This work is made available online in accordance with publisher policies. Please refer to the repository record for this item and our Policy Document available from the repository home page for further information.

To see the final version of this work please visit the publisher's website. Available access to the published online version may require a subscription.

**Link to Publisher's version:** http://dx.doi.org/10.1016/j.carpath.2014.03.004

**Citation:** Sedgwick B, Riches K, Bageghni SA, O'Regan DJ, Porter KE and Turner NA (2014) Investigating inherent functional differences between human cardiac fibroblasts cultured from non-diabetic and type 2 diabetic donors. Cardiovascular Pathology. 23(4): 204-210.

**Copyright statement:** © 2014 Elsevier. Reproduced in accordance with the publisher's self-archiving policy. This manuscript version is made available under the CC-BY-NC-ND 4.0 license.
Investigating Inherent Functional Differences between Human Cardiac Fibroblasts Cultured from Non-Diabetic and Type 2 Diabetic Donors

Bryony Sedgwick¹, Kirsten Riches¹,², Sumia A. Bageghni¹,², David J. O’Regan²,³, Karen E. Porter¹,², Neil A. Turner¹,²

¹Division of Cardiovascular and Diabetes Research, School of Medicine, University of Leeds, Leeds, UK; ²Multidisciplinary Cardiovascular Research Centre (MCRC), University of Leeds, Leeds, UK; ³Department of Cardiac Surgery, The Yorkshire Heart Centre, Leeds General Infirmary, Leeds, UK.

Running title: Type 2 diabetes and human cardiac fibroblast function

Correspondence address:
Dr Neil A. Turner, Division of Cardiovascular and Diabetes Research, School of Medicine, Worsley Building, Clarendon Way, University of Leeds, Leeds LS2 9JT, UK.
Tel: +44(0)113-3435890. Fax: +44(0)113-3434803. E-mail: n.a.turner@leeds.ac.uk
Abstract

Introduction: Type 2 diabetes mellitus (T2DM) promotes adverse myocardial remodelling and increased risk of heart failure; effects that can occur independently of hypertension or coronary artery disease. As cardiac fibroblasts (CF) are key effectors of myocardial remodelling, we investigated whether inherent phenotypic differences exist in CF derived from T2DM donors compared with cells from non-diabetic (ND) donors.

Methods: Cell morphology (cell area), proliferation (cell counting over 7-day period), insulin signalling (phospho-Akt and phospho-ERK Western blotting) and mRNA expression of key remodelling genes (real-time RT-PCR) were compared in CF cultured from atrial tissue from 14 ND and 12 T2DM donors undergoing elective coronary artery bypass surgery.

Results: The major finding was that type I collagen (COL1A1) mRNA levels were significantly elevated by 2-fold in cells derived from T2DM donors compared with those from ND donors; changes reflected at the protein level. T2DM cells had similar proliferation rates but a greater variation in cell size and a trend towards increased cell area compared with ND cells. Insulin-induced Akt and ERK phosphorylation were similar in the two cohorts of cells.

Conclusion: CF from T2DM individuals possess an inherent pro-fibrotic phenotype that may help to explain the augmented cardiac fibrosis observed in diabetic patients.
Mini Summary

We investigated whether inherent phenotypic differences exist between cardiac fibroblasts cultured from donors with or without Type 2 diabetes. Cell morphology, proliferation, insulin signalling and gene expression were compared between multiple cell populations. The major finding was that type I collagen levels were elevated in fibroblasts from diabetic donors, which may help explain the augmented cardiac fibrosis observed with diabetes.

**Key words:** cardiac fibroblasts; type I collagen; human; heart; type 2 diabetes
1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease associated with sedentary lifestyles and obesity which manifests as a result of insulin resistance. Currently T2DM affects over 250 million people worldwide and its prevalence is predicted to rise significantly over the coming years [1]. Overt T2DM is often preceded by several years of insulin resistance during which time the pancreas maintains glycaemic control by secreting increased levels of insulin. Consequently, diagnosis of T2DM is often made only when pancreatic insulin secretion becomes insufficient to maintain normoglycaemia and symptoms of hyperglycaemia ensue. Diabetic patients therefore experience long-term metabolic disturbances that can have detrimental effects well before diagnosis. T2DM manifests with a heightened inflammatory and pro-fibrotic state [2,3] and is an independent risk factor for cardiovascular disease in both men and women [4]. The association between T2DM and heart failure occurs irrespective of aetiology (ischaemic or non-ischaemic) [5], suggesting that T2DM confers detrimental effects directly on the heart. Indeed, the term ‘diabetic cardiomyopathy’ is used to describe a pathology that occurs independently of coronary artery disease and hypertension, and manifests as increased left ventricular mass and reduced ventricular contractility, together with interstitial fibrosis and increased diastolic stiffness [6,7]. Although often manifesting as changes in ventricular remodelling, T2DM is also strongly associated with atrial fibrillation [8] which can drive subsequent atrial remodelling [9]. The cellular and molecular mechanisms underlying the heightened inflammatory and fibrotic states associated with T2DM are yet to be fully elucidated.

Cardiac fibroblasts (CF) [10] are the most prevalent cell type in the heart. In health they are relatively quiescent cells responsible for maintaining cardiac extracellular matrix (ECM) homeostasis through regulated collagen and matrix metalloproteinase (MMP) synthesis [10].
cardiovascular disease, increased mechanical wall stress and biochemical stimuli induce phenotypic differentiation of CF into hyper-secretory myofibroblast cells [10-12]. Fibroblasts and myofibroblasts regulate many aspects of cardiac pathophysiology, and as well as being important for the repair of the heart after injury (e.g. myocardial infarction) they also contribute to fibrosis and heart failure progression [10-12].

Despite the significance of CF in regulating cardiac remodelling there is relatively little known about whether diabetes can directly modulate CF function. There are several reports describing in vitro effects of elevated glucose concentration on CF function [13-16], however hyperglycaemia represents only one component of the complex diabetic milieu. Very recent evidence from rat and mouse models of T2DM has suggested that there are functional differences in CF derived from T2DM hearts compared with control hearts [17,18], however there is no current evidence that such differences exist in human CF.

The aim of the present study was to determine whether there are phenotypic differences in cultured CF derived from T2DM patients compared with cells from donors without diagnosed diabetes; differences that may contribute to adverse cardiac remodelling in T2DM individuals. Accordingly, we compared cell morphology, cell proliferation, insulin signalling and specific gene expression responses in CF derived from a cohort of 14 non-diabetic (ND) and 12 T2DM donors.

2. Materials and Methods

2.1. Cell culture

CF were isolated and cultured as described previously [19-21] by collagenase digestion of right atrial appendage samples from 26 patients without left ventricular dysfunction undergoing
coronary artery bypass graft surgery at Leeds General Infirmary. Local ethical committee approval and informed patient consent were obtained. CF were cultured in full growth medium (FGM) comprising Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal calf serum (FCS), 100 µg/ml penicillin-streptomycin and 2 mM L-glutamine, as described previously [19-21]. Experiments were performed on CF from passages 3-6. Experiments were conducted on cells from a total cohort of 14 ND control donors (86% male, mean age 64.8±1.7, range 50-75) and 12 T2DM donors (92% male, mean age 68.2±3.1, range 42-83). Of the diabetic individuals, 5 were receiving oral therapy, 5 were receiving insulin plus oral therapy and 2 were diet-controlled.

2.2. Cell area calculation

Images were taken of sub-confluent cell populations from individual ND and T2DM donors (10 patients per cohort) at x100 magnification under light microscopy using Ulead Photo Explorer 7.0. Outlines of the first 50 cells per donor were drawn using Image J software (http://imagej.nih.gov/ij) and cell areas calculated and averaged from the number of encompassed pixels before conversion to µm².

2.3. Proliferation assays

Proliferation assays were conducted as described previously [20]. Briefly, cells from ND and T2DM donors (8 patients per cohort) were plated at 1x10⁴ cells per well in 1 ml FGM and incubated overnight prior to being growth-arrested in serum-free medium (SFM) for 72 h. FGM was refreshed on days 0, 2, 4 and 7 and quadruplicate cell counts were conducted using a haemocytometer and Trypan Blue.
2.4. Western blotting

Serum-starved cells from 5 ND and 5 T2DM donors were incubated with 100 nM insulin for 5-60 min before preparing whole cell homogenates by scraping the cell layer into SDS-PAGE sample buffer supplemented with phosphatase and protease inhibitors [22]. Equal protein concentrations of samples (typically 10 µg/lane) were resolved by SDS-PAGE prior to Western blotting with primary antibodies (Cell Signaling Technology) for phospho-Akt(Ser-473) or phospho-ERK(Thr-202/Tyr-204) [22]. Densitometric analysis was performed using Image J software and data expressed relative to a loading control included on each gel.

For assessment of type I collagen protein levels, equal protein concentrations (10 µg) of control samples from signalling experiments were resolved on the same gel before probing membranes with anti-COL1A1 antibody (sc-8784; Santa Cruz Biotechnology). Membranes were reprobed with anti-β-actin antibody (ab8226; Abcam) as a loading control, and densitometric data expressed as the ratio of type I collagen to β-actin levels.

2.5. Quantitative real time-PCR

Serum-starved cells from 6 ND and 6 T2DM donors were incubated with low-serum medium (SFM supplemented with 0.4% FCS) containing 10 ng/ml interleukin (IL)-1α for 6-24 h. This concentration of IL-1α was selected based on our previous study of cytokine gene expression in human CF [23]. RNA extraction and reverse transcription was performed as described previously [24]. Real-time PCR was performed in triplicate using the Applied Biosystems ABI-7500 System and Taqman primer/probes for IL1β (Hs00174097_m1 primer/probe set), IL6 (Hs00174131_m1), IL8 (Hs009999034_m1), COL1A1 (Hs00164004_m1), ACTA2
(Hs00426835_g1), MMP2 (Hs01548727_m1) and MMP3 (Hs00233962_m1). Data are expressed relative to GAPDH mRNA expression (Hs99999905_m1) using the formula \( 2^{-\Delta CT} \times 100 \) [24].

### 2.6. Statistical analysis

Results are presented as mean ± standard error, and the number of experiments on cells from different patients is represented by n. Data were analysed with GraphPad Prism 6 software using Student t-tests or two-way ANOVA with Holm-Sidak’s post-hoc test, as appropriate. P<0.05 was considered statistically significant.

### 3. Results

#### 3.1. Influence of T2DM on cell morphology

We investigated whether there was an association between diabetic status and cellular morphology in CF cultured from multiple ND and T2DM donors. Mean spread cell areas were calculated from 50 cells per patient, with representative images shown in Fig. 1A. The combined data showed that there was more variation in cell size in the T2DM group compared with ND controls (Fig. 1B). Moreover, there was a strong trend towards increased cell size in cells from T2DM donors compared with ND (Fig. 1B), although this did not quite reach statistical significance (P=0.079).

#### 3.2. Influence of T2DM on proliferation rate

Cell proliferation profiles for CF from 8 ND and 8 T2DM donors were constructed by culturing cells in FGM and counting cells over a 7-day period (Fig. 1C). A 3.3-fold and 2.7-fold increase
in cell number was demonstrated over the course of the experiment for ND and T2DM populations respectively (ANOVA: both P<0.001). There was no significant difference in proliferation rates between the two cohorts of cells (ANOVA: P=0.429) (Fig. 1C).

3.3. Influence of T2DM on insulin receptor signalling

At the molecular level, insulin resistance is attributable to defective insulin receptor signalling, in particular impaired phosphorylation of the insulin receptor substrate protein IRS-1 [25]. This results in selective down-regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, but not the extracellular signal-regulated kinase (ERK) mitogen-activated kinase pathway in skeletal muscle [26]. We measured activity of these pathways in CF from ND and T2DM donors following insulin treatment for 5-60 min. ND cells exhibited relatively high basal levels of Akt and ERK phosphorylation and insulin was unable to stimulate them further (Fig. 2). In contrast, in T2DM cells basal levels of Akt and ERK phosphorylation appeared to be lower than in ND cells, although this was not statistically significant. Moreover, the response to insulin was more apparent in T2DM cells with marked increases in Akt and ERK phosphorylation maintained for at least 60 min and 30 min respectively (Fig. 2).

3.4. Influence of T2DM on basal and IL-1α induced gene expression

IL-1α is a proinflammatory cytokine whose levels are increased in the heart early after myocardial infarction and can modulate many cellular functions of CF that are pertinent to cardiac remodelling [27]. Using real-time RT-PCR we assessed the influence of T2DM on mRNA expression of several pertinent genes at basal level and following treatment with IL-1α. Specifically, we investigated expression of the proinflammatory cytokines IL1β, IL6 and IL8, the
myofibroblast differentiation marker ACTA2 (the gene encoding \(\alpha\)-smooth muscle actin [\(\alpha\)SMA]), type I collagen (COL1A1) and the matrix metalloproteinases MMP2 and MMP3; genes that we have previously studied in this cell type [23,28-30].

IL-1\(\alpha\) substantially increased expression of IL1\(\beta\), IL6 and IL8 mRNA after 6 h in both T2DM and ND cells, and levels remained significantly elevated after 24 h (Fig. 3A-C). Although there was a suggestion of reduced IL6 and IL8 expression after 6 h, there was no statistically significant difference in the overall profile of proinflammatory cytokine expression between T2DM and ND cells (Fig. 3A-C).

The mean basal ACTA2 mRNA expression in T2DM cells was 80% higher than that of ND cells (Fig. 3D), however the high level of variation in the T2DM data meant that this difference was not statistically significant (\(P=0.492\)). IL-1\(\alpha\) treatment reduced ACTA2 expression after 24 h, in agreement with our recent report [30], but there was no difference between T2DM and ND CF (Fig. 3D).

Basal and IL-1\(\alpha\)-induced MMP2 mRNA expression was similar in CF from ND and T2DM donors (Fig. 3E). In contrast, there was a trend towards reduced basal mRNA expression of MMP3 in T2DM compared with ND cells (0.004±0.001% GAPDH vs. 0.026±0.013% GAPDH respectively, \(P=0.055\)) (Fig. 3F). Moreover, the extent of IL-1\(\alpha\)-stimulated MMP3 mRNA expression in T2DM CF was generally lower than that of controls over the time course studied (\(P=0.079\)) (Fig. 3F).

The major difference we observed was at the level of COL1A1 gene expression (Fig. 4A). Basal COL1A1 mRNA expression in T2DM CF was 2-fold higher than that observed in ND cells (131.4±26.8% GAPDH compared with 66.2±7.4% GAPDH respectively, \(P=0.033\)). IL-1\(\alpha\) treatment suppressed COL1A1 expression in both T2DM and ND cells. Remarkably though,
even 24 h after IL-1α incubation, COL1A1 mRNA levels in T2DM cells remained greater than those in untreated ND cells (Fig. 4A). This result was confirmed at the protein level by Western blotting (Fig. 4B). There was a larger degree of variation in collagen I protein expression in the T2DM cells and the median basal expression level in T2DM cells was 2.6-fold greater than that of ND cells (P=0.05; Fig. 4B).

4. Discussion
In this study we investigated whether T2DM could confer inherent changes in human CF function at the levels of morphology, proliferation, insulin signalling and mRNA expression of key remodelling genes. Our principal finding was that COL1A1 mRNA levels were elevated in CF derived from T2DM donors compared with cells from ND donors; a change reflected at the protein level. In addition, there was a trend towards reduced MMP3 expression in these T2DM cells. Together these changes suggest that CF from T2DM patients adopt a pro-fibrotic phenotype, in keeping with the increased fibrosis evident in diabetic hearts [6]. CF from T2DM donors had a greater variation in cell size than ND cells and an overall trend towards increased cell area, although cell proliferation rates were similar for the two cohorts. Finally, whilst basal Akt and ERK phosphorylation were lower in T2DM cells, they showed generally increased responses to insulin.

Currently there is relatively little known about whether diabetes can directly modulate CF function. Although there are several reports describing in vitro effects of glucose concentration on CF function [13-16], the diabetic milieu is far more complex than hyperglycaemia alone and other factors such as hyperlipidaemia, advanced glycation end products, oxidative stress, proinflammatory cytokines, adipokines and profibrotic cytokines must also be considered [31].
Using fibroblasts cultured from the hearts of Zucker diabetic rats, a laboratory model of T2DM, it was recently demonstrated that diabetic cells adopt a more myofibroblast-like phenotype compared with cells derived from lean control hearts [17]. Changes were relatively small, but included increased contractile behaviour, increased αSMA and type I collagen expression and increased cell proliferation in the diabetic cells [17]. Very recently, in a study using a murine model of T2DM (db/db mouse), CF derived from db/db hearts were shown to have elevated expression of several pro-fibrotic markers (type I collagen, PAI-1, TGF-β, TIMP-2) compared with cells derived from db/wt littermates [18]. In contrast to previous reports, altering glucose concentration in the culture medium had no modulatory effects on expression of any of the proteins studied [18]. It is worth noting that the Zucker diabetic rat and db/db mouse models, both of which result from a mutation in the leptin receptor, are unlikely to reflect all the changes observed in human T2DM. Indeed, several differences have been noted between the cardiac phenotype of these models compared with that observed in diabetic patients [32].

Despite these inter-species differences, our data add to an emerging consensus that T2DM can drive CF to adopt a pro-fibrotic phenotype (increased type I collagen expression) that is maintained throughout long-term in vitro culture i.e. in the absence of the initiating diabetic stimulus. Increased expression of ECM proteins, including type I and III collagen, is observed in a range of organs affected by diabetes including the heart, kidney, liver and pancreas [6]. Whether the changes we observed were due to increased differentiation of cells to a myofibroblast phenotype, as has been proposed [17], is not clear at this stage as increases in αSMA expression (a marker of the myofibroblast phenotype) observed in diabetic rat CF [17] were not observed in diabetic mouse CF [18]. In our human cells, although mean ACTA2 mRNA expression in T2DM-derived CF was higher than that of ND donors, the variation
between patients meant that this was not statistically significant. Similarly, we observed a trend
towards increased cell size in T2DM cells compared with ND controls, but again the variation in
the data for the T2DM cells meant that these changes were not statistically significant.

In addition to upregulation of COL1A1 mRNA expression, we observed a trend towards
decreased MMP3 mRNA expression in T2DM cells. CF express several members of the MMP
family [33], and importantly MMP3 can stimulate a cascade of ECM degradation by cleaving
and activating other downstream MMPs [34]. Therefore decreased MMP3 expression in T2DM
CF may indicate a broader reduction in downstream MMP activity which, in combination with
increased collagen expression, would predict T2DM CF activity that favours ECM deposition.

Despite the heightened inflammatory state that is associated with T2DM [2], CF from T2DM
donors had a similar profile of proinflammatory cytokine expression compared to ND controls.
This may indicate that fibroblasts are not the major source of increased inflammatory molecules
in T2DM and that other cell types, such as monocytes, are more important.

Our data revealed that T2DM CF exhibit similar proliferation rates to ND cells, which
contrasts with previous studies using CF from rat models of Type 1 [35] and Type 2 [17]
diabetes. Such discrepancies are likely to be multi-factorial and include inter-species variation,
differences in duration of hyperglycaemia or age differences. Previous studies have reported that
high glucose concentrations increase the proliferation rate of human atrial CF [13] which, when
taken in combination with our findings, suggests that glucose can acutely increase cell
proliferation, but that these changes may not be maintained in the absence of the initiating
stimulus.

Basal phosphorylation levels of Akt and ERK were generally lower in CF from T2DM
donors compared with ND cells, and T2DM cells appeared more responsive upon addition of
insulin, although none of these differences between the two cohorts were statistically significant. These results were surprising given previous reports that T2DM impairs PI3K/Akt signalling without affecting ERK signalling [25,26]. However, most of the evidence for this comes from studies on skeletal muscle and it is unknown whether similar mechanisms occur in CF. Interestingly, increased IRS-PI3K-Akt signalling has been reported in left ventricular biopsies from T2DM patients and the myocardium of ob/ob diabetic mouse hearts [36], suggesting that diabetes affects insulin signalling differently in diverse tissues and cells. Our data show no overt deficit in insulin signalling (Akt or ERK) in CF derived from T2DM donors.

We have previously reported that human vascular smooth muscle cells cultured from T2DM donors exhibit altered morphology, increased migration rate and decreased proliferation compared with cells from ND donors [37]. The maintenance of phenotypic features throughout long-term cell culture is likely to be indicative of epigenetic mechanisms that confer persistent changes in cellular function. There is increasing evidence that such epigenetic factors, including specific microRNAs, contribute to the pathogenesis of diabetic cardiomyopathy [38]. Further investigations are needed to determine whether diabetes stimulates epigenetic modification of CF that drives the pro-fibrotic phenotype.

5. Study limitations
Our study has a number of important limitations that should be noted. Firstly, as this was not a prospective study, only limited clinical and demographic information was available on the patient donors i.e. age, gender, diabetic status and diabetes therapy. T2DM is associated with a higher prevalence of coronary artery disease, heart failure and atrial fibrillation, and all of these confounding variables could potentially contribute to the differences we observed. However, it is
worth noting that all of the donors in the study (both ND and T2DM) had coronary artery disease and were undergoing elective coronary artery bypass graft surgery. Therefore the presence or absence of diabetes was the sole known discriminator between the two cohorts. A larger study would be needed to evaluate the impact of other co-morbidities in this regard. Although it would have been informative to have a further control group of donors without coronary artery disease, ethical constraints prevented us from obtaining cardiac biopsies from healthy individuals.

Diabetic cardiomyopathy is often characterised by changes in ventricular remodelling, so our use of atrial fibroblasts rather than ventricular fibroblasts could also be viewed as a shortcoming. However, the atria also undergo remodelling in T2DM patients and so our findings may also be of relevance to T2DM-associated atrial remodelling.

Finally, the size of our study cohort was limited due to restricted availability of donor tissue and cells. Although we were able to use cells from a total of 26 donors, we could not perform all assays on all cell populations.

Despite these limitations, a major strength of our study was the use of CF derived from human heart tissue from multiple diabetic and non-diabetic patients. Our data therefore support a role for T2DM underlying the differential responses of the fibroblasts.

6. Conclusion

CF derived from T2DM individuals express inherently higher levels of type I collagen compared with cells from individuals without diabetes; differences that are maintained throughout long-term cell culture. These findings may help to explain the augmented cardiac fibrosis observed in diabetic patients.
Acknowledgements

This work was supported by undergraduate research project funding from the University of Leeds Intercalated BSc in Clinical Sciences (Cardiovascular Medicine) programme. We are grateful to Phil Warburton for technical support.
References


[24] Turner NA, Mughal RS, Warburton P, O'Regan DJ, Ball SG, Porter KE. Mechanism of
TNFα-induced IL-1α, IL-1β and IL-6 expression in human cardiac fibroblasts: Effects of

phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia

RA, Kahn CR, Mandarino LJ. Insulin resistance differentially affects the PI 3-kinase- and

[27] Turner NA. Effects of interleukin-1 on cardiac fibroblast function: Relevance to post-

[28] Turner NA, Warburton P, O'Regan DJ, Ball SG, Porter KE. Modulatory effect of
interleukin-1α on expression of structural matrix proteins, MMPs and TIMPs in human

[29] Turner NA, Das A, O'Regan DJ, Ball SG, Porter KE. Human cardiac fibroblasts express
ICAM-1, E-selectin and CXC chemokines in response to proinflammatory cytokine

interleukin-1α and transforming growth factor-β1 on modulation of human cardiac

[31] Mandavia CH, Aroor AR, Demarco VG, Sowers JR. Molecular and metabolic

[33] Turner NA, Porter KE. Regulation of myocardial matrix metalloproteinase expression and activity by cardiac fibroblasts. IUBMB. Life 2012;64:143-150.


Figure Legends

**Fig. 1. Influence of T2DM on cell size and proliferation.** (A) Representative photomicrographs of CF cultured from non-diabetic (ND) or T2DM donors, showing traced cell outlines. Scale bar = 100 µm. (B) Outlines of 50 cells from each of 10 ND and 10 T2DM populations were traced and mean cell areas calculated. Box depicts 25\(^{\text{th}}\)-75\(^{\text{th}}\) interquartile range, whiskers indicate full range and horizontal line is median. (C) Cells from 8 ND donors (filled circles) or 8 T2DM donors (open squares) were plated at 1x10\(^4\) cell/well in full growth medium and cell proliferation measured by counting cells over a 7-day period. 2-way ANOVA: P<0.001 for effect of time, P=0.429 for effect of diabetic status.

**Fig. 2. Influence of T2DM on insulin-induced Akt and ERK phosphorylation.** Cells cultured from 5 non-diabetic donors (filled circles) and 5 T2DM donors (open squares) were treated with 100 nM insulin for 5-60 min prior to quantification of phospho-Akt (left panel) and phospho-ERK (right panel) by Western blotting. Pooled densitometry results (mean ± SEM; n=5) are expressed relative to loading control (Ctrl) that was added to every gel. Molecular weights in kDa are shown to the right of the blots.

**Fig. 3. Influence of T2DM on mRNA expression levels of remodelling genes.** Cells cultured from 6 ND donors (filled circles) and 6 T2DM donors (open squares) were treated with 10 ng/ml IL-1α for 6-24 h before measuring IL1β (A), IL6 (B), IL8 (C), ACTA2 (D), MMP2 (E) and MMP3 (F) mRNA levels by real time RT-PCR. Results are expressed as % GAPDH mRNA levels. P values indicate influence of diabetic status on mRNA expression across all time points (2-way ANOVA). *P<0.05 for comparison between ND and T2DM data at a particular time.
point (Holm-Sidak’s post-hoc test); all others non-significant.

**Fig. 4. Influence of T2DM on type I collagen mRNA and protein expression.** (A) Cells cultured from 6 ND donors (filled circles) and 6 T2DM donors (open squares) were treated with 10 ng/ml IL-1α for 6-24 h before measuring COL1A1 mRNA levels by real time RT-PCR. Results are expressed as % GAPDH mRNA levels. P values indicate influence of diabetic status on mRNA expression across all time points (2-way ANOVA). *P<0.05 for comparison between ND and T2DM data at a particular time point (Holm-Sidak’s post-hoc test). (B) Control samples from signalling experiments were analysed for expression of COL1A1 by Western blotting. Expression of β-actin was used as a loading control. Each lane of the gel represents cells from a different patient, grouped according to diabetic status. Scatter plot (with horizontal line representing median) depicts densitometry data obtained from the 5 ND donors and 5 T2DM donors normalised to β-actin levels.
Figure 1

A. Non-diabetic

B. T2DM

B. Cell area (μm²)

C. Cell count (thousands)

P = 0.079
Figure 2

A.  

<table>
<thead>
<tr>
<th>Insulin (min):</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>60</th>
<th>Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>T2DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65</td>
</tr>
</tbody>
</table>

B.  

<table>
<thead>
<tr>
<th>Insulin (min):</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>60</th>
<th>Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>44</td>
<td>42</td>
<td>44</td>
<td>42</td>
<td></td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>T2DM</td>
<td>42</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42</td>
</tr>
</tbody>
</table>
Figure 3

A. IL-6 mRNA (% GAPDH)

B. IL-8 mRNA (% GAPDH)

C. IL1β mRNA (% GAPDH)

D. MMP3 mRNA (% GAPDH)

E. MMP2 mRNA (% GAPDH)

F. ACTA2 mRNA (% GAPDH)

ANOVA: P=0.166

ANOVA: P=0.554

ANOVA: P=0.870

ANOVA: P=0.079

ANOVA: P=0.072

ANOVA: P=0.692
Figure 4

A. 

**COL1A1** mRNA (% GAPDH) over time (h) for non-diabetic (ND) and T2DM groups. The graph shows a significant decrease in **COL1A1** mRNA expression over time, with ANOVA: P=0.018. 

B. 

Western blot analysis of Type I collagen and β-actin expression in non-diabetic (ND) and T2DM groups. The protein bands for Type I collagen are around 70 kDa, and for β-actin around 42 kDa. The figure indicates a comparison of protein expression levels with a significance level of P<0.05.