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Vascular smooth muscle cells in atherosclerosis

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1 **Abstract**

2
3 Vascular smooth muscle cells (VSMCs) are a major cell type present at all stages in
4 atherosclerotic plaques. According to the ‘response to injury’ and ‘vulnerable plaque’
5 hypotheses, contractile VSMCs recruited from the media undergo phenotypic conversion to
6 proliferative synthetic cells that elaborate extracellular matrix to form the fibrous cap and
7 hence stabilise plaques. However, recent lineage tracing studies have highlighted flaws in the
8 interpretation of former studies, revealing these to have underestimated both the content and
9 functions of VSMCs in plaques, and have thus challenged our view on the role of VSMCs in
10 atherosclerosis. It is now evident that VSMCs are even more plastic than previously
11 recognised, and can adopt alternative phenotypes including cells resembling foam cells,
12 macrophages, mesenchymal stem cells, and osteochondrogenic cells, which could contribute
13 both positively and negatively to disease progression. In this review, we present the evidence
14 for VSMC plasticity and summarise the roles of VSMCs and VSMC-derived cells in
15 atherosclerotic plaque development and progression. Correct attribution and spatio-temporal
16 resolution of clinically beneficial and detrimental processes will underpin the success of any
17 therapeutic intervention aimed at VSMCs and their derivatives.
18

19 **Introduction**

20
21
22 Atherosclerosis is the formation of plaques containing lipid, cells, debris and scar tissue in
23 the intima of arteries. As the main pathological process underlying myocardial infarction,
24 angina, heart failure and stroke, atherosclerosis has been the leading cause of morbidity and
25 mortality in the Western world for over half a century and is now the top cause of death
26 globally¹. A significant role for vascular smooth muscle cells (VSMCs) in atherosclerosis
27 was established in the 1960s - as soon as electron microscopy made it possible to identify
28 smooth muscle-like cells in the media of normal arteries², and it was ascertained that the
29 majority of cells in atherosclerotic plaques had characteristics of VSMCs but with altered
30 phenotypes³⁻⁵. However, the perception of how VSMCs contribute to plaque development,
31 remodelling and stabilisation has changed substantially over the last half-century (Box 1),
32 and recent studies have questioned long-standing assumptions about the identity of cells in
33 plaques, demanding a re-evaluation of the role of VSMCs in atherosclerosis.
34
35

36 **Identification of VSMCs**

37
38 VSMCs are defined based upon anatomical localisation (i.e. within the vasculature) and
39 functionality; in healthy arteries VSMCs are located in the medial layer where they are
40 responsible for arterial contraction and production of extracellular matrix (ECM), and play
41 important roles in compliance and elastic recoil in response to changing haemodynamic
42 conditions. VSMC functions are key determinants of the properties of vessels throughout the
43 arterial tree; VSMC-derived elastin is crucial for elastic recoil in large elastic arteries (such as
44 the aorta), whilst VSMC contraction is largely responsible for modulating arterial diameter in
45 muscular arteries and arterioles (the latter being of great importance to systemic arterial
46 resistance). Functionality is usually inferred from a combination of characteristics, including
47 morphology and expression of ‘VSMC-specific’ function-associated markers (which are
48 typically proteins and glycosaminoglycans). In healthy arteries, VSMCs are fusiform-shaped
49 cells that express contractile proteins (including smooth muscle alpha actin (α SMA) and
50 smooth muscle myosin heavy chain (SMMHC) which are organised into myofilaments) and

1 secrete ECM macromolecules (including elastins, collagens and proteoglycans). Most
2 studies to date have relied on these markers⁶⁻⁹ or gene expression profiles¹⁰ for identification
3 of VSMCs. However, as a necessary corollary of their role in tissue homeostasis and repair,
4 VSMCs exhibit considerable phenotypic plasticity in atherosclerosis, in response to injury,
5 and upon culture *in vitro*, which is often accompanied by marked changes in cell morphology
6 and expression of 'VSMC-specific' markers. Hence, definition of cell-type based on
7 functionality or 'specific' markers as a proxy for cell identification is problematic, and has
8 confounded studies on the true extent of the role of VSMCs in atherosclerosis¹¹.

9
10 Developments in genetic engineering have enabled specific labelling of VSMCs in mice,
11 making fate mapping and lineage tracing of VSMCs possible. For example, inducible VSMC
12 labelling systems (such as a tamoxifen inducible-recombinase driven by 'VSMC-specific'
13 gene promoters (typically *MYH11*¹² or *TAGLN*¹³⁻¹⁵)¹⁶ combined with reporter proteins^{17,18}),
14 result in specific and stable labelling of VSMCs at baseline and enable unambiguous tracing
15 of VSMCs and VSMC-derived cells during atherogenesis, even when VSMC characteristics
16 may otherwise be lost or gained^{11,17-24}. This elegant approach has led to important advances
17 in our understanding of the functional consequences of developmental origin, plasticity,
18 clonality and ultimately the fate of VSMCs in plaques, providing evidence for a more
19 complex and prominent role for VSMCs and VSMC-derived cells in atherosclerosis.

20 21 22 **Origin of VSMCs**

23
24 VSMCs are derived from multiple distinct progenitors in embryogenesis (detailed in Box 2),
25 with little or no mixing between different lineages²⁵⁻²⁷, resulting in anatomical segmentation
26 across the arterial tree. Furthermore, there is evidence for positional identity among VSMCs
27 along the anterior-posterior, dorso-ventral, and right-left axes of the embryo²⁸⁻³⁰. Embryonic
28 lineage can have important functional consequences; for example, VSMCs show lineage-
29 dependent responses to important signalling pathways such as TGF- β ^{31,32}, PDGF³³,
30 MRTFB^{34,35}, NF- κ B³⁶ and angiotensin II³⁷. These findings exemplify a fundamental
31 limitation in defining VSMCs on the basis of 'VSMC-specific' function-associated markers,
32 which may be similarly expressed in all VSMC lineages (potentially evoked through different
33 pathways that converge on the same set of 'VSMC-specific' genes, as detailed in Box 3),
34 whilst different VSMC lineages may have distinct functional characteristics.

35
36 Lineage tracing studies have unambiguously demonstrated that VSMCs contribute
37 substantially to plaque formation in murine models of atherosclerosis, generating 30-70% of
38 all plaque cells^{11,18-20,22,23}. In particular, most α SMA positive cells within the fibrous cap are
39 VSMC lineage label positive, refuting earlier ideas^{38,39} that bone marrow-derived cells
40 generate α SMA positive cells⁴⁰⁻⁴². VSMC-derived cells that express mesenchymal stem cell
41 markers (in particular Sca1) have also been identified in the healthy media⁴³ and in
42 plaques^{11,43}, and may represent a plastic intermediate population that is readily responsive to
43 inflammation and capable of generating contractile or phenotypically switched VSMCs⁴³.
44 However, these studies do not rule out a contribution from other sources of progenitors to
45 plaque VSMCs (Box 2).

46
47 Evidence for clonality (discussed below) of VSMCs and VSMC-derived cells in plaques
48 indicates that the majority of plaque cells derive from recruitment and proliferation of local
49 VSMCs, while the anatomical distribution of different developmental origins of VSMCs (and
50 perhaps other cell types, such as pericytes and endothelial cells) may contribute to the

1 anatomical distribution of atherosclerosis susceptibility⁴⁴. This idea is supported by the
2 finding that segments of aorta from atherosclerosis-prone and -resistant regions maintain their
3 atherosclerosis susceptibility upon transplantation to alternative sites⁴⁵. Definitive evidence
4 of similar anatomical segmentation of VSMCs populations in humans is currently lacking,
5 but supported in part by studies showing that human arteries are composed of clonal patches
6 of VSMCs⁴⁶⁻⁴⁸. Furthermore, advances in understanding development of different VSMC
7 lineages *in vivo* have led to generation of VSMCs from stem cells⁴⁹, which will facilitate
8 better disease modelling in human cells *in vitro*⁵⁰.

11 **Plasticity of VSMCs**

13 VSMCs display a fully functional, differentiated phenotype in healthy vessels, yet retain
14 remarkable plasticity. De-differentiation, modulation, or phenotypic switching of VSMCs is
15 characterised by reduced myofilament density and lower expression of contractile proteins.
16 De-differentiated VSMCs upregulate expression of ECM components and ECM-remodelling
17 enzymes, have increased levels of secretory organelles, and express pro-inflammatory
18 cytokines⁵¹. Consequently, phenotypically-switched VSMCs are often referred to as
19 'synthetic', whilst VSMCs expressing high levels of contractile proteins are generally
20 described as 'contractile' (although these definitions imply explicit functional changes that
21 are usually only inferred and very rarely quantified). Activation of VSMC proliferation and
22 migration has also been associated with the synthetic, de-differentiated state, but coordinated
23 regulation of these processes has not been documented and mitotic VSMCs with high levels
24 of contractile proteins have been observed^{52,53}.

26 Phenotypic switching is a reversible process, at least in the early stages. For example, a
27 general, transient loss of contractile protein expression is observed after vascular injury,
28 followed by reestablishment of the contractile phenotype after vessel repair⁵⁴. VSMCs
29 displaying phenotypes ranging from contractile to synthetic states have also been observed
30 both *in vivo*⁵³ and in VSMC cultures *in vitro*^{55,58}, illustrating that phenotypic switching is not
31 a binary process. VSMC heterogeneity in morphology and gene expression^{43,56} is also seen
32 in healthy vessels, including detection of rare atypical VSMCs marked by Sca1/Ly6a, that
33 express phenotypic switch-associated genes⁴³. At the molecular level, VSMC phenotype is
34 governed by regulatory transcription factors (including myocardin/SRF⁵⁷ and KLF4¹¹), which
35 integrate input from the environment (including growth factors, cytokines, lipid mediators,
36 contact with the ECM and other cells) and is regulated at multiple levels, including epigenetic
37 mechanisms (summarised in Box 3).

39 Lineage tracing studies have revealed that VSMCs exhibit greater than anticipated plasticity
40 in atherosclerosis (Table 1). Within plaques a large proportion of reporter-expressing
41 VSMC-derived cells do not have detectable levels of the contractile smooth muscle cell
42 marker α SMA^{11,20}. Instead, some plaque reporter-expressing cells were positive for Mac-3²⁰,
43 Lgals3¹¹ and CD68¹⁷ - markers that have been previously used to study macrophages in
44 atherosclerosis. Stimulation of VSMCs *in vitro* with cholesterol similarly induces expression
45 of macrophage-associated genes^{58,59} and promotes a phagocytic phenotype¹¹. Human
46 VSMC-derived plaque cells were also found to express CD68¹¹, consistent with previous
47 studies co-detecting CD68 and α SMA in human plaque cells^{60,61}. These results support the
48 hypothesis proposed by Wissler in 1968⁶² that at least a subset of foam cells are VSMC-
49 derived. This should be considered when interpreting studies on macrophage function, which
50 rely only on marker expression. Similarly, VSMCs have been proposed to generate

1 osteochondrogenic and mesenchymal stem cell-like plaque cells based on expression of
2 mineralising ECM proteins^{63,64} and Sca1/Eng¹¹ respectively. Expanded plasticity of VSMCs
3 in atherosclerosis was confirmed by transcriptional profiling of individual VSMC-lineage
4 plaque cells, revealing subpopulations of cells expressing Ly6a/Sca1, CD68 and
5 Sox9/Chad⁴³.

8 **Clonality of VSMCs**

10 The combination of multi-colour recombination markers (such as the confetti or rainbow
11 system^{18,22}) with genetic lineage tracing of VSMCs has demonstrated that, surprisingly,
12 mouse VSMC-derived plaque cells are generated by clonal expansion of relatively few cells
13 within the vessel wall^{17,20,22,23}. In contrast, most medial cells do not contribute to mouse
14 plaque formation and the role of VSMC migration independent of proliferation is limited²⁰.
15 Indeed, phenotypically distinct VSMC-derived plaque cells are generated from a common
16 ‘ancestor’. Observations of plaques at different timepoints suggest that, in mice, VSMCs
17 first generate the cap followed by adoption of switched phenotypes in the lesion core²³, but
18 this remains to be experimentally tested.

20 The molecular mechanisms underlying clonality are yet to be established, but macrophage
21 secreted factors have been implicated. For example, bone-marrow transplantation from
22 integrin β 3-deficient mice into ApoE null mice results in polyclonal plaque VSMCs and
23 VSMC-derived cells²³, whilst conditioned media from integrin β 3-deficient macrophages is
24 more mitogenic to VSMCs than conditioned media from wild-type macrophages²³. Early
25 stage cap VSMCs are highly proliferative and express α SMA, SMMHC, and importantly
26 PDGFR β ²³, akin to the primed PDGFR β -positive VSMC progenitors reported in models of
27 hypoxia-induced pulmonary hypertension, which clonally expand in a PDGF-dependent
28 manner^{65,66}. This highlights a potential role for PDGF signalling in clonal expansion of
29 VSMCs, and demonstrates that the study of VSMC clonal expansion in other vascular
30 conditions^{20,65,67} may be relevant for further mechanistic dissection in atherosclerosis.

32 The small number of VSMCs contributing to lesion formation raises the question of whether
33 disease-associated proliferation results from activation of specific cells that are primed to
34 respond to injury (discussed in ref⁶⁸). Supporting this idea, transcriptional profiling of
35 VSMCs from healthy blood vessels revealed significant heterogeneity in expression of genes
36 associated with vascular disease, suggesting the existence of VSMC subtypes^{43,56}.
37 Alternatively, clonality may rely on selection of VSMCs with equal plasticity, based on
38 location (e.g. proximal to breaks in the internal elastic lamina and/or mitogenic signals) or
39 differential capacity for survival or senescence (see below). It has also been speculated that
40 pathways of lateral inhibition may be operating, as is common in development²².
41 Importantly, these possibilities are not mutually exclusive, and the underlying mechanism is
42 likely genetic (somatic mutations) and/or epigenetic changes in the expanded VSMCs relative
43 to non-expanded VSMCs.

45 It is well documented that somatic mutations underlie clonal expansion both in malignancy
46 and in non-malignant tissues as a consequence of aging⁶⁹. Indeed, the acquisition of a
47 particular set of somatic mutations, linked to clonal expansion, in myeloid progenitor cells
48 has recently been shown to be associated with increased risk of atherosclerosis⁷⁰. Therefore,
49 it is reasonable to suggest that similar mechanisms may underlie clonal expansion of VSMCs
50 in atherosclerosis. Indeed, when clonal expansion of VSMCs was first described in plaques it

1 was likened to a smooth muscle cell tumour⁴⁶. Epigenetic changes may influence clonal
2 expansion of VSMCs secondary or independently of somatic mutations. Such changes may
3 reflect differences in VSMC lineage, environmental stimuli, or stochastic events.

4
5 Whilst lineage tracing has provided the most robust evidence yet for clonality of VSMCs in
6 plaques, the concept that most plaque VSMCs derive from clonal expansion, attributed to
7 Benditt and Benditt⁴⁶, has long been discussed⁴⁷, particularly in the context of replicative
8 senescence⁷¹.

11 **VSMC Senescence**

12
13 Senescence is a protective mechanism that induces cell cycle arrest to prevent transmission of
14 defects to progeny cells, particularly to stop malignant transformation⁷²⁻⁷⁴. Replicative
15 senescence occurs after repeated cell division, typically after telomere erosion or damage,
16 while induced senescence arises after oncogene activation, mitochondrial deterioration, DNA
17 damage, or oxidative stress. A persistent DNA damage response (DDR) is the most unified
18 pathway leading to senescence, with sensing by the Ataxia Telangiectasia Mutated (ATM)
19 protein leading to p53 phosphorylation and upregulation of cell cycle inhibitors⁷²⁻⁷⁴. The
20 cyclin-dependent kinase inhibitor (cdki) p21 drives initial cell cycle arrest, allowing repair of
21 moderate DNA damage and re-entry into the cell cycle. However, prolonged arrest
22 upregulates the cdki p16^{Ink4a}, leading to dephosphorylation of retinoblastoma protein pRB,
23 causing permanent cell cycle arrest⁷²⁻⁷⁴.

24
25 With every somatic cell division approximately 20bp or more is lost from the telomere ends
26 of chromosomes. Thus, repeated cell division leads to critical shortening, telomeric erosion
27 and loss of the protective Shelterin complex, which results in a persistent DDR that instigates
28 senescence. VSMC senescence *in vivo* is likely driven by multiple pathways including DNA
29 damage, mitochondrial deterioration, and oxidative stress – all present during atherosclerosis.
30 Loss of autophagy can also drive VSMC senescence⁷⁵. Replicative senescence is highly
31 relevant in the context of plaque VSMC clonality, as to generate all the VSMC-derived cells
32 in advanced plaques by clonal expansion would likely cause replicative senescence. In
33 keeping with this, the telomeres of VSMCs in human plaques are markedly shortened, which
34 correlates with disease severity⁷⁶.

35
36 Most senescent cells develop altered secretory activities known as a senescence-associated
37 secretory phenotype (SASP)^{77,78}. Cells with SASPs release proinflammatory cytokines (such
38 as IL-6, IL-1) and chemokines (such as IL-8, CCL2, CXCL1), growth factors (such as G-
39 CSF, bFGF), and proteases (including MMPs, PAI-1), conferring diverse activities⁷⁸. IL-1 α
40 is the key driver of the SASP^{79,80}, with upstream expression controlled in part by ATM/ATR-
41 mediated liberation of GATA4 from p62-directed autophagy⁸¹ and/or an mTORC1-dependent
42 pathway⁸². In a physiological setting SASPs act as a molecular beacon that recruits and
43 instructs immune cells to remove senescent cells (senescent surveillance⁸³) before further
44 mutation enables senescence bypass and, for example, re-initiation of tumour formation.
45 However, uncleared senescent cells accumulate during aging and disease (perhaps due to a
46 dysfunctional immune system or a suppressive milieu), and these generate chronic
47 inflammation that could worsen outcome and/or drive atherosclerosis⁸⁴.

48
49 Although VSMC senescence occurs in human plaques, proving the effects of senescent
50 VSMCs is difficult, and hampered by technical difficulties in mouse models. For example,

1 telomeres are approximately 10 times longer in mice than in humans, studying mouse SASPs
2 *in vitro* is problematic⁸⁵, and detecting senescence with the classic markers p16 and
3 senescence associated β -galactosidase (SA β G) is also notoriously difficult in mice,
4 particularly when both markers are expressed by macrophages in atherosclerotic plaques.
5 Two main experimental approaches have been used to study the effect of VSMC senescence
6 in atherosclerosis; modulation of senescence induction via the DDR, and clearance of
7 naturally occurring senescent cells with 'senolytics'. For example, VSMC-specific
8 expression of loss-of-function mutant TRF2 (a Shelterin subunit) led to increased DNA
9 damage and VSMC senescence, with bigger plaques and necrotic cores, while gain-of-
10 function TRF2 produced opposite effects⁸⁶. Similarly, VSMCs that lack base excision repair
11 activity have increased oxidative DNA damage and cell senescence, and promote increased
12 plaque size⁸⁷. In contrast, an intriguing recent study utilised electron microscopy to identify
13 crystals proposed to be the product of X-Gal cleavage by SA β G⁸⁴. This study reported more
14 than 50% of all plaque cells to be senescent, including VSMCs, macrophages and endothelial
15 cells⁸⁴. Senescent cells appeared within 9 days of fat feeding, and both genetic and
16 pharmacological elimination of p16 positive cells reduced plaque formation and
17 progression⁸⁴. Although it is unclear which cells were senescent and removed by these
18 treatments, this approach may open a new paradigm for atherosclerosis treatment.

21 **VSMCs in different stages of atherosclerosis**

23 Studies of plaque histology from human autopsy tissues have culminated in a scheme for
24 classification of plaques that encapsulates the progression of atherosclerosis^{88,89} and, based
25 on careful observations of plaque composition from human autopsy and animal models, it is
26 clear that VSMCs are major contributors to plaque development at all stages (summarised in
27 FIG. 1). However, their role and effects of VSMC proliferation or loss may differ according
28 to the stage of atherogenesis.

31 *Pre-atherosclerosis*

33 Diffuse intimal thickenings (DITs), and intimal xanthomas (i.e. fatty streaks) are considered
34 pre-atherosclerotic plaques, because they are common from birth^{90,91} and likely represent
35 physiological adaptation to blood flow⁹². However, the relationship between intimal
36 xanthomas and atherosclerosis is controversial because, although they localise to
37 atherosclerosis-prone regions and some intimal xanthomas develop into atherosclerotic
38 plaques, they are also found elsewhere and sometimes regress⁹³⁻⁹⁵. In contrast, DIT
39 distribution in the young is similar to that of atherosclerotic plaques in later life^{90,96} and DITs
40 are widely considered the most likely precursor to atherosclerotic plaques⁸⁸.

42 Human DITs comprise VSMCs, proteoglycans and elastin, and lack macrophages and
43 thrombus^{88,91,92}. VSMCs in DITs exhibit clonality^{47,91}, and are thought to originate from
44 local medial VSMCs⁵⁶. However, the latter is difficult to prove as many techniques for
45 lineage tracing (e.g. reporter gene expression from a lineage-specific promoter), are limited to
46 animal models, and most mammals (including mice) do not develop DITs⁹⁷. VSMCs in DITs
47 are heterogeneous, but most exhibit increased synthetic organelles (rough endoplasmic
48 reticulum, ribosomes and mitochondria) compared to medial VSMCs⁹⁸, consistent with
49 switching to a synthetic phenotype, which is supported by decreased expression of contractile
50 genes⁹⁹ and increased expression of ECM components¹⁰⁰. VSMCs are thought to be the

1 major source of the ECM in DITs, which accounts for much of the increase in thickness of
2 the intima but, importantly for progression to atherosclerosis, DITs are rich in proteoglycans
3 that are crucial for retention of apolipoproteins¹⁰¹. Furthermore, synthetic phenotype VSMCs
4 metabolise lipid differently to contractile VSMCs, in part through decreased expression of
5 cholesterol esterase and reduced cholesterol efflux transporter ABCA1^{60,102}, resulting in
6 increased tendency towards foam cell formation¹⁰³.

7 8 9 *Early atherosclerosis*

10
11 The first stage in atherosclerosis is the formation of pathological intima thickenings (PITs);
12 the earliest recognised atherosclerotic plaque, which is characterised by the formation of an
13 extra-cellular lipid pools deep in the intima, underlying abundant VSMCs and ECM^{88,89}.
14 DITs can, but do not always, progress to PITs (FIG. 2)¹⁰⁴. Progression is promoted through a
15 complex interplay between retention and oxidation of lipid, induction of inflammation, and
16 VSMC proliferation, phenotype switching, and death.

17
18 The lipid pools(which is distinct from the necrotic pool of more advanced plaques) comprises
19 lipids (including free cholesterol) amidst a proteoglycan (notably biglycan, versican and
20 perlecan) and glycosaminoglycan (GAG, including hyaluronan) -rich ECM. As the
21 predominant cell-type present in DITs, intimal VSMCs are regarded as the most important
22 source of the ECM, and this is supported by analysis of the secretome of VSMCs *in vitro*^{105–}
23 ¹⁰⁹. The ECM has a central role in initiation of atherosclerosis, primarily through the
24 interaction between the negatively charged side chains of proteoglycans (particularly
25 chondroitin sulphate of biglycan and versican and heparin sulphate of perlecan¹¹⁰) with
26 positively charged apolipoproteins (especially apolipoprotein B), which leads to the retention
27 of plasma-derived lipoproteins^{101,111} - as described in the ‘response to retention
28 hypothesis’^{112,113}. Transgenic mice over-expressing biglycan in VSMCs show more lipid
29 retention and increased atherosclerosis than wild-type litter-mates¹¹⁴. Once retained in the
30 intima, lipoproteins undergo modifications, including oxidation to OxLDL, which precedes
31 the recruitment of macrophages¹¹⁵ and initiates the inflammatory response characteristic of
32 atherosclerosis¹¹². Further evidence for this series of events was provided by a recent study
33 comparing DITs to PITs, in which extra-cellular lipid was found deep in the plaque,
34 colocalising with α SMA-positive cells, ApoB, biglycan and versican, but not the more
35 superficial (closer to the lumen) CD68 positive cells (likely macrophages)¹¹⁶.

36
37 Progression to PITs is accompanied by loss of α SMA, which is likely due to a combination
38 of phenotypic switching of VSMCs^{11,18,23} and loss of VSMCs through cell death^{117,118}. For
39 example, uptake of OxLDL and formation of VSMC-derived foam cells has been linked to
40 induction of VSMC death by apoptosis¹¹⁸, and free cholesterol in the lipid pool may be
41 derived from dead VSMC¹¹⁹. The micro-calcification (speckles of 0.5-15 μ m) sometimes
42 observed within the lipid pool of PITs, typically close to the border with the media, may also
43 be a consequence of VSMC apoptosis⁵¹.

44
45 Macrophages may be absent from early PITs⁸⁹, but are a defining characteristic of late stage
46 PITs and crucial to the progression of PITs to fibroatheromas. Lineage tracing studies have
47 shown the macrophage marker-positive cells of early lesions in mice (which resemble intimal
48 xanthomas) are mostly derived from recruited circulating monocytes^{23,120}, and may also
49 involve local resident macrophages^{121,122}. However, co-expression of α SMA and CD68 in
50 human plaques indicate that VSMCs also likely contribute significantly to the macrophage

1 marker-positive cells in early plaques^{5,61}. Monocytes are recruited to PITs through the
2 expression of adhesion molecules (including selectins, ICAM1, VCAM1, CD31¹²³) and
3 chemo-attractants, including chemokines (such as CCL5, CXCL1 and CCL2^{120,124}, which are
4 secreted by VSMCs and ECs stimulated with inflammatory cytokines or OxLDL, *in vitro*¹²⁵)
5 and modified lipids (such as OxLDL¹²⁶). Studies in animal models have collectively revealed
6 an essential requirement for macrophages in the progression of atherosclerosis^{120,122,127,128},
7 which is likely to involve effects on VSMC migration, proliferation (through production of
8 factors such as PDGF¹²⁹) and phenotype switching¹³⁰.

11 *Late atherosclerosis*

13 PITs can progress to fibroatheromas (FIG. 3), characterised by the presence of a fibrous cap
14 and a necrotic core, the origins of which are the extra-cellular lipid pool and insufficient
15 efferocytosis (of dead VSMCs and macrophages)¹³¹⁻¹³³. This phase of atherosclerosis (late
16 PIT/early fibroatheroma) is dependent on extensive accumulation of macrophages on the
17 luminal side of the lipid pool, where they phagocytose deposited lipids to become foam cells.
18 In the absence of resolution, the ensuing inflammatory reaction is self-perpetuating;
19 macrophages and VSMCs become foam cells, which die (mostly by apoptosis but potentially
20 through other mechanisms, Box 4). Since the plaque milieu suppresses efferocytosis¹³³⁻¹³⁶,
21 uncleared apoptotic cells subsequently undergo secondary necrosis with release of further
22 inflammatory material, such as damage-associated molecular patterns (DAMPs)¹³⁷. The
23 accompanying healing response involves the formation of the fibrous cap, which, at least in
24 the early stages, is a highly cellular region, rich in VSMC-derived α SMA-positive cells^{22,40-}
25 ⁴², amongst an altered ECM that has decreased proteoglycan expression and an increase in
26 the proportion of collagens (mostly type I and III).

28 In mice, the fibrous cap VSMCs are derived from medial VSMCs^{22,138} that have undergone
29 migration and proliferation in response to cytokines and growth factors, such as PDGF,
30 derived from macrophages and activated ECs^{23,129,139}. This initial stage of VSMC
31 recruitment is, at least in part, Oct4 dependent²¹. In humans, both pre-existing intimal and
32 medial VSMCs may contribute to plaque VSMCs⁴⁸. Definitive proof that VSMCs are
33 responsible for the production of the fibrous cap ECM is lacking. However, this hypothesis
34 is consistent with co-localization of collagen synthesis to VSMCs in the fibrous cap¹⁴⁰,
35 correlation of fibrous cap thickness with VSMC phenotype in mice^{11,21,141}, and the correlation
36 of fibrous cap stability with VSMC cell number in humans¹⁴². In addition, a recent study of
37 VSMC-specific deletion of Col15a resulted in a greater than 70% reduction in Col15a,
38 supporting VSMCs as the major source of this collagen¹⁴³. Further evidence that VSMCs are
39 the major source of collagens comes from studies *in vitro*, including proteomic analysis of the
40 secretome of lipid-loaded VSMCs¹⁰⁹ and induction of collagen synthesis by VSMCs in
41 culture by TGF- β , PDGF, IL-1, AngII, cholesterol, homocysteine and mechanical
42 stretch^{144,145}.

44 VSMCs in the later stages of atherosclerosis have previously been thought to be entirely
45 beneficial, for example by stabilising the plaque through elaborating the fibrous cap.
46 However, lipid loading of VSMCs and altered interactions with the ECM lead to altered
47 VSMC phenotype, and increased macrophage markers⁵⁹. Indeed, VSMCs contribute between
48 30-70% of the macrophage marker-positive cells^{11,20} and similarly to foam cells¹⁴⁶ in mouse
49 plaques, and around 30-40% of CD68 positive cells and 50% of foam cells in humans^{11,60}.
50 VSMC-specific deletion of the transcription factor KLF4 reduces VSMC switching to

1 macrophage marker-positive cells, and results in a marked increase in the thickness and
2 α SMA-positive cell content of the fibrous cap¹¹. Although these studies have shown that
3 VSMCs can express macrophage markers, *in vitro* studies of the transcriptomes of VSMCs
4 and macrophage-derived foam cells indicate they are functionally distinct, and that VSMC-
5 derived foam cells exhibit reduced phagocytic and efferocytic responses⁵⁹. VSMCs have
6 long been known to contribute to the inflammatory milieu of the plaque through recruitment
7 of macrophages; however, these studies strongly suggest that VSMC-derived macrophage-
8 like cells also directly affect plaque progression.

9
10 In early fibro-atheromas, calcification is observed as large granules in the necrotic core and
11 surrounding ECM, resulting from a number of interrelated processes, including macrophage
12 and VSMC-derived calcifying micro-vesicles^{147–149}, release of apoptotic bodies¹⁵⁰ or the
13 activity of osteochondrogenic cells¹⁵¹. As the fibro-atheroma develops, micro-calcifications
14 can coalesce into larger speckles and fragments that can form sheets or plates¹⁴⁹ visible by
15 tomography. Fragmentation of these sheets and fibrin encapsulation can lead to the
16 formation of calcium nodules, which protrude into the vessel lumen and precipitate
17 thrombosis⁸⁸. The extent of plaque calcification varies according to the vascular bed, and a
18 recent study linked this to the different propensities of the local, developmentally distinct,
19 VSMCs to undergo calcification^{152,153}. VSMCs have long been linked to calcification^{150,154}
20 and osteochondrogenic conversion *in vitro* is enhanced by plaque-like environmental cues,
21 including phenotypic conversion¹⁵⁵, apoptotic bodies¹⁵⁰, oxLDL¹⁵⁶, and inflammatory
22 cytokines such as TNF α ¹⁵⁷, IL-1¹⁵⁸ and IL-18¹⁵⁹. Furthermore, specific genetic modulation
23 of VSMC osteochondrogenesis *in vivo* leads to altered calcification in models of
24 atherosclerosis^{160–162}. Most convincingly, however, recent studies have established that most
25 of the osteochondrogenic precursors (Runx2/Cbfa1+ cells) and chondrocyte-like (type II
26 collagen+) cells of murine plaques are again VSMC-derived¹³⁸.

27 28 29 *Clinical sequelae*

30
31 The major clinical sequelae of atherosclerosis are dependent on the anatomical site of the
32 vascular bed involved (angina and myocardial infarction in coronary arteries; stroke in
33 carotid arteries) and typically manifest as a result of thrombosis. The primary cause
34 (accounting for around 60% to 70% of cases) of thrombosis is plaque rupture¹⁶³ and the
35 remaining cases are predominantly the result of plaque erosion (the latter being much more
36 frequent in young individuals, particularly women) (FIG. 4). A minority (typically around
37 5%) are due to thrombosis forming on calcified nodules. However, thrombosis and clinical
38 sequelae are not an inevitable consequence of atherosclerosis; analysis of autopsies has
39 shown that plaques often show evidence of silent (non-occlusive) thrombi which have
40 undergone repair and healing. Furthermore, the widespread uptake of clinical interventions,
41 including lipid-lowering, are changing the clinical presentation of atherosclerosis in
42 association with changes in the characteristics of the ‘vulnerable plaque’¹⁶⁴.

43
44 As the fibroatheroma develops, so does the necrotic core; the free cholesterol content and
45 calcification increases, and there is breakdown and remodelling of the fibrous cap ECM. The
46 latter is thought to be principally due to the actions of proteases (in particular
47 metalloproteinases¹⁶⁵), but also by sulphatases and exoglycosidases that are predominantly
48 released by macrophages¹⁶⁶, but may also come from VSMCs¹⁶⁷. Concomitantly, VSMCs
49 are depleted through cell death, and so the cap diminishes, whilst the growing necrotic core
50 extends outwards, leading to thinning of the fibrous cap^{168,169}. Thin-cap fibroatheromas

1 (TCFA) are defined by a fibrous cap of less than 65µm, and are also known as ‘vulnerable
2 plaques’ because studies have shown that these plaques are highly susceptible to rupture.
3 The underlying mechanisms are ill-defined, but proteolytic activity^{166,167}, mechanical stress¹⁷⁰
4 and micro-calcification of the fibrous cap^{149,171} have all been linked to plaque rupture.

5
6 Plaque rupture is inversely correlated with VSMC number¹⁴², which is determined by
7 proliferation, migration and death of VSMCs. Advanced human lesions show little VSMC
8 proliferation^{172,173}, but VSMC death, through apoptosis and necrosis (Box 4), is increased
9 compared to normal vessels^{174–176}, and in unstable versus stable plaques¹⁷⁷. Indeed, VSMC
10 apoptosis has been postulated to be key to plaque instability¹⁷⁸. Seminal work showed plaque
11 VSMCs to spontaneously undergo apoptosis *in vitro*, with IGF-1 and PDGF acting as
12 survival factors¹⁷⁹, and plaque VSMCs expressing less IGF-1R¹⁸⁰. Similarly, cell to cell
13 contact via N-cadherin promotes survival¹⁸¹. Conversely, numerous factors that induce
14 VSMC apoptosis have been described, including cell-directed killing (by macrophages, T
15 lymphocytes and mast cells), ROS, DNA damage, anoikis and cholesterol. Studies of VSMC
16 apoptosis *in vivo* have utilised mice that have either alterations to apoptotic pathways or
17 systems to induce apoptosis. Early work with adenoviral p53 expression in plaques led to
18 VSMC apoptosis and cap thinning¹⁸². Similarly, VSMC-specific diphtheria toxin (DT)-
19 induced apoptosis revealed short term VSMC apoptosis within established plaques to have no
20 effect on plaque size, but to result in vulnerable plaques with small fibrous caps and a paucity
21 of VSMCs and structural matrix¹⁷⁸ – a finding subsequently corroborated many times in
22 studies that have promoted or inhibited VSMC death,^{167,181,183–187}. Strikingly, DT induction
23 of VSMC apoptosis alongside high fat feeding during atherogenesis resulted in larger
24 plaques⁵¹, showing that the consequences of VSMC death are more than cell loss alone, and
25 in fact actively drives plaque growth - another well replicated finding^{167,185,187,188}. A key
26 controller of VSMC apoptosis *in vivo* appears to be the survival kinase Akt1^{183,186,187};
27 conditional ablation of Akt1 during atherogenesis induces VSMC apoptosis and larger
28 plaques, and Akt1 ablation in established plaques leads to a reduced fibrous cap. The
29 contribution of VSMC death to plaque stability is complex and extends beyond direct cell
30 loss; with further consequences on the local milieu (such as initiating calcification¹⁵⁰), and
31 wider effects in activating the immune system. The plaque environment is known to inhibit
32 phagocytosis^{133–136}, and defective efferocytosis of apoptotic cells leading to secondary
33 necrosis and leakage of intracellular contents has been proposed to exacerbate the
34 inflammatory milieu^{131,132,137}. Indeed, necrotic VSMCs potently drive inflammation via IL-
35 1α due to a lack of IL-1R2 that normally binds and inhibits IL-1α^{133,189}. Thus, a consensus
36 appears whereby functional VSMCs are essential to maintain the fibrous cap and thus plaque
37 stability, but death of VSMCs is a potent driver of atherogenesis.

38
39 A recent study of the VSMC transcriptome in symptomatic versus asymptomatic carotid
40 plaques has also highlighted the importance of VSMC senescence¹⁹⁰. Unstable mature
41 plaques show low VSMC proliferation and clear evidence of VSMC senescence¹⁹¹.
42 Senescent VSMCs were originally thought to promote plaque instability through inaction -
43 i.e. a lack of VSMC proliferation and matrix production leads to weakening of the fibrous
44 cap. However, senescent VSMCs establish a robust IL-1α-driven SASP containing multiple
45 inflammatory cytokines, chemokines, MMPs and osteogenic factors^{80,192}. Thus, the VSMC
46 SASP can recruit mononuclear cells, induce endothelial cell adhesion receptor expression and
47 activate adjacent normal VSMCs⁸⁰, effectively amplifying the effect of a small number of
48 senescent VSMCs. Senescent VSMCs also produce less collagen and release active
49 MMP9⁸⁰, while BMP2 and osteoprotegerin within the SASP drive calcification¹⁹². Thus,

1 senescent VSMCs can have a negative impact on plaques through both loss of normal
2 function and a direct effect on the local plaque milieu.

3
4 An alternative route to thrombosis and clinical sequelae is through plaque erosion. Erosion
5 refers to the formation of a thrombus in the absence of rupture at sites of endothelial
6 denudation or disruption. The underlying plaque may be an intimal thickening or
7 fibroatheroma^{88,169}, but VSMCs are often abundant, amidst a proteoglycan-rich ECM,
8 enriched in type III collagen, versican and hyaluronan¹⁹³. Recent studies have identified an
9 important role for hyaluronan, which activates TLR2 signalling upon degradation¹⁹⁴ and this
10 combined with altered shear stress, leads to endothelial cell activation and apoptosis¹⁹⁵,
11 neutrophil recruitment and thrombosis¹⁹⁴. Thus VSMCs are implicated in the events leading
12 to plaque erosion, in particular as the major source of hyaluronan¹⁹⁶.

15 **Future perspectives**

17 *Difficulties in extrapolating studies from mice to man*

18
19 Reconciling the results of studies of animal models with those of human atherosclerosis can
20 be challenging, as there are some important differences in how the disease progresses in
21 humans and animal models. This is exemplified in the case of DITs, which are absent in
22 most animal models. Another fundamental difference is that fibroatheromas rarely progress
23 to rupture in animal models, exemplified by the recently reported effects of a neutralising IL-
24 1 β antibody, which were deleterious on the fibrous cap in mice¹⁴¹, but beneficial in reducing
25 cardiovascular events in the CANTOS trial in humans¹⁹⁷. Nonetheless, animal models have
26 been instructive in delineating important pathways and basic principles that might underlie
27 plaque development in humans. This is particularly true of the lineage tracing studies in
28 mouse models of atherosclerosis, which have unambiguously established the importance of
29 clonality and phenotype switching of VSMCs. Combinatorial genetic depletion models will
30 likely be instrumental in assessing whether biasing the phenotype of VSMC-derived cells
31 could be a potential treatment avenue. Recently developed techniques, including mass
32 cytometry (CyToF) and single-cell omics (genomics, transcriptomics and epigenomics), hold
33 great promise for high resolution, spatio-temporal analysis of plaque cells *in situ*, and are
34 likely to provide the conclusive human counterpart and mechanistic data for the
35 aforementioned studies.

39 *VSMCs and genetics of atherosclerosis*

40
41 Over 150 CAD loci have been identified from GWAS and other genetic association
42 studies¹⁹⁸, many of which are associated with disease independently of other known risk
43 factors. Thus, elucidation of the underlying molecular mechanisms may reveal novel
44 pathways and hence targets for therapeutic intervention. However, identification of causal
45 variants is usually far from trivial; CAD loci are often located in non-coding regions, where
46 the causal variant is predicted to effect regulation of gene expression, which may operate
47 over large distances and be cell-type or context specific. Studies are ongoing to identify and
48 functionally characterise the causal variants responsible for each of the CAD loci, and *in vitro*
49 studies of VSMCs are proving an invaluable resource in this quest. Integration of
50 transcriptomic and epigenomic maps from VSMCs (and other plaque cells) with those of the

1 genetic architecture of CAD can be very informative for prioritising variants (and potential
2 pathways) for functional characterisation^{199,200}. Unsurprisingly, given the key role of VSMCs
3 in atherosclerosis, a number of loci have been predicted to modulate disease risk through
4 mechanisms specific to VSMCs²⁰⁰. Thus, studies in cultured VSMCs, and more recently
5 VSMCs derived from stem cells^{49,201}, are likely to be instrumental in the functional
6 characterisation of CAD variants. Recent pioneering examples of such studies include the
7 characterisation of the SMAD3 and TCF21 loci²⁰².

10 **Conclusion**

12 The role of VSMCs in atherosclerosis extends far beyond that perceived for decades.
13 VSMCs and VSMC-derived cells comprise a (if not the) major source of plaque cells, and
14 contribute to numerous plaque cell phenotypes, including macrophage-like and foam cells, in
15 addition to cells responsible for producing the atherogenic and or athero-protective ECM
16 throughout the disease. Thus, VSMCs are implicated mechanistically at all stages of
17 atherosclerosis, and recent studies have established the extent and importance of VSMC
18 clonality and phenotype switching in plaque progression. These concepts have been around
19 for decades, but it is only very recently that technologies for genetic engineering and imaging
20 have converged with a deeper understanding of developmental processes to generate
21 conclusive data in animal models. The era of single cell omics promises to deliver the
22 evidence as to if and how these processes contribute to the disease in humans. It is clear that
23 a better understanding of the biology of VSMCs is required if we are to fulfil aspirations of
24 selectively targeting ‘culprit’ cells or manipulating cell phenotype to enhance clinical benefit
25 and/or avert processes that are detrimental in disease.

28 **Key points:**

- 29 - VSMCs and VSMC-derived cells are a major source of plaque cells and ECM at all stages
30 of atherosclerosis
- 31 - VSMCs contribute to many different plaque cell phenotypes, including ECM-producing
32 cells of the fibrous cap, macrophage-like cells, foam cells, mesenchymal stem cell-like and
33 osteochondrogenic cells
- 34 - Recently progress has been made regarding the source of plaque VSMCs and VSMC-
35 derived cells, which highlights the importance of developmental origin, clonal expansion and
36 phenotype switching of VSMCs in atherosclerosis

1 **Box 1: Historical perspective on VSMCs in atherosclerosis**

2
3 The development of antibodies for ‘VSMC-specific’ function-associated markers, such as
4 smooth muscle alpha actin (α SMA)^{6–9}, greatly facilitated immuno-histological studies of
5 VSMCs in plaques of animal models^{203,204} and humans^{98,103}. These studies, alongside *in vitro*
6 culture models⁵⁵ and models of arterial injury, such as balloon angioplasty, revealed that
7 VSMCs are capable of great phenotypic plasticity, and undergo ‘phenotypic switching’ from
8 contractile to proliferative synthetic phenotypes^{205–207}. Phenotype switching and proliferation
9 of VSMCs in response to arterial injury and lipid infiltration were considered the main
10 pathological processes underlying plaque development²⁰⁷.

11
12 Studies in the 1990s characterised the role of VSMC proliferation, migration, apoptosis, and
13 phenotype switching in atherogenesis²⁰⁸, and revealed that VSMCs can give rise to foam
14 cells^{4,5,102} and osteochondrogenic cells¹⁵⁴. However, detailed post-mortem analyses of culprit
15 plaques in sudden cardiac death established that the integrity of the fibrous cap, comprising
16 mostly α SMA-positive cells and associated extracellular matrix (ECM), is critical to stabilise
17 and protect plaques from rupture, a major cause of the clinical sequelae of
18 atherosclerosis^{142,163,168}. These studies also highlighted the role of immune cells, particularly
19 macrophages, and inflammation as the main driver of plaque development¹⁶⁹. Thus, the
20 prevailing model has been that VSMCs contribute to the cellularity and inflammation of the
21 developing plaque, but have a predominantly beneficial role in its stabilisation though
22 elaborating the fibrous cap²⁰⁹.

23
24 In the last decade, studies applying fate mapping and lineage tracing techniques have
25 revealed the limitations of relying on ‘VSMC-specific’ function-associated markers to infer
26 VSMC identity, and exposed the extent to which this can lead to false negative and false
27 positive identification of VSMCs, as well as oversimplification of VSMC heterogeneity and
28 functions in plaques^{11,17,18}.

29
30 Text boxes (for timeline):

31
32 pre 1900s histology on morbid specimens, including by Virchow (1856) who proposed
33 atherosclerosis to result from inflammation and proliferation as a consequence of arterial
34 injury by mechanical forces

35
36 Marchand coins ‘atherosclerosis’

37
38 Ignatowsky describes relationship between protein/lipid-rich diet and experimental
39 atherosclerosis, these studies were extended by Anichkov in 1913, who discovered the
40 importance of cholesterol

41
42 Foam cells observed in human and experimental atherosclerosis studies by light
43 microscopy^{210,211}

44
45 Pease describes VSMC as the only cell-type in the healthy media by electron microscopy².
46 Studies of experimental and human atherosclerosis quickly followed, revealing VSMC
47 derived cells as prominent cell type in plaques^{3–5}

1 Wissler proposes VSMC are the primary cell type involved in atherosclerosis, assimilating
2 many studies (including Wolinsky & Glagov²¹²) that VSMC are the contractile and ECM-
3 producing cells of the media and, furthermore, contribute to plaque foam cells⁶²
4

5 Ross further develops ‘**response to injury hypothesis**’, emphasizing the role of PDGF
6 mediated VSMC proliferation²⁰⁷ (firstly due to EC injury and platelet activation²¹³ and later
7 updated to incorporate a role for macrophage derived PDGF¹²⁹)
8

9 Benditt & Benditt propose plaque VSMC arise from **clonal expansion**⁴⁶
10

11 Chamley-Campbell et al identify phenotype switching in cultured VSMCs⁵⁵
12

13 ‘**vulnerable plaque**’ concept developed; studies of culprit plaques in cardiac deaths identify
14 fibrous cap integrity essential to plaque stability^{163,168,214}
15

16 ApoE and LDLR mouse models of atherosclerosis developed^{203,204}
17

18 ‘**response to retention hypothesis**’ proposed¹¹³ and supported by identification of the central
19 role of ApoB containing lipoproteins¹⁰¹
20

21 first lineage tracing studies^{11,17,18} which collectively revealed VSMC contribution much more
22 substantial than previously thought, giving rise to macrophage marker positive cells, foam
23 cells, osteochondrogenic and mesenchymal stem cell like cells
24

25 multi-colour lineage tracing studies demonstrate multiple plaque phenotypes are derived from
26 common ancestor – revealing the true extent of VSMC clonality in plaques^{20,22}
27

28 CANTOS trial establishes causal role for inflammation in pathogenesis of atherosclerosis¹⁹⁷
29
30
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1 **Box 2: Embryonic origins of VSMCs and sources of VSMC progenitors in adults**

2
3 During embryonic development, medial VSMCs (and in some instances pericytes²¹⁵) arise
4 from local progenitor cells, of which there are multiples distinct lineages distributed across
5 the arterial tree. In mice, more than eight distinct progenitor populations have been
6 identified^{44,216,217}. The aortic root and outer medial layers of the ascending aorta derive from
7 the secondary heart field^{26,28}; the inner medial layer of the ascending aorta, aortic arch, ductus
8 arteriosus, innominate and right subclavian arteries, right and left common carotid arteries
9 derive from the neural crest²⁵; the descending aorta derives from paraxial (somatic)
10 mesoderm²¹⁸; and the coronary arteries are derived from pro-epicardium, which derives from
11 lateral plate mesoderm²¹⁹.

12
13 Potential VSMC progenitor populations have also been identified in the media in the adult
14 mouse, including VSMC-derived cells expressing Sca1 and other mesenchymal stem cell
15 markers^{11,43}. These cells may be an intermediate population derived from phenotypic
16 switching, which can give rise to different VSMC-derived cell phenotypes⁴³. Other potential
17 progenitor cells include a population of adventitial cells located close to the medial boundary
18 that express mesenchymal stem cell markers (e.g. Sca1) and are sonic hedgehog signalling-
19 responsive (Gli1 positive)^{27,220–222}, and pericytes^{223,224}, which are VSMC-like cells of the
20 microvasculature.

21
22 Importantly, studies have shown that progenitors with distinct origins may achieve a common
23 VSMC fate with respect to expression of ‘VSMC-specific’ function-associated markers
24 (through pathways discussed in Box 3), but are nonetheless distinct with respect to other
25 functional characteristics, such as responses to growth factors.

1 **Box 3: Molecular mechanisms underlying VSMC plasticity**

2 3 **Transcription factors:**

4
5 **Myocardin (MYOCD)** family proteins drive expression of contractile genes⁵⁷.
6 MYOCD is a co-factor for serum response factor (SRF), which binds CArG-box
7 elements within contractile gene promoters. Most environmental cues and signalling
8 pathways affecting VSMC function impact the expression and/or activity of
9 MYOCD^{225,226}

10
11 **KLF4** represses contractile gene expression through several mechanisms, including
12 binding to G/C repressor elements and inhibiting SRF binding to CArG-boxes. KLF4
13 inhibits proliferation; VSMC specific deletion of CHOP leads to decreased VSMC
14 proliferation through increased expression of KLF4²²⁷. Importantly, VSMC
15 phenotype switching is KLF4 dependent. KLF4 is required for induction of
16 progenitor cells prior to clonal expansion of pulmonary VSMCs in hypoxia^{65,66} and
17 VSMC-specific deletion of KLF4 in ApoE^{-/-} animals results in reduced numbers of
18 VSMC-derived macrophage and mesenchymal stem cell marker positive plaque
19 cells¹¹.

20
21
22 **Extracellular stimuli:** the contractile phenotype is promoted by TGF- β , whereas PDGF
23 induces KLF4 expression, VSMC proliferation and phenotypic switching. Other growth
24 factors including WNT signalling also promote proliferation and migration of VSMCs. Pro-
25 inflammatory cytokines (e.g. IL-1 and TNF- α) perturb VSMC phenotype via NF- κ B and AP-
26 1 mediated gene regulation, including MYOCD downregulation. Cholesterol-induced
27 activation of macrophage-associated gene expression in VSMC occurs via microRNA-
28 143/145, involves MYOCD and inflammatory signalling and is affected by KLF4^{59,228}.

29
30 **Cell interactions:** ECM proteins and heparin affect VSMC phenotype²²⁹. Notably, deletion
31 of integrin β 3 results in larger lesions and affects VSMC clonality in atherosclerosis²³.
32 Differences in how cells communicate with the environment may also explain the
33 documented effect of stretch and shear stress on VSMC phenotype²³⁰.

34
35 **Epigenetic regulation:** the reversibility of VSMC phenotypic switching indicates a cellular
36 memory of the contractile state. Indeed, contractile genes remain marked by H3K4me2
37 (generally associated with actively transcribed genes) after phenotypic switching¹⁸ and
38 manipulation of DNA methylation and histone modifying enzymes directly affect VSMC
39 behaviour in murine models of vascular injury and atherosclerosis²³¹⁻²³³, whilst levels of
40 epigenetic markers are altered in human plaques²³⁴. Non-coding RNAs also control VSMC
41 plasticity^{235,236} evidenced by the effect of specific miRNAs and long non-coding RNAs on
42 VSMC biology and function^{237,238}.

1 **Box 4: Mechanisms of cell death**
2
3

4 **Apoptosis:** the commonest form of programmed cell death (PCD) utilised throughout
5 development and day-to-day physiology. Executed by apoptotic caspases (e.g. 3, 7), with
6 main initiation pathways controlled via the mitochondria (via Bcl-2 family members) or
7 external death receptors (e.g. Fas, TNFR). Apoptotic cells must be phagocytosed, or
8 secondary necrosis with leakage of inflammatory contents (including DAMPs) will occur.
9 All major cell types within the plaque are witnessed to undergo apoptosis.

10
11 **Autophagic cell death:** a mechanism for the organised degradation and recycling of
12 intracellular components within double membraned autophagosomes that fuse with
13 lysosomes. Can be a response to stress that enables the cell to survive, but is also witnessed
14 as PCD. VSMC specific deficiency in autophagy leads to increased VSMC death and
15 enhanced features of vulnerable plaques¹⁸⁸.

16
17 **Necrosis:** An un-programmed form of cell death characterized by catastrophic loss of plasma
18 membrane integrity and leakage of cell contents. Uncleared dying cells default to secondary
19 necrosis. Difficult to prove in vivo, but ultrastructural evidence suggests necrotic plaque
20 macrophages and VSMCs occur.

21
22 **Necroptosis:** A programmed form of necrosis allowing cell suicide when apoptosis is
23 blocked (e.g. viral caspase inhibitors). Utilises RIPK1/3 to form the ripoptosome which
24 activates MLKL that destroys the plasma membrane. Increased RIP3 and MLKL reported in
25 human plaques, but difficult to specifically detect necroptosis.

26
27 **Pyroptosis:** Inflammatory form of cell death that occurs in concert with inflammasome
28 activation and IL-1 production, often in response to intracellular infection. Leads to
29 activation of inflammatory caspases (e.g. 1, 4, 5, 11) that activate IL-1 and/or the pore-
30 forming protein GSDMD, and subsequent membrane permeabilisation. Likely happens in
31 plaques after cholesterol crystal activation of macrophage NLRP3 inflammasomes.

32
33 **Paraptosis:** caspase-independent cell death leading to cytoplasmic vacuolation and eventual
34 osmotic lysis. Not currently described in atherosclerotic plaques.
35
36
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40

1 **Table 1: Lineage tracing studies in atherosclerosis**

2

Cell type studied	Cell tracing*	Mouse model	contribution of labelled cells to plaque?	VSMCs	aSMA negative?	Macrophage-like	Osteochondrogenic	MSC-like	Ref
VSMC	Tagln-CreERT2/R26R-LacZ or R26R-mT/mG or R26R-Confetti	ApoE ^{-/-} chow (52 weeks) or HFD (16 weeks)	yes, clonal patches	aSMA+	Yes	Lgals3+, CD68+ (62, 54% respectively of labelled cells)	NA	NA	17
VSMC	Myh11-CreERT2/R26R-EYFP	ApoE ^{-/-} HFJ 18 weeks	yes	aSMA+	>95% of labelled cells	NA	NA	NA	18
VSMC	Myh11-CreERT2/R26R-EYFP	ApoE ^{-/-} HFJ 18 weeks	yes	16% of labeled cells aSMA+	12% of labeled cells Pdgfbr+, 32-51% of labeled cells unknown identity	30% of labeled cells Lgals3+	NA	7% of labeled cells Sca1+	11
VSMC	Myh11-CreERT2/R26R-Confetti	ApoE ^{-/-} HFD 16-19 weeks	70 (40-90)% of plaque cells, oligoclonal	30-100% of labelled cells aSMA+, 70-100% of aSMA+ cells labelled	yes	5-50% of labelled cells Lamp2+, 70% of Lamp2+ cells were labelled	NA	NA	20
VSMC	Myh11-CreERT2/R26R-Confetti	PCSK9-D377Y AAV, 12-36 week HFD	oligo clonal VSMC contribution to plaque cap and core	aSMA+	yes	Oil Red O+, no Lgals3+ cells detected	yes	yes	22
VSMC	Myh11-CreERT2/Brainbow	ApoE ^{-/-} HFD 5-12 weeks	monoclonal VSMC contribution to plaque cap and core	aSMA+	yes	NA	NA	NA	23
VSMC	Myh11-CreERT2/R26R-Confetti	ApoE ^{-/-} HFD 16-19 weeks	yes, clonal patches	aSMA+	yes	yes	Sca1+ (rare)	Sca1+ (rare)	43
Unknown	Chimeras	ApoE ^{-/-} Chow diet 10 months	oligoclonal patches in plaque cap	clonal aSMA+	NA	NA	NA	NA	22
Tcf21+ (Adventitial)	TCF21-MerCreMer/R26R-dTomato	ApoE ^{-/-} HDF 12 weeks, Ldlr ^{-/-} HFD 16-20 weeks	yes	Tagln+	Periostin+	NA	NA	NA	239
Adventitial cells	transplant of cultured Sca1+ adventitial cells from SM-LacZ/ApoE ^{-/-} donor animals into ApoE ^{-/-} hosts	vein graft	yes	LacZ+ cells in plaque	NA	NA	NA	NA	220
Adventitial MSC	Gli1-CreERT2/R26R-dTomato	ApoE ^{-/-} with subtotal (5/6) nephrectomy and HFD 10-16 weeks	observed (40 cells/high power field)	Calponin+ (20-80% of lineage traced cells)	yes	no CD68+ cells detected	calcium tracer+, Runx2+ (10-25% of lineage traced cells)	calcium tracer+, Runx2+ (10-25% of lineage traced cells)	221
BM-derived	BM from GFP+ donor animals transplanted into GFP- hosts	ApoE ^{-/-} HFD 20-32 weeks	Mac2+ foam cells in plaque core	No	yes	Lgals3+	NA	NA	41
BM-derived	BM from GFP+ donor animals transplanted into GFP- hosts	Healing plaque (ApoE ^{-/-} with spontaneous or mechanically disrupted hemorrhagic plaque)	Mac2+ foam cells in plaque core	No	yes	Lgals3+	NA	NA	40
BM-derived	BM from MYH11-Cre/R26R-LacZ/ApoE ^{-/-} donor animals transplanted into ApoE ^{-/-} hosts	ApoE ^{-/-} HFD 6-22 weeks	0.7% of cells in advanced plaque were LacZ+	very rare (0.4% of plaque cells were aSMA+LacZ+)	very rare	NA	NA	NA	42
EC	end.ScCreERT/R26R-EYFP	ApoE ^{-/-} HFD 8-30 weeks	yes	Yes, low contribution (aSMA+SMMHC)	yes, 32-45% of FAP+ fibroblasts are labelled	NA	NA	NA	240
ND	not detected								
NA	not analysed								
HFD	high fat diet								
*	tamoxifen-induced recombination prior to induction of atherosclerosis								
R26R-	ROSA26 locus reporter -								

3

4

5

Fig 1

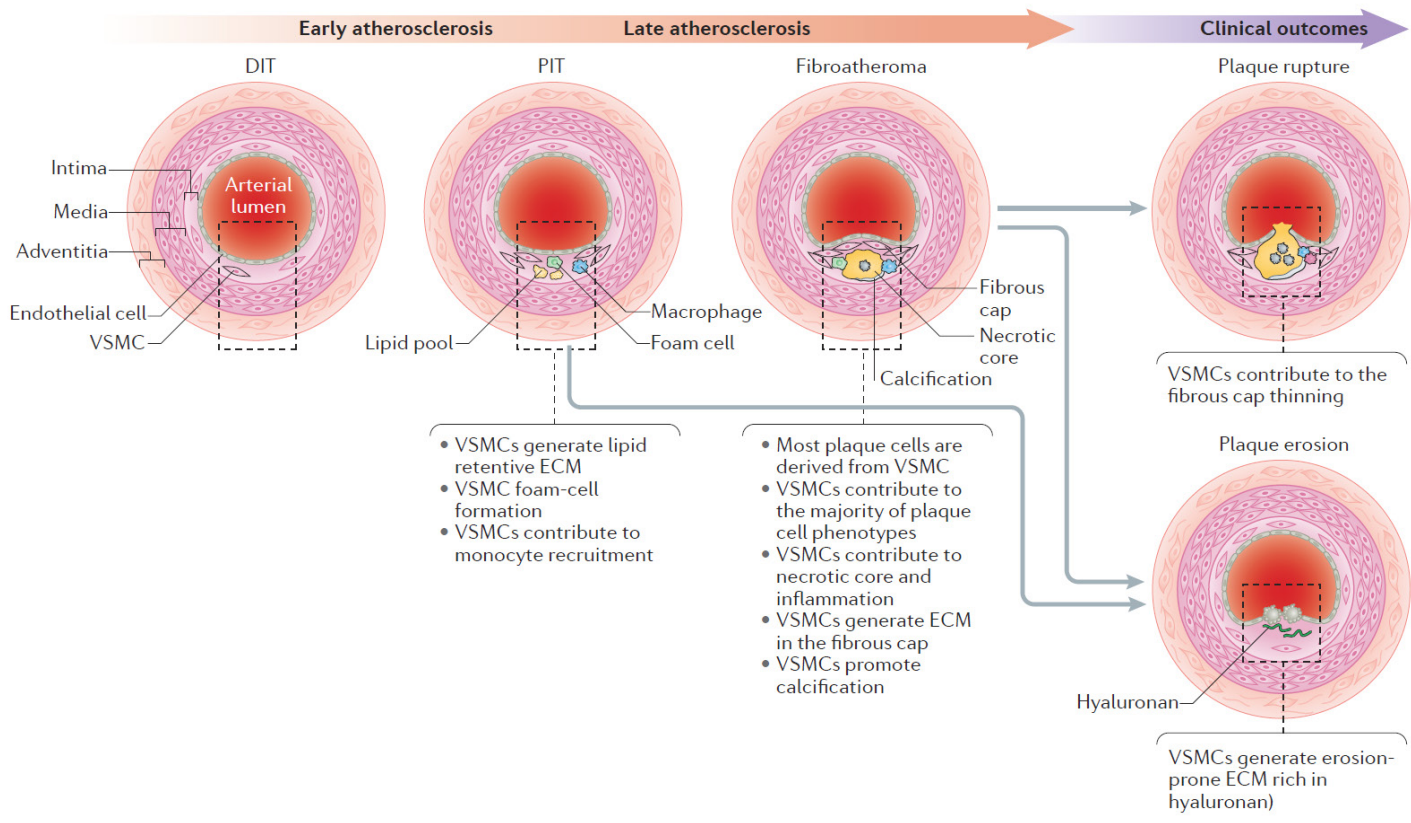


Fig 2

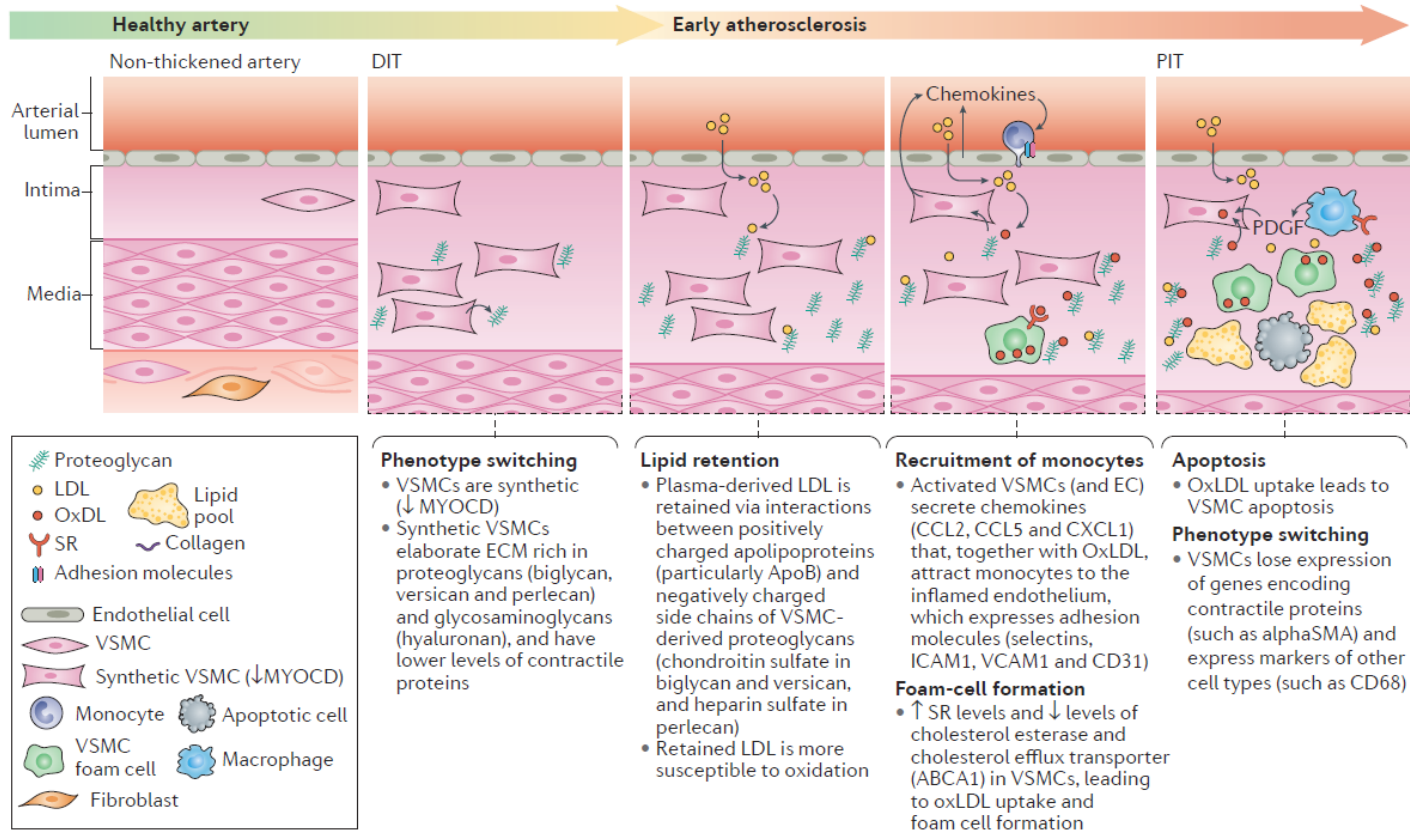


Fig 3

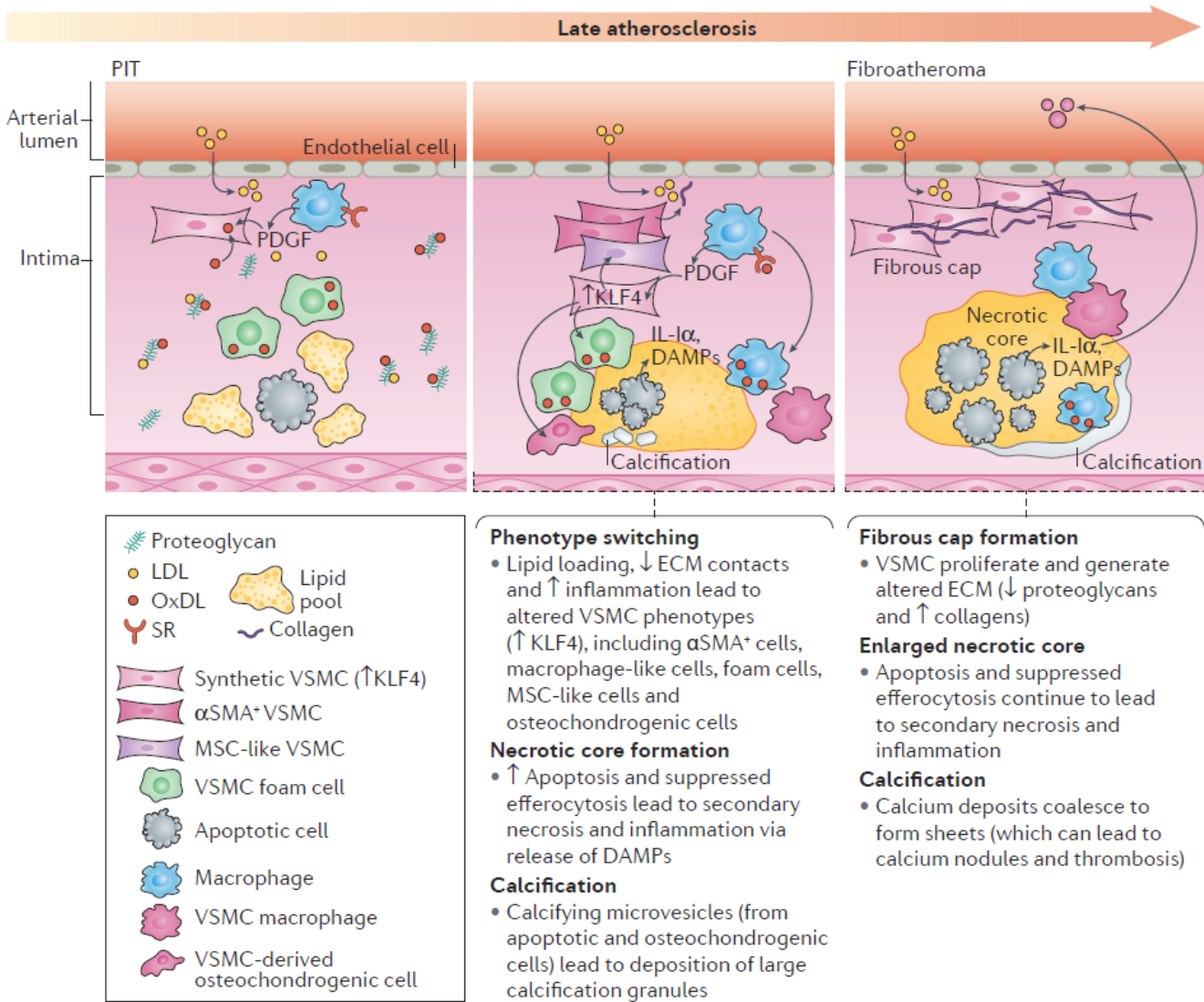
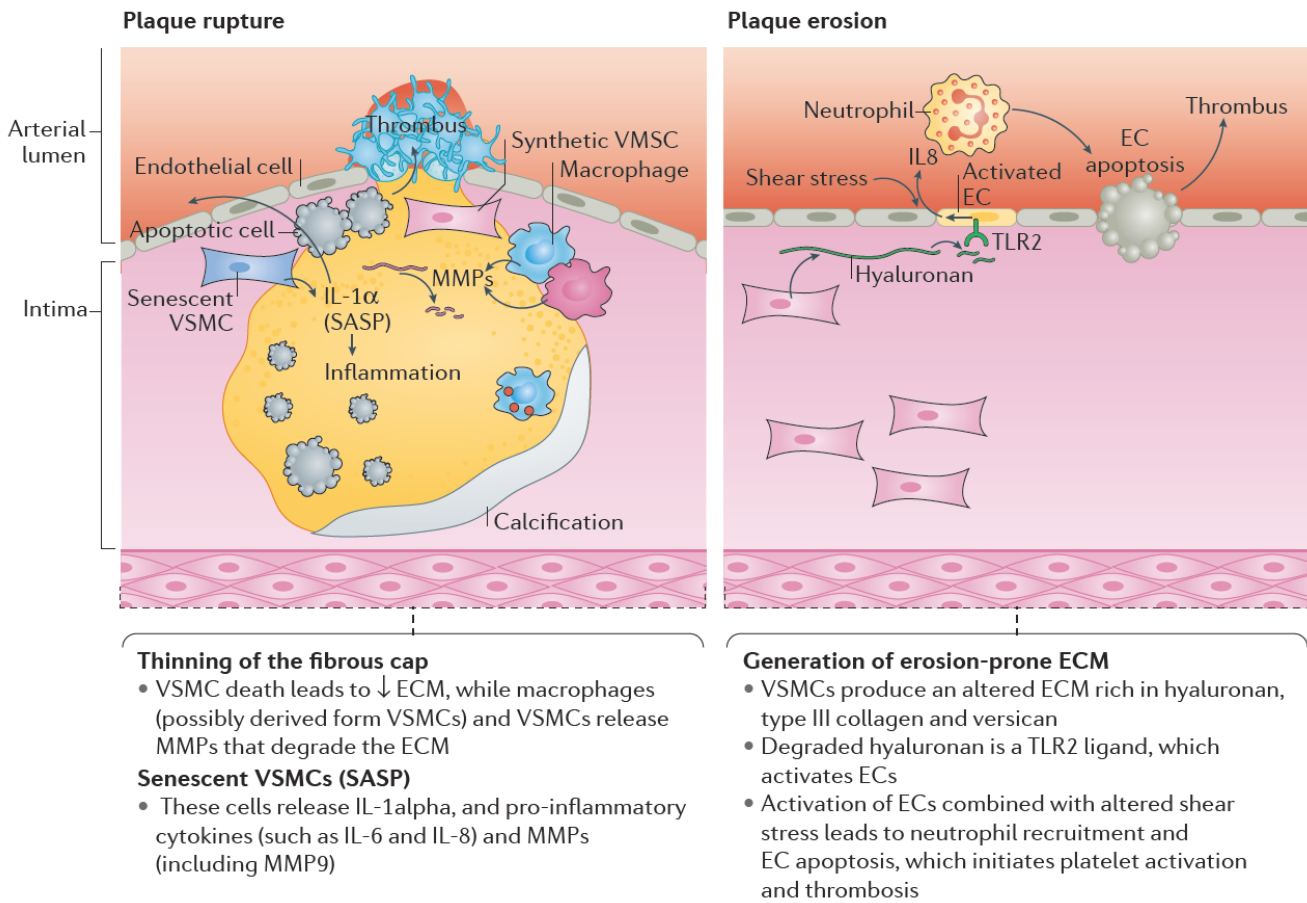
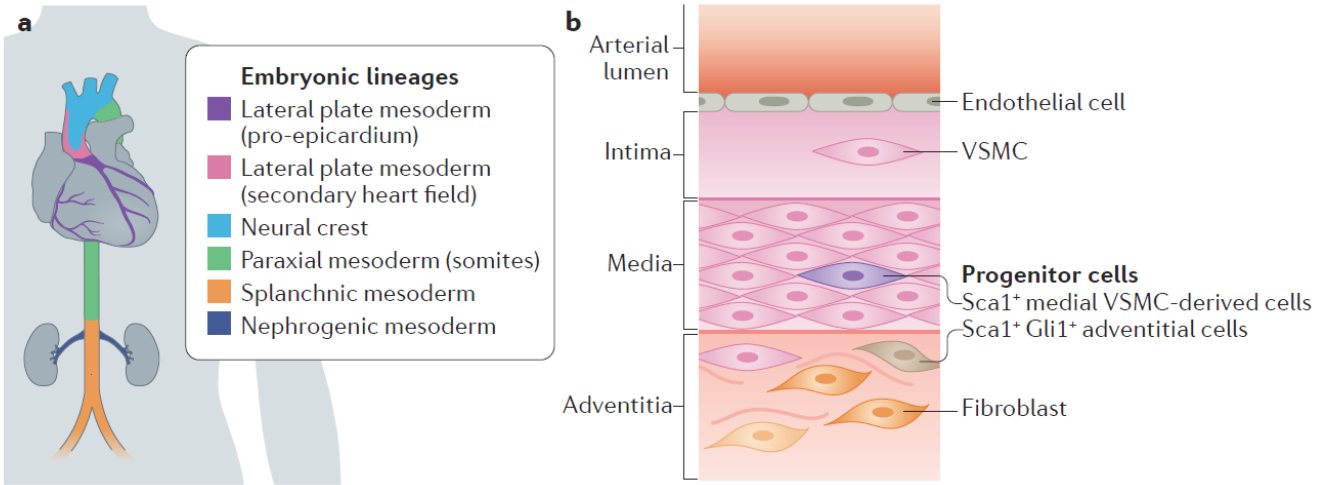


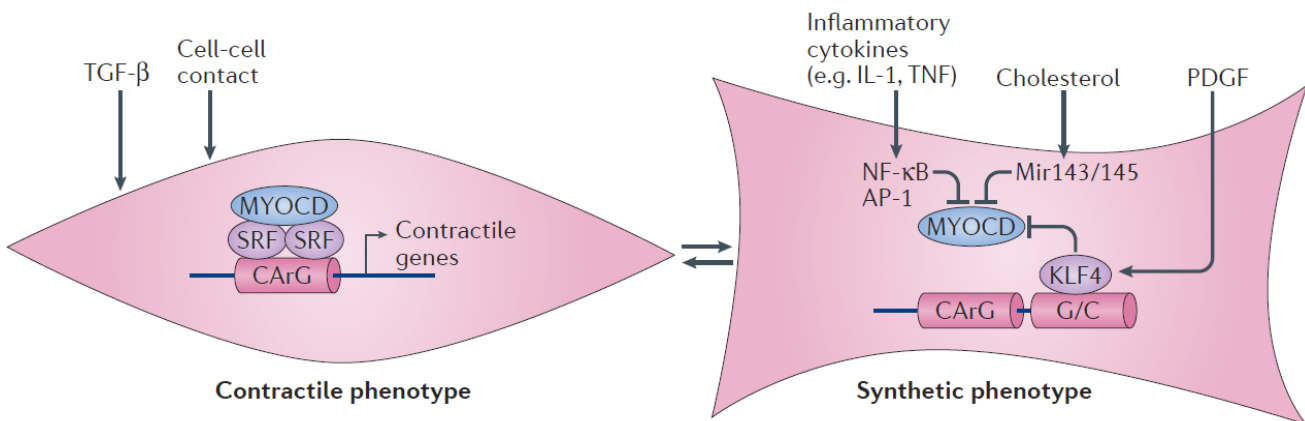
Fig 4



Box 2



Box 3



References

Highlighted references

Gomez 2013, Feil 2014, Shankman 2015 - these were the first lineage tracing studies of VSMCs in the context of atherosclerosis

Chappell 2016 – this article demonstrates that different VSMC phenotypes arise from the same ancestral cell in atherosclerosis

Misra 2018 – This article provides evidence that secreted factors affect clonality

Childs 2016 – This article demonstrates the impact of senescence in atherosclerosis

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Figure legends

Figure 1: Overview of the role of vascular smooth muscle cells (VSMCs) in atherosclerosis

VSMCs are a major source of plaque cells and extra-cellular matrix (ECM) at all stages of atherosclerosis and contribute to numerous processes throughout the disease.

Figure 2: VSMCs in early atherosclerosis

Summary of the role of VSMCs in early atherosclerosis (progression from diffuse intimal thickening to pathological intimal thickening). VSMCs are the predominant cell type and source of atherogenic, lipid (particularly LDL)-retentive extra-cellular matrix in early atherosclerosis. Retained LDL is susceptible to modifications, such as oxidation (to OxLDL). Uptake of OxLDL by VSMCs leads to foam cell formation and death by apoptosis. Activated VSMC secrete chemokines and contribute to recruitment of monocytes, which differentiate to macrophages. Progression to PITs is typically associated with decreased VSMC marker positive cell content (such as smooth muscle alpha actin positive cells, α SMA+) and increased macrophage marker positive cells (such as CD68+ cells), likely reflecting a combination of VSMC death and VSMC phenotype switching to macrophage like cells (as a consequence of decreased MYOCD and increased KLF4).

Abbreviations: ABCA1, ATP-binding cassette transporter 1 ApoB, apolipoprotein B; CCL2, CC motif chemokine 2 (also known as MCP-1); CCL5, CC motif chemokine 5 (also known as RANTES); CXCL1, CXC motif chemokine 1 (also known as GRO α); DIT, diffuse intimal thickening, ECM, extra-cellular matrix; ECs, endothelial cells; ICAM1, intercellular adhesion molecule 1; KLF4, Krüppel like factor 4; LDL, low density lipoprotein; MYOCD, myocardin; PIT, pathological intimal thickening; SR, scavenger receptor; VCAM1, vascular cell adhesion molecule 1; VSMCs, vascular smooth muscle cells.

Figure 3: VSMCs in late atherosclerosis

Summary of the role of VSMCs in late atherosclerosis (progression from pathological intimal thickening to fibroatheroma). This phase of atherosclerosis is characterised by the elaboration of the fibrous cap by VSMCs, and the necrotic core, which is the consequence of defective efferocytosis of apoptotic cells (mostly VSMCs and macrophages). Through phenotype switching, VSMCs contribute to many different plaque cell phenotypes, including the extra-cellular matrix -producing cells of the fibrous cap, macrophage-like cells, foam cells, mesenchymal stem cell-like and osteochondrogenic cells. VSMC also contribute to calcification through a number of mechanisms, including apoptosis and osteochondrogenic conversion.

Abbreviations: α SMA, smooth muscle alpha actin; DAMPs, damage associated molecular patterns; ECM, extra-cellular matrix; IL-1 α , interleukin-1 alpha; KLF4, Krüppel like factor 4; LDL, low density lipoprotein; MSC-like, mesenchymal stem cell-like; MYOCD, myocardin; PDGF, platelet derived growth factor; PIT, pathological intimal thickening; VSMCs, vascular smooth muscle cells.

Figure 4: VSMCs in clinical sequelae of atherosclerosis

Summary of the role of VSMCs in plaque rupture and plaque erosion, the two major processes underlying thrombosis and hence the clinical sequelae of atherosclerosis.

1 Abbreviations: ECM, extra-cellular matrix; EC, endothelial cell; IL-, interleukin-; MMPs, matrix
2 metalloproteinases; SASP, senescence associated secretory phenotype; TLR, Toll like receptor; VSMCs,
3 vascular smooth muscle cells.

5 **Table 1: Lineage tracing studies in atherosclerosis**

8 **Glossary**

10 **Clonal expansion** – proliferation of a single or limited number of ancestral cells

11 **Foam cell** – lipid laden cells with a foamy appearance

12 **Lineage tracing** – technique of following the fate of labelled cells to enable identification of
13 progeny cells

14 **Mesenchymal stem cells** – multipotent stromal cells

15 **Osteochondrogenic cells** – cells capable of generating osteocytes and or chondrocytes

16 **Phenotype switching** – process by which VSMCs alter phenotype, often inferred through
17 decreased expression of VSMC-specific contractile genes and or increased expression of
18 markers typical of synthetic VSMCs or other cell-types

19 **Response to retention hypothesis** – hypothesis that sub-endothelial retention of lipid, in the
20 form of lipoproteins, is the initial step in atherogenesis

21 **Shelterin complex** – multi-protein complex (including TRF2) which binds the repetitive
22 sequences of telomeric DNA, protecting against DNA damage

23 **Vulnerable plaque** – plaque with a phenotype associated with increased risk of rupture, also
24 known as thin-cap fibroatheromas, defined by a thin fibrous cap (of less than 65µm) and
25 large necrotic core

27 **Abbreviations**

28 AngII

29 ApoB

30 aSMA

31 CAD

32 CArG box

33 CyToF

34 DIT

35 DDR

36 DT

37 ECM

38 GAG

39 GWAS

40 MSC

41 MMPs

42 oxLDL

43 ROS

44 SASP

45 SaβG

46 Shelterin

47 X-Gal

49 **Author contributions**

1 G.L.B., H.F.J. and M.C.H.C. wrote the manuscript. H.F.J. and M.C.H.C. contributed equally.
2 All the authors researched data for the article, discussed its content, reviewed the manuscript
3 for important intellectual content, and edited the manuscript before submission.
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Competing interests

7 The authors declare no competing interests.
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