telomeres are evident in atherosclerosis, observed in plaque VSMCs<sup>62</sup> and ECs<sup>81</sup> relative to the normal vessel wall, and in circulating endothelial progenitor cells.<sup>82</sup> Telomeres are also shorter in leukocytes in patients with atherosclerosis compared with control subjects<sup>83,84</sup> and are also inversely correlated to cardiovascular disease risks in patients with subclinical diseases.<sup>85,86</sup> Short telomeres and low levels of telomerase expression and activity are functionally important in VSMC senescence, as ectopic telomerase expression can dramatically increase lifespan of both plaque and normal VSMCs.<sup>62</sup> However, some of these effects may be independent of telomeres, as telomeres continue to shorten in these

cells and cells replicate with critically short telomeres.<sup>62</sup> In addition, although telomere length mostly reflects previous replication, arterial segments resistant to atherosclerosis, such as internal mammary artery or ascending aorta, have longer telomeres than the aortic regions prone to the disease.<sup>87,88</sup> This difference is age-independent, suggesting the existence of intrinsic genetic or developmental variations in telomere regulation may underlie location-specific predisposition in atherogenesis.

VSMCs in human plaques or derived from plaques show early senescence and increased susceptibility to apoptosis. These properties would reduce the ability to repair plaques that undergo rupture. Aged rodent aortas also show increased levels of IL-6 and aged aortic VSMCs have a higher basal secretion of IL-6 than young VSMCs. Indeed, secretion of a common set of secreted proteins as cells age is a widespread phenomenon, known as the senescence-associated secretory phenotype or senescence-associated secretory phenotype. Moreover, aged VSMCs exhibit upregulation of chemokines (eg, MCP-1), adhesion molecules (eg, intercellular adhesion molecule 1 [ICAM-1]), and innate immune receptors (eg, Toll-like receptor 4).<sup>89</sup> These properties generate a proinflammatory environment, further promoting migration of inflammatory cells. Indeed, experimental induction of VSMC senescence has been shown to promote both plaque progression and features of unstable plaques.<sup>90</sup>

Although we have discussed these processes separately to review their consequences, many processes occur simultaneously (Figure 2). For example, VSMC phenotypic switching to a macrophage-like cell, VSMC death, and senescence all promote inflammation, monocyte recruitment, and subsequent secretion of VSMC mitogens. VSMC proliferation ultimately generates cell senescence, as do defects in cell death or cell clearance, and lack of clearance of senescent VSMCs may promote cell death. The complex structure of the atherosclerotic plaque, therefore, reflects the complex cellular and extracellular environment, and both the complementary and competing nature of these processes.

## Summary and Conclusions

The role of VSMCs in atherosclerosis has evolved remarkably in the past 30 years. Previously, it was thought that aberrant proliferation of VSMCs after phenotypic switching drove atherogenesis, although VSMCs were also protective in advanced lesions, preventing fibrous caps from rupturing and promoting plaque repair. VSMCs expressed VSMC markers or no markers, and macrophages expressed macrophage makers.

More recent studies using lineage tracing and VSMC-specific manipulation of both specific genes and pathways have changed our view. It is highly likely that VSMCs and macrophages within lesions have been mis-identified in many previous studies in the field. This is because VSMC marker-positive cells within lesions can be derived from multiple cell types including macrophages and possibly various adventitial cells, and the majority of VSMC-derived cells within lesions lose expression of SMC markers. Similarly, macrophage marker-positive cells within plaques may not be macrophages or even of myeloid origin, and a large fraction of foam cells dual-positive for SMC and macrophage markers are of VSMC

not myeloid origin. A further level of complexity is introduced by findings that cellular proteins and mRNAs can be passed between cells through exosomes or microvesicles, such that a cell can acquire a marker by passive transfer.<sup>91,92</sup> This problem is not just confined to VSMCs, or even vascular biology. Clearly, if a marker can be induced in a different lineage or lost in the same cell type, it is not a marker of that lineage, or implies a property of that cell. Otherwise we end up with a circular argument that a marker is identified if it is expressed in a lineage, and a lineage identified if it expresses a marker, when neither are necessarily true. For example, expression of Sca1 alone does not mean that that cell is a progenitor cell. However, we can conclude that the majority of cells expressing SMC markers in atherosclerosis or after arterial injury derive from the vessel wall and not the bone marrow, although the contribution of endogenous progenitor populations within the media or adventitia is unclear.

Recent studies have also clarified the role of VSMCs in disease. VSMC proliferation in atherosclerosis seems to be predominantly reparative, even in atherogenesis, and not the primary driver of plaque formation. The role of VSMC migration per se in atherosclerosis is still unclear, including adventitial progenitor populations. In contrast, VSMC cell death and cell senescence promote both atherogenesis and multiple features of plaque instability.

A critical challenge for future studies will be to identify the environmental cues within advanced atherosclerotic lesions that regulate phenotypic transitions of VSMCs, as well as each of the major cell types within lesions, and to determine how these might be manipulated therapeutically to reduce plaque burden and increase plaque stability. Our rationale is that development of novel therapeutic approaches for treating atherosclerosis, and reducing major clinical consequences such as MI or stroke, will be dependent on a rigorous understanding of the biology of each of the major cell types that contribute to the pathogenesis of late-stage lesions. We are assuming that certain pathways and targets may have opposing effects on one cell type versus another and that an ideal therapeutic target would promote beneficial changes in multiple cell types. This represents a paradigm shift for the atherosclerosis field because therapies to date have largely been focused on drugs such as statins that control blood lipids, which do modestly reduce disease prevalence, and anti-inflammatory strategies targeting macrophages and other immune cells, which to date are unproven. An important goal for the future is to identify the factors and mechanisms that can promote beneficial changes in VSMC phenotype and processes that can either augment or replace these more conventional antiatherosclerotic therapies.

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## Disclosures

None.



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