Effects of extracellular matrix glycation on cell and tissue function

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Abstract

Methylglyoxal (MG) is a reactive dicarbonyl derived as a by-product of glycolysis. If MG is not metabolized by glyoxalase-1 (Glo1), it glycates macromolecules producing advanced glycation end products (AGEs); these have been linked to larger infarct sizes and poorer cardiac function after myocardial infarction (MI). Proteins of the extracellular matrix (ECM) are prime targets for glycation by MG, but it is unknown if MG modification of the ECM may be a mechanism that contributes to the poor repair and function of the post-MI heart. This study sought to examine if MG-induced modifications of ECM proteins negatively affect fibroblast and endothelial cell function. Analysis with an MG-derived hydroimidazolone 1 (MG-H1) antibody confirmed MG modification of laminin and collagen type (Col) 1, 3, and 4. MG modifications decreased endothelial cell (EC) adhesion on Col3, Col4, and laminin and angiogenesis on ECMatrix. Furthermore, alpha smooth muscle actin staining indicated increased myofibroblast differentiation of fibroblasts on MG-modified proteins. Following induction of MI, extracted mouse hearts were decellularized and compared to healthy controls. Perhaps a result of technical challenges, both western blot and immunohistochemistry contrasted previous data by displaying a marked decrease in MG-H1 modifications post-MI. Overall, these results indicate that MG modifications of the ECM negatively influence EC and fibroblast function, requiring more research on their impact in cardiovascular disease progression.

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List of Abbreviations

AGE	advanced glycation end-product
α-SMA	alpha smooth muscle actin
Ang	angiopoietin
Bcl-2	B-cell lymphoma 2
CEL	carboxyethyllysine
Col1	collagen type 1
Col3	collagen type 3
Col4	collagen type 4
CVD	cardiovascular disease
DCF	dichloro-dihydro-fluorescein diacetate
DDR1	discodin domain receptor 1
DDR2	discodin domain receptor 2
DHAP	dihydroxyacetonephosphate
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase

f-actin	filamentous actin
FBS	fetal bovine serum
FGF	fibroblast growth factor
GA3P	glyceraldehyde 3-phosphate
Glo1	glyoxalase 1
Glo2	glyoxalase 2
GLUT1	glucose transporter 1
GPVI	glycoprotein VI
GSH	glutathione
GSK3β	glycogen synthase kinase 3 beta
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HRP	horseradish peroxidase
ICAM-1	intracellular adhesion molecule 1
IL-1	interleukin 1
iNOS	inducible nitric oxide synthase
IR	ischemia/reperfusion

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MG	methylglyoxal
MG-H1	hydroimidazolone N_{δ}-(5-methyl-4-imidazolon-2-yl)-L-ornithine
MG-H2	2-amino-5-(2-amino-5-hydro-5- methyl-4-imidazolon-1-yl) pentanoic acid
MG-H3	2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl) pentanoic acid
MI	myocardial infarction
MMP	matrix metalloproteinase
MODIC	(2-ammonio-6-({2–[4-ammonio-5-oxido-5-oxopently)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate)
MOLD	6-{1-[(5S)-5-ammonio-6-oxido-6-oxohexyl]-4-methyl-imidazolium-3-yl}-L-norleucine
NADPH	nicotinamide adenine dinucleotide phosphate
NC1	noncollagenous domain 1

NF-κB	nuclear factor kappa beta
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule 1
RAGE	receptor for advanced glycation end-products
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription
TBST	tris-buffered saline with Tween 20
TGF-β	transforming growth factor beta
THP	N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule 1
VE	vascular endothelial
VEGF	vascular endothelial growth factor

VSMC	vascular smooth muscle cell

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CHAPTER 1: INTRODUCTION

1.1 Coronary artery disease and myocardial infarction

Coronary artery disease is described as the impedance or blockage of one or more arteries that supply blood to the heart. In Canada, ischemic heart disease is a major cause of concern, being responsible for over thirty thousand deaths each year.(Statistics Canada 2014). The process of atherosclerosis drives CAD, and it is defined by plaque buildup causing the narrowing and eventual blockage of vessels. Chronic occlusions lead to ischemia, increasing overall stress and load on the heart. Furthermore, the outcome of complete occlusion is a myocardial infarction (MI), where the resulting ischemia causes damage and cell death to the area downstream of the occlusion.

Cardiomyocyte apoptosis and necrosis leads to a loss of contractile tissue as well as triggering a series of post-MI events. Overlapping inflammatory, proliferative, and maturation phases are one way of categorizing and describing the events that unfold after MI (Frangogiannis 2006). Immediately after MI, neutrophils and macrophages are attracted to the infarct and border zone, working to resorb extracellular matrix, necrotic tissue and, perpetuate the inflammatory healing process (Nahrendorf et al. 2007). Following the clearance of the majority of damaged tissue, the heart undergoes infarct expansion. The process is described as loss of muscle thickness, not due to further necrosis, but because of muscle fiber rearrangement (Hutchins and Bulkley 1978). Expansion occurs due to the physical stress placed on the infarct by the continuous need to provide contraction in addition to the breakdown of collagen struts in the time of ECM clearance and remodelling. During the physical expansion of the infarct, both cardiac

fibroblast and endothelial cell populations begin to proliferate. These two cell types work to structurally reinforce and perfuse the infarct and border zone (Virag and Murry 2003). With scar maturation, there is a loss of vasculature and cells within the infarct leading to a scarred and mostly collagenous area of the myocardium which cannot participate in proper contraction. This repair process reinforces the ventricle wall to prevent it from rupturing, but it does not regenerate the lost myocardial cells. This leads to an increase in demand and complex signalling leading to compensatory remodelling. Ventricular hypertrophy and dilation are two ways that the heart attempts to maintain cardiac output and adjust to the increased load. Eventually, the dilation of the ventricle and thinning of the infarct can lead to heart failure.

1.2 Endothelial cells post-MI

Throughout the body, endothelial cells (ECs) have a large impact on tissue functionality due to their intermediary role between the blood and organ tissue. ECs line all blood vessels, maintaining their selectivity through cellular adherens junctions (Dejana, Corada, and Lampugnani 1995). An important protein in such junctions is vascular endothelial (VE) cadherin, modulating EC adhesion, permeability and angiogenesis through connections with the cytoskeletal protein filamentous actin (f-actin). Specifically differential expression of VE-cadherin, as driven by notch and vascular endothelial growth factor (VEGF) receptor signalling, influences proper EC arrangement during angiogenesis (Benn et al. 2015; Bentley et al. 2014). The permeability of the endothelium can be altered by the expression of matrix metalloproteinases (MMPs) during

extracellular matrix (ECM) remodelling (Partridge, Jeffrey, and Malik 1993). Another important junction protein, platelet endothelial cell adhesion molecule (PECAM-1) is distributed in focal adhesions and throughout the surface of ECs, serving to determine permeability and affect cell signalling (Ilan et al. 2000). Similar to VE-cadherin, PECAM-1 interacts with f-actin through beta catenin; and blocking studies have shown the importance of both of these adhesion molecules in angiogenesis (Matsumura, Wolff, and Petzelbauer 1997; Delisser et al. 1997). In addition to regulating permeability, ECs can alter the circulation's hemodynamics. Vasodilation via the nitric oxide (NO) system is the best known such mechanism; ECs can respond to many different agents (hormones, local regulators, etc.) to increase blood flow (Furchgott and Vanhoutte 1989). Vasodilation is also regulated by shear stress, whereby EC signalling allows vascular smooth muscles to respond to dynamic changes in perfusion (Stepp, Nishikawa, and Chilian 1999). An equally important vascoactive molecule is endothelin, opposing the actions of NO, and similarly responding to various agents and physical stress to regulate blood flow (Rubanyi and Botelho 1991). An imbalance in NO and endothelin, specifically a higher endothelin/NO ratio, has been linked to vascular disease (Bourque, Davidge, and Adams 2011).

In the post-MI environment, as summarized by **Fig. 1**, ECs play an important role in myocardial repair by participating in the initial inflammatory actions and through revascularization of the infarct and border zone. ECs contribute to the inflammatory response by producing cytokines (**Table 1**) and chemokines (eg. tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and monocyte chemoattractant protein-1 (MCP-1)) (G Krishnaswamy et al. 1999). These EC actions lead to increased blood flow and

perpetuate the inflammatory response through increased recruitment of inflammatory cells. ECs help recruit inflammatory cells through the upregulation of cell capture adhesion molecules (e.g. intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin) at their surface (Collins et al. 1995). Through cytokine release, ECs also stimulate MMP production from fibroblasts, which are involved in remodeling of the infarct area.

Revascularization of ischemic myocardial tissue is particularly important considering that cardiomyocytes have a high dependence on oxidative metabolism and produce large amounts of waste by-products due to their high metabolic rate. ECs participate in repair through angiogenesis which is defined as sprouting, or the growth of vasculature from pre-existing vessels towards a signal. In a post-MI heart, angiogenesis can be stimulated by growth factors, hypoxia, and nitric oxide synthases (NOS). Angiopoietin (Ang), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and VEGF are some of the primary growth factors that have been implicated in post-MI vascular repair. They have been shown to be involved in promoting EC proliferation, migration and tube formation, as well as further stimulating angiogenic growth factor production (Yoon Shin Park et al. 2016; Seghezzi et al. 1998; H.-K. Chang et al. 2016; Ono et al. 1997; Guo et al. 1995; Gille et al. 2000; Yancopoulos et al. 2000).

Table 1. Summary of cytokines produced by endothelial cells and their impact on cellular processes

Cytokine	Target	Effect	References
CCL2 (MCP-1)	Monocytes	Chemotaxis	(Takahara et al. 1996)
CCL5	T cells, monocytes	Chemotaxis	(Marfaing-Koka et al. 1995)
G-CSF	Monocytes	Survival, proliferation, differentiation, binding, migration	(Rajavashisth et al. 1990)
GM-CSF	Neutrophils, monocytes, progenitors	Survival, proliferation, differentiation, binding, migration	(Guha Krishnaswamy et al. 1998)
M-CSF	Monocytes	Survival, proliferation, differentiation, binding, migration	(Rajavashisth et al. 1990)
IL-1	All cells	Inflammation (activation, proliferation)	(Guha Krishnaswamy et al. 1998; Prabhu and Frangogiannis 2016)
IL-3	Hematopoietic cells	Growth, differentiation	(Nilsen et al. 1998)
IL-6	All cells	Inflammation (activation, proliferation)	(Sterpetti et al. 1993)
IL-8	Neutrophils, T cells, monocytes	Chemotaxis, inflammation	(Zeuke et al. 2002)
IL-11	Hematopoietic progenitors, megakaryocytes	Hematopoiesis, platelet elevation	(Suen et al. 1994)
IL-15	T cells, neutophils	Proliferation, survival	(X. Liu et al. 2009)
TNF-α	TNF-R1 or TNF-R2 expressed differentially on all cells	Apoptosis vs proliferation, preservation, and hypertrophy	(Imaizumi et al. 2000)

Hypoxia-inducible factors (HIF) have significant cardioprotective roles in a post-MI setting; under lowered oxygen conditions, the activation of HIFs can modulate over 100 genes (Weidemann and Johnson 2008). HIF1-α and HIF2-α were previously demonstrated to have their expression increased up to 4 weeks post-MI in rats (Jürgensen et al. 2004). As such, their ability to stimulate vascular regeneration improves coronary blood flow and leads to a reduction in infarct size as well as improved cardiac function (Tekin, Dursun, and Xi 2010). An example of an implicated pathway is the pro-angiogenic transcriptional upregulation of VEGF post-MI (Y. Liu et al. 1995). Thus, HIF responses are crucial in the post-MI EC generated repair processes. The NOS pathway is important in regulating blood vessel dilation/constriction, but it has also been shown to play a role in post-MI angiogenesis (Landmesser et al. 2004). For example, inducible NOS (iNOS) is increased post-MI leading to increased capillary density and improved LV function (Shimazu et al. 2011). As demonstrated by endothelial NOS (eNOS) protein inducers HSPA12B and Apelin, this isoform also serves an angiogenic and antifibrotic function in the infarcted myocardium thereby promoting the healing process and improvement of LV function (Azizi et al. 2015; Jingjin Li et al. 2013).

1.3 Fibroblast function post-MI

Throughout the body, fibroblasts are associated with connective tissues and the production of ECM proteins, mainly collagen Type 1 and Type 3 (Col1 and Col3, respectively), as well as fibronectin (Kanekar et al. 1998). Typically, they are characterized by an oval nucleus, cytoplasmic extensions, and granular material in the

cytoplasm (Baudino et al. 2006). Fibroblasts contribute to cardiac remodelling through their influence on ECM deposition and resorption. Fibroblasts also generate a variety of ECM-degrading enzymes, the MMPs, as well as tissue inhibitors of MMPs (TIMPs) to provide balance. Thus, there is considerable interest in fibroblast mechanical and signalling responses when it comes to physiological and pathological remodelling of the heart (MacKenna, Summerour, and Villarreal 2000; Leask 2007).

The interstitially dispersed fibroblast lacks phenotypic homogeneity such that, in certain conditions, the cell can acquire smooth muscle cell characteristics. The transition to what is known as a myofibroblast, produces a more mobile and contractile cell (Tomasek et al. 2002). The phenotypic change, which is strongly induced by transforming growth factor beta (TGF-β), is important in proper wound closure and healing throughout the body. Myofibroblasts were first described by Gabbiani, Ryan, & Majno (1971), as cells with both fibroblast and smooth muscle cell characteristics containing fibrillar bundles, nuclear irregularity and intracellular connections. The fibrillar bundles resemble stress fibers present in fibroblasts but, are organized like smooth muscle cell microfilaments. Under normal physiological conditions (healthy tissue), fibroblasts produce small amounts of ECM required for turnover and maintenance of structure (Kanekar et al. 1998). However, in cardiac tissue post-MI (**Fig. 1**), myofibroblasts are important in the formation of granulation tissue and the integrity of the collagenous scar (Yao Sun and Weber 2003). Following the inflammatory phase of MI, and in response to cytokines, mechanical stretch and a change in O_2 levels, fibroblasts transition to the more active myofibroblast phenotype. Post-MI, many cytokines that modulate fibroblast function are up-regulated: TGF-β, TNF-α, IL-1β, and

IL-6 are some examples. The pro-fibrotic molecule TGF-β causes fibroblasts to undergo the transition to myofibroblasts; it also increases the expression of Col1, Col3, and fibronectin (Eghbali et al. 1991; Villarreal et al. 1996; Lijnen, Petrov, and Fagard 2000). TNFα causes increased migration, proliferation, and expression of MMPs while it opposes TGF-β in that collagen synthesis is decreased (Jacobs et al. 1999; Mitchell et al. 2007; Siwik, Chang, and Colucci 2000). IL-1β and IL-6 both upregulate migratory capacity, expression of MMPs, and downregulate fibrillar collagen synthesis (Mitchell et al. 2007; Siwik, Chang, and Colucci 2000). Under pathological conditions, changes to the ECM result in altered mechanical properties, which are detected by fibroblasts, triggering compensatory pathways. In particular, the expression of α-SMA is increased with stretch and in fibrotic lesions (Shiojima et al. 1999). Additionally, the expression of Col1, Col3, and certain MMPs is induced by stretch (W Carver et al. 1991; Butt and Bishop 1997; Tyagi et al. 1998; Husse et al. 2007). The myofibroblast phenotype is further perpetuated in mechanical loaded conditions by an increase in TGF-β expression (van Wamel et al. 2001). Lastly, oxygen is an important consideration as its levels are reduced in MI, which can affect fibroblast function. For example, hypoxia stimulates an increase in collagen production in fibroblasts, along with TGF-β expression (Agocha, Sigel, and Eghbali-Webb 1997; Grobe et al. 2007). Furthermore, myofibroblasts can adapt to tolerate lower oxygen levels, so when reoxygenation occurs, this creates a state of hyperoxia which stimulates proliferation and the transition to myofibroblasts (Roy et al. 2003; Lin et al. 1995; Roy et al. 2007). This then leads to an increase in the synthesis of Col1, Col3, MMPs, and TIMPs (Grobe et al. 2007; K. Chen et al. 2004; Makino et al. 2006), further propagating ECM remodeling.

There is a balancing act in terms of the input stimuli working on the cardiac fibroblast in the MI environment. In the early inflammatory phase, TNFα and interleukins dominate, maintaining the normal fibroblast phenotype while providing increased ECM degradation. After resolution of acute inflammation, the proliferation and differentiation of myofibroblasts is stimulated, as described above. The myofibroblasts are able to better migrate into damaged areas and, following tissue clearance by monocytes, begin synthesizing large amounts of ECM proteins. As mentioned previously, they also have an increased expression of MMPs leading to increased turnover and a more plastic remodelling process (Brown et al. 2007). The myofibroblast phenotype is important because it provides stability through ECM deposition and its contractile ability helps to close the wound. Post-MI, myofibroblast contraction causes infarct shrinkage, which is followed by myofibroblast apoptosis and the formation of the mature collagenous scar. However, one of the isoforms of TGF-β remains prominently elevated 4 weeks after MI, so that the myofibroblast phenotype does not completely disappear (Y Sun et al. 1998). This can lead to an unbalanced proliferation of myofibroblasts, further deposition of collagen and eventual cardiac fibrosis. The process impairs cardiac function by increasing stiffness and causing pathological signaling to the myocardium (Díez et al. 2002).

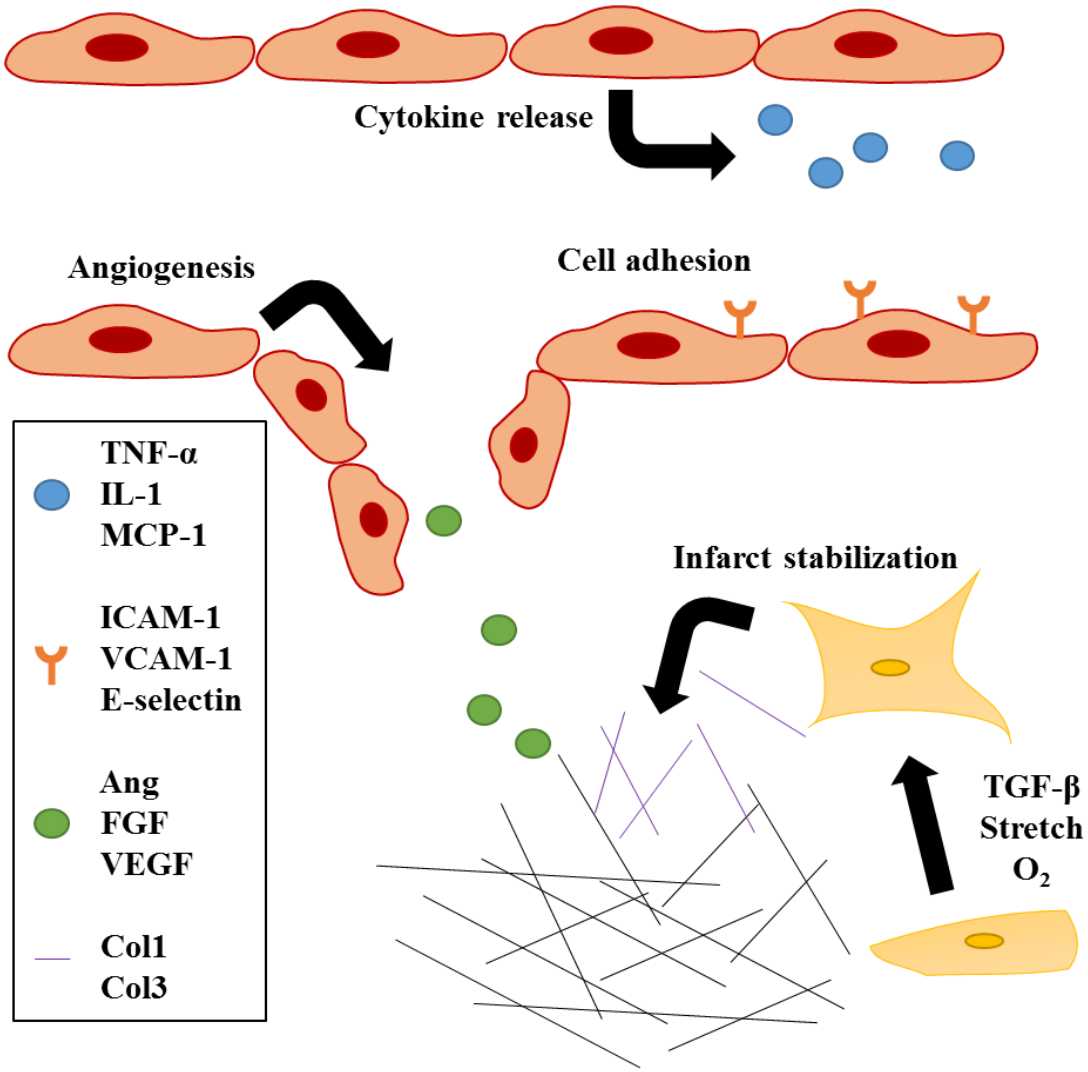

Figure 1. **Post-MI function of ECs and fibroblasts**
Initially, ECs release cytokines which stimulate an inflammatory response and attract immune cells. In order to better facilitate inflammatory cell homing ECs upregulate the expression of surface adhesion molecules. Finally, ECs are stimulated to undergo angiogenesis and improve perfusion to the border zone and infarct. Fibroblasts initially work to break down the ECM but, ultimately their main role is infarct stabilization through deposition of collagen.

1.4 Lysyl Oxidase

An important copper dependent amine oxidase expressed in fibroblasts is lysyl oxidase (LOX). The enzyme is vital in ECM organization due to its ability to facilitate the formation of covalent pyridinoline cross-links between collagen fibers. A study evaluating LOX overexpressing VSMCs cultured on collagen gels showed that both the strength and elastic moduli were two-fold increased as compared to the control (Elbjeirami et al. 2003). Demonstrating its importance in repair, decreased LOX expression and activation in mice was correlated with post-MI rupture and death (Ma et al. 2013). This ECM fortifying enzyme appears to be increased in hypertensive rat myocardium and can be downregulated through inhibition of TGF-β signalling; along with correlations to extent of fibrosis implicating LOX in the process (Hermida et al. 2009). LOX is induced by hypoxia, and pathways suggest advanced glycation end-products (AGEs) may exert the same effect as well (Papachroni et al. 2010). Further, LOX expression is upregulated in heart failure patients with correlations to LV stiffness (López et al. 2009). Thus, the expression of LOX is similar to the myofibroblast phenotype, it is required for infarct stability but, it seems that if unregulated it can contribute to fibrosis and the advancement of heart failure.

1.5 The extracellular matrix

The ECM is the scaffolding that supports cells throughout the entire body. It is made up of many different components including proteoglycans, fibronectin, laminin, collagens and elastin, with different physical characteristics (e.g. adhesive, rigid, and flexible),

depending on the tissue. The ECM's function is not only to provide physical support, as it is also involved in signalling, providing the cells with important chemical and mechanical cues (Fan, Creemers, and Kassiri 2014).

Collagen is the predominant ECM protein in the body, and is the most abundant component in the ECM of heart tissue. There are 28 types of collagen, and all collagens are composed of a right handed polypeptide triple helix, with the Gly-X-Y repeat being consistent throughout; the structure providing great tensile strength (Hulmes 2008). Col1 composes the majority of cardiac tissue, with Col3 being the second most common structural protein, both of which are fibrillar and produced by fibroblasts (Weber 1989). While Col3 is composed of identical trimers, Col1 is a collagen whose alpha chains are heterotrimeric. Col1 is found to pack into thicker fibers as compared to Col3 which provides more flexibility to its supporting structure. Both of these collagens are crucial to cardiac integrity during regular physiology, and perform such a task through longevity with an estimated half-life of 80 to 120 days (Weber 1989). Following MI, most ECM deposition in remodeling results from Col1 and Col3 synthesis, the proteins being crucial to resist cardiac forces and prevent rupture of the weakened infarct area. Conversely, in fibrosis these Col1 and Col3 increases cause cardiac stiffness and reduce cardiac function (Bishop et al. 1990).

Laminin and collagen type 4 (Col4) are proteins that make up a specialized part of the ECM that is referred to as the basement membrane. In cardiac tissue, the basement membrane forms the underlying ECM layer that provides support and interacts with ECs as well as vascular smooth muscle cells (VSMCs). Laminin is composed of three

polypeptides with different sequences but, with some parallels in domain organization, it polymerizes through terminal globular domains (Engel et al. 1981). Col4 like other collagens forms a polypeptide triple helix, its structure uniqueness is provided by the carboxyl terminal end of the protein. The end is of globular structure and is referred to as the noncollagenous (NC1) domain. The NC1 domain, as well as the amino terminus "7S" domain, are crucial to Col4 polymerization into a lateral semiregular hexagonal network which forms the major structural component of the basement membrane (Yurchenco and Furthmayr 1984). In addition to self-binding characteristics, laminin and Col4 interact with each other. Laminin's globular domains are able to bind at two distinct sites on the triple helical part of the Col4 molecule (Charonis et al. 1985). The basement membrane is required for proper tissue function and is involved in many aspects of cellular homeostasis such as proliferation, adhesion, migration, growth and differentiation (Erickson and Couchman 2000). Thus, it is easy to see that in a regenerative environment these proteins can have a great impact on outcome.

The ECM partakes in each of the three MI repair phases. During the inflammatory phase, ECM proteins are cleaved by MMPs, which are largely produced by fibroblasts. However, leukocytes produce additional MMPs that further degrade these fragments to produce inflammatory signal molecules. One example is the Col4 fragment tumstatin which functions to inhibit angiogenesis in this phase of "tissue clean-up" (Yamamoto et al. 2004). Further, the ECM acts on fibroblasts to stimulate the proliferative phase and myofibroblast transition through biomechanical stimuli as outlined above (see section 1.3). As ECM proteins are deposited, the extracellular protein osteopontin has proven to beintegral for proper remodelling (Trueblood et al. 2001). In the maturation phase, the

collagenous scar becomes more rigid with many factors involved in its formation (e.g. LOX, syndecans, periostin), acting on the ECM to maintain infarct stability (Dobaczewski, De Haan, and Frangogiannis 2012; Shimazaki et al. 2008; Matsui et al. 2011).

The ECM has now long been recognized for its role in cell signalling and its impact on homeostasis. For example, in mechano-sensing, it is known that integrins (cellular ECM receptors) help transduce external stimuli from the ECM leading to a multitude of signalling pathways, such as those involved in the outcome of myofibroblast transition (MacKenna, Summerour, and Villarreal 2000). Additionally, the ECM impacts adhesion as well as cell shape and overall tissue organization (Weidenhamer et al. 2015). Integrins are the major route through which cell signalling is influenced by the ECM. Each of these integral membrane proteins is made of an α and a β subunit of which there are 18 and 8, respectively, combining to make a variety of heterodimers. On either side of the membrane, integrins have a large extracellular domain for ligand binding as well as a cytoplasmic domain for cytoskeletal and cytoplasmic protein interaction (Ross and Borg 2001).

Col1 and Col3 both contain the amino acid sequences GFOGER, GLOGER, and GMOGER which interact with high affinity to integrins α1β1 and α2β1 (Y. Xu et al. 2000). α1β1 plays a role in fibroblast migration, while both integrins are important for proper 3D remodelling of collagen gels (Wayne Carver et al. 1995). In addition to expressing α1β1 and α2β1 integrins, cardiac fibroblasts also express α11β1, which is recognized as a key integrin in binding of fibrillar collagens, and an increased expression

of α11 has been shown to be necessary for myofibroblast differentiation (Talior-Volodarsky et al. 2012). Likewise, the ablation of β1 in fibroblasts impairs wound repair by decreasing the myofibroblast transition and formation of granulation tissue (S. Liu et al. 2010). Besides integrins, Col1 and Col3 can bind to cells through discodin domain receptor 1 (DDR1), DDR2, glycoprotein VI (GPVI), and leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1). The DDRs are tyrosine kinases that bind GVMGFO (O=hydroxyproline) motifs on Col1 and Col3; DDR1 is expressed by ECs, while both DDR1 and DDR2 are expressed by cardiac fibroblasts (Goldsmith et al. 2004; Konitsiotis et al. 2008). As for GPVI and LAIR-1, the receptors only bind to Col3 and do so through longer peptide binding motifs (Lebbink et al. 2009; Jarvis et al. 2008). LAIR-1 is expressed on leukocytes, while GPVI is mainly known for its presence in platelets but, it is also expressed in endothelial cells and may impact their ECM interaction (B. Sun et al. 2003; Meyaard et al. 1997).

Like the fibrillar collagens, Col4 and laminin interact with integrins and other specific membrane proteins. Integrin α1β1 binds to Col4 stronger than it does to Col1 and Col3, and it does so via an aspartic acid and an arginine residue (Eble et al. 1993). Fibroblasts lacking α1 were demonstrated to have a deficiency in migration and spreading on Col4 (Gardner et al. 1996). In ECs, the Col4 terminal NC1 promotes an antiangiogenic effect that is mediated by α1β1 (Sudhakar et al. 2005). Unlike the other collagens, Col4 is bound exclusively by DDR1 and not DDR2 (H. Xu et al. 2011). Again, LAIR-1 is a receptor for Col4 but, it is not expressed in fibroblasts or ECs (Tang et al. 2009). Laminin interacts with many integrins: α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4, through LG domains with RGD having been identified as one of the residue sequences

involved (Sasaki and Timpl 2001). The specific outcomes of binding are still to be determined but, genetic analyses have shown the importance cell-ECM interactions in many processes throughout the body (Yurchenco 2011). Dystroglycan forms the dystrophin-glycoprotein complex which can link to laminin, with a prominent role in cytoskeletal anchoring, which has been studied primarily in muscle cells, but, also seen in ECs and fibroblasts (Belkin and Smalheiser 1996). It has been implicated in EC adhesion, migration, and angiogenesis, suggesting a possible significance when considering cardiac regeneration post-MI (Hosokawa et al. 2002; Galvagni et al. 2016). **Fig. 2** provides a summary graphic of cell-ECM interactions that have been detailed above.

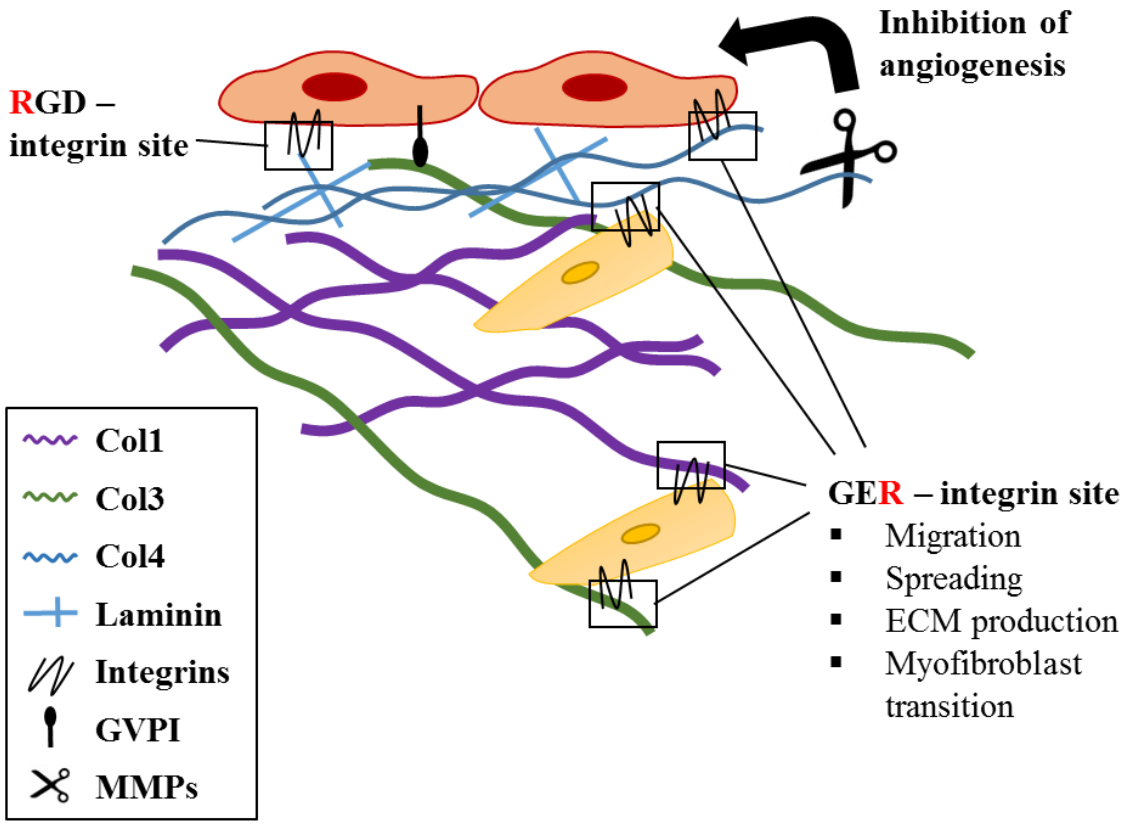

Figure 2. ECM interaction with ECs and fibroblasts
The ECM impacts cell function and homeostasis through a variety of interactions with cell surface receptors. In particular, ECs make connections through integrins with both laminin and Col4. Further, GVPI impacts ECs interaction with Col3 and cleavage products of Col4 result in an anti-inflammatory effect on ECs. Fibroblasts interact with collagens 1, 3, and 4 through integrins; the signaling generating through the contact impacts migration, ECM deposition, and myofibroblast differentiation. Highlighted in red is the arginine residue, which is a primary target for modification by methylglyoxal.

1.6 Methylglyoxal

Glycation is the process by which sugars modify macromolecules such as proteins, DNA, and lipids, thereby altering their structure and function. The process can occur due to a number of causative agents, and in our case we focus on the dicarbonyl methylglyoxal

(MG), a principal glycating agent with alternative names of acetylformaldehyde, pyruvaldehyde, pyruvic aldehyde, and 2-oxopropanal. In the human blood it is estimated that more than 99% of MG is bound to protein (Lo et al. 1994). The mechanism by which MG modifies the macromolecules is known as the Maillard reaction, which leads to the generation of AGEs. More specifically, MG is a small molecule containing two highly reactive oxygen groups and thus AGEs are formed through non-enzymatic glycation of amino acids, fatty acids or nucleotide bases (Prasad et al. 2014). Initially, a reversible Shiff base is formed, but over time this becomes a stable Amadori product, which can be rearranged into the products we call AGEs.

The synthesis of MG depends largely on triosephosphates derived from glycolysis, which normally occurs in minute amounts, but synthesis can be increased in conditions of hyperglycemia or by impairments in the enzymatic pathway responsible for metabolizing MG. Deriving from glucose or fructose, glyceraldehyde 3-phosphate (GA3P) and dihydroxyacetonephosphate (DHAP) are interchangeable through triosephosphate isomerase and both non-enzymatically degrade to form MG (Phillips and Thornalley 1993; Richard 1993). MG production can also occur through other minor pathways, such as from ketone bodies by lipid degradation, from aminoacetate by threonine degradation, or through the diet (Kalapos 2013).

MG is primarily detoxified by the glyoxalase system, comprised of glyoxalase-1 (Glo1) and Glo2. The initial and rate determining step involves Glo1 converting MG to (S)-delta-lactoylglutathione in a glutathione (GSH)-dependent manner. This product is further converted to D-lactate by Glo2, releasing the cofactor GSH (Thornalley 1990).

Aldo-keto reductase isozymes 1A and 1B are alternative mechanisms of MG metabolism; in particular aldo-keto reductase 1B has been found to be the most active in the absence of Glo1 (Baba et al. 2009).

Focusing on proteins, MG glycation occurs most often at Arginine and Lysine residues with cysteine being involved to a lesser extent. The changes exacted on these residues, typically thought to be irreversible, alter the structural characteristics of the peptide or protein as a whole, which may cause functional changes. Constituting 90% of all adducts, the main AGE formed by MG is hydroimidazolone N_{δ}-(5-methyl-4-imidazolon-2-yl)-L-ornithine (MG-H1). Arginine can also be transformed into 2-amino-5-(2-amino-5-hydro-5- methyl-4-imidazolon-1-yl) pentanoic acid (MG-H2) and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl) pentanoic acid (MG-H3) which are both interconvertible with MG-H1 (Klöpfer, Spanneberg, and Glomb 2011). Additional AGEs that can be produced with arginine occur when two MG molecules are added to the amino acid: N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine (THP) and N_{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidine) (Oya et al. 1999; Shipanova, Glomb, and Nagaraj 1997). When glycation of lysine occurs there is the formation of carboxyethyllysine (CEL), or if two lysine residues are involved then 6-{1-[(5S)-5-ammonio-6-oxido-6-oxohexyl]-4-methyl-imidazolium-3-yl}-L-norleucine (MOLD) is formed (Odani et al. 1999). Arginine and lysine modified structures can also come together to form MODIC (2-ammonio-6-({2–[4-ammonio-5-oxido-5-oxopently)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate) (Lederer and Klaiber 1999). Lastly, MG can modify cysteine to generate S-carboxyethyl cysteine (Lo et al. 1994). Information that is found within this

section is simplified below in **Fig. 3**, highlighting the important aspects involved in MG metabolism and action.

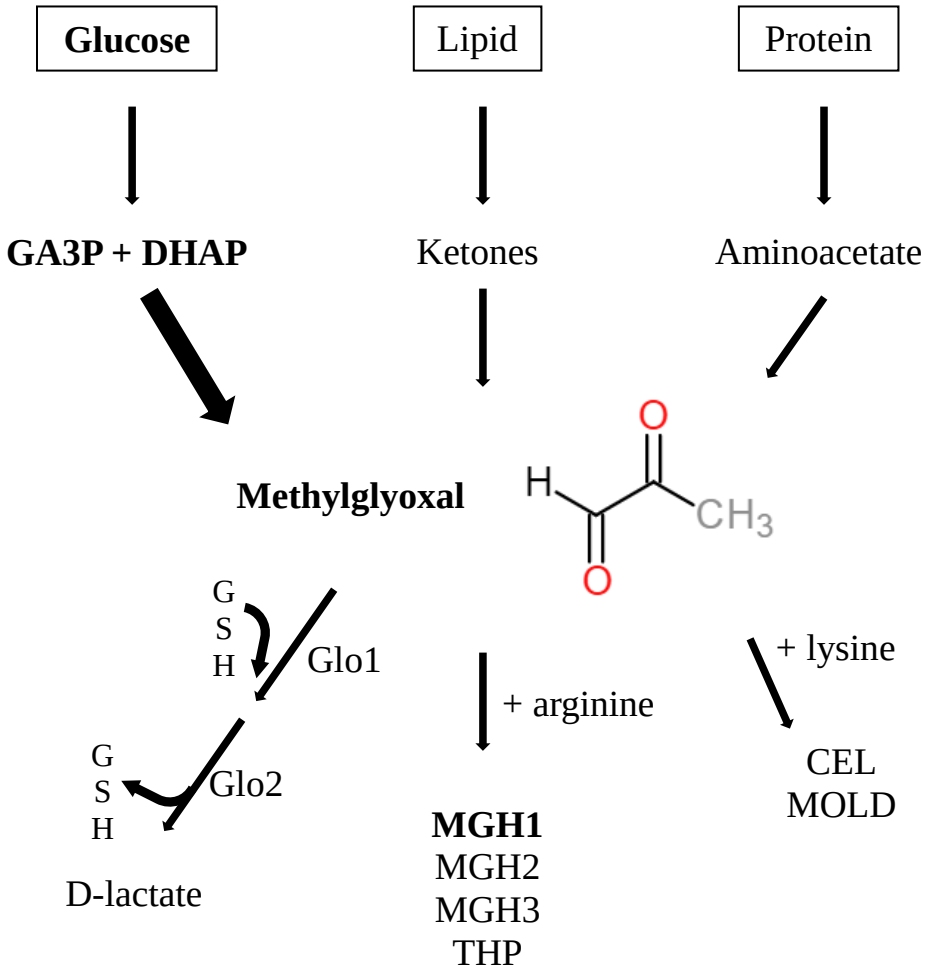

Figure 3. Summary of methylglyoxal pathway
Methylglyoxal is derived from each of the three macromolecule groups listed. The key pathway responsible for its generation is glycolysis, through the production of triosephosphates. Once produced, MG is quickly converted to D-lactate by the glyoxalase system; comprising of Glo1 and Glo2 with the need for co-factor glutathione. Non-detoxified or free MG can modify protein residues. In particular, MG-H1 derived from arginine accounts for 90% of MG adducts.

1.7 Methylglyoxal and advanced glycation end product accumulation

As outlined above, MG production depends on a buildup of its precursor triosephosphates, and thus glucose initially. Both ECs and VSMCs are known as "glucose blind" because their intake does not depend on insulin signaling. They express glucose transporter 1 (GLUT1) which means that in times of hyperglycemia these cells will allow their cytoplasmic glucose concentrations to match the extracellular hyperglycemic state causing an excess of precursors for MG synthesis. Indeed when ECs were treated *in vitro* with high glucose it was demonstrated that this led to an increase in MG levels (Dhar et al. 2010). In diabetes, this accumulation can be further exacerbated by the fact that MG causes β-cell dysfunction. Impaired insulin secretion then causes reduced glucose intake by adipose and muscle tissue, thus further elevating the amount entering ECs and VSMCs (Bo et al. 2016).

Less is known about MG's impact in cardiovascular disease in the absence of diabetes or excessive glucose as a cofactor. A study done in dialysis patients measured AGEs through autofluorescence and was able to inversely relate them to the patients' levels of diastolic function (Hartog et al. 2008). Furthermore, the same group looked at AGE autofluorescence in patients with hypertension and diastolic dysfunction. Following hypertensive pharmacological therapy, patients with higher initial AGE autofluorescence showed an attenuated improvement in diastolic function (Hartog et al. 2010). Nożyński et al. (2012) looked at cardiomyopathy patients and showed a 3-fold increase in AGE accumulation as compared to the control group. Similarly, the severity of coronary artery disease has been shown to be related to increased amounts of AGEs in patients without

diabetes (Kanauchi, Tsujimoto, and Hashimoto 2001). In other work, an ischemia/reperfusion (IR) study with isolated mouse and rat hearts determined that MG levels are increased after IR (Bucciarelli et al. 2006). This was then reproduced *in vivo* through temporary left anterior descending artery occlusion in mice, and showed a 6-fold increase in MG compared to sham animals. An increase in AGE levels followed, which with the activation of the receptor for AGEs (RAGE) was then correlated with larger infarct size (Aleshin et al. 2008). Considering the collective results of animal and human studies, the MG detoxification system seems to be compromised at times of vascular stress allowing for AGE accumulation. Alternatively, high oxidative stress can produce peroxynitrite which is proposed to lead to an accumulation of AGEs by accelerating the final step of their formation, from Amadori product to AGEs (Nagai et al. 2002). In either case, AGE accumulation within the scope of the cardiovascular system is an issue that needs further clarification.

1.8 Methylglyoxal and the receptor for AGEs

MG causes cellular and molecular dysfunction by altering the targeted molecule's composition and thus affecting its function. AGEs may crosslink at an increased rate and this is of particular interest regarding structural protein arrangement and physical properties (Ulrich and Cerami 2001). However, as much emphasis should be put on the signaling abilities of AGEs. MG adducts and modifications can influence both apoptotic and proliferative pathways through their receptor: RAGE. The pro-apoptotic signal was associated with p38 mitogen-activated protein kinase (MAPK)/c-Jun N-terminal kinase

(JNK) signaling in osteoblasts, while in human cardiomyocytes this was related to decreased B-cell lymphoma 2 (Bcl-2) levels (Tsoporis et al. 2010; Alikhani et al. 2007). JNK and glycogen synthase kinase 3 beta (GSK3β) pathways have also been linked to RAGE mediated apoptosis in mouse cardiomyocytes (Shang et al. 2010). MAPKs control cell proliferation, and thus RAGE modulation may impact the cell cycle. In VSMCs, low doses of AGEs increased proliferation while high doses did the opposite, and for ECs, low doses inhibit proliferation (Satoh et al. 1997). Further, RAGE can activate the Janus kinase/ signal transducer and activator of transcription (JAK/STAT) pathway which has an increased proliferative effect on interstitial fibroblasts and VSMCs (Huang et al. 1999; Sakaguchi et al. 2003).

Nuclear factor kappa beta (NF-κB), a transcriptional factor that stimulates inflammation, can be activated by RAGE, causing increases in inflammatory cytokines such as TNF-α and IL-1β. NF-κB can also be activated directly by MG, giving two synergistic mechanisms of inflammation. In retinal pericytes, MG caused an increased rate of apoptosis through NF-κB signaling (J. Kim et al. 2004). NF-κB can also increase the expression of adhesion molecules: ICAM-1, VCAM-1 and E-selectin, for the attraction of immune cells (Albelda, Smith, and Ward 1994). MG stimulation of ICAM-1 through NF-κB was implicated *in vitro* and *in vivo* with hypertensive rat VSMCs (Wu and Juurlink 2002). Similarly, in ECs, VCAM-1 and monocyte chemoattractant protein-1 were upregulated in response to RAGE and likely mediated by NF-κB (Feng et al. 2005). Additionally, NF-κB can deactivate Glo1 and increase RAGE transcription; both serving as positive feedback for MG-AGE effects (J. Li and Schmidt 1997).

1.9 Methylglyoxal induced oxidative stress

Reactive oxygen species (ROS) are reduced by many different mechanisms in the body to prevent oxidation, which has a deleterious effect on many cellular functions. One important molecule in these processes is GSH, which, consequently, is also required for the detoxification of MG. Thus, the use of the same pathway makes MG a pro-oxidative molecule. This may be of particular importance in a post-MI environment due to the reperfusion that occurs physiologically or through surgery. A study using human ECs showed, by cationic dye, 80% more ROS when they were cultured in 0.4 mM of MG (Figarola et al. 2014). Looking at rat cardiomyocytes, MG-AGEs are able to activate the PKC signaling pathway which leads to increased ROS production and cell apoptosis (L. Zhang et al. 2014). ROS quantification through an dichloro-dihydro-fluorescein diacetate (DCF) assay demonstrated that MG caused a 6-fold increase in rat cardiomyocytes (Dhar et al. 2016). Likewise, a DCF assay showed a 67% increase in ROS when VSMCs were treated with 0.1 mM of MG, and an increase of peroxynitrite was identified using an NO probe and an O_2 scavenger (T. Chang, Wang, and Wu 2005). Thus, MG demonstrates a pro-oxidative role throughout the cardiovascular system.

MG promotes ROS production through many pathways, some of which involve increasing the activity of oxidant producing enzymes, disrupting the mitochondria or inactivating antioxidants. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been implicated in atherosclerosis and is a known producer of superoxides through the transfer of electrons from NADPH to O_2 (Konior et al. 2014). An investigation using rat kidney cells provided evidence that NADPH pathway has a role in

MG pro-oxidation (Ho et al. 2007). This pathway was further solidified with similar results of MG-induced NADPH oxidase activation and production of ROS in human ECs (Dhar et al. 2010). In the vasculature, nitric oxide synthase (NOS) which makes NO for vascular dilation, also provides a key precursor of peroxynitrite. This oxidative pathway was upregulated in VSMCs treated with MG (H. Wang et al. 2006). The mitochondria are the primary site of ROS production. As such, MG was used to treat isolated mitochondria and shown to inhibit, in an NADP dependent manner, the normal function of the tricarboxylic acid cycle and electron transport chain. MG's effects on the mitochondria lead to a decreased use of O_2 by these respiratory pathways and thus a buildup of ROS (Rosca et al. 2002). Antioxidant superoxide dismutase is able to turn superoxides into hydrogen peroxide, through catalase or GSH peroxidase hydrogen peroxide to produce water and oxygen (Halliwell 1974). Consequently, MG exerts an inhibitory effect on each of these enzymes resulting in the accumulation of ROS (Yong Seek Park et al. 2003; Choudhary, Chandra, and Kale 1997). Mitochondrial failure and the cellular toxicity that comes with increased ROS has long been linked to CVD etiology, especially given that the tissue is highly oxidative, thus the above evidence implicates MG in CVD progression (Sugamura and Keaney 2011).

1.10 MG, ECM, possible outcomes

Given that MG and AGEs can accumulate extracellularly, it may be important to consider how this affects the ECM and ultimately, the function of the heart. A lot of structural proteins have long turnover rates, which is particularly true for collagen, and this makes

them an easy target for non-enzymatic changes (Paul and Bailey 1996). Considering the previous section on the ECM (see 1.4), Col1, Col3, Col4, and laminin have integrin binding sites that contain an arginine residue, which is the main target of MG (Y. Xu et al. 2000; Sasaki and Timpl 2001). This is also true for the peptide sequence involved in the binding of GPVI to Col3 (Jarvis et al. 2008). The same cannot be said for the binding sites of DDRs to collagens, however there still remains a possibility of a structural alteration to the site (Konitsiotis et al. 2008). Finally, dystroglycan binding of laminin involves many residues with arginine and lysine being key residues in the structure of the domain (Tisi et al. 2000). Thus, glycation modifications may occur and disrupt many sites required for cell-ECM interaction thereby affecting the function of cardiac cells.

A key cell type to consider are ECs: due to the fact that MG is pro-inflammatory and pro-apoptotic, it is possible that ECM-AGEs may lead to their dysfunction. Changes in ECs interaction with the ECM in the injured heart environment (e.g. post-MI) may impact their important functions of revascularization and paracrine signaling to cardiomyocytes. With studies already showing that the arrangement and density of the ECM can have a significant impact on angiogenesis, glycation may have a similar outcome (Edgar et al. 2014). Fibroblasts are also important as they are involved in mediating remodeling of the ECM. Their function is likely to change in response to a glycated matrix environment both due to receptor effects and mechanical changes. Fibroblasts are important in mediating post-MI repair through the deposition of ECM proteins (van Nieuwenhoven and Turner 2013). The fact that AGEs can cause greater cross-linking may lead to a stiffer ECM, which in a post-MI heart can lead to fibrosis by inducing further transition of resident fibroblasts to the myofibroblast phenotype (Galie,

Westfall, and Stegemann 2011). Recently, our lab demonstrated that MG-AGEs are produced post-MI and that their accumulation is deleterious to the repair and function of the myocardium (unpublished). These effects were reduced by Glo1 overexpression, which was attributed, in part, to reduced MG modification of the cardiac ECM. However, the mechanisms underlying these observations remain to be better elucidated; how MG-mediated changes to the cardiac ECM can affect the function of various cell types in the heart post-MI is important to better our understanding and treatment of cardiovascular disease.

1.11 Rationale, hypothesis and objectives

ECM proteins have a long half-life and contain residues that are primary targets for glycation by MG. Given the importance of cell-ECM signaling, MG modification of the ECM may have wide reaching effects. Given that MG is elevated post-MI and that the ECM, fibroblasts and ECs play crucial roles in cardiac stability and reperfusion, **we hypothesize that MG-induced ECM modifications negatively affect the proper functions of fibroblasts and ECs**.

This project aims to elucidate the involvement of MG-mediated glycation of ECM proteins in heart function using human cell cultures, primary mouse cell cultures, and a mouse model of myocardial infarction. Specifically, the aims were to:

1. Establish which key ECM proteins are glycated by MG, as well as determine an approach to be used for glycation of those proteins.
2. Test the effect of MG-modified versus un-modified protein on the function (e.g. viability, adhesion) of cultured endothelial cells and fibroblasts
3. Investigate ECM protein glycation in the post-MI mouse heart.

Ultimately, we hope to determine whether glycation of the ECM may be a mechanism that contributes to the poor repair and function of the post-MI heart.

CHAPTER 2: MATERIALS AND METHODS

2.1 ECM protein coating and MG treatment

ECM proteins were obtained commercially and coated according to manufacturers' instructions. In brief, rat tail Col1 (Corning) was diluted to 85.75 µg/mL using 0.02 N acetic acid, human placenta Col3 (Advanced Biomatrix) was diluted to 80 µg/mL using 0.01 M HCl, human placenta Col4 (Sigma) was diluted to 100 µg/mL using 0.5 M acetic acid and, mouse laminin (Corning) was diluted to 50 µg/mL using PBS. The diluted solutions were used to coat culture dishes (eg. 400 µl per well of 12-well plate) for 1 hour at room temperature. The protein coated culture dishes were stored at 2-8 °C and, PBS washed prior to use.

MG (Sigma) was diluted to 0.01, 0.1, and 1.0, mM concentrations in PBS. Previously coated ECM protein culture wells were then covered in the diluted solution or PBS alone and incubated overnight at 37 °C. A PBS wash followed the incubation. The concentration of 1 mM MG was used for all experiments, with the exception of initial ECM glycation testing and the angiogenesis assays.

Additional troubleshooting experiments for protein glycation involved treatment of proteins in Eppendorf tubes (alternative to coating dishes), and the modification of non-structured collagen, attachment factor (Thermo Scientific), for comparison.

2.2 Animal procedures

All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Experimental MI was induced in C57BL/6J (Charles River) female mice as previously described (Ahmadi, McNeill, et al. 2014; Blackburn et al. 2015), and performed by Rick Seymour, an expert animal technician. Briefly, mice were anaesthetized under 2.5% isofluorane, intubated and kept under mechanical ventilation. Buprenorphine was administered for perioperative analgesia. A fourth intercostal thoracotomy exposed the heart and, a left anterior descending artery ligation was performed below the atrium and with a silk suture. MI was confirmed through blanching of the area. The non-MI group of animals consisted of normal healthy mice that received no procedure. At 5 days post-MI mice were sacrificed and the hearts were collected under sterile conditions; further details on collection and processing can be found below (sections 2.4 and 2.5).

2.3 Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs; Life Technologies) were cultured in medium 200 with low growth serum supplement (Life Technologies) under normoxic conditions (21% O_2, 5% CO_2) at 37 °C. The media was changed every 2-3 days. Lifting cultured cells was done with 0.25% trypsin in PBS, with passages 4-12 being used in the *in vitro* assays.

2.3.1 Viability assay: HUVECs were seeded at 5×10^4 in 12-well plates with MG-modified or unmodified ECM proteins, and cultured at 37 °C for 48 hours under normoxic or hypoxic (1% O_2, 5% CO_2) conditions. Viability was evaluated using the trypan blue exclusion assay. Briefly, culture media was collected along with lifted cells, and the cell suspension was then automatically processed by a ViCell cell viability analyzer (Beckman Coulter) or combined with equal parts trypan blue solution (Sigma) and counted with a hemocytometer.

2.3.2 Adhesion assay: 5×10^4 HUVECs were seeded for 1 or 4 hours under normoxic conditions at 37 °C and on MG-modified or unmodified ECM protein coated 12-well dishes. Following the incubation, the cells were fixed with paraformaldehyde (PFA), stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma), and quantified by microscopy from 4 random fields-of-view.

2.3.3 Angiogenesis assay: The ECMatrix™ angiogenesis assay (Chemicon) was used according to manufacturer's instructions. Briefly, 15 μL of ECMatrix™ was added to each well of a 15-well μ-slide (ibidi), allowed to gel at 37 °C for 1 hour, and was then treated with 0.1 mM MG overnight. The next day, 5×10^3 HUVECs were seeded per well, 50 μL of m200 media was added, and plates were placed in a 37 °C incubator under normoxic conditions. Pictures were taken at 6 and 20 hours, and the total tube network length was quantified with ImageJ.

2.3.4 Adhesion protein expression: HUVECs were seeded at a density of 5×10^4 cells/well in MG-modified or unmodified ECM protein coated 12-well culture dishes. These

cultures were maintained in normoxia and at 37 °C for 48 hours. Following the incubation, cultures were collected for western blot analysis (section 2.6).

2.4 Fibroblast cell culture

Cardiac fibroblasts were isolated from the hearts of C57BL/6J mice after sacrifice, as previously described (Ahmadi, Vulesevic, et al. 2014). Briefly, hearts were extracted and perfused with PBS under sterile conditions. Next, hearts were minced and added to Hanks balanced salt solution (Life Technologies). A digestion buffer (2.4 U/mL Dispase II Roche, 0.1% Collagenase B Roche, 5M NaCl, 1M HEPES pH 7.5) was then added and the samples were incubated for 30 minutes at 37 °C with mixing by inversion every 5 minutes. Centrifugation for 5 minutes at 530 g allowed for removal of the supernatant and resuspension of the cellular pellet in DMEM/F-12 with 10% fetal bovine serum (FBS; Life Technologies). The cells were cultured under normoxic conditions and at 37 °C, with a thorough PBS wash being done 6 hours after initial seeding or the day after. Culture media was changed every 2-3 days.

2.4.1 α-SMA expression: Primary fibroblast passages 2-4 were used to seed 7×10^4 cells onto MG-modified or unmodified ECM protein coated culture dishes. These cultures were maintained in normoxia and at 37 °C for 48 hours. Following the incubation, the fibroblasts were fixed with PFA, washed with TBST, and stained overnight at 2-8 °C in 10% FBS/PBS with rabbit anti-alpha smooth muscle actin antibody (abcam). The samples were then washed with TBST and 10% FBS/PBS with secondary Alexa Fluor® anti-rabbit 568 (Life Technologies) was applied for an hour at room temperature.

Mounting was done with ProLong™ antifade and images were captured by fluorescence microscopy. Cultured cells were also collected for western blot analysis (section 2.6).

2.4.2 Lysyl oxidase assay: Primary fibroblast passages 2-4 were used to seed 7×10^4 cells onto MG-modified or unmodified collagen coated culture dishes. These cultures were maintained in normoxia and at 37 °C for 48 hours. Media was collected from each well and stored at −20 °C or utilized as described by the Lysyl Oxidase Activity Assay Kit (abcam). Briefly, in a 96-well plate 50 µL of media was combined with equal parts of assay reaction mixture (HRP Substrate, Assay Buffer, HRP, DMSO), and the plate was incubated in the dark at 37 °C for 10 to 30 minutes. Recombinant human lysyl oxidase homolog 2 (R&D Systems) was used as the standard. With a plate reader, fluorescence was monitored at Ex/Em = 540/590 nm and absorbance at 576 nm.

2.5 Decellularization and cardiac ECM evaluation

2.5.1 Decellularization: Under sterile conditions, hearts from C57BL/6J mice were excised and perfused with cold PBS. The decellularization technique was adapted from a protocol previously described by Singelyn et al. (2009). In brief, hearts from MI and healthy mice underwent a 3-day wash in 1% SDS/PBS, followed by a 2-day wash in 1% TritonX-100/PBS and then a final overnight wash in distilled water. Confirmation of decellularization was done through Toponin I analysis as described below (section 2.6). The tissue was analyzed by immunohistochemistry (section 2.5.2) or by western blot (section 2.6) as described below.

2.5.2 Immunohistochemistry: Decellularized hearts were frozen in liquid nitrogen in VWR® Clear Frozen Section compound. Hearts were stored at −80 °C until slide preparation with 10 μm tissue cryo-sections. Slides underwent a 20 minute air dry at room temperature followed by fixation for 20 minutes in ice-cold acetone. Following washes in TBST and PBS, the sections underwent a 2.5 hour blocking period at room temperature in 10% FBS/PBS with goat anti-mouse IgG (Jackson). Next, slides were washed in TBST and incubated with primary antibodies overnight at 2-8 °C: mouse anti-MG-H1 monoclonal antibody (Cell Biolabs) or rabbit anti-collagen 1 polyclonal antibody (abcam) were used. Again washing with TBST was done, followed by application of Alexa Fluor® secondary antibodies (in 10% FBS/PBS) for an hour at room temperature: anti-mouse 488 or anti-rabbit 568 (Life Technologies). Subsequently, the slides were washed with TBST and mounted with ProLong™ antifade for image capture by fluorescence microscopy.

2.5.3 Collagen isolation: Under sterile conditions, hearts from C57BL/6J mice were excised and perfused with cold PBS. The collagen isolation technique was adapted from a protocol previously described by Pacak, Powers, and Cowan 2011. In brief, the heart was minced and subjected to 7 washes (5 minutes of agitation per wash) with 0.5 M Sodium Acetate. This was followed by one wash with dH_2O and one wash with 0.075M Sodium Citrate. At this point 40 minutes of agitation was done without changing the Sodium Citrate buffer. Centrifugation was done at 3200 g for 10 min, followed by an additional 30 min at 3200 g with a MWCO Amicon Ultra-15 centrifugal filter device (Millipore). The sample was then extracted and analyzed with a collagen type 1 antibody (section 2.6).

2.6 Western Blot

2.6.1 ECM protein: Wells were coated in protein and treated ± MG as previously described. Sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 45 mM 1,4-dithiothreitol, 1.5 μM bromophenol blue) was added to each well, the wells were scraped and the samples collected. Samples were incubated at 50 °C for 30 minutes and then processed as described below.

2.6.2 Hearts: Decellularized tissue was minced and suspended in ice-cold RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) with a proteinase inhibitor cocktail (Roche) for 30 minutes and then processed as described below.

2.6.3 Cell culture: Culture media was discarded from each well and RIPA buffer with proteinase inhibitors was added. The cultures were scraped, collected, and kept on ice for 30 minutes and then processed as described below.

Lysates obtained as described above were ready to use or were stored at −20 °C. Lysate concentrations were quantified through a BCA protein assay or with A280 on a nanodrop spectrophotometer (Thermo Scientific). Protein separation was done on a 12% SDS-PAGE gel at 35 mA per gel. Gel to nitrocellulose membrane protein transfer was done at 100 V for 1.5 hours. Following transfer, blocking was done for an hour at room temperature with 5% milk in TBST; although 3% BSA in TBST was used for membranes undergoing anti-MG-H1 blotting. Primary antibody incubation was done overnight with the use of: mouse anti-MG-H1 monoclonal antibody (Cell Biolabs), rabbit anti-collagen 1 polyclonal antibody (abcam), rabbit anti-cardiac troponin I polyclonal antibody (abcam),

rabbit anti-VE cadherin polyclonal antibody (abcam), rabbit anti-CD31 polyclonal antibody (abcam), rabbit anti-alpha smooth muscle actin polyclonal antibody (abcam), or rabbit anti-tubulin (Cell Signalling). Following TBST washing, the membrane was then incubated for an hour at room temperature with anti-mouse and anti-rabbit secondary antibodies (Cell Signalling). For multiple antibody blotting, the membrane was treated with Restore western blot stripping buffer (Thermo Scientific) for 15 minutes. After secondary incubation, membranes were treated with Clarity Western ECL substrate and exposed to CL-Xposure film (Bio-Rad). Following development, image blots were quantified with ImageJ.

2.7 Statistical analysis

Data values are presented as mean ± standard error of mean, percent change as compared to control ± standard error of mean, or fold-change of treatment to control ± standard error of mean. Normality of data sets was confirmed by the Shapiro-Wilk normality test. Two group comparisons were done with the use of a two-tailed paired student's t-test. Statistical significance was given in the case of $p<0.05$.

CHAPTER 3: RESULTS

3.1 MG glycates ECM proteins *in vitro*

After coating different ECM proteins (laminin, and collagen types 1, 3, and 4) onto tissue culture plates, they were then treated with 0, 0.1 or 1.0 mM MG in PBS overnight. Evaluation by spectrophotometry revealed that equivalent amounts of protein were plated for the treatment and control groups (**Fig. 4A**). Western blot analysis of the samples using an anti-MG-H1 antibody showed a lack of protein glycation in all PBS lanes and a dose-dependent increase in MG-H1 in all the MG-treated samples (**Fig. 4B**). Additionally, there was an absence in MG alone loaded lane (Appendix **Fig. A1**). For analysis of protein glycation by use of a dot blot, Col1 and Col3 were treated with 0, 0,01, 0.1 or 1.0 mM MG and the generation of MG-H1 was evaluated. Results of the dot blot confirm that MG dose-dependently glycates Col1 and Col3 proteins (**Fig. 4C**).

To exclude protein degradation during coating, ECM proteins were treated with 1.0 mM MG in an Eppendorf without being subjected to the coating process. The outcome (Appendix **Fig. A2**) was indistinguishable to that seen in proteins collected from wells (**Fig. 4B**). Lastly, MG modified attachment factor gelatin was used as a degradation comparison to Col1. The blot (Appendix **Fig. A2**) displayed more of a smeared signal with the gelatin as compared to Col1, suggesting Col1 sample is more intact.

3.2 HUVEC viability is not altered by MG-modified ECM

Endothelial cells and angiogenesis are important for restoring perfusion in the post-MI heart. Our previous work has demonstrated increased MG production and accumulation post-MI, and that the cardiac ECM is a target for glycation by MG. Therefore, we sought to determine if MG-modification of ECM proteins has an effect on the viability of endothelial cells. HUVECs were cultured on MG-modified and unmodified laminin and collagens 1, 3, and 4 for 48 hours, after which viability was assessed. Under normoxic conditions, HUVEC viability was equivalent for all tested substrates, and MG modification had no effect for any condition (**Fig. 5A**). To more closely mimic the ischemic post-MI environment, HUVEC viability was then assessed on the different MG-modified and unmodified ECM proteins in hypoxic conditions. No difference in viability was observed between any of the substrates, and MG modification did not affect the ECM protein's ability to support viable HUVECs (**Fig. 5B**). Therefore, MG modification of laminin and collagens 1, 3 and 4 does not affect the viability of HUVECs in the normoxia and hypoxia conditions tested.

3.3 MG-modified ECM reduces HUVEC adhesion

We also investigated if MG modification of ECM proteins affected the adhesion of ECs. MG modification of laminin, Col3 and Col4 reduced the number of HUVECs that adhered after 1 hour compared to their respective unmodified controls. However, the glycation of Col1 by MG had no effect on HUVEC adhesion (**Fig. 6A**). Also, we looked at a later time point in order to explore whether the ECs could compensate for the

decrease in initial adhesion. After 4 hours, the reduced adhesion of DAPI positive cells persisted (**Fig. 7B**); MG modified Col3, Col4, and laminin had less HUVECs adhered as compared to the cultures on respective unmodified ECM proteins (**Fig. 7A**). Thus, with the exception of Col1, ECM protein glycation reduces the ability of HUVECs to adhere.

3.4 Evaluating adhesion molecules PECAM-1 and VE-Cadherin

EC adhesion and function is dependent on junction proteins such as PECAM-1 and VE-cadherin. Given that we observed reduced adhesion of HUVECs to MG-modified ECM proteins, we sought to determine if this may be, at least in part, due to changes in the expression of these adhesion molecules. When cultured on modified Col1, Col4 or laminin, there was no identifiable difference in the expression of either PECAM-1 (**Fig. 8**) or VE-cadherin (**Fig. 9**) as compared to the control culture counterpart. The MG modified Col3 coating also did not seem to influence PECAM-1 expression but, it appears to be slightly influencing VE-cadherin expression as there was about a 7% decrease (**Fig. 9A**) when compared to the control. Despite reduced adhesion, EC interaction with MG-modified ECM proteins did not lead to altered expression of these adhesion and angiogenic molecules.

3.5 Modified ECM Matrigel has decreased angiogenic properties

To determine if MG modification of the ECM affects the angiogenic potential of ECs, HUVECs were cultured using the commercially available ECMatrix angiogenesis assay. ECMatrix was treated with MG or PBS, rinsed and then HUVECs were added to the

surface and cultured for 6 or 20 hours. The images taken at 6 hours (**Fig. 10A**) showed cells to be more clustered on MG treated matrix as compared to the control PBS. The network length was decreased by about 36±4% (**Fig. 10C**), however this was only a trend (p<0.07). At the 20 hour time point, both tube networks retracted in size compared to their 6 hours levels (**Fig. 10B**). Furthermore, at the 20 hour time point (**Fig. 10D**) there was a significant decrease in the total length of capillary-like structure formations by HUVECs on the MG-modified ECMatrix (by 35±5%; p<0.05).

3.6 α-SMA expression in fibroblasts cultured on MG-modified ECM proteins

The phenotypic transition from fibroblast to myofibroblast is important in infarct stability during MI repair. A main identifying factor for myofibroblasts is the presence of α-SMA positive stress fibres, which allow the cells to undergo wound contraction. However, the maintenance of the myofibroblast state is generally associated with fibrosis and progression of heart failure due to increasing cardiac stiffness (van Nieuwenhoven and Turner 2013). To evaluate the impact of MG AGEs, primary cardiac fibroblasts, freshly isolated from mouse hearts, were cultured on MG-modified and unmodified laminin and collagens 1, 3 and 4 for 48 hours. The myofibroblast transition was assessed by α-SMA expression, both through the use of western blot and immunohistochemistry (**Fig. 11**). The two methods gave different results. Western blot quantification (**Fig. 11A, B**) demonstrated no change in protein expression levels. In contrast, staining of the fixed cultures appeared to indicate an increased presence of α-SMA in fibroblasts cultured on MG modified ECM proteins (**Fig. 11C**).

3.7 Lysyl oxidase expression in fibroblasts cultured on MG-modified ECM proteins

Previous evidence identified that LOX is upregulated in hypoxia as well as possibly in response to AGEs (Papachroni et al. 2010). Additionally, it has been implicated in fibrosis (Hermida et al. 2009). As such, we attempted to evaluate the effect of MG-modified and untreated Col1 and Col3 on the expression of LOX in primary cardiac fibroblasts. Following 48 hours of culture, fibroblast media was evaluated by fluorescence and absorbance using a commercial lysyl oxidase activity kit. Unfortunately, negative control values consistently displayed value ranges similar to the standard and samples, thus the data was unusable (Appendix **Fig. A1**).

3.8 Mouse heart decellularization and MG modification

Following the significant results that were seen *in vitro* with MG-ECM, we proceeded to attempt measurement of MG ECM protein AGEs *in vivo*. MI was induced in mice by left anterior descending artery ligation, followed by harvesting of the heart tissue 5 days later. Decellularization was done through a series of detergent (SDS, Triton) washes in order to isolate the ECM and thus give us the ability to distinguish ECM MG-H1 modifications apart from other MG-H1 modified proteins.

Initial western blot analysis was done for the cardiomyocyte marker Troponin I, and the absence of this signal in tissues (**Fig. 12A**) suggested a successful decellularization process. Additionally, *in vitro* treatment of decellularized hearts with MG produced a positive MG-H1 signal (**Fig. 12A**), indicating the ability of the ECM to be glycated post-process along with serving as a positive control for further analysis.

Western gel bands suggest a decrease in ECM MG-H1 post-MI (**Fig. 12C**). Upon quantification, the signal proved to be approximately 3-fold lower in post-MI decellularized hearts as compared to their healthy counterparts (**Fig. 12B**; $p<0.05$). IHC results showed a similar trend, however many sections for both experimental groups lacked any MG-H1 signal (**Fig. 12D**).

A collagen pulldown protocol previously described by Pacak, Powers, and Cowan 2011 was attempted in order to evaluate MG-H1 modifications in more detail. Extracted hearts were subject to decellularization, washes and molecular weight isolation. The sample's integrity was evaluated by blotting for Col1 however, the signal produced was not banded (Appendix **Fig. A3**), suggesting a degradation or lack of isolation of collagen through the process.

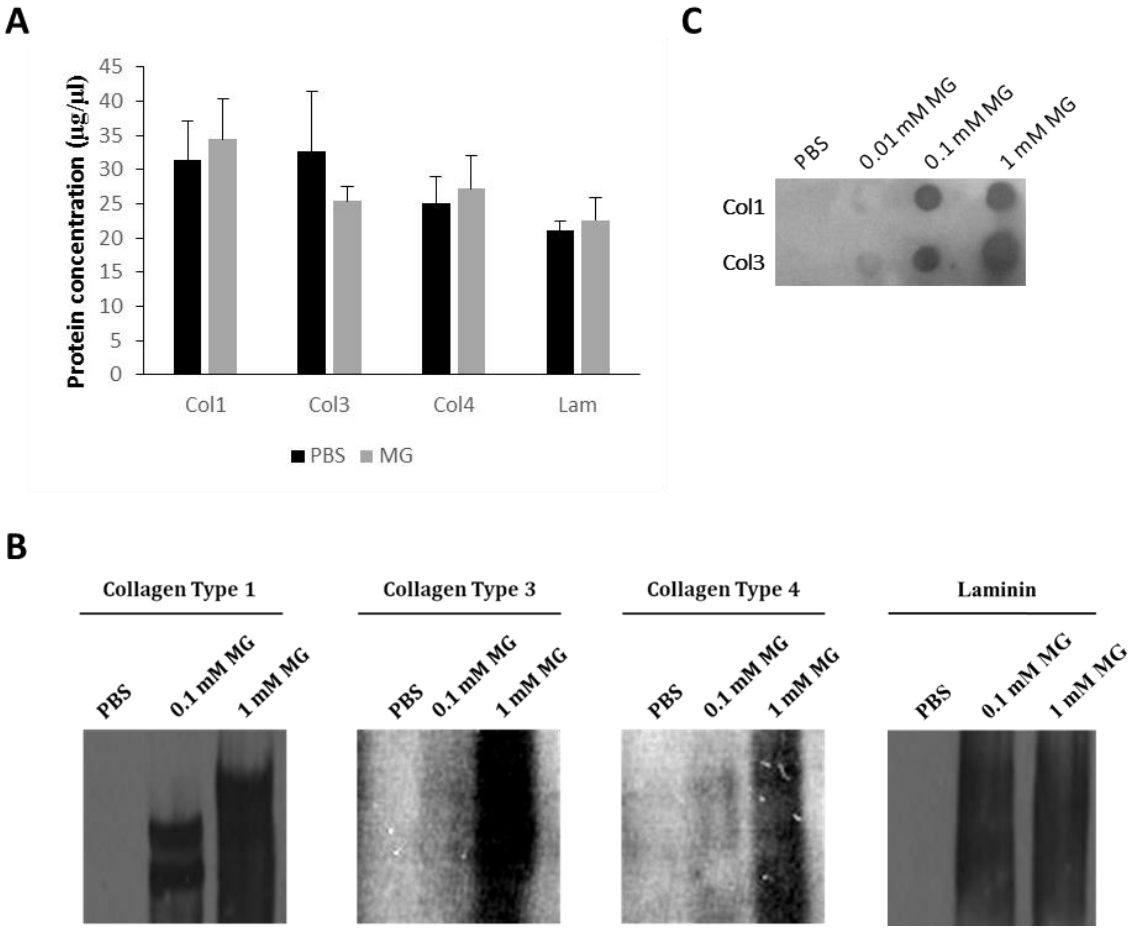

Figure 4. **Assessment of the ability of MG to glycate ECM proteins *in vitro***
Collagen types 1, 3, and 4, and laminin were used to coat culture dishes. The proteins were modified overnight with 1) methylglyoxal in PBS or 2) PBS. Spectrophotometry (A) demonstrated no difference in protein concentration between MG and PBS treated samples, *n*=3. Western blots (B) *n*=3 and dot blots (C) *n*=2, had positive MG-H1 signals for samples treated with MG and absence in PBS treated samples.

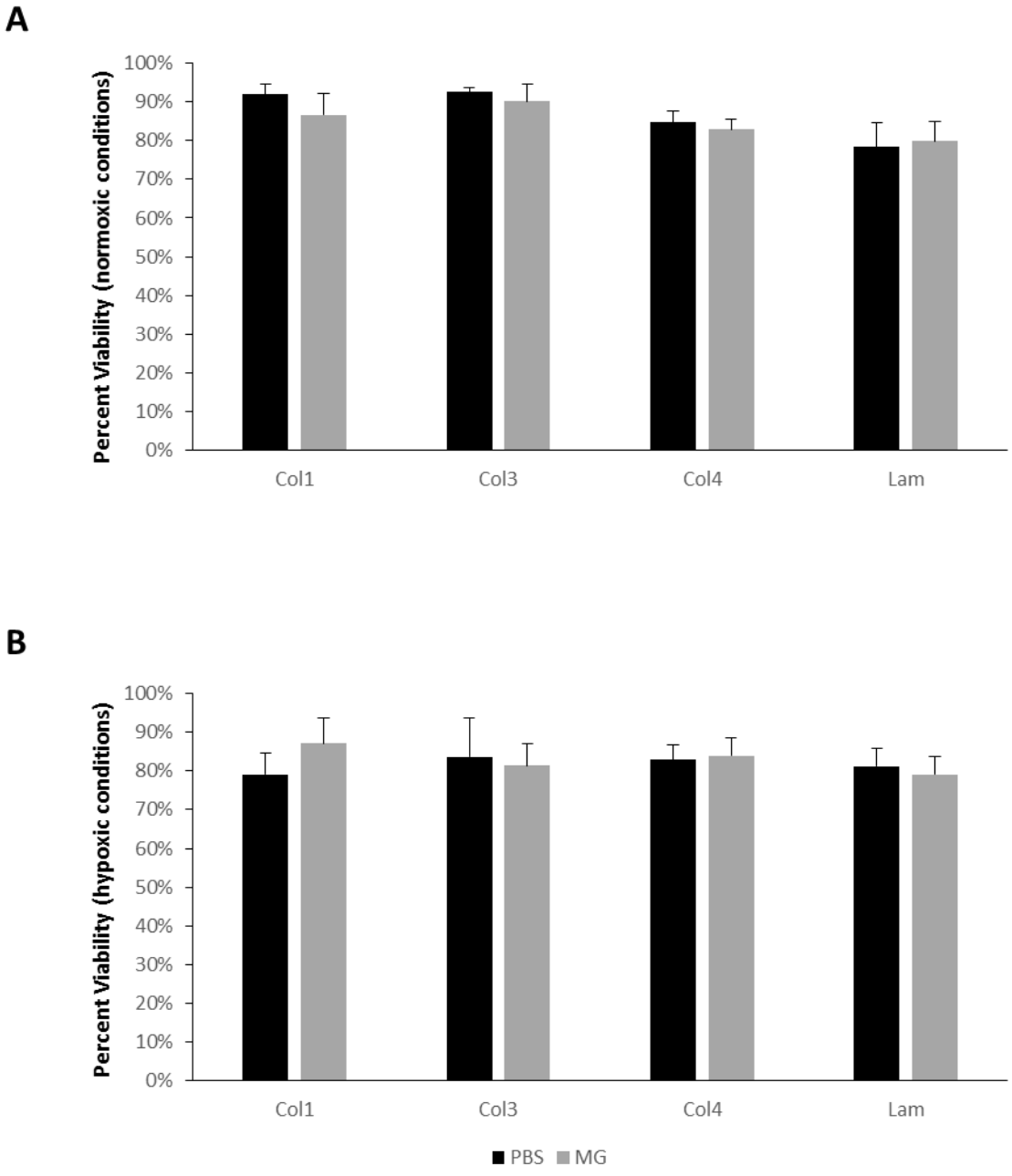

Figure 5. **Impact of MG modifications on HUVEC viability**
HUVEC viability was assessed after 48 hour culture on either MG modified or unmodified ECM protein coated culture dishes. ViCell and hemocytometer percent viability counts were done after incubation in normoxic (A) and hypoxic (B) conditions showing no difference between cultures for MG and PBS treated ECM proteins *n*=3.

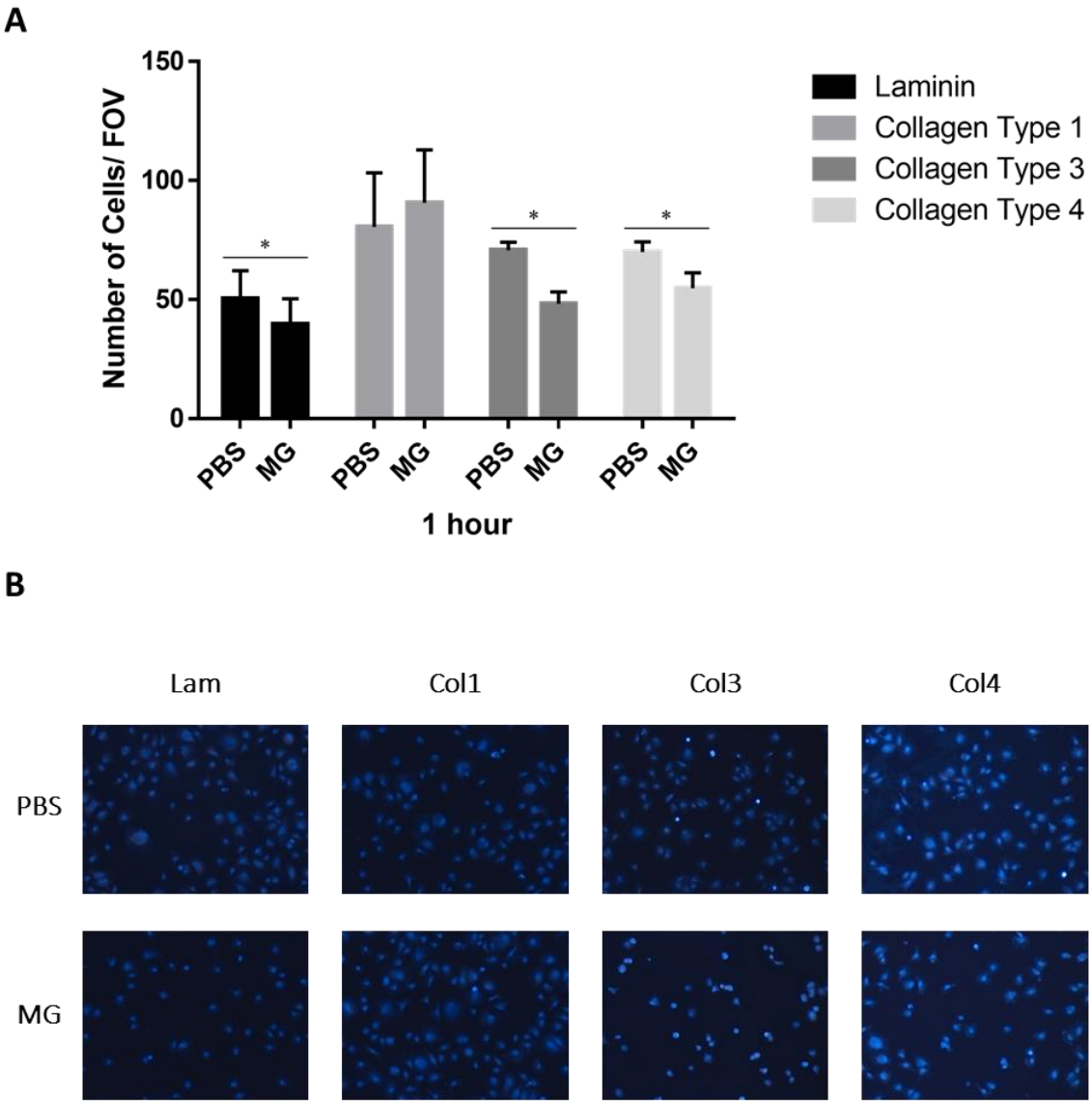

Figure 6. **Effects of MG modification on initial HUVEC adhesion**
Laminin, and collagen types 1, 3, and 4 were used to coat culture dishes and treated with MG or PBS. HUVEC adhesion was assessed after 1 hour, with cultures on MG treated laminin, Col3, and Col4 displaying a decrease in cells/FOV (A). Adhesion was determined by DAPI stained nuclei, shown by the representative images (B) *p<0.05, n=4-5.

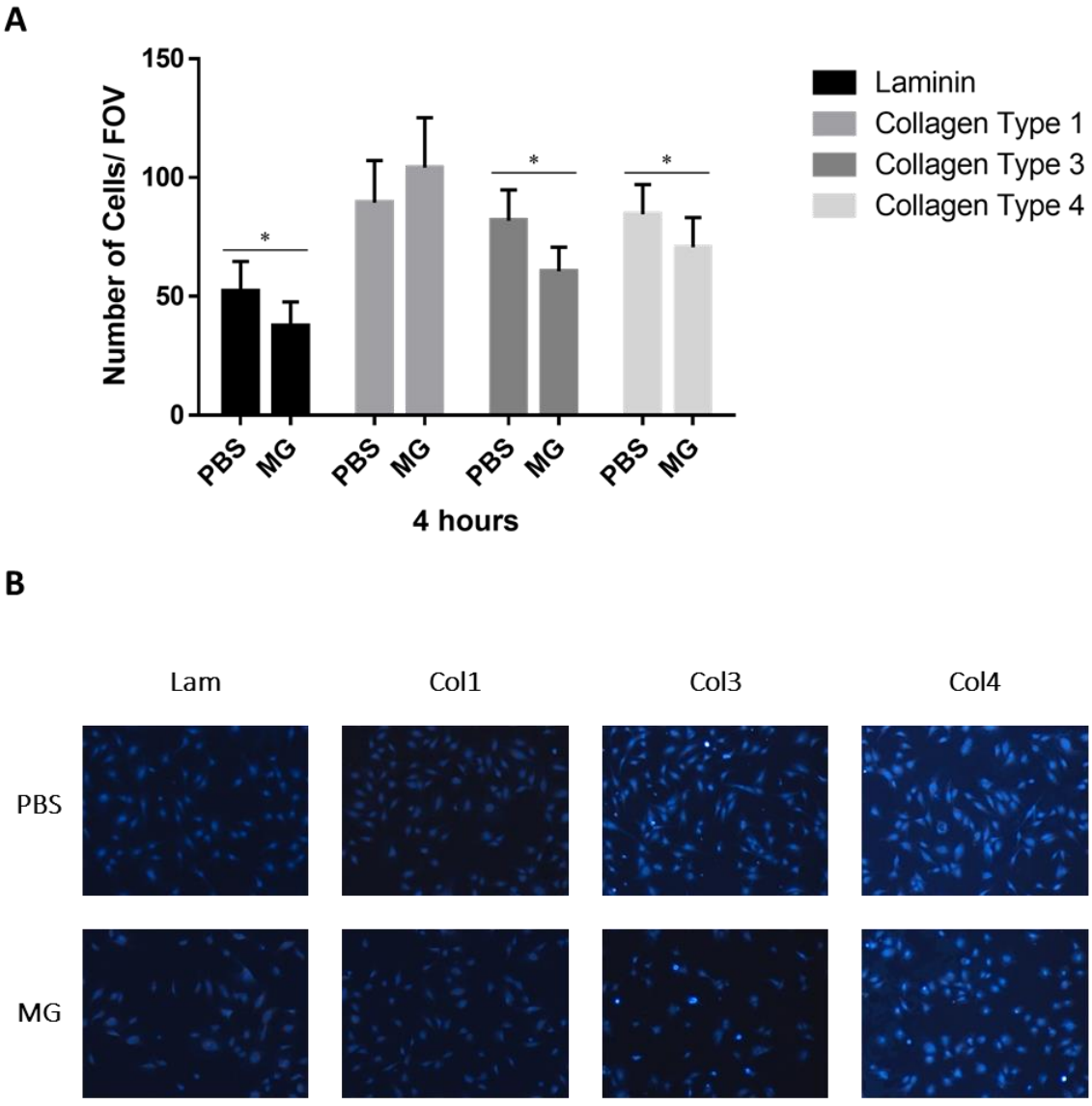

Figure 7. **Effects of MG modification on maintained HUVEC adhesion**
Laminin, and collagen types 1, 3, and 4 were used to coat culture dishes and treated with MG or PBS. HUVEC adhesion was assessed after 4 hours, with cultures on MG treated laminin, Col3, and Col4 displaying a decrease in cells/FOV (A). Adhesion was determined by DAPI stained nuclei, shown by the representative images (B) *p<0.05, n=4-5.

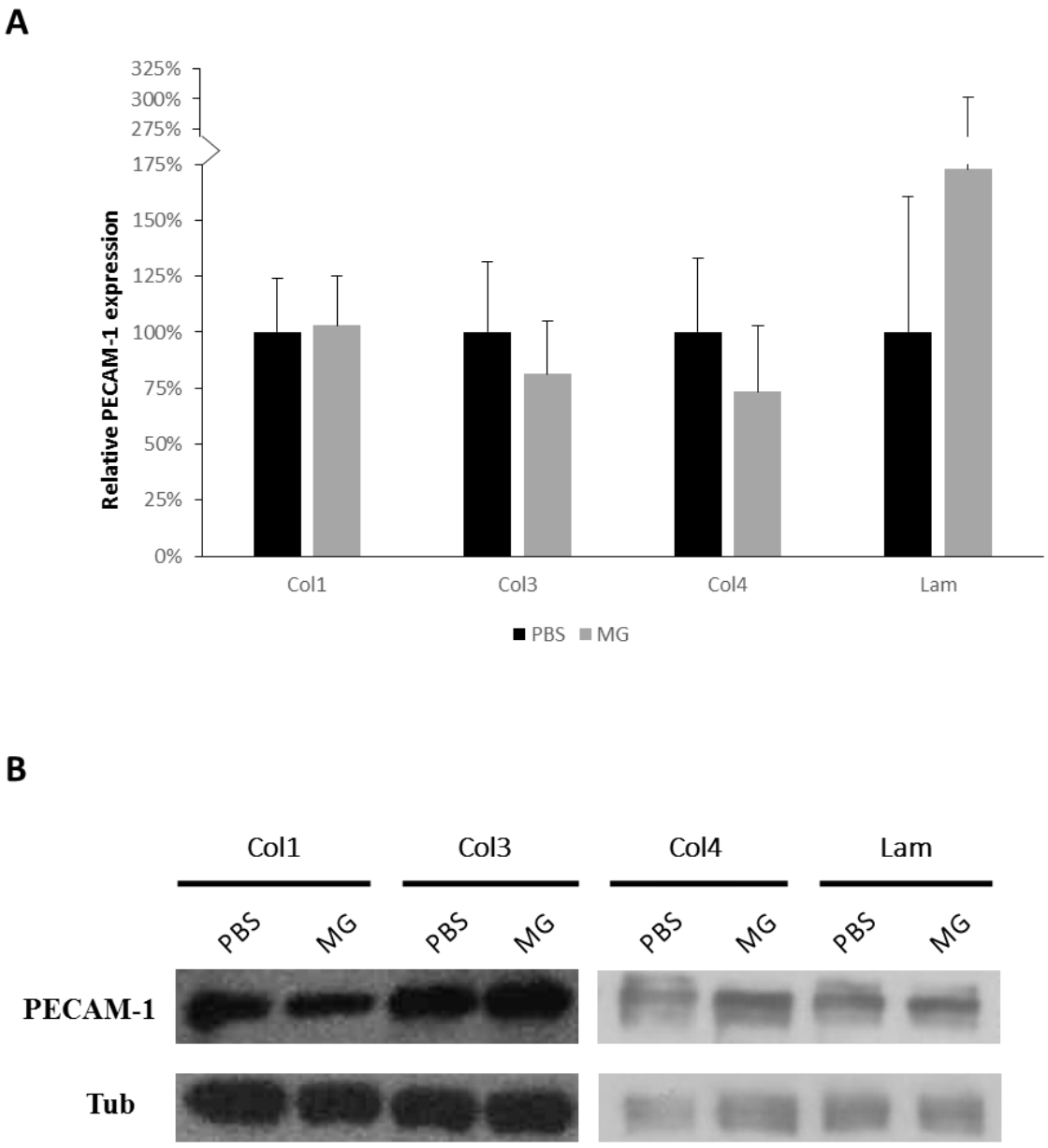

Figure 8. **Effects of MG modification on PECAM-1**
HUVECs were cultured for 48 hours on MG modified or unmodified laminin, and collagen type 1, 3 or 4. PECAM-1 expression was quantified through western blot showing no significant difference in expression of the adhesion molecule *n*=4.

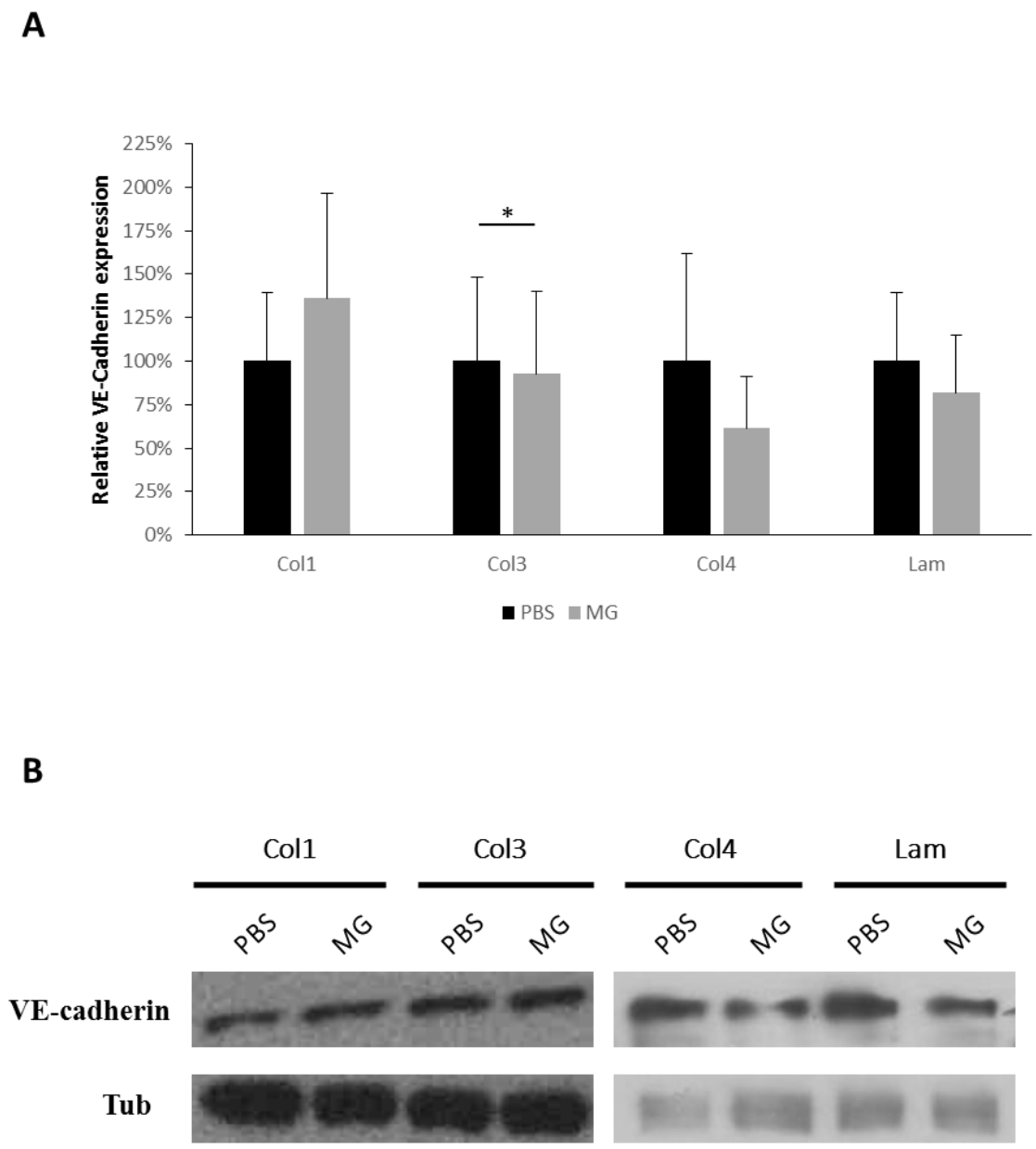

Figure 9. **Effects of MG modification on VE-cadherin**
HUVECs were cultured for 48 hours on MG modified or unmodified laminin, and collagen type 1, 3 or 4. VE-Cadherin expression was quantified through western blot showing a slight decrease on MG modified Col3 but, no significant difference in Col1, Col4, and laminin *p<0.006 n=4.

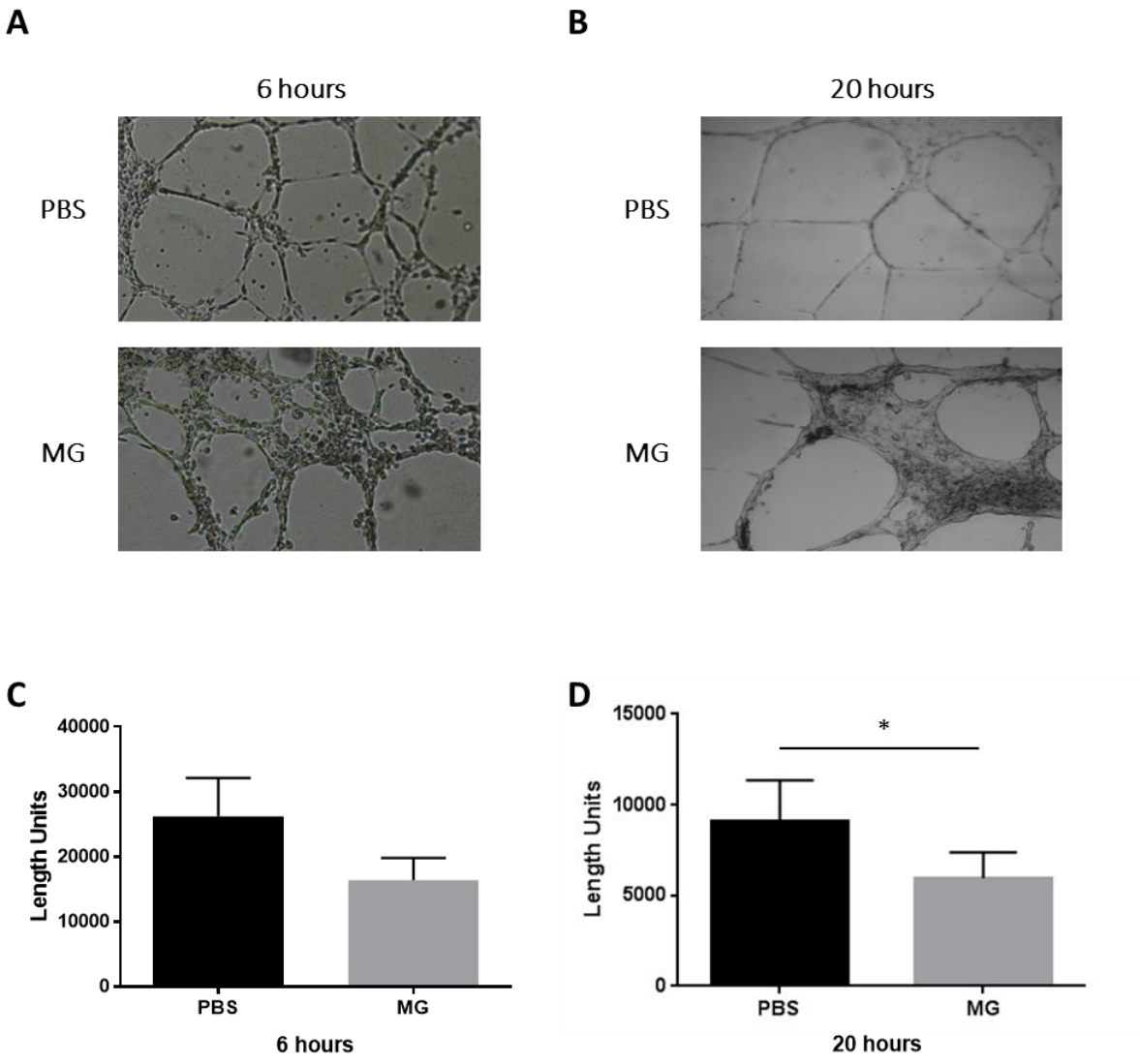

Figure 10. **Impact of MG modification on HUVEC angiogenesis**
ECMatrix was treated overnight with MG or PBS. HUVECs were seeded and capillary-like networks were imaged as shown by representative images at 6 (A) and 20 hours (B). Capillary-like network length quantification showed a trend of less extensive development with MG treated matrix at 6 hours (C), with a significant decrease observed at 20 hours (D) *p<0.05, n=3.

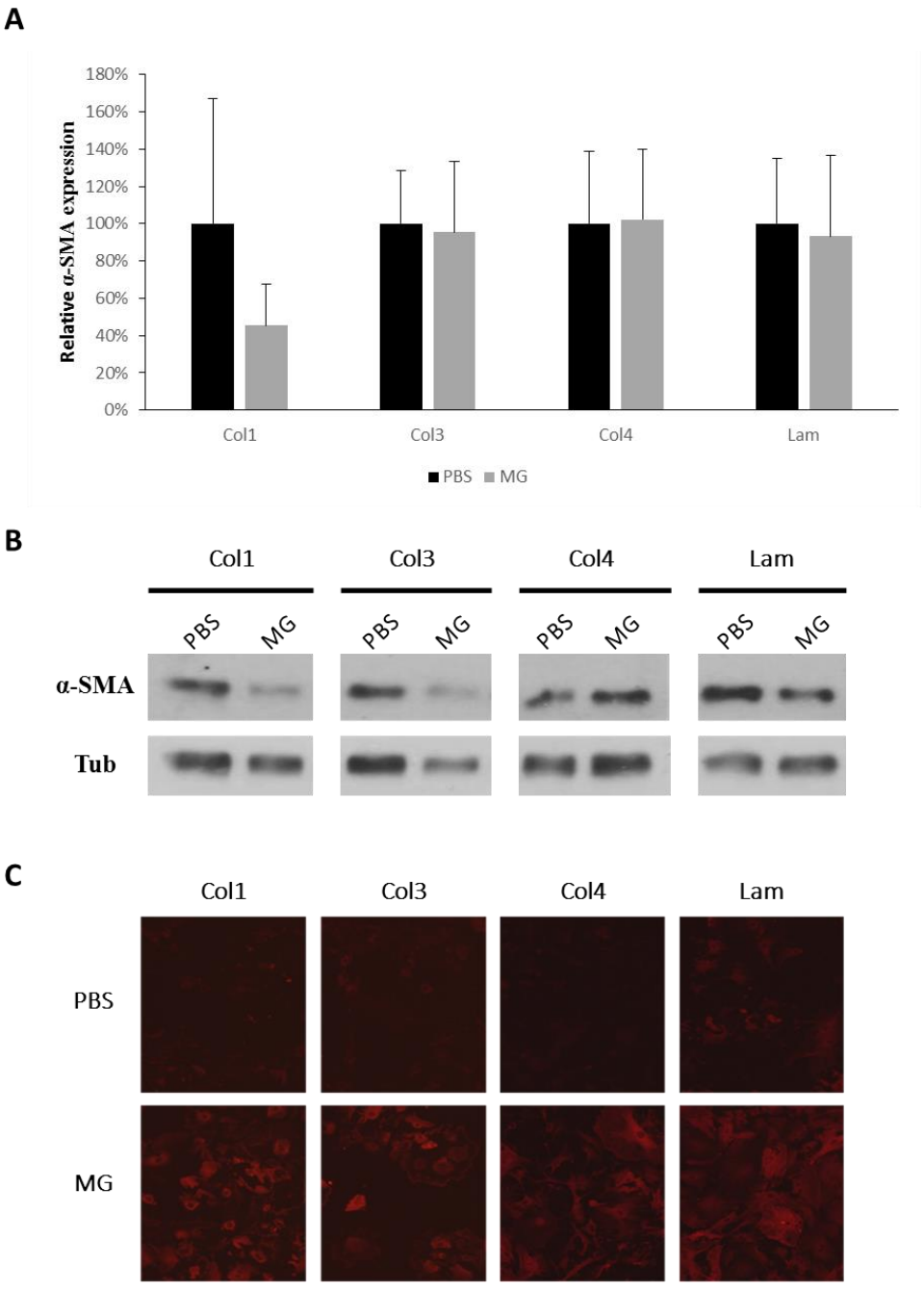

Figure 11. **Effects of MG modification on fibroblast α-SMA expression**
Primary mouse cardiac fibroblasts were cultured on MG modified or unmodified ECM proteins. Lysates were collected for western blot analysis (A) which displayed no significant difference in α-SMA expression, as also seen through representative gel bands (B) *n*=5. Cultures were fixed for immunofluorescence (C), displaying a higher signal for α-SMA expression in cells cultured on MG modified protein *n*=2.

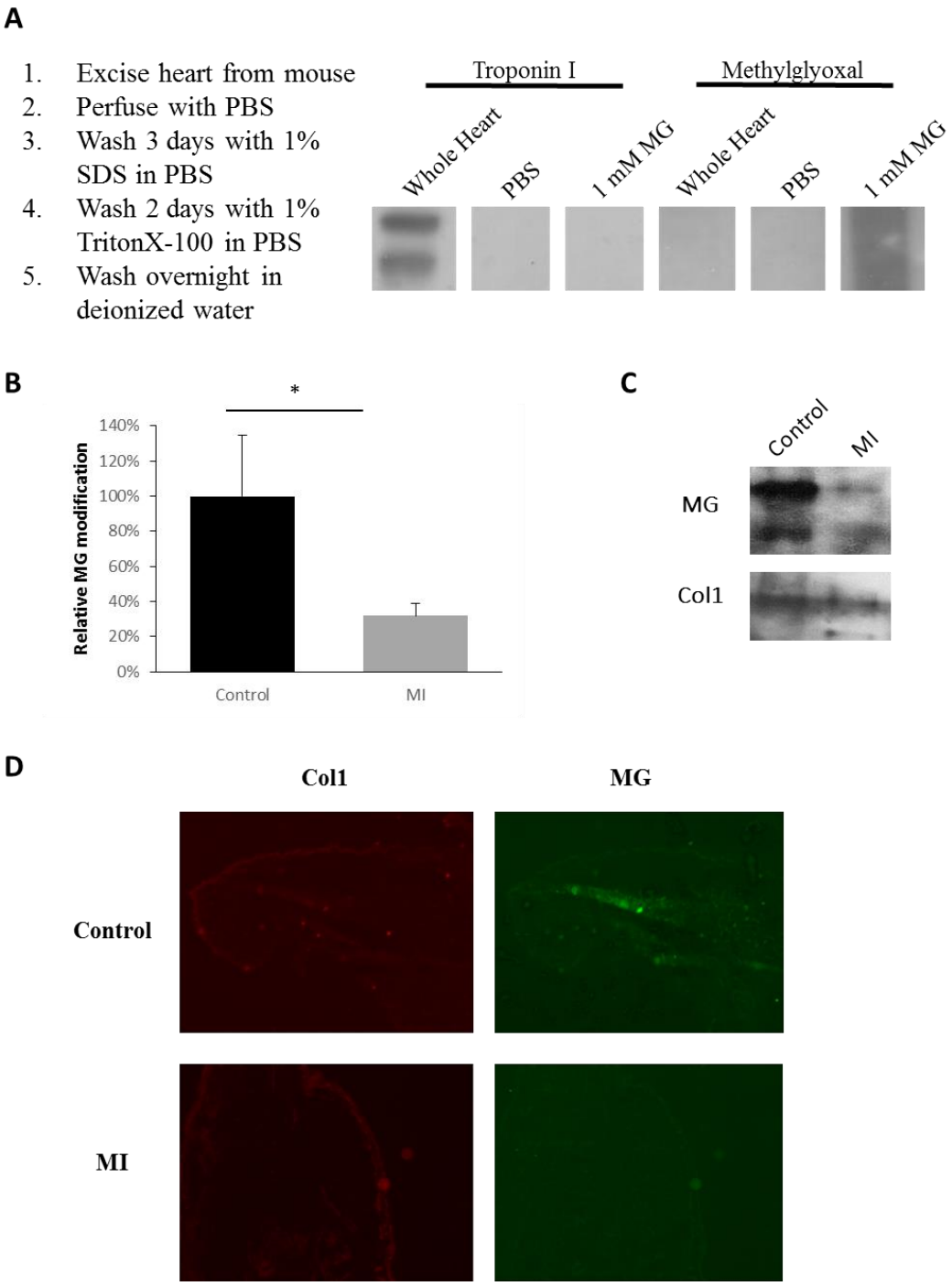

Figure 12. MG modifications of cardiac ECM are downregulated post MI
Following the protocol shown in (A), confirmation of decellularization (troponin expression) and positive control (MG-treated cardiac ECM) were done first n=3. (B) Western blot analysis revealed reduced MG-H1 expression in MI hearts *p<0.05, n=3-5. (C) Representative images of Westerrn blots. Immunohistochemistry on cryosections (D) also show reduced MG-H1 expresson in MI hearts n=3.

CHAPTER 4: DISCUSSION

MG accumulation is well-documented in the settings of hyperglycemia and diabetes. The formation of AGEs has been attributed to various complications associated with the progression of diabetes. Although many of MG's impacts are to vasculature (ECs, VSMCs), studies of MG in the cardiovascular setting without the overt causative agent of high glucose, are few. The thought that MG could reach beyond its high-glucose niche has been explored in mice with IR: one study showed an increase in AGEs (which indirectly suggests a role for MG) and the other directly implicating MG having observed a 6-fold increase of the dicarbonyl compound after IR injury (Bucciarelli et al. 2006; Aleshin et al. 2008),. In a clinical study, patients with ischemic cardiomyopathy were shown to have faster accumulation of AGEs (Nożyński et al. 2012). In all, this incited our interest in a role for MG in a post-MI environment. It is the importance of the ECM in cellular homeostasis and post-MI repair, along with the knowledge that ECM proteins can be prime targets for glycation by MG, which prompted us to look at the effect of MG on these proteins. Our findings can be divided into the initial work with MG and ECM proteins, *in vitro* work with ECs, *in vitro* work with fibroblasts, and the post-MI effect on the cardiac ECM. The main takeaway from the *in vitro* glycation experiments would be that MG can glycate these proteins, as was expected. Furthermore, MG appears to have a negative impact on ECs, with its disruption of angiogenesis it has the potential to greatly alter post-MI repair. In addition, immunohistochemistry findings suggest that MG-modified ECM proteins can promote the fibroblast-to-myofibroblast transition. Finally, post-MI analysis of ECM MG modification levels unexpectedly revealed reduced MG-H1 in the infarcted heart compared to healthy hearts, which is the opposite of previous

findings. Altogether, it was identified that several ECM proteins can be glycated by MG, which can have an effect on the phenotype and/or function of ECs and fibroblasts.

4.1 Glycation, *in vitro*

Given the fact that proteins contain arginine, lysine, or cysteine residues, which are prime targets for MG modification, MG is expected to have a wide reaching effect on the proteome, with the ECM proteins being particularly vulnerable due to their long half-life (Weber 1989; Price and Spiro 1977; Urich et al. 2011). *In vitro* experiments, whereby ECM proteins were treated with MG, found signals with the major fibrillar collagens Col1 and Col3 as well as proteins key to the basement membrane, Col4 and laminin. Signal evaluation did pose an issue due to a western blot result that presented as an intense smeared signal. Although more banded at times, concentration and loading alterations did not seem to clarify the signal. Rejection of a false positive was done by evaluating MG alone and showing an absence of signal. Also, equal coating was suggested by spectrophotometry, and degradation during coating excluded by a comparable signal when proteins were used straight from the bottle. Published literature with this antibody showed a similar smeared result (Dafre et al. 2015).

Ultimately the MG-H1 positive signal was as expected, since previous groups have used similar methods to glycate a multitude of proteins with comparable concentrations of MG, and often using mass spectrometry as confirmation (Pedchenko et al. 2005; Chong et al. 2007; Dobler et al. 2006). We determined that major ECM proteins

of the post-MI heart are susceptible to glycation by MG, and confirmed that our glycated proteins could be used for subsequent cell studies.

4.2 Endothelial cell response to MG modifications of ECM proteins

With the use of HUVECs we examined the impact MG-modified ECM had on ECs, an important cardiovascular cell type. Firstly, we noticed that MG-modified ECM had no effect on cell viability in both normoxic and hypoxic conditions. It is know that MG can lead to cell death through TNF-α (H. Zhang et al. 2009). Interestingly, TNF-α plays a role in anoikis, a type of apoptosis that occurs in the absence of ECM contact or erroneous adhesion (S. I. Park and McCauley 2012). In addition, integrin signaling, which is important for cell-matrix interaction, can be disrupted by MG glycation (Chong et al. 2007), suggesting that MG may be involved in causing anoikis. However, these reported effects are caused by MG glycation within the cell, which was not considered in the present study. It may be that MG modification of ECM proteins alone is not sufficient to affect EC viability. Further experimentation in which both cells and ECM are exposed to MG would be needed to determine if glycation of proteins within the cell and the ECM synergize to negatively impact cell viability. Additionally, when we consider the 48 hour incubation time used in our study, the possibility of refractory proliferation (Ferrari et al. 2012), may counter any loss of cells, and so the outcome of no difference in viability may have been expected. An assessment of proliferation in our cultures would be necessary to make this determination.

For the adhesion studies performed at 1 and 4 hours after seeding, it appeared that the MG modification of ECM proteins had an effect on the ability of ECs to bind. Three out of the four proteins tested, when MG-modified, were determined to be poorer surfaces of adhesion for ECs. Domains on laminin and collagens, containing RGD and GER, interact with integrins through sites which contain an arginine residue (Barczyk, Carracedo, and Gullberg 2010; J. K. Kim et al. 2005; Y. Xu et al. 2000), a key target of MG (Rabbani and Thornalley 2012). As such, it was expected that adhesion would be disrupted when the interacting domains were altered by MG. The glycation of Col1 may not have had an impact on EC adhesion due to the fact that ECs typically form connections with Col1 through their DDR1 receptor and the motif GVMGFO, which lacks arginine, lysine or cysteine for MG to target (Konitsiotis et al. 2008).

Another graduate student in the lab (Nick Blackburn) previously identified that MG levels in the infarcted myocardium increase significantly immediately post-MI (6 hours), and accumulate over time (4 weeks), which was associated with increased MG modification of ECM surrounding the vasculature and a decrease in neovascularization (unpublished). Thus, in the present study, the angiogenic potential of ECs was evaluated *in vitro* to better assess the role of MG-modification of the ECM on vascular growth. Results revealed a 35% decrease in tube network length when the assay was performed using MG-modified ECMatrix, which support the previous *in vivo* observations. The findings are also in line with experiments done using MG-modified vitronectin, which showed that the MG-modified protein resulted in the uncoupling of VEGFR-2 and integrin αvβ3 leading to decreased angiogenesis (L. Wang et al. 2015). In another study, MG modification of Col4 was shown to reduce EC attachment and inhibit angiogenesis,

which was linked to a loss of binding of ECs through integrins α1β1 and α2β1 (Dobler et al. 2006). Ultimately, MG-mediated ECM protein changes have an effect on EC functionality.

To further understand the effect of MG-modified ECM protein on EC adhesion and angiogenic potential, we examined the adhesion molecules VE-Cadherin, which interacts with VEGFR-2 (Vestweber 2008),and PECAM-1 (or CD31), which when inhibited leads to reduced angiogenesis and ECs displaying rounded shapes due to lack of proper adhesive connections (Yang et al. 1999). Additionally, in the post-MI environment, ECs are important in establishing the proper inflammatory response, in part through the promotion of PECAM-1 and VE-Cadherin mediated leukocyte transmigration (Mamdouh et al. 2003; Schulte et al. 2011). In the present study, no physiologically relevant difference was detected in the expression of PECAM-1 and VE-Cadherin by ECs cultured on MG-modified vs. unmodified ECM proteins. One possible explanation for this observation may be related to the coating efficiency of the plates with the different ECM proteins. Perhaps, the ECM proteins did not cover the entire surface of the plate, so that seeded ECs still have areas of TCPS to adhere to. This could result in overall reduced adhesion in MG-modified ECM protein plates, as there is less area to adhere to, but it may not affect the levels of PECAM-1 and VE-Cadherin in the ECs that do adhere between the conditions. Possibly explaining the minor difference observed with VE-Cadherin on modified Col3. A method to confirm the coverage of the plate by the coated proteins and/or an analysis of PECAM-1 and VE-Cadherin expression in both the adherent and non-adherent ECs may provide a better understanding of how MG-modified ECM proteins can affect the levels of these adhesion molecules. Furthermore,

with VEGF and mechanical forces being altered post-MI, and being regulators of VE-Cadherin and PECAM-1 expression, (Gavard 2014; Privratsky and Newman 2014), our *in vitro* assays may not appropriately mimic the conditions present *in vivo* to affect the ECs in the same way.

4.3 Modified ECM appears to promote myofibroblast differentiation

Previous studies have shown that fibroblasts are less adherent on MG-glycated collagen (Talior-Volodarsky et al. 2015). As well, fibroblasts cultured on MG-modified Col1 undergo a transition to a myofibroblast phenotype displaying increased migratory capacity and α-SMA expression (Yuen et al. 2010). The myofibroblast phenotype is generally associated with reparative and later pathological fibrosis during MI resolution (van Nieuwenhoven and Turner 2013). Thus, we looked at the response of fibroblasts cultured on laminin and collagens 1, 3, and 4 to further evaluate the involvement of MG-modified ECM on fibroblast function. Results from the immunofluorescence studies showed increased α-SMA expression in MG-modified ECM protein cultured fibroblasts, which was in line with the literature. However, protein expression by western blot analysis showed no difference between the conditions. A possible explanation for this may be related to antibody specificity and use. Additionally, loading was an issue at times with a 3-fold difference between the highest and lowest tubulin signal with certain sets. This could have made a difference, as work by Yuen et al. 2010 indicated no total difference in α-SMA expressing cells between cultures on control Col1 and MG-Col1 but, rather a proportional difference. Although efforts were made to improve this issue,

culturing on protein coated dishes appeared to significantly disrupt efforts of equal loading.

4.4 Decellularization and glycation of cardiac ECM

To measure MG modifications of the ECM in the infarcted heart *in vivo*, we adapted a heart decellularization protocol from Singelyn et al., (2009). The absence of troponin I suggested that the decellularization procedure resulted in a cell-free matrix. Furthermore, overnight treatment of the cardiac ECM with MG provided a positive control for our subsequent experiments, as it confirmed that the native cardiac ECM is susceptible to MG glycation. As mentioned before, unpublished data from our lab (Nick Blackburn), as well as previous studies (Wendt et al. 2002; Aleshin et al. 2008) have demonstrated an increase in MG and/or AGE levels after myocardial injury and thus prompted us to examine if this translates to increased AGEs on ECM proteins. Since every cell makes contacts with the ECM, a MG-glycated ECM mechanism could help explain the previously described wide-reaching effects of MG (Vulesevic, Milne, and Suuronen 2014; Maessen, Stehouwer, and Schalkwijk 2015). Contrary to the previous findings, our experiments showed that the cardiac ECM of post-MI hearts had reduced MG glycation compared to the hearts of non-operated mice. It is difficult to explain this observation, other than to propose some technical issue prevented proper detection of MG-H1 levels. In the work performed previously by Nick Blackburn, infarct and peri-infarct tissue was collected from hearts 6 hours after MI and mass spectrometry analysis revealed a 52% increase in myocardial MG-H1 content compared to non-infarct controls (unpublished).

Furthermore, immunohistochemistry on tissues at 28 days post-MI showed an accumulation of MG-H1. In the present study, tissues were collected at 5 days post-MI, which falls between the previous study's analysis at 6 hours and 28 days post-MI. Perhaps MG-H1 levels change over the course of infarct evolution. It may be possible that MG-H1 levels that are initially high in the acute inflammatory phase become reduced by remodelling of the ECM during the proliferation phase (samples in the present study were harvested during this time), and then accumulate again as the ECM scar tissue is established. A more comprehensive time course study would be required to test this hypothesis. Alternatively, technical issues may have led to the observed results. During solubilisation of decellularized hearts, for western blotting, their remained undissolved portions which could have altered the results seen. Troubleshooting for a buffer that can properly dissolve the decellularized hearts without impacting the present MG modifications may have yielded results in line with previous findings. In terms of IHC, a low or lack of signal points to the need of proper optimization with the possible use of antigen retrieval strategies in order to properly visualize the modifications that have occurred. Lastly, the low IHC signal suggests the possibility that MG modifications are affected by the decellularization protocol and more sophisticated methods of ECM protein isolation may be required in order to determine their true modification levels.

4.5 Future directions

Overall, the results of this project suggest that MG modifications of cardiac ECM proteins have some negative effects on ECs and fibroblasts, which may contribute to

negative remodeling and poor cardiac repair and function post-MI. It would be worthwhile to explore the mechanisms responsible for the observations gathered thus far on ECs. In terms of adhesion, integrin α1β1 expression would be of interest due to its ability to interact with both Col4 and laminin. The inhibition of this integrin has been shown to decrease adhesion of ECs (Senger et al. 1997), and it is needed for proper transmigration of inflammatory cells (Bazan-Socha et al. 2014), a crucial part of MI repair. ECs also express GPVI (B. Sun et al. 2003), which interacts with Col3, the third protein that was found to impact adhesion when modified. Despite the fact that not much is known about GPVI in ECs, its primary role in platelets is adhesion to collagen (H. Chen et al. 2002) and thus it would not be unrealistic to expect that it serves the same function in ECs. As for our observation of decreased angiogenesis, this also may be due to integrins. The inhibition of α1β1 was demonstrated to cause decreased HUVEC tube formation (Bolas et al. 2014). Similarly, a study found that inhibition of either α1β1 or α2β1 has an antiangiogenic effect on ECs (Momic et al. 2014). A greater understanding of adhesion molecules and surface receptors and how they interact with MG-modified ECM proteins may provide insight into how glycation of the cardiac ECM may control the function and reparative potential of ECs. In addition to evaluating interacting receptors, the expression of angiogenic molecules such as VEGF, FGF, or Ang1 would also be necessary in determining how EC paracrine signaling may be impacted. Finally, exploring markers of endothelial cell inflammation such as ICAM-1, VCAM-1, and E-selectin would be a way to evaluate MG's impact on the early inflammatory stages of EC-mediated MI repair (Heier et al. 2015; Hwang et al. 1997).

Adhesion, collagen deposition, and LOX expression by fibroblasts are aspects to be studied that could play a significant part in the role that MG glycated ECM may play in fibrosis and heart failure. Although studies have implicated integrin subunit β1 (Yuen et al. 2010), and α11 (Talior-Volodarsky et al. 2015) in the myofibroblast transition by MG-AGE signaling, there appears to be a lack of studies considering the mechanistic impact of integrins on adhesion to modified ECM. As such, the study of α1β1 and α2β1 integrin expression, showed to be important in collagen 1, 3, and 4 binding (Y. Xu et al. 2000; Gardner et al. 1996), as well as the effect of their inhibition in an MG glycated ECM setting could clarify their function in fibroblast adhesion. A central factor influencing the progress of pathological fibrosis is the rate of collagen deposition (Bishop et al. 1990). Thus, evaluating the impact that MG glycated ECM has on the rates of collagen protein production would further explain the role of these modifications in the myofibroblast driven pathology. With LOX expression being implicated in fibrosis and progression of heart failure (López et al. 2009), as well as its key part in ECM cross-linking, LOX has a proven significance in fibroblast-ECM interaction. Thus, experiments evaluating the impact of MG glycated ECM proteins on the expression of this enzyme within fibroblasts are of equal importance.

4.6 Conclusions

The ECM is a crucial regulator of homeostasis as well as an active participant in the heart's response to MI. In this study, the *in vitro* glycation of major the ECM constituents Col1 and Col3, which are deposited post-MI, resulted in increased α-SMA expression in

fibroblasts. This suggests that MG-modified ECM proteins may help promote the differentiation of myofibroblasts, the cell type that is primarily responsible for remodeling of the ECM post-MI. Col4 and laminin, making up the basal lamina, were also glycated *in vitro* by MG. These proteins are known to impact EC function, and MG-modified Col4 and laminin was demonstrated to reduce EC adhesion and angiogenesis. Additional studies will be needed to determine if technical issues or temporal changes in MG-H1 expression post-MI may have been responsible for the lack of MG-modified ECM observed in the post-MI heart in the present study. Overall, the results indicate that MG-mediated ECM modifications may play a role in reducing EC function and angiogenesis, and promoting myofibroblast differentiation and fibrosis in the heart after MI. A better understanding of this mechanism may help make MG a novel target for preventing damage and improving cardiac function post-MI.

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Appendix – supplementary figures

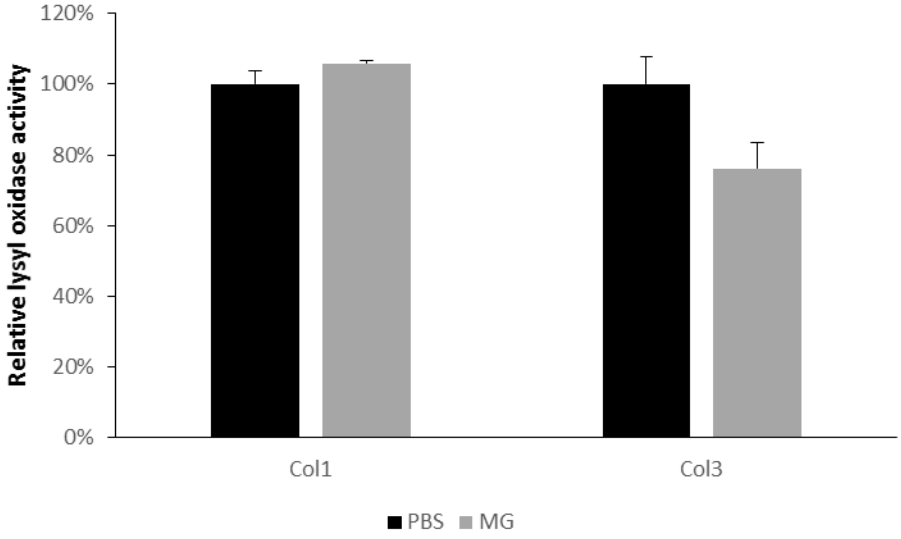

Figure A1. **Impact of MG glycated collagen on fibroblast LOX activity**
Primary mouse cardiac fibroblasts were cultured on MG-modified or unmodified Col1 and Col3. The lysyl oxidase activity assay kit (abcam) was used in conjunction with a microplate reader to provide relative absorbance readings. Unfortunately, negative control samples consistently provided a signal and as such the data could not be used.

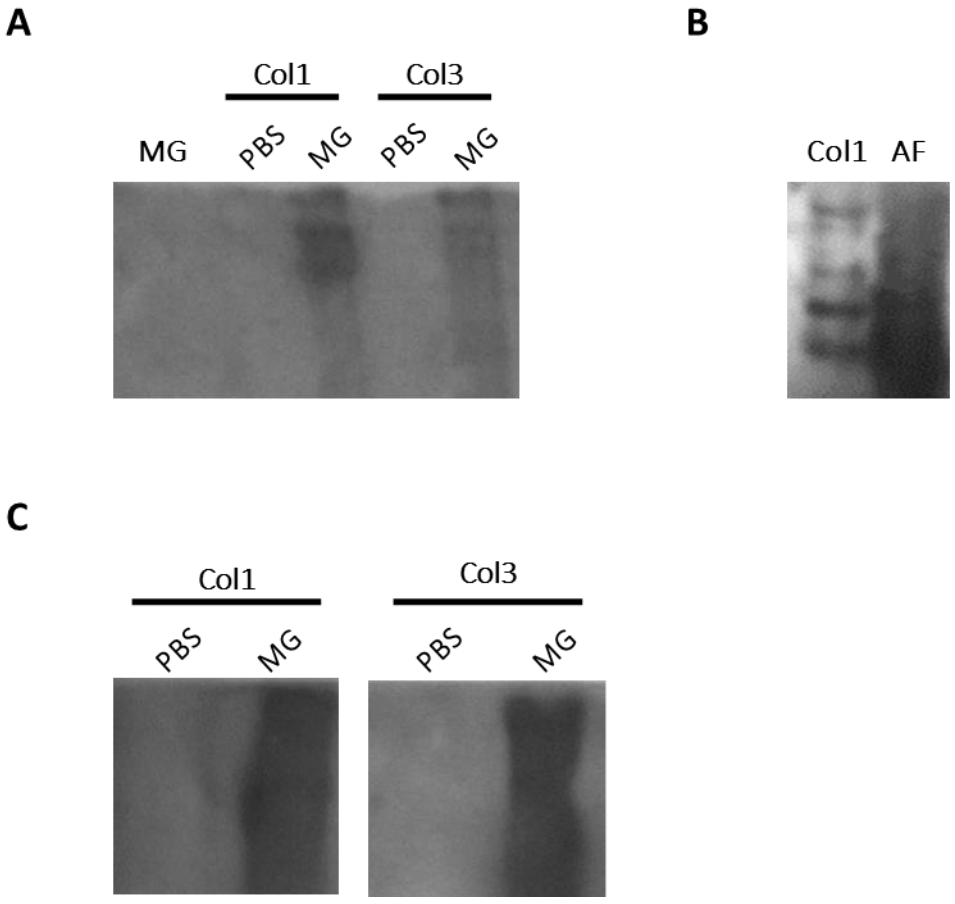

Figure A2. **MG signal assessment troubleshooting**
(A) Loading of MG alone to ensure no false positive, alongside modified (signal present) and unmodified (no signal) Col1 and Col3. (B) Western blot comparison of MG-H1 signal between Col1 and attachment factor (gelatin), both treated overnight with MG. AF demonstrates more uniform and smeared signal due to lack of structure. (C) Loading of ECM proteins without prior coating of cell culture dishes, demonstrates no alteration in signal as compared to those seen in Fig. 4, indicating that protein degradation after plating is not likely occurring.

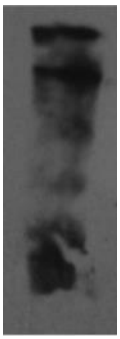

Figure A3. **Collagen isolation from whole heart**
A protocol developed by Pacak, Powers, and Cowan 2011 was used in an attempt to isolate collagen. The purpose of this was to follow-up with a pulldown assay for the evaluation of *in vivo* glycation; the blot above represents the signal with the Col1 antibody signifying the unsuccessful isolation/preservation of collagen.