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CONTEMPORARY REVIEW

Phenotypic Switching of Vascular Smooth Muscle Cells in Atherosclerosis

Runji Chen, MSc; David G. McVey, PhD; Daifei Shen, PhD; Xiaoxin Huang, MSc; Shu Ye, MD, PhD

ABSTRACT: The medial layer of the arterial wall is composed mainly of vascular smooth muscle cells (VSMCs). Under physiological conditions, VSMCs assume a contractile phenotype, and their primary function is to regulate vascular tone. In contrast with terminally differentiated cells, VSMCs possess phenotypic plasticity, capable of transitioning into other cellular phenotypes in response to changes in the vascular environment. Recent research has shown that VSMC phenotypic switching participates in the pathogenesis of atherosclerosis, where the various types of dedifferentiated VSMCs accumulate in the atherosclerotic lesion and participate in the associated vascular remodeling by secreting extracellular matrix proteins and proteases. This review article discusses the 9 VSMC phenotypes that have been reported in atherosclerotic lesions and classifies them into differentiated VSMCs, intermediately dedifferentiated VSMCs, and dedifferentiated VSMCs. It also provides an overview of several methodologies that have been developed for studying VSMC phenotypic switching and discusses their respective advantages and limitations.

Key Words: atherosclerosis ■ cell lineage ■ dedifferentiation ■ phenotypes ■ vascular smooth muscle cells

Atherosclerosis is a leading cause of morbidity and death worldwide. In the past few decades, a number of hypotheses have been proposed for the pathogenesis of atherosclerosis.[1–4] The current prevailing view is that atherosclerosis is a chronic inflammatory disease of the blood vessel wall, whose key processes include endothelial cell dysfunction, lipid deposition, leukocyte infiltration, vascular smooth muscle cell (VSMC) changes, and extracellular matrix accumulation.[4,5] In this review, we focus on findings from recent studies regarding VSMC phenotypic changes in atherosclerosis.

In the normal physiological state, VSMCs hardly proliferate, have low synthetic activity, and assume a contractile phenotype to regulate the vasomotor tone of blood vessels.[6] Therefore, they have been described as differentiated VSMCs, quiescent VSMCs, or contractile VSMCs. However, under pathological conditions such as atherosclerosis, VSMCs can be activated and become what have been traditionally named as proliferative VSMCs or synthetic VSMCs, which have higher proliferation rates and protein synthesis activities.[6]

In addition to the 2 types of VSMCs mentioned above, recent studies have uncovered a number of other phenotypes of VSMCs, which appear to exert diverse roles in atherosclerosis. These findings are important for a better understanding of the complex pathogenesis of atherosclerosis, which in turn can potentially inspire novel therapeutic strategies.

Several previous review articles have discussed various aspects of VSMC phenotypic switching.[7–9] Here, we aim to provide an update to include more recent findings, discussing the 9 different VSMC phenotypes that have so far been reported in atherosclerotic lesions, and endeavor to classify them into 3 categories: differentiated VSMCs, intermediately dedifferentiated VSMCs, and dedifferentiated VSMCs.

In addition, this article summarizes several methodologies that have been developed and used to study

Correspondence to: Shu Ye, MD, PhD, Department of Medicine, Cardiovascular-Metabolic Disease Translational Research Programme, Centre for Translational Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Block MD6, 14 Medical Drive, 117599 Singapore.
Email: shuye68@nus.edu.sg

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Nonstandard Abbreviations and Acronyms

CArG	CC(A/T-rich)6GG cis-regulating elements
Itga8	integrin alpha 8
KLF4	Krüppel-like factor 4
Lgals3	galectin 3
Ly6c1	lymphocyte antigen 6 family member C1
Myh11	myosin heavy chain 11
Oct4	octamer binding transcription factor 4
SEM-like cells	stem cell/endothelial cell/monocyte cell-like cells
SRF	serum response factor
SMC	smooth muscle cell
TCF21	transcription factor 21
TET2	Tet methylcytosine dioxygenase 2
VSMC	vascular smooth muscle cell

VSMC phenotypic switching and discusses their respective advantages and limitations.

VSMC PHENOTYPES REPORTED IN ATHEROSCLEROTIC LESIONS

The various VSMC phenotypes that have been reported in atherosclerotic lesions are summarized in Figure 1[10] and Table 1[11–38] and further discussed below, while reported extracellular stimuli and intracellular mediators of VSMC phenotypic transitions are schematically summarized in Figure 2.

There is evidence from in vitro cell culture experiments and in vivo cell lineage tracing studies, indicating that these different types of cells can derive from the VSMC lineage, as discussed below, but it is currently unclear regarding their anatomic origins and whether they can also derive from non-VSMC lineages.

Differentiated VSMCs

In the healthy mature artery, VSMCs are in a differentiated state and show a contractile phenotype. Markers of the contractile phenotype include MYH11 (also known as smooth muscle myosin heavy chain 11), calponin, transgelin (also known as SM22α), myocardin, and α-smooth muscle actin.[11]

Expression of VSMC contractile genes is regulated by a number of transcription factors, including myocardin, serum response factor (SRF), and Krüppel-like factor 4 (KLF4),[39,40] and modulated by epigenetic factors including DNA methylation and histone modifications[41] as well as noncoding RNAs (including microRNA, lncRNA, and circRNA).[42,43] It has been shown that Tet methylcytosine doxygenase 2 (TET2, also known as ten-eleven translocation-2) binds, and 5-hydroxymethylcytosine is enriched, in 6GG cis-regulating elements (CArG)-rich regions of the myocardin and SRF genes, and that augmented expression of TET2 induces a contractile phenotype of VSMCs whereas TET2 knockdown causes a decrease in the expression of VSMC contractile genes, with concomitant transcriptional upregulation of KLF4.[10]

During VSMC phenotype transitions, the expression of contractile phenotype markers is reduced or absent, while markers associated with other VSMC phenotypes are expressed.[11]

Intermediately Dedifferentiated VSMCs

Mesenchymal-Like Cells

Shankman et al reported a stem cell antigen-1 positive cell population derived from VSMCs in mouse atherosclerotic plaques.[13] Stem cell antigen-1 (also known as lymphocyte antigen 6 family member A) is a cell surface marker of mesenchymal cells and therefore the VSMC subtype identified by Shankman et al may be referred to as mesenchymal-like cells.[13] In agreement, Dobnikar et al showed that stem cell antigen-1 expression is upregulated when VSMCs undergo phenotypic switching and that Sca1-positive VSMC-lineage cells are present in mouse atherosclerotic plaques.[12]

The origin and fate of mesenchymal-like cells in atherosclerotic plaques is unclear. One hypothesis is that during atherogenesis, contractile VSMCs in the media transition into mesenchymal-like cells, then migrate into the intima, and subsequently differentiate into other phenotypes.[8] This view is supported by the findings from a study by Chen et al, which showed that VSMCs differentiated into mesenchymal-like cells, which in turn differentiated into adipocytes-, chondrocytes-, osteoblasts-, and macrophages-like cells in a mouse model of aortic aneurysm.[44] However, Shankman et al found that such mesenchymal-like cells grew slowly after cell sorting from *Apoe*$^{-}$ mice and could not be induced to directional differentiation into adipocytes or osteoblast-like cells.[13] These discrepant findings could potentially be due to the differences in the experimental procedures as Chen et al examined the differentiation of mesenchymal-like cells in mice in vivo,[44] whereas Shankman et al studies cells in culture.[13]

Mechanistically, studies have suggested that during atherogenesis, KLF4 mediates VSMC transition from the contractile phenotype to mesenchymal-like phenotype cells.[8,13,44] KLF4 inhibits the expression of sex-determining region Y-box 9, transient receptor potential cation channel subfamily V member 4, and S100

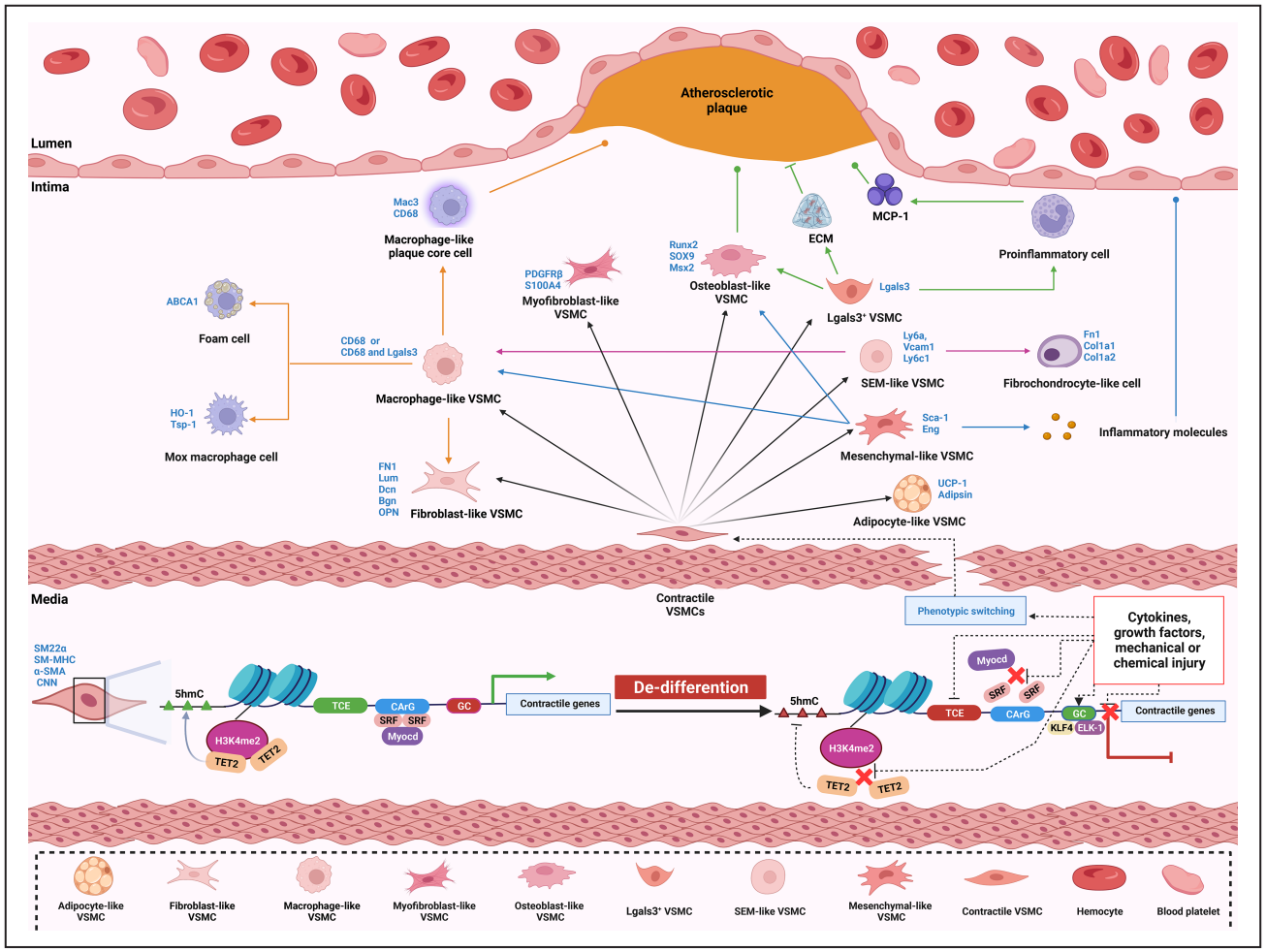

Figure 1. VSMC phenotypic switching in atherosclerosis.
The DNA-modifying enzyme Tet methylcytosine doxygenase 2 (TET2, also known as ten-eleven translocation-2) binds in CArG-rich regions of the *MYOCD* and *SRF* genes, and thereby upregulates the expression of these and downstream VSMC contractile genes.[10] TET2 and its product 5-hydroxymethylcytosine are enriched in contractile VSMCs, while they are reduced in dedifferentiated VSMCs, where the TET2 reduction causes a decrease in the expression of VSMC contractile genes, with concomitant transcriptional upregulation of KLF4.[10] During atherogenesis, VSMCs may transition to intermediately dedifferentiated VSMCs (mesenchymal-like VSMCs, SEM-like cells, or Lgals3$^+$ VSMCs) and then further to dedifferentiated VSMCs (macrophage-like VSMCs, or osteoblast-like VSMCs) or transform directly to dedifferentiated VSMCs (fibroblast-like VSMCs, myofibroblast-like VSMCs, macrophage-like VSMCs, osteoblast-like VSMCs, or adipocyte-like VSMCs). Markers typical or potential for the contractile VSMCs are SM22a, SM-MHC, α-SMA, and calponin[11]; for the fibroblast-like phenotype FN1, Lum, Dcn, Bgn, and OPN[20]; for the adipocyte-like phenotype UCP-1 and Adipsin[26,27]; for the macrophage-like phenotype CD68 alone or CD68 and Lgals3[18,19]; for the myofibroblast-like phenotype PDGFRβ, and S100A4[13,21,22]; for the mesenchymal-like phenotype Sca-1 and Eng[13]; for the osteoblast-like phenotype Cbfa1, Msx2, Runx2, and Sox9[25]; for the Lgals3$^+$ cells phenotype Lgals3[15]; for the SEM-like cells phenotype Ly6a, Vcam1 and Ly6c1[14]; for the Mox macrophage subtype HO-1 and Tsp-1[18]; for the foam cells phenotype ABCA1[28,29]; for the macrophage-like phenotype Mac3 and CD68[17]; for the fibrochondrocyte-like phenotype FN1, Col1a1, Col1a2.[14] ↑ represents VSMC phenotype transformation, ↑ represents exacerbation, and T represents inhibition of atherosclerosis. The blue font represents the marker of each phenotype. 5hmC indicates 5-hydroxymethylcytosine; α-SMA, α-smooth muscle actin; ABCA1, ATP-binding cassette transporter 1; Bgn, biglycan; CArG, CC(A/T-rich)6GG cis-regulating element; cbfa1, core-binding factor α1; CD68, cluster of differentiation 68; CNN, calponin; Col1a1, collagen type I alpha 1 chain; Col1a2, collagen type I alpha 2 chain; Dcn, decorin; ECM, extracellular matrix; ELK; ETS transcription factor ELK1; Eng, endoglin; Fn1, fibronectin 1; GC, G/C repressor element; H3K4me2, histone H3 lysine 4 di-methylation; HO-1, heme oxygenase 1; KLF4, Krüppel-like factor 4; Lgals3, galectin 3; Ly6a, lymphocyte antigen 6 family member A; Ly6c1, lymphocyte antigen 6 family member C1; Lum, lumican; Mac3, lysosomal-associated membrane protein 2; MCP1, monocyte chemoattractant protein 1; Msx2, Msh homeobox 2; Myocd, myocardin; PDGFRβ, platelet-derived growth factor receptor beta; OPN, osteophosphorin; Runx2, Runt-related transcription factor 2; S100A4, S100 calcium binding protein A4; Sca-1, stem cell antigen-1; SEM-like VSMC, stem cell/endothelial cell/monocyte cell-like VSMC;SM22α, transgelin; SM-MHC, myosin heavy chain 11; SOX9, SRY-box transcription factor 9; SRF, serum response factor; TCE, transforming growth factor-beta control elements; TET2, Tet methylcytosine dioxygenase 2; Tsp-1, thrombospondin-1; UCP-1, uncoupling protein 1; Vcam1, vascular cell adhesion molecule 1; and VSMC, vascular smooth muscle cell.

Table 1. Vascular Smooth Muscle Cell Phenotypic Markers

Species	VSMC phenotype	Phenotypic markers	Cell surface markers	References
Mouse	Differentiated VSMCs	Mhy11, calponin, Tagln, Myocd, ACTA2	…	[11]
	Mesenchymal-like	Mhy11, Sca1	Sca1$^+$	[12]
	Mesenchymal stem cell-like	Mhy11, Acta2$^-$, Sca1$^+$	Sca1$^+$, endoglin (CD105)$^+$	[13]
	SEM-like	Myh11$^+$, Ly6a, VCAM1, Ly6c1	Ly6a$^+$, Ly6c1$^+$	[14]
	Lgals3$^+$	Lgals3	…	[15]
	Lgals3$^+$/osteoblast	Myh11$^+$, Runx2, Sox9, Cytl1, S100B, Trpv4		[16]
	Macrophage-like	Myh11$^-$, Acta2$^-$, Lgals3$^+$	…	[13]
		α-SMA, Mac3	…	[17]
		Lgals3, CD68	…	[18]
		Myh11$^-$, CD36, CD68	CD36$^+$, CD68$^+$	[19]
		Myh11$^-$, CD45, CD11b	CD45$^+$, CD11b$^+$	[20]
	Myofibroblast-like	Myh11$^-$, ACTA2, Pdgfβr, S100A4	Pdgfβr$^+$	[13,21,22]
	Fibromyocyte	Fibronectin 1, Tnfrsf11b, Col1a1, lumican, decorin, biglycan	…	[23]
	Fibroblast-like	Fsp1		[24]
	Fibroblast–/pericyte-like	Myh11$^-$, Vimentin, Pdgfra, pdgfrb, NG2	CD45$^+$, CD11b$^+$, F4/F80$^+$, Pdgfra$^+$, pdgfrb$^+$	[20]
	Osteoblast-like	Runx2, Msx2, Wnt, Osx	…	[25]
	Chondrocyte-like	Sox9	…	[25]
	Adipocyte-like	UCP1	…	[26]
Human	Differentiated VSMCs	MYH11, calponin, transgelin, myocardin, ACTA2	…	[11]
	Lgals3$^+$/osteoblast	MYH11, RUNX2, SOX9, CYTL1, S1008, Trpv4		[16]
	Fibromyocyte	Fibronectin 1, TNFSF11B, COL1A1, lumican, decorin, biglycan	…	[23]

α-SMA indicates α-smooth muscle actin; ACTA2, smooth muscle actin alpha 2; CD36, cluster of differentiation 11b; CD36, cluster of differentiation 68; CD36, cluster of differentiation 45; CD68, Col1a1, collagen type I alpha 1 chain; Cytl1, cytokine like 1; F4/F80, cell surface glycoprotein F4/80; Mac3, lysosomal-associated membrane protein 2; Fsp1, fibroblast-specific protein 1; Mhy11, myosin heavy chain 11; Msx2, Msh homeobox 2; NG2, chondroitin sulfate proteoglycan 4; Lgals3, galectin 3; Ly6a, lymphocyte antigen 6 family member A; Lum, lumican; Ly6c1, lymphocyte antigen 6 family member C1; Myocd, myocardin; NG2, chondroitin sulfate proteoglycan 4; Osx, osterix; Pdgfra, platelet-derived growth factor receptor alpha; Pdgfrβ, platelet-derived growth factor receptor beta; Runx2, runt-related transcription factor 2; S100A4, S100 calcium-binding protein A4; S100B, S100 calcium-binding protein B; Sca-1, stem cell antigen-1; Sox9, sex-determining region Y-box 9; SEM-like, stem cell/endothelial cell/monocyte cell-like; Tagln, transgelin; Tnfrsf11b, TNF receptor superfamily member 11b; Trpv4, transient receptor potential cation channel subfamily V member 4; UCP-1, uncoupling protein 1; VCAM1, vascular cell adhesion molecule 1; VSMC, vascular smooth muscle cell; and Wnt, proto-oncogene Wnt.

calcium-binding protein B. Downregulation of these proteins promotes VSMC phenotype switching to an intermediate phenotype with multidirectional differentiation ability, leading to enlargement of atherosclerotic lesions.[16]

Stem Cell/Endothelial Cell/Monocyte Cell-Like Cells

Stem/endothelial/monocyte-like (SEM-like) cells were first reported by Pan et al,[14] who studied a VSMC-lineage tracing murine model and human carotid artery atherosclerotic plaques, and observed the transition of VSMCs to a multipotent intermediate cell state in atherosclerosis. Distinct from mesenchymal-like cells, SEM-like cells do not exhibit expression of mesenchymal cell marker genes (such as *Nt5e* [encoding CD73] and *Eng* [encoding CD105]) but rather take on the phenotypic characteristics of endothelial cells and monocyte-macrophages.[14] Among the differentially expressed genes in this SEM-like cell type is the enrichment of lymphocyte antigen 6 family member A, vascular cell adhesion molecule 1, galectin 3 (Lgals3) and lymphocyte antigen 6 family member C1.[14] SEM-like cells accounted for the most significant proportion among smooth muscle cell (SMC) lineages in this study.[14] In response to stimulation with cytokines, SEM-like cells could be induced into macrophage-like cells, fibrochondrocytes, and even VSMCs.[14]

Mechanistically, the study of Pan et al[14] suggests that cellular retinoic acid binding protein 2, a transducer of retinoic acid signaling, is a master regulator of vascular cell adhesion molecule 1 and lymphocyte antigen 6 family member C1. Altered expression of

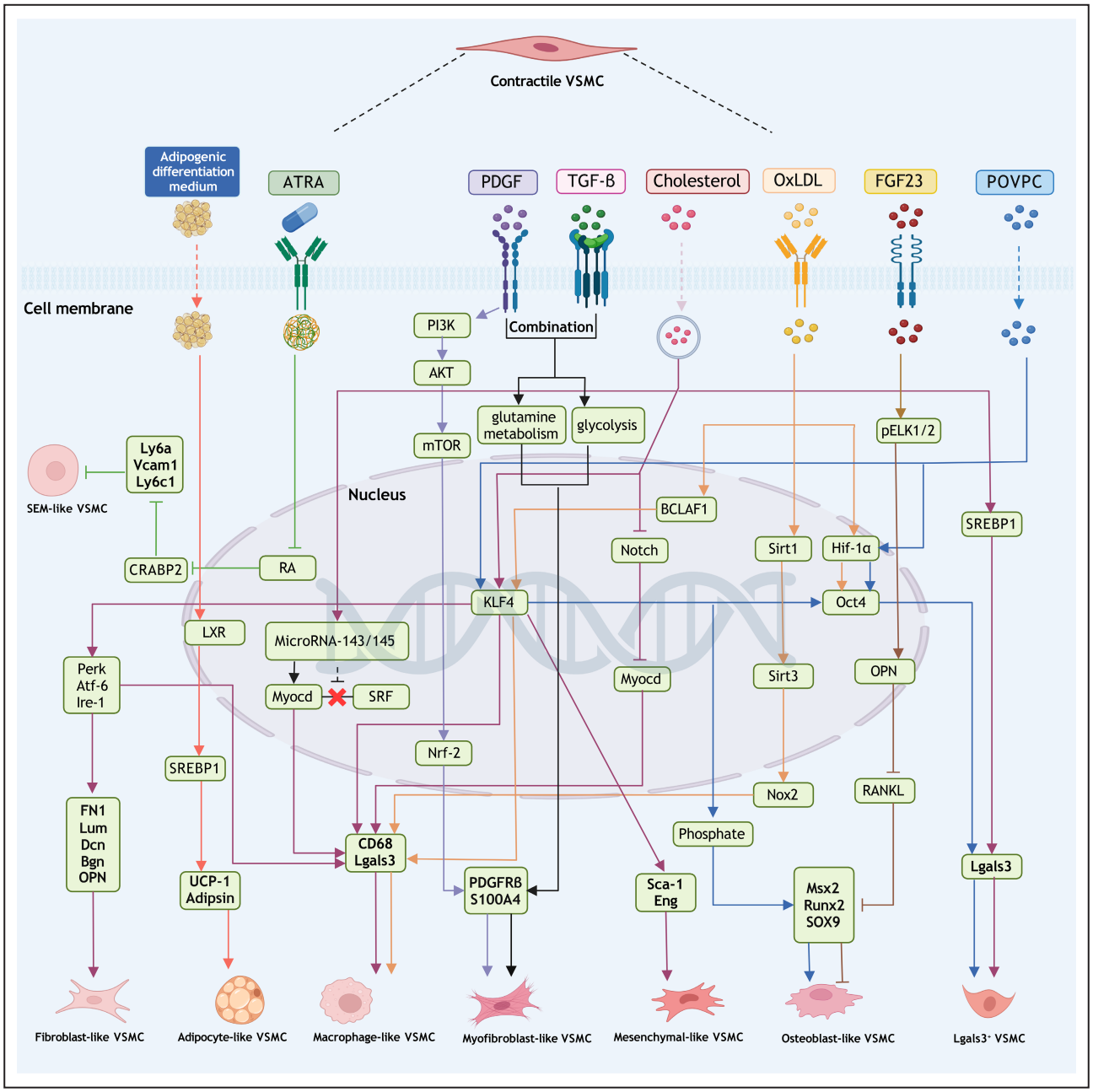

Figure 2. Extracellular stimuli and intracellular mediators of VSMC phenotypic transitions.
The colored lines indicate the respective pathways from the different extracellular stimuli (PDGF,[21,30] TGF-β,[21] cholesterol,[13,31–33] OxLDL,[34–36] FGF23,[37] ATRA,[14] POVPC,[34,38] and adipogenic differentiation medium[27]) via the highlighted intracellular mediators to induce the transition of contractile VSMCs to various dedifferentiated VSMCs (fibroblast-like VSMCs, adipocyte-like VSMCs, macrophage-like VSMCs, myofibroblast-like VSMCs, mesenchymal-like VSMCs, osteoblast-like VSMCs, and Lgals3⁺ VSMCs). ↑ indicates activation, and T represents inhibition. AKT indicates AKT serine/threonine kinase 1; Atf-6, activating transcription factor 6; ATRA, all-trans-retinoic acid; BCLAF1, BCL2-associated transcription factor 1; Bgn, biglycan; CRABP2, cellular retinoic acid-binding protein 2; CD68, cluster of differentiation 68; Dcn, decorin; Eng, endoglin; FGF23, fibroblast growth factor 23; Hif-1α, hypoxia inducible factor 1 subunit alpha; FN1, fibronectin 1; KLF4, Krüppel-like factor 4; Ire-1, serine/threonine-protein kinase 1; Lgals3, galectin 3; Lum, lumican; Ly6a, lymphocyte antigen 6 family member A; Ly6c1, lymphocyte antigen 6 family member C1; LXR, liver X receptor; Msx2, Msh homeobox 2; Nrf-2, nuclear factor-erythroid 2-related factor 2; OxLDL, oxidized low-density lipoprotein; PDGF, platelet-derived growth factor; mTOR, mechanistic target of rapamycin kinase; Oct-4, POU class 5 homeobox 1; OPN, osteophosphorin; Myocd, myocardin; OPN, osteophosphorin; PDGFRβ, platelet-derived growth factor receptor beta; Pert, PKR-like ER kinase; PI3K, phosphatidylinositol 3-kinase; pELK1/2, phospho-ETS transcription factor 1 and 2; RA, retinoic acid; Runx2, runt-related transcription factor 2; S100A4, S100 calcium-binding protein A4; Sca-1, stem cell antigen-1; SEM-like VSMC, stem cell/endothelial cell/monocyte cell-like VSMC; Sox9, sex-determining region Y-box 9; SRF, serum response factor; Sirt1, sirtuin 1; Sirt3, sirtuin 3; SREBP1, sterol regulatory element-binding protein 1; TGF-β, transforming growth factor-β; POVPC, 1-palmitoyl-2-(5′-oxo-valeroyl)-sn-glycero-3-phosphocholine; UCP-1, uncoupling protein 1; Vcam1, vascular cell adhesion molecule 1; and VSMC, vascular smooth muscle cell.

cellular retinoic acid binding protein 2 may induce the phenotypic switch of contractile VSMCs to SEM-like cells, thereby promoting atherosclerosis progression.[14]

Lgals3⁺ Cells

Lgals3 (also referred to as galectin-3) is considered a marker of macrophage-like cells.[13,31] Rong et al detected a population of VSMCs that expressed Lgals3 following cholesterol loading in vitro.[31] Recently, Alencar et al found that *Lgals3* activation is not a specific marker of the differentiation of VSMCs to a macrophage-like state but rather it is a marker of VSMCs entering a transitional state, with increased expression of genes associated with stem cells that are capable of extracellular matrix remodeling.[16] Of note, similar to SEM-like cells, Lgals3⁺ cells also have increased expression of lymphocyte antigen 6 family member A and vascular cell adhesion molecule 1. Further studies to investigate if SEM-like cells are derived from Lgals3⁺ cells are warranted.

Using mouse, rat, and human models of cholesterol-loading in VSMCs, Li et al found that SREBP1 (sterol regulatory-element binding protein-1) and Krüppel-like factor-15 induced up- and downregulation of Lgals3, respectively, via binding to the *Lgals3* gene promoter (albeit at different sites).[45] Likewise, Lgals3 promoted *SREBP1* gene expression, producing a feedforward loop upregulated by cholesterol loading.[45] Moreover, Lgals3 and SREBP1 downregulated myocardin-related transcription factor A expression in VSMCs.[45] In another study, Owsiany et al used a dual lineage tracing model and found that Lgals3⁺ VSMCs produce monocyte chemoattractant protein 1, a proinflammatory chemokine.[15] Knockout of monocyte chemoattractant protein 1 specifically in Lgals3⁺ VSMCs resulted in the formation of atherosclerotic lesions with a greater ACTA2 content in the fibrous cap and decreased Lgals3⁺ cell content, a feature of stable plaque.[15] Another study showed that deletion of the *Has3* (smooth muscle cell hyaluronan synthase 3) gene in mouse promoted VSMC transition to a Lgals3⁺ state.[46]

Dedifferentiated VSMCs

Macrophage-Like VSMCs

Macrophage-like VSMCs were first reported by Faggiotto et al, who observed lipid-laden VSMCs in nonhuman primate plaque sections.[47] Subsequently, Rong et al found that with an increase in cholesterol load, the expression of contractile genes in cultured VSMCs, decreased while the expression of macrophage markers (CD68 and Lgals3) was upregulated.[31] Using a lineage tracing approach, Feil et al demonstrated that VSMCs could differentiate into a macrophage-like phenotype in vivo.[18]

It is long believed that during atherogenesis, circulating monocytes migrate through the endothelium into the arterial wall where they differentiate into macrophages and can subsequently internalize lipids to become lipid-laden foam cells.[4,5] However, more recent studies have now indicated that ≈50% of foam cells found in human atherosclerotic plaques are derived from VSMCs and not from myeloid cells,[28] a finding also replicated in a mouse model of atherosclerosis.[48] As such, many of the intimal foam cells that were previously thought of being derived from monocytes/macrophages are probably instead VSMCs in a macrophage-like state. Interestingly, compared with macrophages, macrophage-like VSMCs differ in their transcriptome and functional properties, and their ability to destabilize atherosclerotic plaques is significantly lower.[29,48] Further, as macrophage-like VSMCs have low level of lysosomal acid lipase, they have lower ability to catabolize lipoproteins and therefore are more likely to become foam cells.[29,48] However, it is unknown whether VSMCs acquire a macrophage-like phenotype and then become foam cells or whether they express macrophage markers after becoming foam cells.

Li et al generated a dual orthogonal recombination mouse model using *Dre-rox* and *Cre-loxP* to specifically trace VSMCs and discovered a VSMC subtype expressing macrophage markers (CD45, CD11b, F4/80, and CD68) that may revert to VSMCs or transition to fibroblasts or pericytes during atherosclerosis progression.[20] As such, multiple cell lineages can be derived from macrophage-like VSMCs.[20]

Studies have indicated that macrophage-like VSMCs undergo a series of gene expression changes during the transition, with upregulation of lysosome- and inflammation-associated genes.[32] There is evidence suggesting that during atherogenesis, ATP-binding cassette transporter-1 expression is reduced and therefore intracellular cholesterol efflux is hampered, resulting in intracellular lipid overload, leading to the formation of macrophage-like VSMCs.[32,33] Sustaining the expression levels of SMC contraction-related genes (such as myocardin, miR-143/145, and cardiac mesoderm enhancer-associated noncoding RNA) has been shown to arrest and even reverse this pathological process.[48,49]

Myofibroblast-Like VSMCs

Hao et al found a subpopulation of VSMCs in the intima of human atherosclerotic lesions that had reduced or completely lost expression of MYH11 and SMTN, and displayed characteristics of myofibroblasts.[50] The myofibroblast cell is phenotypically intermediate between fibroblasts and VSMCs.[51]

To date, there are no specific markers to distinguish myofibroblast-like VSMCs from VSMCs. Some potential markers include platelet-derived growth factor

receptor beta and S100A4.[13,21,22] Of note, the contraction mechanism of myofibroblasts is different from that of VSMCs.[52,53] Thus, it is possible that analyzing the contractile characteristics can help distinguish myofibroblast-like VSMCs from VSMCs.

In vitro studies have shown that VSMCs can be dedifferentiated to a myofibroblast-like VSMC state by stimulating VSMCs with platelet-derived growth factor and transforming growth factor-β.[21,54] In vivo studies have suggested that myofibroblast-like VSMCs are derived from a subset of tenascin C VSMCs recruited from the tunica media.[55]

Myofibroblasts are rarely found in healthy humans, but studies indicate that VSMCs can dedifferentiate into myofibroblast-like VSMCs during atherogenesis and the accumulation of such cells is associated with thrombotic complications.[50,56] A study by Benavente et al suggests that VSMC dedifferentiation into myofibroblast-like cells is harmful at the late stages of human atherosclerotic plaque development, possibly by becoming a scaffold for calcification.[57]

Fibroblast-Like VSMCs

With the use of the single-cell RNA-sequencing technique, Wirka et al detected the presence of fibroblast-like VSMCs (referred to as fibromyocytes) in human and mouse atherosclerotic lesions, which exhibited increased expression of fibroblast-related genes.[23] In contrast to myofibroblast-like VSMCs, fibroblast-like VSMCs express markers associated with both myofibroblasts (Acta2) and intermediate state VSMCs (stem cell antigen-1 and Lgals3).[23] Furthermore, fibronectin 1, TNF receptor superfamily member 11b (also referred to as osteoprotegrin), and small leucine-rich proteoglycans, such as lumican, decorin, and biglycan, are significantly increased during phenotype switching to a fibroblast-like cell type.[23] Interestingly, instead of reflecting division into different lineages, these markers seem to suggest evolution along a single path of phenotypic switching. The authors further demonstrated that VSMC-specific knockout of transcription factor 21 (TCF21) inhibited VSMC phenotypic transition in mice and consequently resulted in fewer fibromyocytes in atherosclerotic lesions, revealing an important role of TCF21 in VSMC phenotypic switching to fibroblasts.[23] Furthermore, the authors found that there was a strong association between TCF21 expression and VSMC phenotypic modulation in human atherosclerotic coronary arteries, with higher TCF21 expression levels being associated with lower risk of coronary artery disease, indicating that TCF21 and SMC phenotypic modulation play a protective role.[23]

A recent study indicates that intracellular lipid overload induces the formation of fibroblast-like VSMCs through pathways involving Perk, Ire 1α, and ATF6, and thereby promotes atherogenesis.[33] Additionally, another recent study suggests that basic helix–loop–helix ARNT like 1 promotes the differentiation of VSMCs to fibroblast-like cells by upregulating the expression of Yes1 associated transcriptional regulator, thereby stabilizing atherosclerotic plaques in humans and mice.[24]

Whether myofibroblast-like VSMCs are an intermediate phenotype of fibroblast-like VSMCs transitioning from VSMCs or that myofibroblast-like VSMCs are an alternative path taken by cells on the VSMC to fibroblast-like cell transition remains to be clarified.

Osteoblast-Like VSMCs and Chondrocyte-Like VSMCs

Vascular calcification is a process of ectopic deposition of hydroxyapatite crystals into the intima and media. It is prevalent in advanced atherosclerotic lesions, with microcalcifications (calcium deposits <50 mm) being more often detected in the fibrous cap, while macrocalcifications (calcium deposits >200 mm) are more often seen in the deep intima adjacent to the internal elastic lamella and tunica media.[58–60] Microcalcification is associated with an increased risk of plaque rupture, whereas macrocalcification increases plaque stability.[61]

Speer et al reported bone-like tissue in calcified vascular lesions associated with VSMC transitioning to osteoblast-like and chondrocyte-like cells in mice.[25] Similarly, chondrocyte-like VSMCs have been detected in areas surrounding the necrotic cores of human atherosclerotic plaques.[62]

The change of VSMCs from the contractile to chondrocyte and osteoblast-like phenotype is characterized by the appearance of calcifying vesicles, reduced expression of mineralization inhibitory molecules and production of a calcification-prone matrix.[63] This process is accompanied by the loss of expression of contractile markers and upregulation of chondrocyte-like and osteoblast-like markers, including Runt-related transcription factor 2, SRY-box transcription factor 9, osteopontin, osteocalcin, alkaline phosphatase, osterix, and types II and X collagen.[64,65]

Mechanistically, VSMC transition to osteoblast-like cells is influenced by several factors including the calcium–phosphorus balance, the amounts of calcification inhibitors, mitochondrial dysfunction, endoplasmic reticulum stress, and glucose metabolism.[66–70]

Adipocyte-Like VSMCs

Davies et al reported that VSMCs could undergo phenotypic transition from a contractile to an adipocyte-like phenotype in vitro.[27] Cultured VSMCs were exposed to an adipogenic differentiation medium for 21 days, which resulted in reduced expression of contractile markers, while the expression of adipocyte markers

(such as uncoupling protein 1, adipsin, adipocyte fatty acid-binding protein, CCAAT enhancer binding protein alpha) was upregulated.[27]

Long et al, using an in vivo fate mapping approach, found that at least a subset of beige fat cells originated from VSMCs.[26] A mouse model exposed to cold was found to undergo VSMC to beige adipocyte-like cell differentiation, with the expression of thermogenic adipocyte markers.[26] Further, overexpression of PR domain containing 16, a regulator of beige adipocyte differentiation, in VSMCs in vitro resulted in the differentiation of VSMCs into beige adipocyte-like cells. PR domain containing 16 inhibits the expression of SRF and thereby promotes transition of VSMCs to beige adipocyte-like cells expressing the thermogenic gene *UCP-1*, which aggravates the inflammatory response and vascular remodeling after injury.[26,71] During the development of atherosclerotic lesions, VSMCs take up lipoprotein through sterol regulatory-element binding protein-1/LXR and MAPK pathways and develop an adipocyte-like phenotype with increased expression of proinflammatory and proliferative markers.[27,72]

To some extent, the phagocytosis of lipids by VSMCs may be a marker of VSMC to foam cell phenotype transition. Therefore, it cannot be ruled out that adipocyte-like VSMCs may be a transitional stage of VSMC differentiation to foam cells.

METHODOLOGIES FOR STUDYING VSMC PHENOTYPIC SWITCHING

The research field of VSMC phenotypic switching has been facilitated by the development and availability of a number of techniques, each with its advantages and limitations as discussed below.

Cell Culture

Induction of phenotype switching of VSMCs in culture can be achieved by incubating cells with relevant stimuli (Figure 3). For example, VSMC transition to macrophage-like VSMCs can be induced by serum starvation followed by treatment with 10 μg/mL methyl-β-cyclodextrin cholesterol for 48 to 72 hours, as well as by treatment with soluble cholesterol or oxidized low-density lipoprotein,[31,32] while switching to osteoblast-like VSMCs can be stimulated by culturing VSMCs in calcification medium (containing 10 mmol/L β-glycerophosphate, 100 μmol/L insulin, and 50 μg/mL ascorbic acid) for 14 days.[73–75] A limitation of in vitro experiments of cultured cells is that they cannot recapitulate the in vivo environment.

Mouse Models for Cell Lineage Tracing

Several mouse lines for cell lineage tracing have been developed and used for studying VSMC phenotypic switching. The advantages and disadvantages of these different lines are summarized in Table 2 and discussed below. Some useful features of some of these lines are also highlighted.

Myosin Heavy Chain 11-Cre Mice

Myosin heavy chain 11 (Myh11) is considered to be a specific marker for the smooth muscle lineage in humans. Similarly, it is suggested that Myh11 is highly specific to VSMCs in embryonic and adult mice[88]; however, some studies have shown that Myh11 is also expressed in some non-SMC cells (such as alveoli myofibroblasts and pericytes), although these cells might originate from VSMCs.[77,78]

A *Myh11-CreER*^{T2} mouse model has been used in many studies for VSMC-specific lineage tracing. This model has been used to successfully identify fibroblast-like VSMCs, mesenchymal-like VSMCs, and myofibroblast-like VSMCs.[13,21,23] In this model, *Myh11*-driven Cre is linked with an estrogen T2 receptor[76] and the expression of MYH11 is induced by tamoxifen.[89] A limitation of the *Myh11-CreER*^{T2} mouse model is that the *Myh11* transgene is located on the Y chromosome, and therefore it is not useful for studies of female mice. Liao et al sought to address this issue by building an X-linked *Myh11-CreER*^{T2} mouse model; however, X-linked inactivation led to mosaicism in the labeling of SMCs in female mice.[90] The issue has recently been overcome by Deaton et al who created a chromosome 2 *Myh11-CreER*^{T2} mouse (referred to as *Myh11-CreER*^{T2}-RAD) that can be used in both male and female mice.[79]

Tagln-Cre Mice

Tagln-Cre mice, also known as *SM22α-Cre* mice, has also been used in many studies of VSMC phenotypic switching.[18,25,91] Osteoblast-like VSMCs and macrophage-like VSMCs were identified using this model.[18,24] A limitation of this model is that *Tagln* is transiently expressed in cardiomyocytes and skeletal myocytes during embryogenesis[80] and has also been detected in myeloid cells and primary human adipose stromal vascular cells.[81,82] Its expression in adipose stromal vascular cells can be particularly confounding in examination of VSMC lineage in phenotype switching studies. In response to the nonspecific expression of *Cre* recombinase, He et al reported a dual recombinase-mediated genetic lineage tracing technique.[92] The combination of the Dre-rox recombination system permits rigorous control of potential unintentional *Cre-loxP* recombination, effectively improving the accuracy of the traditional *Cre-loxP* approach in lineage tracing.

ACTA2-Cre Mice

ACTA2 is a key actin involved in cell contraction and is currently considered as a marker for myofibroblasts.[93]

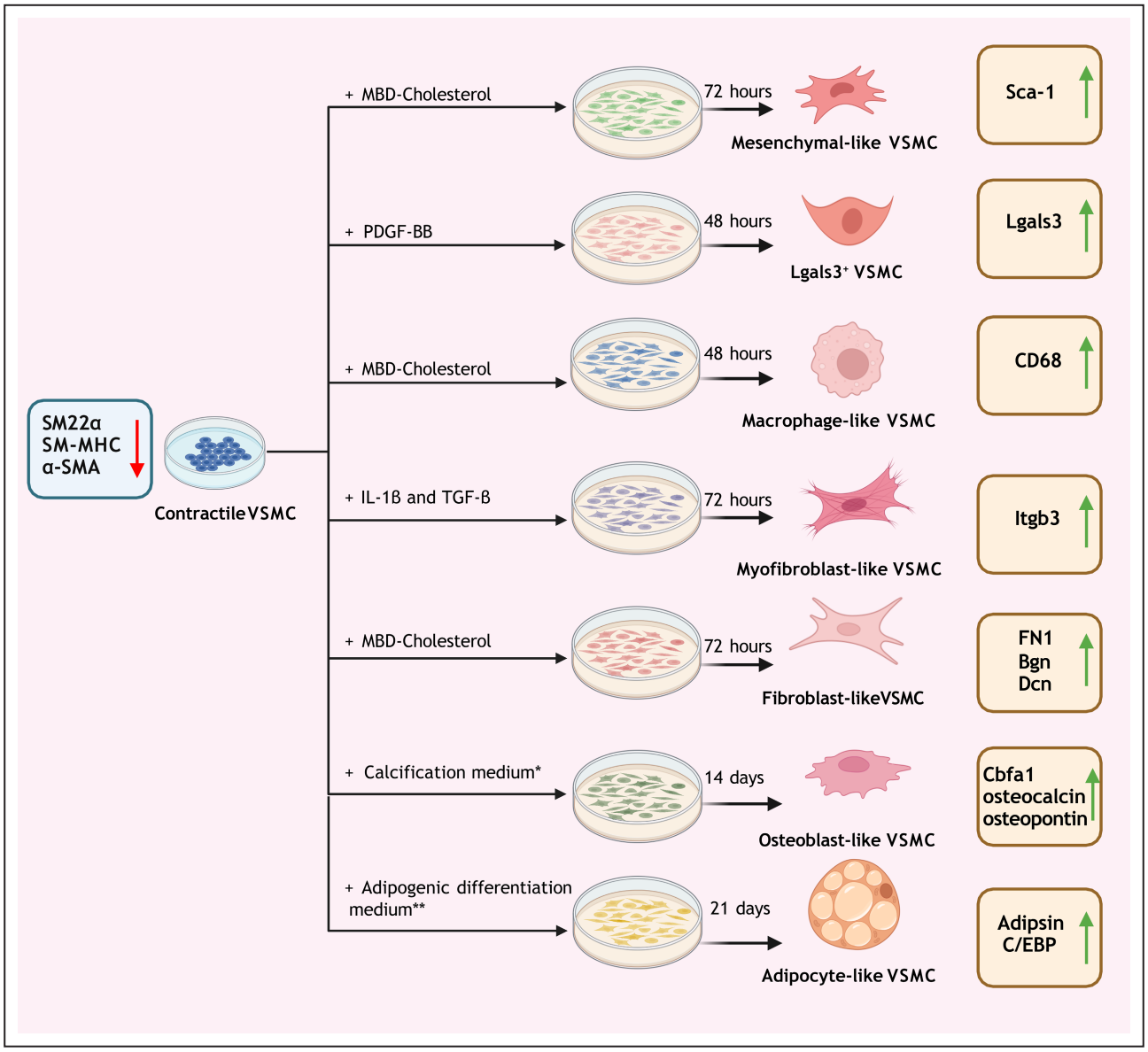

Figure 3. Inducers of VSMC phenotypic transitions in vitro.
Induction conditions for transformation of VSMC phenotypes (mesenchymal stem cell-like,[13] Lgals3+,[49] macrophage-like,[31–33] myofibroblast-like,[21] fibroblast-like,[23] osteoblast-like,[70] and adipocyte-like[27]). *The calcification medium consists of Dulbecco's Modified Eagle Medium containing sodium pyruvate, 15% fetal bovine serum, 10 mmol/L β-glycerophosphate, 100 μmol/L insulin, and 50 μg/mL ascorbic acid.[73–75] **The adipogenic differentiation medium consists of Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1, by volume) or Medium 199, containing 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 33 μmol/L biotin, 17 μmol/L pantothenate, 1.2 μmol/L human insulin, 100 nmol/L dexamethasone, 1 nmol/L triiodothyronine, 0.25 mmol/L 3-isobutyl-1-methylxanthine, and antibiotics.[27] α-SMA indicates α-smooth muscle actin; Lgals3, galectin 3; Bgn, biglycan; Cbfa1, runt-related transcription factor 2; CD68, cluster of differentiation 68; C/EBP, CCAAT/enhancer binding protein; Dcn, decorin; FN1, fibronectin 1; IL-1β, interleukin-1β; Itgb3, integrin beta 3; MBD-Cholesterol, methyl-beta-cyclodextrin cholesterol; PDGF-BB, platelet-derived growth factor-BB; SM22α, transgelin; TGF-β, transforming growth factor-β; Sca-1, stem cell antigen-1; SM-MHC, myosin heavy chain 11; and VSMC, vascular smooth muscle cell.

Compared to *Myh11-CreER*^{T2} mice, *Acta2-CreER*^{T2} mice exhibit a more rigorous labeling ability on specific organs (eg, pulmonary arterioles and alveoli).[77] In general, due to ACTA2 not being a decisive marker of the smooth muscle lineage, it is rarely used in tracing smooth muscle lineage and mainly used in fibrosis-related studies.[83,84]

Integrin Alpha 8-Cre Mice

Based on some of the aforementioned limitations, the *Itga8-CreER*^{T2} mouse has been developed. Warthi et al generated vascular smooth muscle-specific *Itga8-CreER*^{T2} mice.[87] Integrin alpha 8 (Itga8), a 160-kDa transmembrane protein, is highly expressed in

Table 2. Advantages and Disadvantages of Mouse Models for Vascular Smooth Muscle Cell Lineage Tracing in Atherosclerosis

Mouse line	Advantages or potentially useful features	Disadvantages
Myh11-Cre[76–78]	• Myh11 has a relatively high specificity as a VSMC marker. • Consistent expression level of Myh11 in VSMCs	• The *Myh11* transgene is located on the Y chromosome and so this line is unsuitable for studying female mice. • Some non-VSMCs (such as alveoli myofibroblasts and pericytes) may express Myh11.
Myh11-CreER^{T2}-RAD[79]	• Myh11 has a relatively high specificity as a VSMC marker. • Consistent expression level of Myh11 in VSMCs • Suitable for studying both male and female mice	• Some non-VSMCs (such as alveoli myofibroblasts and pericytes) may express Myh11.
Tagln-Cre[80–82]	• Low recombination leakage • Consistent expression level of Tagln in VSMCs • Suitable for studying both male and female mice	• Transgelin is expressed in some non-VSMCs such adipose stromal vascular cells and cardiomyocytes.
Acta2-Cre[77,83,84]	• Suitable for studying both male and female mice	• ACTA2 is expressed in several other cell-types such as myofibroblasts.
Itga8-Cre[79,85–87]	• Itga8 regulates VSMC contractile gene expression independently of the myocardin-SRF-CArG complex. • Low recombination leakage • Suitable for studying both male and female mice	• Itga8 is expressed in glomerular cells of the kidney. • Variable recombination leakage

ACTA indicates α-smooth muscle actin; CArG, CC(A/T-rich)6GG cis-regulating element; Itga8, integrin alpha 8; Myh11, myosin heavy chain 11; SRF, serum response factor; Tagln, transgelin; and VSMC, vascular smooth muscle cell.

VSMCs and VSMC-like cells but rarely expressed in mouse-, rat-, and human-derived visceral tissues containing smooth muscle. Itga8 has been reported to promote the VSMC contractile phenotype and inhibits vascular smooth muscle cell migration.[94] Genomic analysis of the *Itga8* gene revealed that it contains a proximal CArG box and a distal conserved CArG box in the region adjacent to the 5′ promoter. Most SMC contraction genes contain CArG elements, to which SRF/myocardin binds, but data indicates that neither SRF nor myocardin could activate the *Itga8* promoter in vitro, suggesting that Itga8 regulates VSMC contractile gene independently of the myocardin-SRF-CArG complex.[85] In addition, knockout of SRF in *Itga8-Cre*RE^{T2} mice reduced contractile protein expression, while *Itga8* expression was not affected, suggesting that *Itga8* was an ideal locus for targeting with an inducible *Cre*.[87] In a comprehensive comparative evaluation of recombination efficiency, recombination leakage, and expression localization with the widely used *Myh11-CreER*^{T2} mouse, *Itga8-CreER*^{T2} mice showed similar Cre activity in VSMCs but no significant myeloid Cre activity.[87] Further, because of its location on chromosome 2, the *Itga8-CreER*^{T2} construct can be used in lineage tracing and functional gene studies in both male and female mice.[87]

However, there are still some limitations to the application of *Itga8-CreER*^{T2} mice. Above all, *Itga8* expression has been detected in glomerular cells of the kidney.[86] Second, variable recombination of a reporter gene may produce more ambiguous findings in visceral VSMCs.[87] Third, there needs to be more documentation of the VSMC specificity of *Itga8* gene expression throughout the life span of the mouse.[79]

Immunofluorescence

Immunofluorescence staining can be used to detect the types of VSMCs in tissues. A novel immunofluorescence technique known as in situ hybridization/proximity ligation assay warrants highlighting. This technique is capable of visualizing histone modifications in genes of interest in individual cells in tissue samples.[95] It has been used to study VSMC phenotypic switching as histone 3 lysine four dimethylation enrichment in the CArG region of the *MYH11* gene is considered a specific epigenetic feature of the VSMC lineage.[96] This technique has an advantage over some other methods, such as chromatin immunoprecipitation, which is unable to detect histone modifications in individual cells.

Flow Cytometry

This technique can be used to sort cells according to the levels of VSMC phenotype protein markers (Table 1). A limitation of this technique is that there is no known cell surface marker for VSMCs. Another limitation is that some antibodies lack specificity or are not amenable to cell sorting by cytometry. Furthermore, some cell types have overlapping marker protein expression patterns and are therefore difficult to separate by cytometry.

Single-Cell RNA Sequencing

The single-cell RNA sequencing technique provides transcriptomic data of individual cells and therefore can be applied to identify VSMCs of different types according to their gene expression profiles. A limitation of this technique is that it requires lysing cells and therefore does not allow further molecular and functional

analyses on the same cells. This issue can be overcome by the living cell transcriptome sequencing (Live-seq) technique recently described by Chen et al, which preserves cell viability during RNA extraction.[97] This method can be used to relate transcriptomic data with further functional analyses of VSMC phenotypic transitions.

Single-cell RNA sequencing has some other limitations: the procedure for the preparation of single cells (by, eg enzymatic digestion) can potentially lead to changes in gene expression; the levels of transcripts may not reflect the levels of proteins; the RNA content in single cells is low, and therefore amplifications by many folds is required with potential introduction of bias; and this technique does not provide information about the spatial locations of cells.[98] The latter limitation could potentially overcome by the spatial transcriptomics technique.[99]

SUMMARY

Studies to date have provided substantial insights into VSMC plasticity and phenotypic switching, and its roles in atherosclerosis.

Currently, there is no consensus on a classification of VSMC phenotypes. This review summarizes the 9 VSMC phenotypes that have been reported in atherosclerotic lesions and has attempted to classify them into 3 categories: differentiated VSMCs, intermediately dedifferentiated VSMCs, and dedifferentiated VSMCs. We acknowledge that this tentative classification may be too simplistic, and it is possible that there are overlaps between the different phenotypes; for example, Wirka et al used the term *fibromyocyte* to indicate cells with a gene expression profile intermediate between VSMCs and fibroblasts.[23]

It is presently unclear as to whether the various types of dedifferentiated VSMCs discussed here have the biological functions of the implied cell types (eg, if, and to what extent, macrophage-like VSMCs can function as macrophages) and whether they can progressively transition to eventually lose their VSMC characteristics and become a completely different cell type (such as adipocyte). More research into these questions is required.

Further, more studies are needed to better clarify the roles of the different types of dedifferentiated VSMCs in the development and progression of atherosclerosis, and the relative contributions of these different types of cells to these processes. A more complete understanding of the role of VSMC phenotype transition and the relative contributions of the different VSMC phenotypes can potentially aid the identification of new therapeutic targets and the development of new drugs that can modulate VSMC phenotypes and inhibit atherosclerosis progression.

Affiliations

Shantou University Medical College, Shantou, China (R.C., X.H., S.Y.); Department of Cardiovascular Sciences, University of Leicester, Leicester, United Kingdom (D.G.M., S.Y.); Research Center for Translational Medicine, The Second Affiliated Hospital of Shantou University Medical College, Shantou, China (D.S.); and Cardiovascular-Metabolic Disease Translational Research Programme, National University of Singapore, Singapore (S.Y.).

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Disclosures

None.

REFERENCES

1. Ross R. The pathogenesis of atherosclerosis—an update. *N Engl J Med*. 1986;314:488–500. doi: 10.1056/NEJM198602203140806
2. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809. doi: 10.1038/362801a0
3. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol*. 1995;15:551–561. doi: 10.1161/01.atv.15.5.551
4. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999;340:115–126. doi: 10.1056/NEJM199901143400207
5. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874. doi: 10.1038/nature01323
6. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res*. 2012;95:156–164. doi: 10.1093/cvr/cvs115
7. Liu M, Gomez D. Smooth muscle cell phenotypic diversity. *Arterioscler Thromb Vasc Biol*. 2019;39:1715–1723. doi: 10.1161/ATVBAHA.119.312131
8. Yap C, Mieremet A, de Vries CJM, Micha D, de Waard V. Six shades of vascular smooth muscle cells illuminated by KLF4 (Kruppel-like factor 4). *Arterioscler Thromb Vasc Biol*. 2021;41:2693–2707. doi: 10.1161/ATVBAHA.121.316600
9. Cao G, Xuan X, Hu J, Zhang R, Jin H, Dong H. How vascular smooth muscle cell phenotype switching contributes to vascular disease. *Cell Commun Signal*. 2022;20:180. doi: 10.1186/s12964-022-00993-2
10. Liu R, Jin Y, Tang WH, Qin L, Zhang X, Tellides G, Hwa J, Yu J, Martin KA. Ten-eleven translocation-2 (TET2) is a master regulator of smooth muscle cell plasticity. *Circulation*. 2013;128:2047–2057. doi: 10.1161/CIRCULATIONAHA.113.002887
11. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995;75:487–517. doi: 10.1152/physrev.1995.75.3.487
12. Dobnikar L, Taylor AL, Chappell J, Oldach P, Harman JL, Oerton E, Dzierzak E, Bennett MR, Spivakov M, Jorgensen HF. Disease-relevant transcriptional signatures identified in individual smooth muscle cells from healthy mouse vessels. *Nat Commun*. 2018;9:4567. doi: 10.1038/s41467-018-06891-x
13. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, Swiatlowska P, Newman AA, Greene ES, Straub AC, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nat Med*. 2015;21:628–637. doi: 10.1038/nm.3866
14. Pan HZ, Xue CY, Auerbach BJ, Fan JX, Bashore AC, Cui J, Yang DAY, Trignano SB, Liu W, Shi JT, et al. Single-cell genomics reveals a novel cell state during smooth muscle cell phenotypic switching and potential therapeutic targets for atherosclerosis in mouse and human. *Circulation*. 2020;142:2060–2075. doi: 10.1161/circulationaha.120.048378
15. Owsiany KM, Deaton RA, Soohoo KG, Tram Nguyen A, Owens GK. Dichotomous roles of smooth muscle cell-derived MCP1 (monocyte chemoattractant protein 1) in development of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2022;42:942–956. doi: 10.1161/ATVBAHA.122.317882
16. Alencar GF, Owsiany KM, Karnewar S, Sukhavasi K, Mocci G, Nguyen AT, Williams CM, Shamsuzzaman S, Mokry M, Henderson CA, et al. Stem cell pluripotency genes Klf4 and Oct4 regulate complex SMC phenotypic changes critical in late-stage atherosclerotic

lesion pathogenesis. *Circulation*. 2020;142:2045–2059. doi: 10.1161/CIRCULATIONAHA.120.046672
17. Chappell J, Harman JL, Narasimhan VM, Yu H, Foote K, Simons BD, Bennett MR, Jorgensen HF. Extensive proliferation of a subset of differentiated, yet plastic, medial vascular smooth muscle cells contributes to neointimal formation in mouse injury and atherosclerosis models. *Circ Res*. 2016;119:1313–1323. doi: 10.1161/CIRCRESAHA.116.309799
18. Feil S, Fehrenbacher B, Lukowski R, Essmann F, Schulze-Osthoff K, Schaller M, Feil R. Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. *Circ Res*. 2014;115:662–667. doi: 10.1161/CIRCRESAHA.115.304634
19. Rykaczewska U, Zhao Q, Saliba-Gustafsson P, Lengquist M, Kronqvist M, Bergman O, Huang Z, Lund K, Waden K, Pons Vila Z, et al. Plaque evaluation by ultrasound and transcriptomics reveals BCLAF1 as a regulator of smooth muscle cell lipid transdifferentiation in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2022;42:659–676. doi: 10.1161/ATVBAHA.121.317018
20. Li Y, Zhu H, Zhang Q, Han X, Zhang Z, Shen L, Wang L, Lui KO, He B, Zhou B. Smooth muscle-derived macrophage-like cells contribute to multiple cell lineages in the atherosclerotic plaque. *Cell Discov*. 2021;7:111. doi: 10.1038/s41421-021-00328-4
21. Newman AAC, Serbulea V, Baylis RA, Shankman LS, Bradley X, Alencar GF, Owsiany K, Deaton RA, Karnewar S, Shamsuzzaman S, et al. Multiple cell types contribute to the atherosclerotic lesion fibrous cap by PDGFRbeta and bioenergetic mechanisms. *Nat Metab*. 2021;3:166–181. doi: 10.1038/s42255-020-00338-8
22. Brisset AC, Hao H, Camenzind E, Bacchetta M, Geinoz A, Sanchez JC, Chaponnier C, Gabbiani G, Bochaton-Piallat ML. Intimal smooth muscle cells of porcine and human coronary artery express S100A4, a marker of the rhomboid phenotype in vitro. *Circ Res*. 2007;100:1055–1062. doi: 10.1161/01.RES.0000262654.84810.6c
23. Wirka RC, Wagh D, Paik DT, Pjanic M, Nguyen T, Miller CL, Kundu R, Nagao M, Coller J, Koyano TK, et al. Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis. *Nat Med*. 2019;25:1280–1289. doi: 10.1038/s41591-019-0512-5
24. Shen Y, Xu LR, Yan D, Zhou M, Han TL, Lu C, Tang X, Lin CP, Qian RZ, Guo DQ. BMAL1 modulates smooth muscle cells phenotypic switch towards fibroblast-like cells and stabilizes atherosclerotic plaques by upregulating YAP1. *Biochim Biophys Acta Mol Basis Dis*. 2022;1868:166450. doi: 10.1016/j.bbadis.2022.166450
25. Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, Frutkin A, Dichek D, Giachelli CM. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res*. 2009;104:733–741. doi: 10.1161/CIRCRESAHA.108.183053
26. Long Jonathan Z, Svensson Katrin J, Tsai L, Zeng X, Roh Hyun C, Kong X, Rao Rajesh R, Lou J, Lokurkar I, Baur W, et al. A smooth muscle-like origin for beige adipocytes. *Cell Metab*. 2014;19:810–820. doi: 10.1016/j.cmet.2014.03.025
27. Davies JD, Carpenter KL, Challis IR, Figg NL, McNair R, Proudfoot D, Weissberg PL, Shanahan CM. Adipocytic differentiation and liver x receptor pathways regulate the accumulation of triacylglycerols in human vascular smooth muscle cells. *J Biol Chem*. 2005;280:3911–3919. doi: 10.1074/jbc.M410075200
28. Allahverdian S, Chehroudi AC, McManus BM, Abraham T, Francis GA. Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation*. 2014;129:1551–1559. doi: 10.1161/CIRCULATIONAHA.113.005015
29. Dubland JA, Allahverdian S, Besler KJ, Ortega C, Wang Y, Pryma CS, Boukais K, Chan T, Seidman MA, Francis GA. Low LAL (lysosomal acid lipase) expression by smooth muscle cells relative to macrophages as a mechanism for arterial foam cell formation. *Arterioscler Thromb Vasc Biol*. 2021;41:e354–e368. doi: 10.1161/ATVBAHA.120.316063
30. Zhang P, Guan Y, Chen J, Li X, McConnell BK, Zhou W, Boini KM, Zhang Y. Contribution of p62/SQSTM1 to PDGF-BB-induced myofibroblast-like phenotypic transition in vascular smooth muscle cells lacking Smpd1 gene. *Cell Death Dis*. 2018;9:1145. doi: 10.1038/s41419-018-1197-2
31. Rong JX, Shapiro M, Trogan E, Fisher EA. Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proc Natl Acad Sci U S A*. 2003;100:13531–13536. doi: 10.1073/pnas.1735526100
32. Zhang Z, Huang J, Wang Y, Shen W. Transcriptome analysis revealed a two-step transformation of vascular smooth muscle cells to macrophage-like cells. *Atherosclerosis*. 2022;346:26–35. doi: 10.1016/j.atherosclerosis.2022.02.021
33. Chattopadhyay A, Kwartler CS, Kaw K, Li Y, Kaw A, Chen J, LeMaire SA, Shen YH, Milewicz DM. Cholesterol-induced phenotypic modulation of smooth muscle cells to macrophage/fibroblast-like cells is driven by an unfolded protein response. *Arterioscler Thromb Vasc Biol*. 2021;41:302–316. doi: 10.1161/ATVBAHA.120.315164
34. Cherepanova OA, Gomez D, Shankman LS, Swiatlowska P, Williams J, Sarmento OF, Alencar GF, Hess DL, Bevard MH, Greene ES, et al. Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. *Nat Med*. 2016;22:657–665. doi: 10.1038/nm.4109
35. Burger F, Baptista D, Roth A, da Silva RF, Montecucco F, Mach F, Brandt KJ, Miteva K. NLRP3 inflammasome activation controls vascular smooth muscle cells phenotypic switch in atherosclerosis. *Int J Mol Sci*. 2021;23:340. doi: 10.3390/ijms23010340
36. Chen Z, Xue Q, Cao L, Wang Y, Chen Y, Zhang X, Xiao F, Yang Y, Hayden MR, Liu Y, et al. Toll-like receptor 4 mediated oxidized low-density lipoprotein-induced foam cell formation in vascular smooth muscle cells via Src and Sirt1/3 pathway. *Mediators Inflamm*. 2021;2021:6639252. doi: 10.1155/2021/6639252
37. Nakahara T, Kawai-Kowase K, Matsui H, Sunaga H, Utsugi T, Iso T, Arai M, Tomono S, Kurabayashi M. Fibroblast growth factor 23 inhibits osteoblastic gene expression and induces osteoprotegerin in vascular smooth muscle cells. *Atherosclerosis*. 2016;253:102–110. doi: 10.1016/j.atherosclerosis.2016.08.010
38. Yoshida T, Yamashita M, Hayashi M. Kruppel-like factor 4 contributes to high phosphate-induced phenotypic switching of vascular smooth muscle cells into osteogenic cells. *J Biol Chem*. 2012;287:25706–25714. doi: 10.1074/jbc.M112.361360
39. Miano JM, Long X, Fujiwara K. Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *Am J Physiol Cell Physiol*. 2007;292:C70–C81. doi: 10.1152/ajpcell.00386.2006
40. Salmon M, Gomez D, Greene E, Shankman L, Owens GK. Cooperative binding of KLF4, pELK-1, and HDAC2 to a G/C repressor element in the SM22alpha promoter mediates transcriptional silencing during SMC phenotypic switching in vivo. *Circ Res*. 2012;111:685–696. doi: 10.1161/CIRCRESAHA.112.269811
41. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. *Ann Rev Physiol*. 2012;74:13–40. doi: 10.1146/annurev-physiol-012110-142315
42. Zhang F, Guo X, Xia Y, Mao L. An update on the phenotypic switching of vascular smooth muscle cells in the pathogenesis of atherosclerosis. *Cell Mol Life Sci*. 2021;79:6. doi: 10.1007/s00018-021-04079-z
43. Fasolo F, Paloschi V, Maegdefessel L. Long non-coding RNAs at the crossroad of vascular smooth muscle cell phenotypic modulation in atherosclerosis and neointimal formation. *Atherosclerosis*. 2022;374:34–43. doi: 10.1016/j.atherosclerosis.2022.11.021
44. Chen PY, Qin LF, Li GX, Malagon-Lopez J, Wang Z, Bergaya S, Gujja S, Caulk AW, Murtada SI, Zhang XB, et al. Smooth muscle cell reprogramming in aortic aneurysms. *Cell Stem Cell*. 2020;26:542–557.e11. doi: 10.1016/j.stem.2020.02.013
45. Li J, Shen H, Owens GK, Guo LW. SREBP1 regulates Lgals3 activation in response to cholesterol loading. *Mol Ther Nucleic Acids*. 2022;28:892–909. doi: 10.1016/j.omtn.2022.05.028
46. Hartmann F, Gorski DJ, Newman AAC, Homann S, Petz A, Owsiany KM, Serbulea V, Zhou YQ, Deaton RA, Bendeck M, et al. SMC-derived hyaluronan modulates vascular SMC phenotype in murine atherosclerosis. *Circ Res*. 2021;129:992–1005. doi: 10.1161/CIRCRESAHA.120.318479
47. Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis*. 1984;4:323–340. doi: 10.1161/01.atv.4.4.323
48. Vengrenyuk Y, Nishi H, Long X, Ouimet M, Savji N, Martinez FO, Cassella CP, Moore KJ, Ramsey SA, Miano JM, et al. Cholesterol loading reprograms the microRNA-143/145-myocardin axis to convert aortic smooth muscle cells to a dysfunctional macrophage-like phenotype. *Arterioscler Thromb Vasc Biol*. 2015;35:535–546. doi: 10.1161/ATVBAHA.114.304029
49. Vacante F, Rodor J, Lalwani MK, Mahmoud AD, Bennett M, De Pace AL, Miller E, Van Kuijk K, de Bruijn J, Gijbels M, et al. CARMN loss regulates smooth muscle cells and accelerates atherosclerosis in mice. *Circ Res*. 2021;128:1258–1275. doi: 10.1161/CIRCRESAHA.120.318688
50. Hao H, Gabbiani G, Camenzind E, Bacchetta M, Virmani R, Bochaton-Piallat ML. Phenotypic modulation of intima and media smooth muscle

cells in fatal cases of coronary artery lesion. *Arterioscler Thromb Vasc Biol*. 2006;26:326–332. doi: 10.1161/01.ATV.0000199393.74656.4c
51. Kacem K, Sercombe R. Similar pathological effects of sympathectomy and hypercholesterolemia on arterial smooth muscle cells and fibroblasts. *Acta Histochemica*. 2008;110:302–313. doi: 10.1016/j.acthis.2007.11.007
52. Hinz B, Gabbiani G. Mechanisms of force generation and transmission by myofibroblasts. *Curr Opin Biotechnol*. 2003;14:538–546. doi: 10.1016/j.copbio.2003.08.006
53. Gan Q, Yoshida T, Li J, Owens GK. Smooth muscle cells and myofibroblasts use distinct transcriptional mechanisms for smooth muscle alpha-actin expression. *Circ Res*. 2007;101:883–892. doi: 10.1161/circresaha.107.154831
54. Salamon A, Serbulea V, Deaton R, Owens G. Glutamine metabolism contributes to smooth muscle-to-myofibroblast transitions and enriched extracellular matrix production. *Free Radic Biol Med*. 2020;159:S74. doi: 10.1016/j.freeradbiomed.2020.10.194
55. Shi X, Zhu S, Liu M, Stone SS, Rong Y, Mao K, Xu X, Ma C, Jiang Z, Zha Y, et al. Single-cell RNA-seq reveals a population of smooth muscle cells responsible for atherogenesis. *Aging Dis*. 2022;13:1939–1953. doi: 10.14336/ad.2022.0313
56. Kendall RT, Feghali-Bostwick CA. Fibroblasts in fibrosis: novel roles and mediators. *Front Pharmacol*. 2014;5:123. doi: 10.3389/fphar.2014.00123
57. Benavente ED, Karnewar S, Buono M, Mili E, Hartman RJG, Kapteijn D, Slenders L, Daniels M, Aherrahrou R, Reinberger T, et al. Female gene networks are expressed in myofibroblast-like smooth muscle cells in vulnerable atherosclerotic plaques. *bioRxiv*. 2023. doi: 10.1101/2023.02.08.527690.
58. Hutcheson JD, Goettsch C, Bertazzo S, Maldonado N, Ruiz JL, Goh W, Yabusaki K, Faits T, Bouten C, Franck G, et al. Genesis and growth of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. *Nat Mater*. 2016;15:335–343. doi: 10.1038/nmat4519
59. Otsuka F, Sakakura K, Yahagi K, Joner M, Virmani R. Has our understanding of calcification in human coronary atherosclerosis progressed? *Arterioscler Thromb Vasc Biol*. 2014;34:724–736. doi: 10.1161/atvbaha.113.302642
60. Reutelingsperger C, Schurgers L. Coronary artery calcification: a Janus-faced biomarker? *JACC Cardiovasc Imaging*. 2018;11:1324–1326. doi: 10.1016/j.jcmg.2017.04.009
61. Shioi A, Ikari Y. Plaque calcification during atherosclerosis progression and regression. *J Atheroscler Thromb*. 2018;25:294–303. doi: 10.5551/jat.RV17020
62. Bobryshev YV. Transdifferentiation of smooth muscle cells into chondrocytes in atherosclerotic arteries in situ: implications for diffuse intimal calcification. *J Pathol*. 2005;205:641–650. doi: 10.1002/path.1743
63. Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res*. 2011;109:697–711. doi: 10.1161/CIRCRESAHA.110.234914
64. Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. *Cardiovasc Res*. 2018;114:590–600. doi: 10.1093/cvr/cvy010
65. Tyson KL, Reynolds JL, McNair R, Zhang Q, Weissberg PL, Shanahan CM. Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification. *Arterioscler Thromb Vasc Biol*. 2003;23:489–494. doi: 10.1161/01.ATV.0000059406.92165.31
66. Rocha-Singh KJ, Zeller T, Jaff MR. Peripheral arterial calcification: prevalence, mechanism, detection, and clinical implications. *Catheter Cardiovasc Interv*. 2014;83:E212–E220. doi: 10.1002/ccd.25387
67. Lee SJ, Lee IK, Jeon JH. Vascular calcification-new insights into its mechanism. *Int J Mol Sci*. 2020;21:2685. doi: 10.3390/ijms21082685
68. Hu CT, Shao YD, Liu YZ, Xiao X, Cheng ZB, Qu SL, Huang L, Zhang C. Oxidative stress in vascular calcification. *Clin Chim Acta*. 2021;519:101–110. doi: 10.1016/j.cca.2021.04.012
69. Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest*. 1994;93:2393–2402. doi: 10.1172/jci117246
70. Shi J, Yang Y, Cheng A, Xu G, He F. Metabolism of vascular smooth muscle cells in vascular diseases. *Am J Physiol Heart Circ Physiol*. 2020;319:H613–H631. doi: 10.1152/ajpheart.00220.2020
71. Adachi Y, Ueda K, Nomura S, Ito K, Katoh M, Katagiri M, Yamada S, Hashimoto M, Zhai B, Numata G, et al. Beiging of perivascular adipose tissue regulates its inflammation and vascular remodeling. *Nat Commun*. 2022;13:5117. doi: 10.1038/s41467-022-32658-6
72. Nguyen Dinh Cat A, Briones AM, Callera GE, Yogi A, He Y, Montezano AC, Touyz RM. Adipocyte-derived factors regulate vascular smooth muscle cells through mineralocorticoid and glucocorticoid receptors. *Hypertension*. 2011;58:479–488. doi: 10.1161/HYPERTENSIONAHA.110.168872
73. Shioi A, Nishizawa Y, Jono S, Koyama H, Hosoi M, Morii H. Beta-glycerophosphate accelerates calcification in cultured bovine vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1995;15:2003–2009. doi: 10.1161/01.atv.15.11.2003
74. Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, Schinke T, Karsenty G, Giachelli CM. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circ Res*. 2001;89:1147–1154. doi: 10.1161/hh2401.101070
75. Wada T, McKee MD, Steitz S, Giachelli CM. Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. *Circ Res*. 1999;84:166–178. doi: 10.1161/01.res.84.2.166
76. Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, Orsy P, Horvath B, Maser-Gluth C, Greiner E, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med*. 2008;14:64–68. doi: 10.1038/nm1666
77. Sheikh AQ, Lighthouse JK, Greif DM. Recapitulation of developing artery muscularization in pulmonary hypertension. *Cell Rep*. 2014;6:809–817. doi: 10.1016/j.celrep.2014.01.042
78. Wu Z, Yang L, Cai L, Zhang M, Cheng X, Yang X, Xu J. Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse. *Respir Res*. 2007;8:1. doi: 10.1186/1465-9921-8-1
79. Deaton RA, Bulut G, Serbulea V, Salamon A, Shankman LS, Nguyen AT, Owens GK. A new autosomal Myh11-CreER(T2) smooth muscle cell lineage tracing and gene knockout mouse model-brief report. *Arterioscler Thromb Vasc Biol*. 2023;43:203–211. doi: 10.1161/atvbaha.122.318160
80. Chakraborty R, Saddouk FZ, Carrao AC, Krause DS, Greif DM, Martin KA. Promoters to study vascular smooth muscle. *Arterioscler Thromb Vasc Biol*. 2019;39:603–612. doi: 10.1161/ATVBAHA.119.312449
81. Shen Z, Li C, Frieler RA, Gerasimova AS, Lee SJ, Wu J, Wang MM, Lumeng CN, Brosius FC III, Duan SZ, et al. Smooth muscle protein 22 alpha-Cre is expressed in myeloid cells in mice. *Biochem Biophys Res Commun*. 2012;422:639–642. doi: 10.1016/j.bbrc.2012.05.041
82. Sui Y, Park SH, Xu J, Monette S, Helsley RN, Han SS, Zhou C. IKKβ links vascular inflammation to obesity and atherosclerosis. *J Exp Med*. 2014;211:869–886. doi: 10.1084/jem.20131281
83. McGowan SE, McCoy DM. Regulation of fibroblast lipid storage and myofibroblast phenotypes during alveolar septation in mice. *Am J Physiol Lung Cell Mol Physiol*. 2014;307:L618–L631. doi: 10.1152/ajplung.00144.2014
84. Chu X, Lingampally A, Moiseenko A, Kheirollahi V, Vazquez-Armendariz AI, Koepke J, Khadim A, Kiliaris G, Shahriari Felordi M, Zabihi M, et al. GLI1+ cells are a source of repair-supportive mesenchymal cells (RSMCs) during airway epithelial regeneration. *Cell Mol Life Sci*. 2022;79:581. doi: 10.1007/s00018-022-04599-2
85. Kitchen CM, Cowan SL, Long X, Miano JM. Expression and promoter analysis of a highly restricted integrin alpha gene in vascular smooth muscle. *Gene*. 2013;513:82–89. doi: 10.1016/j.gene.2012.10.073
86. Valdivielso JM, Rodríguez-Puyol D, Pascual J, Barrios C, Bermúdez-López M, Sánchez-Niño MD, Pérez-Fernández M, Ortiz A. Atherosclerosis in chronic kidney disease: more, less, or just different? *Arterioscler Thromb Vasc Biol*. 2019;39:1938–1966. doi: 10.1161/atvbaha.119.312705
87. Warthi G, Faulkner JL, Doja J, Ghanam AR, Gao P, Yang AC, Slivano OJ, Barris CT, Kress TC, Zawieja SD, et al. Generation and comparative analysis of an Itga8-CreER (T2) mouse with preferential activity in vascular smooth muscle cells. *Nat Cardiovasc Res*. 2022;1:1084–1100. doi: 10.1038/s44161-022-00162-1
88. Miano JM, Cserjesi P, Ligon KL, Periasamy M, Olson EN. Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. *Circ Res*. 1994;75:803–812. doi: 10.1161/01.res.75.5.803
89. Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun*. 1997;237:752–757. doi: 10.1006/bbrc.1997.7124
90. Liao M, Zhou J, Wang F, Ali YH, Chan KL, Zou F, Offermanns S, Jiang Z, Jiang Z. An X-linked Myh11-CreER(T2) mouse line resulting from Y to

X chromosome-translocation of the Cre allele. *Genesis*. 2017;55:55. doi: 10.1002/dvg.23054
91. Feil S, Hofmann F, Feil R. SM22alpha modulates vascular smooth muscle cell phenotype during atherogenesis. *Circ Res*. 2004;94:863–865. doi: 10.1161/01.Res.0000126417.38728.F6
92. He L, Li Y, Li Y, Pu W, Huang X, Tian X, Wang Y, Zhang H, Liu Q, Zhang L, et al. Enhancing the precision of genetic lineage tracing using dual recombinases. *Nat Med*. 2017;23:1488–1498. doi: 10.1038/nm.4437
93. Mifflin RC, Pinchuk IV, Saada JI, Powell DW. Intestinal myofibroblasts: targets for stem cell therapy. *Am J Physiol Gastrointest Liver Physiol*. 2011;300:G684–G696. doi: 10.1152/ajpgi.00474.2010
94. Zargham R, Touyz RM, Thibault G. Alpha 8 integrin overexpression in de-differentiated vascular smooth muscle cells attenuates migratory activity and restores the characteristics of the differentiated phenotype. *Atherosclerosis*. 2007;195:303–312. doi: 10.1016/j.atherosclerosis.2007.01.005
95. Gomez D, Shankman LS, Nguyen AT, Owens GK. Detection of histone modifications at specific gene loci in single cells in histological sections. *Nat Methods*. 2013;10:171–177. doi: 10.1038/nmeth.2332
96. McDonald OG, Wamhoff BR, Hoofnagle MH, Owens GK. Control of SRF binding to CArG box chromatin regulates smooth muscle gene expression in vivo. *J Clin Invest*. 2006;116:36–48. doi: 10.1172/JCI26505
97. Chen W, Guillaume-Gentil O, Rainer PY, Gäbelein CG, Saelens W, Gardeux V, Klaeger A, Dainese R, Zachara M, Zambelli T, et al. Live-seq enables temporal transcriptomic recording of single cells. *Nature*. 2022;608:733–740. doi: 10.1038/s41586-022-05046-9
98. Slenders L, Tessels DE, van der Laan SW, Pasterkamp G, Mokry M. The applications of single-cell RNA sequencing in atherosclerotic disease. *Front Cardiovasc Med*. 2022;9:826103. doi: 10.3389/fcvm.2022.826103
99. Sun S, Zhu J, Zhou X. Statistical analysis of spatial expression patterns for spatially resolved transcriptomic studies. *Nat Methods*. 2020;17:193–200. doi: 10.1038/s41592-019-0701-7