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REVIEW ARTICLE

# The role of smooth muscle cells in plaque stability: Therapeutic targeting potential

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Events responsible for cardiovascular mortality and morbidity are predominantly caused by rupture of "vulnerable" atherosclerotic lesions. Vascular smooth muscle cells (VSMCs) play a key role in atherogenesis and have historically been considered beneficial for plaque stability. VSMCs constitute the main cellular component of the protective fibrous cap within lesions and are responsible for synthesising strength-giving extracellular matrix components. However, lineage-tracing experiments in mouse models of atherosclerosis have shown that, in addition to the fibrous cap, VSMCs also give rise to many of the cell types found within the plaque core. In particular, VSMCs generate a substantial fraction of lipid-laden foam cells, and VSMC-derived cells expressing markers of macrophages, osteochondrocyte, and mesenchymal stem cells have been observed within lesions. Here, we review recent studies that have changed our perspective on VSMC function in atherosclerosis and discuss how VSMCs could be targeted to increase plaque stability.

## 1 | INTRODUCTION

Atherosclerosis, the leading cause of death worldwide, is a chronic and progressive inflammatory disease of large- to medium-sized blood vessels (Libby, Ridker, & Hansson, 2011; Tabas, Garcia-Cardena, & Owens, 2015; http://www.who.int/mediacentre/factsheets/fs310/en/). Atherosclerotic plaques consist of lipids and extracellular matrix (ECM) and involve several cell types, including bone marrow-derived cells, vascular smooth muscle cells (VSMCs), and endothelial cells. The process of atherogenesis is complex and can be characterised by the following main stages (recently reviewed by Basatemur et al. (2019)). Firstly, endothelial cell damage and dysfunction stimulates the accumulation and oxidation of LDL within the vessel wall. Oxidised LDL (oxLDL) attracts monocytes from the blood into the subendothelial intima where they transform into macrophages, which ingest lipoproteins to become foam cells. The subsequent production of inflammatory mediators and cytokines stimulates VSMCs to migrate from the media to the intima where they proliferate and secrete ECM proteins. Importantly, VSMC accumulation in the intimal space (referred to as diffuse intimal thickening) occurs at sites of aberrant flow in humans already in utero and is thought to predispose for plaque development (Basatemur et al., 2019). In progressing plaques, macrophages and VSMCs undergoing cell death release lipids, which accumulate within the centre of the plaque to form the necrotic core. VSMCs are thought to migrate and proliferate to encage the necrotic core and create a fibrous cap that stabilises the plaque. Thinning of the fibrous cap in advanced plaques increases the risk of rupture, which triggers thrombus formation and subsequent clinical complications including heart attack and stroke (Libby et al., 2011; Tabas et al., 2015). In non-lethal cases, VSMCs are thought to accumulate at the rupture site and secrete strength-giving ECM proteins to restore the integrity of the plaque surface (Bentzon, Otsuka, Virmani, & Falk, 2014; Bentzon, Sondergaard, Kassem, & Falk, 2007; Davies, Bland, Hangartner, Angelini, & Thomas, 1989). However, this healing process may also have adverse effects such as constrictive remodelling of the vascular wall (Bentzon et al., 2014; Burke et al., 2001). It is therefore

**Abbreviations:** ABCA1, ATP-binding cassette transporter; AP-1, activator protein-1; APOE, apolipoprotein E; CArG, $CC(A/T\text{-rich})_6GG$; ECM, extracellular matrix; H3K27me3, histone H3 lysine 27 trimethylation; H3K4me2, histone H3 lysine 4 dimethylation; H3K9me2/3, histone H3 lysine 9 dimethylation/trimethylation; KLF4, Krüppel-like factor 4; Ldlr, LDL receptor; MYH11, myosin heavy chain 11; oxLDL, oxidised LDL; Sca1, stem cell antigen 1; SMA, smooth muscle actin; SMMHC, smooth muscle myosin heavy chain; SRF, serum response factor; TCF21, transcription factor 21; VSMC, vascular smooth muscle cell; YFP, yellow fluorescent protein.

  

of considerable therapeutic importance to understand the mechanisms that regulate plaque stability.

Post-mortem and clinical imaging studies have shown that vulnerable atherosclerotic plaque typically displays a thin fibrous cap, often containing micro-calcifications, covering a lipid-rich necrotic core, which is infiltrated by large numbers of bone marrow-derived cells (Bennett, Sinha, & Owens, 2016; Durham, Speer, Scatena, Giachelli, & Shanahan, 2018; Shankman et al., 2015; Figure 1a). In contrast, stable lesions are thought to have a thick collagen-rich fibrous cap covering a plaque core, which contains a high ratio of αSMA-positive to CD68-positive cells and possibly macro-calcified deposits (Bennett et al., 2016; Durham et al., 2018; Shankman et al., 2015). The many cell types contributing to atherosclerotic lesions each influence plaque stability. However, an increasing body of evidence, including many genetic lineage-tracing studies, has demonstrated that VSMCs play a substantial role in atherogenesis. This review discusses newly discovered aspects of VSMC biology, which could be targeted to detect, prevent, or treat vulnerable atherosclerotic plaques.

## 2 | PHENOTYPIC MODULATION OF VSMCs

In the healthy blood vessel, VSMCs exhibit a low rate of proliferation and low synthetic activity and express a unique set of contractile proteins, essential for the contraction and relaxation of the vascular wall (Bennett et al., 2016). As reviewed by Owens, Kumar, and Wamhoff (2004), many VSMC-specific genes that encode contractile proteins, including αSMA/ACTA2, Calponin1/CNN1, SM22α/TAGLN, and SMMHC/MYH11, are controlled by CC(A/T-rich)$_6$GG (CArG) *cis*-regulatory elements in their promoter, which are bound by the widely expressed transcription factor, serum response factor (SRF). To achieve cell-type-specific expression of CArG-dependent contractile

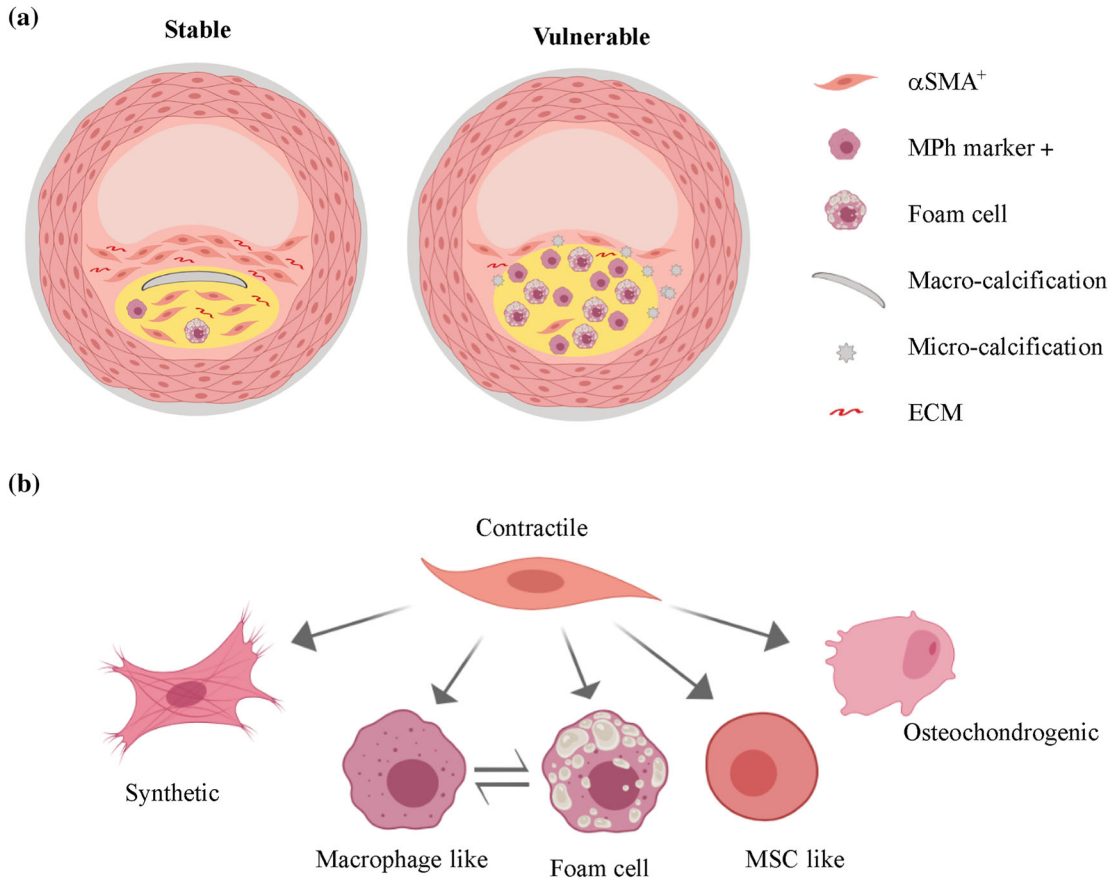


**FIGURE 1** Stable versus vulnerable atherosclerotic plaque and vascular smooth muscle cell (VSMC)-derived plaque cell phenotypes. (a) A simplified scheme showing a stable lesion with a thick collagen-rich (extracellular matrix [ECM]) fibrous cap covering a plaque core (yellow area), which contains a high ratio of αSMA-positive (αSMA$^+$) cells compared with cells expressing macrophage-associated markers (MPh-marker +) and macro-calcified deposits. In contrast, vulnerable plaques have a thin fibrous cap, which often contains micro-calcified deposits, fewer cells, and less ECM. The lipid-rich core (yellow area) of vulnerable lesions includes numerous foam cells as well as a high ratio of cells expressing macrophage-associated markers compared with αSMA-positive cells. Please note that details such as endothelial cells, adventitial cells, and internal and external elastic lamina are not displayed. (b) Contractile VSMCs can alter their phenotype to a more active "synthetic" state in which they up-regulate selective gene sets important for vascular remodelling, including cytokines, chemokines, proteases, and adhesion proteins. Within plaques, VSMCs also give rise to foam cells or express markers traditionally associated with other cell types, such as macrophages, mesenchymal stem cells (MSCs), or osteochondrocytes. The relative contribution of VSMC-derived plaque cell phenotypes in stable versus vulnerable plaque remains unknown

VSMC genes, SRF associates with myocardin, which is only expressed in the vasculature by VSMCs (Wang et al., 2001).

Despite being a highly differentiated and specialised cell type, VSMCs retain remarkable plasticity and can alter their quiescent "contractile" phenotype to a more active "synthetic" state (Figure 1b; Chamley-Campbell, Campbell, & Ross, 1979; Alexander & Owens, 2012). Synthetic VSMCs can re-acquire many characteristics of the contractile phenotype, suggesting that the phenotypic switch is reversible (Aikawa et al., 1997; Christen et al., 2001; Manderson, Mosse, Safstrom, Young, & Campbell, 1989; Sottiurai et al., 1989; Thyberg, Blomgren, Hedin, & Dryjski, 1995; Thyberg, Blomgren, Roy, Tran, & Hedin, 1997). The synthetic VSMC phenotype is characterised by loss of contractile marker expression and up-regulation of selective gene sets, including pro-inflammatory cytokines and **MMP**s, leading to increased cell migration, proliferation, and secretion of pro-inflammatory cytokines (Alexander & Owens, 2012; Clarke, Talib, Figg, & Bennett, 2010; Owens et al., 2004). Such phenotypic switching is required to maintain vascular homeostasis and regulate vascular response to injury and inflammation but can become dysregulated in disease.

## 3 | ORIGIN OF VSMC-LIKE CELLS WITHIN ATHEROSCLEROTIC PLAQUES

VSMCs down-regulate contractile VSMCs genes in response to injury and inflammation, which may be linked to the reduced expression of myocardin, a key regulator of the contractile VSMC state, during plaque development (Ackers-Johnson et al., 2015). Furthermore, contractile VSMC markers can be induced in other cell types (Gomez & Owens, 2012). For example, myofibroblasts express αSMA (Darby, Skalli, & Gabbiani, 1990), and adventitial cells have been reported to express contractile VSMC markers in vitro (Hu et al., 2004). This promiscuous expression of proposed lineage-specific cell markers has prompted heated debate regarding the origin of VSMC-like cells within atherosclerotic plaque.

Originally, VSMC-like cells within plaque were suggested to be of myeloid origin (Sata et al., 2002). However, these claims have later been refuted with the emergence of genetic lineage-tracing studies. For example, Iwata et al. (2010) transplanted bone marrow cells from mice expressing LacZ under the control of the *Myh11* promoter into *Apoe*$^{-/-}$ mice and showed that while bone marrow-derived cells contribute to atherogenesis, they do not differentiate into *Myh11*-expressing cells. Several groups have suggested that adventitial cells generate contractile marker-expressing plaque cells (Chen et al., 2013; Hu et al., 2004). In 2016, Kramann et al. demonstrated that adventitial Gli1$^{+}$ MSC-like cells can generate αSMA-positive cells and proposed that migration of these cells into atherosclerotic lesions contributes to plaque calcification. A subpopulation of progenitor cells within healthy blood vessels that express the transcription factor TCF21 have also been described (Nurnberg et al., 2015). The authors of this study observed that cells in the media and adventitia, which are TCF21 positive prior to injury, give rise to VSMC marker-positive cells within the fibrous cap (Nurnberg et al., 2015). It has also been hypothesised that progenitor cells within the medial layer are responsible for the VSMC-like cells observed within plaque. For example, *Myh11*-negative cells within the media were found to express multipotency-associated factors, including Sox17, Sox10, and S100b when cultured (Tang et al., 2012). In this study, the authors used the VSMC-specific *Myh11* promoter to express a constitutively active Cre recombinase (Myh11–Cre) combined with a Rosa26–loxP–GFP recombination reporter and found an absence of GFP-positive cells but the presence of S100b-positive cells within the neointima following wire endothelial denudation injury to conclude that the identified cells are involved in disease-associated cell accumulation (Tang et al., 2012). However, this observation was at odds with lineage-tracing studies using temporally inducible recombinases (Nemenoff et al., 2011), which are key for analysis of cell fate, and the conclusions made by Tang et al. were subsequently challenged in a paper co-authored by a number of experts in the field (Nguyen et al., 2013).

In 2013, Gomez et al. combined a **tamoxifen**-inducible recombinase driven by the VSMC-specific *Myh11* promoter (Myh11–CreERt2) with the Rosa26–STOP–flox–YFP reporter (Figure 2a) for lineage tracing of VSMCs in the *Apoe*$^{-/-}$ mouse model of atherosclerosis (Gomez et al., 2013). In such experiments, animals are treated with tamoxifen to induce recombination-mediated expression of YFP-reporter in healthy VSMCs before being fed a high-fat diet. Since YFP reporter expression is stably maintained, independent of *Myh11* expression, this model allows tracing the fate of mature VSMC during atherosclerosis (Figure 2a). This study demonstrated that a large proportion of cells within the plaque is VSMC-derived and that most plaque cells of VSMC origin do not express the classical contractile marker αSMA (Gomez et al., 2013). Since then, several other genetic fate mapping studies have confirmed this using transgenic mice with similar tamoxifen-regulated, VSMC-specific recombinase, and Cre-dependent reporter genes to label mature VSMCs (Albarran-Juarez, Kaur, Grimm, Offermanns, & Wettschureck, 2016; Chappell et al., 2016; Feil et al., 2014; Gomez et al., 2013; Jacobsen et al., 2017; Misra et al., 2018; Shankman et al., 2015). More recently, VSMC lineage tracing using multicolour reporters (Figure 2b) has definitively shown that atherosclerotic lesions are oligoclonal in terms of VSMC-derived cells (Chappell et al., 2016; Feil et al., 2014; Jacobsen et al., 2017; Misra et al., 2018). Therefore, VSMC accumulation within plaque arises from proliferation of a very small number of mature yet plastic *Myh11*-expressing VSMCs (Chappell et al., 2016; Feil et al., 2014; Jacobsen et al., 2017; Misra et al., 2018). It is tempting to extrapolate this oligoclonal nature of atherogenesis to human lesions, especially when considering studies on X-chromosome inactivation patterns that suggest clonal cell expansion in human plaque (Benditt & Benditt, 1973).

## 4 | VSMC BEHAVIOUR WITHIN ATHEROSCLEROTIC PLAQUE

Genetic lineage-tracing studies have shown that VSMCs modulate their phenotype in response to signals within the surrounding milieu (Chappell et al., 2016; Feil et al., 2014; Gomez et al., 2013; Jacobsen

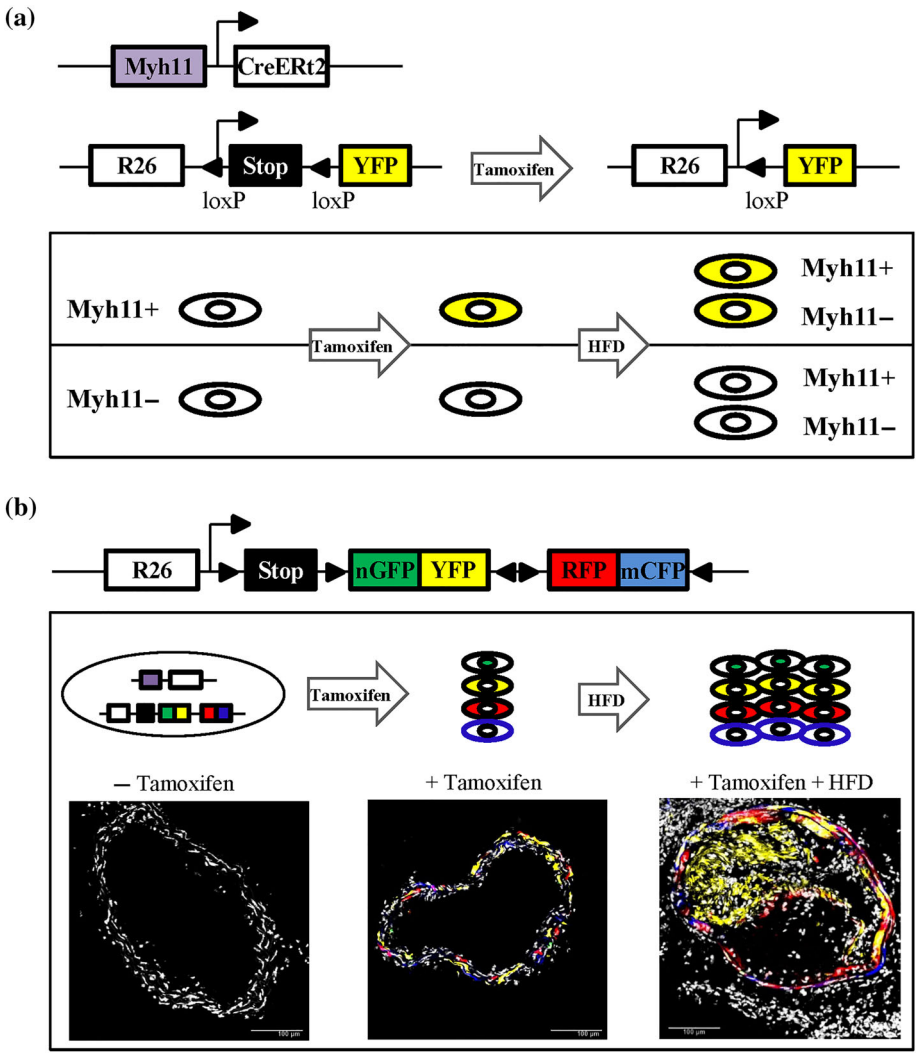


**FIGURE 2** Genetic lineage labelling of vascular smooth muscle cells (VSMCs). (a) Diagram of the Myh11–CreERt2 transgene and the ROSA26–YFP reporter allele. Tamoxifen-induced recombination at the ROSA26–YFP locus results in the expression of YFP protein, which is stably propagated within progeny after high-fat diet (HFD)-induced atherogenesis independent of Myh11 expression levels (b) Schematic of the ROSA26–Confetti reporter allele. Tamoxifen-induced recombination at the ROSA26–Confetti locus results in stochastic expression of one of four fluorescent proteins (nuclear GFP, YFP, RFP, or membrane-associated CFP), which are stably propagated within progeny after HFD-induced atherogenesis. Confocal images show non-labelled and Myh11-lineage Confetti-labelled VSMCs in carotid arteries before and after HFD-induced atherogenesis. Scale bars are 100 μm

et al., 2017; Misra et al., 2018 ; Shankman et al., 2015). With the progression of atherosclerosis, VSMCs down-regulate contractile proteins, adopt a more proliferative state, become more migratory, and respond to inflammatory signals (Allahverdian, Chaabane, Boukais, Francis, & Bochaton-Piallat, 2018; Bennett et al., 2016).

In addition to adopting a "synthetic" state, VSMCs within atherosclerotic lesions can also alter their gene expression profile to resemble various other cell types (Figure 1b). For example, VSMC-derived plaque cells have been reported to express markers that are traditionally associated with macrophages (*LAMP2*/MAC3, *LGALS3*/MAC2, and *CD68*; Chappell et al., 2016; Dobnikar et al., 2018; Feil et al., 2014; Misra et al., 2018; Shankman et al., 2015), mesenchymal stem cells (*Sca1*; Dobnikar et al., 2018; Shankman et al., 2015), and myofibroblasts (*Acta2* and *Pdgfrb*; Misra et al., 2018; Shankman et al., 2015). A number of studies also suggest that VSMCs can acquire an osteochondrocytic transcriptional repertoire (*Alp*, *Bglap*, *Opn*, *Runx2*, and *Bmp2*) as reviewed by Durham et al. (2018). These phenotypically distinct cells of VSMC origin might influence multiple processes underlying atherosclerotic plaque stability, including lesion growth, lipid retention, inflammation, and ECM composition (Allahverdian et al., 2018; Bennett et al., 2016; Durham et al., 2018; Johnson, 2017). Therefore, it has been proposed that VSMC-derived cells can both improve plaque stability and exacerbate plaque rupture (Allahverdian et al., 2018; Bennett et al., 2016; Durham et al., 2018; Johnson, 2017).

## 5 | TARGETING A VSMC PROGENITOR

The existence of specialised VSMCs "response" population could explain the selective clonal VSMC expansion within plaque observed

in mouse models of atherosclerosis (Chappell et al., 2016; Feil et al., 2014; Jacobsen et al., 2017; Misra et al., 2018). Several studies have reported subpopulations of VSMCs, which express multipotency-associated genes (Cherepanova et al., 2016; Dobnikar et al., 2018; Sainz et al., 2006; Shankman et al., 2015). These observations have led to discussions regarding the similarities between atherosclerosis and cancer (DiRenzo, Owens, & Leeper, 2017). Like emerging pro-efferocytic therapies that appear to target the root of cancer, selective elimination of hyper-proliferative VSMC subpopulations has been proposed as a strategy to limiting atherosclerotic plaque growth (DiRenzo et al., 2017). However, studies have shown that plaque size is not a suitable surrogate for plaque stability. Rather than lesion size, the lipid content of the necrotic core, the thickness of the fibrous cap, the composition of the ECM, and the presence of calcification appear to provide a clearer indication of plaque vulnerability (Baylis, Gomez, & Owens, 2017). Targeting other aspects of VSMC-progenitor function, such as migration, transdifferentiation, or synthetic activity, might therefore be a more feasible approach to treat vulnerable plaque.

The nature of VSMC-progenitor populations and the mechanisms regulating their behaviour in atherogenesis is currently being investigated. Cherepanova et al. (2016) found that VSMCs express the pluripotency factor Oct4/Pou5f1. The authors show that Oct4 expression in VSMCs promotes migration and investment of VSMCs into the fibrous cap and improves lesion stability (Cherepanova et al., 2016). Therefore, it may be beneficial to encourage the survival or proliferation of Oct4-positive VSMC-derived cells or force expression of Oct4 in VSMCs to treat vulnerable plaque. Furthermore, a rare population of VSMCs express the multipotent progenitor marker stem cell antigen 1 (Sca1/Ly6a; Dobnikar et al., 2018; Sainz et al., 2006). Single cell transcriptomics showed that Sca1 expression in VSMCs correlates negatively with expression of contractile VSMC genes and is associated with up-regulation of genes that are induced by VSMCs in response to inflammation and growth factors (Dobnikar et al., 2018). As the proportion of VSMCs expressing Sca1 is up-regulated upon exposure to stimuli known to induce phenotypic switching (Dobnikar et al., 2018), it is tempting to hypothesise that Sca1 expression might mark a primed VSMC state that can readily respond to environmental cues (Dobnikar et al., 2018). In support of this hypothesis, Majesky et al. (2017) used lineage tracing to show that differentiated VSMCs generate a subpopulation of Sca1-positive cells in the adventitia, which could transdifferentiate into several cell types in vitro, including macrophages. The generation of VSMC-derived Sca1-positive cells was dependent on the induction of the pluripotency factor KLF4 (Majesky et al., 2017), which has previously been implicated in the regulation of VSMC plasticity.

KLF4 negatively regulates VSMC contractility by interacting with SRF to repress myocardin-induced activation of contractile VSMC genes (He et al., 2015; Liu et al., 2005; Liu, Sinha, & Owens, 2003). In addition, KLF4 has been shown to repress contractile VSMC gene expression by binding to the **TGF-β** control element within their promoter, blocking the recruitment of activating complexes (Liu et al., 2003; Guo & Chen, 2012). Interestingly, VSMC-specific conditional knockout of *Klf4* in a mouse model of atherosclerosis partially suppressed macrophage-like cell conversions and significantly reduced VSMC proliferation and apoptosis resulting in a thicker fibrous cap (Shankman et al., 2015). These results therefore suggest that KLF4 could be targeted to block VSMC transdifferentiation to a macrophage-like state and possibly inhibit VSMCs-derived foam cell accumulation.

## 6 | TARGETING VSMC CONTRIBUTION TO THE FIBROUS CAP

It has long been recognised that atherosclerotic plaque stability depends on the thickness and composition of the fibrous cap and the lineage-tracing studies described above have shown that the fibrous cap predominately contains cells derived from VSMCs. These VSMC-derived cap cells are thought to be the primary source of collagen within the fibrous cap, providing mechanical tensile strength and resistance to rupture (Adiguzel, Ahmad, Franco, & Bendeck, 2009). Consistent with these findings, in vitro studies demonstrated that cultured VSMCs produce collagens in response to pro-inflammatory stimuli such as **IL-1β** (Adiguzel et al., 2009; Amento, Ehsani, Palmer, & Libby, 1991). Therefore, promoting VSMC collagen production may reduce cardiovascular events caused by plaque rupture. Knockout of *Col15a* specifically in VSMCs has been reported to reduce proliferation and result in smaller lesions, which lack a VSMC and ECM-rich fibrous cap (Durgin et al., 2017). However, this study investigated plaque development rather than the regression or stability of established plaque. More research is required to investigate whether expression of specific ECM proteins by VSMCs similarly affects stability in established or healing plaque.

VSMCs also secrete proteases that degrade components of the ECM, including MMPs (Johnson, 2017). **MMP2** is constitutively expressed in VSMCs (Newby, 2005), and several other MMPs exhibit enhanced expression within VSMCs in diseased blood vessels (Choudhary et al., 2006). VSMCs from human atherosclerotic plaque shoulder regions and areas of foam cell accumulation display increased **MMP3**, **MMP9**, and **MMP12** activity compared with their medial counterparts (Galis, Sukhova, Lark, & Libby, 1994; Muller et al., 2014). This increased protease activity corresponds to regions containing higher levels of pro-inflammatory cytokines released from necrotic VSMCs and macrophages (Galis, Sukhova, et al., 1994; Muller et al., 2014). Indeed, pro-inflammatory cytokine-induced expression of MMP genes has be observed in vitro (Galis et al., 1994).

MMPs play complex roles in late stage plaque and, as reviewed by Johnson, can both stabilise plaques and promote rupture (Johnson, 2017). MMPs affect plaque stability directly by degrading major components of the ECM, thereby weakening cap stability. For example, a selective MMP12 inhibitor has been shown to slow atherosclerotic plaque rupture in *Apoe*$^{-/-}$ mice (Johnson et al., 2011). However, matrix degradation also has indirect effects via modulating the migration and proliferation of VSMCs and infiltration of inflammatory cells into tissue, which incidentally affect the stability of atherosclerotic plaque (Johnson, 2017). Indeed, MMP activity is thought to stabilise

atherosclerotic plaques by increasing contractile VSMC migration and proliferation. For instance, loss of either *Mmp2* or *Mmp9* in Apoe-deficient animals reduces lesion stability, with plaques containing fewer VSMCs than macrophages compared with Apoe single knockout control animals (Johnson, George, Newby, & Jackson, 2005; Kuzuya et al., 2006). Due to the dual effect of MMPs on plaque stability and rupture, the development of broad spectrum MMP inhibitors to treat vascular disease has been problematic (Galis & Khatri, 2002; Newby, 2012). Given the effect of selective MMP12 inhibition, specific targeting of the activity or expression of individual proteases might be a more viable approach (Johnson, 2017) to stabilise established or healing plaque.

## 7 | TARGETING VSMC-DERIVED MACROPHAGE-LIKE AND FOAM CELLS

VSMC-derived cells not only influence the structural integrity of the fibrous cap but also substantially contribute to the generation of the plaque core. Genetic lineage-tracing studies have shown that a large proportion of VSMC-derived cells within the plaque express macrophage markers including CD68 (54%), Mac2 (62%), and Mac3 (Albarran-Juarez et al., 2016; Chappell et al., 2016; Feil et al., 2014; Shankman et al., 2015). Like macrophages, some VSMC-derived plaque cells actively participate in lipid ingestion and processing. Indeed, 81% of VSMC-derived cells in atherosclerotic plaque take up oxLDL to form foam cells (Feil et al., 2014), and up to 75% of all foam cells within lesions of Apoe$^{-/-}$ animals are VSMC derived (Wang et al., 2019). Furthermore, cholesterol loading of VSMCs in vitro induces macrophage-related gene expression (e.g., *Cd68* and *Lgals3*/*Mac2*; Rong, Shapiro, Trogan, & Fisher, 2003), and VSMC treatment with oxLDL up-regulates the expression of **ACAT1**, an intracellular enzyme that breaks down excess free cholesterol to facilitate storage in cytoplasmic lipid droplets (Yin et al., 2014). Although this process helps clearing atherogenic lipoprotein complexes from the vessel wall, excessive oxLDL uptake has deleterious effects (Maguire, Pearce, & Xiao, 2018). Lipid-rich foam cells secrete a variety of pro-inflammatory mediators, elicit pro-apoptotic pathways, and attenuate clearance of dying cells leading to increased growth and destabilisation of the necrotic core (Maguire et al., 2018).

Importantly, transdifferentiation of VSMCs to a macrophage-like state has also been observed in human atherosclerotic lesions (Allahverdian, Chehroudi, McManus, Abraham, & Francis, 2014; Chappell et al., 2016; Feil et al., 2014; Shankman et al., 2015). In human coronary artery plaques, 18% of CD68$^{+}$ cells were marked by dimethylation of histone H3 at lysine 4 (H3K4me2) within the *MYH11* promoter (Shankman et al., 2015), an epigenetic mark retained by VSMCs even after loss of the contractile state (Gomez et al., 2013). Interestingly, a large proportion of plaque cells marked by the VSMC lineage label (mouse) or αSMA (human) showed evidence of lipid ingestion (Feil et al., 2014). Moreover, Allahverdian et al. (2014) found that 50% of foam cell populations in human atherosclerotic lesions express αSMA but not CD45, suggesting that they are generated from VSMCs, although the origin of these cells remains to be verified. These αSMA-positive foam cells exhibit lower levels of the **cholesterol exporter ABCA1** compared with CD45-positive cells in late stage plaque (Allahverdian et al., 2014). With these observations in mind, it is tempting to speculate that VSMC-derived foam cells contribute significantly to lipid retention within the necrotic core in human lesions.

Perhaps blocking VSMC transdifferentiation to a macrophage-like state would slow foam cell accumulation. However, it is important to remember that macrophages are also a very heterogeneous cell type and contribute to numerous processes in addition to foam cell formation (Gibson, Domingues, & Vieira, 2018). For example, M2 macrophage subpopulations are known to stimulate remodelling and anti-inflammatory responses and hence promote plaque stability (Gibson et al., 2018; Maguire et al., 2018). It will be important to test whether VSMCs expressing macrophage markers also adopt such athero-protective properties in order to understand how these cells affect plaque stability.

## 8 | TARGETING VSMC-DERIVED OSTEOCHONDROGENIC CELLS

As reviewed by Durham et al. (2018), arterial calcification is caused in part by transdifferentiation of VSMCs within the vessel wall. Inflammation, apoptosis, and oxidative stress are all thought to drive VSMC differentiation to an osteochondrogenic state and stimulate calcification (Durham et al., 2018). Lineage tracing of SM22α-expressing cells (Tagln–Cre) in the *Apoe*$^{-/-}$ murine model of atherosclerosis revealed that ~80% of osteochondrogenic-like (Runx2/Cbfa1$^{+}$) cells and 98% of chondrocyte-like (Sox9 + Col2a1$^{+}$) cells within plaque are VSMC-derived cells that have lost contractile VSMC marker gene expression (Naik et al., 2012). The location of these osteochondrogenic/chondrocyte-like VSMC-derived cells was consistent with areas of calcification within the fibrous cap and core observed in human lesions (Hutcheson et al., 2016; Otsuka, Sakakura, Yahagi, Joner, & Virmani, 2014). In support of these findings, single-cell RNA sequencing of VSMC lineage cells (Myh11–CreERt2) from atherosclerotic plaque in *Apoe*$^{-/-}$ mice found that a subset of VSMC lineage cells express chondrocytic genes (*Sox9*, *Ibsp*, and *Chad*), consistent with a calcifying phenotype (Dobnikar et al., 2018). Together, these observations suggest a positive role for VSMCs in promoting vascular calcification. Indeed, deficiency of pro-osteogenic transcription factors Msx1 and Msx2 in VSMCs within atherosclerotic *Ldlr*$^{-/-}$ mice reduced vascular calcification (Cheng et al., 2014). Moreover, VSMC-specific deletion of Runx2 attenuated vascular osteochondrogenesis and calcification without influencing lipid metabolism, monocyte/macrophage recruitment, or atherosclerotic lesion size (Lin et al., 2016). This finding implies that the factors regulating vascular calcification are distinct from those that govern lipid storage.

Like many processes underlying atherogenesis, the effects of calcification on plaque stability are context dependent. Whereas micro-calcifications, particularly of the fibrous cap, are associated with

greater risk of plaque rupture (Kelly-Arnold et al., 2013), macro-calcification is widely considered to increase plaque stability (Imoto et al., 2005; Lin et al., 2006; Wong, Thavornpattanapong, Cheung, Sun, & Tu, 2012). Macrophage-derived pro-inflammatory cytokines such as **TNF-α**, IL-1β, **IL-6**, and **oncostatin M** have been shown to promote VSMC calcification (Ceneri et al., 2017; Parhami, Basseri, Hwang, Tintut, & Demer, 2002; Shioi et al., 2002; Tintut et al., 2002). Furthermore, molecular imaging studies have identified a link between the presence of inflammation and calcification (Aikawa et al., 2007; Dweck et al., 2016). It has therefore been hypothesised that VSMCs undergo early stages of osteogenic differentiation in inflammatory plaques (Shanahan, 2007; New & Aikawa, 2011; Pugliese, Iacobini, Blasetti Fantauzzi, & Menini, 2015). Moreover, inflammatory signals that induce VSMC apoptosis promote matrix calcification, primarily through the release of the calcifying membrane-bound matrix vesicles, which act as nucleation sites of calcification (Kapustin et al., 2011; Proudfoot et al., 2000). Together, these processes are thought to produce micro-calcified deposits detrimental to the structural integrity of the plaque (Pugliese et al., 2015; Shanahan, 2007; Shioi & Ikari, 2018). However, on the resolution of inflammation, macroscopic calcium deposition is proposed to be facilitated through induction of osteoblastic differentiation and maturation of VSMCs (Pugliese et al., 2015; Shanahan, 2007; Shioi & Ikari, 2018). Macro-calcification is associated with organised structures similar to that observed in authentic bone tissue and is therefore thought to stabilise atherosclerotic lesions (Shanahan, 2007; Pugliese et al., 2015; Shioi & Ikari, 2018).

Consequently, it is not clear how simply preventing VSMC conversion to an osteochondrocytic cell state will affect the risk of plaque rupture. However, the presence of micro-calcification can instead be used to detect vulnerable plaques. Non-invasive detection of micro-calcification, as opposed to macro-calcification, within high-risk plaques is presently possible using $^{18}$F-sodium fluoride PET (Irkle et al., 2015). This clinical imaging platform could therefore be used to test the efficacy of pharmacological therapies targeting vulnerable plaque.

## 9 | TARGETING VSMC INFLAMMATORY ACTIVATION

Many experimental and clinical studies have associated inflammation with atherogenesis and increased risk of cardiovascular events (Libby, 2017). Indeed, C-reactive protein blood concentration, an inflammatory biomarker, improves the prediction of cardiovascular events beyond traditional risk algorithms alone (Ridker, 2016).

Vascular inflammation involves bidirectional interaction between resident vascular cells and inflammatory cells, which is governed in part by pro-inflammatory cytokines. Similar to other cell types within the plaque, VSMCs can both produce pro-inflammatory cytokines and respond to those secreted from other cells by activating NF-κB, AP-1, **JAK–STAT**, and SMAD signalling pathways (Schober, 2008; Sprague & Khalil, 2009). IL-1β, a key cytokine in vascular inflammation, is known to induce VSMCs to switch from a contractile to a synthetic phenotype, stimulating NF-κB-dependent transcription of cytokines (e.g., IL-6), chemokines (e.g., **CCL2**), and MMPs (Nagase, Visse, & Murphy, 2006; Lim & Park, 2014). IL-6 has been observed to up-regulate VSMC migration, proliferation, and vascular calcification while attenuating VSMC contraction (Kurozumi et al., 2016; Lee et al., 2016; Watanabe et al., 2004). Also, IL-6 treatment of cultured VSMCs activates the JAK–STAT pathway, which induces the expression of CCL2 (Watanabe et al., 2004). CCL2 plays a major role in the recruitment of monocytes and T cells to the vessel wall and has been shown to stimulate VSMC migration and proliferation (Schober, 2008; Selzman et al., 2002).

Many inflammation-responsive genes, including those induced by IL-1β, are under NF-κB transcriptional control (Chistiakov, Melnichenko, Grechko, Myasoedova, & Orekhov, 2018). Activated NF-κB transcription factors have been observed in VSMC-like nuclei within the intima of human atherosclerotic lesions (Bourcier, Sukhova, & Libby, 1997). Furthermore, selective inhibition of NF-κB in VSMCs attenuated loss of the contractile state and reduced neointima formation after vascular injury (Yoshida, Yamashita, Horimai, & Hayashi, 2013), highlighting a key role of NF-κB in regulating VSMC phenotype. Many studies have also shown that NF-κB plays a direct role in regulating the expression of contractile VSMC genes. Activation of the NF-κB signalling pathway in VSMCs results in binding of the NF-κB–p65 subunit to the *MYOCD* promoter, decreasing the expression of myocardin and myocardin-dependent contractile genes (e.g., *αSMA/ACTA2*, *SM22α/TAGLN*, and *SMMHC/MYH11*; Tang et al., 2008; Yoshida et al., 2013; Singh & Zheng, 2014). Together, these studies suggest that NF-κB inhibition, which specifically affects VSMCs could be a potential therapeutic target.

Alternatively, plaque stability could also be promoted by directly inhibiting VSMC exposure to inflammatory cytokines. Promisingly, the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study trial showed that reducing inflammation by treatment with **canakinumab**, an IL-1β-neutralising monoclonal antibody, significantly lowered the rate of cardiovascular events compared with placebo (Ridker et al., 2017). However, IL-1β antibody treatment of Apoe-deficient mice where VSMCs were labelled by a tamoxifen-inducible *Myh11*-driven Cre recombinase prior to inducing advanced atherosclerosis by a high-fat diet promoted multiple characteristics of unstable plaque (Gomez, Baylis, Durgin, & Newman, 2018), such as a 40% decrease in contractile VSMC content, a 30% decrease in collagen content, and a 50% increase in M2 ($Arg1^+$) macrophage content within the fibrous cap (Gomez et al., 2018). This study also used VSMC-specific genetic deletion of *Il1r1* combined with VSMC lineage tracing before feeding mice a high-fat diet to demonstrate that IL-1β signalling is required for VSMC investment into lesions and the fibrous cap (Gomez et al., 2018).

Like IL-1β, the role of many other cytokines in VSMC behaviour remains unclear (Lim & Park, 2014). For example, Singh and Zheng (2014) report dual regulation of myocardin expression by TNF-α in VSMCs. The authors show that, in cultured VSMCs, TNF-α activates the NF-κB signalling pathway and decreases the expression of myocardin and myocardin-dependent genes by direct binding of NF-κB (p65) to the *MYOCD* promoter (Singh & Zheng, 2014). In

contrast, TNF-α treatment of cultured VSMCs overexpressing myocardin significantly potentiates the expression of myocardin and CArG box-containing contractile VSMC genes by stabilising myocardin mRNA via the NF-κB and **MAPK** pathway (Singh & Zheng, 2014). These findings suggests that the effect of TNF-α on myocardin activity depends on the VSMC phenotypic state. Therefore, blocking VSMC exposure to inflammatory stimuli or inhibiting the VSMC response to such stimuli may promote plaque stability or rupture in a context-dependent manner.

## 10 | EPIGENETIC TARGETS

As discussed, much is known regarding the signals that regulate VSMCs within atherosclerotic plaque. However, it is becoming increasingly clear that it is also important to understand the molecular mechanisms underlying VSMC behaviour at the epigenetic level. Recent studies have demonstrated that histone modifications, DNA methylation, and microRNAs all influence how VSMCs contribute to plaque (Alexander & Owens, 2012).

Interestingly, VSMCs within atherosclerotic lesions exhibit perturbed levels of histone methylation, including H3K27me3 (Greißel et al., 2015; Wierda et al., 2015) and H3K9me2/3 (Chen et al., 2017; Greißel et al., 2015). Additionally, VSMCs from diabetic patients display reduced levels of H3K9me2 compared with non-diabetic controls, which suggests that dysregulation of H3K9me2 might underlie the vascular complications associated with diabetes (Chen et al., 2017; Villeneuve et al., 2008). Several studies also demonstrate that DNA methylation regulates VSMC behaviour in atherosclerotic plaque. Hiltunen et al. (2002) were the first to report DNA hypomethylation in advanced human atherosclerotic lesions. Azechi, Sato, Sudo, and Wachi (2014) observed that inhibiting DNA methyltransferases with **5-aza-2′-deoxycytidine** potentiates inorganic phosphate-induced mineralisation in human aortic VSMCs, possibly through demethylation of the alkaline phosphatase promoter. However, it is important to consider local as well as global levels of histone and DNA modifications. For example, the DNA demethylase TET2 positively regulates the expression of *SRF* and contractile genes, including *MYOCD* and *MYH11*, in human VSMCs (Liu et al., 2013). Furthermore, TET2 expression levels are inversely correlated with severity of atherosclerosis in patients, and knock-down of TET2 in mouse exacerbates vascular response to injury (Liu et al., 2013). Targeting the enzymes that regulate these de novo changes could be a promising strategy to inhibit the expression of inflammation-responsive VSMC genes associated with plaque instability.

There is a growing body of evidence demonstrating that microRNAs regulate VSMC phenotype (Lu, Thavarajah, Gu, Cai, & Xu, 2018). For example, cultured VSMCs have been shown to switch to a macrophage-like state after cholesterol loading by down-regulating the microRNA (miR)-143/145–myocardin axis (Vengrenyuk et al., 2015), a key pathway that is essential for maintaining the contractile VSMC state. Maintaining the expression of myocardin or miR-143/145 prevented and reversed these phenotypic changes induced by cholesterol loading (Vengrenyuk et al., 2015). Manipulation of miRNA activity in vulnerable plaque could therefore be considered as a potential therapeutic strategy. As unstable plaque typically displays thin fibrous caps with a high ratio of macrophage-like to VSMC-like cells (Kolodgie et al., 2004), addition of miR-143/145 mimics to block VSMC transdifferentiation to a macrophage-like state could perhaps be used to stabilise plaque. Multiple miRNA-based agents have now moved into the clinic to treat a range of diseases (Rupaimoole & Slack, 2017), suggesting that this might be a feasible strategy.

## 11 | CONCLUSIONS

In recent years, our understanding of VSMCS within atherosclerotic plaque has changed dramatically. Historically, VSMCs were thought to be entirely beneficial by forming the fibrous cap, protecting the plaque from rupture. However, numerous genetic lineage mapping studies have definitively shown a wide-ranging plasticity of VSMCs and suggested that these cells can modulate their behaviour in response to a variety of stimuli. Consequently, we now know that VSMCs have complex roles within atherosclerotic lesions and may act to both promote plaque stability and rupture depending on the context. To develop efficient therapeutic strategies to limit cardiovascular risk, additional knowledge about how specific VSMC-derived cell types function in mature plaque is therefore needed. Additionally, mechanistic insight into the regulation of VSMC plasticity is required to enable specific interventions. Additionally, many murine studies focus on prevention of atherosclerosis in young healthy animals rather than regression or treatment of established plaque. As discussed by Baylis et al. (2017), there is therefore a need for implementing preclinical murine models of regression or treatment of established plaque that better mimic therapeutic intervention in humans to study VSMC function.

### 11.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

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### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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