BRIEF REVIEW

Six Shades of Vascular Smooth Muscle Cells Illuminated by KLF4 (Krüppel-Like Factor 4)

Carmen Yap, Arnout Mieremet, Carlie J.M. de Vries, Dimitra Micha, Vivian de Waard

ABSTRACT: Multiple layers of vascular smooth muscle cells (vSMCs) are present in blood vessels forming the media of the vessel wall. vSMCs provide a vessel wall structure, enabling it to contract and relax, thus modulating blood flow. They also play a crucial role in the development of vascular diseases, such as atherosclerosis and aortic aneurysm formation. vSMCs display a remarkable high degree of plasticity. At present, the number of different vSMC phenotypes has only partially been characterized. By mapping vSMC phenotypes in detail and identifying triggers for phenotype switching, the relevance of the different phenotypes in vascular disease may be identified. Up until recently, vSMCs were classified as either contractile or dedifferentiated (ie, synthetic). However, single-cell RNA sequencing studies revealed such dedifferentiated arterial vSMCs to be highly diverse. Currently, no consensus exist about the number of vSMC phenotypes. Therefore, we reviewed the data from relevant single-cell RNA sequencing studies, and classified a total of 6 vSMC phenotypes. The central dedifferentiated vSMC type that we classified is the mesenchymal-like phenotype. Mesenchymal-like vSMCs subsequently seem to differentiate into fibroblast-like, macrophage-like, osteogenic-like, and adipocyte-like vSMCs, which contribute differentially to vascular disease. This phenotype switching between vSMCs requires the transcription factor KLF4 (Krüppel-like factor 4). Here, we performed an integrated analysis of the data about the recently identified vSMC phenotypes, their associated gene expression profiles, and previous vSMC knowledge to better understand the role of vSMC phenotype transitions in vascular pathology.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis • cardiovascular disease • myocytes • phenotype • smooth muscle

Cardiovascular diseases are the leading cause of death worldwide. Many of these pathologies are characterized by progressive cellular modulations derived from genetic predisposition, aging, or lifestyle. The 3 main cellular layers forming the vessel wall are the adventitia, media, and the intima, surrounding the lumen. In the media, the middle layer, vascular smooth muscle cells (vSMCs) are the major cellular component. These vSMCs contribute to the integrity of the vessels and are able to adequately respond to stimuli of vasoconstriction and vasodilation. In healthy vessels, vSMCs are regarded as quiescent, differentiated cells, which display remarkable plasticity. The contractile vSMCs are subject to context-dependent changes and studies have shown that a subset of vSMCs in healthy tissue express reduced levels of the contractile vSMC markers, capable of phenotypic switching. There is a growing body of evidence that multiple vSMC phenotypes exist in healthy vessels. Single-cell RNA sequencing (scRNA-seq) studies identified the presence of specific vSMC-derived cell populations. However, there is still limited understanding of the role of these vSMC phenotypes in physiological and pathological conditions.

For decades, vSMC activation and dedifferentiation has been regarded as the adoption of a single synthetic, proliferative phenotype. However, as revealed by recent scRNA-seq analyses, the diversity of vSMC phenotypes is far more sophisticated. The power of scRNA-seq is that it provides detailed, unbiased information on distinct cell populations within healthy or...
diseased tissue. Distinct cell populations were identified by scRNA-seq in the vessel wall, with the majority concerning immune cells, such as T cells, B cells, myeloid cells, and mast cells. Nonimmune cells in atherosclerotic plaques and aortic aneurysm tissue included endothelial cells and vSMCs. The combination of scRNA-seq and lineage tracing is extremely useful as it allows in-depth vSMC phenotypic characterization. With the development of lineage tracing in mouse models, the fate of vSMCs can be accurately tracked and provides detailed information of their phenotypic modulation and contribution to diseased tissue. In this review, we highlighted the information obtained about vSMCs and used the recent scRNA-seq literature to classify 6 different vSMC phenotypes. Besides a contractile phenotype, we distinguish the mesenchymal-like, fibroblast-like, macrophage-like, osteogenic-like, and adipocyte-like phenotypes. In addition, we address the current insights about stimulating and inhibitory cues mediating vSMC phenotype switching. From that analysis, we inferred that transcription factor KLF4 (Krüppel-like factor 4) plays a pivotal role in the initial dedifferentiation of vSMCs to the mesenchymal-like phenotype enabling further cellular changes toward the other 4 vSMC phenotypes.

**Nonstandard Abbreviations and Acronyms**

| Abbreviation | Description |
|--------------|-------------|
| AHR          | aryl hydrocarbon receptor |
| ATF4         | activating transcription factor 4 |
| BMP2         | bone morphogenetic protein 2 |
| C/EBPβ       | CCAAT-enhancer-binding protein beta |
| CVD          | cardiovascular disease |
| DKK3         | dickkopf 3 |
| DOCK2        | dedicator of cytokinesis 2 |
| ECM          | extracellular matrix |
| ER           | endoplasmic reticulum |
| HDAC9        | histone deacetylase 9 |
| HDL          | high-density lipoprotein |
| JNK          | c-Jun N-terminal kinase |
| LDL          | low-density lipoprotein |
| MEF2C        | myocyte enhancer factor 2C |
| MGP          | matrix gla protein |
| MSX2         | Msh Homeobox 2 |
| MYOCD        | myocardin |
| NKx2-5       | NK2 transcription factor related locus 5 |
| OCT4         | octamer-binding transcription factor 4 |
| Olfm2        | olfactomedin 2 |
| PDGF-BB      | platelet-derived growth factor BB |
| PKB          | protein kinase B |
| PLK1         | polo-like kinase-1 |
| PRKG1        | cyclic GMP–dependent protein kinase 1 |
| PRMT5        | protein arginine methyltransferase 5 |
| RUNX2        | runt-related transcription factor 2 |
| SCA1         | stem cell antigen-1 |
| scRNA-seq    | single-cell RNA sequencing |
| SOX2         | SRY-BOX transcription factor 2 |
| Sp1          | stimulating protein-1 |
| SRF          | serum response factor |
| TCF21        | transcription factor 21 |
| TET2         | ten-eleven translocation-2 |
| TGF-β        | transforming growth factor beta |
| UPR          | unfolded protein response |
| vSMC         | vascular smooth muscle cell |
| Wnt          | wingless-related integration site |
| ZFP148       | zinc finger protein 148 |

**Highlights**

- Single-cell RNA sequencing and lineage tracing analyses revealed that vascular smooth muscle cells can be identified beyond the synthetic and contractile profile.
- We discuss the phenotypic switching of vascular smooth muscle cells from a contractile state to a mesenchymal-like, fibroblast-like, macrophage-like, osteogenic-like, or adipocyte-like phenotype.
- The transcription factor KLF4 (Krüppel-like factor 4) plays a pivotal role in regulation of vascular smooth muscle cells phenotype switching and emerges as a potential drug target.
- The current challenge is to pinpoint the diverging roles of vascular smooth muscle cell phenotypes and their impact on the broad range of cardiovascular diseases.

**CONTRACTILE VSMCS**

In a healthy state, vSMCs in the media of the arterial vessel wall actively synthesize, secrete, modulate, and maintain the extracellular matrix (ECM) to provide elasticity and strength to the blood vessel. In the vasculature, the vSMCs work in close collaboration with other cells to counterbalance the strong mechanical forces that the vessel wall experiences. A single layer of endothelial cells at the luminal side of the vessel forms the tight barrier between vascular lumen and the vessel wall, providing cues for vSMC relaxation and contraction. Endothelial cell function is determined by shear stress, stretching of the vessel due to heart pulse, and by circulating factors. In the adventitia, fibroblasts produce and maintain collagen fibers, thereby forming a peripheral structure to preserve vascular integrity at high pressures. In the adventitia of larger blood vessels, a microvasculature bed is present, termed the vasa vasorum. This structure protrudes into...
the outer layers of the media to provide sufficient oxygen and nutrients to the multi-layered vSMCs in the media.13

Contractile vSMCs are regarded as differentiated and quiescent cells under physiological conditions, expressing a panel of typical contractile proteins that is crucial to maintain vascular tension.15 Although the embryonic origin of vSMCs is diverse, the contractile vSMC phenotype is considered universal throughout the arterial vasculature.16 Contractile vSMCs are embedded in an intricate structure of ECM composed of elastin and collagens as key fibers.13,14,17 The ECM is produced by vSMCs themselves and sequesters growth factors, such as those belonging to the TGF-β (transforming growth factor beta) family.18 Upon damage of the vessel wall, the sequestered growth factors are released to induce a local repair response.19

Contractile vSMCs exhibit an elongated, spindle-shaped morphology and express a well-characterized set of contractile markers including smooth muscle actin (ACTA2), smooth muscle myosin heavy chain (MYH11), smooth muscle protein 22-alpha (SM22α/SMCα), smoothelin (SMTN), and calponin (CNN1).20–22 Expression of these proteins is controlled by the transcription factors MYOCD (myocardin) and SRF (serum response factor), both of which are involved in the regulation of these proteins is controlled by the transcription factors as those belonging to the TGF-β (transforming growth factor beta) family.23 Upon damage of the vessel wall, the sequestered growth factors are released to induce a local repair response.19

Induction of KLF4 in vSMCs results in a phenotypic switch from contractile to a mesenchymal-like phenotype.29,30 A mesenchymal-like vSMC is characterized by the ability to proliferate and self-renew and is marked by reduced expression of contractile proteins. This phenotype overlaps with that of mesenchymal stem cells, which are defined as stromal cells that have the capacity to differentiate into multiple lineages.29,30 Recently, a significant number of scRNA-seq studies confirmed that among the various vSMC phenotypes identified, mesenchymal-like vSMCs are also present (Table). The selected scRNA-seq studies were performed on human and murine vascular tissue of atherosclerotic plaques or aortic aneurysms. Distinct vSMC clusters were specified, and based on their gene expression profile and available knowledge of upstream modulators, we aimed to identify the main drivers of phenotypic switches. In this review, we classified stem cell marker positive vSMCs as belonging to the mesenchymal-like phenotype. This included the pioneer cell phenotype of Alencar et al.33 and vSMC-derived intermediate cells (SEM) by Pan et al.8

Dedifferentiation of vSMCs from a contractile to a mesenchymal-like phenotype is driven by external stimuli that induce repair and/or proliferation. A key initiating factor for this phenotypic switch is transcription factor KLF4,29,34–38 which regulates cellular proliferation and dedifferentiation. KLF4 also has a crucial function in the induction of cellular pluripotency.39 In fact, generation of induced pluripotent stem cells from a wide range of somatic cells requires KLF4 and 3 other, so-called Yamanaka, factors: OCT4 (octamer transcription factor), SOX2 (SRY-BOX transcription factor 2) and cMYC (MYC proto-oncogene, bHLH transcription factor).39

Induction of KLF4 in vSMCs results in a phenotypic switch from contractile to mesenchymal-like and initiates the expression of mesenchymal markers such as stem cell antigen-1 (SCA1)/LY6A, CD34, and CD44.40,41 During this transition, while gaining expression of mesenchymal markers, the contractile vSMCs lose expression of their contractile markers.12,13,15 Under physiological conditions, a subpopulation of mesenchymal-like vSMCs resides in the medial and adventitial layer of the arterial wall.35,43,44 Upon injury, mesenchymal-like vSMCs proliferate and migrate into the media and intima, to support tissue repair, possibly leading to neointimal thickening.45–47 Expression of the mesenchymal-like vSMC marker SCA1/LY6A increases in vSMCs cultured in vitro, after carotid artery ligation and in a subset of vSMCs in

**MESENCHYMAL-LIKE VSMCS**

Extensive lineage tracing studies revealed that contractile vSMCs can switch from a contractile status to a mesenchymal-like phenotype.29–31 A mesenchymal-like vSMC is characterized by the ability to proliferate and self-renew and is marked by reduced expression of contractile proteins. This phenotype overlaps with that of mesenchymal stem cells, which are defined as stromal cells that have the capacity to differentiate into multiple lineages.29,30 Recently, a significant number of scRNA-seq studies confirmed that among the various vSMC phenotypes identified, mesenchymal-like vSMCs are also present (Table). The selected scRNA-seq studies were performed on human and murine vascular tissue of atherosclerotic plaques or aortic aneurysms. Distinct vSMC clusters were specified, and based on their gene expression profile and available knowledge of upstream modulators, we aimed to identify the main drivers of phenotypic switches. In this review, we classified stem cell marker positive vSMCs as belonging to the mesenchymal-like phenotype. This included the pioneer cell phenotype of Alencar et al.33 and vSMC-derived intermediate cells (SEM) by Pan et al.8

Dedifferentiation of vSMCs from a contractile to a mesenchymal-like phenotype is driven by external stimuli that induce repair and/or proliferation. A key initiating factor for this phenotypic switch is transcription factor KLF4,29,34–38 which regulates cellular proliferation and dedifferentiation. KLF4 also has a crucial function in the induction of cellular pluripotency.39 In fact, generation of induced pluripotent stem cells from a wide range of somatic cells requires KLF4 and 3 other, so-called Yamanaka, factors: OCT4 (octamer transcription factor 4), SOX2 (SRY-BOX transcription factor 2) and cMYC (MYC proto-oncogene, bHLH transcription factor).39

Induction of KLF4 in vSMCs results in a phenotypic switch from contractile to mesenchymal-like and initiates the expression of mesenchymal markers such as stem cell antigen-1 (SCA1)/LY6A, CD34, and CD44.40,41 During this transition, while gaining expression of mesenchymal markers, the contractile vSMCs lose expression of their contractile markers.12,13,15 Under physiological conditions, a subpopulation of mesenchymal-like vSMCs resides in the medial and adventitial layer of the arterial wall.35,43,44 Upon injury, mesenchymal-like vSMCs proliferate and migrate into the media and intima, to support tissue repair, possibly leading to neointimal thickening.45–47 Expression of the mesenchymal-like vSMC marker SCA1/LY6A increases in vSMCs cultured in vitro, after carotid artery ligation and in a subset of vSMCs in
Table. Overview of scRNA-Seq Studies That Identified vSMC Phenotypes

| Diseased tissue | Species | Number of vSMC phenotypes | vSMC phenotype classification | Driver of switch | Marker of switch | References |
|----------------|---------|---------------------------|-------------------------------|------------------|----------------|------------|
| Ascending aorta atherosclerotic plaque and aneurysm | Mouse (ApoE<sup>−/−</sup>; Myh11CreERT2; mGf/f;Tgfbr2<sup>fl/fl</sup>) | 6 | Contractile, mesenchymal, fibroblast, macrophage, 2× osteogenic, adipocyte | KLF4 | | Chen et al<sup>21</sup> |
| Carotid atherosclerotic plaque | Human | 3 | Contractile, mesenchymal, macrophage | KLF4 | | Depuydt et al<sup>25</sup> |
| Brachiocephalic atherosclerotic plaque | Mouse (Myh11-CreERT2; Rosa-eYFP; ApoE<sup>−/−</sup>) | 5 | Contractile, mesenchymal, fibroblast, macrophage, 2× osteogenic | KLF4 | | Alencar et al<sup>33</sup> |
| Carotid atherosclerotic plaque | Human | 5 | Contractile, mesenchymal, fibroblast, macrophage, 2× osteogenic | KLF4 | | Alencar et al<sup>33</sup> |
| Marfan syndrome aortic aneurysm | Mouse (Fbn1-C1041G/+)| 2 | Contractile, fibroblast | KLF4 | | Pedroza et al<sup>32</sup> |
| Marfan syndrome aortic aneurysm | Human (Fbn1c;7988G>A) | 2 | Contractile, fibroblast | KLF4 | | Pedroza et al<sup>32</sup> |
| Carotid artery atherosclerotic plaque | Human | 1 | Contractile | KLF4 | | Hartman et al<sup>31</sup> |
| Brachiocephalic artery plaque | Mouse (Myh11-CreERT2;eYFP; ApoE<sup>−/−</sup>;Klf4<sup>Δ/Δ</sup>) | 8 | Unspecified | KLF4 | | Hartman et al<sup>31</sup> |
| Infrarenal abdominal aortic aneurysm | Mouse (C57BL/6J) | 3 | 2× contractile, mesenchymal, macrophage | KLF4 | | Zhao et al<sup>34</sup> |
| Aorta atherosclerotic plaque | Mouse (ROSA26Zs-Green1<sup>−/−</sup>; Ldrl<sup>−/−</sup>; Myh11-CreERT2) | 4 | Contractile, mesenchymal, fibroblast, macrophage | RAR | | Pan et al<sup>9</sup> |
| Carotid and coronary artery atherosclerotic plaque | Human | 4 | Contractile, mesenchymal, fibroblast, macrophage | RAR | | Pan et al<sup>9</sup> |
| Aorta atherosclerotic plaque | Mouse (Myh11-CreERT2/Confetti, ApoE<sup>−/−</sup>) | 4 | Contractile, mesenchymal, fibroblast, macrophage | SCA1 | | Dobnikar et al<sup>34</sup> |
| Aortic root and ascending aorta atherosclerotic plaque | Mouse (TgMyh11-CreERT2; ROSAtdT/tdT; ApoE<sup>−/−</sup>) | 3 | Contractile, fibroblast, osteogenic | TCF21, AHR | | Kim et al<sup>35</sup> |
| Aortic root and ascending aorta atherosclerotic plaque | Mouse (TgMyh11-CreERT2; ROSAtdT/tdT; ApoE<sup>−/−</sup>) | 2 | 2× contractile, fibroblast | TCF21 | | Wirka et al<sup>7</sup> |
| Coronary artery atherosclerotic plaque | Human | 2 | Contractile, 2× mesenchymal, fibroblast | TCF21 | | Wirka et al<sup>7</sup> |
| Ascending aortic aneurysm | Human | 3 | 2× contractile, 2× mesenchymal, fibroblast | ERQ1 | | Li et al<sup>35</sup> |

*2× indicates 2 different subtypes described; ERG, erythroblast transformation–specific related gene; KLF4, Krüppel-like factor 4; RAR, retinoic acid receptor; SCA1, stem cell antigen-1; scRNA-seq, single-cell RNA sequencing; TCF21, transcription factor 21; and vSMC, vascular smooth muscle cell.

*Number and classification of vSMC phenotypes are indicated according to the categorization used in this review.

Atherosclerotic plaques. Interestingly, a recent genetic fate mapping study reported only minimal involvement of SCA1/LY6A-positive vSMCs in atherosclerotic neo-intima formation, suggesting an injury- and/or context-dependent role of the SCA1/LY6A vSMC population. To further investigate the plasticity of human vSMCs switching to mesenchymal-like vSMCs as a source of reparative vSMCs, it is important to identify the human SCA1/LY6A orthologue. This will establish whether the mesenchymal-like vSMC population is also a prerequisite for tissue regeneration in human vascular disease.

In several mouse vascular disease models, KLF4 signaling has been shown to induce vSMC differentiation toward a mesenchymal-like vSMC type in a context-dependent manner. In the adventitia, a population of vSMC-derived SCA1/LY6A-positive cells is formed upon induction of KLF4. Targeted deletion of KLF4 in vSMCs resulted in the elective loss of these vSMC-derived adventitial SCA1/LY6A-positive cells, but not of non-vSMC-derived adventitial SCA1/LY6A-positive cells. Consistent with these in vivo findings, KLF4 overexpression in cultured vSMCs promotes the formation of a progenitor cell phenotype with loss of vSMC differentiation markers. Moreover, KLF4 overexpression inhibits expression of vSMC contractile markers, possibly by repressing the expression of MYOCD.

KLF4 is regulated by various signaling complexes at transcriptional and posttranslational levels. After exposure to PDGF-BB (platelet-derived growth factor BB), a stimulus of vSMC proliferation and phenotype switching, elevated levels of KLF4 were identified. PDGF-BB induces KLF4 expression via its receptor and subsequent transcription factor Sp1 (stimulating protein-1) activation. Interestingly, Sp1 also enhances ACTA2 expression via TGF-β1 signaling. TGF-β1 is known to induce vSMC contractile proteins via activation of specific SMAD transcription factor family members, promoting the vSMC contractile phenotype.
Indeed, deficiency of SMAD3 disrupts TGF-β signaling and decreases gene expression of the contractile vSMC phenotype markers. Thus, there seems to be a dual role for Sp1 in vSMC phenotype modulation. PDGF-BB also induced DOCK2 (dedicator of cytokinesis 2) and Olfm2 (olfactomedin 2). DOCK2 inhibits MYOCD-induced vSMC marker promoter activity. In addition, DOCK2 and KLF4 cooperatively inhibit MYOCD-SRF interaction. Olfm2 promotes the interaction of SRF with RUNX2 (runt-related transcription factor 2), leading eventually to reduction of vSMC marker gene transcription and consequent vSMC phenotypic modulation.

KLF4 activity is modulated by retinoic acid as well. Retinoic acid and PDGF-BB have opposite effects on vSMC proliferation and differentiation by changing the phosphorylation and acetylation state of KLF4 in different ways, causing it to preferentially bind to different regions within the TAGLN promoter. Retinoic acid receptor activation leads to the phosphorylation of KLF4, thereby facilitating its acetylation and subsequent translocation to different transcriptional activation domains, alleviating the repression of contractile gene expression.

Furthermore, it has been reported in human vSMCs, human tissue, and mouse models that the DNA-modifying enzyme TET2 (ten-eleven translocation-2) is upregulated in contractile vSMCs and reduced in dedifferentiation vSMCs. Knockdown of TET2 inhibited expression of MYOCD and SRF with transcriptional upregulation of KLF4, thus preventing vSMC differentiation to contractile vSMCs. Another member of the Krüppel family, namely ZFP148 (zinc finger protein 148) also modulates vSMC phenotype transition by inhibiting vSMC contractile marker expression in a neurofibromin 1-dependent manner.

In line with the observation that vSMC phenotype switching is context-dependent, sex differences have also been detected in a recent scRNA-seq study comparing atherosclerotic plaque tissue of males and females. Different gene expression profiles were observed with a more pronounced KLF4-driven pattern in females compared with males. In a vSMC-specific KLF4 knockout mouse model, expression of several female-biased genes was observed (FN1 was downregulated; MFP4, CNN1, NDRG2, GAS6, and OSR1 were upregulated). Such sex-specific differences in vSMC phenotype are undervalued and require more attention in future studies.

The role of miRNAs in the (de)differentiation of vSMCs has been highlighted in dedicated reviews. MiR-143 and miR-145 are crucial for the fate of vSMCs, facilitating vSMC differentiation and inhibiting proliferation. MiRNA-143/145 are controlled by transcription factors NKx2-5 (NK2 transcription factor related locus 5) and SRF with its coactivator MYOCD and target KLF4, KLF5, and Sp1. Deficiency of miRNA143/145 in mice resulted in thinning of the aortic wall and a reduced number of contractile vSMCs, indicating that miR-143/145 are important in maintaining the contractile vSMC phenotype. In addition, miR-1 repressed KLF4 to regulate vSMC differentiation. Specifically, miR1 expression was increased during differentiation of pluripotent embryonic stem cells toward vSMCs, and inhibition of miR1 repressed vSMC differentiation.

To prevent or reverse the phenotypic transition to mesenchymal-like vSMCs, the expression of KLF4 can be suppressed by TGF-β or miR-143/145 to maintain the contractile vSMC phenotype. Treatment of mesenchymal-like vSMCs with TGF-β for 3 days increased ACTA2 expression, indicating an adoption of a more contractile phenotype. Also, the transcription factor erythroblast transformation-specific related gene, which is involved in handling reactive oxygen species and endoplasmic reticulum (ER) signaling, was decreased in mesenchymal-like vSMCs. Increasing the expression of erythroblast transformation-specific related gene, which plays an important role in maintaining normal aortic wall function, promotes the contractile vSMC phenotype. Alternatively, mesenchymal-like vSMC may undergo further changes into other vSMC phenotypes.

**FIBROBLAST-LIKE VSMCS**

Fibroblasts are cells, which are primarily responsible for the production and modification of ECM proteins, such as collagens and fibronectin throughout tissues. In response to injury, fibroblasts can transition into myofibroblasts, which generate collagen-rich scar tissue to repair a wound. Subsequently, extensive remodeling will take place to resolve the scar. Most scRNA-seq studies have reported fibroblasts-like vSMCs as a phenotype (Table). Generally, the fibroblast-like vSMC phenotype is observed following vascular injury, such as during atherosclerotic progression or aortic aneurysm formation. This phenotype is also referred to as myofibroblasts-like vSMCs or fibrocytes.

Fibroblast-like vSMCs shift toward, but cluster separately from, authentic fibroblasts in scRNA-seq analyses performed in atherosclerotic lesions of murine and human arteries. Fibroblast-like vSMCs express ACTA2, SCA1/LY6A, and the fibroblast markers lumican (LUM), biglycan (BGN), and decorin (DCN). The cells show substantially reduced levels of contractile markers (TAGLN, CNN1) as compared with contractile vSMCs. Pheno-type switching to a fibroblast-like vSMC was observed in the thoracic aortic aneurysm of a Marfan syndrome mouse model. Profiling of gene expression in that cluster revealed enhanced expression of genes involved in adhesion, ECM organization, cellular proliferation, and deposition of collagen. The latter is considered a hallmark for aortic aneurysm formation, which involves aortic fibrosis and stiffness.
A number of factors have been identified that induce this fibroblast-like vSMC phenotype, summarized below. It has been shown that adventitial SCA1/LY6A-positive fibroblast-like vSMCs arise in a KLF4-dependent manner. The adventitial SCA1/LY6A-derived fibroblast-like vSMCs may even migrate into the intima where they promote a fibrotic response, which stiffens the vessel wall.63 Interestingly, cholesterol or oxidized phospholipid exposure can also induce a phenotypic switch of vSMCs as shown by induced expression of fibroblast and macrophage markers.40 Fibroblast markers upregulated under these conditions were fibronectin 1 (FN1), Ecrg4 augurin precursor (ECRG4), proteoglycan 4 (PRG4), secreted phosphoprotein 1 (SPP1), lipocalin-2 (LP2), metalloproteinase inhibitor 1 (TIMP1), BGN, and DCN.40

Interestingly, the ER unfolded protein response (UPR) can promote phenotypic switching in vSMCs toward the fibroblast-like phenotype as well.40 The ER normally has a low cholesterol content, while accumulation of free cholesterol in the ER induces membrane dysfunction and subsequent ER stress, causing UPR.84,85 Moreover, the UPR promotes a macrophage-like vSMC phenotype, which may explain the appearance of both fibroblast-like and macrophage-like vSMCs in atherosclerotic plaques.83 Even without cholesterol exposure, chemically induced UPR is sufficient to cause phenotype switching of vSMCs to fibroblast-like or macrophage-like vSMCs.40 The underlying mechanism of this phenotypic switch is linked to the UPR effector ATF4 (activating transcription factor 4). ATF4 prevents proteasomal degradation of KLF4, and this enhanced KLF4 expression promotes atherosclerotic plaque formation.40

The vSMC-specific knockout of TCF21 (transcription factor 21) in hyperlipidemic apolipoprotein E deficient (ApoE−/−) mice led to fewer fibroblast-like vSMCs in the protective fibrous cap of the atherosclerotic lesions.9 Moreover, high TCF21 expression is associated with decreased coronary artery disease risk in humans, possibly due to a more stable and fibroblast-like vSMC-rich plaque. In addition, TCF21 is activated early on in coronary artery disease and directly inhibits SMAD3-mediated gene expression, thereby reducing expression of the contractile markers.96 Together, these studies point to TCF21 as a regulator of vSMC phenotype switching toward a protective fibroblast-like vSMC population in atherosclerosis.96

The vSMC phenotypes distinct from the contractile type are often described as modulated vSMCs.78 Gene expression profiles of these modulated vSMCs comprise markers of mesenchymal-like and fibroblast-like but also of macrophage-like or osteogenic-like cells.78,80 Many fibroblast-like vSMCs originate from the mesenchymal-like pool of vSMCs, although it is challenging to distinguish between these phenotypes.6 It is also not clear if all fibroblast-like vSMCs first, and entirely, transition via the mesenchymal-like vSMC state. Therefore, it cannot be concluded that the fibroblast-like vSMCs are part of mesenchymal-like vSMCs or an entirely separate cell type without additional research. However, as the gene expression patterns still differ, currently, a distinction between the 2 cell states has been made.

Reverse differentiation from a fibroblast-like phenotype back to the contractile vSMCs phenotype has been described for DKK3 (dickkopf 3), which is a Wingless-related integration site (Wnt) inhibitor. DKK3 was shown to be involved in the differentiation of stem and progenitor cells to contractile vSMCs in ApoE−/− mice.88 DKK3 induces differentiation of SCA1/LY6A-positive mesenchymal-like and fibroblasts-like vSMCs to contractile vSMCs via activation of TGF-β in a Wnt-dependent manner. While TCF21 seems to reduce atherogenesis severity by promoting the fibroblast-like vSMC, DKK3 promotes protective atherosclerotic plaque stabilization by increasing the number of contractile vSMCs. Therefore, it is not yet clear which vSMC phenotype is actually beneficial in the context of atherosclerosis.

**MACROPHAGE-LIKE VSMCs**

Macrophages play a central role in all stages of inflammation and healing and recruit other immune cells to initiate an appropriate immune response to clear debris and combat pathogens.89 In atherosclerosis, macrophages are known to clear the vascular wall of oxidized LDL (low-density lipoprotein), in the process becoming foam cells.90 As stated earlier, vSMCs can acquire a macrophage-like phenotype, even with phagocytic properties,91 as reported in 6 out of eleven selected scRNA-seq studies (Table). Phenotype switching from a contractile vSMC, via the mesenchymal-like to a macrophage-like vSMC, is associated with development of atherosclerosis.99,192

Macrophage-like vSMCs are typically indicated by expression of LGALS3 and classical macrophage markers such as CD11b, CD45, CD68, CD11c, and F4/80 (for murine macrophages).93 Phenotype switching to macrophage-like vSMCs also involves KLF4 signaling.99,192 A conditional KLF4 knockout in an atherosclerotic mouse model results in reduced vSMC-derived mesenchymal-like cells and macrophage-like cells, plus a marked reduction in lesion size and increased plaque stability.90,92 Similar macrophage-like vSMCs have been identified in human atherosclerotic plaques.5,8,201 A low number of cells within the vSMC group was KLF4 positive, indicating that vSMCs probably only have transient KLF4 expression. One of these vSMC clusters was characterized by ACTA2, LGALS3, and CD68 expression, typical for the macrophage-like phenotype.6 However, identification of macrophage-like cells is often based on different macrophage or foam cell markers between the scRNA-seq studies, with LGALS3 and CD68 being the most common markers.
KLF4 gene expression in vSMCs facilitates foam cell formation by enhancing the uptake of cholesterol-rich lipoproteins.94 While classically lipid-laden foam cells were considered to be solely derived from monocytes/macrophages, it has become evident that vSMCs with a macrophage-like phenotype are abundantly present in the atherosclerotic plaque.94 Upon high cholesterol exposure, expression of KLF4 was induced. This transforms contractile vSMCs into macrophage-like vSMCs, a cell population contributing to disease progression.96 Switching of vSMCs to a macrophage-like phenotype coincides with loss of the contractile vSMC factor MYOC.D.91,94,97 Conversely, gain of MYOC expression inhibits macrophage-like vSMC accumulation in atherosclerotic lesions in vivo.91 One of the Yamanaka factors, OCT4, also plays a role in regulating vSMC phenotypic transition, but interestingly in a contrasting way compared with KLF4. Deficiency of KLF4 or OCT4 resulted in consistent with increased plaque stability.33,98

Various scRNA-seq analyses have been performed on atherosclerotic plaques of mice.47,31,33,99 In ApoE−/− mice fed a western diet, vSMCs expressing Lgals3 compose up to two thirds of all vSMCs in the atherosclerotic lesion.23 This macrophage-like phenotype was similarly found by others in ApoE−/− mice.29,31,33,90,93 as well as in LDL receptor knockout (LDLR−/−) mice.8 Through fate mapping, it appears that these macrophage-like vSMCs were derived from a multipotent vSMC-derived intermediate cell state,8 which bear similarity to the mesenchymal-like vSMCs. Recent evidence even suggests that a considerable subset of the plaque may originate from dedifferentiated vSMCs, which proliferate in a clonal fashion.100

Other extracellular stimuli playing a role in phenotypic switching to macrophage-like vSMCs are nitric oxide and natriuretic peptides. In response to these compounds, vSMCs generate cyclic GMP, which induces vasodilation, enhancing blood flow.101 Interestingly, PRKG1 (cyclic GMP-dependent protein kinase 1) activation also contributes to the formation of macrophage-like vSMCs that reside within the atherosclerotic plaque.101 Under atherogenic conditions, vSMCs migrate to the atherosclerotic plaque (intima), which in Ptkg1-deficient mice remain in the medial layer. Using cell-fate mapping, it was shown that Ptkg1 is involved in phenotype switching of vSMCs to macrophage-like vSMCs in the plaques. In line with this, postnatal ablation of Ptkg1 in murine vSMCs resulted in smaller lesions.101 This study also demonstrates that macrophage-like vSMCs are derived from mature vSMCs that migrated into the plaque.101

Reverse differentiation of the macrophage-like vSMC phenotype is accomplished by HDL (high-density lipoprotein). HDL is responsible for the efflux of cholesterol from cells and transport to the liver to remove cholesterol from the periphery. Exposure to HDL reduces the macrophage-like vSMC phenotype by increasing MYOC and miR-143/145 expression in vSMCs in vitro.95 The question remains whether macrophage-like vSMCs have similar functions as monocyte-derived macrophage subsets in atherosclerosis.8

**OSTEOMI-TIC-LIKE VSMCS**

The natural function of chondrocytes is to form and maintain cartilage, while that of osteoblasts is to generate bone tissue.102,103 During ossification, hypertrophic chondrocytes produce a unique ECM that mineralizes, enabling cells to differentiate into osteoblasts. Both chondrocytes and osteoblasts are of mesenchymal origin and share a common precursor.104 Switching from a mesenchymal-like vSMC phenotype to a chondrocyte-like or osteoblast-like vSMC has been identified in 4 out of the 11 scRNA-seq studies (Table). In this review, we classified chondrocyte-like and osteoblast-like vSMC phenotypes together as osteogenic-like vSMCs.

Deposition of calcium phosphate can drive vSMC phenotype switching to contribute to various cardiovascular diseases.105,106 Calcification of the intimal layer is associated with arterial obstruction and atherosclerotic plaque rupture, while calcification of the medial layer is associated with vessel stiffening leading to heart failure.106 In addition, vascular calcification has been associated with hypertension, osteoporosis, rheumatoid arthritis, chronic kidney disease, diabetes type II, and aortic aneurysm formation.106–108 Interestingly, overexpression of the Twist family BHLH transcription factor 1 (TWIST1), a coronary artery disease risk gene, in rat aortic vSMCs increases cell proliferation and decreases calcification, whereas TWIST1 knockdown has the opposite effect.109 Of note, TWIST1 was also found to be differentially expressed in several vSMC clusters in ascending aorta aneurysm tissue analyzed by scRNA-seq.15

The osteogenic-like vSMC phenotype is characterized by a loss of contractile markers (SM22α and ACTA2) and an increase in calcification markers, such as osteogenic transcription factors MSX2 (Msh Homeobox 2), Cbfa1 (core-binding factor α-1, also known as RUNX2), and Sp7/Osterix, as well as the chondrogenic transcription factor SOX9.106,107,109 Other markers such as osteopontin, osteocalcin, alkaline phosphatase, collagen II, and collagen X were reported as markers of this osteogenic-like vSMC phenotype as well.106,111 The expression of RUNX2 and SOX9 is a main determinant of the osteogenic-like vSMC phenotype, since these 2 transcription factors drive the osteoblast or chondrocyte phenotype, respectively.106,112

Deficiency of RUNX2 in vSMCs prevents differentiation into the osteogenic-like vSMC phenotype, shown both in vitro and in vivo.107,113,114 In vitro, RUNX2, Osterix,
and alkaline phosphatase expression is required to drive the calcification process. This process also involves the PKB (protein kinase B) or AKT and c-JNK (Jun N-terminal kinase) signaling pathways. Human vSMCs exposed to a calcifying medium show decreased AKT phosphorylation and a transient increase in JNK activity. This is in line with the observation that elevated levels of AKT and JNK protect from vascular calcification. Another pathway involves Wnt signaling, which promotes vSMC differentiation to osteogenic-like cells primarily through RUNX2. Activation of the Wnt cascade in osteogenic-like vSMCs increases BMP2 (bone morphogenetic protein 2) signaling, which plays a significant role in vascular calcification. BMPs are part of the TGF-β superfamily, which signal via the SMAD transcription factors -1 and -5, regulating RUNX2. Interestingly, in disease models associated with vascular calcification, KLF4 was demonstrated to decrease expression of vSMC differentiation makers and induced osteogenic genes. KLF4 was also demonstrated to regulate RUNX2 transcription, with knockdown of KLF4 inhibiting upregulation of RUNX2 and vSMC calcification. In addition, KLF4 enhanced chondrocyte differentiation as characterized by upregulation of SOX9 and downregulation of the chondrocyte dedifferentiation marker Col1α1. In the context of scRNA-seq, loss of KLF4 in vSMCs coincided with reduction of an osteogenic phenotype. However, the exact link between KLF4 and osteogenic-like cells is not fully clear and requires further research.

An in vivo study recapitulated vSMC differentiation into an osteogenic-like phenotype, by demonstrating that MGP (matrix gla protein)-deficient mice develop severe calcification of vSMCs in arterial blood vessels. Interestingly, MGP-deficient mice also lacking HDAC9 (histone deacetylase 9) had a 40% reduction in aortic calcification with improved survival. Thus the presence of HDAC9 causes progression toward the osteogenic-like vSMC phenotype induced by the absence of MGP.

Another pathway involved in the suppression of calcification is based on aryl hydrocarbon receptor (AHR)-mediated gene expression. This transcription factor is involved in stem cell maintenance and cellular differentiation and is a downstream target of TCF21. TCF21 promotes vSMC phenotype switching to fibroblast-like cells and knockdown of AHR resulted in switching of fibroblast-like vSMCs to the osteogenic-like vSMCs. This is in line with the ability of AHR to suppress SOX9 and RUNX2 expression. Interestingly, TCF21 knockdown prevented both the fibroblast-like and osteogenic-like vSMC differentiation, indicating that the fibroblast-like phenotype may first be required before transition toward the osteoblast-like phenotype can occur. Taken together, TCF21 can be considered a driver and AHR an inhibitor of these vSMC phenotypes.

**ADIPOCYTE-LIKE VSMCs**

An adipocyte-like vSMC phenotype has only been described in a single scRNA-seq study (Table). Using an in vivo fate mapping approach, with either a constitutive or inducible Myh11-driven Cre mouse model, it has been demonstrated that vSMCs are able to adapt toward this adipocyte-like phenotype. These adipocyte-like vSMCs were classified as beige adipocytes, which are also known as inducible brown adipocytes. Brown/beige adipocytes regulate thermogenesis by producing heat when burning fatty acids in the presence of an UCP-1 (uncoupling protein-1), while white adipocytes store triglycerides to save energy. Sustained thermogenic activation leads to the browning of white adipose tissue, in which a population of adipocytes differentiate into beige adipocytes. Given that only 1 scRNA-seq study reports adipocyte-like vSMCs, this may indicate that a higher barrier exists for differentiation towards an adipocyte-like vSMC type or that cues to induce this phenotype are not widespread in atherosclerotic and aneurysm tissue.

Adipocyte-like vSMCs express the crucial beige adipocyte marker UCP1, as mentioned earlier, as well as PPARG (peroxisome proliferator-activated receptor gamma) coactivator 1 alpha (PPARGC1A), transmembrane protein 26 (TMEM26), PR-domain containing 16 (PRDM16), and the temperature-sensitive ion channel transient receptor potential cation channel subfamily V member 1 (TRPV1). Upon 7-day cold exposure, these cells are able to mature further to brown adipocyte-like vSMC expressing UCP1, adiponectin (ADIPQ), cell death inducing DFFA like effector A (CIDEA), and iodothyronine deiodinase 2 (DIO2). Furthermore, KLF4 is suggested to regulate early adipogenesis to induce C/EBPβ (CCAAT-enhancer-binding protein beta). C/EBPβ stimulates expression of PPARG, which is required for adipocyte differentiation. However, if this pathway is also activated in vSMC by KLF4 has yet to be determined.

Conversion of contractile vSMCs to adipocyte-like vSMCs within the vessel wall is presumably not without consequences, but as this phenotype is least studied, this will require more research to understand its relevance.

**EXPANDING THE VIEW ON VSMC PHENOTYPES**

Recent scRNA-seq analyses of human and mouse vascular tissues disclose a previously not well-defined diversity of arterial vSMC phenotypes. We reasoned that the classification of vSMC phenotypes should be expanded beyond the traditional exclusive division of contractile and synthetic vSMCs. In this review, we summarized the current evidence for the presence of 6 distinct vSMC phenotypes in (diseased) vascular tissue: contractile, mesenchymal-like, fibroblast-like, macrophage-like, osteogenic-like, and adipocyte-like vSMCs (Figure 1A).
Combining the scRNA-seq data on vSMC subtypes with knowledge on vSMC function, one may speculate that there is an initial transition of the contractile vSMC dedifferentiating into mesenchymal-like vSMCs, followed by differentiation towards the other 4 vSMC phenotypes. A remarkable characteristic of vSMCs is their plasticity, as none of the phenotypes seem to be a final state of cellular differentiation. Rather, vSMCs can go back and forth between different phenotypes, triggered by specific stimuli (Figure 1B). The exact phenotypic composition of vSMCs in the vessel wall may determine vascular pathology in various cardiovascular diseases, and at present...
KLF4 AND ITS CRUCIAL ROLE IN VSMC PHENOTYPE SWITCHING

The transcription factors KLF4, retinoic acid receptor, TCF21, AHR, and erythroblast transformation-specific related gene are all to some extent involved in vSMC phenotype switching in cardiovascular diseases, with a key role for KLF4. Therefore, we summarize the current knowledge on modulation of KLF4 to interfere with vSMC phenotype switching. KLF4 is known to regulate gene expression via different mechanisms, not merely as a DNA-binding transcription factor, which may in part explain its involvement in the different vSMC phenotypes.

The sections above substantiate the importance of KLF4 in relation to vSMC phenotype switching. The distinct role of KLF4 per vSMC phenotype with respect to gene regulation and transcription factor cooperation is context-dependent and affected by additional stimuli, as summarized in Figure 2. In the contractile vSMC phenotype, the activated transcription factor complex MYOD-SRF induces the expression of contractile proteins.

In this situation, KLF4 expression is balanced by protein ubiquitination and subsequent degradation. Posttranslational modification through acetylation of KLF4 changes its binding to preferential DNA sites to alleviate the repression of contractile gene expression. In the mesenchymal-like vSMC phenotype, KLF4 expression is upregulated, which reduces MYOCD-SRF complex formation and thereby inhibits expression of contractile proteins. KLF4 then increases mesenchymal marker expression: SCA1, CD34, and CD44. When KLF4 signaling is enhanced in the fibroblast-like vSMC phenotype, this is linked to the UPR and its effector factor ATF4. KLF4 has also been associated with macrophage foam cell formation by enhancing uptake of cholesterol-rich lipoproteins. Switching of vSMCs toward a macrophage-like phenotype may be induced by KLF4 through enhanced UPR or lipid uptake, which can lead to foam cell formation. The transition toward an osteogenic-like phenotype requires the induction of RUNX2 or SOX9 to induce vascular calcification by releasing extracellular vesicles.

Activation of KLF4 may be required for switching toward an adipocyte-like phenotype, which could lead to adipogenesis through C/EBPβ and PPARγ signaling.

In atherosclerosis, Alencar et al identified a reduction in lesion size and increased plaque stability in mice with a vSMC-specific conditional deficiency of KLF4. These mice show an increase in the number of contractile vSMCs, and a substantial reduction of macrophage-like and osteogenic-like vSMCs. Moreover, Chen et al observed a decrease in overall atherosclerotic burden and a reduction in the development of aortic aneurysms (regardless of cholesterol levels), in vSMC-specific KLF4 knockout mice. A KLF4 signature was also identified in atherosclerotic mice. Furthermore, using an in vitro approach, it was shown that KLF4 regulates the expression of genes involved in vSMC phenotype switching, including MYOCD, MYOD, and SRF.
induced pluripotent stem cell–derived vSMC model with distinct FBN1 mutations from Marfan syndrome patients, knockdown of KLF4 restored Fbn1 fiber deposition, vSMC proliferation defects, and contractile function.129 In contrast, a marked reduction in KLF4 expression was observed in the aorta of very young Fbn1 hypomorphic (mgR/mgR model) Marfan mice, which was concomitant with an increase in contractile vSMCs.130 Together, these data may indicate that the contractile population of vSMCs is unable to undergo phenotype switching during early stages of aneurysm formation.5,130 However, vSMC-specific KLF4-deficient Marfan mice are yet to be generated to precisely determine the effect of KLF4 in aortic aneurysm formation.

These findings emphasize KLF4 as an interesting potential drug target to diminish vascular disease. As there are no highly selective KLF4 antagonists, KLF4 expression can be targeted by indirect inhibitors, of which several are in preclinical development. One of these inhibitors is designed to interfere with the methylation of KLF4 by PRMT5 (protein arginine methyltransferase 5).131 Since KLF4 has a short half-life, governed by VHL-VBC ubiquitin-protein ligase, methylation of amino acids R374, R376, and R377 prolong protein stability. The small molecule antagonist WX2–43 was designed to interfere with the PRMT5-KLF4 interaction, preventing the methylation, thus promoting degradation. Another indirect inhibitor of KLF4 targets its interaction with PLK1 (polo-like kinase-1). This kinase phosphorylates KLF4 at Ser234, promoting protein stability, previously linked to hyperplastic intima of injured vessels.132 The experimental drug BI6727, originally developed as an anticancer drug, is a highly selective, potent PLK1 inhibitor. Exposure to BI6727 results in reduced expression of KLF4 and increased protein turnover due to reduced stability.133 Given that the translation of miRNAs into clinical medicine will become more common in the future, it is of importance that several miRNAs have been reported to interact with KLF4 mRNA, and may serve as blueprint for therapeutic oligonucleotides.134 The miRNAs miR-1, miR-25, miR-29, miR-143, miR-145, and miR-375 induce RNAi-mediated silencing of KLF4 protein expression.92,135–138 Further research will establish the potential of targeting KLF4 in the context of the different cardiovascular diseases, where KLF4 may be beneficial in one, but detrimental in another vascular disease type. In addition, potential side effects of repressed KLF4 should not be overlooked, as KLF4 inhibition has been shown to delay wound healing and elevate insulin resistance.139,140

**DISCUSSION AND PERSPECTIVES**

The scRNA-seq technique, together with lineage tracing, opens up a whole new world of possibilities to study vSMCs in the normal and diseased vessel wall. Until now, the scRNA-seq has been performed on (diseased) tissues collected at a single point in time, especially in human end-stage disease. To understand the dynamics of vSMC phenotype plasticity between the 6 vSMC phenotypes, phenotype switching over time needs to be explored in detail. To date, only Pan et al8 performed scRNA-seq and lineage tracing in mice over various time points and suggested that this method provided both sensitive and specificity for tracking vSMC behaviors during atherosclerosis.

In addition, we argued that the data point in the direction of the mesenchymal-like cells giving rise to the other vSMC phenotypes; however, further experimental validation is needed to fully support this hypothesis. Furthermore, it remains challenging to recognize the vSMC origin of a cell population, especially in nonlineage traced human tissue samples. However, even conventional lineage tracing studies have their limitations; they can only mark the fate of the contractile vSMC but not the fate of once contractile vSMCs that underwent phenotype switching with the loss of their contractile markers.

Limitations and potential biases should also be considered when analyzing and interpreting different scRNA-seq datasets. As often, the data rely on a single-cell suspension representative of all cell populations present within tissue samples exposed to tissue disruption. scRNA-seq also lacks spatial information about the distribution of the different subpopulations within tissues, and different processes can cause variations in transcript levels that might not reflect differences in protein levels or cellular functions. Moreover, the lack of depth of scRNA-seq also makes it difficult to investigate low-expression genes, which are often transcription factors.10

Although the transcriptomic signature in defining vSMC states and transitions offers a wealth of information, it is yet to be determined what this means on a protein level and whether these vSMC-derived cells have similar functionality to their classical counterparts (eg, macrophage-like vSMC compared with a bone marrow-derived macrophage). The vSMC-derived cells seem to be heavily influenced by their environment to trigger the transition and may not always provide positive consequences.

As for the function of the different vSMCs phenotypes, their diverging roles in the broad range of CVDs remain to be discovered. It will eventually pinpoint which type is beneficial or harmful in specific vascular disorders. This knowledge is crucial for the development of innovative disease intervention strategies.

**ARTICLE INFORMATION**

Received June 3, 2021; accepted August 20, 2021.

**Affiliations**

Department of Medical Biochemistry, Amsterdam Cardiovascular Sciences, University of Amsterdam, Amsterdam UMC, Location Academic Medical Center, The Netherlands (C.Y., A.M., C.J.M.d.V., V.d.W.). Department of Clinical Genetics, Amsterdam Cardiovascular Sciences, Vrije Universiteit Amsterdam,
REFERENCES

1. Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abersa SF, Abyu G, Ahmed M, Aksut B, Alam T, Alam K, et al. Global, regional, and national burden of cardiovascular diseases for 10 causes, 1990 to 2015. J Am Coll Cardiol 2017;69:1–38. doi:10.1016/j.jacc.2017.04.052

2. Quyyumi AA, Dakak N, Andrews NP, Gilligan DM, Panza JA, Cannon RO 3rd. None.

3. Sources of Funding

We thank Dr Dave Speijer for critical reading of the article.

4. Disclosures

None.

5. REFERENCES

1. Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abersa SF, Abyu G, Ahmed M, Aksut B, Alam T, Alam K, et al. Global, regional, and national burden of cardiovascular diseases for 10 causes, 1990 to 2015. J Am Coll Cardiol 2017;69:1–38. doi:10.1016/j.jacc.2017.04.052

2. Quyyumi AA, Dakak N, Andrews NP, Gilligan DM, Panza JA, Cannon RO 3rd. None.

3. Sources of Funding

We thank Dr Dave Speijer for critical reading of the article.

4. Disclosures

None.

5. REFERENCES
drives phenotypic modulation in vivo. J Pharmacol Exp Ther. 2010;333:34–42. doi: 10.1124/pept.109.163949
36. Majesky MW, Horita H, Ostriker A, Lu S, Regan JN, Bagchi A, Dong XR, Poczobutt J, Nemenoff RA, Weiser-Evans MC. Differentiated smooth muscle cells generate a subpopulation of resident vascular progenitor cells in the adventitia regulated by Klf4. Circ Res. 2017;120:296–311. doi: 10.1161/CIRCRESAHA.116.309222
37. Yoshida T, Kastner KH, Owens GK. Conditional deletion of Krüppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. Circ Res. 2008;102:1548–1557. doi: 10.1161/CIRCRESAHA.107.169974
38. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 2012;74:13–40. doi: 10.1146/annurev-physiol-012811-135215
39. Karagiannis F, Takahashi K, Saito M, Yoshida Y, Okita K, Watanabe A, Inoue H, Yamashita JK, Todani M, Nakagawa M, et al. Induced pluripotent stem cells and their use in human models of disease and development. Physiol Rev. 2019;99:79–114. doi: 10.1152/physrev.00003.2017
40. Chattopadhyay A, Kawralt CS, Kaw K, Li Y, Kaw A, Chen J, LeMaire SA, Shen YH, Milewicz DM. Cerebrox-induced phenotypic modulation of smooth muscle cells to macrophage/fibroblast-like cells is driven by an unfolded protein response. Arterioscler Thromb Vasc Biol. 2021;41:302–316. doi: 10.1161/ATVBAHA.120.315164
41. Zhao G, Lu H, Chang Z, Zhao Y, Zhu T, Chang L, Guo Y, Garcia-Barrio MT, Chen YE, Zhang J. Single-cell RNA sequencing reveals the cellular heterogeneity of aneurysmal infrarenal abdominal aorta. Cardiovasc Res. 2020;109:402–416. doi: 10.1093/ckb/fcz014
42. Wu Y, Liu X, Guo L, Zhang L, Zheng F, Li S, Li X-Y, Yuan Y, Liu Y, Yan Y-W. S100B is required for maintaining an intermediate state with double-positive sca-1+ progenitor and vascular smooth muscle cells during neointimal formation. Stem Cell Res Ther. 2019;10:1–16. doi: 10.1186/s13287-019-1400-0
43. Tang J, Wang H, Huang X, Li F, Zhu H, Li Y, He L, Zhang H, Pu W, Liu K, et al. Adaptive sca-1+ vascular stem cells generate de novo smooth muscle cells during neointimal formation. Stem Cell Stem Regen Med. 2020;26:681–696.e84. doi: 10.1016/j.stemcr.2019.11.010
44. Sartore S, Chiavegato A, Faggini E, Franch R, Puato M, Ausoni S, Pauletto P. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. Circ Res. 2001;89:1111–1121. doi: 10.1161/hc0501.100644
45. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest. 2004;113:1258–1265. doi: 10.1172/JCI16928
46. Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW. A sonic hedgehog signaling domain in the arterial adventitia supports resident sca1+ smooth muscle progenitor cells. Proc Natl Acad Sci U.S.A. 2008;105:9349–9354. doi: 10.1073/pnas.0711821105
47. Kramm R, Goerttsch C, Wongboonsin J, Iwata H, Schneider RK, Kuppe C, Kaeusler N, Chang-Paranesso M, Machado FG, Gratwohl S, et al. Adversarial MSC-like cells are progenitors of vascular smooth muscle cells and drive vascular calcification in chronic kidney disease. Cell Stem Cell. 2016;19:626–642. doi: 10.1016/j.stem.2016.08.001
48. Wang H, Zhao H, Zhu H, Li Y, Tang J, Li Y, Zhou B. Sca1+ cells minimize to smooth muscle cells in atherosclerosis. Circ Res. 2021;128:135–135. doi: 10.1161/CIRCRESAHA.120.317972
49. Holmes C, Stanford WL. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells. 2007;25:1339–1347. doi: 10.1634/stemcells.2006-0644
50. Johnston WF, Mokry M, Kovacic JC, Pasterkamp G, et al. Sex-stratified gene regulatory network regulates smooth muscle gene expression and vascular remodeling. Circ Res. 2013;112:139–150. doi: 10.1161/CIRCRESAHA.112.309022
51. Liu R, Jin Y, Tang WH, Qin L, Zhang X, Tellides G, Hwa J, Yu J, Martin KA. Ten-eleven translocation-2 (TET2) is a master regulator of smooth muscle cell plasticity. Circulation. 2013;128:1047–1056. doi: 10.1161/CIRCULATIONAHA.113.002887
52. Salmon M, Schaheen B, Spinosa M, Montgomery W, Pope NH, Davis JP, Johnston WF, Sharma AK, Owens GK, Merchant JL, et al. ZFP148 (Zinc-Finger Protein 148) binds cooperatively with NF-1 (Nuclear Factor 1) to inhibit smooth muscle marker gene expression during abdominal aortic aneurysm formation. Arterioscler Thromb Vasc Biol. 2019;39:73–83. doi: 10.1161/ATVBAHA.118.311136
53. Hartman RJG, Ovsyannik K, Ma L, Kopelev S, Hao K, Slenders L, Civelek M, Mokry M, Kovic JC, Pasterkamp G, et al. Sex-stratified gene regulatory networks reveal female key driver genes of atherosclerosis involved in smooth muscle cell phenotype switching. Circulation. 2021;143:713–726. doi: 10.1161/CIRCULATIONAHA.120.051291

Yap et al. Six Shades of vSMCs Illuminated by KLF4

Arterioscler Thromb Vasc Biol. 2021;41:2693–2707. DOI: 10.1161/ATVBAHA.121.316600 November 2021 2705
Yap et al Six Shades of vSMCs Illuminated by KLF4

72. Elia L, Quintavalle M, Zhang J, Contu R, Cossu L, Latronico MV, Peterson KL, Indolfi C, Cataldiucci D, Chen J, et al. The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death Differ. 2009;16:1590–1598. doi: 10.1038/cdd.2009.153

73. Davis-Dusenberg BN, Chan MC, Reno KE, Weisman AS, Layne MD, Lagina G, Hatta A. Down-regulation of kruppel-like factor-4 (klf4) by microrna-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-β and bone morphogenetic protein 4. J Biol Chem. 2011;286:28097–2811. 10.1074/jbc.M111.236950

74. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell. 2009;137:647–658. 10.1016/j.cell.2009.02.038

75. Li Y, Ren P, Dawson A, Vasquez HG, Ageedi W, Zhang C, Luo W, Chen R, Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell. 2009;137:647–658. 10.1016/j.cell.2009.02.038

76. D’Urso M, Kurniawan NA. Mechanical and physical regulation of fibroblast-myofibroblast transition: from cellular mechanoresponses to tissue pathology. Front Bioeng Biotechnol. 2020;6:609653. 10.3389/fbioe.2020.609653

77. Burke RM, Burgos Villar KN, Small EM. Fibroblast contributions to ischemic cardiac remodeling. Cell Sci Int. 2021;77:109824. 10.1042/cellsig.2020.109824

78. Reichardt IM, Robeson KZ, Regnier M, Davis J. Controlling cardiac fibrosis. Adv Drug Deliv Rev. 2021;300147:1–50. 10.1016/j.addr.2021.102707

79. Henderson NC, Rieder F, Wynn TA. Fibrosis: from mechanisms to medicines. Adv Drug Deliv Rev. 2021;300147:1–50. 10.1016/j.addr.2021.102707

80. Li Y, Kim S, et al. Single-cell transcriptome analysis reveals dynamic cell populations and differential gene expression patterns in control and human aortic tissue. Cell. 2009;137:647–658. 10.1016/j.cell.2009.02.038

81. Wang Y, Nanda V, Dierroz D, Ye J, Xia, S, Kojima Y, Howe KL, Jarru K, Flores AM, Tsantilas P, et al. Clnally expanding smooth muscle cells promote atherosclerosis by escaping effector/cytosis and activating the complement cascade. Proc Natl Acad Sci USA. 2020;117:15818–15826. 10.1073/pnas.2006848117

82. Lehnars M, Dobrowskis H, Feil S, Feil R. cGMP signalization and vascular smooth muscle cell plasticity. J Cardiovasc Dev Dis. 2018;5:401–404. 10.1007/s12265-018-09961-y

83. Li Y, Xiao, H, Huang L, Zhou F, Chen LH, Zhao YY, Qu SL, Zhang C. Role of kruppel-like factor 4 in atherosclerosis. Clin Chim Acta. 2021;512:135–141. 10.1016/j.cca.2020.11.002

84. Kedi X, Ming Y, Yongping W, Yi Y, Xiaoxiang Z. Free cholesterol overloading alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death Differ. 2020;27:109824. 10.1016/j.cdd.2020.109824

85. Karamariti E, Zhai C, Yu B, Qiao L, Wang Z, Potter CMF, Wong MM, et al. Genomic profiling of human vascular cells identifies TWIST1 as a causal gene for common vascular diseases. PLoS Genet. 2020;16:e1008538. 10.1371/journal.pgen.1008538

86. Nicoll R, Henein MY. The predictive value of arterial and valvular calcification during hyperphosphatemia. Cell Mol Life Sci. 2019;76:2077–2091. 10.1007/s00018-019-03054-z

87. Nekolla S, Henein MY. The predictive value of arterial and valvular calcification for mortality and cardiovascular events. Int J Cardiovasc Heart Vessel. 2014;3:1–5. 10.1016/j.jcvh.2014.02.001

88. Nurnberg ST, Guerreya MA, Wirka RC, Rao HS, Piraj M, Norton S, Serrano F, Perisic L, Elwyn S, Pluta J, et al. Genomic profiling of human vascular cells identifies TWIST1 as a causal gene for common vascular diseases. PLoS Genet. 2020;16:e1008538. 10.1371/journal.pgen.1008538

89. Oishi Y, Manabe I. Macrophages in inflammation, repair and regeneration. Int Immunol. 2018;30:511–528. 10.1093/intimm/dxy054

90. Jinnouchi I, Kato T, Akasaka T, Sato H, Kajiwara M, Komori H, Kawasaki K, Kawanami T, Fukuyama R, Mori M, Yamana K, Nakamura K, et al. SP7 inhibits osteoblast differentiation as a systemic regulator. J Bone Miner Res. 2015;30:1566–1577. 10.1002/jbmr.2332

91. Bennett MR, Sinha S, Owens GK. Vascular smooth muscle cells in atherosclerosis. Circ Res. 2016;118:692–702. 10.1161/CIRCRESAHA.115.306361
patterns of expression in human arterial calcification. Arterioscler Thromb Vasc Biol 2003;23:489–494. doi: 10.1161/01.ATV.0000054046.92165.31

112. Loebel C, Czekanowska EM, Bruderer M, Salzmann G, Alini M, Stoddart MJ. In vitro osteogenic potential of human mesenchymal stem cells is predicted by Runx2/Sox9 ratio. Tissue Eng Part A. 2015;21:115–123. doi: 10.1089/ten.TEA.2014.0096

113. Lin ME, Chen TM, Walldorf MC, Nyugen NB, Yamada S, Savangmalee C, Zhang J, Speer MY, Giachelli CM. Runx2 deletion in smooth muscle cells inhibits vascular osteochondrogenesis and calcification but not atherosclerotic lesion formation. Cardiovasc Res. 2016;112:606–616. doi: 10.1093/cvr/crv205

114. Lin ME, Chen T, Leaf EM, Speer MY, Giachelli CM. Runx2 expression in smooth muscle cells is required for arterial medial calcification in mice. Am J Pathol 2015;185:1958–1969. doi: 10.1016/j.ajpath.2015.03.020

115. da Silva RA, da S Feltran G, da C Fernandes CJ, Zambuzzi WF. Osteogenic

116. Tyson J, Bundy K, Roach C, Douglas H, Ventura V, Segars MF, Schwartz O,

117. Yoshida T, Yamashita M, Hayashi M. Krüppel-like factor 4 contributes to

118. Zhu L, Zhang N, Yan R, Yang W, Cong G, Yan N, Ma W, Hou J, Yang L, Jia S.

119. Gurusinghe S, Bandara N, Hilbert B, Trope G, Wang L, Strappe P. Lentiviral

120. Long JZ, Svensson KJ, Tsai L, Zeng X, Roh HC, Kong X, Rao RR, Lou J,

121. Gaspar RC, Pauli JR, Shulman GI, Muñoz VR. An update on brown adipose

122. Villarroya F, Cereijo R, Villarroya J, Giralt M. Brown adipose tis-

123. Shamsi F, Piper M, Ho LL, Huang TL, Gupta A, Streets A, Lynes MD,

124. Birsoy K, Chen Z, Friedman J. Transcriptional regulation of adipogenesis by

125. Xie XQ, Wan Y. A novel small-molecule antagonizes PRMT5-mediated KLF4 methylation for targeted therapy. EBioMedicine. 2019;4:498–111. doi: 10.1016/j.ebiom.2019.05.011

126. Sur S, Swier VJ, Radwan MM, Agrawal DK. Increased expression of phospho-

127. Hien TT, Garcia-Vaz E, Stenkula KG, Sjögren J, Nilsson J, Gomez MF,

128. Mao Q, Quan T, Luo B, Guo X, Liu L, Zheng Q. MiR-375 targets KLF4

129. Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, Paruchuri K,

130. Schwill S, Seppelt P, Jugold M, Ruhparwar A, Robinsson PN, Karck M, Kallenbach K. The fibrilin-1 hypomorph mgf/mgf murine model of Marfan syndrome shows severe elastolysis in all segments of the aorta. J Vasc Surg. 2013;57:1629–36, 1636.e1. doi: 10.1016/j.jvascsurg.2012.10.007

131. Zhou Z, Feng Z, Hu D, Yang P, Gur M, Bahar I, Cristofanilli M, Gradishar WJ,

132. Klein D, Weisshardt P, Kleff V, Jastrow H, Jakob HG, Ergün S. Vascular

133. Mai J, Zhong ZY, Guo GF, Chen XX, Xiang YQ, Li X, Zhang HL, Chen YH,

134. Hanna J, Hossain GS, Kocerha J. The potential for microRNA therapeu-

135. Hien TT, Garcia-Vaz E, Stenkula KG, Sjögren J, Nilsson J, Gomez MF,

136. Kuhn AR, Schlauch K, Lao R, Halayko AJ, Gerthoffer WT, Singer CA.

137. Hien TT, Garcia-Vaz E, Stenkula KG, Sjögren J, Nilsson J, Gomez MF,

138. Mao Q, Quan T, Luo B, Guo X, Liu L, Zheng Q. MiR-375 targets KLF4 and impacts the proliferation of colorectal cancer. Tumour Biol. 2016;37:463–471. doi:10.1007/s13277-015-3809-0

139. Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, Paruchuri K, Mahabeshwar GHT, Dalmas E, Venteflec N, et al. Krüppel-like factor 4 regulates macrophage polarization. J Clin Invest. 2011;121:2736–2749. doi: 10.1172/JCI45444

140. Ou L, Shi Y, Dong W, Liu C, Schmidt TJ, Nagarkatti P, Nagarkatti M, Fan D, Al W. Kruppel-like factor KLF4 facilitates cutaneous wound healing by promoting fibrocyte generation from myofibro-dened suppressor cells. J Invest Dermatol. 2015;135:1345–1343. doi: 10.1097/jid.2015.3

141. Dale M, Fitzgerald MP, Liu Z, Messinger T, Karpisek A, Purcell LN, Carlson JS, Harding P, Lang H, Koutakis P, et al. Premature aortic smooth muscle cell differentiation contributes to matrix dysregulation in Marfan Syndrome. PLoS One. 2017;12:e0186603. doi:10.1371/journal.pone.0186603

142. Crossas-Molist E, Meirelles T, López-Luque J, Serra-Peinado C, Selva J, Caja L, Gorbenko Del Blanco D, Uriarte JJ, Bertran E, Mendizábal Y, et al. Vascular smooth muscle cell phenotypic changes in patients with Marfan syndrome. Arterioscler Thromb Vasc Biol. 2015;35:960–972. doi: 10.1161/ATVBAHA.114.304412

143. Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: evidence for CD34 as a common marker for diverse progenitors. Stem Cells. 2012;30:1380–1389. doi: 10.1002/stem.1661

144. Klein D, Weisshardt P, Klett V, Jastrow H, Jakob HG, Ergün S. Vascular wall-resident CD44+ multipotent stem cells give rise to pericytes and smooth muscle cells and contribute to new vessel maturation. PLoS One. 2011;6:e20540. doi: 10.1371/journal.pone.0020540

145. dos Anjos Cassado A. F4/80 as a major macrophage marker: the case of the peritoneum and spleen. In: Kloc M, ed. Macrophages: Origin, functions and biointerfaces. Springer International Publishing; 2017:161-179.