Smooth muscle cells in atherosclerosis: clones but not carbon copies

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ABSTRACT

Our knowledge of the contribution of vascular smooth muscle cells (SMCs) to atherosclerosis has greatly advanced in the previous decade with the development of techniques allowing for the unambiguous identification and phenotypic characterization of SMC populations within the diseased vascular wall. By performing fate mapping or single-cell transcriptomics studies, or a combination of both, the field has made key observations: SMCs populate atherosclerotic lesions by the selective expansion and investment of a limited number of medial SMCs, which undergo profound and diverse modifications of their original phenotype and function. Thus, if SMCs residing within atherosclerotic lesions and contributing to the disease are clones, they are not carbon copies and can play atheroprotective or atheropromoting roles, depending on the nature of their phenotypic transitions. Tremendous progress has been made in identifying the transcriptional mechanisms biasing SMC fate. In the present review, we have summarized the recent advances in characterizing SMC investment and phenotypic diversity and the molecular mechanisms controlling SMC fate in atherosclerotic lesions. We have also discussed some of the remaining questions associated with these breakthrough observations. These questions include the underlying mechanisms regulating the phenomenon of SMC oligoclonal expansion; whether single-cell transcriptomics is reliable and sufficient to ascertain SMC functions and contributions during atherosclerosis development and progression; and how SMC clonality and phenotypic plasticity affects translational research and the therapeutic approaches developed to prevent atherosclerosis complications. Finally, we have discussed the complementary approaches the field should lean toward by combining single-cell phenotypic categorization and functional studies to understand further the complex SMC behavior and contribution in atherosclerosis. (JVS—Vascular Science 2021;2:136-48.)

Keywords: Cell differentiation; Cell plasticity; Coronary artery disease; Transcriptomics; Vascular cell; Vascular disease

Vascular smooth muscle cells (SMCs) are highly specialized contractile cells located in the vascular wall medial layer. They are essential for blood vessel integrity and tone control. However, SMCs remain highly responsive to acute and chronic modifications of their environment, adapting their phenotypic characteristics during physiologic and pathologic vascular remodeling. SMCs' capacity to modulate their phenotype, or phenotypic switching, was first conceptualized by Chamley-Campbell et al[1] in 1979. With a variety of stimuli, remarkable morphologic changes occur in SMCs, consisting of the loss of myofilaments and contractile apparatus expression and a shift from an elongated, spindle shape to a flat and rounded cell shape, similar to that of a fibroblast. This process, largely studied in vitro, has been challenging to investigate and validate in vivo owing to the lack of reliable methods to identify dedifferentiated SMCs. The development of lineage tracing and fate mapping systems has revolutionized SMC research in vivo by allowing for the tracking of these cells over time, irrespective of the changes in their phenotype and the expression of traditional cell-specific markers (Fig 1, *A*).[2,3] This tracking relies on the inducible and conditional expression of reporters by combining SMC-specific promoter-controlled Cre-ER^{T2} (tamoxifen-inducible estrogen receptor Cre recombinase) and floxed-STOP reporter alleles.[4–8] Although several promoter-controlled Cre-ER^{T2} systems have been developed (eg, *Tagln*, *Acta2*),[5,9] the use of *Myh11*-Cre ER^{T2} transgenic mice has become the reference standard for SMC fate mapping owing to the greater specificity in Myh11 expression in the SMC lineage.[3] Murine fate mapping models have permitted specific and definitive SMC tagging during a given time window and have been instrumental in investigating the fate and behavior of medial SMCs during atherogenesis, including their

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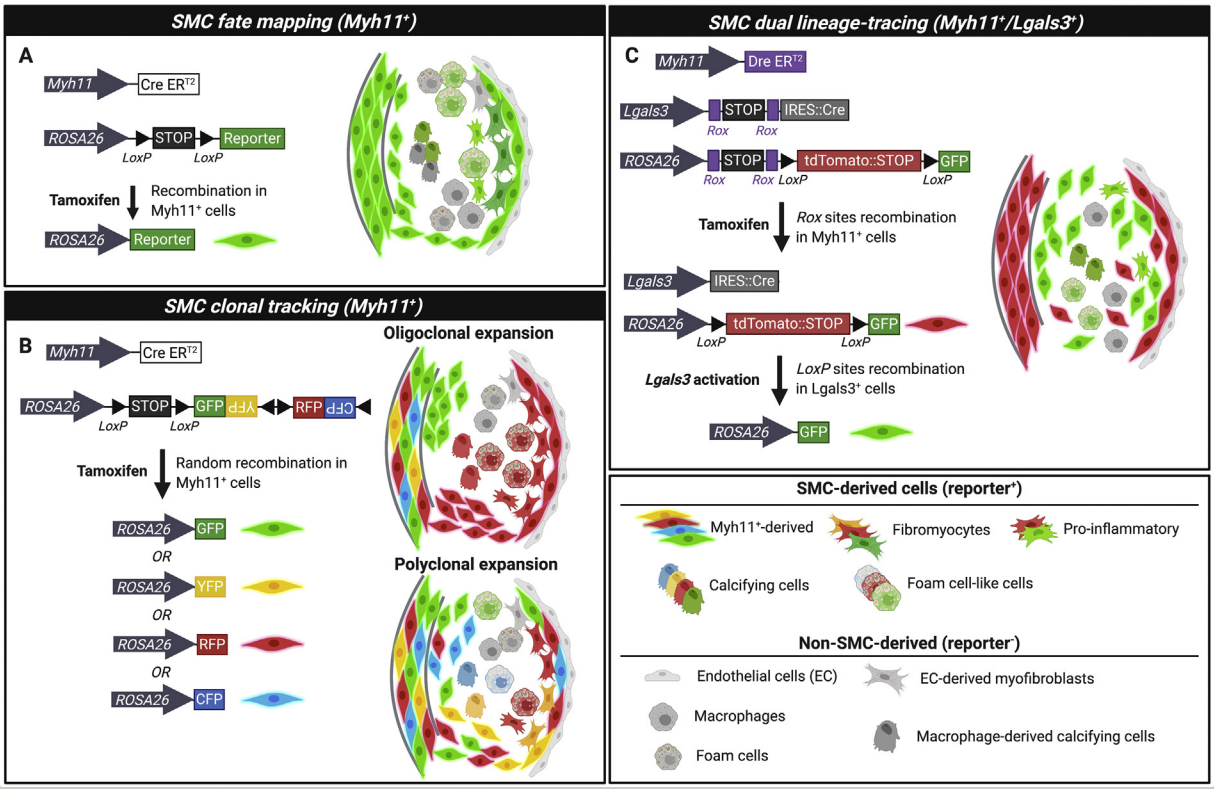

Fig 1. In vivo smooth muscle cell (*SMC*) tracking systems for investigation of SMC phenotypic transitions and clonality. **A,** SMC fate mapping with a combination of highly specific and tamoxifen-inducible *Myh11*-Cre-ER^{T2} (tamoxifen-inducible estrogen receptor Cre recombinase) transgene with a floxed-STOP reporter allowing for rigorous tracking of Myh11^{+} SMCs during atherosclerotic plaque formation and progression. Tamoxifen treatment induced definitive expression of the reporter in Myh11^{+} medial SMCs and their progeny and tracking of SMC investment and downstream phenotypic transitions. **B,** SMC clonality tracking. Random tamoxifen-induced recombination of multicolor reporter systems (eg, rainbow, confetti) in Myh11^{+} cells permitted determination of clonal pattern of SMCs investing the lesion. Oligoclonal expansion was characterized by the predominant presence of a few monocolor patches of SMCs within the lesion. In contrast, an intermixing of SMCs and equal representation of the different reporters were associated with polyclonal expansion. **C,** Dual SMC-lineage tracing (example of dual lineage tracing used by Alencar et al[10]). Dual lineage tracing systems are useful to study the transitions between phenotypic states and to characterize intermediary states. The system developed by Alencar et al[10] allows for the precise tracking of all Myh11^{+} SMCs (*red*) and the identification of the subset of these cells activating the gene *Lgals3* at any time during atherosclerosis progression (*green*). This dual lineage tracking requires the combination of Dre ER^{T2}-and Cre-mediated excision of Rox and LoxP sites, respectively, and a multicolor reporter.

abundance and repartition within the plaque, their phenotypic and functional evolution, and their proliferative capacities and clonal profile (Fig 1, *A*). In the present review, we have summarized and discussed these processes and mechanisms and the evidence of the versatile contribution of the SMC lineage to atherosclerosis pathogenesis.

OLIGOCLONAL EXPANSION IS DRIVING SMC INVESTMENT OF ATHEROSCLEROTIC LESIONS

SMC clonal profile in atherosclerotic lesions. Early seminal studies extensively analyzed the SMC clonal profile in neointimal proliferation but led to contradictory observations. Based on the presence of patches of cells with similar X-chromosome inactivation patterns, Benditt[11] hypothesized that SMCs' focal intimal proliferation originated from the monoclonal expansion of medial SMCs. This theory was opposed by the "response to injury" hypothesis presented by Virchow,[12] who stated that the atherosclerotic response is triggered by proinflammatory cytokines and toxic lipid accumulation, affecting homogenously medial SMCs that would first migrate and then expand after undergoing phenotypic switching. Finally, growth fraction studies by Clowes et al[13] and Clowes and Schwartz[14] showed that most medial SMCs will switch from quiescent to proliferative active cells after vascular injury, suggesting that a large proportion of medial SMCs will proliferate and participate in lesion formation. Although remarkably designed, all these studies included inherent limitations owing to

the lack of reliable SMC identification and clonal tracking systems. The derivation of SMC lineage tracing methods using multicolor reporters (R26R-confetti and R26R-rainbow) has allowed for the assessment of the SMC clonal profile in further detail. These clonality tracking approaches allow for the random recombination of the multicolor reporter cassette and the random labeling of medial SMCs (Fig 1, *B*). The presence of patches with cells labeled with the same color indicates that they originated from the same precursor. In contrast, a mixed distribution of cells labeled with different colors and an absence of patches indicates polyclonal expansion. Several studies have used these tools and found that most atherosclerotic plaques will only contain one or two colors, suggesting the oligoclonal expansion from a few medial SMCs.[15–17] SMC clonality is not unique to atherosclerosis development and occurs in other pathologic entities involving vascular remodeling, including vascular injury, aortic dissection, and pulmonary arterial hypertension (PAH).[9,15,18–20] SMC clonal expansion mechanisms during vascular remodeling likely evolved from the need to maintain the vascular wall integrity. However, SMC expansion during aortic wall morphogenesis and development does not follow the same pattern. Clonal tracking has revealed that healthy embryonic and adult aortic wall SMCs arise from multiple precursors stochastically intermixed.[15,16]

Mechanisms driving SMC clonal expansion: priming vs environment. Although evidence has shown that a limited number of dominant SMC clones populate atherosclerotic lesions, the exact nature of this process remains to be determined. A plausible hypothesis is that only a few primed and transcriptionally distinct medial SMCs will respond to proproliferative cues. By combining SMC lineage tracing and small conditional RNA (scRNA) sequencing, Dobnikar et al[21] investigated the transcriptomic profile of medial SMCs and identified seven distinct clusters within healthy media. A small subset of these SMCs expressed the mesenchymal stem cell (MSC) marker stem cell antigen 1 (Sca1), the expression of which has also been reported in a SMC subpopulation within atherosclerotic plaque.[22,23] It has been, thus, postulated that medial Sca1$^+$ SMCs are primed to undergo clonal expansion. However, uncertainties and missing evidence remain to allow for full validation of this hypothesis. First, the proliferative and clonal capacities of medial Sca1$^+$ SMCs have not been rigorously established in vivo. Second, no evidence is available to adjudicate whether the lesion Sca1$^+$ SMCs originate from medial Sca1$^+$ SMCs or acquire Sca1 expression after their investment within the atherosclerotic plaque. Third, recent lineage tracing studies using a Sca1-Cre-ER^{T2} reporter mouse model found a minimal contribution of resident Sca1$^+$ cells to the development of atherosclerotic lesions.[24] SMC priming has also been suggested in the development of aortic aneurysms. An scRNA trajectory and network analysis identified a SMC cluster with increased expression of the transcription factor Kruppel-like factor 4 (KLF4) responsible for SMC clonal expansion and phenotypic modulation promoting disease development.[18] As previously stated, there is no definitive evidence of causality between these medial clusters and the selective SMC clonal expansion. The development and use of dual lineage tracing systems (Fig 1, *C*) to permit tracking and selective knockout (KO) of these particular SMC subsets is necessary to investigate further the functional relevance of medial SMC diversity in the development and progression of vascular diseases.[25,26]

Another plausible explanation for SMC oligoclonal expansion in atherosclerosis is the influence of environmental factors. A hypoxic environment will prime cells for migration and clonal expansion sequentially on the small arterioles in PAH.[9,27] We can also hypothesize that if all medial SMCs have similar proliferative capacities, a given medial SMC would have to be at the right location, in the right environment, and at the right time to become a dominant clone. SMC migration into the intima can be influenced by endothelial cells and reorganization of the elastic lamina.[28] Endothelial cell-specific deletion of the transcription factor Mef2c diminished SMC migration through internal elastic lamina fenestrations after vascular injury.[29] Another speculation is that medial SMCs proliferating first, in response to environmental cue modifications, inhibit their neighbors' expansion, similar to the tip and stalk endothelial formation during angiogenesis.[30,31] This process is controlled by the Notch pathway and follows the Notch lateral inhibition model.[32] This raises the question of whether Notch lateral inhibition might apply to medial SMC oligoclonal expansion. The expression of Notch ligands and receptors has been characterized in atherosclerotic lesions, with Notch2 and Notch3 staining observed on the media and intima.[33] Similar studies of PAH have shown that Notch3$^+$ cells clonally expand, causing vessel occlusion.[20] However, the involvement of Notch-dependent lateral inhibition of medial SMCs by dominant clones in atherosclerosis must be experimentally tested.

It is important to note that in vivo clonality tracking systems permit the precise identification of cells deriving from a common precursor but cannot elucidate the processes leading to the formation of clones nor whether the appearance of clonal patches is a consequence of clonal expansion or clonal selection, or both, or whether the precursors of dominant clones are the only medial cells to retain proliferative capacities. Interestingly, atherosclerotic plaque clonal profile can be reversed from oligoclonal to polyclonal, suggesting that a larger proportion of medial SMCs will maintain their ability to undergo cell division and that environmental pressure could mediate the expansion or selection of a dominant clone. Misra et al[16] showed that the crosstalk between

SMCs and macrophages controls SMC oligoclonality. The deletion of integrin-β3 in macrophages leads to the polyclonal investment of atherosclerotic lesions by SMCs.[16] In a recent study, Wang et al[23] observed that Sca1$^+$ SMCs within atherosclerotic plaque expressed a higher level of complement C3, making them resistant to efferocytosis by macrophages.[23] C3 produced by Sca1$^+$ SMCs served two roles: (1) an autocrine loop that induced SMC proliferation; and (2) a paracrine effect on macrophages that altered the macrophage "appetite" and increasing "do not eat me" signals. C3 release gives SMCs a survival advantage and the ability to escape immunosurveillance and efferocytosis by macrophages. Inhibition of the "do not eat me" signals, like CD47, led to the reduction of clonal dominance and increased the number of medial SMCs populating the neointima. These studies have provided compelling evidence of the critical role of environmental factors in regulating the SMC clonal profile in atherosclerosis and have highlighted the complexity of the mechanisms at play. Beyond the identification of these upstream mechanisms, our understanding of the significance of clonality on disease progression has not yet been evaluated. Moreover, it is unclear whether cardiovascular disease (CVD) risk factors, such as aging, can modify the SMC clonal profile. SMC clonality inhibition has not appeared, however, as a relevant new therapeutic avenue for the treatment of atherosclerosis. Although SMCs contribute to plaque formation and represent a large fraction of lesion cells, clear evidence has shown that a lack of SMC investment does not lead to the formation of smaller plaques, but rather, to more unstable plaques.[34,35]

SMC PHENOTYPIC CHARACTERIZATION IN ATHEROSCLEROTIC PLAQUE

As discussed, lineage tracing systems have allowed for the unbiased characterization of SMC phenotypic evolution in atherosclerosis owing to some key characteristics[2,3]: (1) the reliability, specificity, and efficacy in labeling SMCs; (2) the temporally controlled tagging of differentiated SMCs in healthy mice before disease development; and (3) the stable labeling of these cells and their progeny, irrespective of their phenotype and expression of SMC marker genes. These systems are especially suitable for long-term tracking of the SMC population in chronic diseases such as atherosclerosis. Lineage tracing studies, combined with high-resolution microscopy or flow cytometry, have led to multiple central findings. First, they demonstrated that previously differentiated medial SMCs populate atherosclerotic lesions and neointima proliferation.[7,8,22] In addition to its abundance, the distribution of SMCs is ubiquitous within the plaque and not restricted to the fibrous cap. Second, the vast majority (>80%) of the SMCs present in atherosclerotic plaques lack expression of SMC marker genes, including ACTA2 and MYH11, traditionally used to identify SMCs.[22] Finally, although these cells lose the expression of the SMC contractile gene repertoire, they acquire new phenotypic characteristics and express genes associated with other cell types such as MSCs (Sca1$^+$), foam cells and macrophages (Lgals3$^+$, Mac3$^+$, F4/80$^+$), chondrocytes and osteoblasts (Sox9$^+$, Runx2$^+$), and myofibroblasts (PDGFBR$^+$).[22,36–38] These studies have robustly demonstrated the ability of SMCs to modulate their phenotype during atherosclerosis and led to a new classification of SMC phenotypic transitions into macrophage-like cells, MSC-like cells, and myofibroblast-like cells. However, the full characterization of the SMC phenotypic changes was markedly limited in these studies owing to the low number of cell markers analyzed using immunostaining or flow cytometry.

SMC phenotypic categorization by single-cell transcriptomics. The combination of scRNA sequencing and lineage tracing has recently provided a wealth of data that could help identify and characterize phenotypically modulated SMCs and classify the SMC phenotypic transitions (Fig 2 and the Table).[21,39,10] These data have posited a new understanding of how SMC gene expression is affected in atherosclerosis. By performing scRNA sequencing after isolating reporter-positive cells from brachiocephalic arteries (BCAs) in *Apoe*$^{-/-}$ SMC lineage-tracing mice, Dobnikar et al[21] found that SMC-derived cells were divided into nine different clusters, including three expressing a low level of SMC contractile genes. These three clusters regrouped cells with enriched expression of osteochondrogenic genes, macrophage-associated genes, and the MSC gene, Sca1. These results align with previously cited reports.[22,36,37] In contrast, Wirka et al[39] established a different SMC classification in aortic root atherosclerotic lesions consisting of two main clusters of SMC-derived cells: differentiated SMCs and modulated SMCs transitioning to fibroblast-like cells or "fibromyocytes."[39] Fibromyocytes lack expression of SMC contractile gene transcripts (eg, Tagln, Cnn1) but express specific markers usually not expressed by differentiated SMCs, including *Lum* and *Tnfrs11b*. That study also concluded that SMCs do not transdifferentiate into classic macrophages because of the following observations[39]: (1) the ubiquitous expression of Lgals3 in the fibromyocyte population; (2) the expression of CD68 was restricted to myeloid-derived macrophages and not found in SMC-derived cells; and (3) the modulated SMC transcriptomic profile greatly differs from macrophages. These last results confirmed previous observations showing the transcriptomic discrepancy between cholesterol-loaded SMCs and macrophages.[40] Finally, evidence has shown that SMCs that transition to fibromyocytes, controlled by the transcription factor TCF21, play a protective role in atherosclerosis. SMC-specific KO of TCF21, not only markedly reduced the number of

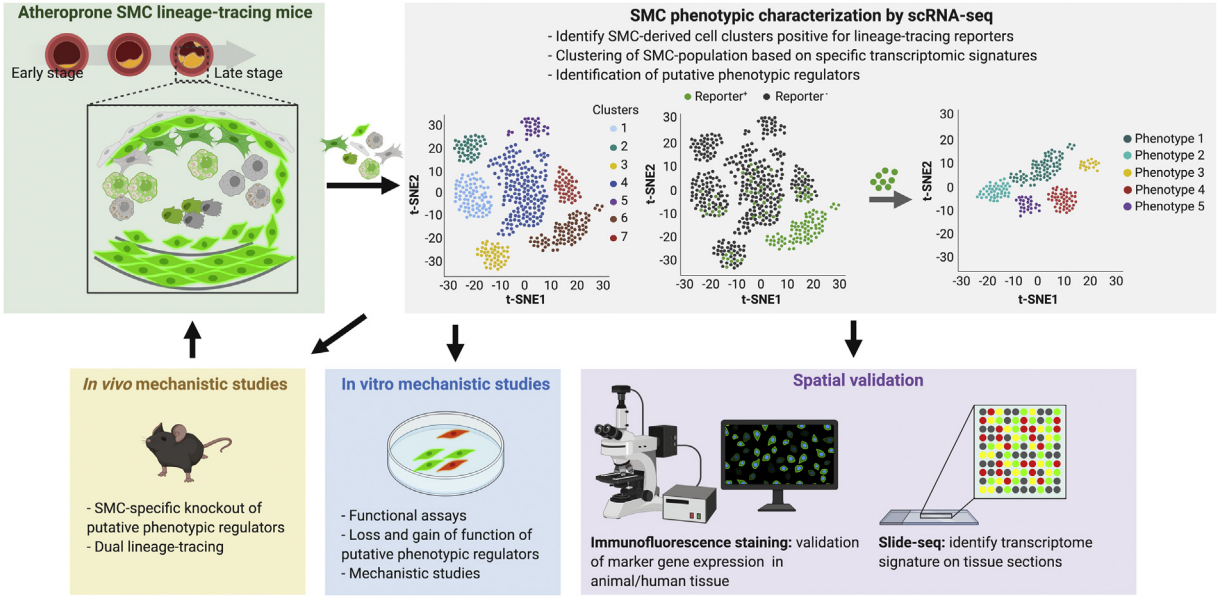

Fig 2. Proposed experimental pipeline for studying smooth muscle cell (*SMC*) phenotypes and function in atherosclerosis. This experimental pipeline is based on SMC lineage tracing animal models and the integration of multiple technical platforms, including single cell RNA sequencing, spatial and temporal studies by high-resolution microscopy and slide-sequencing, and in vitro and in vivo studies investigating the functional relevance of putative phenotypic regulators in SMCs.

fibromyocytes within the plaque, but also decreased the indexes of plaque stability, including a reduction in fibrous cap thickness. A subsequent study by the same group showed that a subset of the SMC fibromyocyte cluster comprises "chondromyocytes," which express genes associated with the osteochondrogenic differentiation program (Sox9, Runx2, Col2a1, Tgfb2) and participate in lesion calcification.[41] Alencar et al[10] performed scRNA sequencing on micro-dissected BCA atherosclerotic lesions and found that Lgals3^{+} SMCs result in a similar chondrocyte-like population. Using an innovative dual lineage tracing *Myh11*-DreER^{T2} *Lgals3*-Cre *Rosa26*-tdTomato/eGFP system, which allows for the differential tracking of the global SMC population and SMC-expressing Lgals3 at any point after tamoxifen injection during atherogenesis, they also demonstrated that more than two thirds of lesion SMCs had expressed Lgals3 during their investment within the lesion (Fig 1, *C*).[10] Alencar et al[10] postulated that the activation of Lgals3 is the "pioneer" phenotype that SMCs acquire before giving rise to other phenotypes.

Considerations for SMC scRNA sequencing study interpretation. Although these studies have provided a wealth of information regarding the modifications of the SMC transcription profile in atherosclerotic lesions, a consensus has not yet been reached regarding the SMC phenotype classification and categorization (Table). Several possibilities can explain the divergence between these studies. First, these transcriptomic analyses have been differently designed and performed on different vascular beds (eg, aortic root[39] or BCAs[21,10]) composed of SMCs from different embryonic origins.[49,50] Thus, it is unclear whether the results obtained from a given vascular bed and atherosclerotic plaque location should be generalized to other vascular beds and locations. Second, differences in the experimental designs and procedures (eg, tissue isolation and digestion) could have resulted in the differences observed. Alencar et al[10] performed scRNA sequencing on microdissected lesions and avoided the possible dilution of rare phenotypically modulated SMC populations by the predominance of medial SMCs. However, scRNA sequencing has its own inherent challenges and limitations that should be acknowledged and carefully considered.[51] Tissue digestion can lead to an artificial bias against rare or fragile populations.[52,53] For example, foam cells are particularly sensitive to digestion and single-cell suspension procedures and could easily be underrepresented.[42] Overall, these limitations could explain some of the discrepancies observed. Of particular interest, the lack of CD68 expression reported by Wirka et al[39] is difficult to reconcile with the multiple studies providing compelling evidence of CD68 expression by SMCs in SMC lineage-tracing mouse models or human atherosclerotic lesions and could reflect depletion of the CD68^{+} population.[22,36,54] Despite these divergent analysis and conclusions, we have highlighted the central and corroborating findings of these transcriptomic studies. First, general agreement has been reached that SMCs

Table. Marker genes associated with SMC phenotypic transitions[a]

Cell type	Gene marker	Experimental design
Macrophage-like/ phagocytic cells		
	Mouse studies	
	Cd74, yz2, C1qa, C1qb, H2-Aa, H2-Ab1, C1qC, H2-Eb1	SMC-derived cells in macrophage cell cluster by scRNA sequencing with SMC-lineage tracing of ApoE$^{-/-}$ mice[38]
	Itgam, Mrc1, Adgre1, Ptprc, Cd68, Lgals3	SMC-derived cells in macrophage cell cluster by scRNA sequencing with SMC-lineage tracing of ApoE$^{-/-}$ mice[39]
	CD68, Lyz2, Fcer1g	SMC-derived cells in macrophage cell clusters by scRNA sequencing with SMC-lineage tracing of ApoE$^{-/-}$ mice[21]
	Lgals3, CD68	Immunofluorescent staining in vivo; oxidized low-density lipoprotein staining and morphologic analysis by electron microscopy[35]
	Human studies	
	CD68	Immunofluorescent staining in vivo; phagocytosis assay in vitro with cholesterol loading[22]
	ACTA2, CD68, Abca1	Immunofluorescent staining[42]
	In vitro studies	
	Mac2, CD68, Abca1	In vitro cholesterol loading; phagocytosis assay in vitro[10,43]
MSC-like cells		
	Mouse studies	
	Ly6a/Sca1	scRNA sequencing with SMC-lineage tracing of ApoE$^{-/-}$ mice; immunofluorescent staining in vivo[21]
	Sca1	Immunofluorescent staining in vivo[22]
	Sca1, Lgals3, Vcam1	SMC-derived transitioning cluster by scRNA sequencing with SMC lineage tracing of ApoE$^{-/-}$ mice; Lgals3 SMC dual lineage tracing validation[39]
	Human studies	
	Human homolog Sca1 has not been identified	
Osteochondrogenic/ chondrocyte-like cells		
	Mouse studies	
	Runx2, RANKL	*Runx2*-deficient mice consuming high-fat diet showed decreased vascular calcification in vivo[44]
	Sox9, Ibsp, Chad	SMC-derived cells present in chondrocyte cell cluster by scRNA sequencing with SMC lineage tracing of ApoE$^{-/-}$ mice[21]
	Sox9, Runx2, Cytl1, Ibsp, Alpl	SMC-derived osteogenic cluster by scRNA sequencing with SMC-lineage tracing mice; Sox9 staining on mouse plaque[39]
	Human studies	
	TRPV4, S100 B, Sox9	Validation in ruptured human coronary atherosclerotic lesions[39]
	In vitro studies	
	PiT-1, Dmp1, E11, SOST	In vitro calcification, as measured by Alizarin red staining and alkaline phosphatase activity[48]
	Runx2, BMP-2	Hypoxic conditions and $HiPO_4$ treatment drove increased mineralization of SMCs[45]
	ALPL, Runx2, MGP, COL1A2	Alkaline phosphatase activity and deposition of calcified matrix in vitro[46]
	Sox9, Agg, Col II, TG2	In vitro chondrogenesis of SMCs[47]
Fibromyocyte		

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Table. Continued.

Cell type	Gene marker	Experimental design
	Mouse studies	
	Lgals3, Tnfrsf11b, TCF21, Lum, Dcn, Bgn, Mgp	SMC-derived fibromyocyte cluster by scRNA sequencing with SMC lineage tracing of ApoE$^{-/-}$ mice; in situ hybridization for *Lum* mRNA on mouse fibrous cap; TCF21 SMC-specific knockout reduced SMC contribution to fibrous cap by SMC-lineage tracing[38]
	Human studies	
	TCF21, Tnfrsf11b, Mgp, Bgn, Lum, MMP2, Dcn	Human atherosclerotic coronary arteries scRNA sequencing; in situ hybridization of *Tnfrsf11b* mRNA within human coronary lesion[38]

MSC, Mesenchymal stem cell; *scRNA*, small conditional RNA; *SMC*, smooth muscle cell.
[a]Some markers such as Sox9 seem specific to the osteochondrogenic transition; however, others such as Lgals3 appear to not be expressed by different SMC subpopulations.

undergo a process of dedifferentiation and loss of contractile phenotype. Second, SMC-derived cells divide into various transcriptionally distinct states and express transcriptomic signatures associated with both protective (eg, fibromyocyte) or detrimental (eg, osteochondrogenic-like, foam cell-like) behaviors, confirming the versatile and complex roles of SMCs in atherosclerosis. Finally, scRNA sequencing is a remarkable tool for assessing the relevance and reliability of so-called cell-specific or function-specific markers. The example of *Lgals3* is especially striking in that regard.[10] The categorization of SMC phenotypes in the transitions to alternative cell types (eg, macrophage-like cells or MSC-like cells) also seems problematic owing to the major differences in the transcriptomic profiles between modulated SMCs and other lineages.[39] All these considerations underscore the importance of unbiased transcriptomic studies and also the need for combinatorial functional studies (Fig 2). Such approaches could help ascertain whether SMCs acquire new functions that might be shared with different cell types (rather than transdifferentiation), which might be more effective for identifying targetable pathways for atherosclerosis treatment.

Slide-sequencing: toward spatial resolution of single-cell transcriptomics and multidimensional evaluation of SMC transitions. Current scRNA sequencing studies partially capture the chronicity of SMC phenotypic modulation. However, a spatially nuanced understanding of vascular SMCs in atherosclerosis still eludes us. Profiling cells from various locations within the plaque where they are subject to varying levels of inflammation, hypoxia, or other environmental cues could elucidate the specific biologic factors that dictate SMC phenotype. For example, hypoxia and hypoxia-induced factor-1α (HIF-1α) have emerged as key regulators of SMC migration, proliferation, and clonal expansion.[9,27,55] However, downstream mechanisms suggest a possibly larger effect for hypoxia and HIF-1α in regulating the SMC phenotype and function. Hypoxia induces the expression of two key regulators of the SMC phenotype, the pluripotency factors KLF4 and OCT4.[34] OCT4 and KLF4 mediate very distinct gene subsets and pathways and drive SMCs to transition to atheroprotective or atheropromoting phenotypes, respectively.[22,34] Hypoxia could trigger versatile responses that vary with disease stage, such as SMC location (media vs lesion) and state but also could promote migration, lipid uptake and accumulation, and osteochondrogenic differentiation.[56,57] It will be exciting to see future studies using new technologies such as slide-sequencing to answer some of these remaining questions. With an efficiency in transcript detection similar to that for scRNA sequencing, slide-sequencing maps single-cell gene expression in tissue sections and maintains the spatial information about the precise in situ location of cell populations.[43] In brief, the tissue sections are placed on a grid of thousands of randomly DNA-barcoded beads, permitting retention of cell spatial information during transcript sequencing.[58] This technique provides fine three-dimensional mapping of single-cell transcriptional activity within the context of whole tissue and neighboring cells. The power of slide-sequencing to spatially resolve transcript expression represents a remarkable tool that will likely play a major role in characterizing SMC phenotypic heterogeneity in atherosclerotic lesions.

FUNCTIONAL DIVERSITY OF SMC IN ATHEROSCLEROTIC LESIONS

Considering the lack of consensus of scRNA sequencing studies in defining lesion SMC subpopulations, we have summarized the evidence of SMC functional diversity in atherosclerosis and discuss the following questions. In addition to the expression of given markers, do SMCs acquire the biologic functions of other lineages? If so, how do these functions influence

the overall contribution of modulated SMCs in atherosclerosis pathogenesis?

SMCs contribute to formation of the protective fibrous cap. SMCs have been classically described as a protective cell type in atherosclerosis by forming the fibrous cap.[44,59,60] One bold study induced SMC depletion by SMC-specific expression of diphtheria toxin receptor (*Tagln-h*DTR) and diphtheria toxin (DT) to assess the overall contribution of SMCs in advanced lesions.[61] No significant changes were observed in normal vessels, although 50% to 70% of SMCs were lost within the media. However, features of vulnerable plaques, including a thinner fibrous cap, a decreased collagen percentage, an increased necrotic core size, and total inflammation loci, were detected after the injection of DT in mice with advanced atherosclerotic lesions. These results suggest that SMCs play an overall protective role in forming the fibrous cap and stabilizing the plaque. However, because DT receptor was delivered after atherosclerotic plaque formation, only TAGLN$^+$ SMCs (mainly localized in the fibrous cap) were cleared. In contrast, TAGLN$^-$-modulated SMCs were not depleted, and their effect was not assessed in these experiments.[61]

SMCs accumulate lipids and become poorly efficient foam cells. A large body of research has reported that SMCs can display multiple functions in addition to fibrous cap maintenance. Rong et al[62] provided the first evidence of the ability of SMCs to uptake lipids and acquire the functions of foam cells traditionally attributed to macrophages.[62] In addition to upregulation of key macrophage and phagocytosis marker genes (CD68 and Lgals3) and downregulation of SMC contractile markers (ACTA2 and MYH11), Rong et al[62] showed the accumulation of intracellular lipid using Oil Red O staining and phagocytotic activity using latex beads in cultured SMCs.[40,62] In vivo studies using SMC lineage-tracing mice have shown that SMCs can perform efferocytosis of apoptotic cells[22] and that SMCs will uptake lipids and become foam cells.[17,36,38] Wang et al[38] also provided evidence that SMCs contribute the most foam cells in advanced atherosclerosis. They demonstrated that leukocyte marker CD45 expression could reliably discriminate monocyte- and SMC-derived foam cells, with the latter lacking CD45 expression in atherosclerotic lesions. By comparing the proportion of foam cells (BODIPY$^+$) derived from leukocytes (CD45$^+$) and nonleukocytes (CD45$^-$) using flow cytometry, they found that nonleukocyte–derived foam cells (BODIPY$^+$ CD45$^-$) constituted >80% of total foam cells within advanced lesions.[38] These findings, obtained in atheroprone SMC lineage-tracing mice,[38] extend remarkably to human lesions.[54] Costaining of intracellular lipid (Oil Red O) and SMC marker genes (ACTA2) in the intima of human coronary artery atherosclerotic lesions revealed a high proportion of Oil Red O$^+$ foam cells expressing ACTA2, suggesting their potential origin could be SMCs.[54] However, cholesterol-loaded SMCs have reduced phagocytosis and efferocytosis capacities compared with bone marrow-derived macrophages.[40] They also reported that SMC-derived foam cells have a reduced level of ABCA1, the rate-limiting exporter of excess intracellular cholesterol, suggesting defective foam cell function and capability to export cholesterol. Overall, the results from these functional studies provide compelling evidence that SMCs acquire foam cell functions of lipid uptake, phagocytosis, and efferocytosis. They also suggest that SMC-derived foam cells are not as efficient as macrophage-derived foam cells and could, thus, be more detrimental during disease progression and might constitute a key target for therapy.

SMC are key drivers of plaque calcification. Accumulating evidence has indicated that vascular calcification is a complex and active process resembling the osteogenesis initiated and perpetuated by macrophages and SMCs. Calcification is a risk factor for CVD and increases atherosclerotic plaque instability.[63] Cell fate mapping studies have demonstrated that SMCs participate in vascular calcification through their transition into osteochondrogenic and osteoblastic-like cells, including the following. First, SMCs present in calcified blood vessels upregulate the expression of bone-related transcription factors (Msx2, Sox9, and Runx2) and mature chondrogenic markers such as collagen X and MMP13.[37,64] Second, SMCs can enact a cellular program that mediates deposition of bone matrix in blood vessels. Finally, SMCs form most chondrocyte-like precursors (98%) in the atherosclerotic lesion.[64] Deletion of Runx2 uniformly prevented calcification in different atherosclerotic mouse models.[65,66] Rather than changing the number of SMCs differentiating into chondrocyte-like cell precursors, Runx2 deficiency reduced the presence of SMC-derived mature osteochondrogenic-like cells, calcium deposition, and mineralization in the deep intima layers.[66,67] These studies have provided compelling evidence that SMCs and SMC phenotypic modulation drive calcification within atherosclerotic plaques.

Evidential and hypothetical role of Sca1$^+$ SMCs. Finally, it has been hypothesized that SMCs acquire an MSC-like phenotype and regain pluriplasticity determined by Sca1 expression in a subset of medial and lesional SMCs and the activation of the pluripotency factors KLF4 and OCT4. A recent hypothesis postulated that the SMC transition to an MSC-like state would be a necessary intermediate state before SMCs transition to other lesion phenotypes.[21] However, the function and contribution of SMC-derived Sca1$^+$ cells in driving SMC investment and phenotypic transitions in vivo have remained poorly understood. A part of the challenge in investigating Sca1$^+$ SMCs in

atherosclerotic lesions is that other cell types residing in the vascular wall express this MSC marker and contribute to the disease (eg, resident adventitial MSCs).[68–70] In vitro, lineage-tagged Sca1$^+$ SMCs isolated from atherosclerotic lesions displayed a low capacity to transdifferentiate into other lineages compared with non–SMC-derived MSCs.[22] In contrast, Sca1$^+$ SMCs isolated in healthy vessels presented an enhanced proliferation profile and plastic properties, including expression of macrophage and endothelial cell markers, and were able to redifferentiate into mature SMCs in Matrigel experiments performed with Sca1$^+$ SMCs implanted within syngeneic mice.[71] Fate mapping studies in vivo have demonstrated that Sca1$^+$ SMCs residing at the border between the media and adventitia populate the adventitial connective tissue and are a source of Sca1$^+$ MSC-like cells in this compartment.[71,72] This population expands during vascular remodeling. The results from these studies have provided compelling evidence of the presence of a Sca1$^+$ population within the vessel wall. We wonder whether these adventitial Sca1$^+$ SMCs are the same population as the medial Sca1$^+$ SMC population described by Dobnikar et al.[21] Again, this question could be answered by implementing technologies such as slide-sequencing, combining depth in phenotypic characterization and spatial information.[43]

Using a novel *Sca1*-ER^{T2} Cre lineage tracing mouse model, a recent study showed that resident Sca1$^+$ cells contribute minimally to atherosclerotic lesions and barely transition to SMCs expressing phagocytosis and foam cell markers.[24] These data suggest that a resident SMC-derived Sca1$^+$ population would not significantly contribute to atherosclerosis formation, contradicting the theory that media Sca1$^+$ SMCs are primed to undergo clonal expansion and populate the lesion massively. However, these Sca1 fate mapping studies did not discriminate between SMC-derived and non–SMC-derived Sca1$^+$ cells. Determination of Sca1$^+$ SMC relevance to lesion investment and SMC phenotypic plasticity will only be revealed using dual lineage tracing systems to rigorously and precisely track SMC-derived Sca1$^+$ cells and studies investigating the effect of SMC-specific Sca1 KO. Sca1 KO studies have only been performed of cardiac progenitor cells, which showed an early decrease in proliferation, survival, and contractility.[73] However, the functional roles of Sca1$^+$ SMCs in atherosclerosis remains to be experimentally determined.

Overall, SMCs play a complex role within atherosclerotic lesions and can actively participate in protective (extracellular matrix production, fibrous cap formation) or detrimental (calcification, phagocytosis, efferocytosis) processes. This functional SMC characterization seems critical and, somehow, more pertinent than the phenotypic categorizations established by immunofluorescent staining, flow cytometry, and scRNA sequencing. Rather than categorizing SMC according to a transdifferentiation model (eg, macrophage-like cells), we propose that implementing a functional classification would be more accurate and relevant to understanding the SMCs' contribution to disease and identifying targetable pathways.

TRANSCRIPTIONAL CONTROL OF SMC PHENOTYPIC TRANSITIONS IN ATHEROSCLEROSIS

A large number of mechanisms, including transcriptional factors, chromatin modifications, and noncoding RNAs, have been implicated in controlling SMC dedifferentiation, SMC contractile gene repression, and phenotypic transitions. Although an exhaustive enumeration of the transcription factors involved in regulating the SMC phenotype was beyond the scope of the present review, we have focused on summarizing and discussing the role of two transcriptional nexuses, KLF4 and TCF21, in regulating SMC function in atherosclerosis. Recent studies, including SMC-specific KO of TCF21 and KLF4, coupled with scRNA sequencing, have provided remarkable evidence of the central, yet complex, contribution of these transcription factors in biasing the SMC fate in atherosclerosis (Fig 3).

KLF4 promotes SMC dedifferentiation and transition to atheropromoting phenotypes. SMC contractile gene expression and differentiation programs are tightly and dynamically regulated.[74] SMC contractile gene expression, including that of MYH11, ACTA2, TAGLN, and CNN1, is dependent on the formation of an activating complex composed of the transcriptional factor, serum response factor (SRF) and the cofactor myocardin and its binding to CArG box DNA motifs present in the promoter-enhancer regions of these genes.[75–77] KLF4 disrupts the CArG-SRF-myocardin complex by binding to adjacent DNA motifs (including TGF-β control elements and G-/C-rich repressor elements), mediating epigenetic silencing of the SMC contractile genes and inducing direct repression of myocardin.[78–82] Moreover, KLF4 is responsive to hypoxia as a direct target of the transcription factor HIF-1α and acts as a mediator on hypoxia-induced SMC migration.[55] Combined fate mapping and SMC-specific KLF4 KO studies have suggested that KLF4 could be involved in several SMC phenotypic transitions in atherosclerosis. First, Shankman et al[22] showed that SMC-specific deletion of KLF4 resulted in a higher number of ACTA2$^+$ SMCs within the fibrous cap and a reduction in SMCs expressing Lgals3, interpreted as a decrease in the number of SMC-derived foam cells. Further evidence supported a role for KLF4 in mediating the formation of SMC-derived foam cells. Chromatin immunoprecipitation assays demonstrated a direct positive regulation of phagocytic and macrophage marker genes by KLF4 in lesional SMCs. Moreover, KLF4 deficiency led to a marked reduction in SMC phagocytic capacities in vitro. KLF4 has also been implicated in the

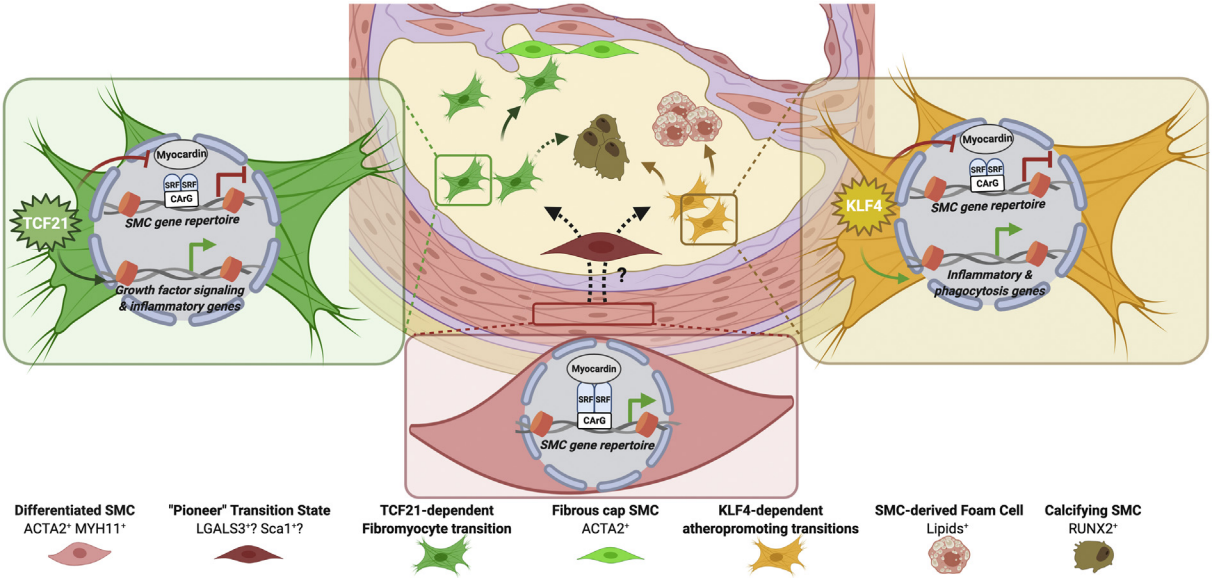

Fig 3. TCF21 and KLF4 mediate vascular smooth muscle cell (*SMC*) phenotypic transitions in atherosclerosis. The transcription factors TCF21 and KLF4 play central and opposite roles in regulating SMC phenotypic modulation in atherosclerosis. TCF21 mediates SMC transition to fibromyocytes and population of the fibrous cap by ACTA2$^+$ SMCs. In contrast, KLF4 controls transitions to foam and chondrogenic cells. It has been postulated that the expression of LGALS3 and/or SCA1 marks an early and intermediate state mediating SMC investment within the lesion and further transitions. The exact interdependence between these states and transitions requires further investigation.

SMC transition into adventitial Sca1$^+$ cells and maintenance of their multipotent state.[71,72] Finally, a recent scRNA sequencing study showed that SMC-specific deletion of KLF4 was associated with an increase in the abundance of SMCs expressing SMC contractile genes and a reduction in SMCs displaying a chondrogenic phenotype.[10] The same study used a *Myh11/Lgals3* dual lineage tracing system and found that Lgals3 is activated, sometimes transiently, in a large subset of SMCs undergoing different phenotypic transitions, suggesting that Lgals3 activation could be an early, intermediate, and common step in SMC phenotypic transition.[10] Overall, KLF4 appears to be a critical regulator of SMC fate in atherosclerosis, although key questions regarding its role remain. These include which steps of SMC investment and participation in atherosclerotic lesion are regulated by KLF4; whether KLF4 regulates the activation of Lgals3 expression; and whether KLF4 has a distinct role at different stages of the disease and in different SMC subpopulations. These questions highlight the limitations of cross-sectional studies and the difficulties in implementing feasible and comprehensive longitudinal analyses.

TCF21 promotes SMC investment and transition to fibromyocytes in atherosclerotic plaques. TCF21 also remarkably influences SMC differentiation state. Similar to KLF4, TCF21 induces the profound repression of the SMC contractile gene repertoire.[83] TCF21 blocks SMC marker gene expression by directly interacting with myocardin, inhibiting the formation of the SRF—myocardin complex and repressing myocardin expression. Genomewide and post-genomewide association functional studies have identified genomic variants at the human chromosome 6q23.2 locus, containing *TCF21,* as a risk locus associated with CVD.[45,46,48] Polymorphisms in this locus causing alteration in TCF21 expression were associated with greater CVD risk.[46] A large body of evidence has demonstrated that TCF21 plays a beneficial role in atherosclerosis by promoting SMC population of plaque and their transition to atheroprotective phenotypes. LacZ reporter studies in atheroprone mice showed that, although not expressed ubiquitously in the media of healthy vessels, TCF21 is expressed in a subset of medial SMCs and a large number of lesional SMCs in early-stage plaques.[47] In advanced atherosclerotic lesions, TCF21 expression was mainly restricted to the fibrous cap. Tamoxifen-induced TCF21 lineage tracing demonstrated that TCF21$^+$ cells constituted a significant fraction of lesion and fibrous cap cells, including ACTA2$^+$ fibrous cap cells. However, these cells' original lineage (SMC vs adventitial cells) could not be rigorously determined in these studies. In vitro studies have provided further evidence of the role of TCF21 in positively regulating SMC proliferation and migration, again suggesting that TCF21 could play a central role in mediating SMC investment within atherosclerotic plaques.[47] SMC-specific TFC21 KO, combined with single-cell transcriptomics, indicated that TCF21 is a major

driver of SMC transition to a fibromyocytic phenotype.[39] Together, these results have provided strong evidence that TCF21 plays a protective role by controlling SMC dedifferentiation, proliferation, migration, plaque investment, and fibrous cap population. However, it seems that TCF21 has more versatile action on SMC gene expression, because it has also been reported that TCF21 can partner with the aryl-hydrocarbon receptor to activate the expression of proinflammatory genes (eg, IL-1α) implicated in disease progression.[84]

KLF4, TCF21, and other transcription factors (eg, OCT4,[34] HIF-1α,[34,85] SMAD3[86]) remarkably control the SMCs' fate and behavior in atherosclerosis, making them potentially attractive therapeutic targets. However, further investigation regarding these factors' precise role at different stages of atherosclerosis and selectively in different SMC subpopulations is required, considering the versatility in these factors' actions, transcriptional partners, and downstream targets.

CONCLUSIONS AND PERSPECTIVES

Although the field has made tremendous progress in the phenotypic characterization of SMCs, our understanding of phenotypic transition dynamics (spatio-temporal information and interdependence) and their respective contribution in atherosclerosis remains limited. As compelling evidence of SMC phenotypic and functional diversity has been provided, an interesting hypothesis has emerged that a subgroup of medial SMCs could transiently transition to a "pioneer" or "first responder" state owing to priming or environmental selection. In a reductionist manner, the difficulty in "observing" the SMC phenotypic state dynamics in atherosclerosis could be compared with the Schrödinger's cat paradox and the superposition theory. In Erwin Schrödinger's imagined experiment, if a cat is in an opaque box in the presence of poison, the cat could be simultaneously dead and alive. When the experimenter opens the box, it reduces the cat state to either alive or dead. SMCs in the atherosclerotic plaque exist in multiple states simultaneously, and the current technologies can only show a screenshot of their location, phenotypic identity, and function. The field is attempting to solve this issue by combining fate studies, single-cell transcriptomics, and biocomputational predictions. Timeline projections have allowed for the approximation of the evolution of these transitions and the progression of SMC phenotypic clusters. However, several limitations were encountered, such as the lack of a specific location and functional significance of these different SMC intermediate states. Novel technologies, such as slide-sequencing, could account for the characterization of spatial gene expression and heterogeneous populations' location. Still, these approaches should be combined with a detailed functional categorization of these SMC states. By taking advantage of dual-lineage tracing, the SMC-specific loss of function of master regulators (KLF4, Sca1, TCF21), and transcriptomic analysis (scRNA sequencing and slide-sequencing), we might, not only achieve depth and accuracy in our definition of SMC phenotypic plasticity in atherosclerosis, but also be able to design effective therapeutic approaches for the prevention and treatment of advance atherosclerosis development. However, these novel therapeutic axes should target both SMC intrinsic functions and SMC interactions with other cells populating the plaque (ie, macrophages, fibroblasts, endothelial cells, hematopoietic cells), such as a selective increase in proatherogenic SMC immunogenicity and clearance by macrophages.

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REFERENCES

1. Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. Physiol Rev 1979;59:1-61.
2. Liu M, Gomez D. Smooth muscle cell phenotypic diversity. Arterioscler Thromb Vasc Biol 2019;39:1715-23.
3. Chakraborty R, Saddouk FZ, Carrao AC, Krause DS, Greif DM, Martin KA. Promoters to study vascular smooth muscle. Arterioscler Thromb Vasc Biol 2019;39:603-12.
4. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P. Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A 1996;93:10887-90.
5. Kuhbandner S, Brummer S, Metzger D, Chambon P, Hofmann F, Feil R. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15-22.
6. Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. Nat Med 2008;14:64-8.
7. 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;10:171-7.
8. Herring BP, Hoggatt AM, Burlak C, Offermanns S. Previously differentiated medial vascular smooth muscle cells contribute to neointima formation following vascular injury. Vasc Cell 2014;6:21.
9. Sheikh AQ, Misra A, Rosas IO, Adams RH, Greif DM. Smooth muscle cell progenitors are primed to muscularize in pulmonary hypertension. Sci Transl Med 2015;7:308ra159.
10. Alencar GF, Owsiany KM, Karnewar S, Sukhavasi K, Mocci G, Nguyen A, et al. The stem cell pluripotency genes Klf4 and Oct4 regulate complex SMC phenotypic changes critical in late-stage atherosclerotic lesion pathogenesis. Circulation 2020;142:2045-59.
11. Benditt EP. Evidence for a monoclonal origin of human atherosclerotic plaques and some implications. Circulation 1974;50:650-2.
12. Virchow R. Cellular pathology: as based upon physiological and pathological histology. Lecture XVI—atheromatous affection of arteries. 1858. Nutr Rev 1989;47:23-5.
13. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Lab Invest 1983;49:327-33.

14. Clowes AW, Schwartz SM. Significance of quiescent smooth muscle migration in the injured rat carotid artery. Circ Res 1985;56:139-45.
15. Chappell J, Harman JL, Narasimhan VM, Yu H, Foote K, Simons BD, et al. 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;119:1313-23.
16. Misra A, Feng Z, Chandran RR, Kabir I, Rotllan N, Aryal B, et al. Integrin beta3 regulates clonality and fate of smooth muscle-derived atherosclerotic plaque cells. Nat Commun 2018;9:2073.
17. Jacobsen K, Lund MB, Shim J, Gunnersen S, Fuchtbauer EM, Kjolby M, et al. Diverse cellular architecture of atherosclerotic plaque derives from clonal expansion of a few medial SMCs. JCI Insight 2017;2:e95890.
18. Chen PY, Qin L, Li G, Malagon-Lopez J, Wang Z, Bergaya S, et al. Smooth muscle cell reprogramming in aortic aneurysms. Cell Stem Cell 2020;26:542-57.e11.
19. Clement M, Chappell J, Raffort J, Lareyre F, Vandestienne M, Taylor AL, et al. Vascular smooth muscle cell plasticity and autophagy in dissecting aortic aneurysms. Arterioscler Thromb Vasc Biol 2019;39:1149-59.
20. Steffes LC, Froistad AA, Andruska A, Boehm M, McGlynn M, Zhang F, et al. A Notch3-marked subpopulation of vascular smooth muscle cells is the cell of origin for occlusive pulmonary vascular lesions. Circulation 2020;142:1545-61.
21. Dobnikar L, Taylor AL, Chappell J, Oldach P, Harman JL, Oerton E, et al. Disease-relevant transcriptional signatures identified in individual smooth muscle cells from healthy mouse vessels. Nat Commun 2018;9:4567.
22. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat Med 2015;21:628-37.
23. Wang Y, Nanda V, Direnzo D, Ye J, Xiao S, Kojima Y, et al. Clonally expanding smooth muscle cells promote atherosclerosis by escaping efferocytosis and activating the complement cascade. Proc Natl Acad Sci U S A 2020;117:15818-26.
24. Wang H, Zhao H, Zhu H, Li Y, Tang J, Li Y, et al. Sca1(+) cells minimally contribute to smooth muscle cells in atherosclerosis. Circ Res 2020;128:133-5.
25. He L, Li Y, Li Y, Pu W, Huang X, Tian X, et al. Enhancing the precision of genetic lineage tracing using dual recombinases. Nat Med 2017;23:1488-98.
26. Anastassiadis K, Fu J, Patsch C, Hu S, Weidlich S, Duerschke K, et al. Dre recombinase, like Cre, is a highly efficient site-specific recombinase in E. coli, mammalian cells and mice. Dis Model Mech 2009;2:508-15.
27. Sheikh AQ, Saddouk FZ, Ntokou A, Mazurek R, Greif DM. Cell autonomous and non-cell autonomous regulation of SMC progenitors in pulmonary hypertension. Cell Rep 2018;23:1152-65.
28. Schwartz SM, Virmani R, Majesky MW. An update on clonality: what smooth muscle cell type makes up the atherosclerotic plaque? F1000Res 2018;7:1969.
29. Lu YW, Lowery AM, Sun LY, Singer HA, Dai G, Adam AP, et al. Endothelial myocyte enhancer factor 2c inhibits migration of smooth muscle cells through fenestrations in the internal elastic lamina. Arterioscler Thromb Vasc Biol 2017;37:1380-90.
30. Chen W, Xia P, Wang H, Tu J, Liang X, Zhang X, et al. The endothelial tip-stalk cell selection and shuffling during angiogenesis. J Cell Commun Signal 2019;13:291-301.
31. Pitulescu ME, Schmidt I, Giaimo BD, Antoine T, Berkenfeld F, Ferrante F, et al. Dll4 and Notch signalling couples sprouting angiogenesis and artery formation. Nat Cell Biol 2017;19:915-27.
32. Bray SJ. Notch signalling in context. Nat Rev Mol Cell Biol 2016;17:722-35.
33. Davis-Knowlton J, Turner JE, Turner A, Damian-Loring S, Hagler N, Henderson T, et al. Characterization of smooth muscle cells from human atherosclerotic lesions and their responses to Notch signaling. Lab Invest 2019;99:290-304.
34. Cherepanova OA, Gomez D, Shankman LS, Swiatlowska P, Williams J, Sarmento OF, et al. Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. Nat Med 2016;22:657-65.
35. Newman AA, Baylis RA, Hess DL, Griffith SD, Shankman LS, Cherepanova OA, et al. Irradiation abolishes smooth muscle investment into vascular lesions in specific vascular beds. JCI Insight 2018;3:e121017.
36. Feil S, Fehrenbacher B, Lukowski R, Essmann F, Schulze-Osthoff K, Schaller M, et al. Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. Circ Res 2014;115:662-7.
37. Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, et al. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. Circ Res 2009;104:733-41.
38. Wang Y, Dubland JA, Allahverdian S, Asonye E, Sahin B, Jaw JE, et al. Smooth muscle cells contribute the majority of foam cells in ApoE (apolipoprotein E)-deficient mouse atherosclerosis. Arterioscler Thromb Vasc Biol 2019;39:876-87.
39. Wirka RC, Wagh D, Paik DT, Pjanic M, Nguyen T, Miller CL, et al. Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis. Nat Med 2019;25:1280-9.
40. Vengrenyuk Y, Nishi H, Long X, Ouimet M, Savji N, Martinez FO, 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;35:535-46.
41. Juyong BK, Quanyi Z, Trieu N, Milos P, Paul C, Robert W. Environment-sensing aryl hydrocarbon receptor inhibits the chondrogenic fate of modulated smooth muscle cells in atherosclerotic lesions. Circulation 2020;142:575-90.
42. Winkels H, Ehinger E, Ghosheh Y, Wolf D, Ley K. Atherosclerosis in the single-cell era. Curr Opin Lipidol 2018;29:389-96.
43. Rodriques SG, Stickels RR, Goeva A, Martin CA, Murray E, Vanderburg CR, et al. Slide-seq: a scalable technology for measuring genome-wide expression at high spatial resolution. Science 2019;363:1463-7.
44. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801-9.
45. Nikpay M, Goel A, Won HH, Hall LM, Willenborg C, Kanoni S, et al. A comprehensive 1,000 genomes-based genome-wide association meta-analysis of coronary artery disease. Nat Genet 2015;47:1121-30.
46. Miller CL, Anderson DR, Kundu RK, Raiesdana A, Nurnberg ST, Diaz R, et al. Disease-related growth factor and embryonic signaling pathways modulate an enhancer of TCF21 expression at the 6q23.2 coronary heart disease locus. PLoS Genet 2013;9:e1003652.
47. Nurnberg ST, Cheng K, Raiesdana A, Kundu R, Miller CL, Kim JB, et al. Coronary artery disease associated transcription factor TCF21 regulates smooth muscle precursor cells that contribute to the fibrous cap. PLoS Genet 2015;11:e1005155.
48. Deloukas P, Kanoni S, Willenborg C, Farrall M, Assimes TL, Thompson JR, et al; CARDIoGRAMplusC4D Consortium. Large-scale association analysis identifies new risk loci for coronary artery disease. Nat Genet 2013;45:25-33.
49. Majesky MW. Vascular development. Arterioscler Thromb Vasc Biol 2018;38:e17-24.
50. Majesky MW. Developmental basis of vascular smooth muscle diversity. Arterioscler Thromb Vasc Biol 2007;27:1248-58.
51. Lahnemann D, Koster J, Szczurek E, McCarthy DJ, Hicks SC, Robinson MD, et al. Eleven grand challenges in single-cell data science. Genome Biol 2020;21:31.
52. van den Brink SC, Sage F, Vertesy A, Spanjaard B, Peterson-Maduro J, Baron CS, et al. Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. Nat Methods 2017;14:935-6.
53. McArdle S, Mikulski Z, Ley K. Live cell imaging to understand monocyte, macrophage, and dendritic cell function in atherosclerosis. J Exp Med 2016;213:1117-31.
54. 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;129:1551-9.
55. Shan F, Huang Z, Xiong R, Huang QY, Li J. HIF1alpha-induced upregulation of KLF4 promotes migration of human vascular smooth muscle cells under hypoxia. J Cell Physiol 2020;235:141-50.
56. Wada Y, Sugiyama A, Yamamoto T, Naito M, Noguchi N, Yokoyama S, et al. Lipid accumulation in smooth muscle cells under LDL loading

is independent of LDL receptor pathway and enhanced by hypoxic conditions. Arterioscler Thromb Vasc Biol 2002;22:1712-9.
57. Balogh E, Toth A, Mehes G, Trencsenyi G, Paragh G, Jeney V. Hypoxia triggers osteochondrogenic differentiation of vascular smooth muscle cells in an HIF-1 (hypoxia-inducible factor 1)-dependent and reactive oxygen species-dependent manner. Arterioscler Thromb Vasc Biol 2019;39:1088-99.
58. Stickels RR, Murray E, Kumar P, Li J, Marshall JL, Di Bella DJ, et al. Highly sensitive spatial transcriptomics at near-cellular resolution with Slide-seqV2. Nat Biotechnol 2021;39:313-9.
59. Raines EW, Ross R. Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. Br Heart J 1993;69(Suppl):S30-7.
60. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000;20:1262-75.
61. Clarke MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. Nat Med 2006;12:1075-80.
62. 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;100:13531-6.
63. Leopold JA. Vascular calcification: mechanisms of vascular smooth muscle cell calcification. Trends Cardiovasc Med 2015;25:267-74.
64. Naik V, Leaf EM, Hu JH, Yang HY, Nguyen NB, Giachelli CM, et al. Sources of cells that contribute to atherosclerotic intimal calcification: an in vivo genetic fate mapping study. Cardiovasc Res 2012;94:545-54.
65. Sun Y, Byon CH, Yuan K, Chen J, Mao X, Heath JM, et al. Smooth muscle cell-specific runx2 deficiency inhibits vascular calcification. Circ Res 2012;111:543-52.
66. Lin ME, Chen TM, Wallingford MC, Nguyen NB, Yamada S, Sawangmake C, et al. Runx2 deletion in smooth muscle cells inhibits vascular osteochondrogenesis and calcification but not atherosclerotic lesion formation. Cardiovasc Res 2016;112:606-16.
67. Lin ME, Chen T, Leaf EM, Speer MY, Giachelli CM. Runx2 expression in smooth muscle cells is required for arterial medial calcification in mice. Am J Pathol 2015;185:1958-69.
68. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, et al. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest 2004;113:1258-65.
69. Kramann R, Goettsch C, Wongboonsin J, Iwata H, Schneider RK, Kuppe C, et al. Adventitial MSC-like cells are progenitors of vascular smooth muscle cells and drive vascular calcification in chronic kidney disease. Cell Stem Cell 2016;19:628-42.
70. Ni Z, Deng J, Potter CMF, Nowak WN, Gu W, Zhang Z, et al. Recipient c-kit lineage cells repopulate smooth muscle cells of transplant arteriosclerosis in mouse models. Circ Res 2019;125:223-41.
71. Majesky MW, Horita H, Ostriker A, Lu S, Regan JN, Bagchi A, et al. Differentiated smooth muscle cells generate a subpopulation of resident vascular progenitor cells in the adventitia regulated by Klf4. Circ Res 2017;120:296-311.
72. Lu S, Jolly AJ, Strand KA, Dubner AM, Mutryn MF, Moulton KS, et al. Smooth muscle-derived progenitor cell myofibroblast differentiation through KLF4 downregulation promotes arterial remodeling and fibrosis. JCI Insight 2020;5:e139445.
73. Bailey B, Fransioli J, Gude NA, Alvarez R Jr, Zhang X, Gustafsson AB, et al. Sca-1 knockout impairs myocardial and cardiac progenitor cell function. Circ Res 2012;111:750-60.
74. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004;84:767-801.
75. Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, Olson EN. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature 2004;428:185-9.
76. Wang Z, Wang DZ, Pipes GC, Olson EN. Myocardin is a master regulator of smooth muscle gene expression. Proc Natl Acad Sci U S A 2003;100:7129-34.
77. Yoshida T, Sinha S, Dandre F, Wamhoff BR, Hoofnagle MH, Kremer BE, et al. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. Circ Res 2003;92:856-64.
78. Liu Y, Sinha S, Owens G. A transforming growth factor-beta control element required for SM alpha-actin expression in vivo also partially mediates GKLF-dependent transcriptional repression. J Biol Chem 2003;278:48004-11.
79. 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;111:685-96.
80. Yoshida T, Gan Q, Owens GK. Kruppel-like factor 4, Elk-1, and histone deacetylases cooperatively suppress smooth muscle cell differentiation markers in response to oxidized phospholipids. Am J Physiol Cell Physiol 2008;295:C1175-82.
81. Liu Y, Sinha S, McDonald OG, Shang Y, Hoofnagle MH, Owens GK. Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. J Biol Chem 2005;280:9719-27.
82. Turner EC, Huang CL, Govindarajan K, Caplice NM. Identification of a Klf4-dependent upstream repressor region mediating transcriptional regulation of the myocardin gene in human smooth muscle cells. Biochim Biophys Acta 2013;1829:1191-201.
83. Nagao M, Lyu Q, Zhao Q, Wirka RC, Bagga J, Nguyen T, et al. Coronary disease-associated gene TCF21 inhibits smooth muscle cell differentiation by blocking the myocardin-serum response factor pathway. Circ Res 2020;126:517-29.
84. Kim JB, Pjanic M, Nguyen T, Miller CL, Iyer D, Liu B, et al. TCF21 and the environmental sensor aryl-hydrocarbon receptor cooperate to activate a pro-inflammatory gene expression program in coronary artery smooth muscle cells. PLoS Genet 2017;13:e1006750.
85. Mokas S, Lariviere R, Lamalice L, Gobeil S, Cornfield DN, Agharazii M, et al. Hypoxia-inducible factor-1 plays a role in phosphate-induced vascular smooth muscle cell calcification. Kidney Int 2016;90:598-609.
86. Iyer D, Zhao Q, Wirka R, Naravane A, Nguyen T, Liu B, et al. Coronary artery disease genes SMAD3 and TCF21 promote opposing interactive genetic programs that regulate smooth muscle cell differentiation and disease risk. PLoS Genet 2018;14:e1007681.

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