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SPOTLIGHT REVIEW

Smooth muscle cell fate and plasticity in atherosclerosis

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Abstract Current knowledge suggests that intimal smooth muscle cells (SMCs) in native atherosclerotic plaque derive mainly from the medial arterial layer. During this process, SMCs undergo complex structural and functional changes giving rise to a broad spectrum of phenotypes. Classically, intimal SMCs are described as dedifferentiated/synthetic SMCs, a phenotype characterized by reduced expression of contractile proteins. Intimal SMCs are considered to have a beneficial role by contributing to the fibrous cap and thereby stabilizing atherosclerotic plaque. However, intimal SMCs can lose their properties to such an extent that they become hard to identify, contribute significantly to the foam cell population, and acquire inflammatory-like cell features. This review highlights mechanisms of SMC plasticity in different stages of native atherosclerotic plaque formation, their potential for monoclonal or oligoclonal expansion, as well as recent findings demonstrating the underestimated deleterious role of SMCs in this disease.

Keywords Smooth muscle cells • Atherosclerosis • Phenotype • Foam cells • Fibrous cap

This article is part of the Spotlight Issue on Novel concepts for the role of smooth muscle cells in vascular disease.

1. Introduction

Smooth muscle cells (SMCs) are the primary cell type in the pre-atherosclerotic intima, a state known as diffuse intimal thickening (DIT), and in all stages of human atherosclerotic plaque development.[1] SMCs are not terminally differentiated and can change their phenotype in response to environmental cues including growth factors/inhibitors, mechanical influences, cell-cell and cell-matrix interactions, extracellular lipids and lipoproteins, and various inflammatory mediators in the injured artery wall.[2] In pre-atherosclerotic DIT, SMCs exhibit a low proliferative rate and maintain a stable phenotype.[3] With development of atherosclerotic plaque, however, phenotypic modulation of intimal SMCs occurs, including a more proliferative state, loss of contractility, increased synthesis of proteoglycans, and reduced expression of SMC markers including α-smooth muscle actin (α-SMA) and smooth muscle myosin heavy chains (SMMHCs).[2,4] Within the atherosclerotic plaque SMCs express variations in their phenotype including foam cell formation,[2,5–7] while SMCs forming the fibrous cap retain SMC markers including α-SMA.[8–11]

Phenotype switching of SMCs to less differentiated forms with reduction or total absence of SMC markers has led to an underestimation of their role in the development of atherosclerosis in both humans and animal models of this disease. Moreover, less differentiated SMCs often express other cell type's markers including those of macrophages, which leads to their misidentification.[12] In this review, we discuss the current knowledge regarding SMC phenotypic variation (*Figure 1*), as well as transcriptional and epigenetic regulation of SMC differentiation. The contribution of intimal SMCs originating from diverse sources including circulating blood, adventitia and endothelium is covered by other reviews in this Spotlight Issue.

2. Contractile vs. synthetic SMC phenotype

SMCs constituting the medial layer of healthy arteries are primarily quiescent and highly differentiated. However, in contrast to skeletal and heart muscle cells, they retain a high degree of dedifferentiation potential and plasticity and can shift from a contractile to a so-called synthetic phenotype. This phenomenon is known as SMC phenotypic modulation or switching, typical of SMCs accumulating in the intima during the formation of atherosclerotic plaques, as well as in restenotic lesions following angioplasty/stent placement in humans. SMCs accumulated in the intima during pre-atherosclerotic DIT also exhibit phenotypic dedifferentiation

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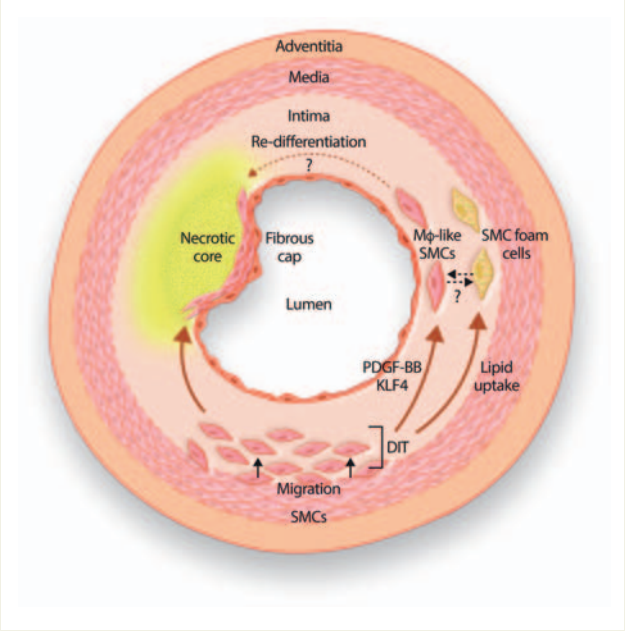

Figure 1 Model of SMC phenotypic diversity in atherosclerotic intima. In humans, medial SMCs migrate into the intima starting *in utero* in atherosclerosis-prone arteries, forming the DIT layer, likely by monoclonal or oligoclonal expansion. Intimal SMCs can take up lipids to become foam cells, which *in vitro* data indicate causes a loss of SMC markers and expression of macrophage markers. In addition, SMCs can de-differentiate and express macrophage markers under the influence of factors and epigenetic regulators including PDGF-BB and KLF4. Whether initial lipoprotein loading is required for SMCs to express macrophage markers requires further investigation. It is also not yet known whether SMCs first have to assume a macrophage-like phenotype in order to allow or enhance SMC foam cell formation. SMCs in the fibrous cap that cover the necrotic core have a more differentiated phenotype. Whether some de-differentiated intimal SMCs re-differentiate to supply fibrous cap SMCs remains to be determined.

from medial SMCs. Although species other than humans do not exhibit prominent pre-atherosclerotic DIT,[13] intimal SMCs of experimentally induced intimal thickening in rat, rabbit and pig arteries after endothelial injury or hypercholesterolemic diet show phenotypic changes similar to those in human intimal SMCs.[14,15] In these pathological and experimental situations, SMCs migrate from the media toward the intima where they proliferate and acquire a synthetic phenotype. The transition toward a synthetic phenotype results in a gradual shifting from a differentiated to a dedifferentiated state, defined by the decrease or loss of several SMC-specific cytoskeletal proteins. Expression of α-SMA (corresponding to the gene *ACTA2*), the actin isoform typical of vascular SMCs and most general marker of SMC lineage from early stages of development, is reduced or lost during this dedifferentiation process. Additional SMC markers lost during dedifferentiation include SMMHC (corresponding to the gene *MYH11*) isoforms SM1 and SM2, SM22α (or transgelin corresponding to the gene *TAGLN*), serum response factor (SRF), calponin (corresponding to the gene *CNN1*), h-caldesmon and meta-vinculin. Proteins known to be up- or downregulated in contractile and synthetic SMCs are summarized in *Table 1*.[16–18]

Although SMCs placed in culture have a tendency to become synthetic, several groups have been able to isolate and culture morphologically distinct SMCs typical of the contractile and synthetic phenotypes. First, using rat carotid artery and aorta, spindle-shaped (S) SMCs with the classical 'hill and valley' growth pattern were isolated from the normal media, and epithelioid (E) SMCs, which grow as a monolayer and exhibit a cobblestone morphology at confluence, were obtained from the experimental intimal thickening induced 15 days after endothelial injury.[19–22] S-SMCs display features typical of the contractile phenotype, and E-SMCs are typical of the synthetic phenotype. Additional SMC phenotypes have been described in vessels of other species such as mouse and dog aorta, cow pulmonary artery and porcine coronary artery; in larger animals these include rhomboid [R]-SMCs that are similar to rat E-SMCs.[23,24] The isolation of distinct SMC subpopulations in humans has been reported sporadically over the last decade.[16] From the media of human carotid endarterectomy specimens, we have recently isolated two SMC phenotypes with different morphologies, large and small SMCs. The latter, which exhibit features similar to that of E- and R-SMCs, was obtained only after coculture with macrophage-derived foam cells isolated from the plaque, suggesting that macrophage foam cells promote the dedifferentiation and recruitment of a particular medial SMC population by yet unidentified factors.[25] E- and R-SMCs are characterized by reduced SMC markers and increased proliferative, migratory, and proteolytic activities[16,17] typical of the synthetic phenotype. The high migratory activity of E and R-SMCs correlates with high expression of metalloproteinase-2 (MMP-2), urokinase- and tissue-type plasminogen activators.[16]

Integrins, transmembrane receptors linking the intracellular cytoskeleton to the extracellular matrix, play a significant role in SMC phenotypic changes.[26,27] α1β1 and α7β1 integrins, which bind to Collagen IV and laminin, respectively are highly expressed in contractile SMCs; their deletion induces SMC phenotypic modulation from a contractile and spindle phenotype to a synthetic, epithelioid state, as well as SMC proliferation and accumulation in experimental intimal thickening. In contrast α2β1 (binding Collagens I and VIII), α5β1 and αvβ3 (both binding fibronectin) integrins are increased in synthetic SMCs. Of note α5β1 integrin, which is increased in intimal SMCs, is required for collagen deposition through increased fibronectin fibrillogenesis.[28] Likewise, αvβ3 integrin is involved in fibronectin deposition and prevents oxidized low-density lipoprotein (Ox-LDL)-induced SMC apoptosis.[29] α5β1 and αvβ3 integrins are believed to contribute to plaque stability.[27]

3. Challenge in identifying markers of synthetic/intimal SMCs

The loss of cytoskeletal markers typical of mature, differentiated SMCs observed in the intima can occur to such an extent that they are no longer recognized as SMCs. Therefore, the need for specific markers of intimal SMCs was crucial. Comparison of the S- and E- or R-phenotype, mainly by means of proteomic approaches, has led to the identification of proteins typical of the synthetic phenotype and possibly involved in the phenotypic changes that occur *in vivo*. Among the genes specifically expressed by E-SMCs, osteopontin, an extracellular matrix protein involved in bone mineralization, is one of the most studied, and has led to a better understanding of the role of SMCs in atherosclerotic calcification ('reviewed by Shanahan and Giachelli in this Spotlight issue'). *In vivo*, osteopontin is transiently up-regulated in rat experimentally-induced intimal thickening, and accumulates in calcified areas of human atheromatous plaque.[30] We identified another protein, S100A4, as a marker of R-SMCs *in vitro* and of intimal SMCs in both pigs and humans.[31] Recently

Table 1 Expression of proteins typical of contractile and synthetic SMCs

Proteins	Abbreviations	Expression		References
		Contractile	Synthetic	
Cytoskeletal proteins				
α-smooth muscle actin *(ACTA-2)*	α-SMA	+++	+	2,16–18
Smooth muscle myosin heavy chains *(MYH11)*	SMMHCs	+	–	2,16–18
Transgelin or Smooth muscle 22α *(TAGLN)*	SM22α	+	–	2,16–18
Desmin	–	+	–	2,16–18
Smoothelin		+	–	2,16–18
Calponin *(CNN1)*	–	+	–	2,16–18
SM α-tropomyosin	–	+	–	2,16–18
Heavy-caldesmon	H-caldesmon	+	–	2,16–18
Metavinculin	–	+	–	2,16–18
Leiomodin-1	LMOD1	+	–	9,126
Cytokeratin 8 and 18	CK8 and 18	–	+	127–129
Zonula occludens-2 protein	ZO-2	–	+	127,130
Cingulin	–	–	+	127
Extracellular matrix-related components				
Collagen VIII	–	–	+	114
Osteopontin	OPN	–	+	30
Matrix metalloproteinases-2, -9	MMP-2 and -9,	–	+	131,132
Urokinase-type plasminogen activators	u-PA	–	+	133,134
Tissue-type plasminogen activators	t-PA	–	+	24
Calcium-binding protein				
S100A4	–	–	+	31,32,135
Calmodulin	CaM	–	+	25
Cytokine				
Monocyte chemoattractant protein 1	MCP-1	–	+	101,102
Interleukins-1β and -6	IL-1β and -6	–	+	101,102
Tumor necrosis factor-α	TNF-α	–	+	101,102
Transmembrane molecule				
α1β1, α7β1, α8β1 Integrins	–	+	–	27
α2β1, α5β1, αvβ3 Integrins	–	–	+	27–29
Intercellular adhesion molecule-1	ICAM-1	–	+	101,102
Vascular cell adhesion molecule-1	VCAM-1	–	+	101,102
Connexin 43	Cx43	–	+	35
Receptor				
Toll-like receptors 2 and 4	TLR-2 and -4	–	+	101,102
Receptor for advanced glycation end products	RAGE	–	+	101,102

we have shown that the extracellular form of S100A4 is essential for the establishment of the R-phenotype and acts, to some extent, through the receptor for advanced glycation end products (RAGE). Remarkably, S-SMCs treated with S100A4-rich conditioned medium collected from S100A4-transfected SMCs acquire pro-inflammatory properties.[32] Other proteins such as Cytokeratin 8 and 18, zonula occludens-2 protein, cingulin, and Connexin 43 are upregulated in E- or R-SMCs *in vitro*, in experimentally induced intimal thickening, and human atherosclerotic plaques.[33–35] We also found that the small synthetic phenotype SMCs isolated from human carotid artery predominantly express the calcium-binding protein calmodulin (CaM), and that CaM was overexpressed in SMCs in atherosclerotic plaque.[25] We observed that inhibition of CaM resulted in a striking reduction in human SMC proliferation.[25] These observations suggest that CaM is a marker of a specific human intimal SMC population and plays a role in SMC behaviour. Even though these markers are instrumental in characterizing intimal SMCs and give further insight into the mechanisms of SMC phenotypic modulation, it is not proven that they persist in all intimal SMCs. For that reason these individual markers are not suitable to clearly identify cells of SMC lineage in the intima. This issue has been recently addressed by Owens and co-workers by using epigenetic markers specific for SMCs[12,36] (see below).

4. Transcriptional regulation of SMC differentiation markers

The expression of SMC-specific cytoskeletal proteins is dependent on different regulatory elements in their gene promoter regions (*Table 2*, *Figure 2*). Myocardin (MYOCD) is the main mediator driving the SMC contractile phenotype.[37,38] It forms a complex with SRF, a ubiquitous DNA-binding transcription factor, which binds the CArG (i.e. CC[A/T-rich]$_6$GG) sequence motif. SRF thereby serves as a docking platform for

Table 2 Regulation of transcription and epigenetic factors during SMC transition to a synthetic phenotype

Proteins	Abbreviations	Up/Down	References
Transcription factor			
Serum response factor	SRF	↓	37,38
Myocardin	MYOCD	↓	37,38
Myocardin-related transcription factor	MRTF-A and B	↓	37,38
Phosphatase and tensin homolog	PTEN	↓	136
Specificity protein-1	Sp-1	↑	40
Nuclear factor-κB	NF-κB	↑	101,102
Nuclear factor of activated T-cells	NFAT	↑	101,102
Forkhead box protein O4	FOXO4	↑	50
ETS domain-containing protein-1	Elk-1	↑	49
Pluripotency transcription factor			
Krüppel-like factor 4	KLF4	↑	12,46,137
Octamer-binding transcription factor 4	Oct4	↓	138
Epigenetic factor			
microRNA-143/-145, -29,-210,-663	miR-143/-145, -29, -210, -663	↓	59
microRNA -221/-222,-21	miR-221/-222,-21	↑	59
Histone acetyltransferase	HAT	↓	36,54
Histone deacetylase	HDAC	↑	36,56
Ten–eleven translocation-2	TET2	↓	57,58
Miscellaneous			
Dedicator of cytokinesis 2	DOCK2	↑	51

Gene acronyms are indicated in brackets.

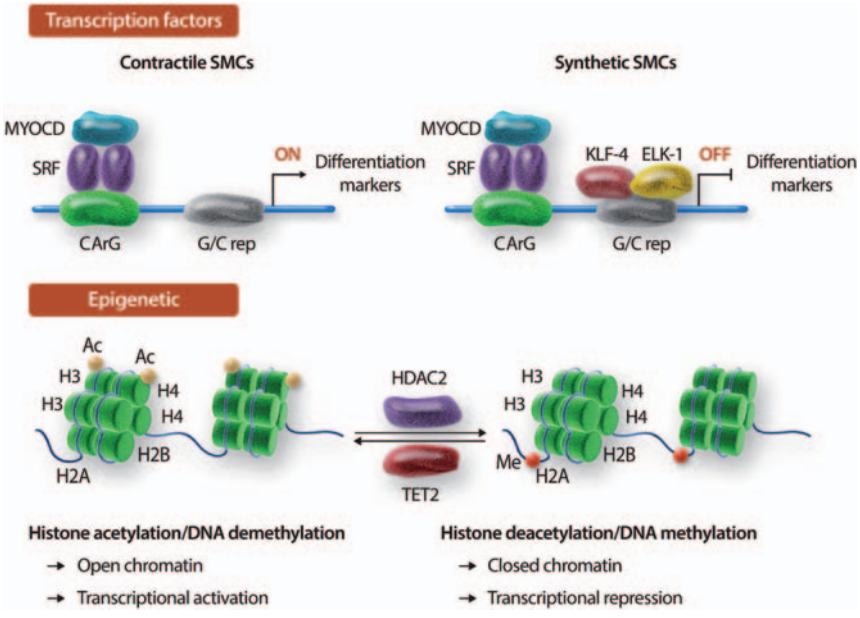

Figure 2 Schematic model of transcriptional and epigenetic regulation of SMC differentiation markers. In contractile SMCs, the complex MYOCD/SRF binds to CArG box and increases expression of SMC differentiation markers. In contrast, KLF4, a pluripotency transcription factor, absent in contractile SMCs, is increased in intimal SMCs. KLF-4 and ELK-1 bind to G/C repressor element and inhibit the MYOCD/SRF complex leading to decreased expression of SMC differentiation markers. In addition deacetylation of histones by HDAC2 closes chromatin leading to transcriptional repression of SMC differentiation markers. By contrast, DNA demethylation by TET2 increases DNA accessibility to transcription factors resulting in increased SMC differentiation marker expression. (SRF, serum-response factor; MYOCD, Myocardin; KLF 4, Krüppel-like factor 4; ELK1, ETS domain-containing protein-1; CArG, CArG box; G/C rep, G/C repressor elements; HDAC2, histone deacetylase 2; TET2, ten-eleven translocation-2; H, histones; Ac, acetyl group; Me, methyl group).

MYOCD activity. MYOCD-related transcription factor (MRTF)-A and B have been identified as coactivators of SRF as well. In contrast, Krüppel-like factor 4 (KLF4), a pluripotency transcription factor absent in contractile SMCs, promotes SMC dedifferentiation.[39] KLF4 is upregulated *in vitro* in response to platelet-derived growth factor (PDGF)-BB,[40,41] PDGF-DD,[42] cholesterol loading,[43] and oxidized-phospholipids.[41,44,45] After endothelial injury in mouse carotid artery, KLF4 expression is increased in intimal SMCs,[45,46] and its deletion leads to delayed SMC dedifferentiation even if SMC proliferation is enhanced.[46] KLF4 binds to G/C repressor elements localized in close vicinity of CArG boxes and interacts with SRF.[47] It acts on SMC dedifferentiation through multiple mechanisms, including suppression of MYOCD expression, disruption of the MYOCD/SRF complex, dissociation of MYOCD/SRF from SMC promoters, and modification of chromatin structure (see below).[39] Other transcription factors have been shown to play a crucial role in SMC differentiation/dedifferentiation, mainly by acting on the MYOD/SRF complex dynamics. Recently, phosphatase and tensin homologue (PTEN) has been shown to maintain SMC contractile phenotype via its interaction with SRF and MYOCD.[48] In contrast, phosphorylated ETS domain-containing protein-1 (Elk-1) promotes dedifferentiation of SMCs by competing with MYOCD for interaction with SRF.[49] Sp-1 mediates the PDGF-BB- or vascular injury-induced SMC phenotypic modulation via its interaction with the KLF4 promoter.[40] Forkhead box protein O4 (FOXO4), which is increased in proliferating SMCs of experimentally induced intimal thickening in mouse carotid artery, promotes dedifferentiation of SMCs through its interaction with the MYOCD/SRF complex thereby inhibiting MYOCD activity.[50] Recently, dedicator of Cytokinesis 2 (DOCK2), an activator of the small G protein Rac, has been identified as a novel regulator of SMC phenotypic modulation. DOCK2 is upregulated by PDGF-BB *in vitro* and in experimentally induced intimal thickening in rats and mice and its deletion inhibits intimal thickening formation and restores SMC differentiation marker expression. It interacts with MYOCD thereby weakening the MYOCD/SRF interaction and promoting SMC dedifferentiation. Moreover, DOCK2 and KLF4 cooperatively inhibit the MYOCD/SRF interaction.[51] It should keep in mind that molecular pathways responsible for SMC phenotypic transition have been unravelled mainly *in vitro* and in animal experimental models, and most of these changes are related to studies of chronic atherosclerosis rather than acute plaque rupture. These observations provide important clues to SMC differentiation and dedifferentiation mechanisms for confirmation in chronic atherosclerosis and acute coronary syndromes in humans.[52]

5. Epigenetic regulation of SMC differentiation markers

In addition to gene regulation, programmed chromatin remodelling may dictate distinct SMC phenotypes and identify cells of SMC lineage (studied extensively by Owens and co-workers, *Table 2*, *Figure 2*).[36,39,53] Chromatin is composed of nucleosomes, constituted of 146 base-pairs of DNA wrapped around an octamer of histone proteins (two copies of histones H2A, H3B, H3, and H4). Chromatin is present in two distinct conformations: euchromatin and heterochromatin, corresponding to a non-condensed and condensed form of DNA, thus resulting in the activation or silencing of gene transcription, respectively. Chromatin conformation depends on histone acetylation and methylation of lysine residues.[53] Histones H3 and H4 associated with CArG containing regulatory elements of SM-MHCs, SM22α, and α-SMA have been shown to be acetylated (a characteristic of chromatin accessibility) in contractile SMCs, leading to SRF binding to the CArG box. In contrast, decreased H4 acetylation takes place in PDGF-BB-treated SMCs and in balloon-injured rat carotid artery.[54] Increased histone acetyltransferase (HAT) activity stimulates SM22α expression whereas increased histone deacetylase (HDACs) prevents SM22α expression.[36] H3 histone dimethylation in lysine position 4 (H3K4me2) is a marker of differentiated SMCs and is maintained even if SMCs undergo phenotypic modulation.[36,39,53] Owens and co-workers have used this property to trace SMC lineage in mouse and human histologic sections (see below).[55] PDGF-BB and oxidized phospholipid-induced KLF4 recruits HDACs at the CArG box region, thereby decreasing histone acetylation and accessibility to MYOCD, MRTF-A, and SRF.[36,53] KLF4, ELK-1, and HDAC isoform 2 form a complex, which binds to a G/C repressor element leading to decreased H3 acetylation in the SM22α promoter and consequently to the downregulation of SM22α expression.[56]

More recently DNA demethylation has attracted much interest. Ten–eleven translocation-2 (TET2) is a DNA demethylase, which oxidizes 5-methylcytosis (5mC) to 5-hydroxymethylcytosis and hence increases accessibility of DNA to transcription factors. TET2 is downregulated in dedifferentiated SMCs *in vitro* (e.g. when SMCs are treated with PDGF-BB), in experimentally induced intimal thickening and in human coronary artery atherosclerotic plaque. Knockdown of TET2 in cultured SMCs leads to increased 5mC and reduces chromatin accessibility at key SMC contractile promoters such as MYOCD, SRF, SMMHCs and α-SMA.[57,58]

MicroRNA (miR) also play a key role in SMC phenotypic modulation ('reviewed by Leeper and Maegdefessel in this Spotlight Issue'), in particular the miR-143/145 cluster, which is a potent promoter of the contractile SMC phenotype. Other miRNAs such as miR-21, miR-221, and miR-222 generate opposite effects, driving SMCs towards proliferation.[59]

6. Monoclonal/oligoclonal expansion of intimal SMCs

Despite numerous studies on the factors modulating SMC phenotypes, a question that remains open is whether SMCs in the media can undergo phenotypic modulation upon environmental cues (i.e. the 'response-to-injury' or polyclonal hypothesis)[60] or whether a pre-existing medial SMC subpopulation is prone to migrate and accumulate in the intima **(**monoclonal/oligoclonal hypothesis**)**. The second possibility has been raised on the basis of the original work of Benditt and Benditt[61] in the 1970s, who reported that human atherosclerotic plaque SMCs have the features of monoclonal or oligoclonal expansion. By means of microdissection of different portions of human plaques followed by PCR amplification of the DNA of an X-inactivated gene (the human androgen receptor gene) allele, Schwartz and Murry[62] later demonstrated that SMCs of the fibrous cap have a monoclonal origin.

In vitro, by means of tissue explantation or production of SMC clones, E- or R-SMCs can be isolated from healthy media, supporting the hypothesis that this particular population pre-exists in the media and is prone to migrate into and accumulate in intimal thickening.[19,23,24,63] By implanting S- and E-SMCs into rat carotid artery previously subjected to endothelial injury, we observed that these two SMC populations maintained their distinct features.[64] This is an indicator that the phenotype of SMCs is more dependent on intrinsic features rather than on their environment, thereby reinforcing the notion of SMC heterogeneity. One of

the most conclusive works demonstrating the presence of distinct SMC populations in humans is based on the finding that E-SMCs can be cloned from the media of undiseased arteries.[65]

Very recently Chappell *et al.*[11] have been able to convincingly trace mature SMCs of the media by generating ApoE$^{-/-}$ mice expressing tamoxifen-inducible Cre recombinase under the MYH11 (SMMHC) promoter in conjunction with the ROSA26-Confetti multicolour (four fluorescent proteins) reporter allele.[66] In this transgenic mouse tamoxifen treatment induces recombination of the Rosa26-Confetti allele only in SMCs resulting in the random expression of one of the four fluorescent proteins, which is retained within progeny. These authors elegantly demonstrated that the media was stochastically multicoloured whereas SMCs in atherosclerotic lesions and in injury-induced intimal thickening were monochromatic, suggesting that clonal intimal SMCs derive from a subset of medial SMCs. Previously Feil *et al.*,[8] used a similar model with SM22α as a SMC-specific gene and the ROSA26-β-galactosidase or Confetti as reporter genes; they generated transgenic mice with only a small fraction of the medial SMCs labelled and observed a clonal expansion of highly proliferating SMCs in atherosclerotic plaques. These two studies have led to renewed interest in the monoclonal/oligoclonal hypothesis as highlighted in a recent review by DiRenzo, Owens and Leeper.[67] In their editorial related to the Chappell paper,[68] Gomez and Owens pointed out the seminal observation of Thomas *et al.*[69] and Clowes *et al.*[70] showing that 100 and 40% of medial SMCs re-enter the cell cycle in rat carotid artery after endothelial injury or hyperlipidemic swine models of atherosclerosis, respectively. To reconcile these data with the oligoclonal origin of intimal SMCs, they suggest that numerous medial SMCs proliferate but only a subset of them survive in the intima.

7. SMC phenotype in diffuse intimal thickening

DIT is the presence of a thickened intima composed primarily of SMCs that forms early in life in humans before atherosclerosis development.[71] DIT is initiated *in utero* and is present in 100% of human atherosclerosis-prone arteries such as the coronary arteries and abdominal aorta by the age of 2 years.[13,72] It is localized in the non-branching long segments of arteries where shear stress is low.[73] DIT precedes the appearance of lipid accumulation and inflammatory cell infiltration by years.[71,72,74] The correlation of DIT with atherosclerosis is indicated by its consistent presence in atherosclerosis-prone arteries,[13,75,76] and by the positive correlation of the extent of DIT in infants with the presence of coronary disease in family members.[77]

DIT is composed essentially of SMCs, proteoglycans, and elastin.[72,76] The origin of SMCs in DIT has been suggested to be from SMCs in the media.[62,78] Consistent with the monoclonal theory of intimal SMC proliferation, Murry *et al.*[62] reported that SMCs in both DIT and atherosclerotic plaques are monoclonal in origin. SMCs in DIT show a phenotypic shift when compared with medial SMCs, exhibiting higher expression of genes involved in cell migration, proliferation, and production of extracellular matrix such as heparin-binding epidermal growth like,[79] αvβ3 integrin,[80] and transforming growth factor (TGF-β),[81] and a decrease in expression of SMMHCs.[82]

Importantly, DIT is not present in small laboratory mammals like mice, rats and rabbits.[13,83] This differentiates the pathogenesis of human atherosclerosis, which develops over decades on a bed of pre-existing intimal SMCs, from the accelerated development of atherosclerotic lesions induced by atherogenic diets in animal models lacking DIT.

8. SMC phenotype in athero-prone vs. athero-resistant arteries

The tendency to develop atherosclerosis varies markedly between different vascular beds. Internal mammary arteries (IMAs), also known as internal thoracic arteries, are resistant, whereas coronary and carotid arteries and the ascending and abdominal aorta are prone to the development of atherosclerosis. This property of IMA has made it the most common graft material in coronary artery bypass grafting (CABG), with significantly better long-term outcomes and lower complication rates compared with saphenous vein, radial artery, and gastroepiploic artery, other vessels used in CABG.[84,85] Many factors including hemodynamic properties,[86,87] structural features of the internal elastic lamina,[88] and histological and functional characteristics of the endothelial layer[89] of IMA have been implicated in its resistance to development of atherosclerosis.

Differences in SMC characteristics between athero-prone and athero-resistant arteries have been less well studied. It is known that SMCs of the IMA are resistant to proliferation and keep their contractile phenotype for decades.[89] Archacki *et al.*[90] performed microarray analysis to identify genes differentially expressed between IMA and the left anterior descending (LAD) coronary artery. Twenty-nine genes showed significant differences in expression levels between IMA and LAD. Using genome-wide transcriptome analysis of IMA and aorta other investigators found 19 pathways differentially expressed, with IMA expressing lower levels of genes encoding pro-atherosclerotic proteins and inflammatory markers.[91] Both studies used the whole artery for analysis. In a more specific manner, Qin *et al.*[92] compared gene expression of porcine coronary arteries and IMA after removal of the endothelium and adventitia; they found genes associated with tight and intermediate junctions were more highly expressed in IMA whereas genes associated with pro-atherosclerotic processes including lipid retention and metabolism such as extracellular matrix proteins, Caveolin-1 and 2, proteolipid protein, colipase, inflammation, and cell growth were more highly expressed in coronary arteries. Others have shown that SMCs in atherosclerosis-prone regions of aorta in ApoE$^{-/-}$ mice exhibit a proatherogenic gene expression pattern when compared with athero-resistant regions, even prior to the development of atherosclerosis.[93]

It is possible that biomechanical stimuli and paracrine mediators induce an altered phenotype of SMCs between athero-prone and athero-resistant arteries. Intrinsic differences in phenotype of SMCs in these arteries may also be responsible for their different tendency to develop atherosclerosis. Further understanding of the characteristics of SMCs between these vascular beds may provide useful targets for the prevention of ischemic vascular disease.

9. Loss of SMC markers and expression of non-SMC markers by plaque SMCs

SMCs display a spectrum of phenotypes with contractile and synthetic phenotypes being the extremes and many forms in between.[94] Although SMCs show a remarkable plasticity in the artery wall, the real challenge comes when they have significantly reduced or total loss of expression of their markers and become hard to identify. This phenomenon has been known for decades but has never been quantified until recently when, by using a SMC-specific lineage marker, Shankman *et al.*[12]

reported that >80% of SMC-derived cells in atherosclerotic lesions of ApoE$^{-/-}$ mice lack expression of SMC markers including α-SMA. Different stimuli present in the plaque microenvironment including cholesterol,[95] oxidized phospholipids,[44] growth factors like PDGF-BB,[96] and hemodynamic factors[97] have been implicated in downregulation of SMC differentiation markers.

A striking feature of SMCs in atherosclerotic plaque is that they can express markers of other cell types like macrophages, and therefore be misidentified. Two decades ago Andreeva *et al.*[98] showed the presence of cells expressing both α-SMA and CD68, previously thought to be a macrophage/leukocyte-specific marker, in 'en face' preparations of human aortic intima and in primary cultures of SMCs prepared from grossly normal and atherosclerotic intima. Later on, studies from Fisher's laboratory showed that cultured mouse SMCs express macrophage markers including CD68 and Mac2 upon cholesterol loading.[95] Follow-up studies by their laboratory indicated that in spite of expressing some macrophage markers after cholesterol loading, their transcriptome profile and phagocytic capacity shows that they remain clearly distinct from macrophages and exhibit only weak macrophage-like function.[43] They found that cholesterol loading of SMCs induces a SMC-to-macrophage transdifferentiation by downregulating the miR-143/145-MYOCD axis. Notably, they have reported that KLF4 is increased after cholesterol loading due to downregulation of the miR-143/145/SRF/MYOCD complex.[43] We found that 40% of CD68+ cells in advanced lesions of human coronary atherosclerosis also strongly express α-SMA. Consistent with this estimate we observed that ∼34% of CD68+ cells in advanced lesions do not express CD45, a leukocyte-specific lineage marker. Our data suggest that ∼40% of CD68-expressing cells in advanced human coronary atherosclerosis are SMC- rather than leukocyte-derived.[7] Consistent with our findings, by combining *in situ* hybridization and proximity ligation assay in histological human specimens, allowing the detection of the H3K4me2 permanent epigenetic marker of SMC lineage in the SMMHC promotor, Shankman *et al.*[12] estimated that ∼30% of macrophage marker-positive cells are derived from SMCs in advanced human coronary artery atherosclerotic plaques. This finding has now been confirmed in mouse atherosclerotic lesions using several SMC lineage tracing models.[8,10–12] Although it has been shown that cultured macrophages can express SMC markers including α-SMA after stimulation with TGF-β or thrombin,[99,100] there is no evidence for α-SMA expression by H3K4me2 negative cells (non-SMCs, including monocyte-derived macrophages) in human or mouse tissues *in vivo*.[12]

Not only can SMCs express macrophage markers, SMC lineage-tracing mice indicate they can also express markers of mesenchymal stem cells (MSCs), and myofibroblasts, which strongly express PDGF receptor-β (PDGFR-β) and share with SMCs the expression of α-SMA.[12] Overall this study found that 30% of SMCs in a mouse model of atherosclerosis are macrophage-like, 7% are MSC-like, and 12% are myofibroblast-like cells.

Furthermore, SMCs have the capacity to acquire inflammatory cell markers and release inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1.[101] TNF-α or IL-1β induce expression of inflammatory molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 and release of MMP-9, an MMP related to plaque vulnerability, by SMCs. ICAM-1 is also upregulated on the surface of SMCs in human atherosclerotic plaque and promotes adhesion to monocyte/macrophages and T cells. Additionally, factors within the atherosclerotic plaque such as Ox-LDL, oxidized phospholipids, high-mobility group box 1, Angiotensin II, or Toll-like receptor (TLR) ligands are also able to induce activation of transcription factor-mediated inflammation by SMCs. These include nuclear factor-κB (NF-κb) and NF of activated T-cells (NFAT) through TLR-2 and -4 and the receptor for advanced glycated-end product (RAGE).[15,102]

10. SMC foam cells

Proteoglycans secreted by DIT SMCs are thought to be responsible for the initial retention of lipoproteins in the deep intimal layer of the artery wall, due to an interaction between positively-charged apolipoprotein B on lipoproteins and the negatively charged proteoglycans.[103] The ability of intimal SMCs to accumulate excess lipid and become foam cells has been documented by multiple studies.[6,104–108] By quantifying SMC foam cells in human coronary lesions we found for the first time that, conservatively, SMCs contribute >50% of total foam cells, a much larger fraction than previously known.[7]

SMCs express scavenger receptors and can therefore take up intimal lipoproteins like macrophages.[109] Other mechanisms including micropinocytosis of serum lipoproteins [110] and uptake of aggregated LDL via LDL receptor-related protein-1[111] have been implicated in foam cell formation by SMCs. As noted above, free cholesterol loading induces expression of macrophage markers and loss of expression of SMC markers by cultured SMCs,[43,95] features that are reversible by removal of the excess cholesterol.[43] Several factors including transcription factor KLF4[12] and miR-143/145/SRF/MYOCD axis[43] have been shown to mediate the phenotypic change of SMCs after cholesterol loading. Contrary to this, neither RAW 264-7 mouse macrophage cells or human monocytes exhibit H3K4me2 in the *Myh11* promoter after exposure to POVPC, an oxidative product of LDL.[12]

An important question is whether prevention of the phenotypic shift in vascular SMCs by knocking down KLF4 or overexpression of miR-143/145/SRF/MYOCD complex can prevent SMC foam cell formation. Whether DIT SMCs *in vivo* take up sufficient lipoproteins to become foam cells in their native state, or first have to be converted to a more macrophage-like phenotype, e.g. with increased scavenger receptor expression, also remains to be determined (*Figure 1*).

11. SMC phenotype in the fibrous cap

Rupture of atherosclerotic plaque and subsequent luminal thrombosis is the most common cause of acute coronary syndromes and sudden coronary death. Erosion and calcified nodules are the two other mechanisms of luminal thrombosis.[112] The fibrous cap consists of SMCs in a collagen-proteoglycan matrix with varying degrees of macrophage and lymphocyte infiltration, lying between the necrotic core and luminal surface of the plaque. The fibrous cap has a critical role in maintenance of the integrity of the plaque. The number of SMCs in fibrous caps is directly correlated with plaque stability,[18] with SMCs being the main producer of collagen and proteoglycans in the fibrous matrix.[113] Of note, Collagen VIII deficiency in ApoE$^{-/-}$ mice results in thinning of the fibrous cap; this is due to decreased SMC proliferation and migration whereas macrophage accumulation is not affected. These results suggest that Collagen VIII plays an important role in plaque stabilization by protecting the plaque from rupture.[114,115] Unlike SMCs in the deeper intima, SMCs of the fibrous cap have been shown to strongly express differentiated SMC markers including α-SMA,[8–11]

The origin of the differentiated SMCs in the fibrous cap is not fully understood. Nonetheless, as noted above most evidence suggests that intimal SMCs derive locally from the media. Benditt and Benditt[61] found that SMCs in fibrous caps were monoclonal in nature, whereas other intimal and medial SMCs were a mixture of phenotypes. In a recent study, using lethally irradiated ApoE$^{-/-}$ mice reconstituted with sex-mismatched bone-marrow cells from eGFP+ ApoE$^{-/-}$ mice, Bentzon *et al.*[116] did not find eGFP+ SMCs in the fibrous cap, indicating these SMCs originate from the local intimal SMCs and not from circulating progenitor cells.

PDGF is an important mediator of vascular SMC proliferation and migration. Sano *et al.*[117] showed that inhibition of the PDGFR-β pathway markedly suppresses fibrous cap formation *in vivo*. Environmental stimuli that shift the phenotype of SMCs towards a differentiated phenotype may also lead to formation of the fibrous cap. Induction of a more differentiated phenotype in SMCs by an increase in TGF-β signalling or over-expression of insulin growth factor-1 in SMCs of ApoE$^{-/-}$ mice results in increased fibrous cap thickness.[118,119] Further, SMC-specific knockout of KLF4 resulted in an increase in fibrous cap area and an increase in the percentage of α-SMA positive cells in the fibrous cap.[12] Unexpectedly SMC-specific conditional knockout of another pluripotency transcription factor, octamer-binding transcription Factor 4 (Oct4), in ApoE$^{-/-}$ mice was shown to enhance atherosclerotic plaque formation, reduce SMCs in the fibrous cap and increase plaque instability.[138] This highlights the complexity of atherosclerotic plaque formation and progression.

It has been shown that lipid loading of SMCs impairs their ability to assemble fibrillar extracellular matrix,[120] which may contribute to reducing plaque stability. On the other hand enhancement of cholesterol efflux preserves the assembly of fibrillar collagen and fibronectin.[121] In a similar manner when mouse plaques were placed into an atherosclerosis regression environment with elevated HDL, macrophage content of the plaque decreased and SMC content in the subendothelial layer increased.[122,123] As such, SMCs may not leave the plaque similar to macrophages[124] in a regression environment, but may become more differentiated and contribute to increased plaque stability. The capacity of intimal SMCs to release excess lipids, however, given their reduced expression of the cholesterol exporter ABCA1,[7,125] remains to be determined.

12. Conclusions and perspectives

In recent years our understanding of the role of SMCs in atherosclerotic plaque formation has changed greatly. SMCs appear to be much more versatile than expected. The classical view is that intimal SMCs are beneficial by forming the fibrous cap, protecting the plaque from rupture. In this situation they share features with myofibroblasts and are hence dedicated to the healing-like process. Despite this, it has long been recognized that SMCs can become foam cells and acquire features of inflammatory cells. Human atherosclerosis studies as well as SMC-lineage tracing mouse models and epigenetic studies show that transition of intimal SMCs into macrophage-like cells is a major process during atherosclerotic plaque formation, further demonstrating the unexpected deleterious role of SMCs. Therefore, SMCs exhibit dual and antagonistic roles in atherosclerosis. It is worthy of note to mention that most of the studies related to SMC phenotypic modulation, as well as to their monoclonal/oligoclonality, have reinforced the concept that intimal SMCs in native atherosclerosis originate locally from the media.

The study of epigenetic signatures will be instrumental in characterizing the distinct SMC subpopulations in atherosclerotic plaque. Alternatively, a critical challenge for future studies will be to identify persistent markers specific to beneficial and/or deleterious SMCs. Finally, recent findings have shed light on the mechanisms of SMC fate and plasticity in the intima that should be instrumental to develop tools targeting more precisely the distinct SMC subpopulations and their influence on the evolution of atherosclerotic plaque.

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