α11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy

Ilana Talior-Volodarsky1, Kim A. Connelly2, Pamma D. Arora1, Donald Gullberg3, and Christopher A. McCulloch1*

1Matrix Dynamics Group, University of Toronto, Room 244, Fitzgerald Building, 150 College Street, Toronto, Ontario, Canada M5S 3E2; 2Keenan Research Centre, Li Ka Shing Knowledge Institute, St Michael's Hospital and University of Toronto, Toronto, Ontario, Canada; and 3Department of Biomedicine, University of Bergen, Bergen, Norway

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Aims
Diabetic cardiomyopathy is characterized by the production of a disorganized fibrotic matrix in the absence of coronary atherosclerosis and hypertension. We examined whether adhesion of cardiac fibroblasts to glycated collagens mediates the differentiation of pro-fibrotic myofibroblasts, which may contribute to cardiac fibrosis.

Methods and results
By microarray, we found that methylglyoxal-treated collagen selectively enhanced α11 integrin expression in human cardiac fibroblasts, while levels of other collagen-binding integrins (α1, α2, and α10) were unchanged. Similar increases in α11 integrin mRNA and protein expression were observed in cardiac fibroblasts from streptozotocin (STZ)-treated Sprague–Dawley rats. In human cardiac fibroblasts plated on methylglyoxal-treated collagen and in cardiac fibroblasts from diabetic rats, transforming growth factor (TGF)-β2 but not TGF-β1 or TGF-β3 was increased compared with controls. Knock-down of α11 integrin and TGF-β receptors with small interfering RNA blocked the increased expression of TGF-β2, α-smooth muscle actin (α-SMA), and α11 integrin that were induced in cells plated on methylglyoxal-treated collagen. Further, inhibition of Smad3 signalling blocked methylglyoxal-collagen up-regulation of α11 integrin and α-SMA expression. Rats with STZ-induced diabetes exhibited increased phosphorylation of Smad3 in cardiac tissues compared with control rats.

Conclusion
Interactions between α11 integrins and the Smad-dependent TGF-β2 signalling may contribute to the formation of pro-fibrotic myofibroblasts and the development of a fibrotic interstitium in diabetic cardiomyopathy.

Keywords
Collagen • Fibrosis • TGF-β • Myofibroblasts

1. Introduction
Diabetic cardiomyopathy is associated with structural and functional abnormalities that lead eventually to left ventricular hypertrophy, diastolic and systolic dysfunction,1 and the formation of a fibrotic cardiac interstitium that is enriched in poorly organized, glycated collagen.2,3 This disorganized matrix disrupts the structural organization and electrical conductivity of myocytes, increases myocardial stiffness, and inhibits diastolic function.4–6

Fibroblasts are abundant cells in the myocardium7 and adhere to extracellular matrix proteins through integrins.8,9 In fibrosis, a collagen-rich matrix is formed by fibroblasts and by pro-fibrotic myofibroblasts, which can differentiate from cardiac fibroblasts10 and also from circulating, bone marrow-derived cells.11,12 Cardiac fibroblasts may play a role in the fibrosis of diabetic cardiomyopathy13 by increased proliferation14 elevated remodelling of collagen15,16 and by switch to a pro-fibrotic, highly contractile, myofibroblast phenotype.17 Myofibroblasts express abundant alpha-smooth muscle actin (α-SMA), which is not found in fibroblasts of normal adult hearts but are seen in fibrotic myocardium10,18 at sites of myocardial infarction19,20 and in failing human hearts.21

The α11 integrin is a major collagen receptor on fibroblastic cells,22–24 which controls myofibroblast differentiation and may be involved in collagen reorganization mediated by myofibroblasts.25 Currently, the role of the α11 integrin in diabetic cardiomyopathy and its impact on the differentiation of cardiac fibroblasts into myofibroblasts are not defined. We used streptozotocin (STZ)-induced diabetes in rats and human cardiac fibroblasts plated on methylglyoxal-treated collagen to examine myofibroblast differentiation in response to glycated collagen.

* Corresponding author. Tel: +1 416 978 1258; fax: +1 416 978 5956. E-mail: christopher.mcculloch@utoronto.ca

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2. Methods

2.1 Animals

Sprague–Dawley male rats (10–11 weeks of age; Jackson Laboratory, Bar Harbor, ME, USA) were housed for 1 week and then divided into two diabetic groups (diabetic and diabetic + insulin) and one control group. Rats in the diabetic groups were injected intravenously (lateral tail vein) with STZ (0.5 mL; 40 mg/kg body weight, freshly prepared in saline); controls were injected with saline (0.5 mL). The diabetic + insulin group was treated with insulin (0.5 U per 100 g of body weight) followed by intraperitoneal insulin injections the day after STZ treatment. The insulin injections were performed every other day for the duration of the experiment. Rats were maintained for 12 weeks with food and water ad libitum. Blood glucose levels, body weights, and blood pressure were measured weekly. Blood glucose levels were estimated from glucometer readings. Final data processing used the quantile normalization method. Data were exported into Excel files with cut-off of adjusted P-values < 0.05 at a >2-fold change. Assays were performed in triplicate for each treatment.

2.2 Rat cardiac fibroblast isolation

Rats were euthanized with CO2. Hearts were excised immediately and placed in ice-cold PBS containing antibiotics. Ventricles were washed, minced (1 mm2 pieces), and digested with trypsin and bacterial collagenase to produce cell suspensions that were used for purification of rat cardiac fibroblasts. Cells were incubated with mouse anti-rat antibody to CD90 (recognizing Thy-1) coupled to magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) to purify fibroblasts.

2.3 Cell culture

Human cardiac fibroblasts (ScienCell, Carlsbad, CA, USA; obtained from discarded ventricle biopsies), C2C12-WT, and C2C12-α11 over-expressing cells were cultured as described11 and passaged with trypsin.

2.4 Collagen gels and glycation

Collagen gels were prepared from a collagen type I solution (1 mg/mL; Advanced BioMatrix, San Diego, CA, USA) and were treated with PBS or glycated with 1 mM methylglyoxal (MGO, Sigma) overnight. Different MGO concentrations were used to assess the effect of collagen glycation on expression of α-SMA, a marker of myofibroblastic cells.27

2.5 Collagen and AGE staining in gels

Collagen was localized by immunostaining using a rabbit polyclonal antibody against collagen type I and a Texas Red-conjugated goat anti-rabbit secondary antibody. Control (PBS-treated) and MGO-treated collagen gels were incubated with mouse monoclonal antibody against AG (Arg-pyrimidine) followed by FITC-conjugated goat anti-mouse secondary antibody. Collagen was localized by immunostaining using a rabbit polyclonal antibody against collagen type I and a Texas Red-conjugated goat anti-rabbit secondary antibody. Gels were imaged by laser scanning confocal microscopy and the staining intensities in 5 μm2 grids were measured for collagen and AGE.

2.6 Immunohistochemistry and histochemistry

Cardiac ventricles were fixed with formalin, embedded in paraffin, and transverse sections were stained with Masson Trichrome for general tissue structure or picrosirius red for collagen28 (Centre for Phenomics, Toronto, ON, USA). Immunostaining of cardiac tissues for gly-collagen was performed as described.29

2.7 Microarray

HCFs plated on PBS- or MGO-treated collagen type I were analysed with an Affymetrix 1.0ST Gene Chip Array (Microarray Centre, University Health Network, Toronto, ON, USA). Only transcripts with at least 1 sample having intensity >20% percentile of raw intensities were analysed. Unpaired t-tests were performed between the two groups and the Benjamini & Hochberg analytical method was used for multiple testing corrections. Final data processing used the quantile normalization method. Data were exported into Excel files with cut-off of adjusted P-values < 0.05 at a >2-fold change. Assays were performed in triplicate for each treatment.

2.8 siRNA transfection

Human cardiac fibroblasts were plated on control collagen (PBS) or 1 mM MGO-collagen (MGO)-coated plates 1 day before transfection and trans- fected as described.25 Cells were either not transfected (NT) or transfected with 100 nM ON-TARGET plus siRNA to human α11 integrin (Dharmacon; Lafayette, CO, USA) #1 target sequence—GGACUCA-GACGGUGAAGAUU or with a second target sequence—UCAAAUA-CAUCGGCAUGUA or with 100 nM ON TARGET plus SMART pools for TGF-βRI/TGF-βRII or with an irrelevant siRNA sequence (100 nM siNR) using HiPerfect transfection reagent (Qiagen, Mississauga, ON, USA). Cells were incubated for 48 h and processed for immunoblotting or qPCR as described below.

2.9 Immunoblotting and immunoprecipitation

For intact rat hearts, ventricles were dissected, cut into small pieces, and homogenized in SDS-sample buffer with protease and phosphatase inhibitors (Sigma). Lysates of heart fragments and cells were resolved by 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked BSA and immunoblotted for α1, α2, α10, and α11 integrins, α-SMA, vimentin, desmin, or GAPDH overnight at 4°C and bound antibodies were detected by chemiluminescence. For immunoprecipitations, cells were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma). Lysates were immuno-precipitated with Smad3 antibody and immune complexes were eluted from beads followed by immunoblotting for phospho-Smad3 or Smad3.

2.10 Quantitative PCR

RNA from cardiac tissues and from human cardiac fibroblasts was isolated using the RNeasy Mini Kit (Qiagen, Mississauga, ON, USA). cDNA was generated using reverse transcriptase. Real-time PCR was performed using iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) with validated human or rat primers (Table 1). Relative quantification was calculated using the ΔΔCt method normalized to GAPDH.

2.11 Cell and bead attachment assays

Cell attachment to collagen was measured with a colorimetric assay (Promega, Madison, WI, USA). Fibroblasts that were separated and purified from diabetic and control rat ventricles were plated on control collagen. Human cardiac fibroblasts or C2C12-WT or C2C12-α11 over-expressing cells were plated on PBS or MGO-treated collagen. Cells were plated for 0–60 min, washed, stained with dye, and absorbance recorded at 570 nm.

For bead attachment assays to recombinant human α11β1 integrin, 2 μm green or blue latex beads were coated with collagen or BSA, treated with PBS (green) or MGO (blue), plated on recombinant
human α11β1 integrin for 1 h, and jet-washed with PBS. The numbers of attached beads in 1000 μm² grids were counted in a fluorescent microscope with filters that allowed separate counts of blue and green beads.

2.12 Statistical analysis
For all studies, experiments were repeated at least three times. Quantitative data are reported as mean ± SEM and/or shown as x-fold values relative to the indicated control conditions. For quantitative data, Student’s t-test was used for two samples and ANOVA for multiple samples; P < 0.05 was considered to be statistically significant.

3. Results
3.1 Rat diabetes model
The mean blood glucose level prior to treatment (day 0) for all animals was 5.8 ± 0.28 mmol/L. STZ treatment caused increased blood glucose, decreased insulin level, and failure to gain weight, that were maintained for the 12 weeks of observation (Table 2). Systolic blood pressures were measured over 8 weeks and remained unchanged in STZ-treated rats compared with controls (systolic blood pressure measurements at the end of the measurement period: controls: 109.3 ± 11.48 mm Hg; STZ-diabetics: 130 ± 15.36 mm Hg (P > 0.2)).

Analysis of rat ventricles showed collagen deposition in the cardiac interstitium of diabetic rats but not controls (Figure 1A and B). We found increased immunostaining for AGE and collagen in the ventricles of diabetic animals compared with controls (Figure 1C). Merged images of collagen and AGE staining (glycated collagen) showed marked co-localization in diabetic but not in control rats.

We compared the extent of glycation generated with the in vitro model (Figure 1D) to that of the in vivo model by measuring immunofluorescence staining intensity of AGE adjusted for the staining intensity of type I collagen. Quantification of AGE staining (FITC fluorescence) and collagen staining (Texas red fluorescence) in fixed areas (5 μm²) were used to compute fluorescence intensity ratios. For the in vivo model, the ratios of AGE:collagen staining intensities were 0.34 ± 0.04 for controls and 0.68 ± 0.05 for diabetics (P < 0.05). For the in vitro model, the AGE:collagen ratios were 0.24 ± 0.07 for controls and 0.69 ± 0.12 for MGO-treated collagen gels (P < 0.05).

3.2 Collagen glycation enhances α11 integrin and α-SMA expression
By microarray, we screened for candidate genes that are differentially expressed in diabetic conditions, may be involved in fibroblast-to-myofibroblast conversion, and are associated with cardiac fibrosis.10 RNA from human cardiac fibroblasts plated on PBS- and MGO-treated collagen was extracted and analysed with the Affymetrix Human Genome 1.0ST Gene Chip Array. Of the 17 880 genes screened, 79 genes were differentially expressed in MGO collagen compared with PBS-collagen-treated cells. Of the 79 differentially expressed genes, 29 were up-regulated and 50 were down-regulated. Based on the previous results showing that collagen glycation may affect cardiac fibroblast binding to collagen,11 we focused on the collagen receptors α1, α2, α10, and α11 integrins for further study. Cells plated on MGO-collagen showed increased (2.1-fold) expression of the collagen-binding α11 integrin vs. PBS-treated cells while the expression of α1 (1.3-fold), α2 (1.0-fold), and α10 (1.1-fold) integrins was unchanged (Supplementary material online, Table S1).

We examined the role of the α11 integrin in diabetes-associated fibrosis by examining fibroblasts from control and diabetic rats. We
separated fibroblasts from other types of cells in the cell suspensions prepared from rat hearts and tested the purity of the extraction procedure. With vimentin as an intermediate filament marker of fibroblasts and desmin as a marker of muscle cells, we found that the purification procedure provided discrete cell populations that were enriched either with fibroblasts or with cells expressing desmin (Supplementary material online, Figure S1). Quantitative real-time PCR and immunoblot analysis showed that fibroblasts purified from rat ventricles exhibited two-fold higher mRNA and protein for \( \alpha \)-SMA and \( \alpha_1 \) integrin compared with controls (Figure 2A; \( P < 0.05 \)). Analysis of whole rat ventricles showed a similar, two-fold increase in \( \alpha \)-SMA and \( \alpha_1 \) integrin expression (Figure 2B; \( P < 0.05 \)). Treatment of diabetic animals with insulin every other day reduced cardiac mRNA levels of \( \alpha \)-SMA and \( \alpha_1 \) integrin (Figure 2C).

We assessed whether collagen glycation in vitro would affect \( \alpha \)-SMA expression in vitro. When the concentration of MGO was increased during the collagen glycation preparation step and the cells were plated on these substrates, we found a dose-dependent relationship between the concentrations of MGO used to glycate the collagen and the abundance of \( \alpha \)-SMA (Supplementary material online, Figure S2). In cells plated on MGO-collagen, there was a two-fold increase in mRNA for \( \alpha \)-SMA and \( \alpha_1 \) integrin compared with controls (Figure 2D and E; \( P < 0.02 \)).

### 3.3 Cell attachment to collagen

As fibroblasts from diabetic rats exhibited increased \( \alpha_1 \) integrin expression, we examined attachment of rat and human cardiac fibroblasts to collagen. There was enhanced attachment to collagen of cells isolated from control animals compared with cells from diabetic animals (\( P < 0.05; 1–60 \text{ min}; \) Figure 3A). Cells adhered better to native than to glycated collagen (Figure 3B). Unexpectedly, knockdown of \( \alpha_1 \) integrin expression in human cardiac fibroblasts plated on MGO-treated collagen showed enhanced cell attachment compared with controls (Figure 3B). Further, latex beads coated with native collagen bound to recombinant \( \alpha_1 \)\( \beta_1 \) integrin more avidly (two-fold) than beads coated with glycated collagen (Figure 3C). Finally, C2C12 cells over-expressing the \( \alpha_1 \) integrin, which is the only fibrillar collagen receptor in these cells, demonstrated greater attachment to native collagen than to MGO-treated collagen (Figure 3D) and exhibited increased expression of \( \alpha \)-SMA (Supplementary material online, Figure S3C).
Figure 2 STZ-induced diabetes upregulate α-SMA and α11 integrin mRNA and protein expression in purified rat cardiac fibroblasts, whole heart, and human cardiac fibroblasts. Separated rat cardiac fibroblasts (A and C), whole rat heart (B), or human cardiac fibroblasts (D and E) were examined. Rat cardiac fibroblasts were separated from other types of cardiac cells by MACS separation (A, C; described in Methods). Analyses of α-SMA and α11 integrin in rat cardiac fibroblasts (A and C), whole heart samples (B), or human cardiac fibroblasts (D and E). For mRNA analyses, real-time PCR was conducted. Ct values were GAPDH-corrected and analysed by t-tests to determine statistical difference between samples. Data are the mean ± SE relative to control animals from five independent experiments; *P < 0.05. For immunoblot analysis, samples were resolved by SDS–PAGE and immunoblotted for α11 integrin (150 kDa) or α-SMA (42 kDa). GAPDH (36 kDa) was used as a loading control.
3.4 α11 integrin regulates MGO-collagen-induced α-SMA expression

We assessed the role of the α11 integrin in myofibroblast formation by knocking down α11 integrin (with siRNA) as previously done in human fibroblasts32 and then measuring α-SMA. Two different siRNAs were used, both of which produced large (>90%) knockdown of the α11 integrin (Figure 4; Supplementary material online, Figure S3). Knockdown of the α11 integrin did not affect the expression of the other collagen-binding integrins, α1, α2, and α10. As expected, there was enhanced α-SMA expression in cells plated on MGO-collagen compared with PBS-collagen when α11 integrin was expressed (i.e. control siRNA conditions). After α11 integrin knockdown, there was marked reduction in α-SMA expression, indicating that the α11 integrin may be important for α-SMA expression in diabetes-induced myofibroblast differentiation (Figure 4B).

3.5 Interactions between α11 integrin and TGF-β2

As integrin expression levels can affect TGF-β expression, an important cytokine in cardiac fibrosis,25,33,34 we examined expression levels of three TGF-β isoforms in fibroblasts from diabetic and control rats and in human cardiac fibroblasts plated on MGO- or PBS-treated collagen. There were increased levels of TGF-β2 mRNA in cells, whole ventricles from diabetic animals and from cells plated on MGO-
Figure 4 Effect of knockdown of α11 integrin on integrin and α-SMA expression in human cardiac fibroblasts. Human cardiac fibroblasts were either NT or transfected with 100 nM of irrelevant siRNA (siNR) or with two different siRNAs (#1 set is shown here) to the α11 integrin. Cell lysates were separated by SDS–PAGE and immunoblotted for α1 (190 kDa), α2 (145 kDa), α10 (145 kDa), α11 (150 kDa) integrins or α-SMA (42 kDa) (A) GAPDH (36 kDa) was used as loading control. Representative blots from five independent experiments are shown. (B and C) Histograms show mean ± SE of α11 integrin and α-SMA blot densities for cells transfected with siRNA for α11 integrin and plated on MGO- or PBS-treated collagen vs. untreated (NT) cells treated with the same conditions. Data are from five independent experiments (B); *P < 0.05.

3.6 TGF-β regulation of α11 integrin in cardiac fibroblasts through Smad
TGF-β regulates cell function through Smad proteins.36 We examined whether α11 integrin and α-SMA expression were regulated through Smads with SIS3, a specific inhibitor of Smad3 phosphorylation.37 SIS3 reduced the expression level of α-SMA and α11 integrin in cells plated on PBS- or MGO-collagen (Figure 6E). Further, Smad3 phosphorylation was increased markedly in diabetic hearts compared with control animals (Figure 6F).
Figure 5 Expression of TGF-β2 in human fibroblasts, whole rat heart, and rat cardiac fibroblasts. Human cardiac fibroblasts plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) for 2 days (A) or whole heart tissue (B, left panel) or isolated rat cardiac fibroblasts (B, right panel) from control and diabetic rats were used for RNA preparation. The effect of MGO collagen or diabetic conditions on TGF-β2 expression was examined by real-time quantitative PCR. (C) PBS or MGO-treated-human cardiac fibroblasts were either NT or transfected with 100 nM irrelevant siRNA (siNR) or siRNA to α11 integrin. The effect of α11 siRNA on TGFβ2 expression was examined by real-time quantitative PCR. (D and E) HCFs plated on collagen were serum-starved for 48 h before treatment with TGF-β2 for an additional 48 h. The effects of TGFβ2 on α-SMA and α11 integrin mRNA and protein expression level were estimated by western blot (D) or real-time quantitative PCR (E). Data are from five independent experiments each; *P < 0.05.
4. Discussion

Our main finding is that the α11 integrin and TGF-β2 mediate myofibroblast differentiation in cardiac fibroblasts in the diabetic cardiac interstitium. Myofibroblasts are strongly associated with fibrosis in a wide variety of organs, including the heart, and could be involved in the fibrosis of diabetic cardiomyopathy. While oxidative/nitrative stress, inflammation, and apoptosis may contribute to the fibrosis of diabetic hearts, myofibroblasts, which produce poorly organized, fibrotic matrices, may also contribute to impaired diastolic function. Notably, cell adhesions and increased mechanical stiffness of the ECM may signal myofibroblast formation, which could explain the development of disorganized matrix in the diabetic myocardium.

Collagen fibril-binding integrins are important for mediating cell–matrix interactions. They provide adhesion of cardiac fibroblasts to type I collagen, the principal matrix protein of the cardiac interstitium. We found very low expression of α11 integrin in fibrosis, including the heart, and could be involved in the fibrosis of diabetic cardiomyopathy. While oxidative/nitrative stress, inflammation, and apoptosis may contribute to the fibrosis of diabetic hearts, myofibroblasts, which produce poorly organized, fibrotic matrices, may also contribute to impaired diastolic function. Notably, cell adhesions and increased mechanical stiffness of the ECM may signal myofibroblast formation, which could explain the development of disorganized matrix in the diabetic myocardium.

Figure 6 TGF-β regulates α11 integrin and α-SMA expression in human cardiac fibroblasts through the Smad pathway. Human cardiac fibroblasts plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) for 2 days were either non-transfected (NT), transfected with 100 nM irrelevant siRNA (siNR), or with siRNA to TGFβRI/TGFβRII (A–D). The effect of TGFβRI/TGFβRII siRNA on α11 integrin and α-SMA expression was examined by real-time quantitative PCR as described above and analysed by t-test to determine statistical differences between samples. Data are the mean ± SE relative to untreated (NT) cells plated on PBS-collagen or to untreated cells plated on MGO-collagen from five independent experiments. (E) HCFs plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) for 2 days were either untreated or treated with 10 μM SIS3. The effect of SIS3 on α11 integrin and α-SMA expression was examined by real-time quantitative PCR. Data are the mean ± SE relative to cells treated on PBS/MGO without SIS3 from five independent experiments. (F) Whole heart lysates from diabetic and control rats were prepared and immunoprecipitated (IP) with Smad3 antibody as described in Methods. Samples were resolved by SDS–PAGE and immunoblotted (IB) with phospho-Smad3 (52 kDa) or Smad3 (52 kDa) antibodies. TGFβ-treated HCFs were used as positive control. Representative blots from five independent experiments are shown.
TGF-β2 was selectively up-regulated in diabetic rat hearts. Knockdown of TGF-βR1 and TGF-βR2 or treatment with S13 strongly reduced α11 integrin and α-SMA expression in HCF, indicating that glycated collagen mediates myofibroblast differentiation through Smad3. Collectively, these results indicate an important role for TGF-β2 in regulating α11 integrin expression through the Smad pathway in the diabetic heart.

Increased collagen production by cardiac fibroblasts is a prominent feature of heart failure. While increased integrin levels can promote TGF-β expression, which is an important cytokine in cardiac fibrosis, it is not known how the strength of attachment or that inhibit the TGF-β integrin signalling pathway could then block this differentiation process.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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