Chapter 13

The Dichotomy of Vascular Smooth Muscle Differentiation/De-Differentiation in Health and Disease

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Abstract

Vascular smooth muscle cells (SMCs) are thought to display cellular plasticity by alternating between a quiescent 'contractile' differentiated phenotype and a proliferative 'synthetic' de-differentiated phenotype in response to induction of distinct developmental pathways or to local micro-environmental cues. This classic de-differentiation and re-programming process is associated with a significant loss in the expression of key SMC differentiation marker genes for a large number of proliferative vascular diseases *in vivo* and in sub-cultured cells *in vitro*. Regarded as essential for vascular regeneration and repair *in vivo*, phenotypic modulation represents a critical target for therapeutic intervention. However, recent evidence now suggests that this process of vascular regeneration may also involve differentiation of resident vascular stem cells and the accumulation of stem cell-derived myogenic, osteochondrogenic and macrophage-like phenotypes within vascular lesions *in vivo* and across sub-cultured SMC cell populations *in vitro*. This review summarises our current knowledge of vascular regeneration, de-differentiation and re-programming of vascular SMCs, and focuses on the accumulating evidence of a putative role for stem cell-derived progeny and the evolving dichotomy of the origin of SMC-like cells during intimal-medial thickening and the progression of arteriosclerotic disease.

Keywords: smooth muscle cells, differentiation, re-programming, vascular stem cells, atherosclerosis, arteriosclerosis, intimal-medial thickening, Cre-LoxP, lineage tracing, epigenetics

1. Introduction

Vascular smooth muscle cells (SMCs) comprise the medial layer of arterial and venous blood vessels. Through their contraction or relaxation, SMCs control vascular tone and blood flow, thereby playing a fundamental role in the regulation of blood pressure and the delivery of dietary nutrients and oxygen throughout the body [1]. Historically, SMCs are widely reported to have significant cellular plasticity. They are capable of de-differentiation to a more synthetic state and undergo re-programming to myogenic, osteochondrogenic, and macrophage-like phenotypes [2, 3]. This phenotypic switch also occurs when SMCs are cultured *in vitro* [4], and is commonly associated with vascular injury and disease [5], and hypertension [6] *in vivo* that leads to a (re)stenosis of the vessel lumen. In particular, SMCs within arteriosclerotic lesions exhibit proliferative, migratory and extracellular matrix (ECM) secretory capacities, indicative of their phenotypic switch to a de-differentiated phenotype [7]. In diseases such as atherosclerosis, SMCs can also assume a foam cell phenotype, typical of the sub-intimal macrophage-derived foam-like cells following exposure to cholesterol and oxidised LDL *in vitro* [8] and *in vivo* [9]. This display of different phenotypic identities is due to an inherent ability to respond effectively to numerous micro-environmental cues and extracellular and intracellular stimuli [5]. Moreover, this phenotypic switch can occur in response to aberrant signalling inputs and changes in contractile SMC gene expression associated with vascular pathology and requires integration of key transcriptional, metabolic and ultrastructural programs [10]. Indeed, several growth factors [platelet-derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor beta 1 (TGF-β1) are important regulators of SMC phenotype in part by modulating autophagic activity [11].

Notwithstanding the 40-year-old phenomenon of SMC de-differentiation and phenotypic switching and the significant evidence of a putative role for de-differentiated SMCs in vascular disease [12, 13], there is now compelling evidence to suggest that lesional cells within the adventitial, medial and (neo)intimal layers of arteriosclerotic vessels may also be derived from resident vascular stem cells following iatrogenic injury in rodent models [14–18] and in human arteriosclerotic tissue [19, 20]. Indeed, recent lineage tracing analysis of genomic marked stem cells supports this contention [18, 21, 22]. There is also evidence that circulating progenitor cells home to sites of vascular injury with subsequent differentiation into various cell lineages [16, 17, 23]. Within the early vasculature, progenitor cells are recruited to an endothelial tube and traverse intermediate stages of phenotypic adaptation during development from embryonic to adult SMCs [24]. These progenitors remain within the vessel wall throughout life with the potential to become synthetic SMC-like cells and other cell types within the local vascular micro-environment of diseased vessels [25].

Therefore, a major driver of phenotypic change (whether de-differentiation/re-programming of differentiated SMCs and/or differentiation of stem cells and the generation of stem-cell derived progeny) is the relative level of various transcriptional and post-transcriptional regulatory effectors of myogenic, vasculogenic, osteochondrogenic and macrophage-like phenotypes [13, 26–28]. This is further influenced by the modulatory role of various microRNAs (miRs) [29], and other lineage-restricted regulatory effectors [30] that impact on SMC differentiation *in vivo* and in cultured cells *in vitro* [31].

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2. Differentiated vascular smooth muscle cells (SMCs)

SMCs provide important structural integrity for stabilisation during embryonic and postnatal development and facilitate the distribution of blood throughout the adult circulation (**Figure 1**) [32]. Differentiated adult contractile SMC express a number of cell-specific contractile genes for this purpose including myosin heavy chain 11 (Myh11), calponin 1 (Cnn1) and alpha actin (Acta2) that encode for proteins critical to regulating vessel diameter dynamically via contraction and relaxation in response to key vasoactive stimuli [13]. They have a low rate of turnover (less than 0.1%) within the normal healthy vessel wall [32] and are closely associated with a dynamic ECM that provides important structure thereby promoting a quiescent, non-migratory phenotype that facilitates cell contraction [33]. SMCs themselves provide all the surrounding matrix proteins (collagen, elastin) required for this structural support. Endothelial cells (ECs) connect to SMCs through myoendothelial junctions (MEJs) that penetrate the internal elastic lamina [34] and facilitate transport of solutes and other molecular mediators between SMC and the overlying endothelium [34].

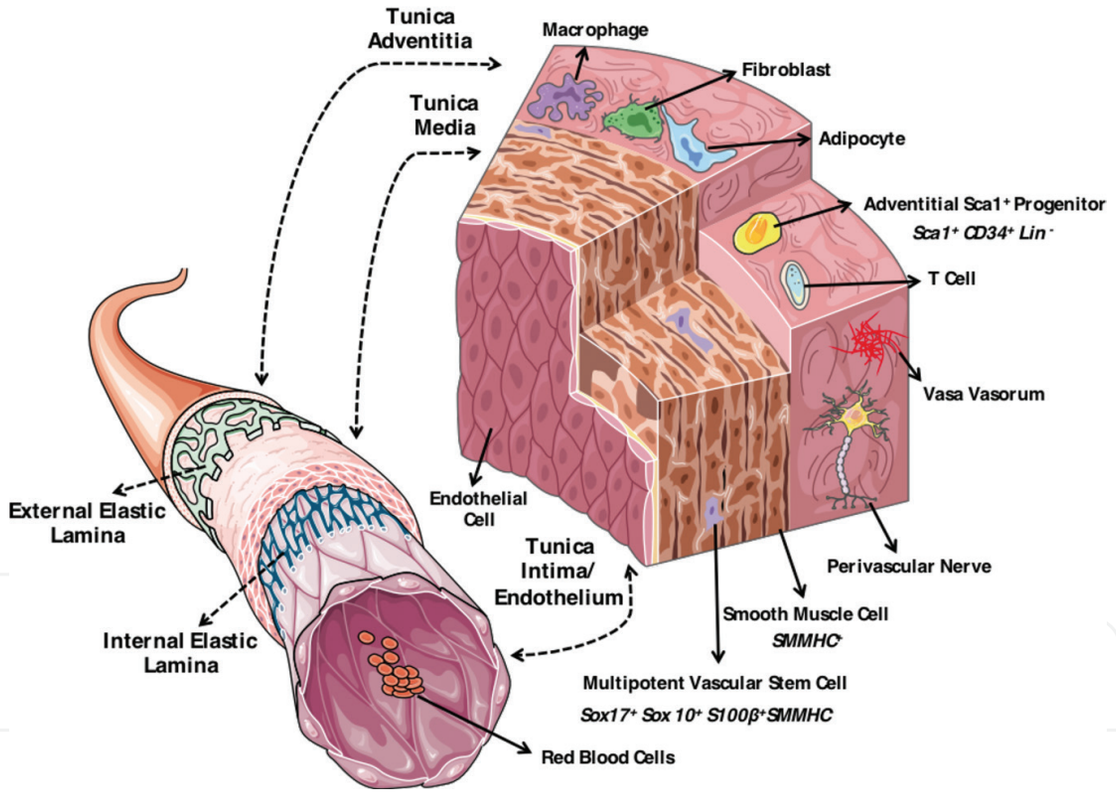

Figure 1. Schematic diagram of a murine blood vessel wall consisting of a tunica intima (endothelial), tunica media (SMCs) and tunica adventitia (fibroblasts) housing differentiated cells in addition to various undifferentiated resident stem cell populations including medial Sox10/Sox17/S100β$^+$ multipotent vascular stem cells (MVSCs) and adventitial Sca1$^+$ progenitors [19, 23].

3. Transcriptional control of SMC differentiation genes

Control of SMC differentiation is a complex process involving the co-operative interaction of many different key factors [10]. Vascular SMC differentiation is determined primarily by serum

response factor (SRF) and myocardin (MYOCD) (**Figure 2**) [36]. They collectively directly promote SMC differentiation through binding to CArG elements and activation of the corresponding SMC differentiation genes. The level and activity of SRF-MYOCD dictates the transcriptional switch for a growing number of SMC differentiation genes [37]. A common feature of these genes is the presence of a 10 base pair cis-element known as a CArG box (consensus is CCW6GG, where W can be either A or T). These CArG sequences are recognised and bound by SRF and many of the genes restricted to SMC are dependent upon SRF activity. SRF recruits a number of coactivators that modulate the binding of CArG-SRF around those SMC genes to maintain cellular homeostasis. MYOCD, a cardiac- and SMC-restricted gene selectively transactivates CArG-containing contractile genes through a physical association with the MADS domain of SRF [38]. SRF-MYOCD is highly active over genes containing multiple CArG elements, yet the presence of multiple CArG sites does not always indicate functionality in SRF-MYOCD [39] as additional coding information in DNA may attenuate SRF-MYOCD transcriptional activation, probably through structural changes in CArG-SRF leading to sub-optimal MYOCD binding. The putative role of MYOCD in dictating SMC phenotype is confirmed by several loss-and-gain-of-function studies that demonstrate any non-SMC type overexpressing MYOCD is converted into a SMC-like state [40]. There are two other related myocardin genes [Myocardin-related transcription factor A (MRTFA) and B (MRTFB)] that have similar SRF-dependent functions and are widely expressed but are under different control processes than MYOCD [41].

In addition to SRF-MYOCD complexes, microRNAs (miRs) represent another class of regulatory effectors of SMC differentiation (**Figure 2**) [40, 42]. These non-coding RNAs function as molecular regulators by lowering protein levels through mRNA degradation, mRNA de-adenylation, or translational repression [43]. Several miRs have been defined in SMCs (miR-143/145, miR-1, miR-21, miR-221, miR-146a, miR-24, and miR-26a) [44] but the major regulatory effector for SMC differentiation is the miR-143/145 bicistronic cluster [31]. The miR-145 targets include the Kruppel-like factor 4 (KLF4) and 5 (KLF5) to direct SMC differentiation and are sufficient to promote myogenic differentiation while reducing the proliferative response to growth factors [31]. In addition, there are several parallel transcriptional

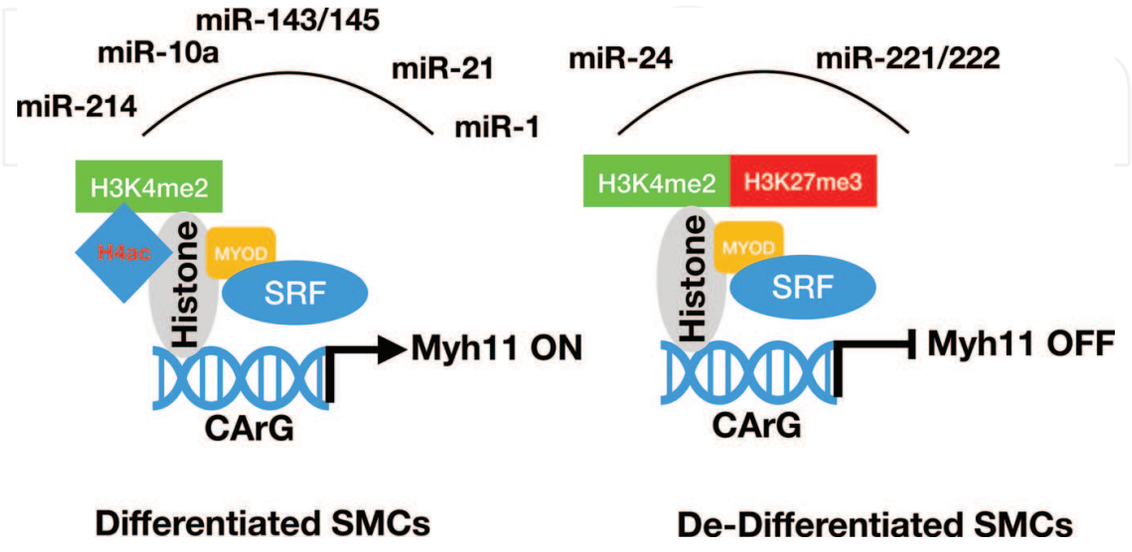

Figure 2. Cartoon summarising the transcriptional regulation of the Myh11 promoter by SRF, MYOCD and miRs at CArG sites in mature SMCs and phenotypically modulated de-differentiated SMCs [35].

(or post-transcriptional) pathways that control SMC differentiation including the histidine-rich calcium-binding protein (HRC) gene that lacks functional CArG elements but contains a conserved binding site for the MEF2 family of transcription factors [45]. Similarly, (Heart And Neural Crest Derivatives Expressed 2 (HAND2), intracellular Notch receptor domains (NICD), SMADs and a large number of zinc finger-containing transcription factors have also been implicated in SRF-MYOCD independent control of SMC-specific genes [42, 46].

4. Signalling pathways that control SMC differentiation

The process of SMC differentiation is dependent on diverse stimuli that include growth factors, ECM, miRs, epigenetic modifiers, and mechanical forces [14]. The most widely recognised stimulators of SMC differentiation are TGF-β1 [47], PDGF-BB [48] and the Notch [49] and Hedgehog [50, 51] signalling pathways. TGF-β1 is a potent multifunctional soluble cytokine that exists in at least three isoforms, TGF-β1, -2 and -3. *In vivo*, loss-and-gain-of function studies clearly associate TGF-β ligands with early embryogenesis, vasculogenesis, angiogenesis, haematopoiesis and cell adhesion [47]. TGF-β signalling can also cause nonhereditary disorders like atherosclerosis and cardiac fibrosis [47]. *In vitro*, TGF-β1 promotes vascular myogenic differentiation of embryonic stem cells (ESCs) and the maturation of mural cells by positive regulation of SMC differentiation genes through the Smad2 and Smad3 dependent pathways and the Notch signalling pathway [52–54]. PDGF is another key modulator of SMC differentiation. It exists in five isoforms (PDGF-A, PDGF-B, PDGF-C, PDGF-D and PDGF-AB homo- or hetero-dimmer including PDGF-AA, PDGF-BB and PDGF-AB), and is mainly derived from platelets upon activation. PDGF-BB transduces its signal via specific tyrosine kinase receptors, PDGFR-α and PDGFR-β and acts as a potent mitogen. PDGF-BB promotes myogenic differentiation of stem cell antigen 1 $(Sca1)^+$ progenitor stem cells via PDGFR-β-mediated signalling [23]. Both TGF-β1 and PDGF-BB can also have a negative effect on SMC differentiation by the repression of SMC markers [48, 55]. These effects are cell density dependent and are mediated by Smad3 and ETS1, respectively.

Notch and hedgehog ligands are integral to SMC differentiation and arterial identity during development [56, 57]. Notch signalling can either promote [52, 53] or inhibit [54, 58] SMC differentiation depending on the origin of the SMC and/or progenitor [59]. Although the precise mechanism(s) of Jagged-1/Notch-induced SMC differentiation is still poorly understood, a number of studies have systematically investigated the molecular pathways leading to the pro-differentiation and pro-proliferative effects of Notch signalling in SMCs [49, 60]. In a similar manner, Hedgehog signalling promotes SMC growth via a Notch-dependent mechanism [51], while hedgehog ligands promote SMC differentiation of SMC progenitors [50, 55, 61, 62].

5. Epigenetic control of SMC differentiation genes

Epigenetics refers to heritable changes in gene expression that occur independent of changes in the genomic sequence due to environmental influences [63]. Epigenetic mechanisms play an important role in the regulation of chromatin structure and remodelling during SMC

differentiation (**Figure 3**). Chromatin is composed primarily of genomic DNA and protein. The nucleosome is the fundamental unit of chromatin encompassing 146 base pairs of DNA wrapped around an octamer of histone proteins. This octamer contains two copies each of histones H2A, H2B, H3, and H4. The histone N-terminal tails are not bound to the nucleosome core and may undergo post-translational modifications including acetylation, phosphorylation, ubiquitination, and ADP-ribosylation [63]. Post-translational modifications of histone proteins alter chromatin conformation and thereby control the manner in which key transcription factors bind DNA, resulting in the activation or silencing of gene transcription. Two of the most extensively studied epigenetic changes during SMC differentiation are histone modifications (which alter the packaging of the chromatin) and DNA methylation (occurring at the 5′-cytosine in CpG dinucleotides).

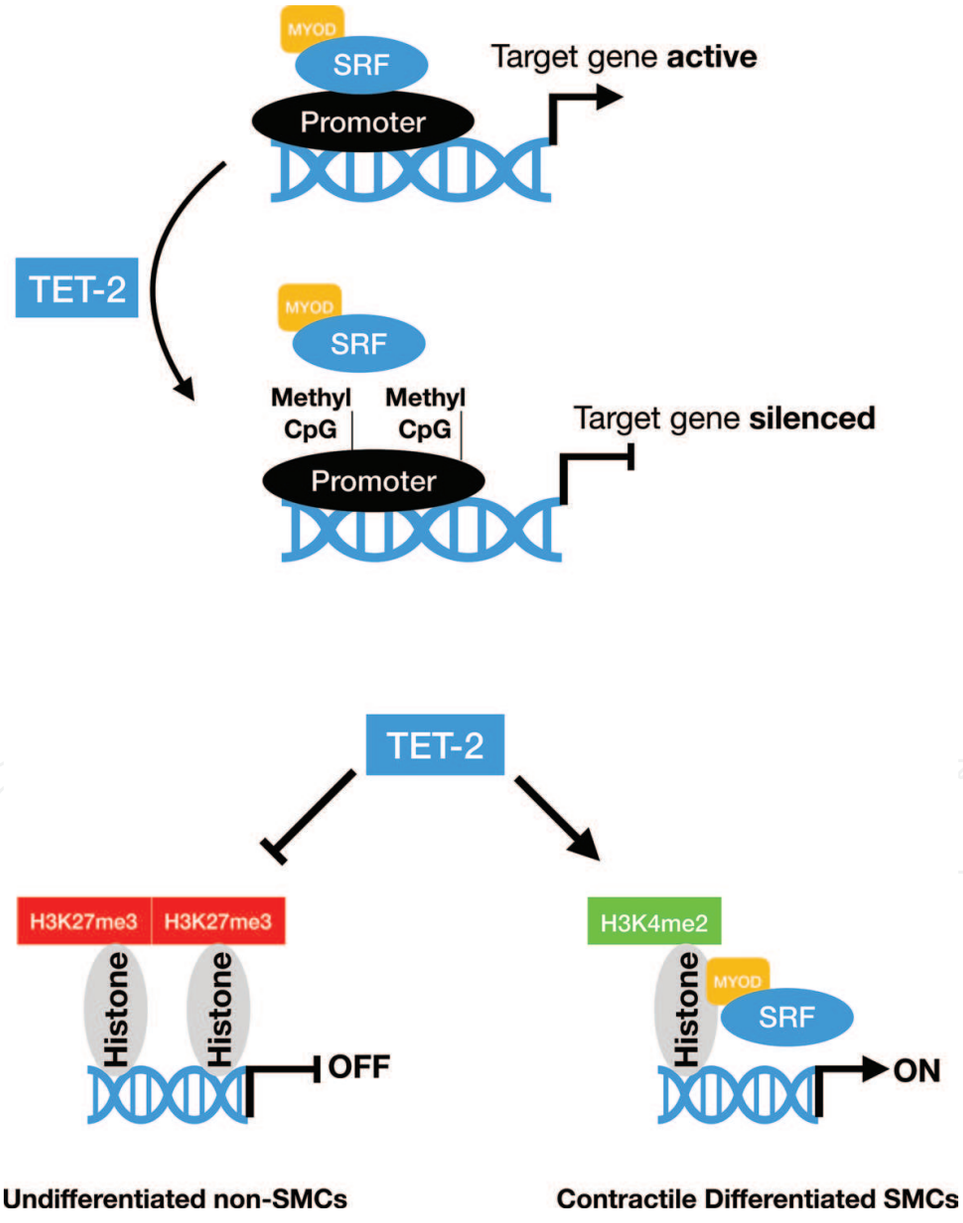

Figure 3. Cartoon summarising the epigenetic regulation (histone modifications and demethylation of DNA) of SMC promoters at CArG sites in mature SMCs and undifferentiated non-SMCs [10].

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Histone modifications alter the higher order chromatin structure through attraction or repulsion of charged histone tails thereby regulating nucleosome density and the accessibility of various cis promoter–enhancer control elements. For instance, changes in chromatin accessibility, mediated in part by histone modifications, render SMC marker genes permissive for subsequent activation by the SRF/myocardin complex during SMC differentiation [64]. Histone acetylation is a potent mechanism of gene activation and occurs on Myh11 and Acta2 gene loci early in the process of myogenic differentiation from SMC precursors. SRF binds only to the SMC marker genes that have been enriched in histone acetylation. Moreover, inhibition of histone acetyl transferases (HATs) or expression of histone deacetylases (HDACs) leads to decreased SMC marker gene promoter activity in cultured SMCs [58, 65]. In addition, there is also a marked enrichment of the histone modification, di-methylation of lysine 4 on H3 (H3K4me2), on key SMC marker genes, including Myh11, Acta2, and Transgelin (Sm22α), in both mature SMC [66] and more notably SMC progenitor cells committed to myogenic differentiation [60]. The methylation of H3K4 is partially attributed to the recruitment of WD repeat-containing protein 5 (WDR5) and the associated histone lysine methyltransferase SET/MLL by paired-like homeodomain transcription factor 2 (Pitx2) to SMC promoters in early stages of differentiation [67]. In contrast, this enrichment was absent in ESCs and non-SMC cells [66]. Moreover, H3K4me2 enrichment may occur in the absence of binding of SRF to CArG elements facilitating a tethering of MYOCD to H3K4me2-modified histone tails that stabilise binding of the SRF–MYOCD to CArG regions (**Figure 3**) [66]. A novel *in vivo* assay combining in situ hybridization and a proximity ligation assay provides further evidence that the H3K4me2 mark at the Myh11 locus is restricted to differentiated SMCs *in vivo* [68]. Similarly, a histone H3 lysine 4 mono-methylation (H3K4me1) catalysed by the Set7 lysine methyltransferase is considered a further hallmark of transcriptionally active chromatin [69]. Recent transcriptional network analyses has revealed that SMC differentiation genes are also subject to Set7-mediated regulation [69]. Hence, several cell-specific epigenetic mechanisms govern the expression of cellular markers during SMC differentiation.

DNA methylation is the most widely characterised epigenetic modification linked to gene silencing [70]. In mammalian cells, DNA methylation occurs at the 5′ position of the cytosine ring through the actions of the DNA methyltransferases DNMT1, DNMT3A and DNMT3B [70]. Several *in vivo* and *in vitro* studies have reported a role for DNA methylation and gene silencing of some key SMC differentiation genes during disease progression and changes in SMC phenotype. However, more evidence is required to determine whether DNA methylation directly plays a causal role in this process (**Figure 3**) [71]. Nevertheless, the mechanisms of DNA demethylation, albeit controversial have recently been described during SMC differentiation and centre on the putative role of the ten-eleven-translocation (TET) family of enzymes that oxidise 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) in SMC [72]. Through the DNA repair pathway and thymine-DNA glycosylase (TDG), 5hmC is then converted to unmethylated cytosine, leading to DNA demethylation and gene activation. TET-2 is the predominant isoform in human coronary artery SMC cultures, and is highly enriched in smooth muscle tissues. TET-2 expression increased following myogenic differentiation and was significantly reduced following PDGF-BB-stimulated de-differentiation. Parallel loss-and-gain-of-function studies confirm the putative role TET-2 induction of SMC differentiation genes (MYOCD, Myh11, Acta2, and Sm22α) by modifying histone methylation

(H3K4me2) contributing to regulation of these genes [72]. Notably, while 5hmC was initially thought to serve only as a transient intermediate in the process of demethylation [73], this epigenetic mark may persist in quiescent, differentiated SMC *in vivo* to play a role in self-renewal and lineage commitment [71].

6. De-differentiated vascular smooth muscle cells (SMCs)

When vascular regeneration occurs after iatrogenic injury, de-differentiated SMCs are thought to promote Intimal-medial thickening (IMT) and participate in the formation of neointima by decreasing the expression of contractile proteins and increasing proliferation, migration and matrix protein synthesis [74, 75]. Similarly, during various disease states such as arteriosclerosis and atherosclerosis, the recruited SMCs also acquire a synthetic de-differentiated phenotype in the course of lesion formation [12]. Indeed, IMT is present in human arteries before atherosclerosis develops, particularly in the atherosclerosis-prone arteries such as coronary arteries and aorta [76].

The paradigm of de-differentiation, phenotypic switching and re-programming of SMCs was first proposed to explain the phenotypic changes that occur when differentiated contractile medial SMCs are isolated and grown in culture [2, 3].

PDGF-BB is considered the major stimulus for SMC de-differentiation *in vitro* [48]. Sub-cultured SMCs from a variety of species lose their expression of SMC differentiation markers (MYH11, CNN1 and SM22α) and acquire an extensive rough endoplasmic reticulum/Golgi system to facilitate SMC migration and proliferation concomitant with an increase in cytoskeletal proteins such as non-muscle myosin (Myh10) and vinculin [2]. Decreases in microRNAs such as miR-143/145 can also occur resulting in a less contractile phenotype [31]. The expression of MYOCD is further consistently reduced in several *in vitro* models of cultured SMC when compared to fresh aortic tissue [36] while ectopic expression of MYOCD results in partial recovery of the SMC differentiated phenotype in phenotypically modified SMCs [77]. SMC phenotypic switching is therefore primarily a function of reduced MYOCD expression with little understanding of the molecular mechanisms underlying this phenomena *in vitro* or *in vivo*.

Epigenetically, SMCs in culture have been extensively characterised following treatment with PDGF-BB and other stimulators of SMC de-differentiation [10]. Although the acetylation of histones is diminished during the de-differentiation process, the H3K4me2 epigenetic mark at the Myh11 locus persists through SMC phenotypic modulation [68], albeit at a lower level when compared to fresh aorta [60], concomitant with reduced expression of the SMC differentiation marker, Myh11 (**Figure 3**) [60]. Importantly, the maintenance of this epigenetic mark in cultured SMCs *in vitro* has been widely used to purport that SMCs in culture are derived from a differentiated parent population *ex vivo* [68].

Another important factor that dictates SMC phenotypic switching is the transcription factor KLF4. KLF4 factors are expressed in phenotypically modulated SMCs and bind to G/C-rich cis elements found in SMC marker gene promoters. KLF4 is not expressed in differentiated SMCs in normal blood vessels but is rapidly induced following vascular injury [78]. The effects of KLF4 in suppressing SMC differentiation marker gene expression are mediated through

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epigenetic changes associated with transcriptional silencing, including reduced H4 acetylation mediated through KLF4-dependent recruitment of HDAC2 and HDAC5, and nearly complete loss of SRF binding to the SMC promoter CArG elements within intact chromatin. PDGF-BB mediates compaction of chromatin at SMC differentiation gene loci through KLF4 recruitment of HDAC2, HDAC4, or HDAC5 to CArG regions on the Acta2 and Myh11 promoters, thereby reducing histone acetylation and inhibiting the accessibility of this region to the transcription factors MYOCD, SRF, and MRTF [48, 66]. Overexpression of KLF4 in cultured SMCs also results in profound activation of multiple induced pluripotential stem (iPS) cell pluripotency factors, including the POU domain transcription factor, Oct4 and the transcription factor, SRY (sex determining region Y)-box 2 (Sox2), but not Nanog, suggesting that SMC phenotypic switching involves the activation of multiple pluripotency genes in addition to KLF4 [79].

Differentiated medial SMCs also undergo dramatic phenotypic changes following vascular injury comparable to the changes observed in sub-cultured SMCs (e.g., reduced SMC differentiation marker expression) [2, 3, 80]. Following injury, the cytoskeleton becomes perturbed resulting in defective organisation of cytoskeletal-contractile proteins, SMC apoptosis and the release of SMC- and matrix-associated growth factors concomitant with plasma- and platelet-derived factors that impact on the surviving SMCs [81]. Platelet-derived factors (e.g., PDGF-BB) are considered crucial to SMC phenotypic switching and re-programming of differentiated SMCs *in vivo* through binding and activation of surface receptors present on SMCs [35]. These structural, molecular and physiological changes in medial SMCs are considered pivotal to the development of neointimal lesions following vascular injury.

It is clear that SRF levels do not significantly change in SMCs following arterial injury but reflect an association with different coactivators (such as the ETS domain-containing protein ELK1) that direct new programmes of immediate early gene expression (e.g., c-fos, jun) [42, 82]. These genes are not normally expressed in differentiated SMCs as SRF is bound to MYOCD but result in the secondary activation of delayed response genes, including growth factors that act in an autocrine/intracrine fashion to stimulate SMC cell cycle entry and migration [82]. The significant decrease in SMC differentiation marker gene expression following vascular injury results from a decrease in MYOCD and miR-145 both of which are reduced following vascular injury [83, 84]. This change in MYOCD stabilises ELK1 binding to SRF and is thought to facilitate SRF binding to a different set of CArG-dependent genes that promote phenotypic switching of the remaining medial differentiated SMCs following injury. Thus, SRF in neointimal de-differentiated cells may engage a new set of CArG-dependent genes different from those in medial SMCs [42]. Indeed, over-expression of MYOCD mitigates against SMC phenotypic switching following vascular injury [77]. Similarly, KLF4 mediates its effects at least in part by inducing epigenetic changes of SMC marker gene loci associated with the formation of heterochromatin and transcriptional silencing [10]. Moreover, gene expression profiling of differentiated SMCs versus neointimal 'de-differentiated' cells has revealed distinct molecular phenotypes between these two cell populations [85]. In particular, a subset of differentiated SMCs is thought to revert and re-programme to a more primitive phenotype characterised by the expression of so-called 'embryonic' genes (e.g., tropoelastin, osteopontin, PDGF-BB) that promote growth and migration of SMCs typical of intimal medial thickening [85, 86].

Vascular injury-induced de-differentiation *in vivo* is accompanied by significant changes in the epigenetic profile of these cells. Specifically, there is a decrease in H3 acetylation at the Sm22α

promoter concomitant with the binding of a complex consisting of KLF4, ELK1, and HDAC2 [87]. This complex was contingent on a G/C repressor element found in many CArG-dependent SMC genes. Similarly, a transient decrease in H4 acetylation has been reported at the Acta2 and Myh11 promoters following injury [66]. The importance of this epigenetic change is confirmed using HDACs inhibitors that attenuated SMC dedifferentiation and neointima formation following injury [88]. A further common feature of SMC phenotypic switching following vascular injury *in vivo* in murine models is the persistence of H3K4me2 mark on specific SMC gene promoter loci despite silencing of these genes following injury due to the loss of SRF-MYOCD binding, the formation of heterochromatin, and the loss of H3/H4 hyper-acetylation [10]. Recent studies have since confirmed that neointimal cells from human lesions retain the H3K4me2 mark at the Myh11 promoter suggesting that these cells may also be derived from a differentiated medial SMC that re-programmed following phenotypic switching [68]. This raises the possibility, if true, that H3K4me2 may serve as a mechanism of epigenetic cell lineage memory, i.e., a mechanism for phenotypically modulated SMCs to remain permissive for de-differentiation and re-programming during reversible phenotypic switching [89].

Indirect evidence from several groups has strongly supported the apparent contribution of mature differentiated SMCs that undergo phenotypic switching during the progression of IMT and arteriosclerotic lesions, including neointima formation after endothelial injury, vein graft arteriosclerosis and native atherosclerosis [10]. The more compelling recent lineage tracing studies using tamoxifen-inducible Myh11-CreER mice to mark Myh11 differentiated SMCs before injury have provided further evidence of SMC phenotypic switching and re-programming *in vivo* [7, 90, 91]. These data are consistent with the longstanding view that differentiated SMCs undergo injury-induced SMC phenotypic switching with onset of cell proliferation. However, many cells not of SMC origin have also been identified within atherosclerotic lesion [9].

Collectively, these data suggest that SMCs may acquire mechanisms that reactivate certain pluripotency gene networks as a means of increasing their cellular plasticity and enhancing regenerative processes critical for survival following injury [10].

7. The role of resident vascular stem cells in intimal-medial thickening (IMT)

The accumulation of 'de-differentiated' SMC-like cells within the intima was initially proposed as a key event in the development of arteriosclerosis [92]. As outlined above, compelling support for this view comes from recent lineage tracing studies of genomic marked Myh11 differentiated SMCs using Cre-LoxP transgenic animals (**Figure 4**) [7, 68, 90, 91]. A discrete subpopulation of Myh11 medial SMCs appear responsible for the vast majority of neointimal cells within the vessel wall following injury or disease [7]. If correct, the existence of such a subpopulation is to be expected since the majority (>70%) of medial cells are lost by apoptosis following mechanical-induced injury [93, 94]. This early apoptotic response is considered vital for the progression of IMT since inhibition of SMC apoptosis results in a significant reduction in IMT, independent of re-endothelialization [95].

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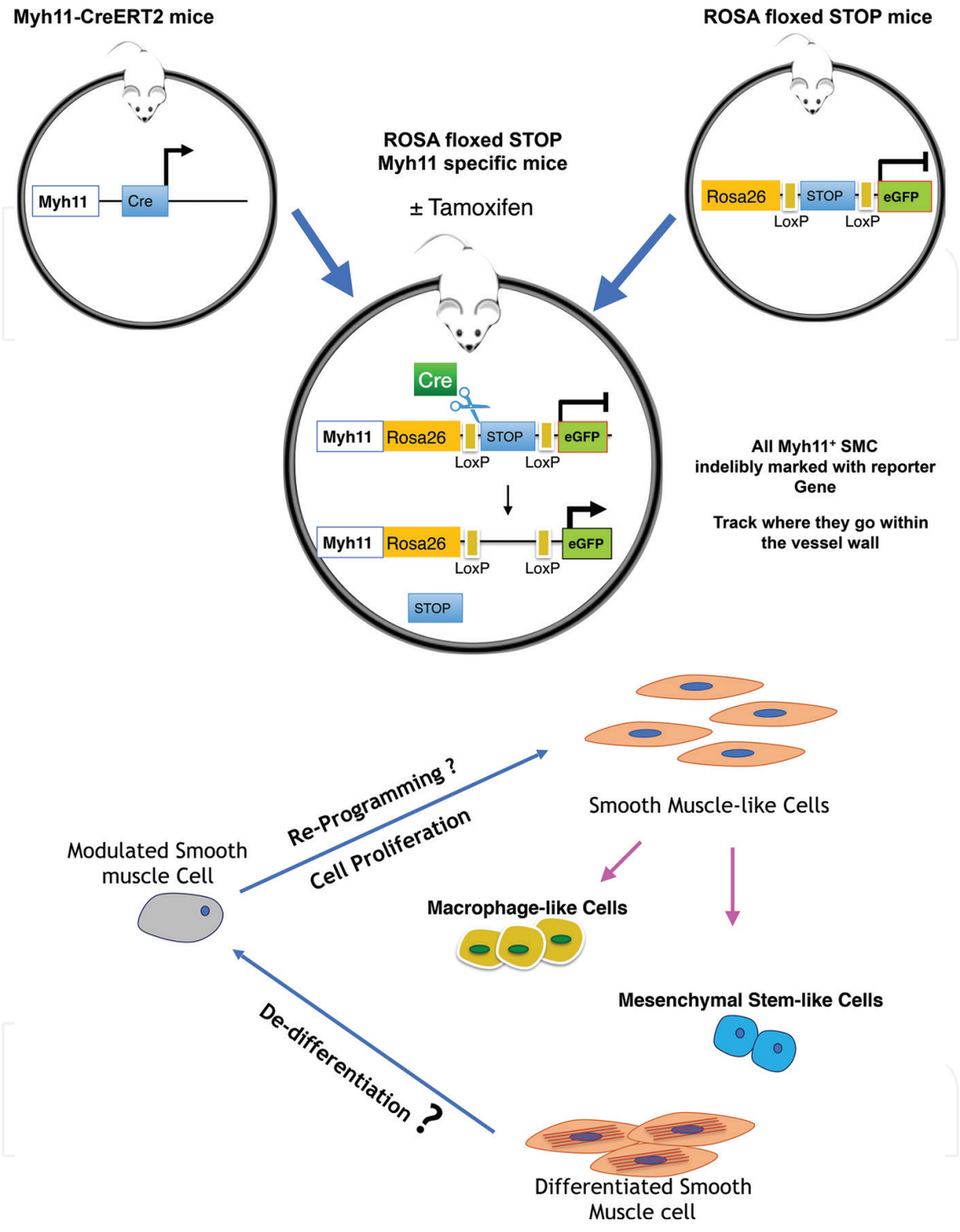

Figure 4. Cartoon summarising lineage tracing analysis of Myh11 marked differentiated SMCs using inducible Myh11-CreERT2-Rosa26-eGFP transgenic reporter mice in support of de-differentiation of mature SMCs and the paradigm of de-differentiation and phenotypic switching of SMCs *in vivo* to explain IMT [7, 91].

A key unanswered question is whether it is solely the differentiated subpopulation of SMC within the vessel, or whether there is also a contribution from resident vascular stem cells? If the latter, resident vascular stem cell niche(s) could give rise to alternative cell types during IMT and arteriosclerotic disease progression, and in doing so re-programme chromatin architecture

to facilitate the appearance of new cell lineages [89]. This stem cell hypothesis remained speculative due to the absence of robust genetic fate mapping data. However, recent studies whilst controversial, provide compelling evidence that the mobilisation and recruitment of

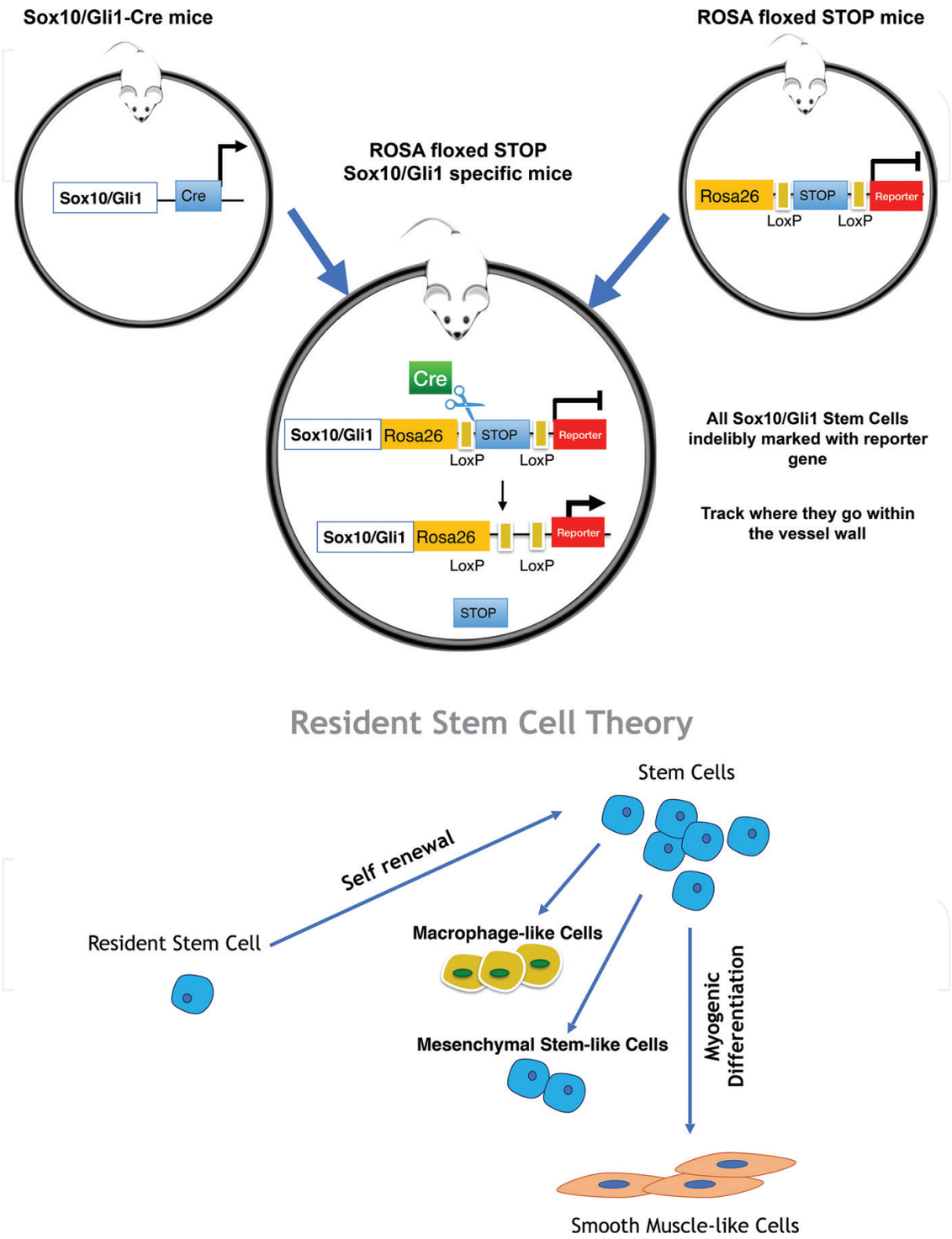

Figure 5. Cartoon summarising lineage tracing analysis of Sox10 and Gli1 marked stem cells using constitutive Cre-LoxP transgenic reporter mice in support of stem cell derived myogenic progeny and the paradigm of myogenic differentiation of resident vascular stem cells *in vivo* to explain IMT [18, 21, 22].

stem/progenitor cells present within the vessel wall and accumulation of their progeny also significantly contribute to IMT and vascular remodelling (**Figure 5**) [18, 19, 21, 22].

Initial transplant studies using green fluorescent protein (GFP) bone-marrow (BM) from GFP transgenic mice addressed the potential role of circulating progenitors and the contribution of BM-derived SMC-like cells to lesion formation [96]. Further human transplant studies demonstrate that SMC-like cells of donor origin enrich in coronary atherosclerotic plaques when compared with the healthy controls. These data initially supported the contention a circulating progenitor origin for neointimal cells in primary atherosclerosis [97]. However, subsequent studies using apolipoprotein E $(ApoE)^{-/-}$ mice transplanted with BM from Myh11–Cre/ROSA26R/$ApoE^{-/-}$ transgenic mice clearly demonstrated that very few neointimal SMC-like cells of atherosclerotic lesions were marked as Myh11 positive, thereby ruling out a BM origin for neointimal SMC-like cells at least in atherosclerotic lesions [98].

Meanwhile, several studies have since demonstrated the presence of multipotent vascular stem cells within the normal vessel wall [19, 23, 99–104] and the appearance of discrete stem cell markers, stem cell antigen-1 (Sca1), SRY-related HMG-box 10 (Sox10), SRY-box 17 (Sox17), S100 calcium binding protein B (S100β), and haematopoietic cell E- And L-Selectin ligand (CD44) on neointimal and medial cells *in vivo* following vascular injury and IMT [18, 19, 105], and in human lesions [106]. Moreover, sub-cultured SMC express these same stem cell markers *in vitro* [107, 108]. While de-differentiation and re-programming of SMCs to a more plastic phenotype could account for some of these observations, recent lineage tracing studies that selectively genomic marked resident vascular stem cells provide compelling evidence in support of the 'stem cell hypothesis' [18, 21, 22].

8. The role of adventitial stem cells

The outermost connective tissue surrounding blood vessels is called the adventitia [109]. It contributes significantly to a variety of disease pathologies, including IMT, atherosclerosis and restenosis [109, 110]. In 2004, Hu et al., were first to describe Sca1, CD117/stem cell factor receptor (c-kit), CD34 and Flk1 vascular progenitor cells within the adventitia, particularly in the region of the aortic root, that differentiate into SMCs and participate in lesion formation in vein grafts [23]. Subsequent studies identified resident vascular progenitor cells in the border zone of adventitia and media in human arteries and veins (**Figure 1**) [99, 102]. Adventitial Sca1 stem cells differentiate into other types of cells participating in vascular lesions, including osteogenic progeny [111] and macrophage colony-forming units [112]. More recent studies using lineage tracing analysis have suggested that these adventitial Sca1 cells are derived from Myh11 genomic marked differentiated SMCs and are major contributors to adventitial remodelling [113]. Indeed, the generation of adventitial vascular progenitor cells from differentiated SMCs may be a normal physiological process that contributes to the vascular stem cell pool and plays an important role in arterial homeostasis and disease [113]. The vasa vasorum and surrounding connective tissue in adult thoracic aortic adventitia is considered a niche for these progenitor cell populations in human vessels that facilitates their role as myogenic progenitors with the potential for multi-lineage progression in atherosclerosis and

IMT following injury [20]. Importantly, recent lineage tracing studies that genomic mark and track these adventitial cells following iatrogenic injury using Gli1-Cre transgenic mice provide compelling evidence for their specific role in IMT and neointimal formation in mice [21, 22]. These studies clearly demonstrate that Sca1, CD105, CD29, and CD34 positive adventitial progenitors cells contribute to neointima formation after acute femoral artery injury and support the idea that resident perivascular mesenchymal stem cell (MSC)-like cells represent a major source of SMC-like cells in vascular lesions [21, 22].

9. The role of medial stem cells

The presence of resident vascular stem cells within the medial layer of the vessel wall has also been recently established (**Figure 1**). These cells are Sca1, c-kit(−/low) Lin-CD34(−/low) and undergo myogenic (SMC) and vasculogenic (EC) differentiation *in vitro* in response to PDGF-BB/TGF-β1 and VEGF, respectively [104]. Similarly, Sca1, Oct4, Stro-1 and Notch-1 positive mesenchymal-like stem cells have been reported that lack haematopoietic or endothelial markers but exhibit myogenic, adipogenic and chondrogenic potential [103]. In 2012, Tang et al., reported on the existence of a Myh11 negative, Sox10, Sox17, S100β, nestin (Nes) positive neuroectodermal medial stem cell population in various human and rodent vessels that may give rise to the majority of SMC-like cells within lesions following injury [19]. Sox10 is expressed in neural crest stem cells during embryonic development and controls their multipotency [114]. Moreover, in normal vessels, Sox10 stem cells are sparse and primarily located within the medial and adventitial layer [115]. Using Myh11-Cre transgenic mice to mark and track medial SMCs, these lineage tracing studies purported that medial SMCs are in fact terminally differentiated and incapable of phenotypic transition during vascular injury and disease [19]. However, these studies proved controversial since they relied on tracking a Myh11 negative population that might represent failed cre-mediated recombination, silencing of the lineage tracing gene, and/or technical loss of the reporter marker [116]. In addition, since isolation of these cells required complete removal of the adventitia prior to medial explant, it is possible that residual adventitial Sox10 cells remained on the external elastic lamina (EEL) and migrate from medial explants during culture. Nevertheless, follow-up lineage tracing analysis using Sox10-cre/Rosa-loxP-LacZ mice supported their original conclusion and confirmed that a resident $Sox10^{+}$ multipotent vascular stem cell (whether from the medial or adventitial layer) is an important source of SMC-like cells during IMT following iatrogenic injury [18].

10. The evolving dichotomy about the origin of Neointimal cells

Numerous research groups, some mentioned above, have attempted to define the specific cell population that gives rise to IMT and the progression of arteriosclerotic disease [117]. Data in support of the classic theory of SMC de-differentiation and re-programming has provided compelling evidence that neointimal cells are derived from a discrete subpopulation of medial differentiated SMCs; those studies employed robust lineage tracing analysis [7, 91], clonal SMC expansion in aggregation chimeras [118], and *in situ* epigenetic profiling of the

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stable SMC epigenetic mark, H3K4me2 [9, 68]. However, parallel lineage analysis of marked resident vascular stem cells has clearly demonstrated that adventitial and/or medial progenitor stem cells also play a significant contributory role [18, 21, 22]. Given the innate heterogeneity of medial SMCs in culture, it is not surprising that there exists such a dichotomy. Although significant progress has been made in understanding the molecular mechanisms and signalling pathways that dictate myogenic differentiation of stem cells into SMCs [59], a key issue of molecular switching of differentiated SMCs has yet to be fully established because most of the molecular analysis that controls SMC differentiation was performed on stem cells and/or sub-cultured SMCs *in vitro* [89]. This may explain some of the controversy about the origin of neointimal cells since the acute changes in phenotype of freshly isolated SMCs over time and the fate of genomic marked medial SMCs in culture as models of SMC de-differentiation have both been recently investigated [4, 18].

Sandison and colleagues tracked native medial SMCs continuously post isolation for up to 4 days in culture using time-lapse imaging to determine if de-differentiation and phenotypic switching can give rise to different functional behaviours of SMCs *in vitro* [4]. Their studies indicate that differentiated SMCs are capable of altered functional behaviour by acutely converting from a contractile phenotype to a migratory one capable of phagocytosis [4]. However, when genomic marked differentiated medial SMCs from Myh11-Cre/Rosa-loxP-RFP transgenic mice were isolated, grown and sub-cultured *in vitro* using standard protocols, Myh11 marked cells were lost over time and replaced by a $Sox10^{+}$ population [18]. These data strongly suggest that sub-cultured SMCs routinely used in culture are not in fact derived from medial differentiated SMCs that undergo de-differentiation and re-programming [18], but instead are derived from a Sox 10 positive population that outgrows medial SMCs [18]. Interestingly, several commercial SMC lines also exhibit a similar stem cell phenotypic expression profile in culture (i.e., Sox10, Sox17, S100β) [107, 108]. Moreover, when sub-cultured SMCs were interrogated using vibrational Raman spectroscopy, their photonic signature was notably similar to stem cell-derived myogenic progeny *in vitro* [60]. Collectively, these data suggest that sub-cultured SMCs routinely used to assess the process of SMC de-differentiation *in vitro* are not derived from differentiated medial SMCs but rather from a Sox10 progenitor stem cell present in primary isolates that predominates the culture population over time [18].

Further evidence for this paradigm comes from epigenetic profiling of medial SMCs in culture [10]. Medial SMCs are enriched for the stable SMC epigenetic mark, H3K4me2, at the Myh11 locus by chromatin immunoprecipitation (ChIP) analysis of cell populations [60] and by using an *in situ* hybridization and proximity ligation assay of individual cells [68]. However, when these cells are isolated and grown in culture (up to passage 3), the level of H3K4me2 enrichment within the population is significantly lower compared with fresh aortic SMCs [60] with up to 80% of individual cells in early passage reported negative for the H3K4me2 mark at the Myh11 promoter [64, 68]. If this H3K4me2 epigenetic mark is truly stable in de-differentiated cells, as previously reported [66], these data further reinforce the likelihood that sub-cultured SMCs lacking this mark are not derived from medial differentiated SMCs as originally thought [66]. The presence of this stable H3K4me2 epigenetic mark at the Myh11 promoter has also been used to confirm the presence of de-differentiated SMCs *in vivo* within murine and human arteriosclerotic lesions [9, 68]. However, since the acute reduction in Myh11 promoter activity recovers in SMC-like cells following injury [119] and because stem

cell-derived progeny acquire this H3K4me2 mark at the Myh11 promoter following myogenic differentiation *in vitro* [60, 66], it is likely that that stem cell-derived myogenic progeny also enrich for the H3K4me2 mark *in vivo*.

The ultimate dichotomy arises from the outcomes of the most recent lineage tracing analyses that independently marked and tracked both Myh11/SM22α/Acta2 differentiated SMCs [7, 91, 120], and Sox10/Gli1 stem cells [18, 21, 22] following vascular injury. Both series of cell fate mapping studies concluded that the vast majority of neointimal cells are derived from either one source or the other. Differences in the animal models deployed (carotid ligation vs. femoral injury vs. ApoE mice) [121], the extent of the endothelial damage and the level of disruption to the internal elastic lamina to facilitate movement of differentiated SMCs, may in part account for these differences [59, 122]. However, recent studies assessing tamoxifen (Tm)-inducible Cre recombinases activity in mice may also offer some important clues [123]. While the efficiency of inducible Cre-loxP recombination is readily evaluated with reporter strains, the precise length of time that Tm induces nuclear translocation of CreER(Tm) and subsequent recombination of a target allele following cessation of Tm treatment is rarely assessed. It is clear that the doses of Tm commonly used to induce Cre-loxP recombination in transgenic mice to track differentiated SMCs may continue to label a significant number of cells for weeks after Tm treatment if the Myh11 promoter is active, thereby confounding the interpretation of time-sensitive studies using Tm-dependent models [123]. It is widely accepted that Myh11 promoter activity is initially lost after vascular injury but recovers in neointimal cells to near normal levels within 7–14 days [119]. Hence, it is highly likely that stem cells that acquire an active Myh11 promoter following myogenic differentiation will also be marked as Tm may still be present with the tissue [123]. In this context, resident vascular stem cells that are not originally marked following Cre-loxP recombination using Myh11-CreERT2 transgenic mice could become marked when the Myh11 promoter becomes active 7 days post injury and Tm is still present with the vessel wall to drive the recombination and mark the stem cell. Importantly, most studies using Tm to induce Cre-loxP recombination and mark differentiated SMCs wait 5–14 days before injury; yet Tm is known to remain in tissues for up to 4 weeks [123]. For most studies using $ApoE^{-/-}$ mice, the high fat diet-induced vascular injury is routinely initiated immediately after the Tm treatment ceases [9, 79] raising the likelihood that stem cells acquiring an active Myh11 promoter following myogenic differentiation will also be marked as Tm may still be present with the tissue [123]. Hence, the length of time that Tm-induced Cre-LoxP recombination occurs following cessation of Tm treatment needs to be empirically and routinely evaluated before these possibilities can be disregarded. In a similar manner, medial differentiated SMCs that are not marked using the constitutively active Sox10-Cre or Gli1-Cre transgenic mice to track stem cells could in theory also acquire the mark if the respective promoters driving the cre recombinases become active post injury during a re-programming event. It would be prudent to clarify this in future studies, since many of these elements are widespread in experimental arteriosclerosis research.

There is a clear need for more rigorous lineage tracing studies using Tm-inducible Cre recombinases to track resident stem cells. In this context, we have recently reported using Sca1-eGFP transgenic mice that eGFP positive cells predominate the lesion following injury [105] and that these cells also express S100β [124]. Moreover, lineage tracing analysis using a Tm-inducible

Cre recombinase driven by S100β promoter confirms that the majority of neointimal cells post injury are derived from a S100β parent population present within the adventitia prior to injury and not present within the medial layer where differentiated SMCs are located [124].

11. Concluding remarks

Our understanding of de-differentiation and re-programming of SMCs continues to evolve since the seminal work of Julie Chamley-Campbell and Gordon Campbell [2, 3]. Initially it was thought that aberrant proliferation of SMCs after phenotypic switching exclusively drove IMT. At the same time, it was acknowledged that SMCs were also protective in advanced lesions, preventing fibrous cap rupture and promoting plaque repair. However, more recent studies using lineage tracing, loss-and-gain-of-function, and epigenetic profiling have changed the landscape [12]. In this context, SMC differentiation and re-programming may also account for the appearance of osteochondrogenic and macrophage-like phenotypes within vascular lesions [17, 92].

Furthermore, whilst still controversial, resident vascular stem/progenitor cells are beginning to be recognised as potentially important players in the development of IMT and the pathogenesis of atherosclerosis. Regardless of the source of the progenitor stem cell (circulating, adventitial, or medial), the impact of the local micro-environment and the relevant cues dictating the pattern of gene expression and behaviour of these cells warrants further investigation. In this context, many of the signalling molecules and molecular switches that are known to impact on the generation of stem cell-derived myogenic, osteochondrogenic and macrophage-like phenotypes have all been implicated in lesion development [42]. For instance, Notch and Hedgehog signalling proteins are known to control stem cell fate and are also upregulated within vascular lesions [49, 51, 125] when either SMC re-programming and/or stem cell-derived differentiation down various lineages is presumed to occur. Inhibition of these pathways ameliorates IMT confirming their putative role in vascular pathology [125, 126]. While Notch-dependent lateral inhibition signalling may promote a particular fate but prevent surrounding cells from doing the same ensuring that not all medial SMCs de-differentiate and re-programme, its putative role in controlling adventitial and/or medial stem fate cannot be disregarded [52]. Further studies that define the specific cell populations contributing to IMT under different circumstances, and the molecular controls involved in their regulation, will add greatly to our overall understanding of vascular pathology and our ability to successfully target these cells therapeutically.

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