Collagen Glycation Promotes Myofibroblast Differentiation

by

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Abstract

The incidence of cardiomyopathy and cardiac fibrosis is markedly increased in patients with diabetes mellitus. As cardiac fibrosis is mediated by myofibroblasts, we investigated the effect of diabetes-associated collagen glycation on the conversion of cardiac fibroblasts to myofibroblasts. Collagen glycation was modeled by the glucose metabolite, methylglyoxal (MGO). Cells cultured on MGO-treated collagen exhibited increased activity of the α-smooth muscle actin (SMA) promoter, elevated levels of collagen I, α-SMA mRNA, and enhanced protein expression of α-SMA, ED-A fibronectin and cadherins. Increased expression of α-SMA was dependent on β1 integrins and on TGF-β. In collagen gel assays, MGO-collagen promoted faster contraction and cell migration was increased by MGO-collagen. In shear-force detachment assays, cells on MGO-collagen were less adherent, and β_1 integrin activation and focal adhesion formation were inhibited. We conclude that collagen glycation augments the formation and migration of myofibroblasts, critical processes in the development of cardiac fibrosis in diabetes.

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Emily Won designed the AFM experiments and analyzed the respective data.

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This research project is dedicated to my father, Michael Yuen, who passed away suddenly from cardiac failure. Despite his early departure, he has taught me to live life to the fullest and to love those God has blessed us with. I hope I have made him proud.

Table of Contents

List of Tables

List of Figures

Chapter 1: Literature Review

i) Background: Understanding Diabetic Cardiomyopathy

Historically, the heart was considered to be a simple mechanical pump; hence congestive heart failure was considered as a problem of circulatory insufficiency. Heart failure was believed to be caused by severe and irreversible injury to the heart. However, after decades of investigation, heart failure is now widely accepted as a complex, progressive process rather than a single event. Heart failure is currently defined as a deficiency in the ability of the heart to adequately pump blood in response to systemic demands, which results in premature fatigue or dyspnea, and peripheral edema *(*Heineke *et al.*, 2006*)*. Heart failure is the leading cause of death in developed countries and is the leading cause of hospitalization in elderly patients; it affects more than five million people in the United States and extracts a very large economic burden from societies ($50 billion US per year) *(*Fedak *et al.*, 2005*)*. Data from the Framingham Heart Study *(*Dawber *et al.*, 1958*)* showed that patients with congestive heart failure have more hospitalizations than controls. This observation may due in part to the rising prevalence of other diseases in the general population that increase risk and, in particular diabetes *(*Kannel *et al.*, 2000*)*. Indeed, the World Health Organization in 2003 estimated that 194 million individuals were diagnosed with diabetes, which implies a global prevalence of 3% (5.1% for those aged 20-79). The number is expected to reach 333 million (6.3%) by the year 2025 *(*Boutayeb *et al.*, 2004*)*. Diabetes mellitus is an endocrine and metabolic disorder characterized by hyperglycemia. Poor long-term control of diabetes may result in complications such as nephropathy, neuropathy, retinopathy, and cardiomyopathy.

Cardiovascular diseases are responsible for 80% of deaths among diabetic patients. These deaths have been largely attributed to coronary artery disease. The term ‘diabetic cardiomyopathy’ was suggested for a disease originally identified in 1972 in four diabetic

patients who presented with heart failure but without evidence of hypertension, coronary artery disease, valvular or congenital heart disease *(*Rubler *et al.*, 1972*)*. A follow-up of the Framingham cohort showed increased incidence of cardiac heart failure in diabetic males (2.4:1) and females (5:1), independent of age, hypertension, obesity, coronary artery disease and hyperlipidemia *(*Kannel *et al.*, 1974*)*. Some of the major molecular abnormalities of diabetic cardiomyopathy include perturbations of the renin-aldosterone system, impaired endothelial function and interference with fatty acid production, which leads to increased extracellular matrix deposition. The increased deposition results in fibrosis in the myocardium and increased stiffness, which is exacerbated by the formation of advanced glycation end products.

ii) The extracellular matrix of the myocardium and its role in cardiac function

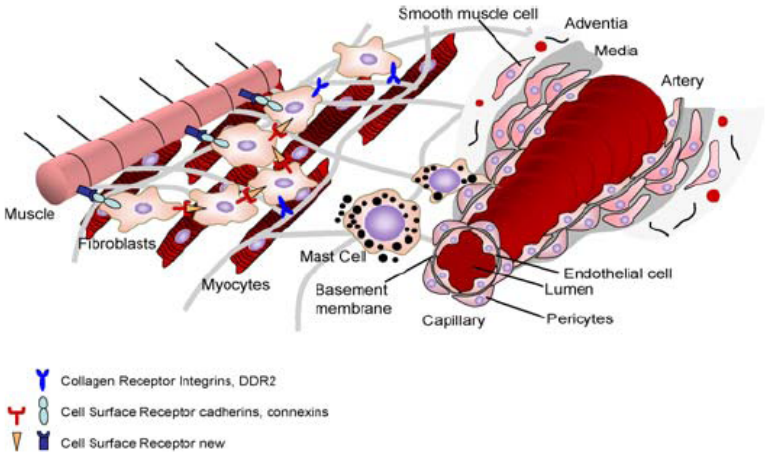


Figure 1: Model of the intimate interaction between cells and the extracellular matrix in the heart. *(*Banerjee *et al*, 2006*,)*

The extracellular matrix (ECM) of the heart is comprised of fibrillar collagen, elastin, microfibrillar proteins, proteoglycans and adhesive proteins such as laminin and fibronectin. The ECM provides a scaffold for the three dimensional organization of cells but is no longer viewed as a passive structure; instead it can influence the activity of cells, particularly when tissue architecture is altered. More recent data point to potentially important functions of the ECM in

cardiac function *(*Baudino *et al.*, 2006*)*, including provision of a scaffold function for transmitting mechanical signals to individual cells via cell surface receptors.

The cardiac ECM is a network that surrounds and supports myocardial cells and is comprised of structural proteins (collagen and elastin), adhesive proteins (laminin and fibronectin), anti-adhesive proteins and proteoglycans *(*Jane-Lise *et al.*, 2000*)*, as well as cells such as cardiomyocytes and fibroblasts (Figure 1). The structure and function of cardiomyocytes have been investigated in depth. However, cardiomycytes represent only 1/3 of all cells in the myocardium while fibroblasts comprise nearly 2/3. *(*Camelliti *et al.*, 2006*)* Fibroblasts synthesize and remodel the ECM and their abundance in the cardiac interstitium is consistent with the notion that the ECM undergoes turnover. Further, the ECM and its resident fibroblast population may be important for the regulation of overall cardiac function by virtue of the ability of fibroblasts to sense mechanical signals and to transmit contractile force to the ECM *(*Baudino *et al.*, 2006*)*.

iii) Collagen

Collagen is the most abundant protein in mammals *(*Perez-Tamayo *et al.*, 1978*)* and is by far the most abundant protein of extracellular matrices. Collagen transmits mechanical signals and contains molecular motifs that regulate the metabolism of fibroblasts. Collagen is also the major structural element of connective tissues and contributes to the form and stability of tissues and organs. Many connective tissue disorders such as chrondrodysplasia, osteogenesis imperfecta, Alport syndrome and Ehler's-Danlos Syndrome, are the secondary result of a defect in collagen structure.

'Collagen' is a term that describes proteins containing 3 polypeptide chains organized into a characteristic triple helix. All collagens exhibit the repeating tri-peptide (Gly-X-Y) motif but with variations in size and function. Currently, at least 26 different types of collagen have been identified *(*Gelse *et al.*, 2003*)*. The right-handed triple helix of all members of the collagen family is composed of three α chains, which may be homotrimers (found in collagen types II, III,

VII, VIII, X) or heterotrimers (found in collagen types I, IV, V, VI, IX, VIII, X). Each of the three α chains exhibits an extended left-handed helix structure with a pitch of 18 amino acids per turn *(*Hofmann *et al.*, 1978*)*. The three chains are staggered by one residue relative to another and are right-handed supercoiled around a central axis to form the triple helix *(*Fraser *et al.*, 1979*)*. To enable the formation of a highly compact triple helical structure, the glycine in every third residue of the triple helix (hence the Gly-X-Y) enables tight packing of the α chain wrap around a central axis. Glycine faces towards the inner axis of the molecule while amino acids with more bulky side chains comprise the other residues. The proline and lysine residues in collagen are modified by post-translational hydroxylation. Notably, 4-hydroxylproline is needed for formation of intramolecular hydrogen bonds and is important for the stability of triple helical conformation. Hydroxylysine is modified by glycosylation, which is important in cell recognition of collagen and in cross-linking.

Collagens can be classified into eight categories based on their structural organization, splice variants, presence of non-helical domains and assembly or function. These categories are: fibril-forming collagens (Types I, II, III, V and XI), basement membrane collagen (Type IV), microfibrillar collagen (Type VI), anchoring fibrils (Type VII), hexagonal network forming collagens (Type VIII and X), FACIT (Fibril Associated Collagens with Interupted Triple helices) collagens (Types IX, XII, XIV, XIX, XX and XXI), transmembrane collagens (XIII, and XVII) and multiplexins (Types XV, XVI and XVIII). The most abundant collagens are the fibril-forming collagens (I, II, III, V, XI).

The helix-forming portion (Gly-X-Y) is the main repeated motif in fibril-forming collagens (I, II, III), which produces triple helical domains of 300 nm in length, corresponding to ~1000 amino acids. Fibrillar collagens have a characteristic, quarter-staggered fibril array with a diameter of between 35-40 nm when visualized by electron microscopy that results in a banding

pattern of approximately 67 nm (called the D period) due to the staggered arrangement of individual collagen monomers *(*Hulmes *et al.*, 1981*)*

Type I collagen, COL1A1, is the most abundant collagen and comprises >90% of the organic mass of bone, major collagen in tendons, skin, ligaments, cornea and the heart. Type I collagen is usually a heterotrimer composed off two α1(I) chains and one α2(I) chain. Type II collagen is the main collagen found in hyaline cartilage, as well as in the vitreous body, corneal epithelium, notochord and the nucleus pulposus of the intervertebral disc. Type II collagen is composed of three α1 chains and hence is a homotrimeric collagen. Compared to type I collagen, type II collagen exhibits a higher proportion of hydroxylysine residues and glycosyl and galactosyl groups, which mediate interactions with proteoglycans. Type III collagen is a homotrimer of three α1 chains and is found in many collagen I-containing tissues with the exception of bone. Type III collagen is enriched in the reticular fibers of the interstitial tissues of lungs, liver, vessels, spleen and dermis.

iv) The Cardiac Fibroblast and Myofibroblast Differentiation

a) Role of fibroblasts in matrix remodeling and wound healing

In studies of heart failure, cardiomyocytes have been intensively investigated in view of their central role in contraction and their abundance in the myocardium. However, non-cadiomyocytes such as fibroblasts, smooth muscle cells, endothelial cells and mast cells may also contribute to pathological states *(*Fedak *et al*, 2005*)*. Fibroblasts form up to two-thirds of the cell population of the heart, mostly interspersed in a collagen network *(*Camelliti *et al.*, 2006*)*, *(*Grove *et al.*, 1969*)*. Fibroblasts were traditionally defined as cells of mesenchymal origin that produce interstitial collagen. In contrast to myocytes that form collagen type IV as part of their basement membrane, fibroblasts produce mainly types I, III and VI collagens *(*MacKenna *et al.*, 2000*)*.

Fibroblasts are widely distributed in vertebrate organisms and are found associated with various forms of soft connective tissues. The ability of fibroblasts to synthesize collagen is no

longer used for identification of these cells. Instead, identification is based on morphological characteristics that vary with the location and metabolic activity of a cell *(*Baudino *et al.*, 2006*)*. Fibroblasts do not reside on basement membranes but instead are dispersed through the interstitium. They generally exhibit an ovoid nucleus, extensive rough endoplasmic reticulum, a prominent Golgi apparatus and abundant cytoplasmic granular material. A characteristic phenotypic marker for fibroblasts is vimentin, an abundant intermediate filament protein *(*Camelliti *et al.*, 2006*)*.

Fibroblasts arise from various sources at different stages of development. In the embryonic heart, cells derived from the pro-epicardial organ generate progeny that undergo epithelial mesenchymal transformation to form fibroblasts *(*Border *et al.*, 1994*)*. The relative abundance of fibroblasts increases with normal development and aging. During early human development, following the completion of neonatal development, fibroblast proliferation returns to a low level, unless stimulated by either physiological or pathological processes.

Since cardiac fibroblasts are mainly responsible for the deposition of extracellular matrix, the role of the fibroblast is of considerable interest in pathological volume or pressure overload secondary to excessive deposition of extracellular matrix proteins. In this context the cardiac fibroblast may be able to "sense", "integrate" and "respond" to mechanical stimuli. *(*MacKenna *et al.*, 2000*)*. Further, cardiac fibroblasts may convert into myofibroblasts, a process that greatly contributes to the hypertrophic state since myofibroblasts synthesize and secrete extracellular matrix proteins that further stiffen the myocardium *(*Chien *et al.*, 1999*)*.

b) Definition of Myofibroblasts

Myofibroblasts were first described by Gabbiani (1971) using electron microscopy of the granulation tissue of healing wounds. He found cells with features of both smooth muscle cells and fibroblasts that exhibited prominent bundles of microfilaments, dense bodies and numerous gap junctions *(*Gabbiani *et al.*, 1971*)*. Morphologically, the contractile components of

myofibroblasts in vivo are organized as bundles of microfilaments, which are analogous in part to the stress fibers of cultured fibroblasts. The actin bundles terminate at the myofibroblast surface in an adhesive structure denoted the fibronexus in vivo, which may resemble the supermature focal adhesion in cultured cells *(*Dugina *et al.*, 2001*)*.

c) Myofibroblast function in matrix remodeling and wound healing

Myofibroblasts play a central role in tissue repair after injury and in the fibrosis seen in hypertrophic scars, scleroderma, Dupuytren's disease and in the failing heart and kidney. In normal tissues fibroblasts have few or no actin-associated intercellular or cell-matrix contacts, and they produce limited ECM *(*Grinnell *et al.*, 2003*).* After tissue injury, fibroblasts migrate into the damaged tissue and synthesize abundant new ECM proteins in response to locally released cytokines. Mechanical alterations to cells and the matrix can also stimulate phenotypic changes *(*Arora *et al.*, 1999*).*

In normally functioning, intact tissues, the ECM protects the fibroblast from direct mechanical perturbations (stress shielding). The critical steps in the formation and contraction of granulation tissue *(*Gabbiani *et al.*, 1971*)* include the migration of fibroblasts into the wound site and their deposition of an ECM rich in collagen and fibronectin. As they migrate along collagen fibers, fibroblasts exert tractional forces to mediate collagen reorganization *(*Hinz *et al.*, 2001*).* This process in turn increases mechanical tension and stimulates fibroblasts to develop arrays of contractile actin filaments, structures which have been modeled in vitro by the formation of stress fibers. However, after injury, it has been proposed that when fibroblasts acquire contractile stress fibers they should be denoted as "protomyofibroblasts" in order to differentiate them from quiescent fibroblasts that are devoid of a contractile apparatus. Stress fibers are connected to fibrous ECM proteins at sites of integrin-containing cell-matrix junction or at junctions between cells, stress fibers can anchor into cadherin-type adherens junctions *(*Dugina *et al.*, 2001*)*, *(*Grinnell *et al.*, 1994*).* With the development of increasing levels of tensile force by cells

attached to the ECM, proto-myofibroblasts can further develop into differentiated myofibroblasts, as assessed by expression of α–SMA *(*Skalli, *et al.* 1986*)*, an actin isoform that is typically found in vascular smooth muscle cells.

The incorporation of α-SMA into stress fibers increases the contractile activity of these cells and facilitates the contraction phase of connective tissue remodeling. Simultaneously, myofibroblasts synthesize collagen and other ECM proteins, as well as proteases, which facilitate wound closure. In normal wound healing, myofibroblasts disappear by apoptosis and variable sized scars may be formed when epithelialization is complete. However, in pathological situations, myofibroblasts persist and continue to remodel the ECM, which results in connective tissue contracture, inappropriate remodeling, increased tendency to scar formation and frequently, fibrosis.

Under appropriate conditions, relatively quiescent fibroblasts can acquire an overtly synthetic, contractile phenotype and express several smooth muscle cell markers that are not typical of fibroblasts. The enhanced expression of contractile proteins by relatively undifferentiated fibroblasts can manifest in vitro as more rapid contraction of collagen gels and is an important feature of wound closure and the structural integrity of healing scars *(*Tomasek, *et al,.* 2002*)*, *(*Grinnell *et al.*, 2000*)*. After cardiac injury, myofibroblasts appear in the myocardium and are thought to arise from progenitor cells in the cardiac interstitium, but they may also derive from cells in the systemic circulation *(*Olson *et al.*, 1998*)*.

c) Markers of the myofibroblast

The major ultrastructural features that distinguish quiescent fibroblasts from myofibroblasts include bundles of contractile microfilaments, extensive cell-to-matrix attachments, intercellular adherens junctions and gap junctions. However, these defining features do not permit clear-cut differentiation from other contractile cell types; hence there is a need for specific molecular markers *(*Hinz *et al*, 2007*)*, *(*Chambers *et al*, 2003*)*. Cytoskeletal markers for

the myofibroblast include *de novo* expression of α−SMA, the increased expression of the ED-A fibronectin isoform *(*Serini *et al.*, 1998*)* and increased assembly of stress fibers and focal adhesions.

d) Regulation of myofibroblast differentiation

Several soluble factors induce the formation of myofibroblasts including TGF-β1, an important wound healing cytokine that promotes the expression of α-SMA *(*Desmouliere *et al.*, 1993*)*. TGF-β1 also increases expression of ED-A FN, which precedes α-SMA actin expression by fibroblasts during granulation tissue formation *(*Serini *et al.*, 1998*)*. Only a few factors can induce myofibroblast differentiation independent of TGF-β1, including endothelin, interferon-γ, IL- 6 *(*Gallucci *et al.*, 2006*)* and nerve growth factor *(*Micera *et al.*, 2001*)*.

Myofibroblast development is profoundly influenced by the mechanical properties of the environment, particularly the organization and stiffness of the ECM *(*Hinz *et al.*, 2003*)*. TGF-β1 induction of αSMA depends on the compliance of the collagen substrate *(*Arora *et al.*, 1999*)*. More compliant collagen substrates block the expression of SMA induced by TGF-β_1, while rigid substrates may enhance SMA expression *(*Merryman *et al.*, 2006*)*

The ECM in early wounds has an elastic modulus on the order of 1 kPa, which is comparable to that of a polymerized collagen gel *(*Kaufman *et al.*, 2005*)*. Hence these collagen gels have been used extensively as in vitro models of tissue repair *(*Grinnell *et al.*, 2003*)*. In mechanically constrained gels, cell-generated tension is increased during the culture period. In turn, increased tension induces the formation of α-SMA negative stress fibers and the cells transform into putative proto-myofibroblasts. At this stage collagen gels have an elastic modulus of ~3-6 kPa *(*Discher *et al.*, 2005*)*. To induce differentiation of myofibroblasts, the stiffness of collagen gels increases after 2-3 days of remodeling by proto-myofibroblasts *(*Hinz *et al.*, 2006*)*. At ~20 kPa, *de novo* expression of α-SMA stress fibers is initiated. Accordingly, α-SMA may be

a mechanically sensitive protein that only localizes to stress fibers under significant mechanical loads, which suggests a rapid mechanism to control myofibroblast function *(*Hinz *et al.*, 2006*)*.

v) Collagen receptors

Cardiac fibroblasts, like other cell types, respond to mechanical stimuli by altering patterns of gene expression. The exact mechanism of how cardiac fibroblasts sense applied and cell-generated forces is not defined, however it is known that forces can be transmitted via cell surface matrix adhesion receptors *(*MacKenna *et al.*, 2000*)*. Cell adhesion in many cell types is mediated by binding of transmembrane molecules such as integrins to extracellular matrix ligands such as fibronectin, vitronectin, collagen and laminin *(*Turner *et al.*, 2000*)*. Binding of ECM ligands results in clustering of integrins, which leads to a series of intracellular responses including changes in protein phosphorylation, intracellular pH, intracellular free calcium ion concentration, and activation of mitogen-activated protein kinases. These signals also result in re-organization of the actin cytoskeleton, which precedes changes in cell shape, motility and gene expression *(*Turner *et al.*, 2000*)*.

Cells interact with collagen in a variety of ways. Some cell types can tightly adhere to collagen while others may migrate through a collagen-rich stroma. A simple definition of a collagen receptor is a transmembrane protein that directly interacts with triple helical fibrillar collagen *(*Leitinger *et al.*, 2007*)*. Different classes of collagen receptors are displayed in Table I.

	Receptor structure	Collagen specificity	Selected biological functions
Integrin α1β1	Heterodimer of large α and β subunits; collagen binds to MIDAS motif in α subunit I domain	Preferentially collagens IV and VI, but also fibril-forming collagens; specific sites in collagen I and IV identified	Fibroblast proliferation; regulation of collagen synthesis and MMP expression; response to renal injury
Integrin α2β1	(as above)	Preferentially fibril-forming collagens; specific site in collagen I and III identified	Platelet adhesion; branching morphogenesis; mast cell activation; keratinocyte adhesion
Integrins α10β1	(as above)	Preferentially collagens IV and VI, but also collagen II	Growth plate morphogenesis and function
Integrin α11β1	(as above)	Preferentially fibril-forming collagens; specific site in collagen I identified	Not well defined
DDR1	Homodimer; ectodomain consists of a collagen-binding discoidin domain followed by ~200 residues of unknown structure	Fibril-forming collagens and collagen IV; collagen VIII	Mammary gland development; arterial wound repair; regulation of cell proliferation and MMP expression; kidney function, differentiation and function of leukocytes
DDR2	(as above)	Fibril-forming collagens, collagen X; specific site in collagen II identified	Chondrocyte proliferation and bone growth; regulation of cell proliferation and MMP expression
Glycoprotein VI	Heterotetramer with FcRγ chain; ectodomain contains two IG domains, the first of which binds collagen	Fibril-forming collagens; (GPO)10	Platelet adhesion and activation
LAIR-1	Ectodomain consists of a single IG domain	Fibril-forming collagens; (GPO)10	Immune cell regulation
Mannose receptor family (MR, PLA2R, DEC-205, Endo180)	Large ectodomain consisting of cysteine-rich domain, a collagen-binding F2 domain, and eight to ten C-type lectin domains	Fibril-forming collagens and collagen IV, as well as gelatin; may lack specificity	Innate immunity; clearance of serum glycoproteins; collagen endocytosis

Table 1 – Mammalian Collagen receptors *(*Gelse *et al.*, 2003*)*

Integrins are the major mammalian receptors for cell adhesion to the extracellular matrix. The term "integrin" was coined as they are both integral membrane proteins and are also involved in the maintenance of cellular and extracellular matrix integrity *(*Tamkun *et al.*, 1986*)*. There are eight β-integrin sub-units (which range in size from 120-180 kDa) and 18 α-sub-units (size from 90-110 kDa) subunits, which, in various combinations, can form at least 24 different integrin heterodimers. Structurally, integrins exhibit a large modular extracellular domain formed by 700-1100 amino acids (to which extracellular ligands bind), a single transmembrane helix and a short cytoplasmic domain that interacts with cytoskeletal proteins. There are five main β1 integrins that act as collagen receptors- α1β1, α2β1, α3β1, α10β1 and α11β1, all of which are expressed by a broad array of mesenchymal cells. The α2β1 integrin is also expressed by epithelial cells and platelets. The main collagen receptors for fibrillar collagens are the α2β1 and α11β1 integrins. Specific GFOGER sequences in fibrillar collagen are high affinity binding sites for α1β1 and α2β1 integrins *(*Knight *et al.*, 1998*)*.

Integrins are subject to allosteric control *(*Ross *et al.*, 2001*)* meaning that different integrin conformations exist. These conformations permit conversion between low and high affinity binding states that regulate the relative attachment strength of integrins to their cognate ligands. The balance between the affinity states can be influenced by cytosolic integrin binding partners that regulate affinity from the cytoplasmic domains (i.e. inside-out signaling leading to integrin activation) or ligand binding to the extracellular head which can lead to the generation of intracellular signals (i.e. outside-in signaling). With inside-out signaling, intracellular signals can cause integrins to change their affinity for binding to the extracellular matrix. For example, an agonist may initiate signaling events that trigger cytoskeletal changes, which then lead to conformational changes of the integrin and enhance binding. In outside-in signaling, binding of a ligand to an inactive integrin results in conformational changes that result in the initiation of intracellular signaling events (e.g., increased pH or intracellular free calcium ion concentration).

Other collagen receptors include discoidin domain receptors (DDR), glycoprotein IV, leukocyte associated Ig-like receptor-1, Endo 180 and the mannose receptor family**.** DDR1 and DDR2 are a subfamily of receptor tyrosine kinases. They are collagen receptors independent of β1 integrins *(*Vogel *et al.*, 1999*)* and are special amongst receptor tyrosine kinases in that they are activated by extracellular signals while other receptor tyrosine kinases can be activated by myriad other signals including growth factors *(*Vogel *et al.*, 2006*)*. DDR1 is activated by all forms of collagen while DDR2 is only activated by fibrillar collagen. Activation only occurs when collagen is in its native, triple helical state since heat-denatured collagen (i.e., gelatin) lacks the triple helical structure of collagen and does not increase kinase activity.

vi) Cell Adhesion to Collagen

In cultured cells, the specific sites where clustered integrins engage extracellular matrix proteins are known as focal adhesions. These structures were first identified by electron microscopy and were described as electron-dense regions of the plasma membrane that make intimate contact with the substratum of cultured cells *(*Abercrombie *et al.*, 1971*)*. Focal adhesions form the structural link between the extracellular matrix and the actin cytoskeleton and may manifest as various types including focal contacts, focal adhesions, fibrillar adhesions, focal complexes and podosomes. Focal adhesions require the aggregation of integrin heterodimers, which bind to extracellular matrix proteins. Integrins do not directly bind actin filaments or exhibit enzymatic activities; hence all the structural and signaling events associated with integrin signaling are mediated by proteins that bind the cytoplasmic tails of integrins and the molecules they recruit. More than 50 focal adhesions proteins have been identified *(*Zamir *et al.*, 2001*)* and more are likely to be added to the list. Focal adhesion proteins can be divided into 3 main groups: extracellular, transmembrane and cytoplasmic.

Location		Focal adhesion proteins
Extracellular		Collagen, fibronectin, heparan sulfate, laminin, proteoglycan, vitronectin
Transmembrane		Integrins 18 α and 8 β (24 combinations in humans), LAR-PTP receptor, layilin, syndecan-4
Cytoplasmic	Structural	Actin, α-actinin, EAST, ezrin, filamin, fimbrin, kindling, lasp-1, LIM nebulette, MENA, meosin, nexilin, paladin, parvin, profilin, ponsin, radixin, talin, tensin, tenuin, VASP, vinculin, vinexin
	Enzymatic	Protein tyrosine kinase: Abl, Csk, FAK, Pyk2, Src Protein serine/threonine kinase: ILK, PAK, PKC Protein phosphatase: SHP-2, PTP-1B, ILKAP Modulators of small GTPase: ASAP1, DLC-1, Graf, PKL, PSGAP, RC-GAP72 Others: calpain II, PI3-K, PLCγ
	Adapters	p130Cas, caveolin-1, Crk, CRP, cten, DOCK180, DRAL, FRNK, Grb 7, Hic-5, LIP.1, LPP, Mig-2, migfilin, paxillin, PINCH, syndesmos, syntenin, tes, Trip 6, zyxin

Known focal adhesion components.

Table 2 – Focal Adhesion proteins*(*Zamir *et al.*, 2001*)*

From the initiation of cell adherence to the substrate, nascent adhesions known as focal complexes mature to become focal adhesions *(*Geiger *et al.*, 2001*)*. Focal complexes are precursors of focal contacts which typically form at the edges of lamellipodia. The maturation of these structures is mediated by Rho-family GTPase binding proteins such as Rac. Attachment to extracellular matrix proteins induces recruitment of activated Rac to membranes, particularly in lamellipodia. These are sites where actin polymerization and branching of actin filaments is initiated. Rapid actin flow promotes clustering of integrin molecules. From mature focal adhesions there may be a further transition to the so-called super-mature focal adhesions. *(*Dugina *et al.*, 2001*)*. On compliant substrates, fibroblasts exhibit immature focal adhesions with few stress fibers. With increased cell-generated tension, proto-myofibroblasts develop more mature adhesions and contractile stress fibers. Contractile forces arising from stress fibers increase tensile forces applied to the matrix and this may lead to the formation of 'super-mature focal adhesions'*(*Dugina *et al.*, 2001*)*. Supermature focal adhesions are found in cultured myofibroblasts and are ~8-30 μm long; they are enriched with vinculin, paxillin, tensin, αvβ3 and α5β1 integrins, and α-actinin. Supermature focal adhesions increase adhesion, effectively immobilizing the myofibroblast and enhancing force generation.

vii) Collagen Gel Contraction Assays

Collagen gel contraction assays are used as in vitro models for studying fibroblast-collagen interactions that are important in wound healing, fibrosis, scar contraction, and connective tissue morphogenesis. These models can provide insights into biological phenomena that are not easily obtained with commonly used, two-dimensional protein-coated substrates. Notably, in vivo, cell adhesion occurs in three dimensions to attachment sites that are distributed around the periphery of the cell. *(*Pelham *et al.*, 1997*)* At the cellular level, cells explore the elasticity of their environment as they anchor to the substrate and also "pull" on these adhesions. These activities

are dependent in part on myosin-based contractility, as well as on transcellular adhesions such as integrins and cadherins, which respond to cytoskeletal organization.

Bell *et al.* (1979) noted that myoblasts, myofibroblast and fibroblasts could contract fibrous protein gels, particularly those involving collagen. Since their development, collagen gels have been used to measure fibroblast contractility under different conditions *(*Erlich *et al.*, 1990*)*. A newly polymerized collagen gel is a flexible substrate with an elastic modulus of 1 kPa, whereas a normal culture dish has a rigidity of ~1 GPa *(*Wang *et al.*, 2003*)*. Collagen gels, but not substrates coated with thin collagen gels, down regulated focal adhesion proteins in all cell lines that were examined *(*Wang *et al.*, 2003*)*.

There are three major variants of collagen gel models including floating, anchored and stress-relaxed gels (Grinnell *et al.*, 2000). For the floating gel, the collagen matrix is released from the culture dish immediately after polymerization, hence the gel can contract in all three dimensions. In this system the cells remain more or less spherical, and extend fine protrusions as they contract the matrix. In contrast, anchored collagen gels are attached to the surface of the plate. As a result of cell-mediated contraction, there is only a reduction in gel height, not in gel diameter. For the stress-relaxed collagen gels, the collagen matrix is cultured with embedded cells for 1-2 days before the gel is released from the plate. After the first few hours, the cells attach to the matrix and then they develop isometric tension and form stress fibers. When the collagen matrix is released, the cell extensions collapse and stress fibers disappear *(*Ehrlich *et al.*, 1990*)*.

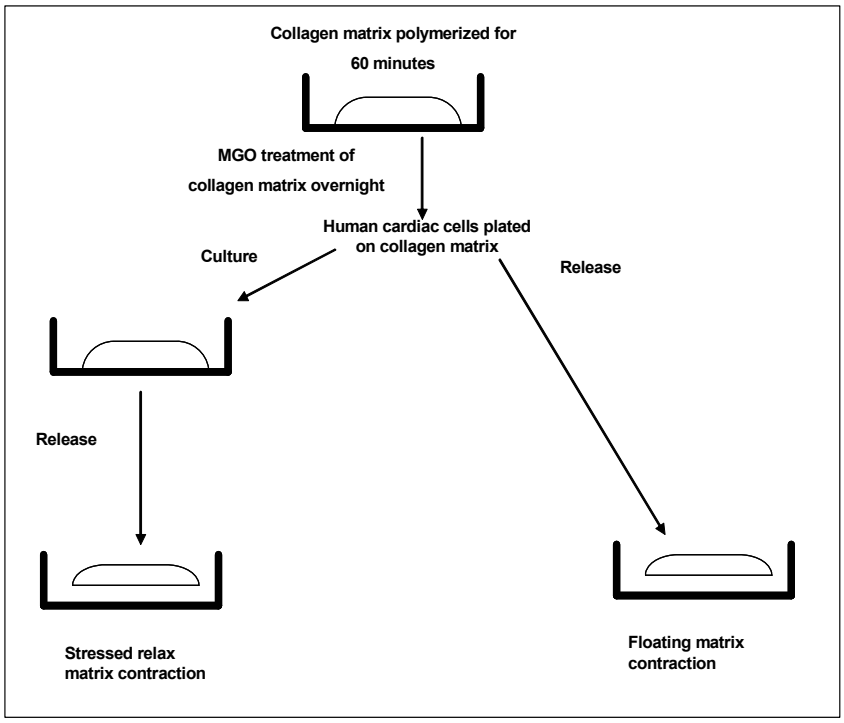


Figure 2: Schematic for Stress relaxed and floating gel models

There are two proposed mechanisms for how cells contract and re-organize the collagen gel matrix. First, isometric tension applied to the collagen fibrils by the cells can result in contraction of the collagen gel *(*Dodd *et al.*, 1982*)*. The second mechanism is by generation of a tractional force arising from the combined motility and contractility of the cells; they exert strong shear forces tangential to their surface *(*Harris *et al.*, 1981*)*. Actin and myosin molecules underlie most cellular motile process involving the cortical cytoskeleton. With binding of extracellular matrix proteins, integrins are activated and initiate signals that enable cell movement *(*Mitchison *et al.*, 1996*)*. Fibroblast lamellipodia extend along attached collagen fibers; the cells bind and retract the collagen fibers in a 'hand over hand' cycle *(*Meshel *et al.*, 2005*)*.

viii) Diabetes Metabolism and Glycation

Long-standing diabetes results in glycation of extracellular matrix proteins. Collagen is particularly susceptible (in part because of its abundance and its relatively slow turnover) and glycation can affect various properties including its ability to form precise, supra molecular

aggregates. Glycation alters the charge profile of collagen and hence interaction with cells; its biomechanical functions are also affected *(*Paul *et al.*, 1996*)*. One of the mechanisms that decreases myocardial wall compliance in diabetics is a non-enzymatic reaction between glucose and proteins in the arterial wall known as the Maillard reaction *(*Aronson *et al.*, 2003*)*.

The Maillard reaction was first cited in the field of nutritional science and is also known as the browning reaction. Maillard (1912) observed that when glucose was incubated with amino acids, yellow-brown pigments were formed due to non-enzymatic glycosylation of glucose with proteins *(*Aronson *et al.*, 2003*)*. Briefly, the reaction involves the formation of a Schiff base when glucose reacts with a reactive amino group. The formation of Schiff bases is fast and highly reversible. However, over a longer period of time (i.e., days), the Schiff base rearranges to form a more stable, Amadori product. Proteins with Amadori adducts are known as glycated proteins. Ultimately, the Maillard reaction was linked to age and diabetic-related changes in the mechanical properties of vascular tissues *(*Monnier *et al.*, 1981*)*. It was then observed that some of the early glycated proteins continue to undergo changes and rearrangements in vivo to form complex compounds and cross-links known as advanced glycation end products (AGEs) (Figure 3).

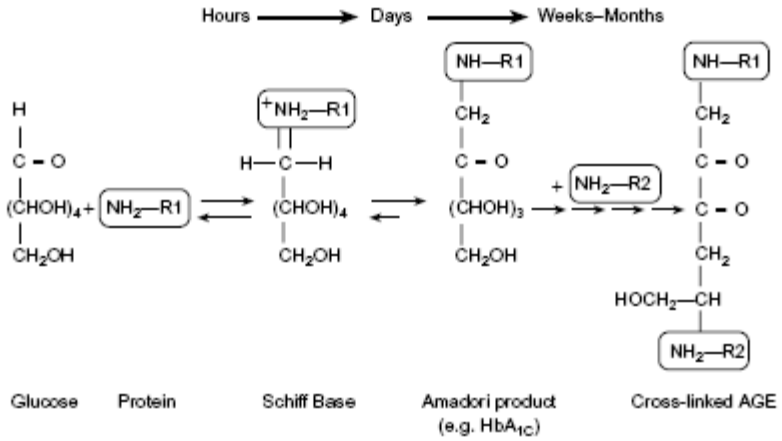


Figure 3: The formation of advanced glycation end products (AGE)

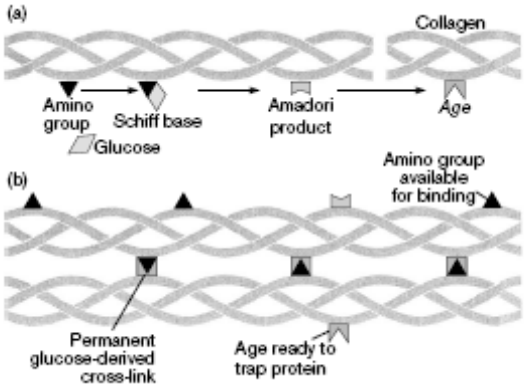


Figure 4: Schematic representation of formation of collagen crosslinks. *(*Avery *et al.*, 2006*)*

In normal collagen, cross-linking is limited to the amino and carboxy terminals of the molecule, however in the diabetic state, AGEs form cross-links throughout the collagen molecule. The cross-linking occurs between two adjacent molecules and frequently involves adjacent lysine or arginine residues (Figure 4). The linkages are thought to be located between the triple helical domains of adjacent collagen fibers *(*Avery *et al.*, 2006*)*. The increased number of linkages may be responsible for the changes in vascular tissue and loss of elasticity and flexibility in tissues observed with age. However, this process is greatly accelerated in diabetes *(*Bailey *et al.*, 1998*)*.

Pentosidine is the best characterized glycation cross-linker, however it is in very low concentrations in cardiac tissues and thus is unlikely to mediate diabetes-induced changes of collagen. Other cross-linkers of specific amino acid residues that are abundant in collagen (i.e., arginine and lysine) include glucosepane and 3-deoxyglucosone. Glycated collagen can accelerate oxidation of unsaturated lipids leading to the formation of malondialdehyde, 4-hydroxynonenal, glyoxal and methylglyoxal (MGO) *(*Thornalley *et al.*, 1999*)*. MGO and glyoxal can also be formed from triosephosphate, acetone from ketone, and degradation of unsaturated lipids *(*Paul *et al.,* 1996*)*. These compounds are only present in trace amounts but their reactivity is several thousand times higher than that of glucose. While glucose is the most abundant sugar in normal metabolism, only 0.002% of glucose is present in an open-chain, reactive form.

Consequently, the effective reactive glucose concentration is ~100 nM in normal conditions and ~200-500 nM in diabetes. In contrast, the MGO concentration in health is 256 nM and is ~500 nM in diabetics *(*Shipanova *et al.*, 1997*)*. In the experiments described in this thesis, MGO was chosen as the glycating agent because the reaction time required for an in vitro model was <24 hours as opposed to the 5 days required for other glycation agents such as glucose *(*Chong *et al.*, 2007*)*.

The intermolecular cross-linking of collagen caused by glycation can explain many of the modifications to the physical properties of collagen, however glycation also affects specific amino acids and can lead to the formation of stable AGEs, which may lead to modification of collagen interaction with cells. As noted above, cells interact with collagen through the binding of integrins to specific amino acid sequences in collagen. The presence of glycated modifications of collagen at lysine and arginine residues alters the charge profile as well as the binding sites to cells, resulting in reduction of cell-collagen interactions *(*Avery *et al.*, 2006*)*. As described above, integrins $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha11\beta1$ are important receptors for fibrillar collagen. These integrins recognize the GFOGER amino acid sequence as a high affinity binding site in collagen types I and IV. Evidently, glycation of the arginine residues at these specific molecular sites on collagen inhibits integrin interaction *(*Chong *et al.*, 2007*)*. In MG63 and HT1080 cells, modification of the arginine by MGO resulted in decreased adhesion and spreading of the cells *(*Paul *et al.*, 1996*)*.

AGE-mediated cross-links also increase resistance to enzymatic proteolysis and decrease degradation rates. Hence the balance between synthesis and degradation of collagen is shifted to favor net increase of collagen and enhanced tissue stiffness *(*Schnider *et al.*, 1981*)*. Mechanisms that confer resistance to enzymatic degradation are thought to be due to the synergistic effect of intermolecular cross-linking and modification of side chains *(*Avery *et al.*, 2006*)*. This occurs first by the blockage of enzymatic sites such as lysine and arginine, and second by the closer packing of molecules due to enhanced cross-linking.

ix) Diabetes-induced alterations of the myocardial matrix: impact on cardiac cell function

Cardiovascular complications are a common cause of morbidity and mortality in diabetics. Diabetes mellitus induces pathological changes in the structure of the heart, including cell membranes, organelles of cardiomyocytes, and increased interstitial fibrosis. The interstitial fibrosis is due in part to an increase of the relative quantity of collagen, indicating an abnormality in the remodeling of the extracellular matrix. When measured biochemically, fibrosis is likely to manifest as an increase of hydroxyproline concentration and increased collagen cross-linking, resulting in increased myocardial stiffness. Collagen is a stable protein whose turnover by cardiac fibroblasts is slow in health (estimated to be 80 to 120 days) *(*Shirwany *et al.*, 2006*)*. The balance between synthesis and degradation is easily disrupted, particularly when synthesis outweighs degradation. Morphologically, accumulation of collagen in the heart takes two forms: 1) reactive fibrosis, resulting in adverse accumulation of perimysial collagen; 2) reparative fibrosis, restricted to sites where scar tissue replaces cardiomyocytes lost due to necrotic cell death. Reparative fibrosis helps to preserve the structural integrity of the myocardium after myocardial infarction or injury. Both forms of fibrosis restrict the ability of the heart to pump blood and to provide adequate systemic perfusion.

AGE can also act as ligands for various AGE receptors which are present on macrophages, epithelial cells, mesangial cells and endothelial cells. These interactions result in enhanced inflammatory responses, angiogenesis, synthesis of pro-fibrotic factors such as TGF-β, and continued increase of extracellular matrix production *(*Raj *et al.*, 2000*)*. This amplifying, feed-forward loop further contributes to the fibrosis of the cardiac interstitium.

The detrimental effects of AGE formation and inter-protein cross-linking can be inhibited by aminoguianidine *(*Brownlee *et al.*, 1986*)*, a molecule that is an analogue of the arginine residue in collagen side chains. By reacting with Amadori products it prevents the generation of

more advanced products, thereby reducing the formation of cross links. Aminoguanidine can inhibit the formation of AGE in animal models and has demonstrated clinical efficacy in clinical trials *(*Vlassara *et al.*, 2002*) (*Ulrich *et al.*, 2001*)*.

Statement of the Problem:

Diabetes mellitus is a high prevalence metabolic disease that is associated with extensive morbidity. Metabolic alterations associated with diabetes lead to altered gene expression and signal transduction that have globally deleterious effects on organ function (Ruderman *et al.*, 1992). Notably, the prolonged hyperglycemia of poorly controlled diabetes has a major impact on the structure and function of the extracellular matrices of the myocardium. Diabetes-induced modifications of matrix proteins alter the biomechanical integrity of the myocardium and strongly increase risk of myocardial infarction and cardiomyopathy (Marwick *et al.*, 2006). Indeed, the formation of advanced glycation end products in high abundance interstitial proteins such as collagen, leads to excessive collagen cross-linking, a key pathological change that plays an important role in the increased myocardial stiffness and loss of compliance that is observed in diabetic hearts (Aronson *et al.*, 2003).

The healthy myocardium contains cardiac myocytes but there are much higher numbers of cardiac fibroblasts (~35% of cardiac cells are fibroblasts, 20% are myocytes; the remaining cells are endothelial cells and various types of immune and neural cells (Eghbali *et al*, 1992)). Fibroblasts are essential for the synthesis and remodeling of the extracellular matrix of the myocardium and mediate the fibrotic response to chronic volume or pressure overload. These conditions lead to loss of organization of myocardial connective tissues, excessive fibrous tissue formation, increases in the numbers of cardiac fibroblasts, and the switch of cardiac fibroblasts to a myofibroblastic phenotype in which cells express α-smooth muscle actin (SMA) (Wang *et al.*, 2003). Myofibroblasts are thought to play a critical role in the conversion of the healthy myocardium to the stiff and non-compliant myocardium that is seen in pressure overload and cardiomyopathy. Notably, in failing hearts, myofibroblasts produce a densely fibrotic

extracellular matrix that reduces cardiac contractility and inhibits cardiac filling in diastole (Weber *et al*, 1997).

Despite the greatly increased prevalence of heart failure in diabetics (Kannel *et al.*, 1974) and the well-defined role of the myofibroblast in the formation of fibrous tissue in the heart (Campbell *et al.*, 1997), the impact of diabetes-induced glycation of extracellular matrix proteins in myofibroblast formation has not been defined. Further, as glycation of extracellular matrix proteins such as collagen greatly increases the stiffness of the extracellular matrix in the myocardium (Ulrich *et al.*, 2001), it is important to understand how diabetic metabolites and mechanical alterations to the matrix regulate the formation and metabolism of fibroblasts and their conversion to myofibroblasts.

Hypothesis:

Differentiation of myofibroblasts is regulated by glycation-induced cross-linking of collagen.

Overall Goal:

Use an in vitro model to define how diabetes-induced glycation of collagen affects cardiac fibroblast function.

Specific Experimental Objectives:

1) Validate MGO-induced glycation model of collagen
2) Determine mechanical properties of glycated/cross-linked collagen gels.
3) Characterize the phenotype of human cardiac myofibroblasts (i.e., α-smooth muscle actin, type I collagen, vimentin, desmin).
4) Use collagen gel contraction models to evaluate the effect of MGO-induced glycation of collagen on fibroblast function.
5) Assess if α-SMA expression is affected by changes in the chemical and mechanical characteristics of collagen gels.
6) Measure cell migration on and adhesion to glycated collagen.

7) Evaluate focal adhesions formed in response to MGO-treated collagen.

8) Examine effect of collagen glycation on activated cell surface β1 integrins.

9) Measure apoptosis in cells on MGO-treated collagen.

10) Examine effect of collagen glycation on SMA transcription.

11) Examine effect of collagen glycation on other adhesion receptors such as cadherins.

12) Examine effect of collagen glycation on ED- A fibronectin and collagen-1 expression.

13) Examine whether collagen glycation affects the Rho kinase or the TGF-β pathway.

Chapter 2: Collagen Glycation Promotes Myofibroblast Differentiation

Introduction

Heart failure is a high prevalence disorder (Fedak *et al.,* 2005) of substantial morbidity (Heineke *et al.*, 2006) that is common in diabetics (Kannel *et al*.1974). Cardiovascular complications are strongly associated with diabetes-related mortality (Jay *et al.,* 2006) but the contribution of hyperglycemia-induced changes of the cardiac extracellular matrix to diabetic cardiomyopathy has not been defined. Hyperglycemic episodes cause increased glycation of extracellular matrix proteins, including collagens. Non-enzymatic processes such as the Maillard reaction (Aronson *et al.,* 2003) mediate the formation of advanced glycation end products and increased collagen cross-linking (Avery *et al.,*2006), which alters cellular interactions with collagen and its biomechanical functions (Paul *et al.,* 2006).

The cardiac interstitium is populated by fibroblasts, which comprise two-thirds of the cell population of the ventricular wall (Camelliti *et al,* 2006). Homeostasis of the cardiac interstitium requires precise regulation of matrix remodeling and tight control of the migration, proliferation and differentiation of cardiac fibroblasts (Fedak *et al.,* 2005). The differentiation of fibroblasts into myofibroblasts is strongly upregulated in failing hearts (Weber *et al.,* 2000) and is characterized by *de novo* expression of α-smooth muscle actin (Gabbiani *et al.,* 1971) and by increased formation of a disorganized collagen matrix (Sappino *et al.,* 1990).

Currently, very little is known about how post-translational modifications to collagen influence myofibroblast differentiation. We treated collagen with methylglyoxal, which is a widely used (Chong et al., 2007; Dobler et al., 2006; Goh and Cooper, 2008; Paul and Bailey, 1999; Pedchenko et al., 2005; Shamsi et al., 1998) to model the effect of diabetes-induced glycation of the extracellular matrix on the cell function. Our main findings are that collagen

glycation inhibits integrin activation and focal adhesion formation, while enhancing the expression of α-smooth muscle actin, ED-A fibronectin and P-cadherin, and cell migration over collagen. This phenomenon suggests a novel mechanism for cardiac fibrosis in which glycated collagen enables myofibroblast differentiation.

Material and Methods

Reagents

Mouse monoclonal antibodies to type I bovine collagen (clone 1319), α-SMA (clone 1A4), desmin (clone DE-U-10), vinculin and vimentin (clone VIM 13.2) were purchased from Sigma-Aldrich (Oakville, ON). Antibody to human pro-collagen type I was obtained from J. Sodek, University of Toronto. Antibody to methylglyoxal-AGE (Arg-pyrimidine) was from Biologo (Germany). Mouse monoclonal antibody to paxillin (clone 5H11) was obtained from Upstate (Lake Placid, NY). Mouse monoclonal blocking antibody to β1 integrin (clone 4B4) was purchased from Beckman-Coulter (Burlington, ON). Antibodies to activated β1 integrin (clone 12G10) and to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Chemicon-Millipore (Bilerica, MA). Antibodies to pan-cadherin (CH-19) from Abcam, Cambridge, MA and β-catenin were from Transduction-BD Biosciences (Mississauga, ON) and from Invitrogen (Mississauga, ON) and to ED-A fibronectin were from Serotec (Oxford, UK). Neutralizing antibody to TGF-β1 was from R&D (Minneapolis, MN). Transwell 8.0 μm pore-size polycarbonate membranes were obtained from Corning Inc. (Corning, NY). Purified type I calf skin collagen was from Inamed (Fremont, CA).

Collagen glycation

Methylglyoxal (MGO) was used to glycate collagen instead of glucose or ribose as this procedure reduces the time required for collagen glycation and facilitates more consistent cross-linking of collagen gels (Paul *et al,* 1999*)*. Collagen gels were incubated overnight with 1 mM, 0.1 mM or 0.01 mM MGO at 37ºC and rinsed with PBS as described (Chong *et al.,* 2007).

Mechanical characteristics of collagen gels

The effect of MGO-induced glycation on the rigidity of collagen gels was examined using *in situ* scanning probe microscopy. Force spectroscopy data were acquired on a Digital Instruments Nanoscope IIIa Bioscope scanning probe microscope using the Nanoscope software to control the Z-directed piezo motion. The force curves were acquired using the Bioscope fluid cell using 115 μm long oxide-sharpened silicon nitride V-shaped cantilevers (model DNP-S, Veeco, Plainview, NY; 35° half angle) with a measured force constant (k), as determined from the thermal resonance peak of the cantilever of ~0.06 N/m ($k \sim (k_B T)/\langle A^2 \rangle$) on samples placed in plastic well plates and immersed in buffer solution. The vertical deflection of the AFM tip was monitored separately at a sampling frequency of 60 KHz using a National Instruments DAQ card (model PCI-6251) and in-house written LabView software (version 7.1, National Instruments). All force curves were acquired at a tip approach rate of ~4 μm/s and were collected continuously. During the force curve measurement, the maximum force applied to the sample surface by the tip was ~ 0.625 nN, as defined by the force curve deflection trigger set-point. Ten force curves were collected at one or two locations on each gel and averaged. AFM force-displacement curves were fit to a Hertz contact model by nonlinear regression (NLREG Version 6.3 Advanced). Elastic moduli of the gels were estimated based on the curve fits for the portion of the curve up to 200 nm of indentation, assuming a Poisson's ratio of 0.5.

Cell Culture

Human cardiac fibroblasts (HCF, ScienCell, Carlsbad, CA) were plated in DMEM/F-12 medium containing fetal bovine serum, fibroblast growth supplement (ScienCell) and penicillin/streptomycin (100 U/ml and 100ug/ml). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 and passaged with trypsin and EDTA. For experiments on the effect of MGO-collagen on cell behavior, cells were plated at 3.0×10^4 cells per 28 mm^2 of collagen substrate (low density) or, for most experiments, at 6.0×10^4 cells per 28 mm^2 of collagen

substrate (high density). DDR1 null fibroblasts (generously provided by W. Vogel, University of Toronto) were cultured in α-minimal essential medium containing 10% (v/v) fetal bovine serum and penicillin/streptomycin (100 U/ml and 100ug/ml). β1 integrin null mouse fibroblasts (GD25 cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin–streptomycin and puromycin (10 ug/ml).

Fibroblast phenotype

Human cardiac fibroblasts were immunostained for vimentin, SMA, type I collagen and desmin (Sappino *et al.,* 1990*)*. Passage-3 cultures were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with primary antibodies and counter-stained with FITC-conjugated goat anti-mouse antibody. Cell nuclei were stained with DAPI or propidium iodide and imaged by fluorescence or confocal microscopy (SP, Leica, Germany).

Collagen gels

The effect of glycation on collagen gel contraction was examined in floating and stress-relaxed collagen gels. Sterile collagen gels (1.875 mg/mL) were polymerized at 37ºC and treated with MGO or PBS overnight. Gels were rinsed before plating with cells on the upper gel surface. To optimize plating densities for collagen gel contraction experiments, assays were performed at cell densities ranging between 10^4-10^7 cells/mL. Contraction of floating collagen gels was measured daily for 4 days by microscopy using an intraocular grid to estimate gel diameters. MGO and control collagen gels were initially of the same diameter, prior to contraction assays. For stress-relaxed collagen gels, collagen matrices were polymerized for 60 min at 37 °C and cells were plated on top of the collagen gels at 10^7 cells/mL of collagen solution. To initiate contraction, mechanically stressed matrices were gently released from the culture dish with a spatula.

Analysis of cross-linked collagen

SDS-PAGE sample buffer was added to control or MGO-treated collagen. Samples were heated to 50°C for 30 min and analyzed by SDS-PAGE (7.5% acrylamide) followed by silver staining.

SMA expression and stress fiber organization

As fibroblasts respond to endogenously generated forces by reorganization of actin filaments (Petroll *et al,* 1993) and increased expression of SMA (Gabbiani *et al,* 1999), we determined whether collagen glycation affects SMA expression and actin filament organization. Cells were plated on control or glycated collagen gels. After fixation, cells were immunostained for SMA or stained for actin filaments with rhodamine phalloidin.

Cell morphology

Scanning electron microscopy was used to visualize cell morphology. Gels were fixed in formaldehyde on poly-L-lysine-coated glass slides and dehydrated through an ethanol series. The samples were critical point dried in a Polaron CPD7501. Samples were mounted on aluminum stubs and plasma sprayed with a 5 nm thick coat of platinum in a Polaron SC 515 SEM coating system and examined with a Hitachi C-2500 scanning electron microscope.

Migration assays

Migration assays were performed with Transwell chambers (8.0 μm diameter pore size). Membranes were coated with collagen and incubated with either PBS or MGO (1 mM) overnight. To avoid clogging of the pores with collagen, vacuum was applied to the chambers and the patency of the pores was verified by light microscopy after Coomassie blue staining. Cells (1 X 10^4) were seeded on top of the membrane insert in serum-free DMEM and with DMEM containing 10% FBS in the bottom chamber as a chemoattractant. After 24 hours, cells were washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with DAPI and migrating cells were counted on the lower surface at 250X.

Adhesion Assay

Adhesion strength under shear force was measured with parallel arrays of micro-channels mounted on glass slides (Young *et al,* 2007). The glass was coated with collagen and treated with PBS or MGO at different concentrations directly in the micro-channels. To measure relative

adhesion strengths, cells were subjected to 110 dyn/cm^2 flow-induced shear stress for four minutes. The number of attached cells per unit area after shearing was quantified by ImageJ.

SMA expression

Cell lysates were prepared by adding first buffer A (2% Triton X-100, 160 mM KCL, 40 mM TrisHCl, 20 mM EGTA, 10 mM PMSF, 1 mM leupeptin, 1 mM benzamidine) and then an equal volume of Laemmli sample buffer. The protein content of samples was estimated with Bio-Rad assays. Equal amounts of protein were separated on 10% SDS gels, transferred to nitrocellulose filters blocked with milk, incubated with SMA antibody, horseradish peroxidase-conjugated second antibody, and developed with ECL reagents (Amersham, Oakville, ON). The equivalence of protein loading of individual samples was examined by immunoblotting for GAPDH.

Apoptosis

FITC-annexin binding (Roche, Montreal, QB) was used to identify apoptotic cells in cultures of fibroblasts growing on MGO- or untreated collagen.

Analysis of SMA Promoter Activity

Cells were transiently transfected with a luciferase reporter plasmid for SMA obtained from R. Nemenoff (Denver, CO). Cells were co-transfected with a β-galactosidase Rous sarcoma virus (RSV) expression plasmid to normalize for equal loading using LipofectAMINE 2000 (Invitrogen). Following transfection (20 h), cells were plated on PBS or MGO-treated collagen and grown for 24 hours. Cell extracts were prepared with a detergent lysis method and luciferase assays and β–galactosidase reporter enzyme activity were measured.

Quantitative Real-Time PCR.

Human cardiac fibroblasts were plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) overnight, or for 2 or 5 days. RNA was isolated using the RNeasy Mini Kit (Qiagen,Missisauga, ON, Canada). cDNA was generated using Reverse Transcriptase (Fermentas, Burlington, ON,

Canada). Real-Time PCR was performed using iQ™SYBR® Green Supermix (BioRad, Hercules, CA) with validated human Collagen-I primers:

F'- TACCATGACCGAGACGTGTGGAAA; R'- AGATCACGTCATCGCACAACACCT;

or with α-Smooth Muscle Actin primers:

F'- TGACAATGGCTCTGGGCTCTGTAA; R'- TTCGTCACCCACGTAGCTGTCTTT;

or with P cadherin primers:

F'- ATGACGTGGCACCAACCAT; R'- GTTAGCCGCCTTCAGGTTCTC

or with TGF-β1 primers:

F'- CCCAGCATCTGCAAAGCTC; R'- GTCAATGTACAGCTGCCGCA

or with TGF-β2 primers:

F'- TAACATCTCCAACCCAGCGCTACA; R'- GCATCAGTTACATCGAAGGAGAGCCA

or with TGF-β3 primers

F'- ACTGGCTGTCTGCCCTAAAGGAAT; R'- AAGACCCGGAATTCTGCTCGGAATA.

Relative quantification was calculated using the ΔΔCt method normalized to human GAPDH using the primers (F'- AATCCCATCACCATCTTCCA; R'- TGGACTCCACGACGTACTCA).

β1 integrin activation

As the α2β1 integrin is the principal collagen receptor that mediates collagen gel contraction (Schiro *et al,* 1991), we measured the effect of collagen glycation on β1 integrin activation by immunostaining with 12G10, which recognizes a neo-epitope in activated β1 integrins (Mould *et al.,* 1995*),*

Statistical analysis

For all data sets, experiments were repeated at least three times and each repeat contained three replicates. For continuous variables, means and standard errors of the mean were computed. Comparisons between two groups were made with the unpaired Student's *t*-test. For multiple

comparisons ANOVA with *post-hoc* analysis Bonferroni analysis was performed. Statistical significance was set at $p<0.05$.

Results

Collagen glycation and cardiac fibroblast phenotype

We modeled diabetes-induced alteration of the extracellular matrix with the diabetes metabolite methylglyoxal (MGO), which mediates collagen glycation (Chong *et al.,* 2005). MGO induced the formation of both higher and lower molecular mass collagen molecules (Fig. 5A), reflecting the presence of MGO-collagen adducts with altered charge profiles and consequent variations of electrophoretic mobility. Immunoblotting of samples with an antibody that recognizes MGO-advanced glycation end products (Arg-pyrimidine) showed that MGO strongly increased glycation (Fig. 5B), which was blocked by aminoguanidine. As myofibroblast differentiation is affected in part by the stiffness of the substrate (Arora *et al.* 1999), we characterized the mechanical properties of collagen gels by atomic force microscopy. We found no differences in the elastic moduli of collagen gels treated with vehicle or MGO at different concentrations (Fig. 5C).

Phenotyping of human cardiac fibroblasts by immunostaining showed that in passage 3 cells that had been maintained on tissue culture plastic for 2 days, 90% and 70% exhibited staining for vimentin and pro-collagen respectively, but there was no staining for desmin (Figs. 5D-G); ~20% of the cells stained for SMA. Scanning electron micrographs of human cardiac fibroblasts plated at identical densities on PBS-treated collagen gels or 1 mM MGO-treated collagen gels showed that cells plated on MGO-treated collagen were clustered and not evenly distributed over the gels (Figs. 5H,I).

Collagen Gel Contraction

We determined the optimal cell densities for the stress-relaxed and floating gel contraction assays (Grinnell *et al.,* 2003) to analyze the effect of MGO treatment on collagen contraction. For stress-relaxed assays in which measurements were made over 2-3 hours, a density of 10^7 cells/mL was optimal while in contrast, for floating collagen gels, we used densities of 10^6 cells/mL since

at this concentration the changes of gel diameter were most readily measured over a 4 day sampling period. Cells were incubated on gels so that equivalent numbers of cells were attached for collagen and MGO-collagen conditions, although the distribution of the cells on the gels was not homogeneous on glycated collagen (Fig. 5H,I). For the stress-relaxed contraction assays, measurements over 2.5 hours showed that 1 mM MGO-treated collagen gels contracted significantly more than untreated controls (Fig. 6A: $p<0.05$, slope of best fit linear curve for 1 mM MGO-treated collagen (slope = 4.0) was 1.2 fold higher than PBS-collagen (3.3)). There were similar differences attributable to MGO in the floating collagen gel assays (Fig. 6B: $p<0.025$; slope of best fit linear curve for 1 mM MGO-treated collagen (4.5) was 1.4 times higher than PBS-collagen (3.1)).

SMA expression

As there was enhanced contraction of MGO-treated collagen gels compared to controls, we examined whether there was an increase of the percentage of cells expressing α-smooth muscle actin (SMA) since SMA expression by fibroblasts is associated with enhanced collagen gel contraction (Arora *et al,* 1994). In cells incubated on control collagen or MGO-collagen substrates for 2 days, there were equivalent numbers of SMA-stained cells in the different conditions but there were relatively fewer cells that did not express SMA on the MGO-treated collagen. Consequently, there were higher relative proportions of SMA-stained cells as a result of MGO treatment (Fig. 7A). Consistent with these data, immunoblotting for SMA and GAPDH (as loading control) in cells plated on collagen or MGO collagen (0.1 mM) and cultured for 2 days showed that there was markedly more SMA in isolated colonies of cells that had been plated on MGO-collagen. In these colonies SMA was incorporated into well-developed bundles of actin filaments that appeared to be connected to adjacent, possibly contacting cells (Fig. 7B).

Some of the increased SMA expression was likely due to enhanced SMA transcription since cells plated on MGO-collagen for 2 days showed 1.6-fold higher ($p<0.02$) SMA luciferase

activity than cells plated on control collagen (data were normalized to a loading control plasmid, RSV) (Fig. 7C). Notably, quantification of immunoblots for SMA and GAPDH followed by densitometry indicated that plating cells on MGO-collagen that were co-incubated with 20 mM aminoguanidine (a treatment that prevents the formation of glycated proteins (Lo *et al*, 1994) including collagen (Chong *et al,* 2005)) inhibited the MGO-collagen-induced enhancement of SMA expression (Fig. 7D). The effect of aminoguanidine on MGO-collagen treatment indicated that the enhanced expression of SMA by MGO was likely due to collagen glycation.

As a result of the relatively smaller percentages of non-SMA expressing cells that adhered to MGO-collagen, we considered that the increased percentage of cells expressing SMA may be attributable to differential susceptibility to apoptosis in the SMA-negative and SMA-expressing cell populations. Accordingly, we quantified apoptosis by FITC-annexin binding, a marker of early stages of apoptosis in 1 day cultures. One day cultures were used instead of 2 day cultures because dying cells detached from the substrate could not be accurately analyzed for apoptosis and SMA staining. For the whole cell population there were increased percentages of annexin-positive cells when plated on MGO-collagen (Fig. 7E; all MGO-collagen conditions were $p<0.01$ compared to PBS controls). In cells plated for 1 day on 0.1 M MGO-collagen, stained with FITC-annexin and immunostained for SMA, there was a significantly lower percentage of annexin-positive cells expressing SMA compared to SMA null cells ($3.1\pm2.2\%$ vs $28.2\pm14.1\%$; $p<0.05$). In contrast, for cells plated on control collagen there were equivalent % of annexin-positive SMA-expressing cells and SMA negative cells (annexin-positive cells: SMA-expressing cells=8.23 ± 4.27; SMA-null cells=$5.4\pm5.4\%$; $p>0.2$). These data indicate that culture of human cardiac fibroblasts on MGO-treated collagen favors survival of SMA-expressing cells.

Quantitative real-time PCR showed that collagen glycation increased mRNA expression of α-SMA (Fig. 8B) and that over time, there was an increase of the myofibroblast marker collagen-1 (Fig. 8AB). We found no detectable N-cadherin by real-time PCR but there was

increased expression of the mRNA for P-cadherin, which was detected after 5 days culture (Fig. 8C). Analysis of TGF-β isoform expression showed no detectable expression for TGF-β1 (data not shown), a marked decrease of TGF-β3 and a 5-fold increase of TGF-β2 after 2 days of cell plating on MGO-collagen compared to collagen.

Migration

The observed differences of collagen gel contraction attributable to MGO described above could be due in part to differences in the rates of cell migration. There was enhanced migration of cells plated on 1 mM MGO-treated collagen compared to vehicle controls and of cells plated on lower concentrations of MGO-collagen compared to control collagen (Fig. 9A; $p<0.025$).

Migration of cells involves a complex series of cellular events and one of the rate-determining factors is adhesion strength to the substrate (Loftis *et al,* 2003). We compared adhesion strength of cells plated on control or MGO-collagen using a microfluidics flow chamber that generates prescribed shear force (Fig. 9B). Cells plated on control collagen were more strongly adherent than cells plated on all the MGO-treated collagen conditions ($p<0.05$), indicating that MGO treatment of collagen inhibits resistance to shear and likely, high affinity cell adhesions to collagen.

Collagen receptors

Since attachment of fibroblasts to collagen is mediated by β1 integrins, we first quantified the effect of MGO treatment of collagen on the formation of high affinity β1 integrins. Cells were immunostained with 12G10, an antibody which recognizes a neo-epitope in ligand-bound, activated β1 integrins. Cells that were plated and allowed to spread on MGO-collagen showed no 12G10 staining at their leading edges compared to cells plated on vehicle-treated collagen (Fig. 10). Notably, staining for cortical actin (i.e. not associated with focal adhesions) was unchanged by plating cells on MGO-collagen.

The α2β1 integrin is important for collagen gel contraction by fibroblasts (Schiro *et al,* 1991). As MGO treatment of collagen appeared to affect the contractile capacity of human cardiac fibroblasts, we determined the functional importance of the β1 integrin in mediating the effects of MGO-collagen on gel contraction. Cells were pre-incubated with irrelevant antibody or a β1 integrin-inhibiting antibody (4B4), which effectively blocks all β1 integrin-forming heterodimers. Cells were then plated on MGO-treated or control floating collagen gels as described above. For antibody controls, there was faster contraction for cells cultured on MGO-treated collagen gels compared to control collagen ($p<0.05$; Table 3). Treatment of cells with an antibody that binds to and inhibits β1 integrin interaction with collagen (4B4) greatly reduced contractility of cells plated on control or MGO collagen and the differences between the three conditions were not statistically significant ($p>0.2$). We also examined whether the differences of contraction rates of cells plated on MGO-treated collagen or control collagen could be dissipated by an activating antibody to the β1 integrin subunit. Cells were pre-incubated with a β1 integrin activating antibody (12G10) and plated on control collagen or MGO-treated collagen gels. There was faster contraction for cells cultured on 1mM MGO-treated collagen gels than PBS collagen controls ($p<0.05$; Table 3) independent of the activating antibody treatment. These results were not due to reduced surface expression of β1 integrins since flow cytometry analysis of non-permeabilized cells stained with the 4B4 antibody to detect cell surface integrin abundance showed no difference (cells on collagen, 14.0±1.0 fluorescence units; cells on 0.1 mM MGO-collagen, 12.7±1.0 fluorescence units; $p>0.2$). Collectively these findings indicate that the effect of MGO on collagen gel contraction is due in part to β1 integrin interactions with collagen but does not require β1 integrin activation.

We examined the effect of β1 integrin function on SMA content in cells plated on control or MGO-treated collagen. Human cardiac fibroblasts exhibited increased SMA content when

plated on MGO-collagen compared to control collagen (Fig. 11A). Similarly, after incubation with β1 integrin-activating antibodies, there was increased SMA expression with MGO treatment of collagen (Fig. 11B) but there was no detectable difference in SMA content between the untreated versus β1 integrin-activating antibody, which corresponded to the collagen gel contraction data described above. When β1 integrin-inhibiting antibodies were used, SMA expression was not detected, whether or not collagen was treated with MGO (Fig. 11C). We also examined SMA expression in cells which do not express β1 integrins (GD25 cells (Fassler *et al,* 1995)). There was minimal SMA expression (Fig. 11D), confirming the role of the β1 integrin in MGO-collagen-induced expression of SMA. As an additional control, we examined cells that did not express DDR1, an important but separate receptor for fibrillar collagens that acts independently of β1 integrins (Vogel *et al,* 1999). These cells exhibited similar patterns of α-SMA expression after MGO treatment of collagen as controls (Fig. 11E), indicating that the β1 integrin and not DDR1 was important in mediating the MGO-collagen effect on SMA expression.

Focal adhesions

Immunostaining for vinculin and paxillin showed characteristic focal streaks in cells plated on control collagen but these structures were barely visible in cells plated on 0.1 mM or 1 mM MGO-treated collagen (Fig. 12A,B). Quantification of these structures by morphometric analysis indicated that MGO treatment of collagen substantially reduced the abundance of paxillin and vinculin-stained focal adhesions in cells (Fig. 12C). ($p<0.01$).

Intercellular adhesions

The lack of effect of β1 integrin activation on SMA expression, the inhibition of focal adhesion formation by MGO collagen and the requirement for β1 integrin interactions with collagen indicated that other adhesion receptors may cooperate with β1 integrins to mediate SMA expression in human cardiac fibroblasts. Accordingly, we examined the effect of 1.0 mM MGO

collagen using antibodies against OB-cadherin, pan-cadherin and β-catenin, which are expressed by fibroblasts undergoing differentiation to myofibroblasts (Hinz *et al,* 2004*)*,(Pittet *et al,* 2008). MGO-collagen strongly increased the density of immunoblots prepared with pan-cadherin and β–catenin antibodies (Fig. 13A) after 2 days of plating but we were unable to detect reactivity with OB-cadherin antibody in these cells. This was not because of technical limitations since lysates of osteoblastic cells (SAOS2) showed strong immunoreactivity with the antibody to OB-cadherin. Confocal immunofluorescence analysis of cells also showed that staining with the pan-cadherin antibody was detected in cells plated on MGO-collagen but not on control collagen (Fig. 13B). Staining with the pan-cadherin antibody disappeared when cells plated on 1.0 mM MGO-collagen were co-incubated with aminoguanidine (20 mM) to prevent the formation of glycated proteins. Further, if cells were plated at lower densities (i.e. $3.0x10^4$ cells per 28 mm^2 of collagen substrate instead of the usual $6.0x10^4$ cells per 28 mm^2 of collagen substrate) staining with the pan-cadherin antibody was undetectable (Fig. 13B, right panel). By immunoblotting there were 1.85-fold higher ratios of SMA/GAPDH for cells on $6.0x10^4$ cells per 28 mm^2 of collagen substrate compared to cells plated at $3.0x10^4$ cells per 28 mm^2 of collagen substrate.

Plating cells on MGO-collagen also induced the expression of another protein that is associated with myofibroblast differentiation, ED-A fibronectin (Fig. 13C). The expression of ED-A fibronectin, cadherin and SMA was not inhibited if cells plated on MGO-collagen were co-incubated with 10 μM Y27632, an inhibitor of the Rho kinase.

As our quantitative RT-PCR data showed no detectable TGF-β1, a reduction of TGF-β3 and a 5-fold increase of TGF-β2 after 2 days of culture (Fig. 8D), we determined if absorption of all TGF-β isoforms with an inhibitory antibody may affect MGO-collagen-induced SMA expression. Cells were plated on MGO-collagen or control collagen and co-incubated with TGF-

β neutralizing antibody (50 μg/ml, changed daily for 3 days (Walker *et al,* 2004)). The MGO-collagen-induced increase of SMA was blocked compared to controls (Fig. 13D; $p>0.2$).

Discussion

Despite the greatly increased prevalence of heart failure in diabetics (Kannel *et al,* 1974) and the well-defined role of the myofibroblast in the formation of fibrous tissue in the heart, (Campbell *et al,* 1997*)* the impact of diabetes-induced glycation of extracellular matrix proteins in myofibroblast formation has not been defined. Our central findings indicated that treatment of collagen with the diabetic metabolite MGO enhances the differentiation, survival and migration of myofibroblasts, which in turn facilitates collagen matrix contraction. These results arise in part from the effect of MGO-induced glycation of collagen on β1 integrin adhesions and point to a central role for diabetic metabolites in regulating the migratory behavior and persistence of myofibroblasts in the cardiac interstitium of diabetics.

Integrins and collagen glycation

Integrin-dependent interactions with matrix proteins mediate cell polarization, extension of lamellipodia and filopodia, formation and stabilization of attachments, and generation of tractional forces (Sheetz *et al,* 1994) and cell migration (Lauffenburger *et al,* 1996). In migrating cells integrins are concentrated in contacts at the leading edge (Burridge *et al,* 1988). For migration and remodeling by fibroblasts in type I collagen, the principal integrin is the α2β1 (Klein *et al,* 1991). We found by immunostaining that MGO-collagen inhibits formation of high affinity β1 integrin adhesions, as detected with an activation-dependent, neo-epitope antibody. Consistent with these data, shear adhesion assays showed decreased adhesion to MGO-collagen. MGO interacts with lysine, cysteine and arginine residues to form glycation adducts that perturb integrin-collagen interactions (Paul *et al,* 1999). In particular, MGO preferentially reacts with arginine to form imidazolones, advanced glycation end products that exhibit loss of associated side-chain positive charge (Ahmed *et al.,* 2002). Notably, the α2β1 integrin recognizes and binds the GFOGER sequence in fibrillar collagens (Emsley *et al,* 2000*).*

MGO adduct formation with the arginine in the GFOGER sequence of collagen appears to be particularly important in modifying and inhibiting β1 integrin-dependent cell attachment and spreading on collagen (Chong *et al,* 2005), processes that may be critical for inhibition of focal adhesion formation observed here. We used an antibody to Arg-pyrimidine and immunoblotting to confirm the efficacy of MGO adduct formation and aminoguanidine to block adduct formation by MGO.

Cell Migration

Initial formation of cell adhesions at the leading edge of migrating cells is marked by the involvement of actin binding proteins such as vinculin, a regulator of cell migration and adhesion (DePasquale *et al,* 1987), (Zimerman *et al,* 2004). After vinculin recruitment to cell adhesions the cell-to-matrix distance is reduced, which leads to the formation of higher affinity (DePasquale *et al,* 1987) and stable adhesions (Geiger *et al,* 2001). The anchorage provided by these adhesions facilitates transmission of cell-generated forces to enable migration (Lauffenburger *et al,* 1996) but if the cells are highly adherent, migration is impeded. We found that the relative abundance of vinculin and paxillin in cell adhesions was markedly reduced in cells plated on MGO-collagen. Apparently, the observed decrease in β1 integrin activation impedes focal adhesion maturation. These results are consistent with findings that show vinculin null cells form few focal adhesions and do not spread well, but migrate rapidly. (Rodriguez Fernandez *et al,* 1992). Accordingly, our data on MGO-collagen enhancement of cell migration in Boyden chamber assays may be explained by the inability of cells to form mature focal adhesions on glycated collagen.

Collagen remodeling

After prolonged hyperglycemia the extracellular matrix of the cardiac interstitium can be profoundly affected in diabetic patients, which may manifest as increased cross-linking of collagen and alteration of its functional properties. We found that for both stress-relaxed and floating gels (Grinnell *et al,* 2000) collagen glycation by MGO enhanced gel contraction. Two

proposed mechanisms may account for cell-mediated contraction and re-organization of collagen matrices. The first mechanism suggests that isometric tension applied to collagen fibrils by cells results in gel contraction (Dodd *et al,* 1982), which is in agreement with our observations that MGO-collagen enhanced SMA expression, and that SMA content of fibroblasts increases collagen gel contraction (Arora *et al,* 1994). The second mechanism suggests that tractional forces arising from the combined motility and contractility of cells exert shear forces tangential to their surface (Harris *et al,* 1981). Since we also observed that MGO treatment increased cell migration on collagen, at least part of the more rapid collagen gel contraction can be attributed to migration through the gels (Harris *et al,* 1981), (Roy *et al,* 1999) while for the stress-relaxed collagen gels, which depend on factors that impact cell contractility (Tarpila *et al,* 1998), SMA expression may be more important.

SMA expression

The formation of myofibroblasts is a critical process in heart failure since myofibroblasts produce a densely fibrotic extracellular matrix that reduces cardiac contractility and inhibits filling in diastole (Weber *et al,* 1997). Myofibroblasts are characterized by *de novo* expression of collagen, SMA and, under certain conditions, ED-A fibronectin, as well as the formation of actin stress fibers in vitro (Tomasek *et al,* 2002). We found that SMA, type I collagen and ED-A fibronectin expression in human cardiac fibroblasts was enhanced by MGO treatment of collagen, in spite of the reduced adhesion strength to the glycated extracellular matrix. The MGO-induced glycation of collagen was evidently a key factor in this conversion process since co-incubation with aminoguanidine (Lo *et al,* 1994) blocked MGO-collagen-induced SMA expression.

When cultured on MGO-collagen substrates, SMA was found in well-developed actin bundles that appear to terminate in adhesions proximal to adjacent cells, suggesting that some type of cell adhesion was involved in providing the anchorage point for the stress fibers. Previous reports have indicated that N- and OB-cadherins are important for myofibroblast differentiation

(Hinz *et al,* 2004), (Pittet *et al,* 2008) and our data show that the increased expression of cadherins (possibly P-cadherin as detected by quantitative RT-PCR, but not OB-cadherin) and β-catenin, important intercellular adhesion molecules, may be involved in enhanced myofibroblast differentiation in response to MGO-collagen. If cells were cultured at lower densities, which reduced the likelihood of intercellular adhesion contacts between adjacent cells, the MGO-collagen-induced expression of SMA dissipated. The induction of SMA expression by plating cells on MGO-collagen evidently does not involve the Rho kinase pathway since incubation of cells on MGO-collagen and treatment with Y27632 did not affect SMA expression, indicating that cell-generated tension (Tomasek *et al,* 2002) may not be an important factor in mediating this process.

The MGO-collagen induction of SMA expression involved selectively increased expression of TGF-β2 and required TGF-β since blockade of this cytokine with specific neutralizing antibodies inhibited MGO-collagen-induced enhancement of SMA expression. TGF-β-neutralizing antibodies have been used earlier in studies of the differentiation of heart valve myofibroblasts (Walker *et al,* 2004). Further, SMA expression in high density cultures was strongly linked to survival from MGO-collagen-induced apoptosis, consistent with the notion that intercellular adhesions, possibly mediated by cadherins and β-catenin, can enable the persistence of myofibroblasts when cultured on MGO-collagen. Indeed, the selective inhibition of apoptosis in SMA-expressing myofibroblasts by MGO-collagen may suggest a mechanism by which these cells persist in glycated extracellular matrices.

Stress fiber formation, cadherins, SMA, and ED-A fibronectin expression are considered to be important markers for myofibroblasts (Hinz *et al,* 2004), (Tomasek *et al,* 2002). In addition to the apparent role for intercellular adhesions and TGF-β in myofibroblast formation, our data on the role of β1 integrins in this process also show that attachment to collagen is

important for enhanced SMA expression. Otherwise, if β1 integrin-collagen interactions were inhibited by the 4B4 antibody, then SMA expression was blocked. Hinz and co-workers (Hinz *et al,* 2004) have suggested a model for myofibroblast formation in which some cells in the fibroblast population attach to the extracellular matrix while other cells attach strongly to each other by intercellular adhesions. They consider that the formation of fibrogenic fibroblasts requires the formation of intercellular adhesions to tether and provide critical signals for myofibroblast formation (Follonier *et al,* 2008). These fibrogenic cells, which express SMA and ED-A fibronectin, evidently do not need to be tightly adherent to the extracellular matrix, which might explain our results indicating that inhibition of the Rho kinase does not interfere with myofibroblast formation.

Our findings indicate that myofibroblast formation in response to collagen glycation is not a β1 integrin-dependent, cell-induced force effect and contrasts with the classical model of myofibroblast differentiation model *(*Tomasek *et al*., 2002). Instead, the impact of collagen glycation appears to rely on a novel pathway, which induces the expression of TGF-β2 and activates myofibroblast differentiation, as shown by the expression of collagen-1, SMA, ED-A fibronectin and cadherin. Identification of the receptors that mediate this signaling pathway is needed to understand the factors that induced the formation, persistence and spread of myofibroblasts through the diabetic myocardium.

Figure Legends

Fig. 5. (A) SDS-PAGE analysis of control and MGO-treated collagen. Note higher and lower molecular mass aggregates that are detected as a result of glycation-induced adduct formation and alteration of charge profiles of collagen molecules due to MGO treatment. (B) Immunoblot analysis of collagen, 1.0 mM MGO-treated collagen, 20 mM aminoguanidine and 1.0 mM MGO-treated collagen, or 20 mM aminoguanidine-treated collagen. Samples were separated by electrophoresis, transferred to a nitrocellulose membrane and immunoblotted for Arg-pyrimidine residues. (C) Elastic moduli of MGO-collagen and collagen gels, measured by atomic force microscopy and presented as mean ± standard error (n = 3 gels per condition), were not significantly different ($p>0.8$). (D-G) Fluorescence micrographs of human cardiac fibroblasts (passage 3) cultured on glass coverslips overnight and immunostained for vimentin (D), SMA (E), desmin (F) and pro-collagen (G). (H,I) Scanning electron micrographs of human cardiac fibroblasts plated on PBS-treated collagen gels and 1 mM MGO-treated collagen gels. Cells plated on MGO-treated collagen did not attach as well as controls and exhibited lower plating densities.

Fig. 6. (A) Stress-relaxed collagen gel contraction assays for control and MGO-collagen. Each data point is the mean±standard errors of the mean and the data are based on 4 replicates. (B) Mean±standard errors from floating collagen gels for control and MGO-collagen.

Fig. 7. (A) Morphometric analysis of cells immunostained for SMA. Cells were categorized as stained or not stained based upon fluorescence threshold intensities determined after staining with irrelevant antibody. Cells were plated on collagen or MGO-collagen gels for 2 days. Data are mean±standard error of number of cells counted in 250X power microscope fields (3 fields per culture from 4 separate cultures). (B) Immunoblots of SMA and GAPDH of cells cultured on control collagen or MGO-collagen (1.0 mM MGO) for 2 days. Right panel shows cells immunostained for SMA after 2 days plating. (C) Cells were co-transfected with luciferase SMA

promoter and β-galactosidase Rous sarcoma virus (RSV) constructs. RSV was used as a loading control. Data are mean±standard error of mean of normalized luciferase activity. Cells plated on 1.0 mM MGO-collagen showed 1.6 fold increase of SMA promoter activity ($p<0.05$). (D) Cells whether plated on collagen only, MGO-treated collagen, collagen co-incubated with aminoguanidine or MGO-treated collagen co-incubated with aminoguanidine. Quantification of SMA and GAPDH expression was measured by immunoblotting. The effect of aminoguanidine on MGO collagen treatment indicated that enhanced expression of SMA by MGO was likely due to collagen glycation. * indicates $p<0.01$. (E) Percentages of annexin-positive cells by fluorescence microscopy. Data are mean % increases±standard error of mean above % of annexin positive cells plated on PBS-collagen.

Fig. 8. MGO treatment increases mRNA expression for collagen-I, α-smooth muscle actin and P-cadherin. Human cardiac fibroblasts were plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) for over night, 2 days or 5 days. The effect of MGO on α-smooth muscle actin (A), collagen-I (B), P-cadherin (C) and TGF-β2 and TGF-β3 (D) mRNA levels were estimated by real-time quantitative PCR. For real-time PCR analysis, Ct values were GAPDH corrected and subjected to a t-test to determine statistical differences between samples. Data are the means±SE relative to PBS-collagen treated cells from 3-5 independent experiments; *, indicates $p \leq 0.05$

Fig. 9. (A) Cell migration on collagen or MGO-collagen examined using Transwell assays. Data are mean±standard error of mean number of cells that had migrated on to lower side of membrane. Each unit area was a 250X power microscope field (4 field per membrane; 4 separate cultures). * indicates $p<0.01$ and ** indicates $p<0.001$ different than collagen controls. (B) Adhesion after application of shear force. Data are mean±standard error of mean proportion of cells remaining per mm^2 of micro-channel surface for different indicated coating conditions. Cells plated on collagen remain attached more tightly than cells plated on MGO-collagen ($p<0.05$ for control collagen versus 1 and 10 mM MGO).

Fig. 10. Fluorescence micrographs of rhodamine phalloidin staining for actin filaments and for activated, ligand binding-induced neo-epitope on β1 integrin subunits using the 12G10 antibody. Integrin activation is clearly seen only in cells plated on control collagen. Note that rhodamine actin staining in cortical regions is not markedly affected by MGO-collagen.

Fig. 11 Immunoblotting for SMA and GAPDH in human cardiac fibroblasts (A-C), GD-25 cells and DDR1 null cells as indicated. Cells were cultured on control collagen, MGO-collagen or in suspension. (A) Cells on MGO-collagen express higher levels of SMA than control collagen. (B) Treatment with activating antibody (12G10) does not substantially alter SMA expression compared to cells in A. (C) Cells incubated with β1 integrin inhibiting antibody (4B4) show no SMA expression in spite of plating on MGO-collagen. (D) GD-25 cells, which are null for β1 integrin, also did not express SMA. (E) DDR-1 null cells show similar levels of SMA as HCF cells plated on MGO-collagen.

Fig. 12. (A,B) Fluorescence micrographs of immunostaining for paxillin and vinculin. Cells were counter-stained with rhodamine phalloidin for actin filaments in human cardiac fibroblasts 1 day after culture on slides coated with control collagen or 0.1 mM or 1 mM MGO-collagen. Plating on MGO-collagen inhibited formation of paxillin and vinculin-stained adhesions. (C) Morphometric analysis of number of vinculin or paxillin-stained focal adhesions per cell. Data are mean±standard error of number of focal adhesions counted in 10 cells from 4 different cultures. * indicates $p<0.05$ and ** indicates $p<0.01$ different than collagen controls.

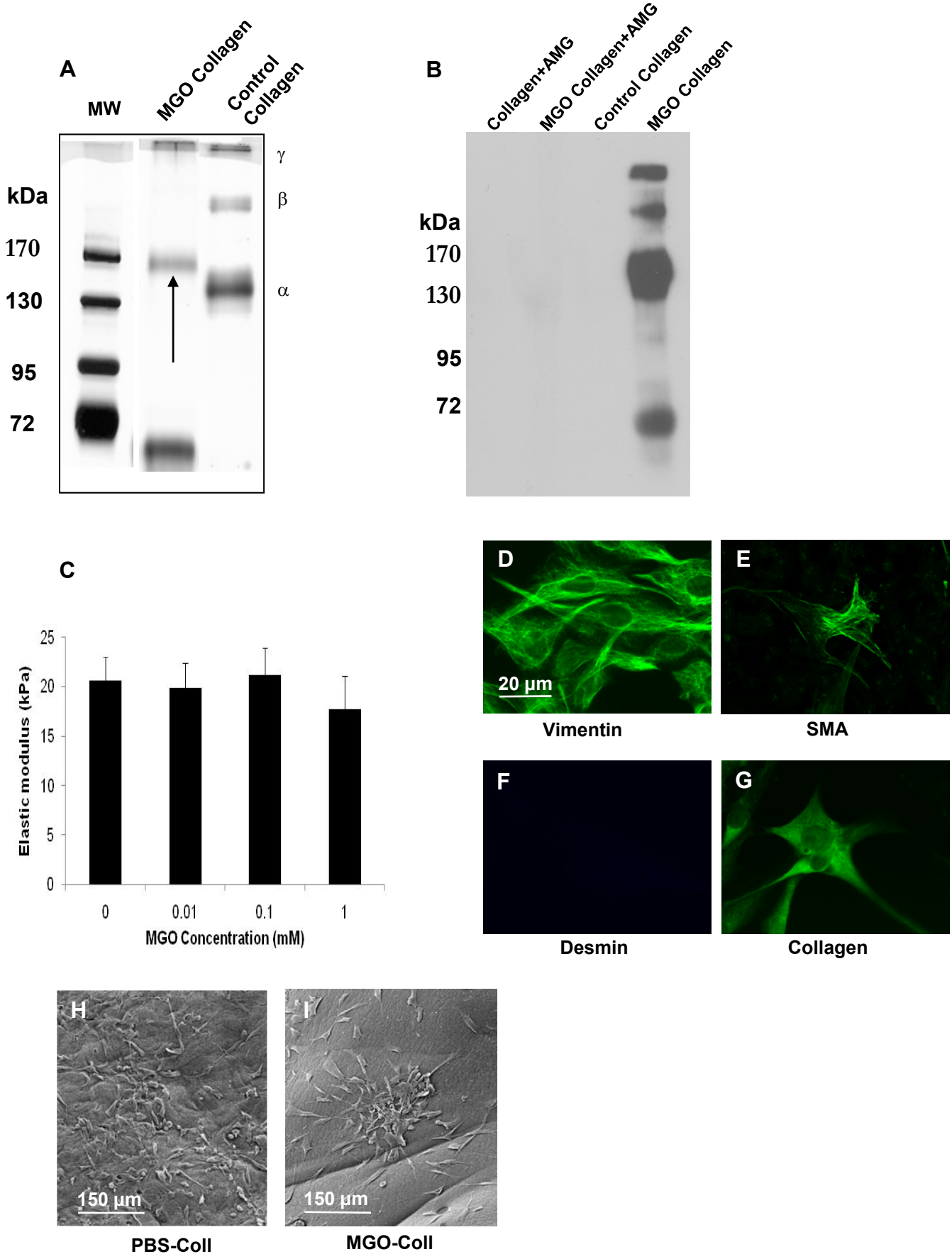
Fig. 13. (A) Immunoblotting of human cardiac fibroblasts plated on control or 1.0 mM MGO-collagen for indicated intercellular adhesion proteins. SAOS2 cells lysates were used as a positive control for OB-cadherin. (B) Human cardiac fibroblasts were plated at high density on control collagen (Col), 1.0 mM MGO-collagen (MGO-Col), or co-incubated with 20 mM aminoguanidine and 1.0 mM MGO-collagen (AMG MGO-Col) or plated at low density on 1.0 mM MGO-collagen (MGO-Col; Low Density). Cells were immunostained with antibody to pan-

cadherin and counterstained with propidium iodide to visualize nuclei. Note that cadherin staining fills the cytoplasm of cells on MGO-collagen and extends to the adjacent cell membrane. (C) Human cardiac fibroblasts were plated on control collagen or 1.0 mM MGO-collagen in the presence or absence of 10 μM Y27632, an inhibitor of the Rho kinase. Cells were immunoblotted for the indicated myofibroblast markers. The Rho kinase is evidently not required for MGO-collagen-induced myofibroblast differentiation. (D). Ratios of mean immunoblot densities of SMA and GAPDH normalized to control cell values of 1.0. Data are normalized densities±standard errors of the mean. Data are for cells plated on collagen (Col), or on MGO-collagen (MGO-Col), or cells pre-incubated with a TGF-β neutralizing antibody on collagen (Col+Ab) or cells pre-incubated with the neutralizing antibody and plated on MGO-collagen (MGO-Col+Ab).

Table

Table 3: Floating collagen gel contraction assays - Slopes of best fit linear curve

Conditions Slopes of best fit curve	PBS control	0.1 mM MGO	1 mM MGO
Integrin Control	4.5±0.07	4.6±0.09	6.1±0.10
Integrin Activator	4.9±0.13	6.07±0.12	6.2±0.18
Integrin Inhibitor	0.81±0.06	0.77±0.07	1.0±0.22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Control Collagen
kDa
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130
95
72
γ
β
α
B
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Control Collagen
MGO Collagen
kDa
170
130
95
72
C
Elastic modulus (kPa)
25
20
15
10
5
0
0
0.01
0.1
1
MGO Concentration (mM)
D
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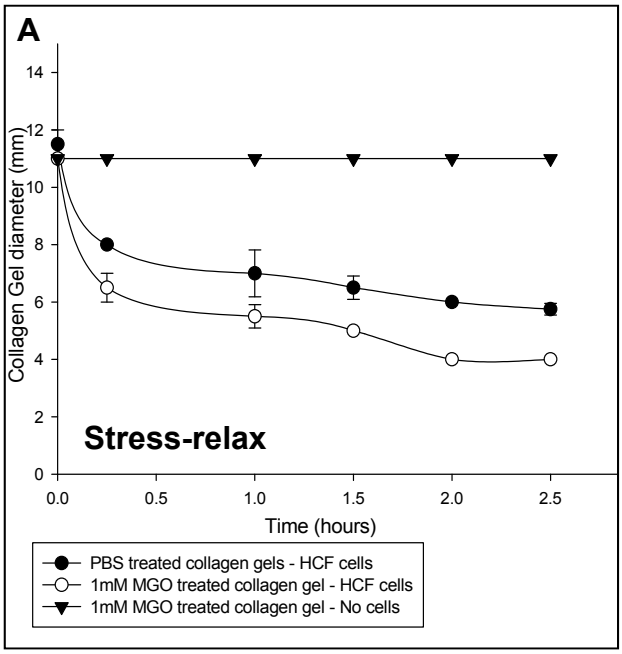
Vimentin
E
SMA
F
Desmin
G
Collagen
H
150 μm
PBS-Coll
I
150 μm
MGO-Coll

Figure 5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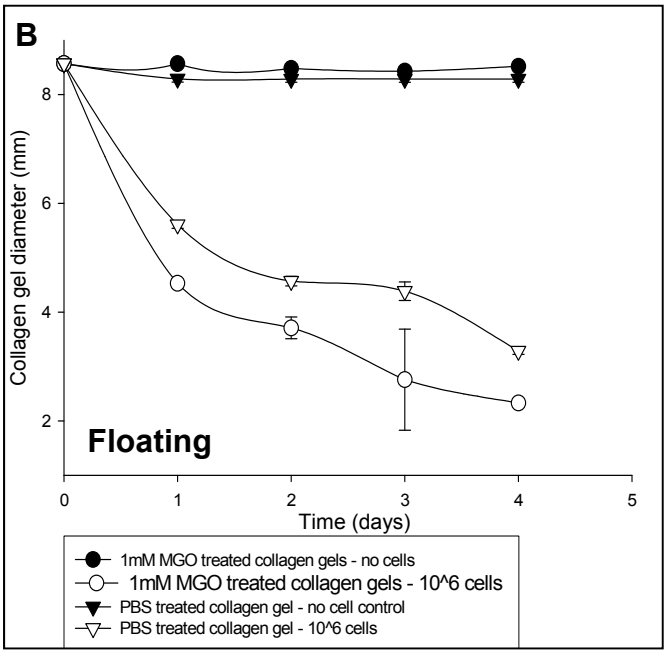
Collagen Gel diameter (mm)
Time (hours)
Stress-relax
PBS treated collagen gels - HCF cells
1mM MGO treated collagen gel - HCF cells
1mM MGO treated collagen gel - No cells


B
Collagen gel diameter (mm)
Time (days)
Floating
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1mM MGO treated collagen gels - 10^6 cells
PBS treated collagen gel - no cell control
PBS treated collagen gel - 10^6 cells

Figure 6

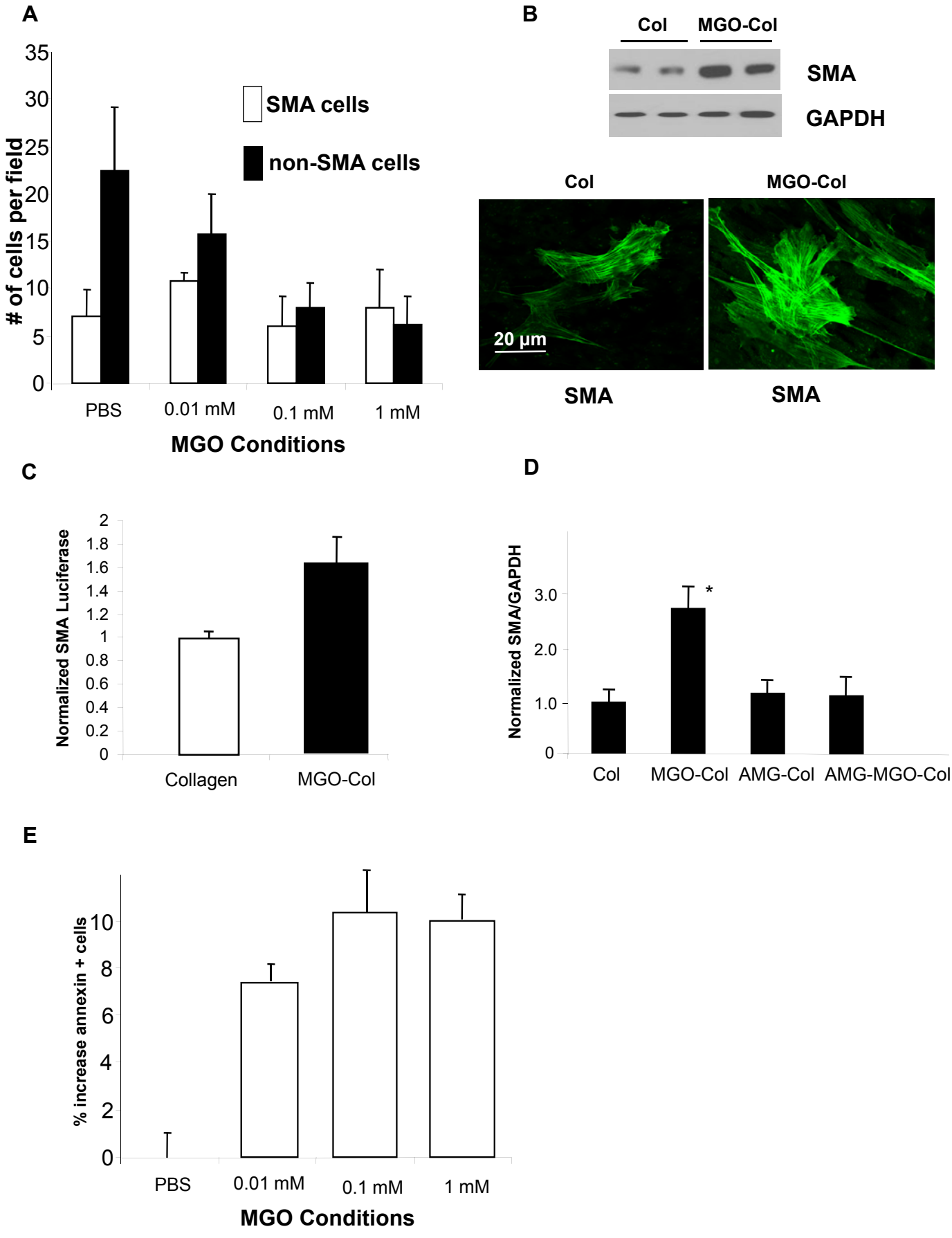
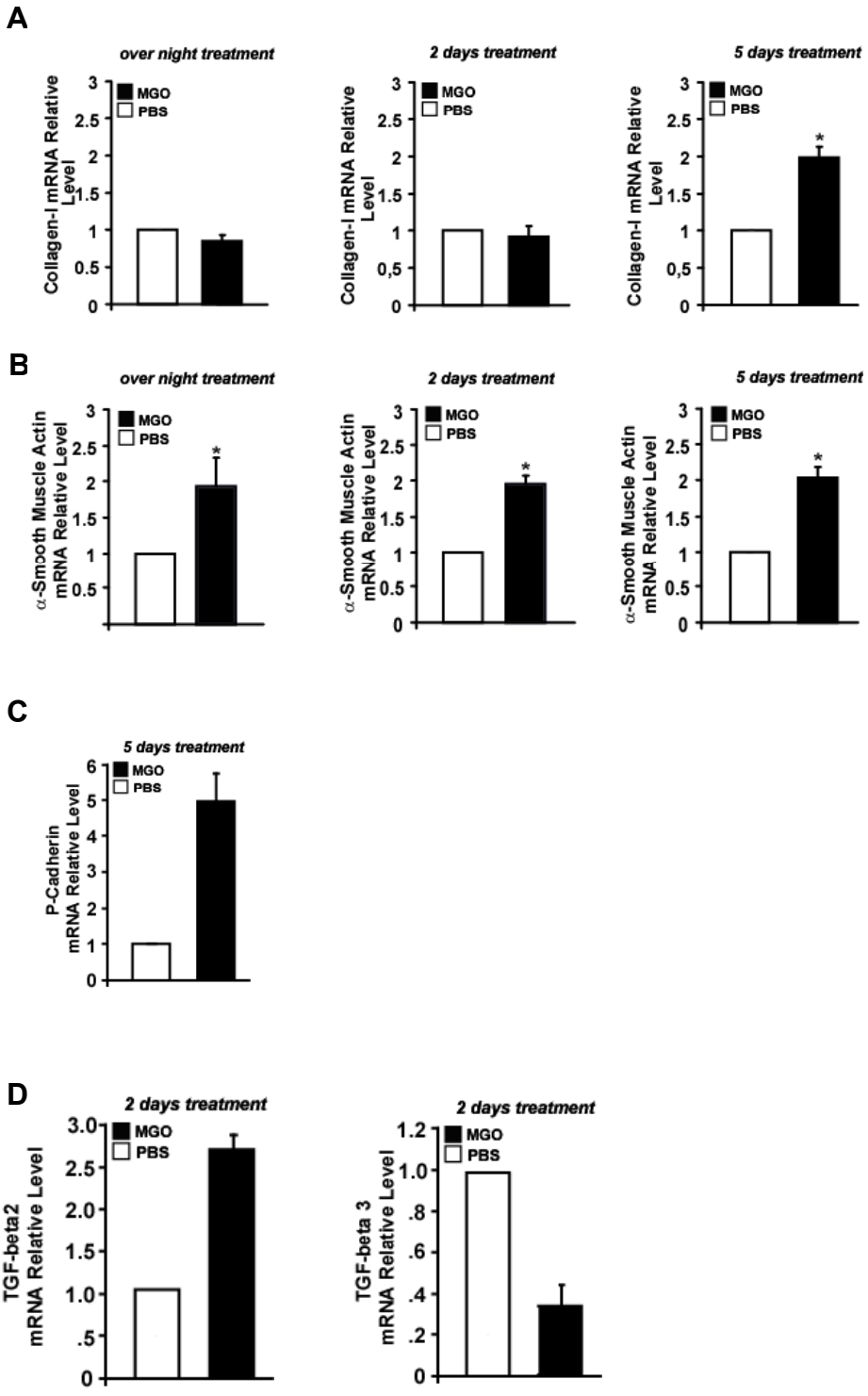

A
35
30
25
20
15
10
5
0
# of cells per field
SMA cells
non-SMA cells
PBS
0.01 mM
0.1 mM
1 mM
MGO Conditions
B
Col
MGO-Col
SMA
GAPDH
Col
MGO-Col
20 µm
SMA
SMA
C
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Normalized SMA Luciferase
Collagen
MGO-Col
D
3.0
2.0
1.0
0
Normalized SMA/GAPDH
*
Col
MGO-Col
AMG-Col
AMG-MGO-Col
E
10
8
6
4
2
0
% increase annexin + cells
PBS
0.01 mM
0.1 mM
1 mM
MGO Conditions

Figure 7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5 days treatment
MGO
PBS
Collagen-I mRNA Relative Level
B
α-Smooth Muscle Actin mRNA Relative Level
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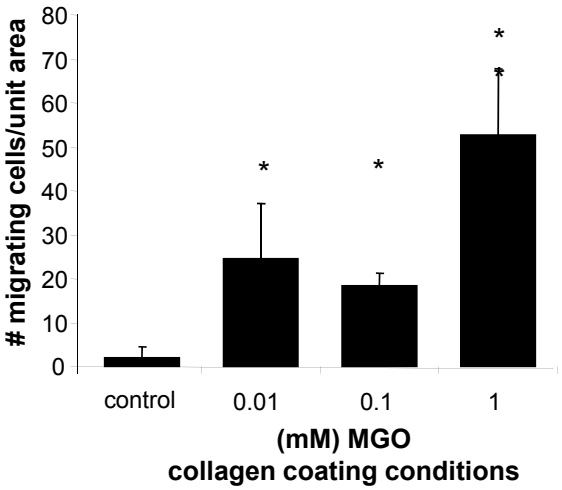
D
2 days treatment
TGF-beta2 mRNA Relative Level
2 days treatment
TGF-beta 3 mRNA Relative Level

Figure 8

A


migrating cells/unit area
80
70
60
50
40
30
20
10
0
*
*
*
control
0.01
0.1
1
(mM) MGO
collagen coating conditions

B

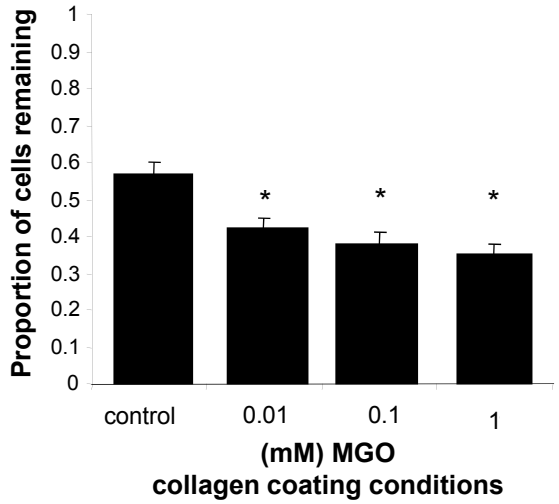

Proportion of cells remaining
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
control
0.01
0.1
1
(mM) MGO
collagen coating conditions

Figure 9

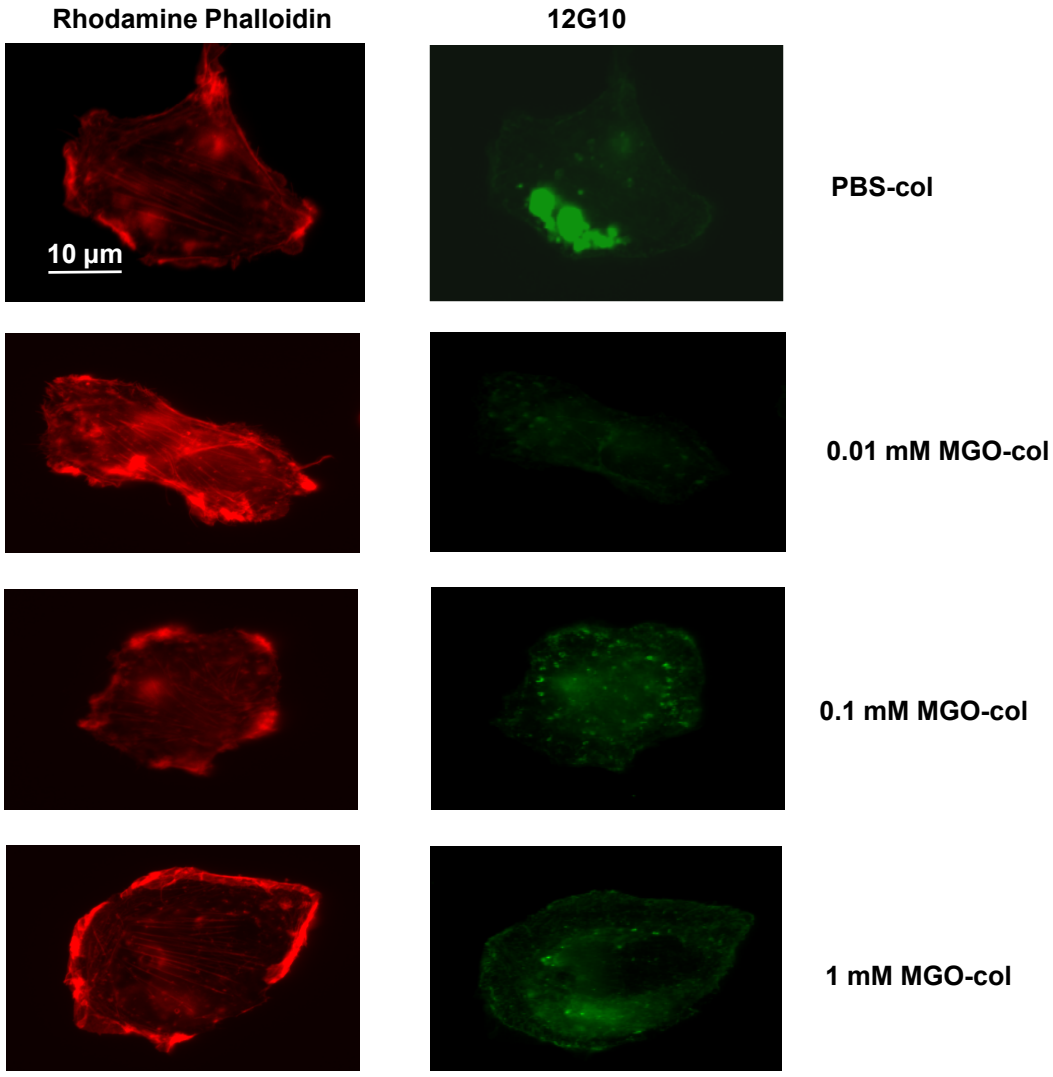

Rhodamine Phalloidin
12G10
10 µm
PBS-col
0.01 mM MGO-col
0.1 mM MGO-col
1 mM MGO-col

Figure 10

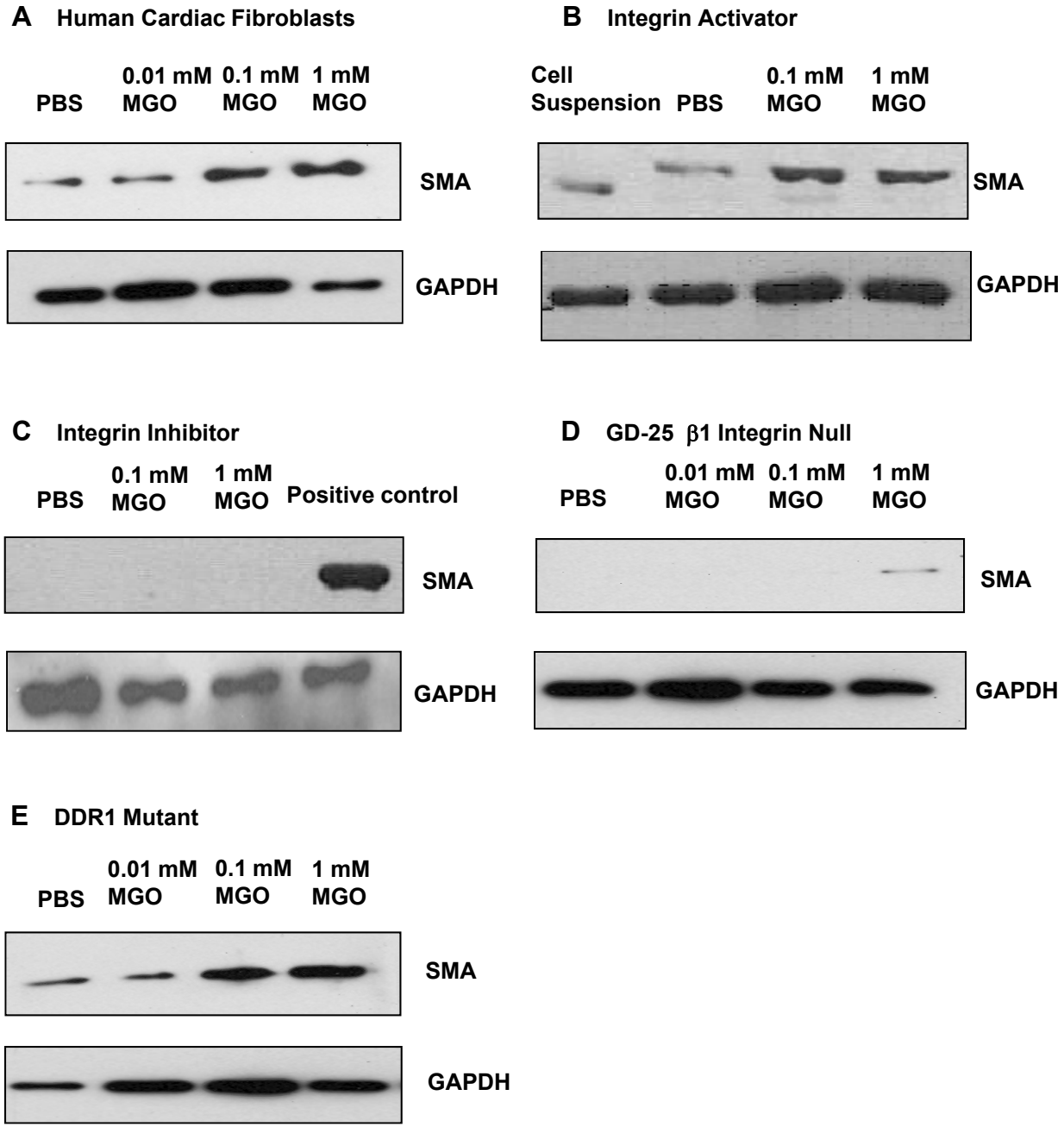
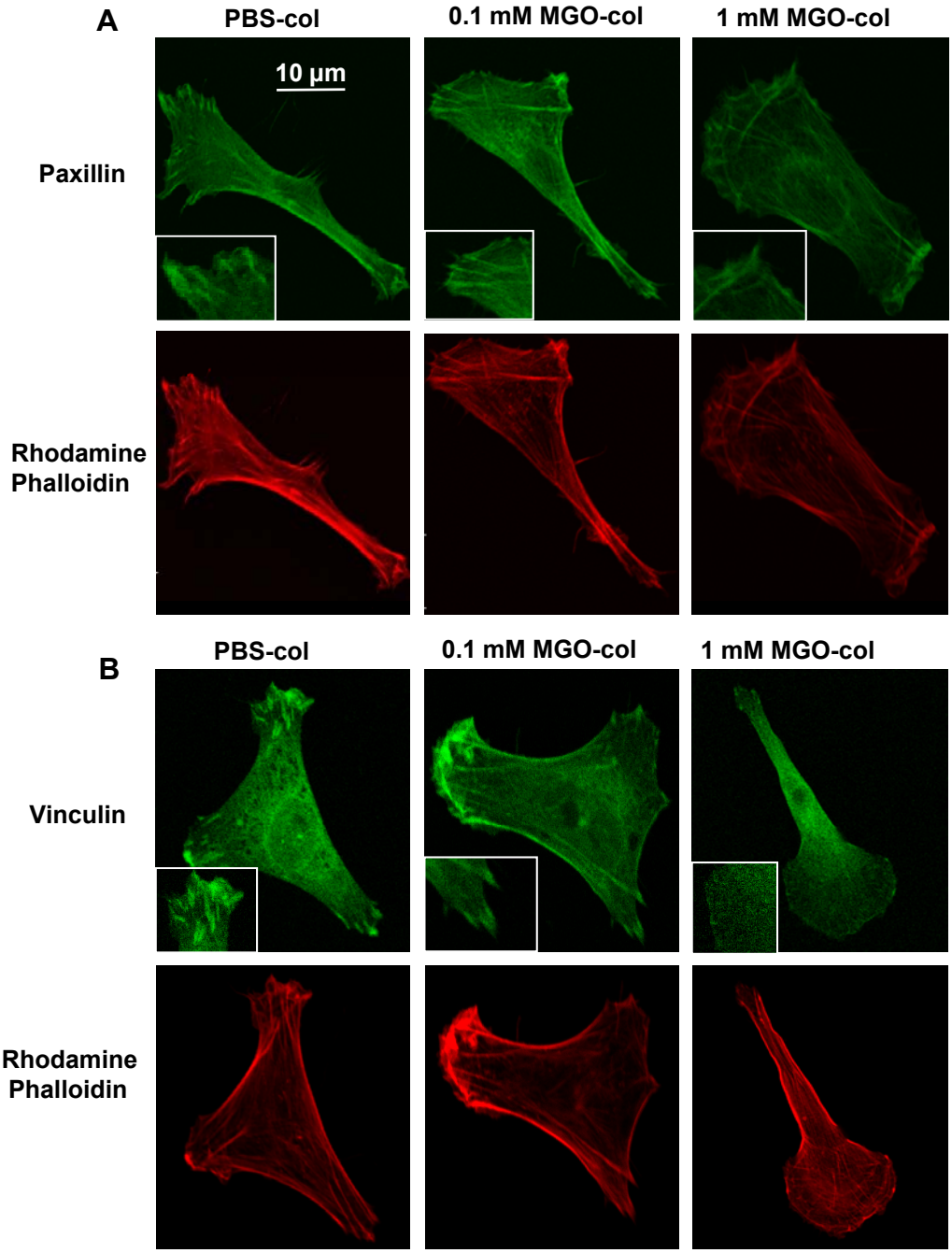

A Human Cardiac Fibroblasts
PBS
0.01 mM MGO
0.1 mM MGO
1 mM MGO
SMA
GAPDH
B Integrin Activator
Cell Suspension
PBS
0.1 mM MGO
1 mM MGO
SMA
GAPDH
C Integrin Inhibitor
PBS
0.1 mM MGO
1 mM MGO
Positive control
SMA
GAPDH
D GD-25 β1 Integrin Null
PBS
0.01 mM MGO
0.1 mM MGO
1 mM MGO
SMA
GAPDH
E DDR1 Mutant
PBS
0.01 mM MGO
0.1 mM MGO
1 mM MGO
SMA
GAPDH

Figure 11


A
PBS-col
0.1 mM MGO-col
1 mM MGO-col
10 μm
Paxillin
Rhodamine Phalloidin
B
PBS-col
0.1 mM MGO-col
1 mM MGO-col
Vinculin
Rhodamine Phalloidin

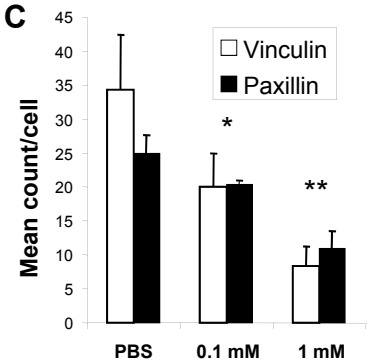
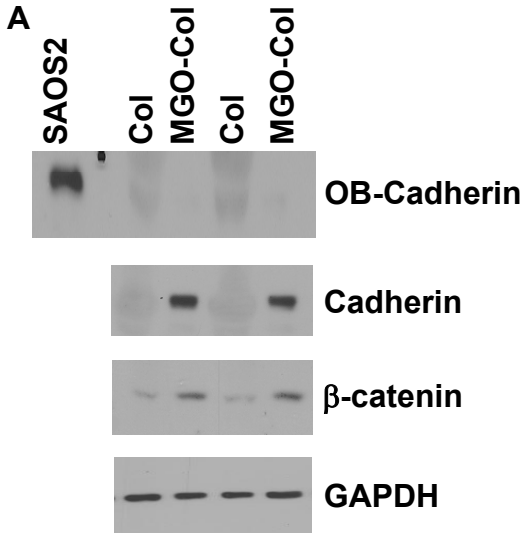
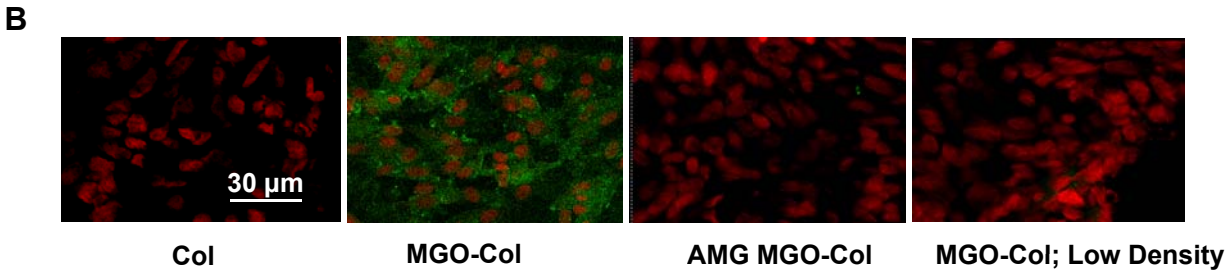
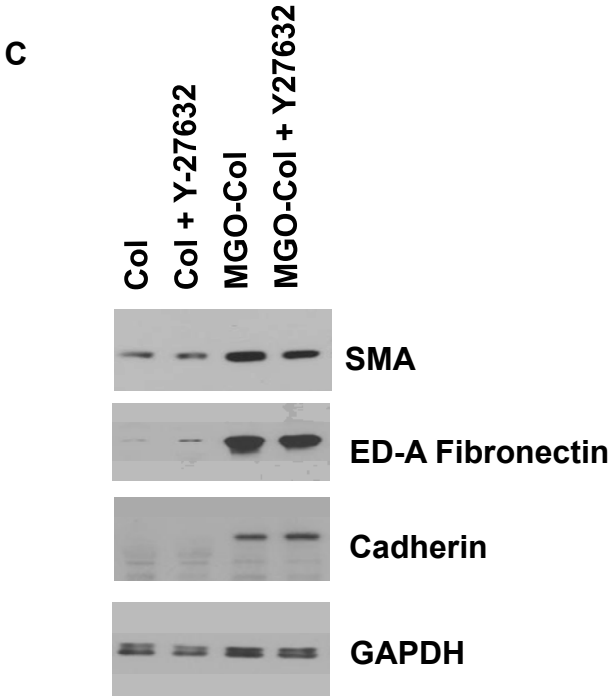

C
Mean count/cell
45
40
35
30
25
20
15
10
5
0
Vinculin
Paxillin
*
**
PBS
0.1 mM
1 mM

Figure 12


A
SAOS2
Col
MGO-Col
Col
MGO-Col
OB-Cadherin
Cadherin
β-catenin
GAPDH


B
30 µm
Col
MGO-Col
AMG MGO-Col
MGO-Col; Low Density


C
Col
Col + Y-27632
MGO-Col
MGO-Col + Y27632
SMA
ED-A Fibronectin
Cadherin
GAPDH

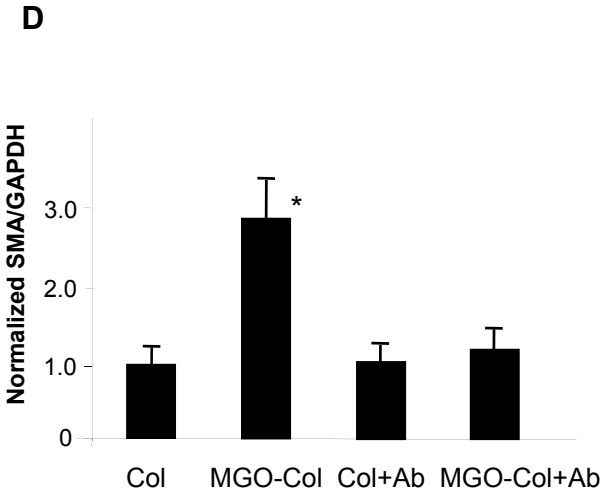

D
Normalized SMA/GAPDH
3.0
2.0
1.0
0
*
Col
MGO-Col
Col+Ab
MGO-Col+Ab

Figure 13

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