TSpace

The University of Toronto's research repository

Accepted Manuscript of Glycated collagen induces $\alpha 11$
integrin expression through TGF- $\beta 2$ and Smad3

How to cite TSpace items

Always cite the published version, so the author(s) will receive recognition through services that track citation counts, e.g. Scopus. If you need to cite the page number of the TSpace version because you cannot access the published version, then cite the Tspace version¹ **in addition to** the published version².

Published version citation:

Talior-Volodarsky I, Arora PD, Wang Y, Zeltz C, Connelly KA, Gullberg D, McCulloch CA. Glycated collagen induces $\alpha 11$ integrin expression through TGF- $\beta 2$ and Smad3. J Cell Physiol. 2014 Jun 24.

TSpace version citation:

Talior-Volodarsky I, Arora PD, Wang Y, Zeltz C, Connelly KA, Gullberg D, McCulloch CA. Glycated collagen induces $\alpha 11$ integrin expression through TGF- $\beta 2$ and Smad3. TSpace. 2014. Available at <http://hdl.handle.net/XXXX/XXXXX>. Replace the 'XXXX/XXXXX' with the item ID from the URL.

tspace.library.utoronto.ca

¹ TSpace version: includes the pre-print/original manuscript (version before peer review) and post-print/accepted manuscript (version after peer-review and editing).

² Published version: the publisher's final PDF.

Glycated collagen induces alpha-11 integrin expression through TGF-beta and Smad3

Journal:	<i>Journal of Cellular Physiology</i>
Manuscript ID:	JCP-14-0281.R1
Wiley - Manuscript type:	Original Research Article
Date Submitted by the Author:	20-Jun-2014
Complete List of Authors:	Talior-Volodarsky, Ilana; University of Toronto, Matrix Dynamics Group Arora, Pamma; University of Toronto, Matrix Dynamics Group Wang, Yongqiang; University of Toronto, Matrix Dynamics Group Zeltz, Cédric; University of Bergen, Department of Biomedicine and Centre of Cancer Biomarkers, Norwegian Centre of Excellence Connelly, Kim; St. Michael Hospital, Keenan Research Centre for Biomedical Science Gullberg, Donald; University of Bergen, Department of Biomedicine and Centre of Cancer Biomarkers, Norwegian Centre of Excellence McCulloch, Christopher; Faculty of Dentistry, University of Toronto, Matrix Dynamics Group
Key Words:	cardiomyopathy, fibrosis, alpha11beta1 integrin, collagen glycation, diabetes

SCHOLARONE™
Manuscripts

Glycated collagen induces $\alpha 11$ integrin expression through TGF- $\beta 2$ and Smad3

Ilana Talior-Volodarsky¹, Pamma D. Arora¹, Yongqiang Wang¹,
Cédric Zeltz³, Kim A. Connelly², Donald Gullberg³,
Christopher A. McCulloch^{1*}

¹Matrix Dynamics Group, University of Toronto
Toronto, Ontario, Canada

² Keenan Research Centre for Biomedical Science of St. Michael Hospital,
Toronto, Ontario, Canada

³Dept. of Biomedicine and Centre of Cancer Biomarkers, Norwegian Centre of Excellence,
University of Bergen, Bergen, Norway

*Corresponding Author: C.A. McCulloch
Room 244, Fitzgerald Building, 150 College Street,
University of Toronto
Toronto, ON M5S 3E2
Phone: 416-978-1258; FAX: 416-978-5956;
e-mail: christopher.mcculloch@utoronto.ca

Running title: glycated collagen-induced TGF- $\beta 2$ signalling

Abbreviations used:

DCM – Diabetic Cardiomyopathy

PBS- phosphate buffered saline

MGO- methylglyoxal

TGF- β - transforming growth factor beta

INT11 – $\alpha 11\beta 1$ integrin

α - SMA – alpha smooth muscle actin

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

Smad2/3 – mammalian homolog of mothers against decapentaplegic 2/3

SBE – Smad binding element

SIS3 - specific inhibitor of Smad3

RNA - ribonucleic acid

mRNA - messenger RNA

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

BSA - bovine serum albumin

NP-40 - Tergitol-type NP-40 (nonyl phenoxypolyethoxylethanol).

qRT-PCR – quantitative real-time polymerase chain reaction

GFOGER motif - glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine motif

ABSTRACT

The adhesion of cardiac fibroblasts to the glycated collagen interstitium in diabetics is associated with *de novo* expression of the $\alpha 11$ integrin, myofibroblast formation and cardiac fibrosis. We examined how methylglyoxal-glycated collagen regulates $\alpha 11$ integrin expression. In cardiac fibroblasts plated on glycated collagen but not glycated fibronectin, there was markedly increased $\alpha 11$ integrin and α -smooth muscle actin expression. Compared with native collagen, binding of purified $\alpha 11\beta 1$ integrin to glycated collagen was reduced by >4-fold, which was consistent with reduced fibroblast attachment to glycated collagen. Glycated collagen strongly enhanced the expression of TGF- $\beta 2$ but not TGF- $\beta 1$ or TGF- $\beta 3$. The increased expression of TGF- $\beta 2$ was inhibited by triple helical collagen peptides that mimic the $\alpha 11\beta 1$ integrin binding site on type I collagen. In cardiac fibroblasts transfected with $\alpha 11$ integrin luciferase promoter constructs, glycated collagen activated the $\alpha 11$ integrin promoter. Analysis of $\alpha 11$ integrin promoter truncation mutants showed a novel Smad2/3 binding site located between -809 and -1300 nt that was required for promoter activation. We conclude that glycated collagen in the cardiac interstitium triggers an autocrine TGF- $\beta 2$ signalling pathway that stimulates $\alpha 11$ integrin expression through Smad2/3 binding elements in the $\alpha 11$ integrin promoter, which is important for myofibroblast formation and fibrosis.

Key words: cardiomyopathy, fibrosis, diabetes, collagen glycation, $\alpha 11\beta 1$ integrin

INTRODUCTION

Despite significant therapeutic advances, the worldwide prevalence of heart failure is substantial (1-3% of the general population) and represents a large economic burden on health care systems (Burchfield et al., 2013). Currently available data indicate that diabetes is associated with greatly increased risk of heart failure, particularly in patients under 65 years (Nichols et al., 2004) and with increased risk of death (Yeung et al., 2012). Diabetic cardiomyopathy (DCM), which is defined as heart failure in the absence of overt coronary artery disease or hypertension, accounts in part for the adverse prognosis and increased mortality seen in diabetic subjects (Fang et al., 2004). While the exact mechanisms underlying the cause of DCM are unknown, it is thought that a critical step in the pathogenesis of DCM is the formation of a disorganized, fibrotic interstitium. This abnormal extracellular matrix is enriched with poorly organized, glycated collagen (Herrmann et al., 2003; Kohda et al., 2009) that disrupts the structural organization and electrical conductivity of myocytes, increases myocardial stiffness and impairs ventricular diastolic function (Aneja et al., 2008; Nicoletti and Michel, 1999).

The most abundant cells in myocardium are cardiac fibroblasts, which comprise up to one half of the cells in the ventricular wall (Eghbali, 1992). Cardiac fibroblasts adhere to extracellular matrix proteins through integrins (Gullberg et al., 1992; MacKenna et al., 1998), cell surface receptors that anchor cells to the actin cytoskeleton and help to mediate normal matrix remodelling by contraction and reorganization of collagen fibrils. In cardiac tissues altered by fibrosis, a collagen-rich matrix is formed by myofibroblasts, cells which contribute to the development of heart failure. Indeed cardiac fibroblasts and myofibroblasts play critical roles in the development of the fibrotic response in diabetic cardiomyopathy (Kelly et al., 2007) that in turn affects cardiac function.

The attachment of cardiac fibroblasts to glycated collagen causes increased cell proliferation (Arora et al., 1999), collagen remodelling (Tang et al., 2007) and differentiation of a highly contractile myofibroblastic phenotype, which is characterized by expression of abundant alpha-smooth muscle actin (α -SMA). This actin isoforms is not found in fibroblasts of the normal heart but is expressed by cardiac myofibroblasts in fibrotic myocardium (Campbell and Katwa, 1997; Leslie et al., 1991). Myofibroblasts are increased locally at sites of myocardial infarction (Campbell et al., 1995; Katwa et al., 1997) and more globally in heart failure (Suurmeijer et al., 2003). Collectively these observations provide a rationale for defining the mechanisms by which glycated collagen promotes myofibroblast differentiation in diabetic hearts.

Integrins are a large family of heterodimeric cell surface receptors (Calderwood et al., 2000; Shai et al., 2002) that can form extracellular matrix adhesions. Four different integrins bind collagen ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$ (Popova et al., 2007)). In particular, the $\alpha 11$ integrin is a major fibrillar collagen receptor of fibroblastic cells (Gullberg et al., 1995; Popova et al., 2007) that contributes to myofibroblast differentiation and may be involved in collagen reorganization (Carracedo et al., 2010). While the $\alpha 11$ integrin was initially identified in differentiating human fetal muscle cells (Gullberg et al., 1995), subsequent mRNA expression analyses of human embryos showed that $\alpha 11$ integrin expression was localized to mesenchymal non-muscle cells at sites with well-organized interstitial collagen networks. Later work showed that the $\alpha 11$ integrin is expressed by odontoblasts and by several different types of fibroblasts (Popova et al., 2007). Consistent with the expression of $\alpha 11$ integrin at sites enriched with fibrillar collagens, the $\alpha 11$ integrin binds more tightly to type I collagen than to type IV collagen (Tiger et al., 2001).

The $\alpha 11$ integrin controls myofibroblast differentiation in human corneal fibroblasts and may play important roles in collagen reorganization mediated by myofibroblasts (Carracedo et al., 2010). While the role of the $\alpha 11$ integrin in myofibroblast formation in diabetic cardiomyopathy is not defined, we previously demonstrated that in cardiac fibroblasts, collagen glycation weakens the adhesion of cells to collagen, which in turn promotes a compensatory induction of $\alpha 11$ integrin expression that is mediated by one of the TGF- β isoforms, TGF- $\beta 2$ (Talior-Volodarsky et al., 2012). TGF- β isoforms are important regulators of integrin expression and function (Carver et al., 1995; Heino and Massague, 1989) that bind to cell surface receptors, which dimerize and lead to phosphorylation of receptor-regulated Smads (Massagué et al., 2005; Ross and Hill, 2008) and ultimately to the transcriptional regulation of target genes. Notably, TGF- $\beta 2$ is associated with cardiovascular complications in patients with Kawasaki disease (Shimizu et al., 2011) and TGF- $\beta 2$ levels are elevated in the myocardium of patients with dilated cardiomyopathy (Pauschinger et al., 1999). Further, disruption of TGF- $\beta 2$ signaling in mice leads to myocardial defects (Sanford et al., 1997) and TGF- $\beta 2$ is the only ligand with a clear-cut role in Epithelial-Mesenchymal Cell Transformation in the embryonic mouse heart (Mercado-Pimentel and Runyan, 2007).

To define how $\alpha 11$ integrin expression by cardiac fibroblasts is regulated by glycosylated collagen we examined Smad-dependent and TGF β -mediated transcriptional regulation of the $\alpha 11$ integrin in human cardiac fibroblasts plated on glycosylated collagen and transfected with $\alpha 11$ integrin promoter constructs (Lu et al., 2006; Zhang et al., 2002). Our data indicate a novel Smad binding site in the $\alpha 11$ integrin promoter that regulates $\alpha 11$ integrin expression.

MATERIALS and METHODS

Reagents

Rabbit antibodies to the human $\alpha 11$ integrin peptide sequence CRREPGLDPTPKVLE were produced as described (Carracedo et al., 2010; Popova et al., 2007) (Innovagen AB, Lund, Sweden). Rabbit polyclonal antibodies to Smad3, phospho-Smad3 and GAPDH, and horseradish peroxidase-conjugated (HRP) goat anti-rabbit antibodies, were from Cell Signaling Technology (Beverly, MA). Monoclonal antibody to α -smooth muscle actin (α -SMA; clone 1A4), goat anti-mouse IgG2a antibody and human fibronectin were from Sigma (Oakville, ON). Goat anti-mouse Dylight 488 or Dylight 549 goat anti-rabbit conjugated secondary IgGs antibodies were from Jackson ImmunoResearch (West Grove, PA). Rabbit TrueBlot®-anti-Rabbit IgG-HRP was from eBioscience (San Diego, CA). Recombinant human TGF- $\beta 1,2$ recombinant human integrin $\alpha 11\beta 1$, neutralizing antibodies to TGF $\beta 1$ and TGF $\beta 2$ and Quantikine ELISA Kits for TGF- $\beta 1$, TGF- $\beta 2$ and DuoSet ELISA Kit for TGF- $\beta 3$ were from R&D Systems (Minneapolis, MN). SIS3 (a Smad3 inhibitor) was from Calbiochem (San Diego, CA). Carboxylate-modified blue latex beads (2 μ m) were from Invitrogen (Eugene, OR). Rat tail collagen type 1 was from BD Biosciences (Bedford, MA). X-treme GENE HP DNA Transfection Reagent was from Roche (Mississauga, ON). Dual Luciferase Reporter Assay System Kit was from Promega (Madison, WI). Alexa Fluor 488 Protein Labeling Kit was from Invitrogen (Burlington, ON). NR191 and GFOGER-containing triple helical collagen peptides were provided by Richard Farndale of the Department of Biochemistry, Cambridge University (Great Britain). The pcCMV5B-Smad3 construct was provided by Andras Kapus (St. Michaels Hospital Research Institute; Toronto, ON).

Cell culture

Human adult ventricular cardiac fibroblasts (ScienCell, Carlsbad, CA; obtained from human biopsy materials) were cultured in DMEM (with 4.5 g/L glucose), F-12, 10% fetal bovine serum, fibroblast growth supplement and 10% antibiotics, as described (Yuen et al., 2010) and passaged with trypsin. A transfected mink lung epithelial cell (TMLEC) stably expressing firefly luciferase under the control of a TGF- β -sensitive portion of the PAI-1 promoter was a gift from D.B. Rifkin (New York University, New York City, NY) and cultured as described (Abe et al., 1994).

Collagen gels and glycation

Collagen gels prepared from rat type I collagen (1 mg/ml; Advanced BioMatrix, SanDiego, CA) or fibronectin (10 μ g/ml) coated on plates were treated with PBS or were glycated with 1 mM methylglyoxal (MGO, Sigma) overnight as described (Chong et al., 2007; Yuen et al., 2010) and MGO was washed out. MGO was used to glycate collagen instead of glucose and ribose as this procedure reduces the time required for collagen glycation and facilitates more consistent crosslinking of collagen gels (Chong et al., 2007; Yuen et al., 2010). MGO modification of collagen on Arg residues, which has been described previously (Chong et al., 2007), was used to assess the effect of collagen glycation on expression of α 11 integrin and SMA, a marker of the myofibroblast (Hinz B, 2007).

Immunoblotting and immunoprecipitation

Proteins in cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with BSA and immunoblotted for α 11 integrins, α -SMA, or GAPDH overnight at 4°C; bound antibodies were detected by chemiluminescence. For immunoprecipitations, cells were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma) and lysates were incubated with Smad3 antibody. Immune complexes were eluted from beads followed by immunoblotting for phospho-Smad3 or total Smad 3.

Immunofluorescence

Cells were cultured on PBS- or MGO-treated collagen or fibronectin that had been coated on to glass coverslips for 2h. Cells were then fixed with methanol for 5 min at -20°C. Following rehydration in PBS, cells were blocked with 10% goat serum in PBS for 45 min at 37°C. Fixed cells were incubated with antibody to α -SMA or with rabbit antisera to the α 11 integrin (diluted 1:400 and 1:200 respectively) or counter-stained with rhodamine phalloidin for 1h at 37°C. Antibody-labeled cells were washed three times for 10 min with PBS and incubated in goat anti-mouse Dylight 488 or Dylight 549 goat anti-rabbit conjugated secondary IgGs (diluted 1:200, 1:1000, respectively) for 1h at RT. Cells were visualized with a Zeiss Axioscope fluorescence microscope (Oslo, Norway). Images were acquired with an AxioCam MRm camera.

Quantitative PCR

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

Bead attachment assays

For assessment of the effect of glycation on collagen- $\alpha 11\beta 1$ integrin binding, recombinant human $\alpha 11\beta 1$ integrin was labeled with Alexa488 according to the manufacturer's protocol and attached to non-tissue culture plastic. Blue latex beads (2 μm diameter; 350 nm excitation, 410 nm emission) were coated with collagen or BSA, treated with PBS or MGO overnight and incubated on Alexa488-labeled $\alpha 11\beta 1$ integrin for 1 hour at 37°C. The beads were washed extensively with PBS (x1) to remove non-specifically bound beads. The fluorescence associated with the beads attached to the Alexa88 $\alpha 11\beta 1$ integrin was measured by fluorimetry (Photon Technology International, London, ON). Bead attachment data were normalized to the number of BSA-coated control beads that were bound to the $\alpha 11\beta 1$ integrin.

$\alpha 11$ promoter-luciferase reporter constructs

$\alpha 11$ promoter-luciferase reporter constructs, which contain serial deletions of the $\alpha 11$ promoter region: -76/+25, -127/+25, -176/+25, -330/+25, -400/+25, -616/+25, -809/+25, -1317/+25, -1598/+25, -2962/+25 in the pGL3 luciferase vector have been described previously (Lu, 2006; Zhang et al., 2003; Zhang et al., 2002). The $\alpha 11$ promoter-luciferase reporter construct that contains deletion of the predicted Smad-binding site in the ITGA11 promoter region [(-920)GTCTGGGACT(-911)] was generated by QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) as described previously (Liu and Naismith, 2008). The primers used for mutagenesis were designed according to Ensembl (www.ensembl.org) gene ID ENSG00000137809: (F5'-TATCAGCATGTCAACCTGGTGGGGACGGGAAGAGGGGGTGAAGG-3' and R 5'-GGTTGACATGCTGATATTCCCCAGCCCGCTCTCCCTCATTCTC-3'). Deletion of the -920 to -911 site in the promoter construct was verified by sequencing.

$\alpha 11$ integrin promoter assays

Cells were seeded on to plates one day prior to transfection and transiently co-transfected with one of the following luciferase reporters – SV40, 3b, -76, -127, -176, -330, -400, -616, -809, -1317, -1598, -2962 in pGL3 luciferase vector, and with pcCMV5B-Smad3, pcDNA3-Smad2, pcDNA3-Smad7 and with the *Renilla* construct – pRL-TK, using X-treme GENE HP DNA Transfection Reagent according to the manufacturer's protocol. Luciferase reporter assays were performed using 1.5 μg /well luciferase constructs, 1.5 μg /well pRL-TK. Twenty four hours after transfection, cells were lysed and luciferase activity was determined using the Dual Luciferase Reporter Assay System Kit. For each condition, treatments were performed in duplicate, and experiments were repeated at least three times. From each sample, the firefly luciferase activity corresponding to a specific promoter construct was normalized to the *Renilla* luciferase activity of the same sample. Results are expressed as fold changes compared with the mean *Renilla* ratio of the untreated controls taken as a unit.

Statistical analysis

For all studies, experiments were repeated at least three times. Quantitative data are reported as mean $\pm$ SEM and are 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; differences of $p<0.05$ were considered to be statistically significant.

RESULTS

$\alpha 11$ integrin expression in response to collagen glycation

We found earlier that collagen glycation by MGO weakens integrin-collagen interactions in intact cells in a MGO dose-dependent fashion (Chong et al., 2007; Yuen et al., 2010). The glycation of collagen by MGO promotes compensatory, TGF- $\beta 2$ -induced expression of the $\alpha 11$ integrin and the myofibroblast marker α -SMA (Talior-Volodarsky et al., 2012). To define the contribution of the $\alpha 11$ integrin in collagen glycation-induced myofibroblast formation, we examined $\alpha 11$ integrin immunostaining in the adhesions of human cardiac fibroblasts adherent to native or glycated collagen. In cells plated on MGO-treated collagen, $\alpha 11$ integrin-stained adhesions were not as distinct and were smaller than in cells plated on native (control) collagen (Fig. 1A). Immunostaining for the myofibroblast marker α -SMA showed that in contrast to cells plated on native collagen, cells plated on glycated collagen exhibited fewer but more strongly stained α -SMA-containing stress fibers. We examined whether binding of purified, recombinant $\alpha 11 \beta 1$ integrin to collagen was affected by collagen glycation. Compared with glycated collagen beads, there were >4-fold more native collagen-coated beads bound to the $\alpha 11 \beta 1$ integrin (Fig. 1B), consistent with previous data indicating that fibroblasts adhere more strongly to native than to glycated collagen (Talior-Volodarsky et al., 2012).

We determined whether total $\alpha 11$ integrin expression was affected by plating cells on glycated collagen. Immunoblots of cell lysates showed markedly increased expression of $\alpha 11$ integrin and SMA in cells plated on MGO-collagen compared with native collagen (Fig. 1C). We used fibronectin as an alternative matrix protein to determine whether glycated fibronectin also affects $\alpha 11$ integrin and SMA expression. Cells plated on fibronectin with or without glycation showed abundant and equivalent levels of $\alpha 11$ integrin and SMA protein expression, indicating that fibronectin glycation does not affect the expression of these proteins (Fig. 1C). Experiments of similar design were analyzed by qRT-PCR to assess transcriptional regulation mediated by collagen or fibronectin glycation. These data showed that $\alpha 11$ integrin and α -SMA mRNA expression levels were increased in cells plated on MGO-collagen compared with native collagen while there was no significant difference in expression between cells plated on native or glycated fibronectin (Fig. 1D). Further, we wondered whether fibronectin or collagen glycation would affect stress fiber formation and cell spreading. In cells plated on native or glycated collagen or fibronectin, there were fewer well-defined stress fibers in cells plated on glycated collagen compared with native collagen but with no obvious effect on stress fibers for cells spread on glycated fibronectin. There was no effect of glycation on cell spreading area (Supplementary Fig. 1) although cells spread on fibronectin exhibited a 15% greater spreading area than cells plated on collagen ($p < 0.01$).

Glycated collagen-induced activation of the $\alpha 11$ integrin promoter

We examined whether collagen glycation activates the $\alpha 11$ integrin promoter with the use of luciferase promoter constructs containing 3 kb of the $\alpha 11$ integrin promoter (Lu et al., 2006). In human cardiac fibroblasts transfected with pGL-2962 (which contains most of the proximal $\alpha 11$ integrin promoter) and plated on native or MGO collagen, there was >40% increase of luciferase activity in cells plated on MGO collagen compared with native collagen, but there was no change in cells plated on glycated fibronectin compared with native fibronectin (Fig. 2A).

We assessed MGO-collagen stimulation of the $\alpha 11$ integrin promoter by the use of luciferase constructs in which there were systematic truncations of the 3 kb promoter. In

comparisons of cells plated on native or glycated collagen, measurement of luciferase activity showed no significant change when cells were transfected with constructs containing nt-809 or less of the full-length promoter (Fig. 2B) whereas promoter activity was increased by glycated collagen in cells transfected with constructs extending to nt-1317 or longer ($p<0.01$). Notably, application of the PROMO transcriptional analysis software (Farre et al., 2003) for analysis of the human $\alpha 11$ integrin gene promoter (ITGA11; GenBank acc. No. AF109681.1) indicated a potential Smad3 binding site (TGTCTGGGAC) at nt-921 to nt-912. We deleted this putative Smad3 binding site (nt-920 to nt-911; GTCTGGGACT) in the -2962 wild type promoter construct; luciferase activity was measured in cells transfected with the full-length wild type promoter and this mutant promoter construct. Promoter activity in cells transfected with the wild type construct was significantly decreased when cells were plated on glycated collagen ($p<0.01$; Fig. 2C) whereas cells transfected with the mutant promoter construct demonstrated no change of promoter activity when cells were plated on MGO-treated collagen compared to native collagen.

Role of GFOGER collagen domain on $\alpha 11$ integrin promoter activity

Binding of fibrillar collagen to the $\alpha 11$ integrin is dependent on the sequence glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine (GFOGER) in fibrillar collagens (Zhang et al., 2003). This binding sequence is common to the collagen receptors $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$ (Knight et al., 1998; Knight et al., 2000). With the use of a triple-helical collagen mimetic peptide that incorporates the GFOGER binding sequence (Reyes and Garcia, 2003), we examined whether this peptide can block glycated collagen-induced activation of the $\alpha 11$ integrin promoter. In human cardiac fibroblasts transfected with the pGL-2962 construct and plated on native or glycated collagen, the GFOGER peptide reduced the effect of glycated collagen on $\alpha 11$ integrin promoter activity to control levels (Fig. 3). In contrast to cells transfected with the full-length promoter, plating cells on glycated collagen did not increase the activity of the pGL-330 promoter, which includes a TGF- β -sensitive control element and two Smad-binding elements (Lu et al., 2010). A control, triple helical collagen peptide that contains an irrelevant peptide binding sequence (NR 191) did not affect glycated collagen-induced $\alpha 11$ integrin promoter activity.

Since TGF- β isoforms and especially TGF- $\beta 2$ play important roles in cardiac development (Doetschman et al., 2012; Mercado-Pimentel and Runyan, 2007) we examined the levels of total TGF- β isoforms in HCF-conditioned media. The level of total TGF- $\beta 2$ was 4-fold higher than the level of TGF- $\beta 1$ ($p<0.001$; Figure 4A), but total levels of TGF- $\beta 1$ and TGF- $\beta 2$ were not changed in the media of HCF plated on glycated collagen compared with PBS-treated collagen (PBS-collagen: TGF- $\beta 1$ - 1435 $\pm$ 426 pg/ml; TGF- $\beta 2$ - 5696 $\pm$ 1661 pg/ml. MGO-collagen: TGF- $\beta 1$ - 1419 $\pm$ 403 pg/ml; TGF- $\beta 2$ - 5833 $\pm$ 782 pg/ml). The level of TGF- $\beta 3$ was below the detection level of the assay (minimal detection limit is 31.2 pg/ml) and further analysis of this isoform was not pursued. We determined whether collagen glycation stimulates activation of TGF- β using co-cultures of HCFs and TMLEC indicator cells, which provide proportional estimates of the levels of active TGF- β in the medium (Abe et al., 1994). When HCFs were pre-plated on PBS- or MGO-treated collagen and co-cultured with the TMLEC cells, there was ~2-fold increase of luciferase activity in the cells co-cultured on MGO-treated collagen ($p<0.05$; Figure 4B).

We examined whether plating cells on glycated collagen affected mRNA expression of the $\alpha 11$ integrin, α -SMA (Talior-Volodarsky et al., 2012) and TGF- $\beta 2$, and whether these effects

were dependent on cell binding to fibrillar collagen through the GFOGER sequence. Plating cells on glycated collagen in the presence of the GFOGER peptide blocked MGO-collagen enhanced mRNA expression of $\alpha 11$ integrin and α -SMA (Fig. 4D) and TGF- $\beta 2$ (Fig. 4C). Collagen glycation did not affect expression of TGF- $\beta 1$ or TGF- $\beta 3$ and there was also no impact of the GFOGER peptide on the expression of these TGF- β isoforms (Fig. 4C).

Effect of TGF- β isoforms on $\alpha 11$ integrin promoter activity

As TGF- β regulates gene expression through Smads (Derynck and Zhang, 2003), we examined Smad-dependent TGF- β regulation on $\alpha 11$ integrin mRNA expression. We used two $\alpha 11$ promoter constructs (pGL-330 and pGL-2962), both of which contain two previously identified Smad-binding elements in the first 330 nt of the promoter sequence (Lu et al., 2010). In HCFs that were transfected with pGL-330 and then treated with TGF- $\beta 1$, there was 4-fold higher $\alpha 11$ integrin promoter activity than untreated controls (Fig. 5A), whereas cells treated with TGF- $\beta 2$ showed no increase of promoter activity. In contrast, both TGF- $\beta 1$ and TGF- $\beta 2$ strongly increased activity of the pGL-2962 promoter (Fig. 5A). Smad 3 phosphorylation was increased >5-fold in human cardiac fibroblasts treated with TGF- $\beta 2$ or in cells plated on glycated collagen compared with controls (Fig. 5B). Further, HCFs plated on PBS-treated or glycated collagen that had been pre-incubated with TGF- β neutralizing antibodies (all TGF- β isoforms or TGF- β isoform-specific), showed greatly reduced α -SMA protein (Fig. 5C) and $\alpha 11$ integrin mRNA expression (Figure 5D).

Role of Smad3 in $\alpha 11$ integrin promoter activity

We examined whether the activity of the $\alpha 11$ integrin promoter was regulated through Smads with the use of SIS3, a specific inhibitor of Smad 3 phosphorylation (Jinnin et al., 2006). SIS3 reduced $\alpha 11$ integrin -2962 promoter activity to background levels in cells plated on PBS- or MGO-collagen (Fig. 6A). There was no significant effect of SIS3 on the low levels of luciferase activity in cells transfected with the $\alpha 11$ integrin -330 promoter construct and then plated on native or glycated collagen. Co-transfection of human cardiac fibroblasts with the $\alpha 11$ integrin -2962 promoter construct and with Smad2 and Smad3 constructs were used as positive controls and separate cell preparations were co-transfected with a Smad7 construct as a negative control. These experiments showed the expected Smad 2/3 promotion and Smad7 inhibition of $\alpha 11$ integrin promoter activity.

We co-transfected human cardiac fibroblasts with a constitutively active Smad3 construct and with -330, -809, -1598 or -2962 $\alpha 11$ integrin promoter constructs. Only cells co-transfected with the -1598 or -2962 promoters exhibited increased activity arising from constitutively active Smad3 while cells transfected with the -330 and -809 promoters showed no change of activity as a result of transfection with the active Smad3 (Fig. 6B).

DISCUSSION

While collagen binding integrins and TGF- β are known to regulate myofibroblast differentiation (Hinz et al., 2007), little is known of how cardiac fibrosis is induced in diabetic hearts. Our major finding is that in response to adhesion to glycated collagen, TGF- $\beta 2$ and Smad3 in cardiac fibroblasts are involved in an autocrine signalling pathway that induces the expression of the $\alpha 11$

integrin, a subunit of a key fibrillar collagen adhesion receptor implicated in myofibroblast formation (Carracedo et al., 2010). This autocrine signalling system evidently involves bi-directional integrin-TGF- β 2 interactions because blockade of cell binding through the GFOGER domain of fibrillar collagen interfered with glycosylated collagen-induced expression of TGF- β 2. Further, TGF- β 2 appears to signal through a third putative Smad3 binding site in the α 11 integrin promoter that is located between -809 and -1317 nt and that appears to regulate α 11 integrin expression differently than TGF- β 1 (Lu et al., 2010). These data provide new insights into the central and cooperative roles of α 11 β 1 integrin and TGF- β 2 in promoting myofibroblast formation in response to glycosylated matrix proteins in diabetic cardiomyopathy.

TGF- β isoforms are important pro-fibrogenic growth factors that induce the expression of extracellular matrix proteins and promote the differentiation of fibroblasts into myofibroblasts, cells that are involved in fibrotic lesions of the myocardium in heart failure (Doetschman et al., 2012; Ross and Borg, 2001). We found earlier that the α 11 integrin and TGF- β 2 may contribute to myofibroblast differentiation in the diabetic rat cardiac interstitium (Talior-Volodarsky et al., 2012). Based on these previous data and our current findings we propose that collagen glycation associated with chronic hyperglycaemic episodes weakens integrin-collagen interactions, which in turn drives a compensatory, autocrine enhancement of α 11 integrin expression through TGF- β 2 that appears to be regulated differently than TGF- β 1. In this current report we demonstrate that pan TGF- β or TGF- β 2 neutralizing antibodies inhibit the glycosylated collagen-induced expression of α 11 integrin and α SMA by HCFs. Consistent with these data, earlier studies showed that pan TGF- β or TGF- β 2 neutralizing antibodies reduced post-operative scarring after surgery in animals and humans (Cordeiro et al., 2003; Siriwardena et al., 2002) and that blockade of inhibition of TGF- β reduces fibrosis in rodent models of cardiac hypertrophy, pulmonary fibrosis and glomerulonephritis (Isaka et al., 1999; Kuwahara et al., 2002). Our data implicating TGF- β 2 specifically in regulating the expression of α 11 integrin in response to glycosylated collagen, underlines the importance of this signaling pathway in myofibroblast formation (Carracedo et al., 2010) and in the fibrosis observed in DCM (Fang et al., 2005).

For obtaining a more in-depth understanding of how the α 11 integrin and TGF- β 2 cooperatively interact to induce myofibroblast differentiation, we examined the mechanism by which glycosylated collagen enhances the expression of α 11 integrin and SMA. Initially we compared α 11 integrin expression by human cardiac fibroblasts in response to glycosylated collagen or glycosylated fibronectin. There was no induction of α 11 integrin when cells were plated on glycosylated fibronectin, indicating that adhesive interactions with glycosylated collagen are required for increased α 11 integrin expression. Consistent with these data, blockade of the α 2 β 1 and α 11 β 1 integrin binding sites in fibrillar collagen with the use of triple helical collagen peptides containing the GFOGER sequence (Zhang et al., 2003), consistently inhibited glycosylated collagen-induced expression of the α 11 integrin, TGF- β 2 and SMA. While the GFOGER sequence is a common recognition motif that is also involved in collagen binding to the α 1 β 1 and α 10 β 1 integrins, these adhesion receptors preferentially bind collagen monomers and not fibrillar collagen (Zeltz et al., 2014).

Previous analyses of the α 11 integrin promoter employing serial deletions, demonstrated two TGF- β 1-responsive Smad binding elements in the proximal promoter (nt-176/+25) that extended up to nt-330 (Lu et al., 2010). In this previous study, transfection and expression of inhibitory Smad7 reduced TGF- β 1-dependent α 11 integrin mRNA and protein expression. Further, with mutation and deletion analyses, a Smad-binding element at nt-182/-176 was

considered to be an important Smad3-binding site in this part of the promoter. With the use of PROMO (Farre et al., 2003), a transcription factor binding site prediction tool, we found a putative third Smad3 binding site (TGTCTGGGAC) located at nt-921 and nt-912 in the $\alpha 11$ integrin promoter. The position of this sequence in the $\alpha 11$ promoter was consistent with our analyses of deletion constructs and with the $\Delta 2962$ mutant construct in transfected cells plated on native or glycated collagen. There was no significant change of luciferase activity when cells were transfected with constructs containing nt- 809 or less of the full-length promoter. Further, $\alpha 11$ integrin promoter activity was strongly increased by collagen glycation in cells transfected with constructs extending to nt-1317 or longer but was decreased with the $\Delta 2962$ mutant construct in which nt- 911 to nt- 920 were deleted. Collectively these data indicate a potential role for a third Smad3 binding element in regulating glycated collagen-induced expression of the $\alpha 11$ integrin in addition to the two, previously-demonstrated Smad binding elements that respond to TGF- $\beta 1$ (Lu et al., 2010).

TGF- β and integrins cooperatively interact to regulate transcription of several critical genes that mediate matrix structure and remodeling (Margadant and Sonnenberg, 2010). Our current data strongly implicate the $\alpha 11$ integrin subunit in these processes and describe a TGF- $\beta 2$ -dependent signaling pathway that employs a novel regulatory site in the $\alpha 11$ integrin promoter compared with TGF- $\beta 1$ -dependent $\alpha 11$ integrin promoter activation (Lu et al., 2010). In response to TGF- $\beta 1$, the $\alpha 11$ integrin promoter is activated by Sp1 and Smad2/3 binding to the promoter, which extends up to nt-330. For glycated collagen-induced $\alpha 11$ integrin promoter activation, Smad3 is clearly important as the inhibitory Smad7 and SIS3 both blocked activation and there was robust phosphorylation of Smad3 after plating cells on glycated collagen. Collectively our data are consistent with the notion that glycated collagen mediates stimulation of $\alpha 11$ integrin transcription through a Smad3-dependent, TGF- $\beta 2$ -regulated signaling pathway. This pathway is evidently important for myofibroblast formation in response to glycated collagen and implicates TGF- $\beta 2$ as a specific TGF- β isoform in the pathogenesis of diabetic cardiomyopathy.

ACKNOWLEDGEMENTs

We are grateful to Andras Kapus (St. Michael Hospital, Toronto) for provision of the pcCMV5B-Smad3 construct and helpful comments on the manuscript. We thank Daniel Rifkin and Boris Hinz for provision of the TMLEC cells. We thank Rachael James and Richard Farndale (Department of Biochemistry, Cambridge University, UK) for provision of NR191 and GFOGER triple helical collagen peptides, Ning Lu (Department of Biomedicine, University of Bergen, Norway) for providing integrin $\alpha 11$ promoter constructs and Rainer Heuchel (Karolinska Hospital, Sweden) for providing pcDNA3-Smad2, pcDNA3-Smad7 vectors.

FUNDING

This work was supported by Heart and Stroke Foundation of Ontario operating grant #NA-6736 to KC and to CM, who is also supported by a Canada Research Chair (Tier 1) and grants to DG from Nasjonalforeningen for folkehelsen (No 6716) and EEA grand Poland Norway MOMENTO (ID 202952).

Disclosure: The authors have declared that no conflict of interest exists.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

LITERATURE CITED

Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. 1994. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 216:276-284.

Aneja A, Tang, WH, Bansilal S, Garcia MJ, and Farkouh ME. 2008. Diabetic cardiomyopathy: insights into pathogenesis, diagnostic challenges, and therapeutic options. *Am J Med* 121:748-757.

Arora PD, Narani N, McCulloch CA. 1999. The compliance of collagen gels regulates transforming growth factor-beta induction of alpha-smooth muscle actin in fibroblasts. *Am J Pathol* 154(3):871-882.

Burchfield JS, Xie M, Hill J. 2013. Pathological ventricular remodeling: mechanisms: part 1 of 2. *Circulation* 128(4):388-400.

Calderwood DC, Shatti S, Ginsberg MH. 2000. Integrins and actin filaments: reciprocal regulation of cell adhesion and signalling. *J Biol Chem* 275:22607-22610.

Campbell SE, Janicki JS, Weber KT. 1995. Temporal differences in fibroblast proliferation and phenotype expression in response to chronic administration of angiotensin II or aldosterone. *J Mol Cell Cardiol* 27(8):1545-1560.

Campbell SE, Katwa LC. 1997. Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol* 29(7):1947-1958.

Carracedo S, Lu N, Popova SN, Jonsson R, Eckes B, Gullberg D. 2010. The fibroblast integrin alpha11beta1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. *J Biol Chem* 285(14):10434-10445.

Carver W, Molano I, Reaves TA, Borg TK, Terracio L. 1995. Role of the alpha 1 beta 1 integrin complex in collagen gel contraction in vitro by fibroblasts. *J Cell Physiol* 165(2):425-437.

Chong SA, Lee W, Arora PD, Laschinger C, Young EW, Simmons CA, Manolson M, Sodek J, McCulloch CA. 2007. Methylglyoxal inhibits the binding step of collagen phagocytosis. *J Biol Chem* 282(11):8510-8520.

Cordeiro MF, Mead A, Ali RR, Alexander RA, Murray S, Chen C, York-Defalco C, Dean NM, Schultz GS, Khaw PT. 2003. Novel antisense oligonucleotides targeting TGF-beta inhibit in vivo scarring and improve surgical outcome. *Gene Ther* 10(1):59-71.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425:577-584.

- Doetschman T, Barnett JV, Runyan RB, Camenisch TD, Heimark RL, Granzier HL, Conway SJ, Azhar M. 2012. Transforming growth factor beta signaling in adult cardiovascular diseases and repair. *Cell Tissue Res* 347(1):203-223.
- Eghbali M. 1992. Cardiac fibroblasts: function, regulation of gene expression, and phenotypic modulation. *Basic Res Cardiol* 87 Suppl 2:183-189.
- Fang ZY, Prins JB, Marwick TH. 2004. Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. *Endocr Rev* 25(4):543-567.
- Fang ZY, Schull-Meade R, Downey M, Prins J, Marwick TH. 2005. Determinants of subclinical diabetic heart disease. *Diabetologia* 48(2):394-402.
- Farre D, Roset R, Huerta M, Adsua JE, Rosello L, Alba MM, Messeguer X. 2003. Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Res* 31(13):3651-3653.
- Gullberg D, Gehlsen KR, Turner DC, Ahlen K, Zijenah LS, Barnes MJ, Rubin K. 1992. Analysis of alpha 1 beta 1, alpha 2 beta 1 and alpha 3 beta 1 integrins in cell-collagen interactions: identification of conformation dependent alpha 1 beta 1 binding sites in collagen type I. *EMBO J* 11(11):3865-3873.
- Gullberg D, Velling T, Sjoberg G, Sejersen T. 1995. Up-regulation of a novel integrin alpha-chain (alpha mt) on human fetal myotubes. *Dev Dyn* 204(1):57-65.
- Heino J, Massague J. 1989. Transforming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. *J Biol Chem* 264:21806-21811.
- Herrmann KL, McCulloch AD, Omens JH. 2003. Glycated collagen cross-linking alters cardiac mechanics in volume-overload hypertrophy. *Am J Physiol Heart Circ Physiol* 284(4):H1277-1284.
- Hinz B. 2007. Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127(3):526-537.
- Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. 2007. The myofibroblast: one function, multiple origins. *Am J Pathol* 170:1807-1816.
- Isaka Y, Akagi Y, Ando Y, Tsujie M, Sudo T, Ohno N, Border WA, Noble NA, Kaneda Y, Hori M, Imai E. 1999. Gene therapy by transforming growth factor-beta receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 55(2):465-475.
- Liu H, Naismith JH. 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnol* 8:91.
- Jinnin M, Ihn H, Tamaki K. 2006. Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression. *Mol Pharmacol* 69:597-607.
- Katwa LC, Campbell SE, Tyagi SC, Lee SJ, Cicila GT, Weber KT. 1997. Cultured myofibroblasts generate angiotensin peptides de novo. *J Mol Cell Cardiol* 29(5):1375-1386.
- Kelly DJ, Zhang Y, Connelly K, Cox AJ, Martin J, Krum H, Gilbert RE. 2007. Tranilast attenuates diastolic dysfunction and structural injury in experimental diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol* 293(5):H2860-2869.
- Knight CG, Morton LF, Onley DJ, Peachey AR, Messent AJ, Smethurst PA, Tuckwell DS, Farndale RW, Barnes MJ. 1998. Identification in collagen type I of an integrin alpha2

- beta1-binding site containing an essential GER sequence. *J Biol Chem* 273(50):33287-33294.
- Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. 2000. The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* 275(1):35-40.
- Kohda Y, Kanematsu M, Kono T, Terasaki F, and Tanaka T. 2009. Protein O-glycosylation induces collagen expression and contributes to diabetic cardiomyopathy in rat cardiac fibroblasts. *J Pharmacol Sci* 111(446-450).
- Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K, Imaizumi T. 2002. Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. *Circulation* 106(1):130-135.
- Leslie KO, Taatjes DJ, Schwarz J, vonTurkovich M, Low RB. 1991. Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am J Pathol* 139(1):207-216.
- Lu N, Carracedo S, Ranta J, Heuchel R, Soininen R, Gullberg D. 2010. The human alpha11 integrin promoter drives fibroblast-restricted expression in vivo and is regulated by TGF-beta1 in a Smad- and Sp1-dependent manner. *Matrix Biol* 29(3):166-176.
- Lu N, Heuchel R, Barczyk M, Zhang WM, Gullberg D. 2006. Tandem Sp1/Sp3 sites together with an Ets-1 site cooperate to mediate alpha11 integrin chain expression in mesenchymal cells. *Matrix Biol* 25(2):118-129.
- MacKenna DA, Dolfi F, Vuori K, Ruoslahti E. 1998. Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J Clin Invest* 101(2):301-310.
- Margadant C, Sonnenberg A. 2010. Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO Rep* 11(2):97-105.
- Massagué J, Seoane J, Wotton D. 2005. Smad transcription factors. *Genes Dev* 19(23):2783-2810.
- Mercado-Pimentel ME, Runyan RB. 2007. Multiple transforming growth factor-beta isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. *Cells Tissues Organs* 185(1-3):146-156.
- Nichols GA, Gullion CM, Koro CE, Ephross SA, Brown JB. 2004. The incidence of congestive heart failure in type 2 diabetes: an update. *Diabetes Care* 28(8):1879-1884.
- Nicoletti A, Michel JB. 1999. Cardiac fibrosis and inflammation: interaction with hemodynamic and hormonal factors. *Cardiovascular research* 41:532-543.
- Pauschinger M, Knopf D, Petschauer S, Doerner A, Poller W, Schwimmbeck PL, Kuhl U, Schultheiss HP. 1999. Dilated cardiomyopathy is associated with significant changes in collagen type I/III ratio. *Circulation* 99(21):2750-2756.
- Popova SN, Lundgren-Akerlund E, Wiig H, Gullberg D. 2007. Physiology and pathology of collagen receptors. *Acta Physiol (Oxf)* 190(3):179-187.
- Reyes CD, Garcia AJ. 2003. Engineering integrin-specific surfaces with a triple-helical collagen-mimetic peptide. *J Biomed Mater Res A* 65(4):511-523.
- Ross RS, Borg TK. 2001. Integrins and the myocardium. *Circ Res* 88(11):112-1119.
- Ross S, Hill CS. 2008. How the Smads regulate transcription. *Int J Biochem Cell Biol* 40(3):383-408.

- Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 124(13):2659-2670.
- Shai SY, Harpf AE, Babbitt CJ, Jordan MC, Fishbein MC, Chen J, Omura M, Leil TA, Becker KD, Jiang M, Smith DJ, Cherry SR, Loftus JC, Ross RS. 2002. Cardiac myocyte-specific excision of the beta1 integrin gene results in myocardial fibrosis and cardiac failure. *Circ Res* 90(4):458-464.
- Shimizu C, Jain S, Davila S, Hibberd ML, Lin KO, Molkara D, Frazer JR, Sun S, Baker AL, Newburger JW, Rowley AH, Shulman ST, Burgner D, Breunis WB, Kuijpers TW, Wright VJ, Levin M, Eleftherohorinou H, Coin L, Popper SJ, Relman DA, Fury W, Lin C, Mellis S, Tremoulet AH, Burns JC. 2011. Transforming growth factor-beta signaling pathway in patients with Kawasaki disease. *Circ Cardiovasc Genet* 4(1):16-25.
- Siriwardena D, Khaw PT, King AJ, Donaldson ML, Overton BM, Migdal C, Cordeiro MF. 2002. Human antitransforming growth factor beta(2) monoclonal antibody-a new modulator of wound healing in trabeculectomy: a randomized placebo controlled clinical study. *Ophthalmology* 109(3):427-431.
- Suurmeijer AJ, Clement S, Francesconi A, Bocchi L, Angelini A, Van Veldhuisen DJ, Spagnoli LG, Gabbiani G, Orlandi A. 2003. Alpha-actin isoform distribution in normal and failing human heart: a morphological, morphometric, and biochemical study. *J Pathol* 199(3):387-397.
- Talior-Volodarsky I, Connelly KA, Arora PD, Gullberg D, McCulloch CA. 2012. α 11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. *Cardiovascular Research* 96(2):265-275.
- Tang M, Zhang W, Lin H, Jiang H, Dai H, Zhang Y. 2007. High glucose promotes the production of collagen types I and III by cardiac fibroblasts through a pathway dependent on extracellular-signal-regulated kinase 1/2. *Mol Cell Biochem* 301(1-2):109-114.
- Tiger CF, Fougereousse F, Grundstrom G, Velling T, Gullberg D. 2001. α 11 β 1 integrin is a receptor for interstitial collagens involved in cell migration and collagen reorganization on mesenchymal nonmuscle cells. *Dev Biol* 237(1):116-129.
- Yeung DF, Boom NK, Guo H, Lee DS, Schultz SE, Tu JV. 2012. Trends in the incidence and outcomes of heart failure in Ontario, Canada: 1997 to 2007. *CMAJ* 184(14):E765-E773.
- Yuen A, Laschinger C, Talior I, Lee W, Chan M, Birek J, Young EW, Sivagurunathan K, Won E, Simmons CA, McCulloch CA. 2010. Methylglyoxal-modified collagen promotes myofibroblast differentiation. *Matrix Biol* 29(6):537-548.
- Zeltz C, Orgel J, Gullberg D. 2014. Molecular composition and function of integrin-based collagen glues-Introducing COLINBRIs. *Biochim Biophys Acta* in press.
- Zhang WM, Kapyla J, Puranen JS, Knight CG, Tiger CF, Pentikainen OT, Johnson MS, Farndale RW, Heino J, Gullberg D. 2003. α 11 β 1 integrin recognizes the GFOGER sequence in interstitial collagens. *J Biol Chem* 278(9):7270-7277.
- Zhang WM, Popova SN, Bergman C, Velling T, Gullberg MK, Gullberg D. 2002. Analysis of the human integrin α 11 gene (ITGA11) and its promoter. *Matrix Biol* 21(6):513-523.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

FIGURE LEGENDS

Figure 1. α 11 integrin expression in cells plated on glycated collagen.

A - Immunofluorescence staining for α 11 integrin in human cardiac fibroblasts grown on PBS or MGO-treated collagen for 2 days. Cells were fixed and stained for α 11 integrin (a, b), and for α -SMA (c, d) as described in the Experimental section. e, f – are merged images including DAPI staining. Initial magnification - $\times 40$. Scale bars- 10 μ m. B - Alexa 488-labeled recombinant human α 11 β 1 integrin was incubated with blue latex beads, which were previously coated with native or glycated collagen or BSA. The beads then were washed extensively with PBS to remove non-specifically bound beads. Bead attachment data were obtained by fluorimetry and were adjusted to non-specifically bound BSA control beads. * indicates $p < 0.05$. C - Human cardiac fibroblasts plated on collagen or fibronectin for 2 days were lysed and lysates were separated by SDS-PAGE and immunoblotted for α 11 integrin (150 kDa) or α -SMA (42 kDa). GAPDH (36 kDa) was used as loading control. Blots are representative of three independent experiments. D - α 11 integrin and SMA mRNA expression in human cardiac fibroblasts treated as indicated. Real-time PCR was conducted as indicated in the Experimental section. Ct values were GAPDH-corrected and analyzed by t-tests to determine statistical difference between samples. Data are mean $\pm$ SEM relative to control samples from 3 independent experiments; * indicates $p < 0.05$.

Figure 2. α 11 integrin promoter activity.

A - Promoter activity of α 11 integrin in human cardiac fibroblasts plated on PBS or MGO-treated collagen or fibronectin. Luciferase was measured 48 hours after transfection and normalized against *Renilla* luciferase activity for same sample. The activity of the positive control pGL-SV40 was arbitrarily set at 1. All assays were performed in duplicates and were repeated at least three times. All data are the mean $\pm$ SEM. Comparisons are between PBS and MGO-treated cells. * indicates $p < 0.05$. B - Luciferase activity of truncation mutants of α 11 integrin promoters as indicated in transfected cells plated on PBS or MGO-treated collagen. C – Luciferase activity of the predicted Smad-binding site in the ITGA11 promoter region [(-920)GTCTGGGACT(-911)] mutant (Δ 2962) and full-length (2962) integrin promoter in transfected cells plated on PBS or MGO-treated collagen. * indicates $p < 0.05$ difference between paired control.

Figure 3. Effect of collagen-mimetic GFOGER peptide on α 11 integrin promoter activity.

GFOGER peptide effect on activity of full-length (2962) or truncated (330) $\alpha 11$ integrin promoters transfected into human cardiac fibroblasts. Cells were plated on PBS or MGO-treated collagen. Luciferase activity was measured 48 hours after transfection. NR191 was used as a non-collagen binding, control triple helical peptide. Luciferase activity was normalized against *Renilla* luciferase activity for each sample. The activity of the positive control pGL-SV40 was arbitrarily set at 1. All assays were performed in duplicates and repeated at least three times. All data are the mean $\pm$ SE. Comparisons are between PBS/MGO or GFOGER peptide and NR191-treated cells. * and ** indicates $p < 0.05$ and $p < 0.01$ respectively.

Figure 4. Effect of collagen-mimetic GFOGER peptide on mRNA expression of $\alpha 11$ integrin, α -smooth muscle actin and TGF- β isoforms.

A – Total TGF β 1 and TGF β 2 concentration was detected in HCF culture media by using Quantitative ELISA Kits. B – TMLEC cells were pre-transfected with *Renilla* construct and co-cultured with HCF on PBS- or MGO-treated collagen. Luciferase activity was measured 48 hours after transfection and normalized against *Renilla* luciferase activity of the same sample. For positive control cells were treated with TGF β . The activity of PBS-treated cells was arbitrarily set at 1. All assays were performed in duplicates and repeated more than three times. All data are the mean $\pm$ SE. Comparisons are between PBS or MGO treated cells. * indicates $p < 0.05$. D – For $\alpha 11$ integrin and α -smooth muscle actin expression, human cardiac fibroblasts were grown on PBS- or MGO-treated glycogen and co-incubated with GFOGER or NR191 control triple helical peptide. Quantitative real-time PCR was conducted. Ct values were GAPDH-corrected and analyzed by t-test to determine statistical difference between samples. Data are the mean $\pm$ SE relative to control samples from 3 independent experiments. Comparisons are between PBS/MGO or GFOGER peptide and NR191-treated cells. * and ** indicates $p < 0.05$ and $p < 0.01$ respectively. C - Same analytical approach as A was used for analysis of mRNA expression levels of TGF- β isoforms as indicated.

Figure 5. Effect of TGF- β isoforms on $\alpha 11$ integrin promoter activity.

A – Human cardiac fibroblasts were transfected with full-length (2962) or truncated (330) $\alpha 11$ integrin promoters and treated with TGF- β 1 or TGF- β 2. Promoter activity was measured 48 hours after transfection and normalized against *Renilla* luciferase activity of the same sample. The activity of the positive control pGL-SV40 was arbitrarily set at 1. All assays were performed in duplicates and repeated more than three times. All data are the mean $\pm$ SE. Comparisons are between TGF- β 1 or TGF- β 2 treated and un-treated cells. *, ** indicates $p < 0.05$ and $p < 0.01$ respectively. B – Human cardiac fibroblasts plated on PBS or MGO-coated collagen were lysed. Lysates were immunoprecipitated with *Smad3* antibody as described in Experimental section. Samples were resolved by SDS-PAGE and immunoblotted with phospho-Smad3 (52kDa) or Smad3 (52 kDa) antibodies. TGF- β 2-treated human cardiac fibroblasts were used as positive control. C – Human cardiac fibroblasts plated on PBS or MGO-treated collagen with or without TGF- β neutralizing antibody (PBS+TGF- β NA and MGO+TGF β NA, respectively) were lysed. Samples were resolved by SDS-PAGE and immunoblotted for α -SMA and GAPDH (as loading control). Blots are representative of three independent experiments. D - For $\alpha 11$ integrin expression, human cardiac fibroblasts were grown on PBS- or MGO-treated glycogen and co-incubated with TGF β , TGF β 1- or TGF β 2-neutralizing antibodies (TGF β NA, TGF β 1NA and TGF β 2NA, respectively). Quantitative real-time PCR was conducted. Ct values were GAPDH-corrected and analyzed by t-test to determine statistical difference between samples. Data are the

mean $\pm$ SE relative to control samples from 3 independent experiments. Comparisons are between PBS/MGO treated cells. * indicates $p < 0.05$.

Figure 6. Impact of Smad3 on $\alpha 11$ integrin promoter activity.

A – Smad3 inhibitor SIS3 was used to examine impact of Smad3-dependent regulation of $\alpha 11$ integrin promoter activity. Full length (2962) or truncated (330) $\alpha 11$ integrin promoters were transfected into human cardiac fibroblasts plated on PBS or MGO-treated collagen. In the indicated samples, transfected cells were co-transfected with pcCMV5B-Smad3, pcDNA3-Smad2 or pcDNA3-Smad7 constructs. Luciferase activity was measured 48 hours after transfection and SIS3 treatment. Data were normalized against *Renilla* luciferase activity of the same sample. Comparisons are between PBS/MGO and SIS3 treated and untreated cell. * and ** indicate $p < 0.05$ and $p < 0.01$ respectively. B - Human cardiac fibroblasts were transfected with $\alpha 11$ promoter integrin constructs only (full-length or truncation mutants as indicated) or were co-transfected with pcCMV5B-Smad3 construct as indicated. The activity of the positive control pGL-SV40 was arbitrarily set at 1. All assays were performed in duplicates and repeated at least 3 times. All data are mean $\pm$ SE. Comparisons are between cells with and without transfected Smad3. * and ** indicate $p < 0.05$ and $p < 0.01$ respectively.

Figure 1

171x99mm (300 x 300 DPI)

Figure 2

241x328mm (300 x 300 DPI)

Figure 4

265x393mm (300 x 300 DPI)

Figure 5

127x111mm (300 x 300 DPI)

Figure 6

246x340mm (300 x 300 DPI)

Table 1. Primer sequences used for qPCR

Gene		Primer sequences	Amplicon size (bp)
α11 integrin	Forward (F)	5'-ACCTTCAACATGGACACCAGGAA-3'	101
	Reverse (R)	5'-CACTTATTGCCACTGATGTCGT-3'	
α-smooth muscle actin	F	5'-TGACAATGGCTCTGGGCTCTGTAA-3'	142
	R	5'-TTCGTCACCCACGTAGCTGTCTTT-3'	
TGF-β1	F	5'-CCCAGCATCTGCAAAGCTC-3'	101
	R	5'-GTCAATGTACAGCTGCCGCA-3'	
TGF-β2	F	5'-TAACATCTCCAACCCAGCGCTACA-3'	87
	R	5'-CATCAGTTACATCGAAGGAGAGCCA-3'	
TGF-β3	F	5'-ACTGGCTGTCTGCCCTAAAGGAAT-3'	103
	R	5'-GACCCGGAATTCTGCTCGGAATA-3'	
GAPDH	F	5'-AATCCCATCACCATCTTCCA-3'	82
	R	5'-TGGACTCCACGACGTACTCA-3'	