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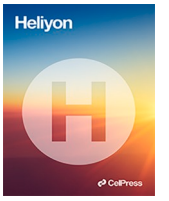

Review article

Vascular smooth muscle cell phenotypic switching in atherosclerosis

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ABSTRACT

Atherosclerosis (AS) is a complex pathology process involving intricate interactions among various cells and biological processes. Vascular smooth muscle cells (VSMCs) are the predominant cell type in normal arteries, and under atherosclerotic stimuli, VSMCs respond to altered blood flow and microenvironment changes by downregulating contractile markers and switching their phenotype. This review overviews the diverse phenotypes of VSMCs, including the canonical contractile VSMCs, synthetic VSMCs, and phenotypes resembling macrophages, foam cells, myofibroblasts, osteoblasts/chondrocytes, and mesenchymal stem cells. We summarize their presumed protective and pro-atherosclerotic roles in AS development. Additionally, we underscore the molecular mechanisms and regulatory pathways governing VSMC phenotypic switching, encompassing transcriptional regulation, biochemical factors, plaque microenvironment, epigenetics, miRNAs, and the cytoskeleton, emphasizing their significance in AS development. Finally, we outline probable future research directions targeting VSMCs, offering insights into potential therapeutic strategies for AS management.

1. Introduction

Atherosclerosis (AS), stands as the leading cause of cardiovascular diseases, evolves through a continuum of intricate histologic changes within the arterial wall, centered on inflammation and lipid metabolism dysfunction [1]. Under pro-atherosclerosis circumstances, the endothelial cells (ECs) were injured and activated, which set into motion a multitude of pathological process including increased permeability and the secretion of adhesion molecules, inflammatory cytokines, and chemokines. Consequently, this cascade facilitates the infiltration of leukocytes and lipoproteins into the vessel wall. Subendothelial oxidation of low-density lipoprotein (LDL) to oxidized LDL (ox-LDL) further activates ECs, promoting leukocyte recruitment. In addition, the monocytes transform to macrophages, produce inflammatory cytokines, take up lipoproteins, and become "cholesterol-engorged" foam cells, and their apoptosis contributes to a necrotic core. Moreover, the lipoproteins resident in the arterial wall and cytokines stimulate proliferation and recruitment of the vascular smooth muscle cells (VSMCs) from the media to intima, where they synthetic extracellular matrix (ECM) and form the fibrous cap, thus generating the atherosclerotic lesion.

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Since proposed in 1960s that VSMC-derived cells are the predominant cell type within atherosclerotic plaques, VSMC has been a major area of interest [2,3]. In health, VSMCs take responsible for maintaining vasodilation and contraction, and supporting the normal physiological function of the vessels. In disease, VSMCs play a vital role in the progression of AS, contributing to both plaque stability and plaque healing. VSMCs retain extensive phenotypic plasticity such that it undergoes structural and functional transition in response to environmental stimuli. During AS, VSMCs proliferate, migrate into intimal layer of the arterial wall, and undergo dedifferentiation to other phenotypes. Lineage tracing studies have confirmed that a large fraction of cells present in the atherosclerotic lesion are derived from medial differentiated VSMCs. These VSMCs-derived cells are characterized by the loss of VSMC-specific contractile markers and upregulation of markers associated with other cell types. Recent studies have further demonstrated a multi-potent effect of these dedifferentiated VSMCs in either stabilizing or destabilizing the evolving atherosclerotic lesions [4,5].

Given the multiple pivotal roles of VSMCs throughout AS, targeting the VSMCs may presents a novel breakthrough. While controversy lingers over the role of multiple phenotypic states of VSMC, and the precise regulatory mechanism in VSMC phenotypic switching. In this review, we will trace the progress of VSMC phenotypic switching in different pathological states of AS, focusing on the cellular and molecular mechanisms that underlie the pivotal roles of VSMCs in the formation and progression of the atherosclerotic lesion; explore its relationship to clinical risk factors of CAD; consider approaches to the detection of atherosclerotic lesion progression; and outline some promising new treatment modalities for AS.

2. Plasticity of VSMCs

Conventional protein staining methods inadequately capture the dynamic variations of VSMCs in AS pathology. Fate mapping systems and single-cell RNA sequencing (scRNA-seq) techniques offer more precise tracking of VSMC fate. VSMCs exhibit remarkable phenotypic plasticity in AS. A significant proportion of VSMC-derived cells within atherosclerotic lesions undergo a phenotypic transition, characterized by the loss of VSMC-specific contractile marker genes. These cells adopt phenotypes resembling various other cell lineages, including macrophages, foam cells, osteoblasts/chondrocytes, myofibroblasts, and mesenchymal cells (Table 1 and Fig. 1).

2.1. Differentiated VSMCs

Under physiological conditions, medial VSMCs displayed limited proliferative, migratory, and synthetic activities, primarily manifesting a contractile phenotype essential for regulating vasomotor tone in blood vessels. Referred to as differentiated, quiescent, or contractile VSMCs, these cells express specific proteins essential for contractility, including smooth muscle α-actin (SMA-α or ACTA2), smooth muscle myosin heavy chain (SM-MHC or MYH11), smooth muscle protein 22α (SM22α or TAGLN), and calponin. Collectively, these proteins contribute to vascular stability and tone maintenance.

2.2. Dedifferentiated VSMCs

2.2.1. Synthetic VSMCs

Normally quiescent, differentiated VSMCs become activated in response to environmental cues such as hemodynamic changes, inflammation, hyperlipidemia, oxidized phospholipids, and platelet-derived growth factor (PDGF), transitioning from a contractile to a dedifferentiated, synthetic phenotype [6–8]. This phenotypic modulation of VSMCs is commonly observed during vascular injury

Table 1
Characteristics of VSMC phenotypes.

Phenotype	Morphological feature	Function	Marker gene
Contractile VSMC	Spindle-like morphology	Regulate vasomotor tone	SMA-α, MYH11, SM22α
Synthetic VSMC	Reduced myofilament density, increased secretory organelles, sparse myofilaments with a rhombic shape	Enhanced ability of proliferation and migration, capable of further phenotypic transformation	Detailed in phenotypes as follows
Macrophage-like VSMC	Extended pseudopodia, phagocytic vesicles, phagocytosed debris within cytoplasm	Uptake, storage, and efflux of lipid, secretion of pro-inflammatory mediators and MMPs, further transformation to foam cell-like VSMCs	CD68, LGALS3
Foam cell-like VSMC	Enlarged size with intracellular lipid droplets	Uptake, storage, and efflux of lipid, secretion of pro-inflammatory mediators	CD68, LGALS3
Myofibroblast-like VSMC	Elongated pseudopodia and cell shrinkage	Collagen and ECM synthesis, fibrous cap formation	FN1, LUM, DCN, BGN, FSP-1
Osteoblast/chondrocyte-like VSMC	Containing calcifying vesicles	Promoting calcification	OPN, RUNX2, SOX9
Mesenchymal-like VSMC	Altered cytoskeleton with reduced F-actin	Further differentiation into multiple cell types	LGALS3, LY6A, SCA1

Abbreviations: VSMC, vascular smooth muscle cell; ECM, extracellular matrix; MMP, matrix metalloproteinases.

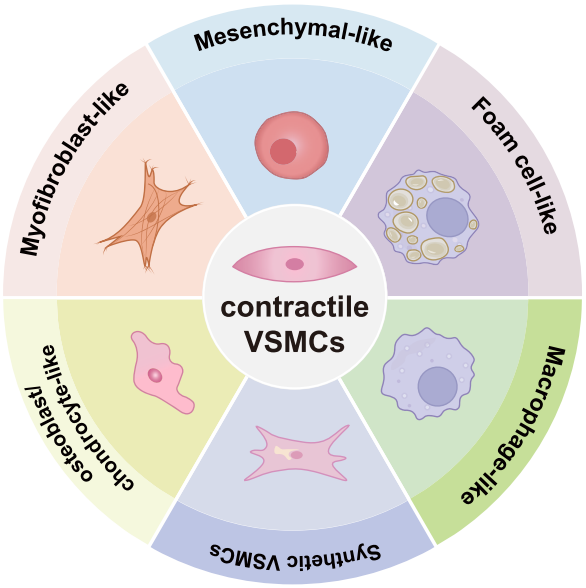

Fig. 1. Overview of the phenotype of VSMCs within media and atherosclerotic lesions.
Under physiological conditions, medial VSMCs primarily exhibit a differentiated, quiescent, contractile phenotype, which is essential for regulating vasomotor tone in blood vessels. When exposed to atherosclerotic environment, these contractile VSMCs become activated, exhibiting significant variability in terms of the degree of dedifferentiation and the phenotypic changes induced by exposure to diverse environmental signals.
VSMC, vascular smooth muscle cell.

repair, with the atherosclerotic environment further promoting this process. Importantly, these dedifferentiated VSMCs retain the potential to revert back to a contractile phenotype.

Synthetic VSMCs are characterized by reduced myofilament density and increased secretory organelles, notably the rough endoplasmic reticulum and Golgi complexes, with sparse myofilaments [9]. Functionally, these synthetic VSMCs exhibit a downregulation of canonical VSMC contractile proteins, an upregulation of synthetic proteins, heightened expression of ECM-remodeling enzymes, and increased synthesis of ECM components [10]. Moreover, they demonstrate enhanced proliferation and migration capacities. These cells migrate from the media to the neointima, actively participating in AS plaque formation. Lineage tracing studies have identified approximately 30 % of cells within atherosclerotic lesions as VSMC-derived [11].

Notably, dedifferentiated VSMCs within atherosclerotic plaques exhibit significant variability, both in terms of the degree of dedifferentiation and the phenotypic changes induced by exposure to diverse environmental signals. These dedifferentiated, synthetic VSMCs secrete chemokines and cytokines, constituting an inflammatory plaque microenvironment which further activate and promote the VSMC phenotypic transition.

2.2.2. Macrophage-like VSMCs

Within the intricate context of AS progression, VSMCs exhibit remarkable plasticity, as evidenced by their expression of macrophage markers such as CD68 and LGALS3/MAC2, predominantly in response to cholesterol accumulation. Intriguingly, research reveals a dual expression of CD68 and SMA-α within plaques, highlighting the extent of VSMC plasticity in pathological conditions [12]. The macrophage-like phenotype is prevalent in mature, late-stage atherosclerotic plaques. This phenotype robustly induces inflammatory responses and releases matrix metalloproteinases (MMPs), significantly contributing to plaque instability. In-vivo studies have indicated the presence of non-myeloid-origin $MAC2^{+}$ cells, constituting about 30 % of the total cell population within the plaque, indicating a derivation beyond traditional monocyte lineage [13]. Concurrently, in-vitro findings of coronary arteries from CAD patients revealed that approximately 40 % of $CD68^{+}$ cells are VSMC-derived, further corroborating the in vivo observations [14].

However, it is essential to recognize that these VSMC-derived macrophage-like cells, despite their macrophage marker expression, do not attain the full spectrum of macrophage functionality. Notably, they exhibit deficient phagocytosis and efferocytosis capacities, and are predominantly located close to the necrotic core [15]. This functional divergence from classical macrophages is significant, shedding light on the complexity and heterogeneity of cell functions within atherosclerotic lesions.

2.2.3. Foam cell-like VSMCs

Foam cells are pivotal constituents of atherosclerotic plaque. While traditionally considered predominantly of macrophage origin, recent findings have unveiled the substantial contribution of VSMCs to over half of the foam cells within plaques [14]. These VSMC-derived foam cells exhibit characteristics of both VSMCs and macrophages, as evidenced by the expression of specific markers [12]. For instance, CD68, extensively used to characterize macrophages, has been shown to be expressed by VSMCs upon cholesterol loading [16], suggesting that some foam cells may indeed originate from VSMCs that expressing macrophage markers.

Functionally, foam-cell like VSMCs acquire phagocytic properties and upregulate pro-inflammatory cytokines [17]. These cells also exhibit enhanced expression of MMPs [18] and impaired collagen and fibronectin assembly [19], thereby contributing to vulnerable plaques.

Ox-LDL has been shown to promote the formation of foam cell in both murine [20] and human [21] arterial VSMCs. It binds with apolipoprotein (Apo) B to create new antigenic epitopes that evade recognition by typical LDL receptors. VSMCs express increased level of ox-LDL receptors such as scavenger receptors and Toll-like receptors, facilitating the internalization of these modified lipoproteins and their conversion into foam cell-like phenotypes within atherosclerotic lesions [22–24].

Additionally, VSMC take up lipoproteins via phagocytosis [25] or micropinocytosis [26], independent of scavenger receptors, subsequently leading to the formation of foam cell-like VSMCs. Modified forms of LDL, such as enzymatic nonoxidizing modifications of LDL (eLDL) generated by exposure to cholesterol esterase and trypsin, have also been shown to induce VSMC-derived foam cell formation [26]. In vitro studies have demonstrated that VSMCs assimilate aggregated low-density lipoprotein (agg-LDL) by upregulating LDL receptor-related protein-1 (LRP1) [27], promoting foam cell formation characterized by cholesteryl ester lipid droplets accumulation. Remarkably, the formation of foam cell-like VSMCs via LRP-1 and agg-LDL treatment appears to occur independently of the conventional endocytic processing pathway, suggesting that exhibit distinct mechanisms for lipoprotein uptake and storage between VSMCs and macrophages, potentially contributing to divergent pathways of foam cell formation.

In addition, ox-LDL may trigger an immune response, leading to the production of autoantibodies and immune complexes containing LDL [28], thereby increasing cholesterol accumulation in human aortic intimal VSMCs [29] and inducing the phenotypic transition to foam cell-like VSMCs [30].

The VSMC-derived foam cell formation is also attributed to their reduced ability to efflux excess cholesterol via the ATP-binding cassette transporter A1 (ABCA1), a key component of the reverse cholesterol transport pathway [31]. Initial exposure to lipid induces overexpression of ABCA1 in VSMCs, but prolonged cholesterol loading leads to decreased ABCA1 levels in intimal VSMCs, promoting their transformation into foam cell-like phenotypes compared to medial VSMCs [32,33]. Moreover, mechanical forces and chemical modifications, including oxidation, enzymatic and nonenzymatic reactions, promote lipoprotein aggregation and fusion [34], crucial for generating foam cell-like VSMCs.

2.2.4. *Myofibroblast-like VSMCs*

Fibroblasts, exist in the interstitial tissue of mammalian organs, play a crucial role in ECM synthesis, organization, and remodeling. Following vessel injury, fibroblast undergo activation and transition to myofibroblast, characterized by their contractile properties and expression of ACTA2 in cytoskeleton. Generally, myofibroblasts are regarded as an intermediate state between fibroblasts and VSMCs. The phenotype of myofibroblast-like VSMCs, also referred to as 'fibromyocytes' or 'fibroblasts', reflects their origin from VSMCs and their adoption of a myofibroblast-like phenotype [35], manifested by co-expression of VSMC and fibroblast marker gene [36]. These myofibroblast-like VSMCs exhibit elongated pseudopodia and cell shrinkage compared to contractile VSMCs [37].

The transition to myofibroblast-like VSMCs maintains the expression of ACTA2, displays a decrease in other contractile VSMC gene expression, and initiates the expression of fibroblast markers such as fibronectin 1 (FN1), osteoprotegerin/Tnfrsf11b, lumican (LUM), decorin (DCN), biglycan (BGN), platelet-derived growth factor beta receptor (PDGFβR), and fibroblast-specific protein (FSP)-1 [37]. Additionally, other matrix components such as collagen 1a1 (COL 1α1), COL 1a2, and COL 3a1, and the macrophage marker LGALS3 were also reported [4].

Most studies suggest an AS protective role of myofibroblast-like VSMCs, as this cluster of cells is capable of encoding genes regarding extracellular matrix organization and collagen deposition. Notably, these myofibroblast-like VSMCs contribute to the thick, protective fibrous cap and prevent plaque rupture. Approximately 70 % of $ACTA2^{+}$ fibroblast-like cells are derived from VSMCs [38]. Whereas some studies regard this phenotype as detrimental. ECM-producing fibroblast-like VSMCs contributes to fibrous cap, while they are related to vulnerable plaques in women, serving as a scaffold for calcification [39]. This might be realized by further shifting towards a continuous, linear trajectory to osteoblast-like cells. Hence fibroblast-like VSMCs might not represent a most dedifferentiated status of VSMCs.

2.2.5. *Osteoblast/chondrocyte-like VSMCs*

Osteochondrogenesis markedly affects the stability of atherosclerotic plaques, with certain patterns of coronary calcification linked to myocardial infarction [40]. Notably, superficial microcalcifications within fibrous caps are associated with increased inflammation and believed to augment local stress, thereby elevating the risk of plaque rupture and predicting subsequent cardiovascular events [41]. Conversely, macrocalcifications, which frequently accumulate in the deep intima or the necrotic core, are thought to enhance plaque stability [42].

Osteoblast/chondrocyte-like VSMCs in plaques are major contributors to calcification. VSMCs adopting an osteoblast/chondrocyte phenotype progressively lost the expression of contractile VSMC markers and upregulate osteoblastic and chondrocytic differentiation markers including osteopontin (OPN), sex-determining region Y-related high-mobility group box 9 (SOX9), and Runt-related transcription factor 2 (RUNX2) [5]. Genetic fate mapping studies have proved that most cells with osteoblastic or chondrocytic properties in AS plaques are of VSMC lineage [43]. Osteoblast/Chondrocyte-like VSMCs display typical calcifying vesicles and develop a matrix prone to calcification [44]. These cells are predominantly located in the fibrous cap or areas of cartilaginous metaplasia and calcification with AS plaques [45], as identified by electron microscopy around the necrotic cores in human aortic and carotid plaques [46]. Speer et al. [47] identified osteoblast/chondrocyte-like VSMCs in mouse vessels prior to calcium deposition by means of SM22α-Cre recombinase and Rosa26-LacZ alleles, supporting the viewpoint that VSMC transdifferentiated into an osteoblast/chondrocyte-like phenotype drives vascular calcification.

2.2.6. Mesenchymal-like VSMCs

VSMCs also converts to mesenchymal-like cells during AS, characterized by their functional relevance and capacity to differentiate into multiple cell types. These mesenchymal-like cells resemble mesenchymal cells and are characterized by the loss of canonical

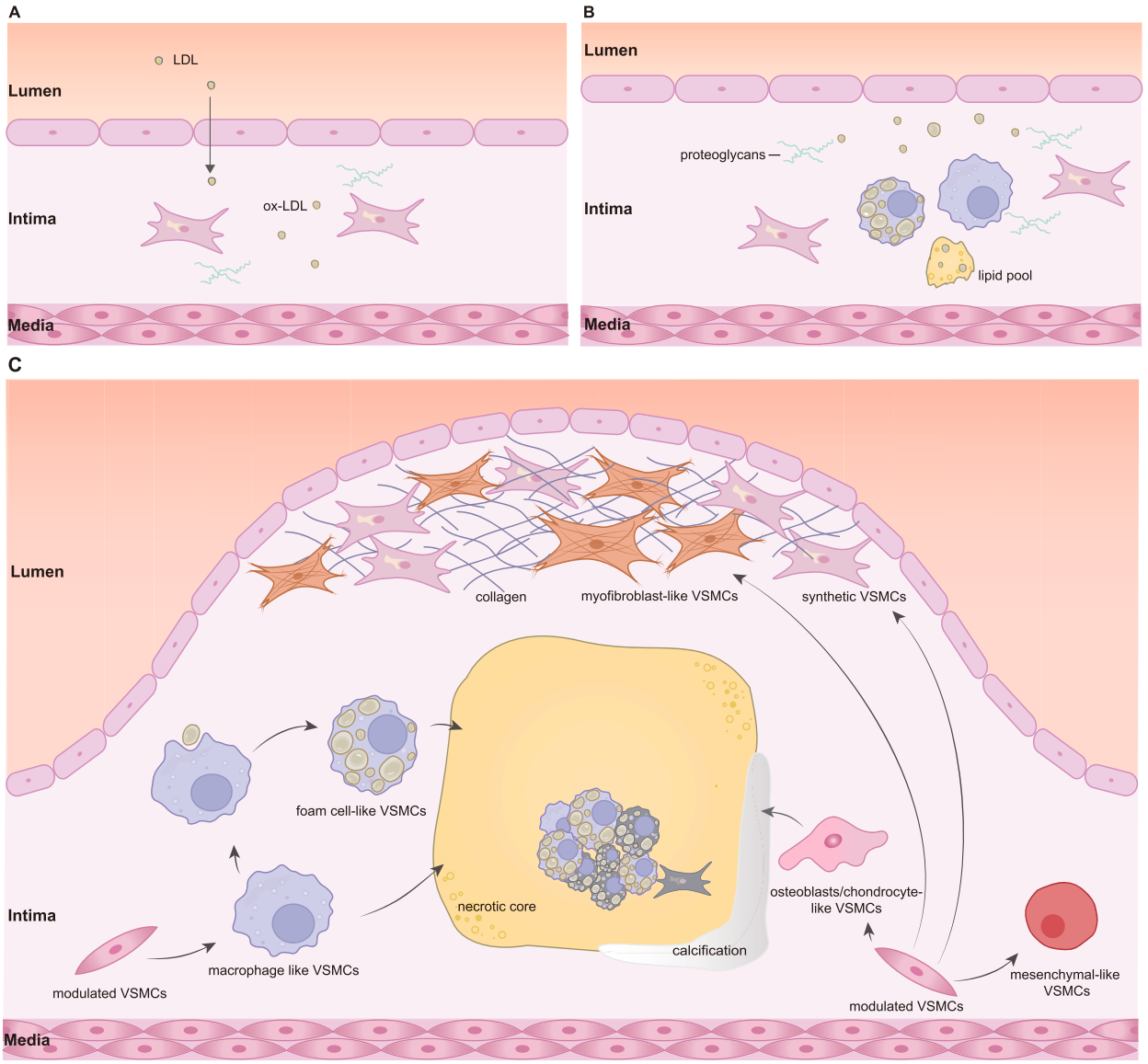

Fig. 2. The role of VSMCs in different stages of AS.

A VSMCs in pre-atherosclerosis.

During pre-atherosclerosis, VSMCs are the predominant cell type within the plaque. They drive intima thickening through their synthesis of extracellular matrix (ECM), which is predominantly composed of proteoglycans and elastin. Low-density lipoprotein (LDL) from the circulation is retained in the intima through interaction between LDL apolipoproteins and VSMC-derived proteoglycans, subsequently undergoing oxidation to form oxidized LDL (ox-LDL). VSMCs exhibit enhanced proliferation and migration capabilities during this phase due to phenotypic modulation.

B VSMCs in PIT.

During pathological intimal thickening (PIT), plasma lipids accumulate abundantly to form lipid pools, which are initially observed beneath the foam cells. VSMCs upregulate macrophage markers CD68, acquire phagocytic ability, and present scavenger receptors, which enable them to influx ox-LDL and transform into VSMC-derived foam cells.

C VSMCs in late AS.

During fibroatheroma and calcification stages, VSMCs differentiate into various phenotypes within the plaque, including synthetic VSMCs, macrophage-like, foam cell-like, mesenchymal-like, osteoblast/chondrocyte-like, and myofibroblast-like VSMCs. Synthetic VSMCs and myofibroblast-like VSMCs synthesize ECM and form the fibrous cap, which increase the stability of atherosclerotic plaques. Osteoblast/chondrocyte-like VSMCs contribute to calcifying microvesicles, potentially leading to large calcification granules and calcium nodules. Mesenchymal-like VSMCs are pluripotent and capable of transdifferentiating into other cell types.

contractile markers and an increase in the mesenchymal-stem cell marker Ly6a/Sca1 [4,5].

Recent studies utilizing lineage tracing and scRNA-seq have identified transitional mesenchymal-like VSMCs. Dobnikar et al. [48] performed scRNA-seq on healthy mouse aortic media and reveal 7 distinct VSMC clusters, one of which expressed the multipotent progenitor marker Stem Cell Antigen 1 (Sca1). These mesenchymal-like cells progressively increase genes associated with wound healing, migration, and activation of growth factor signaling, while decreasing canonical contractile VSMC markers. Enhanced Sca1 represents a hallmark of phenotypic transition, and VSMC-derived $Sca1^{+}$ cells have been identified in atherosclerotic plaques. Similarly, utilizing VSMC fate mapping and single-cell genomics to interrogate the trajectories of VSMC transdifferentiation during AS, Pan et al. [4] identified a cluster of unique, intermediate VSMC-lineage cells in intima. These cells are multipotent and capable of differentiate into macrophage-like and fibroblast-like cells or reverse back towards contractile VSMC. They express Ly6a, Vcam1 and Ly6c1, which are considered markers of stem cell, endothelial cell, and monocytes/macrophage, respectively. Moreover, study by Gabriel F. Alencar et al. [5] provides evidence that the activation of Lgals3, Ly6a/Sca1 along with Vcam1 in VSMCs marks a transitional state, with these cells serving as precursors for other VSMC-derived cell types. While 60 to 80 percentage of VSMC-derived cells in the advanced lesion had expressed Lgals3, only 25 % maintain Lgals3 expression over time.

3. VSMCs in different stages of AS plaques

The progression of atherosclerotic plaques is a dynamic process, marked by distinct stages reflecting changes in their composition and volume. VSMCs play a crucial role as the primary source of plaque cells and ECM throughout all stages of atherosclerosis, contributing significantly to various processes involved in the disease progression (Fig. 2).

3.1. Pre-atherosclerosis

The journey into AS begins with diffuse intimal thickening (DIT), characterized by VSMCs and ECM primarily proteoglycans and elastin accumulate in the intima without lipid deposits (Fig. 2A). DIT, typically emerging spontaneously from birth, sets the stage for initial lesion development and is prevalent in human arteries susceptible to AS. DIT evolves over years on pre-existing intimal VSMCs [49]. As the process progresses, circulating macrophages infiltrate the deep intima, phagocytizing lipids and transforming into foam cells, leading to the formation of xanthomas or fatty streaks [50]. DIT and fatty steak are categorized as nonatherosclerotic intimal lesions. In some pathologic situations, such as disturbed blood flow, they evolve further into early AS phase by persistent retention of LDL and foam cells in pre-existing intimal proteoglycans [51].

VSMCs in DIT drive the thickening of the artery through their synthesis of ECM, which is predominantly composed of proteoglycans and elastin. The structure of DIT is characterized by two distinct layers: a proteoglycan-rich inner layer, contains VSMCs and ECM composed primarily of proteoglycans; and the musculoelastic outer layer, denses with high concentration of VSMCs and elastin but fewer proteoglycans [52].

VSMCs in DIT are believed to undergo phenotypic modulation, characterized by increased synthetic organelles compared to VSMCs in the media [53]. Moreover, these cells exhibit enhanced proliferation and migration capabilities, upregulating genes responsible for synthesis and migration [54]. A notable example is the overexpression of $\alpha_v\beta_3$ integrin by VSMCs in DIT, facilitating their migration from the media to the intima [55].

The gene expression profile of VSMCs in arteries prone to AS is distinct from those in atherosclerotic-resistant arteries before AS. Assche et al. [56] utilized whole-genome mouse microarrays and real-time quantitative polymerase chain reaction (RT-PCR) to examine the gene expression of VSMCs in lesion-free atherosclerotic-prone and atherosclerotic-resistant arteries of $ApoE^{-/-}$mice. They identified several gene associated with cell proliferation, differentiation, and inflammation in AS that displayed altered expression in the atherosclerotic-prone arteries.

3.2. Pathological intimal thickening

Considered the earliest stage of AS, pathological intimal thickening (PIT) emerges when lipid pools are initially observed beneath the foam cells layers without altering the normal intima structure [57]. This phase is typified by lipid pools, macrophage-foam cells, and VSMCs residue in the ECM, accompanied by extracellular lipid accumulation and oxidation (Fig. 2B).

It is noteworthy that DIT is a unique feature observed solely in human atherosclerotic plaques, which accounts for the paucity of information regarding the underlying molecular mechanisms contributing to the pathogenesis of DIT. In mammal animals like mice and rabbits, VSMCs are recruited and migrate from the media to intima during PIT, which could be induced by a western-type diet. Furthermore, proteoglycans have been shown to play a role in the initiation of AS by promoting lipid retention according to the 'response to retention' hypothesis, which elicits macrophages migration and leads to PIT formation [51]. This process is possibly achieved by linking the plasma-derived lipoproteins to collagen fibers [58]. The interaction between the protein component of lipoproteins, particularly ApoB, and the sulfate groups present on the proteoglycan sugars is considered the most probable retention mechanism. These sulfate groups carry a negative charge, while the protein component of lipoproteins is positively charged, leading to their interaction [59,60]. Interesting, the quantity of VSMCs remains constant throughout the transition from DIT to PIT [61].

3.3. Fibroatheromas

The hallmark of late, advanced AS lesion is the formation of fibroatheromas, characterized by a fibrous cap and necrotic core.

Infiltration of macrophages into the lipid pool, coupled with apoptosis and necrosis of VSMCs and foam cells, as well as diminished efferocytotic activity of macrophages, significantly contributes to the formation of the necrotic core [62–64] (Fig. 2C).

During early and late fibroatheromas, VSMCs produce ECM as the major structural component of the fibrous cap. The fibrous cap, primarily composed of VSMC and ECM, wraps around the necrotic core, serving to harbor the contents of the necrotic core and thus shielding it from exposure to the arterial lumen.

The atherosclerotic plaque could be stratified into two categories in terms of its stability: stable plaques, distinguished by a thick, ECM-rich fibrous cap, and vulnerable plaques characterized by the presence of a large necrotic core and an overlying thin fibrous cap, known as the thin-cap fibroatheroma [65]. It is believed that the thickness and component of the fibrous cap might influence the stability of plaque. With the progression of the AS, the overlying fibrous cap is prone to thinning and rupture due to multiple factors including MMPs, inflammation, and VSMC apoptosis [66]. As VSMCs are the primary producers of collagen in the fibrous cap, the content of collagen and VSMCs within the cap determines the plaque stability.

The composition and content of ECM in fibroatheromas remain dynamic. In healthy or mature arteries, VSMC remain contractile and adhere to basement membrane (BM), which prevents their phenotypic modulation [67]. However, with the development of AS and VSMC phenotypic modulation, the ECM undergoes constant changes. The content of fibronectin largely increased during fatty streak, and Stermran et al. [68] had proposed fibronectin as an indicator of early AS. With AS progression, the synthesis of BM including laminin, fibronectin and heparan sulphates gradually decreases, while the synthesis and distribution of interstitial membrane, mainly including type I and III collagen, increase [69]. A mature AS plaque contains few fibronectin but a large amount of collagen [70].

Several studies have elucidated the clonal characteristics and origins of VSMCs in the fibroatheromas. VSMCs in plaque are oligoclonal that only a small number of VSMCs proliferate and contribute to the plaque formation [71,72]. In particular, VSMCs within fibrous cap are $ACTA2^{+}$, and similarly deriving from a limited number of medial VSMCs, which give rise to almost all VSMC-derived cells in both the cap and core [71]. Based on the genetic recombination Cre-loxP technique, Misra et al. [73] conducted a $SMMHC\text{-}CreER^{T2}$, $ROSA26R^{(mTmG/+)}$ $ApoE^{-/-}$ mouse model to describe the trajectory of VSMC-derived cells in AS. They demonstrated that with high fat feeding, $ACTA2^{+}$ VSMC-derived cells initially infiltrate the plaque at the shoulder of fibrous cap after a high-fat diet at 5.5 weeks. By week 6, these cells cover the majority of the cap and express PDGFR-β, the marker of myofibroblast. By week 8, the core of the plaque presents VSMC-derived cells with progressively downregulated VSMC markers. This process continues until week 12 when the expression of VSMC markers is scarcely apparent. Similarly, Worssam et al. [74] observed a subpopulation of VSMCs exhibit enhanced proliferative ability and present clonal expansion at early stage of AS. However, their study holds opposite view on the trajectory of VSMC invasion that the medial VSMCs proliferate and contribute to the atherosclerotic plaque before invading the fibrous cap.

VSMCs with enhanced proliferative capacity give rise to various phenotypes. In view of that $SCA1^{+}$ VSMCs serve as "precursors" in the media that give rise to a large number of VSMC-derived cells in the atherosclerotic plaque, and they are prone to respond to pro-atherosclerotic stimulus, we have to agree with the concept recently proposed by Pan et al. that AS act as a tumor-like disease [75]. A key area for further exploration will be identifying how VSMC fate is determined. Moreover, the selective extension of VSMCs from the media to intima depends on differences among each VSMC clones in migration speed and the distance with the migrating channel (i.e. fenestrations in the internal elastic lamina) [76]. This may underlie why only a small number of medial VSMC clones contribute to atherosclerotic plaques.

3.4. Calcification

The AS plaque may undergo calcification as they mature, which is likely driven by factors including specific osteogenic cells, inflammation, high phosphate, and calcifying vesicles [77–79]. VSMCs also play a significant role in the calcification process, primarily through the mechanisms involving apoptosis and osteochondrogenic transformation (Fig. 2C). Apoptotic macrophages and VSMCs in lesions release considerable amounts of minerals that form calcium crystal granules, initiating microcalcification. These microcalcifications amplify and coalesce into macrocalcifications, which contribute to plaque instability by intensifying stress within the ECM of the fibrous cap [80]. Additionally, microcalcifications initiate inflammatory responses that further expedite the calcification process. This cascade eventually culminates in the formation of calcified nodules, which coexist alongside atherosclerotic plaques, marking a critical step in the progression of AS.

Over decades, these developments in AS plaques narrow artery lumina and obstruct blood flow, causing life-threatening thrombosis primarily through plaque rupture or erosion, and eventually trigger clinical events. Therefore, we could make a conclusion that, the proliferation, migration, senescence, apoptosis of VSMC, as well as the synthesis and degradation of ECM, are crucial in determining the stability of lesions and subsequent clinical events. Intimal thickening and the formation of fibrous cap are the net overall result of VSMC proliferation, migration, senescence, and death.

4. Mechanisms of phenotypic modulation

The phenotypic transformation of VSMCs during AS is driven by a complex interplay of molecular and environmental factors that collectively contribute to plaque formation. Understanding these mechanisms is essential for elucidating the role of VSMCs in the pathogenesis of AS and identifying potential therapeutic targets. Recent studies have highlighted several key mechanisms governing VSMC phenotypic modulation, including transcriptional regulation, biochemical influences, microenvironmental cues, epigenetic modifications, and the role of microRNAs (Fig. 3).

4.1. Transcriptional regulation

The phenotypic transformation of VSMCs during AS is driven by a complex interplay of molecular and environmental factors that collectively contribute to plaque formation. Understanding these mechanisms is essential for elucidating the role of VSMCs in the pathogenesis of AS and identifying potential therapeutic targets. Recent studies have highlighted several key mechanisms governing VSMC phenotypic modulation, including transcriptional regulation, biochemical influences, microenvironmental cues, epigenetic modifications, and the role of microRNAs (Fig. 3).

4.1.1. MYOCD-SRF-CArG box

The VSMC-specific myocardin (MYOCD)/serum response factor (SRF)/CArG complex plays a crucial role in maintaining the contractile phenotype of VSMCs. Disruption of this complex downregulates the genes coding for contractile proteins, which triggers VSMC dedifferentiation.

SRF is a DNA-binding transcription factor that governs the gene expression relevant to muscle movement and growth, while MYOCD acts as its co-activator [81,82]. SRF functions through binding to a DNA motif known as CArG box, which is commonly located in the promoter regions of downstream target genes. This subsequently leads to the transcriptional activation of various downstream genes including Sm22α, Acta2, and Myh11. MYOCD has been identified as a crucial regulator of VSMC phenotype, as it impedes VSMC

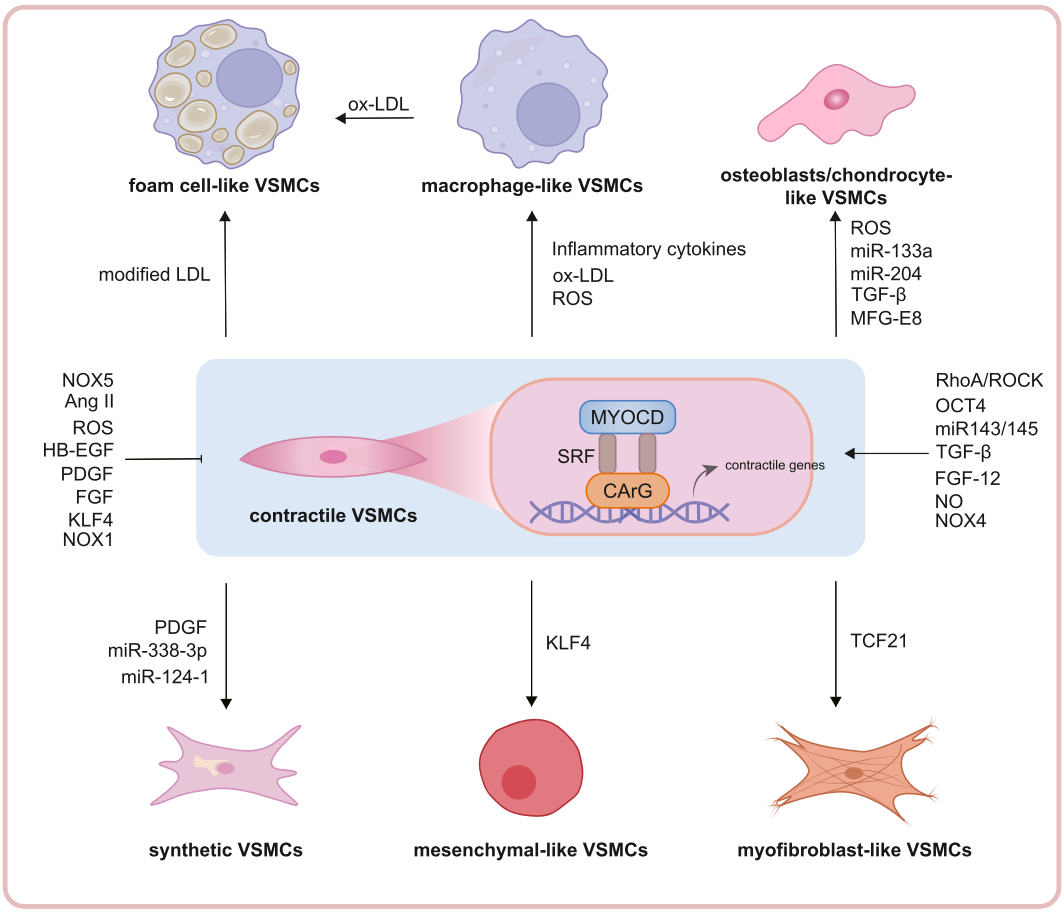

Fig. 3. Mechanisms of VSMC phenotypic transition in atherosclerosis.
The MYOCD-SRF-CArG box plays a crucial role in maintaining the contractile phenotype of VSMCs. MYOCD acts as a cofactor of SRF, which binds to the CArG-box element within the promoter of VSMC marker genes to promote the expression of VSMC contractile gene. Various transcription factors (e.g. KLF4, OCT4) function through the MYOCD-SRF-CArG box to regulate the expression of VSMC contractile genes. Biochemical factors such as LDL, growth factors (e.g. PDGF, FGF and HB-EGF), inflammatory factors and cytokines, Ang II and oxidative stress are involved in VSMC proliferation, migration, and differentiation. MicroRNAs also participate in the regulation of VSMC phenotypic transition.
Ang II, angiotensin II; FGF, fibroblast growth factor; HB-EGF, heparin-binding epidermal growth-factor-like growth factor; KLF4, krüppel-like factor 4; LDL, low-density lipoprotein; MFG-E8, milk fat globule-epidermal growth factor 8; MYOCD, myocardin; NOX, nicotinamide adenine dinucleotide phosphate oxidases; OCT, octamer binding transcription factor 4; ox-LDL, oxidized LDL; PDGF, platelet-derived growth factor; ROCK, Rho-associated kinase; ROS, reactive oxygen species; SRF, serum response factor; TCF21, transcription factor 21; TGF-β, transforming growth factor-β; VSMC, vascular smooth muscle cell.

proliferation and migration [82]. Additionally, MYOCD serves as a marker of contractile gene expression in VSMCs [83]. VSMCs from human atherosclerotic arteries have decreased level of MYOCD and α-SMA [84], and it has also been proved in animal model that MYOCD deficient mice exhibit decreased expression of VSMC-specific contractile proteins and ultrastructural features [85].

MYOCD functions through both SRF-dependent and -independent manners. For example, MYOCD plays a crucial role in regulating transforming grown factor (TGF)-β1 signaling by direct interaction with Smad3, facilitating its binding to the Smad-binding element (SBE) region [86]. This results in the activation of promoters of contractile protein such as SM22α. Additionally, MYOCD competes with ternary complex factors (TCFs) such as ELK-1 for SRF-binding sites in the promoters of SM22α, thereby suppressing myogenic gene activation and promoting a transition to a dedifferentiated phenotype [87].

4.1.2. KLF4

Krüppel-like factors 4 (KLF4) is a crucial transcription factor responsible for the plasticity of VSMC. KLF4 regulating the contractile VSMC transition to multiple phenotypes including osteoblast-like cells [5], macrophage-like [11], and likely to be detrimental for plaque destabilization [5]. VSMC-specific knockout of KLF4 in ApoE$^{-/-}$ mice results in reduced lesion size along with thicker VSMC-enriched ACTA2^{+} fibrous cap, indicative of enhanced plaque stability [11].

KLF4 is overexpressed in dedifferentiated VSMCs, while its level remains quite low in quiescent, contractile VSMCs the level of KLF4 [88]. Most evidence supports that KLF4 promotes the VSMC dedifferentiation. Lineage tracing conducted by Shankman et al. [11] demonstrated that conditional deletion of KLF4 in VSMCs did not completely prevent VSMC phenotypic switching but significantly restricted the atherosclerotic lesions size and enhanced fibrous cap stability. Interestingly, the overall number of VSMCs remained consistent by the deletion of KLF4. However, a notable decrease was observed in the population of VSMC-derived macrophage-like and mesenchymal-like cells. Their findings highlight a novel and critical role for KLF4 in regulating the transition of VSMCs into detrimental macrophage-like cells, shedding light on the intricate interplay between VSMCs and macrophages within the atherosclerotic context [11].

Mechanistically, KLF4 inhibits VSMC differentiation and represses the expression of VSMC marker genes by binding to specific G/C repressive elements within the promoter regions of these contractile genes, thereby effectively suppressing their transcription [89]. Additionally, KLF4 competes with SRF for binding, thus preventing SRF from interacting with CArG elements [90]. KLF4 also disrupts the cooperative interaction of paired CArG elements by either binding to their paired elements or blocking their interaction with SRF, leading to further inhibition of gene expression [91].

4.1.3. OCT4

Octamer binding transcription factor 4 (OCT4), the stem cell pluripotency factor, plays a protective role in AS by preserving the contractile phenotype of VSMC. In contrast to KLF4, conditional knockout of OCT4 in VSMC leads to increased atherosclerotic lesion size, thinner fibrous cap, and larger necrotic core [92].

4.1.4. TCF21

Identified as a pivotal gene at the CAD-associated locus, the basic helix–loop–helix transcription factor TCF21 assumes a significant role in the orchestration of VSMC phenotypic regulation. Occasionally, TCF21 expression is downregulated in VSMCs from healthy arteries. However, an intriguing phenomenon unfolds in atherosclerotic lesions, where Tcf21+ cells accumulate and contribute to the formation of a protective fibrous cap or subcapsular structure by transforming into contractile VSMCs [93].

In cultured human coronary artery smooth muscle cells (HCASMCs), TCF21 knock-down leads to the upregulation of canonical VSMC lineage markers and inhibition of apoptosis. Whereas the over-expression of Tcf21 in HCASMCs induces proliferation [93]. A recent scRNA-Seq in VSMCs from human coronary vessels and ApoE$^{-/-}$ mice conducted by Robert C. Wirka et al. [35] further elucidated the impact of TCF21. VSMC-specific TCF21 knock-out markedly inhibited VSMC phenotypic modulation towards a fibroblast-like phenotype, resulting in fewer fibroblast-like VSMCs in the lesion and fibrous cap. The overexpression of Tcf21, conversely, upregulated the fibroblast-like VSMC markers in vitro [35]. Mechanistically, TCF21 directly binds to MYOCD, blocking the association of MYOCD and SRF, thereby suppressing VSMC canonical markers [94].

4.2. Biochemical factors

4.2.1. Growth factors

Growth factors play crucial roles in regulating VSMC proliferation, migration, and gene expression thereby playing critical roles in AS progression. Growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and Heparin-binding epidermal growth-factor-like growth factor (HB-EGF) are involved in VSMC proliferation, migration from media to intima, and the rapid downregulation of multiple VSMC differentiation marker genes [95,96].

The effects of PDGF on VSMCs are contingent upon the extent of PDGF receptor (PDGFR) exposure at the VSMC surface. The level of PDGFR mRNA significantly increased in VSMCs within the AS lesions, and the blockade of PDGFR lead to the reduced AS lesion size and VSMC counts [97].

PDGF-BB represses contractile genes expression through various mechanisms, mainly including increasing the KLF4 expression [98], repressing the expression of MYOCD directly, dissociation of MYOCD from the CArG-containing region of VSMC marker genes promoters [99], and by phosphorylating ELK1 which subsequently competes with MYOCD for binding with SRF [100]. Similarly, PDGF induces the expression of Olfactomedin 2, which prompts RUNX2 to bind SRF competitively with MYOCD, leading to the decreased interaction between SRF and MYOCD and inhibition of VSMC marker gene transcription [101]. Moreover, PDGF-BB induces

the expression of dedicator of cytokinesis 2 (DOCK2) [102] and surfactant protein A (SPA) [103], downregulates VSMC marker genes via repressing MYOCD.

TGF-β serves as a potent regulator of VSMC phenotype and function. Markedly reduced plasma levels of active TGF-β1 have been observed in patients with advanced AS compared to healthy controls [104]. TGF-β drives the expression of VSMC contractile genes. Activation of TGF-β in ApoE$^{-/-}$ mice leads to decreased inflammatory cell infiltration, increased VSMC collagen secretion and αSMA expression, and enhanced stability of the atherosclerotic lesions [105,106].

The underlying mechanisms through which TGF-β preserves the VSMC contractile phenotype are intricate. TGF-β suppress KLF4 to maintain the contractile VSMC phenotype [107]. TGF-β upregulates the expression of transcription factor Smad2/3, which binds to SBEs in the promoters of VSMC-specific gene, thereby contributing to the activation of VSMC contractile gene [108,109]. Additionally, TGF-β induces the expression of SRF and facilitates its binding to CArG element within the promoters of VSMC marker genes [110]. Moreover, TGF-β stimulates ECM production, transactivates the gene expression of collagen and proteoglycan, and play a part in the inhibition of pro-inflammatory cytokines induced by MMPs [111].

However, conflicting evidence also suggests a role for TGF-β in AS pathogenesis. For example, patients with triple vessel disease exhibit almost twice the level of active TGF-β1 in the plasma compared with those with mild CAD and non-CAD [112]. Similarly, immunostaining studies of human AS plaques indicate activation of TGF-β signaling pathway in more than 50 % of VSMCs in early lesions [113]. Interestingly, TGF-β has been shown to variably stimulate or inhibit VSMC proliferation and migration [114]. In balloon-injured rat carotid arteries, overexpression of Smad3, the principal intracellular mediators of TGF-β signaling, markedly accelerated intimal thickening after 14 days. This was linked to the activated p-ERK/MAPK signaling in both whole arteries and isolated VSMCs [115]. Activation of TGF-β signaling is also demonstrated to drive the osteogenic transdifferentiation.

Additionally, Other growth factors such as fibroblast growth factor 12 (FGF12) contribute to maintaining a contractile phenotype of VSMCs via upregulating MYOCD and SRF [116], leading to upregulation of VSMC contractile genes and inhibition of VSMC proliferation and migration.

In summary, understanding the interplay of these growth factors provides valuable insights into the complex regulation of VSMC phenotype. A comprehensive comprehension of these regulatory networks will undoubtedly pave the way for novel therapeutic interventions.

4.2.2. Inflammatory factors and cytokines

Inflammation is a hallmark of AS. The activated, dedifferentiated VSMCs secrete cytokines such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), IL-1β, tumor necrosis factor-α (TNF-α), as well as chemotactic factors to recruit inflammatory cells to the arteries. These factors contribute to vascular inflammation and oxidative stress, influencing VSMCs phenotype and function, as well as vascular remodeling in cardiovascular diseases.

Inflammatory factors can induce VSMC phenotypic transition to macrophages-like cells. For instance, IL-1β treatment resulted in a profound increase of lipids accumulation in VSMCs, as evidenced by the amplification of the Myh11^{+} cells expressing LipidTOX, an indicator of lipids cell accumulation and subsequently foam cells formation [117]. The dedifferentiated inflammatory VSMCs further aggravate local inflammation, which creates a vicious cycle.

Inflammatory signaling pathways, including nuclear factor kappa B (NF-κB) signaling [118], Toll-like receptor signaling [119,120], TNF-α signaling [121], mitogen-activated protein kinase (MAPK) signaling [122], signal transducer and activator of transcription 3 (STAT3) signaling [123], promote VSMC inflammation, migration, proliferation, dedifferentiation, and AS progression. Moreover, inflammatory stimuli inhibit collagen synthesis and promote the production of MMPs by VSMCs. Anti-inflammatory therapies counteract these effects by increasing VSMC coverage, enhancing collagen content, and reducing MMPs and pro-inflammatory cytokines [124]. This demonstrates the potential of anti-inflammatory therapies in mitigating the atherosclerotic pathological changes by targeting the inflammatory-induced VSMC phenotype.

4.2.3. Redox signaling

The function of VSMCs is regulated by reactive oxygen species (ROS) and redox dependent signaling. Under normal physiological conditions, antioxidant enzymes effectively quench ROS, maintaining a balance between antioxidant and pro-oxidants following oxidative stress. However, in the context of AS, the upregulation of pro-oxidants and the downregulation of antioxidants results in an imbalance, leading to heightened ROS level [125], and disturbed cellular redox balance in turn mediated the pathogenesis of AS [126].

Superoxide anion and hydrogen peroxide are major sources of ROS in VSMCs, with nicotinamide adenine dinucleotide phosphate oxidases (NOX) being the most widely studied enzymatic source of superoxide anion in the vessel wall. VSMCs express multiple NOX isoforms mainly including NOX1, NOX4, and NOX5. Within the NOX family, NOX1 induces the VSMC phenotypic transformation, as evidenced by the reduced proliferation, migration, and fibronectin secretion in NOX1-deficient VSMCs [127]. Similarly, NOX5 promotes VSMC phenotypic transformation and knockdown of NOX5 upregulates contractile VSMC markers and downregulates vascular calcification [128]. Conversely, NOX4 is essential for maintaining a differentiated, contractile phenotype of VSMCs. During the transformation to a synthetic phenotype, VSMC progressively downregulates NOX4. VSMC-specific knockdown of NOX4 results in decreased levels of VSMC contractile markers [129]. Notably, NOX4 has also been demonstrated to act an atheroprotective role. Reduced Nox4 mRNA expression has been observed in both human and mouse atherosclerotic plaques, and Nox4$^{-/-}$ mice exhibit enhanced level of proinflammatory cytokines [130,131].

Oxidative stress significantly influences the abnormal proliferation and migration of VSMCs. Mechanistically, ROS affect several key molecular targets involved in VSMC migration, such as proteins linked to ECM breakdown, focal adhesion complexes assembly, and cytoskeleton dynamics [132,133]. ROS can directly or indirectly activate MMP2 and MMP9 in VSMCs, and induce VSMC

dedifferentiation in response to inflammatory cues through the activation of key transcriptional factor NF-κB [134]. Additionally, ROS promotes the VSMC switch to an osteoblast-like phenotype in a defined osteogenic medium via AKT-activated RUNX2 [135].

4.2.4. Angiotensin II

Angiotensin II (Ang II) profoundly influences VSMCs, upregulating genes associated with proliferation, migration, collagen synthesis, and pro-inflammatory processes [136–139].

The atherogenic stimuli induce expression of Ang II. Ang II exerts pro-atherosclerotic effects on VSMCs via type 1 angiotensin II receptor (AT1R), a G protein-coupled receptor [140]. The expression of AT1R in VSMC is modulated by pro-inflammatory cytokines and other factors. For instance, rabbits fed a high cholesterol diet exhibited a five-fold increase in AT1R expression in both the medial and intimal VSMC [141]. Similarly, immunohistochemical co-localization in humans have determined expression of both Ang II and AT1R in atherosclerotic plaque from patients with unstable angina [142].

In Mechanism, Ang II induces cellular damage in VSMCs by producing ROS and exerting paracrine effects. Additionally, Ang II activates MAPK activity through the modulation of MAPK phosphatases, which leads to pathological responses in VSMCs [143]. Furthermore, Ang II promotes VSMC migration while concurrently reducing levels of MMPs such as MMP2 and MMP9, which are responsible for degrading the surrounding ECM and facilitates VSMC migration [144]. Collectively, this intricate interplay highlights the multifaceted impact of Ang II on VSMC behavior, and its significant role in the development and progression of AS.

4.3. Plaque microenvironmental cues and cellular crosstalk

4.3.1. ECM

The ECM is constituted predominantly composed of matricellular proteins alongside classical proteins, including collagen, proteoglycan, glycoprotein, and elastin. ECM in arteries has distinct functions upon the phenotype of VSMC depending on their type or cellular origin.

For instance, the proteoglycan DCN mitigates VSMC migration, proliferation, and cytokine expression induced by PDGF and interferon-γ,thus preserving a contractile phenotype [145]. BGN, a leucine-rich repeat proteoglycan synthesized by various cells such as endothelial cells, fibroblasts, and VSMCs, enhances the structural integrity of the artery wall under physiological conditions [146]. However, BGN is implicated in disease progression, particularly in trapping and retaining lipoproteins, leading to macrophage infiltration and accumulation [52]. Consequently, elevated BGN content in arteries is associated with an augmented risk of AS and lipid retention [147]. Furthermore, BGN exerts a pro-atherosclerotic effect on VSMCs by promoting VSMC proliferation and migration both in vivo and vitro [148]. Hyaluronan, a linear unbranched glycosaminoglycan synthesized by VSMCs and other cells during AS, is thought to contribute to AS by promoting VSMC proliferation and migration of VSMCs [149]. Fibronectin (FN), which deposits in areas predisposed to AS prior to the formation of atherosclerotic lesions, fosters a pro-atherogenic microenvironment in early AS [150]. FN induces proliferate and migratory effect on VSMCs, associated with the formation of a protective fibrous cap [151]. Milk fat globule-epidermal growth factor 8 (MFG-E8), a glycoprotein expressed in VSMCs, drives the osteogenic transdifferentiation of VSMCs through promoting β1 integrin-dependent MMP2 expression and subsequent TGF-β1 activation [152].

Additionally, some ECM exert an influence on maintaining the contractile VSMC phenotype. Nidogen-2, a glycoprotein in BM, maintains the contractile phenotype of VSMCs through directly binding to Jagged1 and activating Notch3 signaling [153]. The normal BM proteins, such as collagen type IV and laminin, retain VSMCs in a contractile state [154].

Moreover, structural alterations in the artery, such as the inverted ratio of elastin to collagen, the nonenzymatic glycosylation of collagen, contribute to the pathogenesis of AS by increasing mechanical artery rigidity and stiffness [155]. These changes, characterized by a decrease in elastin content and an increase in collagen cross-linking, lead to arterial remodeling and diminished arterial compliance, which are known predisposing factors for AS development. The resulting alterations in arterial biomechanics not only exacerbate hemodynamic stress on the vessel wall but also promote endothelial dysfunction and VSMC phenotypic switching, ultimately facilitating AS progression.

Overall, ECM remodeling significantly impacts VSMC de-differentiation by promoting adhesion, decreasing migratory resistance, and encouraging VSMC proliferation. Likewise, dedifferentiated VSMCs may modify the pericellular matrix through production and secretion as a feedback effect. The intricate interplay between ECM components and VSMCs underscores the multifaceted nature of AS pathogenesis. Targeting ECM-VSMC interactions may offer novel therapeutic strategies for mitigating AS progression [156].

4.3.2. EC-VSMC interaction

Reports have underscored the direct interplay between ECs and VSMCs in atherogenesis. The erythropoietin-producing hepatoma receptor (Eph) family, activated through binding to ephrin ligands, is integral to the contact-dependent interactions between ECs and VSMCs [157]. Both Eph receptors and ephrin ligands have been identified within atherosclerotic plaques. Notably, $EphA_2$ activation has been linked to enhanced EC inflammatory responses. In vivo studies reveal elevated $EphA_2$ and $ephrinA_1$ in murine and human atherosclerotic plaques [158]. Knockdown of $EphA_2$ has been shown to mitigate AS progression, with increased expression of EphA2 observed in plaque-associated dedifferentiated VSMCs compared to contractile VSMCs [159]. Remarkably, $EphA_2$ knockdown impedes VSMC-mediated deposition and remodeling of ECM [159]. On the other hand, Notch signaling is essential for getting a $ACTA2^{+}$, quiescent fibrous cap VSMC phenotype and impeding AS lesion development [160]. ECs inhibit the transition of VSMCs to a synthetic phenotype by binding to Notch3 receptors on VSMCs via the Notch ligand Jagged1 [161].

Indirect interactions between EC and VSMC also significantly contribute to the AS progression. Under physiological conditions, ECs release endothelium-derived relaxing factors (EDRFs) such as nitric oxide (NO), which not only maintain the contractile phenotype of

VSMC but also facilitates vasorelaxation [162]. Endothelial dysfunction significantly diminishes NO production and bioavailability, thereby escalating the activity of MMPs, further inducing VSMC dedifferentiation [163].

Moreover, in the AS context, EC activation leads to an upsurge in inflammatory cytokines, growth factors, and adhesion molecules. These elements orchestrate the migration of VSMCs into the intimal layer and trigger their phenotypic transformation, marking a pivotal step in AS development. Hergenreider et al. [164] also provided compelling evidence of a paracrine mechanism by which ECs influence VSMC phenotype. The overexpression of KLF2 in ECs leads to the upregulation of the miR-143/145 cluster, which are then transferred to VSMCs via extracellular vesicles and lead to the repression of KLF4 and a contractile VSMC phenotype.

4.3.3. VSMC-macrophage interaction

The macrophages within stable AS plaques that clear apoptotic cells produce anti-inflammatory molecules such as IL-10, and specialized pro-resolving lipid mediators (SPMs) [165]. In turn, these pro-resolving molecules binds to receptors on the surface of VSMCs, which drive the binding of MYOCD/SRF to CArG boxes and consequently upregulating contractile VSMC genes [166].

In advanced AS, there exists an imbalance between SPMs and proinflammatory factors. Within vulnerable plaques, macrophages exhibit impaired efferocytosis, leading to the release of proinflammatory cytokines and chemokines [167]. These molecules, upon binding to their cognate receptors in VSMCs, induce the downregulation of MYOCD expression, thus driving the phenotypic transition [168]. For example, macrophages isolated from stroke patients exhibit elevated expression of chemokine ligand 5 (CCL5). Notably, the interaction between CCL5 and its cognate receptor, chemokine receptor 5 (CCR5), on VSMCs stimulates VSMC proliferation, dedifferentiation, and vascular remodeling [169,170]. This underscores the significance of cell-to-cell communication within atherosclerotic plaques.

4.4. Epigenetics

Epigenetic mechanisms play a crucial role in the regulation of the contractile gene in VSMC. Various histone modifications remodel chromatin conformation, thereby modulating the accessibility of transcription factors to genes and regulating gene expression in a specific manner, thus influencing VSMC phenotypic plasticity.

Histone acetylation and histone deacetylation are pivotal processes in gene expression regulation, dynamically maintaining chromatin structure balance. These processes are tightly regulated by the coordinated activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), and have been demonstrated to be crucial in VSMC differentiation [171]. Histone acetylation promotes the expression of VSMC contractile gene and inhibits phenotypic switching. For instance, although KLF4 act as a potent transcriptional repressor of VSMC differentiation markers, acetylation of KLF4 induced by HDAC2 enhances its binding to the SM22α promoter, leading to increased SM22α expression in VSMCs [172]. Notably, during phenotypic transition of VSMCs, the HAT p300 is repressed and downregulated [173]. In a study by Manabe et al. [174], a novel P19-derived clonal cell line called A404 effectively differentiation into VSMCs upon retinoic acid treatment. Notably, following differentiation, these cells exhibit significantly increased expression of VSMC markers such as SM-MHC and SMA-α, without significant alteration in SRF expression levels or its binding activity to CArG elements. Chromatin immunoprecipitation analyses revealed increased SRF binding to CArG elements of endogenous VSMC differentiation marker genes located within intact chromatin, regulated by histone H3 and H4 hyperacetylation. These findings underscore the importance of chromatin acetylation in facilitating SRF binding and subsequent activation of endogenous VSMC differentiation marker genes.

Conversely, accumulating evidence have suggested that deacetylation inhibits VSMC differentiation, suppressing contractile gene activation and promoting phenotypic modulation [175,176]. A genome-wide association study indicated that HDAC9 prevented the RUNX2 expression in VSMCs under calcifying conditions, inhibiting phenotype switching towards osteoblast-like cells [177].

On the contrary, emerging evidence have suggested that histone methylation plays a distinct role in VSMC differentiation. Specifically, the DNA demethylase ten eleven transformation 2 (TET2) has been identified as a key regulator promoting VSMC differentiation and inhibiting phenotypic switching by converting DNA methylation into hydroxy methylation on VSMC contractile genes. Notably, decreased TET2 was observed in human atherosclerotic plaques [178], and TET2 knockdown in VSMCs led to reduced chromatin accessibility and VSMC contractile markers expression, further supporting the crucial role of TET2-mediated histone methylation in VSMC differentiation [178]. Additionally, H3 histone di-methylation at lysine position 4 (H3K4me2) serves as a marker of differentiated VSMCs and has been proposed as an epigenetic signature of VSMCs [179]. H3K4me2 is enriched at the promoter of contractile genes during VSMC phenotypic modulation and associated with increased binding of MYOCD. Liu et al. [180] constructed a loss-of-function study on H3K4me2 to assess its role in VSMC phenotypic switching. Inducing H3K4me2 demethylation of MYOCD-dependent VSMC contractile gene in vitro resulted in significantly, irreversibly decreased expression of contractile genes, heightened lipid uptake capacities, and overexpression of phagocytosis markers. They further proved that H3K4me2 functions through the recruitment of TET2 on the VSMC contractile gene. Demethylation of H3K4me2 reduced its interaction with TET2, leading to highly DNA methylation of VSMC differentiation genes.

4.5. MicroRNAs

MicroRNAs constitute a class of small non-coding RNA molecules pivotal in regulating target gene expression. They operate by binding to specific target mRNAs, thereby inducing their degradation or repressing protein translation. Their capacity to post-transcriptionally modulate gene expression renders them important regulators in diverse biological and pathological processes. Moreover, they have been demonstrated to serve as versatile regulators in VSMC phenotypic modulation.

The miR-143/145 cluster emerges as a pivotal regulator of VSMC lineage differentiation during embryonic development. MiR-145 directly targets TGFβRII, inhibiting TGF-β-dependent synthesis of ECM and inducing VSMC differentiation [181]. Notably, the downregulation of miR-145 and miR-143 has been observed in VSMCs derived from atherosclerotic arteries. MiR-145 and miR-143 collaboratively target transcription factors, including KLF4 and MYOCD, synergistically facilitating VSMC differentiation while suppressing proliferation [182]. Interestingly, Hergenreider et al. [164] also demonstrated a paracrine signaling mechanism underlying this regulation. They revealed that the overexpression of KLF2 in ECs lead to robust upregulation of miR-143/145 cluster. Notably, these miRNAs are packaged into extracellular vesicles that released by ECs and subsequently transported to VSMCs. This transfer of miR-143/145 from ECs to VSMCs results in the downregulation of KLF4 in VSMCs. By repressing KLF4 expression and other dedifferentiation-related genes, miR-143/145 induces an atheroprotective VSMC phenotype and mitigating atherosclerotic lesion formation. Their study underscores the cell-dependent effects of KLF family members on VSMC phenotype switch and highlights the complex regulatory network involving miRNAs and transcription factors like KLF4 in VSMC phenotypic transformation.

Additionally, several other miRNAs have been implicated in the VSMC phenotypic modulation. For example, miR-133a [183], miR-204 [184] and miR-34b/c [185] inhibit VSMC phenotypic modulation to osteoblast-like cells and protect against calcification. MiR-338-3p overexpress under AS circumstance and promotes the phenotypic switching into synthetic VSMCs [186], whereas miR-124 protect against this process [187]. Overall, these findings highlight the intricate regulatory role of miRNAs in VSMC biology and underscore their potential as therapeutic targets for the management of AS.

4.6. Cytoskeleton

The cytoskeleton, composed of actin microfilaments (F-actin), microtubules, and intermediate filaments, provides the structural integrity and plasticity of VSMCs. It transports mechanotransduction from external forces, activating downstream signaling pathways to modulate VSMC phenotypes [188].

VSMCs perceive mechanical stimuli and the mechanical properties of ECM, translating these into biochemical signals that induce phenotype changes. For instance, the arterial elastic modulus increases dramatically during AS, which promoting the dedifferentiation of VSMCs to a pro-inflammatory phenotype [189]. The transmission of mechanical stress applied to VSMC relies on focal adhesions that link integrins to the actin cytoskeleton. These mechanical loads promote the assembly of the cytoskeleton into actin stress fibers and focal adhesions [190], driving various signaling cascades including the nuclear translocation of MYOCD-associated transcription factors megakaryoblastic acute leukemia (MKL), which induce the expression of VSMC contractile genes [191]. Additionally, the unpolymerized globular form of F-actin (G-actin) negatively regulates the SRF co-activator MKL1, promoting the sequestration of MKL1 away from the nucleus and leading to the reduced transcription of VSMC contractile markers [192].

Small GTPases are key modulators of actin cytoskeletal dynamics in VSMCs. The Rho family of small GTPases, including RhoA, Rac1, and Cdc42, orchestrates VSMC migration and phenotypic transition through their effects on the actin cytoskeleton [193]. RhoA and its effector kinase Rho-associated kinase (ROCK) are the integral regulator of VSMC contraction. They collectively enhance VSMC contractility [194], promote proliferation and migration [195], and induce dedifferentiation [196]. Overactivation of Rho/ROCK signaling by Ang II leads to actin polymerization, subsequent downregulation of VSMC contractile genes, and VSMC migration [196]. Conversely, inhibition of RhoA and ROCK effectively suppress VSMC proliferation and migration [197]. However, RhoA also stabilizes actin filaments, controls actomyosin contractility [198], and promotes the nuclear translocation of MKL and the MKL-dependent transcription of contractile genes, which leading to the contractile phenotype of VSMCs [199].

Rac1 has been indicated to promote actin polymerization, and specifically, Rac1 facilitates lamellipodia formation and increases cytoskeletal dynamics to promote membrane remodeling [200,201]. Rac1 is essential for Rho-regulated actin stress fiber and focal adhesion complex formation [202]. Interestingly, Rac1 and RhoA play antagonistic roles in regulating cytoskeleton dynamics and VSMC phenotype. The negative control of Rac1 activity by RhoA has been broadly described [203,204]. In vivo study also proved that VSMC-specific $Rac1^{-/-}$ mice displayed overexpression of RhoA/ROCK signaling [205]. Additionally, the study of Shefali Talwar et al. [206] integrated mathematical modeling with experimentation and revealed that under the stimuli of ECM stiffness, Rho contributed to a contractile VSMC phenotype, whereas Rac was associated with both differentiated and dedifferentiated states. The homeostasis between Rac and Rho ultimately determined the VSMC phenotypic outcome.

The influence of these small GTPases extends to their downstream effectors, such as YAP and TAZ. These mechanosensitive transcriptional coregulators significantly affect VSMC proliferation and differentiation [207,208]. The absence of YAP/TAZ in VSMCs lead to an osteogenic phenotype [209] and activation of the transcription factor SOX9 [210]. Interestingly, the study of Talwar et al. [206] demonstrated that Rac increased the relative abundance of nuclear YAP while decreased it for TAZ. They also pointed out that TAZ inhibited VSMC proliferation, whereas YAP promoted both contractile and proliferative phenotypes of VSMCs. This differential regulation highlights the complex role of YAP and TAZ in VSMC phenotypic modulation, indicating that the balance between these transcriptional regulators, influenced by Rac and Rho GTPases, is crucial for determining the phenotypic outcome of VSMCs.

5. Conclusion and future perspective

Studies to date have provided substantial insights into the VSMC plasticity and phenotypic switching and its roles in AS. Nevertheless, there are still restrictions in accurately identifying the origin and fate of VSMCs in the context of AS.

Firstly, VSMC-targeting Cre lines driven by the Myh11, Tagln, and Acta2 promoters not only express in VSMCs but also confer expression in non-muscular cells, exhibiting complex expression patterns. For instance, approximately 20 % of $ACTA2^{+}$ cells within the fibrous cap are of EC- and not VSMC-origin [211–213]. ScRNA-seq analyses and VSMC-lineage tracing studies have demonstrated that,

a quarter of the ACTA2^{+} fibrous cap cells originate from a non-VSMC source [38]. These indicating a tapestry of cell types undergoes phenotypic transition to an myofibroblast-like state, which accounts for ACTA2^{+} cells in both human and mouse atherosclerotic lesions.

Secondly, the linear trajectory of phenotypic transition has not been clearly revealed. Apart from the terminal contractile VSMCs, some dedifferentiated VSMCs also had a certain level of contractile activity [214]. Hence there might be intermediate phenotypes, and the intermediate phenotypes between contractile and dedifferentiated VSMCs suggest the existence of overlaps between different phenotypes. For example, Lgals3^{+} VSMCs are generally regarded as macrophage-like VSMCs. However, several research have demonstrated these cells in an intermediate state [4,5,215], which give clue that there are overlaps between the different phenotypes. Moreover, in the study of Talwar et al. [206], a unique 'null state' was identified, which is characterized by low expression of SMA-α, low proliferative rate, and relatively small cell areas. This phenotype appeared when cultured on a soft ECM and could transform to either a proliferative or contractile phenotype under ECM stiffness. These findings raise important questions for us: Do VSMCs undergo phenotypic transformation in a predetermined route, and to which extent do they gain the function of other cell types? Further research into these questions is required.

In the future, strategies aimed at limiting matrix proteolysis, enhancing ECM deposition in the fibrous cap, and tuning VSMC phenotype hold significant potential for effectively managing AS. However, they are accompanied by notable challenges that require careful consideration, emphasizing the need for interdisciplinary collaborations, advanced technologies, and extensive research efforts.

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No data was used for the research described in the article.

CRediT authorship contribution statement

Yanqiao Yu: Writing – original draft. **Yajie Cai:** Visualization. **Furong Yang:** Visualization. **Yankai Yang:** Visualization. **Zhuorui Cui:** Visualization. **Dazhuo Shi:** Funding acquisition. **Ruina Bai:** Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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