The Effect of Ageing on Vascular Smooth Muscle Cell Behaviour – A Mini-Review

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Key Words
Vascular smooth muscle · Ageing · Atherosclerosis · Cardiovascular disease · Proliferation · Apoptosis · Signalling

Abstract
Ageing is a prominent risk factor for atherosclerosis and cardiovascular disease. Vascular smooth muscle cells (VSMCs) are an integral part of atherosclerotic plaque formation, progression and subsequent rupture. Emerging evidence suggests that VSMC behaviour is modified by age, which in turn may affect disease outcome in the elderly. In this review, we discuss the effect of age on VSMC behaviour, proliferation, migration, apoptosis, inflammation, extracellular matrix synthesis and calcification. In addition, we discuss the multiple signalling factors underlying these behavioural changes including angiotensin-II, matrix metalloproteinases, monocyte chemotactic protein-1, and transforming growth factor-β1. Understanding the molecular processes underpinning altered VSMC behaviour with age, may lead to the identification of novel therapeutic targets for suppressing atherosclerosis in the elderly population.

Introduction

Ageing of the Blood Vessel Wall and Atherosclerosis
Ageing is a prominent risk factor for cardiovascular diseases [for review, see 1]. As the proportion of the global population over 60 years old rises [2], it becomes increasingly important to understand the molecular mechanisms underlying the relationship between ageing and cardiovascular pathology. With age, the vasculature undergoes various structural and molecular changes which are thought to predispose or accelerate cardiovascular disease. Detailed reviews of changes in blood vessel structure are available elsewhere [for example 3 or 4]. Briefly, with age the blood vessel wall broadens and develops a thickened intima consisting of infiltrating vascular smooth muscle cells (VSMCs) and inflammatory cells resulting in local inflammation and protease expression. In addition, the vascular wall stiffens due to increased collagen deposition, elastin degradation, production of advanced glycation end products (AGEs) and calcification [for review, see 3].

Importantly, these adaptations in the aged vascular wall closely reflect early atherosclerosis, the vascular pathology that underpins many cardiovascular diseases including coronary heart disease and stroke [discussed in detail in 4]. Atherosclerosis is characterised by the formation of fatty inflammatory lesions enclosed by a layer of VSMCs and fibrotic extracellular matrix (ECM) within the intimal layer [for review, see 5]. The causal relationship between ageing and atherosclerosis has been established in multiple animal models demonstrating advanced vascular pathology in aged rabbits [6], aged low-density lipoprotein receptor-deficient mice [7] and senescence-accelerated mice [8], compared to young controls. Moreover, accelerated atherosclerosis has been observed in patients with the premature ageing disease...
In support of this, increased proliferative rates with age, many studies have suggested that expression of cell cycle regulators and senescence-associated β-galactosidase (SAβG) staining, were observed in primary VSMCs isolated from aged human donors compared to younger controls [24]. It is proposed that the presence of senescent VSMCs in atherosclerotic plaques may promote plaque progression either by an impaired ability to repair plaque damage or increased expression of inflammatory and pro-osteogenic molecules [see more detailed discussion in review article by Wang and Bennett 22]. However, the exact mechanisms and direct effects of VSMC senescence in blood vessel ageing and atherosclerosis remain unclear and require further investigations since it appears to only be a small proportion of the VSMC population that has become senescent.

Ageing and VSMC Proliferation

The majority of studies investigating the effect of ageing on VSMC behaviour have been performed in isolated rat VSMCs or in arteries of aged rats in vivo. Multiple in vitro studies employing rat VSMCs have demonstrated that VSMC proliferation is increased with age [15, 18, 20–21, 25]. In support of this, increased proliferative markers, PCNA and Ki-67, were observed in intact aortas from old rats [21]. However, studies in rat vessels injured in vivo contradict these findings. Torella et al. [26] reported reduced proliferation of VSMCs and impaired neointima formation following balloon injury of aged rat carotid arteries compared to young controls. Additionally, diminished injury-induced proliferation of VSMCs was reported in aged rat VSMCs in vitro [27]. Interestingly, although increased proliferation with age has been replicated in primary rabbit and mouse VSMCs [28, 29], in two human VSMC studies and one mouse VSMC study reduced proliferation with age has been reported [30–32]. In support of this finding, a clinical study by Hugl et al. [33] reported reduced prevalence of restenosis following carotid endarterectomy in patients over 70 years, compared to younger controls. The exact reason for these conflicting proliferative data is unclear but may be due to (a) species variation, (b) differences in uninjured or injured blood vessels, (c) altered VSMC isolation or (d) methodological differences. Importantly, such contradictions suggest we should be cautious when extrapolating findings from studies employing rat VSMCs to human physiology.

In support of altered proliferative rates with age, many studies have suggested that expression of cell cycle regulators is also affected by ageing. For example, increased ex-
pression of cyclin-A and cyclin-dependent kinase-2 in aged rabbit VSMCs compared to young controls was observed [28]. Conversely, decreased proliferation of aged mouse VSMCs in response to α-thrombin was accompanied by reduced upregulation of cyclin-D1 and impaired downregulation of the cell cycle inhibitor p27kip1 in another study [32]. Similarly, impaired injury-induced VSMC proliferation with age was accompanied by failure of injury to induce Akt activation, subsequent telomerase activation and downregulation of p27kip1 in rats [26]. Additionally, Guntani et al. [31] suggested reduced proliferation of aged human VSMCs was accompanied by decreased expression of the cell cycle checkpoint regulator BubR1; however, multiple in vitro passages were necessary before age-related differences in BubR1 and proliferation were observed.

Many studies have investigated the molecular mechanisms underlying modified VSMC proliferation with age in rat VSMCs. Published data suggest that the secretion and responsiveness to pro-proliferative factors is altered with ageing. For instance, increased proliferation of aged rat VSMCs was at least in part due to increased expression of milk fat globule protein epidermal growth factor-8 (MFG-EGF8) [21], a protein previously shown to increase in the aged rat aorta [34]. MFG-EGF8 promotes cell cycle progression through a pathway involving integrin αvβ3, extracellular signal-regulated kinases 1/2 (ERK1/2) and platelet-derived growth factor (PDGF) [21]. Moreover, this study also demonstrated increased PDGFRα and PDGFRβ expression in aged rat VSMCs, indicative of enhanced responsiveness to pro-proliferative PDGF [21]. Similarly, enhanced proliferation in response to PDGFBB via increased expression of PDGFRα has been demonstrated in aged mouse VSMCs [29]. A summary of studies investigating VSMC proliferation with age can be found in table 1.

### Ageing and VSMC Migration

Similar to proliferation, many studies employing rat VSMCs have reported enhanced cell migration with age. For example, in one study increased invasion through a matrigel-coated membrane was observed in aged rat VSMCs [35], whilst in another increased invasion of aged rat VSMCs across filters coated with collagen and fibronectin or matrigel was reported [20]. Improved understanding of the molecular mechanisms underlying the increased migration in rat VSMCs has been achieved by numerous studies over the last decade. Spinetti et al. [20] indicated that the chemokine MCP-1 underpinned the increased migration of rat VSMCs with age, as inhibition of the MCP-1 receptor, chemokine (C-C motif) receptor-2 (CCR2), eliminated age-induced differences in VSMC migration, whilst MCP-1 addition induced migration in young VSMCs. Furthermore, these researchers corroborated their conclusions in the ageing rat aorta in vivo by the detection of increased MCP-1 expression and CCR2 expression [20]. In addition to MCP-1, numerous studies have reported increased MMP expression and ac-

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tivity in the ageing vascular wall which may also facilitate increased VSMC migration. Specifically, MMP-13 [36], MMP-9 [10, 37] and MMP-2 expression and activity were elevated in the ageing vascular wall [10, 12–13, 36, 37]. It has been proposed that increased MMP-2 activation is due to a combination of elevated levels of the MMP-2 activator MMP-14 and reduced levels of tissue inhibitor of metalloproteinase-2 in the ageing intima [13]. MMP-2 has previously been implicated in promoting VSMCs migration via digestion of ECM [38] and is upregulated in the neo-intima following injury [39]. Likewise, a gradient of MCP-1 and MMP-2/MMP-9 in human aortas with age was demonstrated, which the authors proposed would promote migration into the expanding intima [10]. Furthermore, increased binding of MMP-2 with transforming growth factor-β1 (TGFβ1) was observed in aged rat aortic lysates which may facilitate MMP-2-mediated activation of TGFβ1 and its downstream signalling pathway [40]. Intense research into the role of these factors in VSMC migration culminated in a paper by Wang et al. [35] demonstrating a positive feed-back loop to promote and maintain augmented migration in aged VSMCs consisting of MCP-1, MMP-2 and TGFβ1 (as illustrated in fig. 1). Briefly, this study demonstrated using multiple loss-of-function and gain-of-function experiments that MCP-1 expression by aged VSMCs enhances MMP-2 expression and activity, which activates TGFβ1 which in turn positively regulates MCP-1 and MMP-2 [35]. Evidence for this cycle was later supported in vivo as chronic MMP inhibition in ageing rats resulted in reduced MCP-1 and TGFβ1 activation alongside reduced age-associated vascular remodelling [36]. Furthermore, emerging evidence suggests that components of this cycle may be modulated by angiotensin-II (Ang-II), MFG-EGF8 and the enzyme calpain-1 all of which have been shown to increase in the ageing vasculature [13, 34, 41, 42]. In addition, increased collagen VIII expression was detected in male non-human primates with ageing [43], which has previously been shown to enhance VSMC migration [44]. Changes in MMPs with age appear to be species specific as studies employing mouse VSMCs have demonstrated no difference [29] or reduced MMP-9 and MMP-2 levels with age [45]. Intriguingly, there is contradictory evidence regarding the effect of age on human VSMC migration. One study demonstrated that VSMCs isolated from an aged human donor (53 years old) had increased invasion across a matrigel-coated membrane compared to young VSMCs (25 years old) [10]. Conversely, in a similar model reduced basal, insulin-like growth factor-1-stimulated and insulin-stimulated invasion of primary human VSMCs with age was observed [46]. However, as only small sample numbers were employed in both reports, larger studies incorporating more donors may be necessary to clar-
ify the effect of age on human VSMC migration. A summary of studies investigating VSMC migration with age can be found in table 2.

### Ageing and VSMC Apoptosis

The effect of age on VSMC apoptosis has been less extensively studied in comparison to proliferation and migration, and there is conflicting evidence within the available literature. Increased VSMC apoptosis with age has been observed in mouse VSMCs in vitro [47]. Furthermore, the authors identified increased phosphodiesterase-5 (PDE-5) mRNA expression in aged VSMCs and demonstrated that augmenting nitric oxide signalling, by inhibition of PDE-5 mediated cGMP degradation, reduced the enhanced VSMC apoptosis with age [47]. Similarly, increased apoptosis with age has been reported in rat carotid arteries following balloon injury [26]. On the other hand, VSMC apoptosis was reduced in ageing mice, both in vitro and in vivo following wire-induced carotid artery injury [29]. Additionally, reduced vulnerability to apoptosis has been reported in isolated rat VSMCs challenged with elevated glucose [48]. Therefore, it is unclear how ageing affects VSMC apoptosis. Contradicting reports in vitro may reflect differing methods of VSMC isolation, assessment of apoptosis and method of apoptosis induction. A summary of studies investigating changes in the rate of VSMC apoptosis with age can be found in table 3.

### Ageing and VSMC Inflammation

Published data suggest that in the ageing aortic intima there is increased expression of inflammatory cytokines including MCP-1, adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and proteases including MMPs [10–12, 49]. As many studies have reported VSMCs as the predominant cell type in the aged intima, with minimal inflammatory cell infiltration, it seems likely VSMCs are at least in part responsible for this enhanced intimal inflammation [10, 12]. In support of this, many inflammation-associated factors have been reported in primary aged VSMCs. Specifically, the pro-inflammatory chemokine MCP-1, its receptor CCR2 [20] and the adhesion molecule VCAM-1 [18] are increased with age in isolated rat VSMCs. Similarly, increased expression of the cytokine interleukin-6 and chemokines MCP-1, che...

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**Table 2.** Summary of studies investigating the effect of age on VSMC migration

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<td>Ferlosio et al. [18]</td>
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<td>Fisher344 × Brown Norway rats</td>
<td>in vitro</td>
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<td>Wang et al. [10]</td>
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<td>in vitro</td>
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<td>↓ VSMC migration</td>
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<td>Ruiz-Torres et al. [46]</td>
<td>human</td>
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**Table 3.** Summary of the studies investigating the effect of age on VSMC apoptosis

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kine (C-C motif) ligand 5 and chemokine (C-X-C motif) ligand 2, as well as ICAM-1 and immune receptor toll-like receptor 4, have been reported in aged mouse VSMCs [50]. Importantly, the authors postulated that due to the involvement of these inflammatory molecules in atherosclerosis, this change in VSMC phenotype would promote vascular disease [20, 50].

Evidence suggests that in aged VSMCs, dysregulation of aldosterone and Ang-II may contribute to increased inflammation. Krug et al. [49] demonstrated upregulation of mineralocorticoid receptor expression in aged rat VSMCs was responsible for increased TGFβ and ICAM-1 expression in these cells. Moreover, increased Ang-II, angiotensin-converting enzyme (ACE) and Ang-II receptor type I (AT1 receptor) expression has been demonstrated in the VSMC-infiltrated aged intima [10, 13]. In vitro, Ang-II treatment has been shown to increase VSMC expression of MCP-1, MMP-2, calpain-1, MFG-EGF, mineralocorticoid receptor expression and reactive oxygen species (ROS) [31, 34, 41, 49, 51, 52]. Furthermore, Ang-II treatment has been shown to induce senescence in primary human VSMCs through increased mitochondrial and NADPH-dependent superoxide production [53]. Taken together, these data suggest that Ang-II promotes an inflammatory VSMC phenotype in addition to the migration discussed above. In support of this conclusion, an elegant study by Wang et al. [52] demonstrated that chronic infusion of young rats with Ang-II induced age-associated vascular remodelling including the development of a VSMC-rich neointima and increased expression of TGFβ1, PDGF and MMP-2. Conversely, aortic wall thickening with age can be inhibited by chronic AT1 receptor or ACE inhibition [54].

On top of the inflammatory molecules listed above, evidence suggests that superoxide radicals are increased in the ageing aorta and co-localise with VSMCs [55, 56]. Similarly, enhanced hydrogen peroxide production has been observed in proliferating aged mouse VSMCs in vitro [32]. Evidence suggests that increased ROS may be at least in part due to increased NAD(P)H oxidase expression and activity with age. For instance, expression of the NAD(P)H oxidase subunit p22phox was elevated in the ageing rat aortic media [57], whilst increased NADH-induced superoxide radical synthesis was detected in ageing rat carotid arteries [56]. Intriguingly, both studies demonstrated that NAD(P)H oxidase inhibition reduced superoxide production in aged blood vessels [56, 57]. Moreover, a study by McCrann et al. [58] demonstrated that NAD(P)H oxidase-4 expression was increased in polyplloid rather than diploid VSMCs isolated from the ageing rat aorta, suggesting that increased ROS production with ageing may originate from a specific VSMC subpopulation. Importantly in VSMCs, high ROS levels are associated with oxidative damage to cell components [32], cell senescence [23] and cell death [59]. Although ROS are increased, studies indicate that antioxidant enzymes, which would reduce the oxidative damage to VSMCs, are not likewise upregulated with age. An enhanced ROS to superoxide dismutase (SOD) ratio was reported in the ageing rat aorta [60], whilst reduced manganese-SOD and glutathione expression was observed in aged mouse primary VSMCs [32]. Importantly, in both studies impaired antioxidant defences were associated with increased oxidative damage or inflammation [32, 60]. Similarly, increased oxidative damage and peroxynitrite formation have been reported in the ageing artery [11, 55, 56]. One possible explanation of reduced antioxidant levels in aged VSMCs could be increased insulin-like growth factor-1 receptor signalling with age which results in increased inhibition of the transcription factor forkhead box O3A (FOXO3a) and reduced FOXO3a-mediated expression of antioxidants including catalase and manganese-SOD [61]. For a full review of modifications in redox signalling with ageing, see review by Li and Fukagawa [62].

Ageing and VSMC ECM Secretion and Calcification

It is well established that blood vessels stiffen with advancing age [63, 64]. Evidence suggests that modification of the vascular ECM including elastin, collagen and proteoglycan content along with calcification is responsible for age-induced changes in vessel mechanics [for review, see 3]. Data suggest that within the aortic wall, the ratio of collagen to elastin is increased with age, resulting in reduced vessel elasticity [64]. Interestingly in the ageing rat aorta, degraded medial elastin co-localises with MMP-2, suggesting a role for this protease in this process [12]. Further to this, Li et al. [12] demonstrated that cytokine stimulation of rat VSMCs in vitro was necessary to reveal differences in MMP-2 activity with age, suggesting that local inflammation contributed to enhanced MMP-2 activity and subsequent elastin degradation in vivo. The pivotal role for MMPs in vascular ECM modification with age was demonstrated in ageing rats treated with an MMP inhibitor, which reduced elastic digestion and collagen accumulation compared to untreated aged controls [36]. Additionally, TGFβ1 expression and signalling via SMAD2/3 and SMAD4 increases in the aged rat aorta and has been reported to result in increased expression of fi-
bronectin and collagen [40]. Similarly, increased TGFβ3, fibronectin and biglycan have been reported in the ageing rat aorta [12, 34]. Evidence also suggests that collagen and elastin are modified in the ageing vascular wall by AGEs which correlate with increased vascular stiffness [65]. Furthermore, AGEs have been shown to modulate VSMC behaviour, as AGE modifications of bovine serum albumin have been shown to increase TGFβ-induced fibronectin production in porcine primary aortic VSMCs [66], whilst inhibition of receptor for AGEs has been shown to reduce neointimal formation and VSMC proliferation following femoral artery injury [67].

As well as these ECM changes with age, evidence suggests that with age rat VSMCs become more osteogenic with an increased propensity to calcify in vitro [42]. A study demonstrated that increased in vitro calcification, increased expression of calcification factors including TGFβ1 and collagen-II and reduced expression of anti-calcifying factors osteopontin and osteonectin could be achieved in young rat VSMCs overexpressing calpain-1, indicating a role for this protein in VSMC-mediated calcification with age [42].

In addition, studies into premature vascular ageing in HGPS have implicated a role for prelamin-A in VSMC-mediated calcification. In HGPS, mutations in the LMNA gene result in incomplete processing of the nuclear lamina protein lamin-A, resulting in the accumulation of an unprocessed prelamin-A, called progerin [68]. Accumulation of this mutated precursor occurs in the vasculature of HGPS patients [69] but has also been detected in VSMCs in ageing and atherosclerotic human blood vessels [24]. Interestingly, Liu et al. [70] also detected prelamin-A accumulation in VSMCs within the calcified vasculature of young patients (under 18 years) on dialysis, suggesting a role for progerin in VSMC-mediated calcification. The authors found that adenoviral overexpression of mutated prelamin-A in human VSMCs induced osteogenic differentiation at least in part through increased DNA damage signalling [70]. Together, these studies suggest that prelamin-A accumulation in VSMCs with age promotes osteogenic differentiation, calcification and vascular stiffness.

Moreover, studies suggest that in combination with increased ECM stiffness, VSMCs themselves may stiffen with age. Qiu et al. [71] demonstrated that in vitro assemblies of isolated VSMCs from aged non-human primates seeded onto a collagen ring had increased stiffness compared with those seeded with young VSMCs. Furthermore, studies employing atomic force microscopy and primary non-human primate VSMCs demonstrated that single VSMCs became stiffer with age and demonstrated increased cell-ECM adhesion, specifically increased β1-integrin-dependent adhesion to fibronectin [71, 72].

**Conclusions**

The effect of age on VSMC behaviour has been widely studied, and multiple molecules have been implicated in the altered regulation of VSMC proliferation, migration,
apoptosis, inflammation and vascular stiffness with age. These factors are summarised in figure 2. Interestingly, some factors including Ang-II, MCP-1, TGF-β, calpain-1 and MFG-EGF8 have been shown to affect more than one aspect of VSMC behaviour. Moreover, MMPs have been associated with the age-associated modulation of VSMC migration, inflammation and vascular stiffness. In addition, MMPs have previously been implicated in VSMC viability [73], allowing speculation of an involvement in VSMC apoptosis with ageing also. The contribution of MMPs to multiple aspects of VSMC behaviour suggests that MMP inhibition could be an effective target to limit age-related changes in VSMC behaviour and thereby atherosclerosis. In fact, emerging studies have already demonstrated the ability of chronic MMP inhibition to reduce vascular remodelling in aged rats, leading to speculation of their therapeutic effects in humans [36].

This review highlights the array of animal models employed by researchers to assess VSMC behaviour with ageing. The findings of studies in different animal models are summarised in figure 3. The majority of studies have been performed in rat VSMCs and suggest that with age, VSMC proliferation, migration, inflammation and stiffness are increased [for example 18, 20, 21, 25, 42]. On the other hand, studies in human VSMCs are fewer in number and indicate reduced VSMC proliferation with age [30, 31]. These discrepancies provoke the questions: which animal model is most appropriate to study VSMC ageing? And how translational are findings from isolated rat VSMCs to human physiology? It would be preferable to begin more and larger studies employing human VSMCs and blood vessels. However, human studies are hindered by (a) limited availability of samples from both young and old patients of defined age groups, (b) interference of co-morbidities and additional cardiovascular risk factors in samples from aged patients, and (c) inability to perform in vivo analyses. Alternatively, an animal model must be identified which closely mimics ageing human vascular physiology.

In addition to contradictory findings in differing animal models, there are also conflicting results in studies employing the same animal model. For example, evidence supporting both increased and reduced mouse VSMC proliferation with age has been published [29, 32]. The exact reasons for these discrepancies remain unclear, but may reflect the use of differing VSMC isolation techniques, VSMC passage in vitro or methods of assessing VSMC behaviour. Furthermore, conflicting proliferative data from isolated rat VSMCs [for instance 15, 21, 25] with VSMCs in injured vessels in vivo [26] suggest that study of VSMCs in vitro may not always reflect the in vivo situation if vessel injury is involved. In support of this,
Urano et al. [27] reported age-related differences in the proliferative rates of VSMCs isolated from injured, but not uninjured, rat aortas. It is also questionable whether VSMC isolation modifies VSMC phenotype, or indeed if an age-associated phenotype is maintained with passaging. Bochaton-Piallat et al. [16] reported that although cultured VSMCs maintained age-related expression of contractile proteins when seeded into middle-aged denuded rat carotid arteries, the increased proliferation of aged cells was not mirrored by greater neointimal formation in vivo, suggesting that some but not all aspects of age-related phenotypes were maintained. Alternatively, other factors of VSMC behaviour including age-related β-adrenoreceptor signalling [74], proliferative rate [31] and differentiated contractile protein expression [15] have been reported to be altered with passaging. This observation suggests that low passage VSMCs should be employed in in vitro studies and all observations should be supported by in vivo evidence.

An alternative explanation for these conflicting results, both in vitro and in vivo stems from the continuing debate regarding the origin of neointimal VSMCs or VSMCs in culture. A study by Tang et al. [75] proposes that VSMCs populating the injury-induced neointima do not arise from the dedifferentiation of contractile medial VSMCs, as the current dogma suggests, but instead arise from a small quiescent population of medial derived multi-potent vascular cells (MVSCs) which when activated by injury proliferate and differentiate into synthetic VSMCs. Similarly, this study demonstrated that due to the proliferative nature of MVSCs, these cells populated in vitro cultures, rather than mature medial VSMCs [75]. However, this theory remains under debate [75, 76] and other studies employing fate tracing have produced directly contradictory results [77]. The exact contribution of mature VSMCs and vascular progenitor cells to the neointima is currently unclear, as is the effect of age, species or injury on this contribution, which may account for some of the conflicting VSMC behavioural data discussed in this review.

Overall, although the effect of age on VSMC behaviour has been extensively researched, the literature is peppered with contradiction. Studies in rat VSMCs dominate the current literature, and suggest that with age a more proliferative synthetic VSMC phenotype is observed, mimicking the phenotypic switch observed in early atherosclerosis. However, whether this switch occurs in ageing humans remains to be established. This review has discussed multiple molecular factors involved in altered VSMC phenotype with age which may be exploited as therapeutic targets. In particular, inhibition of MMPs, which regulate multiple adverse VSMC behaviours, may be most effective to suppress atherosclerosis in the elderly population. However, prior to further investigation into these target leads, it would be preferable to clearly define the phenotypic changes in ageing human VSMCs.

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