Lineage tracking of origin and fate of smooth muscle cells in atherosclerosis

Jacob F. Bentzon,[1,2] MD, PhD
Mark W. Majesky,[3,4*] PhD

Affiliations:
[1]Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain
[2]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[3]Center for Developmental Biology & Regenerative Medicine, Seattle Children's Research Institute, Seattle, WA 98011
[4]Departments of Pediatrics and Pathology, University of Washington, Seattle, WA 98195

*Correspondence:
Mark W. Majesky, Ph.D.
Center for Developmental Biology & Regenerative Medicine
Room 525; M/S C9S-5
Seattle Children's Research Institute
Seattle, WA 98101
USA

Email: mwm84@uw.edu
Phone: +1-206-884-3661
Fax: +1-206-884-1407

Sources of funding:
J.F.B. is supported by research grants from the Danish Council for Independent Research (Sapere Aude II, 4004-00459B) and the Ministerio de Economía, Industria y Competitividad (MEIC; Programa RETOS, SAF2016-75580-R) with cofunding by Fondo Europeo de Desarrollo Regional (FEDER). The CNIC is supported by MEIC and the Pro CNIC Foundation, and is a Severo Ochoa Center of Excellence (MINECO award SEV-2015-0505).

M.W.M. is supported by research grants from the National Institutes of Health (RO1-HL123650, and RO1-HL121877), the Loie Power Robinson Stem Cell & Regenerative Medicine Fund, and the Seattle Children's Research Institute, Seattle, WA.

Dedication:
We would like to dedicate this article to the memory of Elaine W. Raines, Research Professor of Pathology, University of Washington. Elaine was a wonderful colleague, teacher, and friend who enriched our understanding of the complex roles played by smooth muscle cells, macrophages, and the platelet-derived growth factors in atherosclerosis.

Abstract
Advances in lineage tracking techniques have provided new insights into the origins and fates of smooth muscle cells (SMCs) in atherosclerosis. Yet new tools present new challenges for data interpretation that require careful consideration of the strengths and weaknesses of the methods employed. At the same time, discoveries in other fields have introduced new perspectives on longstanding questions about steps in atherogenesis that remain poorly understood. In this article, we attempt to address both the challenges and opportunities for a better understanding of the mechanisms by which cells appearing as or deriving from SMCs accumulate in atherosclerosis.

Introduction

There is little argument that focal accumulation of cells with the characteristics of smooth muscle cells (SMCs) contribute abundantly to clinically relevant atherosclerotic lesions. Such cells can be recognized by their content of myofilaments or SMC contractile proteins, and they, among other roles, build the fibrous cap of plaques that protect fibroatheromas from undergoing rupture and causing thrombosis.[1] Yet although bearing SMC characteristics, and typically being denoted *plaque SMCs*, the phenotype of these cells still differs from that of SMCs in normal arteries. Myofilaments are typically more scarce and differently distributed in the cytoplasm,[2] and some of them acquire additional characteristics that are unusual for SMCs, such as lipid engorgement.[3] This phenotypic ambiguity has played well with ideas presented over the last decade that plaque SMCs may not be SMC lineage cells at all, but cells derived from other sources that are masquerading for SMCs by their expression of SMC marker proteins.

Recent studies employing novel lineage tracking techniques have provided a clearer view of this question, laying out the sources and hereditary relationships of different cell types in the atherosclerotic plaque. These studies have – almost - settled the dispute regarding the origin of plaque SMCs, showing that most, if not all, plaque cells expressing SMC contractile proteins are progeny of local, pre-existing arterial SMCs.[4,5] Moreover, they have revealed that plaque cells expressing SMC contractile proteins are but a small part of a larger and phenotypically heterogeneous population of SMC lineage cells in plaques, many of which fully lose distinguishing SMC features.[6–8] Finally, they have confirmed the longstanding hypothesis that clonal expansion is an important mechanism involved in SMC accumulation in plaques.[9–11]

Here, we critically discuss some of these lineage tracking experiments and the conceptual advances that have resulted from them, which promise to provide new insights into the key roles played by SMCs in atherosclerosis.

Lineage tracking strategies

Lineage tracking is based on a simple principle. A tracking marker, which stably labels a cell population and its progeny, is combined with detection of one or more phenotypic markers that are characteristic for the cell type(s) of interest. The tracking marker is most often a reporter transgene encoding a fluorescent protein or β-galactosidase with expression limited to the intended cell population by cell type-specific Cre/LoxP recombination or transplantation. The phenotypic markers are most often cell type-specific or –selective proteins detected by immunofluorescence staining, but other combinations of tracking and phenotypic markers are possible.

If looking for the source of a particular group of cells, *A*, these cells need to be defined by one or more phenotypic markers. Candidate cell populations (*B*,*C*,*D*,..) from which cells in *A* might originate can then be successively labeled with a tracking marker and it can be determined whether labeled cells turn up among *A* cells **(Figure 1)**. In fate mapping, the starting point is different, but the principle is the same. Here a fixed group of cells *E* are labeled with a tracking marker, and their migration patterns and potential modulation or differentiation into other cell types (*F*,*G*,*H*,..) are followed using a panel of phenotypic markers.

During the last decades, research applying these principles to study SMCs in arterial diseases, including in particular atherosclerosis, has fostered new insight, but also a considerable amount of confusion.[12] Attempts to delineate the source of SMCs in atherosclerotic plaques, defined as cells with expression of contractile proteins such as ACTA2 (smooth muscle α-actin), have produced a number of conflicting reports,[4,5,12,13] some claiming and others disclaiming that ACTA2+ cells can originate from sources other than local medial SMCs, such as hematopoietic cells, progenitor cells in the media or adventitia, and endothelial cells undergoing endothelial-to-mesenchymal transition. To further complicate the picture, fate mapping has shown that local medial SMCs not only give rise to ACTA2+ cells in lesions, but also multiple other

phenotypes in the intima and adventitia that express no or low levels of contractile proteins and undertake other, some yet poorly understood, functions.[6,7,14,15]

If all reports in this field were taken at face value, it would suggest a highly complex pattern of SMC recruitment to atherosclerotic plaques. It is, however, important to appreciate that techniques for lineage tracking, although deceptively simple, have many methodological pitfalls, and that the raw data are rarely of a clarity to not necessitate some interpretation on the part of the researcher. For readers not accustomed with lineage tracking techniques, it must be difficult to decide what to believe and why, but this is essential to make sense of the literature.

Technical challenges

Technical requirements for fate mapping and tracking of cell origin are the same. Tracking and phenotypic markers - and the methods to detect them – need to be sensitive and specific, and there must be a technique that can clearly determine if markers are present in the same cell. Fluorescence microscopy is commonly used because of its ability to detect and distinguish multiplex fluorochrome-labeled markers in tissues at cellular resolution. The steps involved in optimizing lineage tracking with fluorescence microscopy have been discussed in detail previously.[4,16] As a reader, one typically has little chance to evaluate the methodology in great detail and most works contain limited description of the positive and negative controls that researchers must have performed to evaluate sensitivity and specificity of their technique. However, there is a short list of quality criteria that one should be able to evaluate and tick off for any high-quality lineage tracking paper.

Has the tracking marker been fixed in situ, not to float out of labeled cells after sectioning? For eGFP and similar freely diffusible fluorescent proteins, sectioning without prior fixation will produce this kind of problem.

Do tracking and phenotypic markers produce clear signal? In fluorescence microscopy, intensity of signal is arbitrary being ultimately determined by the brightness and contrast adjustments of the raw image data. This means that even faint differences in real signal intensity can be boosted to all-or-none contrasts in final images. Properly controlled against negative and positive controls this poses a minor problem, but if not managed correctly, subtle variations in autofluorescence or unspecific staining can be boosted to mimic clear signal. A telltale sign that brightness and contrast settings have been set aggressively to amplify small differences in signal is the appearance of pixelated noise in the image.

Can tracking and phenotypic markers be clearly distinguished from autofluorescence? Atherosclerotic plaques and many other tissues contain rich amounts of autofluorescent material, e.g. lipofuscin, which is excited and emits at a broad range of wavelengths over the visible spectrum. Consequently, autofluorescent material will produce signal with microscope settings for many different fluorophores, making it easy to confuse it for specific signal. The presence of exact overlapping signal in different channels, which is typically not expected for the specific tracking and phenotypic marker signals, should raise concern over autofluorescence problems.

Can single cells be identified? It is a common misconception that cells positive for tracking and phenotypic markers can be identified by looking for pixels with co-localizing color. First of all, the tracking and phenotypic marker are often not localized at the same site in cells. Second, closely apposed cells will often give rise to some signal overlap, even when the signals arise from different cells. Third, as already noted, autofluorescence will produce exactly this kind of appearance. The key is to identify *single* cells having the *expected* intracellular distribution of tracking and phenotypic markers. This also means that without good tissue preservation and clear single cell resolution of the microscopy, there is no hope for lineage tracking analysis. For the most common combination of tracking (eGFP expression) and phenotypic markers (ACTA2 expression) used to analyze the origin of plaque SMCs, the expected appearance of double positive cells is shown in **Figure 2**.

Vascular smooth muscle: A developmental mosaic

A natural starting point for understanding SMC origin and fates in atherosclerosis is to understand the developmental history of SMCs in normal arteries. The embryonic vascular system first forms as a highly branched network composed of endothelial cells and their basement membrane.[17,18] Continued growth of the embryo beyond E10.5 in the mouse requires building layers of circumferentially arranged SMCs to provide mechanical strength and vasomotor regulation to the vessel wall, and to normalize wall stress.[19–21] As the embryo increases in size and blood vessels increase in length, the circumferential orientation of SMCs transitions to a helical one characteristic of SMCs in mature arteries.[22–24] Investment of developing blood vessels with SMCs is a local process driven by flow and pressure-dependent mechanotransduction, cell contact-mediated signaling and short-range paracrine factors.[19,25,26] By the time SMC investment begins around E10.5, the vascular network has reached all parts of the growing embryo making it unlikely that a single anlage for vascular SMCs exists. In fact, lineage-tracking studies in avian and mammalian embryos have shown that vascular smooth muscle is a developmental mosaic that is derived from a surprising number of different primary origins *in utero* **(Figure 3)**.[27,26] In the mouse, at least eight independent SMC lineages are used to build the walls of blood vessels as they emerge from the heart and branch into complex networks that ramify throughout the developing embryo.[20] Evidence for a similar mosaic pattern of vascular smooth muscle in human development has also been reported.[28,29]

Segmental architecture of blood vessels

The axial borders of vascular segments containing SMCs of different embryonic origins appear abrupt with little or no intermixing.[30–32] Once these borders are formed, they appear to be maintained throughout adulthood in the majority of cases studied.[30,32,33] This means that, at an anatomical level, the arterial system is composed of a series of origin-specific segments that may predict segment-specific functional properties. In addition, vascular SMCs also possess differing positional identity within the three major axes of the embryo (anterior-posterior, dorsal-ventral, right-left).[34,35] One important consequence of this mosaic pattern of lineage diversity and positional identity is that common systemic risk factors for atherosclerosis (hypertension, elevated plasma lipids, inflammation) do not act upon a single generic type of vascular SMC, but rather act on different kinds of SMCs depending on their location in the arterial tree with potentially different outcomes. For example, Trigueros-Motos et al showed that SMCs in adult mouse descending aorta (DA) (paraxial mesoderm-derived) express relatively high levels of *HoxA9* whereas ascending thoracic and transverse aortic (AA) SMCs (neural crest-derived) *HoxA9* levels are comparatively low.[36] Since HoxA9 can repress expression of the p65 subunit of NF-kB, then inflammatory stimuli such as TNFα elicit NF-kB activation responses that are position-dependent (low in DA, and high in AA). This anatomical variance in NF-kB activation mirrors the severity of aortic atherosclerotic lesion development in *Apoe-/-* mice.[36] The aortic segment-dependent differences in TNFα-mediated NF-kB activation in adult mice are reminiscent of the lineage-specific differences in TGFβ-mediated signaling and extracellular matrix-sensing in developing chick embryo aortic SMCs.[37,38] By extension, therefore, SMC lineage diversity is an organizing principle in vascular development that has important implications for where vascular disease manifests and how it progresses in the adult vascular system.

Sources of atherosclerotic plaque ACTA2+ SMCs

Until the start of the millennium, lineage-tracking studies had not tested the origin of atherosclerotic plaque SMCs **(Figure 4A)**. The longstanding paradigm in the field had considered plaque SMCs to be derived exclusively from local SMCs, but this was based on indirect evidence. Medial SMC proliferation was known to precede neointimal formation after balloon injury in primates and rats, and by electron microscopy, SMCs could sometimes be seen in fenestrae of the internal elastic lamina apparently moving from the media to the intima.[39,40] Furthermore, cell culture studies had shown the capacity of local arterial SMCs to undergo phenotypic modulation to a proliferative, matrix-producing SMC phenotype with low expression of contractile proteins, which aligned well with the ultrastructural type of SMCs

seen in atherosclerotic lesions.[41] Feil et al were the first to use lineage tracking to show that migration of medial SMCs into developing atherosclerotic plaques in mice do, indeed, occur and that this is a quantitatively important source of plaque SMCs.[42] Others have confirmed this conclusion many times subsequently, using different SMC-restricted labeling strategies.[7,9,10,43]

Circulatory origin of plaque SMCs – an idea undone

The first paper to suggest that other cell populations than local arterial SMCs could be a source of plaque cells with SMC characteristics was published in 2002 by Sata et al.[44] *Apoe−/−* mice were BM transplanted using donor cells from transgenic mice expressing either enhanced green fluorescent protein (eGFP) or bacterial β-galactosidase. Atherosclerosis was then allowed to develop, and eGFP, β-galactosidase, and ACTA2 tracking and phenotypic markers were identified in plaques by immunofluorescence staining. Based on overlap of the eGFP/β-galactosidase staining with that of ACTA2, it was concluded that approximately half of ACTA2+ cells in atherosclerotic plaques were of donor BM origin, presumably differentiating from hematopoietic stem cells. Soon after, Caplice et al. examined coronary atherosclerotic plaques from deceased patients who had received a sex-mismatched BM transplant for hematological disease. Using sex chromosomes as tracking markers and ACTA2, MYH11 (smooth muscle myosin heavy chain), and calponin to identify plaque SMCs, it was concluded that ~20% of such cells originated from the transplanted BM cells.[45]

Others have largely failed to replicate these findings in mouse models. In one study, one of us with colleagues repeated part of the experiments of Sata et al. with technical alterations to improve fidelity of the lineage tracking.[46] More than 10000 ACTA2+ cells were examined in plaques from BM-transplanted *Apoe-/-* mice without encountering a single hematopoietically-derived ACTA2+ cell. In subsequent studies, where atherosclerosis was induced in transplanted isogenic vessel segments, not only BM-derived but all circulating cell types could be excluded as a contributing source to plaque ACTA2+ cells.[46] Healing of mechanical plaque ruptures in BM- and vessel-transplanted *Apoe-/-* mice produced analogous results.[47] Moreover, Yu et al. found little evidence for BM-derived plaque SMCs in *Apoe-/-* mice transplanted with BM from a SM22α-reporter mouse.[48] These results in atherosclerosis are consistent with multiple reports investigating SMC origin in other vascular pathologies.[49,50]

Recent studies, including a re-investigation of the question by the group behind the seminal 2002 paper, reported that while blood cells may not be an important source of bona fide plaque SMCs, they may still give rise to ACTA2 expressing cells.[51,52] The contribution to plaque ACTA2+ cells is probably at a level where it is difficult to distinguish it effectively from background events caused by unspecific staining signals or closely apposed cells, and the few examples of circulatory-derived ACTA2 presented do not pass as typical plaque SMCs with the expected intracellular distribution of ACTA2 and tracking markers (**Figure 2**). This interpretation is also supported by the lower expression levels of SMC characteristic genes in blood-derived versus medial SMC-derived plaque ACTA2 cells.[51]

Controversy continues to surround the topic, but it seems reasonable to conclude that the idea is today largely undone that cells outside the local arterial wall contribute to plaque SMCs in mouse models of atherosclerosis in any functionally important manner. Further studies are still needed to clarify the potential contribution of circulation cells to plaque SMCs in humans.

Other local sources within the vascular wall

Other sources within the arterial wall may contribute to neointimal SMCs in vascular pathologies, although direct evidence that this occurs in atherosclerosis is sparse. Importantly, these ideas are not at odds with those studies that have used SMC-restricted Cre/LoxP lineage tracking to identify SMC origin, since neither medial SMCs nor plaque SMCs are completely labeled in these experiments.[42] It has been found by several groups that progenitor cells exist at the interface of the arterial media and adventitia, which have the ability to differentiate into SMCs and multiple other cell types in vitro.[32,53] The contribution of these progenitors to

neointimal SMCs may be highly context dependent. Seeding to irradiated vein grafts in *Apoe-/-* mice led to some progenitor-derived neointimal SMCs,[53] but direct contribution to atherosclerotic plaque SMCs have not yet been shown. Furthermore, endothelial to mesenchymal transition has recently been reported to underlie a small number of ACTA2+ cells in mouse atherosclerosis.[54] More research is required to definitively confirm or rule out sizeable contributions to plaque SMCs from local non-SMC sources in different settings.

Fate mapping of medial SMCs in atherosclerosis

Fate mapping of medial SMCs by Cre/LoxP activation of reporter transgenes in mice have shown that in addition to being the predominant source of ACTA2+ plaque cells, medial SMCs beget a large number of highly modulated SMC phenotypes in plaques that have low or no expression of contractile proteins and therefore escape recognition as SMCs in standard immunohistochemistry (**Figure 4B**).[6,43] Several studies have shown that these ACTA2- SMC-derived cells in plaques even significantly outnumber ACTA2+ progeny.[6,7,43] Using an epigenetic modification in the *MYH11* gene promoter as a persistent SMC lineage marker, the existence of ACTA2- SMC-derived cells has also been demonstrated in human lesions.[43] The ACTA2- SMC lineage cells is a heterogeneous population comprising a number of cell types that are yet sparsely defined with respect to the genes they express, the mechanisms underlying their derivation from medial SMCs, and their function in the atherosclerotic lesion.

Some ACTA2- SMC-derived cells acquire a characteristic chondrocyte-like morphology and express the osteo-chondrogenic transcription factors SOX9 and RUNX2.[14] These modulated SMCs, which are also present in human atherosclerosis, are likely partakers in plaque calcification.[55] Other SMC-derived cells in the plaque and adventitia express the progenitor marker SCA1.[7,15] Isolated from the adventitia, such cells exhibit multipotent differentiation potential being able to give rise to mature SMCs as well as cells with resident macrophage and endothelial-like phenotypes.[15] Whether the SCA1-positive SMC progeny that are present within plaques have similar progenitor capabilities has been addressed, but not yet confirmed.[7] Yet other SMC lineage cells in the plaque have been shown to express proteins that have been considered characteristic of macrophages, such as CD68, LGALS3/Mac-2, and LAMP2/Mac-3, and accordingly are sometimes referred to as macrophage-like SMCs.[6,7,9] Similar conversion of medial SMCs can be induced by cholesterol loading in vitro, which provide clues to their appearance in atherosclerosis, but on the global gene expression level they remain distinct from authentic macrophages and monocytes.[8] Intermediate cell phenotypes co-expressing ACTA2 and CD68, or ACTA2 and SOX9, have also been described in human and mouse atherosclerosis.[3,55] Lastly, there is a large residual population of medial SMC-derived cells in the murine plaque that do not fall into either of the categories above.

One should be very cautious in trying to infer the function of such modulated SMCs by their expression of marker proteins, e.g. macrophage-like function because of the presence of certain macrophage markers. The very assumption underlying the use of markers such as CD68 or LGALS3 to identify macrophages is that they are not expressed by other cell lineages in the tissue of interest. When this assumption cannot be upheld, as is the case in the atherosclerotic plaque, they lose their power to predict macrophage phenotype. The simplest conclusion that can be drawn from the expression of CD68, LGALS3, and LAMP2 in SMC-derived cells is thus that these proteins are not good macrophage markers in atherosclerosis. Indeed, each of them is also expressed in some non-macrophage cells in other tissues.[56]

The expression of these proteins could still in their own right reflect important new functions acquired by the SMC-derived cells, even if they are not useful as cell type markers, but this has yet to be addressed. Even in macrophages the biochemical function of CD68, LGALS3, and LAMP2 and potential effects in atherogenesis remain incompletely understood. CD68 is a scavenger receptor, which in macrophages is mainly found localized to endosomes. It was initially believed to be involved in scavenging of extracellular material including modified LDL, but this has not been confirmed in studies of *Cd68-/-* mice, which rather indicated a regulating role of CD68 for MHC-II mediated antigen presentation.[57] Potential involvement of CD68 in atherosclerosis is unknown. LGALS3/Mac-2 is a β-galactose binding lectin that is

involved in a broad range of biological functions, including phagocytosis, cell turnover, and apoptosis. LGALS3 facilitates development of atherosclerosis in *Apoe-/-* mice, but is unknown what mechanism and which cell type(s) carry the effect.[58] LAMP2 is located in the lysosomal membrane of macrophages, as well as other cell types, and is important for autophagy.[59] Its potential role in atherosclerosis has not been explored.

Inasmuch as the modulation of SMCs in the arterial wall may represent some degree of reversal to a less differentiated phenotype (followed by re-differentiation to another) it is tempting to speculate that the developmental origin of SMCs in different regions (see **Figure 3**) could influence the spectrum of modulated SMC phenotypes in plaques. Going forward it will be crucial to further characterize the diversity of SMC progeny in atherosclerosis, and in particular to explore the functional consequences of SMC recruitment and modulation in atherosclerosis, e.g. through SMC-specific gene manipulation as recently described.[7,60,61] This is a growing research field, which is covered in a separate review in this Spotlight issue.[62]

SMC clonality in atherosclerosis

In 1973, Benditt reported that human atherosclerotic plaques are monoclonal lesions analogous to a benign smooth muscle tumor.[11] This was a striking finding, repeated by multiple groups at the time, and yet was ignored for many years as clonality was not predicted by popular models of atherogenesis based on disorders of lipid metabolism or chronic inflammation. As methods for single cell fate mapping in embryos and adult animal models of injury and disease have advanced in recent years,[9,21,63] the clonality of atherosclerotic lesions has come back into the picture as reflective of some fundamental property of blood vessels that we have very little understanding of.

While the original hypothesis of a mutational origin of SMC clonal growth[11] has not been ruled out, more recent studies have sought to understand clonality of the lesion in terms of the patch size of SMC clones produced in normal vascular development. If the clonal patch size in normal aortic wall is large, for example, then localized focal injuries or signals might produce spatially restricted proliferative responses by multiple medial SMCs from the same developmental clone leading to an apparent "clonal" expansion into the intima. Evidence for such a comparatively large size of clonal patches in human artery walls has been reported by techniques to analyze X chromosome inactivation skewing in homogenates of micro-dissected arteries.[64,65] In contrast, experiments with single-cell resolution in multicolor reporter or aggregation chimeric mice, have shown a small clonal patch size in developing pulmonary arteries[21] and mature aortas[10] consistent with extensive mixing of the progeny of single SMCs during development. The caveat to using these mouse studies to infer clonal patch structure in normal human arteries is the significant size scale differences. Human arteries are much larger in diameter and consist of many more layers of SMCs and elastin than their counterparts in the mouse.[23,25] It is conceivable that patch size could be different in this setting, and the possibility of a large preexisting SMC clones in arteries of humans effectively preclude conclusions on whether clonal expansion of SMCs in human atherosclerosis occurs during development and body growth or is part of atherogenesis.

In the mouse, however, it has now been shown using multicolor reporter transgenes and aggregation chimeras that intimal SMCs accumulating in atherosclerosis or after arterial injury are the product of a mono- or oligo-clonal expansion process.[9,10] Remarkably few, if any, atherosclerotic plaques in *Apoe-/-* or PCSK9-overexpressing mice fed a high fat diet were produced by a truly polyclonal proliferation of SMCs.[9,10]

While the evidence for intimal lesion clonality is now quite strong, a consensus mechanism for how clonal SMC proliferation arises is lacking. One possibility is that clonal populations derive from a subgroup of SMCs in the intima or inner media with a cell-autonomous propensity for proliferation. Such a specialized type of SMC was proposed by Sheikh et al to explain the distal muscularization of terminal arterioles in pulmonary hypertension.[63] Second, the clone precursors may be ordinary SMCs that are simply located at the right place and the right time, e.g. where the barrier of the internal elastic lamina is breached or a local signal is present to initiate their expansion. Possibly SMCs recruited in this way could even actively inhibit surrounding SMCs from taken the same path. Such a lateral inhibition process is a pattern

commonly seen in embryo development when few cells from an initially homogenous cell population are led by a particular developmental path,[66] and could preclude large numbers of SMCs from modulating and leaving the arterial intima at the same time.

Is SMC clonal expansion an emergent property within normal artery walls?
An entirely different possibility is that clonal expansion of SMCs may be the result of an emergent property among a group of cells interacting by a process known as distributed sensing or "quorum" sensing.[67] This is a systems level property that was discovered in bacteria and is now known to also be active in mammalian cells.[68–70] In this model, vascular SMCs exist in groups or collectives in which "sensing" the external environment is a function that is shared by individual SMCs that differ in their sensitivity to, for example, inflammatory cytokines. This proposal is based on recent work studying the responses of large vessel endothelial cells to acetylcholine (Ach).[69] Using an intact vessel perfusion system and an intracellular fluorescent calcium indicator, Wilson et al found that any given endothelial cell responds to Ach with a concentration-dependent increase in intracellular calcium indicator fluorescence over only one order of magnitude. Some endothelial cells are very sensitive to low concentrations of Ach and exhibit a full response over the range of 10^{-10}-10^{-9} M Ach. Other endothelial cells are much less sensitive and fully respond only over a much higher range of 10^{-7}–10^{-6} M Ach. The former do not distinguish signal from noise very well, while the latter are practically insensitive to baseline noise intrinsic to all signaling systems. Only the combination of these differentially responding endothelial cells gives the entire appropriate Ach dose-response curve over four orders of magnitude characteristic of endothelial-dependent relaxation *in vivo*. If the response were SMC migration in a mature artery wall and the conditions were to (a) overcome inhibitors of migration present at high concentrations and (b) respond to migration promoting factors present at low concentrations then there may be only a small number of cells that are even capable of meeting these conditions in a normal artery wall. If that were true, then a mono/oligo-clonal expansion of plaque SMCs would be the predicted outcome. This model is, of course, highly speculative. Yet our current models for atherogenesis are mostly sequential modifications of older hypotheses that, in some cases, go back well over a century. The lack of a clear consensus for how SMC clonality arises in plaque formation encourages consideration of different approaches to the problem.

Comparative aspects – mouse versus human
Genetically engineered mice have been central to investigation of SMC origin and fate in atherosclerosis; the reason being that this is the only species in which both induction of atherosclerosis and Cre/Lox based cell tracking is practical. However, while lineage tracking can be performed with high fidelity in the mouse, the structure of atherosclerosis-prone arteries and the pathogenesis of atherosclerotic lesion formation show important differences between mice and humans.
The murine intima in mice at atherosclerosis-prone sites is thin consisting only of the endothelium and scattered dendritic cells,[71] whereas in humans, the normal intima contains variable amounts of intimal SMCs and connective tissue.[72] Especially at atherosclerosis-prone sites, intimal SMCs can be abundant and the thickness of the intima as large as the adjacent media. It is formally unknown if SMCs in human atherosclerotic plaques descend from pre-existing intimal SMCs or recruited medial SMCs, but it appears highly plausible that the SMCs already in the human intima play an important role. This underscores the need to be careful in extrapolating from mouse models to human with respect to plaque SMCs. The presence of intimal SMCs from the very onset of atherosclerosis, the differences in the phenotype of pre-existing intimal and medial SMCs,[73] and the different mechanisms involved in their recruitment could well distinguish human from murine atherosclerosis with respect to the pathophysiological roles undertaken by SMCs, their clonal relationships, and the modulated phenotypes to which they contribute.
In humans, the possibilities for SMC lineage and clonality tracking are limited to using the telltales that are naturally present in tissues. One such method, developed by Gomez et al,[43] identifies SMC lineage cells by an epigenetic modification in the *Myh11* locus, which is

preserved during - at least some types of - phenotypic modulation. Development of X chromosome inactivation pattern analysis with cellular resolution is a potential approach for clonal analysis. Finally, emerging single-cell gene expression techniques promise to increase understanding of cell types in many biological systems,[74] and could also shed new light over the identity and relationships of SMC lineage cells in human atherosclerotic plaques.

References

1. Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J, Finn A V, Virmani R. The thin-cap fibroatheroma: a type of vulnerable plaque: the major precursor lesion to acute coronary syndromes. *Curr Opin Cardiol* 2001;**16**:285–292.
2. Stary HC. *Atlas of atherosclerosis — progression and regression.* Parthenon Publishing Group; 1999.
3. Allahverdian S, Chehroudi AC, Mcmanus BM, Abraham T, Francis GA. Coronary heart disease contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation* 2014;**129**:1551–1559.
4. Bentzon JF, Falk E. Circulating smooth muscle progenitor cells in atherosclerosis and plaque rupture: current perspective and methods of analysis. *Vascul Pharmacol* 2010;**52**:11–20.
5. Hoofnagle MH, Thomas JA, Wamhoff BR, Owens GK. Origin of neointimal smooth muscle: We've come full circle. *Arterioscler Thromb Vasc Biol* 2006;**26**:2579–2581.
6. 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.
7. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, Swiatlowska P, Newman A a C, Greene ES, Straub AC, Isakson B, Randolph GJ, Owens GK. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nat Med* 2015;**21**:628–637.
8. Vengrenyuk Y, Nishi H, Long X, Ouimet M, Savji N, Martinez FO, Cassella CP, Moore KJ, Ramsey SA, Miano JM, Fisher EA. 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.
9. 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 contribute to neointimal formation in mouse injury and atherosclerosis models. *Circ Res* 2016;**119**:1313–1323.
10. Jacobsen K, Lund MB, Shim J, Gunnersen S, Füchtbauer E, Kjolby M, Carramolino L, Bentzon JF. Diverse cellular architecture of atherosclerotic plaque derives from clonal expansion of a few medial SMCs. *JCI insight* 2017;**2**:e95890.
11. Benditt EP, Benditt JM. Evidence for a monoclonal origin of human atherosclerotic plaques. 1973;**70**:1753–1756.
12. Bennett MR, Sinha S, Owens GK. Vascular smooth muscle cells in atherosclerosis. *Circ Res* 2016;**118**:692–703.
13. Nguyen AT, Gomez D, Bell RD, Campbell JH, Clowes AW, Gabbiani G, Giachelli CM, Parmacek MS, Raines EW, Rusch NJ, Speer MY, Sturek M, Thyberg J, Towler DA, Weiser-Evans MC, Yan C, Miano JM, Owens GK. Smooth muscle cell plasticity: Fact or fiction? *Circ Res* 2013;**112**:17–22.
14. Naik V, Leaf EM, Hu JH, Yang HY, Nguyen NB, Giachelli CM, Speer MY. Sources of cells that contribute to atherosclerotic intimal calcification: an in vivo genetic fate mapping study. *Cardiovasc Res* 2012;**94**:545–554.
15. Majesky MW, Horita H, Ostriker A, Lu S, Regan JN, Bagchi A, Dong XR, Poczobutt J, Nemenoff RA, Weiser-Evans MCM. Differentiated smooth muscle cells generate a subpopulation of resident vascular progenitor cells in the adventitia regulated by Klf4. *Circ Res* 2017;**120**:296–311.
16. Brazelton TR, Blau HM. Optimizing techniques for tracking transplanted stem cells in vivo. *Stem Cells* 2005;**23**:1251–1265.
17. Herbert SP, Stainier DYR. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol* 2011;**12**:551–564.
18. Lazarus A, Del-Moral PM, Ilovich O, Mishani E, Warburton D, Keshet E. A

perfusion-independent role of blood vessels in determining branching stereotypy of lung airways. *Development* 2011;**138**:2359–2368.
19. Wagenseil JE, Mecham RP. Vascular extracellular matrix and arterial mechanics. *Physiol Rev* 2009;**89**:957–989.
20. Majesky MW, Dong XR, Regan JN, Hoglund VJ. Vascular smooth muscle progenitor cells: Building and repairing blood vessels. *Circ Res* 2011;**108**:365–377.
21. Greif DM, Kumar M, Lighthouse JK, Hum J, An A, Ding L, Red-Horse K, Espinoza FH, Olson L, Offermanns S, Krasnow MA. Radial construction of an arterial wall. *Dev Cell* 2012;**23**:482–493.
22. Cliff W. The aortic tunica media in growing rats studied with the electron microscope. *Lab Invest* 1967;**17**:599–614.
23. Wolinsky H, Glagov S. A lamellar unit of aortic medial structure and function in mammals. *Circ Res* 1967;**20**:99–111.
24. Spronck B, Megens RTA, Reesink KD, Delhaas T. A method for three-dimensional quantification of vascular smooth muscle orientation: application in viable murine carotid arteries. *Biomech Model Mechanobiol* 2016;**15**:419–432.
25. Shadwick RE. Mechanical design in arteries. *J Exp Biol* 1999;**202**:3305–3313.
26. Manderfield LJ, Aghajanian H, Engleka KA, Lim LY, Liu F, Jain R, Li L, Olson EN, Epstein JA. Hippo signaling is required for Notch-dependent smooth muscle differentiation of neural crest. 2015;2962–2971.
27. Majesky MW. Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol* 2007;**27**:1248–1258.
28. Cheung C, Bernardo AS, Trotter MWB, Pedersen RA, Sinha S. Generation of human vascular smooth muscle subtypes provides insight into embryological origin-dependent disease susceptibility. *Nat Biotechnol* 2012;**30**:165–173.
29. Sinha S, Iyer D, Granata A. Embryonic origins of human vascular smooth muscle cells: Implications for in vitro modeling and clinical application. *Cell Mol Life Sci* 2014;**71**:2271–2288.
30. Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. *Development* 2000;**127**:1607–1616.
31. Waldo KL, Hutson MR, Ward CC, Zdanowicz M, Stadt HA, Kumiski D, Abu-Issa R, Kirby ML. Secondary heart field contributes myocardium and smooth muscle to the arterial pole of the developing heart. *Dev Biol* 2005;**281**:78–90.
32. Passman JN, Dong XR, Wu S-P, Maguire CT, Hogan KA, Bautch VL, Majesky MW. A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A* 2008;**105**:9349–9354.
33. Sawada H, Rateri DL, Moorleghen JJ, Majesky MW, Daugherty A. Smooth muscle cells derived from second heart field and cardiac neural crest reside in spatially distinct domains in the media of the ascending aorta. *Arterioscler Thromb Vasc Biol* 2017;**37**:1722–1726.
34. Chang HY. Anatomic Demarcation of cells: Genes to patterns. *Science* 2009;**326**:1206–1207.
35. Pruett ND, Hajdu Z, Zhang J, Visconti RP, Kern MJ, Wellik DM, Majesky MW, Awgulewitsch A. Changing topographic Hox expression in blood vessels results in regionally distinct vessel wall remodeling. *Biol Open* 2012;**1**:430–435.
36. Trigueros-Motos L, Gonzalez-Granado JM, Cheung C, Fernandez P, Sanchez-Cabo F, Dopazo A, Sinha S, Andrés V. Embryological-origin-dependent differences in homeobox expression in adult aorta: Role in regional phenotypic variability and regulation of NF-kB activity. *Arterioscler Thromb Vasc Biol* 2013;**33**:1248–1256.
37. Topouzis S, Majesky MW. Smooth muscle lineage diversity in the chick embryo two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-beta. *Dev Biol* 1996;**178**:430–445.
38. Thieszen SL, Dalton M, Gadson PF, Patterson E, Rosenquist TH. Embryonic lineage of vascular smooth muscle cells determines responses to collagen matrices and

integrin receptor expression. *Exp Cell Res* 1996;**227**:135–145.
39. Stemerman MB, Ross R. Experimental arteriosclerosis. I. Fibrous plaque formation in primates, an electron microscope study. *J Exp Med* 1972;**136**:769–789.
40. Clowes AW, Reidy M, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest* 1983;**49**:327–333.
41. Campbell JH, Campbell GR. Smooth muscle phenotypic modulation - a personal experience. *Arterioscler Thromb Vasc Biol* 2012;**32**:1784–1789.
42. Feil S, Hofmann F, Feil R. SM22alpha modulates vascular smooth muscle cell phenotype during atherogenesis. *Circ Res* 2004;**94**:863–865.
43. 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.
44. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* 2002;**8**:403–409.
45. Caplice NM, Bunch TJ, Stalboerger PG, Wang S, Simper D, Miller D V, Russell SJ, Litzow MR, Edwards WD. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. *Proc Natl Acad Sci U S A* 2003;**100**:4754–4759.
46. Bentzon JF, Weile C, Sondergaard CS, Hindkjaer J, Kassem M, Falk E. Smooth muscle cells in atherosclerosis originate from the local vessel wall and not circulating progenitor cells in ApoE knockout mice. *Arterioscler Thromb Vasc Biol* 2006;**26**:2696–2702.
47. Bentzon JF, Sondergaard CS, Kassem M, Falk E. Smooth muscle cells healing atherosclerotic plaque disruptions are of local, not blood, origin in apolipoprotein E knockout mice. *Circulation* 2007;**116**:2053–2061.
48. Yu H, Stoneman V, Clarke M, Figg N, Xin HB, Kotlikoff M, Littlewood T, Bennett M. Bone marrow-derived smooth muscle-like cells are infrequent in advanced primary atherosclerotic plaques but promote atherosclerosis. *Arterioscler Thromb Vasc Biol* 2011;**31**:1291–1299.
49. Hagensen MK, Shim J, Falk E, Bentzon JF. Flanking recipient vasculature, not circulating progenitor cells, contributes to endothelium and smooth muscle in murine allograft vasculopathy. *Arterioscler Thromb Vasc Biol* 2011;**31**:808–813.
50. Daniel JM, Sedding DG. Circulating smooth muscle progenitor cells in arterial remodeling. *J Mol Cell Cardiol* Elsevier Ltd; 2011;**50**:73–279.
51. Albarran-Juarez J, Kaur H, Grimm M, Offermanns S, Wettschureck N. Lineage tracing of cells involved in atherosclerosis. *Atherosclerosis* 2016;**16**:445–453.
52. Iwata H, Manabe I, Fujiu K, Yamamoto T, Takeda N, Eguchi K, Furuya A, Kuro-o M, Sata M, Nagai R. Bone marrow-derived cells contribute to vascular inflammation but do not differentiate into smooth muscle cell lineages. *Circulation* 2010;**122**:2048–2057.
53. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest* 2004;**113**:1258–1265.
54. Evrard SM, Lecce L, Michelis KC, Nomura-kitabayashi A, Pandey G, Purushothaman K, Escamard V, Li JR, Hadri L, Fujitani K, Moreno PR, Benard L, Rimmele P, Cohain A, Mecham B, Randolph GJ, Nabel EG, Hajjar R, Fuster V, Boehm M, Kovacic JC. Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. *Nat Commun* 2016;**7**:11853.
55. Bobryshev Y V. Transdifferentiation of smooth muscle cells into chondrocytes in atherosclerotic arteries in situ: Implications for diffuse intimal calcification. *J Pathol* 2005;**205**:641–650.
56. Rehg JE, Bush D, Ward JM. The utility of immunohistochemistry for the identification of hematopoietic and lymphoid cells in normal tissues and interpretation

of proliferative and inflammatory lesions of mice and rats. *Toxicol Pathol* 2012;**40**:345–374.
57. Song L, Lee C, Schindler C. Deletion of the murine scavenger receptor CD68. *J Lipid Res* 2011;**52**:1542–1550.
58. MacKinnon AC, Liu X, Hadoke PW, Miller MR, Newby DE, Sethi T. Inhibition of galectin-3 reduces atherosclerosis in apolipoprotein E-deficient mice. *Glycobiology* 2013;**23**:654–663.
59. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lüllmann-Rauch R, Janssen PM, Blanz J, Figura K von, Saftig P. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature* 2000;**406**:902–906.
60. Cherepanova OA, Gomez D, Shankman LS, Swiatlowska P, Williams J, Sarmento OF, Alencar GF, Hess DL, Bevard MH, Greene ES, Murgai M, Turner SD, Geng Y, Bekiranov S, Connelly JJ, Tomilin A, Owens GK. Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. *Nat Med* 2016;**22**:657–665.
61. Sui Y, Park S, Xu J, Monette S, Helsley RN, Han S, Zhou C. IKKβ links vascular inflammation to obesity and atherosclerosis. 2014;**211**:869–886.
62. Bochaton-Piallat M-L, Francis GA. Smooth muscle cell fate/plasticity and plaque vulnerability. *Cardiovasc Res* 2018;Under preparation.
63. 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.
64. Murry CE, Gipaya CT, Bartosek T, Benditt EP, Schwartz SM. Monoclonality of smooth muscle cells in human atherosclerosis. *Am J Pathol* 1997;**151**:697–705.
65. Chung IM, Schwartz SM, Murry CE. Clonal architecture of normal and atherosclerotic aorta: implications for atherogenesis and vascular development. *Am J Pathol* 1998;**152**:913–923.
66. Bray SJ. Notch signalling in context. *Nat Rev Mol Cell Biol* 2016;**9**:722–735.
67. Widelitz R, Chuong CM. Quorum sensing and other collective regenerative behavior in organ populations. *Curr Opin Genet Dev* 2016;**40**:138–143.
68. Chen CC, Wang L, Plikus M V., Jiang TX, Murray PJ, Ramos R, Guerrero-Juarez CF, Hughes MW, Lee OK, Shi S, Widelitz RB, Lander AD, Chuong CM. Organ-level quorum sensing directs regeneration in hair stem cell populations. *Cell* 2015;**161**:277–290.
69. Wilson C, Saunter CD, Girkin JM, McCarron JG. Clusters of specialized detector cells provide sensitive and high fidelity receptor signaling in the intact endothelium. *FASEB J* 2016;**30**:2000–2013.
70. McCarron JG, Lee MD, Wilson C. The endothelium solves problems that endothelial cells do not know exist. *Trends Pharmacol Sci* 2017;**38**:322–338.
71. Paulson KE, Zhu SN, Chen M, Nurmohamed S, Jongstra-Bilen J, Cybulsky MI. Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circ Res* 2010;**106**:383–390.
72. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W, Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1992;**85**:391–405.
73. Adams LD, Geary RL, Li J, Rossini A, Schwartz SM. Expression profiling identifies smooth muscle cell diversity within human intima and plaque fibrous cap: Loss of RGS5 distinguishes the cap. *Arterioscler Thromb Vasc Biol* 2006;**26**:319–325.
74. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nat Rev Immunol* 2017;Advance online publication.

Figure 1

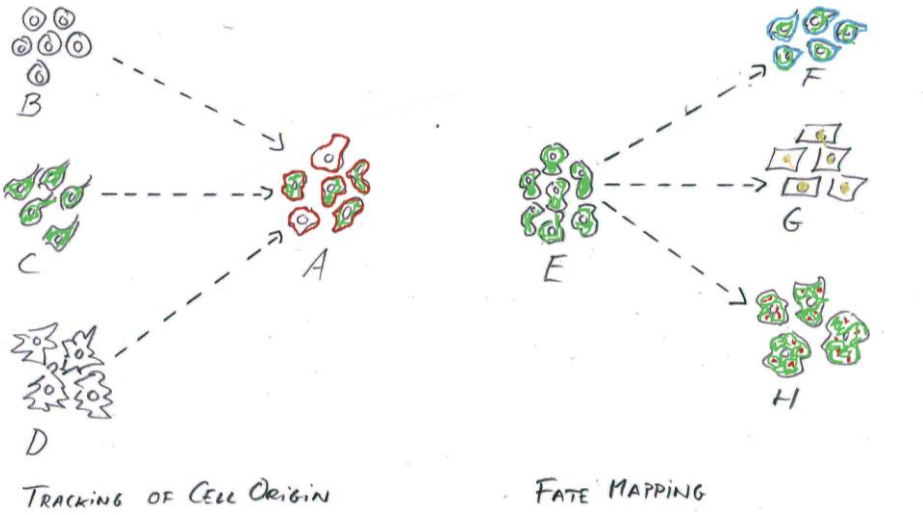

Figure 1. Lineage tracking strategies. A. For investigating the origin of a set of cells, *A*, several different, mutually exclusive, candidate populations can be successively labeled with a tracking marker, and it can be determined if labeled cells turn of among *A* cells. In the example shown, the phenotypic marker used to define *A* is a red membrane stain and the candidate population *C* is labeled with a green cytoplasmic tracking marker. *C* contributes to *A*, but other sources for *A* exist. **B.** In fate mapping, a group of cells, *E*, is labeled with a tracking marker, and the migration and phenotypic changes (modulation, differentiation) of *E* and their progeny is characterized. The example shows that tracking marker-labeled *E* cells develop into *F* and *H* cells defined by blue membrane and cytoplasmic red dots. Success with both strategies is crucially dependent on the sensitivity and specificity of the tracking and phenotypic markers used to define the populations.

Figure 2

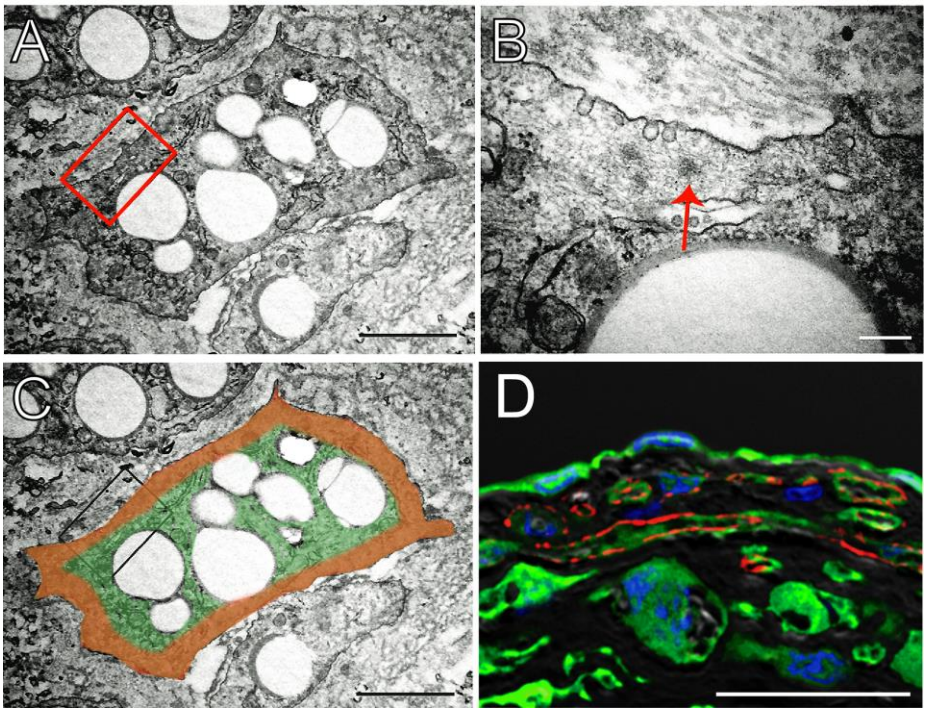

Figure 2. Example of intracellular distribution of tracking and phenotypic markers. **A.** ACTA2 is the most common first-line marker for SMCs in atherosclerosis. In murine and human plaque SMCs, the contractile filaments containing ACTA2 are typically confined to the sub-plasmalemmal space, whereas the cell interior is occupied by organelles of the synthetic machinery and common lipid droplets. Shown in A is the ultrastructure of an SMC from an *Apoe-/-* mouse plaque. **B**. Greater magnification of the demarcated area in A showing the faint myofilaments and their anchor points (dense bodies, red arrow). **C.** Drawing showing the expected appearance of signals in plaque SMCs expressing eGFP and stained for ACTA2 with a red fluorochrome. **D**. An actual red fluorescent ACTA2 staining of a plaque in an eGFP+ *Apoe-/-* mouse artery showing the expected distribution of signal. Scale bars 2 μm (A and C); B (0.2 μm); D (25 μm).

Figure 3

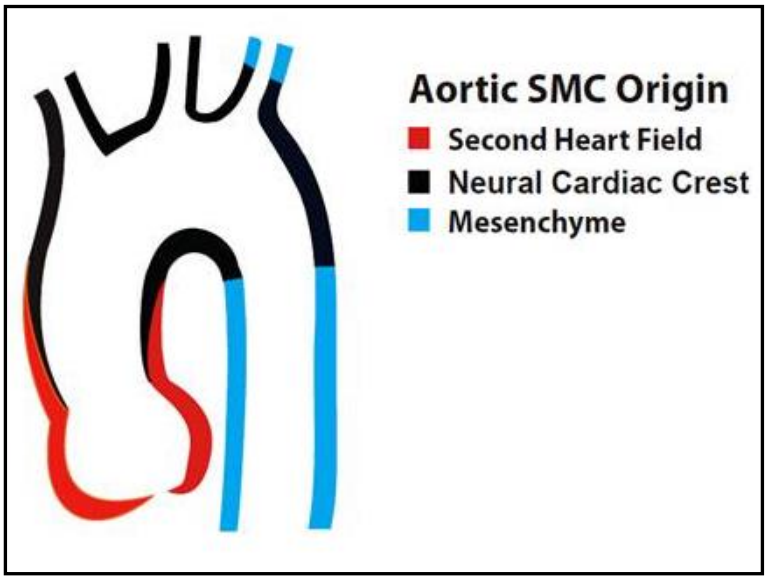

Figure 3. Developmental origins of arterial SMCs. Aortic SMCs arise from multiple, independent origins in the embryo. Three distinct types of SMC progenitor cells (color coded) produce a mosaic architecture of the thoracic aortic media. Note the inner media of ascending aorta is neural crest-derived while outer media is produced by progenitors from second heart field.[33] Fate-mapping studies have shown that here are at least eight independent origins for vascular smooth muscle that have been identified to date.[27]

Figure 4

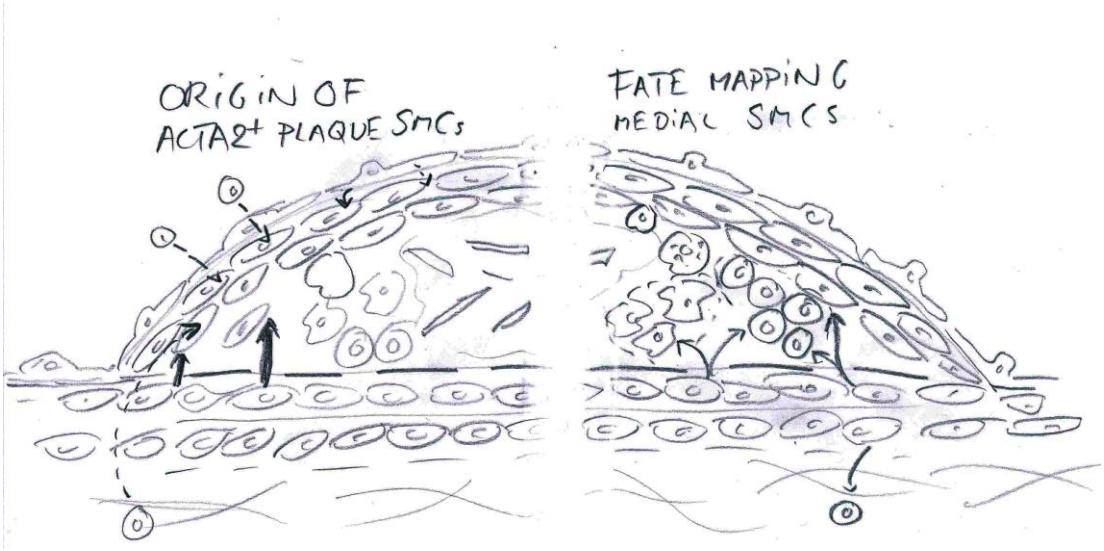

Figure 4. Sources and fates of SMCs in atherosclerosis. A. An illustration of proven and suggested sources of ACTA2+ SMCs in atherosclerosis. Medial smooth muscle cells (1) is the quantitatively dominant, and possibly only, source in atherosclerosis. Additional candidate populations that may contribute are circulating progenitor cells (2), advential progenitor cells (3) and endothelial cells (4) through the process of endothelial to mesenchymal transition. **B.** Fate mapping of medial SMCs in atherosclerosis. Medial SMCs produce classical ACTA2+ SMCs (1), mainly located in the subendothelial region of plaques, but also several other types of cells, including chondrocyte-like cells (2), SMCs expressing macrophage markers (3), and adventitial progenitor cells (4).