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Type 2 diabetes impairs venous, but not arterial smooth muscle cell function: possible role of differential RhoA activity

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Abstract

Background/Purpose: Coronary heart disease is the leading cause of morbidity in patients with type 2 diabetes mellitus (T2DM), frequently resulting in a requirement for coronary revascularization using the internal mammary artery (IMA) or saphenous vein (SV). Patency rates of SV grafts are inferior to IMA and further impaired by T2DM whilst IMA patencies appear similar in both populations. Smooth muscle cells (SMC) play a pivotal role in graft integration; we therefore examined the phenotype and proliferative function of IMA- and SV-SMC isolated from non-diabetic (ND) patients or those diagnosed with T2DM.

Methods/Materials: SMC were cultured from fragments of SV or IMA. Morphology was analyzed under light microscopy (spread cell area measurements) and confocal microscopy (F-actin staining). Proliferation was analyzed by cell counting. Levels of RhoA mRNA, protein and activity were measured by real-time RT-PCR, western blotting and G-LISA respectively.

Results: IMA-SMC from T2DM and ND patients were indistinguishable in both morphology and function. By comparison, SV-SMC from T2DM patients exhibited significantly larger spread cell areas (1.5-fold increase, $P<0.05$), truncated F-actin fibers and reduced proliferation (33% reduction, $P<0.05$). Furthermore, lower expression and activity of RhoA was observed in SV-SMC of T2DM patients (37% reduction in expression, $P<0.05$ and 43% reduction in activity, $P<0.01$).

Conclusions: IMA-SMC appear impervious to phenotypic modulation by T2DM. In contrast, SV-SMC from T2DM patients exhibit phenotypic and functional changes accompanied by reduced RhoA activity. These aberrancies may be epigenetic in nature, compromising SMC plasticity and SV graft adaptation in T2DM patients.

Keywords: Type 2 diabetes, smooth muscle cell, saphenous vein, internal mammary artery, RhoA; cell phenotype

Summary

The internal mammary artery (IMA) is the conduit of choice for bypass grafting and is generally successful in all patients, including those with Type 2 diabetes (T2DM). By contrast, saphenous vein (SV) is inferior to IMA and furthermore patients with T2DM suffer strikingly poorer outcomes than their non-diabetic (ND) counterparts. We discovered that SV-SMC from T2DM patients exhibit altered persistent morphology and function compared to ND SV-SMC, with differential expression and activity of the small GTPase RhoA, yet ND and T2DM IMA-SMC were indistinguishable. These data offer an explanation for the superior patency of IMA grafting independent of the presence of diabetes.

Introduction

Type 2 diabetes mellitus (T2DM) is an escalating global epidemic, and in the UK alone the number of patients with diagnosed T2DM has almost doubled over the past 15 years (diabetes.org.uk). Importantly, treatment of patients with T2DM and its resultant complications now accounts for approximately 10% of the entire UK National Health Service budget. One of the leading causes of morbidity and mortality in patients with T2DM is accelerated atherosclerosis and coronary heart disease (1) that often precedes clinical diagnosis of T2DM (2).

The surgical approach to revascularizing atherosclerotic coronary arteries is coronary artery bypass grafting (CABG) using autologous internal mammary artery (IMA) or saphenous vein (SV) to restore blood supply to the ischemic heart. Whilst the IMA is known to be a superior conduit with patency rates significantly higher than SV (3), due to its limited availability and the need for multiple grafts the SV is routinely the conduit of choice in many patients. Furthermore, patients with T2DM have poorer SV graft outcomes compared to their non-diabetic counterparts (4). Interestingly, this is not the case with IMA, in which patency rates are comparable between patients with or without T2DM (reviewed recently in (5)).

IMA and SV are structurally distinct vessels (3) that respond differently to alterations in pressure and cyclical stretching that are evident when vessels are implanted following CABG. This leads to changes in vessel structure and function, e.g. altered distensibility and stiffness (reviewed in (6)) and intimal abnormalities (7), due at least in part to the orientation and behaviour of smooth muscle cells (SMC). Successful integration of grafts early after implantation requires efficient adaptive remodeling (8), a process that involves phenotypic switching of SMC in terms of co-ordinated migration, proliferation and cytoskeletal rearrangement. This functional capacity to adapt is temporally distinct from the subsequent neointimal thickening that underlies narrowing and restenosis. The signaling cascades regulating these processes are complex and include mitogen activated protein kinases

(MAPK) such as extracellular signal related kinase (ERK) and p38 MAPK (9), amongst others. Small GTPases and in particular RhoA / Rho kinase are also well recognized effectors of such adaptive changes (10).

RhoA is an archetypal member of the Rho family of small GTPases, activation of which promotes formation of F-actin stress fibers and focal adhesions which link stress fibers to the plasma membrane, thereby affecting SMC contractility and adhesion (11). RhoA regulates many cellular functions including migration and proliferation (11), dysregulation of which are implicated in cardiovascular disorders such as hypertension, coronary artery vasospasm and neointimal hyperplasia (reviewed in (10)). RhoA is reportedly activated by hyperglycemia, and accordingly aberrant RhoA activity has been demonstrated in rodent models of diabetic nephropathy (12) and myocardial fibrosis (13).

We have previously reported inherent differences in the morphology and function of SV-SMC from non-diabetic (ND) and T2DM patients; specifically that SV-SMC from patients with diabetes exhibited rhomboid-like morphology, altered cytoskeletal arrangement and impaired proliferative capacity compared to those isolated from patients without diabetes (14). Of particular interest was our observation that the cellular disparities were maintained throughout culture and serial passaging and not influenced by glucose concentration. We therefore speculated that through prior exposure to the metabolic milieu, SMC from diabetic patients show evidence of “memory”. The aim of this study was therefore to investigate any influence of T2DM on IMA-SMC phenotype and to determine a potential role for RhoA.

Materials and Methods

SMC isolation and culture

SMC were obtained from IMA and SV fragments from a total 63 different patients undergoing CABG at Leeds General Infirmary, UK, and were cultured by explant technique as described

previously (15). Local ethical committee approval and informed patient consent was obtained. This study conformed to the principles outlined in the Declaration of Helsinki. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-Glutamine and 1% penicillin / streptomycin / fungizone (full growth media – FGM) at 37°C in 5% CO_2 in air. SMC were serially passaged using trypsin/EDTA as necessary. All cells were isolated and sub-cultured for up to 6 passages; individual experiments were performed on SMC of equivalent passage from age-matched donors with diagnosed T2DM or without (ND), characteristics of whom are documented in Table 1. All investigations were performed on cells between passages 2-6, an interval over which we have previously identified a stable phenotype (14).

Cell area measurements

SMC were seeded at a density of $2x10^5$ cells per 75 cm^2 flask, cultured for 96 h and then examined under phase contrast microscopy at x100 magnification. Fifteen random fields of view were captured and the spread areas of 50 cells per patient were measured using ImageJ software (http://imagej.nih.gov/ij/). In addition to calculating the average cellular area per patient population, data was used to record the distribution of cell sizes (5000 μm^2 increments) within each population.

Immunocytochemistry

SMC were seeded at a density of $2x10^3$ per well in Lab-Tek chamberslides, cultured for 96 h in FGM and then fixed in 4% paraformaldehyde. Immunostaining for smooth muscle myosin heavy chain (SM-MHC; AbCam 1:100), alpha smooth muscle actin (α-SMA; Sigma-Aldrich 1:200), F-actin (Invitrogen, 1:50) and vinculin (Sigma-Aldrich, 1:400) was performed essentially as previously described (14). Images were visualized using a Zeiss LSM 510 confocal microscope at x400 magnification.

Proliferation assays

Proliferation assays were performed essentially as we described previously (14). Briefly, cells were seeded at $1x10^4$ cells per ml in 24-well plates, cultured for 24 h, then quiesced in serum-free medium for 72 h prior to making triplicate 'day 0' counts using trypan blue and a hemocytometer. SMC were maintained in FGM for up to 7 d. Media was replenished on days 2 and 4 and triplicate viable cell counts taken on days 2, 4 and 7 to generate a growth curve from which area under curve (AUC) analysis was performed.

RhoA real-time RT-PCR

SMC were seeded at a density of $2x10^5$ in 25 cm^2 flasks and cultured in FGM for 96 h before total mRNA was isolated as previously described (16). Real-time reverse transcription PCR was used to determine RhoA mRNA expression using specific TaqMan assays (Life Technologies, Hs00357608_m1). RhoA expression was calculated as a percentage of GAPDH (Hs99999905_m1) using the formula $2^{-\Delta CT}$ x 100.

RhoA Western blotting

Reduced whole cell lysates were prepared and immunoblotted for RhoA (Santa Cruz, 1:100) or GAPDH (Sigma-Aldrich, 1:4000) as previously described (17). Expression of RhoA and GAPDH was normalized to a single sample that was included on all membranes to allow comparison between experiments.

RhoA activity assay

SMC were seeded in FGM at a density of $2x10^5$ cells in 25 cm^2 tissue culture flasks and cultured for 96 h before preparing non-reduced whole cell lysates. RhoA activity was measured using a commercial RhoA G-LISA™ according to manufacturer's instructions (Cytoskeleton, Inc).

Statistical analyses

All results are expressed as mean ± SEM with n representing the number of experiments on cells from different patients. Differences between treatment groups were analyzed using unpaired ratio t-tests or ratio two-way ANOVA with Bonferroni post-hoc test as appropriate (GraphPad Prism software). $P<0.05$ was considered statistically significant.

Results

Morphology of IMA and SV-SMC from ND and T2DM patients

Differences between IMA and SV-SMC were evident soon after explant (typically between 3-8 days). IMA-SMC presented a wide distribution of spread cell area but ND and T2DM IMA-SMC were not significantly different (9586 ± 814 μm^2 versus 11969 ± 2728 μm^2, respectively) (Fig. 1A,B).

In ND patients, venous cells were ~33% smaller in area than arterial cells (6454 ± 947 μm^2 versus 9586 ± 814 μm^2). However, when comparing SV-SMC between ND and T2DM patients, the latter were significantly larger (1.5-fold increase, 9838 ± 814 μm^2, Fig. 1C,D) consistent with our previous report (14). Moreover this phenotype was maintained throughout all passages studied (Fig. 1E,F).

Cytoskeletal morphology of IMA and SV-SMC from ND and T2DM patients

All cells co-expressed SM-MHC and α-SMA, confirming their identity as SMC (Fig. 2A,B). In IMA-SMC, SM-MHC formed distinct fibers within the cytoplasm and at the cell periphery in cells from both ND and T2DM patients (Fig. 2A). In contrast, localization was diffuse throughout the cytoplasm of SV-SMC (Fig. 2B) irrespective of diabetic status. This suggested that SM-MHC distribution and localization was related to the source of SMC rather than being associated with diabetic status.

All cells stained positively for α-SMA and although arrangement into fibers was infrequent, when present they were visible only in ND SV-SMC (Fig. 2A,B). F-actin formed thin fibers in IMA-SMC in both ND and T2DM patients (Fig. 2A). In contrast, SV-SMC from ND patients exhibited long stress fibers traversing the cell (Fig. 2B), but this pattern was absent in T2DM SV-SMC in which fibers were visibly truncated and disorganized (Fig. 2B).

Vinculin-positive focal adhesions were sparse in all cell populations, and there were no apparent differences between IMA- and SV-SMC from ND or T2DM patients (Fig. 2A,B).

Proliferative capacity of IMA and SV-SMC from ND and T2DM patients

In agreement with our previous work (18), ND IMA-SMC exhibited a 50% lower proliferation rate than ND SV-SMC (AUC 104.8 ± 22.8 vs. 209.8 ± 18.6 respectively). Importantly, IMA-SMC proliferation did not differ between the patient cohorts (Fig. 3A,B), unlike SV-SMC where it was significantly lower in T2DM patients (32% reduction, Fig. 3C,D), concurrent with our previous report (14).

Cellular RhoA expression

The small GTPase RhoA is a known regulator of the F-actin cytoskeleton, focal adhesion formation and cell proliferation (10;11); we therefore quantified expression (mRNA and protein) and activity (GTP-bound) of RhoA in IMA-SMC and SV-SMC. RhoA mRNA and protein expression were comparable in IMA-SMC from both ND and T2DM patients and RhoA activity, whilst variable between patients, was similar regardless of diabetic status. (Fig. 4A-C). In contrast, whilst RhoA mRNA levels were comparable between patient groups in SV-SMC (Fig. 4D), protein expression and basal activity of RhoA was significantly lower (>40%) in SV-SMC from T2DM compared with ND patients (Fig. 4E,F).

Discussion

In this study we demonstrated that IMA-SMC from T2DM patients were morphologically indistinguishable from those of ND patients. In contrast, SV-SMC from T2DM patients displayed a distinct phenotype (aberrant morphology, cytoskeletal disarray and impaired proliferative capacity) that was accompanied by reduced levels of RhoA expression/activity. Effective adaptation to arterial environments early after implantation is a key determinant of the long-term patency of SV grafts (8). The ability of vascular SMC to retain plasticity and respond (for example through increased cellular proliferation) during adaptation and "arterialization" of the grafted vein is therefore of major importance for the success of the graft. The distinct T2DM SMC phenotype that we identified appears specific to the SV and likely compromises the capacity to remodel and arterialize the venous graft. Crucially, this adaptive phase is temporally distinct from subsequent maladaptive intimal thickening and occlusion (19). Whilst IMA grafts are superior to SV, importantly they are equally effective in patients with and without T2DM, whereas SV grafts in diabetic patients are additionally compromised (4;5). The phenotypic differences accompanied by altered RhoA activity highlighted in our study may reveal a molecular basis for this clinical phenomenon.

Cytoskeleton

We previously described a distinct SV-SMC phenotype from patients with T2DM (14) and in the present study, we quantified spread cell areas from IMA and SV sources. IMA-SMC were large and more spread than SV-SMC but they did not differ between ND and T2DM patients. ND SV-SMC were typically smaller than ND IMA-SMC, and additionally SV-SMC from T2DM patients whilst more heterogeneous, were significantly larger than their ND counterparts. Because IMA are more successful grafts overall, our data suggest that cell size *per se* does not impair the ability of SMC to form an efficient arterial conduit, but that molecular regulation of cytoskeletal structure may be essential.

In line with similar spread cell area profiles, the cytoskeleton of IMA-SMC was similar in both ND and T2DM patients although all IMA-SMC consistently exhibited a fiber-like arrangement of SM-MHC rather than a diffuse staining as observed in SV-SMC. This pattern of staining has previously been demonstrated in rabbit aortic SMC where it was associated with cells of a contractile phenotype (20), suggesting that under routine culture conditions, IMA-SMC exhibit a more contractile phenotype compared to SV-SMC; a phenomenon that is unrelated to diabetic status. We previously demonstrated that SV-SMC from patients with T2DM exhibited diffuse α-SMA immunoreactivity, truncated F-actin fibers and increased frequency of large vinculin-positive focal adhesions compared to those from non-diabetic patients (14). The perceived resistance of IMA-SMC to cytoskeletal modulation by T2DM as seen in SV-SMC may confer functional properties that explain the superior efficacy of IMA grafts.

Proliferation

Consistent with our previous findings (18), IMA-SMC proliferated more slowly than SV-SMC and here we additionally demonstrate this was not influenced by diabetic status. It appears that IMA-SMC may be generally less susceptible to growth factor stimulation than SV-SMC. For example, altered PDGF receptor subtype expression (21) and reduced phosphorylation levels of ERK in IMA-SMC (18) are possible explanations. Interestingly, inhibition of RhoA has been demonstrated to decrease proliferation in SMC (22), supporting a hypothesis that impaired cellular RhoA activity as we observed in T2DM-SV-SMC may underlie their reduced proliferation capacity with a potentially deleterious effect on graft remodeling.

RhoA expression and activity

Rho family GTPases contribute to vascular disorders through modulating vascular cell behaviour at least in part via altered F-actin dynamics and proliferation (10;11). Truncated F-actin fibers and impaired proliferation that we observed in T2DM SV-SMC led us to hypothesize that differential RhoA activity may be evident between SV and IMA-SMC, and additionally may vary according to diabetic status. Indeed, a previous study reported that the

Rho kinase inhibitor, fasudil, induced greater relaxation responses in intact SV from T2DM patients compared to SV from non-diabetic patients. Importantly, this differential sensitivity was not evident in IMA from the same patients (23).

Basal RhoA activity in ND SV- and IMA-SMC were similar. Previous reports comparing RhoA mRNA expression between IMA-SMC and SV-SMC have yielded conflicting results, reporting either increased (24), or no change in SV expression (21). Our data supports the latter, but importantly the G-LISA approach permitted quantification, specifically of RhoA activity.

Published reports relating to the influence of T2DM on RhoA activity and / or expression are also at variance. Whilst RhoA is generally upregulated in animal (12;13) and human (23) models of diabetes, there are also reports of no differences (25) or marked reductions (26). These inconsistencies may arise from both variation in experimental technique and divergent expression of RhoA in target tissues. In the current study, expression and activity of RhoA in IMA-SMC was similar between the two patient groups. In direct contrast, SV-SMC from T2DM patients displayed significantly lower RhoA activity than ND cells. Interestingly, RhoA mRNA levels did not vary, suggesting that a mechanism of regulation involving altered mRNA stability and/or translational inhibition may exist in vascular SMC. MicroRNAs (miRs) are small, non-coding RNAs that negatively regulate target genes in a species- and cell-specific manner (27). Of particular note, miR-133a is known to target RhoA (28) and is reportedly increased in skeletal muscle of a murine model of Type 1 diabetes (29). It is therefore possible that differential expression of miRs in T2DM SV-SMC may impact RhoA and is an area worthy of further investigation.

Involvement of SMC phenotypic switching

Phenotypic switching of SMC from contractile to synthetic states is an area of intense research and has been extensively reviewed (30). Increased spread cell area and reduced proliferation in SV-SMC from T2DM individuals suggests that the SMC have acquired a more

contractile state yet the defective stress fibers and impaired RhoA activation also demonstrated are not features of the contractile phenotype. This almost certainly supports the concept of complex mechanisms that underlie these changes, some of which may include transcription factors, RhoA guanine exchange factors or cell cycle regulators that are not associated with differentiation status *per se* but more related to the acquisition of a distinct 'diabetes' SMC phenotype. Indeed, we have recently reviewed the evidence for existence of such a phenotype from diabetes models (31).

Clinical relevance

Whilst initially symptomless, the long-term risks to T2DM patients are those of debilitating cardiovascular complications. In the clinical setting, the United Kingdom Prospective Diabetes Study (UKPDS) reported the advantage of early diagnosis of diabetes and prompt glycaemic control leading to a sustained reduction in cardiovascular events (32). Conversely, other large clinical trials reported minimal impact of glycaemic control on cardiovascular benefit in patients with diabetes and existing clinical CAD (33;34). Such phenomena have been termed "metabolic memory" (35). The importance of our data is underscored by the observation that the T2DM SV-SMC phenotype cannot be rescued and persists throughout culture. We propose that SV-SMC "remember" previous metabolic disturbance and hyperglycaemia, and display a particular signature that is maintained even after removal of a damaging stimulus. The molecular mechanisms underlying this are undoubtedly complex although there is emerging evidence for epigenetic mechanisms (36), a fuller understanding of which would be potentially valuable.

Conclusions

It is unequivocal that *in vivo*, the structural and mechanical differences between the SV and IMA, together with significant endothelial dysfunction impact vascular function. The novelty of this study is the observation of a distinct SV-SMC phenotype in T2DM whereas IMA-SMC do not appear vulnerable to T2DM-induced phenotypic alterations (cell morphology,

cytoskeleton and proliferation). In contrast, SV-SMC isolated from patients with T2DM exhibit distinct aberrancies accompanied by reduced levels of RhoA expression and activity. Our data may help to explain why IMA grafts are equally robust in both ND and T2DM patients. Exploration of epigenetic mechanisms of RhoA expression and activity is an avenue potentially amenable to therapeutic manipulation.

Acknowledgements

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Figure legends

Figure 1: SMC morphology. Cells were imaged (x100 magnification) and spread cell areas measured using ImageJ. (A) Distribution of cell areas in IMA-SMC with representative phase contrast images of ND and T2DM cells (2 each), and (B) mean cell area. (C) Distribution of cell areas in SV-SMC with representative phase contrast images of ND and T2DM cells (2 each), and (D) mean cell area (all n=10 ND and 10 T2DM, *p<0.05, ns = non-significant vs. ND, scale bar = 100 µm). The enlarged phenotype observed in T2DM SV-SMC was maintained throughout passaging (P2-P6). (E) Representative images (scale bar = 100 µm) and (F) mean cell area from cells at each passage (n=4 ND and 4 T2DM). Two-way ANOVA p = non-significant for the effect of passaging, *p<0.05 for effect of T2DM.

Figure 2: SMC cytoskeletal morphology. Cells were stained for SM-MHC, α-SMA, F-actin and vinculin, and imaged at x400 magnification. (A) Representative images of cytoskeletal morphology in ND and T2DM IMA-SMC and (B) ND and T2DM SV-SMC. Images acquired from quadruplicate fields of a minimum of 4 patients for both SV and IMA. Scale bar = 50 µm.

Figure 3: SMC proliferation. Growth curves were constructed from viable cell counts made over a 7-day period. (A) Proliferation time course of IMA-SMC from ND and T2DM patients; (B) area under curve analysis. (C) Proliferation time course of SV-SMC from ND and T2DM patients and (D) area under curve analysis. All n=10 ND and 10 T2DM, *p<0.05, ns = non-significant vs. ND.

Figure 4: RhoA mRNA, protein and activity in ND and T2DM SMC. (A) RhoA mRNA expression (RT-PCR), (B) protein (immunoblotting) and (C) activity (G-LISA) in ND and T2DM IMA-SMC. Comparative analysis performed on SV-SMC for (D) RhoA mRNA, (E) protein and (F) activity. In western blots, RhoA protein levels were normalized to GAPDH levels from the corresponding sample. All n=10 ND and 10 T2DM, **p<0.01, *p<0.05, ns = non-significant vs. ND.

Figure 1

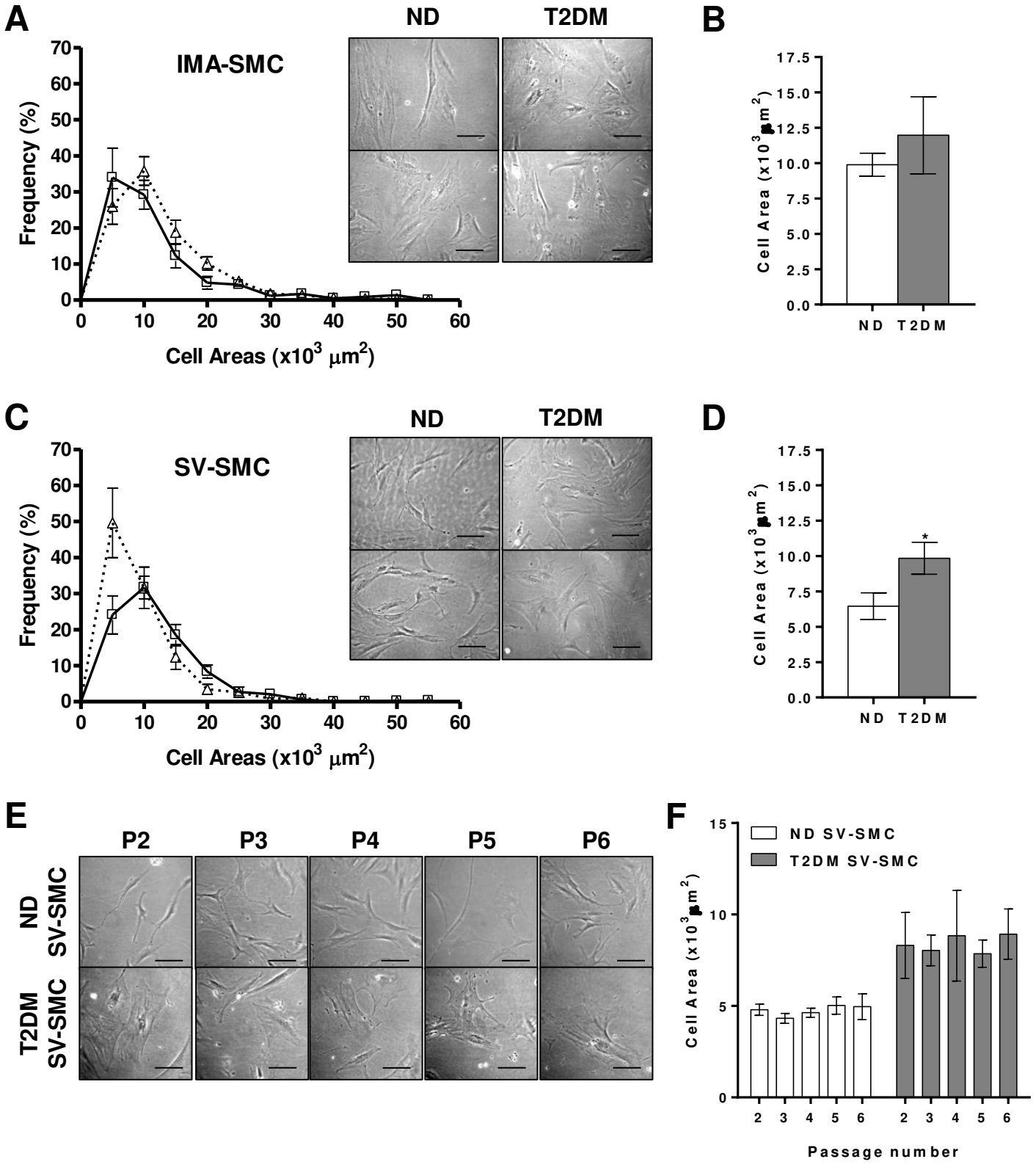
A
ND
T2DM
IMA-SMC
Frequency (%)
Cell Areas (x10³ μm²)
B
Cell Area (x10³ μm²)
ND
T2DM
C
SV-SMC
D
*
E
P2
P3
P4
P5
P6
ND SV-SMC
T2DM SV-SMC
F
ND SV-SMC
T2DM SV-SMC
Passage number

Figure 2

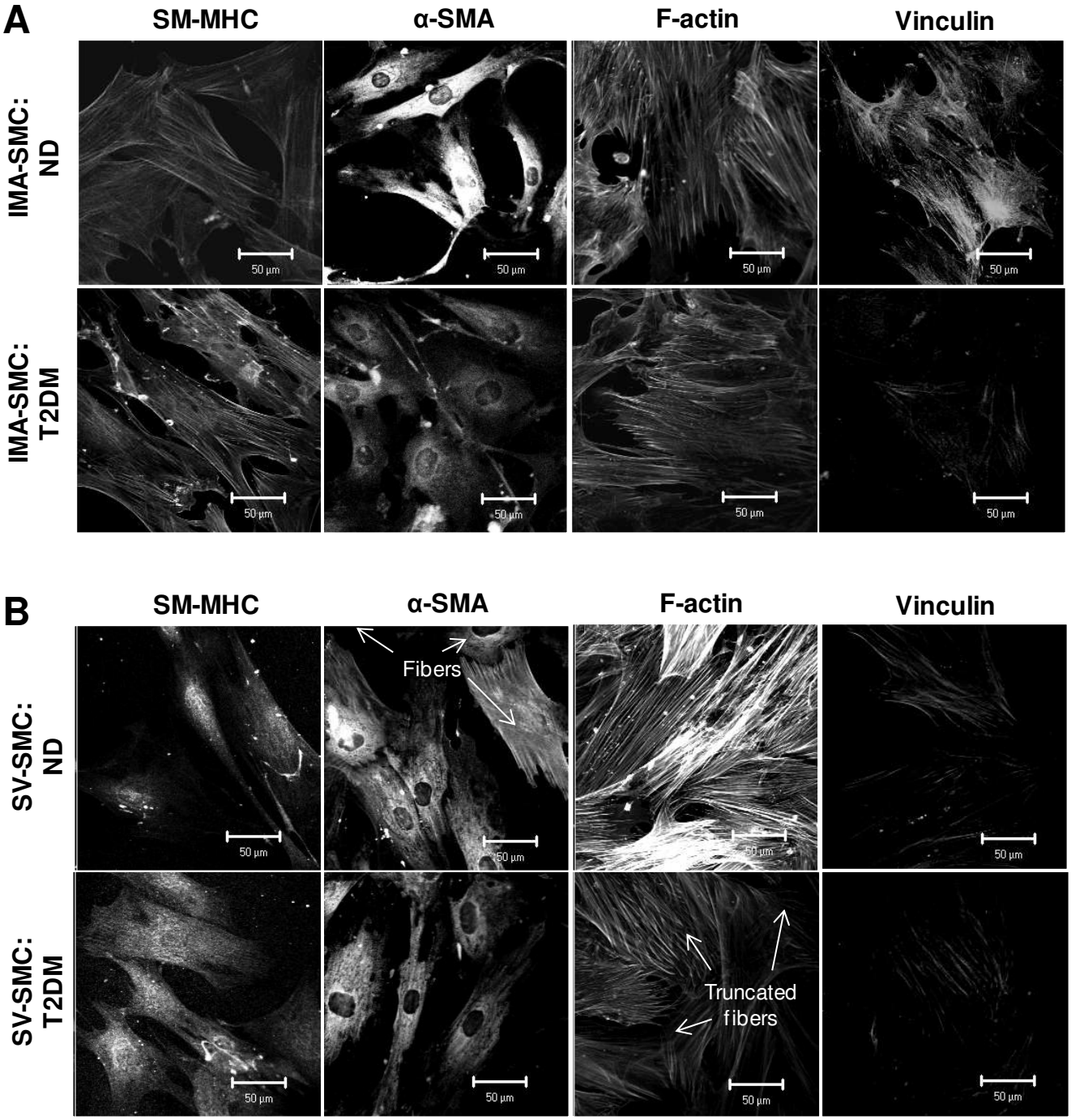
A
SM-MHC
α-SMA
F-actin
Vinculin
IMA-SMC: ND
IMA-SMC: T2DM
50 µm
B
SM-MHC
α-SMA
F-actin
Vinculin
SV-SMC: ND
SV-SMC: T2DM
Fibers
Truncated fibers
50 µm

Figure 3

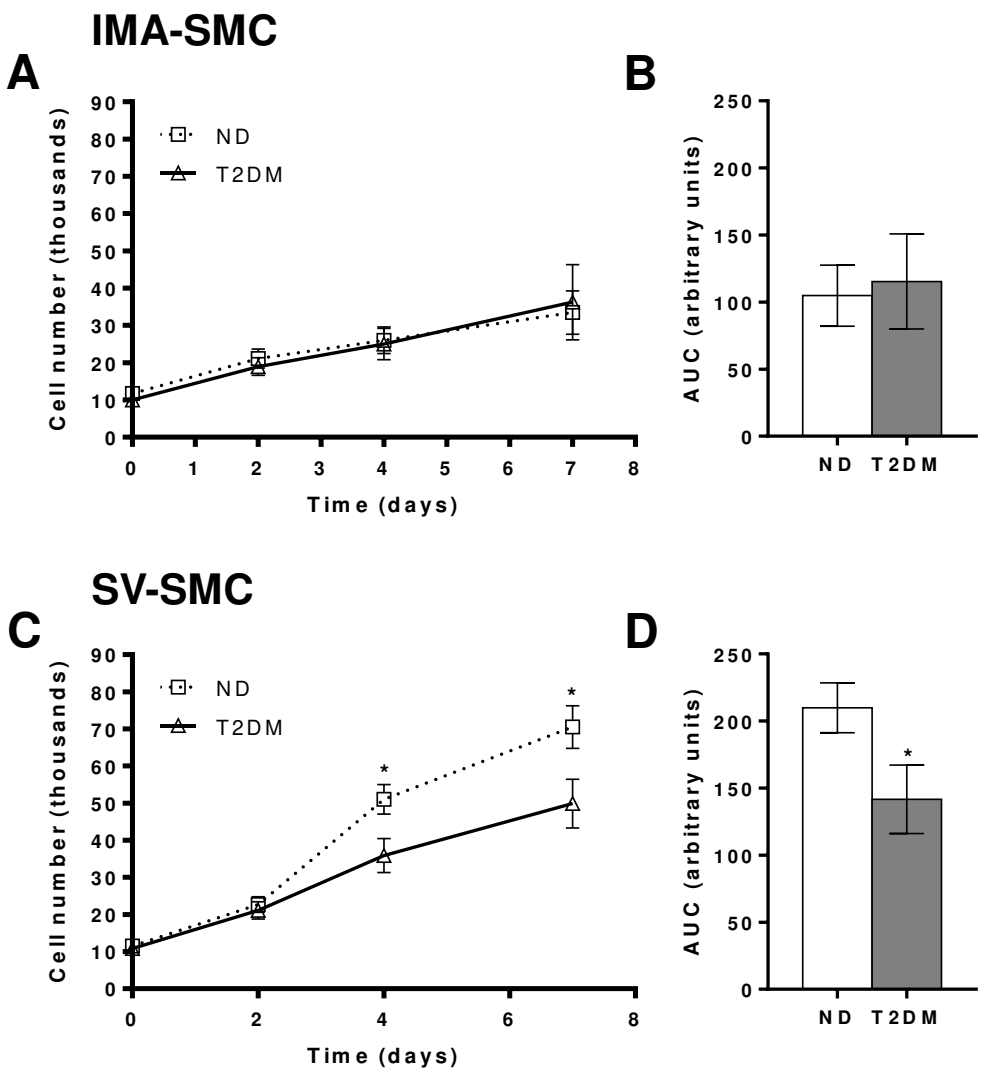
IMA-SMC
A
B
Cell number (thousands)
ND
T2DM
Time (days)
AUC (arbitrary units)
ND
T2DM
SV-SMC
C
D
Cell number (thousands)
ND
T2DM
Time (days)
AUC (arbitrary units)
ND
T2DM

Figure 4

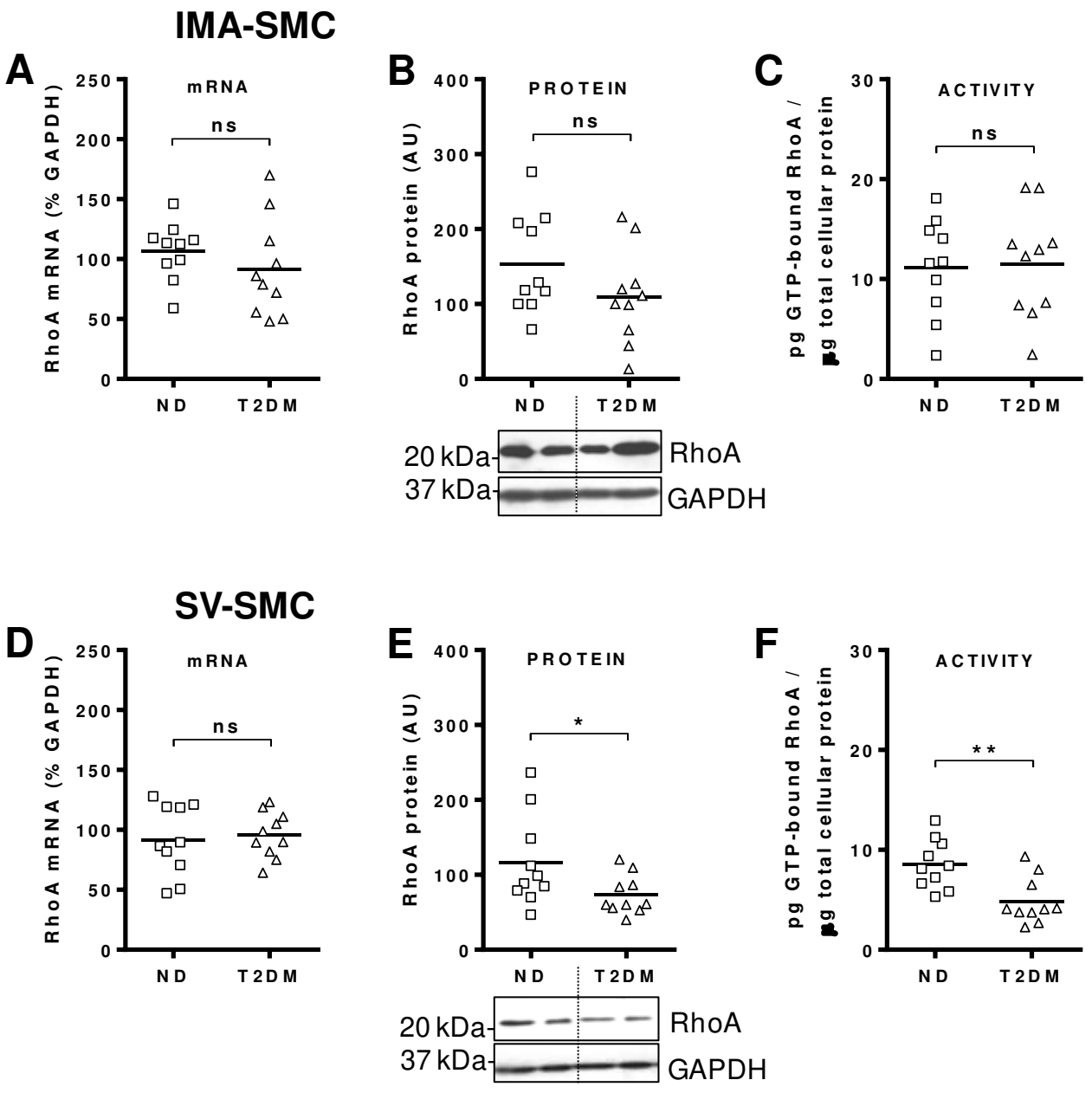
IMA-SMC
A
mRNA
RhoA mRNA (% GAPDH)
250
200
150
100
50
0
ns
ND
T2DM
B
PROTEIN
RhoA protein (AU)
400
300
200
100
0
ns
ND
T2DM
20 kDa
37 kDa
RhoA
GAPDH
C
ACTIVITY
pg GTP-bound RhoA / µg total cellular protein
30
20
10
0
ns
ND
T2DM
SV-SMC
D
mRNA
RhoA mRNA (% GAPDH)
250
200
150
100
50
0
ns
ND
T2DM
E
PROTEIN
RhoA protein (AU)
400
300
200
100
0
*
ND
T2DM
20 kDa
37 kDa
RhoA
GAPDH
F
ACTIVITY
pg GTP-bound RhoA / µg total cellular protein
30
20
10
0
**
ND
T2DM

Table 1

1

Tables

		Mean Age (Range)	Sex	T2DM Treatment
IMA-SMC ND	n = 19	64.5 years (49-82)	89% Male	N/A
IMA-SMC T2DM	n = 16	61.8 years (34-82)	94% Male	12.4% diet controlled 43.8% oral therapy 43.8% oral plus insulin therapy
SV-SMC ND	n = 25	64.8 years (49-82)	92% Male	N/A
SV-SMC T2DM	n = 23	61.4 years (34-85	100% Male	4.3% diet-controlled 43.5% oral therapy 52.2% oral plus insulin therapy

Table 1: Donor patient characteristics. All SMC groups were from age-matched donor patients, predominantly (>90%) male. The majority of T2DM patients were receiving oral or oral plus insulin therapies and a smaller percentage were on diet management programmes.