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The Vascular Smooth Muscle Cell: Therapeutic Target in Type 2 Diabetes?

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ABSTRACT

The rising epidemic of Type 2 diabetes (T2DM) worldwide is of significant concern. The inherently silent nature of the disease in its early stages precludes early detection; hence cardiovascular disease is often established by the time diabetes is diagnosed. This increased cardiovascular risk leads to significant morbidity and mortality in these individuals. Progressive development of complications as a result of previous exposure to metabolic disturbances appears to leave a long-lasting impression on cells of the vasculature that is not easily reversed and termed “metabolic memory”.

Smooth muscle cells (SMC) of blood vessel walls, through their inherent ability to switch between a contractile quiescent phenotype and an active secretory state, maintain vascular homeostasis in health and development. This plasticity also confers SMC with the essential capacity to adapt and remodel in pathological states. Emerging clinical and experimental studies propose that SMC in diabetes may be functionally impaired and thus contribute to the increased incidence of macrovascular complications. Although this idea has general support, the underlying molecular mechanisms are currently unknown and hence the subject of intense research.

The aim of this review is to explore and evaluate current literature relating to the problem of vascular disease in T2DM and discuss the critical role of SMC in vascular remodelling. Possibilities for therapeutic strategies specifically at the level of T2DM-SMC, including recent, novel advances in the areas of microRNAs and epigenetics will be evaluated. Since restoring glucose control in diabetes patients has limited effect in ameliorating their cardiovascular risk, discovering alternative strategies that restrict or reverse disease progression is vital. Current research in this area will be discussed.
1. Introduction

1.1 Prevalence of type 2 diabetes and cardiovascular risk

Gradual decline of vascular function is a consequence of normal aging that manifests as structural and biochemical changes in blood vessel walls that gradually compromise vascular health [1]. Insulin resistance leading to type 2 diabetes (T2DM) is a chronic metabolic and inflammatory condition [2] and a recognised cause of accelerated vascular aging [3]. Although T2DM is initially symptomless and likely present for years, in the long term it is associated with debilitating cardiovascular complications and premature death. It is perceived that up to half of patients have evidence of cardiovascular complications by the time diabetes is diagnosed [4] and that mortality from T2DM-related cardiovascular disease reportedly confers a risk equivalent to 15 years of aging [5]. In the UK alone, the number of people diagnosed with T2DM has increased from 1.4 million in 1996 to 2.9 million in 2011 (diabetes.org.uk), with an inevitable impact on healthcare costs reportedly accounting for £9 billion per annum - approximately 10% of the entire NHS budget. These alarming figures are attributable not only to an aging population but importantly to the rising epidemic of obesity and physical inactivity.

The complications of T2DM encompass a diverse range of pathologies of the large and small arteries. In addition, diabetes patients are vulnerable to a distinctive cardiomyopathy independent of coronary artery disease that underlies progression to heart failure [6]. Microvascular (principally retinopathy, nephropathy) and neuropathic complications can, to a significant degree be retarded by early, intensive control of hyperglycaemia by insulin or oral therapies (reviewed recently in [7]). Macrovascular pathologies are numerous and coronary artery disease (CAD) is common, regularly manifesting itself earlier in life than in individuals without diabetes [8]. The anatomy of atherosclerosis in patients with diabetes is distinct, regularly presenting as multivessel disease characterised by diffuse and rapidly progressive lesions [9] that predispose to myocardial ischaemia, infarction and stroke [10]. Peripheral arterial disease leads to critical limb ischaemia and, together with impaired capacity to develop collateral vessels in T2DM, increases the risk of lower limb amputations [11]. In addition, both coronary and peripheral revascularisation procedures in patients with diabetes are problematic and the long-term results are disappointing [12;13]. Increased arterial stiffness is a characteristic of T2DM vessels [14] that undoubtedly impedes essential, adaptive remodelling in response to vascular interventions. Vessel stiffening, together with increased thrombotic risk [15], augmented intimal hyperplasia and restenosis [16;17] are key contributors to inferior prognosis and poor outcomes after revascularisation in diabetes patients.

1.2 Glycaemic control and vascular complications

Individuals with insulin resistance and T2DM generally have coexistent conditions of hyperglycaemia and hyperlipidaemia together with hypercoagulable tendency, all of which impart vulnerability to complications [12]. Plasma levels of glycated haemoglobin (HbA1c) provide a marker of average blood glucose levels over 8-12 weeks; in healthy individuals this is typically 4.0 - 6.0%. In addition to being a diagnostic indicator of diabetes, HbA1c serves as a marker of glycaemic control in individuals with established diabetes. Current guidelines suggest that in T2DM patients HbA1c is ideally maintained therapeutically by insulin and/or oral therapies ≤ 7.0% [7]. The DCCT and UKPDS studies concluded that this level of control was effective in retarding and preventing microvascular complications [7], yet the beneficial effects on macrovascular complications were not evident, at least in the medium-term. Indeed, early indications from the ADVANCE, VADT and ACCORD trials suggested that intensive glycaemic treatment had little, if any additional benefit in patients with diabetes and clinical CAD [7;18]. The ACCORD trial aimed specifically to achieve near-normal HbA1c levels.
(<6.0%) but unexpectedly this led to higher mortality in intensively treated patients (3.5 year period) with established cardiovascular disease [19]. Nevertheless, it does appear that beneficial macrovascular benefits may be achieved through intensive glucose lowering in patients without established disease and followed up over a longer time frame. The continued follow-up of UKPDS reported a sustained (10 years) reduction in cardiovascular events, notably in patients in whom early glycaemic management was achieved [20]. Modest benefits of intensive glycaemic control were also visible beyond 5 years of follow-up in both ADVANCE and ACCORD [19;21]. Hence it appears that the advantage of glucose control early in the course of T2DM may reduce cardiovascular events in the long term, and that the conferred benefits are retained, even after a return to often inferior glycaemic control. Importantly, the beneficial effect of previous glycaemic treatment was still evident 12 years after the end of the DCCT trial; patients with type 1, insulin-dependent diabetes who received prior intensive treatment exhibited significantly less macrovascular disease than those allocated conventional treatment [22]. It is perhaps too simplistic to consider that glucose alone is responsible for the vascular complications of T2DM and that successful management of other risk factors of the metabolic syndrome such as obesity, hyperlipidaemia and hypertension may lead to improvement of cardiovascular outcomes. Indeed, the outcome of the Steno-2 trial [23] supported such a multifactorial approach.

2. Role of endothelial dysfunction in T2DM
The vascular manifestations associated with T2DM can be attributed to dysfunction of the cellular components of the vasculature in a complex response to environmental stimuli [24]; the endothelium and smooth muscle cells (SMC) being key players. In health, the endothelium plays a central role in vessel wall homeostasis by synthesising a critical balance of vasodilators (of which nitric oxide (NO) is essential), and vasoconstrictors such as angiotensin II, endothelin-1 and reactive oxygen species (ROS). NO generation in endothelial cells (EC) is dependent on intact insulin signalling through the phosphoinositide 3-kinase (PI3-K) /Akt pathway [25], and as such exerts beneficial vasorelaxant, anti-inflammatory and antioxidant effects on the vasculature. NO production maintains blood pressure within physiological ranges through promoting relaxation of medial SMC lying beneath the endothelium. Endothelial dysfunction, a common early event in insulin resistance (“pre-diabetes”) and T2DM, is characterised by a range of abnormalities, the most extensively studied being decreased NO synthesis (reviewed in [26]). In these circumstances, atherosclerosis is accelerated, blood pressure is elevated and a paradoxical coronary vasoconstriction occurs. Indeed, reduced NO bioavailability is observed prior to the onset of atherosclerotic structural changes and has been shown to predict the development of coronary artery disease [27] and future cardiovascular events [28].

Endothelial function that precedes and contributes to the progression of cardiovascular disease is a subject of global interest; its association with complications of T2DM is also well studied (see [29-31] for recent reviews). By comparison, despite the pivotal role of the vascular SMC in vessel wall remodelling and homeostasis, its function in the setting of T2DM is less well explored. The purpose of this manuscript therefore is to review current opinion specifically relating to vascular SMC phenotype and function in diabetes, and evaluate new and novel targets that may be amenable to therapeutic manipulation.

3. The smooth muscle cell: critical effector of vascular function
3.1 SMC plasticity
Vascular SMC of blood vessel walls exhibit remarkable plasticity, switching between differentiated and dedifferentiated phenotypes in response to changes in the local environment (reviewed in [32]). In health, through signals from the endothelium, SMC maintain a
predominant differentiated “contractile” phenotype. The importance of SMC plasticity not only in vascular development but also in responding rapidly to adapt to environmental cues is unequivocal. In the adult, this highly differentiated cell regulates vessel tone, diameter and blood pressure through its key function – that of contractility. In these circumstances SMC have a very low turnover and negligible synthetic activity. They express a specialised repertoire of contractile proteins and cellular signalling molecules that is unique from other cell types and is necessary to maintain vessel homeostasis (thoroughly reviewed in [32;33]). Conversely, SMC dedifferentiation to a synthetic phenotype is an early event in numerous cardiovascular pathologies including atherosclerosis [34], restenosis [35] and aortic aneurysm disease [36]. These SMC are susceptible to proatherosclerotic stimuli that induce alterations in organelle distribution, aberrant matrix metabolism and increased proliferation and migration [32]. Such responses are in direct opposition to features observed in EC, that of early senescence and apoptosis [37]. Within the vasculature the proportions of contractile and secretory SMC are reported to vary, with the presence of distinct subpopulations that may have implications for arterial disease [35]. Therefore, whilst phenotypic modulation is vital to embryogenesis, vascular adaptation, remodelling and repair, it also augments progression of vascular diseases such as atherosclerosis, hypertension, restenosis and bypass graft failure [32]. The ability of SMC to maintain plasticity and respond appropriately is therefore critical in this respect.

3.2 Influence of phenotype on SMC morphology and function
During the phenotypic modulation that is characteristic of a number of cardiovascular disorders, SMC undergo morphological reorganisation. Differentiated SMC exhibit a highly organised cytoskeleton with defined F-actin filaments that maintain contractile function, expressing smooth muscle α-actin (SMA-α), smooth muscle myosin heavy chain (MHC), h1-calponin, SM22α and smoothelin [38]. Stress fibre formation is promoted by binding of the accessory protein SM22α to actin filaments, inducing bundling and cell contractility. In contrast, dedifferentiated SMC exhibit loss of fibres and a diffuse, loose F-actin network, concomitant with a reduction in SM22α [39]. The balance of SMA isoform expression in dedifferentiated cells therefore lies in favour of increased non-muscle β-actin and reduced SMA-α content and fibres [40]. Other key characteristics of dedifferentiated SMC are nuclear enlargement, increased ribosomal content and enlarged Golgi apparatus [41;42]. The ability of SMC to spontaneously adopt a dedifferentiated phenotype in culture has greatly facilitated laboratory research into such disorders [41].

Functionally, SMC dedifferentiation imparts proliferative and migratory capacity to the cells through increasing sensitivity to stimulation by serum-derived mitogenic factors [41]. Importantly, SMC alternate between contractile and synthetic states according to the perceived environmental stimulus or external signal, indicating that phenotypic switching is fully reversible. For example, in a rat carotid injury model, contractile SMC adopted a synthetic phenotype after balloon injury, migrated to form a neointima and over time were noted to revert to a contractile state [42].

3.3 Molecular regulation of SMC phenotype
Regulation of SMC phenotype is complex and has recently been thoroughly reviewed [34]. Switching of SMC between the differentiated and dedifferentiated state is regulated by a variety of environmental stimuli, and as mentioned above, dedifferentiation is accompanied by loss of SMC-specific marker genes such as SMA-α, and increased synthetic gene expression (reviewed in [32]). Most SMC marker genes are regulated by CArG box motifs within their promoters which are bound by serum-response factor (SRF) that induces transcription and differentiation. Principal co-activators of SRF are myocardin (MyoD) and
myocardin-related factors that are crucially involved in SMC marker gene expression. The kruppel-like factor (KLF) family of transcription factors are central regulators of SMC phenotypic modulation and in particular KLF4, a key transcription factor in SMC switching, can reduce the expression of MyoD leading to a reduction in SMC marker gene expression. The KLF4 – MyoD – SRF axis is thus a central regulator of SMC differentiation/dedifferentiation [43]. However, whilst much is known about the factors and mechanisms that control SMC plasticity in cell culture conditions, in vivo evidence, for example in native atherosclerosis in man or animal models, is still far from complete.

SMC phenotype in vitro is influenced by multiple factors but typically platelet-derived growth factor BB (PDGF) promotes dedifferentiation [43] and transforming growth factor beta (TGF-β) maintains cells in a differentiated state [44]. Recent evidence indicates that microRNAs (miRs) play a central role in the mechanisms determining SMC phenotype, not only in cardiovascular development and function [45;46] but also linked to cellular dysfunction in vascular diseases (reviewed recently in [47]). Whilst miR-1, miR-10a and miR-145 are involved in directing the differentiation of stem cells into SMC [48-50], miR-21 was the first to be identified as a regulator of SMC differentiation and proliferation [51], but later shown also to respond to TGF-β by promoting differentiation [52]. Furthermore, inhibition of miR-21 by PDGF promotes SMC migration [53]. Elevated miR-21 levels are observed in rat neointimal lesions [51], human atherosclerotic peripheral arteries [54] and hypoxic pulmonary artery SMC [55], driving proliferative and migratory responses. Conversely, PDGF-induced expression of miR-221 [56;57] or miR-24 [58] promotes dedifferentiation, reduced SMC marker gene expression and increased proliferation. Perhaps the most widely studied miRs at the level of the vascular SMC are miR-143/145, products of the same bicistronic gene that are known to be highly expressed in the vascular wall, particularly in SMC where they are reported to regulate SMC homeostasis and differentiation to a contractile phenotype [59;60]. Downregulation of miR-145 is associated with neointima formation in murine models of arterial injury [59;61]; conversely increased miR-145 levels reduce proliferation by promoting SMC differentiation [48]. A recent report also demonstrated miR-133 as a key regulator of SMC phenotype, inhibition of which exacerbated proliferation and migration in vivo and in vitro [62]. In another study, transfection of miR-195 precursor reduced human SMC proliferation, migration and proinflammatory cytokine secretion, conversely miR-195 was downregulated in balloon-injured rat arteries [63]. Given the complexity of regulatory control of SMC phenotype (see Figure 1), it is therefore unsurprising that multiple miRs appear to be involved and it is highly likely that others will be discovered.

4. Can T2DM directly influence vascular SMC phenotype?

Although an immediate connection between T2DM per se and SMC phenotype is not clear, the metabolic milieu of insulin resistance and T2DM can progress for a decade or more prior to diagnosis [64]. During this gradual progression, “diabetic stimuli” particularly hyperglycaemia and hyperinsulinaemia, can exert direct effects on all vessel wall cells, potentially inflicting detrimental changes in phenotype and function and acceleration of cardiovascular complications. Increased susceptibility to cardiovascular diseases in individuals with T2DM suggests that a pathological “diabetes phenotype” may exist in vascular SMC that is worthy of detailed study.

4.1 Disparities in SMC structure and function

Human studies are by their very nature usually restricted to ex vivo or in vitro investigation, yet despite this restraint, studies in man have revealed important findings. In SMC cultured from primary tissues in vitro, those of T2DM origin are morphologically distinct from ND
cells; specifically arterial and venous SMC of T2DM patients tend to lose the typical hill-and-valley, spindle-shaped appearance and adopt a more rhomboid phenotype [65;66]. Rhomboid morphology appears to be characteristic of dedifferentiated, proliferative SMC that are prevalent in vascular neointimal lesions [35], correlating with those reported in animal models of type 1 diabetes [40;67]. However, the concept of a global switch from differentiated to a dedifferentiated phenotype is unlikely. Co-existence of both synthetic (increased collagen secretion) and contractile (expressing SMA-α) SMC populations in atherosclerotic lesions of diabetic mice support this notion [68], suggesting that the critical factor directing vascular function is the relative proportion of each SMC phenotype.

Saphenous vein (SV)-SMC from T2DM patients exhibit a marked disorganisation of the F-actin cytoskeleton, a feature reproduced by Rho kinase (ROCK) inhibition in non-diabetic (ND) cells [66]. Inhibition of ROCK suppresses neointimal formation in balloon-injured rat arteries [69], an observation consistent with aberrant RhoA/ROCK signalling being detrimental to the vasculature. Concordant with rhomboid SMC being more proliferative, arterial SMC from T2DM patients also exhibit increased proliferative capacity compared to their ND counterparts [65;70]. Interestingly, conditioned media from T2DM SMC was able to promote proliferation of ND SMC, suggesting that the increased proliferation observed in T2DM arterial SMC is dependent on a mitogenic factor secreted from the cells themselves [70]. A more recent study demonstrated enhanced rate of cell cycle entry in arterial SMC from patients with T2DM together with increased basal phosphorylation of p38 and ERK1/2 [71], signalling pathways that are associated with cell proliferation. Enhanced proliferation has also been observed in SMC from both arterial (infrageniculer) and venous (SV) sources [65]. However, there is divided opinion in this respect; our studies observed consistently less proliferation in T2DM SV-SMC compared with ND cells and no discernible difference in ERK phosphorylation between the two populations [66]. The discrepant observations are perceivably due to passage number and protocol employed to quantify proliferation (total DNA fluorescence microscopy versus direct cell counting). It is also known that intrinsically different proliferative capacities are apparent between SMC cultured from arterial and venous sources, but also from different vascular beds [72].

Examination of intact human native vessels in vivo using ultrasound [73] and ex vivo using functional and histological techniques [74] has indicated increased wall stiffness in T2DM compared with ND individuals. Interestingly, the extent of structural abnormalities observed in harvested SV grafts of T2DM patients was inversely correlated with the efficiency of glycaemic control [74]. Consistent with increased tissue stiffness we reported that SV-SMC from T2DM patients display increased numbers of large, vinculin-positive focal adhesions. Notably, increased propensity to form focal adhesions is associated with both cell stiffness and adhesion and is reportedly more prevalent in rhomboid SMC [66;75;76]. Accordingly, studies using arterial and venous SMC have reported increased adhesion in T2DM-derived cells [65].

Early diagnosis of T2DM is difficult and up to half of newly diagnosed patients have evidence of cardiovascular complications [4]. Owing to the essentially silent and progressive nature of insulin resistance leading to T2DM, the vasculature is potentially exposed to a variety of circulating metabolic disturbances for a prolonged period. Even transient exposure to high glucose levels has been reported to inflict persistent phenotypic changes and altered gene expression in cultured vascular cells [77]. Whilst hyperglycaemia per se is undoubtedly a key trigger, hyperinsulinaemia, advanced glycation end products (AGEs) and raised levels of proinflammatory cytokines and elevated plasma lipid levels, all of which are associated with the diabetes phenotype [78], may also be important.
4.2 Insulin
Elevated plasma levels of insulin and glucose promote early senescence and apoptosis in EC [37;79], and conversely vascular SMC are susceptible to growth-stimulatory effects of insulin and insulin-like growth factors (IGF). Insulin induced primate aortic SMC proliferation in a concentration-dependent manner [80] and, through a mechanism involving insulin-stimulated release of IGF-1, induced proliferation of human arterial SMC [81;82]. Another study showed a mitogenic effect of IGF-1 itself on human aortic SMC [83]. There is, however, speculation that serially passaged SMC gradually lose the mitogenic response to insulin [84]. In our laboratory, and in agreement with previous reports, we discovered that supplementation of SV-SMC cultures with insulin increased proliferation concentration-dependently, whilst in contrast, internal mammary artery (IMA)-SMC cultured from the same patients were entirely resistant to insulin’s growth-promoting effects [85]. The autologous SV is routinely used to revascularise diseased coronary vessels and, although the IMA is proven to be a more robust conduit, its use is limited by availability. SV patency rates are generally poorer than IMA grafts, and significantly inferior in the diabetic population, yet IMA patency rates are comparable in both ND and T2DM patients [86;87]. Clearly the pathogenesis of graft intimal hyperplasia is multifactorial; however the apparent lack of mitogenic effect of insulin on IMA-SMC may offer an explanation for the superior patency rates of IMA grafts, even in diabetic patients.

In a study of bovine aortic SMC, insulin-induced migration was mediated via mitogen activated protein kinase (MAPK) signalling [88]. In our own studies, we demonstrated that insulin also promoted chemotaxis of human SV-SMC [66;85]. In a separate study we observed that cells of diabetic origin exhibited consistently higher migratory capacities than those of non-diabetic origin when maintained in the presence of insulin [66]. It is tempting to speculate that in the diabetic state the perceived “beneficial” effects of insulin signalling via PI3-K are impaired, whilst the “detrimental” (proatherogenic, pro-restenosis) pathways may predominate via preserved MAPK signalling. Treatment with exogenous insulin in T2DM patients may potentially aggravate the already heightened susceptibility to cardiovascular disease through its actions on SMC.

4.3 Glucose
In contrast to insulin, SMC responses to hyperglycaemia are reportedly variable. Glucose was shown to increase proliferation of cultured human infragenicular SMC and together with insulin it was synergistic [89]. Some other studies have reported a growth-promoting effect of high glucose alone on vascular SMC from human aorta and umbilical artery (25 mM) [90], and rat aorta (20 mM) [91;92]. A separate study reported that glucose-induced accelerated SMC proliferation was attributable to downregulation of protein kinase C [93]. Other studies, however, did not concur that glucose is mitogenic. For example, in porcine and human SMC high levels of glucose did not modulate proliferation either alone or via synergism with other SMC mitogens such as PDGF [94]. In agreement, our own studies using SMC cultured from multiple patients under conditions of normal (5.5mM) and elevated (25mM) glucose, did not identify any growth-modulating properties of glucose itself [66].

Irrespective of the direct influence of glucose on SMC function, it appears that glycaemia and its capacity to inflict biochemical modifications to proteins may influence the efficacy of pharmacological therapies, particularly anti-platelet therapies. Indeed, this was exemplified by the Primary Prevention Project trial [95] in which cardiovascular risk reduction with aspirin was ineffective in patients with diabetes. Several mechanisms have been suggested that include glucose-induced inhibition of aspirin-mediated NO activity [96] and modulating the activity of components of the coagulation cascade [97]. It is therefore not
without precedent that glycaemia-induced modulation of therapeutic agents could similarly modify vascular SMC function, either beneficially or detrimentally.

4.4 Advanced glycation end products
Whilst glucose itself can directly impact SMC behaviour, it is important to consider other consequences of chronic hyperglycaemia and importantly, that of formation of advanced glycation end products (AGEs). These appear to contribute to development and progression of cardiovascular disease in patients with T2DM through modification of the structure, function and mechanical properties of tissues, cross-linking cellular proteins such as collagen and thus inflicting tissue stiffening [98]. The composition of the extracellular matrix which also impacts on tissue flexibility is predominantly regulated by SMC. Studies using intact IMA from T2DM patients revealed a decrease in matrix-degrading metalloproteinases MMP-1 and MMP-3, paralleled by increased matrix deposition [71]. Taken together, altered SMC secretory function and aberrancies of ECM metabolism together could impact on vessel wall rigidity. AGEs also activate specific receptors (e.g. RAGE) on numerous vascular cell types including macrophages, EC and SMC, stimulating release of inflammatory cytokines, cell adhesion molecules and profibrotic growth factors [99]. Vascular SMC derived from insulin-resistant and diabetic db/db mice were shown to express elevated levels of RAGE, increased inflammatory gene expression and increased cell migration that were attenuated by a RAGE antibody [100]. AGE also increased proliferation and migration of human aortic SMC through activation of the MAPK pathway and NFκB [101]. In arterial injury models, blockade of RAGE-ligand interaction reduced SMC proliferation and neointima formation in Zucker diabetic rats [102] and similarly RAGE-deficient knockout mice exhibited less neointimal hyperplasia, decreased SMC proliferation and reduced collagen deposition [103].

4.5 Lipids
Oxidised LDL (oxLDL) promotes a switch towards a synthetic SMC phenotype with increases in proliferation, migration and apoptosis [63;104;105], cytoskeletal disruption [105] and induction of inflammatory cytokines and MMPs [63;106] being reported. Elevated levels of cholesterol and free fatty acids can also influence SMC phenotype. Interestingly, cholesterol and oleic acid have been shown to induce atheromatous foam cell formation from SMC, suggesting that not all foam cells are of macrophage origin [107;108]. Palmitic and oleic acids also induce aberrant cellular function either directly or through paracrine mechanisms by modulating SMC proliferation, migration and apoptosis [109-111]. The combination of oxidative stress and dyslipidaemia common to T2DM patients likely impacts on SMC through enhanced ability of lipid moieties in the oxidised state to activate key signal transduction pathways [112].

4.6 Inflammatory cytokines
A key hallmark of T2DM is a state of chronic inflammation; proinflammatory cytokines, amongst which interleukins (IL-1, IL-6), monocyte chemoattractant protein (MCP-1) and tumour necrosis factor alpha (TNF-α) are prominent, are detected at elevated circulating levels in obese and diabetic individuals [113]. These stimuli induce SMC dedifferentiation, increase proliferation and migration, and can induce further secretion of pro-inflammatory mediators from the SMC themselves [114;115], exacerbating vascular dysfunction. Persistent SMC inflammatory gene expression observed in murine models of T2DM appears to be mediated through epigenetic modifications, through de-repression of the gene promoters (reviewed in [116]). SMC established in vitro from these models exhibit a heightened response to cytokine stimulation that appears to be retained in culture [117].
Although the key metabolic disturbances of T2DM have been briefly described here, this is by no means an exhaustive account and importantly, these stimuli do not exist or act in isolation in the vasculature. It is clear that overlapping and complex interaction between cellular and non-cellular components can influence SMC phenotype and function through a diversity of intracellular signalling cascades and molecular mechanisms.

In adults the vascular SMC is a highly specialised cell whose key role is that of contraction. The remarkable plasticity of the SMC is evidenced by its ability to undergo phenotypic modulation during remodelling responses to vascular injury and in a variety of disease states. This intriguing cell should perhaps not be categorised either as “contractile” or “synthetic” as it can exist as a range of phenotypes with distinguishing features and regulatory pathways (reviewed in [38]). Following on from our earlier description of a “T2DM SMC phenotype” we have started to explore this further and have observed evidence of molecular mechanisms that appear to discriminate between SMC of T2DM and ND origin (unpublished). There is clearly a need to explore whether such mechanisms have an enduring nature, and are apparent in vivo before considering their possible value as targets for “correcting” the T2DM phenotype. A most challenging aspect is the existence of variable degrees of heterogeneity between SMC populations from different patients and furthermore, that the severity of the “T2DM phenotype” we have previously described [66] is almost certainly influenced by other variables, including for example, degree of insulin resistance, duration of T2DM and effectiveness of glycaemic control.

5. Evaluating SMC phenotype and function in animal models
The ideal animal model of metabolic syndrome and T2DM would present with obesity, hypertension, dyslipidaemia and insulin resistance. T1DM and T2DM are unmistakable and formally defined human diseases whereas it is clear that hyperglycaemia and its attendant consequences are of multiple aetiological origins in animal models [118]. Therefore, whilst no single animal model encompasses all the characteristics of human T2DM, many have proven valuable in understanding pathophysiological mechanisms relevant to man (reviewed in [118]). The utility of the high-fat fed C57/BL6 mouse has been acknowledged for close to two decades [119], and from a practical point of view, rodent models are popular in the study of both the pathogenesis and vascular complications of T2DM.

5.1. Murine models
Use of the db/db mouse model has increased understanding of vascular SMC function/dysfunction. In this model which harbours mutations of the leptin receptor [120], increased Ca\textsuperscript{2+} influx in cerebral arterial SMC led to vascular dysfunction, a phenomenon that was mimicked by hyperglycaemic culture of rat cerebral artery SMC [121]. Another recent study also reported disturbed Ca\textsuperscript{2+} homeostasis in SMC of db/db mice [122]. Disrupted SMC contractile responses through disturbed clock gene expression [123] and increased COX-2 have been reported to induce SMC contractile hypereactivity [124]. Increased SMC accumulation and matrix formation was reported to underlie augmented neointima formation after vein bypass grafting in this murine model [125]. Importantly, SMC cultured from diabetic db/db mouse aorta exhibited an increased inflammatory gene expression profile compared to non-diabetic db/+ SMC and this was retained throughout several cell culture passages [117;126], suggestive of a persistent “memory” of previous metabolic disturbance.

5.2 Rat models
The obese Zucker rat exhibits symptoms of metabolic syndrome; those of hyperlipidaemia, obesity and insulin resistance, although with only mild hyperglycaemia. The Zucker diabetic
fatty rat, (ZDF), which like the db/db mouse carries leptin mutations, rapidly becomes obese and is hyperinsulinaemic, insulin resistant and hyperglycaemic [118]. Consistent with observed vascular dysfunction in human T2DM, ZDF rats exhibit impaired resistance artery remodelling [127] and also develop larger neointimas after arterial injury than lean (control) Zucker animals [128]. The enhanced neointimal hyperplasia observed in the ZDF rat suggests acquisition of a synthetic SMC phenotype – an assumption backed up by two separate studies in the Goto-Kakizaki (GK) rat [129;130], a T2DM model exhibiting hyperglycaemia, hyperinsulinaemia and accelerated atherosclerosis. In those studies, increased vascular SMC proliferation and migration was attributable to increased ERK phosphorylation, an observation confirmed in a separate study showing that both basal and IL-1β-stimulated levels of ERK activity were significantly higher in SMC from GK rats than in those from control rats [131]. Perhaps of significant interest is the report that SMC cultured from the GK rat exhibit both increased contractility and aberrant Rho kinase activation [132] which supports the notion of a “mixed” SMC phenotype.

5.3 Porcine models
Rodent models are clearly most practical for laboratory study, yet large animal models of T2DM, particularly porcine, are invaluable as they share greater compatibility with man in terms of size and physiology. Enhanced atherosclerosis has been reported in two different porcine models fed a high-fat diet, congruent with events in humans [133;134]. Ossabaw pigs with diet-induced metabolic syndrome and coronary artery disease are reported to develop augmented intimal hyperplasia after stent implantation, and isolated coronary SMC from these animals exhibit marked dysfunction in Ca\(^{2+}\) influx [133]. Whilst enhanced intimal hyperplasia would suggest a synthetic phenotype and aberrant Ca\(^{2+}\) handling indicative of a contractile cell, these data together also support the idea of T2DM-SMC possessing some characteristics of each phenotype.

6. Metabolic Memory: concept and evidence

6.1 Clinical trials
Reducing the significant healthcare burden of T2DM complications is a challenging task. Although tight glycaemic control has been shown to ameliorate microvascular complications, the reported benefits on cardiovascular events are controversial. As mentioned earlier, progressive development of vascular complications as a result of prior exposure to hyperinsulinaemia and hyperglycaemia appears to confer a persistent alteration of vascular gene expression that has been termed “metabolic memory” [135]. Indeed, this proposal is underpinned in the clinical setting in that ADVANCE, VADT and ACCORD; large clinical trials that reported minimal impact of glycaemic control on cardiovascular benefit in patients with diabetes and existing clinical CAD [7;18]. However, as discussed earlier, these trials included individuals with a long history of diabetes with previously poor glycaemic control, such that a “negative” metabolic memory theory would explain a reduced impact of subsequent improved glycaemic control. This concept is corroborated by the UKPDS study, in which only subjects with newly diagnosed diabetes were studied and early glycaemic control led to a long-term improvement in both micro- and macrovascular complications [20]. The UKPDS investigators termed this phenomenon a “legacy” effect, thus supporting the need for early control, not only of glycaemia but other associated metabolic abnormalities. Follow-up studies of the DCCT trial revealed that early, intensive glycaemic control in type 1 diabetic patients led to sustained benefits and better macrovascular outcomes [135]. Indeed, 12 years on from the end of DCCT, a beneficial effect of prior glycaemic control was still evident for atherosclerosis in much the same way as identified for microvascular disease [22].

It has recently been proposed that minimising early exposure to hyperglycaemia in
T2DM is paramount [136], underpinning the idea that a change in cellular phenotype is not easily and rapidly reversible by restoring glycaemic control at later time points. Therefore, in the absence of early diagnosis of hyperglycaemia then alternative strategies to beneficially modulate cell phenotype would be necessary and of considerable value.

6.2 Experimental studies
Experimental studies have revealed that transient high glucose exposure can induce persistent phenotypic changes and altered gene expression in the vasculature. For example, in diabetic mice progressive atherosclerosis was noted after restoration of normoglycaemia following a period of hyperglycaemia [137]. Brief hyperglycaemia induced proinflammatory gene expression in aortic EC of non-diabetic mice \textit{in vivo} and \textit{in vitro}, which was maintained even after restoration of normal glycaemia [77]. Furthermore, SMC cultured from diabetic \textit{db/db} mouse aorta exhibited an increased inflammatory gene expression profile compared to non-diabetic \textit{db/+} SMC that was retained throughout several passages [117;126]. In accordance, we have observed a distinct phenotype in human SV-SMC of T2DM origin (reduced proliferative capacity, rhomboid morphology, F-actin fragmentation) that is retained throughout culture and passaging [66]. Taken together, these studies lend support to the idea that loss of SMC plasticity in T2DM may compromise vascular function through an inability to respond to environmental changes.

Thus, emerging perception is that prior metabolic disturbance and hyperglycaemia leaves an early imprint on target cells of the vasculature and is potentially the origin of epigenetic changes that favour vascular dysfunction that is difficult to reverse. Whilst hyperglycaemia \textit{per se} appears to be a key trigger, hyperinsulinaemia, AGEs and raised levels of proinflammatory cytokines and lipids are hallmarks of a diabetes phenotype [78], and likely to be of importance in this regard.

7. Current therapeutics
Although epidemiological studies advocate that tight metabolic control should impact favourably on cardiovascular risk in diabetes (reviewed in [24]), it is clear that this goal is not achieved effectively using current drug therapies (reviewed in [7;18]). It is interesting, however, that some cardiovascular therapies are reported to influence SMC phenotype, at least to some degree.

7.1 Statins
The cholesterol independent effects of statin therapy are clear [138]. It is reported that statin therapy impairs SMC phenotypic modulation from contractile to synthetic state [139], and the antiproliferative effect of statins on SMC improves SV bypass grafting [140]. On a cautionary note, in the CORALL study, high doses of rosvuastatin modestly impaired glycaemic control (HbA1c) in diabetic patients [141]; whilst another study concluded that in non-diabetic individuals statin therapy reduced the incidence of cardiovascular events but conversely was associated with an increase in the onset of T2DM [142].

7.2 Angiotensin Receptor Blockers (ARBs)
These widely used antihypertensive agents appear to improve vascular complications in diabetes patients (reviewed in [143]), some effects being attributable to modulation of SMC phenotype. Through blocking the effect of angiotensin II, ARBs reportedly regulate SMC contractile function by preventing SMC dedifferentiation in atherosclerotic lesions, through a mechanism involving NFAT5 [144]. SMC phenotype is also reportedly regulated by KCa3.1; a calcium-activated potassium channel expressed by synthetic, rather than contractile, SMC [145]. In that particular study of rodent arterial injury and also in a porcine model [146], inhibition of KCa3.1 reduced neointima formation, thereby proposing a mechanism by which
ARBs, through inhibition of KCa3.1, may prevent SMC dedifferentiation. Interestingly, insulin treatment in rats has been recently shown to increase expression of KCa3.1 in SMC, conferring a dedifferentiated phenotype and increased migration and proliferation [147]. Determining whether ARB therapy could reverse these effects would further inform their capacity to control SMC phenotype.

In general, despite the routine use of drugs such as aspirin, statins, antiplatelet drugs, ARBs, ACE inhibitors and other antihypertensive agents, increased cardiovascular disease in patients with T2DM remains consistently higher than that of individuals without diabetes but receiving similar therapies. The search for novel, alternative interventions is therefore a rapidly expanding field.

Selective targeting of SMC phenotype holds promise for a variety of vascular pathologies characterised by a remodelling response. These include hypertension, aneurysm disease, atherosclerosis, restenosis, angiogenesis and wound healing [148]. Elucidating distinct mechanisms that are responsible for particular features of SMC behaviour in vascular diseases would provide exciting prospects for future therapies; recent advances in which will now be briefly considered.

8. Moving towards novel therapies

Traditional risk factors for the development of T2DM are obesity, lack of exercise, smoking, ethnicity; with an HbA1c above 6.0% being a useful marker. Although modulating the risk factors can delay or prevent the development of T2DM [149], once established, reversing the detrimental effect on the vasculature appears a challenging task. As mentioned above, and in support of the reported “legacy” effect in the ACCORD, ADVANCE and VADT clinical trials [7;18], we have observed that cultured SMC from T2DM individuals retain a distinct phenotypic profile in vitro and throughout several weeks to months of subculturing [66]. Such observations suggest that SMC are able to retain memory of previous metabolic disturbance [150], whereby induced epigenetic changes persist even after the damaging stimulus is removed, for example when normal plasma glucose levels are restored.

Current interests in molecular biomarkers encompass the fields of genomics, transcriptomics, proteomics and metabolomics (reviewed in [151]). A variety of markers have been suggested that may precede the development of cardiovascular disease, for example plasminogen-activator-inhibitor-1 (PAI-1), interleukin-6 and phospholipase A2 [152] and are all implicated in T2DM [153-155]. However, it is likely that these are effectors of SMC dysfunction, rather than markers that originate directly from the SMC themselves that are indicative of their phenotype.

8.1 MicroRNAs

(i) miRs as biomarkers of disease

Extensive research has shown that miRs are important in the regulation of diverse cellular functions including those in the vasculature; hence aberrant expression may lead to pathological states [156]. MiRs are also associated with multiple aspects of T2DM, with changes in expression being reported in the liver, pancreatic beta cells, white adipose tissue and skeletal muscle [157]. The potential for miRs as biomarkers has been exemplified in studies whereby the plasma miR profile of T2DM and ND patients was examined. One particular study reported reduced levels of a number of miRs including miR-21 and endothelial miR-126 and importantly, the reduction of miR-126 in the plasma was observed prior to the development of overt T2DM [158]. A different study focussed on several miRs involved in the regulation of insulin gene expression, all of which were found to be increased in the plasma of T2DM subjects [159]. However, these differences were not apparent in the
pre-diabetes period, hence restricting their use as early biomarkers. Identifying miRs that are directly linked to the T2DM SMC phenotype and subsequently investigating whether these can be reliably detected in plasma samples is potentially of greater use.

(ii) miRs as therapeutic targets in T2DM

The evidence for miRs as regulators of SMC phenotype (introduced in section 3.3 above) is well regarded and rapidly accumulating. Importantly, whilst several studies have implicated roles for miRs in the pathogenesis of T2DM (reviewed in [157;160]), their role in diabetic vascular complications is not widely studied. Reports are now emerging that associate altered levels of a number of identified miRs with several diabetic cardiovascular complications (reviewed in [116;160]); however, very little is known about how diabetes per se may modulate phenotype and function of the SMC themselves and indeed, in vivo data is limited.

A recent study reported that enhanced levels of miR-125b were expressed in SMC of diabetic db/db mice relative to control db/+ mice, suggesting that a miR-dependent mechanism promotes a diabetic phenotype [161]. Levels of miR-143 and miR-145 are increased in genetic- and diet-induced mouse models of obesity-associated insulin resistance [162], yet a link with SMC phenotype has not been made to date. There is some evidence for a role of miR-21 in diabetic complications, although both contributory [163] and protective roles [164] in nephropathy are reported in mouse models. Increased miR-200 was very recently shown to enhance inflammatory gene expression in vascular SMC from db/db mice [165]. In that study the authors proposed that disruption of the negative regulatory loop between miR-200 and a transcriptional repressor, Zeb 1 was a direct result of “diabetic conditions”. Abundant expression of miR-195 has been demonstrated in human and rat vascular SMC, that appears to be vasculoprotective in terms of reducing proliferation, migration and inflammatory gene expression as a result of oxLDL exposure [63]. As mentioned earlier, one of the hallmarks of T2DM is elevated levels of oxLDL, suggesting that evaluation of miR-195 in SMC in the setting of T2DM is undoubtedly worthy of further investigation. From evidence accrued to date, it seems likely that dysregulation of miRs induced by the metabolic milieu may therefore feasibly contribute to altered gene expression and aberrancies of SMC function in T2DM individuals, hence augmenting their cardiovascular risk. Whilst some miRs may be perceived beneficial, yet others appear harmful, detailed exploration of cell and species-specific expression and activity is therefore imperative.

The clinical potential of miR therapies is substantial, and they have already been explored in detail in a variety of cancers. Molecular tools for manipulating miR levels (through inhibition or mimicry) have been an area of intense interest, rapid development and ongoing refinement [166]. It is clear that miRs lend themselves to in vivo manipulation and are currently in translational studies in oncology (reviewed recently in [167]). The future potential of miRs with respect to cardiovascular complications in T2DM may lie in restoring a functionally “normal” phenotype to SMC in the macrovasculature. However, it will be essential to understand how particular miRs may direct the fate of the T2DM-SMC and develop cell-targeted delivery strategies to explore them further. Importantly, consolidating this knowledge using in vitro and in vivo studies, and subsequently translating to therapies in man will be a challenging but potentially powerful approach.

8.2 Epigenetics

Whilst genetic factors are known to contribute to the pathogenesis of diabetes, the study of epigenetic mechanisms - that of complex interactions between genes and the environment is rapidly gaining momentum. Chromatin is a dynamic polymer into which genomic DNA is packaged and its remodelling plays a key role in determining cellular
phenotype. As mentioned earlier, this is a varied and complex process for which there is now substantial evidence of epigenetic regulation, particularly with respect to acetylation and methylation of histones in DNA (recently extensively reviewed in [38]). Chromatin remodelling therefore appears to be central to determination of SMC fate through controlling transcriptional access and recruitment of regulatory enzymes [168]. MiRs themselves can be regulated epigenetically, for example through DNA methylation, although evidence accrued to date is derived predominantly from cancer rather than cardiovascular studies (reviewed in [169]). Epigenetic influences can have profound effects on gene expression that control cell phenotype and function [170] and are believed to play roles in diabetes and its attendant complications [171].

Changes in post-translational modifications on histones have been linked to gene expression changes in diabetes [172]. Altered DNA methylation and histone acetylation/methylation have all been observed in animal and cell models of diabetes and can be induced by transient exposure to hyperglycaemia [77;117;137;172;173]. Hyperglycaemia in EC has recently been shown to confer chromatin methylation signatures, resulting in transcriptional modification of genes involved in EC dysfunction [174]. At the level of vascular SMC, those derived from a murine model of diabetes retain proinflammatory and proatherogenic features in culture [117], and in our own laboratory, the phenotypic anomalies we observed in human T2DM-SMC of T2DM were stable over several passages in culture [66], consistent with an underlying epigenetic mechanism that is inheritable through mitosis, and requires further study.

It is simplistic to perceive diabetes as a state of hyperglycaemia. Rather, it is complicated by multiple risk factors (discussed above), any or all of which can conceivably induce epigenetic changes in chromatin structure, gene expression and ultimately modulate SMC phenotype and function. Of relevance to the topic of this article, understanding how epigenetic changes in SMC might underlie metabolic memory would be of extreme value.

The ongoing Human Epigenome project [175] should reveal greater levels of understanding of how such changes regulate features of health and disease. Exploring the relationship between epigenetics and miRs in diabetes complications is also an expanding area of intense research activity [176], fuelled by recent discoveries that are beyond the scope of this review. Their intricate interactions represent a means to not only understand molecular mechanisms but to uncover novel therapeutic targets.

9. Summary and Conclusions
There is emerging support for a distinct and persistent vascular SMC phenotype that occurs with T2DM (Fig. 2 & Table 1) although the evidence is tentative. Further detailed exploration in this area to unambiguously define the characteristics of a T2DM-SMC phenotype will be valuable in identifying new therapeutic targets. In vivo and in vitro studies indicate that reinstating glucose control in the short to medium-term is itself insufficient to restore vascular homeostasis and the scenario is clearly more complex. Cardiovascular complications of T2DM are potentially driven by cellular dysfunction/aberrancies induced by metabolic memory.

Substantial evidence suggests that early detection of T2DM is critical for prevention of diabetes-related macrovascular disease, yet the natural history of the condition makes this difficult. As a result, cardiovascular disease is already evident in around half of T2DM patients by the time of diagnosis. Recent studies propose that plasma biomarkers are useful for early detection of changes in SMC phenotype and function; a complete understanding of the regulation of SMC dysfunction and identification of specific molecular markers would be valuable. Therapies that target SMC to a reparative phenotype hold promise to effectively “erase” metabolic memory and ameliorate cardiovascular disease in this population at risk.
MicroRNA and epigenetic studies are an area of intense interest and these novel targets already demonstrate significant promise for the future.

References


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<th>Contractile</th>
<th>Synthetic</th>
<th>Diabetes</th>
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<tbody>
<tr>
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<td>Rhomboid 32</td>
<td>Rhomboid [66]</td>
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<tr>
<td>Nuclear heterochromatin</td>
<td>Abundant [41]</td>
<td>Minimal [41]</td>
<td>Minimal [40]</td>
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<tr>
<td>Organellos (Rough endoplasmic reticulum, Golgi apparatus, ribosomes)</td>
<td>Minimal [41]</td>
<td>Abundant [41, 42]</td>
<td>Enlarged [40]</td>
</tr>
<tr>
<td>Migratory capacity</td>
<td>Minimal [42]</td>
<td>High [42]</td>
<td>Inconsistent [65, 66]</td>
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Table 1: Characteristics of SMC phenotypes. SMC phenotypes are broadly classed as differentiated “contractile” or dedifferentiated “synthetic”, distinguished by divergent morphological and functional characteristics (see main text). SMC derived from animal models of diabetes and human diabetes patients exhibit a phenotype with similarities predominantly those of dedifferentiated cells, yet with distinct differences in organelle morphology and cell function. Citations as for main text.
Figure 1: Established roles of microRNAs in regulating SMC phenotype and function. MicroRNAs interact with the myocardin – serum-response factor (SRF) – Kruppel-like Factor-4 (KLF4) pathway, modulating SMC phenotype through stimulatory or inhibitory mechanisms. MiR-24, -221 and -222 promote a dedifferentiated synthetic phenotype (marked green) whilst miR-1, -21, -143, -145 and -195 promote a differentiated contractile phenotype (marked red). Cellular functional effects are indicated.
Figure 2. Characterisation of T2DM-SMC phenotype. Mature, differentiated SMC acquire a unique phenotype that may be induced by the metabolic disturbances of T2DM. This functionally impaired, aberrant SMC phenotype contributes to vessel wall dysfunction and increased cardiovascular complications.

Images: Upper panels, phase contrast images (mag. x100, scale bar = 100µm); Lower panels, Rhodamine-phalloidin fluorescence images of F-actin (mag. x630, scale bar = 50µm).