



## Original article

# Elevated expression levels of miR-143/5 in saphenous vein smooth muscle cells from patients with Type 2 diabetes drive persistent changes in phenotype and function



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## ABSTRACT

Type 2 diabetes (T2DM) promotes premature atherosclerosis and inferior prognosis after arterial reconstruction. Vascular smooth muscle cells (SMC) respond to patho/physiological stimuli, switching between quiescent contractile and activated synthetic phenotypes under the control of microRNAs (miRs) that regulate multiple genes critical to SMC plasticity. The importance of miRs to SMC function specifically in T2DM is unknown. This study was performed to evaluate phenotype and function in SMC cultured from non-diabetic and T2DM patients, to explore any aberrancies and investigate underlying mechanisms. Saphenous vein SMC cultured from T2DM patients (T2DM-SMC) exhibited increased spread cell area, disorganised cytoskeleton and impaired proliferation relative to cells from non-diabetic patients (ND-SMC), accompanied by a persistent, selective up-regulation of miR-143 and miR-145. Transfection of premiR-143/145 into ND-SMC induced morphological and functional characteristics similar to native T2DM-SMC; modulating miR-143/145 targets Kruppel-like factor 4, alpha smooth muscle actin and myosin VI. Conversely, transfection of anti-miR-143/145 into T2DM-SMC conferred characteristics of the ND phenotype. Exposure of ND-SMC to transforming growth factor beta (TGF $\beta$ ) induced a diabetes-like phenotype; elevated miR-143/145, increased cell area and reduced proliferation. Furthermore, these effects were dependent on miR-143/145. In conclusion, aberrant expression of miR-143/145 induces a distinct saphenous vein SMC phenotype that may contribute to vascular complications in patients with T2DM, and is potentially amenable to therapeutic manipulation.

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## 1. Introduction

Insulin resistance leading to type 2 diabetes mellitus (T2DM) confers a risk equivalent to 15 years of aging [1]. Early diagnosis is difficult as the disease is initially symptomless; hence up to half of patients have evidence of cardiovascular complications by the time diabetes is confirmed [2]. Such individuals are vulnerable to accelerated atherosclerosis and premature coronary heart disease (CHD), and revascularisation procedures are also problematic with disappointing long-term outcomes [3]. Despite this, coronary artery bypass grafting (CABG) using autologous saphenous vein (SV) remains the optimal treatment for diabetes patients with multivessel disease, following which graft failure is a

significant problem [4,5]. Importantly, structural abnormalities in SV of diabetic subjects are evident pre-operatively, the severity of which appears to be associated with poor glycaemic control [6].

Clinical trials revealed that intensive control of hyperglycaemia is effective in retarding and preventing the microvascular complications of diabetes [7] yet medium term, macrovascular complications persist particularly in patients with diabetes and active CHD [7,8]. Development of vascular complications as a result of prior exposure to hyperinsulinaemia and hyperglycaemia in diabetes appears to confer persistent alterations in vascular gene expression, indicative of epigenetic modulation that is referred to as metabolic memory (reviewed in [9]). Elucidating molecular mechanisms that underlie this phenomenon is therefore of great interest.

Smooth muscle cells (SMC) of blood vessel walls switch between differentiated and dedifferentiated phenotypes in response to local cues. Phenotypic switching is essential during vascular development, repair and adaptation, but also contributes to progression of atherosclerosis

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and bypass graft failure [10]. Effective adaptation to arterial environments early after implantation is a determinant of the long-term patency of SV grafts [11]; hence the ability of SMC to retain plasticity during adaptation and “arterialisation” is vital. Failure of SMC to respond dynamically to conditions of increased flow and pressure early after grafting conceivably jeopardises the longer-term patency of SV used as arterial bypass grafts [12].

We previously reported that human SV-SMC cultured from patients with T2DM were phenotypically and functionally distinct from those of non-diabetic individuals [13]. Key features were rhomboid morphology, F-actin fragmentation and reduced proliferation capacity [13], any of which can conceivably contribute to impaired vessel remodelling in diabetic patients following bypass grafting.

Recent evidence suggests that changes in SMC phenotype and function during vascular remodelling are controlled by epigenetic mechanisms, including microRNAs (miRs) (reviewed in [14]). These small non-coding RNAs act in a tissue- and cell-specific manner, regulating target genes by inducing mRNA degradation or translational repression [15]. Altered levels of miRs have been associated with a number of cardiovascular complications in diabetes (reviewed in [16,17]), however little is known about how diabetes *per se* modulates phenotype and function of vascular SMC. Dysregulation of miRs induced by the metabolic milieu may contribute to altered gene expression and SMC aberrancies in individuals with T2DM [18].

In this study, we discovered that SMC cultured from T2DM patients expressed increased levels of miR-143 and –145 and elucidated roles for these miRs in driving cellular dysfunction.

## 2. Materials and methods

### 2.1. Cell culture

SMC were cultured as we previously described from explants of SV [19] obtained from patients without known diabetes (ND-SMC), or with diagnosed type 2 diabetes (T2DM-SMC) receiving oral therapy alone or oral therapy plus insulin. All patients were undergoing elective CABG surgery at the Leeds General Infirmary. Local ethical committee approval and informed, written patient consent was obtained and the study conformed to the principles outlined in the Declaration of Helsinki.

### 2.2. Cell area measurements

Cells were imaged at x100 magnification and the boundaries of 50 subconfluent individual cells per patient were traced. Spread cell areas were calculated using Image J software (<http://imagej.nih.gov/ij/>). For each patient population, cell areas were ordered (1000  $\mu\text{m}^2$  increments) from which a distribution profile and average cell area was determined.

### 2.3. Rhodamine phalloidin immunofluorescence

Cells were cultured for 48 h in full growth medium (FGM) and fixed in 4% paraformaldehyde. F-actin fibres were labelled using rhodamine phalloidin (1:40) as previously described [20].

### 2.4. Quantitative real-time RT-PCR

Cellular RNA was extracted and real-time RT-PCR was performed using intron-spanning human ACTA2 primers and Taqman probes (Applied Biosystems, Foster City, California) and Applied Biosystems 7500 Real-Time PCR System. Data are expressed as percentage of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control mRNA expression using the formula  $2^{-\Delta\text{CT}} \times 100$ .

### 2.5. Proliferation assays

Proliferation assays were performed over 7 days as previously described [13].

### 2.6. Quantification of miR expression levels

RNA was isolated and relative expression levels of miR-125b, –133a, –143 and –145 determined using specific TaqMan microRNA assays (Applied Biosystems, Foster City, California) and real-time PCR according to manufacturer's protocols. Data analysis was performed using the comparative  $C_T$  method, and values normalised to U6 expression.

### 2.7. Transfection of premiRs and antimiRs

Subconfluent cells were transfected with premiRs (30 nM) or antimiRs (60 nM) for miR-143 and miR-145, and associated negative controls. Endpoints were measured 72 h later (cell morphology, RNA isolation, F-actin staining) or proliferation assays performed.

### 2.8. Immunoblotting

SMC were transfected with relevant premiRs, antimiRs or negative controls. Following transfection, whole cell homogenates were sequentially prepared 3, 5, 7 and 10 days later. Protein lysates were immunoblotted for alpha-smooth muscle actin ( $\alpha$ -SMA), calcineurin (PPP3CA), calcium/calmodulin-dependent protein kinase 2 delta (CaMKII $\delta$ ), kruppel-like factor 4 (KLF4), insulin-like growth factor-1 receptor (IGF-1R), insulin receptor substrate-1 (IRS-1), myosin VI or protein kinase C epsilon (PKC $\epsilon$ ), as described previously [21]. GAPDH acted as a loading control. Selected proteins were also investigated from native ND- and T2DM-SMC cultured under identical conditions.

### 2.9. Induction of miR expression

SMC were treated with high glucose (25 mM), insulin (100 nM), pro-inflammatory cytokines (a combination of interleukin (IL)-1 $\alpha$  and tumour necrosis factor (TNF)- $\alpha$ , both 10 ng/mL), or TGF $\beta$  (0.1–10 ng/mL) in low serum (0.4%)-containing medium for 48 h. Cells maintained in 5.5 mM glucose for the same timeframe acted as controls. RNA was extracted and miR-143 and miR-145 expression levels determined.

### 2.10. TGF $\beta$ signalling blockade

For functional experiments, T2DM-SMC were treated with 10  $\mu\text{g}/\text{ml}$  anti-TGF $\beta$ RII neutralising antibody or control polyclonal goat IgG for 48 h after which end-points were measured (cell morphology, RNA isolation, F-actin staining). For proliferation, media was refreshed after 48 h and final cell counts performed after 96 h. Data were expressed as increase in cell number between days 0 and 4.

### 2.11. Chronic TGF $\beta$ treatment

ND-SMC were treated daily with 1 ng/ml TGF $\beta$  or vehicle for 7 d in media containing 2.5% FCS. TGF $\beta$  was then withdrawn and culture was continued for a further 7 d. Cell area measurements were performed and RNA was extracted on days 7, 11 and 14. Data were expressed as fold change in miR-143/145 levels in cells treated with TGF $\beta$  versus vehicle at each time point.

### 2.12. Statistical analysis

Results are presented as mean  $\pm$  SEM with *n* representing the number of experiments on cells from different patients. Any potential differences in cardiovascular therapies between ND and T2DM-SMC

were evaluated using contingency tables and two-sided Fishers exact test. All experimental data were tested for normality and analysed using parametric or non-parametric unpaired ratio *t*-tests, one-way ratio ANOVA or two-way ANOVA with post-hoc test as appropriate (GraphPad Prism software). *P* < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Subject characteristics

SV-SMC were cultured from a total of 130 patients recruited between August 2008 and January 2014. The mean age of non-diabetic individuals (*n* = 77, 84% male) was  $65.2 \pm 1.05$  (range 42–83) years. For T2DM patients (*n* = 53, 100% male) the mean age was  $63.0 \pm 1.4$  (range 34–85) years. The lack of female subjects in the T2DM group was a reflection of the predominance of male gender undergoing CABG surgery and greater prevalence of T2DM in male subjects, together with the retrospective analysis of subject characteristics.

More detailed data were acquired for 57 of the non-diabetic and 44 of the T2DM patients. Whilst plasma levels of LDL-cholesterol and creatinine were similar between cohorts, fasting glucose and HbA1c were significantly elevated in the diabetic patients (Table 1). All T2DM patients were receiving oral therapy (metformin/sulfonylureas/gliptins), and 30% of these were also receiving insulin (Table 2). Routine cardiovascular medications were similar in both cohorts (Table 2).

Plasma levels of glucose, glycated haemoglobin (HbA1c), LDL cholesterol (LDL-C) and creatinine in non-diabetic and T2DM patients at the time of surgery. Data are expressed as median (range). Whilst cholesterol and creatinine levels were similar, both fasting glucose and HbA1c were significantly elevated in the diabetic patients (Mann–Whitney unpaired *t*-test, NS - not statistically significant).

Comparison of drug therapies between the patient cohorts. Typical cardiovascular treatments: cholesterol-lowering drugs (statins), angiotensin-modulating agents (ACE-I, ARBs), beta adrenergic receptor blockers ( $\beta$ -blockers), anticoagulation therapies and diuretics were common to both patient groups. All diagnosed T2DM patients were receiving oral therapies (predominantly metformin) with some patients also receiving sulfonylureas or gliptins. Of these, 30% were also receiving insulin therapy.

#### 3.2. SV-SMC from T2DM donors exhibit distinct morphology and impaired proliferation

In contrast to the spindle morphology of ND-SMC, cells cultured from T2DM patients were predominantly rhomboid (Fig. 1A), consistent with our previous report [13]. The majority of ND-SMC had spread areas < 10,000  $\mu\text{m}^2$  (Fig. 1B). In contrast, T2DM-SMC displayed greater morphological heterogeneity (Fig. 1B) reflected by mean spread cell area ~60% larger than ND-SMC (Fig. 1C). There was a trend towards increased ACTA2 mRNA levels (the gene encoding  $\alpha$ -SMA, a marker of SMC differentiation) in T2DM-SMC although this did not reach

**Table 1**  
Biochemical characteristics of patients from whom SV-SMC were cultured.

	No diabetes	Type 2 diabetes	P value
Fasting glucose (mmol/L)	5.4 (4.9–9.9)	8.1 (4.3–15.7)	*** <i>P</i> < 0.001
HbA1c (mmol/mol)	42.5 (32.0–56.0)	66.0 (32.0–94.0)	*** <i>P</i> < 0.001
HbA1c (%)	6.04 (5.08–7.27)	8.19 (5.08–10.75)	*** <i>P</i> < 0.001
LDL-C (mmol/L)	3.9 (2.4–7.8)	3.9 (2.2–6.6)	<i>P</i> = 0.97
Creatinine ( $\mu\text{mol/L}$ )	97.0 (50.0–147.0)	100.7 (54.0–203.0)	<i>P</i> = 0.57

Plasma levels of glucose, glycated haemoglobin (HbA1c), LDL cholesterol (LDL-C) and creatinine in non-diabetic and T2DM patients at the time of surgery. Data are expressed as median (range). Whilst cholesterol and creatinine levels were similar, both fasting glucose and HbA1c were significantly elevated in the diabetic patients (Mann–Whitney unpaired *t*-test, NS - not statistically significant).

**Table 2**  
Clinical characteristics of patients from whom SV-SMC were cultured.

	No diabetes	Type 2 diabetes	P value
Oral therapy			
Metformin	-	85.6%	-
+ Sulfonylurea	-	21.4%	-
+ Gliptin	-	7.1%	-
Oral + insulin therapy	-	30.8%	-
Statins	80.0%	78.6%	<i>P</i> = 1.00
ACE inhibitors/ARBs	91.0%	73.3%	<i>P</i> = 0.37
$\beta$ -blockers	92.8%	80.0%	<i>P</i> = 0.37
Antiplatelet/anticoagulant	84.6%	93.8%	<i>P</i> = 0.61
Diuretics	33.3%	40.0%	<i>P</i> = 1.00

Comparison of drug therapies between the patient cohorts. Typical cardiovascular treatments: cholesterol-lowering drugs (statins), angiotensin-modulating agents (ACE-I, ARBs), beta adrenergic receptor blockers ( $\beta$ -blockers), anticoagulation therapies and diuretics were common to both patient groups. All diagnosed T2DM patients were receiving oral therapies (predominantly metformin) with some patients also receiving sulfonylureas or gliptins. Of these, 30% were also receiving insulin therapy.

statistical significance (Fig. 1D). Additionally, T2DM-SMC exhibited a disrupted F-actin cytoskeleton with truncated fibres compared to ND-SMC (Supplementary Fig. 1A) and proliferated more slowly than ND-SMC (Supplementary Fig. 1B), consistent with our previous observations [13]. These divergent profiles appear specific to SV-SMC since we recently observed no disparity in proliferation rate of SMC cultured from internal mammary artery of ND and T2DM patients [22].

#### 3.3. Expression of miR-143 and miR-145 is elevated in T2DM-SMC

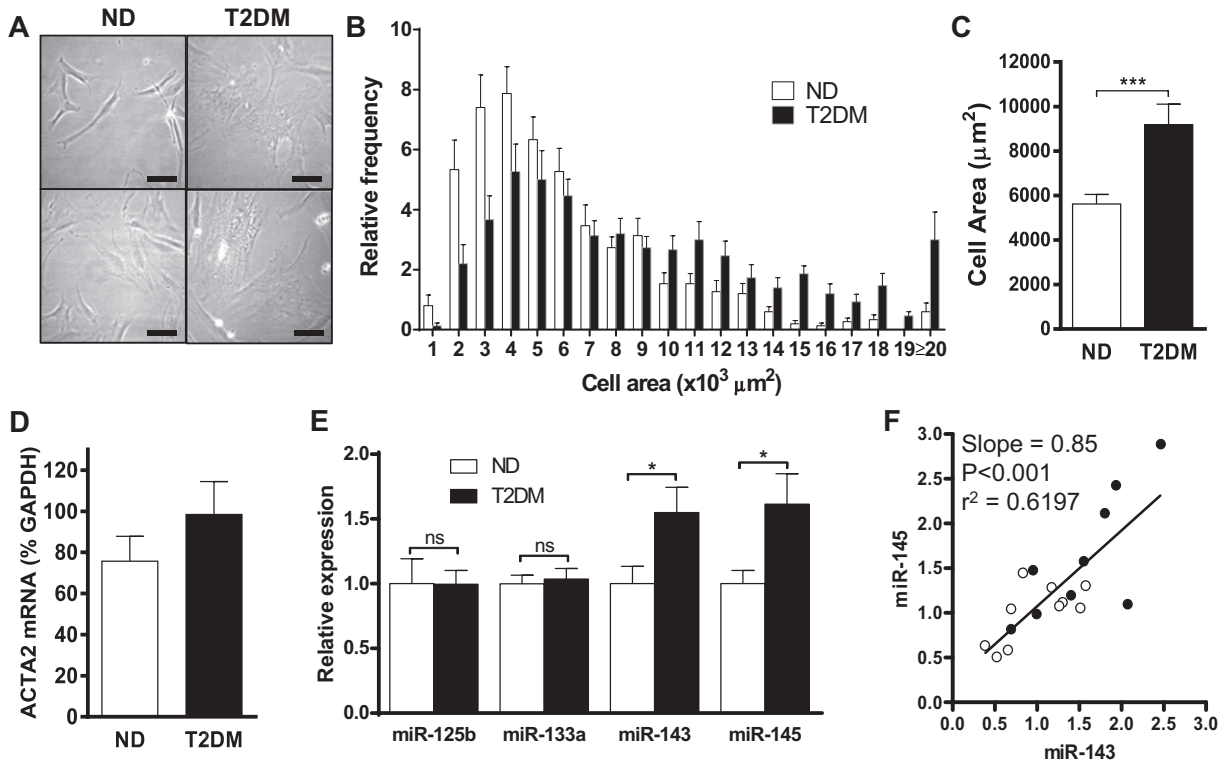
Dysregulated expression of miRs has been implicated in T2DM complications [16,17] Therefore we determined expression levels of “candidate” miRs (125b, 133a, 143 and 145) using quantitative RT-PCR (Fig. 1E). MiR-143 and – 145 are highly expressed in SMC in vascular walls where they reportedly regulate SMC homeostasis and differentiation [23,24]. MiR-125b and miR-133a were investigated given the reported increase in miR-125b in vascular SMC of *db/db* mice [25] and the contribution of miR-133a to abnormal cardiac function in a rabbit model of diabetes [26]. Neither miR-125b nor miR-133a levels differed between SMC populations, but miR-143 and miR-145 were both elevated (~60%) in T2DM-SMC (Fig. 1E). Absolute miR-145 levels were ~10-fold higher than miR-143 in both ND- and T2DM-SMC, but there was a strong correlation between them (Fig. 1F). Expression levels of miR-143 and – 145 remained stable throughout passaging (Supplementary Fig. 2).

#### 3.4. MiR-143 and miR-145 regulate SV-SMC morphology

To determine whether miR-143 and/or – 145 were driving changes in SV-SMC morphology, we artificially manipulated them by transfection with premiRs or antimiRs (Fig. 2). As miR-143 and miR-145 were proportionally higher in T2DM-SMC we used antimiRs to reduce expression levels in T2DM-SMC. Conversely, we used premiRs to increase expression in ND-SMC. Transfection of ND-SMC with premiR-143 and – 145 (alone or combined) led to a 36% increase in spread cell area (Fig. 2A) and the appearance of truncated F-actin fibres similar to those in native T2DM-SMC (Fig. 2B). In T2DM-SMC the combination of antimiR-143 + 145 together was required to modulate spread cell area (~36% reduction) (Fig. 2C) and also visibly reduced the abundance of truncated F-actin fibres (Fig. 2D).

#### 3.5. MiR-145 regulates SMC proliferation

To determine whether altered miR-143/145 expression contributed to the divergent SMC proliferation between non-diabetic and T2DM patients, appropriately transfected cells were incorporated into proliferation assays. Overexpression of premiR-143 + 145 reduced



**Fig. 1. Differential morphology and miR expression of SV-SMC from non-diabetic and type 2 diabetic patients.** (A) Representative phase contrast images of SV-SMC. Cells from non-diabetic patients (ND) show typical spindle-shaped morphology whereas those from patients with type 2 diabetes (T2DM) exhibit a more rhomboid morphology. Scale bar = 100 μm. (B) Frequency distribution of cell areas in 1000 μm<sup>2</sup> intervals and (C) mean cell areas (\*\*P < 0.001, n = 15 patient donors each). (D) RT-PCR analysis of relative expression of ACTA2 mRNA in SV-SMC. Normalized to GAPDH, P = 0.286, n = 20 patient donors each. (E) Relative expression of miRs in SV-SMC from 10 non-diabetic (open bars) and 10 diabetic (closed bars) patients. Normalized to U6 (\*P < 0.05, ns = not significant). (F) Two-tailed Pearson correlation of relative expression of miR-143 and miR-145 in SV-SMC from same 10 nondiabetic (open circles) and 10 diabetic (closed circles) patients. P < 0.001, r<sup>2</sup> = 0.6197.

proliferation of ND-SMC by 35% (P < 0.05, Fig. 2E). Conversely, transfection of T2DM-SMC with anti-miR-143 + 145 increased cell proliferation by 50% (P < 0.05, Fig. 2F). In both cases, the functional effect on proliferation was attributable solely to miR-145; manipulating miR-143 alone was ineffective (Figs. 2E,F).

### 3.6. Identification of miR-143/145 targets in SV-SMC

We used immunoblotting to investigate protein targets potentially responsible for mediating the effects of altered miR-143/145 expression on SMC function. Six validated miR-143 and -145 target genes whose activities are known to regulate SMC function and/or phenotype (KLF4, myosin VI, CaMKIIδ, IGF-1R, IRS-1 and PKCε) [27–30] were explored. α-SMA was studied because endogenous ACTA2 mRNA levels were generally higher in T2DM-SMC (Fig. 1D) and there was a strong correlation between miR-145 and ACTA2 mRNA levels (P < 0.001, r<sup>2</sup> = 0.6324, n = 20). Initial experiments revealed that protein levels of CaMKIIδ, IRS-1 and PKCε were unaffected by miR-143 + 145 modulation at any time point (Supplementary Fig. 3). For targets where some regulation was evident, day 7 post-transfection was optimum for evaluating protein expression following premiR transfection in ND-SMC, and day 10 for anti-miR transfection in T2DM-SMC (Supplementary Fig. 4).

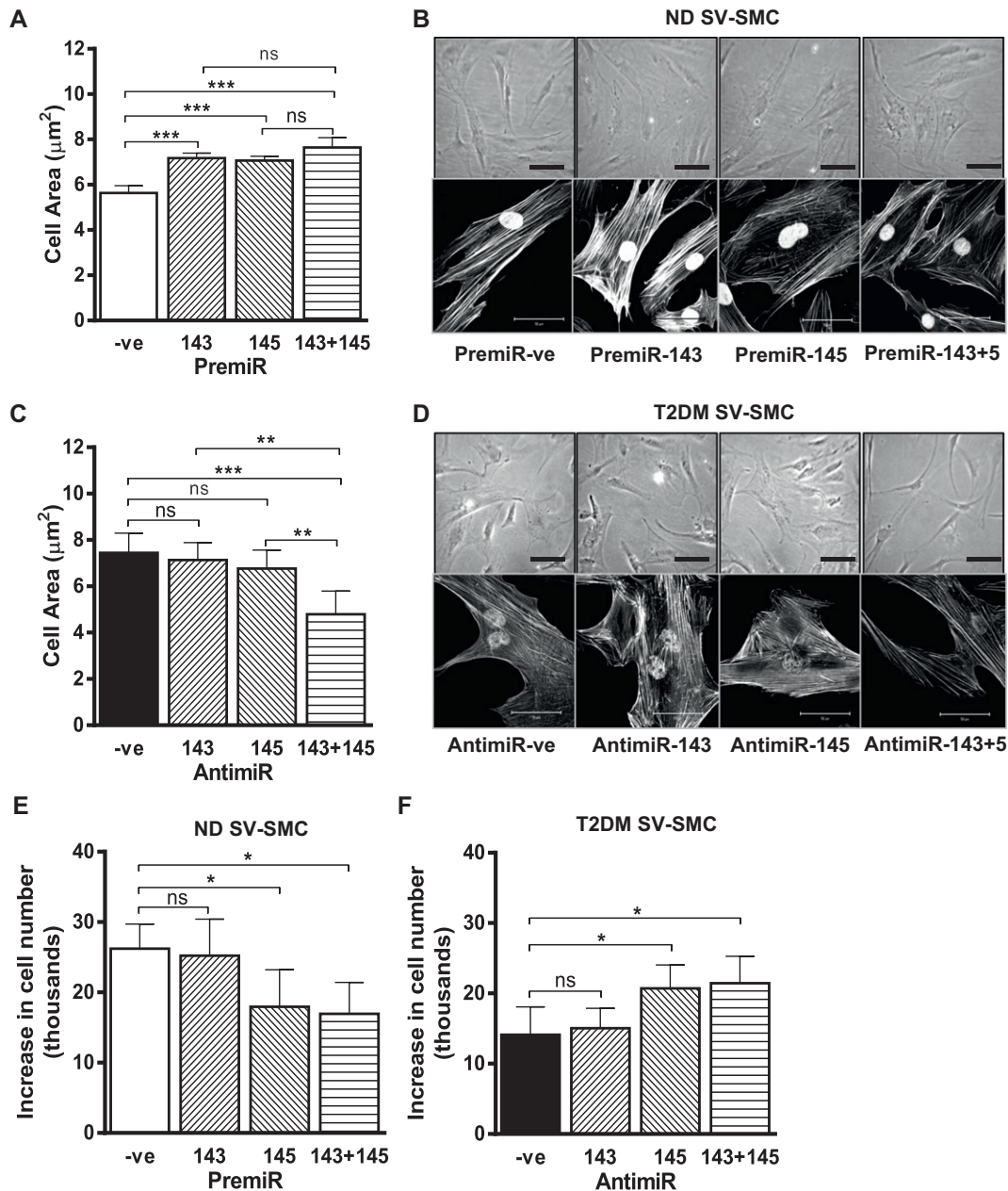
In ND-SMC, protein levels of α-SMA (a marker of differentiation) increased in response to elevating miR-143/145 levels (premiR-143/145) alone or combined, whilst miR inhibition using anti-miR-143 + 145-transfected T2DM-SMC exhibited >40% decrease in α-SMA expression. In each case, modulation appeared predominantly attributable to miR-145 (Fig. 3A). KLF4 (a potential direct target of miR-145) protein levels followed the opposite pattern; they were reduced by either premiR-143 or premiR-145 in ND-SMC and increased following transfection of either anti-miR-143 or -145 (Fig. 3A). Although both premiR-143 and

-145 induced IGF-1R protein expression, neither anti-miR reduced IGF-1R protein levels (Fig. 3A). For myosin VI, a small yet consistent reduction was observed following premiR transfection with a comparable increase in response to anti-miRs (Fig. 3A).

Immunoblotting of lysates from native ND- and T2DM-SMC revealed inherent variability in target protein levels between samples, although there was a trend towards increased α-SMA and decreased KLF4 protein levels in T2DM-SMC (Figs. 3B,C), in agreement with the premiR/anti-miR studies (Fig. 3A). There were no discernible differences in IGF-1R expression (Fig. 3D), but myosin VI expression was significantly lower in native T2DM-SMC than in ND-SMC (Fig. 3E) compatible with the effect of artificially manipulating miR-143 and -145 levels (Fig. 3A). Expression levels of miR-143 and miR-145 were quantified in RNA extracted from cells from the same patient donors as the protein samples; demonstrating that miR-143 and miR-145 were significantly elevated in the T2DM-SMC used specifically in these experiments (Figs. 3F,G), and in agreement with data in Fig. 1E and Supplementary Fig. 2.

### 3.7. TGFβ modifies miR-143/145, cell area and proliferation

In order to identify the molecular mechanisms underlying elevated miR-143/145 in T2DM-SMC, we used a variety of “diabetogenic” stimuli to investigate their ability to increase miR-143/145 levels in ND-SMC (Fig. 4). Whilst glucose, insulin and inflammatory cytokines (IL-1α and TNFα) did not induce miR-143 or -145 expression over the timeframe studied, TGFβ (10 ng/mL) provoked a 2.5-fold increase in miR-143 and -145 after 48 h (Figs. 4A,B). This effect was concentration-dependent (data not shown), being maximal at 1 ng/mL TGFβ. Treatment of ND-SMC with 1 ng/mL TGFβ increased spread cell area by 57% (Figs. 4C,D), increased fragmented F-actin fibres (Fig. 4E), and impaired cell proliferation by 37% (Fig. 4F).



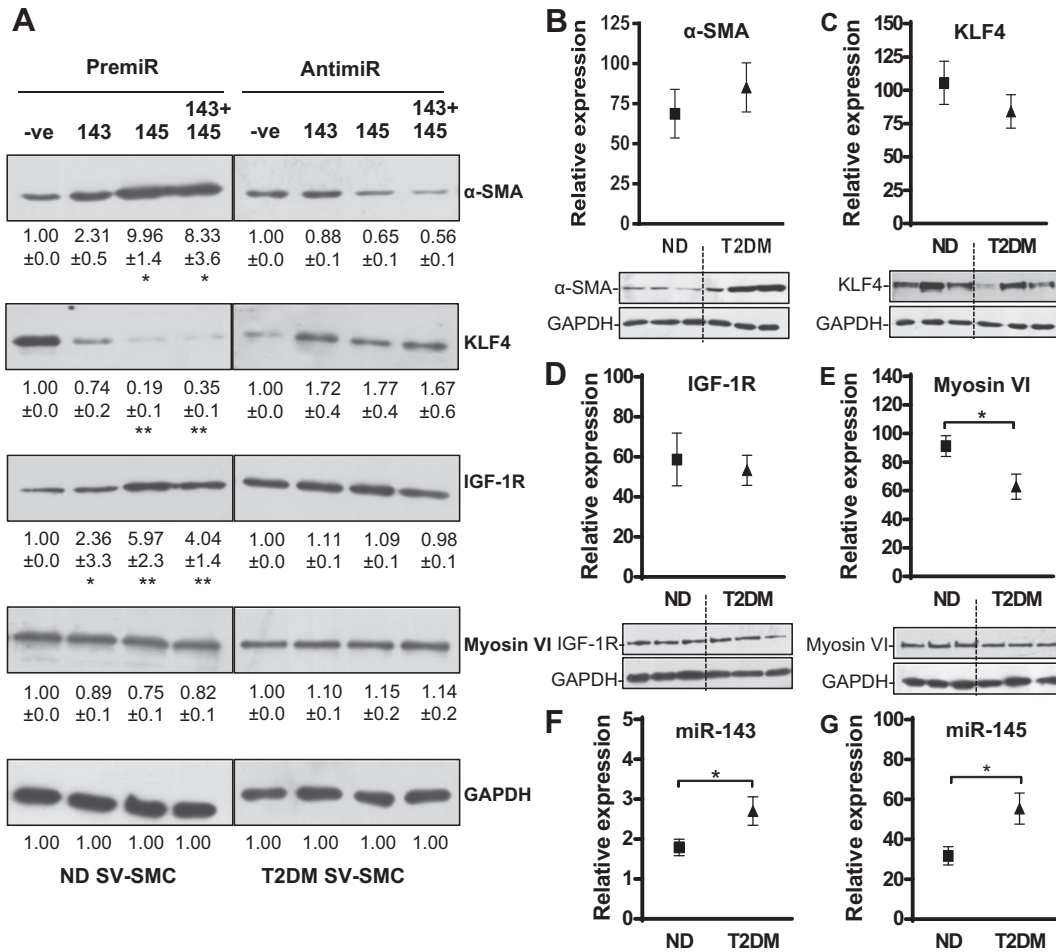
**Fig. 2.** Effect of altering miR-143 and miR-145 levels on SV-SMC morphology and function. (A) Cell areas of ND-SMC transfected with premiR negative (–ve) or premiR-143/145 separately and in combination (\*\*\* $P < 0.001$ , ns = not significant,  $n = 4$  patient donors). (B) Representative phase contrast images (upper panel, scale bar = 100  $\mu\text{m}$ ) or rhodamine phalloidin staining (lower panel, scale bar = 50  $\mu\text{m}$ ) of SV-SMC from 2 different non-diabetic patients transfected with premiR negative (–ve) or premiR-143/145 separately and in combination. (C) Cell areas of T2DM-SMC transfected with anti-miR negative (–ve) or anti-miR-143/145 separately and in combination (\*\*\* $P < 0.001$ , \*\* $P < 0.01$ , ns = not significant,  $n = 4$  patient donors). (D) Representative phase contrast images (upper panel, scale bar = 100  $\mu\text{m}$ ) or rhodamine phalloidin staining (lower panel, scale bar = 50  $\mu\text{m}$ ) of SV-SMC from 2 different diabetic patients transfected with anti-miR negative (–ve) or anti-miR-143/145 separately and in combination. (E,F) SV-SMC were transfected with negative control premiR/anti-miR (–ve) or relevant premiRs or anti-miRs, and cultured for 3 days prior to determining initial cell counts. Cells were then placed into full growth media for a further 4 days before final cell counts (day 4). Data expressed as difference in absolute cell number between final counts and initial counts. (E) Proliferation of premiR-transfected ND cells. (F) Proliferation of anti-miR-transfected T2DM cells (\* $P < 0.05$ , ns = not significant,  $n = 6$  patient donors each).

### 3.8. MiR-143/145 modulate effects of TGF $\beta$ on ND-SMC function

To investigate the relationship between miR-143 + 145 and TGF $\beta$ -mediated cellular effects, ND-SMC were transfected with anti-miRs prior to TGF $\beta$  stimulation. In control (anti-miR negative) cells, TGF $\beta$  induced a 48% increase in cell area whilst in anti-miR-143 + 145-transfected cells this increase was prevented ( $P < 0.01$ , Figs. 5A,B). Similarly, TGF $\beta$  reduced proliferation in control cells by 65%, an effect

completely negated in anti-miR-145-transfected cells ( $P < 0.01$ , Fig. 5C). These data suggest that increased cell area and reduced proliferation rate in response to TGF $\beta$  is mediated via miR-143/5.

We then explored whether inhibition of TGF $\beta$  signalling *per se* could reinstate ND characteristics to T2DM-SMC. A TGF $\beta$ RII neutralising antibody used at a concentration that abrogated TGF $\beta$ -induced Smad3 phosphorylation (Fig. 5D) did not modulate T2DM-SMC cell morphology (Figs. 5E,F), F-actin organisation (Fig. 5G), proliferation (Fig. 5H) or



**Fig. 3. Identification of miR-143/145 targets in SV-SMC.** (A) SMC were transfected with premiRs (ND-SMC) or anti-miRs (T2DM-SMC) for miR-143, miR-145, alone or in combination, or negative controls, and whole cell homogenates prepared after 7 days (premiRs) or 10 days (anti-miRs). Representative immunoblots and pooled densitometry data for  $\alpha$ -SMA, KLF4, IGF-1R, myosin VI and GAPDH loading control ( $n = 4$  patient donors, \* $P < 0.05$ , \*\* $P < 0.01$  vs. premiR -ve). (B) Whole cell lysates were prepared from 20 ND- and 20 T2DM-SMC populations and immunoblotted for  $\alpha$ -SMA, (C) KLF4, (D) IGF-1R and (E) myosin VI (\* $P < 0.05$ ). Densitometry data normalized to GAPDH loading control. (F) miR-143 and (G) miR-145 expression was analysed in RNA from the same patients as the protein samples in panels B-G (\* $P < 0.05$ ).

expression of miR-143 or – 145 (Fig. 5I). Thus although we show that TGF $\beta$  modifies cellular properties through miR-143 and – 145, these effects are not readily reversible in cultured cells.

### 3.9. Chronic exposure to TGF $\beta$ exerts persistent effects on ND-SMC phenotype

To investigate whether long-term application of TGF $\beta$  could induce persistent changes in cellular phenotype even after its removal, ND-SMC were treated for 7 d with TGF $\beta$  and then cultured for an additional 7 d without TGF $\beta$  treatment. TGF $\beta$  induced a 1.9-fold increase in cell area after 7 days (Figs. 6A,B), which was maintained after the removal of TGF $\beta$  for at least one further week (Figs. 6A,B). In parallel, TGF $\beta$  induced a 2.5-fold and 2.2-fold increase in miR-143 and miR-145 expression respectively after 7 days (Figs. 6C,D). However in contrast to the persistent effect on cell morphology, expression of miR-143 and miR-145 returned towards basal levels following withdrawal of TGF $\beta$ .

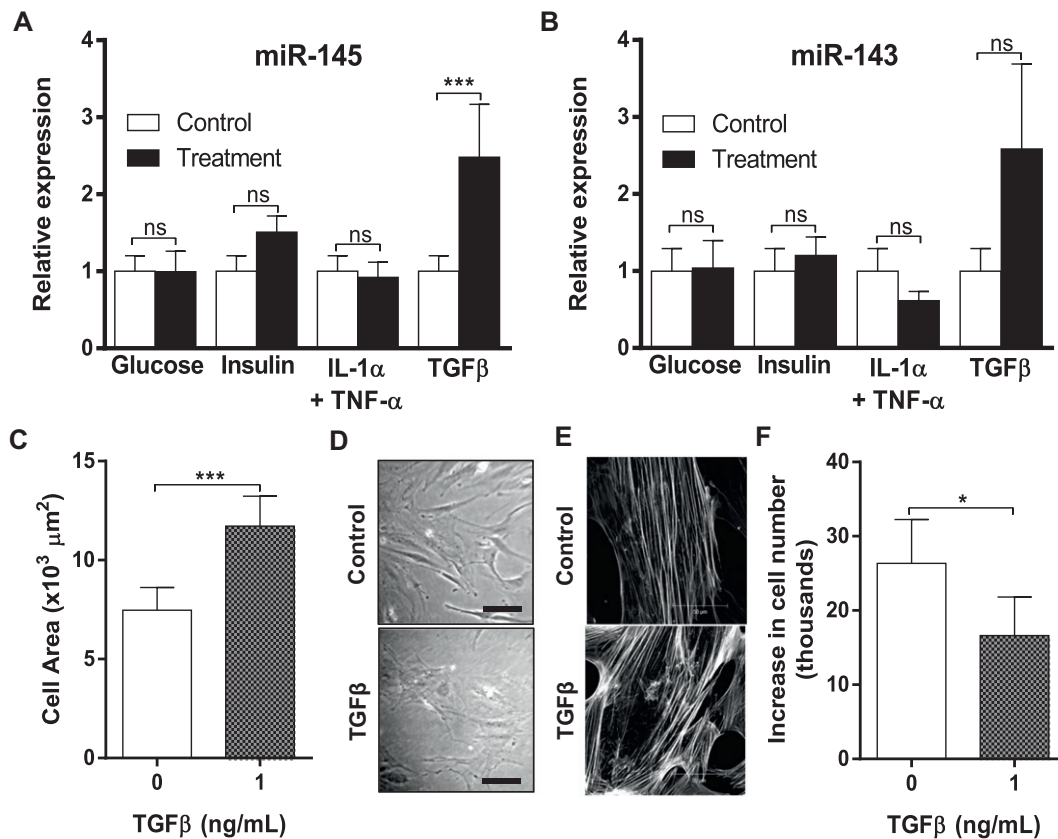
## 4. Discussion

### 4.1. Human saphenous vein smooth muscle cells from diabetic patients exhibit a distinct and persistent phenotype

Structural anomalies and varying degrees of fibrotic thickening have been observed in intact SV prior to use as bypass grafts [31–33]. In

addition, pre-operative abnormalities have been detected in SV harvested from T2DM patients [6] but characterisation at the level of the SMC has not previously been reported. To our knowledge this is the first study to associate dysregulation of miR-143/145 with aberrancies of SV-SMC phenotype and function specific to patients with T2DM.

The principal function of differentiated vascular SMC is contraction, yet their remarkable plasticity confers an ability to undergo phenotypic switching. Vessel remodelling in response to altered blood flow, and repair mechanisms following vascular injury are characterised by dedifferentiated, synthetic SMC with reduced expression of differentiated contractile markers (reviewed in [10]). These classical phenotypes represent extremes of the differentiation scale although SMC may exist in intermediate states [34]. Our data indicate a distinct phenotype in T2DM-SMC, exhibiting both classical differentiated and dedifferentiated characteristics that likely compromise remodelling of venous grafts by preventing dynamic structural changes that are necessary early after implantation to withstand arterial haemodynamic forces (“arterialisation”) [11]. Importantly, this adaptive phase is temporally distinct from ensuing maladaptive intimal thickening and occlusion [35], and requires maintenance of SMC plasticity to execute appropriate cellular function, including proliferation [12]. The data we present therefore is in the context of early graft failure through inadequate arterialisation rather than the subsequent development of intimal hyperplasia. In support of this proposal, recent translational studies demonstrated that loss of primary patency in



**Fig. 4.** Effect of TGF $\beta$  on miR-143/145 expression, cell morphology and proliferation. (A,B) ND-SMC were treated with high glucose (25 mM), insulin (100 nM), IL-1 $\alpha$  and TNF- $\alpha$  (both 10 ng/mL) or TGF $\beta$  (10 ng/mL) for 48 h, and expression of miR-145 and miR-143 analyzed by RT-PCR (\*\*\*)  $P < 0.001$ , ns = not significant,  $n = 5$  patient donors). (C) Quantification of cell areas of ND-SMC treated with 1 ng/mL TGF $\beta$  (\*\*\*)  $P < 0.001$ ,  $n = 5$  patient donors). (D) Representative phase contrast images of cells from same experiment (scale bar = 100  $\mu\text{m}$ ). (E) Rhodamine phalloidin staining of control and TGF $\beta$ -treated cells (scale bar = 50  $\mu\text{m}$ ). (F) Proliferation of ND-SMC cells treated with or without 1 ng/ml TGF $\beta$  (\* $P < 0.05$ ,  $n = 5$  patient donors).

SV grafts was associated with a failure to remodel efficiently within the first 30 days [36].

We discovered increased levels of miR-143 and -145 in SV-SMC cultured from T2DM patients that persisted in long-term culture and in the absence of deleterious stimuli. This modest (1.6-fold) elevation is comparable with other studies that have investigated miR expression in atherosclerosis in native tissues and cells [37–39]. Over-expression of miR-145 (and to a lesser degree miR-143) in ND-SMC increased cell area, disturbed F-actin dynamics and impaired proliferation reminiscent of native T2DM-SMC. Conversely, inhibition of miR-143 + 145 in T2DM-SMC reduced cell area, restored F-actin organisation and increased proliferation to a level indistinguishable from native ND-SMC. Patients with T2DM have elevated plasma levels of TGF $\beta$  [40] and we demonstrated that TGF $\beta$  modulated miR-143/145 expression, cell area and proliferation akin to the native phenotype of T2DM-SMC; effects mediated in part via miR-143 + 145. Our main findings are summarised in Fig. 7.

#### 4.2. MiR expression and function is cell type and species-specific

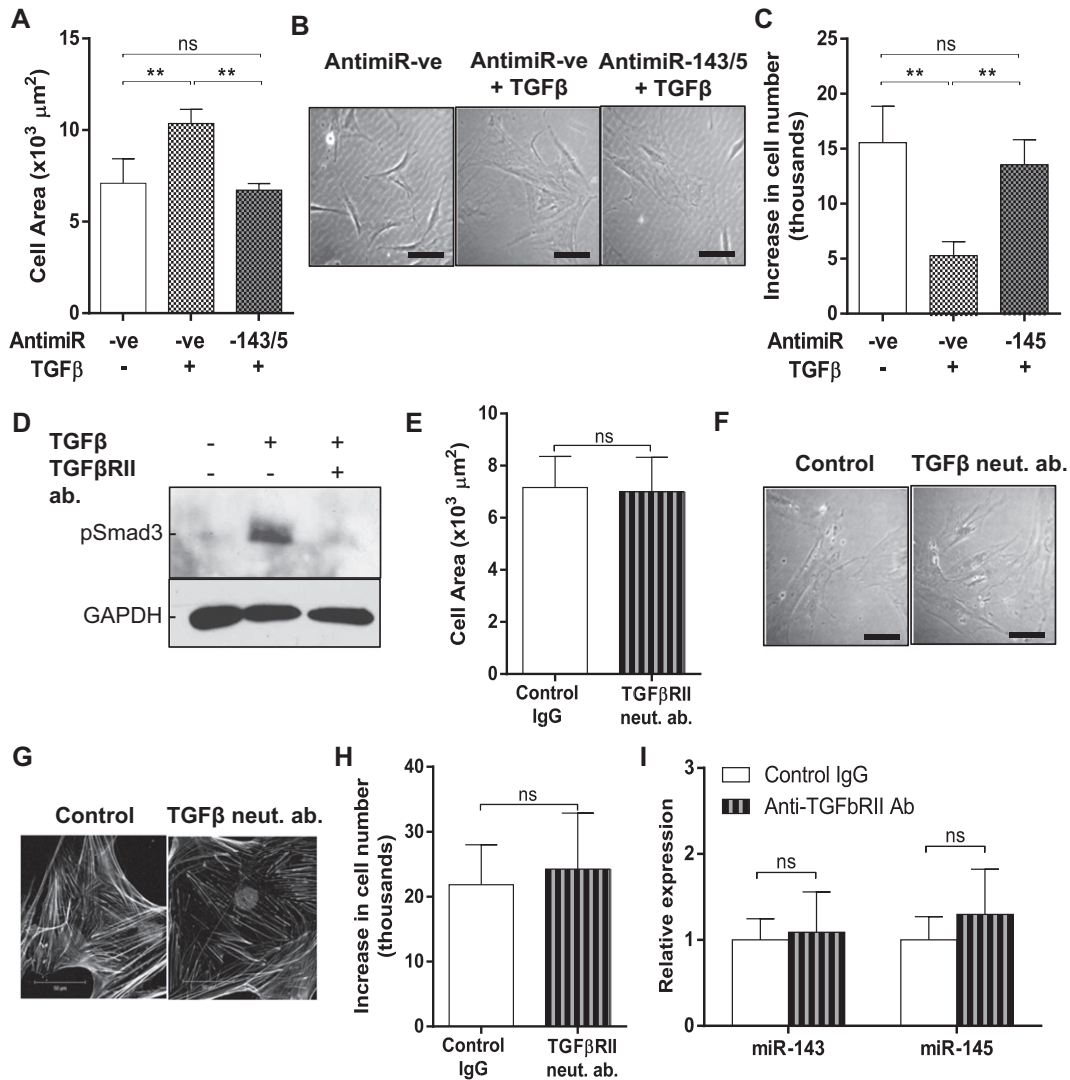
Animal studies have shown that miR-143/145 expression is upregulated in the liver of mouse models of obesity [41], and downregulated in SMC of rats with metabolic syndrome [42]. No differences in plasma levels of miR-143/145 were observed in a study of T2DM and non-diabetic patients [43]; however the source of plasma miRs is most likely circulating and endothelial cells rather than SMC. Our study is therefore the first to discover elevated expression levels of miR-143 and -145, specifically in SV-SMC from T2DM patients, conferring characteristics that persist in culture and throughout passaging.

MiR-143 and -145 are SMC-enriched miRs, yet expression is reported in other tissues such as liver, adipose tissue and plasma [41, 43]. Both are transcribed from the same locus in a bicistronic transcript unit [27] and accordingly we observed similar fold increases in expression of miR-143 and -145. Relative expression levels of miR-145 were 10-fold higher than miR-143, consistent with previous reports [44].

We observed no difference in expression of miR-125b and miR-133a between SV-SMC from human non-diabetic and T2DM patients, despite previous associations with T2DM in cardiovascular cells of animal models [25,26,45]. These anomalies may therefore highlight species-, tissue- or cell type-specific differences. Of particular relevance is a recent report that miR-145 expression was reduced in coronary SMC of rats with metabolic syndrome and associated with impaired collateral growth [42]. Of critical importance however, and of direct relevance to our study, was a demonstration that whilst reinstatement of physiological miR-145 levels completely restored collateral growth, overexpression of miR-145 severely compromised this function, underscoring the necessity for “physiological” levels of miR-145 for normal vascular structure and function [42].

#### 4.3. miR-143 and -145 modulate SV-SMC morphology and function

We observed an association between T2DM, larger SMC size and increased expression levels of miR-143 and -145, concurring with reports that aortic SMC from diabetic rats have larger cytoplasmic volumes than their non-diabetic counterparts [46], and that vascular SMC of miR-143/145 knockout mice are smaller than those of wild-type littermates [47]. We also showed that manipulation of miR-143 and miR-145 regulated actin expression and organisation. Interestingly, a number of regulators of actin dynamics are known targets of these



**Fig. 5.** Effect of modulating TGF $\beta$  signaling on cell morphology and function. (A) ND-SMC were transfected with anti-miR negative (–ve) or anti-miR-143 + 145. After 72 h, transfected cells were treated with TGF $\beta$  (1 ng/mL in 10% FCS media) for 48 h, and cell area measured (\*\* $P$  < 0.001, ns = not significant,  $n$  = 6 patient donors). (B) Representative images of cells from same experiment (scale bar = 100  $\mu$ m). (C) Cells were transfected with anti-miR-ve or anti-miR-145. After 72 h, cells were counted (day 0) and remaining cells treated with TGF $\beta$  (1 ng/ml) or vehicle for 96 h, and cells counted again. Data are represented as the increase in cell number (\*\* $P$  < 0.01, ns = not significant,  $n$  = 6 patient donors). (D) TGF $\beta$  signaling was blocked using TGF $\beta$ RII neutralizing antibody (ab). Confluent cells were treated with TGF $\beta$  (1 ng/mL) for 20 min following a 1 h pre-treatment with anti-TGF $\beta$ RII ab (10  $\mu$ g/mL). Cells were harvested and immunoblotted for phosphorylation of Smad3 or GAPDH (loading control). (E) T2DM-SMC were treated with TGF $\beta$ RII neutralizing ab (10  $\mu$ g/mL) or control IgG for 48 h in 10% FCS media, and cell area measurements taken (ns = not significant,  $n$  = 4 patient donors). (F) Representative images from the same experiment (scale bar = 100  $\mu$ m). (G) Following the same treatment, F-actin cytoskeleton was visualized using rhodamine phalloidin. Representative images, scale bar = 50  $\mu$ m. (H) For proliferation, quiesced cells were treated with 10  $\mu$ g/mL TGF $\beta$ RII neutralizing ab or control IgG for 96 h in 10% FCS media prior to cell counting (ns = non-significant,  $n$  = 4 patient donors). (I) Expression levels of miR-145 and miR-143 following 48 h treatment with 10  $\mu$ g/mL TGF $\beta$ RII neutralizing ab or control IgG (ns = non-significant,  $n$  = 4 patient donors).

miRs [43,47]. Through the promotion of contractile gene expression, miR-145 overexpression has been shown to inhibit the development of neointimal hyperplasia in injured murine arteries [23,48]. Interestingly in a very small human study, miR-145 downregulation in “traditionally” harvested SV was associated with poorer patency than in veins harvested using a “no touch” technique where miR-145 levels were reportedly higher [49].

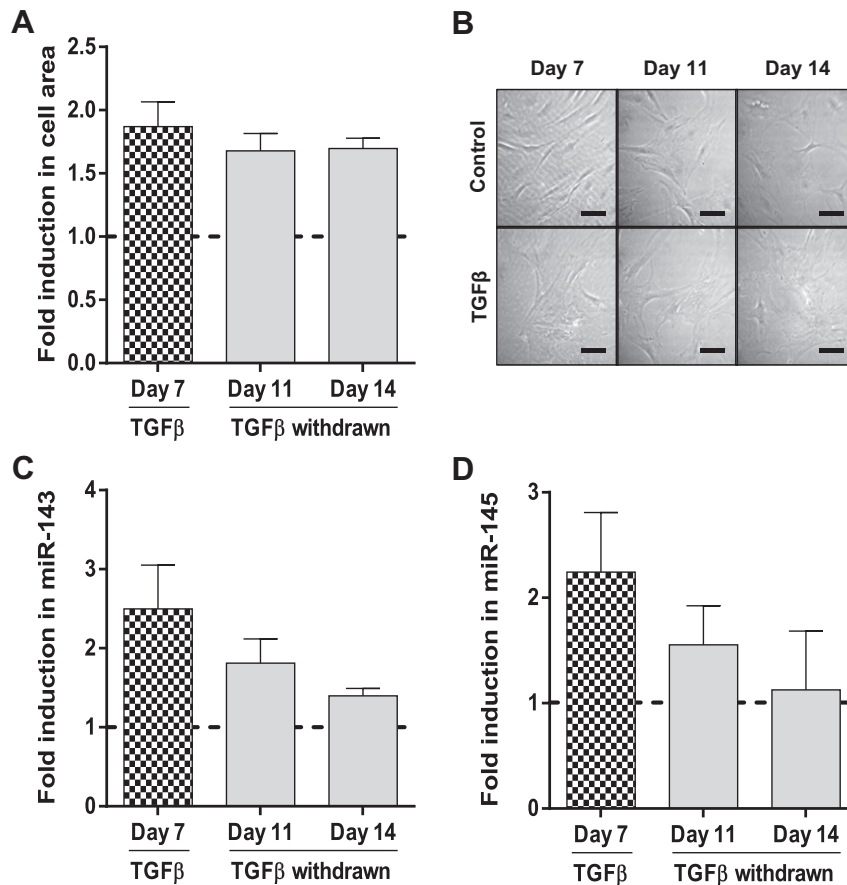
We previously reported that T2DM-SMC exhibit impaired proliferation rate [13]; results corroborated in the present study and additionally found to be characteristic of SV, but not internal mammary artery. This impairment was effectively rescued by transfection with anti-miR-145 and conversely, premiR-145 transfection into ND-SMC reduced proliferation. Manipulation of miR-143 in either SMC population was ineffective, indicating that specifically miR-145 modulates proliferative activity. In accordance with our findings, overexpression of miR-145 and to a lesser degree miR-143, suppressed proliferation of rat aortic

[27] and human pulmonary artery SMC [50]. As the most abundantly expressed in the vasculature, miR-145 is associated with differentiated SMC [23] with apparent greater influence than miR-143 [44]. Reduced levels of miR-143/145 were observed in proliferative vascular SMC following carotid injury in a murine model [27]. However, no such differences were evident in cardiac muscle in the same animals, again highlighting the cell and tissue-type specificity of miR expression, features that are potentially attractive for therapeutic miR manipulation.

#### 4.4. Identification of miR-143/145 targets in SV-SMC

Our data suggest that overexpression of miR-145 reduced expression of KLF4 and increased expression of the differentiation marker  $\alpha$ -SMA. In human pulmonary artery SMC, miR-143 or –145 overexpression down-regulated KLF4, although the magnitude of effect was greater with miR-145 [44]. Lentiviral transfer of miR-145 into murine carotid arteries





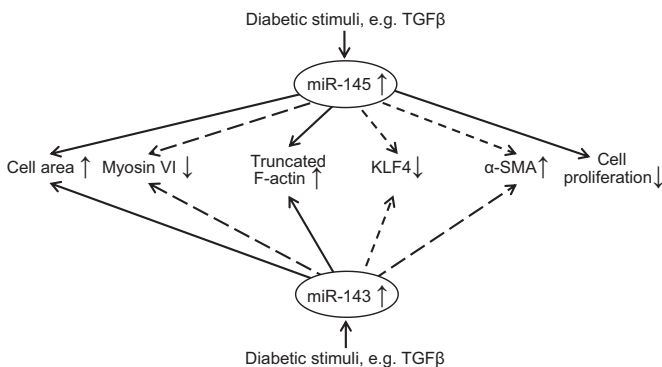
**Fig. 6. Prolonged effect of TGFβ on ND-SMC phenotype.** ND-SMC were cultured for 7 days with 1 ng/mL TGFβ or vehicle control, and then for a further 7 days in media with no treatment. Cell areas, miR-143 and miR-145 expression were monitored on days 7, 11 and 14. Data are expressed as fold increase in cells cultured with TGFβ for 7 days relative to vehicle-treated cells maintained for the same time period (dotted line). (A) Fold-induction in spread cell area at each time point (n = 4). (B) Representative phase contrast images of ND-SMC from the same experiment (scale bar = 100 μm). (C) Induction of miR-143 expression (n = 4) and (D) miR-145 expression in the same cells (n = 4).

*in vivo* increased expression of markers of SMC differentiation, including α-SMA [27]. KLF4 is not expressed in healthy blood vessels but is rapidly induced following vascular injury where it is associated with transcriptional repression of SMC differentiation marker genes including α-SMA [51]. Recent studies provide new evidence that loss of KLF4 and subsequent SMC differentiation is driven at least in part by miR-143/145 [27, 47]. In the current study, whilst a trend towards increased α-SMA and decreased KLF4 in T2DM-SMC was apparent, this was not statistically significant. It is likely that the high inter-patient variation together with

the semi-quantitative nature of immunoblotting were contributing factors. Myosin VI regulates cellular adhesion, endocytosis and gene transcription and of particular relevance to this study, has been shown to stabilise F-actin fibres [52,53]. It is plausible that the persistent disorganised F-actin cytoskeleton that we observed in T2DM-SMC may be related to reduced expression of myosin VI, although a more detailed study of the time course of this effect would be required.

#### 4.5. TGFβ up-regulates miR-143/145 and alters SV-SMC phenotype and function

Upregulation of miR-143/145 could conceivably be caused by any number of factors within the diabetic milieu. Elevated concentrations of glucose, insulin and inflammatory cytokines reportedly modulate expression of various microRNAs [25,54,55]. We examined the effect of several stimuli on miR-143/145 expression and discovered TGFβ as a candidate, concurring with reports in pulmonary and coronary artery SMC [44,56]. One of these studies clearly demonstrated rapid transcriptional induction of miR-143/145 and increased contractile gene expression by TGFβ in human pulmonary artery SMC, and importantly, this was specific to SMC of human, but not rodent origin [44]. Furthermore, in our study, treatment of ND-SMC with TGFβ increased spread cell area and reduced proliferation similar to levels observed in T2DM-SMC; effects rescued by miR-143 + 145 knockdown. Elevated plasma levels of TGFβ in T2DM patients *in vivo* [40,57,58] may therefore augment vascular dysfunction evident in this patient group, at least in part through elevating miR-143/145 expression. Inhibition of TGFβ signalling through Smad3 failed to re-establish a ND phenotype in T2DM-SMC, indicating that continued TGFβ signalling in T2DM cells is not required for



**Fig. 7. Summary Figure.** Stimuli associated with type 2 diabetes (e.g. TGFβ) drive persistent increases in expression of miR-143 and – 145 in SV-SMC. Increased miR-145 levels reduce SV-SMC proliferation, whilst increases in either miR-143 or – 145 levels lead to increased cell area and truncated F-actin. Broken arrows indicate a proposed association based upon our data and literature within the discussion.

these sustained phenotypic differences that more likely arose from earlier *in vivo* TGF $\beta$  exposure. This concept was confirmed by our data revealing a persistent effect of TGF $\beta$  on cell morphology that was retained for a week following its withdrawal. Additionally, secretion of TGF $\beta$  from cultured ND- and T2DM-SMC was similar (data not shown).

To examine the persistent nature of TGF $\beta$  stimulation further we also monitored miR-143/145 levels following the removal of TGF $\beta$ . In this case, and in contrast to the effects on cell morphology, expression of miR-143/145 returned towards baseline levels. It is possible that exposure time of greater than a week would be necessary to confer a prolonged upregulation of miR-143/145. Indeed, in terms of the progression of T2DM it is likely that patients endure elevated TGF $\beta$  levels for months or years (a scenario that is difficult to model *in vitro*), which may inflict persistent upregulation of miR-143/145. Whilst in our study we clearly demonstrated that miR-143/145 does drive TGF $\beta$ -mediated cellular effects (i.e. they were abrogated by anti-miR transfection), the diabetic milieu is complex and other factors are likely to act in concert with TGF $\beta$  *in vivo* in maintaining elevated miR-143/145 expression as we observed in native T2DM cells.

#### 4.6. Clinical Perspective

Effective adaptation to arterial environments after grafting is a key determinant of SV graft patency [11] and hence the ability of SMC to retain plasticity during this early phase of adaptation and “arterialisation” is of major importance. We have revealed a distinct and persistent phenotype of T2DM-SMC possessing characteristics in common with both classically differentiated and dedifferentiated SMC that would potentially compromise this plasticity. Owing to the silent, progressive nature of insulin resistance leading to T2DM, the vasculature is exposed to circulating metabolic disturbances for a prolonged period. Clinical data unequivocally illustrate the harmful nature of metabolic memory and have led to intense activity aimed at deciphering the underlying molecular mechanisms [59]. For example, even transient exposure to high glucose appears sufficient to impose long-term changes in gene expression in cultured vascular endothelial cells [60]. Our data indicate that elevated levels of miR-143/145 in human SV-SMC induced by diabetogenic stimuli such as TGF $\beta$  may represent one such mechanism (summarised in Fig. 7). Species and tissue specificity is undoubtedly an important aspect of miR expression; defining potential disparate functions of individual miRs according to pathophysiology and/or cell type are key considerations [24]. Therapeutic manipulation of miR-143/-145 in SV-SMC may therefore provide a novel opportunity to erase metabolic memory and restore vascular function in T2DM patients.

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**Disclosures:** None

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmcc.2014.05.018>.

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## Glossary

- $\alpha$ -SMA: alpha smooth muscle actin  
 CABG: coronary artery bypass grafting  
 CaMKII $\delta$ : calcium/calmodulin-dependent protein kinase 2 delta  
 CHD: coronary heart disease  
 DMEM: Dulbecco's modified eagle medium  
 FCS: foetal calf serum  
 FGM: full growth medium  
 GAPDH: glyceraldehyde 3-phosphate dehydrogenase  
 IGF-1R: insulin-like growth factor receptor 1  
 IL-1 $\alpha$ : interleukin-1 alpha  
 IRS-1: insulin receptor substrate 1  
 KLF4: Kruppel-like factor 4  
 miR: microRNA  
 ND: non-diabetic  
 PKC $\epsilon$ : protein kinase C epsilon  
 SMC: smooth muscle cell  
 SV: saphenous vein  
 T2DM: type 2 diabetes  
 TGF $\beta$ : transforming growth factor beta  
 TNF- $\alpha$ : tumour necrosis factor alpha