Differentiation of Primary Human Pro-Fibrotic Mesenchymal Cells Influenced by Extracellular Matrix Environment Determined by Micro-RNA Expression

by

Alison Louise Müller

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

University of Alberta

Abstract: Fibrosis, a multi-faceted process that exacerbates numerous cardiovascular pathologies leading to heart failure, is the result of excessive extracellular matrix protein deposition. Although fibrosis is classically thought to be the result of myofibroblasts activated from interstitial fibroblasts endogenously present within the heart, recent research has indicated another significant pro-fibrotic cell source which resides in the bone marrow. These mesenchymal progenitor cells are recruited to the heart in response to inflammation in a variety of cardiovascular diseases and have been directly implicated in extracellular matrix protein deposition. The fibrotic extracellular matrix environment significantly influences the differentiation of mesenchymal progenitor cells. These cells continuously deposit extracellular matrix proteins worsening fibrosis and further recruiting circulating bone marrow-derived mesenchymal progenitor cells. This hostile fibrotic environment has also stunted and further complicated the success of regenerative cell therapy which aims to stimulate cardiac repair and healing. In the many cardiovascular disorders that lead to fibrosis, these endogenous fibroblasts and bone marrow-derived progenitor cells differentiate rapidly to the myofibroblast phenotype in response to injury. This pro-fibrotic differentiation process has been shown to be facilitated and guided by microRNA. The putative role of microRNA has been implied in both the differentiation of bone marrow-derived stem cells and interstitial fibroblasts to myofibroblasts. The human body has a myriad of different environments that contribute to cell differentiation, which includes stiff pathological fibrotic environments. Pathological fibrosis occurs in response to injury which alters the microenvironment experienced by mesenchymal cells by promoting a pro-fibrotic phenotype which is influenced, in part, by miRNA expression. The significant potential for a link between miRNA, the extracellular matrix environment, and the differentiation of various progenitor cell-types and their contribution to cardiac fibrosis will be explored in this thesis. We hypoethsize that mesenchymal cells, both bone marrow-derived progenitor cells and atrial fibroblasts, are susceptible to changes in their surrounding environment that influences their behaviour. As decellularization techniques can be utilized to isolate native extracellular matrix (ECM), it is possible to evaluate mesenchymal cell phenotypic changes on an endogenous pro-fibrotic environment. Therefore, this hypothesis will be tested

using physiological, native ECM isolated from human left ventricular cardiac tissue. This pro-fibrotic influence may be attenuated by over-expression of miRNA-301a.

This shall be accomplished by first evaluating the effects of statin therapy on the mesenchymal progenitor cell population to establish how they interact with potential cell sources that would be utilized in clinical applications. It is recognized that mesenchymal cells that contribute to pathological fibrosis undergo a proliferative phenotype change upon exposure to inflammation as a result of injury. We identified a miRNA involved in this phenotype switch and determined how it influences the progression of mesenchymal cells as they exhibit myofibroblast-like properties under normal culture conditions. Understanding that progenitor cell differentiation is determined by their niche microenvironment, including extracellular stiffness, we evaluated how human mesenchymal progenitor cells (hMPCs) respond to varying surface tensions while measuring possible changes in miRNA expression. In order to observe physiologically accurate phenomena, it is possible to decellularize native tissue to isolate ECM. With access to human cardiac tissue, both diseased and healthy, we focused on the influence of pathologically remodeled ECM on hMPCs and human atrial fibroblasts (hAFs), by using decellularization techniques to isolate fibrotic-rich areas of left ventricular (LV) myocardium and subsequently recellularizing them with hMPCs and hAFs. Although studying the effects of hMPCs and hAFs on human LV, we also evaluated the effects of these cells on commercially available decellularized porcine matrix, called CorMatrix®, which is used in a variety of cardiac surgery procedures. Finally, in addition to being able to evaluate molecular differences between ECM products, native diseased and healthy ECM, we assessed the efficacy of miR-301a in attenuating differentiation into a pro-fibrotic phenotype on hAFs and hMPCs seeded in profibrotic decellularized LV ECM.

Dedication

I dedicate this PhD thesis to Osiris, who I could always depend on making wherever I lived a home, whether in Winnipeg or Edmonton. Regardless of the time or day, you always made me smile.

Acknowledgements

To the Department of Physiology at both the University of Manitoba and University of Alberta. Thank you for supporting my research and providing me with the opportunity to succeed in your department. I would like to especially thank the amazing administrative assistants Gail (U of M) and Sharon (U of A) for your patience with me while we navigate the university graduate student system. It's not easy to transition between different universities and you made the experience seamless. I would also like to thank Dr. Gregory Funk for making my time as a graduate student at the U of A a wonderful, supportive environment. I loved our conversations and your appreciation of the science communication work that I do.

To my committee members, Drs. Consolato Sergi and Zamaneh Kassiri. Thank you for your guidance and appreciation of my project. Your feedback and input into my thesis helped round out my experiments and provided useful thoughtful perspectives on my work.

To my wonderful friends in Winnipeg. After establishing a home and a loving, supportive community in Winnipeg, it was tough to come to Edmonton not knowing a soul. Krista and Riley, you both encouraged me to be the best that I can be and remain enthusiastic and high-spirited throughout my academic career. I have appreciated your advice, friendship, both in Winnipeg, and after I moved. I love you both very much!

To the new-found friends in Edmonton, thank you for making the transition so easy. To my board game crew, our weekly get-togethers kept me sane during the hard times and helped me get through some very tough times during my degree. To the Let's Talk Science ladies! We had such a blast teaching science and having wonderful social gatherings. I will never forget all of our wonderful adventures!

To my Vancouver friends, especially Chi-ka, Hae-Young, Katie, and Aiza. You always made an effort to make time for me when I visited, even at very last notice. I loved our "long distance" conversations and your patience with me and my last-minute visits. You have been with me since the start of my academic career, and I couldn't have persevered without your love and encouragement. I love you all very much!

I would like to thank my wonderful family. They have been very supportive of my endeavours and always encouraging me to pursue my dreams. To my dad, who introduced me to the wonderful opportunities medical research has to offer. To my mom, who always encouraged me to keep going and so excited to hear about what I've been up to. To my brother, who could bring me back down to Earth when I was stressed about the little things, emphasizing that what I was doing was wonderful and that I'll finish it, eventually.

And to all the lab mates, technicians, and summer students in both Winnipeg and Edmonton, thank you for making the laboratory such an engaging, fun environment to work in! Finally, this thesis wouldn't have been possible without my amazing supervisor, Dr. Darren Freed. Thank you so much for giving me the opportunity and freedom to pursue my interests during my PhD. I could always count on you to provide constructive feedback and encouragement, even during some of the very trying, stressful times. I am grateful for your patience with me and my project, as well as your support for my non-academic pursuits. I couldn't have asked for a better supervisor!

Table of Contents

List of Figures and Tables

List of Abbreviations

α-SMA – α-smooth muscle actin

3'UTR – three prime untranslated region

2D – two-dimensional

3D – three-dimensional

AFs – atrial fibroblasts

AngII – angiotensin II

ANOVA – one-way analysis of variance

ATP – adenosine triphosphate

BCA – bicinchoninic acid assay

BMP-2 – bone morphogenetic protein-2

BSA – bovine serum albumin

CAV – cardiac allograft vasculopathy

CCR2 – C-C chemokine receptor type 2

CXCR4-CXCL12 – C-X-C chemokine receptor type 4 – C-X-C chemoke ligand type 12

CoA – coenzyme A

Col1 – collagen1

Col1A1 – collagen1A1

Col1A2 – collagen1A2

COPD – chronic obstructive pulmonary disease

CoQ10 – Coenzyme Q10

CVD – cardiovascular disease

DAPI – 4',6-diamidino-2-phenylindole, dihydrochloride

DDW – double-distilled water

DMAPP – dimethylallyl pyrophosphate

DMEM-F12 – Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

ESC – embryonic stem cell

ECM – extracellular matrix

EDA-FN – extracellular domain A fibronectin

ELISA – enzyme-linked immunosorbent assay

EPFH – explanted pro-fibrotic heart

ERK – extracellular signal-regulated kinase

eGFP – enhanced green fluorescent protein

EDTA – ethylenediaminetetraacetic acid

EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EMT – epithelial-to-mesenchymal transition

EndMT – endothelial-to-mesenchymal transition

FAK – focal adhesion kinase

FBS – fetal bovine serum

FGF – fibroblast growth factor

FPP – farnesyl pyrophosphate

FSP-1 – fibroblast-specific protein-1

GAGs - glycosaminoglycans

H&E – hematoxylin and eosin

HA – hyaluronic acid

hAFs – primary human atrial fibroblasts

HBSS – Hank's Buffered Saline Solution

hESCs – human embryonic stem cells

HF – heart failure

HIDS - hyperimmunoglobulinemia and periodic fever syndrome

HLV – human left ventricle

HMDS - hexamethyldisilazane

HMG-CoA – hydroxymethylglutaryl-coenzyme A

hMPCs – primary human bone marrow-derived mesenchymal progenitor cells

HSCs – hematopoietic stem cells

GAPDH – glyceraldehydes 3-phosphate dehydrogenase

GDP – guanosine diphosphate

GFP – green fluorescent protein

GGPP – geranylgeranyl pyrophosphate

GTP – guanosine triphosphate

ICAM – intracellular adhesion molecule-1

IF – immunofluorescence

IFN – interferon

IKKβ – inhibitor of nuclear factor kappa-B kinase-β

IL – interleukin

IPF – idiopathic pulmonary fibrosis

IPP – isopentenyl pyrophosphate

iPSCs – inducible pluripotent stem cells

KLF – Krüppel-like family of transcription factor

LINC – linker of nucleo- and cytoskeleton

LV – left ventricle

LVAD – left-ventricular assist device

MA – mevalonic aciduria

MCP-1 – methyl-accepting chemotaxis protein-1

MHCα – major histocompability complex-α

MI – myocardial infarction

MIP – macrophage inflammatory protein

miRNA – micro-ribonucleic acid

MKD – mevalonate kinase deficiency

MMP – matrix metalloproteinase

MPCs – mesenchymal progenitor cells

mRNA – messenger ribonucleic acid

mTORC – mammalian target of rapamycin complex-1

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor

MYH – myosin heavy chain

NADPH – nicotinamide adenine dinucleotide phosphate

NBEC – normal bronchial epithelial cells

NFκB – nuclear factor-κB

NMMII – non-muscle myosin II

NMMIIA – non-muscle myosin IIA

NMMIIB – non-muscle myosin IIB

NT2 – human model neurons

O/N - overnight

P(#) – passage (#)

PAD – peripheral artery disease

PBS – phosphate-buffered saline

PDGF – platelet-derived growth factor

PFA – paraformaldehyde

P/S – penicillin streptomycin

PTEN – phosphatase and tensin homolog

PVDF – polyvinylidenedifluoride

qRT-PCR – quantitative reverse-transcriptase polymerase chain reaction

RAAS – renin angiontensin aldosterone system

RGD – Arginine-Glycine-Aspartate

Rheb – Ras homologue enriched in brain

ROCK – Rho-associate protein kinase

ROS – reactive oxygen species

RT – room temperature

SDS – sodium dodecyl sulphate

SEM – scanning electron microsopy/standard error of the mean [context-dependent]

SFD-1α – stromal cell-derived factor 1α

siRNA – small-interfering ribonucleic acid

SMC – smooth muscle cell

SMM – smooth muscle myosin

Sp1D8 – monoclonal antibody against collagen, type 1A1 pro-peptide

SPARC – secreted protein, acidic and rich in cysteine

SUN – Sad1p, UNC-84

TAC – transaortic constriction

TEM – transmission electron microscopy

TGFβ – transforming growth factor-β

TNFα – tumour necrosis factor-α

VCAM – vascular cell adhesion protein-1

VEGF – vascular endothelial growth factor

WHO – World Health Organization

1. **Introduction**

The international burden of cardiovascular disease is exacerbated by the excessive extracellular matrix (ECM) protein deposition phenomenon known as fibrosis (1). In patients with chronic heart failure, biomarkers linked to pathological fibrosis were strong predictors of pump failure risk, which emphasizes the impact fibrosis has on cardiovascular function and patient outcome (2). This process is multi-faceted and drives numerous cardiovascular pathologies towards heart failure, which is the fastest growing subclass of cardiovascular diseases (1,3,4). Acute cell death that is caused by cardiovascular disease triggers reparative fibrosis by eliciting an inflammatory reaction, similar to that which occurs during a myocardial infarction (MI) (5). Pathological fibrosis, known as reactive fibrosis, also occurs in various clinical complications in the context of systemic hypertension, aortic stenosis, hypertrophic and dilated cardiomyopathies, and heart transplantation, all of which have impaired survival (3-8). In addition, aging results in increased levels of interstitial ECM protein production which can stiffen the myocardium and impair overall cardiac function (9). As the heart has stunted regenerative properties, MI was the initial catalyst in investigating the regenerative potential of progenitor cell therapy. However, understanding how endogenous progenitor cells respond to a pro-fibrotic environment equips the field of tissue regeneration with the knowledge that allows treatments to be more effective by developing cells immune to the influence of a profibrotic extracellular matrix environment in any cardiovascular disease state.

Traditionally, fibrosis was presumed to be solely the result of myofibroblasts derived from interstitial resident fibroblasts within the heart; however, recent research has found that there are numerous cell sources contributing to fibrosis, including various cell lineages found in the bone marrow (7, 10-14). Mesenchymal cells of bone marrow origin contribute to pathological ECM protein deposition in a variety of cardiovascular diseases in both animal models and patients (13, 15-20). Earlier research in our lab identified these cells as myofibroblast-like mesenchymal progenitor cells (MPCs) (20). These cells appear ubiquitously involved in both physiological and pathological fibrosis within the human body, including the cardiovascular system (21-26).

Although a variety of scientific tools have been used to discover the culprits responsible for cardiovascular fibrosis, it is crucial to elucidate activities of bone marrow derived cells in an *in vitro* setting to better interpret their physiology and understand their impact on the wide variety of tissue scaffolds being utilized in cell therapy and tissue engineering prior to patient implantation. This allows easier manipulation and targeted study of these cells to better understand the mechanisms that differ between beneficial fibrosis required for normal wound healing, compared to pathological fibrosis that impairs cardiac functionality. *In vitro* analysis comparing primary human atrial fibroblasts (hAFs) with primary human bone marrow-derived mesenchymal progenitor cells (hMPCs) within the same patient was recently published (20). It was found that hMPCs rapidly acquire a myofibroblastic phenotype in culture, the primary cell responsible for pathological cardiac fibrosis, evidenced by protein and mRNA expression of α-smooth muscle actin, non-muscle myosin isoforms and EDA-fibronectin. These cells also functionally behave like myofibroblasts, evidenced by contraction of collagen gels at baseline, as well as in response to transforming growth factor-β (TGFβ) stimulation (27). Additionally, research groups, including our own, can induce these cells to acquire a myofibroblast-like phenotype in culture, specifically through administration of TGFβ1 (28-30). Understanding the various mechanisms of how bone marrow-derived progenitor cells are influenced by their extracellular environment to differentiate to myofibroblast-like cells could reveal therapeutic targets to prevent overzealous profibrotic differentiation in a cardiovascular disease environment.

A key molecule, discovered to be essential to differentiation from a single embryonic stem cell to a multi-cellular living organism, is microRNA (miRNA) (31). These powerful 20-25 nucleotide-long sequences prevent targeted messenger RNA (mRNA) from forming proteins and significantly influence cell differentiation, including that of cells that contribute to fibrosis (31, 32). Because they have the potential to simultaneously interact with multiple mRNA targets, miRNA molecules can act as a master switch to regulate cellular processes, including the multi-faceted behaviour of differentiation. Many different miRNA molecules have been implicated in cell differentiation; however, miR-21, miR-24, miR-

29, miR-133, miR-145, and miR-208a have been found to be the most influential in regulating cardiac fibrosis either through myofibroblast differentiation or altering ECM protein deposition (33-38). Due to the potential role of miRNA in regulating numerous protein targets, including secreted ECM proteins, miRNA represents an exciting potential avenue for therapeutic targets in preventing, and possibly even reversing, pathological fibrosis in the heart.

Although understanding how intracellular mechanisms influence differentiation into a pro-fibrotic phenotype is important, it would be naïve to assume the cell has an innate knowledge of when to differentiate and what to differentiate into. Cells are signal dependent entities, and, therefore, receive their information on how to behave or respond based on their surrounding environment. The environment surrounding the cells in all solid organs of the body is called the extracellular matrix, which was described earlier as the environment where pathological fibrosis occurs. Pioneering research done by both Taylor's (39) and Discher's (40) groups calls attention to the significant influence of the ECM on organ function and cell differentiation, respectively. These monumental findings not only highlight the importance of the ECM as a operative component of solid organ structure, but also as a signaling element influencing progenitor cell differentiation. Current research focused on tissue regeneration is recognizing the importance of ECM and, in doing so, is merging the fields of biomaterials and stem/progenitor cell therapy leading to the creation of bioscaffolds to optimize cardiac tissue repair (41).

1.1 Hypothesis

Mesenchymal cells, both bone marrow-derived progenitor cells and atrial fibroblasts, are susceptible to changes in their surrounding environment that influences their behaviour. The human body has a myriad of different environments that contribute to differentiation of cells, including pathological fibrosis which provides a stiff environment. Pathological fibrosis occurs in response to injury which alters the microenvironment experienced by mesenchymal cells by promoting a pro-fibrotic phenotype which is influenced, in part, by miRNA expression. As decellularization techniques can be utilized to isolate native

ECM, it is possible to evaluate mesenchymal cell phenotypic changes on an endogenous pro-fibrotic environment. Therefore, this thesis hypothesizes that mesenchymal cells, both bone marrow-derived progenitor cells and atrial fibroblasts, are influenced by its microenviroment, which can be evaluated in physiological, native ECM isolated from human left ventricular cardiac tissue. This pro-fibrotic influence may be attenuated by over-expression of miRNA-301a.

1.2 Objectives

Project 1: As the majority of cardiovascular patients who would be eligible for cell therapy take statin medication, it is important to determine how it effects the mesenchymal progenitor cell population, which is a common source for clinical cell therapy and tissue engineering treatments. It has been observed in patients that statins may be able to mobilize endothelial progenitor cells; however their effect on mobilizing hMPCs is largely unknown. Establishing how statins interact with potential cell sources in clinical applications that would be used to repair damaged cardiac tissue is important in determing how effective either therapy will be when administered in conjunction with the other.

Project 2: Mesenchymal cells that contribute to pathological fibrosis have a tendency to exhibit a dichotomous phenotype, where cells in healthy patients maintain a proliferative phenotype until exposure to inflammation as a result of injury. In response to inflammation, they become more contractile and secretory as they build ECM proteins to stabilize damaged organ infrastructure. Our lab focused on identifying a miRNA involved in this phenotype switch to determine how it influences the progression of mesenchymal cells into myofibroblast-like pro-fibrotic cells. A miRNA target could be used as a potential treatment to target progenitor cells prior when administering them to a pathological fibrotic microenvironment.

Project 3: Differentiation of progenitor cells is significantly determined by their microenvironment, including the stiffness of their surrounding extracellular matrix. MPCs are especially attuned to this as they differentiate into adipogenic, chondrogenic, and osteogenic tissue which have drastically varying

stiffnesses. Identifying how MPCs respond to varying surface tensions while measuring possible changes in miRNA expression could elucidate what environmental signals a miRNA of interest uses to determine whether or not MPCs maintain a proliferative or differentiated phenotype.

Project 4: It is possible to decellularize native tissue to isolate ECM. This provides 3-dimensional scaffolding, more accurately representing an endogenous cell growth environment and provides an ability to observe cells in a physiological environment. With access to human cardiac tissue, by using both diseased and healthy, it is possible to seed mesenchymal cells on fibrotic and control ECM to determine how it influences pro-fibrotic differentiation. However, although decellularization followed by recellularization has been performed on the heart, it has only been published as an entire organ as part of an *ex vivo* circuit. This requires an enormous amount of resources and fails to target isolated fibrotic lesions. In order to focus on the influence of pathologically remodeled ECM, a technique isolating and decellularization smaller, focused pieces of tissue must be established.

Project 5: Although it is ideal to use decellularized human LV tissue, there is a non-cardiac ECM product, called CorMatrix®, which is used in a variety of cardiac surgery procedures. This product is used to strengthen areas of weakness; however, it has a significantly denser ECM protein distribution. This could stimulate a pro-fibrotic phenotype in mesenchymal cells already being recruited to this area as a result of damage sustained post-operation. Using similar recellularizing procedures as with decellularized LV tissue, it is possible to compare how mesenchymal cells respond to the non-cardiac ECM. In addition to being able to evaluate molecular differences between ECM products, native diseased and healthy ECM, it can also be used to test the efficacy of our miRNA of intereset in attenuating differentiation into a pro-fibrotic phenotype.

1.3 Pathological Fibrosis

Within nearly all tissue and organ systems there is a heterogenous population of cells that have potential to stimulate pathological fibrosis. Although collectively termed myofibroblasts, they exhibit

multifarious phenotypes elucidated by their distinct physiological characteristics (42). There have even been degrees of diversity observed in myofibroblasts isolated from distinct areas of the same organ. This is demonstrated by myofibroblasts isolated from different dermis depths which express distinct ECM protein profiles for collagen type I and II, and proteoglycans versican and decorin (43,44). Pathological fibrosis can be triggered by inflammation, radiation, chemical agents, persistent infections, genetic disorders, and autoimmune disease which exacerbates the development of numerous diseases and contributes to high morbidity and mortality (45-48). The pathogenesis of fibrosis commonly occurs in response to wound healing when epithelial and/or endothelial cells release pro-inflammatory cytokines in response to damage (45). Persistant stimuli caused by overexpression of pro-inflammatory cytokines or growth factors can cause abnormal signaling and sustain myofibroblast activation, switching the wound healing process to a pro-fibrotic process (49,50).

1.3.1 Cardiovascular Disease and Fibrosis

Cardiovascular disease (CVD) is defined as "disorders of the heart and blood vessels that includes coronary heart disease, acute MI, cerebrovascular disease (stroke), hypertension, peripheral artery disease (PAD), rheumatic heart disease, congenital heart disease, and heart failure" by the World Health Organization (WHO). Not only has it been responsible for more than 17.7 million premature deaths in 2015, it is projected to exceed 22 million deaths annually by 2030 (51). In 2016, cardiovascular disease directly causes 37% of all-cause mortality internationally making it the number 1 cause of death in the world. There are many identified risk factors of CVD, some of which are influenced by lifestyle whereas others by genetic predisposition or congenital defects. Family history of CVD, age, race, genetic mutations, or congenital defects are risk factors that are unavoidable; however, physical inactivity, obesity, and alcohol, tobacco, and/or illicit drug abuse are modifiable factors that significantly increase the risk of CVD (51-55). Although extensive research has proven that healthy eating habits and moderate physical activity can prevent and/or delay the onset of CVD, these measures are inadequate to cure it, especially as the heart is unable to effectively heal once it has been damaged.

As mentioned previously, MI causes a significant number of deaths in patients with CVD because it contributes to the risk of developing heart failure (HF). One of the primary contributing factors to HF caused by MI is remodeling of the cardiac interstitium: cardiac fibrosis (56). MI causes cardiomyocyte necrosis as a result of being deprived of nutrients and oxygen normally delivered via the coronary vasculature. This damage releases pro-fibrotic signaling factors and signals resident cardiac fibroblasts to differentiate into myofibroblasts that synthesize numerous ECM proteins, predominantly fibrillar collagens, in order to maintain cardiac integrity (56). This acute response forms a collagen based scar that replaces the necrotic cardiomyocytes, providing tensile strength and thus preventing myocardial rupture where sudden death is inexorable (57-59). Unfortunately, this damaged myocardium is never replaced with healthy cardiomyocytes to the extent where the scar is no longer needed; therefore, a positive feedback mechanism can develop as a result of residual myofibroblasts perpetually signaling for continuous interstitial remodeling. Although this response may not begin until 2-3 days post-MI in patients, the inflammatory signal may persist, triggering further deposition of fibrillar collagen which may invade viable myocardium and compromise cardiac function leading to heart failure (60).

1.3.1.1. Cell Sources of Fibrosis

Homeostatic ECM turnover is maintained by flat, spindle-shaped cells called fibroblasts which lack a basement membrane. Developmentally derived from mesenchymal origins, fibroblasts reside within the cardiac interstitium and are considered sentinal cells (61). This is because they remain relatively quiescent until activated by a variety of stimuli including transforming growth factor-β (TGFβ), endothelin-1, angiotensin II, electrical signaling, and mechanical stress (30, 63-70). Upon exposure to the aforementioned stimuli, fibroblasts become slightly larger cells exhibiting an exemplified secretory and synthetic phenotype called a myofibroblast (71). Myofibroblasts are the quintessential cellular source of fibrosis, especially within the cardiovascular system. They are present within the valve leaflets of a healthy heart in order to maintain valve durability and structure from continuous hemodynamic stress caused by contraction and subsequent relaxation (72). After activation, myofibroblasts alter their

secretory profile to include α-smooth muscle actin (α-SMA), fibronectin (FN), non-muscle myosin heavy chain-b (NMMIIB), TGFβ receptors, fibrillar collagens type I and III, vimentin, and periostin (73-79). In addition to these cells, there are circulating cells, derived from the bone marrow, that contribute a significant portion of ECM protein deposition.

Numerous studies have been performed with various cell-tracing techniques and bone marrow transplant models to understand the relative contribution of pro-fibrotic cells originating from the bone marrow compartment (9-12, 23, 80). Using enhanced green fluorescent protein (eGFP)-transgenic mice, two independent groups have demonstrated both the presence and the active contribution of bone marrow-derived cells to cardiac fibrosis (80,81). Both studies found that approximately 20-24% of the myofibroblast cells present within the scar expressed eGFP seven days post-infarct, indicative of their bone marrow origin. Another comprehensive study utilized a gender mismatched bone marrow transplant technique in mice to evaluate the number of Y chromosome-positive cells in aortic banded, via transverse aortic constriction, and sham treated hearts (12). This study revealed that the banded hearts had 13.4% and 21.1% of the cells with a Y-chromosome fibroblast specific protein-1 (FSP-1) and/or α-SMA positive, respectively, indicating that these cells expressing fibroblast and myofibroblast markers originated in the bone marrow. Interestingly, in sham hearts, there were no FSP-1 positive cells with a Y chromosome; however, about 3.4% of cells with a Y chromosome were α-SMA positive which indicates that, even in normal hearts, bone marrow-derived cells mildly furnish the fibroblast population within the heart. It is important to elucidate whether this cellular behaviour is possible in a human population. Excitingly, similar analysis was performed in on autopsy samples of heart transplant patients with gender mismatched hearts who suffered a myocardial infarction post-transplant. The gender mismatching of the hearts allowed for cell tracking in the transplanted heart and, as early as two to four weeks after infarction, almost 6% of all cells presented in the infarcted region were non-inflammatory progenitor cells (15). Although it was unspecified whether these non-inflammatory cells were fibroblasts and/or mesenchymal progenitor cells, it was discovered that 24.1% of all cells carrying a Y-chromosome were cells that were derived from the patients who received the organ transplant and experience an MI, which

compared to only 5.3% of of sex-mismatched cells in control non-infarcted patients (15). Although these numbers are lower than those found in animal studies, they are still comparable and might indicate that it may take longer for cells to be recruited from the bone marrow to contribute to scar formation in transplant patients as a result of additional complications and/or medications that may interfere with the wound healing process. Even though these studies clearly demonstrate that bone marrow-derived cells are present within the heart post-injury, it is also important to determine that these cells directly contribute to pathological fibrosis by participating in ECM protein deposition. To evaluate this, van Amerongen *et al* (80) utilized a bone marrow transplant model with mice expressing a pro-Col1A2 (coding for the α2 chain of pro-collagen I) promoter fused to both β-galactosidase and luciferase. Analysis of *in vivo* luciferase and *in vitro* β-galactosidase activity showed that cells derived from the transplanted pro-Col1A2 transgenic mice bone marrow were present in the post-MI heart and actively expressed collagen.

A common element of these studies previously discussed is that the proportion of bone marrow-derived pro-fibrotic cells significantly increases as a result of injury. Inflammation signals the body that injury has occurred which stimulates fibrosis, indicating these two processes are inextricably affiliated with each other (7). The recruitment of these bone marrow-derived cells to sites of inflammation is the result of a variety of factors. There are unique profiles of chemokines, cytokines, growth factors, and ECM structure in heart tissue that indicate whether it is healthy, injured, inflamed, or fibrotic. These profiles create a specific myocardial signaling milieu that cause varied differentials of administered multi-lineage progenitor cells at different stages of myocarditis (11,81).

Analysis of ischemic heart disease models indicates that bone marrow-derived recruited or administered progenitor cells do not differentiate into cardiomyocytes, but, if hematopoietic, will continue along that same lineage (82-84). One lab has investigated the importance of the chemokine receptor C-C chemokyne receptor type 2 (CCR2) (85), which is found in to be expressed in >80% of bone marrow-derived fibroblast precursors. Their experimentation with CCR2-KO mice given an angiotensin-II (AngII) infusion caused a decrease in collagen accumulation and Col1 and fibronectin mRNA expression in the heart compared to wild-type controls. They also showed that bone marrow-derived fibroblast

$CD34^+/CD45^+$ precursors identified with $Col1^+$ were increased in AngII-treated wild-type mice, but these numbers were significantly lower when compared to CCR2 KO mice. Interestingly, there was found to be no difference in cardiac hypertrophy or hypertension between these two groups indicating the presence of a unique mechanism for recruitment of bone marrow-derived fibroblasts (85). Cells expressing CD133 have also been observed infiltrating the myocardium (86), indicating bone marrow-derived hematopoietic lineage origins. These cells also express stromal cell-derived factor-1 α (SDF-1α) (87), a common chemokine for hematopoietic progenitor cells, which was found to be significantly up-regulated in the myocardium of AngII-exposed animals as early as day one of treatment (17). Finally, the C-X-C chemokine receptor type 4 – C-X-C chemoke ligand type 12 (CXCR4-CXCL12) axis is instrumental in homing bone marrow-derived progenitor cells (88), as CXCR4 is a crucial chemokine receptor in stem cell trafficking, and the differential expression of CXCL12 within various tissues allows for trafficking $CXCR4^+$ cells in a gradient-dependent manner to ensure tissue specificity (88,89). These studies highlight the significance of the immune system in cell recruitment, as immunodeficient mice showed no bone marrow-derived cell contribution to fibrosis, however further parameters regarding outcomes was not assessed (90). This immune system response has been shown to be imperative in facilitating the migration of bone marrow-derived progenitor cells to fibrogenic areas, and encouraging their differentiation into pro-fibrotic cells (91). It is important to mention that clinical trials utilize injections of freely flowing bone marrow-derived progenitor cells as a regenerative cell therapy. Although reported improvements have been modest, they have been attributed to bone marrow-derived MPCs anti-inflammatory properties that have been extensively studied in relevant animal models and correlated with human tissue (92-98).

Mesenchymal Progenitor Cells (MPCs)

There are two distinct lineages of progenitor cells residing within the bone marrow that have the potential to differentiate into myofibroblast-like cells *in vitro*: hematopoietic stem cells (HSCs) and mesenchymal progenitor cells (MPCs) (99). MPCs have been shown to share many features with myofibroblasts, which is why their contribution to fibrosis has been studied extensively (27,28,85,100-102). They are a rare population of cells that make up an exiguous 0.001-0.01% of bone marrow (103)

where their contribution to fibrosis is not limited to responding to injury or acute inflammation, but also as a result of aging (104). MPCs are known precursors for cells, namely fibroblasts and smooth muscle cells that actively build ECM proteins (99). When comparing MPCs to fibroblasts, it is important to note that initially, MPCs were identified as colony-forming unit fibroblast-like cells (9). Known fibroblast cell lines, specifically HS68 and NHDF, were screened using a phenotype panel of 22 CD surface markers against MPCs and determined to be identical (105). A study co-culturing human MPCs with human dermal fibroblasts found that there was MPC-induced fibrosis as a result of the differentiation of the MPCs into myofibroblasts with well-organized α-SMA filaments (106). MPCs have even been found to express basal levels of contraction similar to cardiac fibroblasts and respond in a similar way to TGFβ-1 treated cells by strengthening their ability to contract (107) through co-expression of αSMA (108). During culturing on plastic dishes, MPCs produced collagen, although this did not increase in response to TGFβ-1, which differed from observations utilizing human myofibroblasts obtained from atrial tissue (107, 109,110). TGFβ-1 is an important ligand as it stimulates the differentiation of fibroblasts to collagen-forming myofibroblasts (108). In addition to exposure to a variety of growth factors, including connective tissue growth factor and fibroblast growth factor-2, MPCs have also been found to differentiate into fibroblasts by cyclic mechanical stimulation (111-115). A unique feature of MPCs is their ability to have an intercellular connection with resident cardiomyocytes via connexin 43; however, it this same trait has been observed with cardiac fibroblasts (116-118). This allows for electrical signal transduction throughout the heart that further emphasizes the similarities between these cell types, as well as allowing for the potential for endogenous differentiation of MPCs to myofibroblasts (119, 120).

Fibrocytes

Recently a unique cell type contributing to cardiac fibrosis was identified, sharing the cell markers of leukocytes, hematopoietic progenitor cells, and fibroblasts (collagen$^+$/CD13$^+$/CD34$^+$/CD45$^+$) (22, 121). Within the literature, these cells have been given a variety of names including telocytes, CD34$^+$ stromal cells, and fibrocytes (122). For the purposes of this introduction, we shall refer to these cells as fibrocytes which originate from immature mesenchymal cells within the peripheral blood but have the hematopoietic

lineage marker $CD34^+$ (122). Although they only make up less than 0.5% of mononucleated cells in peripheral blood, they are ubiquitously present throughout the body and have been found to be involved in synthesizing substrates, immunomodulation, providing scaffolding support for other cells, phagocytosis, parenchymal regulation, as well as synthesizing and remodeling ECM (89, 122). Recently, a clinical study investigating biomarker predictors of unstable angina noted that patients had increased levels circulating fibrocytes compared to patients with stable angina, as previous research indicated that concentrations of fibrocytes can increase up to 100-fold in the blood after tissue injury (20, 89, 123). Although fibrocytes are classified as a subset of fibroblasts, they have a distinct progenitor phenotype as they demonstrate the capacity to differentiate into adipogenic, osteoblastic, and chondrogenic lineages (124) and are present in animal models of atherosclerosis (125). In studies evaluating human tissue, fibrocytes are present within the fibrous cap of human carotid artery plaques (126), in patients with atrial fibrosis in the context of chronic atrial fibrillation (127), and in patients with hypertrophic cardiomyopathy (128). Repetitive episodes of ischemia-reperfusion inducing fibrotic cardiomyopathy resulted in an increase in fibrocytes, as indicated by the expression of collagen-1, αSMA, CD34, and CD45 (9). The presence of these cells in various cardiovascular diseases where pathogenic fibrosis occurs indicates that they could be a key target in ameliorating detrimental ECM remodeling. They are robustly active in producing ECM proteins including collagen I, collagen III, and vimentin, and secrete matrix metalloproteinases (MMPs) which are key regulators of cardiac ECM remodeling (129, 130). As previously discussed, immunomodulation is an important factor in promoting fibrosis and fibrocytes which uniquely contribute by being a source of inflammatory cytokines, chemokines, and growth factors. During wound healing, fibrocytes can localize to granuloma formation and ECM matrix protein deposition (129). Research has indicated that fibrocytes are released from the bone marrow into the peripheral blood to hone into zones of inflammation via a CCR2-mediated pathway (85). Fibrocytes have the adaptive capabilities to undergo phenotypic changes by being proliferative, synthetic, and/or contractile and, by expressing markers such as endosialin and integrin receptors, they can bind to fibronectin and fibrin to situate themselves in close proximity with resident fibroblasts (131, 132).

Other Differentiating Pro-Fibrotic Cell Sources

Although bone marrow-derived progenitor cells contribute significantly to fibrosis, they are not the only cell type capable of altering their phenotype to become pro-fibrotic. Endothelial-to-mesenchymal transition (EndMT) contributes to fibrosis through conversion of endothelial cells to fibroblasts (12, 133) which has been observed in the heart with endothelial cells that reside in the endocardium. During development, some of these cells undergo EndMT in order to form valve interstitial cells that then further mature into valve mesenchymal cells and valve leaflets (134). An important factor in triggering this cell transformation is TGFβ (135), which, as previously mentioned, is found extensively in pro-inflammatory and pro-fibrotic areas of the heart and is responsible for fibroblast activation (136). When losartan was used to inhibit activation of the TGFβ/SMAD pathway there was a concurrent significant reduction in EndMT (137). In a lineage tracing analysis utilizing LacZ irreversibly labelled endothelial cells, Ziesber, *et al*., (12) found that mice exposed to pressure-overload for five days had nearly 33% of their cardiac fibroblasts originate from the endothelial layer. Not only do EndMT cells further complicate fibrosis by forming fibroblasts, but there is also a net loss of endothelial cells which impairs normal organ function. In a study investigating the role of endothelial cells in chronic kidney injury, it was found that reduced bioavailability of nitric oxide synthesized by endothelial cells actually causes further endothelial dysfunction where there was up-regulation of collagen, increased TGFβ and rarefaction of capillaries (138). This loss of endothelial cells may also contribute to the decreased stability of atherosclerotic lesions as noted recently in a study investigating human plaques which determined that the extent of EndMT correlates to an unstable plaque phenotype (139). Most recently, myofibroblasts derived from EndMT were found to engulf dead cells post-MI in both human and mice, which is an activity not yet observed in pro-fibrotic cells originating from bone marrow (140).

Another key cellular transformation process that occurs during embryonic heart development is epithelial-to-mesenchymal transition (EMT). In adults, this mechanism has been implicated in pathological ECM remodeling of the heart by up-regulating a number of genes encoding growth factors. VEGF, FGF2, TGFβ2, and MCP1 which promote angiogenesis and might be beneficial in reducing heart

injury post-MI (141). EMT has been established as a key source of fibroblasts in the liver (142), and lungs (143), and epithelial cells of the epicardium have demonstrated the capacity to differentiate into fibroblasts as well (144). Fibrogetic EMT has been observed in the heart by overexpression miR-21, found to be upregulated during EMT (145). This unique pro-fibrotic cell source has yet to be verified to determine if it contributes to human patient pathological fibrosis in the heart.

In addition to progenitor cells found within the bone marrow, a distinct population of cardiac stem cells has been found residing in the heart (146). They are negative for various hematopoietic lineage markers (such as CD34, CD45, CD8) but are positive for c-kit, a canonical stem cell marker. Although these cells are being evaluated for their potential to become functional cardiomyocytes for therapeutic purposes (146, 147), there is currently no information available exploring the potential for these cells to differentiate into myofibroblasts or any other cell type that could contribute to cardiac fibrosis.

1.3.2. Current Anti-Fibrotic Therapies: Focus on Effects of Statins

Despite the prevalence of pathological cardiac fibrosis in a myriad of cardiovascular diseases, there are few effective pharmaceutical options. Inhibitors of the renin angiotension aldosterone system (RAAS) have had minor success in small clinical trials; however cardiac fibrosis still persists in heart failure patients even when treated (148). Although inhibiting TGFβ, a significant factor contributing to fibrosis, seems like a logical target, its effectiveness in animal studies has failed to translate to clinical success. Treatment with tranilast was ineffective in preventing post-percutaneous transluminal coronary angioplasty restenosis clinically and it, as well as pirfenidone, causes liver dysfunction (149,150). An alternative avenue to targeting fibrosis is inhibiting the activation of MMPs, ECM proteases responsible for pathological ECM remodeling. Unfortunately, the PREMIER study of orally active MMP inhibitor, PG-116800, in 253 patients failed to improve clinical outcomes, although this trial neglected to evaluate any parameters of fibrosis (151). Many interventions have been tested to prevent fibrosis; however, the most ubiquitous treatment, not primarily targeting fibrosis but shown to have anti-inflammatory effects, are compounds inhibiting hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase (150). They are

extensively used to treat hypertension as they prevent the downstream formation of cholesterol by inhibiting mevalonate formation (152,153). These compounds are ubiquitously known as statins and share a common active component that competitively inhibits HMG-CoA reductase by forming a β-hydroxyacid *in vivo* to from a lactone ring (152). The base unit of cholesterol production is acetyl CoA which is converted to acetoactyl CoA by condensation. HMG-CoA synthase utilizes the latter to form HMG-CoA which is reduced by HMG-CoA reductase to produce mevalonate (154). Statins prevents mevalonate production by blocking HMG-CoA reductase (154). We shall focus on the effects of statin treatment as 12% of Canadians (ages 20-79) and over 20% of Americans are prescribed HMG Co-A reductase inhibitors (155, 156). In addition, regardless of whether or not novel therapies will effectively target fibrosis, it is projected that 27% of the Canadian population will be recommended for statin treatment within the next decade (155).

Although inhibiting mevalonate production is effective in reducing cholesterol production, there are many downstream compounds dependent on the formation of mevalonate which have been shown to be affected by statin treatment. There are a number of crucial isoprenoid building blocks that are derived from mevalonate including steroids, bile acids, vitamin D, haem A, Coenzyme Q10 (CoQ10) and isopentenyl adenine, to name a few (157). Of particular interest are CoQ10, involved in membrane structure (157-159) and dolichol, which is mandatory for glycoprotein synthesis (160). CoQ10, known as ubiquinol in its reduced form and ubiquinone in its oxidized form, is a lipid molecule found in the inner membrane of the mitochondria. It forms a crucial component of the electron transport chain during adenosine triphosphate (ATP) synthesis (161), and is shown to be decreased with statin treatment (162-164). Dolichols facilitate transportation of glycan precursors required for forming oligosaccharides is vital in forming steroid hormones (160). Preventing mevalonate synthesis prevents the formation of farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) which are crucial lipid attachments for numerous proteins including Ras, Rho, and Rac of the small guanosine triphosphate (GTP) binding protein family (157). Classically, it has also been observed that mevalonate depletion also

prevents the DNA synthesis (S) phase of the cell cycle thereby reducing cell proliferation (165-168). In addition, treatment with mevastatin, or cells lacking either HMG-CoA synthase or HMG-CoA reductase, will not grow in a lipoprotein-free environment unless mevalonate is present (169-171). There have also been numerous studies evaluating the effect of mevalonate inhibition on patient immunology, as mevalonate inhibition also inhibits nuclear factor-κB (NFκB) activity which is responsible for recruiting many pro-inflammatory factors including TNFα and IL-6 (171-175). Unfortunately, complete inhibition of mevalonate downstream products results in manifestation of mevalonate kinase deficiency (MKD) which is the result of genetic mutation of mevalonate kinase. MKD leads to two distinct syndromes, mevalonic aciduria and hyper-IgD syndrome (HIDS) which are characterized by increased inflammasome activity (176). Evidently there are many effects that occur as a result of inhibiting mevalonate formation which will be discussed more thoroughly in this section. It is important to note that currently there are a number of studies that have occurred and are ongoing evaluating the pleiotropic effects of statins, independent of cholesterol formation inhibition, in patients both with and without cardiovascular diseases, which are more elaborately discussed in these noted reviews (177-181).

1.3.2.1 Mevalonate and G-Protein Activation

The mevalonate pathway is a highly conserved metabolic cascade in mammals and yeast which yield isoprenoids, the oldest known biomolecules, which are synthesized from isopentenyl pyrophosphate (IPP), a compound downstream of mevalonate synthesis, and dimethylallyl pyrophosphate (DMAPP) (182,183). Mevalonate is the original substrate from which FPP and GGPP are derived (157). They are formed when mevalonate is metabolized into IPP and its isomer DMAPP. FPP synthase then catalyzes condensation reactions of DMAPP with two units of IPP to form FPP which undergoes further condensation reactions by GGPP synthase to form GGPP (184,185). These compounds are crucial in protein isoprenylation which is important in numerous intracellular signalling events (186,187). Levels of isoprenoids have been shown to be reduced by statin treatment in a variety of cell types (188,189). It is interesting to note that, although there are many proteins that have been discovered to contain farnesyl or

geranylgeranyl groups, it is estimated that there are over 300 prenylated proteins in the human proteome (190). One of the most important functions of isoprenylation is enabling the activation of a few crucial members of the large family of G-proteins. G-proteins utilize and/or bind GTP in order to perform a wide variety of cellular activities. They function as molecular switches that convert GTP to the products guanosine diphosphate (GDP) and inorganic phosphate. When active, these molecules are bound with GTP which then become inactive once the GTP is utilized, which can then be re-activated when the GDP is replaced with another GTP molecule via guanine nucleotide exchange factors (191,192). Three members of the smaller subset of the GTP family, Ras, Rac, and Rho, known as GTPases, are soluble cytosolic proteins and require isoprenylation to anchor themselves to the lipid membrane in order to function (193,194). GTPases are responsible for a wide variety of cellular functions, and the Ras superfamily, which includes, Rac, and Rho, is responsible for regulation of gene expression, in addition to many other functions including reactive oxygen species (ROS) generation and cytoskeletal organization (195,196). The majority of effects observed with mevalonate depletion involving the Ras superfamily focus on ameliorating oxidative stress. For example, in order to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a molecule that is responsible for generating a superoxide anion, Rac1 must undergo isoprenylation which enables it to translocate to the membrane and bring it within proximity of NADPH oxidase. This direct contribution to reactive oxygen species formation is prevented if isoprenylation of Rac1 fails to occur (197). This has a cumulative effect as ROS generation promotes production of proinflammatory cytokines and oxidized low-densitiy lipoprotein which further stimulates Rac1 membrane translocation (198). There are currently 22 known genes of the Rho family, all of which bind GTP, and are primarily responsible for leukocyte adhesion to endothelial cells (ECs). This is done by the clustering of ICAM-1 and VCAM-1 and forming the necessary docking structures around adherent leukocytes (199, 200). The Rho family of proteins require prenylation from either GGPP and/or FPP, which affects their ability to optimally locate themselves on the membranes of leukocytes (201). Inhibition of Rho activation has also shown to be beneficial in promoting vasorelaxation as it increases the bioavailability of endothelium-derived nitric oxide (202,203). Another molecule whose

activation is targeted by the GTPase family is the mammalian target of rapamycin complex 1 (mTORC1), a Ras homologue enriched in brain (Rheb). mTORC1 is known to activate translation of proteins and is involved with numerous aspects of cell physiology including cell growth, protein synthesis, ribosome biogenesis, nutrient metabolism and autophagy (204). With regard to statin treatment, it was found that proliferation of T-cells and IFNγ expression were both blocked as a result of increased KLF-2 expression, which is naturally suppressed by mTORC1 activation (205).

There has also been clinical evidence indicating the influence of altered levels of GTPases. Inhibition of Rac1 isoprenylation has also shown to be effective in improving the environment of saphenous vein grafts in patients who underwent short-term treatment with atorvastatin prior to a coronary artery bypass graft procedure. This was also done by inhibiting the activation of NADPH oxidase (206). It has been consistently observed that endothelial function is improved in patients treated with statins which is a result of reduced ROS production due, in part, to a decrease Rac1-activated NADPH oxidase levels (207-209). Rac1 was also the first of the small GTPases to be linked to cardiac remodelling, where it was demonstrated to mediate cardiac hypertrophy (210,211). Interestingly, in a recent study by Zhu *et al* (212), although mevalonate depletion disabled the ability for Rho to associate with the cell membrane, it accumulated in the cytosol of human cancer cells, with bound GTP, and underwent apoptosis. Further studies evaluating this effect in non-cancer cells would be important in determining whether or not this effect is cancer-cell specific and could provide an effective method of targeting cancer cells. It is evident that inactivation of a few members of the small GTPase family appear to have multiple clinical benefits, not only for patients with hypercholesterolaemia, but also with a wide variety of both cardiovascular and non-cardiovascular-related pathologies.

1.3.2.2 Coenzyme Q10

CoQ10 is a lipid molecule that consists of a 1,4-benzoquinone compound with an additional 50 carbon atoms that helps transport electrons when the cell is synthesizing ATP (161) in the inner

membrane of the mitochondria. It is present in all cells and is involved with redox modulation, membrane stabilization, in addition to electron transport (213,214). Deficiency of CoQ10 has been shown to lead to the decreased functionality of mitochondrial complexes I, II, and IV of the electron transport chain (215). By its proton carrying capacity, it is able to activate the uncoupler proteins (216,217). In addition to its many roles in the mitochondria, studies have revealed that CoQ10 supplementation can act as an antioxidant by reducing oxidative stress biomarkers and increasing the antioxidant efficiency of other antioxidants, such as renegerating α-tocopheroxyl from α-tocopheroxyl radicals (218-220). It is incredibly effective in this regard due to its proximity to oxidative stress environments and its ability to continuously regenerate itself by reduction (221). It also plays an important role in the inflammatory response by suppressing nuclear factor-κB (NF-κB) activation (214).

The interruption of its production by statin administration has been demonstrated in clinical settings (222,223). It has been shown that patients taking statins show a decreased level of CoQ10 in serum (164, 224). Interestingly, in the large clinical trial CORONA (Controlled Rosuvastatin Multinational Study in Heart Failure), it was found that, although serum levels of CoQ10 were lower in older patients with more advanced heart failure, there was little interaction between rosuvastatin and CoQ10 (225). It was determined that, although mortality was higher in patients with less CoQ10, and rosuvastatin further reduced CoQ10 levels, this did not lead to a worse outcome for the patients (226,227). As low CoQ10 was related to poorer prognosis independent of statin treatment, supplementation of CoQ10 has been investigated. It has been found that, in concert with cerivastatin, CoQ10 can improve flow mediated dilation of the brachial artery indicative of improved endothelial function (225). Due to its involvement in the energy cascade, one of the clinically observed side effects of CoQ10 depletion is fatigue (162). Another previously reported side effect from depleted CoQ10 levels is myalgia (162); however a CoQ10 supplementation trial stated that supplementation of CoQ10 had no beneficial effect on muscle pain, muscle strength, or aerobic exercise capacity (223). The more recent study estimated that only one third of patients who claimed to have statin myalgia had true statin myalgia (228). As supplementation with

CoQ10 had a negligible effect on alleviating myalgia, it is perhaps a different missing link of the mevalonate cascade that is causing muscle pain brought on by statin treatment.

1.3.2.3 Anti-Inflammatory Effects of Statins

One of the most investigated and intriguing of the many pleiotropic effects of reduced mevalonate levels has been its effect on inflammation. There have been changes observed in how statin treatment affects B-cells, T-cells, regulatory T-cells, macrophages, and endothelial cells in the immune response (229-235). Although there are a glorious number of factors that can influence the immune response, addition of mevalonate has been observed to reverse statin-mediated effects (229, 236). As initiators of the immune response, T-cell modulation by mevalonate depletion is of particular interest. It has been observed that their proliferation, Th1 differentiation, and migration are decreased when mevalonate is depleted (237-240). This reduced inflammatory response results by decreasing the secretion of pro-inflammatory cytokines including TNFα, and IFN-γ and increased secretion of protective IL-4 (237-240). As previously mentioned in the chapter, mevalonate is responsible for producing factors that enable cell-cell tethering, so it is no surprise that statins interfere with interactions critical in neutrophil tethering and rolling. Simvastatin was used in a study evaluating human neutrophils and their interaction with human umbilical vein endothelial cells (ECs). Histamine was used to simulate a pro-inflammatory environment where increased transient interactions between neutrophils and ECs were reduced upon administration of simvastatin (241). Mevalonate pre-treatment was observed to reverse the statin effect on both tethering and rolling which was found to be affected by the surface expression of P- and E-selectin, which are instrumental in the rolling and tethering processes of neutrophils (241).

A number of studies have been performed evaluating the effect of mevalonate inhibition in attenuating the pro-inflammatory response in a number of pathologies. In a kidney transplant model, atorvastatin was shown to block renal inflammation and fibrosis by decreasing the number of infiltrating mononuclear cells (ED1+, CD8+, CD68+), the mRNA levels of pro-inflammatory cytokines (IL2, IFγ,

IL10), chemokines (RANTES, MCP-1), and pro-fibrotic genes (TGFβ1, collagen III) (242). In intestinal fibrosis, pravastatin was found to induce dose-dependent decreases in inflammatory mediators osteopontin, C-reactive protein, and TGFβ1, in addition to reducing the number of macrophages. This led to abrogation of tubulointerstitial inflammation and fibrosis (243). Attenuated production of pro-inflammatory cytokines, IL-6 and IL-8, has been show to occur in human fibroblast-like synoviocytes derived from patients with rheumatoid arthritis treated with simvastatin. This observation was reversed upon concomitant incubation with mevalonate (244).

These experimental models have also translated to the clinical setting. In patients with non-ischemic heart failure, there have been recorded decreases of inflammatory markers, including high-sensitivity C-reactive protein and IL-6, with atorvastatin treatment (245). A similar reduction in inflammation with simvastatin treatment occurred where there was also a measured increase in left-ventricular ejection fraction (246). In peripheral blood mononuclear cells isolated from patients with hypercholesterolemia, the original patient target for statin treatment, administration of statins led to a reduction in the expression of a number of pro-inflammatory cytokines including IL-6, IL-8, and MCP-1 in both *in vitro* and *in vivo* experiments (247). The JUPITER (Justification for the Use of Statin in Prevention: An Intervention Trial Evaluating Rosuvastatin) trial was designed to objectively evaluate the anti-inflammatory properties of statins. This was done in 17 802 patients with no history of cardiovascular disease and low cholesterol levels. It was found that rosuvastatin was able to lower high-sensitivity C-reactive protein levels which led to decreased incidences of major cardiovascular events (248). These observations indicate that mevalonate produces compounds that enable a pro-inflammatory response; however, in pathologies where inflammation is detrimental, statins have been shown to mitigate a more tolerant response (236). This is a promising active area of research taking advantage of the pleiotropic effect of statins and their modification of the mevalonate pathway.

1.3.2.4 Mevalonate Kinase Deficiency

Although it appears that statin administration has been observed to be more beneficial than detrimental to patients, it is important to keep in mind that there is not a complete cessation of mevalonate production and its downstream products. Mevalonate kinase deficiency (MKD) is a congenital disease resulting from an autosomal recessive error present in the *MVK*-gene responsible for transcribing mevalonate kinase (249-252). There are two manifestations of disease resulting from mutations of this gene: 1) hyperimmunoglobulinemia and periodic fever syndrome (HIDS), which involves brief inflammatory episodes that may be accompanied by lymphadenopathy, hepatosplenomegaly, abdominal pain, vomiting, diarrhea, arthralgia, myalgia, skin rashes, and mucosal ulcers (252,253), and 2) mevalonic aciduria (MA), which recognized as the more severe form of MKD that is further characterized by dysmorphic features and pre- and post-natal growth retardation (250,251,253). These patients have mutations in both *MVK* alleles the majority of which result in a single amino acid change, although other changes involve stop, frame shift, and splice mutants (254). The range of severity of the disease is thought to be a result of the level of inactivity of the product of *MVK,* which is dependent on the types of mutations present. It has been observed that residual enzyme activity of HIDS patients ranges from 1.8%-28%, whereas MA occurs with residual enzyme activity is less than 0.5%; however there are no reports of enzyme activity at 0% which is hypothesized to be lethal in utero (252,255). It is important to keep in mind that these patients produce mevalonate, but not downstream isoprenoids.

Historically, it was thought that the build-up of mevalonate was the primary exacerbator of the disease as it was found in increased levels in the urine during fever attacks and is less prevalent in the urine in HIDS patients compared to MA patients (256). Statins were used to reduce the levels of mevalonate in MA patients, however it was discovered that this further aggravated the symptoms (257). There has been hope; however, as treatment with isoprenoid-like compounds geraniol or farnesol was able to attenuate inflammation in animal models and *ex vivo* patient cells (258,259). The inflammatory aspect of MVD is brought on by formation of an inflammasome complex; however it is still not clearly understood how decreases in FPP or GGPP could be involved. It is hypothesized that, due to the lack of

FPP and/or GGPP, small GTPases (which can be responsible for modulating inflammatory responses) are unable to localize to the membrane. As a result, they accumulate within the cytosol of the cell where they favour the GTP-bound active state and form autophagosomes which has been shown to regulate the maturation and secretion of IL-1β (260-262). Another potential mechanism for increased inflammation in MVD patients is mitochondrial dysfunction. This chapter previously discussed how CoQ10, a molecule important in the mitochondrial electron transport chain, is formed downstream of mevalonate. Severely depleted levels of CoQ10 could lead to mitochondrial dysfunction and release of mitochondrial ROS which can activate inflammasomes (263). It is evident that complete, or even reduction of downstream mevalonate products by less than 30%, can cause pathological pro-inflammatory effects which should be noted when considering inhibiting mevalonate formation to utilize its pleiotropic effects when treating the myriad of diseases statins have shown to positively affect.

Although inhibition of the mevalonate signalling pathway induces pleiotropic effects via statin treatment, there is no doubt that statin treatment has been beneficial in lowering cholesterol and reducing the number of cardiovascular events in the hypercholesterolaemic population. However, its effectiveness in anti-fibrotic treatment is questionable and alternative therapies are required to effectively tackle pathological fibrosis. It is important to note that, as evident in patients suffering from MVD, complete cessation of mevalonate production should be avoided as its downstream products have been shown to be essential in balancing inflammation.

1.4 MicroRNA

The molecular mechanism partially responsible for the phenotype transition of embryonic stem cells differentiating into discrete cell types specific to various tissues throughout the body was discovered to be small non-coding RNA molecules that silence RNA and regulate gene expression post-transcription (264). The non-coding miRNA molecule is a hairpin precursor most commonly derived from introns of protein-coding host genes transcribed by RNA polymerase II (265). The miRNA precursor then migrates

from the nucleus to the cytosol and becomes activated by the enzyme Dicer, an RNase III endonuclease (266).

1.4.1 Influence of miRNA on Pro-Fibrotic Cell Differentiation and Pathological Fibrosis

When injury occurs in the body, cells have to respond expeditiously in order to initiate immediate healing to prevent further damage. As discussed previously, there are numerous cells within the body that undergo a phenotype change in order contribute in fibrosis. These cells are then guided to the damaged area by inflammation which promotes ECM remodeling. In most circumstances, this change occurs rapidly as the cell essentially "switches" its phenotype. It takes time for a cell to undergo complete protein translation based off of extracellular signaling that subsequently causes the activation of transcription factors. Luckily, a type of recently discovered molecule, known as miRNA, could rapidly facilitate this phenotypic "switch", that essentially skips the translation step of protein synthesis. The miRNA molecule is made up of 20-25 nucleotides derived from mostly non-coding regions of the genome that target mRNA to fine-tune protein translation (31, 267). The first miRNA was discovered during the adult stage of *Caenorhabditis elegans* where loss of the miRNA let-7 caused reiteration to larval cell fates, which indicates that let-7 is necessary for the differentiation of adult cells (31). Furthermore, let-7 has also been shown to be important in the reversion of adult fibroblasts back in adult MPCs (268). Activation of miRNA is required for them to function effectively and this is done by the protein Dicer (269). In a study where Dicer was knocked out, MPCs retained their mesenchymal identity indicating that miRNAs do not necessarily govern cell identity but that miRNA processing is required for active differentiation, suggesting its presence is critical for cell state transition (268). These data supports additional findings that demonstrate the importance of miRNAs in regulating cellular transitions and physiological robustness in various model systems (270,271). A specific miRNA has been identified as being crucial in cardiac myofibroblast differentiation, namely miR-145 (38). This particular miRNA was initially identified as a regulator of smooth muscle cell differentiation. Understanding how miR-145 influences myofibroblast differentiation led to research focusing on α-SMA filaments. When treated with miR-145,

α-SMA positive cells readily formed stress filaments and were organized in parallel actin-filament bundles, permitting cellular contractility that was comparable to fibroblasts treated with TGFβ1 (38). In addition, fibroblasts expressing miR-145 migrated to a comparable degree as fibroblasts treated with TGFβ. Expression of the essential ECM protein collagen was also assessed, with miR-145 treated cells showing comparable decreases in pro-collagen 1A1 and pro-collagen 1A2 but significant comparable increases in mature collagen 1A1 and collagen 1A2 production. The natural design of miRNA allows it have the potential to have numerous downstream targets, however researchers were able to identify KLF5 as a critical target for myofibroblast differentiation. KLF5 is instrumental in cardiovascular remodeling, particularly in smooth muscle cells, as it activates platelet-derived growth factor A/B, Egr-1, plasminogen activator inhibitor-1, inducible nitric oxide synthase, and vascular endothelial growth factor (272). Whether miR-145 is the sole miRNA to facilitate the fibroblast to myofibroblast transition is yet to be determined. Not only can miRNAs cause differentiation of fibroblasts to myofibroblasts, they are also critical in the de-differentiation of human fibroblasts. As Nobel Prize winner Yamanaka demonstrated, fibroblasts can be reprogrammed back into an embryonic-like phenotype (273) and this discovery prompted several scientists to look at various aspects of this phenomenon. Two miRNA that were found to promote the reprogramming of human fibroblasts to inducible pluripotent stem cells were miR-302b and miR-372 (274).

The expression of several miRNAs, including several let-7s, miR-1, miR-133a, miR-133b, miR-19a, miR-19b, miR-150, miR-195, miR-199, miR-221, miR-23a, miR-23b, miR-29a, miR-29b, the miR-30 family and miR-320, are either increased or decreased during heart failure which highlights the complexity of this pathology (275). The majority of the research focuses on the most expressed miRNA in the heart is miR-1, which accounts for 40% of all cardiac miRNAs (276). Upon transfection into embryonic stem cells, miR-1 enhanced cardiomyocyte differentiation by modulating the PTEN/AKt pathway (277). It also plays a role in the regulation of the cardiac conduction system, in part by controlling the expression of connexin 43 (278). It has been shown to have differential expression based on the whether or not the heart is succumbing to short-term injury such as ischemic injury, where it is up-

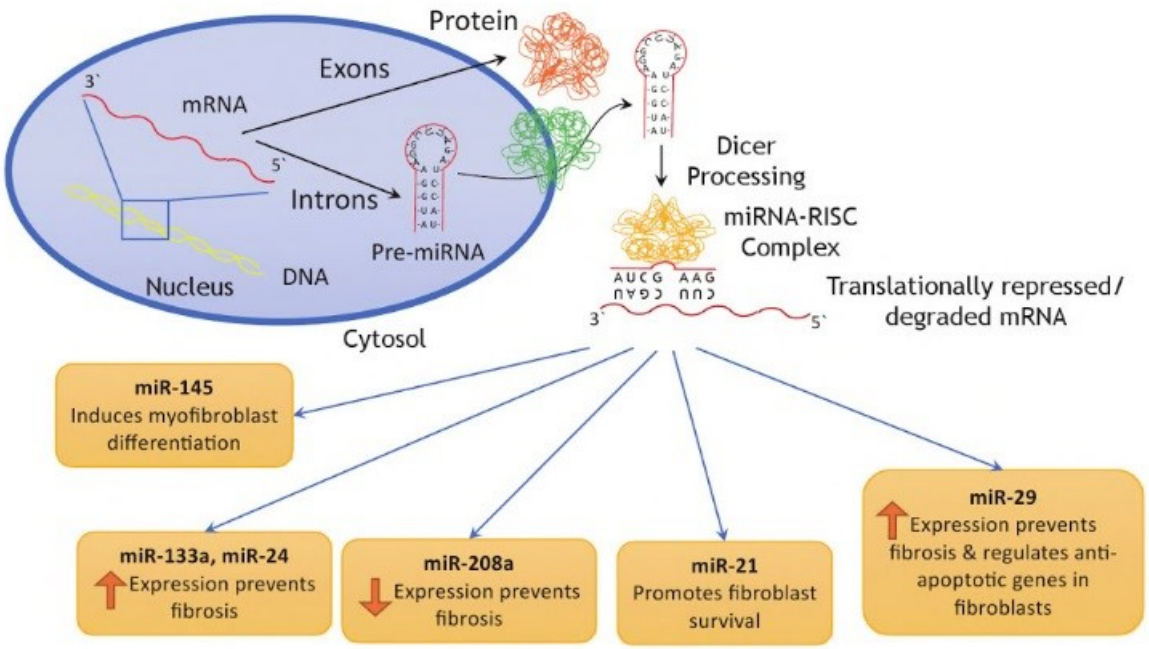
regulated, or long-term injury such as hypertrophy and heart failure, where it is down-regulated (275). One of its downstream targets is the cytoskeletal regulatory protein twinfilin-1 whose inhibition evokes cytoskeleton-regulated hypertrophy (279). Its potential role in fibrosis has yet to be elucidated although, due to its high expression in the heart, it is unlikely that it is a bystander during pathological cardiac ECM remodeling. MiR-133a may be more directly implicated in fibrosis as its increased expression prevented both fibrosis and apoptosis in an animal model of transaortic constriction (TAC) (34). It did not affect hypertrophy in either the TAC model or in isoproterenol-induced hypertrophy. An additional miRNA of interest is miR-208a because, when it is knocked out in a TAC model, there is negligible hypertrophy of cardiomyocytes or fibrosis detected (35). In a different study evaluating miR-208a, it was found that in areas associated with interstitial fibrosis, there was miR-208a expression which occurred concurrently with a decrease in connexin 40, indicating that there might be a link between miR-208a, cardiac conduction, and fibrosis (36). Unfortunately, there is little evidence on how these miRNA vary among patients.

Additional miRNA regulators of cardiac fibrosis, miR-24, miR-21, and miR-29, are found to influence direct pro-fibrotic cell activity (37, 280-283). Expression of miR-24 in fibroblasts has been correlated with the degree of fibrosis in hypertrophic hearts (284). Post-MI, miR-24 is down-regulated only after an initial week of peaking where its increased expression is parallel with the levels of collagen-1, fibronectin, and TGFβ1 (37). When delivered *in vivo,* miR-24 reduces cardiac fibrosis, in part, by decreasing collagen-1 protein expression. Not only is miR-24 expressed in fibrocytes, it is also found to decrease the expression of α-SMA, a myofibroblast marker (37). One of the downstream targets of miR-24 is furin, a regulator of the TGFβ pathway that acts by proteolytically maturing TGFβ. This activity correlates with the observation that TGFβ-1 treatment up-regulates miR-24 expression in a time- and dose-dependent manner (37). Both miR-21 and miR-29 have been identified in cardiac fibroblasts, where their expression is greater than that measured in cardiomyocytes (280,281). Over-expression of miR-21 in the heart results in reduced infarct size after ischemia reperfusion injury with preserved ejection fraction and decreased collagen deposition (284), in addition to promoting fibroblast survival (282,283).

Conversely, knock-down of miR-21 attenuated interstitial fibrosis and cardiac remodeling after aortic banding (283). In the myocardial infarct border zone, miR-21 was upregulated, inhibiting PTEN expression, which resulted in up-regulation of MMP-2 expression (282). As mentioned previously, miR-21 is also involved with fibrogenic EMT of epicardial mesothelial cells (145). Finally, a very popular miRNA of interest in the fibrosis literature is miR-29, which regulates a subset of fibrosis-related gene expression including many collagens, fibrillins, laminins, integrins, and elastin (281). When down-regulated, there is an increase of these ECM proteins found which is then attenuated with over-expression of miR-29. In humans, miR-29 expression was decreased in areas adjacent to the infarct zone of the heart (281). Although inhibiting miR-29 would be an ideal therapeutic target to prevent pathological ECM remodeling, it also regulates several anti-apoptotic genes including *Tcl-1*, *Mcl-1*, *p53*, and *CDC42* (284-286). Figure 1.1 Illustration of the various effects of miRNA in fibrosis. The continuing investigation of how miRNAs are involved in both physiological and pathological ECM remodeling is an additional therapeutic target that could be utilized in combatting fibrosis, not only within the heart, but in other fibrotic diseases as well.

Figure 1.1 Schematic diagram of microRNA formation mechanism and roles of specific microRNAs involved in cardiac fibrosis (*mRNA*: messenger RNA, *Pre-miRNA*: pre-microRNA, *miRNA-RISC*: RNA-induced silencing complex)

Müller AL, Freed DH. Cardiac Fibrosis & Heart Failure: Cause or Effect? Eds Dixon IMC, Wigle JT. Springer 2015.



1.5 Extracellular Matrix

Although archaically viewed as the architectural framework determining the shape and structure of multi-cellular organs, the ECM has proven to be dynamically involved in organ function (and dysfunction) as well. The ECM is a highly complex and organized network composed of a variety of proteins with unique physical properties that complement specific organ function. Structural proteins including proteoglycans, fibronectins, laminin, and fibrillar collagens type I and III provide the scaffolding for the cells residing in the heart (287-291). There are three major components of ECM where varying quantities allow for tunability of the ECM depending on its function within the tissue (292). Proteoglycans, such as heparin and keratin sulfate, are highly viscous and provide cell cushioning. They consist of a core protein where glycosaminoglycans (GAGs) covalently attach. These GAG chains are long, negatively-charged polysaccharides that vary in size depending on the tissue. They are able to interact with growth factors, cell surface factors, cytokines, and chemokines, and various ECM molecules which lends to their dynamic roles within the ECM including signaling, proliferation, migration, and differentiation (292-295). Secondly, the varieties of insoluble collagen fibres provide resilience and strength to the ECM. Finally, a numerous different ECM proteins, including laminin and fibronectin, link proteoglycans and collagen fibers to cells by binding them to receptors on the cell surface. Although cardiomyocytes encompass the majority of spatial volume within the heart, the most numerous cell type in the heart are cardiac fibroblasts (296,297). In healthy myocardium, they are responsible for maintaining the cardiac ECM as well as responding to biochemical, mechanical, and electrical signals (298,299).

Collagens make up the majority of ECM proteins in the heart and require intracellular cleavage processing before being secreted by cardiac fibroblasts and myofibroblasts into the extracellular space. In order to provide sufficient tensile strength, secreted collagens are cross-linked into fibres by lysyl oxidase (300-302). The stiffness of the myocardium is the result of the ratio of collagen type I to collagen type III fibres (303). Collagen type I, a triple helix-shaped protein made up of a 2:1 ratio of Col1α1 to Col1α2, provides the myocardial stiffness. Collagen type III are thinner helices, formed from three molecules of Col 3α1 (304-306). This ratio is significantly skewed during pathological fibrosis as more collagen is

produced to replace necrotic tissues in wound healing. Fibronectin, a glycoprotein, is another major ECM protein whose homeostatic activity alters when exposed to a pro-fibrotic response. It exists as a homodimer of two 250 kDa monomers where alternative splicing may yield more than 20 different isoforms (307). In response to cardiac damage, increased levels of the extracellular domain-A splice variant of fibronectin (ED-A FN) are found and can trigger fibroblast activation into myofibroblasts and wound closure (308). Upon cleavage of fibronectin, an Arg-Gly-Asp (RGD) motif is exposed and subsequently recognized by intregins to alter cell migration, proliferation, adhesion, and even survival. Utilization of this interaction has been used in biomaterial development to create functional, biomimetic research tools (309,310).

The web-like nature of the ECM facilitates storage of cell signaling molecules, including cytokines and growth factors that are released upon proteolytic cleavage or mechanical disruption of ECM components (311). These molecules are store in their associated latency complexes that, upon release, are able to rapidly respond to stimuli rather than depending on starting an intracellular signaling cascade to cause production (312-315).

1.5.1 Extracellular Matrix Influence on Differentiation

ECM proteins have recently come to light as influential in determining cell behavior (40). Much of the influence is a result of mechanical force being projected onto the cells and how this changes in an *in vivo* setting. This is because forces are able to be channeled along discrete molecular filament networks that connect the ECM to molecules within the cell. This allows forces to be concentrated on specific molecules in discrete locations within the cell without necessarily affecting the entire structure of the cell. These stresses have the ability to mechanically alter the conformation of proteins influencing the kinetics of protein-protein or protein-ligand binding in living cells (316). One of the most prominent ways that mechanical stress influences differentiation is the interaction between the ECM and integrins where a molecular “slip-bond” is created by small changes in cytoskeletal tension on closely packed integrins where several bonds are formed to stabilize the focal adhesion complexes that mechanical couple

integrins to the actin skeleton (317,318). Innovative work studying the interaction between ECM and the cardiomyocyte cell surface showed that integrin proteins allow developing cells to attach to specific ECM components (314). Integrin proteins bind to highly conserved ECM protein adhesive sequences, including RGDs (317). The influence of the ECM happens as early as embryogenesis where manipulation of hyaluronan can coax embryonic stem cells (ESCs) to increase expression of cardiac lineage-promoting genes (319). Adherence to specific ECM proteins, such as endogenous collagen, is crucial to ECs development along mesodermal lineages and is not able to be rescued by introduction to exogenous type I collagen (316). Cell-matrix interactions can regulate ESC differentiation. In a pro-fibrotic environment, versican secretion from arterial smooth muscle cells is increased in atherosclerotic and coronary restenotic lesions (320). Versican can actually inhibit fibroblast adhesion and is not found in areas of focal adhesion formation (321,322). When understanding MPCs attraction to fibrotic areas, researchers investigated the CD44-hyaluronic acid (HA) interaction, initially used for activated T-cell extravasation into inflammatory areas (323). Upon deletion of CD44, suppression using an anti-CD44 antibody, and/or by CD44 small-interfering RNA (siRNA), MPCs were unable to interact with HA indicating that the CD44-HA interaction could be part of the interaction that draws MPCs to damaged areas (324). HA is also important in 3D cardiac organoid development as demonstrated by Khademhosseini *et al* who used microfluidic patterning techniques to create HA-based micropatterns that enabled the creation of contractile cardiac organoids (325). In healthy hearts, cardiomyocytes are surrounded by collagen types I, IV, and laminin, although when ECM extracted from cardiac fibroblasts, it consists of collagen types I and III, laminin, fibronectin, proteoglycans, and glycoproteins. During development of human embryoid bodies, there was an increase in both fibronectin and laminin expression within the first 5 days of differentiation (326). Further understanding of the significance of laminin in embryonic cardiomyocyte differentiation was evaluated in a dose-dependent manner by using semi-interpenetrating polymer networks of collagen and laminin. Increased concentration of laminin facilitated a parallel increase in cardiac differentiation (327). This collection of ECM proteins actually encourages structural maturation and beating rates of embryonic stem cell-derived cardiomyocytes (328). An additional ECM protein is

noted to be required for cardiomyocyte development is secreted protein, acidic and rich in cysteine (SPARC), where its administration resulted in nearly 100% of embryonic stem cells increasing gene expression of cardiomyocyte-specific genes, Nkx2.5, BMP-2, and MHCα as well as beating cardiomyocytes (329). Collagen inhibitors used on embryonic bodies prevented cardiomyocyte differentiation, as it provides mechanical stability necessary for contractility (330-332).

Not only are there specific components of the ECM influence pro-fibrotic cell differentiation, the physical properties, such as ECM stiffness, are also important to consider. Mechanotransduction, where cellular and molecular processes are stimulated by mechanical stimuli that have been converted into biochemical signals, describes the phenomenon that influences differentiation. One of the earliest examples of how ECM influences differentiation was noted in mouse mammary epithelial cells where growth on a soft substrate facilitated increased differentiation compared to cells grown on stiff plastic dishes (15). Differentiation of cardiomyocytes on surfaces of 10 kPa, close to native myocardium, demonstrated the largest calcium transients, sarcoplasmic calcium stores, and better aligned sarcomeres (333). In diseased myocardium, there are more pronounced ECM crosslinks and the myocardial elastic modulus increases from 16 kPa to 32 kPa, 53 kPa, and 90 kPa at one, two, and three weeks post-infarction, respectively (334,335). There is an optimal ratio of matrix to cell elasticity that governs stress fiber anisotropy as a result of matrix stiffness influencing acto-myosin assembly and contractile forces (336). Non-muscle myosin II (NMMII), a phosphorylation-regulated filament-forming actin crosslinking protein, can exert force on the ECM as well as receive resistance feedback. This interaction can modify the organization and assembly of bundled actin filaments, called stress fibers, within contractile cell types including stem cells (40,337,338). In addition, a phenomenon coined ‘durataxis’ describes a specific cellular behaviour, originally observed in MPCs, where differential stiffness of a substrate will actually draw cells towards it. This phenomenon is NMMII-dependent where NMMII-B is unpolarized on soft substrates, but polarizes at the rear of the cell upon cell migration to a stiffer substrate that is signaled by the assembly of NMMII-A into stress fibers. This same research determined that MPCs will differentiate in response to ECM elasticity cues (339-341).

Mechanical stress experienced by the cytoskeleton can influence gene expression of influencing chromatin organization. This is achieved by transduction of stress signals by the cytoskeleton of the cell to the nuclear envelope made up of the lamina (342). The nuclear lamina is a network composed of intermediate filament proteins of A- and B-type lamins. This LINC complex ("linker of nucleo- and cytoskeleton") is involved with the mechanical transference of signals from the cytoskeleton via nuclear membrane proteins called nesprins (343,344). Actin filaments in the cytoplasm connect to nesprins through their N-terminal domain and to SUN (Sad1p, UNC-84) proteins through their C-terminal domain (345). Upon disruption/deletion of these LINC complexes there is impaired cell function which can lead to clinical manifestations including Emergy-Dreifuss muscular dystrophy and Hutchinson-Gilford progeria syndrome (346). It is evident that interactions between the ECM and cytoskeletal proteins influence cell function which includes differentiation.

1.5.1.1 Stem Cell Differentiation

The importance of extracellular matrix physical properties on influencing differentiation was observed as early as 1979 by Emerman et al (15). Their study noted that a flexible collagen substrate was a crucial component required for mammary epithelial cells to obtain an optimal shape required for differentiation into secretory cells. The importance of physiologically-relevant ECM is emphasized by anoikis, where cells undergo apoptosis if they lack appropriate ECM signals (347). This is especially crucial for understanding stem cell physiology, as their ability to maintain their differentiation phenotype is dependent upon the signals and ECM components of their niche (348,349). This was eloquently demonstrated by Discher's group in their ground-breaking study which deduced that, by merely altering ECM stiffness, one can influence differentiation of mesenchymal progenitor cells (MPCs) into distinct cell types that reside in physiological environments with vastly different stiffnesses (40). MPCs can sense compressive, shear, or tensile stresses due to the deformation of the surrounding matrix via mechanotransduction pathways (351-352). Dependence on the ECM for differentiation cues starts at development, as embryonic stem cells (ESCs) are more elastic and sensitive to local stresses (353-355). It has also been observed that ECM properties that influence MPC differentiation also influence ESC

differentiation (355,356). The Nobel-prize winning development of the technique creating inducible pluripotent stem cells (iPSCs), which behave similar to ESCs, are also influenced by ECM properties. During their development, iPSCs needed to be co-cultured with fibroblast feeder cells, which are the key cells in ECM formation, degradation, and maintaining its homeostasis (273). In order to differentiate iPSCs into intended cell types, they must receive targeted, controlled signals reminiscent of their *in vivo* environment. For example, key ECM proteins, such as laminin, were used to facilitate differentiation of iPSCs into functional retinal pigmented epithelium (357). Understanding ECM cues that stem cells utilize to determine their differentiation is crucial in the development of tissue regeneration technologies.

Embryonic Stem Cells

ESCs are the source of all the information to build an individual, which originate from the inner cell mass of a blastocyst (358). Their stunning ability to differentiate into the hundreds of different cell types within the body has to be tightly controlled in order to successfully grow into a functioning human organism. The first distinct lineage of differentiation is determined by the formation of three germ layers: endoderm, mesoderm, and ectoderm (359). By recapitulating biochemical cues present in embryogenesis, investigators have effectively differentiated ESCs into specific cell types from each germ layer including pancreatic cells (360), cardiomyocytes (361), motor neurons (362), and hepatocyte-like cells (363) in a laboratory environment. However, many cell types evaluated do not reach their complete, mature phenotype as it is evident that biochemical cues alone are insufficient (359,364). *In vivo*, it is an exquisite combination of biochemical, structural, physical, and sometimes electrical cues that maintain a stem cell's niche and direct its differentiation. ECM is an essential component in providing many of those cues, not only in maintaining the stem cell niche, but also in directing ESC differentiation (365). It has even been observed that mechanical cues of force being applied to the cell surface can affect tissue patterning by reorienting the cell division plane (366).

ESCs and ECM work cohesively in dynamic reciprocity (367), where cells are responsible for secreting ECM proteins while the ECM regulates cells' behaviour and they work together to remodel ECM (368,369). Due to their increased elasticity, ESCs are significantly more sensitive to local stresses so *in vitro* conditions must closely resemble *in vivo* conditions to precisely and accurately influence ESC differentiation (370,371). Inspired by Engler *et al's* (40) pioneering research, it was demonstrated that ESCs can also be influenced by substrate stiffness alone, as highly rigid substrates promoted early mesoderm and cardiomyocyte differentiation in both human and mouse ESCs (372). As mentioned previously, these interactions occur via integrins which are specific to cell and tissue type (373). Integrins nonspecifically bind ECM ligands with intracellular cytoskeletal proteins inducing a conformational change which initiates downstream signaling (374). This mechanism has been shown to be especially crucial in regulating embryogenesis; however, it is incompletely understood (375). The initial step of differentiation determines whether ESCs will become endoderm, mesoderm, or ectoderm. For example, the ECM protein laminin supports definitive endoderm differentiation (376) which results in a difference of expression of 11 integrin α subunits and 8 integrin β subunits. The human fibroblast-generated ECM substrate GoGel supported ESC differentiation into hepatocyte-like cells, an endoderm lineage (377). The integrins that bound to GoGel were α3β1, α6β1, and α7β1, of which α3β1 has a high affinity for laminin. (378-380). In addition, the regulation of TGFβ by integrin αVβ5 is important for the differentiation and maintenance of definitive endoderm differentiation (381) where crosstalk between integrins, hepatocyte growth factor, and TGFβ-SMAD signaling is important to facilitate hepatic differentiation (382).

One of the most highly sought after endoderm tissues for differentiation are islet cells to restore beta-cell mass to treat type I diabetes. One of the key pancreatic differentiation signals was mediated by laminin α5 transduced by integrin receptor β1 (383). In addition, the collagen IV/fibronectin/laminin ratio of 1:3:3 has been observed to be crucial in the differentiation of ESC-derived pancreatic cells into insulin-producing cells (384). In summary, for endoderm cell differentiation, laminin substrates are mediated by integrins αV and β5, and further differentiation towards pancreatic or hepatic lineages relies on integrin

β1 (364). In mesoderm development, it has been observed that fibronectin and laminin are present in the inner cell mass, and that mutation of integrin α5 causes mesoderm defects (389,390). It was also demonstrated that a combination of both α5β1 and α6β1 integrins were required for mesoderm differentiation (363). Cardiomyocytes are derived from the mesoderm lineage and their differentiation from ESCs can be stimulated by upregulation of integrin α6A expression (387). Cardiac fibroblasts are responsible for maintaining cardiac ECM and secrete collagen types I and III, laminin, fibronectin, and proteoglycans, which have been show to significantly influence cardiomyocyte differentiation from ESCs (388,389). The 7:3 ratio of fibronectin:laminin also seems to significantly promote ESC-derived cardiomyocyte differentiation (390,391). Utilizing this specificity of ECM components in cardiomyocyte differentiation and maintenance is significantly more effective relative to gelatin (363), which further stresses the importance of how variances of ECM and the extracellular environment influence ESC differentiation. Furthermore, differentiation of ectoderm tissue into various neural lineages requires unique ECM proteins to encourage specific cell fates. For example, when ESCs are cultured as embryoid bodies on collagen-coated substrate they favour neural differentiation and neurite outgrowth, whereas fibronectin and laminin coated substrates promoted oligodendrocyte formation (391). The α3 integrin has been shown to be crucial in ESC neural differentiation as deficiency of this subunit is correlated with defective neuron migration (392).

In autonomic nervous system formation, interaction between the β1 integrin with laminin and fibronectin is important in the migration and maintenance of neural precursor cells (393). In Goh et al.'s study (394), they found that the ECM components polyornithine-laminin followed by polyornithine-laminin-fibronectin, can bind a specific arrangement of integrins critical for the differentiation of specific neural crest lineages. One technique that has been used to encourage differentiation of ESCs is nano-etching. Neuronal differentiation of ESCs was achieved by growing cells using nanogrooves (350 nm wide ridges/grooves with 500 nm depth) without induction reagants (395). Skin is also derived from the ectoderm, where key interactions in keratinocytes occur between α2β1-collagen IV, -collagen IV, α3β1-

laminin 331/511, and α6β4-laminin 331/511 (396). This differs from melanocytes where α6β1-laminin and α2, α3, and αvβ3-collagen IV (397).

Numerous studies evaluating the effect of the extracellular environment in ESC differentiation highlight their sensitivity to their niches and how important it is in developing the complex tissues of the human body. Understanding the cellular mechanisms involved in determining differentiation is crucial when performing experiments in a laboratory setting in order to optimize stem cell therapy and tissue engineering processes. Understanding and subsequently extrapolating upon how ESCs are influenced by their ECM has advanced laboratory techniques and enabled scientists to expand their research of stem cells without relying on ESCs obtained from controversial sources which was impeding progress. Preliminary research of ESCs has provided the opportunity to better understand and manipulate adult stem cells as well as led to the discovery of embryonic-like inducible pluripotent stem cells.

Inducible Pluripotent Stem Cells

Inducible pluripotent stem cells (iPSCs) behave like ESCs but are derived from differentiated adult cells. In 2006, Yamanaka's group (275) developed iPSCs from skin fibroblasts using transcription factors Oct3/4, Sox2, c-Myc, and Klf4 under ESC conditions. These iPSCs demonstrated ESC behaviour upon transplantation into nude mice resulting in tumours containing tissue from the three developmental germ layers, endoderm, mesoderm, and ectoderm. This ground-breaking discovery opened entirely new possibilities for tissue regeneration and stem cell therapy as it provides the opportunity for autologous transplant. However, the dramatic conversion of somatic cells to iPSCs and then subsequent differentiation into a target tissue type has repercussions. Reprogramming in ESCs and iPSCs causes a metabolic shift from oxidative respiration to oxidative glycolysis. This leads to an increased leakage of electrons into the cytoplasm causing oxidative stress which elevates levels of oxidative DNA damage and ROS (394,395). iPSCs also experience de novo genetic variants as a result of reprogramming that could impact phenotype stability, including chromosomal aberrations, aneuploidy, sub-chromosomal copy

number variations, and single nucleotide variations (400-404). A more thorough review regarding the effect of how genetic instability influences iPSC differentiation was written by Turinetto et al (364), although the main point to note that this variability caused by iPSC generation and subsequent differentiation increases their propensity to become teratomas which has a significant negative clinical impact (405,406).

One of the most influential aspects that contributes to iPSC complications involves techniques for *in vitro* culturing. For example, hypoxic culture conditioning reduced oxidative stress, prevented DNA mutation accumulation and differentiation, promoted cell survival, and improved the efficiency of iPSC generation (407,408). The effectiveness of culturing iPSCs in hypoxic conditions should come as no surprise considering that the preliminary stages of ESC differentiation occur under hypoxic conditions beginning at implantation and continuing throughout fetal development (409). Stem cell niches are located deep within tissue where there are concentration gradients of various influential factors including oxygen, ions, growth factors, chemokines and/cytokines. This organization helps influence differentiation of daughter cells which respond to this gradient which is often more dynamic and complex than the more static ECM of target tissue (410). One of the biggest challenges of iPSC culture is maintaining a naïve state, which is done most effectively by a feeder layer culture or Matrigel® coated surfaces. However, this technique is challenging to translate to a clinically efficient protocol as it is much more costly to achieve a relevant number of iPSCs (often 10-1000 million cells/patient) (411,412). Nano-etching may be an effective alternative as iPSCs grown in nanogrooves (350 wide; 300 nm tall) exhibited an increased expression of neuronal marker expression, indicating the influence of topography in influencing differentiation (413,414). This technique was shown to be effective in cardiomyocyte differentiation of iPSCs where nanotopographic substrates were integrated with the cell adhesion motif Arg-Gly-Asp within a chimeric peptide. Cardiomyocyte structural development and organization was dependent on nanotopographical feature size in a biphasic manner within the 700-1000 nm range (415-417). These studies indicate the importance of niche topography in the differentiation of two unique cell types that

originate from different developmental layers. It was recently published that the pluripotent state of iPSCs could be maintained by small feature size, high wave number, and high feature density while being culture in xeno-free medium (418).

Cell adhesion molecules have also been shown to be important in iPSC self-renewal and pluripotent states. E-cadherin, which is important in regulating the pluripotent state of ESCs, contributes to increased reprogramming efficiency of human keratinocytes already expressing E-cadherin compared to human fibroblasts and also that E-cadherin is necessary for the self-renewal of iPSCs (419,420). Expression of E-cadherin, Sox2, Klf3, and c-Myc were shown to be sufficient for iPSC reprogramming without Oct4 indicating that E-cadherin's contribution to spatial and mechanical input influences cell fate (421). With regards to integrins, human iPSC lines cultured on Matrigel® and vitronectin express integrins comparable to those of hESCs, except for increased expression of α5, α6, αV, β1, and β5 (422). Experimental analysis determined that β1 was essential for adhesion to the Matrigel® ECM and subsequent proliferation while integrin αVβ5 was necessary to attach to vitronectin (422). Myogenic progenitor cells derived from iPSCs also show increased expression of integrin α9β1 (423). In a gelatin scaffold with bone morphogenetic protein-4, iPSCs showed a dramatic loss of α3 and α6 integrins but highlighted the importance of α1 integrin as part of a signaling cascade in early ondotoblast-like cell differentiation (424). Further analysis regarding the interplay between integrins and ECM in determining iPSC differentiation needs to be explored in order to encourage differentiation akin to ESCs to reduce the probability of teratoma formation and increase the efficacy of iPSC differentiation for cell therapy and tissue engineering treatments.

Adult Stem Cells

Sensitivity to the extracellular environment is also important in adult stem cell differentiation as they are responsible for overall maintenance of the body. Stem cells of particular interest in health research are MPCs because they are the easiest to procure clinically, thus making those ideal candidates for stem cell

therapy and tissue engineering applications. MPCs are responsible for maintaining osteogenic, chondrogenic, and adipogenic tissues which vary vastly in both niche biochemistry as well as extracellular stiffness (40).

There are many physical forces within the human body that cells experience including compressive, tensile, and shear stress (425). These various stresses can influence differentiation. For example, it is known that fluid shear stress in rhe vascular system is necessary in differentiating MPCs to vascular endothelial cells which are constantly exposed to shear stress within the cardiovascular system (426). In an experiment evaluating the effect of shear stress on MPC differentiation it was found that both intermittent and continuous shear stress increased expression of cardiomyocyte-related markers without the influence of any growth factors (427). Shear stress also influences osteoblast differentiation from MPCs by modifying the actin cytoskeleton (428). MPCs have the ability to differentiate into osteoblastic, chondrogenic, and adipogenic lineages are known to vastly differ in their actin cytoskeleton alignment. Osteoblasts have a stiffer cytoskeleton with increased focal adhesions compared to the thinner and more dispersed actin cytoskeleton of chondrogenic and adipogenic differentiation which is influenced by mechanical stimulation (429). Mechanical stress alone has also been shown to influence differentiation of MPCs where tensile loading of 2.5% at 17 Hz initiated osteogenic differentiation (430), cyclic tensile loading of low amplitude (0.5%) at 0.5 Hz induced neuronal differentiation (431), and strain magnitudes of 10% at 1 Hz stimulated cardiomyocyte differentiation (432). As mentioned previously, changing the nano-geometry of the surface where stem cells grow also influences their differentiation. MPCs grown on a flat surface encouraged differentiation into osteo-like cells whereas MPCs grown on a grooved surface leaned towards a more smooth muscle cell-like phenotype (433). Compression of MPCs has also been demonstrated to be just as effective as TGF-β treatment when stimulating chondrogenic differentiation of MPCs (434) by regulating p38 kinase phosphorylation in a less “slow and lagged” manner compared to differentiating medium (435). In some cases, the same signaling molecule can yield different progenitor cell differentiation when they are grown on plates of varying stiffnesses. This was demonstrated by Park

et al (436) when MPCs were cultured on soft and stiff substrates and treated with TGFβ. The expression of smooth muscle cell (SMC) markers were increased on cells grown on the stiff substrate whereas chondrogenic markers were increased on cells grown on the softer substrate where there was weaker focal adhesion due to decreased stress-fiber formation. In some situations, the influence of mechanical stresses can supersede growth factors as demonstrated in a study by Kim et al (437). When MPCs are grown in endothelial growth factor medium and exposed to low shear stress (2.5 dyn/cm^2), which regulates MPC differentiation into ECs, and high shear stress (10 dyn/cm^2), which favours SMC differentiation, the MPCs exposed to higher shear stress grown in endothelial growth factor media still differentiated into SMCs. This experiment determined that shear stress was more influential in catalyzing the morphological change and influencing cytoskeletal rearrangements than a chemical signal.

Integrins play a crucial role in determining differentiation of adult stem cells as well. Ratios of integrin binding is important in both 2D and 3D culturing of MPCs where stiffer substrates require integrin binding in order to differentiate into osteogenic lineages; however, integrin binding has been shown to be less important in the differentiation of adipogenic and neurogenic lineages on softer substrates (438,439). When evaluating integrin binding concentrations compared to increased matrix stiffness, there is a bell curve distribution with the hydrogel stiffness and peak integrin bond formation happening optimally for osteogenic differentiation (438). MPCs grown on a stiff substrate is regulated by the α2 integrin which activates the FAK, RhoA/ROCK, and ERK1/2 pathways facilitating osteogenesis whereas the β1 integrin is important in neurogenic differentiation of MPCs grown on softer substrates (40, 438). Inhibition of α2 integrin expression and subsequent FAK inhibition decreased osteogenesis (440). There are also proteins responsible for creating a connection between the nucleus and the actin and microfilaments of the cytoskeleton called the LINC complex (343). In response to extrinsic and intrinsic mechanical signals, the nuclei of MPCs deform which initiates the transduction of signals to the lamins present in the nuclei. These lamins provide support for a variety of nuclear proteins involved with gene expression including DNA replication and transcription (441-443). The mechanism behind this

phenomenon requires further study but does help illustrate the important influence of mechanical stress on the genetic expression of cells, especially stem and progenitor cells whose gene expression facilitates differentiation causing phenotype change.

As indicated by numerous experiments, it is evident that mechanical stresses induced by the ECM and the niche environment of stem cells are important in determining differentiation. Integrins provide a crucial link between ECM proteins and the cellular cytoskeleton and transmit mechanical stresses that, alone, can stimulate differentiation as effectively and sometimes more so, than chemical signaling. This becomes especially important when considering the difference between healthy and damaged ECM and how those environments influence stem cell differentiation.

1.5.2 Extracellular Matrix Scaffolds

Tissue engineering has the potential to repair and replace damaged tissue in order to functionally reconstruct numerous organs throughout the body including skin, bone, nerve, liver, kidney, and other tissues (444). Currently, there are a few commercially available ECM-derived biologic scaffolds derived from a variety of tissue including porcine, fetal bovine and human skin, porcine small intestinal submucosa and urinary bladder matrix, and bovine and equine pericardium (445). One of the largest hurdles for tissue engineering is the 3 dimensional nature of tissue, which differs greatly from conventional 2 dimensional culture methods. Instead of growing tissue on culture dishes, there has been an emergence of using extracellular matrix scaffolds to encourage 3D cell growth and better mimic a physiological environment as it provides mechanical properties and structural support for cell attachment and tissue development (446). This method has significantly grown and improved since the development of a variety of different techniques including 3D printing, which utilizes various ECM protein and/or cellular ink to build 3D scaffolds, or decellularization of native ECM tissue. The flexibility of 3D printing allows for customized scaffolds to be developed, some of which may only utilize one ECM protein while other scaffolds can be built using a variety of ECM proteins in order to closely represent niche

environments of various tissues. Another important feature of ECM matrices is that they have a tunable rate of biodegradation composed of non-toxic byproducts in the body that compliments the rate of tissue regeneration (447). As tissue regeneration will be used to repair/replace damaged tissue, it is important that ECM scaffolding has low immunogenicity that will not exacerbate the healing process (444).

1.5.2.1 Single ECM Protein Scaffolds

Collagen

The most popular protein used in ECM scaffolds is collagen, as it makes 25-35% of the total mass of protein in the human body and is a major component of the ECM (448,449). Because of its fibrillar nature, collagen is able to create networks of varying porosities and fiber diameters where its distribution varies depending on the tissue it is providing structural support to. For example, in small intestinal submucosa, collagen is in a spiral arrangement to support peristaltic movement; however, collagen is linearly aligned with tendons and skeletal muscle to impart strength and stability (450,451). Despite its abundance, compared to other ECM proteins, it forms physical hydrogels with weak mechanical properties unless additional cross-linking occurs after 3D printing either by formaldehyde, glutaraldehyde, or transglutimase. It is also difficult to sterilize as heat alters its fibrillar structure and it also shrinks in response to cell activity (452-455). Because of its subpar mechanical stability, 3D collagen structures are typically printed in preformed molds or inside other materials that serve as a support (456,457). Despite these complications, 3D printed collagen has been used in a variety of applications including printing models for 3D biological studies, tissue assays, modeling disease, and drug testing. It has also been used to build constructs, bone regeneration, skin grafts and mimics, corneal epithelium scaffolds, and neural tissue engineering illustrating its versatility in tissue engineering ventures (458-464). Collagen scaffolds are even able to printed using inkjet printing technology by slightly modifying either Hewlett Packard 550 or Canon Bubble Jet printers using a collagen type 1 solution at 1 mg/mL (465-467). More commonly, in-house developed machines applying a 3D printing system using an electromechanical

microvalve with low pressure is utilized; some have even been integrated with an advanced imaging system to be able to image cells inside 3D printed structures using a mesoscopic fluorescence molecular tomography imaging system (459,468). It should be noted that, due to its weak mechanical properties, pure collagen does not print well and is now commonly printed with soluble polymers or particles to form composite materials (469).

Fibrinogen

Fibrinogen is a glycoprotein popular in the use of building 3D scaffolds as it plays a natural role in wound healing, in addition to cell-matrix interactions and inflammatory reactions (470,471). When activated by serine protease thrombin it becomes fibrin which stabilizes in the presence of calcium ions that facilitate covalent crosslinking further stabilizing the fibrin structure. This is important in tissue engineering as its rate of crosslinking can be controlled by a variety of controllable and physiologically compatible factors by altering the concentration of fibrinogen, thrombin, and calcium (471). It is also fairly easy to isolate from an autologous source as in can be procured by centrifugation of the patient's blood (472). Its primary use in tissue engineering is as vascular grafts but it can also be used to make gels as it has tunable porosity, contains cell binding sites, gelation occurs within seconds, and they are biocompatible and biodegradable (470,472). The most significant complications with fibrinogen derived 3D tissue scaffolds is their low printing accuracy and their rapid degradation rate which may require the use of protease inhibitors in certain *in vivo* clinical settings (473). The unique properties of printed fibrinogen enable it to be used in tissue scaffolds as a pre-engineered vascular system facilitating these scaffolds to exceed 150-200 μm where diffusion of nutrients and O_2 is limited (472). Neuronal 3D structures have also been printed on fibrinogen scaffolds which alternated between fibrin, thrombin, and NT2 neurons. This structure exhibited outgrowth of neurites after 12 days of culturing as the neural cells were able to attach and spread on the fibrin fibers (466). Fibrin has also been used to build scaffolding for MPC transplantation (474).

1.5.2.2 Multi-Protein ECM Scaffolds

Collagen-Fibrin Composites

The combination of collagen and fibrinogen merges the beneficial properties of both ECM matrix proteins, translating to a bioink that has processable viscosities for printing, ~15 s gelation times, and negligible contraction (463). This combination has been used to print *in situ* skin grafts in mice when embedded with amniotic fluid-derived stem cells or bone marrow-derived MPCs (475). Neural cell migration was observed migrating toward the fibrin layer of a 3D scaffold composed of alternating collagen and fibrin layers (476). A commercial collagen-fibrin patch, called Tachosil®, has recently been found to be successful in clinical trials of women with gynecologic malignancies by preventing symptomatic lymphoceles after pelvic lymphadenectomy by up to 66% (477). It was also able to effectively reduce postoperative air leaking in patients undergoing redo thoracic surgery, possibly correlated with an observed decreased mean length of hospital stay (478). However, its use in other surgical procedures including distal pancreatectomy and thyroid surgery has shown to be ineffective in preventing bleeding, reoperation rate, length of hospital stay, or mortality (479,480). Although its effectiveness in preventing air and blood leakage might very among different surgical procedures, more clinical research is required to assess the combination of collagen and fibronectin ECM scaffolds in tissue engineering applications.

Matrigel

Matrigel is a commercially available ECM protein scaffold composed of a variety of collagen, laminin, entactin, heparin sulfate proteoglycans, and growth factors isolated from mouse sarcoma cells, and is intended to be used as a reconstituted basement membrane preparation (481,482). The importance of basement membrane proteins on differentiation has been observed over the past few decades as illustrated by studying the differences between Schwann cells grown on traditional culture dishes versus those cultured on basement membrane-coated plates. Exposed neuronal processes were found to lack

myelin and Schwann cells were not elongate (483). Currently, Matrigel is most widely used as a support matrix for human embryonic stem cells as it is able to maintain self-renewal and pluripotency (484). It is also used to mimic the ECM in both stem cell and cancer cell culture because its composition facilitates cell-ECM interaction. The originally distributed Matrigel had high levels of growth factors including basic fibroblast growth factor β, platelet-derived growth factor, epidermal growth factor, insulin-like growth factor 1, transforming growth factor β, and nerve growth factor 11. GFR (growth factor reduced) Matrigel was released with reduced levels of growth factors using an AS precipitation protocol (482); however, due to differences in tumor sizes in Matrigel extraction animals and tissue preparation, there is variation between batches which encourages experimenters to test between lots for optimal results for their experiments (484).

There have been an extensive variety of uses for Matrigel in *in vitro* experimentation. One of its preliminary uses is in endothelial tube formation as, within a few hours after being plated onto plates coated with Matrigel, endothelial cells migrate to form tubes with a lumen reminiscent of capillary vasculature. This has been ground-breaking in studying angiogenesis mechanisms for processes such as cell adhesion, migration, and protease secretion (485-487). Work in this ,field evaluating stem cells isolated from either neonatal foreskin and adipose tissue, have differentiated into tubular structures with an endothelial cell phenotype (488,489). Since the origin of Matrigel is from sarcoma-derived basement membrane, it has also been used to study a variety of different tumour-developing behaviour including culturing tumour cells in 3D culture, tumor dormancy and invasion assays (490-499). Matrigel has been able to be extruded with epithelial and hepatocyte cells to form connective tissues in a microfluidic device creating an *in vitro* liver analog (411). Despite its extensive use in basic science, there is limited information on its translatability to the clinic; however, it demonstrates the potential of isolating basement membrane from human sources, such as discarded tissue including the placenta or unutilized organ donor tissue, could play a role in tissue engineering in the future.

Decellularized Extracellular Matrix

Exceptional work published in Nature Medicine by Dr. Doris Taylor's group (39) demonstrated that the ECM is powerful enough to signal cardiac and endothelial cells to functionally orientate themselves to generate pump function. They demonstrated that fiber composition, orientation, and the vascular basal membranes were preserved at the end of the decellularization process along with a competent aortic and tricuspid valves. Eight days post-recellularization with neonatal cardiomyocytes, fibrocytes, endothelial cells, and smooth muscle cells, the viability of the cells remained >95% throughout the entire thickness (0.5-1.1mm) with sarcomeric α-actin and cardiac myosin heavy chain being expressed along with cells positive for von Willebrand factor. Synchronous paced contraction of the recellularized whole heart set-up occurred as early as day 4 indicating functional connexin-43 connections between cardiomyocytes reperfused into pure ECM. This study emphasized the crucial role ECM plays in physiological function of cells and broadened the field of tissue engineering to include decellularized ECM as a viable scaffold with the potential to be effective as a clinical therapy.

Although work by Taylor's group evaluated whole organ decellularization, ECM matrices utilized in tissue engineering research originates from ECM secreted by MPCs stimulated by different media components that catalyze differentiation into specific MPC differentiation phenotypes. MPCs are cultured under standard culture conditions or with specific culture medium compositions that promote tissue-specific cell differentiation. For example, tissue-specific ECM can be used from MPCs differentiating into bone, cartilage, and adipose tissue (498-500). A variety of decellularization processes have been used to remove the MPCs while retaining the secreted ECM, but generally NH_4OH and Triton X-100 solutions are used to dissolve cell membranes and then nucleases are used to remove DNA and RNA content (501-505). The influence of decellularized ECM has also been shown to be effective in either maintaining the naïve MPC phenotype by enhancing proliferation or by stimulating MPC differentiation along the phenotypic lineage of cells from which the ECM was derived. Decellularized ECM deposited by MPCs exposed to bone were able to differentiate into osteoblasts and ECM deposited by smooth muscle cells induced naïve MPCs to exhibit smooth muscle cell-like characteristics (498, 504). Umbilical cord-derived

MPCs have been shown to build an ECM that attenuated intracellular reactive oxygen species, enhanced antioxidant enzymes, and improved resistance to exogenous oxidative stress (506) which would be very useful in clinical situations when replacing damaged tissue as the ECM may have the ability to reduce the hostility of those environments. In addition MPC derived ECM has been shown to be supportive in maintaining and expanding human hematopoietic stem and progenitor cells *in vitro* (507).

There have also been a variety of tissues used clinically for decellularized ECM production including skin, pericardium, bladder wall, adipose tissue, vasculature, tendons and ligaments (508-515). A review by Parmaksiz *et al* has an extensive list of decellularized ECM products currently available in the clinic or being tested in clinical trials (516). Sources of ECM include human tissue (Allopatch, GraftJacket, InterGro, etc), porcine tissue (Permacol, Strattice, CorMatrix, MatriStem, etc), bovine tissue (MatriDerm, CuffPatch, Veritas, etc), and even ovine tissue (Endoform). They have been used in orthopedic applications where ECM has been used as grafts for rotator cuff damage, tendon augmentation, transplantation-based repair, and as a material for inter-positional arthroplasty (517,518). One of the first applications of ECM is in skin repair and grafting for burn tissue, which affects about 5 million patients in the USA and has grown to a market of ~10 billion US dollars (516). Acellular products used in skin repair use decellularization techniques on both xeno- and allo-graft products with the current technology focusing on regenerating natural skin and regaining skin functions as current clinical outcomes do not result in scar-free tissue, despite their ability promote healing (516,519). Decellularized ECM products have also been used in cardiovascular applications and there are numerous reconstructive procedures available to patients including aortic, ventricular septal wall, ventricle or atria, and heart valves which benefit from biomaterials used to repair or replace damaged cardiac tissue (520). Cardiac patches are routinely used in cardiac surgery to repair damaged myocardium. One of the most successful cardiac patches available in the clinic is CorMatrix, which has been effective in pericardial tissue repair, congenital cardiac surgery, infective endocarditis, repair of atrial septal defects, pulmonary arterioplasty, and aortoplasty applications (521,522). Since the decellularization method removes the majority of

cellular components and antigens from the tissue, there are decreased risks of inflammatory response and immunological rejection essentially enabling an essentially limitless source of ECM material from xenogeneic sources (523-525). The next step is to develop ECM scaffolding that promotes regenerative remodeling of damaged tissue, either used in conjunction with stem cell therapy or to encourage endogenous healing of the area. The versatility and effectiveness of decellularized ECM demonstrated in the clinic thus far is just the tip of the iceberg when discussing the potential of tissue engineering to repair and replace damaged tissue.

In addition to its practical versatility in clinical applications, decellularized ECM can also be used to effectively analyze cell biology *in vitro* as it provides a true bioactive physiogical microenvironment. Utilizing this technique is especially pertinent in cancer research in biopsies and tumor excision, where excess tissue is normally considered waste, as it can be repurposed to better understand how carcinogenic ECM influences cell physiology. This was demonstrated by Piccoli *et al* (526) where they seeded decellularized colorectal cancer biopsies with HT-29 cells. They determined that both the proteome and secretome differed between healthy and cancerous mucusa, specifically measuring an increase in IL-8 and decreased angiongenic potential in the latter. The beauty of using biopsies as a source for diseased ECM is that it it is already a common, minimally invasive, method of collecting human tissue and is used to diagnose a myriad of diseases where pathogenesis alters the ECM microenvironment. In idiopathic pulmonary fibrosis (IPF), primary fibroblasts seeded on pathological ECM exhibited increased expressions of ECM genes regardless of whether the fibroblasts were isolated from patients with IPF or healthy controls (527). This emphasizes the magnitude of influence that ECM has on determining cell behaviour regardless of the programming it received in a prior microenvironment. In patients diagnosed with chronic obstructive pulmonary disease (COPD), extensive remodeling of airways occurs in COPD which influences normal bronchial epithelial cells (NBEC) by (528). This was shown when comparing decellularized bronchial ECM between patients diagnosed with COPD and those that were not. NBECs seeded on diseased ECM had an increase in gene expression of hepatocyte growth factor, TGFβ, and

platelet growth factor-B, which are all associated with COPD pathophysiology (528). Evaluating the effect of diseased decellularized ECM on cell physiology is a field still in its infancy; however, it provides an exquisitely excellent *in vitro* method to relevantly study cell physiology in a variety of pathologies.

1.6. Conclusions

It is evident that more than endogenously present interstitial fibroblasts within the myocardium contribute to fibrosis, both physiologically and pathologically. This multi-faceted process drives numerous cardiovascular pathologies toward heart failure and occurs via a number of different cell sources, including various cell lineages found in the bone marrow (10-13, 17). These include fibrocytes and MPCs that contribute directly to ECM remodeling and protein deposition, and appear ubiquitously involved in both physiological and pathological fibrosis (20, 22). We have also outlined how similar fibroblasts and MPCs act in culture and under stimulation of pro-fibrotic TGFβ. Additional sources of pro-fibrotic cells are endothelial cells undergoing EndoMT transition (13, 23, 24) and epithelial cells undergoing EMT (28).

A thorough understanding of how these various cell types become pro-fibrotic could reveal therapeutic targets for preventing overzealous ECM deposition and remodeling. We explored the possibility of miRNA in inducing cell differentiation to a pro-fibrotic phenotype and/or contributing directly to fibrosis including miR-21, miR-24, miR-29, miR-133, miR-145, and miR-208a (35, 36, 38-40, 107). miRNA provides a potential therapeutic target to either promote physiological fibrosis for optimal wound healing, or to inhibit pathological cardiac ECM remodeling.

Numerous products of this novel approach are already undergoing clinical trials; however, there exists little understanding of how progenitor cells already present in the body, will respond to these scaffolds and how the ECM influences miRNA to determine differentiation. Understanding how endogenous progenitor cells respond to a pro-fibrotic environment equips the field of tissue regeneration with the knowledge that allows treatments to be more effective by developing cells immune to the influence of a profibrotic extracellular matrix environment in any cardiovascular disease state.

1.7 References

1. Kong P, Christia P, Frangogiannis NG (2014) The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 71: 549-574

2. Ahmad T, Fluzat M, Neely B, et al (2014) Biomarkers of myocardial stress and fibrosis as predictors of mode of death in patients with chronic heart failure. JACC Heart Fail 2: 260-268

3. Elzenaar I, Pinto YM, van Oort RJ (2013) MicroRNAs in heart failure: new targets in disease management. Clin Pharmacol Therap 94: 480-489

4. Kania G, Blyszczuk P, Eriksson U (2009) Mechanisms of cardiac fibrosis in inflammatory heart disease. Trends Cardiovasc Med 19: 247-252

5. Frangogiannis NG (2012) Regulation of the inflammatory response in cardiac repair. Circ Res 100: 159-173

6. Berk BC, Fujiwara K, Lehoux S (2007) ECM remodeling in hypertensive heart disease. J Clin Invest 117: 568-575

7. Ashrafian H, McKenna WJ, Watkins H (2011) Disease pathways and novel therapeutic targets in hypertrophic cardiomyopathy. Circ Res 109: 86-96

8. Pickering JG, Boughner DR (1990) Fibrosis in the transplanted heart and its relation to donor ischemic time. Assessment with polarized light microscopy and digital image analysis. Circulation 81: 949-958

9. Cieslik KA, Trial J, Crawford JR, et al (2014) Adverse fibrosis in the aging heart depends on signaling between myeloid and mesenchymal cells; role of inflammatory fibroblasts. J Mol Cell Cardiol 70: 56-63

10. Haudek SB, Xia Y, Huebener P, et al (2006) Bone marrow-derived fibroblast precursors mediate ischemic cardiomyopathy in mice. Proc Natl Acad Sci USA 103: 18284-18289

11. Blyszczuk P, Kania G, Dieterle T, et al (2009) Myeloid differentiation factor-88/interleukin-1 signaling controls cardiac fibrosis and heart failure progression in inflammatory dilated cardiomyopathy. Circ Res 105: 912-920

12. Kania G, Blyszczuk P, Stein S, et al (2009) Heart-infiltrating prominin-1+/CD133+ progenitor cells represent the cellular source of transforming growth factor beta-mediated cardiac fibrosis in experimental autoimmune myocarditis. Circ Res 105: 462-470

13. Zeisberg EM, Tarnavski O, Zeisberg M, et al (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 13: 952-961

14. Lei PP, Qu YQ, Tao SM, et al. (2013) Fibrocytes are associated with the fibrosis of coronary heart disease. Pathol Res Pract 209: 36-43

15. Korhonen J, Polvi A, Partanen J, et al (1994) The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. Oncogene 9: 395-403

16. Hocht-Zeisberg E, Kahnert H, Guan K, et al (2004) Cellular repopulation of myocardial infarction in patients with sex-mismatched heart transplantation. Eur Heart J 25: 749-758

17. Villarreal FJ, Dillman WH (1992) Cardiac hypertrophy-induced changes in mRNA levels for TGF-beta 1, fibronectin, and collagen. Am J Physiol Heart Circ Physiol 262: H1861-H1866

18. Sopel MJ, Rosin NL, Lee TD, et al (2011) Myocardial fibrosis in response to angiotensin II is preceded by the recruitment of mesenchymal progenitor cells. Lab Invest 91: 565-578

19. Quaini F, Urbanek K, Beltrami AP, et al (2002) Chimerism of the transplanted heart. N Engl J Med 346: 5-15

20. Ngo M, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

21. Keeley EC, Mehrad B, Strieter RM (2009) The role of circulating mesenchymal progenitor cells (fibrocytes) in the pathogenesis of fibrotic disorders. Thromb Haemost 101: 613-618

22. Ebihara Y, Masuya M, Larue AC, et al (2006) Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. Exp Hematol 34: 219-229

23. Keeley EC, Mehrad B, Strieter RM (2010) Fibrocytes: Bringing new insights into mechanisms of inflammation and fibrosis. Int J Biochem Cell Biol 42: 535-542

24. Goumans MJ, van Zonneveld AJ, ten Dijke P (2008) Transforming growth factor beta-induced endothelial-to-mesenchymal transition: a switch to cardiac fibrosis? Trends Cardiovasc Med 18: 293-298

25. Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 2142: 199-210.

26. Serini G, Gabbiani G (1999) Mechanisms of myofibroblast activity and phenotype modulation. Exp Cell Res 250: 273-283

27. Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4: 267-274

28. Covas DT, Panepucci RA, Fontes AM, et al (2008) Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol 36: 642-654

29. Roberts AB, Sporn MB, Assoian RK, et al (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. PNAS 83: 4167-4171

30. Kinner B, Zaleskas JM, Spector M (2002) Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells. Exp Cell Res 278: 4167-4171

31. Reinhart BJ, Slack FJ, Basson M, et al (2000) The 21-nucleotide *let-7* RNA regulated developmental timing in *Caenorhabditis elegans*. Nature 203: 901-906

32. Choi E, Choi E, Hwang KC (2013) MicroRNAs as novel regulators of stem cell fate. World J Stem Cells 5: 172-187

33. Tomé M, López-Romero P, Albo C, et al (2011) miR-355 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. Cell Death Differ 18: 985-995

34. Matkovich SJ, Wang W, Tu Y, et al (2010) MicroRNA-133a protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts. Circ Res 106: 166-175

35. van Rooij E, Quiat D, Johnson BA, et al (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. Science 316: 575-579

36. Callis TE, Pandya K, Seok HY, et al (2009) MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J Clin Invest 119: 2772-2786

37. Wang J, Huang W, Xu R, et al (2012) MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med 16: 2150-2160

38. Wang YS, Li SH, Guo J, et al (2014) Role of miR-145 in cardiac myofibroblast differentiation. J Mol Cell Cardiol 66: 94-105

39. Ott HC, Matthiesen TS, Goh SK, et al (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14(2): 213-221

40. Engler AJ, Sen S, Sweeney HL, et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126(4): 677-689

41. Perea-Gil I, Prat-Vidal C, Bayes-Genis A (2015) In vivo experience with natural scaffolds for myocardial infarction: the times they are a-changin'. Stem Cell Res Ther 6: 248

42. Chang HY, Chi JT, Dudoit S, et al (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci U S A 99:12877-12882

43. Wang J, Dodd C, Shankowsky HA et al (2008) Deep dermal fibroblasts contribute to hypertrophic scarring. Lab Invest 88:1278-1290

44. Varkey M, Ding J and Tredget EE (2011) Differential collagen-glycosaminoglycan matrix remodeling by superficial and deep dermal fibroblasts: potential therapeutic targets for hypertrophic scar. Biomaterials 32:7581-7591

45. Li X, Zhu L, Wang B, et al (2017) Drugs and targets in fibrosis. Front Pharmacol 8:855

46. Gulati A, Jabbour A, Ismail TF, et al (2013) Association of fibrosis with mortality and sudden cardiac death in patients with nonischemic dilated cardiomyopathy. JAMA 309:896-908

47. Hutchinson J, Fogarty A, Hubbard R, et al (2015) Global incidence and mortality of idiopathic pulmonary fibrosis: a systematic review. Eur Respir J 46:795-806

48. Raimundo K, Chang E, Broder MS, et al (2016) Clinical and economic burden of idiopathic pulmonary fibrosis: a retrospective cohort study. BMC Pulm Med 16:2

49. Kim L, Kim DK, Yang WI, et al (2008) Overexpression of transforming growth factor-beta1 in the valvular fibrosis of chronic rheumatic heart disease. J Korean Med Sci 23:41-48

50. Allen JT, Spiteri MA (2002) Growth factors in idiopathic pulmonary fibrosis: relative roles. Respir Res 3:13

51. World Health Organization. Cardiovascular diseases - fact sheet #317. May 2017

52. Yusuf S, Hawken S, Ounpuu S, et al (2004) Effect of potentially modifiable risk factors 167 associated with myocardial infarction in 52 countries (the INTERHEART study): casecontrol study. Lancet 364:937-952

53. Lloyd-Jones DM, Nam BH, D'Agostino RB, et al (2004) Parental cardiovascular disease as a risk factor for cardiovascular disease in middle-aged adults: a prospective study of parents and offspring. JAMA 291:2204-2211

54. Sesso HD, Lee IM, Gaziano JM, et al (2001) Maternal and paternal history of myocardial infarction and risk of cardiovascular disease in men and women. Circulation 104:393-398

55. Goldenberg I, Jonas M, Tenenbaum A, et al (2003) Current smoking, smoking cessation, and the risk of sudden cardiac death in patients with coronary artery disease. Arch Intern Med 163:2301-2305

56. Dobaczewski M, de Haan JJ, and Frangogiannis NG (2012) The extracellular matrix modulates fibroblast phenotype and function in the infarcted myocardium. J Cardiovasc Transl Res 5:837-847

57. Ma Y, Halade GV, and Lindsey ML (2012) Extracellular matrix and fibroblast communication following myocardial infarction. J Cardiovasc Transl Res 5:848- 857

58. Akhmedov AT and Marin-Garcia J (2013) Myocardial regeneration of the failing heart. Heart Fail Rev 18:815-833

59. Santini MP and Rosenthal N (2012) Myocardial regenerative properties of macrophage populations and stem cells. J Cardiovasc Transl Res 5:700-712

60. Willems IE, Havenith MG, De Mey JG, et al (1994) The alpha-smooth muscle actin-positive cells in healing human myocardial scars. Am J Pathol 145:868-875

62. Souders CA, Bowers SL, and Baudino TA (2009) Cardiac fibroblast: the renaissance cell. Circ Res 105:1164-1176

63. Bujak M and Frangogiannis NG (2007) The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res 74:184-195

64. Desmouliere A, Geinoz A, Gabbiani F, et al (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122:103-111

65. Evans RA, Tian YC, Steadman R, et al (2003) TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins. Exp Cell Res 282:90-100

66. Hao J, Wang B, Jones SC, et al (2000) Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and *in vitro*. Am J Physiol Heart Circ Physiol 279:H3020-H3030

67. Kawano H, Cody RJ, Graf K, et al (2000) Angiotensin II enhances integrin and alpha-actinin expression in adult rat cardiac fibroblasts. Hypertension 35:273-279

68. Kawano H, Do YS, Kawano Y, et al (2000) Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. Circulation 101:1130-1137

69. Hafizi S, Wharton J, Chester AH, et al (2004) Profibrotic effects of endothelin-1 via the ETA receptor in cultured human cardiac fibroblasts. Cell Physiol Biochem 14:285-292

70. Masur SK, Dewal HS, Dinh TT, et al (1996) Myofibroblasts differentiate from fibroblasts when plated at low density. Proc Natl Acad Sci U S A 93:4219-4223

71. Baum J and Duffy HS (2011) Fibroblasts and myofibroblasts: what are we talking about? J Cardiovasc Pharmacol 57:376-379

72. Chester AH and Taylor PM (2007) Molecular and functional characteristics of heart valve interstitial cells. Philos Trans R Soc Lond B Biol Sci 362:1437-1443

73. Cunnington RH, Wang B, Ghavami S, et al (2011) Antifibrotic properties of c-Ski and its regulation of cardiac myofibroblast phenotype and contractility. Am J Physiol Cell Physiol. 300:C176-C186

74. Serini G, Bochaton-Piallat ML, Ropraz P, et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-β1. J Cell Biol 142:873-881

75. Sun Y (1997) Local angiotensin II and myocardial fibrosis. Adv Exp Med Biol 432:55-61

76. Lijnen P, Petrov VV, Rumilla K, et al (2002) Stimulation of collagen gel contraction by angiotensin II and III in cardiac fibroblasts. J Renin Angiotensin Aldosterone Syst 3:160-166

77. Lijnen PJ, Petrov VV, and Fagard RH (2001) Angiotensin II-induced stimulation of collagen secretion and production in cardiac fibroblasts is mediated via angiotensin II subtype 1 receptors. J Renin Angiotensin Aldosterone Syst 2:117-122

78. Petrov VV, Fagard RH, and Lijnen PJ (2002) Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 39:258-263

79. Darby I, Skalli O, and Gabbiani G (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing

80. van Amerongen MJ, Bou-Gharios G, Popa E, et al (2008) Bone marrow-derived myofibroblasts contribute functionally to scar after myocardial infarction. J Pathol 214: 377-386

81. Balsam LB, Wagers AJ, Christensen JL, et al (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428: 668-673

82. Endo J, Sano M, Fujita J, et al (2007) Bone marrow derived cells are involved in the pathogenesis of cardiac hypertrophy in response to pressure overload. Circulation 116: 1176-1184

83. Murry CE, Soonpaa MH, Reinecke H, et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428: 664-668

84. Handgretinger R, Gordon PR, Leimig T, et al (2003) Biology and plasticity of CD133+ hematopoietic stem cells. Ann NY Acad Sci 996: 141-151

85. Serini G, Gabbiani G (1999) Mechanisms of myofibroblast activity and phenotype modulation. Exp Cell Res 250: 273-283

86. Mohle R, Bautz F, Rafii S, et al (1998) The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 91: 4523-4530

87. Murdoch C (2000) CXCR: chemokine receptor extraordinaire. Immunol Rev 177: 175-184

88. Phillips RJ, Burdick MD, Hong K, et al (2004) Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 114: 438-446

89. Yano T, Miura T, Ikeda Y, et al (2005) Intracardiac fibroblasts, but not bone marrow derived cells, are the origin of myofibroblasts in myocardial infarct repair. Cardiovasc Pathol 14: 241-246

90. Abe R, Donnelly SC, Peng T, et al (2001) Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol 166: 7556-7562

91. Shah RV, Mitchell RN (2005) The role of stem cells in the response to myocardial and vascular wall injury. Cardiovasc Pathol 14: 225-231

92. Guo J, Lin GS, Bao CY, et al (2007) Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30(3-4): 97-104

93. Lee RH, Pulin AA, Seo MJ, et al (2009) Intravenous hMPCs improve myocardial infarction in mice because cells embolized in lung are activated to secret the anti-inflammatory protein TSG-6. Cell Stem Cell 5(1): 54-63

94. Lee RH, Seo MJ, Pulin AA, et al (2009) The CD34-like protein PODXL and alpha6-integrin (CD49f) identify early progenitor MPCs with increased clonogenicity and migration to infracted heart in mice. Blood 113(4): 816-826

95. Psaltis PJ, Paton S, See F, et al (2010) Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol 223(2): 530-540

96, Du YY, Zhou SH, Zhou T, et al (2008) Immuno-inflammatory regulation effect of mesenchymal stem cell transplantation in a rat model of myocardial infarction. Cytotherapy 10(5): 469-478

97. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105(4): 1815-1822

98. Burchfield JS, Iwasaki M, Koyanagi M, et al (2008) Interleukin-10 from transplanted bone marrow mononuclear cells contributes to cardiac protection after myocardial infarction. Circ Res 103(2): 203-211

99. Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2: 313-319

100. Wagner W, Ho AD (2007) Mesenchymal stem cell preparations – comparing apples and oranges. Stem Cell Rev 3: 239-248

101. Ball SG, Shuttleworth AC, Kielty CM (2004) Direct cell contact influences bone marrow mesenchymal stem cell fate. Int J Biochem Cell Biol 36: 714-727

102. Novotny NM, Ray R, Markel TA, et al (2008) Stem cell therapy in myocardial repair and remodeling. J Am Coll Surg 207: 423-434

103. Crawford JR, Haudek SB, Cieslik KA, et al (2012) Origin of developmental precursors dictates the pathophysiologic role of cardiac fibroblasts. J Cardiovasc Transl Res 5: 749-759

104. Dawn B, Stein AB, Urbanek K, et al (2005) Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. Proc Natl Acad Sci USA 102: 3766-3771

105. Russell JL, Goetsch SC, Gaiano NR, et al (2011) A dynamic notch injury response activates epicardium and contributes to fibrosis repair. Circ Res 108: 51-59

106. Paul D, Samuel SM, Maulik N (2009) Mesenchymal stem cell: present challenges and prospective cellular cardiomyoplasty approaches for myocardial regeneration. Antioxid Redox Signal 11: 1841-1855

107. Ishikawa F, Shimazu H, Shultz LD, et al (2006) Purified human hematopoietic stem cells contribute to the generation of cardiomyocytes through cell fusion. FASEB J 20: 950-952

108. Docheva D, Popov C, Mutschler W, et al (2007) Human mesenchymal stem cells in contact with their environment surface characteristics and the integrin system. J Cell Mol Med 11: 21-38

109. Gronthos S, Simmons PJ, Graves SE, et al (2001) Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 28: 174-181

110. Gnecchi M, He H, Liang OD, et al (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11: 367-368

111. Wang J, Laschinger C, Zhao XH, et al (2005) Mechanical force activates eIF-2alpha phosphor-kinases in fibroblast. Biochem Biophys Res Commun 330(1): 123-130

112. Gurtner GC, Chang E (2008) “Priming” endothelial progenitor cells: a new strategy to improve cell based therapeutics. Arterioscler Thromb Vasc Biol 28: 1034-1035

113. Hahn JY, Cho HJ, Kang HJ, et al (2008) Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. J Am Coll Cardiol 51: 933-943

114. Haider H, Jiang S, Idris NM, et al (2008) IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. Circ Res 103: 1300-1308

115. Valiunas V, Doronin S, Valiuniene L, et al (2004) Human mesenchymal stem cells make cardiac connexins and form function gap junctions. J Physiol 555: 617-626

116. Kohl P (2003) Heterogeneous cell coupling in the heart: An electrophysiological role for fibroblasts. Circ Res 93: 381-383

117. Chilton L, Giles WR, Smith GL (2007) Evidence of intercellular coupling between co-cultured adult rabbit ventricular myocytes and myofibroblasts. J Physiol 583: 225-236

118. Gaudesius G, Miragoli M, Thomas SP, et al (2003) Coupling of cardiac electrical activity over extended distances by fibroblasts of cardiac origin. Circ Res 93: 421-428

119. Miragoli M, Gaudesius G, Rohr S (2006) Electronic modulation of cardiac impulse conduction by myofibroblasts. Circ Res 98: 801-810

120. Bucala R, Spiegel LA, Chesney J, et al (1994) Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med 1: 71-81

121. Díaz-Flores L, Gutiérrez R, García MP, et al (2014) CD34+ stromal cells/fibroblasts/fibrocytes/telocytes as a tissue reserve and a principle source of mesenchymal cells. Location, morphology, function, and role in pathology. Histol Histopathol 29: 831-870

122. Haniffa MA, Wang XN, Hotlick U, et al (2007) Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J Immunol 179: 1595-1604

123. Keeley EC, Schutt RC, Marinescu MA, et al (2016) Circulating fibrocytes as predictors of adverse effects in unstable angina. Transl Res 172: 73-83

124. Zulli A, Buxton BF, Black MJ, et al (2005) CD34 Class III positive cells are present in atherosclerotic plaques of the rabbit model of atherosclerosis. Histochem Cell Biol 124: 517-522

125. Medbury HJ, Tarran SL, Guiffre AK, et al (2008) Monocytes contribute to the atherosclerotic cap by transformation into fibrocytes. Int Angiol 27: 114-123

126. Xie X, Liu Y, Gao S, et al (2014) Possible involvement of fibrocytes in atrial fibrosis in patients with chronic atrial fibrillation. Circ J 78: 338-344

127. Fang L, Beale A, Ellims AH, et al (2013) Associations between fibrocytes and postcontrast myocardial T1 times in hypertrophic cardiomyopathy. J Am Heart Assoc 2: e000270

128. Chesney J, Metz C, Stavisky AB, et al (1998) Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. J Immunol 160: 419-425

129. D'Armiento J (2002) Matrix metalloproteinase disruption of the extracellular matrix and cardiac dysfunction. Trends Cardiovasc Med 12: 97-101

130. McClain SA, Simon M, Jones E, et al (1996) Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. Am J Pathol 149: 1257-1270

131. Xu J, Clark RA (1997) A three-dimensional collagen lattice induces protein kinase C-zeta activity: role in alpha-2 integrin and collagenase mRNA expression. J Cell Biol 136: 473-483

132. Aisagonhi O, Rai M, Ryzhov S, et al (2011) Experimental myocardial infarction triggers canonical Wnt signaling and endothelial-to-mesenchymal transition. Dis Model Mech 4: 469-483

133. von Gise A, Pu WT (2012) Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. Circ Res 110: 1628-1645

134. Goumans MJ, Mummery C (2000) Functional analysis of the TGFβ receptor/Smad pathway through gene ablation in mice. Int J Dev Biol 443: 253-265

135. Khan R, Sheppard R (2006) Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease, and arrhythmia. Immunology 1181: 10-24

136. O'Riordan E, Mendelev N, Patschan S, et al (2007) Chronic NOS inhibition actuates endothelial-mesencymal transformation. Am J Physiol Heart Circ Physioll 292: H285-H294

137. Tan X, Zhu Y, Chen C, et al (2016) Sprouty1 suppresses epithelial-mesenchymal transition of human lens epithelial cells through blockade of SMAD2 and ERK1/2 pathways. PLoS One 11(7): e0159275

138. Zhou B, Honor LB, He H, et al (2011) Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. J Clin Invest 121: 1894-1904

139. Evrard SM, Lecce L, Michelis KC, et al (2016) Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. Nat Commun 7: 11853

140. Nakaya M, Watari K, Tajima M, et al (2017) Cardiac myofibroblast engulfment of dead cells facilitates recovery after myocardial infarction. J Clin Invest 127(1): 383-401

141. Smith CL, Baek ST, Sung CY, et al (2011) Epicardial-derived cell epithelial-to-mesenchymal transition and fate specification require PDGF receptor signaling. Circ Res 295: 507-522

142. Rygiel KA, Robertson H, Marshall HL (2008) Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. Lab Invest 88: 112-123

143. Willis BC, Liebler JM, Luby-Phelps K (2005) Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol 166: 1321-1332

144. Iwano M, Plieth D, Danoff TM, et al (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 110: 341-350

145. Brønnum H, Andersen DC, Schneider M, et al (2013) miR-21 promotes fibrogenic epithelial-to-mesenchymal transition of epicardial mesothelial cells involving Programmed Cell Death 4 and Sprouty-1. PLoS One 8(2): e56280

146. Oh H, Bradfute SB, Gallardo TD (2003) Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. Proc Nat Acad Sci USA 100: 12313-12318

147. Bartel DP, Chen CA (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet 5: 396-400

148. Querejeta R, López B, González A, et al (2004) Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis. Circulation 110: 1263-1268

149. Holmes DR Jr, Savage M, LaBlanche JM, et al (2002) Results of prevention of REStenosis with tranilast and its outcomes (PRESTO) trial. Circulation 106: 1243-1250

150. Fang L, Murphy AJ, Dart AM (2017) A clinical perspective of anti-fibrotic therapies for cardiovascular disease. Front Pharmacol 8: 186

151. Hudson MP, Armstrong PW, Ruzyllo W, et al (2006) Effects of selective matrix metalloproteinase inhibitor (PG-116800) to prevent ventricular remodeling after myocardial infarction: results of the PREMIER (Prevention of Myocardial Infarction Early Remodeling) trial. J Am Coll Cardiol 48: 15-20

152. Tobert JA (1987) New developments in lipid-lowering therapy: the role of inhibitors of hydroxymethylglutaryl-coenzyme A reductase. Circulation 76:534-538

153. Heart Protection Study Collaborative Group (2002) MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 360:7-22

154. Kesh SB, Sikder K, Manna K, et al (2013) Promising role of ferulic acid, atorvastatin and their combination in ameliorating high fat diet-induced stress in mice. Life Sci. 92: 938-949

155. Hennessy DA, Tanuseputro P, Tuna M, et al (2017) Health Reports: Population health impact of statin treatment in Canada. Statistics Canada: <https://www.statcan.gc.ca/pub/82-003-x/2016001/article/14305-eng.htm>

156. US Department of Health and Human Services. Health, United States, 2016. May 2017. < https://www.cdc.gov/nchs/data/hus/hus16.pdf#glance>

157. Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343:425-430

158. Katsuki H, Bloch K (1967) Studies on the biosynthesis of ergosterol in yeast: formation of methylated intermediates. J Biol Chem 242: 222-227

159. Lynen F (1967) Biosynthetic pathways from acetate to natural products. Pure Appl Chem 14:137-167

160. Wolfe LA, Morava E, He M, et al (2012) Heritable disorders in the metabolism of the dolichols: a bridge from sterol biosynthesis to molecular glycosylation. Am J Med Genet C Semin Med Genet 160C:322-328

161. Molyneux SL, Young JM, Florkowski CM, et al (2008) Coenzyme Q10: is there a clinical role and a case for measurement? Clin Biochem Rev 29: 71-82

162. Diechmann R, Lavie C, Andrews S (2010) Coenzyme Q10 and statin-induced mitochondrial dysfunction. Ochsner J 10:16-21

163. Littarru GP, Tiano L (2007) Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Mitochondrion 7 Suppl:S168-S174

164. Rundek T, Naini A, Sacco R, et al (2004) Atorvastatin decreases the coenzyme Q10 level in the blood of patients at risk for cardiovascular disease and stroke. Arch Neurol 61:889-892

165. Brown MS, Goldstein JL (1980) Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res 21:505-517

166. Gil G, Osborne TF, Goldstein JL, et al (1988) Purification of a protein doublet that binds to six TGG-containing sequences in the promoter for hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J Biol Chem 263: 19009-19019

167. Metherall JE, Goldstein JL, Luskey KL, et al (1989) Loss of transcriptional repression of three sterol-regulated genes in mutant hamster cells. J Biol Chem 264:15634-15641

168. Nakanishi M, Goldstein JL, Brown MS (1988) Multivalent control of 3-hyroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J Biol Chem 263:8929-8937

169. Panini SR, Schnitzer-Polokoff R, Spencer TA, et al (1989) Sterol-independent regulation of 3-hydroxy-3-methyl-glutaryl-CoA reductase by mevalonate in Chinese hamster ovary cells. Magnitude and specificity. J Biol Chem 264: 11044-11052

170. Mosley ST, Brown MS, Anderson RG, et al (1983) Mutant clone of Chinese hamster ovary cells lacking 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Biol Chem 258:13875-13881

171. Ortego M, Bustos C, Hernandez-Presa MA, et al (1999) Atorvastatin reduces NF-kappaB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. Atherosclerosis 147:253-261

172. Zelvyte I, Dominaitiene R, Crisby M, et al (2002) Modulation of inflammatory mediators and PPARgamma and NFkappaB expression by pravastatin in response to lipoproteins in human monocytes in vitro. Pharmacol Res 45: 147-154

173. Weis M, Pehlivanli S, Meiser BM, et al (2001) Simvastatin treatment is associated with improvement in coronary endothelial function and decreased cytokine activation in patients after heart transplantation. J Am Coll Cardiol 38:814-818

174. Lazzerini PE, Lorenzini S, Selvi E, et al (2007) Simvastatin inhibits cytokine production and nuclear factor- κ B activation in interleukin 1beta-stimulated synoviocytes from rheumatoid arthritis patients. Clin Exp Rheumatol 25:696-700s

175. Devaraj S, Chan E, Jialal I (2006) Direct demonstration of an anti-inflammatory effect of simvastatin in subjects with metabolic syndrome. J Clin Endocrinol Metab 91:4489-4496

176. Marcuzzi A, Crovella S, Monasta L, et al (2012) Mevalonate kinase deficiency: disclosing the role of mevalonate pathway modulation in inflammation. Curr Pharm Des 18:5746-5752

177. Yeganeh B, Wiechec E, Ande SR, et al (2014) Targeting the mevalonate cascade as a new therapeutic approach in heart disease, cancer, and pulmonary disease. Pharmacol Ther 143: 87-110

178. Barone E, Di Domenico F, Butterfield DA (2014) Statins more than cholesterol lowering agents in Alzheimer disease: their pleiotropic functions as potential therapeutic targets. Biochem Pharmacol 88:605-616

179. Cortes-Bergoderi M, Pineda AM, Santana O (2013) The pleiotropic effects and therapeutic potential of the hydroxyl-methyl-glutaryl-CoA reductase inhibitors in gastrointestinal tract disorders: a comprehensive review. J Gastroinestin Liver Dis 22:199-204

180. Yuan C, Zhou L, Cheng J, et al (2012) Statins as potential therapeutic drug for asthma? Respir Res 13: 108

181. Zeichner S, Mihos CG, Santana O (2012) The pleiotropic effects and therapeutic potential of the hydroxyl-methyl-glutaryl-CoA reductase inhibitors in malignancies: a comprehensive review. J Cancer Res Ther 8:176-183

182. Lombard J, Moreira D (2011) Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. Mol. Biol Evol 28:87-99

183. Lange BM, Rujan T, Martin W, et al (200) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97: 13172-13177

184. Greenwood J, Steinman L, Zamvil SS (2006) Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. Nat Rev Immunol 6: 358-370

185. McTaggart SJ, (2006) Isoprenylated proteins. Cell Mol Life Sci 63:255-267

186. Wolfrum S, Jensen KS, Liao JK (2003) Endothelium-dependent effects of statins. Arterioscler Throm Vasc Biol 23:729-736

187. Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 8:180-186

188. Sinensky M, Beck LA, Leonard S (1990) Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. J Biol Chem 265:19937-19941

189. Takemoto M, Liao JK (2001) Pleiotropic effets of 3-hydroxy-3-methylgutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol 21:1712-1719

190. Sebti SM (2005) Protein farnesylation: implications for normal physiology, malignant transformation, and cancer therapy. Cancer Cell 7:297-300

191. Scheffzek K, Ahmadian MR (2005) GTPase activating proteins: structural and functional insights 18 years after discovery. Cell Mol Life Sci 62:3014-3038

192. Bourne HR, Sanders DA, McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125-132

193. Liao JK, Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45:89-118

194. Walker K, Olson MF (2005) Targeting Ras and Rho GTPases as opportunities for cancer therapeutics. Curr Opin Genet Dev 15:62-68

195. Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. Physiol Rev 81:153-208

196. van Wetering S, van Buul JD, Quik S, et al (2002) Reactive oxygen species mediate Rac-induced loss of cell-cell adhesion in primary human endothelial cells. J Cell Sci 115:1837-1846

197. Wassmann S, Wassmann K, Nickenig G (2004) Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. Hypertension 44:381-386

198. Antonopoulos AS, Margaritis M, Shirodaria C, et al (2012) Translating the effects of statins: from redox regulation to suppression of vascular wall inflammation. Thromb Haemost 108:840-848

199. Carman CV, Jun CD, Salas A, et al (2003) Endothelial cells proactively form microvilli-like membrane projections upon intercellular adhesion molecule 1 engagement of leukocyte LFA-1. J Immunol 171: 6135-6144

200. Wojciak-Stothard B, Williams L, Ridley AJ (1999) Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. J Cell Biol 145:1293-1307

201. Tousoulis D, Psarros C, Demosthenous M, et al (2014) Innate and adaptive inflammation as a therapeutic target in vascular disease: The emerging role of statins. J Am Coll Cardiol 63: 2491-2502

202. Alvarez De Sotomayor M, Herrera MD, Marhuenda E, et al (2000) Characterization of endothelial factors involved in the vasodilatory effect of simvastatin in aorta and small mesenteric artery of the rat. Br J Pharmacol 131: 1179-1187

203. Mital S, Zhang X, Zhao G, et al (2000)Simvastatin upregulates coronary vascular endothelial nitric oxide production in conscious dogs. Am J Physiol Heart Circ Physiol 279:H2649-H2657

204. Sarbassov DD, Ali S, Sabatini DM (2005) Growing roles for the mTOR pathway. Curr Opin Cell Biol 17:596-603

205. Sinclair LV, Finlay D, Feijoo C, et al (2008) Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. Nat Immunol 9:513-521

206. Antoniades C, Bakogiannis C, Tousoulis D, et al (2010) Preoperative atorvastatin treatment in CABG patients rapidly improves vein redox state by inhibition of Rac1 and NADPH-oxidase activity. Circulation 122:S66-S73

207. Nawawi H, Osman HS, Annuar R, et al (2003) Soluble intercellular adhesion molecule-1 and interleukin-6 levels reflect endothelial dysfunction in patients with primary hypercholesterolaemia treated with atorvastatin. Atherosclerosis 169:283-291

208. Tsunekawa T, Hayashi T, Kano H, et al (2001) Cerivastatin, a hydroxymethylglutaryl coenzyme a reductase inhibitor, improves endothelial function in elderly diabetic patients within 3 days. Circulation 104:376-379

209. Tousoulis D, Antoniades C, Bosinakou E, et al (2005) Effects of atorvastatin on reactive hyperaemia and inflammatory process in patients with congestive heart failure. Atherosclerosis 178:359-363

210. Thorburn A, Thorburn J, Chen SY, et al (1993) HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. J Biol Chem 268:2244-2249

211. Takemoto M, Node K, Nakagami H, et al (2001) Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy. J Clin Invest 108:1429-1437

212. Zhu Y, Casey PJ, Kumar AP, et al (2013) Deciphering the signalling networks underlying simvastatin-induced apoptosis in human cancer cells: evidence for non-canonical activation of RhoA and Rac1 GTPases. Cell Death Dis 4:e568

213. Morris G, Anderson G, Berk M (2013) Coenzyme Q10 depletion in medical and neuropsychiatric disorders: potential repercussions and therapeutic interventions. Mol Neurobiol 48:883-903

214. Crane FL (2008) The evolution of coenzyme Q. Biofactors 32:5-11

215. Rodríguez-Hernández A, Cordero MD, Salviati L, et al (2009) Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. Autophagy 5:19-32

216. Echtay KS, Winkler E, Klingenberg M (2000) Coenzyme Q is an obligatory cofactor for uncoupling protein function. Nature 408:609-613

217. Echtay KS, Winkler E, Frischmuth K, et al (2001) Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). Proc Natl Acad Sci USA 98:1416-1421

218. Kon M, Kimura F, Akimoto T, et al (2007) Effect of coenzyme Q10 supplementation on exercise-induced muscular injury of rats. Exerc Immunol Rev 13:76-88

219. Ernster L, Forsmark P, Nordenbrand K (1992) The mode of action of lipid-soluble antioxidants in biological membranes: relationship between the effects of ubiquinol and vitamin E as inhibitors of lipid peroxidation in submitochondrial particles. Biofactors 3:241-248

220. Kagan V, Serbinova E, Packer L (199) Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. Biochem Biophys Res Commun 169:851-857

221. James AM, Smith RA, Murphy MP (2004) Antioxidant and prooxidant properties of mitochondrial coenzyme Q. Arch Biochem Biophys 423:47-56

222. Passi S, Stancato A, Aleo E, et al (2003) Statins lower plasma and lymphocyte ubiquinol/ubiquinone without affecting other antioxidants and PUFA. Biofactors 18:113-124

223. Langsjoen PH, Langsjoen JO, Langsjoen AM, et al (2005) Treatment of statin adverse effects with supplemental coenzyme Q10 and statin drug discontinuation. Biofactors 25:147-152

224. Littarru GP, Langsjoen P (2007) Coenzyme Q10 and statins: biochemical and clinical implications. Mitochondrion 7 Suppl:S168-S174

225. Kuettner A, Pieper A, Koch J, et al (2005) Influence of CoQ10 and cerivastatin on the flow-mediated vasodilation of the brachial artery: results of the ENDOTACT study. Int J Cardiol 98:413-419

226. McMurray JJ, Dunselman P, Wedel H et al (2010) CoQ10, rosuvastatin, and clinical outcomes in heart failure: a pre-specified substudy of CORONA (controlled rosuvastatin multinational study in heart failure). J Am Coll Cardiol 56:1196-1204

227. Felker GM (2010) CoQ10 and statins in heart failure: the dog that didn't bark. J Am Coll Cardiol 56:1205-1206.

228. Taylor BA, Lorson L, White CM, et al (2015) A randomized trial of CoQ10 in patients with confirmed statin myopathy. Atherosclerosis 238:329-335

229. Dunn SE, Youssef G, Goldstein MJ, et al (2006) Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. J Exp Med 203:401-412

230. Lawman S, Mauri C, Jury EC, et al (2004) Atorvastatin inhibits autoreactive B cell activation and delays lupus development in New Zealand Black/White F1 mice. J Immunol 173:7641-7646

231. Zeiser R, Youssef S, Baker J, et al (2007) HMG-CoA reductase inhibitors (statins) provide acute graft-versus-host disease protection by Th2 cytokine induction whlile sparing graft-versus-leukemia activity. Blood 110:4588-4598

232. Raki M, Molberg O, Tollefsen S, et al (2005) The effects of atorvastatin on gluten-induced intestinal T cell responses in celiac disease. Clin Exp Immunol 142:333-340

233. Mausner-Fainberg K, Luboshitss G, Mor A, et al (2008) The effect of HMG-CoA reductase inhibitors on naturally occurring CD4+ CD25+ T cells. Atherosclerosis 197:829-839

234. Broz P, Ben-Haim N, Grzelakowski M, et al (2008) Inhibition of macrophage phagocytic activity by a receptor-targeted polymer vesicle-based drug delivery formuation of pravastatin. J Cardiovasc Pharmacol 51:246-252

235. Xu SZ, Zhong W, Watson NM, et al (2008) Fluvastatin reduces oxidative damage in human vascular endothelial cells by upregulating Bcl-2. J Thromb Haemost 4:692-700

236. Zeiser R, Maas K, Youssef S, et al (2008) Regulation of different inflammatory disease by impacting the mevalonate pathway. Immunology 127:18-25

237. Youssef S, Stuve O, Patarroyo JC, et al (2002) The HMG-CoA reductase inhibitor atorvastatin, promotes the Th2 bias and reverses paralysis in central nervous system in autoimmune disease. Nature 420:78-84

238. Aktas O, Waiczies S, Smorodchenko A, et al (2003) Treatment of relapsing paralysis in experimenting encephalomyelitis by targeting Th1 cells through atorvastatin. J Exp Med 197:725-733

239. Greenwood J, Walters CE, Pryce G (2003) Lovastatin inhibits brain endothelial Rho-mediated lymphocyte migration and attenuates experimental autoimmune encephalomyelitis. FASEB J 17:905-907

240. Nath N, Giri S, Prasad R (2004) Potential targets of 3-hydroxy-3-methylglutaryl Coenzyme A reductase inhibitor for multiple sclerosis therapy. J Immunol 172:1273-1286

241. Eccles KA, Sowden H, Porter KE, et al (2008) Simvastatin alters human endothelial cell adhesion molecule expression and inhibits leukocyte adhesion under flow. Atherosclerosis 200:69-79

242. Zhang W, Liu M, Wu Y (2007) Protective effects of atorvastatin on chronic allograft nephropathy in rats. J Surg Res 143:428-436

243. Li C, Yang CW, Park JH, et al (2004) Pravastatin treatment attenuates interstitial inflammation and fibrosis in a rat model of chronic cyclosporine-induced nephropathy. Am J Physiol Renal Physiol 286:F46-F57

244. Kohl NE, Omer CA, Conner MW, et al (1995) Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat Med 1:792-797

245. Sola S, Mir MQS, Lerakis S, et al (2006) Atorvastatin improves left ventricular systolic function and serum markers of inflammation in nonischemic heart failure. J Am Coll Cardiol 47: 332-337

246. Node K, Fujita M, Kitakaze M, et al (2003) Short-term statin therapy improves cardiac function and symptoms in patients with idiopathic dilated cardiomyopathy. Circulation 108:839-843

247. Rezaie-Majd A, Maca T, Bucek RA, et al (2002) Simvastatin reduces expression of cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. Aterioscler Thromb Vasc Biol 22:1194-1199

248. Ridker PM, Danielson E, Fonseca FA, et al (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Eng J Med 359:2195-2207

249. van der Meer JW, Vossen JM, Radl J, et al (1984) Hyperimmunoglobulinaemia D and periodic fever: a new syndrome. Lancet 1:1087-1090

250. Berger R, Smit GP, Schierbeek H (1985) Mevalonic aciduria: an inborn error cholesterol biosynthesis. Clin Chim Acta 152:219-222

251. Hoffmann G, Gibson KM, Brandt IK, et al (1986) Mevalonic aciduria –an inborn error of cholesterol and nonsterol isoprene biosynthesis. N Engl J Med 314:1610-1614

252. Houten SM, Kuis W, Duran M, et al (1999) Mutations in MVK, encoding mevalonate kinase, cause hyperimmunoglobulinaemia D and periodic fever syndrome. Nat Genet 22:175-177

253. Simon A, Kremer HP, Wevers RA, et al (2004) Mevalonate kinase deficiency: evidence for a phenotypic continuum. Neurology 62:994-997

254. van der Burgh R, ter Haar NM, Boes ML, et al (2013) Mevalonate kinase deficiency, a metabolic autoinflammatory disease. Clin Immunol 147:197-206

255. Cuisset L, Drenth JP, Simon A, et al (2001)) Molecular analysis of MVK mutations and enzymatic activity in hyper-IgD and periodic fever syndrome. Eur J Human Genet 9:260-266

256. Poll-The BT, Frenkel J, Houten SM, et al (2000) Mevalonic aciduria in 12 unrelated patients with hyperimmunoglobulinaeia D and periodic fever syndrome. J Inhert Metab Dis 23:363-366

257. Hoffmann GF, Charpentier C, Myaytepek E, et al (1993) Clinical and biochemical phenotype in 11 patients with mevalonic aciduria. Pediatrics 91:915-921

258. Marcuzzi A, Crovella S, Pontillo A (2011) Geraniol rescues inflammation in cellular and animal models of mevalonate kinase deficiency. In Vivo 25:87-92

259. Marcuzzi A, Tommasini A, Crovella S, et al (2010) Natural isoprenoids inhibit LPS-induced production of cytokines and nitric oxide in aminobisphosphonate-treated monocytes. Int Immunopharmacol 10:639-642

260. Henneman L, Schneiders MS, Turkenburg M, et al (2010) Compromised geranylgeranyl of RhoA and Rac1 in mevalonate kinase deficiency. J Inherit Metab Dis 33:625-632

261. Chua CE, Gan BQ, Tang BL (2011) Involvement of members of the rab family and related small GTPases in autophagosome formation and maturation. Cell Mol Life Sci 68:3349-335826

262. Dupont N, Jiang S, Pilli M, et al (2011) Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. EMBO J 30:4701-4711

263. Zhou R, Yazdi AS, Menu P, et al (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature 469:221-225

264. Reinhart BJ, Slack FJ, Basson M, et al (2000) The 21 nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403 (6772):901-906

265. Grishok A, Pasquinelli AE, Conte D, et al (2001) Genes and mechanisms related to RNA interference regulate expression of small temporal RNAs that control C. elegans developmental timing. 106(1):23-34

266. Bartel GP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2): 281-297

267. Gurtan AM, Ravi A, Rahl PB, et al (2013) Let-7 represses Nr6a1 and a mid-gestation developmental program in adult fibroblasts. Genes Dev 27: 941-954

268. Herranz H, Cohen SM (2010) MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. Genes Dev 24: 1339-1344

269. Bernstein E, Caudy AA, Hammond SM, et al (2001) Role for bidentate ribonuclease in the initiation step of RNA interference. Nature 409(6818): 363-366

270. Ebert MS, Sharp PA (2012) Roles for microRNAs in conferring robustness to biological processes. Cell 149: 515-524

271. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676

272. Port JD, Sucharov C (2010) Role of microRNAs in cardiovascular disease: therapeutic challenges and potentials. J Cardiovasc Pharmacol 56: 444-453

273. Subramanyam D, Lamouille S, Judson RL, et al (2011) Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. Nat Biotechnol 29: 443-448

274. Nagai R, Suzuki T, Aizawa K, et al (2005) Significance of the transcription factor KLF5 in cardiovascular remodeling. J Thromb Haemost 3: 1569-1576

275. Rao PK, Toyama Y, Chiang R, et al (2009) Loss of cardiac miRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res 105: 585-594

276. Yang B, Lin H, Xiao J, et al (2007) The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 13: 468-491

277. Glass C, Singla DK (2011) MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infracted heart. Am J Physiol Heart Circ Physiol 301(5): H2038-H2049

278. Thum T, Gross C, Fiedler J, et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts. Nature 456: 980-984

279. Li Q, Song XW, Zou J, et al (2010) Attentuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. J Cell Sci 123(Pt 14): 2444-2452

280. van Rooij E, Sutherland LB, Thatcher JE, et al (2008) Dysregulation of miroRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105: 13027-13032

281. Roy S, Khanna S, Hussain SR, et al (2009) MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 82: 21-29

282. Thum T, Gross C, Fiedler J, et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts. Nature 456: 980-984

283. Wang J, Xu R, Lin F, et al (2009) MicroRNA: novel regulators involved in the remodeling and reverse remodeling of the heart. Cardiology 113: 81-88

284. Pekarsky Y, Santanum U, Cimmino A, et al (2006) Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res 66: 11590-11593

285. Mott JL, Kobayashi S, Bronk SF, et al (2007) miR-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 26: 6133-6140

286. Park SY, Lee JH, Ha M, et al (2009) miR-29 miRNAs activate p53 by targeting p85α and CDC42. Nat Struct Mol Biol 16: 23-29

287. Czubryt MP (2012) Common threads in cardiac fibrosis, infarct scar formation, and wound healing. Fibrogenesis Tissue Repair 5:19

288. Borg TK and Caulfield JB (1979) Collagen in the heart. Tex Rep Biol Med 39:321-333 173. Caulfield JB and Borg TK (1979) The collagen network of the heart. Lab Invest 40:364-372

289. Rienks M, Papageorgiou AP, Frangogiannis NG, et al (2014) Myocardial extracellular matrix: an ever-changing and diverse entity. Circ Res 114:872-888

290. Banerjee I, Fuseler JW, Price RL, et al (2007) Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. Am J Physiol Heart Circ Physiol 293:H1883-H1891

291. Nag AC (1980) Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. Cytobios 28:41-61

292. Theocharis AD, Skandalis SS, Gialeli C, et al (2016) Extracellular matrix structure. Adv Drug Deliv Rev 97: 4-27.

293. Theocharis AD, Skandalis SS, Tzanakakis GN, et al (2010) Proteoglycans in health and disease: novel roles for pharmacological targeting. FEBS J 277: 3904-3923.

294. Iozzo RV, Sanderson RD (2011) Proteoglycans in cancer biology, tumor microenvironment and angiogenesis. J Cell Mol Med 15: 1013-1031

295. Afratis N, Gialeli C, Nikitovic T, et al (2012) Glycosaminoglycans: key players in cancer cell biology and treatment. FEBS J 279: 1177-1197

296. Camelliti P, Borg TK, and Kohl P (2005) Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res 65:40-51

297. Camelliti P, McCulloch AD and Kohl P (2005) Microstructured cocultures of cardiac myocytes and fibroblasts: a two-dimensional in vitro model of cardiac tissue. Microsc Microanal 11:249-259

298. Prockop DJ and Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64:403-434

299. Kagan HM and Trackman PC (1991) Properties and function of lysyl oxidase. Am J Respir Cell Mol Biol 5:206-210

300. Smith-Mungo LI and Kagan HM (1998) Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 16:387-398

301. Mukherjee D and Sen S (1991) Alteration of collagen phenotypes in ischemic cardiomyopathy. J Clin Invest 88:1141-1146

302. Weber KT (1989) Cardiac interstitium in health and disease: the fibrillar collagen network. JACC 13:1637-1652

303. Cowan PM, McGavin S and North AC (1955) The polypeptide chain configuration of collagen. Nature 176:1062-1064

304. Pauling L and Corey RB (1951) The structure of fibrous proteins of the collagen-gelatin group. Proc Natl Acad Sci U S A 37:272-281

305. Wierzbicka-Patynowski I, Schwarzbauer JE (2003) The ins and outs of fibronectin matrix assembly. J Cell Sci 116 (Pt 16): 3269-3276

306. Serini G, Bochaton-Piallat ML, Ropraz P, et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor β1. J Cell Biol 142: 873-881

307. Mardilovich A, Craig JA, McCammon MQ et al (2006) Design of a novel fibronectin-mimetic peptide-amphiphile for functionalized biomaterials. Langmuir 2006 22(7): 3259-3264

308. Hozumi K, Nakamura K, Hori H, et al (2016) Mixed fibronectin-derived peptides conjugated to a chitosan matrix effectively promotes biological activities through integrins α4β1, α5β1, αvβ1, αvβ3, and Syndecan. Biores Open Access 5(1):356-366

309. Schonherr E and Hausser HJ (2000) Extracellular matrix and cytokines: a functional unit. Dev Immunol 7:89-101

310. Sarrazy V, Koehler A, Chow ML, et al (2014) Integrins αvβ5 and αvβ3 promote latent TGF-β1 activation by human cardiac fibroblast contraction. Cardiovasc Res 102:407-417

311. Wipff PJ and Hinz B (2008) Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. Eur J Cell Biol 87:601-615

312. Wipff PJ, Rifkin DB, Meister JJ, et al (2007) Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. J Cell Biol 179:1311- 1323

313. Ingber DE (2006) Cellular mechanotransduction: putting all the pieces together. FASEB 20(7):811-827

314. Geiger B, Bershadsky A, Pankov R, et al (2001) Transmembrane crosstalk between extracellular matrix-cytoskeleton crosstalk. Nat Rev Mol Cell Biol 2(11):793-805

315. Giancotti FG, Ruoslahti E (1999) Integrin signaling. Science 285(5430:1028-1032

316. Terracio L, Rubin K, Gullberg D, et al (1991) Expression of collagen binding integrins during cardiac development and hypertrophy. Circ Res 68(3): 734-744

317. Ruoslahti E (1996) RGD and other recognition sequences for integrins. Ann Rev Cell Dev Biol 12:697–715

318. Ventura C, Maioli M, Asara Y, et al (2004) Butyric and retinoic mixed ester of hyaluronan. A novel differentiating glyconjugate affording a high throughput of cardiogenesis in embryonic stem cells. J Biol Chem 279(22):23574-23579

319. Chen SS, Fitzgerald W, Zimmerberg J, et al (2007) Behaviour of cardiomyocytes and skeletal muscle cells on different extracellular matrix components – relevance for cardiac tissue engineering. Artif Organs 31(1):4-12

320. Geary RL, Nikkari ST, Wagner WD, et al (1998) Wound healing: a paradigm for lumen narrowing after arterial reconstruction. J Vasc Surg 27:96-106

321. Yamagata M, Suzuki S, Akiyama SK, et al (1989) Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. J Biol Chem 264:8012-8018

322. Yamagata M, Saga S, Kato M, et al (1993) Selective distributions of proteoglycans and their ligands in pericellular matrix of cultured fibroblasts. Implications for their role in cell-substratum adhesion. J Cell Sci 106:55-65

323. DeGrendele HC , Kosfiszer M , Estess P, et al (1997) CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. J Immunol 159:2549–2553

324. Zhu H, Mitsuhashi N, Klein A, et al (2006) The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. Stem Cells 24(4):928-935

325. Khademhoisseni A, Eng G, Yeh J, et al (2007) Microfluidic patterning for fabrication of contractile cardiac organoids. Biomed Microdev 9(2):149-157

326. Horton RE, Millman JR, Colton CK, et al (2009) Engineering microenvironments for embryonic stem cell differentiation to cardiomyocytes. Regen Med 4(5): 721-732

327. Månsson-Broberg A, Rodin S, Bulatovic I, et al (2016) Wnt/β-Catenin stimulation and laminins support cardiovascular cell progenitor expansion from human fetal cardiac mesenchymal stromal cells. Stem Cell Reports 6(4): 607-617

328. Horton RE, Auguste DT (2012) Synergistic effects of hypoxia and extracellular matrix cues in cardiomyogenesis. Biomaterials 33(27): 6313-6319

329. McCurdy S, Baicu CF, Heymans S, et al (2010) Cardiac extracellular matrix remodeling: fibrillar collagens and Secreted Protein Acidic and Rich in Cysteine (SPARC). J Mol Cell Cardiol 48(3):544-549

330. Hollfelder D, Frasch M, Reim I (2014) Distinct functions of the laminin beta LN domain and collagen IV during cardiac extracellular matrix formation and stabilization of alary muscle attachments revealed by EMS mutagenesis in Drosophila. BMC Dev Biol 14:26

331. Fisher SA, Periasamy M (1994) Collagen synthesis inhibitors disrupt embryonic cardiocyte myofibrillogenesis and alter the expression of cardiac specific genes *in vitro* J Mol Cell Cardio 26(6): 721-731

332. Sato H, Takahashi M, Ise H, et al (2006) Collagen synthesis is required for ascorbic-acid enhanced differentiation of mouse embryonic stem cells into cardiomyocytes. Biochem Biophys Res Commun 342(1): 107-112

333. Jacot JG, McCulloch AD, Omens JH (2008) Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. Biophys J 95(7): 3479-3487

334. Pick E, Kroizman T, Abo A (1989) Activation of the superoxide-forming NADPH oxidase of macrophages requires two cytosolic components – one of them is also present in certain nonphagocytic cells. J Immunol 143(12): 4180-4187

335. Fomovsky GM, Holmes JW (2010) Evolution of scar structure, mechanics, and ventricular function after myocardial function in the rat. Am J Physiol Heart Circ Physiol 298(1): H221-H228

336. Zemel A, Rehfeldt F, Brown AE, et al (2010) Optimal matrix rigidity for stress-fibre polarization in stem cells. Nature Physics 6(6):468–473

337. Ivanovska IL, Shin JW, Swift J, et al (2015) Stem cell mechanobiology: diverse lessons from bone marrow. Trends Cell Biol 25(9):523-532

338. Vicente-Manzanares M, Ma X, Adelstein RS, et al (2009) Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol 10:778–790

339. Raab M, Swift J, Dingal PC, et al (2012) Crawling from soft to stiff matrix polarizes the cytoskeleton and phosphoregulates myosin-II heavy chain. J Cell Biol 199(4):669–683

340. Tse JR, Engler AJ (2011) Stiffness gradients mimicking in vivo tissue variation regulate mesenchymal stem cell fate. Plos One 6(1):e15978

341. Isenberg BC, Dimilla PA, Walker M, et al (2009) Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength. Biophys J 97(5):1313–1322

342. Iyer KV, Pulford S, Mogilner A, et al (2012) Mechanical activation of cells induces chromatin remodeling preceding MKL nuclear transport. Biophysic J 103(7):1416–1428

343. Crisp M, Liu Q, Roux K, et al (2006) Coupling of the nucleus and cytoplasm: role of the LINC complex. J Cell Biol 172:41–53

344. Lombardi ML, Jaalouk DE, Shanahan CM, et al (2011) The interaction between nesprins and sun proteins at the nuclear envelope is critical for force transmission between the nucleus and cytoskeleton. J Biol Chem 286(30):26743–26753

345. Banerjee I, Zhang J, Moore-Morris T, et al (2014) Targeted ablation of nesprin 1 and nesprin 2 from murine myocardium results in cardiomyopathy, alter nuclear morphology, and inhibition of the biomechanical gene response. PLoS Genet 10(2): e1004114

346. Hale CM, Shrestha AL, Khatau SB, et al (2008) Dysfunctional connections between the nucleus and the actin and microtubule networks in laminopathic models. Biophys J 95(11):5462-5475

347. Frisch SM, Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124(4):619–626

348. Miner JH, Yurchenco PD (2004) Laminin functions in tissue morphogenesis. Ann Rev Cell Develop Biol 20:255-284

349. Domogatskaya A, Rodin S, Tryggvason K (2012) Functional diversity of laminins. Ann Rev Cell Develop Biol 28:523-533

350. Cameron AR, Frith JE, Cooper-White JJ (2011) The influence of substance creep on mesenchymal stem cell behaviour and phenotype. Biomaterials 32(26):5979-5993

351. Reilly GC, Engler A (2010) Intrinsic extracellular matrix properties regulate stem cell differentiation. J Biomechanics 43(1):55-62

352. Lee DA, Knight MM, Campbell JJ, et al (2011) Stem cell mechanobiology J Cell Biochem 112(1):1-9

353. Guilak F, Cohen DM, Estes BT, et al (2009) Control of stem cell fate by physical interactions with extracellular matrix. Cell Stem Cell 5(1):17-26

354. Hyn es RO (2009) The extracellular matrix: not just pretty fibrils. Science 326(5957):1216-1219

355. Nishimune H, Sanes JR, Carlson SS (2004) A synaptic laminin-calcium channel interaction organizes active zones in motor nerve terminals. Nature 432(7017):580-587

356. Saha K, Keung AJ, Irwin EF, et al (2008) Substrate modulus directs neural stem cell behaviour. Biophys J 95(9):4426-4438

357. Rowland TJ, Blaschke AJ, Buchholz DE, et al (2013) Differentiation of human pluripotent stem cells to retinal pigmented epithelium in defined conditions using purified extracellular matrix proteins. J Tissue Eng Regen Med 7(8):642-653

358. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154-156

359. Wang H, Luo X, Leighton J (2015) Extracellular matrix and integrins in embryonic stem cell differentiation. Biochem Insights 8(Suppl2);15-21

360. Pagliuca FW, Millman JR, Gürtler M, et al (2014) Generation of functional human pancreatic β cells *in vitro*. Cell 159(2):428-439

361. Zhu W-Z, Van Biber B, Laflamme MA (2011) Methods for the derivation and use of cardiomyocytes from human pluripotent stem cells. Methods Mol Biol 767:419-431

362. Jha BS, Rao M, Malik N (2015) Motor neuron differentiation from pluripotent stem cells and other intermediate proliferative precursors that can be discriminated by lineage specific reporters. Stem Cell Rev 11(1):194-204

363. Hannan NFR, Segeritz C-P, Touboul T, et al (2013) Production of hepatocyte-like cells from human pluripotent stem cells. Nat Protoc 8(2):430-437

364. Turinetto V, Orlando L, Giachino C (2017) Induced pluripotent stem cells: advances in the quest for genetic stability during reprogramming process. Int J Mol Sci 18(9),pii:E1952

365. Gattazzo F, Urciuolo A, Bonaldo P (2014) Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta 1840(8):2506-2519

366. Maniotis A, Bojanowski K, Ingber DE (1997) Mechanical continuity and reversible disassembly within intact genomes removed from living cells. J Cell Biochem 65(1):114-130

367. Bornstein P, McPherson J, Sage H, et al (1982) Synthesis and secretion of structural macromolecules by endothelial cells in culture. In:Nossel HL, Vogel HJ, editors. Pathobiology of the Endothelial Cell. New York: Academic Press, pp 215-228

368. Thorne JT, Segal TR, Chang S, et al (2015) Dynamic reciprocity between cells and their microenvironment in reproduction. Biol Reprod 92(1):25

369. Kaul H, Ventikos Y (2015) Dynamic reciprocity revisited. J Theor Biol 370:205-208

370. Wozniak MA, Chen CS (2009) Mechanotransduction in development: a growing role for contractility. Nat Rev Mol Cell Biol 10(1):34-43

371. Chowdhury F, Na S, Li D et al (2010) Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. Nat Mater 9(1):82-88

372. Arshi A, Nakashima Y, Nakano H, et al (2013) Rigid microenvironments promote cardiac differentiation of mouse and human embryonic stem cells. Sci Technol Adv Mater 14(2):025003

373. Barczyk M, Carrecedo S, Gullberg D (2010) Integrins. Cell Tissue Res 339(1):269-280

374. Ridley AJ, Schwartz MA, Burridge K, et al (2003) Cell migration: integrating signals from front to back. Science 302(5651):1704-1709

375. Darribère Te, Skalski M, Cousin H, et al (2000) Integrins: regulators of embryogenesis. Biol Cell 92(1):5-25

376. Wong JCY, Gao SY, Lees JG, et al (2010) Definitive endoderm derived from human embryonic stem cells highly express the integrin receptors alphaV and beta5. Cell Adh Migr 4(1):39-45

377. Farzaneh Z, Pakzad M, Vosough M, et al (2014) Differentiation of human embryonic stem cells to hepatocyte-like cells on a new developed xeno-free extracellular matrix. Histochem Cell Biol 142(2):217-226

378. Lora JM, Rowader KE, Soares L, et al (1998) α3β1-integrin as a critical mediator of the hepatic differentiation of response to the extracellular matrix. Hepatology 28(4):1095-1104

379. Park KS (2011) TGF-β family signaling in embryonic stem cells. Int J Stem Cells 4(1):18-23

380. Weinstein M, Monga SP, Liu Y, et al (2001) Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on β1-integrin to promote normal liver development. Mol Cell Biol 21(15):5122-5131

381. Higuchi Y, Shikari N, Yamane K, et al (2010) Synthesized basement membranes direct the differentiation of mouse embryonic stem cells into pancreatic lineages. J Cell Sci 123(pt16):2733-2732

382. Narayanan K, Lim VY, Shen J, et al (2014) Extracellular matrix-mediate differentiation of human embryonic stem cells: differentiation to insulin-secreting beta cells. Tissue Eng Part A 20(1-2):424-433

383. Richoux V, Darribère T, Boucat J-C, et al (1989) Distribution of fibronectins and laminin in the early pig embryo. Anat Rec 223(1):72-81

384. Yang JT, Rayburn H, Hynes RO (1993) Embryonic mesodermal defects in alpha 5 integrin-deficient mice. Development 119(4):1093-1105

385. Liu B, Lewis AK, Shen W (2009) Physical hydrogels photo-cross-linked from self-assembled macromers for potential use in tissue engineering. Biomacromolecules 10(12):3182-3197

386. Thorsteinsdóttir S, Roelen BAJ, Goumans M-J, et al (1999) Expression of the α6A integrin splice variant in developing mouse embryonic stem cell aggregates and correlation with cardiac muscle differentiation. Differentiation 64(3):173-184

387. Duan Y, Liu Z, O'Neill J, et al (2011) Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors. J Cardiovasc Transl Res 4(5):605-615

388. Baharvand H, Azarnia M, Parivar K, et al (2005) The effect of extracellular matrix on embryonic stem cell-derived cardiomyocytes. J Mol Cell Cardiol 38(3):495-503

389. Sa S, Wong L, McCloskey KE (2014) Combinatorial fibronectin and laminin signaling promote highly efficient cardiac differentiation of human embryonic stem cells. Biores Open Access 3(4):150-161

390. Kattman SJ, Witty AD, Gagliardi M, et al (2011) Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 8(2):228-240

391. Kothapalli CR, Kamm RD (2013) 3D matrix microenvironment for targeted differentiation of embryonic stem cells into neural and glial lineages. Biomaterials 34(25):5995-6007

392. Anton ES, Kreidberg JA, Rakic P (1999) Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuron 22(2):277-289

393. Dhara SK, Stice SL (2008) Neural differentiation of human embryonic stem cells. J Cell Biochem 105(3):633-640

394. Goh KL, Yang JT, Hynes RO (1997) Mesoderm defects and cranial neural crest apoptosis in alpha5 integrin-null embryos. Development 124(21):4309-4319

395. Lee MR, Kwon KW, Jung H, et al (2010) Direct differentiation of human embryonic stem cells into selective neurons on nanoscale ridge/groove pattern arrays. Biomaterials 31: 4360-4366

396. Eckes B, Krieg T, Wickström SA (2014) Role of integrin signaling through integrin-linked kinase in skin physiology and pathology. Exp Dermatol 23(7):453-456

397. Hara M, Yaar M, Tang A, et al (1994) Role of integrins in melanocyte attachment and dendricity. J Cell Sci 107(pt 10):2739-2748

398. Banito A, Rashid ST, Acosta JC, et al (2009) Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 23:2134-2139

399. Esteban MA, Pei DQ (2012) Vitamin c improves the quality of somatic cell reprogramming. Nat Genet 44:366-367

400. Gore A, Li Z, Fung HL, et al (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471:63-67

401. Mayshar Y, Ben-David U, Lavon N, et al (2010) Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell 7:521-531

402. Cheng LZ, Hansen NF, Zhao L, et al (2012) Low incidence of DNA sequence variation in human induced pluripotent stem cells generated by nonintegrating plasmid expression. Cell Stem Cell 10:337-344

403. Hussein SM, Batada NN, Vuoristo S, et al (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471: 58-62

404. Laurent LC, Ulitsky I, Slavin I, et al (2011) Dynamic changes in the copy number of pluripotency and cell proliferation genes in human escs and ipscs during reprogramming and time in culture. Cell Stem Cell 8:106-118

405. Yoshishara M, Hayashizaki Y, Murakawa Y (2017) Genetic instability of ipscs: Challenges towards their clinical applications. Stem Cell Rev 13:7-16

406. Medvedev SP, Shevchenko AI, Zakian SM (2010) Induced pluripotent stem cells: problems and advantages when applying them in regenerative medicine. Acta Naturae 2(5):18-27

407. Yoshida Y, Takahashi K, Okita K, et al (2009) Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 5:237-241

408. Mathieu J, Zhou WY, Xing YL, et al (2014) Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. Cell Stem Cell 14:592-605

409. Rodesch F, Simon P, Donner C, et al (1992) Oxygen measurements in endometrial and trophoblastic tissues during early-pregnancy. Obstet Gynecol 80:283-285

410. Wang PY, Thissen H, Kingshott P (2016) Modulation of human multipotent and pluripotent stem cells using surface nanotopographies and surface-immobilised bioactive signals: A review. Acta Biomaterialia 45:31-59

411. Chen AKL, Reuveny S, Oh SKW (2013) Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction. Biotechnol Adv 31:1032-1046

412. Guo G, von Meyenn F, Santos F, et al (2016) Naïve pluripotent cells derived directly from isolated cells of human inner cell mass. Stem Cell Rep 6:437-446

413. Pan F, Zhang M, Wu G, et al (2013) Topographic effect on human induced pluripotent stem cells differentiation towards neuronal lineage. Biomaterials 34:8131-8139

414. Chan LY, Birch WR, Yim EK, et al (2013) Temporal application of topography to increase the rate of neuronal differentiation from human pluripotent stem cells. Biomaterials 34:382-392

415. Mosqueira D, Pagliari S, Uto K, et al (2014) Hippo pathway effectors control cardiac progenitor cell fate by acting as dynamic sensors of substrate mechanics and nanostructure. ACS Nano 8:2033-2047

416. Wan CR, Frohlich EM, Charest JL, et al (2011) Effect of surface patterning and presence of collagen I on the phenotypic changes of embryonic stem cell derived cardiomyocytes. Cell Mol Bioeng 4:56-66

417. Kim DH, Kshitiz RR, Smith P, et al (2012) Nanopatterned cardiac cell patches promote stem cell niche formation and myocardial regeneration. Integr Biol 4:1019-1033

418. Reimer A, Vasilevich A, Hulshof F, et al (2016) Scalable topographies to support proliferation and Oct4 expression by human induced pluripotent stem cells. Sci Rep 6:18948.

419. Chou YF, Chen HH, Eijpe M, et al (2008) The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. Cell 135:449–461

420. Aasen T, Raya A, Barrero MJ, et al (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26:1276–1284

421. Redmer T, Diecke S, Grigoryan T,et al (2011) E-cadherin is crucial for embryonic stem cell pluripotency and can replace OCT4 during somatic cell reprogramming. EMBO Rep 12:720–726

422. Rowland TJ, Miller LM, Blaschke AJ, et al (2010) Roles of integrins in human induced pluripotent stem cell growth on Matrigel and vitronectin. Stem Cells Dev 19:1231–1240

423. Magli A, Incitti T, Kiley J, et al (2017) PAX7 targets, CD54, Integrin α9β1, and SDC2, allow isolation of human ESC/iPSC-derived myogenic progenitors. Cell Rep 19(13):2867-2877

424. Ozeki N, Hase N, Higuchi N, et al (2017) Gelatin scaffold combined with bone morphogenetic protein-4 induces odontoblast-like cell differentiation involving integrin profile changes, autophagy-related gene 10, and Wnt5 sequentially in human induced pluripotent stem cells. Differentiation 93:1-14

425. Shah N, Morsi Y, Manasseh R (2014) From mechanical stimulation to biological pathways in the regulation of stem cell fate. Cell Biochem Funct 32(4):309-325

426. Cho M, Titushkin I, Sun S, et al (2010) Physicochemical control of adult stem cell differentiation: shedding light on potential molecular mechanisms. J Biomed Biotechnol 2010:743476

427. Huang Y, Fan Y (2013) Effect of intermittent fluid shear stress on cardiomyogenic differentiation of rat bone marrow mesenchymal stem cells. IFMB 39:1953-1956

428. Pavalko FM, Chen NX, Turner CH, et al (1998) Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. Am J Physiol 275:C1591-C1601

429. Mathieu PS, Loboa EG (2012) Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. Tissue Eng Part B Rev 18(6):436-444

430. Kearney EM, Farrell E, Prendergast PJ (2008) Involvement of stretch-activated ion channels in strain-regulated glycosaminoglycan synthesis in mesenchymal stem cell-seeded 3D scaffolds. J Biomech 41(9):2055-2059

431. Leong W, Wu S, Pal M, et al (2012) Cyclic tensile loading regulates human mesenchymal stem cell differentiation into neuron-like phenotype. J Tissue Eng Regen Med 6(Suppl 3):s68-s79

432. Huang Y, Zheng L, Gong X, et al (2012) Effect of cyclic strain on cardiomyogenic differentiation of rat bone marrow derived mesenchymal stem cells. PLoS One 7(4):e34960

433. Shin JW, Jang JY, Lee SW, et al (2011) Combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagents. J Biomed Biotechnol 2011: 860652

434. Li J, Wang J, Zou Y, et al (2012) The influence of delayed compressive stress on TGF-β1-induced chondrogenic differentiation of rat BMPCs through Smad-dependent and Smad-independent pathways. Biomaterials 33(33):8395-8405

435. Li J, Zhao Z, Yang J, et al (2009) p38 MAPK mediate in compressive stress-induced chrondrogenesis of rat bone marrow MPCs in 3D alginate scaffolds. J Cell Physiol 221(3):609-617

436. Park JS, Chu JS, Tsou AD, et al (2011) The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta. Biomaterials 32(16):3921-3930

437. Kim DH, Heo SJ, Kim SH, et al (2011) Shear stress magnitude is critical in regulating the differention of mesenchymal stem cells even with endothelial growth medium. Biotechnol Lett 33(12):2351-2359

438. Huebsch N, Arany PR, Mao AS, et al (2010) Harnessing traction-mediated manipulation of the cell-matrix interface to control stem-cell fate. Nat Mater 9(6):518-526

439. Parekh SH, Chatterjee K, Lin-Gibson S, et al (2011) Modulus-driven differentiation of marrow stromal cells in 3D scaffolds that is independent of myosin-based cytoskeletal tension. Biomaterials 32:2256-2264

440. Shih Y-RV, Tseng K-F, Lai H-Y, et al (2010) Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. J Bone Miner Res 26(4):730.

441. Dahl KN, Ribeiro AJ, Lammerding J (2008) Nuclear shape, mechanics, and mechanotransduction. Circ Res 102(11):1307-1318

442. Meaburn KJ, Misteli T (2007) Cell biology: chromosome territories. Nature 445(7126): 379-381

443. Parnaik VK, Manju K (2006) Laminopathies: multiple disorders arising from defects in nuclear architecture. J Biosci 31:405-421

444. Yi S , Ding F, Gong L, et al (2017) Extracellular matrix scaffolds for tissue engineering and regenerative medicine. Curr Stem Cell Res Ther 12(3):233-246

445. Faulk DM, Johnson SA, Zhang L, et al (2014) Role of the extracellular matrix in whole organ engineering. J Cell Physiol 229(8):984-989

446. Hubbell JA (1995) Biomaterials in tissue engineering. Biotechnology (NY) 13(6):565-576

447. Gunatillake PA, Adhikari R (2003) Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater 5:1-16

448. Di Lullo GA, Sweeney SM, Korkko J, et al (2002) Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 277(6):4223-4231

449. Khoshnoodi J, Cartailler JP, Alvares K, et al (2006) Molecular recognition in the assembly of collagens: terminal noncollagenous domains are key recognition modules in the formation of triple helical protomers. J Biol Chem 281(50):38117-38121.

450. Badylak SF, Freytes DO, Gilbert TW (2009) Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater 5(1):1-13

451. Wolf K, Friedl P (2011) Extracellular matrix determinants of proteolytic and non-proteolytic cell migration. Trends Cell Biol 21(12):736-744

452. Paranteau-Bareil R, Gauvin R, Berthod F (2010) Collagen-based biomaterials for tissue engineering applications. Materials 3(3):1863-1887

453. Glowacki J, Mizuno S (2008) Collagen scaffolds for tissue engineering. Biopolymers 89(5):338-344

454. Loo Y, Lakshmanan A, Ni M, et al (2015) Peptide bioink: self-assembling nanofibrous scaffolds for three-dimensional organotypic cultures. Nano Lett 15(10): 6919-6925

455. Helary C, Bataille I, Abed A, et al (2010) Concentrated collagen hydrogels as dermal substitutes. Biomaterials 31(3):481-490

456. Nöth U, Rackwitz L, Heymer A, et al (2007) Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels. J Biomed Mater Res Part A 83A(3):626-635

457. Liu CZ, Xia ZD, Han ZW, et al (2008) Novel 3D collagen scaffolds fabricated by indirect printing technique for tissue engineering. J Biomed Mater Res B Appl Biomater 85(2):519-528

458. Park JY, Choi JC, Shim JH, et al (2014) A comparative study on collagen type I and hyaluronic acid dependent cell behavior for osteochondral tissue bioprinting. Biofabrication 6(3):035004

459. Lee W, Debasitis JC, Lee VK, et al (2009) Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. Biomaterials 30(8):1587-1595

460. Lee W, Pinckney J, Lee V, et al (2009) Three-dimensional bioprinting of rat embryonic neural cells. Neuroreport 20(8):798-803

461. Lee V, Singh G, Trasatti JP, et al (2014) Design and fabrication of human skin by three-dimensional bioprinting. Tissue Eng Part C Methods 20(6):473-484

462. Wu Z, Su X, Xu B, et al (2016) Bioprinting three-dimensional cell-laden tissue constructs with controllable degradation. Sci Rep 6:24474

463. Inzana JA, Olvera D, Fuller SM, et al (2014) 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. Biomaterials 35(13):4026-4034

464. Xu T, Gregory CA, Molnar P, et al (2006) Viability and electrophysiology of neural cell structure generated by the inkjet printing method. Biomaterials 27(19):3580-3588

465. Roth EA, Xu T, Das M, et al (2004) Inkjet printing for high-throughput cell patterning. Biomaterials 25(17):3707-3715

466. Xu T, Jin J, Gregory C, et al (2005) Inkjet printing of viable mammalian cells. Biomaterials 26(1):93-99

467. Lee W, Lee V, Polio S, et al (2010) On-demand three-dimensional freeform fabrication of multi-layered hydrogel scaffold with fluidic channels. Biotechnol Bioeng 105(6):1178-1186

468. Zhao L, Lee VK, Yoo S-S, et al (2012) The integration of 3-D cell-printing and mesoscopic fluorescence molecular tomography of vascular constructs with thick hydrogel substrates. Biomaterials 33(21): 5325-5332

469. Duarte Campos DF, Blaeser A, Korsten A, et al (2014) The stiffness and structure of three-dimensional printed hydrogels direct the differentiation of mesenchymal stromal cells toward adipogenic and osteogenic lineages. Tissue Eng Part A 21(3-4):740-756

470. Schense JC, Hubbell JA (1999) Cross-linking exogenous bifunctional properties into fibrin gels with factor XIIIa. Bioconjugate Chem 10(1):75-81

471. Rowe SL, Lee S, Stegemann JP (2007) Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels. Acta Biomater 3(1):59-67

472. Wlodarczyk-Biegun MK, Del Campo A (2017) 3D bioprinting of structural proteins. Biomaterials 134:180-201

473. Murphy SV, Skardal A, Atala A (2013) Evaluation of hydrogels for bio-printing applications. J Biomed Mater Res A 101(1):272-284

474. Bensaid W, Triffitt JT, Blanchat C, et al (2003) A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials 24(14):2497-2502

475. Skardal A, Mack D, Kapetanovic E, et al (2012) Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. Stem Cells Transl Med 1(11):792-802

476. Xu T, Binder KW, Albanna MZ, et al (2013) Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications. Biofabrication 5(1):015001

477. Grimm C, Polterauer S, Helmy S, et al (2014) A collagen-fibrin patch (Tachosil®) for the prevention of symptomatic lymphoceles after pelvic lymphadenectomy in women with gynecologic malignancies: a randomized control trial. BMC Cancer 14:635

478. Filosso PL, Ruffini E, Sandri A, et al (2013) Efficacy and safety of human fibrinogen-thrombin patch (TachoSil(®)) in the treatment of postoperative air leakage in patients submitted to redo surgery for lung malignancies: a randomized trial. Interact Cardiovasc Thorac Surg 16(5):661-666

479. Hüttner FJ, Mihaljevic AL, Hackert T, et al (2016) Effectiveness of Tachosil (®) in the prevention of postoperative pancreatic fistula after distal pancreatectomy: a systematic review and meta-analysis. Langenbecks Arch Surg 401(2):151-159

480. Erdas E, Medas F, Podda F, et al (2015) The use of a biologic topical haemostatic agent (TachoSil(®)) for the prevention of postoperative bleeding in patients on antithrombotic therapy undergoing thyroid surgery: a randomized controlled pilot trial. Int J Surg 20:95-100

481. Snyder JE, Hamid Q, Wang C, et al (2011) Bioprinting cell-laden matrigel for radioprotection study of liver by pro-drug conversion in a dual-tissue microfluidic chip. Biofabrication 3(3):034112

482. Hughes CS, Postovit LM, Lajoie GA (2010) Matrigel: a complex protein mixture required for optimal growth for cell culture. Proteomics 10(9): 1886-1890

483. Carey DJ, Todd MS, Rafferty CM (1986) Schwann cell myelination: induction by exogenous basement membrane-like extracellular matrix. J Cell Biol 102:2254-2263

484. Benton G, Amaoutova I, George J, et al (2014) Matrigel: From discovery and ECM mimicry to assays and models for cancer research. Advanced Drug Delivery Reviews 79-80:3-18

485. Mettouchi A (2012) The role of extracellular matrix in vascular branching morphogenesis. Cell Adhes Migr 6(6):528-534

486. Marcelo KL, Goldie LC, Hirschi KK (2013) Regulation of endothelial cell differentiation and specification. Circ Res 112(9):1272-1287

487. Arnaoutova I, Kleinman HK (2010) *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 5(4):628-635

488. Barna J, Princz A, Kosztelnik M, et al (2012) Heat shock factor-1 intertwines insulin/IGF-1, TGF-β, and cGMP signaling to control development and aging. BMC Dev Biol 12:32

489. Shi Z, Neoh KG, Kang ET, et al (2014) Enhanced endothelial differentiation of adipose-derived stem cells by substrate nanotopography. J Tissue Eng Regen Med 8(1):50-58

490. Albini A, Noonan DM (2010) The “chemoinvasion” assay, 25 years and still going strong: the use of reconstituted basement membranes to study cell invasion and angiogenesis. Curr Opin Cell Biol 22(5):677-689

491. Albini A, Iwamoto Y, Kleinman HK, et al (1987) A rapid *in vitro* assay for quantitating the invasion potential of tumor cells. Cancer Res 47(12):3239-3245

492. Benton G, George J, Kleinman HK, et al (2009) Advancing science and technology via 3D culture on basement membrane matrix. J Cell Physiol 221(1):18-25

493. Barkan D, Green JE, Chambers AF (2010) Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. Eur J Cancer 46(7):1181-1188

494. Barkan D, Kleinman H, Simmons JL, et al (2008) Inhibition of metastatic growth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res 68(15):6241-6250

495. Lo AT, Mori H, Mott J, et al (2012) Constructing three-dimensional models to study mammary gland branching morphogenesis and functional differentiation. J Mammary Gland Biol Neoplasia 17(2):103-110

496. Kenny PA, Lee GY, Myers CA, et al (2007) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 1(1):84-96

497. Mehlhorn AT, Niemeyer P, Kaiser S, et al (2006) Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. Tissue Eng 12(10):2853-2862

498. Rao Pattabhi S, Martinez JS, Keller TC 3rd (2014) Decellularized ECM effects on human mesenchymal stem cell stemness and differentiation. Differentiation 88(4-5):131-143

499. Hoshiba T, Kawazoa N, Tateishi T, et al (2010) Development of extracellular matrices mimicking stepwise adipogenesis of mesenchymal stem cells. Adv Mater 22(28):3042-3047

500. Chen XD, Dusevich V, Feng JQ, et al (2007) Extracellular matrix made by bone marrow cells facilitates expansion of marrow-derived mesenchymal progenitor cells and prevents their differentiation into osteoblasts. J Bone Miner Res 22(12):1943-1956

501. Harvey A, Yen TY, Aizman I, et al (2013) Proteomic analysis of the extracellular matrix produced by mesenchymal stromal cells: implications for cell therapy mechanism. PLoS One 8(11):e79283

502. Lai Y, Sun Y, Skinner CM, et al (2010) Reconstitution of marrow-derived extracellular matrix *ex vivo*: a robust culture system for expanding large-scale highly functional human mesenchymal stem cells. Stem Cells Dev 19(7):1095-1107

503. Hoch AI, Mittal V, Mitra D, et al (2016) Cell-secreted matrices perpetuate the bone-forming phenotype of differentiated mesenchymal stem cells. Biomaterials 74:178-187

504. Choi DH, Suhaeri M, Hwang MP, et al (2014) Multi-lineage differentiation of human mesenchymal stromal cells on the biophysical microenvironment of cell-derived matrix. Cell Tissue Res 357(3):781-792

505. Liu X, Zhou L, Chen X, et al (2016) Culturing on decellularized extracellular matrix enhances antioxidant properties of human umbilical cord-derived mesenchymal stem cells. Mater Sci Eng C Mater Biol Appl 61:437-448

506. Prewitz MC, Stiβel A, Friedrichs J, et al (2015) Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding. Biomaterials 73:60-69

507. Chen RN, Ho HO, Tsai YT, et al (2004) Process development of an acellular dermal matrix (ADM) for biomedical applications. Biomaterials 25(14):2679-2686

508. Hülsmann J, Grün K, El Amouri S, et al (2012) Transplantation material bovine pericardium: biomechanical and immunogenic characteristics after decellularization vs glutaraldehyde-fixing. Xenotransplantation 19(5):286-297

509. Morticelli L, Thomas D, Roberts N, et al (2013) Investigation of the suitability of decellularized porcine pericardium in mitral valve reconstruction. J Heart Valve Dis 22(3):340-353

510. Chen F, Yoo JJ, Atala A (1999) Acellular collagen matrix makes a possible "off the shelf" biomaterial for urethral repair. Urology 54:407-410

511. Freytes DO, Badylak SF, Webster TJ, et al (2004) Biaxial strength of multilaminated extracellular matrix scaffolds. Biomaterials 25(12):2353-2361

512. Flynn LE (2010) The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. Biomaterials 31:4715-4724

513. Grandi C, Baiguera S, Martorina F, et al (2011) Decellularized bovine reinforced vessels for small-diameter tissue-engineered vascular grafts. Int J Mol Med 28(3):315-325

514. Cartmell JS, Dunn MG (2000) Effect of chemical treatments on tendon cellularity and mechanical properties. J Biomed Mater Res 49:134-140

515. Woods T, Gratzer PF (2005) Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. Biomaterials 26:7339-7349

516. Parmaksiz M, Dogan A, Odabas S, et al (2016) Clinical applications of decellularized extracellular matrices for tissue engineering and regenerative medicine. Biomed Mater 11(2): 022003

517. Levenda AC, Sanders NR (2015) A simplified approach for arthroscopic repair of rotator cuff tear with dermal patch augmentation. Adv Orthop Surg 2015:423949

518. Longo UG, Lamberti A, Maffuli N, et al (2010) Tendon augmentation grafts: a systematic review. Br Med Bull 94:165-188

519. Mostow EN, Haraway GD, Dalsing M, et al (2005) Effectiveness of an extracellular matrix graft (OASIS Wound Matrix) in the treatment of chronic leg ulcers: a randomized clinical trial. J Vasc Surg 41(5):837-843

520. Lam MT, Wu JC (2012) Biomaterial applications in cardiovascular tissue repair and regeneration. Expert Rev Cardiovasc Ther 10:1039-1049

521. Yanagawa B, Rao V, Yau TM, et al (2013) Initial experience with intraventricular repair using CorMatrix extracellular matrix. Innovations 8(5):348-352

522. Sündermann SH, Müggler O, Caliskan E, et al (2015) Extracellular matrix for reconstruction of cardiac structures after tumour resections. Interact Cardiovasc Thorac Surg 20(1):10-14

523. Badylak SF, Weiss DJ, Caplan A, et al (2012) Engineered whole organs and complex tissue. Lancet 379(9819):943-952

524. Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. Biomaterials 27:3675-3683

525. Parmaksiz M, Elcin AE, Elcin YM (2017) Decellularization of bovine small intestinal submucosa and its use for the healing of a critical-size full-thickness skin defect alone and in combination with stem cells in a small rodent model. J Tissue Eng Regen Med 11(6):1754-1765

526. Piccoli M, D'Angelo E, Crotti S, et al (2018) Decellularized colorectal cancer matrix as bioactive microenvironment for in vitro 3D cancer research. J Cell Physiol 233: 5937-5948

527. Parker MW, Rossi D, Peterson M, et al (2014) Fibrotic extracellular matrix activates a profibrotic positive feedback loop. J Clin Invest 124: 1622-1635

528. Hedström U, Hallgren O, Öberg L, et al (2018) Bronchial extracellular matrix from COPD patients induces altered gene expression in repopulated human bronchiol epithelial cells. Sci Rep 8: 3502

2. **Materials & Methods**

2.1 Ethics approval for collection of primary human tissue

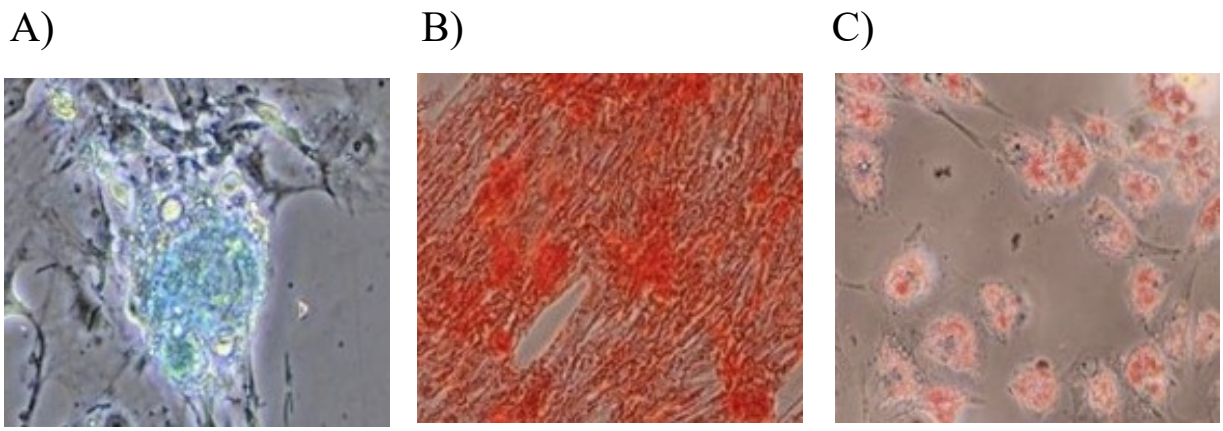
Tissue collection of discarded atrial appendage pieces and bone marrow aspirate from consented patients occurred at the St. Boniface Hospital in Winnipeg, MB, and at the University of Alberta Hospital in Edmonton, AB. The Bannatyne Campus Research Ethics Board of the University of Manitoba approval was obtained for the collection of tissue at St. Boniface Hospital. The Alberta Health Services Ethics Board approval was obtained for the collection of tissue from the University of Alberta Hospital. Written informed consent was obtained from each patient prior to the collection of tissue.

2.2 Procurement, isolation, and culturing of primary human bone marrow-derived mesenchymal progenitor cells (hMPCs)

Human MPCs were procured from patients undergoing open-heart surgery as they are responsible for migrating to pro-fibrotic areas and causing pathological fibrosis. Bone marrow aspirate was taken from the sternum (0.5-3 mL) from patients requiring a full sternotomy, as the bone marrow was easily accessible. Further isolation of hMPCs occurred by plating them on plastic culture dishes to evaluate plastic adherence according to the methods developed by Caplan and Friedenstein (1,2) with some modifications. Fresh bone marrow was placed in a tube containing 10 mL of phosphate-buffered saline (PBS) and subjected to mechanical disaggregation. For hMPCs cultured in Winnipeg, they were then diluted with GIBCO® medium, DMEM-F12, which was supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 100 mM ascorbic acid. hMPCs cultured in Edmonton were grown in GIBCO® medium, DMEM-F12, supplemented with 20% FBS, 100µg/mL of Primocin™ (Invivogen, San Diego CA, USA), an anti-fungal, anti-bacterial agent designed for primary human cell culture, and 100 mM ascorbic acid. In order to verify the progenitor nature of isolated hMPCs, tri-lineage determination of isolated hMPCs was performed with results shown in Fig 2.1. Cells were plated onto 10 cm plastic culture dishes and maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, non-adherent cells (hematopoietic cells) were discarded via media aspiration, and the adherent cells were thoroughly washed twice with PBS. Fresh complete medium was replaced every 3 days. After 10 to 14 days of cultivation, primary cultures were 60-80% confluent. Cells were then

dissociated with TrypLE Express (GIBCO®) and seeded at low densities for expansion through successive passages (P0-P2).

Figure 2.1: Differentiation of isolated P1 hMPCs into A) chondrogenic, B) osteogenic, and C) adipogenic cell types using hMPC Mesenchymal Stem Cell Chondrocyte Differentiation BulletKit™ Medium (Lonza), hMPC Mesenchymal Stem Cell Osteogenic Differentiation BulletKit™ Medium (Lonza), and hMPC Mesenchymal Stem Cell Adipogenic Differentiation BulletKit™ Medium (Lonza), respectively. Identification of chrondrogenesis was identified by Alcian blue staining, osteogenesis by Alizarin red staining, and adipogenesis by Oil Red O staining.



2.3 Procurement, isolation, and culturing of primary human atrial fibroblasts

Superfluous right atrial appendage of the heart, the size of which was based on the discretion of the surgeon, was removed from patients and subjected to collagenase digestion to isolate hAFs. The tissue was transported in a sample container in saline and then finely minced. The minced tissue was left in DMEM-F12 complete media (ThermoFischer Scientific Waltham MA, USA) and left overnight in an incubator at 37 °C to optimize hAF procurement. The following day, the minced tissue was subject to 2 mg/ml collagenase II in SMEM (ThermoFischer Scientific Waltham MA, USA) media and incubated for 3 h at 37 °C. Collagenase was neutralized by the addition of an equal volume of DMEM-F12 media containing 20 % FBS and liberated cells were collected by centrifugation at 2,000 rpm for 7 min. Cells were resuspended in fresh media containing 20 % FBS, seeded onto plastic culture dishes and incubated at 37 °C in 5 % CO2 and 95 % humidity. The digestion was repeated with the remaining tissue pieces. Non-adherent cells were removed the next day and fresh medium was added to the adherent cells and replaced every 3 days. At 60-80% confluency, cells were dissociated with TrypLE Express (GIBCO®) and seeded at low densities for expansion through successive passages. Samples were harvested at early passages (P1, P2) for analysis.

2.4 miR-301a Transfection

Overexpression of miR-301a was obtained by transfection of both hAFs and hMPCs at 50-60% confluency with hsa-miR-301a-3p and Negative Control 1 pre-miR-unconjugated microRNA (Ambion Foster City CA, USA). Cells were washed thoroughly two times with PBS and media replaced with 20% FBS DMEM-F12 void of penicillin/streptomycoin or Primocin™ one hour prior to transfection. A mixture of Dharmafect transfection reagent (Thermoscientific) and a 50nM concentration of pre-miR-301a or negative-scramble control (Ambion) were prepared as per manufacturer's instructions. Cells were incubated for 3 hours with the transfection mixture and subsequently washed two times with PBS. Cells were then maintained in 20% FBS DMEM-F12 without penicillin/streptomycoin or Primocin™ for 24-48 hours, after which further analysis or recellularizing onto decellularized matrix was performed.

2.5 RNA Isolation, Quantification, and Processing from Plate Cultured Cells

Acquisition of mRNA and miRNA from hAFs and hMPCs was performed using the TRIzol reagent (Ambion, Foster City CA, USA). Total RNA was purified using the TRIzol RNA isolation protocol provided by the manufacturer. Briefly, 1 mL of TRIzol was added to 10 cm culture dishes after cells had been rinsed 2x with PBS. TRIzol coated plates were incubated for 5 min at RT and then pipetted into a 1.5 mL centrifuge tube after which 200 µL of chloroform was added. After shaking tubes for 15 sec, they were left to incubate at RT for 2-3 prior to being centrifuged for 15 min at 12 000 x g at 4°C. The colourless aqueous phase containing the RNA was transferred to a new tube and incubated with 500 µL of isopropanol O/N at -20 °C. In order to obtain an RNA pellet, the samples were centrifuged for 20 min at 12 000 x g at 4 °C. The supernatant was removed and the RNA pellet was washed 2x with 75% ethanol and then air dried in a fume hood for 5 minutes. The pellet was re-suspended in 15-30 µL of RNAse-free DDW and stored in -80 °C until further analysis. RNA concentration and quality was analyzed using an Agilent 2100 Bioanalyzer (Santa Clara CA, USA) in Winnipeg and a NanoDrop 8000 Spectrophotometer (ThermoFischer Scientific, Walham MA, USA) in Edmonton.

In order to make cDNA for qRT-PCR experiments, 500 ng of RNA was added to create a total volume of 5 µL of nuclease-free H_2O. The ABI High-Capacity cDNA Reverse Transcription Kit (ThermoFischer) was used according to the manufacturerer's instructions. A total volume of 10 µL was used for the cDNA protocol which underwent a thermocycling program of 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and then held at 4°C. Samples were stored in -20°C under used.

2.6 Protein Isolation and Quantification from Plate Cultured Cells

HMPCs and hAFs cultured for protein isolation, as previously described, were washed twice in cold PBS and lysed in New RIPA lysis buffer (150 mMNaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) and protease inhibitor cocktail (0.1 M phenylmethylsulfonyl fluoride, 5 µg/ml leupepetin, 2 µg/ml aprotinin, and 1 µg/ml pepestatin). The lysed solution was the further processed by sonication of the samples for 30 seconds, 3x with the sample

sitting in an container surrounded by ice to keep the temperature of the sample low. To remove unsoluble cellular debris, samples were centrifuged for 15 min at 14 000 rpm.

Protein concentrations of whole cell lysates were determined using the BCA method using the Bradford Protein Assay kit (Bio-Rad, Hercules CA, USA). Briefly, 5 µL of samples and standards (using serial dilutions of bovine serum albumin ranging from 0.25 µg/mL to 3 µg/mL) was added to a 96-well plate with 25 µL of Reagent A. After the addition of the samples, a multi-channel pipette was used to added 200 µL of Reagent B and then left to incubate for at RT for 15 min. The plate was then read at 570 nm on a Synergy H4 Hybrid Reader Gen5 2.00 Spectrophotometer (BioTek, Winooski VT, USA) to determine protein concentration.

2.7 Decellularization & Recellularization *In Vitro*

2.7.1 Decellularization

A schematic of the decellularization process and protocol is represented by Figure 2a. Cardiac tissue was acquired from un-utilized donor hearts (control) and excised explant hearts from consented patients undergoing cardiac transplant surgery. Large chunks of tissue were placed in containers surrounded by OCT, rapidly frozen in liquid nitrogen, and stored at -80°C. In order to prepare smaller sections, frozen tissue was sliced at a~150-200 nm thickness using a cryostat, put in cold PBS, and warmed to room temperature. The following decellularization procedure was slightly modified from that utilized in Parker et al's paper (12) and performed in a sterile environment. First, tissue slices were placed in histological cassettes within a 100-mm Petri dish to allow flow of solutions with minimal damage to tissue slices. All oscillation rates during this decellularization procedure were at 30 oscillations per minute at room temperature, unless otherwise specified. All solutions were autoclaved prior to use except for the DNAse (20µg/mL) plus 4.2 mM MgCl solution which was vacuum filtered using a 0.22 µm pore membrane. Two 1 hour cycles of the first lysis solution (1% SDS in H_2O) was used before a third cycle was oscillated overnight. The following day, the first lysis solution was removed and replaced with 1% Triton X-100 in

H_2O (the second lysis solution) and oscillated for three 1 hour long cycles of decellularization using fresh solution at the beginning of each cycle. After the final hour cycle using the second lysis solution, the final cellular lysis step used 1% Triton X-100 solution oscillating overnight. The tissue slices were then thoroughly rinsed by using two rounds of PBS followed by 8 rinses of double distilled H_2O (DDW), essentially until no more bubbles were observed during the rinse steps. In order to thoroughly remove any residual nuclei or DNA, the following steps were performed. Firstly, 1 M NaCl (in H_2O), was added to the tissue and oscillated for 1 hour. Afterwards, treatment with DNAse (20μg/mL) plus 4.2 mM MgCl (in H_2O) at 37°C occurred for an hour, oscillating at 25 oscillations per minute. The tissue was subsequently rinsed twice with DDW and then twice with PBS. The final step, prior to storage, was to protect the tissue from bacterial contamination by oscillating it with Hank's Buffered Saline Solution (HBSS) plus 100 U/mL penicillin and 100 μg streptomycin (P/S) for 30 minutes. Afterwards, the tissue was removed from the histology cassettes and stored in HBSS plus P/S at 4°C. Prior to co-incubation with either hMPCs or hAFs, selected decellularized sections were rinsed twice with PBS at RT, and incubated in the same culture media used to grow the corresponding cell type at 37°C overnight. Co-incubation with hMPCs or hAFs proceeded the following day.

2.7.2 Recellularization

At 90-95% confluency, P1 primary hMPCs and hAFs were trypsinized using TrypLE and centrifuged at 2000xG for 7 minutes to pellet cells. They were re-suspended in 1 mL of media (DMEM-F12, 20% FBS, 100 μg/mL of Primocin™, and 100 mM ascorbic acid) and added to 15 mL tubes so that each tube contained ~5,000,000-10,000,000 cells. Five to ten pieces of decellularized HLV were added to each tube and the volume of media brought up to 10 mL. Tubes were placed on a battery powered rotating device and kept in an incubator at 37°C (95% CO_2, 5% O_2) overnight. The following day, recellularized were rinsed 2X in PBS prior to being put in fresh media and returned, rotating, into the incubator where media was changed every 3 days for two to three weeks. Recellularization was deemed complete after media

colour change rate was equivalent to plates at 90% confluency. Slices were fixed in the appropriate fixation solution (either 4% paraformaldehyde or specified for SEM and TEM).

2.8 Statistical Analysis

All data are expressed as the means ± SEM. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism unless otherwise specified. Means between two groups were also compared using the paired two-tailed Student's *t*-test when the hMPCs and hAFs between the same patients were able to be compared. A *p*-value less than 0.05 is considered statistically significant.

2.9 References

1. Friedernstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-274

2. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641-650

3. **Statins Impair Survival of Primary Human Mesenchymal Progenitor Cells via Mevalonate Depletion, NF-κB Signaling, and Bnip3**

By Li Y*, Müller AL*, Ngo MA, Sran K, Bellan D, Arora RC, Kirshenbaum LA, Freed DH

*Authors contributed equally to the work

Li Y: Cultured cells (45%), performed preliminary MTT and WB experiments w/ and w/out mevalonate

Müller AL: Collected & cultured cells (40%), performed addition MTT and WB experiments, wrote and edited manuscript, IF imaging

MA Ngo: Collected and cultured cells (10%)

Sran K: Collected and cultured cells (5%), ran WB experiments

Bellan D: Performed Iκκβ experiments

Arora RC: Edited manuscript

Kirshenbaum LA: Provided Iκκβ inhibitor and IF equipment, experimental design, edited manuscript

Freed DH: Experimental design, edited manuscript

This has been slightly modified from the article published in the Journal of Cardiovascular Translational Research 2015 (8): 96-105.

Abstract: Circulating progenitor cells of bone marrow origin have been implicated in transplant cardiac allograft vasculopathy (CAV) and cardiac fibrosis. HMG-CoA reductase inhibitors, clinically referred to as "statins", have been shown to impair the progression of CAV and improve patient survival. We examined the *in vitro* effects of three HMG-CoA reductase inhibitors atorvastatin, simvastatin and pravastatin on the viability of MPCs and expression of NF-κB. Human bone marrow-derived mesenchymal progenitor cells (MPCs) isolated from human patients were treated with atorvastatin, simvastatin, and pravastatin at 0.1, 1.0 or 10 μM ± mevalonate. Human MPC treatment with 1 and 10 μM simvastatin or atorvastatin resulted in progressively reduced cell viability which was associated with a decline in NF-κB p65. Pravastatin did not affect MPC viability or NF-κB expression. Viability was rescued by co-incubation with mevalonate or by pretreatment with Ikk-β implying that mevalonate depletion through lipophilic HMG-CoA reductase inhibition impairs the viability of primary human MPC via down-regulation of NF-κB.

3.1 Introduction

Development of HMG-CoA reductase inhibitors, herein referred to as "statins", to reduce cholesterol has resulted in the significant decrease in adverse cardiac events in patients with coronary heart disease (1); however, the full potential of their effectiveness in other cardiovascular complications is still being explored. Multiple studies have found that statins can reduce carotid atherosclerosis decreasing stroke risk (2-5), minimize the complications associated with carotid artery stenting (6), improve cardiovascular outcomes in abdominal aortic aneurysm patients (7-9), retard the decline of impaired mobility in peripheral arterial disease (10, 11) and slow the progression of cardiac allograft vasculopathy (CAV) improving patient survival (12-15). When evaluating the effectiveness of statins in alternative cardiovascular related pathologies it is important to keep in mind that there are two classes of statins: hydrophilic and lipophilic. Lipophilic statins diffuse more easily across the lipid bilayer of cells compared to hydrophilic statins. The smaller class of hydrophilic statins includes rosuvastatin and pravastatin and the larger class of lipophilic statins includes atorvastatin, lovastatin, and simvastatin (16). There have been differences shown between lipophilic and hydrophilic statin effectiveness in trials including one that showed that hydrophilic rosuvastatin is more efficacious than milligram-equivalent doses of other statins (17). It is important to note that the vast majority of clinical trials which evaluate both hydrophilic and lypophilic statins focus on their effectiveness of lowering cholesterol in patients with heart disease, without looking at other potential effects that could differ between these two classes of statins. A key difference between lypophilic and hydrophilic statins is their ability to transverse the cell membrane, as lipophilic statins easily transverse the lipid bilayer cellular membrane of various cell types whereas hydrophilic statins, although effective, do not diffuse across the cell membrane as easily (16). Despite have similar efficacy in lowering cholesterol, their differing pharmacokinetics could influence their behaviour with other cell mechanisms. In addition, not only do lipophilic statins bind significantly easier to proteins in the plasma compared to hydrophilic proteins, they are eliminated via metabolism (18-20). This information could be critical in determining which statin would be most effective in treating a patient

and/or which would have minimal or no side-effects. This study aims to further elucidate differences between hydrophilic and lipophilic statins and how they affect primary human bone marrow-derived mesenchymal progenitor cell (hMPC) survival and pro-survival signaling pathways.

One of the side effects of statins, which has been recently elucidated, is their inhibition of the growth of lesions in various vascular pathologies where one of the key common elements is fibrosis. It has recently been observed that cells of bone marrow origin possibly have a key role in the initiation of myocardial fibrosis (21) in addition to myofibroblasts potentially being of bone marrow origin contributing to overall collagen deposition (22, 23). In fact, circulating progenitor cells of bone marrow origin have been implicated in CAV and shown to contribute to neointima formation in vascular injury and cardiac fibrosis (24). In a diseased or injured state, increased levels of progenitor cells contribute to vessel scar formation as well as myocardial fibrosis (25) which is a significant issue as scar tissue lacks effective contractile properties. The increased stiffness caused by fibrosis can impair overall cardiac function, especially after myocardial infarction. A variety of other cell types also contribute to CAV (Figure 3.1) which contribute to pathological fibrosis and vessel scar formation. Pathological fibrosis infiltrates surrounding viable contractile tissue which contributes to cardiac contractile dysfunction, subsequently leading to heart failure (26).

HMPCs are of interest in the fibrotic phenomenon as they have been studied in the context of tissue regeneration in numerous organs, including the heart, with the capacity to differentiate into various cell types including hematopoietic cells, adipocytes, osteocytes, and myocytes (27, 28). There is debate as to whether, when injected into the heart to promote myocardial regeneration, they turn into a cardiomyocyte phenotype or into other cell types (29-33). One of the cell types being proposed has been termed a “fibrocyte” which was found when isolating progenitor cells expressing $CD34^{+}$ from a bone marrow population (34). These cells have been shown to contribute to fibrosis in various cardiovascular complications including hypertension and left ventricular hypertrophy (35), coronary heart disease (36), as well as in situations with increased circulating NaCl (37) and fibrocytes are thought to be the cell type responsible for initiating myocardial fibrosis (21).

As statins are ubiquitously employed in treating cardiovascular disease and mesenchymal stem cells are implicated in contributing to fibrosis, our study aims to examine the *in vitro* effects of three HMG-CoA reductase inhibitors atorvastatin, simvastatin and pravastatin on the viability of hMPCs. The roles of mevalonate and NFκB will also be elucidated as they are crucial in cellular growth, differentiation, and survival and have been observed to be affected by statins (38, 39). We were also interested in assessing the levels of Bnip3 expression, as it is indicative of atypical apoptosis and has yet to be assessed in hMPCs. These results may indicate involvement of the NFκB signaling pathway, which has been established as an important signaling molecule in the cell death pathway of the heart (40).

3.2 Materials and Methods

Isolation and Culture of Human MPCs

The Bannatyne Campus Research Ethics Board of the University of Manitoba approval was obtained for the collection of bone marrow. Human MPC cultures were prepared based on plastic adherence according to the methods developed by Caplan and Friedenstein (41, 42) with some modifications as described previously. All experiments were performed using P2 cells and each n-value indicates the number of unique patients used for each experiment. Culture cells were in 10 cm^2 dishes for immunoblot analysis, in 96 well plate for an optical MTT assay and in 24 well plate with cover glass (12mm) for Live/Dead assay. At the start of each experiment, cells were rendered quiescent by incubation in 1% FBS medium for 24 hours and then stimulated with each HMG-CoA reductase inhibitors atorvastatin, simvastatin and pravastatin with 0.1, 1.0, or 10 μM for 48 hrs and 96 hrs before assessment.

Cell Viability Assessment-MTT Assay

Using a 96-well plate, 500-10,000 cells were seeded in 200μl 20% FBS media per well. Cells were incubated (37°C, 5% CO_2) to allow the cell get 50- 70% confluent. Cells were rendered quiescent by incubation in 1% FBS medium for 24 hours and then stimulated with each of the HMG-CoA reductase inhibitors atorvastatin, simvastatin and pravastatin at concentrations of 0.1, 1.0, or 10 μM ± Mevalonate (or IKKβ) for 48 and 96 hrs (n=3/4). After treatment time expired, 20 μL of 5mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor) was added to each well and allowed to

incubate (37C, 5% CO2) for 2-3 hours to enable the MTT metabolization. The media was carefully removed and resuspended in formazan (MTT metabolic product) in 200µl DMSO. The plate was mix well and absorbance was read at 570nm by using SPECTRAmax Plus.

Western Blot Analysis

Cells were washed twice in cold PBS and lysed in New RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) and protease inhibitor cocktail (0.1 M phenylmethylsulfonyl fluoride, 5 µg/ml leupepetin, 2 µg/ml aprotinin, and 1 µg/ml pepestatin). Protein concentrations of whole cell lysates were determined using the BCA method and equal amounts of each protein sample (20µg) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel at 130 V. Separated proteins were then transferred to a polyvinylidene difluoride membrane for 1 hour at 300 mA. Membranes were blocked with 5% non-fat skim milk in 1xPBS (phosphate buffered saline). Proteins were visualized using ECL Plus (Amersham) after probing with primary and secondary antibodies. Membranes were subsequently stripped and reprobed for β-tubulin as a loading control. Blot densities were measured using Quantity One software and normalized to β-tubulin blot densities.

Live/Dead assay

Cells were seeded on a 24 well plate with sterile coverslips (12mm.VWR). The Live/Dead Viability/Cytotoxicity kit (Invitrogen L3224) was used to detect both live and dead cells using a two-colour fluorescence cell viability assay. When the cells were ready to be assessed, culture media was removed, and then Live/dead mix solution (in 10mL serum free DMEMF12 contain 2µL Calcein and 3µL Ethidium homodimer-1) was added so that enough covered coverslips in each well. The cells were protected from light and incubated (37°C, 5% CO_2) for 30 minutes. These cells were viewed under a fluorescence microscope.

Autophagic Flux

To assess autophagy in cardiomyocytes, cells were infected with an adenovirus encoding green fluorescent protein (GFP)-fused LC3 in the absence and in the presence of chloroquine to assess

autophagic flux. The number of green puncta in cells was counted as an index for autophagic flux compared with control cells, as previously reported (43).

Reagents Used

Cell culture media and reagents used for hMPCs were purchased from GIBCO (Grand Island, NY) unless otherwise specified. FBS was purchased from Hyclone Laboratories Inc. (Logan, UT) and antibiotics (penicillin and streptomycin) were purchased from LONZA (Walkersville, MD). Cell culture plates and coverslips were purchased from BD Falcon VWR (Franklin Lakes, NJ). BCA kit used for protein assay and pre-stained protein ladder for western blot analysis were purchased from BIO-RAD (Hercules, CA), PVDF membrane was obtained from Millipore (Etobicoke, ON). Primary antibodies used were rabbit polyclonal LC3B and monoclonal NF-κB p65 from Cell Signaling (Danvers, MA), rabbit monoclonal β-tubulin from Abcam (Toronto, ON), and Bnip-3 developed in-house as previously described (44). The enhanced chemilumiscence (ECL Plus) detection system was purchased from Amersham Biosciences (Buckinghamshire, UK). MTT, Pravastatin, Simvastatin and Mevalonolactone were purchased from Sigma-Aldrich Canada (Oakville, ON). Atorvastatin was from Pfizer Inc (New York, NY). LIVE/DEAD Viability/Cytotoxicity kit used to assess cell viability was purchased from Molecular Probes (Eugene, OR).

Statistics

Statistical analysis was performed as described in Chapter 2.8.

3.3 Results

Treatment of primary bone marrow-derived hMPCs with hydrophilic pravastatin after both 48 and 96 hrs (Fig 3.2) resulted in no significant difference in cell viability as detected by the Live/Dead assay; however, treatment of hMPCs with lipophilic statins resulted in a decrease of cell viability. Specifically, 10 μM of simvastatin at 48 hrs and all three concentrations at 96 hrs resulted in detectable cell death in P2 cells. Treatment of hMPCs with atorvastatin resulted in a similar pattern as that shown by simvastatin. Cell viability progressively declined when treated with 1 and 10 μM of simvastatin or atorvastatin which

was evident at 48 hrs, decreasing until nearly 50% at 96 hrs. Interestingly, pravastatin treatment appeared to only have a significant decreased on cell viability at 96 hrs (Fig 3.3). As the effects of simvastatin and atorvastatin on cell viability were comparable, experiments to determine the effect of mevalonate and NFκB were performed using atorvastatin. Treatment with mevalonate recovered cell viability at both 0.1 μM and 0.3 μM which was noticeable as early as 48 hrs into treatment and up to 90% at 96 hrs (Fig. 3.4). In order to evaluate how atorvastatin influenced NFκB, western blots detected the quantity of the p65 subunit which was found to decline to 50 and 70% at 48 and 96 hrs, respectively (Fig. 3.5a). To further elucidate the direct nature of NFκB influence, adenoviral infection using Iκκ-β was added. It caused a recovery of 65 - 90% (Fig. 3.5b). To summarize, both mevalonate and NFκB were able to attenuate the observed decrease in hMPC cellular viability when treated with lipophilic statins, whereas treatment with pravastatin, the hydrophilic statin, did not affect hMPC cellular viability. In order to further elucidate the mechanism of NFκB signaling, Bnip3 expression was analyzed and was found to be down-regulated in pravastatin-treated cells when compared to atorvastatin treated cells (Fig. 3.5c). To further elucidate the mechanism of cell death in pravastatin-treated cells, the autophagy marker, LC3 was used to detect formed autophagosomes using IF (Fig. 3.6a). Both IF and protein analysis (Fig. 3.6b) of L3BII subunits found that autophagy was increased substantially in atorvastatin treated hMPCs.

3.4 Discussion

There are numerous observations that contribute to the growing literature in understanding statins and their growing repertoire of additional beneficial effects on cardiovascular disease. The primary observation is that lipophilic statins, of which the majority of statins fall under, substantially decrease hMPC viability. This decrease in hMPC viability is recovered using both mevalonate and Iκκ-β indicating that both the mevalonate and NFκB pathways could be involved in hMPC viability. We further elucidated that Bnip3 expression was found to be involved in this cell death pathway. In order to elucidate which mechanism was involved in cell death, authophagy markers were explored and found to be increased in lipophilic statin treatment. This correlates with recent findings by Wang *et al* (44) where maladaptive

autophagy is linked to Bnip3 expression. When comparing the effects between different types of statins it appears that pravastatin statins do not affect hMPC viability and thus, may not be involved in inhibiting either the mevalonate-isoprenoid or NFκB pathway. Mevalonate depletion through HMG-CoA reductase inhibition impairs the viability of primary human MPC in culture through down-regulating NF-κB p65. This represents an additional pleiotropic effect of statins and may explain the beneficial action of this therapy independent of its effect on lipid metabolism. The lack of effect with pravastatin treatment may be related to the hydrophilic characteristic of the molecule, preventing uptake of the drug by hMPC cells.

Our study focused on both the NFκB and mevalonate-isoprenoid cell survival pathways as they have been shown to be negatively influenced by statin treatment in other cell types (38, 39). Other studies have shown that when NFκB signaling is inhibited pharmacologically in hMPCs, they undergo apoptosis (45-47) whereas when NFκB activity is increased by inhibiting TNF-α, hMPC proliferation is promoted (47) highlighting its importance in hMPC cell viability. The mevalonate-isoprenoid pathway is intricately correlated with statin use as statins inhibit the conversion of HMG-CoA to mevalonate and thus its subsequent conversion to cholesterol, and key signaling proteins such as isoprenyl proteins, dolichol, and ubiquinone (48). Interestingly, there are limited studies evaluating how mevalonate directly influences progenitor cell properties. One study has shown that statins can inhibit the transition from renal epithelial-to-mesenchymal transition which is responsible for renal fibrosis, which is abrogated by addition of mevalonate (49), suggesting maintenance of a more proliferative phenotype whereas another study has shown that, regardless of cholesterol presence, mevalonate can attenuate lipophilic statin-induced inhibitory effects on proliferation (50). These studies indicate that the susceptibility of cells to lipophilic statins has a direct influence on key pathways responsible for maintaining cell proliferative properties, and their overall viability. In addition, the substantial increase of autophagy in cells treated with lipophilic treated statins indicates a maladaptive response that causes cell death via Bnip3. Autophagy has also been shown to occur in the heart under duress (42).

When understanding how statins influence the physiology and pathophysiology properties of hMPCs, it is crucial to consider the apoptotic effects statins have on other progenitor cells, such as endothelial

progenitor cells (EPCs). Interestingly, some statins appear to have different effects on EPC viability and proliferation on EPCs when compared to hMPCs. Simvastatin was found to increase the number of functionally active EPCs (51) and lipophilic statins can reduce senescence and increase proliferation of EPCs (51, 52) through a mechanism thought to involve decreasing oxidative DNA damage and preventing telomere shortening (53). Although, initially these observations appear to oppose what was found with hMPCs, it is important to keep in mind that these effects on EPCs are dose-dependent because increased doses of statins inhibit EPC proliferation and promote apoptosis at concentrations comparable to the doses that were found to decrease hMPC viability in our study (51). When comparing these findings to hMPCs, it has been shown that low doses of simvastatin act with hMPCs to facilitate neovascularization in musculoskeletal ischemia, indicating a similarity with EPCs in increased activity with low-dose statin treatment (54). Unpublished results from our lab also show an increase in cell death of atrial fibroblasts and neonatal cardiomyocytes at the same doses which caused an increase in cell death in hMPCs. A number of other studies have looked at how different statins could affect other cell behaviours, including one that showed that rosuvastatin, a hydrophilic statin initially used at high dosage pre-MI and then halved post-MI, can effectively protect implanted hMPCs in a post-infarct environment in a rat model (55). Interestingly, a study evaluating the effects of atorvastatin on autophagy found that low doses (0.001-0.01 μM) had a neglible effect on apoptosis, but still elevated compared to control under hypoxic and serum-deprived conditions (56). The slight decrease in apoptosis under these conditions appears to be due to an increase in autophagy as a response to the hostile environment which mimics that of an infarct scar. When considered in tandem with our study, it indicates the potential for decreasing cell viability in healthy areas of the body, but the cells that are able to survive the dosage of statins would be resilient against the damaging environment of the infarct. This would result in fewer hMPCs migrating to the infarct zone differentiating into myofibroblasts to cause exemplified scar formation and invasion in viable myocardium, but enough to strengthen the infarct zone to prevent rupture. Further studies would need to be performed in an *in vitro* environment in order to find a balance between reducing hMPCs to prevent superfluous myofibroblast cell differentiation and cardiac structure stability.

In summary, we found that lipophilic statins, simvastatin and atorvastatin, significantly decreased hMPC viability through mevalonate depletion and NFκB signaling and an increase in maladaptive authophagy. Pravastatin, the hydrophilic statin, had no effect on cell viability. As MPCs have been implicated in neointimal formation, cardiac allograft vasculopathy, and tissue fibrosis, these observations may need to be considered when selecting a particular compound for its desired therapeutic effect. This is also important when considered hMPCs as a cellular basis for therapeutic treatment either as cell therapy or as a component of engineered tissue in patients with cardiovascular disease because it is highly probably that they will also be prescribed statins. Maintaining viability of therapeutic cells is important in this patient population as many of them are candidates for cardiac tissue repair and cholesterol inhibition therapy.

3.5 References

1. Mills EJ, Wu P, Chong G (2011) Efficacy and safety of statin treatment for cardiovascular disease: a network meta-analysis of 170 255 patients from 76 randomized trials. Q J Med 104:109-124

2. Liapis CD, Bell PR, Mikhailidis D et al (2009) ESVS guidelines. Invasive treatment for carotid stenosis: indications, techniques. Eur J Vasc Endovasc Surg 37:1-19

3. Liapis CD, Bell PR, Mikhailidis D et al (2010) ESVS guidelines: Section A – prevention in patients with carotid stenosis. Curr Vasc Pharmacol 8:673-681

4. Tahara N, Kai H, Ishibashi M, Nakuara H, Kaida H, Baba K, Hayabuchi N, Imaizumi T (2006) Simvastatin attenuates plaque inflammation: evaluation by fluorodeoxyglucose positron emission topography. J Am Coll Cardiol 48:1825-1831

5. Sillesen H (2009) Statins and their use in preventing carotid disease. Curr Atherscler Rep 11:309-314

6. Verzini F, De RP, Parlani G, Giordano G, Caso V, Cieri E, Isernia G, Cao P (2011) Effects of statins on early and late results of coronary stenting. J Vasc Surg 53:71-79

7. Kertai MD, Boersma E, Westerhout CM, van Domburg R, Klein J, Bax JJ, van Urk H, Poldermans D (2004) Association between long-term statin use and mortality after successful abdominal aortic aneurysm surgery. Am J Med 116:96-103

8. Kalyanasundaram A, Elmore JR, Manazer JR, Golden A, Franklin DP, Galt SW, Zakhary EM, Carey DJ (2006) Simvastatin suppresses experimental aortic aneurysm expansion. J Vasc Surg 43:117-124

9. Schouten O, van Laanen JH, Boersma E, Vidakovic R, Feringa HH, Dunkelgrün M, Bax JJ, Koning J, van Urk H, Poldermans D (2006) Statins are associated with a reduced infrarenal abdominal aortic aneurysm growth. Eur J Vasc Endovasc Surg 32:21-26

10. Giri J, McDermott MM, Greenland P, Guralnik JM, Criqui MH, Liu K, Ferrucci L, Green D, Schneider JR, Tian L (2006) Statin use and functional decline in patients with and without peripheral artery disease. J Am Coll Cardiol 47:998-1004

11. Heart Protection Study Group (2007) Randomized trial of the effects of cholesterol-lowering with simvastatin on peripheral vascular and other major vascular outcomes in 20 536 people with peripheral arterial disease and other high-risk conditions. J Vasc Surg 45:645-654

12. Kobashigawa JA, Katznelson S, Laks H et al (1995) Effect of pravastatin on outcomes after cardiac transplantation. N Engl J Med 333:621-627

13. Nissen SE, Nicholls SJ, Sipahi I et al (2006) Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. JAMA 295:1556-1565

14. Mehra MR, Uber PA, Vivekananthan K, Solis S, Scott RL, Park MH, Milani RV, Lavie CJ (2002) Comparative beneficial effects of simvastatin and pravastatin on cardiac allograft rejection and survival. J Am Coll Cardiol 40:1609-1614

15. Kobashigawa JA, Moriguchi JD, Laks H, Wener L, Hage A, Hamilton MA, Cogert G, Marquez A, Vassilakis ME, Patel J, Yeatman L (2005) Ten-year follow-up of a randomized trial of pravastatin in heart transplant patients. J Heart Lung Transplant 24:1736-1740

16. Schachter M (2005) Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. Fundam Clin Pharmacol 19:117-125

17. Bullano MF,Wertz DA, Yang GW, Kamat S, Borok GM, Gandhi S, McDonough KL, Willey VJ (2006) Effect of rosuvastatin compared with other statins on lipid levels and national cholesterol education program goal attainment for low-density lipoprotein cholesterol in a usual care setting. Pharmacotherapy 26:469-478

18. Lennernas H, Fager G (1997) Pharmacodynamis and pharmacokinetics of the HMG-CoA reductase inhibitors: similarities and differences. Clin Pharmacokinet 32:403-425

19. Singhvi SM, Pan HW, Morrison RA, Willard DA (1990) Disposition of pravastatin sodium, a tissue-selective HMG-CoA reductase inhibitor, in healthy subjects. Br J Clin Pharmacol 20:239-243

20.Zhou Z, Rahme E, Pilote L (2006) Are statins created equal? Evidence from randomized trials of pravastatin, simvastatin, and atorvastatin for cardiovascular disease prevention. Am Heart J 151:273-281

21. Sopel MJ, Rosin NL, Lee TD, Legare JF (2011) Myocardial fibrosis in response to Angiotensin II is preceded by the recruitment of mesenchymal progenitor cells. Lab Invest 91:565-578

22. van Amerongen MJ, Bou-Gharios G, Popa E, van Ark J, Petersen AH, van Dam GM, van Luyn MJ, Harmsen MC (2008) Bone marrow-derived myofibroblasts contribute functionally to scar formation after myocardial infarction. J Pathol 214:377-386

23. Möllmann H, Nef HM, Kostin S, von KC, Pilz I, Weber M, Schaper J, Hamm CW, Elsässer A (2006) Bone marrow-derived cells contribute to infarct remodelling. Cardiovasc Res 71:661-671

24. Salama M, Andrukhova O, Roedler S, Zuckermann A, Laufer G, Aharinejad S (2011) Association of CD14+ monocyte-derived progenitor cells with cardiac allograft vasculopathy. J Thorac Cardiovasc Surg 142:1246-1253

25. Carlson S, Trial J, Soeller C, Entman ML (2011) Cardiac mesenchymal stem cells contribute to scar formation after myocardial infarction. Cardiovasc Res 91:99-107

26. van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J (2010) Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 7:30-37

27. Hombach-Klonisch S, Panigrahi S, Rashedi I, Seifert A, Alberti E, Pocar P, Kurpisz M, Schulze-Osthof K, Mackiewicz A, Los M (2008) Adult stem cells and their trans-differentiation potential-perspectives and therapeutic applications. J Mol Med 86:1301-1314

28. Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 25:2739-2749

29. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 938:221-229

30. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701-705

31. Bittira B, Kuang JQ, Al-Khaldi A, Shum-Tim D, Chiu RC (2002) In vitro preprogramming of marrow stromal cells for myocardial regeneration. Ann Thorac Surg 74:1154-1159

32. Chedrawy EG, Wang JS, Nguyen DM, Shum-Tim D, Chiu RC (2002) Incorporation and integration of implanted myogenic and stem cells into native myocardial fibers: anatomic basis for functional improvements. J Thorac Cardiovasc Surg 124:584-590

33. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428:668-673

34. Bellini A, Mattoli S (2007) The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest 87:858-870

35. Keeley EC, Mehrad B, Janardhanan R, Salerno M, Hunter JR, Burdick MM, Field JJ, Strieter RM, Kramer CM (2012) Elevated circulating fibrocyte levels in patients with hypertensive heart disease. J Hypertens 30:1856-1861

36. Lei PP, Qu YQ, Shuai Q, Tao SM, Bao YX, Wang Y, Wang SW, Wang DH (2013) Fibrocytes are associated with the fibrosis of coronary heart disease. Pathol Res Pract 209:36-43

37. Cox N, Pilling D, Gomer RH (2012) NaCl potentiates human fibrocyte differentiation. PLoS One 7:e45674

38. Buhaescu I, Izzedine H (2007) Mevalonate pathway: a review of clinical and therapeutical implications. Clin Biochem 40:575-584

39. Hilgendorff A, Muth H, Parviz B, Staubitz A, Haberbosch W, Tillmanns H, Hölschermann H (2003) Statins differ in their ability to block NF-kappaB activation in human blood monocytes. Int J Clin Pharmacol Ther 41:397-401

40. Dhingra R, Gang H, Wang Y, Biala AK, Aviv Y, Margulets V, Tee A, Kirshenbaum LA (2013) Bidirectional regulation of nuclear factor- κB and mammalian target of rapamycin signaling functionally links Bnip3 gene repression and cell survival of ventricular myocytes. Circ Heart Fail 6:335-343

41.Friedernstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-274

42. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641-650

43. Sciarretta S, Hariharan N, Monden Y, Zablocki D, Sadoshima J (2011) Is autophagy in response to ischemia and reperfusion protective or detrimental for the heart? Pediatr Cardiol 32:275-281

44.Wang EY, Gang H, Aviv Y, Dhingra R, Margulets V, Kirshenbaum LA (2013) p53 mediates autophagy and cell death by a mechanism contingent on Bnip3. Hypertension 62:70-77

45. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, Yang R, Chen W, Wang S, Shi S (2011) Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-γ and TNF-α. Nat Med 17:1594-1601

46. Peng CF, Han YL, Jie-Deng, Yan CH, Jian-Kang, Bo-Luan, Jie-Li (2011) Overexpression of cellular repressor of E1A-stimulated genes inhibits TNF-α-induced apoptosis via NFκB in mesenchymal stem cells. Biochem Biophys Res Commun 406:601-607

47. Kim JM, Cho HH, Lee SY, Hong CP, yang JW, Kim YS, Suh KT, Jung JS (2012) Role of IRAK1 on TNF-induced proliferation and NFκB activation in human bone marrow mesenchymal stem cells. Cell Physiol Biochem 30:46-60

48. Gauthaman K, Fong CY, Bongso A (2009) Statins, stem cells, and cancer. J Cell Biochem 106:975-983

49. Patel S, Mason RM, Suzuki J, Imaizumi A, Kamimura T, Zhang Z (2006) Inhibitory effect of statins on renal epithelial-to-mesenchymal transition. Am J Nephrol 26:381-387

50. Piotrowski PC, Kwintkiewicz J, Rzepczynska IJ, Seval Y, Cakmak H, Arici A, Duleba AJ (2006) Statins inhibit growth of human endometrial stromal cells independently of cholesterol availability. Biol Reprod 75:107-111

51. Shao H, Tan Y, Eton D, Yang Z, Uberti MG, Li S, Schulick A, Yu H (2008) Statin and stromal cell-derived factor-1 additively promote angiogenesis by enhancement of progenitor cells incorporation into new vessels. Stem Cells 26:1376–1384

52. Assmus B, Urbich C, Aicher A, Hofmann WK, Haendeler J, Rössig L, Spyridopoulos I, Zeiher AM, Dimmeler S (2003) HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. Circ Res 92:1049–1055

53. Satoh M, Minami Y, Takahashi Y, Tabuchi T, Itoh T, Nakamura M (2009) Effect of intensive lipid-lowering therapy on telomere erosion in endothelial progenitor cells obtained from patients with coronary artery disease. Clin Sci (Lond) 116:827–835

54. Zhang Y, Zhang R, Li Y, He G, Zhang D, Zhang F (2012) Simvastatin augments the efficacy of therapeutic angiogenesis induced by bone-marrow-derived mesenchymal stem cells in a murine model of hindlimb ischemia. Mol Biol Rep 39:285-293

55. Xu H, Yang YJ, Qian HY, Tang YD, Wang H, Zhang Q (2011) Rosuvastatin treatment activates JAK-STAT pathway and increases efficacy of allogenic mesenchymal stem cell transplantation in infracted hearts. Circ J 75:1476-1485

56. Zhang Q, Yang YJ, Wang H, Dong QT, Wang TJ, Qian HY, Xu H (2012) Autophagy activation: a novel mechanism of atrovastatin to protect mesenchymal stem cell from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway. Stem Cells Dev 21:1321-1332

3.6 Figures

Figure 3.1: Schematic of non-immune cell involvement that contributes to fibrosis and/or arteriosclerosis in coronary allograft vasculopathy. EMT- endothelial-mesenchymal transition; ECM – extracellular matrix

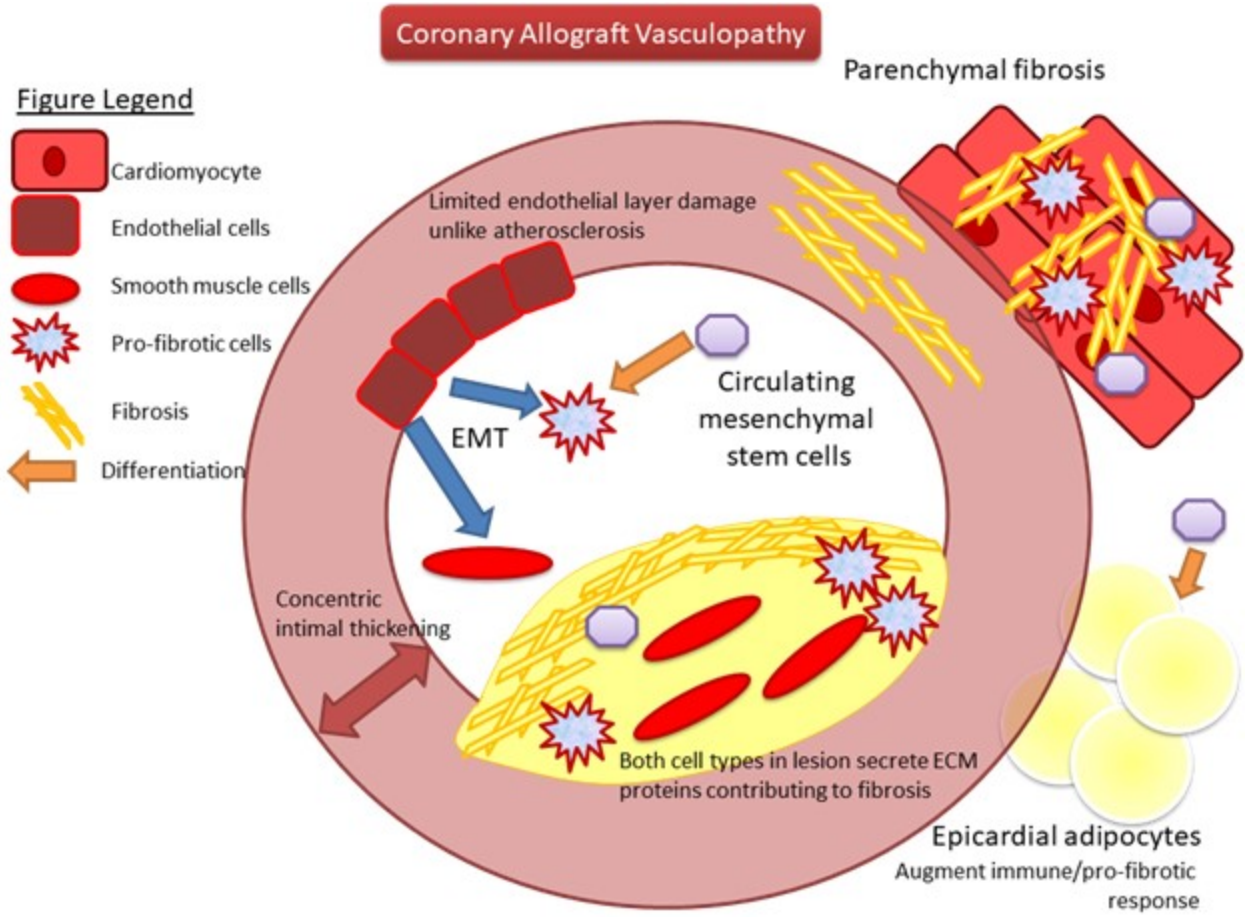


Figure 3.2: Live-dead assay analysis of primary human bone marrow-derived mesenchymal stem cells treated at 0.1 µM, 1µM, and 10 µM concentrations of A) hydrophilic pravastatin, B) lipophilic simvastatin, C) lipophilic atorvastatin at 48 and 96 hours indicating no change across the treatment groups.

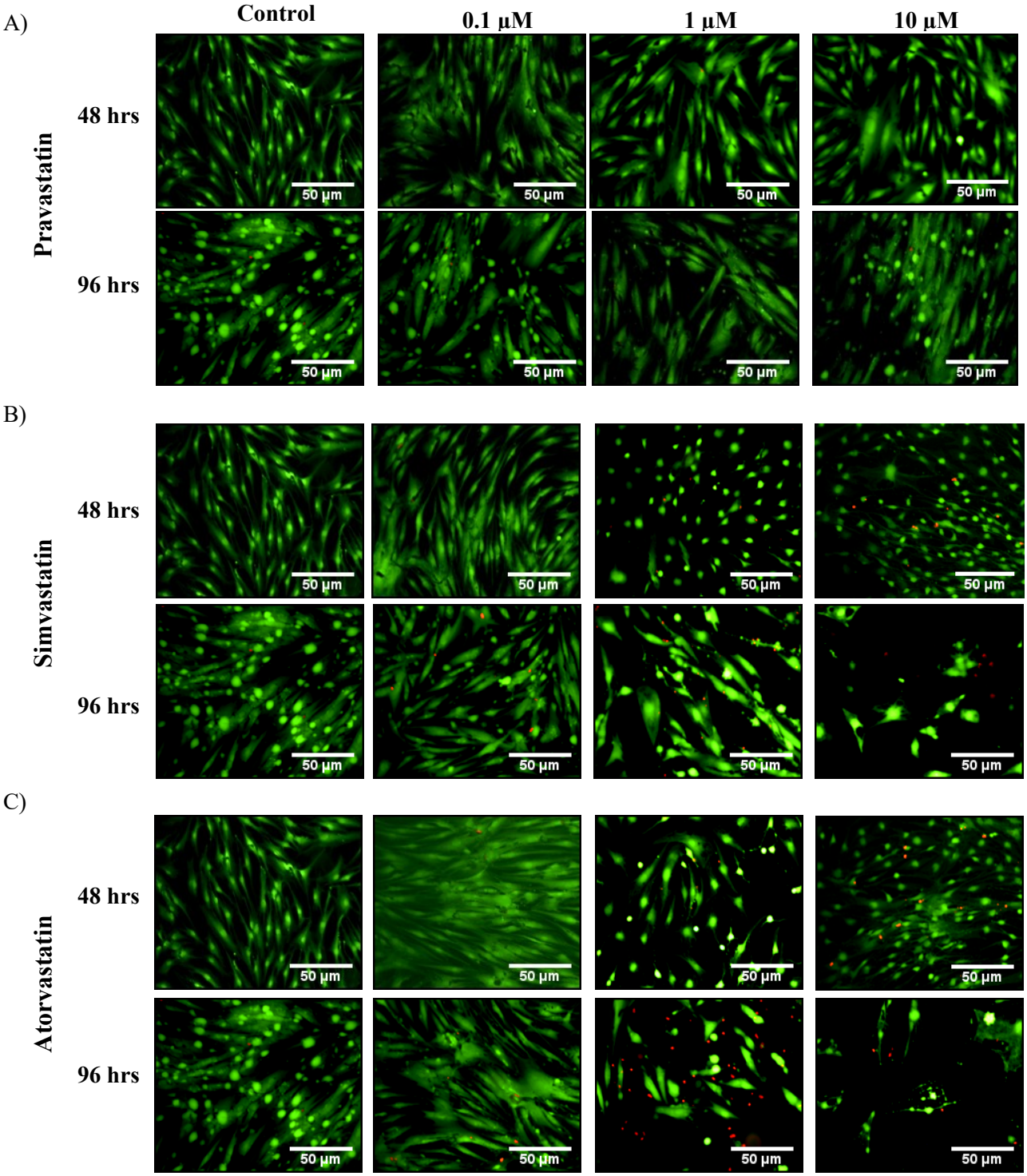
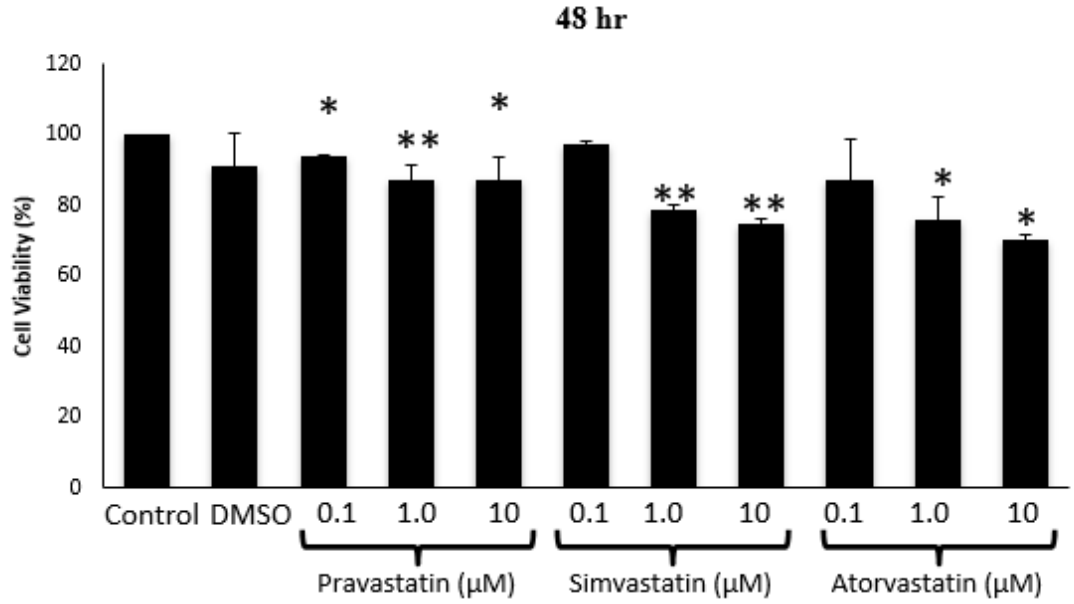


Figure 3.3: MTT cell viability analysis of lipophilic and hydrophilic statin-treated primary human bone marrow-derived mesenchymal stem cells indicating initial decrease in viability at 48 hours (A) with all three statins; however at 96 (B) hours, only lipophilic statins, simvastatin and atorvastatin, show significantly decreased viability at all three concentrations (n=4). One-way ANOVA,* p-value< 0.05 compared to Control, ** p-value < 0.01 compared to Control

A)



B)

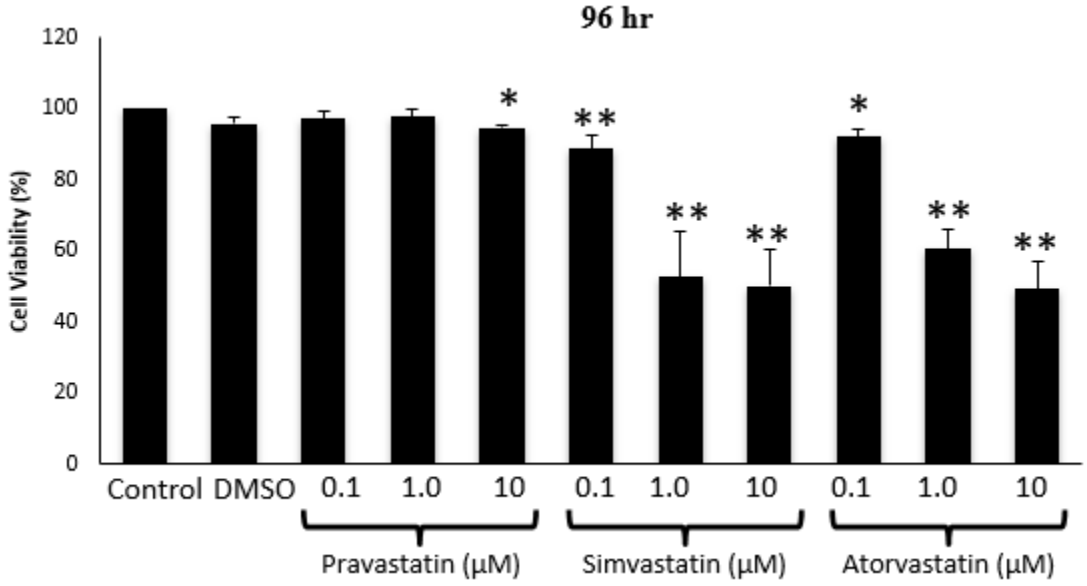


Figure 3.4: Mevalonate treatment of primary human bone marrow-derived mesenchymal stem cells showing recovery of decreased viability on atorvastatin-treated cells using MTT cell viability analysis at A) 48 hrs and B) 96 hrs (n=3, one-way ANOVA,* p-value <0.05, # p-value <0.05 compared to 10 µM atorvastatin).

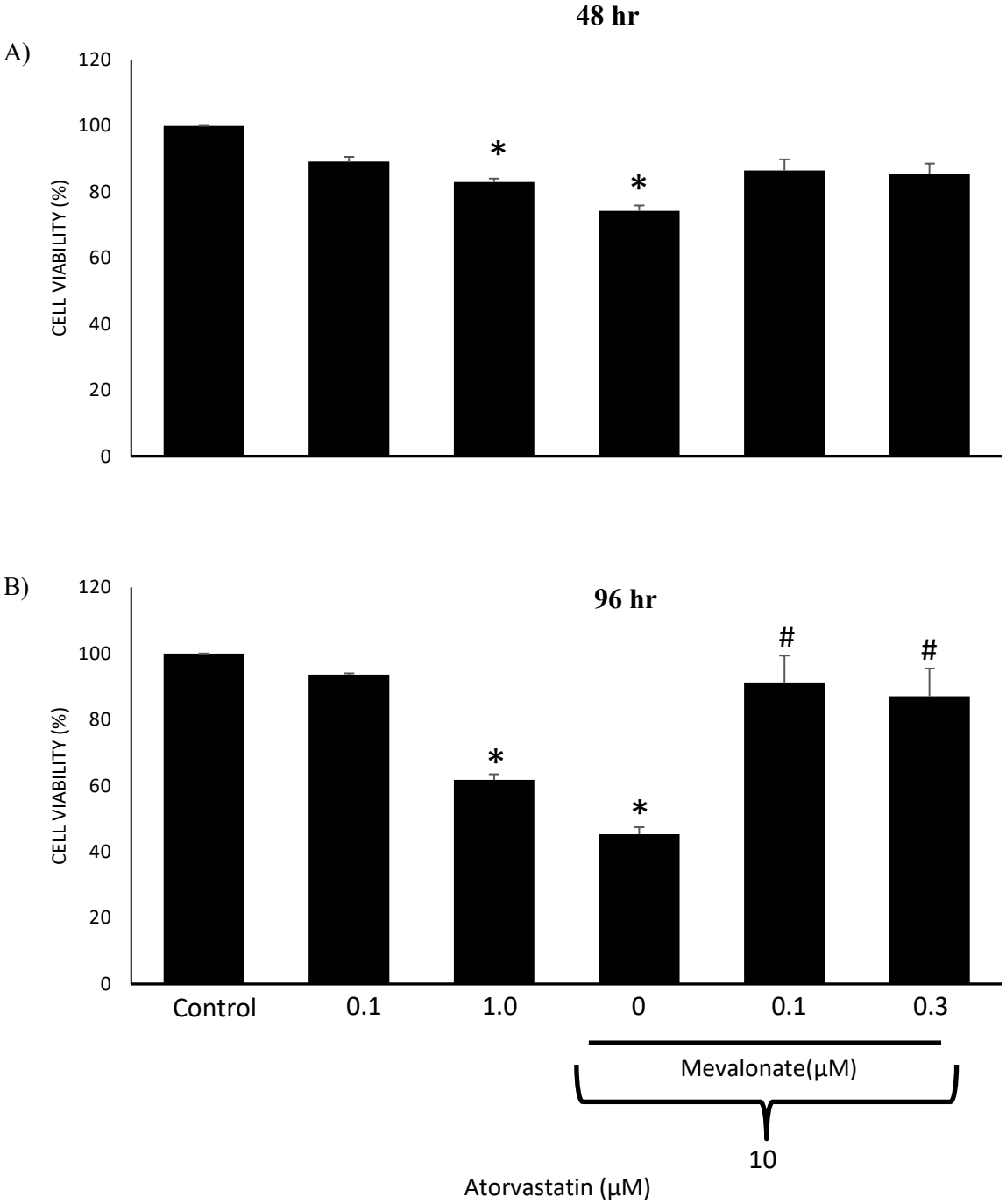


Figure 3.5: A) Representative blot of protein analysis of NF-κB p65 sub-unit in atorvastatin-treated primary human bone marrow-derived mesenchymal stem cells showing a decrease in the p65 subunit of NF-κB with increased treatment of atorvastatin at both 48 hours and 96 hours, (n=6) B) Atorvastatin-treated primary human bone marrow-derived mesenchymal stem cells treated with Iκκ-β (active subunit of NFκB) shows partial recovery of cell viability with treatment at $5x10^8$ MOI at the 96 hour time-point (n=3). C) Representative blot showing Bnip3 expression of Pravastatin (P) and Atorvastatin (A) statin treatment. One-way ANOVA, * p-value <0.05, # p-value <0.05 compared to 10 μM atorvastatin

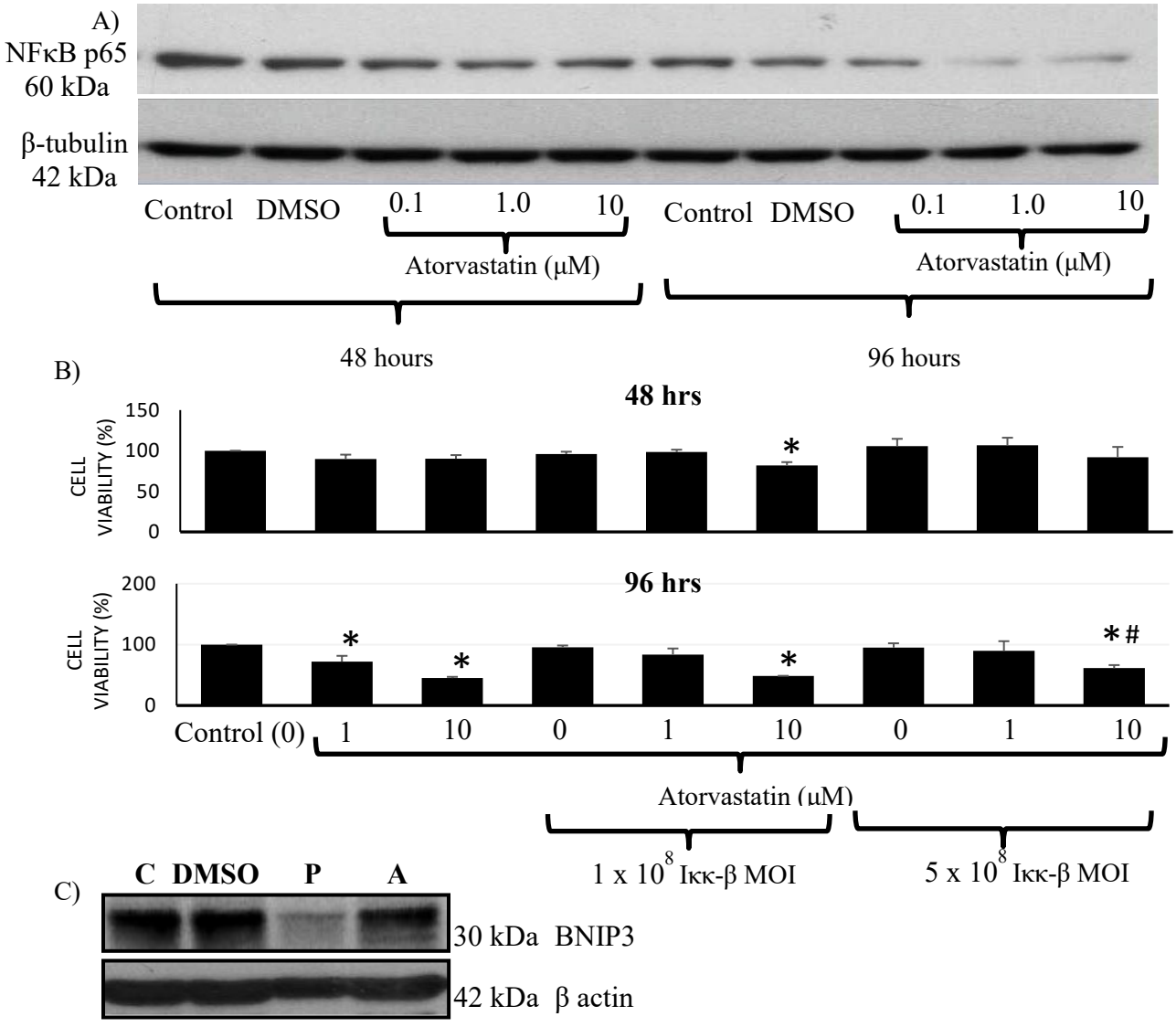
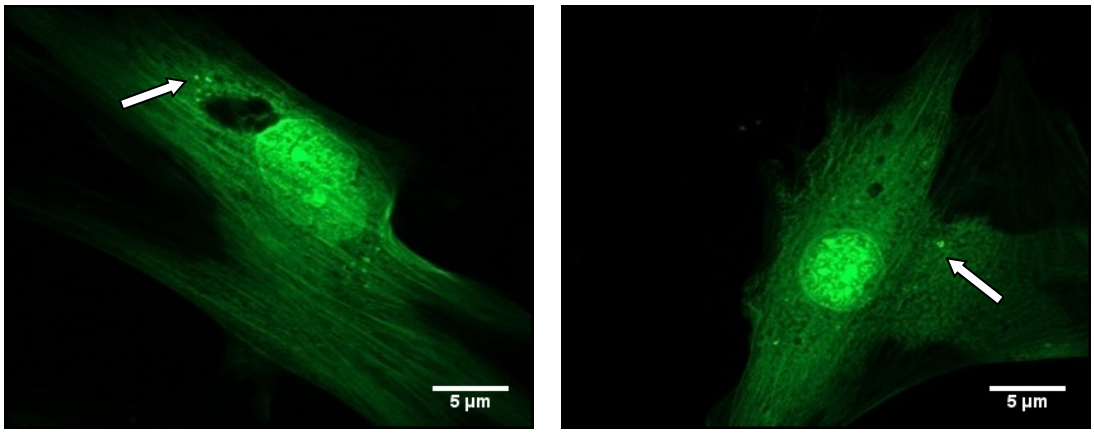


Figure 3.6: A) Autophagy analysis of primary bone marrow-derived mesenchymal progenitor cells (hMPCs) showing increased formation of autophagosomes using LC3 IF imaging (*i-iv*) in atorvastatin-treated cells (*i-iii*) compared to Control, DMSO, and pravastatin-treated cells. Representative images from single patient, arrows indicate LC3. B) Protein analysis of L3B subunits indicate increased authophagy in atorvastatin-treated hMPCs compared to pravastatin (representative blot from single patient)

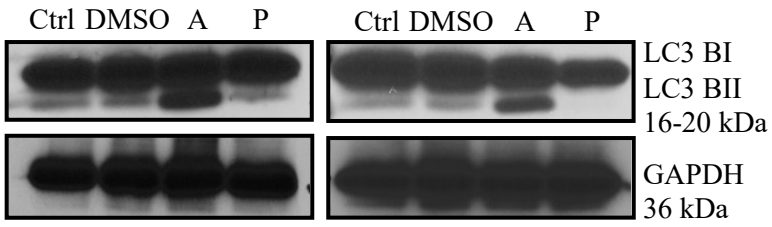
A)



i. Control ii. DMSO

iii. Atorvastatin iv. Pravastatin

B)



4. Identification of miR-301a in Primary Human Atrial Fibroblasts and Bone Marrow-Derived Mesenchymal Progenitor Cells to Attenuate Endogenous Differentiation into Pro-Fibrotic Cells

By Müller AL, Klassen J, Neumann S, Ngo M, Li Y, Freed DH

Müller AL: Collected & cultured cells (60%), evaluated Ska2 and miR-301a expression, performed WB, mRNA, collagen gel contraction and MTT assay experiments of transfected cells, IF imaging, experimental design, wrote manuscript

Klassen J: Collected & cultured cells (30%), standardized miR-301a transfection procedure, performed preliminary WB, mRNA, collagen gel contraction, and MTT assay experiments

Neumann S: Standardized miR-301a transfection procedure, preliminary mRNA analysis

MA Ngo: Collected and cultured cells (10%), standardized collagen gel contraction assay

Li Y: Technical assistance

Freed DH: Experimental design, edited manuscript

Abstract: Cardiac fibrosis manifests in as a result of human atrial fibroblasts (hAFs) and mesenchymal progenitor cells (hMPCs) pathologically depositing extracellular matrix proteins, resulting in significantly impaired cardiac function. The mechanism of hMPC-myofibroblast differentiation is unclear; however, expression of small non-coding RNAs, called microRNA (miRNA), has been shown to influence differentiation of other cell types. We hypothesize that miRNA expression changes coincide and influence both the hAF and the hMPC to pro-fibrotic cell transformation. To elucidate the potential of miRNA as a mechanism influencing hAFs and bone marrow-derived hMPCs becoming pro-fibrotic, these cells were isolated from the atrial appendage and sternum of patients undergoing open heart surgery, respectively. Both cell types were cultured in standard DMEM/F12 with 20% FBS. Cells were transfected using 50nM concentrations of pre-Scrambled and pre-miR-301a. Collagen gel contraction and MTT assays were utilized to evaluate cellular behaviour and qRT-PCR and western blotting was used to analyze expression and content of myofibroblast markers. Both hAFs and hMPCs displayed increased proliferation upon over-expression of miR-301a in hMPCs resulted in reduced collagen gel contraction and. Analysis using qRT-PCR revealed a decrease in non-muscle myosin II (NMMII) A and B isoforms in transfected hMPCs and in NMMIIA in transfected hAFs. Protein analysis of miR-301a transfected hMPCs and hAFs determined decreases in NMMIIA and pro-collagen type 1. These results imply that overexpression of miR-301a has the ability to reduce contractility and reduce pro-fibrotic markers in both hAFs and hMPCs indicating the potential for a common cellular mechanism to be influencing differentiation into a pro-fibrotic phenotype.

4.1 Introduction

Cardiac fibrosis is a prominent pathology that affects cardiovascular function in a myriad of cardiovascular diseases. Both atrial fibroblasts (AFs) and bone marrow-derived mesenchymal progenitor cells (hMPCs) have been shown to contribute to cardiac fibrosis (1). Isolated hMPCs are being evaluated in clinical trials as an effective therapy to combat heart failure (2). Unfortunately, clinical trials hoping to capitalize on their proliferative and multipotent differentiation capacity have reported only minimal improvement of an anti-inflammatory nature, and not necessarily differentiation into a contractile phenotype (3-6). This may be a result of a dichotomy that exists in hMPC biology whereby their transformation from a highly proliferative hMPC phenotype changes into a more differentiated phenotype with a reduced ability to proliferate (7-9). This could significantly impair the reparative capabilities of hMPCs. Originally, hMPCs were thought to have more significant therapeutic potential due to the ease with which they can be expanded *in vitro*, while maintaining the ability to differentiate into various lineages (10). However, it appears that optimizing the potential of hMPCs to be used as a clinical therapy may require a better understanding of the physiology that determines specific stimuli required to alter hMPCs' fate. Although several theories have been formed to explain the molecular mechanisms driving differentiation, the non-coding RNA molecule identified as microRNA (miRNA) provides an elegant explanation to this shift in genetic expression. The ability of miRNA to influence protein expression rapidly has been noted in numerous cell types under a variety of physiological and pathological conditions (11). MicroRNAs (miRNAs) have recently emerged as a novel intrinsic method of gene regulation predominantly located in intronic gene sequences as 21 or 22 nucleotide-polymers. They act by post-transcriptionally binding the 3'UTR of mRNA targets and ultimately decreasing mRNA levels (12). These molecules have been implicated in a variety of cell processes including differentiation (13-15). Previous work from our laboratory (16) has found that primary bone marrow-derived hMPCs readily adopt a myofibroblast-like phenotype over serial passaging in standard culture conditions. These observations were confirmed by collagen gel contraction and expression of myofibroblast markers in a

pattern comparable to isolated primary human cardiac myofibroblasts. Moreover, hMPCs displayed an increase in collagen gel contraction when exposed to TGF-β similar to that of cardiac myofibroblasts, which is known to induce a myofibroblast phenotype.

Myofibroblast differentiation is governed by a variety of factors including interaction with the ECM, and exposure to ED-A fibronectin and TGF-β. Prior to injury in a healthy heart, fibroblasts do not express contractile microfilaments or stress fibers and produce little ECM (17). When injury occurs, differentiation to myofibroblasts involves a transition into proto-myofibroblasts which are characterized by stress fibers linking cells to the ECM (18). Interestingly, in studies evaluating the migration of hMPCs via eGFP mouse bone marrow transplant models, it has been found, a significant proportion of myofibroblasts present in the post-MI heart of the post-transplant mice express eGFP, suggesting a bone marrow origin (19,20). These studies both indicate that bone marrow-derived cells are an important contributor to the myofibroblast and fibroblast population within the infarct area post-MI.

As miRNA is implicated in the transition of many cell types, our study focuses on investigating whether or not miRNAs could play a role in the differentiation process of both hAFs and hMPCs to a pro-fibrotic phenotype. Little work has been done on miRNA-301a, especially in a cardiovascular context; however, its host gene, ska2, is located at17q22-23 in the human genome and the protein is involved in the onset of anaphase during mitosis and also the maintenance of metaphase (21). This implicates a potential role in manipulating proliferative potential. In addition, other studies have shown that endogenous overexpression of miR-301a has been implicated in pancreatic and hepatocellular cancer cell proliferation (22-24). Although miR-301a has become a popular microRNA in cancer research, its role in hAF and hMPC differentiation has not been well studied; however, there is evidence to suggest that miR-301a is heavily involved in proliferation and/or differentiation of human cells.

4.2 Materials & Methods

Isolation, Culture, and Transfection of Primary Human MPCs and Human AFs

Human MPC cultures were prepared based on plastic adherence according to the methods developed by Caplan and Friedenstein (25, 26) with some modifications as previously described in Chapter 2. Cells were then dissociated with TrypLE Express (GIBCO®) and seeded at low densities for expansion through successive passages. HAFs and hMPCs were cultured in 10 cm^2 dishes for immunoblot and qRT-PCR analysis, in 24 well plates for collagen gel contraction assays, and in 96-well plates for optical MTT assays. Cells used for collagen contraction were treated with 0%, 1%, or 20% FBS once plated on collagen gel. Cells used for MTT assays were cultured on a 96-well plate and cultured with 0% FBS or 20% FBS. A third treatment group for MTT assay analysis was initially cultured with 0% FBS for the first 24 hrs and then grown in DMEM-F12 with 20% FBS for 48 hours. Transfection with miR-301a, as described in Chapter 2, was performed on cells at 80-90% confluency.

RNA and qRT-PCR analysis

P1 hAFs and hMPCs were lysed after reaching 90% confluencey using TRIzol reagent 48 hours post-transfection and RNA was isolated. Total RNA was purified using Ambion's DNA-free kit according to the manufacturer's instructions and RNA concentration and quality was analyzed using an Agilent 2100 Bioanalyzer. A more detailed explanation of mRNA isolation and quantification can be found in Chapter 2. Expression of myosin heavy chain 9 (forward (F) primer – ACACCGCCTACAGGAGTATGA; reverse (R) primer – ACACCGCCTACAGGAGTATGA), myosin heavy chain 10 (F – AGGTGGACTATAAGGCAGATGAG; R – CTGTCTGATGACTGGTGCAAAA), collagen1A1 (F – CCAAAGGATCTCCTGGTGAA; R – AGTTTTGCCATCAGGACCAG), and collagen1A2 (F – TTGACCCTAACCAAGGATGC; R- TTCTTGGCTGGGATGTTTTC) were analyzed by quantitative real-time PCR. qPCR results were compared against β-actin which was used as a reference gene. Expression of mRNA was determined using MT Mini Personal Thermal Cycler (Bio-Rad) using Quanta Biosciences B-R 1-Step SYBR® Green qRT-PCR kit. Reverse transcription to make cDNA was performed using the TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystems) according to

the manufacturer's instructions. Amplification of cDNA was used with Taqman®Universal PCR Master Mix (Applied Biosystems). U6 and miR-301a primers were provided by ThermoFischer Scientific and q-RT-PCR quantification was performed using Roche Lightcycler 480 II.

Western Blot Analysis

P1 hAFs and hMPCs were lysed 48 hours post-transfection and processed as indicated in Chapter 2. Equal amounts of each protein sample (20µg) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel at 130 V. Separated proteins were then transferred to a polyvinylidenedifluoride (PVDF) membrane for 1 hour at 300 mA. Membranes were blocked with 5% non-fat skim milk in PBS (phosphate buffered saline) for 1 hour at room temperature. After blocking, antibodies against pro-collagen I (1:1000; Developmental Studies Hybridoma Bank), non-muscle myosin IIA (NMMIIA, 1:2000; Abcam), non-muscle myosin IIB (NMMIIB, 1:2000; Abcam), and smooth muscle myosin (SMM, 1:1000; Sigma-Aldrich) were used to evaluate the protein expression with their corresponding secondary antibody (goat anti-rabbit IgG, rabbit anti-mouse IgG; Jackson ImmunoResearch Laboratories) conjugated with horseradish peroxidase. The loading control used was β-tubulin (1:1000, Abcam) which was probed for after blots were stripped. For ska2 protein analysis, Memcode^{TM} Reversible Protein Stain Kit (Pierce Biotechnology, Rockford IL, USA) according to the manufacturere's instructions. Proteins were visualized using Pierce ECL Western Blotting System (Thermoscientific) according to manufacturer's instructions. Densitometry was performed using GS-800 Calibrated Densitometer (BioRad) and quantified using Quantity One software.

Collagen Gel Contraction Assay

To prepare collagen gels, 1 mL of PureCol (Advanced Biomatrix) bovine type I collagen (3 mg/mL) in 5X DMEM F-12 (powder form from Thermo-Fischer Scientific) was pipetted in 24-well culture dishes and was allowed to set overnight at 37°C prior to plating at a cellular density of $5x10^4$ cells/2 cm^2. P1 hAFs and hMPCs were seeded overnight on the collagen prior to transfection. Transfection proceeded as previously explained. At 24 hours after miR-301a transfection, the gels were detached from the edges of the wells using a custom cutting tool and then digital photos taken at 0 hr and 24 hr post-detachment, or

24 and 48 hours post-transfection, respectively. These photos were used to measure the diameter of the collagen with each treatment group was repeated in triplicate. Photos were analyzed using IDL-based MeasureGel software to determine the change in collagen gel surface area by isolating the collagen gel area on the program and calculating the difference in surface area of the collagen gel between 0 and 24 post-detachment.

Cell Viability Assessment-MTT Assay

Using a 96-well plate, 5000 passage 1 cells/well were seeded in 200µl 20% FBS media per well. Cells were incubated at 37°C and 5% CO_2 and allowed to attach overnight. A plate per patient was set-up and separated into 6 sections where there was a Control and miR-301a transfection section for each time point. At the time of transfection (T0), and 24 and 48 hours post-transfection, 20 µL of 5mg/mL MTT (Thiazolyl Blue Tetrazolium Bromide) was added to each well and allowed to incubate (37C, 5% CO2) for 3 hours to enable the MTT metabolization. The media was carefully aspirated and resuspended in dimethyl sulfoxide (DMSO). Absorbance was read at 570nm by using SPECTRAmax Plus.

Immunofluorescent Imaging

HMPCs were cultured in 24-well plates overlaid with glass coverslips with some wells transfected with miR-301a or a scrambled control as described previously. After 48 hrs, both miR-301a and scrambled control cells were fixed in 4 % PFA for 15 min and permeabilized in 0.2 % Triton X-100 in PBS for 5 min. Cells were subsequently washed with PBS and blocked with 5 % BSA for 30 min at RT. Fixed cells were incubated with 50 µl of either a NMMIIA (1:200 dilution in PBS-T) or EDA-FN (1:100 dilution in PBS-T) primary antibodies overnight at 4 °C and detected with the appropriate biotinylated secondary antibody for 1 h at RT. Cells requiring dual staining for filamentous actin were washed with PBS after incubation with the secondary antibody, and incubated with 50 µl of phalloidin for 30 min at RT. Vectashield with DAPI was used to mount the coverslips and cells were visualized with an epifluorescent microscope with appropriate filters.

Statistics

Statistical analysis was performed as described in Chapter 2.

4.3 Results

Effect of Nutrient Deprivation

HMPCs grown in 20% FBS were significantly more proliferative than hMPCs grown in 0% FBS. Evaluation of the collagen gel contraction assay, it was observed that 1% FBS treated hMPCS exhibited a lower change in surface area than hMPCs grown at 0% FBS (Figure 4.1). After microarray analysis, it was found that this decrease in contractility coincided with an increased expression of miRNA-301a. In hAFs, ska2 expression remained unchanged between the three treatment groups; however, miR-301a expression was significantly increased 48 hrs after a serum-free culture environment became a serum-rich culture environment. This change in expression corresponded with altered ska2 and miR-301a expression where both ska2 and miR-301a were elevated in serum-rich conditions (Figure 4.2).

Functional Assessment of miR-301a Transfection in hAFs and hMPCs

To assess the mechanistic role of miR-301a, overexpression of miR-301a in hMPCs resulted in significantly reduced contractility of 9.7% compared with a negative-scramble control (Figure 4.3). Although a decrease in contractility was not statistically significant in hAFs, there did appear to be a trend towards reduced contractility (Figure 4.3). MTT proliferation assessment determined that both hAFs and hMPCs that there was a significant increase in the rate of cell proliferation determined at 48 hrs post-transfection (Figure 4.4).

Molecular Assessment of miR-301a Transfection in hAFs and hMPCs

To evaluate the observed cause in contractility and delineate this phenotype caused by miR-301a overexpression, the protein and RNA expression of a variety of myofibroblast markers were analyzed. We evaluated the gene expression of myosin isoforms and collagen-1 which revealed a significant decrease in the gene expression of MYH9, MYH10 and Col1A2 in hMPCs (Figure 4.5A) with overexpression of miR-301a. In hAFs (Figure 4.5B) there was a significant decrease in the gene expression of MYH9 and Col1A1 in miR-301a transfected cells. Protein expression of the corresponding proteins was also analyzed

in addition to myofibroblast markers. This resulted in significant decreases evident at the protein level in NMMIIA and pro-collagen 1 (Sp1D8) without significant changes in NMMIIB and SMM in hMPCs (Figure 4.6A). In hAFs, there were more significant changes indicated by a decrease in NMMIIA, Sp1D8, and SMM (Figure 4.6B).

4.4 Discussion

Our study has successfully identified a microRNA that may be influential in preventing a pro-fibrotic phenotype in two primary human cell types that are known to contribute to pathological cardiac fibrosis. As these cells were isolated from patients, it is important to note that isolating hMPCs from a heterogenous bone marrow aspirate involves separating them from both mesenchymal and hematopoietic stem cells in various stages of differentiation. It is known that, although hMPCs comprise merely 0.001-0.01% of the total population of cells isolated (27), hematopoietic cells do not adhere to plastic culture plates and are thus washed away leaving the attached hMPCs behind (28) which is how the hMPCs were isolated. Further isolation procedures were not utilized as this would involve further processing of the hMPCs which could produce experimental artifacts and influence their differentiation. It has also been observed that cell markers used to identify progenitor cells are not always accurate or appropriate (29). We have also included the immunohistology of our isolated hMPCs differentiated into chondrogenic, osteogenic, and adipogenic cell types using the appropriate media in our Supplementary Figures to show that our isolated hMPCs are indeed progenitor cells. The potential for multipotency of these cells has been demonstrated in various studies (9, 30); however, why they have reduced effectiveness in clinical trials has yet to be elucidated. Our study has two primary findings: firstly, hMPCs exhibit a dichotomous phenotype between proliferative versus contractile function which is similar to the hAF phenotype. Both hMPCs and hAFs are able to differentiate into a more contractile myofibroblast cell type, which was observed in our previous study (16). Secondly miR-301a likely plays a mechanistic role in suppressing the contractile phenotype while favouring the proliferative phenotype in both hMPCs and hAFs (Figure 4.3). The initial investigation involved a deprivation of essential nutrients to create an environment

comparable to that of injured cardiac tissue where these conditions trigger the differentiation of various cell types, including hAFs into myofibroblasts (31,32). In order to investigate whether or not these conditions will alter the phenotype of hMPCs, they were grown in both serum-full and serum-free media which caused a drastic change in the functional properties of the hMPCs. The collagen gel contraction assay demonstrated that when hMPCs are cultured in a nutrient-deficient environment, similar to that of an ischemia, there is a significant increase in the ability to contract collagen-rich substrates. This observation correlates with the increase in the differentiation of fibroblasts to myofibroblasts in response to MI where the decreased blood flow prevents nutrient transport and causes an inflammatory response (33, 34). On the other hand, when there are available nutrients, hMPCs do not undergo a contractile phenotype which indicates that there is a switch in the phenotype as a response to extracellular signals. It should be noted that, even though cells treated to 1% FBS are able to proliferate more in culture with the provision of nutrients, the increase in cell number did not contribute to the greater contraction of the collagen substrate. To further understand how this change from a proliferative to a differentiated phenotype is occurring, a micro-array analysis on miRNA was performed to look for potential targets. It was found that miR-301a was highly responsive to the changes in phenotype.

In order to further elucidate the role of miR-301a, additional studies were performed to understand how it responds to changes in mitogen levels. Both miR-301a and the protein product of its host gene, ska2 were found to be increased after initial nutrient deprivation for 24 hours followed by a 48 hr recovery period when FBS was restored. Although ska2 protein expression was found to be increased, it did not reach statistical significance; however its associated miRNA product, miR-301a did. This may be a result of the prolonged time it takes to translate protein in the cell compared to producing microRNA. Interestingly, it was only upon a deprivation and subsequent re-introduction of mitogens that miR-301a expression was altered, indicating that MPCs utilize miR-301a to respond to a phenotypic change to react to their environment. Exposure of hMPCs to nutrients promotes a more proliferative versus a contractile myofibroblastic phenotype, suggesting a dichotomy between the two phenotypes. This concept of a

dichotomy between a proliferative versus contractile phenotype has been well documented in the smooth muscle literature, with microRNA playing a mechanistic role. In smooth muscle cells, miR-145 and miR-143 has been implicated in regulating this process (35). In addition, it has been observed the miR-21 and miR-145 play a role in the differentiation from fibroblasts to myofibroblasts (36,37).

HMPCs have often been compared to fibroblasts with respect to appearance as well as gene and protein expression. This myofibroblast phenotype has not previously been well documented. Phenotypic characteristics of myofibroblasts include an ability to contract collagen gels and expression of markers such as α-SMA, collagen, and NMMIIA as is seen in cardiac myofibroblasts. HMPCs also display decreased proliferative capacity as they are cultured and expanded (38). Transfection of miR-301a resulted in a reduced ability to contract compared to control and the presence of focal adhesions that contain β-actin and γ-actin microfilaments associated with non-muscle myosin commonly found in proto-myofibroblast formation (18). In addition, hMPCs transfected with miR-301a had increased proliferation early on in culture. We observed that exposure to mitogens, such those present in fetal bovine serum, induced a proliferative, less contractile phenotype, whereas serum deprivation induced a phenotype of decreased proliferation and increased contraction, suggesting that the myofibroblast phenotype as the polar opposite of the proliferative phenotype. In addition, this increase in proliferation with miR-301a transfection also occurred in hAFs indicating that a similar molecular mechanism could be acting in both cell types, although further analysis needs to be performed in order to elucidate it. Our results indicate that miR-301a over-expression antagonizes the mitogenic phenotype of early passage hMPCs, as evidenced by decreased collagen gel contractility. This decrease in contractility could be mediated by the observed decrease in the mRNA expression of MYH9 and MYH10. Although a significant decrease in MYH9 occurred in hAFs, the lack of effect on MYH10 could indicate why hAFs did not significantly increase their contractility. The decrease in protein expression of NMMIIA, as evident by western blotting indicates that the protein level of non-muscle myosin in both hMPCs and hAFs is affected by the miR-301a. There is also a significant decrease in pro-collagen type, which was evaluated by protein

quantification, indicating that these cells would not be significantly contributing to extracellular matrix formation which is a characteristic property of myofibroblasts. This is a promising target to further elucidate the mechanism of how various cell types contribute to pathological fibrosis as both hAFs and hMPCs were influenced by miR-301a. This could indicate a common mechanism as this single microRNA is affecting two different cell types in a similar fashion. These results suggest that miR-301a plays a mechanistic role in increasing proliferative potential of hMPCs and hAFs while attenuating expression of a pro-fibrotic phenotype. This correlates with the findings that miR-301a promotes proliferation in various types of malignant cells. Like all microRNAs, it is unlikely that miR-301a has a direct effect on hMPC phenotype, but rather that it suppresses protagonists of the myogenic phenotype, maintaining a predominantly proliferative physiology. Further work is required to determine in miR-301a exerts its effect through targeting these genes in hMPCs, and if these effects translate into altered fibrosis *in vivo*. The use of miRNA has been suggested as a therapeutic intervention, and it may be that miR-301a could be used to treat established fibrosis or to effectively inhibit endogenous atrial fibroblasts and bone marrow-derived progenitor cells from adopting a pathological ECM secreting phenotype in a pro-fibrotic environment.

4.5 References

1. Krenning G, Zeisberg EM, Kalluri R (2010) The origin of fibroblasts and the mechanism of cardiac fibrosis. J Cell Physiol 225: 631-637.

2. Kassem M, Abdallah BM. Human bone-marrow-derived mesenchymal stem cells: Biological characteristics and potential role in therapy of degenerative diseases (2008) Cell and Tissue Research 331: 157-163.

3. Hare JM, Fishman JE, Gerstenblith G, et al (2012) Comparison of allogenic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. JAMA 308: 2369-2379.

4. Yang Z, Xhang F, Ma W, et al (2010) A novel approach to transplanting bone marrow stem cells to repair human myocardial infarction: delivery via a noninfarct-relative artery. Cardiovasc Ther28: 380-385.

5. Perin EC, Dohmann HF, Borojevic R, et al (2003) Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 107: 2994-2302

6. Hare JM, Traverse JH, Henry TD, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am CollCardiol 2009; 54: 2277-2286

7. Reyes M, Lund T, Lenvik T, et al (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98: 2615-2625

8. Niemeyer P, Krause U, Fellenberg J, et al (2004) Evaluation of mineralized collagen and alpha-tricalcium phosphate as scaffolds for tissue engineering of bone using human mesenchymal stem cells. Cells Tissues Organs 177: 68-78

9. Yuge L, Kajiume T, Tahara H, et al (2006) Microgravity potentiates stem cell proliferation while sustaining the capability of differentiation. Stem Cells Dev 15: 921-929

10. Chamberlain G, Fox J, Ashton B, et al (2007) Concise review: mesechymalstelm cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 25: 2739-2749

11. Amiel J, de Pontual L, Henrion-Caude A (2012) miRNA, development and disease. Adv Genet 80: 1-36

12. Hobert O (2008) Gene regulation by transcription factors and microRNAs. Science 319: 1785-1786

13. Inose H, Ochi H, Kimura A, et al (2009) A microRNA regulatory mechanism of osteoblast differentiation. PNAS 106: 20794-20799

14. Jiang J, Gusev Y, Aderca I, et al (2008) Association of microRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. Clin Can Res 14; 419-427

15. Mycko MP, Cichalewska M, Machlanska A, et al (2012) MicroRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. PNAS 109: E1248-E1257

16. Ngo MA, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

17. Tomasek JJ, Gabbiani G, Hinz B, et al (2002) Myofibroblasts and mechano-regulation of connective tissue remodeling. Nature Reviews 3: 349-363

18. Hinz B, Gabbiani G (2003) Mechanisms of force generation and transmission by myofibroblasts. CurrOpin Biotech 14: 538-546

19. van Amerongen MJ, Bou-Gharios G, Popa E, et al (2008) Bone marrow-derived myofibroblasts contribute functionally to scar formation after myocardial infarction. J Pathol 214: 377-386

20. Mollmann H, Nef HM, Kostin S, et al (2006) Bone marrow-derived cells contribute to infarct remodeling. Cardiovasc Res 71: 661-671

21. Hanisch A, Sillje HH, Nigg EA (2005) Timely anaphase onset requires a novel spindle and kinetochore complex comprising Ska1 and Ska2. EMBO J 25: 5504-5515

22. Chen Z, Chen LY, Dai HY, et al (2012) miR-301a promotes pancreatic cancer cell proliferation by directly inhibiting bim expression. J of Cell Biochem 113: 3229-3235

23. Lu Z, Li Y, Takwi A, et al (2011) miR-301a as an NF-kappaB activator in pancreatic cancer cells. EMBO J 30; 57-67

24. Zhou P, Jiang W, Wu L, et al (2012) miR-301a is a candidate oncogene that targets the homeobox gene gax in human hepatocellular carcinoma. Big Diseases Sciences 57: 1171-1180

25. Friedernstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs.Exp Hematol 4: 267-274

26. Caplan AI. Mesenchymal stem cells (1991) J Orthop Res 9: 641-650

27. Pittenger MF, Mackay AM, Beck SC, et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147

28. Sekiya I, Larson BL, Smith JR, et al (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20: 530-541

29. Lin CS, Xin ZC, Dai J, et al (2013) Commonly used mesenchymal stem cell markers and tracking labels: limitations and challenges. Histol Histopathol 28: 1109-1116

30. Battula VL, Treml S, Bareiss PM, et al 2009) Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. Haematological 94: 173-184

31. Larsson O, Diebold D, Fan D, et al (2008) Fibrotic myofibroblasts manifest genome-wide derangements of translational control. PLoS One 3: e3220

32. Kalluri R, Neilson EG (2003) Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 112: 1776-1784

33. Serini G, Bochaton-Piallat ML, Ropraz P, et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. J Cell Biol 142: 873-881

34. Wang J, Chen H, Seth A, et al (2003) Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. Am J Heart Physiol 285: H1871-H1881

35. Cordes KR, Sheehy NT, White MP, et al (2009) miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 460: 705-710

36. Gong C, Nie Y, Qu S, et al (2014) miR-21 induces myofibroblast differentiation and promotes the malignant progression of breast phyllodes tumors. Cancer Res 74: 4341-4352

37. Wang YS, Li SH, Guo J, et al (2014) Role of miR-145 in cardiac myofibroblast differentiation. J Mol Cell Cardiol 66: 94-105

38. Digirolamo CM, Stokes D, Colter D, et al (1999) Propagation and senescence of human marrow stromal cells in culture: A simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Brit J Haem 107: 275-281

4.6 Figures

Figure 4.1: hMPCs contractility was evaluated using collagen gel contraction assays where 1% FBS was found to result in significantly decreased effectiveness in contraction a collagen substrate when compared to the starving 0% FBS cells (n=3 patients). Paired student t-test, ** P-value < 0.01 compared to 0% FBS.

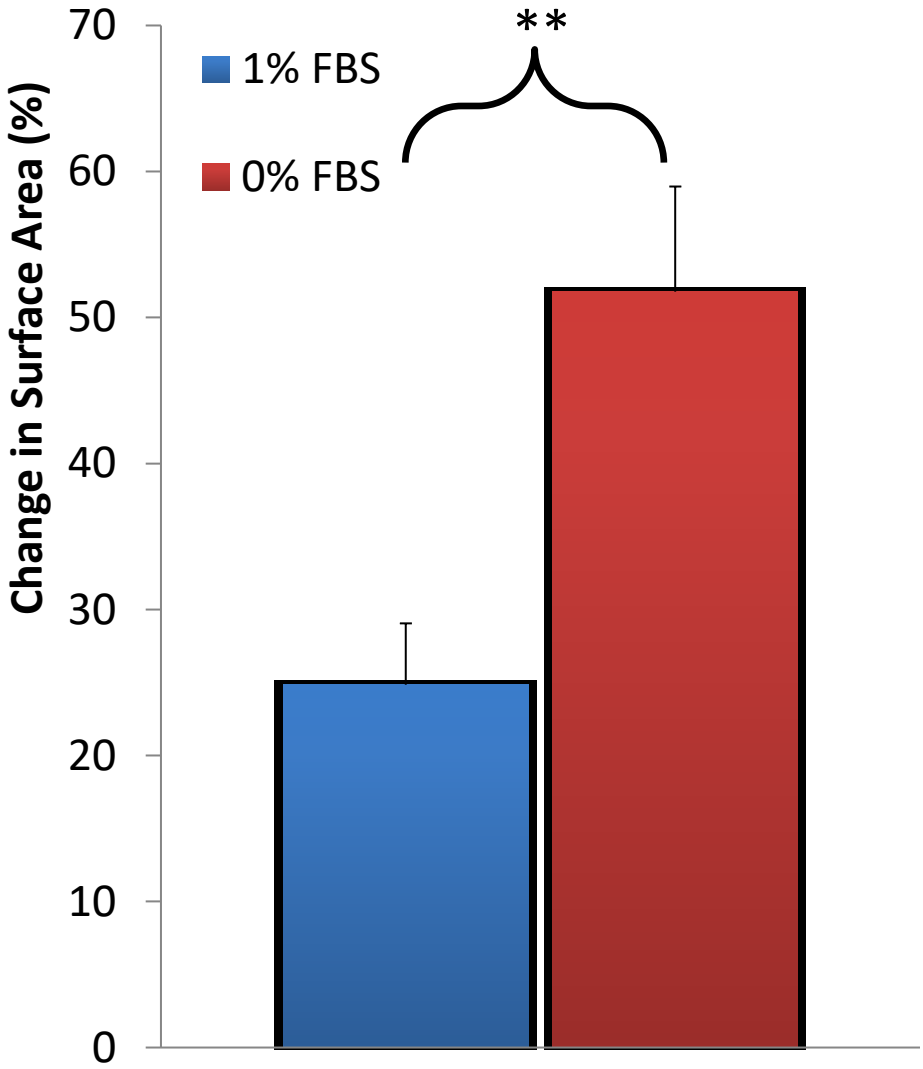


Figure 4.2: Protein expression of Ska2 and expression of miR-301a compared at 0% FBS, 20% FBS, and re-introduction to 20%FBS for 48 hours after 24 hours of 0% FBS a) primary human atrial fibroblasts (n=4) with representative western blot and b) primary human bone marrow-derived mesenchymal progenitor cells (n=5) with representative western blot. Paired student t-test, one-way ANOVA, * p-value < 0.05 compared to 0% FBS, # p-value<0.05 compared to 20% FBS (miR-301a).

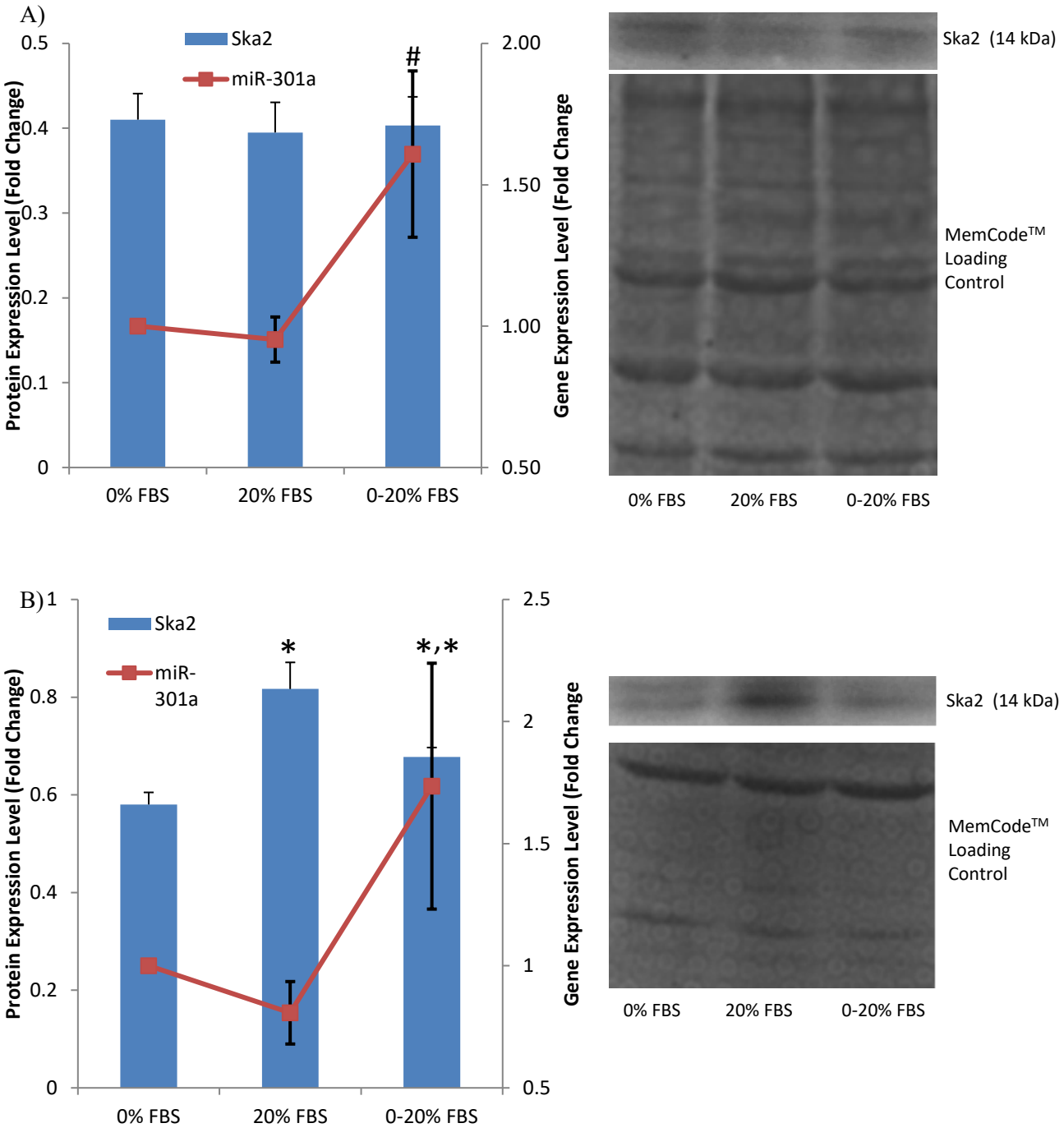


Figure 4.3: Transfection of miR-301a in hMPCs was found to cause a significant decrease in contractility when compared to a Scrambled control in passage 1 (P1) cells, a non-significant decrease in contractility was observed in hAFs (n=4 patients). Paired student t-test for the hMPCs and one-way ANOVA for the hAFs, * p-value< 0.05 compared to Control.

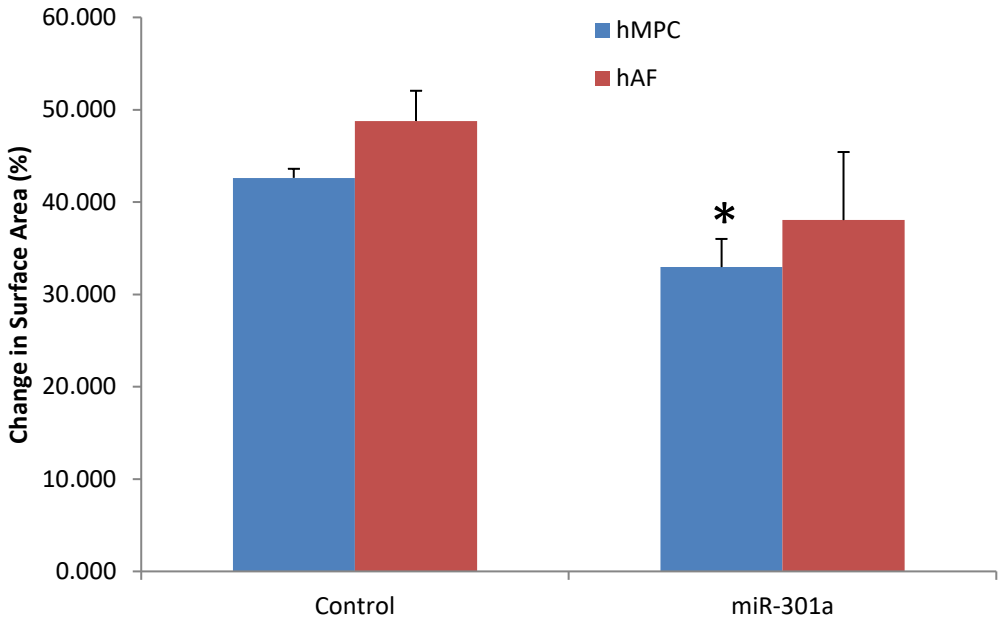


Figure 4.4: Cell proliferation quantification using MTT assay P1 hMPCs (A) and P1 hAFs (B) at t=0 hrs, t=24 hrs, and t=48 hrs, showing a gradual increase in proliferation (n=4). One-way ANOVA, * p-value< 0.05 compared to Control.

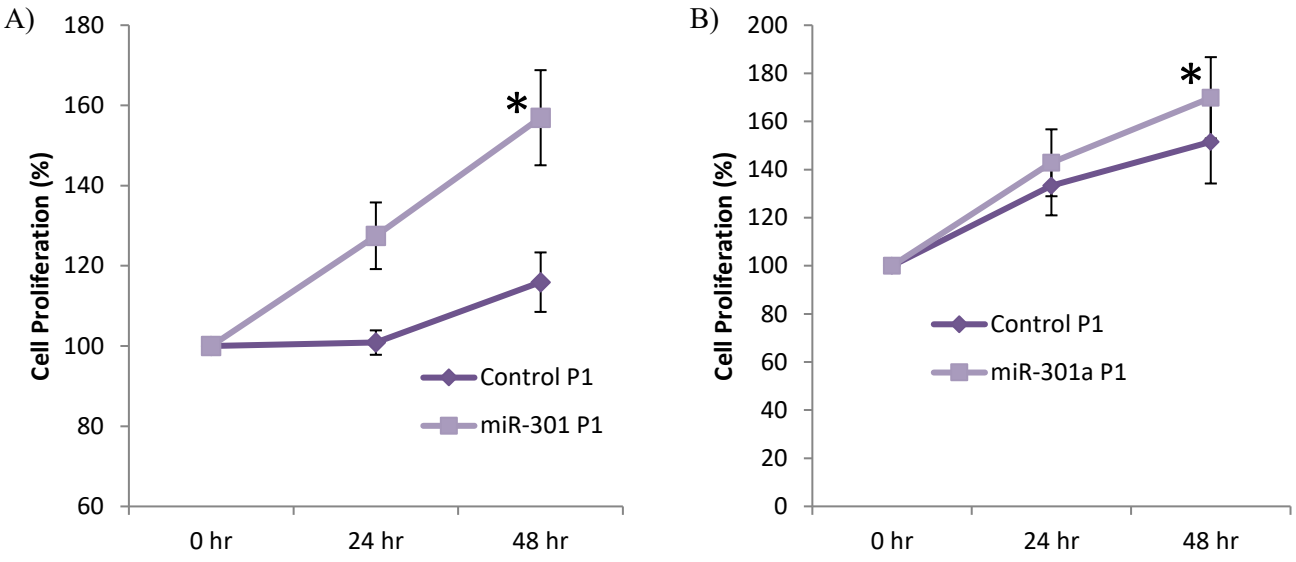


Figure 4.5: mRNA expression of *myosin heavy chain-9 (MYH9), myosin heavy chain-10 (MYH10),* and *collagen1A1 (Col1A1), collagen1A2 (Col1A2)* between scrambled Control and miR-301a transfected (A) hMPCs at P0 showing a significant decrease in transfected cells in all transcripts except *Col1A1* and transfected (B) hAFs at P0 show a significant decreased in *MYH9* and *Col1A1* (n=4-6). Paired student t-test, one-way ANOVA, * p-value< 0.05 and ** p-value < 0.01 when compared to Control.

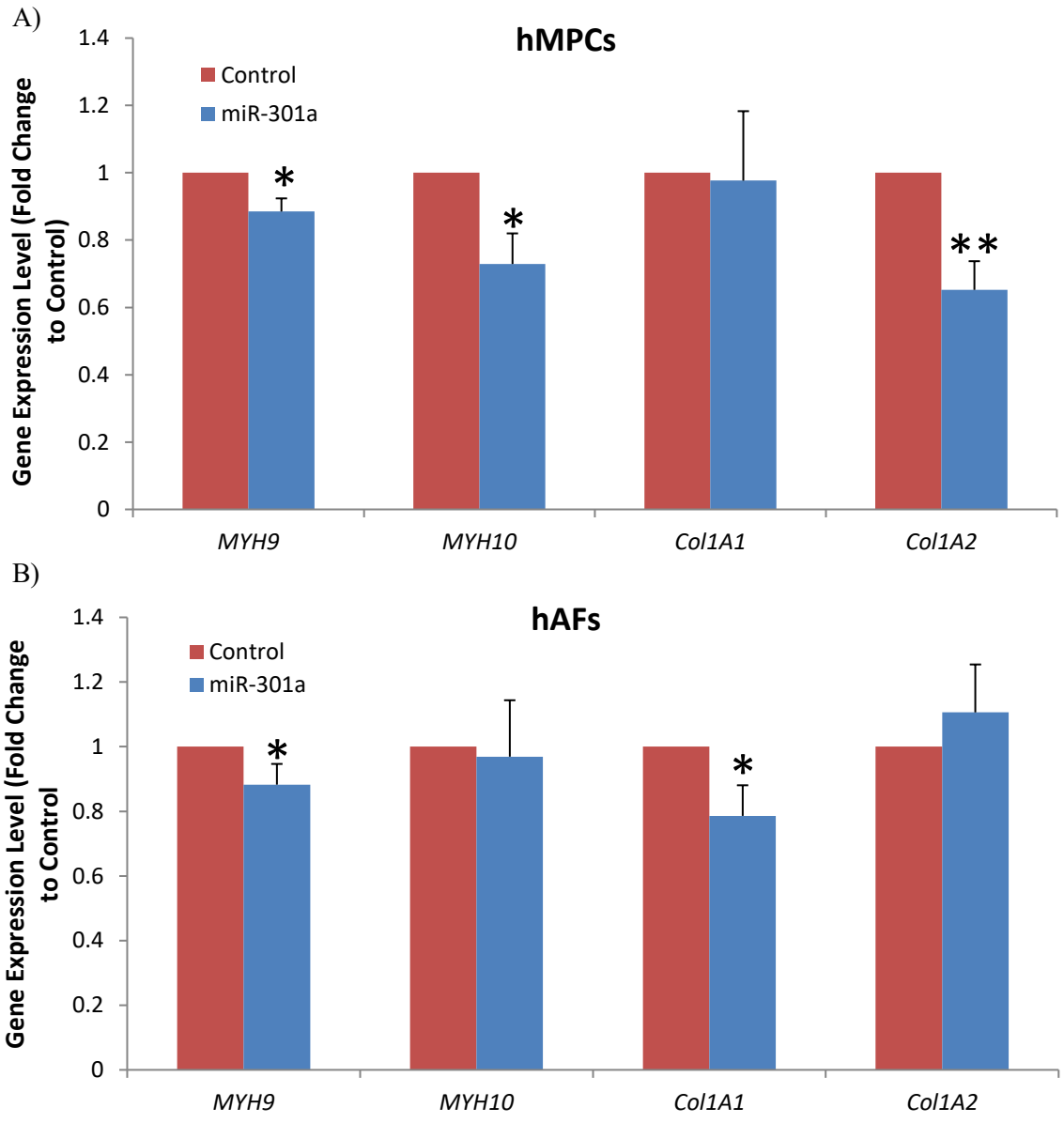


Figure 4.6: Protein level changes of non-muscle myosin IIA (NMMIIA), non-muscle myosin IIB (NMMIIB), pro-collagen type I (Sp1.D8), and smooth muscle myosin (SMM) in A) hMPCs and B) hAFs transfected with miR-301a compared to an Scrambled control (n=4-5) with representative WBs for ii) NMMIIA, iii) NMMIIB, iv) Sp1D8, and v) SMM. One-way ANOVA,* p-value< 0.05 compared to Control

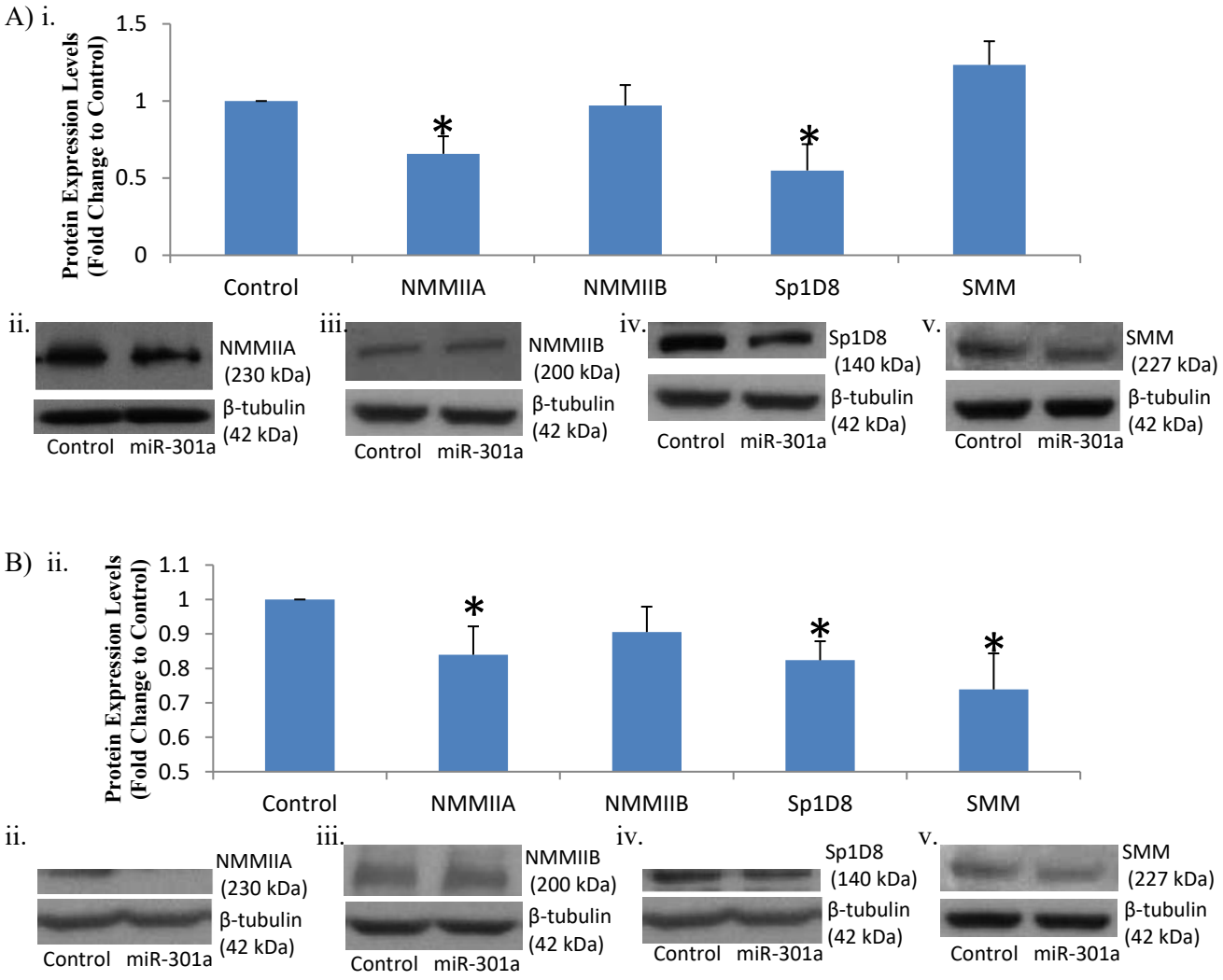
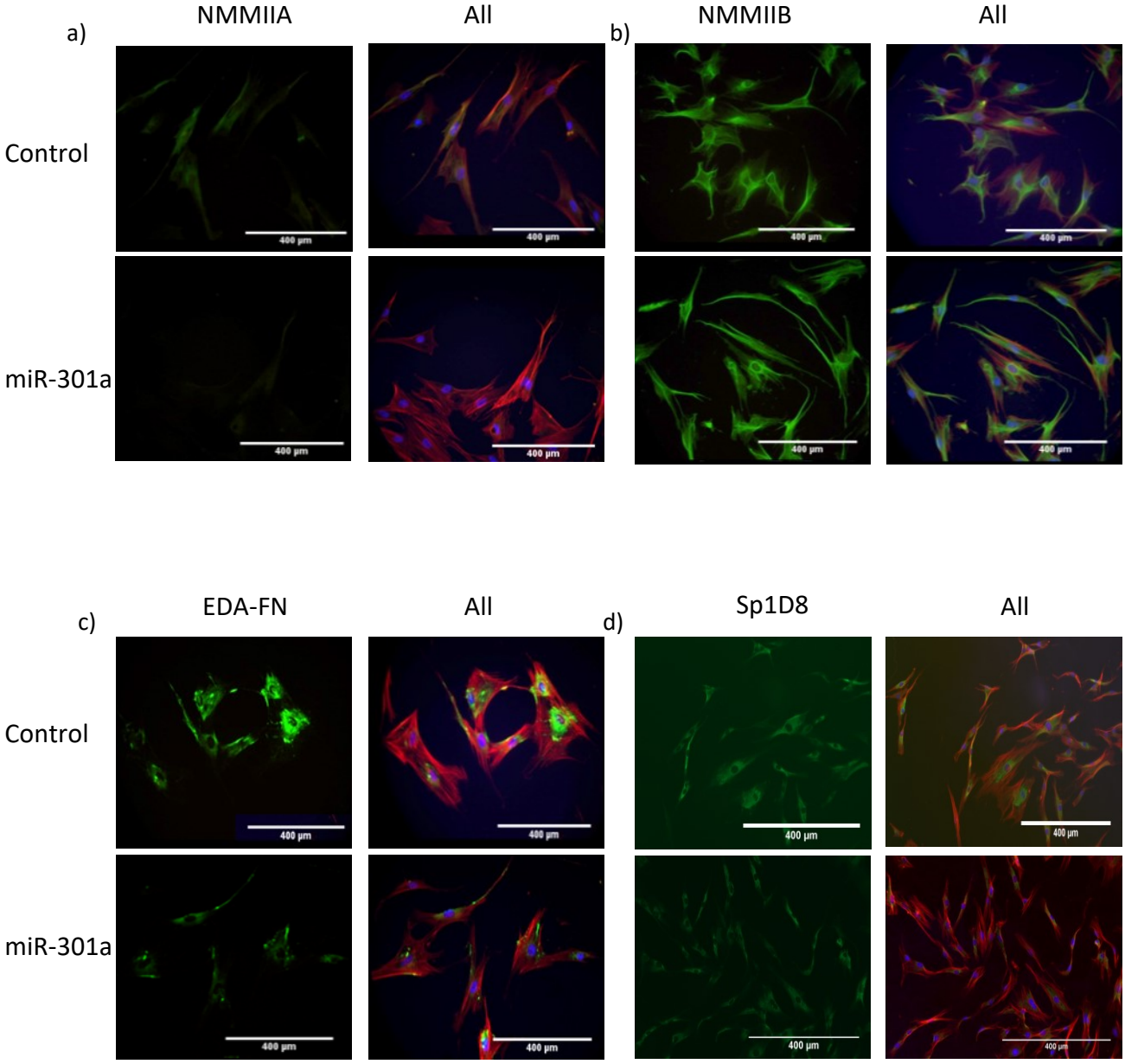


Figure 4.7: Immunofluorescent staining of hMPCs showing decreases in a) NMMIIA c) EDA-fibronectin (FN), d) Sp1D8 (green) and lack of change in b) NMMIIB (green) in cells transfected with miR-301a, with F-actin (red) and nuclei DAPI staining (blue), indicated by "All"



5. Phenotype Changes of Primary Human Bone Marrow-Derived Mesenchymal Progenitor Cells On Fibronectin-Coated Plates of Varying Surface Tensions Influenced by MicroRNA

By Müller AL, Li Y, Freed DH

Müller AL: Collected & cultured cells, performed all experiments except microarray (performed by Dr. Czubyrt's lab), experimental design, wrote manuscript

Abstract: Differentiation of human mesenchymal progenitor cells (hMPCs) has been shown to be influenced by the surrounding microenvironment. It is important to understand the physiological implications of the hMPC microenvironment regarding differentiation within the body, especially in patients with cardiovascular disease. This can be investigated by understanding the influence of differing extracellular matrix (ECM) stiffnesses found within the body, from the bone marrow to the infarct scar, to effectively treat patients recovering from myocardial infarction. As hMPCs must respond rapidly to their environment, the influence of microRNA at different surface tensions was also analyzed. HMPCs were isolated from the bone marrow of patients undergoing open heart surgery and cultured in standard DMEM/F12 with 20% FBS. These cells were plated on fibronectin-coated plates with surface tensions of 2kPa which simulates a bone marrow environment; 15kPa, which simulates a healthy left ventricle environment; and plastic (>1000 kPa), representing a pathological fibrotic environment. Protein and mRNA were collected for further analysis. Our data reveals that softer surface tensions, representing a bone marrow-nondifferentiating environment, cause a decrease in the protein expression of proteins expressed by myofibroblasts including EDA-fibronectin and alpha-smooth muscle actin. There were also increases in mRNA of myosin heavy chain-9 and 10, and collagen-1. Evaluation of miR-301a expression determined that, at 15kPa, it is increased. Interestingly, Dicer1, responsible for processing microRNAs, is upregulated at softer surface tensions and attenuated at 15kPa. These results indicate that ECM stiffness influences hMPC differentiation and the increase in Dicer1 found with softer matrices could represent an umbrella miRNA inhibition effect to effectively suppress hMPC differentiation.

5.1 Introduction

It has previously been shown that ECM surface tension helps influence progenitor cell differentiation (1), which demonstrates the importance of the ECM as a signaling element in determining cell fate. In an elegant study by Hinz's group (2), they showed that the expression of α-smooth muscle actin (α-SMA) altered according to substrate stiffness. In stiff environments simulating a fibrotic scar, the expression of α-SMA increased whereas it was decreased in softer environments. As previously observed, transfection of miR-301a inhibits a pro-fibrotic phenotype, indicating that the influence of ECM could occur during translation. Other miRNAs, including miR-21, miR-24, and miR-29 have been observed to be directly involved influencing a pro-fibrotic phenotype in mesenchymal cells (3-6). The influence of the mechanical nature of ECM is important in tissue engineering where various scaffolds are being developed in order to optimize the healing effect of various cell therapies. Traditionally, cell culturing occurs on plastic surfaces made up of polystyrene and subsequently treated by either corona discharge under atmospheric pressure or gas-plasma under vacuum in order to transform the hydrophobic surface to become more hydrophilic to permit cell binding (7). Although this method is relatively inexpensive and predominantly used for culturing cells, it is not ideal for representing a physiologically accurate environment.

The human body is composed of a variety of environments exhibiting different surface tension pressures. The influence of how extracellular environment affects progenitor cells endogenously is represented by the homeostatic lineages of bone marrow-derived MPCs. Although MPCs are derived from the soft bone marrow niche, they exhibit tri-lineage potential by undergoing adipogenic, chondrogenic, and osteogenic differentiation, which have increasingly stiff ECM (8). The study that initially reported the sensitivity of hMPCs to ECM stiffness showed that they are capable of differentiating into neurogenic, myogenic, and osteogenic lineages. It is important to note that prior to culturing on plates of varying surface tensions, hMPCs were chemically induced using the appropriate induction media to stimulate a targeted differentiation (1). This initial foray into elucidating the

mechanical influence of ECM on stem cell differentiation stimulated hundreds, if not thousands, of subsequent studies to understand the influence of ECM on stem cell differentiation. For example, the manipulation of the ECM protein hyaluronan promotes cardiac gene expression in embryonic stem cells (9). The importance of the structure of ECM was highlighted by an experiment comparing endogenous to exogenous collagen, where EC differentiation along a myogenic lineage was compromised and failed to be rescued by the mere addition of type 1 collagen (10).

Our lab is interested in investigating how various endogenous factors influence a pro-fibrotic response in patients with pathological fibrosis. The study published by Hinz's group (2) highlights the importance of surface tension pressures influencing the activation of hMPCs into ECM protein-secreting pro-fibrotic cells. It was observed that a decrease in surface tension pressures led to a decrease in α-smooth muscle actin (α-SMA); however, further evaluation regarding how decreased surface tension pressures could affect the expression of other pro-fibrotic markers is warranted. Our previous study (11), evaluated a number of different proteins, both intracellular and extracellular, that are involved in a pathological pro-fibrotic cell phenotype. It is important to evaluate the influence hMPCs have on the ECM as their differentiation affects their secretome. We hypothesize that the hMPCs cultured on less stiff environments will have reduced expression of both intracellular and extracellular pro-fibrotic molecules resulting in reduced differentiation into a myofibroblast-like cell phenotype.

Differentiation initiates a change in the miRNA profile in order to alter the expression of protein which ultimately defines the identity of a cell (12). There have been many miRNAs associated with the activation of mesenchymal pro-fibrotic cells, including miR-145, miR-21, miR-24, and miR-29 (13-17). More specifically, increased expression of miR-21 correlated with increased expression of pro-fibrotic mRNA expression of hMPCs on stiff substrates emulating a fibrotic scar. After miR-21 knock-down, the influence of the stiff substrate priming was attenuated, and hMPCs responded to softer substrates, indicating a role of miR-21 in preserving fibrotic mechanical memory (17). As previously observed,

increased miR-301a preserves a progenitor phenotype in hMPCs, so evaluation of how its expression is influenced by varying substrate pressures is warranted.

5.2 Materials & Methods

Isolation and Culture of Primary Human MPCs

Human MPC cultures were prepared based on plastic adherence according to the methods developed by Caplan and Friedenstein (18,19) with some modifications as previously described. Control hMPCs were plated onto 10 cm plastic culture dishes coated with 2ug/cm^2 of fibronectin, whereas experimental hMPCs were plated on 2 ug/cm^2 fibronectin-coated elastic silicone substrate culture plates exhibiting 2 kPa and 15 kPa of surface tension pressure. Plates at 2 kPa closely simulate the surface tension pressures found in the bone marrow whereas plates at 15kPa simulate the surface tension pressures of non-fibrotic cardiac muscle (20). It should be pointed out that each n-value of hMPCs cultured among the varying surface tension pressures were isolated from the same patient to reduce the variability between pressures as small as experimentally possible. After 24 hours, non-adherent cells (hematopoietic cells) were washed off with PBS and discarded, and the adherent cells were thoroughly washed twice with PBS. Fresh medium was added to the adherent cells and replaced every 3 or 4 days. Samples were harvested at P0 for protein and mRNA quantification analysis.

Western Blot Analysis

Passage 0 hAFs and hMPCs were lysed 48 hours post-transfection using the protein isolation and quantification techniques described in Chapter 2.6. Equal amounts of each protein sample (20µg) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel at 130 V. Separated proteins were then transferred to a polyvinylidenedifluoride (PVDF) membrane for 1 hour at 300 mA. Membranes were blocked with 5% non-fat skim milk in PBS (phosphate buffered saline) for 1 hour at room temperature. After blocking, antibodies against alpha-smooth muscle actin (1:1000; Abcam), non-muscle myosin IIA (NMMIIA, 1:2000; Abcam), EDA-fibronectin (1:1000; Santa Cruz), and pro-collagen I (1:1000;

Developmental Studies Hybridoma Bank), were used to evaluate the protein expression with their corresponding secondary antibody (goat anti-rabbit IgG, rabbit anti-mouse IgG; Jackson ImmunoResearch Laboratories) conjugated with horseradish peroxidase. The loading control used was β-tubulin (1:1000, Abcam) which was probed for after blots were stripped using 0.5 M NaOH stripping buffer. Proteins were visualized using Pierce ECL Western Blotting System (Thermoscientific) according to manufacturer's instructions. Densitometry was performed using GS-800 Calibrated Densitometer (BioRad) and quantified using Quantity One software.

RNA and qRT-PCR Analysis

Collection and quantification of mRNA from passage 0 hMPCs was performed utilizing techqniues described in Chapter 2.5. Expression of collagen1A1 (F – CCAAAGGATCTCCTGGTGAA; R – AGTTTTGCCATCAGGACCAG), collagen1A2 (F – TTGACCCTAACCAAGGATGC; R-TTCTTGGCTGGGATGTTTTC), myosin heavy chain 9 (forward (F) primer – ACACCGCCTACAGGAGTATGA; reverse (R) primer – ACACCGCCTACAGGAGTATGA), myosin heavy chain 10 (F – AGGTGGACTATAAGGCAGATGAG; R – CTGTCTGATGACTGGTGCAAAA), Dicer 1a (F – CATCATGTCCTCGCATTTTTG; R – GGTCAGTTGCAGTTTCAGCA), and Dicer 1b (F – GAAGACCAGGTTCCACGAAA; R – GGCTGATCAGGTCTGGGATA) were analyzed by quantitative real-time PCR. qPCR results were compared against β-actin which was used as a reference gene. Expression of mRNA was determined using MT Mini Personal Thermal Cycler (Bio-Rad) using Quanta Biosciences B-R 1-Step SYBR® Green qRT-PCR kit. Quantification of miR-301a was performed using quantitative real-time PCR using Taqman small RNA assay with U6 as a reference gene. Reverse transcription to make cDNA was performed using the TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Amplification of cDNA was used with Taqman®Universal PCR Master Mix (Applied Biosystems). U6 and miR-301a primers were provided by ThermoFischer Scientific.

Microarray Analysis of miR301a Transfected Cells

HMPCs were cultured on uncoated plastic and transfected with miR-301a. These cells were trypsinized and then sent for microarray analysis by Dr. Czubyrt's group in order to determine potential miR-301a targets that could be influencing an anti-pro-fibrotic phenotype. A list of interesting potential targets of miR-301a can be viewed at Table 5.1

Statistics

Statistical analysis was performed as described in Chapter 2 except analysis was done using SigmaStat 3.5 program. Microarray assay statistics performed by Dr. Czubryt's group.

5.3 Results

Varying Surface Tensions Influence Pro-Fibrotic Protein Expression

Plates exhibiting 2 kPa and 15 kPa of surface tension represent the bone marrow and cardiac muscle environmental surface tension pressures, respectively (8) where expression of pro-fibrotic protein markers α-SMA and NMMIIA were found to be significantly decreased (Figure 5.1) compared to a plastic control, representing fibrotic scar tissue. At 15 kPa, expression of α-SMA was decreased by more than 50%; however, there was no significant difference determined between the protein expression of α-SMA or NMMIIA between 2 kPa and 15 kPa. Protein analysis of extracellular matrix proteins EDA-FN and Col1 (Fig 5.2) were significantly decreased when hMPCs were cultured on 2 kPa plates. Interestingly, protein expression of EDA-FN at 15 kPa was not found to be statistically different from either control or 2 kPa culture conditions; however, Col1 protein expression was found to also be significantly decreased at 15 kPa. Col1 also expressed the most significant decrease in expression of the pro-fibrotic proteins being evaluated by only expressing ~20% of its original expression compared to control at both 2 kPa and 15 kPa.

Varying Surface Tensions Alter mRNA Expression of Pro-Fibrotic Markers

Although protein expression of various pro-fibrotic myofibroblast markers were significantly reduced when cultured on plates exhibiting 2 kPa of surface tension pressure, mRNA of Col1s, MYH9, and MYH10 exhibited different expression (Fig 5.3). Genetic expression of Col1A1, Col1A2, MYH9, and MYH10 were all significantly increased compared to control; however when measuring the mRNA levels of these genes at 15 kPa it was calculated that they were significantly decreased. This decrease in mRNA expression was statistically different when compared to both the 2 kPa increase in expression as well as to control when evaluating Col1A1 and Col1A2. Gene expression of MYH9 and MYH10 was noted to be slightly increased at a 15 kPa surface tension pressure compared to control. Overall, it appears that expression of both extracellular collagen and myosin heavy chain expression was increased at softer surface tension pressures simulating a bone marrow environment, but then was reduced when exposed to pressures found in healthy cardiac muscle tissue.

miR-301a Targets Dicer1 Whose Expression Changes by Varying Surface Tensions

As protein expression results mirrored the results previously observed with miR-301a transfection, quantification of miR-301a at various surface tension pressures was measured (Fig 5.4). Evaluation of miR-301a expression at both 2 kPa and 15 kPa were both increased, however, it should be noted that miR-301a expression of hMPCs cultured at 15 kPa was nearly 2-fold higher compared to hMPCs cultured at 2 kPa. Furthermore, microarray analysis of transfected cells indicated that one of the potential inhibitory targets for miR-301a is Dicer1 (Table 5.1) as Dicer1 was found to be decreased by 1.62 fold (p-value<0.005) in miR-301a transfected cells. In order to evaluate whether or not Dicer1 was influenced by changes in surface pressure tension, mRNA expression of Dicer1a and Dicer1b was quantified. Dicer1's pattern of expression mirrored that of Col1 and MYH where there was a significant increase of expression at 2 kPa which was decreased at 15 kPa. It should be noted that although that a significant decrease was only observed with the Dicer1a transcript, the Dicer1b transcript was not statistically different.

Interestingly, the mRNA expression of Dicer1a was also significantly decreased compared to control as well.

5.4 Discussion

As hypothesized, this study further demonstrates that hMPCs are influenced by changes in surface tension pressures. In these experiments, 2 kPa represents the surface tension experienced by hMPCs residing in the bone marrow, their niche environment that maintains their progenitor phenotypic state (21). Surface tension pressure at 15 kPa mimics the pressure of the cardiac muscle environment, whereas the control fibronectin-coated plastic plates represent a stiff, pro-fibrotic scar environment (20). The softer environments led to a decrease in pro-fibrotic protein expression indicating a preference for maintaining a progenitor-like phenotype. At surface tensions reminiscent of bone marrow, proteins responsible for a profibrotic phenotypic change and secreted ECM proteins were decreased. This decrease in protein expression was maintained at pressures simulating healthy cardiac LV tissue. The stiff environment of the control showed increased expression of a pro-fibrotic cell phenotype, as indicated in previously in our lab (11); however, the expression of EDA-FN was not different from either the bone marrow or the stiff scar environment. This may be a result of working with primary human tissue, where there is high variability between patients, but also because EDA-FN is an ECM that is secreted to maintain homeostasis (22).

Interestingly, it is important to note that the changes in protein expression at 2 kPa, representing a bone marrow environment, were completely opposite from the mRNA expression. This indicates that the potential of differentiation into a pro-fibrotic phenotype is readily available as the translation step from DNA to mRNA has already been performed. When measuring mRNA levels, it is possible to quantify mRNA inhibited by miRNA as the primers may attach to a portion of the mRNA different from the where the miRNA is preventing transcription. Therefore, we propose that this difference between mRNA and protein expression is evidence that hMPCs in a progenitor state are “preparing” for responding to an

injury. It is important to note that this discrepancy between mRNA and protein expression occurred in proteins responsible for a phenotype transition from a progenitor to a pro-fibrotic phenotype, as well as with collagen, a secreted ECM protein that make up a significant portion of the proteins found in fibrotic scarring (23). This consistency of expressions between distinct functions in pro-fibrotic hMPCs could indicate a priming of these progenitor cells in response to pro-inflammatory markers. It has been established in a number of studies that hMPCs respond to pro-inflammatory signals by promoting fibrosis in areas of injury (21, 24-28); hence, it would be pragmatic for progenitor cells, when responding to an injury, are ready to differentiate into an ECM protein secretory cell relatively rapidly.

Although mRNA expression at bone marrow simulated surface tension pressure was inversely correlated with protein expression, mRNA levels substantially decreased when cells were cultured on pressures representing a healthy cardiac LV environment. The sensitivity to different pressures highlights the functional importance of ECM with regards to determining progenitor cell fate, including cells responsible for responding to pro-fibrotic stimuli. The expression of collagen was also significantly decreased compared to control, as healthy LV does not require excessive expression of collagen. It is not well understood why there is an increase in mRNA expression of myosins with cells cultured at 15 kPa; however, this was not reflected in the protein expression so this could reflecting a snapshot of an intermediary stage for hMPCs that find themselves on healthy, contractile LV myocardium.

The quantification of miR-301a was performed at various surface tensions in order to investigate the similarities regarding the inhibition of pro-fibrotic phenotypic differentiation upon transfection with miR-301a and the stiffness representing healthy physiological environments. The expression of miR-301a was also influenced by softer substrates, indicating an increased retention of an undifferentiated, non-pro-fibrotic phenotype. Interestingly, the expression of miR-301a was significantly more expressed at a healthy LV tissue pressure than in the un-differentiated bone marrow environment. The reason as to why there is increased expression in a simulated healthy LV environment versus the endogenous bone marrow environment of the bone marrow warrants further investigation. However, as our lab was intrigued by this

result, a miR-301a microarray assay was performed to look for potential targets that may influence the inhibition of a pro-fibrotic phenotype. Among the thousands of potential targets found, a significant portion of them appear to be involved in DNA replication, mitosis, and ECM maintenance (Table 5.1). The one that peaked the most significant interest was that of Dicer1, which was found significantly decreased in miR-301a transfect hMPCs. Dicer1 is an evolutionarily highly conserved protein of interest as it is crucial in miRNA processing by facilitating the activation of miRNAs, first discovered to be responsible for activating miRNAs involved in development (29). It is now known that that majority of miRNAs require Dicer1 as a crucial step in their activation (30), including miR-301a which suggests a unique mechanism of self-regulation. This increase in expression of Dicer1 could indicate a shift in the miRNA profile which has been observed a variety of cell types (31-33). In fact, miRNAs have been found to be able to, not only mediate the differentiation of progenitor cells into a final phenotype, but can also conversely return differentiated cells into a stem-cell like state (12, 34-36). The significance of Dicer1 in embryonic cell differentiation was discovered when disruption of the Dicer1 gene was lethal early in development (12). The functioning of this modular capability of miRNAs is dependent on their activation by Dicer1; hence, the significance of assessing varying expressions of Dicer1 at different surface tension pressures. The increase in Dicer1 mRNA expression at 2 kPa and decrease at 15 kPa, parallel to mRNA expression of genes involved in pro-fibrotic cell differentiation, further illustrates the potential for hMPCs to in preparative status where these cells can quickly respond to damage signaling.

In conclusion, this evaluation of hMPCs indicates that they are solely susceptible to changes in surface tension pressures which influence the expression of pro-fibrotic markers. The softer pressures representing healthy physiological environments did not stimulate differentiation into a myofibroblast-like phenotype; however, hMPCs remaining in their niche environment may be better prepared to respond to damage by increasing the available of pro-fibrotic mRNA. The changes in miR-301a expression in response to healthy environments may influence the progenitor hMPC phenotype by inhibiting Dicer1, responsible for activating miRNAs which are crucial in stem cell differentiation. Further investigation

into how miR-301a and Dicer1 influence phenotype differentiation is warranted. Overall, it is evident that the mechanical properties of healthy ECM environments attenuate hMPC differentiation into myofibroblast-like cells.

5.5 References

1. Engler AJ, Sen S, Sweeney HL, et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126(4): 677-689

2. Talele NP, Fradette J, Davies JE, et al (2015) Expression of α-smooth muscle actin determines fate of mesenchymal stromal cells. Stem Cell Reports 4(6): 1016-1030

3. van Rooij E, Sutherland LB, Thatcher JE, et al (2008) Dysregulation of miroRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105: 13027-13032

4. Roy S, Khanna S, Hussain SR, et al (2009) MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 82: 21-29

5. Wang J, Huang W, Xu R, et al (2012) MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med 16(9): 2150-2160

6. Li CX, Talele NP, Boo S, et al (2017) MicroRNA-21 preserves the fibrotic memory of mesenchymal stem cells. Nat Mater 16(3): 279-289

7. Ryan JA (2008) Evolution of cell culture surfaces. BioFiles 3: 21

8. Dominici M, Le Blanc K, Mueller I, et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy Position Statement. Cytotherapy 8: 315-317

9. Chen SS, Fitzgerald W, Zimmerberg J, et al (2007) Behaviour of cardiomyocytes and skeletal muscle cells on different extracellular matrix components – relevance for cardiac tissue engineering. Artif Organs 31(1):4-12

10. Terracio L, Rubin K, Gullberg D, et al (1991) Expression of collagen binding integrins during cardiac development and hypertrophy. Circ Res 68(3): 734-744

11. Ngo M, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

12. Bernstein E, Kim SY, Carmell MA, et al (2003) Dicer is essential for mouse development. Nat Genet 35: 215-217

13. Möllman H, Nef HM, Kostin S, et al (2006) Bone marrow-derived cells contribute to infarct remodeling. Cardiovasc Res 71: 661-671

14. van Rooij E, Sutherland LB, Thatcher JE, et al (2008) Dysregulation of miroRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105: 13027-13032

15. Roy S, Khanna S, Hussain SR, et al (2009) MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 82: 21-29

16. Thum T, Gross C, Fiedler J, et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts. Nature 456: 980-984

17. Chen X, Talele NP, Boo S, et al (2017) MicroRNA-21 preserves the fibrotic mechanical memory of mesenchymal stem cells. Nature Materials 16: 379-389

18. Friedernstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 1976; 4: 267-274

19. Caplan AI. Mesenchymal stem cells. J Orthop Res 1991; 9: 641-65020. Zimina E, and Hinz B (2015)

20. The stressful life of cardiac myofibroblasts. Cardiac Fibrosis and Heart Failure: Cause or Effect? Eds Dixon IMC, and Wigle, JT. Springer International Publishing Switzerland.

21. Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2: 313-319

22. Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326: 1216-1219

23. Ma Y, Halade GV, and Lindsey ML (2012) Extracellular matrix and fibroblast communication following myocardial infarction. J Cardiovasc Transl Res 5:848- 857

24. Kania G, Blyszczuk P, Valaperti A, et al (2008) Prominin-1+/CD133+ bone marrow-derived heart-resident cells suppress experimental autoimmune myocarditis. Cardiovasc Res 80: 236-245

25. Endo J, Sano M, Fujita J, et al (2007) Bone marrow derived cells are involved in the pathogenesis of cardiac hypertrophy in response to pressure overload. Circulation 116: 1176-1184

26. Guo J, Lin GS, Bao CY, et al (2007) Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30(3-4): 97-104

27. Lee RH, Seo MJ, Pulin AA, et al (2009) The CD34-like protein PODXL and alpha6-integrin (CD49f) identify early progenitor MPCs with increased clonogenicity and migration to infracted heart in mice. Blood 113(4): 816-826

28. van Amerongen MJ, Bou-Gharios G, Popa E, et al (2008) Bone marrow-derived myofibroblasts contribute functionally to scar after myocardial infarction. J Pathol 214: 377-386

29. Wienholds E, Koudijs MJ, van Eeden EJ, et al (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet 35: 217-218

30. Feng Y, Zhang X, Graves P, and Zeng Y (2012) A comprehensive analysis of precursor microRNA cleavage by human Dicer. RNA 18: 2083-2092

31. Stappert L, Borghese L, Roese-Koerner B, et al (2013) MicroRNA-based promotion of human neuronal differentiation and subtype specification. PLoS One 8: e59011

32. Dalmosso G, Nguyen HT, Yan Y, et al (2010) MicroRNAs determine human intestinal epithelial cell fate. Differentiation 80: 147-154

33. Ribeiro AO, Schoof CRG, Izzotti A, et al (2014) MicroRNAs: modulators of cell identity, and their applications in tissue engineering. Microrna 3: 45-53

34. Marson A, Levine SS, Cole MF, et al (2008) Connecting miRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 134: 521-533

35. Eulalio A, Mano M, Dal Ferro M, et al (2012) Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492: 376-381

36. Suh MR, Lee Y, Kim JY, et al (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270: 488-498

5.6 Figures

Figure 5.1: Western blot densitometry analysis of myofibroblast markers a) alpha-smooth muscle actin and b) non-muscle myosin IIA showing a decrease at 2kPa and 15kPa in hMPCs grown on fibronectin-coated plates compared to Control (n=5). One-way ANOVA,* p-value< 0.05 compared to Control

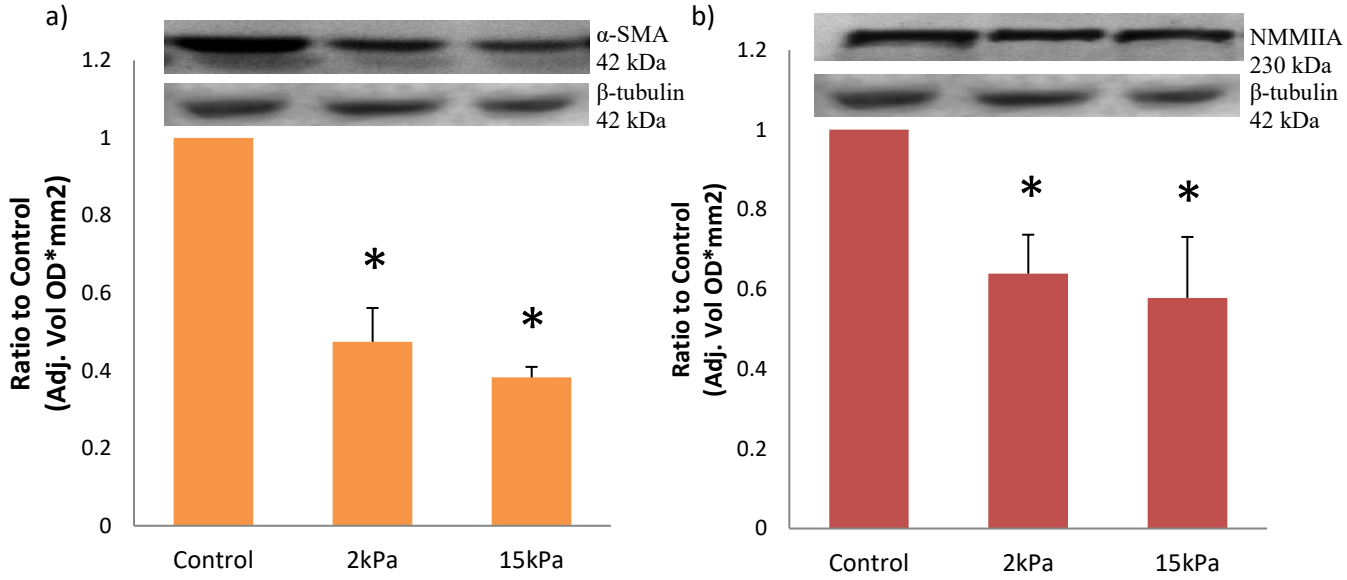


Figure 5.2: Western blot densitometry analysis of extracellular matrix proteins a) EDA fibronectin and b) pro-collagen-type I showing a decrease in 2kPa and 15kPa in hMPCs grown on fibronectin-coated plates compared to Control (n=5). One-way ANOVA* p-value< 0.05 compared to Control

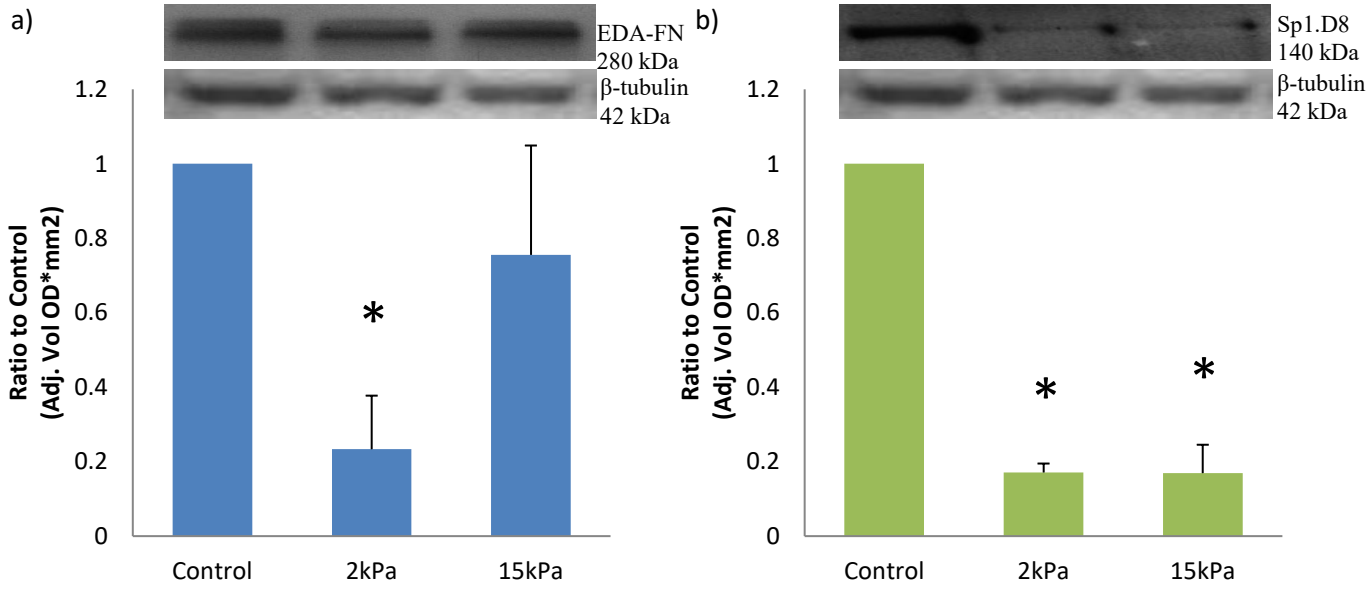


Figure 5.3: mRNA analysis of a) *collagen1-alpha1* and b) *myosin heavy chain-9 and -10* in hMPCs grown on fibronectin-coated plates indicating a significant increase from Control at 2kPa and a significant decrease compared to 15 kPa compared to 2kPa, n=4. Paired student t-test, one-way ANOVA, * p-value < 0.05 compared to Control; ** p-value <0.01 compared to Control; # p-value <0.05 compared to 2 kPa plates; ## p-value < 0.01 compared to 2 kPa plates

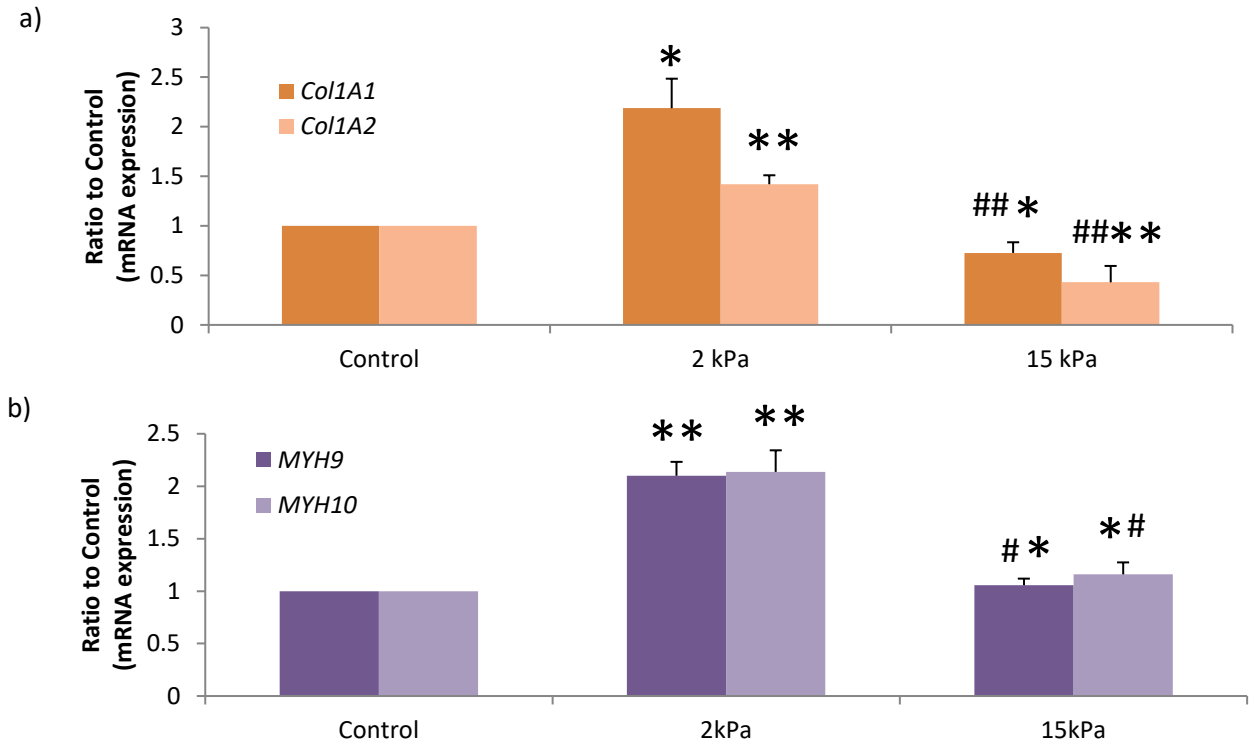


Figure 5.4: Quantification of *miR-301a* expression in hMPCs grown on fibronectin-coated plates indicating increases at both 2 kPa and 15 kPa, n=4. Paired student t-test, one-way ANOVA, * p-value <

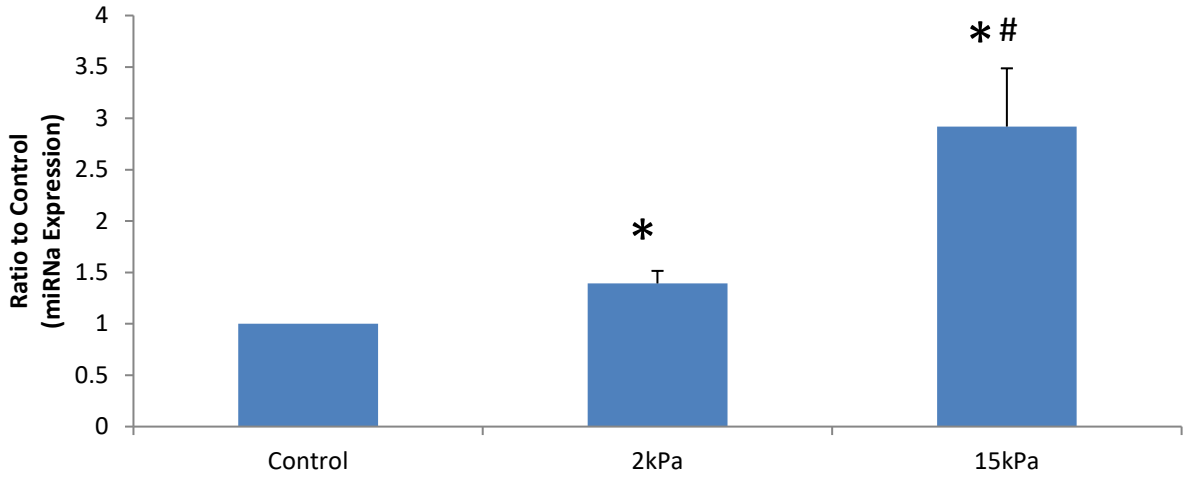


Figure 5.5: mRNA analysis of *Dicer 1a* and *Dicer 1b* transcripts expressed in hMPCs grown on fibronectin-coated plates indicating a increases at both 2 kPa and changes in expression at 15 kPa, n=4 patients. Paired student t-test, one-way ANOVA, * p-value < 0.05 compared to normalized corresponding *Dicer* Control; # p-value < 0.05 compared to 2 kPa plates; ## p-value < 0.01 compared to 2 kPa plates

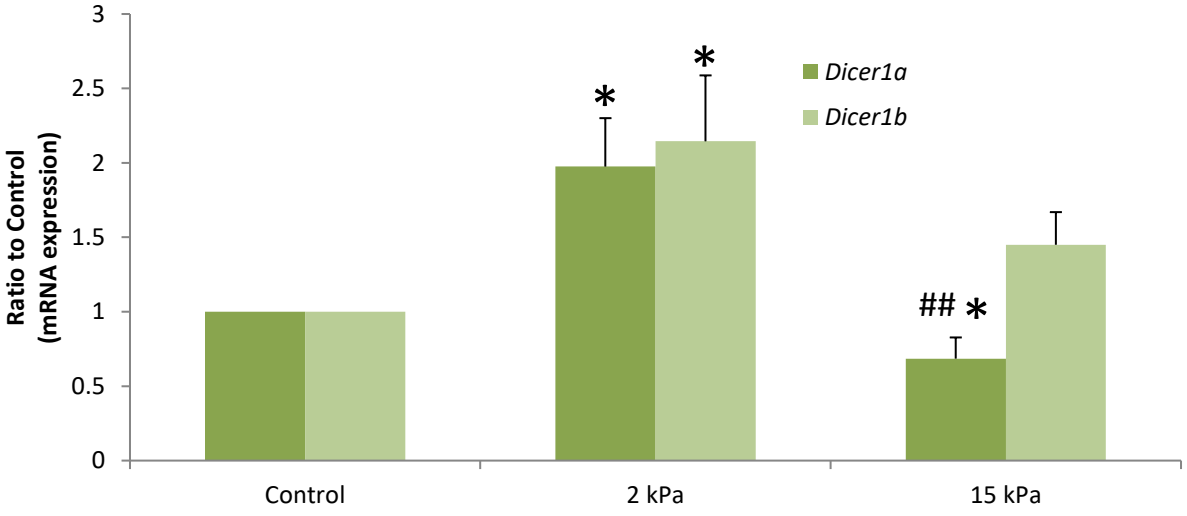


Table 5.1: Potential targets of miR-301a determined by microarray assay of transfected hMPCs cultured on plastic.

General Function	Target Gene	Gene Name	Total Context Score	P_{CT}
	CCND3	cyclin D3	-0.09	0.74
	CCNY	cyclin Y	-0.4	0.87
Cell Cycle	CDK2AP2	cyclin-dependent kinase 2 associated protein 2	-0.28	0.36
	CDK19	cyclin-dependent kinase 19	-0.28	0.94
	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	-0.16	0.58
	PAK6	p21 protein (Cdc42/Rac)-activated kinase 6	-0.24	0.86
	EIF2C1	eukaryotic translation initiation factor 2C, 1	-0.23	0.9
	EIF2C4	eukaryotic translation initiation factor 2C, 4	-0.46	0.91
Protein Synthesis	EIF4E3	eukaryotic translation initiation factor 4E family member 3	-0.17	0.97
	E2F2	E2F transcription factor 2	-0.14	0.86
	E2F7	E2F transcription factor 7	-0.24	0.89
	RPS6KA2	ribosomal protein S6 kinase, 90kDa, polypeptide 2	-0.15	0.6
	DICER1	dicer1, ribonuclease type III	-0.16	0.99
	MEOX2	mesenchyme homeobox 2	-0.37	0.7
	ZEB2	zinc finger E-box binding homeobox 2	-0.23	0.68
	WNT1	wingless-type MMTV integration site family, member 1	-0.24	0.9
	WNT2B	wingless-type MMTV integration site family, member 2B	-0.31	0.75
	WNT10A	wingless-type MMTV integration site family, member 10A	-0.24	0.61
Cell Differentiation	FZD3	frizzled family receptor 3	-0.17	0.64
	FZD6	frizzled family receptor 6	-0.27	0.67
	KLF3	Kruppel-like factor 3 (basic)	-0.17	0.94
	KLF13	Kruppel-like factor 13	-0.16	0.91
	SOX4	SRY (sex determining region Y)-box 4	-0.26	0.73
	SOX5	SRY (sex determining region Y)-box 5	-0.12	0.58
	SOX21	SRY (sex determining region Y)-box 21	-0.13	0.64
	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-0.13	0.81
	IGF1	insulin-like growth factor 1 (somatomedin C)	-0.48	> 0.99
	EDN1	endothelin 1	-0.15	0.37
	TNF	tumor necrosis factor	-0.23	0.57
	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	-0.25	0.83
	BMP3	bone morphogenetic protein 3	-0.28	0.91
	BMPR1B	bone morphogenetic protein receptor, type IB	-0.17	0.55
Signal Transduction	BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	-0.32	0.9
	ACVR1	activin A receptor, type I	-0.46	0.96
	ACVR1C	activin A receptor, type IC	-0.24	0.87
	TGFB2	transforming growth factor, beta 2	-0.1	0.85
	TGFBR1	transforming growth factor, beta receptor 1	-0.16	0.75
	SMAD4	SMAD family member 4	-0.14	0.79
	SMAD5	SMAD family member 5	-0.23	0.88
	SNIP1	Smad nuclear interacting protein 1	-0.21	0.46
	PTEN	phosphatase and tensin homolog	-0.33	0.93

General Function	Target Gene	Gene Name	Total Context Score	P_{CT}
Signal Transduction	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	-0.22	0.83
	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	-0.15	0.35
	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	-0.21	0.88
	S1PR1	sphingosine-1-phosphate receptor 1	-0.19	0.82
	S1PR2	sphingosine-1-phosphate receptor 2	-0.13	0.81
	SOCS5	suppressor of cytokine signaling 5	-0.1	0.46
	SOCS6	suppressor of cytokine signaling 6	-0.18	0.86
	MAPK1	mitogen-activated protein kinase 1	-0.16	0.84
	MAPK10	mitogen-activated protein kinase 10	-0.18	0.35
	MAP3K9	mitogen-activated protein kinase kinase kinase 9	-0.28	0.91
	MAP3K12	mitogen-activated protein kinase kinase kinase 12	-0.17	0.75
	MAP3K14	mitogen-activated protein kinase kinase kinase 14	-0.09	0.78
	MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	-0.04	0.76
	EFNB2	ephrin-B2	-0.17	0.58
	EPHA7	EPH receptor A7	-0.04	0.69
	EPHB4	EPH receptor B4	-0.13	0.78
ECM, Cytoskeleton, and Cell Adhesion	GJA1	gap junction protein, alpha 1, 43kDa	-0.56	0.48
	FNDC4	fibronectin type III domain containing 4	-0.26	0.74
	TIMP2	TIMP metallopeptidase inhibitor 2	-0.14	0.86
	ADAMTS19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	-0.16	0.72
	ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-0.08	0.83
	ITGA11	integrin, alpha 11	-0.14	0.41
	ITGB8	integrin, beta 8	-0.05	0.68
	MYO1D	myosin ID	-0.12	0.45
	MYO10	myosin X	-0.09	0.76
	COL19A1	collagen, type XIX, alpha 1	-0.08	0.71

6. Decellularized Human Ventricular Matrix as a Model for Studying Human Mesenchymal Cell Phenotypes Using Primary Human Mesenchymal Cells

By Müller AL, Freed DH

Müller AL: Performed all experiments, experimental design, wrote manuscript

Freed DH: Experimental design, edited manuscript

Abstract: As a result of pioneering research unveiling the influence of extracellular environment in determining stem/progenitor cell differentiation, a myriad of cellular scaffolds have been developed in order to optimize the delivery of these cells in a diseased environment as a technique to improve their efficacy in stem cell therapy. Although a variety of scaffolds are being studied in different contexts, the most physiologically relevant ones are those which most closely resemble the environment to be treated. Our lab has a keen interest in cardiovascular disease and we have recently optimized a technique that decellularizes and reseeds isolated sections of healthy porcine and human ventricular tissue with primary human atrial cells. Porcine and human ventricular tissue is sliced between 100-200 μm and subject to a multi-step, multi-reagent washing process over a period of four days in order to decellularize the tissue, utilizing various detergents and DNA-destroying reagents. ELISAs of pro-inflammatory markers IL-6 and TNFα were assessed at each washing stage to evaluate washing efficacy and to ensure seeded cells were not placed in a pro-inflammatory environment. After decellularization, tissue slices were co-incubated with isolated primary human atrial fibroblasts (hAFs) or bone marrow-derived mesenchymal progenitor cells (MPCs). Cells were isolated from patients undergoing open heart surgery. HAFs and hMPCs were incubated separately on sections of decellularized human ventricular ECM over a period of 2-3 weeks and then visualized using a variety of microscopy techniques, H&E staining, and IF imaging. Our technique was successful in reseeding decellularized porcine ventricular extracellular matrix determined by the visual presence of cells on recellularized tissue sections compared with decellularized tissue sections. This indicates that the technique was successful in reseeding decellularized human ventricular extracellular matrix and that it can be used to compare differences among cell types and their responses between healthy and damaged or diseased cardiac ECM among different hearts and even within the same heart at a fraction of the resources required to decellularize and recellularize an entire organ.

6.1 Introduction

The field of tissue engineering has led to significant advancements in regenerative technology. One of the most revolutionary approaches, responsible for propelling this field forward, was developed by Dr. Doris Taylor's group when they were successfully able to decellularize and then effectively recellularize rodent hearts which could generate pump function (1). This technique was recently shown to be effective in a porcine animal model (2), representing its potential for becoming a viable clinical option for organ transplant. Since this crucial discovery, decellularizing ECM has become a common method in generating scaffolding for a variety of tissues including skin, bladders, vasculature, adipose tissue, pericardium, ligaments, and tendons, many of which are currently used clinically (3-10). Although currently in the developmental phase, the most recent concept utilizing decellularization involves expanding the donor organ pool by developing a biological artificial heart (11), potentially using recently deceased donor organs as the source of viable, human ECM to then be repopulated by the recipient's own progenitor cells collected at an earlier date.

Despite the ground-breaking impact of decellularization in the tissue engineering field, there has been limited application of this technique to better understand physiological responses in diseased tissues. A paper by Parker et al (12) used this technique to assess mRNA expression in tissue procured from patients with idiopathic pulmonary fibrosis (IPF); however, there is currently no application of this technique exploring cardiac fibrosis. There is significant potential in utilizing decellularized diseased myocardium procured from explanted patients' hearts as it could be the most physiological relevant model when evaluating how primary human cells respond to fibrotic cardiac tissue. Our lab saw an opportunity to use this technology to develop an *in vitro* model as physiologically relevant as possible using human explanted hearts and primary human cells isolated from patients undergoing open heart surgery.

Utilization of this technique can also be used to better understand how progenitor cells endogenously respond to either pro-fibrotic or healthy extracellular environments. Our previous experiments, and others

(13-16), have demonstrated the importance of the extracellular environment in determining progenitor cell fate. This is important when developing ECM-based tissue engineering technologies. Although there have been many basic science and clinical studies evaluating multitudinous ECM environments (3-10), very few of them have published evaluations of the response of circulating and endogenous progenitor cells contributing to the damaged, fibrotic environment being treated. The development of a technique utilizing human cardiac extracellular matrix is important as it represents a physiologically accurate environment to elucidate endogenous cell responses to ECM. In this experiment, a decellularization and recellularization technique is presented that can be used to evaluate physiological responses to human ECM.

6.2 Materials and Methods

Isolation and Culture of Primary Human MPCs and Human AFs

. HMPC and hAF cultures were prepared based on plastic adherence according to the methods described in Chapter 2. HAFs and hMPCs were cultured in 10 cm^2 dishes until 90-95% confluence when they were harvested at P1-P2 for co-incubation with decellularized human left ventricular tissue.

Decellularization Protocol

A schematic of the decellularization process and protocol is represented by Figure 2a. Cardiac tissue was acquired from un-utilized donor hearts (control) and excised explant hearts from consented patients undergoing cardiac transplant surgery. Large chunks of tissue were placed in containers surrounded by OCT, rapidly frozen in liquid nitrogen, and stored at -80°C. In order to prepare smaller sections, frozen tissue was sliced at ~150-200 nm thickness using a cryostat, put in cold PBS, and warmed to room temperature. The following decellularization procedure was slightly modified from that utilized in Parker et al's paper (12) and performed in a sterile environment. First, tissue slices were placed in histological cassettes within a 100-mm Petri dish to allow flow of solutions with minimal damage to tissue slices. All oscillation rates during this decellularization procedure were at 30 oscillations per minute at room temperature, unless otherwise specified. All solutions were autoclaved prior to use except for the DNAse

(20µg/mL) plus 4.2 mM MgCl solution which was vacuum filtered using a 0.22 µm pore membrane. Two 1 hour cycles of the first lysis solution (1% SDS in H_2O) was used before a third cycle was oscillated overnight. The following day, the first lysis solution was removed and replaced with 1% Triton X-100 in H_2O (the second lysis solution) and oscillated for three 1 hour long cycles of decellularization using fresh solution at the beginning of each cycle. After the final hour cycle using the second lysis solution, the final cellular lysis step used 1% Triton X-100 solution oscillating overnight. The tissue slices were then thoroughly rinsed by using two rounds of PBS followed by 8 rinses of double distilled H_2O (DDW), essentially until no more bubbles were observed during the rinse steps. In order to thoroughly remove any residual nuclei or DNA, the following steps were performed. Firstly, 1 M NaCl (in H_2O), was added to the tissue and oscillated for 1 hour. Afterwards, treatment with DNAse (20µg/mL) plus 4.2 mM MgCl (in H_2O) at 37°C occurred for an hour, oscillating at 25 oscillations per minute. The tissue was subsequently rinsed twice with DDW and then twice with PBS. The final step, prior to storage, was to protect the tissue from bacterial contamination by oscillating it with Hank's Buffered Saline Solution (HBSS) plus 100 U/mL penicillin and 100 µg streptomycin (P/S) for 30 minutes. Afterwards, the tissue was removed from the histology cassettes and stored in HBSS plus P/S at 4°C. Prior to co-incubation with either hMPCs or hAFs, selected decellularized sections were rinsed twice with PBS at RT, and incubated in the same culture media used to grow the corresponding cell type at 37°C overnight. Co-incubation with hMPCs or hAFs proceeded the following day.

Recellularization Protocol

At 90-95% confluency, P1 primary hMPCs and hAFs were trypsinized using TrypLE and centrifuged at 2000xG for 7 minutes to pellet cells. They were re-suspended in 1 mL of media (DMEM-F12, 20% FBS, 100 µg/mL of Primocin™, and 100 mM ascorbic acid) and added to 15 mL tubes so that each tube contained ~5,000,000-10,000,000 cells. Five to ten pieces of decellularized HLV were added to each tube and the volume of media brought up to 10 mL. Tubes were placed on a battery powered rotating device and kept in an incubator at 37°C (95% CO_2, 5% O_2) overnight. The following day, recellularized were

rinsed 2X in PBS prior to being put in fresh media and returned, rotating, into the incubator where media was changed every 3 days for two to three weeks. Recellularization was deemed complete after media colour change rate was equivalent to plates at 90% confluency. Slices were fixed in the appropriate fixation solution (either 4% paraformaldehyde or specified for SEM and TEM).

H&E Staining

Staining was performed on paraffin-embedded recellularized healthy and explants HLV myocardial samples after deparaffinization using SafeClear Xylene substitutes (Fisher Scientific) following the manufacturer's instructions. After rehydration, slices were washed briefly in DDW before being stained with Harris hematoxylin solution for 8 minutes. After washing for 5 minutes under running distilled water, specimens were differentiated in 1% acid alcohol for 30 seonds, washed under running water for 1 minute, and put in saturated lithium carbonate solution for 1 minute. After washing under running water for 5 minutes, samples were rinse in 95% ethanol 10x and then counterstained using eosin-phloxine solution for 1 minute. Specimens were then dehydrated, mounted, and viewed using an Olympus SZ61 TR Stereo Light Microscope with an OPLENIC Digital Eyepiece camera.

Quantification of Pro-Inflammatory Markers

Aliquots of solution at the end of each different incubation solution used in the decellularization process (1% SDS, 0.1% Triton X-100, NaCl, DNase, HBSS, and media) were stored at -80°C until later use. Analysis of pro-inflammatory markers tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) was performed using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) following the manufacturer's directions.

Scanning and Transmission Electron Microscopy

Decellularized and recellularized samples for both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were fixed in a solution of 2.5% glutaraldehyde, 2%

paraformaldehyde, and 0.1M phosphate buffer (pH 7.2-7.4). Samples were stored at 4°C until processing. Samples for both SEM and TEM underwent washing 3x with 0.1 M phosphate buffer (15 min/wash) and then incubated with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hr. Dehydration of SEM and TEM started at 30% ethanol, then 50%, 70%, 90%, and 100% three times for 15 min each. At this point, SEM samples were washed with ethanol:HMDS (hexamethyldisilazane) at a 75:25 ratio for 15 min. After that ethanol:HMDS ratio were 50:50, then 25:75, and then twice with pure HMDS, each for 15 min. The SEM specimens were left to dry overnight in a fume hood prior to mounting on SEM stubs and sputter coated with Au/Pd prior to viewing on a Philips/FEI (XL30) Scanning Electron Microscope.

TEM samples were infiltrated with Spur's resin after dehydration at a ethanol:Spurr mixture of 1:1 for three hours, after which the samples were put in pure Spur's resin and left overnight. The following day, the Spur resin was changed and the samples were embedded on flat molds and then cured overnight in a 70°C oven. Sectioning was performed by a Reichart-Jung Ultracut_E Ultramicrotome at 90 nm of thickness and stained with uranyl acetate and then lead citrate. Specimens were viewed on a Philips-FEI Morgagni 268 Transmission Electron Microscope operating at 80 kV using a Gatan Orius CCD camera.

Immunofluorescent Imaging

Fixed recellularized HLV sections were co-incubated with hMPCs were processed in paraffin for further sectioning using a microtome to slice 10 μm thick sections. Deparaffinization was done using SafeClear Xylene substitutes (Fisher Scientific) following the manufacturer's instructions. After rehydration, tissue was stained using primary antibodies anti-integrin beta1 antibody (Abcam, Cambridge UK) and anti-collagen1 antibody (Abcam). Secondary antibodies used were Alexa Fluor IgG dyes 647 and 546, respectively, from Life Technologies (Carslbad, CA, USA). DAPI staining was used to identify nuclei using a concentration of 300 nM after prior to mounting on a coverslip using ProLong Diamond Antifade Mountant (ThermoFischer Scientific, Waltham, MA, USA). Slides were imaged on a Zeiss LSM 710 confocal microscope.

Statistics

Statistical analysis was performed as described in Chapter 2 except analysis was done using SigmaStat 3.5 program.

6.3 Results

Diseased Versus Healthy Cardiac LV Extracellular Matrix

Un-utilized donor cardiac LV tissue exhibits a heterogenous colour indicating a lack of fibrotic lesions and no blatant infiltration of immune cells (Fig 6.1). LV tissue procured from a patient who was supported with a left ventricular assist device (LVAD) shows evidence of extensive scarring and infiltration of pro-inflammatory cells indicated by the deepened purple colour. In order to evaluate the potential for the release of pro-inflammatory cytokines during the decellularization procedure, analysis of pro-inflammatory makers TNFα and IL-6 was performed (Fig 5.2b). This is also important to determine whether these molecules are present during the recellularization process as it may impede its efficacy. ELISA analysis of TNFα and IL-6 show a steep increase after washing with SDS, which remained stable until the final few decellularization steps. Most importantly, the level of TNFα and IL-6 was not significantly different from the start of the decellularization process prior to washing with detergents and significantly decreased from the initial increase in concentration after washing with 1% SDS.

Recellularization of Cardiac LV Extracellular Matrix

There are clear visual differences between decellularized and recellularized LV tissue, even at the macroscope level where recellularized tissue appears more opaque and even slightly yellow (Fig 6.3a). At the cellular level, visualized with both scanning electron microscopy (Fig 6.3b) and transmission electron microscopy (Fig 6.3c), it is evident that cells have successfully infiltrated the decellularized LV tissue. Not only do cells appear to be viable, but also active in mitosis, as evident by the mitosis captured by TEM, indicated with an arrow. Extracellular matrix fibrils at a variety of orientations are visualized as

well. The extent of cellular infiltration is visualized in Fig 6.4 using H&E staining to highlight nuclei and scar tissue present in explants of cardiac tissue. As a result of the nature of recellularization, cells must migrate from the exterior of the tissue towards the center, as is evidenced in the control tissue by the concentration of nuclei on the perimeter of the tissue. Although the explant tissue is more fibrotic, which is a barrier to migration, cells can be observed infiltrating the non-fibrotic portions of the tissue.

Interaction with Extracellular Matrix

TEM was able to capture an image of a cell interacting with the ECM after recellularization (Fig 6.5a). There appears to be vesicles exhibiting either endocytotic or secretory behavior as well as secretion of ECM fibrillar proteins. Immunofluorescent imaging of recellularized tissue (Fig 6.5b) show a positive signal for integrin-β1, a membrane protein that functions as a collagen receptor, in close proximity with the collagen-1.

6.4 Discussion

In order to accurately understand the physiological response to disease and effectively test translational therapies, basic science must use methods that most closely resemble the problem at hand. The ability of the ECM environment to manipulate a pro-fibrotic phenotype of mesenchymal-derived cells (17,18) provides a unique opportunity to use a readily available, physiologically relevant resource to better understand fibrosis pathology. Being able to use healthy and diseased ECM as a scaffold for hAFs and hMPCs provides the most accurate representation of what is occurring endogenously between hAFs, hMPCs, and ECM. This experiment demonstrates that it is possible to recellularize human ECM, regardless of whether the ECM is healthy or riddled with fibrotic lesions, and effectively acquire and analyze molecular data as if it was pieces of tissue.

Although this process involves steps which promote cell lysis, and thus the release of pro-inflammatory cytokines (1), the multitude of decellularization steps effectively remove the influence of these pro-inflammatory cytokines from disrupting the recellularization process. IL-6 and TNFα are

infamous for being the primary culprits for stimulating an anti-inflammatory pro-fibrotic response in cardiovascular disease (19,20). Ensuring that these cytokines are no longer present is important in elucidating the genuine effect of ECM on mesenchymal cells that experience a phenotypic change in response to both molecular and mechanical influences of fibrotic tissue. The increased presence of these cytokines during the decellularization process also show that they are released in response to the process so it is important to ensure that they are removed by the recellularization step so that their influence on pro-fibrotic influence of the ECM are not being exaggerated by molecular mechanisms.

An interesting observation about the nature of recellularization is that cells do cluster around the perimeter of the decellularized tissue. This mechanical barrier must be bypassed in order for cells to be surrounded ECM in a three-dimensional manner. In order for cells to penetrate the border of the decellularized ECM, they must effectively interact with it using a variety of proteases to facilitate further migration. This demonstrates direct interaction with the ECM including molecular connection with the ECM to sense its presence (21), releasing secretory vesicles filled with proteases to degrade ECM proteins to ease migration (22), and continual cellular movement through the ECM via focal adhesion turnover (23). Not only does this demonstrate mesenchymal cell interaction with ECM, but also a biologically active ECM that influences cell behaviour, indicating a physiologically relevant technique for studying the influence of ECM on cells.

These experiments use key elements from a technique that was originally developed on a whole organ rodent model (1); however it requires significantly less resources in order to, not only be effective, but arguably more physiologically accurate and precise. First of all, although this technique can be applied to sections of ECM isolated from various animal models, it is more translational as it can use patient tissue, which is significantly more difficult to come by when wanting to decellularize an entire heart. A significant drawback to using an entire organ is the monstrous amount of cells required to recellularize an entire organ, regardless of the experimental model (11). Our technique uses a fraction of the cells, which, in turn, uses fewer number and volume of reagants, is significantly cheaper, and takes much less time. It

also has the flexibility to be co-incubated with a variety of different cells to evaluate a more focused effect of ECM, for example a direct evaluation of fibrotic lesions on immune cells. Being able to use a fraction of the cells also permits studying the effect of how certain treatments may influence the cellular response to the ECM which could be use to develop effect anti-fibrotic medication. It can also be used as a template to study other types of tissue engineering experiments by evaluating how various scaffolds and/or cell therapy techniques respond to fibrotic environments that would be present endogenously in the patient. Finally, because smaller portions of tissue are acquired from a whole organ, it is possible to excise specific areas of tissue, whether it be fibrotic foci, more diffuse fibrotic areas, or areas with minimal or no presence of fibrosis to be able to compare ECM within the same patient. In conclusion, decellularization and subsequent reseeding of human ECM primary human mesenchymal-derived cells is an effective technique to study genuine physiological interactions with healthy and fibrotic cardiac tissue.

6.5 References

1. Ott HC, Matthiesen TS, Goh SK, et al (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14: 213-221

2. Kitahara H, Yagi H, Tajima K, et al (2016) Heterotropic transplantation of a decellularized and recellularized whole porcine heart. Interact Cardiovasc Thorac Surg 22: 571-579

3. Hülsmann J, Grün K, El Amouri S, et al (2012) Transplantation material bovine pericardium: biomechanical and immunogenic characteristics after decellularization vs glutaraldehyde-fixing. Xenotransplantation 19:286-297

4. Morticelli L, Thomas D, Roberts N, et al (2013) Investigation of the suitability of decellularized porcine pericardium in mitral valve reconstruction. J Heart Valve Dis 22(3):340-353

5. Chen F, Yoo JJ, Atala A (1999) Acellular collagen matrix makes a possible "off the shelf" biomaterial for urethral repair. Urology 54:407-410

6. Freytes DO, Badylak SF, Webster TJ, et al (2004) Biaxial strength of multilaminated extracellular matrix scaffolds. Biomaterials 25:2353-2361

7. Flynn LE (2010) The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. Biomaterials 31:4715-4724

8. Grandi C, Baiguera S, Martorina F, et al (2011) Decellularized bovine reinforced vessels for small-diameter tissue-engineered vascular grafts. Int J Mol Med 28(3):315-325

9. Cartmell JS, Dunn MG (2000) Effect of chemical treatments on tendon cellularity and mechanical properties. J Biomed Mater Res 49:134-140

10. Woods T, Gratzer PF (2005) Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. Biomaterials 26:7339-7349

11. Taylor DA, Parikh RB, Sampaio LC (2017) Bioengineering hearts: simple yet complex. Curr Stem Cell Rep 3: 35-44

12. Parker MW, Rossi D, Peterson M, et al (2014) Fibrotic extracellular matrix activates a profibrotic positive feedback loop. J Clin Invest 124: 1622-1635

13. Engler AJ, Sen S, Sweeney HL, et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126: 677-689

14. Emerman JT, Burwen SJ, and Pitelka DR (1979) Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue Cell 11:109–119

15. Miner JH, Yurchenco PD (2004) Laminin functions in tissue morphogenesis. Ann Rev Cell Develop Biol 20:255-284

16. Wang H, Luo X, Leighton J (2015) Extracellular matrix and integrins in embryonic stem cell differentiation. Biochem Insights 8(Suppl2);15-21

17. Ngo M, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

18. Talele NP, Fradette J, Davies JE, et al (2015) Expression of α-smooth muscle actin determines fate of mesenchymal stromal cells. Stem Cell Reports 4: 1016-1030

19. Dixon IM (2010) The soluble interleukin-6 receptor takes its place in the pantheon of interleukin-6 signaling proteins: phenoconversion of cardiac fibroblasts to myofibroblasts. Hypertension 56: 193-195

20. Porter KE, Turner NA, O'Regan DJ, et al (2004) Tumor necrosis factor alpha induces human atrial myofibroblast proliferation, invasion, and MMP-9 secretion: inhibition by simvastatin. Cardiovasc Res 64: 507-515

21. Cameron AR, Frith JE, Cooper-White JJ (2011) The influence of substance creep on mesenchymal stem cell behaviour and phenotype. Biomaterials 32:5979-5993

22. Salamone M, Carfi Pavia F, Ghersi G (2016) Proteolytic enzymes clustered in specialized plasma-membrane domains drive endothelial cells' migration. PLoS One 11: e0154709

23. Dunlevy JR, Couchman JR (1993) Controlled induction of focal adhesion disassembly and migration in primary fibroblasts. J Cell Sci 105: 489-500

6.6 Figures

Figure 6.1: H&E imaging of human left ventricular cardiac muscle from control heart and explanted pro-fibrotic heart (EPFH) at 2.5x and 10x zoom

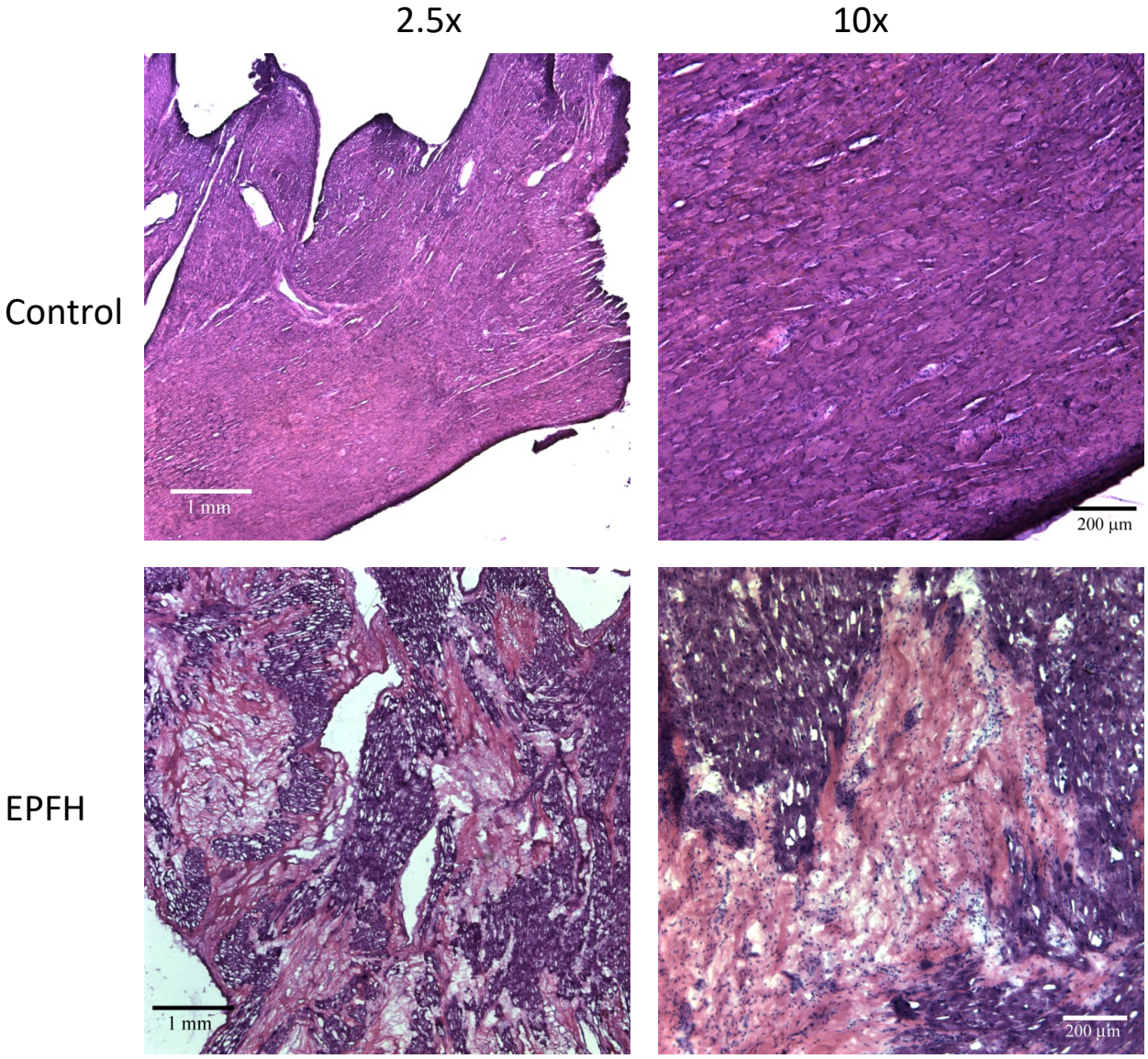


Figure 6.2: a) Schematic of decellularization process (SDS-sodium dodecyl sulfate, O/N-overnight, NaCl-sodium chloride, $MgCl_2$-magnesium dichloride, P/S-penicillin streptomycin, HBSS-Hank's buffered salt solution); b) ELISA analysis of pro-inflammatory markers IL-6 and TNFα during human decellularization wash stages, n=4. One-way ANOVA, * p-value < 0.05 compared to Control; # p-value < 0.05 compared to SDS

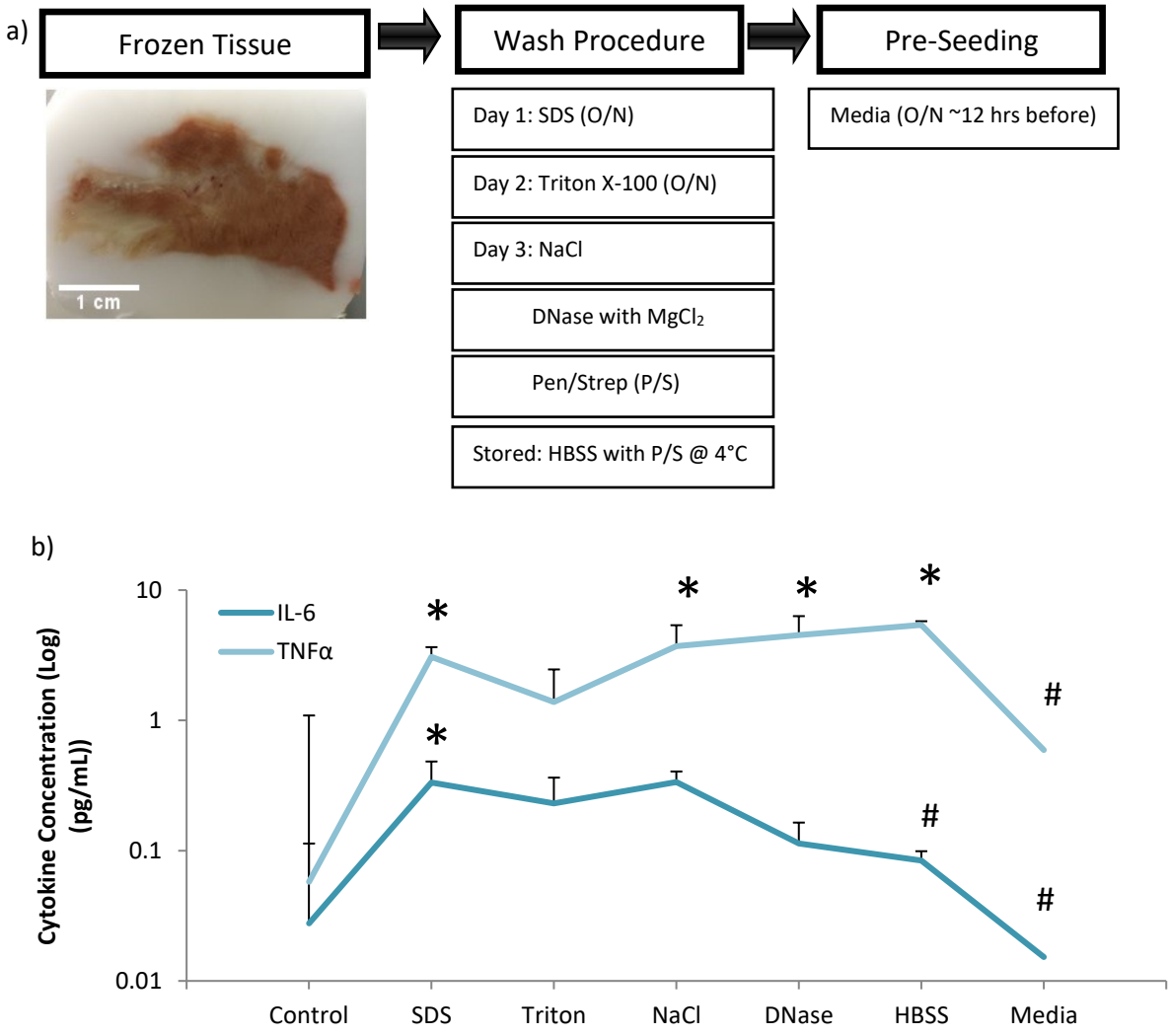


Figure 6.3: Images of decellularized left ventricular (LV) cardiac tissue and recellularized LV cardiac tissue seeded with human bone marrow-derived progenitor cells viewed a) macroscopically, b) with a scanning electron microscope, c) and with a transmission electron microscope. Arrow indicates possible cell mitosis. Collagen fibril orientation highlighted by red circle.

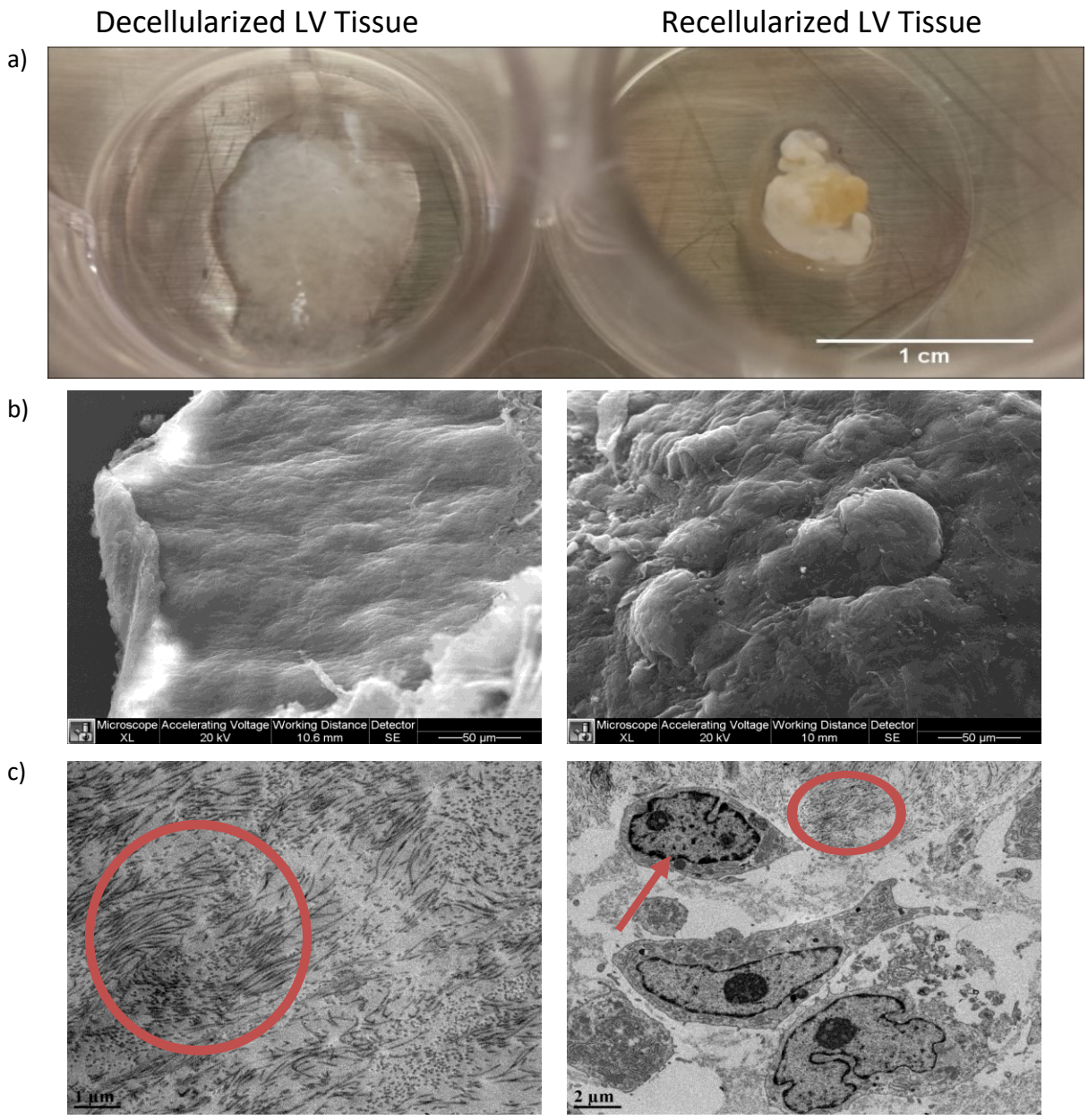


Figure 6.4: Images of recellularized human LV tissue a) with H&E staining indicating extent of recellularization by highlighting nuclei (blue = nuclei)

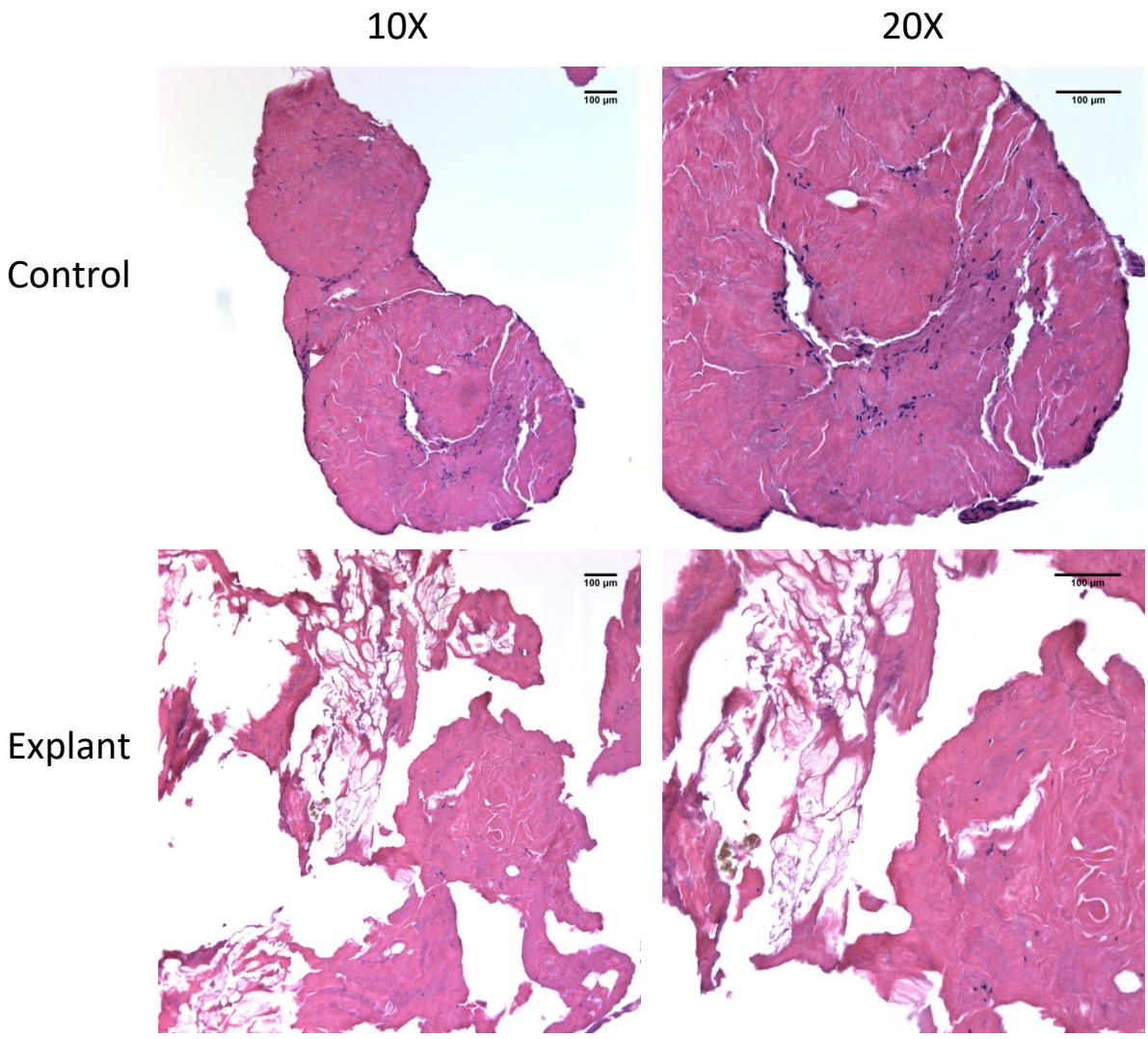
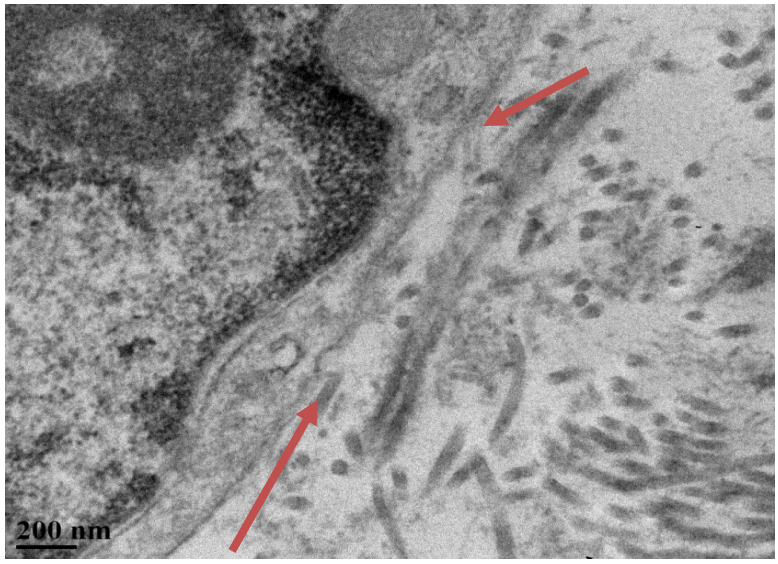
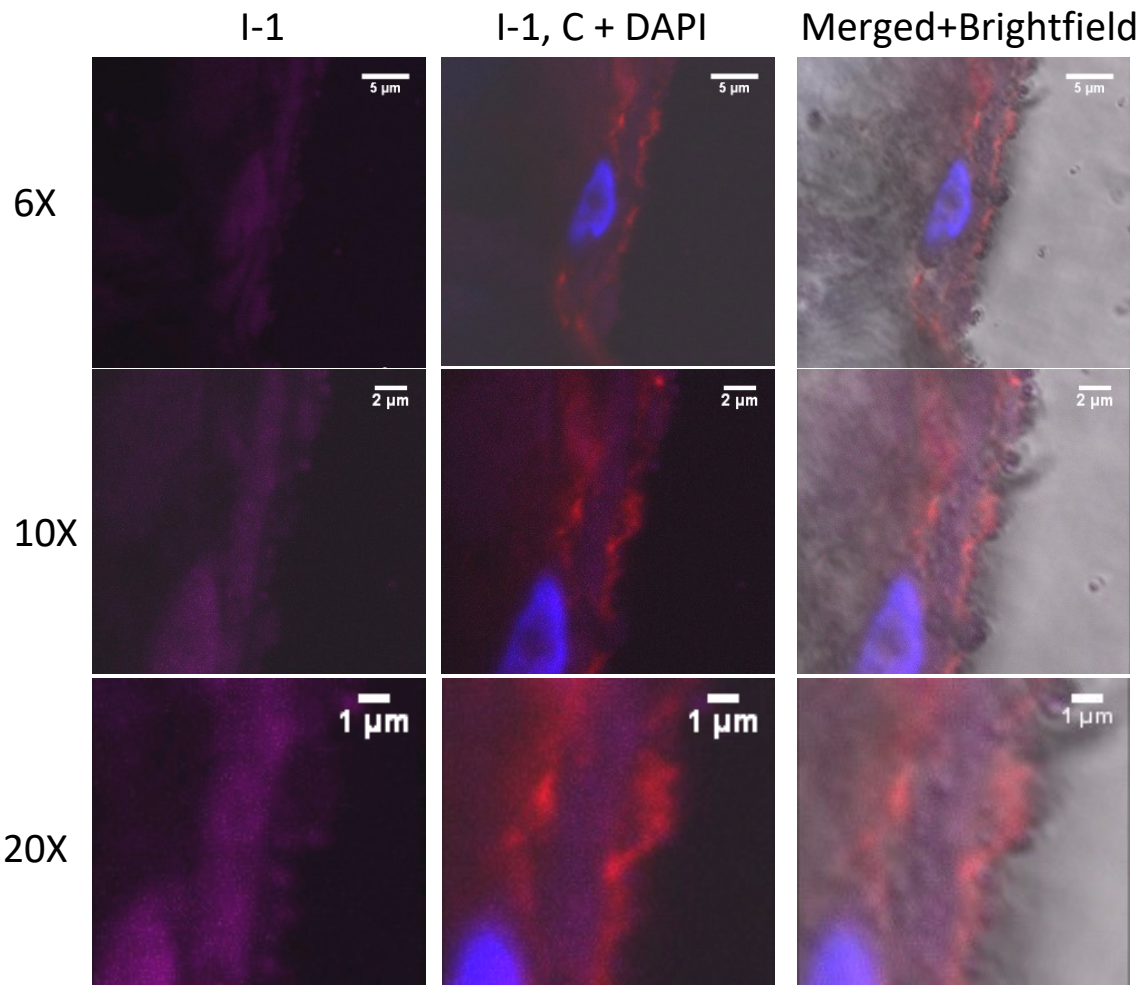


Figure 6.5: a) Transmission electron microscopy tissue highlighting interaction, with ECM at 44 000x zoom, and b) Immunofluorescence imaging of recellularized LV tissue; red = collagen 1 (C), far red = integrin-1 (I1)

a)



b)



7. Increased Expression of Pro-Fibrotic Markers in Primary Human Atrial Fibroblasts and Bone Marrow-Derived Mesenchymal Progenitor Cells Caused by Recellularizing Pathologically Fibrotic Human Extracellular Matrix is Attenuated by Transfection with miR-301a

By Müller AL, Wang X, Freed DH

Müller AL: Performed all experiments except qRT-PCR, experimental design, wrote manuscript

Wang X: Performed qRT-PCR experiments

Freed DH: Experimental design, edited manuscript

Abstract: Decellularized matrix is a material that is increasingly being used in tissue engineering to optimize tissue repair therapies. The heart is an important target for tissue repair as it lacks effective endogenous healing ability after suffering injury. CorMatrix® is a novel extracellular matrix (ECM) bioscaffold developed from decellularized porcine intestine used to promote healing in vascular, pericardial, and cardiac tissue repair. It is currently being used clinically to encourage healing and has been found to be effective, yet there is no research available on how endogenous progenitor cells that can contribute to pathological fibrosis as a result of cardiovascular damage, respond to CorMatrix®. There is also the opportunity to utilize native cardiac ECM to better understand the endogenous response of pro-fibrotic cells to healthy and damaged ECM. Previous research has alluded to over-expression of a pro-proliferative miRNA, miR-301a, inhibiting pro-fibrotic differentiation of endogenous mesenchymal-derived progenitor cells. These experiments investigate the reaction of endogenous hMPCs and hAFs to a variety of decellularized matrices; CorMatrix®, unutilized human donor cardiac LV tissue, and explanted human cardiac LV tissue from a patient who had been equipped with a left ventricular assist device. The effect of these matrices on mesenchymal cell pro-fibrotic differentiation was compared to regular culture methodology using plastic plates and cells at P1. In order to evaluate the effect of these ECMs on pro-fibrotic differentiation, qRT-PCR analysis was performed on a variety of pro-fibrotic markers as well as an ELISA on the protein content of α-SMA, an important indicator of a myofibroblast-like phenotype. In order to evaluate the efficacy of mir-301a on attenuating pro-fibrotic differentiation, miR-301a transfected hAFs and hMPCs were also seeded on healthy or explanted human LV tissue. An increase of pro-fibrotic markers were measured for both hAFs and hMPCs seeded on CorMatrix®. There were also increases of pro-fibrotic markers in cells seeded in the fibrotic explant LV which was attenuated by miR-301a transfection. These results highlight the importance of a physiologically relevant disease model as the effectiveness of a single miR in attenuating the affects of diseased myocardial ECM. Increased expression of miR-301a could be applied to cell therapy techniques when looking to repair damage in fibrotic areas in order to prevent the influence of this pathological environment from impairing reparative processes.

7.1 Introduction

Novel developments in the field of tissue engineering have provided a variety of novel techniques to tackle tissue repair and cell therapy. One of the most significant contributions is the development of three-dimensional matrices for cell culture to better mimic a physiological environment as it provides mechanical properties and structural support for cell attachment and tissue development (1). Not only can a three-dimensional culture environment promote endogenous cell behaviours including proliferation, migration, matrix production, and differentiation, but studies evaluating pathologies using these technologies provide a more accurate representation of what occurs at a physiological level (2). One of the most effective sources of scaffolding that provides a 3D environment is decellularized extracellular matrix (ECM). Since the publication from Taylor's group (3) demonstrating the importance of ECM alone in facilitating cell orientation and function to produce cardiac contraction, a variety of applications of decellularized ECM have been used both in basic science as well as in the clinic. For example, CorMatrix® is a versatile patch developed from decellularized porcine small intestine submucosa (SIS), that is routinely utilized in cardiac surgery for pericardial tissue repair, congenital cardiac surgery, infective endocarditis, repair of atrial septal defects, pulmonary arterioplasty, and aortoplasty applications (4,5). Since the decellularization method removes the majority of cellular components and antigens from the tissue, as demonstrated in the previous chapter, there are decreased risks of inflammatory response and immunological rejection (6-8). However, even though this tissue is being utilized in the clinic, it is currently not well understood how endogenous mesenchymal cells, sensitive to stiff environments, respond to CorMatrix®. This is important as the ECM of SIS is densely packed and provides a stiffer substrate which could promote the differentiation of mesenchymal cells into a pro-fibrotic phenotype (9).

As influential as the effect of the properties of ECM are on promoting a pro-fibrotic phenotype (10,11), our lab previously demonstrated that transfection with miR-301a was able to attenuate the effect, preventing differentiation into a myofibroblast-like cell. MiR-301a is a microRNA, a non-coding RNA molecule that is responsible for shifting in genetic expression. The ability of miRNA to influence protein

expression rapidly has been noted in numerous cell types under a variety of physiological and pathological parameters (12). MicroRNAs (miRNAs) have recently emerged as a novel intrinsic method of gene regulation predominantly located in intronic gene sequences as 21 or 22 nucleotide-polymers. They act by post-transcriptionally binding the 3'UTR of mRNA targets and ultimately decreasing mRNA levels (13). These molecules have been implicated in a variety of cell processes including differentiation (14-16).The effectiveness of miR-301a likely plays a mechanistic role in suppressing the contractile phenotype while favouring the proliferative phenotype in both hMPCs and hAFs. Like all microRNAs, it is unlikely that miR-301a has a direct effect on hMPC phenotype, but rather that it suppresses protagonists of the myogenic phenotype, maintaining a predominantly proliferative physiology. Using the decellularization technique described previously, our lab will compare the effect of CorMatrix®, decellularized human explants left ventricular (LV) tissue, decellularized unutilized human donor LV tissue on the pro-fibrotic phenotype of primary hAFs and primary hMPCs compared to traditional two-dimensional cell culture. We will further investigate the effect of miR-301a transfection to determine if it is able to attenuate a pro-fibrotic phenotype in a three-dimensional culture environment.

7.2 Materials & Methods

Isolation, Culture, and Transfection of Primary Human MPCs and Human AFs

Human MPC cultures were prepared based on plastic adherence according to the methods developed by Caplan and Friedenstein (17, 18) with some modifications. Further details of this procedure and isolation of hAFs can be found in Chapter 2. After 10 to 14 days of cultivation, primary cultures were 60-80% confluent. Cells were then dissociated with TrypLE Express (GIBCO®) and seeded at low densities for expansion through successive passages. HAFs and hMPCs were cultured in 10 cm^2 dishes until 90-95% confluence when they were harvested at P2 for co-incubation with decellularized human left ventricular tissue. Transfection was performed on P1 cells as described in Chapter 2.

Decellularization Protocol

Decellularization occurred as was previously described in the Methods secton. Briefly, cardiac tissue was acquired from un-utilized donor hearts (control) and excised explant hearts from consented patients undergoing cardiac transplant surgery. Large chunks of frozen tissue was sliced at a~150-200 μm thickness using a cryostat, put in cold PBS, and warmed to room temperature. Two 1 hour cycles of the first lysis solution (1% SDS in H_2O) was used before a third cycle was oscillated O/N. The following day, the first lysis solution was removed and replaced with 1% Triton X-100 in H_2O (the second lysis solution) and oscillated for three 1 hour long cycles of decellularization using fresh solution at the beginning of each cycle. After the final hour cycle using the second lysis solution, the final cellular lysis step used 1% Triton X-100 solution oscillating overnight. After rinsing with PBS and DDW, 1 M NaCl (in H_2O), was added to the tissue and oscillated for 1 hour. Afterwards, treatment with DNAse (20μg/mL) plus 4.2 mM MgCl (in H_2O) at 37°C occurred for an hour. The tissue was subsequently rinsed with DDW and then with PBS. The tissue was placed in Hank's Buffered Saline Solution (HBSS) plus 100 U/mL penicillin and 100 μg streptomycin (P/S) for 30 minutes prior to storage in in HBSS plus P/S at 4°C. Prior to co-incubation with either hMPCs or hAFs, selected decellularized sections were rinsed twice with PBS at RT, and incubated in the same culture media used to grow the corresponding cell type at 37°C overnight. Co-incubation with hMPCs or hAFs proceeded the following day.

Recellularization Protocol

The recellularization procedure is more thoroughly explain in the Methods section. Briefly, confluent P1 primary hMPCs and hAFs were trypsinized and centrifuged to produce a pellet. They were re-suspended in media (DMEM-F12, 20% FBS, 100 μg/mL of Primocin™, and 100 mM ascorbic acid) and added to tubes so that each tube contained ~5,000,000-10,000,000 cells. Pieces of decellularized HLV tissue were added to each tube and the volume of media brought up to 10 mL. In order to evaluate the effect of Cormatrix® on hAFs and hMPCs, the same co-incubation steps were taken; however Cormatrix® was re-hydrated according to standard clinical protocol illustrated in the manufacturer's

instructions. Control for the Cormatrix® experiments were aliquoted from the pooled cells and grown in the same culture conditions used to achieve desired confluency for recellulation. Tubes were rotate O/N at 37°C (95% CO_2, 5% O_2). The following day, recellularized tissue pieces were rinsed 2X in PBS prior to being put in fresh media and returned, rotating, into the incubator where media was changed every 3 days for two to three weeks. Recellularization was deemed complete after media colour change rate was equivalent to plates at 90% confluency. Slices were fixed in the appropriate fixation solution (either 4% paraformaldehyde or specified for SEM and TEM) or frozen in liquid N_2 for protein and mRNA isolation.

mRNA and Protein Isolation

Recellularized slices of tissue of from Cormatrix® and the HLV were homogenized in New RIPA lysis buffer (150 mMNaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) and protease inhibitor cocktail (0.1 M phenylmethylsulfonyl fluoride, 5 µg/ml leupepetin, 2 µg/ml aprotinin, and 1 µg/ml pepestatin). For mRNA isolation, a portion of the homogenate was added to 1 mL of TRIzol, frozen overnight, and then thawed the following day to proceed with mRNA isolation as per manufacturer's instructions (Ambion, Foster City CA, USA) previously described in Chapter 2. The remainder of the homogenate was set aside for protein analysis. Homogenate debris was spun down into a pellet using 2000XG for 5 minutes and the remaining liquid supernatant was set aside for protein concentration analysis by the Bradford Lowry Protein Assay method using reagants provided by Bio-Rad (Hercules CA, USA) and following the manufacturer's instructions as described in Chatper 2. Protein samples were stored at -20°C until later use.

qRT-PCR Analysis

Expression of collagen1A1, collagen1A2, fibronectin-1, myosin heavy chain 10, myosin heavy chain 11, α-smooth muscle actin, S100A4, and vimentin (see Table 7.2 for primers) were analyzed by quantitative real-time PCR. qPCR results were compared against β-actin which was used as a reference gene. Expression of mRNA was determined using the Quanta Biosciences (Beverley MA, USA) B-R 1-

Step SYBR® Green qRT-PCR kit according to the manufacturer's instructions and analyzed using the Roche Lightcycler 480II. The PCR thermo-cyling program conditions program was pre-incubation at 95°C for 5 min, a 50-cycle amplification at 95°C for 10 sec followed by 65°C for 30 sec, a melting curve of 95°C for 5 sec, 65°C for 1 min, and then 97°C continuously, after which cooling occurred at 40°C for 15 sec.

Quantification of α-Smooth Muscle Actin

To quantify the protein level of α-smooth muscle actin (α-SMA), an enzyme-linked immunosorbent assay (ELISA) was procured from MyBioSource (San Diego CA, USA). A total of 100 ng of protein homogenate was loaded into each well for quantification. Results were measured using a Synergy H4 Hybrid Reader Gen5 2.00 Spectrophotometer (BioTek, Winooski VT, USA).

Statistics

Statistical analysis was performed as described in Chapter 2 except analysis was done using SigmaStat 3.5 program.

Table 7.2: Forward and reverse primers used for qRT-PCR

Gene	Forward Primer	Reverse Primer
Collagen 1A1	CCAAAGGATCTCCTGGTGAA	AGTTTTGCCATCAGGACCAG
Collagen1A2	TTGACCCTAACCAAGGATGC	TTCTTGGCTGGGATGTTTTC
Fibronectin-1	GTATGAGGGAACACACTTGTGCT	TGACTTCATGCCTCTACTCTCTCA
Myosin Heavy Chain 10	AGGTGGACTATAAGGCAGATGAG	CTGTCTGATGACTGGTGCAAAA
Myosin Heavy Chain 11	CTGCAGGAGAGACAGTAGGC	TACCCCTGAGGCATGACTCC
α-Smooth Muscle Actin	GCAGTAGTGTGGTGTTCTGTATCA	GGCTTGGCTGTAATTGATTCACAAG
S100A4	TGCTCTAGAGCAAGACTGCTG	GGTGGGATTAATAAGACGGTCTCTG
Vimentin	GTTATCAAGACAGACTCAAAAGG GACTT	CATTTCACGCATCTGGCGTT

7.3 Results

Effect of CorMatrix® on hAFs and hMPCs on pro-fibrotic markers

Recellularization of CorMatrix®, a clinical ECM scaffold product isolated from porcine intestines, was found to have varying effects between hAFs and hMPCs (Figure 7.1). Although there appeared to be a significant increase in *Col1A1*expression in hAFs, it was not significant, unlike the comparable, but statistically significant increase in *Col1A1* expression measured in hMPCs when compared to standard culturing conditions. *Col1A2* expression was blunted in both mesenchymal cell types, but more severely in hMPCs. *Fibronectin* mRNA levels were strikingly opposite between hAFs and hMPCs where a significant increase expression occurred in hAFs but vice versa in hMPCs.

Evaluation of intracellular pro-fibrotic markers found a lack of notable differences in the pattern of mRNA expression between hAFs and hMPCs (Fig 7.2b). Neither *MYH10* nor *α-SMA* had a significant change in mRNA expression between hAFs and hMPCs seeded on a CorMatrix® substrate; however, *MYH11*, *S100A4*, and *Vimentin* expression were all significantly elevated in both cell types. Most notably, the increase in expression of *S100A4* and *Vimentin* was several hundred-fold. ELISA analysis of α-SMA, collagen1A1, fibronectin, and MYH11 determined that it is significantly increased in hAFs and hMPCs seeded on CorMatris® compared to traditional culturing (Fig 7.2).

Effect of Healthy and Diseased Human Left Ventricular ECM on Pro-Fibrotic Mesenchymal Cells

The effect of LV tissue isolated from healthy unutilized donor heart, used as a control representing a healthy ECM environment, and LV tissue isolated from diseased myocardium of an explant heart once equipped with an LVAD, representing a pathologically fibrotic ECM environment, were compared between primary hAFs and hMPCs (Fig 7.3). In addition, the change in expression of pro-fibrotic markers among all recellularized tissue was compared to traditional cell culturing methods on plastic plates. The mRNA expression of ECM proteins, *Col1A* and *Col1A2,* were significantly increased in human ECM compared to plastic, with further increased expression occurring in hAFs seeded in explants tissue. The

expression pattern of *Col1A2* of hMPCs mirrored that of hAFs where diseased myocardium induced increased expression; however *Col1A1* in hMPCs was decreased in diseased myocardium. Expression of *Fibronectin* was increased in hAFs seeded in both control and explants tissue; however, only the expression measured in hAFs seeded in control tissue was statistically significant as the variance was too high in the explants tissue. A several hundred-fold increase of *Fibronectin* expression occurred in hMPCs seeded in explants tissue, compared to both the plastic control and healthy ECM.

Although many of the intracellular pro-fibrotic markers measured were noticably impacted by the change of growing on decelluarized human ECM, extensive variability between samples prevented confidence in statistically significant differences among the various treatment groups. Interestingly, no expression of *MYH10* was detectable in hMPCs grown on healthy ECM but there was a several hundred-fold increased measured in hMPCs seeded on diseased tissue. Expression of *MYH11* was only statistically different in hAFs seeded in explants tissue, increased several-thousand fold compared to both plastic and hAFs seeded on healthy ECM. The variability of α-SMA was also observed; however there was a significant increase in expression in hMPCs seeded in explant ECM compared to both the plastic control and the healthy ECM seeded with hMPCs. ELISA analysis of α-SMA, collagen1A1, fibronectin, and MYH11 determined that there are significantly increased protein levels in decellularized explants LV compared to healthy ECM (Fig 7.4). The variability of *S1004A* expression was quite high; however, there does appear to be a trend of increased expression triggered by the pro-fibrotic ECM present in the explant tissue. Finally, *Vimentin* expression was significantly increased in hAFs compared to both plastic culture techniques and healthy ECM; however, there was a decrease in expression observed in explanted ECM. *Vimentin* expression rose by nearly a hundred-fold in hMPCs seeded in explant tissue which was significantly different compared to standard culture conditions as well as to hMPCs seeded on healthy ECM.

Effect of Transfection of miR-301a of Pro-Fibrotic Mesenchymal Cells on Recellularized Human ECM

There is a distinct difference in the expression of pro-fibrotic markers of both hAFs and hMPCs when comparing physiological ECM to standard cell culture conditions. Previous experiments determined that transfection with miR-301a attenuates a pro-fibrotic phenotype in standard cell cultures. The next step is to evaluate if its efficacy translates to decellularized ECM from fibrotic explant LV tissue (Fig 7.5). Expression of *Col1A1* and *Col1A2* of hMPCs on healthy tissue was unaffected by miR-301a transfection, however *Col1A2* was significantly decreased in hAFs. Expression of *Fibronectin* was decreased in miR-301a transfected cells seeded in healthy myocardium compared to the negative scrambled control. No significant change was observed regarding *MYH10* and *MYH11* expression between the healthy ECM groups. Interestingly, when comparing the expression of *α-SMA* it was observed that miR-301a transfected cells seeded in healthy ECM was increased; however, *α-SMA* expression was significantly decreased in hMPCs. *Vimentin* expression was unaltered in miR-301a transfected hMPCs seeded on healthy ECM but was significantly decreased in hAFs. Noticeably, all pro-fibrotic markers had significantly increased expression in explants tissue in both cell types compared to the negative control and miR-301a transfected healthy ECM. Most importantly, this increased expression of mRNA pro-fibrotic markers measured in explant tissue, excluding hMPC expression of MYH10 and MYH11, was significantly attenuated upon miR-301a transfection.

7.4 Discussion

The ECM environment is a key regulator in progenitor cell differentiation (19), as illustrated in our results. Although this has been observed numerous times in various animal models, this study is evidence of genuine fibrotic human ECM influencing a pro-fibrotic phenotype in hAFs and hMPCs. In addition, the evaluation of the interactions of hAFs and hMPCs with CorMatrix® triggers mRNA expression comparable to stimulating a pro-fibrotic phenotype. This is evident by the parallel increases in expression

in previously published paper in our lab of differentiating into a myofibroblast-like cells (10). The appeal of using CorMatrix® in various cardiovascular applications is that it is beneficial in contributing stiffness, especially in high pressure/tension areas (4) but might be less ideal as a scaffold for tissue regeneration or cellular therapy as it may impair healing processes by causing cells to becoming pro-fibrotic and lead to pathological scarring. As CorMatrix® is still a fairly new product, receiving FDA approval for its first clinical trial in 2011, the long-term effects still need to be evaluated, especially regarding the its potential for stimulating a pro-fibrotic environment by providing a stiff ECM triggering endogenous mesenchymal cells to differentiation into myofibroblast-like cells. It is important to note that CorMatrix® is not an ideal substrate for studying the effects of ECM on human mesenchymal cells as tensions promote pathological fibrosis.

Physiologically relevant disease models are important for accurate representations of pathologies to better understand how to manage/treat patients. Our results indicate a striking change of pro-fibrotic mRNA expression when comparing healthy ECM to fibrotic ECM. There were consistent increases of pro-fibrotic markers measured when cells seeded on diseased heart tissue, although not always statistically significant. This may be due to variability between patients' isolated cells or variability within the extent of penetration of the cells into the decellularized LV tissue. Also, there is increased variability among the samples as a result of the heterogenous nature of the decellularized sections as no two sections will have the exact same orientation and extent of fibrosis. Regardless of this variability, there were consistent trends in the data. The lack of expression of MYH10 in hMPCs with no observed statistical difference between intracellular pro-fibrotic markers compared to plastic culturing, indicate that studying hMPCs on plastic in P1 is fairly comparable to how they would respond if seeded on healthy decellularized matrix. This was also observed in hAFs as well; however, the changes in hAFs were highly variable when comparing explant tissue to control tissue, despite observing an increased in expression, for example, with α-SMA. There was consistency between hAFs and hMPCs as evident by the increase in pro-fibrotic markers in hMPCs between explants and control tissue, with the majority of the

measurements being statistically significant. This indicates a definite physiological response to pro-fibrotic ECM alone, as all other factors were unchanged, including the comparisons between hAFs and hMPCs procured from the same patient. These results contribute to the understanding of how endogenous cells genuinely respond to pro-fibrotic environments. This illustrates an important factor when considering tissue engineering and cell therapy techniques, as the application of these will, undoubtedly, involve interacting with the scarred environment in order to rehabilitate it. The involvement of hAFs and hMPCs in pro-fibrotic environments is undeniable so understanding how they interact with diseased, pro-fibrotic ECM environments is important in moving cardiac tissue regeneration forward.

The most exciting discovery is that transfection of miR-301a showed a significant attenuation of pro-fibrotic markers in cells grown on pro-fibrotic explanted tissue. This is crucial as it illustrates the efficacy of a single miRNA in attenuating a powerful mechanical response. This is especially important as this was done using human tissue and mesenchymal cells of patients who are suffering from heart disease. Transfection progenitor cells used in cell therapy techniques with miR-301a may increase their survivability, retention, and efficacy as they would not be influenced by the pathological fibrotic environment surrounding them. The translational application of this technique is feasible in a clinical setting and should be considered when using cell therapy to a repair a fibrotic area.

7.5 References

1. Hubbell JA (1995) Biomaterials in tissue engineering. Biotechnology (NY) 13(6):565-576

2. Duval K, Grover H, Han L-H, et al (2017) Modeling physiological events in 2D vs 3D cell culture. Physiology (Bethesda) 32: 266-277

3. Ott HC, Matthiesen TS, Goh SK, et al (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14(2): 213-221

4. Yanagawa B, Rao V, Yau TM, et al (2013) Initial experience with intraventricular repair using CorMatrix extracellular matrix. Innovations 8(5):348-352

5. Sündermann SH, Müggler O, Caliskan E, et al (2015) Extracellular matrix for reconstruction of cardiac structures after tumour resections. Interact Cardiovasc Thorac Surg 20(1):10-14

6. Badylak SF, Weiss DJ, Caplan A, et al (2012) Engineered whole organs and complex tissue. Lancet 379(9819):943-952

7. Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. Biomaterials 27:3675-3683

8. Parmaksiz M, Elcin AE, Elcin YM (2017) Decellularization of bovine small intestinal submucosa and its use for the healing of a critical-size full-thickness skin defect alone and in combination with stem cells in a small rodent model. J Tissue Eng Regen Med 11(6):1754-1765

9. Andrée B, Bär A, Haverich A, et al (2013) Small intestinal submucosa segments as matrix for tissue engineering: review. Tissue Engin Part B: Reviews 19: 279-291

10. Ngo M, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

11. Talele NP, Fradette J, Davies JE, et al (2015) Expression of α-smooth muscle actin determines fate of mesenchymal stromal cells. Stem Cell Reports 4(6): 1016-1030

12. Amiel J, de Pontual L, Henrion-Caude A. miRNA, development and disease. Adv Genet 2012; 80: 1-36

13. Zeisberg EM, Tarnavski O, Zeisberg M, et al (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 13: 952-961

14. Lei PP, Qu YQ, Tao SM, et al. (2013) Fibrocytes are associated with the fibrosis of coronary heart disease. Pathol Res Pract 209: 36-43

15. Korhonen J, Polvi A, Partanen J, et al (1994) The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. Oncogene 9: 395-403

16. Hocht-Zeisberg E, Kahnert H, Guan K, et al (2004) Cellular repopulation of myocardial infarction in patients with sex-mismatched heart transplantation. Eur Heart J 25: 749-758

17. Friedernstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-274

18. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641-650

19. Engler AJ, Sen S, Sweeney HL, et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126(4): 677-689

7.6 Figures

Figure 7.1: Evaluation of mRNA expression of hMPCs and hAFs co-incubated with Cormatrix evaluating a) extracellular matrix protein gene expression and b) pro-fibrotic marker gene expression; (n=5) NS – not significant, paired student t-test, one-way ANOVA,* p-value < 0.05 compared to Control, ** p-value < 0.01 compared to Control, *** p-value < 0.001 compared to Control; # p-value < 0.05 compared to hAFs

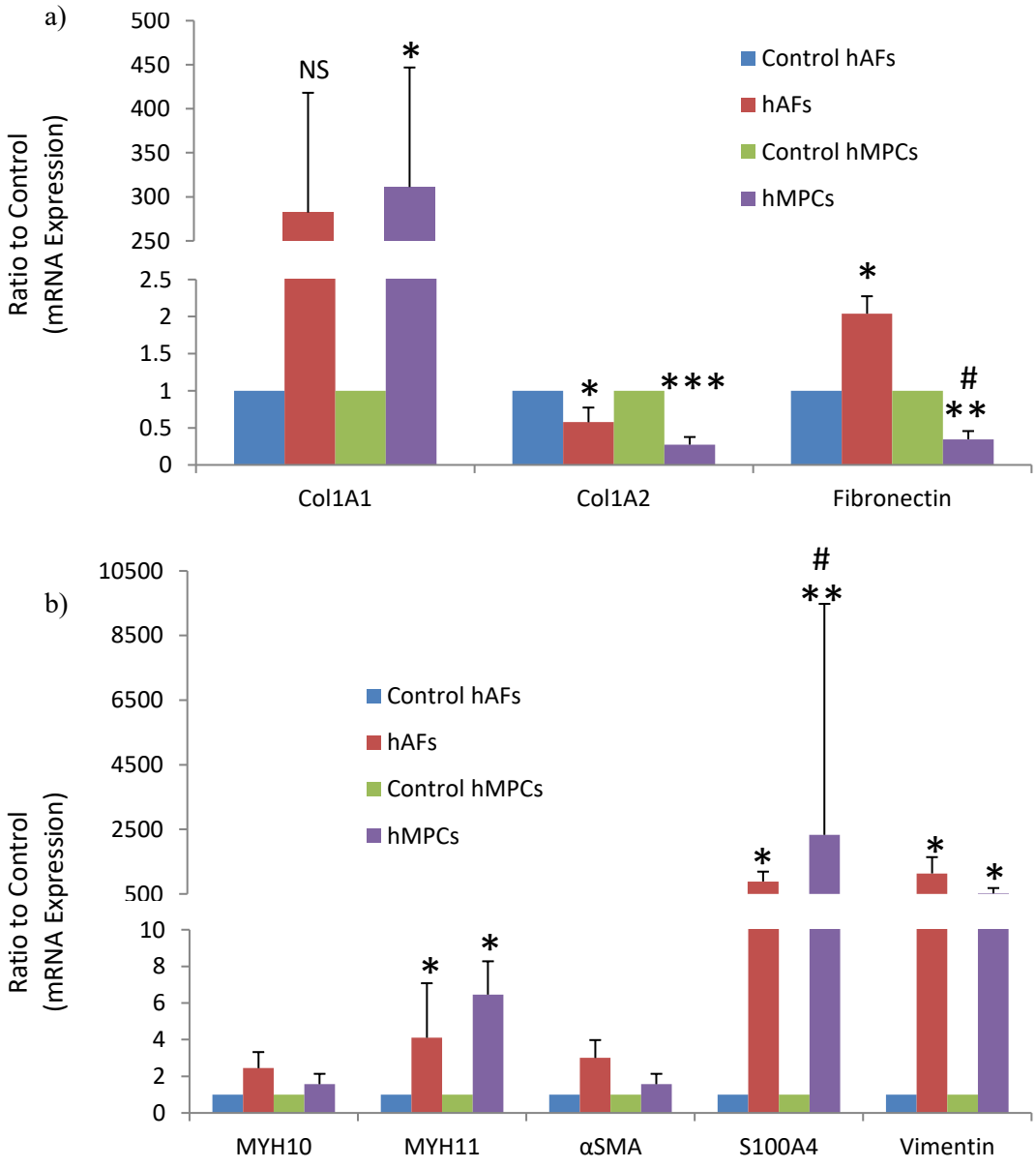


Figure 7.2: ELISA quantification of a) α-smooth muscle actin, b) fibronectin, c) collagen, and d) MYH11 in hMPCs and hAFs co-incubated with Cormatrix; (n=4-6) NS – not significant, paired student t-test, one-way ANOVA,* p-value < 0.05 compared to respective cellular Control (cultured on plastic)

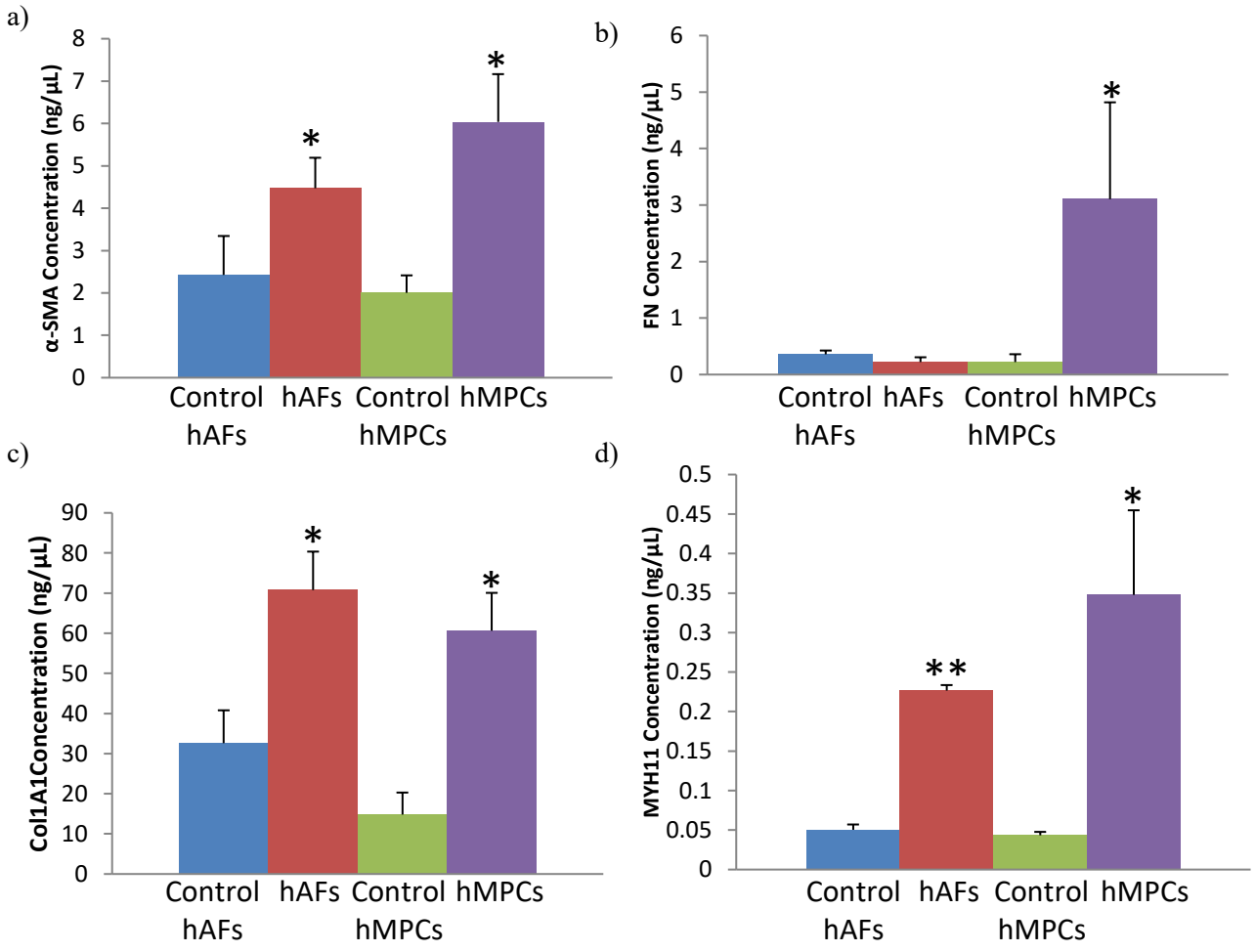


Figure 7.3: Evaluation of mRNA expression of hMPCs and hAFs co-incubated with healthy human left ventricular matrix (Control) or explant human left ventricular matrix; a) *Collagen 1A1*, b) *Collagen 1A2*, c) *Fibronectin*, d) *myosin heavy chain-10*, e) *myosin heavy chain-11*, f) *α-smooth muscle actin*, g) *S100A4,* h) *Vimentin*. (n=6) NE: no expression. one-way ANOVA* p-value < 0.05 compared to Plastic Control; ** p-value < 0.01 compared to Plastic Control; *** p-value < 0.001 compared to corresponding Control tissue; # p-value < 0.05 compared to hAFs; ## p-value < 0.01 compared to corresponding Control tissue

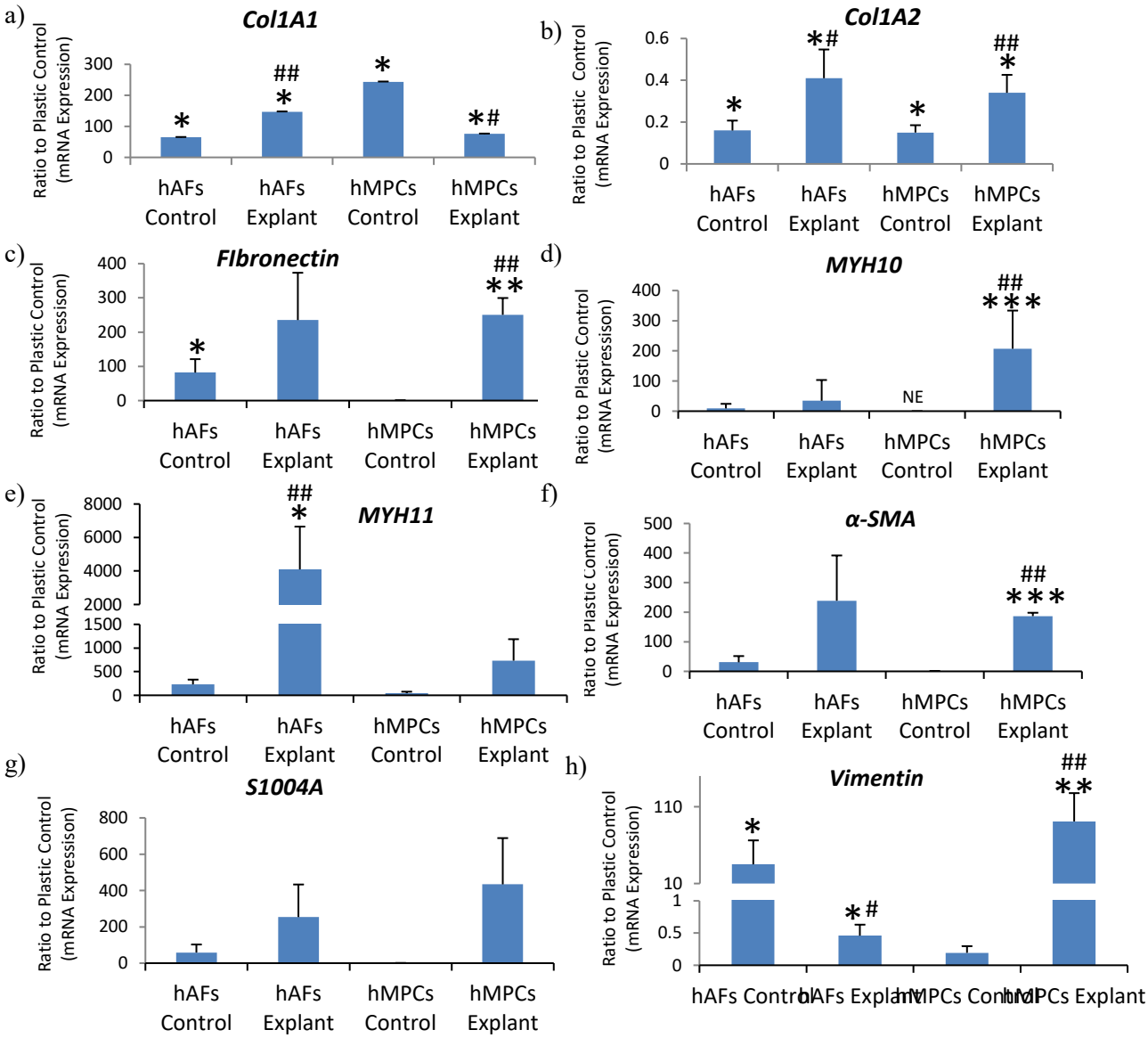


Figure 7.4: ELISA quantification of a) α-smooth muscle actin in hMPCs, and b) fibronectin, c) collagen, and d) MYH11 in hMPCs and hAFs used to recellularize human left ventricular tissue (n=4-6) Note: It was not detectable in hAFs when loading the same concentration of protein as hMPCs, paired student t-test, * p-value <0.05 compared to Control, ** p-value <0.01 compared to Control

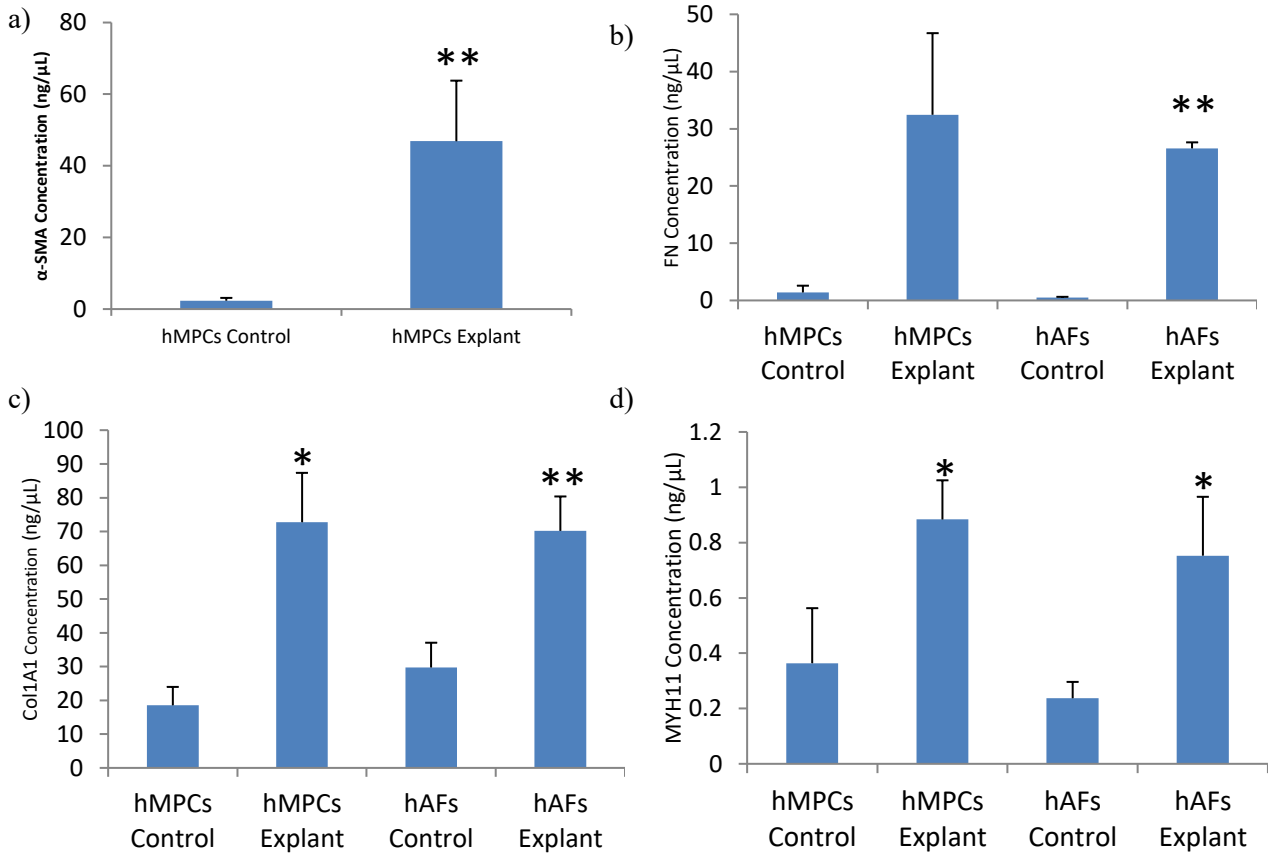
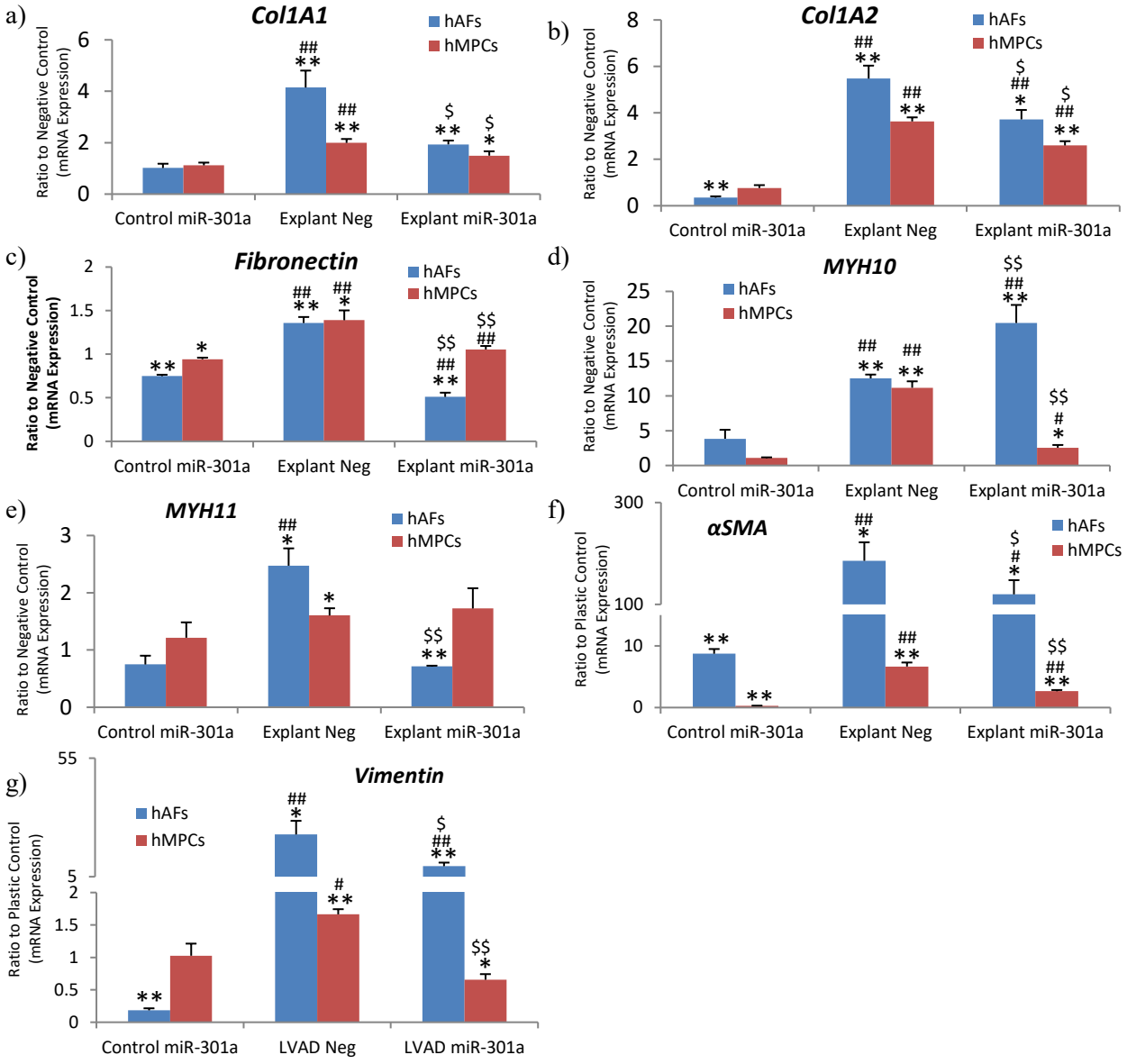


Figure 7.5: Evaluation of mRNA expression of hMPCs and hAFs transfected with miR-301a co-incubated with healthy human left ventricular matrix (Control) or explant human left ventricular matrix; a) *Collagen 1A1*, b) *Collagen 1A2*, c) *Fibronectin*, d) *myosin heavy chain-10*, e) *myosin heavy chain-11*, f) *α-smooth muscle actin,* g) *Vimentin*. (n=5) NE: no expression. Paired Student t-test, 1-way ANOVA* p-value < 0.05 compared to Negative Control; ** p-value < 0.01 compared to Negative Control; # p-value < 0.05 compared to Control miR-301a; ## p-value < 0.01 compared to Control miR-301a; $ p-value < 0.05 compared to LVAD Neg; $$ p-value < 0.01 compared to LVAD



8. Discussion

8.1 DISCUSSION

While exploring the differentiation of mesenchymal cells, both AFs and MPCs, it is evident that their environment lends to a pro-fibrotic phenotype. The hostile, pro-inflammatory environment impairs the success of regenerative cell therapy which aims to stimulate repair and healing, ideally by replacing the damaged/diseased tissue with healthy newly engineered tissue. This method of treatment may be more effective than current pharmacological interventions, such as statins. Although we determined that the proliferation of MPCs under physiological concentrations is significantly decreased, this has failed to lead to effective anti-fibrotic therapy. As transfection with miR-301a has demonstrated the ability to encourage a proliferative phenotype, there is the potential for this to combat the decrease in proliferation from statins. Taking advantage of the effectiveness of miRNA to be able to influence cell phenotype is an important tool that can be utilized to direct preferential differentiation. When evaluating these effects and applying them in a clinically-relevant context, it is optimal to experiment in a physiologically accurate setting; hence the development of a protocol involving decellularizing native tissue to isolate ECM. Having access to both diseased and healthy human cardiac tissue allowed these experiments to evaluate the effect of fibrotic ECM and miR-301a in a physiological setting. Our experiments determined that, not only are human AFs and MPCs influenced by the state of fibrotic ECM of human left ventricular myocardium, their phenotype can be modified by miRNA, which may supersede the influence of the ECM.

Implications of miR-301a in Maintaining a Proliferative Phenotype

As patients with heart conditions are commonly on statin therapy treatment, their negative effect on the viability of progenitor cells is important as this could contribute to decreased survivability of cells used in cell therapy. Transfection of these cells with miR-301a may have a two-fold beneficial effect - first, by inhibiting the influence of stiff ECM in promoting a pro-fibrotic phenotype, and second, by increasing their survivability of after implantation in damaged/diseased tissue. This shall be accomplished

by first evaluating the effects of statin therapy on the mesenchymal progenitor cell population to establish how they interact with potential cell sources that would be utilized in clinical applications.

Identification of miR-301a as a key miRNA involved in preventing a pro-fibrotic phenotype, occurred when exposing cells to an environment comparable to what they would experience under ischemia. Nutrient deprivation, simulated by plating hMPCs without FBS, determined that there is a distinct difference in contractile phenotype between hMPCs with sufficient nutrients to undergo mitosis versus hMPCs responding to an ischemic environment where mitosis is impaired. This suggests a dichotomy between the proliferative and contractile phenotypes, similar to other mesenchymal cells found to contribute to fibrosis, including smooth muscle cells in atherosclerosis and cardiac fibroblasts in both reactive and reparative fibrosis (1,2). Transfection with miR-301a in both hAFs and hMPCs was shown to be effective in preventing a pro-fibrotic phenotype on cells grown on plastic culture dishes, which, as previously determing in our lab (3), normally adopt a pro-fibrotic phenotype. This observation correlates with the increase in the differentiation of fibroblasts to myofibroblasts in response to MI where the decreased blood flow prevents nutrient transport, causes an inflammatory response, and stimulates boisterous ECM protein production (4,5). Interestingly, it was only upon the deprivation and subsequent re-introduction of mitogens that miR-301a expression was altered, indicating that MPCs may utilize miR-301a to respond to a phenotypic change when react to a change in their environment. This would occur more rapidly when responding to an acute environmental change, as the mRNA is already available to be transcribed, as oppose to the cell having to use additional resources to start to build proteins from the beginning to signal DNA transcription.

The consistency of our results found post-transfection, when considering the inherent variability of primary cells isolated from patients, not only because of the heterogeneity within progenitor cells isolated from the bone marrow, but also between patients, both genetically and likely medications taken to treat one or a myriad of medical conditions, emphasizes the effectiveness of miR-301a. The results between hMPCs and hAFs were also consistent, as miR-301a caused a similar phenotype change to occur in hAFs.

Although the decrease in differentiation from hAFs into myofibroblasts was less drastic, the observation that miR-301a still attenuated a pro-fibrotic phenotype indicates the possibility of a common mechanism in maintaining a more progenitor-like state. Because this single microRNA is affecting two different cell types in a similar fashion, these results suggest that miR-301a plays a mechanistic role in increasing proliferative potential of both hMPCs and hAFs, and possibly other pro-fibrotic cell types, while attenuating expression of a pro-fibrotic phenotyp. This correlates with other findings of miR-301a, where it has found to be over-expressed in the pathological proliferation of various types of malignant cells. In a tumour setting, many of these malignant, proliferating cells are exposed to a fibrotic, ischemic environment, so preventing diseased ECM from inhibiting proliferation is a useful survival technique (6). Like the majority of microRNAs, it is unlikely that miR-301a has a direct effect on mesenchymal cell phenotype, but rather, it suppresses protagonists of the myogenic phenotype, maintaining a predominantly proliferative physiology. Further work is required to determine in miR-301a exerts its effect through targeting these genes in hMPCs, especially to determine if malignant proliferation occurs, and then evaluate if these effects translate into altered fibrosis *in vivo*. The use of miRNA has been suggested as a therapeutic intervention, and it may be that miR-301a could be used to treat established fibrosis or to effectively inhibit endogenous AFs and MPCs from adopting a pathological ECM secreting phenotype in a pro-fibrotic environment.

The Extracellular Matrix Environment Influences Mesenchymal Cell Phenotype

There are multitudinous different environments within the human body, and hMPCs experience a variety of them, starting within their progenitor niche and then after settling on their final phenotypic destination, whether it be adipose, chondrogenic, or osteogenic tissue. Although determining that a stiff environment encourages a pro-fibrotic phenotype (3), we found that softer environments led to a decrease in pro-fibrotic protein expression indicating a preference for maintaining a progenitor-like phenotype. This is more representative of their progenitor niche environment within the bone marrow, as the stiffness is reminiscent to that of bone marrow. This decrease in protein expression was maintained at pressures

simulating healthy cardiac LV tissue indicating that, even though the stiffnesses between the soft and ventricular tissue are different, with ventricular tissue being stiffer, it is not at a level that elicits a pro-fibrotic response from hMPCs. This suggests that hMPCs have specific sensitivity when determining whether or not their environment involves a promotion of ECM protein expression, especially as chondrogenic and osteogenic tissue are not only stiffer, but require more ECM protein production as a property of their tissue-type (7).

In addition, we discovered that there was increased mRNA expression of certain pro-fibrotic proteins that did not result in increased protein expression of those same proteins. Therefore, it is possible that this discrepancy between mRNA and protein expression indicates that hMPCs in a progenitor state are "preparing" for responding to an injury. This observation of increased mRNA compared to a lack of change in protein concentration, may indicate a priming of these progenitor cells in response to pro-inflammatory markers. Many studies have established that hMPCs respond to pro-inflammatory signals by promoting fibrosis in areas of injury (8,9); hence, it would be pragmatic for hMPCs, when responding to inflammation, be prepared to differentiate into an ECM protein secretory cell relatively rapidly. This further demonstrates the importance of microRNA as it has the ability to prevent translation of mRNA, while it is still able to be amplified by PCR. This is because the miRNA binds to the 3'-end of the mRNA, whereas the primers utilized in these experiments amplified regions within the center of the mRNA.

After quantification of miR-301a was evaluated at various surface tensions, we determined that miR-301a expression was also influenced by softer substrates, indicating an increased retention of an undifferentiated, non-pro-fibrotic phenotype. Although this would initially seem like a very power application of a single microRNA, after identifying Dicer1 as a potential target of miR-301a, which was found significantly decreased in miR-301a transfect hMPCs. The important of Dicer1 is emphasized by its highly conserved function of being crucial in miRNA processing by facilitating the activation of miRNAs, first discovered to be responsible for activating miRNAs involved in development (10). This proposes a unique mechanism of self-regulation where an increase in Dicer1 could indicate a shift in the

miRNA profile. This phenomenon has been observed a variety of cell types (11-13), and in fact, miRNAs have been found to be able to, not only mediate the differentiation of progenitor cells into a final phenotype, but can also conversely return differentiated cells into a stem-cell like state (14-16).

The Importance of Using Decellularized Human Cardiac Extracellular Matrix

To further evaluate the clinical applicability of miR-301a transfected cells and the influence of the ECM environment in promoting a pro-fibrotic phenotype, we wanted to utilize a more physiologically relevant resource to better understand fibrosis pathology. The availability of diseased and healthy human left ventricular cardiac ECM provided a unique opportunity to decellularize ECM and study hMPCs and hAFs respond to distinct ECM environments while providing an ideal platform to test the efficacy of miR-301a attenuating pro-fibrotic phenotypic differentiation. After determining the relative absence of pro-inflammatory cytokines, that could trigger a pro-fibrotic phenotype, we were able to elucidate the genuine effect of ECM on mesenchymal cells that experience a phenotypic change in response to both molecular and mechanical influences of fibrotic tissue. Once it was observed that recellularization with both hAFs and hMPCs was successful, it was possible to determine that, not only are these surviving in the tissue, but they are responding to their environment as they expressed varying concentrations of pro-fibrotic markers and ECM proteins, depending on the nature of their ECM. As this technique can be used to target a specific area of ECM on the heart, our focus on fibrotic areas allowed us to better evaluate the effect of these fibrotic areas on cell phenotype. Upon comparing our decellularized tissue to CorMatrix®, we determined that triggers mRNA expression comparable to stimulating a pro-fibrotic phenotype. Using CorMatrix® may be beneficial in cardiovascular applications where stiffness is required, especially in high pressure/tension areas (17), but might be less ideal as a scaffold for tissue regeneration or cellular therapy. Because of its increased stiffness compared to healthy myocardial ECM, it is important to note that CorMatrix® may not be an ideal substrate for studying the effects of ECM on hMPCs its ECM environment may promote pathological fibrosis.

When investigating the difference between healthy and fibrotic ECM, there was consistency between hAFs and hMPCs as evident by the increase in pro-fibrotic markers. Upon transfection with miR-301a, both hAFs and hMPCs showed a significant attenuation of pro-fibrotic markers in cells grown on pro-fibrotic explanted tissue. This is crucial as it highlights the efficacy of miR-301a in attenuating a powerful mechanical response, which is essentially overriding the physiological effect of pro-fibrotic ECM. These observations were consistent even when considering the heterogeneous fibrosis between different areas of LV and variability between patients. This contributes to the understanding of how endogenous cells genuinely respond to pro-fibrotic environments which illustrates an important factor when considering tissue engineering and cell therapy techniques. The stiffness of the scaffold or substrate used in tissue engineering will have an effect on phenotype, and could possibly affect endogenous progenitor cells within the patient to migrate to the scaffolding and be influenced by its ECM environment. The involvement of hAFs and hMPCs in pro-fibrotic environments is undeniable so understanding how they interact with diseased, pro-fibrotic ECM environments is important in moving the cardiac tissue regeneration field forward. Transfection progenitor cells used in cell therapy techniques with miR-301a may increase their retention and survivability which imporves their overall efficacy as they would not be influenced by the pathological fibrotic environment surrounding them.

8.2 REFERENCES

1. Cordes KR, Sheehy NT, White MP, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009; 460: 705-710

2. Gong C, Nie Y, Qu S, et al. miR-21 induces myofibroblast differentiation and promotes the malignant progression of breast phyllodes tumors. Cancer Res 2014; 74: 4341-4352

3. Ngo M, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

4. Serini G, Bochaton-Piallat ML, Ropraz P, et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. J Cell Biol 142: 873-881

5. Wang J, Chen H, Seth A, et al (2003) Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. Am J Heart Physiol 285: H1871-H1881

6. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674

7. Bourin P, Gadelorge M, Peyrafitte JA, et al (2008) Mesenchymal progenitor cells: tissue origin, isolation, and culture. Transfus Med Hemother 35: 160-167

8. Endo J, Sano M, Fujita J, et al (2007) Bone marrow derived cells are involved in the pathogenesis of cardiac hypertrophy in response to pressure overload. Circulation 116: 1176-1184

9. Guo J, Lin GS, Bao CY, et al (2007) Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30: 97-104

10. Wienholds E, Koudijs MJ, van Eeden EJ, et al (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet 35: 217-218

11. Stappert L, Borghese L, Roese-Koerner B, et al (2013) MicroRNA-based promotion of human neuronal differentiation and subtype specification. PLoS One 8: e59011

12. Dalmosso G, Nguyen HT, Yan Y, et al (2010) MicroRNAs determine human intestinal epithelial cell fate. Differentiation 80: 147-154

13. Ribeiro AO, Schoof CRG, Izzotti A, et al (2014) MicroRNAs: modulators of cell identity, and their applications in tissue engineering. Microrna 3: 45-53

14. Marson A, Levine SS, Cole MF, et al (2008) Connecting miRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 134: 521-533

15. Eulalio A, Mano M, Dal Ferro M, et al (2012) Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492: 376-381

16. Suh MR, Lee Y, Kim JY, et al (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270: 488-498

17. Sündermann SH, Müggler O, Caliskan E, et al (2015) Extracellular matrix for reconstruction of cardiac structures after tumour resections. Interact Cardiovasc Thorac Surg 20(1):10-14

9. Conclusions

9.1 SUMMARY

These experiments emphasize the importance of ECM in pro-fibrotic phenotype differentiation. The mechanical feedback that mesenchymal cells receive triggers the increased expression of myofibroblast markers and extracellular proteins that contribute to pathological fibrosis. Although miR-301a has been observed to maintain a proliferative phenotype in hMPCs, our data indicates that it is crucial in preventing a pathological secretory phenotype. Encouraging proliferation of hMPCs is also important when evaluating the likelihood of patients being treated both for hypertension with statins as well as requiring cell therapy for cardiac tissue repair. Maintaining cell viability is an important component of effective cell therapy and needs to be considered as patients with cardiovascular disease are likely on a myriad of medications. The ability to explore physiologically relevant environments and evaluate primary human cellular responses was facilitated by developing a technique that merged the importance of ECM architecture with studying the interactions of mesenchymal progenitor cells that contribute to pathological fibrosis by being activated vai the ECM. Decellularization of pathological human cardiac ECM removes the uncertainty of the translable nature of *in vitro* cell culture environments as it is essentially representing the endogenous environment present in patients with cardiovascular disease. Discovering the efficacy of miR-301a in attenuating myofibroblast-like phenotypes in cells triggered by the ECM stiffness of fibrotic tissue is a huge step in optimizing future cell therapy and tissue engineering technology. This research is beautiful and magnificent and the result of many years of troubleshooting, decontamination, determination, character building, evenings, weekends, and blasting EDM music in the lab. It shows that human tissue is super cool and much more relevant than rodent tissue and now we have developed an epic awesome way to figure out how cells naturally react to diseased human environments compared to healthy human environments. How extraordinary is that, eh? GO SCIENCE!

9.2 STUDY LIMITATIONS

There are a few limitations to this study that need to be addressed. The most significant limitation, despite its translatability to a clinical setting, is the variability of responses of hAFs and hMPCs between different patients. In addition to significant genetic variability, patients from which cells were procured were undergoing open heart surgery for different reasons, taking a wide range of medication, and likely had additional health concerns that may have contributed to the large variance among certain measured parameters. Variability between samples could also have occurred as a result of being unable to accurately determine the number of cells successfully integrating into the decellularized nature. Due to the nature of the experiment, quantifying the number of viable cells present on the tissue, and determining functional factors, such as proliferation and contractile ability was not possible. Although we were able to quantify the expression of various pro-fibrotic factors, functional assessment of how these recellularized cells behave, and if it is comparable to 2D behaviour or completely different in their native 3D environment, could not occur. This information would be valuable in determing the extent of differentiation whether or not these cells are contributing to the pathological fibrosis already present on decellularized explants tissue.

9.2 FUTURE DIRECTIONS

These experiments were performed using decellularized cardiac LV tissue; however they could be performed on any decellularized ECM tissue. It would be very interesting to determine if miR-301a is able to inhibit a pro-fibrotic phenotype ubiquitously across all fibrotic tissue, not just cardiovascular. MiR-301a could be a powerful tool to inhibit of influence of diseased and damaged areas of tissue regeneration sites to improve the efficacy of tissue engineering and cell therapies. Because miR-301a has demonstrated an ability increase proliferation in transfected cells, it would be interesting to investigate if it can mitigate the negative effects of statin treatment on progenitor cell survival in order to increase survivability of the cells post-treatment. Transfection with miR-301a could also be applied to cell

therapies looking to rehabilitate cardiac tissue in severely damaged areas as a way to minimize the influence of a fibrotic ECM on progenitor cells focused on repairing the damaged areas. In addition, further exploration of the variety of miR-301a could be explored to better elucidate the mechanism by which this phenomenon occurs. Dicer1 is of particular interest, as this supports a non-tissue specific way of regulating pro-fibrotic differentiation. These experiments have significant translational potential as genetic manipulation of cell therapy techniques is being used in a variety of clinical trials in order to successfully repair diseased tissue, and transfection with miR-301a may help bolster its efficacy across a myriad of physiological systems.

10. Bibliography

1. Kong P, Christia P, Frangogiannis NG (2014) The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 71: 549-574

2. Ahmad T, Fluzat M, Neely B, et al (2014) Biomarkers of myocardial stress and fibrosis as predictors of mode of death in patients with chronic heart failure. JACC Heart Fail 2: 260-268

3. Elzenaar I, Pinto YM, van Oort RJ (2013) MicroRNAs in heart failure: new targets in disease management. Clin Pharmacol Therap 94: 480-489

4. Kania G, Blyszczuk P, Eriksson U (2009) Mechanisms of cardiac fibrosis in inflammatory heart disease. Trends Cardiovasc Med 19: 247-252

5. Frangogiannis NG (2012) Regulation of the inflammatory response in cardiac repair. Circ Res 100: 159-173

6. Berk BC, Fujiwara K, Lehoux S (2007) ECM remodeling in hypertensive heart disease. J Clin Invest 117: 568-575

7. Ashrafian H, McKenna WJ, Watkins H (2011) Disease pathways and novel therapeutic targets in hypertrophic cardiomyopathy. Circ Res 109: 86-96

8. Pickering JG, Boughner DR (1990) Fibrosis in the transplanted heart and its relation to donor ischemic time. Assessment with polarized light microscopy and digital image analysis. Circulation 81: 949-958

9. Cieslik KA, Trial J, Crawford JR, et al (2014) Adverse fibrosis in the aging heart depends on signaling between myeloid and mesenchymal cells; role of inflammatory fibroblasts. J Mol Cell Cardiol 70: 56-63

10. Haudek SB, Xia Y, Huebener P, et al (2006) Bone marrow-derived fibroblast precursors mediate ischemic cardiomyopathy in mice. Proc Natl Acad Sci USA 103: 18284-18289

11. Blyszczuk P, Kania G, Dieterle T, et al (2009) Myeloid differentiation factor-88/interleukin-1 signaling controls cardiac fibrosis and heart failure progression in inflammatory dilated cardiomyopathy. Circ Res 105: 912-920

12. Kania G, Blyszczuk P, Stein S, et al (2009) Heart-infiltrating prominin-1+/CD133+ progenitor cells represent the cellular source of transforming growth factor beta-mediated cardiac fibrosis in experimental autoimmune myocarditis. Circ Res 105: 462-470

13. Zeisberg EM, Tarnavski O, Zeisberg M, et al (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 13: 952-961

14. Lei PP, Qu YQ, Tao SM, et al. (2013) Fibrocytes are associated with the fibrosis of coronary heart disease. Pathol Res Pract 209: 36-43

15. Korhonen J, Polvi A, Partanen J, et al (1994) The mouse tie receptor tyrosine kinase gene: expression during embryonic angiogenesis. Oncogene 9: 395-403

16. Hocht-Zeisberg E, Kahnert H, Guan K, et al (2004) Cellular repopulation of myocardial infarction in patients with sex-mismatched heart transplantation. Eur Heart J 25: 749-758

17. Villarreal FJ, Dillman WH (1992) Cardiac hypertrophy-induced changes in mRNA levels for TGF-beta 1, fibronectin, and collagen. Am J Physiol Heart Circ Physiol 262: H1861-H1866

18. Sopel MJ, Rosin NL, Lee TD, et al (2011) Myocardial fibrosis in response to angiotensin II is preceded by the recruitment of mesenchymal progenitor cells. Lab Invest 91: 565-578

19. Quaini F, Urbanek K, Beltrami AP, et al (2002) Chimerism of the transplanted heart. N Engl J Med 346: 5-15

20. Ngo M, Müller AL, Li Y, et al (2014) Human mesenchymal stem cells express a myofibroblastic phenotype in vitro: comparison to human cardiac myofibroblasts. Mol Cell Biochem 392: 187-204

21. Keeley EC, Mehrad B, Strieter RM (2009) The role of circulating mesenchymal progenitor cells (fibrocytes) in the pathogenesis of fibrotic disorders. Thromb Haemost 101: 613-618

22. Ebihara Y, Masuya M, Larue AC, et al (2006) Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. Exp Hematol 34: 219-229

23. Keeley EC, Mehrad B, Strieter RM (2010) Fibrocytes: Bringing new insights into mechanisms of inflammation and fibrosis. Int J Biochem Cell Biol 42: 535-542

24. Goumans MJ, van Zonneveld AJ, ten Dijke P (2008) Transforming growth factor beta-induced endothelial-to-mesenchymal transition: a switch to cardiac fibrosis? Trends Cardiovasc Med 18: 293-298

25. Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 2142: 199-210.

26. Serini G, Gabbiani G (1999) Mechanisms of myofibroblast activity and phenotype modulation. Exp Cell Res 250: 273-283

27. Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4: 267-274

28. Covas DT, Panepucci RA, Fontes AM, et al (2008) Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol 36: 642-654

29. Roberts AB, Sporn MB, Assoian RK, et al (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. PNAS 83: 4167-4171

30. Kinner B, Zaleskas JM, Spector M (2002) Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells. Exp Cell Res 278: 4167-4171

31. Reinhart BJ, Slack FJ, Basson M, et al (2000) The 21-nucleotide *let-7* RNA regulated developmental timing in *Caenorhabditis elegans*. Nature 203: 901-906

32. Choi E, Choi E, Hwang KC (2013) MicroRNAs as novel regulators of stem cell fate. World J Stem Cells 5: 172-187

33. Tomé M, López-Romero P, Albo C, et al (2011) miR-355 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. Cell Death Differ 18: 985-995

34. Matkovich SJ, Wang W, Tu Y, et al (2010) MicroRNA-133a protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts. Circ Res 106: 166-175

35. van Rooij E, Quiat D, Johnson BA, et al (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. Science 316: 575-579

36. Callis TE, Pandya K, Seok HY, et al (2009) MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J Clin Invest 119: 2772-2786

37. Wang J, Huang W, Xu R, et al (2012) MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med 16: 2150-2160

38. Wang YS, Li SH, Guo J, et al (2014) Role of miR-145 in cardiac myofibroblast differentiation. J Mol Cell Cardiol 66: 94-105

39. Ott HC, Matthiesen TS, Goh SK, et al (2008) Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14(2): 213-221

40. Engler AJ, Sen S, Sweeney HL, et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126(4): 677-689

41. Perea-Gil I, Prat-Vidal C, Bayes-Genis A (2015) In vivo experience with natural scaffolds for myocardial infarction: the times they are a-changin'. Stem Cell Res Ther 6: 248

42. Chang HY, Chi JT, Dudoit S, et al (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci U S A 99:12877-12882

43. Wang J, Dodd C, Shankowsky HA et al (2008) Deep dermal fibroblasts contribute to hypertrophic scarring. Lab Invest 88:1278-1290

44. Varkey M, Ding J and Tredget EE (2011) Differential collagen-glycosaminoglycan matrix remodeling by superficial and deep dermal fibroblasts: potential therapeutic targets for hypertrophic scar. Biomaterials 32:7581-7591

45. Li X, Zhu L, Wang B, et al (2017) Drugs and targets in fibrosis. Front Pharmacol 8:855

46. Gulati A, Jabbour A, Ismail TF, et al (2013) Association of fibrosis with mortality and sudden cardiac death in patients with nonischemic dilated cardiomyopathy. JAMA 309:896-908

47. Hutchinson J, Fogarty A, Hubbard R, et al (2015) Global incidence and mortality of idiopathic pulmonary fibrosis: a systematic review. Eur Respir J 46:795-806

48. Raimundo K, Chang E, Broder MS, et al (2016) Clinical and economic burden of idiopathic pulmonary fibrosis: a retrospective cohort study. BMC Pulm Med 16:2

49. Kim L, Kim DK, Yang WI, et al (2008) Overexpression of transforming growth factor-beta1 in the valvular fibrosis of chronic rheumatic heart disease. J Korean Med Sci 23:41-48

50. Allen JT, Spiteri MA (2002) Growth factors in idiopathic pulmonary fibrosis: relative roles. Respir Res 3:13

51. World Health Organization. Cardiovascular diseases - fact sheet #317. May 2017

52. Yusuf S, Hawken S, Ounpuu S, et al (2004) Effect of potentially modifiable risk factors 167 associated with myocardial infarction in 52 countries (the INTERHEART study): casecontrol study. Lancet 364:937-952

53. Lloyd-Jones DM, Nam BH, D'Agostino RB, et al (2004) Parental cardiovascular disease as a risk factor for cardiovascular disease in middle-aged adults: a prospective study of parents and offspring. JAMA 291:2204-2211

54. Sesso HD, Lee IM, Gaziano JM, et al (2001) Maternal and paternal history of myocardial infarction and risk of cardiovascular disease in men and women. Circulation 104:393-398

55. Goldenberg I, Jonas M, Tenenbaum A, et al (2003) Current smoking, smoking cessation, and the risk of sudden cardiac death in patients with coronary artery disease. Arch Intern Med 163:2301-2305

56. Dobaczewski M, de Haan JJ, and Frangogiannis NG (2012) The extracellular matrix modulates fibroblast phenotype and function in the infarcted myocardium. J Cardiovasc Transl Res 5:837-847

57. Ma Y, Halade GV, and Lindsey ML (2012) Extracellular matrix and fibroblast communication following myocardial infarction. J Cardiovasc Transl Res 5:848- 857

58. Akhmedov AT and Marin-Garcia J (2013) Myocardial regeneration of the failing heart. Heart Fail Rev 18:815-833

59. Santini MP and Rosenthal N (2012) Myocardial regenerative properties of macrophage populations and stem cells. J Cardiovasc Transl Res 5:700-712

60. Willems IE, Havenith MG, De Mey JG, et al (1994) The alpha-smooth muscle actin-positive cells in healing human myocardial scars. Am J Pathol 145:868-875

62. Souders CA, Bowers SL, and Baudino TA (2009) Cardiac fibroblast: the renaissance cell. Circ Res 105:1164-1176

63. Bujak M and Frangogiannis NG (2007) The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res 74:184-195

64. Desmouliere A, Geinoz A, Gabbiani F, et al (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122:103-111

65. Evans RA, Tian YC, Steadman R, et al (2003) TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins. Exp Cell Res 282:90-100

66. Hao J, Wang B, Jones SC, et al (2000) Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and *in vitro*. Am J Physiol Heart Circ Physiol 279:H3020-H3030

67. Kawano H, Cody RJ, Graf K, et al (2000) Angiotensin II enhances integrin and alpha-actinin expression in adult rat cardiac fibroblasts. Hypertension 35:273-279

68. Kawano H, Do YS, Kawano Y, et al (2000) Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. Circulation 101:1130-1137

69. Hafizi S, Wharton J, Chester AH, et al (2004) Profibrotic effects of endothelin-1 via the ETA receptor in cultured human cardiac fibroblasts. Cell Physiol Biochem 14:285-292

70. Masur SK, Dewal HS, Dinh TT, et al (1996) Myofibroblasts differentiate from fibroblasts when plated at low density. Proc Natl Acad Sci U S A 93:4219-4223

71. Baum J and Duffy HS (2011) Fibroblasts and myofibroblasts: what are we talking about? J Cardiovasc Pharmacol 57:376-379

72. Chester AH and Taylor PM (2007) Molecular and functional characteristics of heart valve interstitial cells. Philos Trans R Soc Lond B Biol Sci 362:1437-1443

73. Cunnington RH, Wang B, Ghavami S, et al (2011) Antifibrotic properties of c-Ski and its regulation of cardiac myofibroblast phenotype and contractility. Am J Physiol Cell Physiol. 300:C176-C186

74. Serini G, Bochaton-Piallat ML, Ropraz P, et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-β1. J Cell Biol 142:873-881

75. Sun Y (1997) Local angiotensin II and myocardial fibrosis. Adv Exp Med Biol 432:55-61

76. Lijnen P, Petrov VV, Rumilla K, et al (2002) Stimulation of collagen gel contraction by angiotensin II and III in cardiac fibroblasts. J Renin Angiotensin Aldosterone Syst 3:160-166

77. Lijnen PJ, Petrov VV, and Fagard RH (2001) Angiotensin II-induced stimulation of collagen secretion and production in cardiac fibroblasts is mediated via angiotensin II subtype 1 receptors. J Renin Angiotensin Aldosterone Syst 2:117-122

78. Petrov VV, Fagard RH, and Lijnen PJ (2002) Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 39:258-263

79. Darby I, Skalli O, and Gabbiani G (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing

80. van Amerongen MJ, Bou-Gharios G, Popa E, et al (2008) Bone marrow-derived myofibroblasts contribute functionally to scar after myocardial infarction. J Pathol 214: 377-386

81. Balsam LB, Wagers AJ, Christensen JL, et al (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428: 668-673

82. Endo J, Sano M, Fujita J, et al (2007) Bone marrow derived cells are involved in the pathogenesis of cardiac hypertrophy in response to pressure overload. Circulation 116: 1176-1184

83. Murry CE, Soonpaa MH, Reinecke H, et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428: 664-668

84. Handgretinger R, Gordon PR, Leimig T, et al (2003) Biology and plasticity of CD133+ hematopoietic stem cells. Ann NY Acad Sci 996: 141-151

85. Serini G, Gabbiani G (1999) Mechanisms of myofibroblast activity and phenotype modulation. Exp Cell Res 250: 273-283

86. Mohle R, Bautz F, Rafii S, et al (1998) The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 91: 4523-4530

87. Murdoch C (2000) CXCR: chemokine receptor extraordinaire. Immunol Rev 177: 175-184

88. Phillips RJ, Burdick MD, Hong K, et al (2004) Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 114: 438-446

89. Yano T, Miura T, Ikeda Y, et al (2005) Intracardiac fibroblasts, but not bone marrow derived cells, are the origin of myofibroblasts in myocardial infarct repair. Cardiovasc Pathol 14: 241-246

90. Abe R, Donnelly SC, Peng T, et al (2001) Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol 166: 7556-7562

91. Shah RV, Mitchell RN (2005) The role of stem cells in the response to myocardial and vascular wall injury. Cardiovasc Pathol 14: 225-231

92. Guo J, Lin GS, Bao CY, et al (2007) Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30(3-4): 97-104

93. Lee RH, Pulin AA, Seo MJ, et al (2009) Intravenous hMPCs improve myocardial infarction in mice because cells embolized in lung are activated to secret the anti-inflammatory protein TSG-6. Cell Stem Cell 5(1): 54-63

94. Lee RH, Seo MJ, Pulin AA, et al (2009) The CD34-like protein PODXL and alpha6-integrin (CD49f) identify early progenitor MPCs with increased clonogenicity and migration to infracted heart in mice. Blood 113(4): 816-826

95. Psaltis PJ, Paton S, See F, et al (2010) Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol 223(2): 530-540

96, Du YY, Zhou SH, Zhou T, et al (2008) Immuno-inflammatory regulation effect of mesenchymal stem cell transplantation in a rat model of myocardial infarction. Cytotherapy 10(5): 469-478

97. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105(4): 1815-1822

98. Burchfield JS, Iwasaki M, Koyanagi M, et al (2008) Interleukin-10 from transplanted bone marrow mononuclear cells contributes to cardiac protection after myocardial infarction. Circ Res 103(2): 203-211

99. Bianco P, Robey PG, Simmons PJ (2008) Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2: 313-319

100. Wagner W, Ho AD (2007) Mesenchymal stem cell preparations – comparing apples and oranges. Stem Cell Rev 3: 239-248

101. Ball SG, Shuttleworth AC, Kielty CM (2004) Direct cell contact influences bone marrow mesenchymal stem cell fate. Int J Biochem Cell Biol 36: 714-727

102. Novotny NM, Ray R, Markel TA, et al (2008) Stem cell therapy in myocardial repair and remodeling. J Am Coll Surg 207: 423-434

103. Crawford JR, Haudek SB, Cieslik KA, et al (2012) Origin of developmental precursors dictates the pathophysiologic role of cardiac fibroblasts. J Cardiovasc Transl Res 5: 749-759

104. Dawn B, Stein AB, Urbanek K, et al (2005) Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. Proc Natl Acad Sci USA 102: 3766-3771

105. Russell JL, Goetsch SC, Gaiano NR, et al (2011) A dynamic notch injury response activates epicardium and contributes to fibrosis repair. Circ Res 108: 51-59

106. Paul D, Samuel SM, Maulik N (2009) Mesenchymal stem cell: present challenges and prospective cellular cardiomyoplasty approaches for myocardial regeneration. Antioxid Redox Signal 11: 1841-1855

107. Ishikawa F, Shimazu H, Shultz LD, et al (2006) Purified human hematopoietic stem cells contribute to the generation of cardiomyocytes through cell fusion. FASEB J 20: 950-952

108. Docheva D, Popov C, Mutschler W, et al (2007) Human mesenchymal stem cells in contact with their environment surface characteristics and the integrin system. J Cell Mol Med 11: 21-38

109. Gronthos S, Simmons PJ, Graves SE, et al (2001) Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 28: 174-181

110. Gnecchi M, He H, Liang OD, et al (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11: 367-368

111. Wang J, Laschinger C, Zhao XH, et al (2005) Mechanical force activates eIF-2alpha phosphor-kinases in fibroblast. Biochem Biophys Res Commun 330(1): 123-130

112. Gurtner GC, Chang E (2008) “Priming” endothelial progenitor cells: a new strategy to improve cell based therapeutics. Arterioscler Thromb Vasc Biol 28: 1034-1035

113. Hahn JY, Cho HJ, Kang HJ, et al (2008) Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. J Am Coll Cardiol 51: 933-943

114. Haider H, Jiang S, Idris NM, et al (2008) IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. Circ Res 103: 1300-1308

115. Valiunas V, Doronin S, Valiuniene L, et al (2004) Human mesenchymal stem cells make cardiac connexins and form function gap junctions. J Physiol 555: 617-626

116. Kohl P (2003) Heterogeneous cell coupling in the heart: An electrophysiological role for fibroblasts. Circ Res 93: 381-383

117. Chilton L, Giles WR, Smith GL (2007) Evidence of intercellular coupling between co-cultured adult rabbit ventricular myocytes and myofibroblasts. J Physiol 583: 225-236

118. Gaudesius G, Miragoli M, Thomas SP, et al (2003) Coupling of cardiac electrical activity over extended distances by fibroblasts of cardiac origin. Circ Res 93: 421-428

119. Miragoli M, Gaudesius G, Rohr S (2006) Electronic modulation of cardiac impulse conduction by myofibroblasts. Circ Res 98: 801-810

120. Bucala R, Spiegel LA, Chesney J, et al (1994) Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med 1: 71-81

121. Díaz-Flores L, Gutiérrez R, García MP, et al (2014) CD34+ stromal cells/fibroblasts/fibrocytes/telocytes as a tissue reserve and a principle source of mesenchymal cells. Location, morphology, function, and role in pathology. Histol Histopathol 29: 831-870

122. Haniffa MA, Wang XN, Hotlick U, et al (2007) Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J Immunol 179: 1595-1604

123. Keeley EC, Schutt RC, Marinescu MA, et al (2016) Circulating fibrocytes as predictors of adverse effects in unstable angina. Transl Res 172: 73-83

124. Zulli A, Buxton BF, Black MJ, et al (2005) CD34 Class III positive cells are present in atherosclerotic plaques of the rabbit model of atherosclerosis. Histochem Cell Biol 124: 517-522

125. Medbury HJ, Tarran SL, Guiffre AK, et al (2008) Monocytes contribute to the atherosclerotic cap by transformation into fibrocytes. Int Angiol 27: 114-123

126. Xie X, Liu Y, Gao S, et al (2014) Possible involvement of fibrocytes in atrial fibrosis in patients with chronic atrial fibrillation. Circ J 78: 338-344

127. Fang L, Beale A, Ellims AH, et al (2013) Associations between fibrocytes and postcontrast myocardial T1 times in hypertrophic cardiomyopathy. J Am Heart Assoc 2: e000270

128. Chesney J, Metz C, Stavisky AB, et al (1998) Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. J Immunol 160: 419-425

129. D'Armiento J (2002) Matrix metalloproteinase disruption of the extracellular matrix and cardiac dysfunction. Trends Cardiovasc Med 12: 97-101

130. McClain SA, Simon M, Jones E, et al (1996) Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. Am J Pathol 149: 1257-1270

131. Xu J, Clark RA (1997) A three-dimensional collagen lattice induces protein kinase C-zeta activity: role in alpha-2 integrin and collagenase mRNA expression. J Cell Biol 136: 473-483

132. Aisagonhi O, Rai M, Ryzhov S, et al (2011) Experimental myocardial infarction triggers canonical Wnt signaling and endothelial-to-mesenchymal transition. Dis Model Mech 4: 469-483

133. von Gise A, Pu WT (2012) Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. Circ Res 110: 1628-1645

134. Goumans MJ, Mummery C (2000) Functional analysis of the TGFβ receptor/Smad pathway through gene ablation in mice. Int J Dev Biol 443: 253-265

135. Khan R, Sheppard R (2006) Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease, and arrhythmia. Immunology 1181: 10-24

136. O'Riordan E, Mendelev N, Patschan S, et al (2007) Chronic NOS inhibition actuates endothelial-mesencymal transformation. Am J Physiol Heart Circ Physioll 292: H285-H294

137. Tan X, Zhu Y, Chen C, et al (2016) Sprouty1 suppresses epithelial-mesenchymal transition of human lens epithelial cells through blockade of SMAD2 and ERK1/2 pathways. PLoS One 11(7): e0159275

138. Zhou B, Honor LB, He H, et al (2011) Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. J Clin Invest 121: 1894-1904

139. Evrard SM, Lecce L, Michelis KC, et al (2016) Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. Nat Commun 7: 11853

140. Nakaya M, Watari K, Tajima M, et al (2017) Cardiac myofibroblast engulfment of dead cells facilitates recovery after myocardial infarction. J Clin Invest 127(1): 383-401

141. Smith CL, Baek ST, Sung CY, et al (2011) Epicardial-derived cell epithelial-to-mesenchymal transition and fate specification require PDGF receptor signaling. Circ Res 295: 507-522

142. Rygiel KA, Robertson H, Marshall HL (2008) Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. Lab Invest 88: 112-123

143. Willis BC, Liebler JM, Luby-Phelps K (2005) Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol 166: 1321-1332

144. Iwano M, Plieth D, Danoff TM, et al (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 110: 341-350

145. Brønnum H, Andersen DC, Schneider M, et al (2013) miR-21 promotes fibrogenic epithelial-to-mesenchymal transition of epicardial mesothelial cells involving Programmed Cell Death 4 and Sprouty-1. PLoS One 8(2): e56280

146. Oh H, Bradfute SB, Gallardo TD (2003) Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. Proc Nat Acad Sci USA 100: 12313-12318

147. Bartel DP, Chen CA (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet 5: 396-400

148. Querejeta R, López B, González A, et al (2004) Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis. Circulation 110: 1263-1268

149. Holmes DR Jr, Savage M, LaBlanche JM, et al (2002) Results of prevention of REStenosis with tranilast and its outcomes (PRESTO) trial. Circulation 106: 1243-1250

150. Fang L, Murphy AJ, Dart AM (2017) A clinical perspective of anti-fibrotic therapies for cardiovascular disease. Front Pharmacol 8: 186

151. Hudson MP, Armstrong PW, Ruzyllo W, et al (2006) Effects of selective matrix metalloproteinase inhibitor (PG-116800) to prevent ventricular remodeling after myocardial infarction: results of the PREMIER (Prevention of Myocardial Infarction Early Remodeling) trial. J Am Coll Cardiol 48: 15-20

152. Tobert JA (1987) New developments in lipid-lowering therapy: the role of inhibitors of hydroxymethylglutaryl-coenzyme A reductase. Circulation 76:534-538

153. Heart Protection Study Collaborative Group (2002) MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 360:7-22

154. Kesh SB, Sikder K, Manna K, et al (2013) Promising role of ferulic acid, atorvastatin and their combination in ameliorating high fat diet-induced stress in mice. Life Sci. 92: 938-949

155. Hennessy DA, Tanuseputro P, Tuna M, et al (2017) Health Reports: Population health impact of statin treatment in Canada. Statistics Canada: <https://www.statcan.gc.ca/pub/82-003-x/2016001/article/14305-eng.htm>

156. US Department of Health and Human Services. Health, United States, 2016. May 2017. < https://www.cdc.gov/nchs/data/hus/hus16.pdf#glance>

157. Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343:425-430

158. Katsuki H, Bloch K (1967) Studies on the biosynthesis of ergosterol in yeast: formation of methylated intermediates. J Biol Chem 242: 222-227

159. Lynen F (1967) Biosynthetic pathways from acetate to natural products. Pure Appl Chem 14:137-167

160. Wolfe LA, Morava E, He M, et al (2012) Heritable disorders in the metabolism of the dolichols: a bridge from sterol biosynthesis to molecular glycosylation. Am J Med Genet C Semin Med Genet 160C:322-328

161. Molyneux SL, Young JM, Florkowski CM, et al (2008) Coenzyme Q10: is there a clinical role and a case for measurement? Clin Biochem Rev 29: 71-82

162. Diechmann R, Lavie C, Andrews S (2010) Coenzyme Q10 and statin-induced mitochondrial dysfunction. Ochsner J 10:16-21

163. Littarru GP, Tiano L (2007) Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Mitochondrion 7 Suppl:S168-S174

164. Rundek T, Naini A, Sacco R, et al (2004) Atorvastatin decreases the coenzyme Q10 level in the blood of patients at risk for cardiovascular disease and stroke. Arch Neurol 61:889-892

165. Brown MS, Goldstein JL (1980) Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res 21:505-517

166. Gil G, Osborne TF, Goldstein JL, et al (1988) Purification of a protein doublet that binds to six TGG-containing sequences in the promoter for hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J Biol Chem 263: 19009-19019

167. Metherall JE, Goldstein JL, Luskey KL, et al (1989) Loss of transcriptional repression of three sterol-regulated genes in mutant hamster cells. J Biol Chem 264:15634-15641

168. Nakanishi M, Goldstein JL, Brown MS (1988) Multivalent control of 3-hyroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J Biol Chem 263:8929-8937

169. Panini SR, Schnitzer-Polokoff R, Spencer TA, et al (1989) Sterol-independent regulation of 3-hydroxy-3-methyl-glutaryl-CoA reductase by mevalonate in Chinese hamster ovary cells. Magnitude and specificity. J Biol Chem 264: 11044-11052

170. Mosley ST, Brown MS, Anderson RG, et al (1983) Mutant clone of Chinese hamster ovary cells lacking 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Biol Chem 258:13875-13881

171. Ortego M, Bustos C, Hernandez-Presa MA, et al (1999) Atorvastatin reduces NF-kappaB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. Atherosclerosis 147:253-261

172. Zelvyte I, Dominaitiene R, Crisby M, et al (2002) Modulation of inflammatory mediators and PPARgamma and NFkappaB expression by pravastatin in response to lipoproteins in human monocytes in vitro. Pharmacol Res 45: 147-154

173. Weis M, Pehlivanli S, Meiser BM, et al (2001) Simvastatin treatment is associated with improvement in coronary endothelial function and decreased cytokine activation in patients after heart transplantation. J Am Coll Cardiol 38:814-818

174. Lazzerini PE, Lorenzini S, Selvi E, et al (2007) Simvastatin inhibits cytokine production and nuclear factor- κ B activation in interleukin 1beta-stimulated synoviocytes from rheumatoid arthritis patients. Clin Exp Rheumatol 25:696-700s

175. Devaraj S, Chan E, Jialal I (2006) Direct demonstration of an anti-inflammatory effect of simvastatin in subjects with metabolic syndrome. J Clin Endocrinol Metab 91:4489-4496

176. Marcuzzi A, Crovella S, Monasta L, et al (2012) Mevalonate kinase deficiency: disclosing the role of mevalonate pathway modulation in inflammation. Curr Pharm Des 18:5746-5752

177. Yeganeh B, Wiechec E, Ande SR, et al (2014) Targeting the mevalonate cascade as a new therapeutic approach in heart disease, cancer, and pulmonary disease. Pharmacol Ther 143: 87-110

178. Barone E, Di Domenico F, Butterfield DA (2014) Statins more than cholesterol lowering agents in Alzheimer disease: their pleiotropic functions as potential therapeutic targets. Biochem Pharmacol 88:605-616

179. Cortes-Bergoderi M, Pineda AM, Santana O (2013) The pleiotropic effects and therapeutic potential of the hydroxyl-methyl-glutaryl-CoA reductase inhibitors in gastrointestinal tract disorders: a comprehensive review. J Gastroinestin Liver Dis 22:199-204

180. Yuan C, Zhou L, Cheng J, et al (2012) Statins as potential therapeutic drug for asthma? Respir Res 13: 108

181. Zeichner S, Mihos CG, Santana O (2012) The pleiotropic effects and therapeutic potential of the hydroxyl-methyl-glutaryl-CoA reductase inhibitors in malignancies: a comprehensive review. J Cancer Res Ther 8:176-183

182. Lombard J, Moreira D (2011) Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. Mol. Biol Evol 28:87-99

183. Lange BM, Rujan T, Martin W, et al (200) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97: 13172-13177

184. Greenwood J, Steinman L, Zamvil SS (2006) Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. Nat Rev Immunol 6: 358-370

185. McTaggart SJ, (2006) Isoprenylated proteins. Cell Mol Life Sci 63:255-267

186. Wolfrum S, Jensen KS, Liao JK (2003) Endothelium-dependent effects of statins. Arterioscler Throm Vasc Biol 23:729-736

187. Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 8:180-186

188. Sinensky M, Beck LA, Leonard S (1990) Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. J Biol Chem 265:19937-19941

189. Takemoto M, Liao JK (2001) Pleiotropic effets of 3-hydroxy-3-methylgutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol 21:1712-1719

190. Sebti SM (2005) Protein farnesylation: implications for normal physiology, malignant transformation, and cancer therapy. Cancer Cell 7:297-300

191. Scheffzek K, Ahmadian MR (2005) GTPase activating proteins: structural and functional insights 18 years after discovery. Cell Mol Life Sci 62:3014-3038

192. Bourne HR, Sanders DA, McCormick F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125-132

193. Liao JK, Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45:89-118

194. Walker K, Olson MF (2005) Targeting Ras and Rho GTPases as opportunities for cancer therapeutics. Curr Opin Genet Dev 15:62-68

195. Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. Physiol Rev 81:153-208

196. van Wetering S, van Buul JD, Quik S, et al (2002) Reactive oxygen species mediate Rac-induced loss of cell-cell adhesion in primary human endothelial cells. J Cell Sci 115:1837-1846

197. Wassmann S, Wassmann K, Nickenig G (2004) Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. Hypertension 44:381-386

198. Antonopoulos AS, Margaritis M, Shirodaria C, et al (2012) Translating the effects of statins: from redox regulation to suppression of vascular wall inflammation. Thromb Haemost 108:840-848

199. Carman CV, Jun CD, Salas A, et al (2003) Endothelial cells proactively form microvilli-like membrane projections upon intercellular adhesion molecule 1 engagement of leukocyte LFA-1. J Immunol 171: 6135-6144

200. Wojciak-Stothard B, Williams L, Ridley AJ (1999) Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. J Cell Biol 145:1293-1307

201. Tousoulis D, Psarros C, Demosthenous M, et al (2014) Innate and adaptive inflammation as a therapeutic target in vascular disease: The emerging role of statins. J Am Coll Cardiol 63: 2491-2502

202. Alvarez De Sotomayor M, Herrera MD, Marhuenda E, et al (2000) Characterization of endothelial factors involved in the vasodilatory effect of simvastatin in aorta and small mesenteric artery of the rat. Br J Pharmacol 131: 1179-1187

203. Mital S, Zhang X, Zhao G, et al (2000)Simvastatin upregulates coronary vascular endothelial nitric oxide production in conscious dogs. Am J Physiol Heart Circ Physiol 279:H2649-H2657

204. Sarbassov DD, Ali S, Sabatini DM (2005) Growing roles for the mTOR pathway. Curr Opin Cell Biol 17:596-603

205. Sinclair LV, Finlay D, Feijoo C, et al (2008) Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. Nat Immunol 9:513-521

206. Antoniades C, Bakogiannis C, Tousoulis D, et al (2010) Preoperative atorvastatin treatment in CABG patients rapidly improves vein redox state by inhibition of Rac1 and NADPH-oxidase activity. Circulation 122:S66-S73

207. Nawawi H, Osman HS, Annuar R, et al (2003) Soluble intercellular adhesion molecule-1 and interleukin-6 levels reflect endothelial dysfunction in patients with primary hypercholesterolaemia treated with atorvastatin. Atherosclerosis 169:283-291

208. Tsunekawa T, Hayashi T, Kano H, et al (2001) Cerivastatin, a hydroxymethylglutaryl coenzyme a reductase inhibitor, improves endothelial function in elderly diabetic patients within 3 days. Circulation 104:376-379

209. Tousoulis D, Antoniades C, Bosinakou E, et al (2005) Effects of atorvastatin on reactive hyperaemia and inflammatory process in patients with congestive heart failure. Atherosclerosis 178:359-363

210. Thorburn A, Thorburn J, Chen SY, et al (1993) HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. J Biol Chem 268:2244-2249

211. Takemoto M, Node K, Nakagami H, et al (2001) Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy. J Clin Invest 108:1429-1437

212. Zhu Y, Casey PJ, Kumar AP, et al (2013) Deciphering the signalling networks underlying simvastatin-induced apoptosis in human cancer cells: evidence for non-canonical activation of RhoA and Rac1 GTPases. Cell Death Dis 4:e568

213. Morris G, Anderson G, Berk M (2013) Coenzyme Q10 depletion in medical and neuropsychiatric disorders: potential repercussions and therapeutic interventions. Mol Neurobiol 48:883-903

214. Crane FL (2008) The evolution of coenzyme Q. Biofactors 32:5-11

215. Rodríguez-Hernández A, Cordero MD, Salviati L, et al (2009) Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. Autophagy 5:19-32

216. Echtay KS, Winkler E, Klingenberg M (2000) Coenzyme Q is an obligatory cofactor for uncoupling protein function. Nature 408:609-613

217. Echtay KS, Winkler E, Frischmuth K, et al (2001) Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). Proc Natl Acad Sci USA 98:1416-1421

218. Kon M, Kimura F, Akimoto T, et al (2007) Effect of coenzyme Q10 supplementation on exercise-induced muscular injury of rats. Exerc Immunol Rev 13:76-88

219. Ernster L, Forsmark P, Nordenbrand K (1992) The mode of action of lipid-soluble antioxidants in biological membranes: relationship between the effects of ubiquinol and vitamin E as inhibitors of lipid peroxidation in submitochondrial particles. Biofactors 3:241-248

220. Kagan V, Serbinova E, Packer L (199) Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. Biochem Biophys Res Commun 169:851-857

221. James AM, Smith RA, Murphy MP (2004) Antioxidant and prooxidant properties of mitochondrial coenzyme Q. Arch Biochem Biophys 423:47-56

222. Passi S, Stancato A, Aleo E, et al (2003) Statins lower plasma and lymphocyte ubiquinol/ubiquinone without affecting other antioxidants and PUFA. Biofactors 18:113-124

223. Langsjoen PH, Langsjoen JO, Langsjoen AM, et al (2005) Treatment of statin adverse effects with supplemental coenzyme Q10 and statin drug discontinuation. Biofactors 25:147-152

224. Littarru GP, Langsjoen P (2007) Coenzyme Q10 and statins: biochemical and clinical implications. Mitochondrion 7 Suppl:S168-S174

225. Kuettner A, Pieper A, Koch J, et al (2005) Influence of CoQ10 and cerivastatin on the flow-mediated vasodilation of the brachial artery: results of the ENDOTACT study. Int J Cardiol 98:413-419

226. McMurray JJ, Dunselman P, Wedel H et al (2010) CoQ10, rosuvastatin, and clinical outcomes in heart failure: a pre-specified substudy of CORONA (controlled rosuvastatin multinational study in heart failure). J Am Coll Cardiol 56:1196-1204

227. Felker GM (2010) CoQ10 and statins in heart failure: the dog that didn't bark. J Am Coll Cardiol 56:1205-1206.

228. Taylor BA, Lorson L, White CM, et al (2015) A randomized trial of CoQ10 in patients with confirmed statin myopathy. Atherosclerosis 238:329-335

229. Dunn SE, Youssef G, Goldstein MJ, et al (2006) Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. J Exp Med 203:401-412

230. Lawman S, Mauri C, Jury EC, et al (2004) Atorvastatin inhibits autoreactive B cell activation and delays lupus development in New Zealand Black/White F1 mice. J Immunol 173:7641-7646

231. Zeiser R, Youssef S, Baker J, et al (2007) HMG-CoA reductase inhibitors (statins) provide acute graft-versus-host disease protection by Th2 cytokine induction whlile sparing graft-versus-leukemia activity. Blood 110:4588-4598

232. Raki M, Molberg O, Tollefsen S, et al (2005) The effects of atorvastatin on gluten-induced intestinal T cell responses in celiac disease. Clin Exp Immunol 142:333-340

233. Mausner-Fainberg K, Luboshitss G, Mor A, et al (2008) The effect of HMG-CoA reductase inhibitors on naturally occurring CD4+ CD25+ T cells. Atherosclerosis 197:829-839

234. Broz P, Ben-Haim N, Grzelakowski M, et al (2008) Inhibition of macrophage phagocytic activity by a receptor-targeted polymer vesicle-based drug delivery formuation of pravastatin. J Cardiovasc Pharmacol 51:246-252

235. Xu SZ, Zhong W, Watson NM, et al (2008) Fluvastatin reduces oxidative damage in human vascular endothelial cells by upregulating Bcl-2. J Thromb Haemost 4:692-700

236. Zeiser R, Maas K, Youssef S, et al (2008) Regulation of different inflammatory disease by impacting the mevalonate pathway. Immunology 127:18-25

237. Youssef S, Stuve O, Patarroyo JC, et al (2002) The HMG-CoA reductase inhibitor atorvastatin, promotes the Th2 bias and reverses paralysis in central nervous system in autoimmune disease. Nature 420:78-84

238. Aktas O, Waiczies S, Smorodchenko A, et al (2003) Treatment of relapsing paralysis in experimenting encephalomyelitis by targeting Th1 cells through atorvastatin. J Exp Med 197:725-733

239. Greenwood J, Walters CE, Pryce G (2003) Lovastatin inhibits brain endothelial Rho-mediated lymphocyte migration and attenuates experimental autoimmune encephalomyelitis. FASEB J 17:905-907

240. Nath N, Giri S, Prasad R (2004) Potential targets of 3-hydroxy-3-methylglutaryl Coenzyme A reductase inhibitor for multiple sclerosis therapy. J Immunol 172:1273-1286

241. Eccles KA, Sowden H, Porter KE, et al (2008) Simvastatin alters human endothelial cell adhesion molecule expression and inhibits leukocyte adhesion under flow. Atherosclerosis 200:69-79

242. Zhang W, Liu M, Wu Y (2007) Protective effects of atorvastatin on chronic allograft nephropathy in rats. J Surg Res 143:428-436

243. Li C, Yang CW, Park JH, et al (2004) Pravastatin treatment attenuates interstitial inflammation and fibrosis in a rat model of chronic cyclosporine-induced nephropathy. Am J Physiol Renal Physiol 286:F46-F57

244. Kohl NE, Omer CA, Conner MW, et al (1995) Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat Med 1:792-797

245. Sola S, Mir MQS, Lerakis S, et al (2006) Atorvastatin improves left ventricular systolic function and serum markers of inflammation in nonischemic heart failure. J Am Coll Cardiol 47: 332-337

246. Node K, Fujita M, Kitakaze M, et al (2003) Short-term statin therapy improves cardiac function and symptoms in patients with idiopathic dilated cardiomyopathy. Circulation 108:839-843

247. Rezaie-Majd A, Maca T, Bucek RA, et al (2002) Simvastatin reduces expression of cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. Aterioscler Thromb Vasc Biol 22:1194-1199

248. Ridker PM, Danielson E, Fonseca FA, et al (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Eng J Med 359:2195-2207

249. van der Meer JW, Vossen JM, Radl J, et al (1984) Hyperimmunoglobulinaemia D and periodic fever: a new syndrome. Lancet 1:1087-1090

250. Berger R, Smit GP, Schierbeek H (1985) Mevalonic aciduria: an inborn error cholesterol biosynthesis. Clin Chim Acta 152:219-222

251. Hoffmann G, Gibson KM, Brandt IK, et al (1986) Mevalonic aciduria –an inborn error of cholesterol and nonsterol isoprene biosynthesis. N Engl J Med 314:1610-1614

252. Houten SM, Kuis W, Duran M, et al (1999) Mutations in MVK, encoding mevalonate kinase, cause hyperimmunoglobulinaemia D and periodic fever syndrome. Nat Genet 22:175-177

253. Simon A, Kremer HP, Wevers RA, et al (2004) Mevalonate kinase deficiency: evidence for a phenotypic continuum. Neurology 62:994-997

254. van der Burgh R, ter Haar NM, Boes ML, et al (2013) Mevalonate kinase deficiency, a metabolic autoinflammatory disease. Clin Immunol 147:197-206

255. Cuisset L, Drenth JP, Simon A, et al (2001)) Molecular analysis of MVK mutations and enzymatic activity in hyper-IgD and periodic fever syndrome. Eur J Human Genet 9:260-266

256. Poll-The BT, Frenkel J, Houten SM, et al (2000) Mevalonic aciduria in 12 unrelated patients with hyperimmunoglobulinaeia D and periodic fever syndrome. J Inhert Metab Dis 23:363-366

257. Hoffmann GF, Charpentier C, Myaytepek E, et al (1993) Clinical and biochemical phenotype in 11 patients with mevalonic aciduria. Pediatrics 91:915-921

258. Marcuzzi A, Crovella S, Pontillo A (2011) Geraniol rescues inflammation in cellular and animal models of mevalonate kinase deficiency. In Vivo 25:87-92

259. Marcuzzi A, Tommasini A, Crovella S, et al (2010) Natural isoprenoids inhibit LPS-induced production of cytokines and nitric oxide in aminobisphosphonate-treated monocytes. Int Immunopharmacol 10:639-642

260. Henneman L, Schneiders MS, Turkenburg M, et al (2010) Compromised geranylgeranyl of RhoA and Rac1 in mevalonate kinase deficiency. J Inherit Metab Dis 33:625-632

261. Chua CE, Gan BQ, Tang BL (2011) Involvement of members of the rab family and related small GTPases in autophagosome formation and maturation. Cell Mol Life Sci 68:3349-335826

262. Dupont N, Jiang S, Pilli M, et al (2011) Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. EMBO J 30:4701-4711

263. Zhou R, Yazdi AS, Menu P, et al (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature 469:221-225

264. Reinhart BJ, Slack FJ, Basson M, et al (2000) The 21 nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403 (6772):901-906

265. Grishok A, Pasquinelli AE, Conte D, et al (2001) Genes and mechanisms related to RNA interference regulate expression of small temporal RNAs that control C. elegans developmental timing. 106(1):23-34

266. Bartel GP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2): 281-297

267. Gurtan AM, Ravi A, Rahl PB, et al (2013) Let-7 represses Nr6a1 and a mid-gestation developmental program in adult fibroblasts. Genes Dev 27: 941-954

268. Herranz H, Cohen SM (2010) MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. Genes Dev 24: 1339-1344

269. Bernstein E, Caudy AA, Hammond SM, et al (2001) Role for bidentate ribonuclease in the initiation step of RNA interference. Nature 409(6818): 363-366

270. Ebert MS, Sharp PA (2012) Roles for microRNAs in conferring robustness to biological processes. Cell 149: 515-524

271. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676

272. Port JD, Sucharov C (2010) Role of microRNAs in cardiovascular disease: therapeutic challenges and potentials. J Cardiovasc Pharmacol 56: 444-453

273. Subramanyam D, Lamouille S, Judson RL, et al (2011) Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. Nat Biotechnol 29: 443-448

274. Nagai R, Suzuki T, Aizawa K, et al (2005) Significance of the transcription factor KLF5 in cardiovascular remodeling. J Thromb Haemost 3: 1569-1576

275. Rao PK, Toyama Y, Chiang R, et al (2009) Loss of cardiac miRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res 105: 585-594

276. Yang B, Lin H, Xiao J, et al (2007) The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 13: 468-491

277. Glass C, Singla DK (2011) MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infracted heart. Am J Physiol Heart Circ Physiol 301(5): H2038-H2049

278. Thum T, Gross C, Fiedler J, et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts. Nature 456: 980-984

279. Li Q, Song XW, Zou J, et al (2010) Attentuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. J Cell Sci 123(Pt 14): 2444-2452

280. van Rooij E, Sutherland LB, Thatcher JE, et al (2008) Dysregulation of miroRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105: 13027-13032

281. Roy S, Khanna S, Hussain SR, et al (2009) MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 82: 21-29

282. Thum T, Gross C, Fiedler J, et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts. Nature 456: 980-984

283. Wang J, Xu R, Lin F, et al (2009) MicroRNA: novel regulators involved in the remodeling and reverse remodeling of the heart. Cardiology 113: 81-88

284. Pekarsky Y, Santanum U, Cimmino A, et al (2006) Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res 66: 11590-11593

285. Mott JL, Kobayashi S, Bronk SF, et al (2007) miR-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 26: 6133-6140

286. Park SY, Lee JH, Ha M, et al (2009) miR-29 miRNAs activate p53 by targeting p85α and CDC42. Nat Struct Mol Biol 16: 23-29

287. Czubryt MP (2012) Common threads in cardiac fibrosis, infarct scar formation, and wound healing. Fibrogenesis Tissue Repair 5:19

288. Borg TK and Caulfield JB (1979) Collagen in the heart. Tex Rep Biol Med 39:321-333 173. Caulfield JB and Borg TK (1979) The collagen network of the heart. Lab Invest 40:364-372

289. Rienks M, Papageorgiou AP, Frangogiannis NG, et al (2014) Myocardial extracellular matrix: an ever-changing and diverse entity. Circ Res 114:872-888

290. Banerjee I, Fuseler JW, Price RL, et al (2007) Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. Am J Physiol Heart Circ Physiol 293:H1883-H1891

291. Nag AC (1980) Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. Cytobios 28:41-61

292. Theocharis AD, Skandalis SS, Gialeli C, et al (2016) Extracellular matrix structure. Adv Drug Deliv Rev 97: 4-27.

293. Theocharis AD, Skandalis SS, Tzanakakis GN, et al (2010) Proteoglycans in health and disease: novel roles for pharmacological targeting. FEBS J 277: 3904-3923.

294. Iozzo RV, Sanderson RD (2011) Proteoglycans in cancer biology, tumor microenvironment and angiogenesis. J Cell Mol Med 15: 1013-1031

295. Afratis N, Gialeli C, Nikitovic T, et al (2012) Glycosaminoglycans: key players in cancer cell biology and treatment. FEBS J 279: 1177-1197

296. Camelliti P, Borg TK, and Kohl P (2005) Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res 65:40-51

297. Camelliti P, McCulloch AD and Kohl P (2005) Microstructured cocultures of cardiac myocytes and fibroblasts: a two-dimensional in vitro model of cardiac tissue. Microsc Microanal 11:249-259

298. Prockop DJ and Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64:403-434

299. Kagan HM and Trackman PC (1991) Properties and function of lysyl oxidase. Am J Respir Cell Mol Biol 5:206-210

300. Smith-Mungo LI and Kagan HM (1998) Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 16:387-398

301. Mukherjee D and Sen S (1991) Alteration of collagen phenotypes in ischemic cardiomyopathy. J Clin Invest 88:1141-1146

302. Weber KT (1989) Cardiac interstitium in health and disease: the fibrillar collagen network. JACC 13:1637-1652

303. Cowan PM, McGavin S and North AC (1955) The polypeptide chain configuration of collagen. Nature 176:1062-1064

304. Pauling L and Corey RB (1951) The structure of fibrous proteins of the collagen-gelatin group. Proc Natl Acad Sci U S A 37:272-281

305. Wierzbicka-Patynowski I, Schwarzbauer JE (2003) The ins and outs of fibronectin matrix assembly. J Cell Sci 116 (Pt 16): 3269-3276

306. Serini G, Bochaton-Piallat ML, Ropraz P, et al (1998) The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor β1. J Cell Biol 142: 873-881

307. Mardilovich A, Craig JA, McCammon MQ et al (2006) Design of a novel fibronectin-mimetic peptide-amphiphile for functionalized biomaterials. Langmuir 2006 22(7): 3259-3264

308. Hozumi K, Nakamura K, Hori H, et al (2016) Mixed fibronectin-derived peptides conjugated to a chitosan matrix effectively promotes biological activities through integrins α4β1, α5β1, αvβ1, αvβ3, and Syndecan. Biores Open Access 5(1):356-366

309. Schonherr E and Hausser HJ (2000) Extracellular matrix and cytokines: a functional unit. Dev Immunol 7:89-101

310. Sarrazy V, Koehler A, Chow ML, et al (2014) Integrins αvβ5 and αvβ3 promote latent TGF-β1 activation by human cardiac fibroblast contraction. Cardiovasc Res 102:407-417

311. Wipff PJ and Hinz B (2008) Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. Eur J Cell Biol 87:601-615

312. Wipff PJ, Rifkin DB, Meister JJ, et al (2007) Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. J Cell Biol 179:1311- 1323

313. Ingber DE (2006) Cellular mechanotransduction: putting all the pieces together. FASEB 20(7):811-827

314. Geiger B, Bershadsky A, Pankov R, et al (2001) Transmembrane crosstalk between extracellular matrix-cytoskeleton crosstalk. Nat Rev Mol Cell Biol 2(11):793-805

315. Giancotti FG, Ruoslahti E (1999) Integrin signaling. Science 285(5430:1028-1032

316. Terracio L, Rubin K, Gullberg D, et al (1991) Expression of collagen binding integrins during cardiac development and hypertrophy. Circ Res 68(3): 734-744

317. Ruoslahti E (1996) RGD and other recognition sequences for integrins. Ann Rev Cell Dev Biol 12:697–715

318. Ventura C, Maioli M, Asara Y, et al (2004) Butyric and retinoic mixed ester of hyaluronan. A novel differentiating glyconjugate affording a high throughput of cardiogenesis in embryonic stem cells. J Biol Chem 279(22):23574-23579

319. Chen SS, Fitzgerald W, Zimmerberg J, et al (2007) Behaviour of cardiomyocytes and skeletal muscle cells on different extracellular matrix components – relevance for cardiac tissue engineering. Artif Organs 31(1):4-12

320. Geary RL, Nikkari ST, Wagner WD, et al (1998) Wound healing: a paradigm for lumen narrowing after arterial reconstruction. J Vasc Surg 27:96-106

321. Yamagata M, Suzuki S, Akiyama SK, et al (1989) Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. J Biol Chem 264:8012-8018

322. Yamagata M, Saga S, Kato M, et al (1993) Selective distributions of proteoglycans and their ligands in pericellular matrix of cultured fibroblasts. Implications for their role in cell-substratum adhesion. J Cell Sci 106:55-65

323. DeGrendele HC , Kosfiszer M , Estess P, et al (1997) CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. J Immunol 159:2549–2553

324. Zhu H, Mitsuhashi N, Klein A, et al (2006) The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. Stem Cells 24(4):928-935

325. Khademhoisseni A, Eng G, Yeh J, et al (2007) Microfluidic patterning for fabrication of contractile cardiac organoids. Biomed Microdev 9(2):149-157

326. Horton RE, Millman JR, Colton CK, et al (2009) Engineering microenvironments for embryonic stem cell differentiation to cardiomyocytes. Regen Med 4(5): 721-732

327. Månsson-Broberg A, Rodin S, Bulatovic I, et al (2016) Wnt/β-Catenin stimulation and laminins support cardiovascular cell progenitor expansion from human fetal cardiac mesenchymal stromal cells. Stem Cell Reports 6(4): 607-617

328. Horton RE, Auguste DT (2012) Synergistic effects of hypoxia and extracellular matrix cues in cardiomyogenesis. Biomaterials 33(27): 6313-6319

329. McCurdy S, Baicu CF, Heymans S, et al (2010) Cardiac extracellular matrix remodeling: fibrillar collagens and Secreted Protein Acidic and Rich in Cysteine (SPARC). J Mol Cell Cardiol 48(3):544-549

330. Hollfelder D, Frasch M, Reim I (2014) Distinct functions of the laminin beta LN domain and collagen IV during cardiac extracellular matrix formation and stabilization of alary muscle attachments revealed by EMS mutagenesis in Drosophila. BMC Dev Biol 14:26

331. Fisher SA, Periasamy M (1994) Collagen synthesis inhibitors disrupt embryonic cardiocyte myofibrillogenesis and alter the expression of cardiac specific genes *in vitro* J Mol Cell Cardio 26(6): 721-731

332. Sato H, Takahashi M, Ise H, et al (2006) Collagen synthesis is required for ascorbic-acid enhanced differentiation of mouse embryonic stem cells into cardiomyocytes. Biochem Biophys Res Commun 342(1): 107-112

333. Jacot JG, McCulloch AD, Omens JH (2008) Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. Biophys J 95(7): 3479-3487

334. Pick E, Kroizman T, Abo A (1989) Activation of the superoxide-forming NADPH oxidase of macrophages requires two cytosolic components – one of them is also present in certain nonphagocytic cells. J Immunol 143(12): 4180-4187

335. Fomovsky GM, Holmes JW (2010) Evolution of scar structure, mechanics, and ventricular function after myocardial function in the rat. Am J Physiol Heart Circ Physiol 298(1): H221-H228

336. Zemel A, Rehfeldt F, Brown AE, et al (2010) Optimal matrix rigidity for stress-fibre polarization in stem cells. Nature Physics 6(6):468–473

337. Ivanovska IL, Shin JW, Swift J, et al (2015) Stem cell mechanobiology: diverse lessons from bone marrow. Trends Cell Biol 25(9):523-532

338. Vicente-Manzanares M, Ma X, Adelstein RS, et al (2009) Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol 10:778–790

339. Raab M, Swift J, Dingal PC, et al (2012) Crawling from soft to stiff matrix polarizes the cytoskeleton and phosphoregulates myosin-II heavy chain. J Cell Biol 199(4):669–683

340. Tse JR, Engler AJ (2011) Stiffness gradients mimicking in vivo tissue variation regulate mesenchymal stem cell fate. Plos One 6(1):e15978

341. Isenberg BC, Dimilla PA, Walker M, et al (2009) Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength. Biophys J 97(5):1313–1322

342. Iyer KV, Pulford S, Mogilner A, et al (2012) Mechanical activation of cells induces chromatin remodeling preceding MKL nuclear transport. Biophysic J 103(7):1416–1428

343. Crisp M, Liu Q, Roux K, et al (2006) Coupling of the nucleus and cytoplasm: role of the LINC complex. J Cell Biol 172:41–53

344. Lombardi ML, Jaalouk DE, Shanahan CM, et al (2011) The interaction between nesprins and sun proteins at the nuclear envelope is critical for force transmission between the nucleus and cytoskeleton. J Biol Chem 286(30):26743–26753

345. Banerjee I, Zhang J, Moore-Morris T, et al (2014) Targeted ablation of nesprin 1 and nesprin 2 from murine myocardium results in cardiomyopathy, alter nuclear morphology, and inhibition of the biomechanical gene response. PLoS Genet 10(2): e1004114

346. Hale CM, Shrestha AL, Khatau SB, et al (2008) Dysfunctional connections between the nucleus and the actin and microtubule networks in laminopathic models. Biophys J 95(11):5462-5475

347. Frisch SM, Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124(4):619–626

348. Miner JH, Yurchenco PD (2004) Laminin functions in tissue morphogenesis. Ann Rev Cell Develop Biol 20:255-284

349. Domogatskaya A, Rodin S, Tryggvason K (2012) Functional diversity of laminins. Ann Rev Cell Develop Biol 28:523-533

350. Cameron AR, Frith JE, Cooper-White JJ (2011) The influence of substance creep on mesenchymal stem cell behaviour and phenotype. Biomaterials 32(26):5979-5993

351. Reilly GC, Engler A (2010) Intrinsic extracellular matrix properties regulate stem cell differentiation. J Biomechanics 43(1):55-62

352. Lee DA, Knight MM, Campbell JJ, et al (2011) Stem cell mechanobiology J Cell Biochem 112(1):1-9

353. Guilak F, Cohen DM, Estes BT, et al (2009) Control of stem cell fate by physical interactions with extracellular matrix. Cell Stem Cell 5(1):17-26

354. Hyn es RO (2009) The extracellular matrix: not just pretty fibrils. Science 326(5957):1216-1219

355. Nishimune H, Sanes JR, Carlson SS (2004) A synaptic laminin-calcium channel interaction organizes active zones in motor nerve terminals. Nature 432(7017):580-587

356. Saha K, Keung AJ, Irwin EF, et al (2008) Substrate modulus directs neural stem cell behaviour. Biophys J 95(9):4426-4438

357. Rowland TJ, Blaschke AJ, Buchholz DE, et al (2013) Differentiation of human pluripotent stem cells to retinal pigmented epithelium in defined conditions using purified extracellular matrix proteins. J Tissue Eng Regen Med 7(8):642-653

358. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154-156

359. Wang H, Luo X, Leighton J (2015) Extracellular matrix and integrins in embryonic stem cell differentiation. Biochem Insights 8(Suppl2);15-21

360. Pagliuca FW, Millman JR, Gürtler M, et al (2014) Generation of functional human pancreatic β cells *in vitro*. Cell 159(2):428-439

361. Zhu W-Z, Van Biber B, Laflamme MA (2011) Methods for the derivation and use of cardiomyocytes from human pluripotent stem cells. Methods Mol Biol 767:419-431

362. Jha BS, Rao M, Malik N (2015) Motor neuron differentiation from pluripotent stem cells and other intermediate proliferative precursors that can be discriminated by lineage specific reporters. Stem Cell Rev 11(1):194-204

363. Hannan NFR, Segeritz C-P, Touboul T, et al (2013) Production of hepatocyte-like cells from human pluripotent stem cells. Nat Protoc 8(2):430-437

364. Turinetto V, Orlando L, Giachino C (2017) Induced pluripotent stem cells: advances in the quest for genetic stability during reprogramming process. Int J Mol Sci 18(9),pii:E1952

365. Gattazzo F, Urciuolo A, Bonaldo P (2014) Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta 1840(8):2506-2519

366. Maniotis A, Bojanowski K, Ingber DE (1997) Mechanical continuity and reversible disassembly within intact genomes removed from living cells. J Cell Biochem 65(1):114-130

367. Bornstein P, McPherson J, Sage H, et al (1982) Synthesis and secretion of structural macromolecules by endothelial cells in culture. In:Nossel HL, Vogel HJ, editors. Pathobiology of the Endothelial Cell. New York: Academic Press, pp 215-228

368. Thorne JT, Segal TR, Chang S, et al (2015) Dynamic reciprocity between cells and their microenvironment in reproduction. Biol Reprod 92(1):25

369. Kaul H, Ventikos Y (2015) Dynamic reciprocity revisited. J Theor Biol 370:205-208

370. Wozniak MA, Chen CS (2009) Mechanotransduction in development: a growing role for contractility. Nat Rev Mol Cell Biol 10(1):34-43

371. Chowdhury F, Na S, Li D et al (2010) Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. Nat Mater 9(1):82-88

372. Arshi A, Nakashima Y, Nakano H, et al (2013) Rigid microenvironments promote cardiac differentiation of mouse and human embryonic stem cells. Sci Technol Adv Mater 14(2):025003

373. Barczyk M, Carrecedo S, Gullberg D (2010) Integrins. Cell Tissue Res 339(1):269-280

374. Ridley AJ, Schwartz MA, Burridge K, et al (2003) Cell migration: integrating signals from front to back. Science 302(5651):1704-1709

375. Darribère Te, Skalski M, Cousin H, et al (2000) Integrins: regulators of embryogenesis. Biol Cell 92(1):5-25

376. Wong JCY, Gao SY, Lees JG, et al (2010) Definitive endoderm derived from human embryonic stem cells highly express the integrin receptors alphaV and beta5. Cell Adh Migr 4(1):39-45

377. Farzaneh Z, Pakzad M, Vosough M, et al (2014) Differentiation of human embryonic stem cells to hepatocyte-like cells on a new developed xeno-free extracellular matrix. Histochem Cell Biol 142(2):217-226

378. Lora JM, Rowader KE, Soares L, et al (1998) α3β1-integrin as a critical mediator of the hepatic differentiation of response to the extracellular matrix. Hepatology 28(4):1095-1104

379. Park KS (2011) TGF-β family signaling in embryonic stem cells. Int J Stem Cells 4(1):18-23

380. Weinstein M, Monga SP, Liu Y, et al (2001) Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on β1-integrin to promote normal liver development. Mol Cell Biol 21(15):5122-5131

381. Higuchi Y, Shikari N, Yamane K, et al (2010) Synthesized basement membranes direct the differentiation of mouse embryonic stem cells into pancreatic lineages. J Cell Sci 123(pt16):2733-2732

382. Narayanan K, Lim VY, Shen J, et al (2014) Extracellular matrix-mediate differentiation of human embryonic stem cells: differentiation to insulin-secreting beta cells. Tissue Eng Part A 20(1-2):424-433

383. Richoux V, Darribère T, Boucat J-C, et al (1989) Distribution of fibronectins and laminin in the early pig embryo. Anat Rec 223(1):72-81

384. Yang JT, Rayburn H, Hynes RO (1993) Embryonic mesodermal defects in alpha 5 integrin-deficient mice. Development 119(4):1093-1105

385. Liu B, Lewis AK, Shen W (2009) Physical hydrogels photo-cross-linked from self-assembled macromers for potential use in tissue engineering. Biomacromolecules 10(12):3182-3197

386. Thorsteinsdóttir S, Roelen BAJ, Goumans M-J, et al (1999) Expression of the α6A integrin splice variant in developing mouse embryonic stem cell aggregates and correlation with cardiac muscle differentiation. Differentiation 64(3):173-184

387. Duan Y, Liu Z, O'Neill J, et al (2011) Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors. J Cardiovasc Transl Res 4(5):605-615

388. Baharvand H, Azarnia M, Parivar K, et al (2005) The effect of extracellular matrix on embryonic stem cell-derived cardiomyocytes. J Mol Cell Cardiol 38(3):495-503

389. Sa S, Wong L, McCloskey KE (2014) Combinatorial fibronectin and laminin signaling promote highly efficient cardiac differentiation of human embryonic stem cells. Biores Open Access 3(4):150-161

390. Kattman SJ, Witty AD, Gagliardi M, et al (2011) Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 8(2):228-240

391. Kothapalli CR, Kamm RD (2013) 3D matrix microenvironment for targeted differentiation of embryonic stem cells into neural and glial lineages. Biomaterials 34(25):5995-6007

392. Anton ES, Kreidberg JA, Rakic P (1999) Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuron 22(2):277-289

393. Dhara SK, Stice SL (2008) Neural differentiation of human embryonic stem cells. J Cell Biochem 105(3):633-640

394. Goh KL, Yang JT, Hynes RO (1997) Mesoderm defects and cranial neural crest apoptosis in alpha5 integrin-null embryos. Development 124(21):4309-4319

395. Lee MR, Kwon KW, Jung H, et al (2010) Direct differentiation of human embryonic stem cells into selective neurons on nanoscale ridge/groove pattern arrays. Biomaterials 31: 4360-4366

396. Eckes B, Krieg T, Wickström SA (2014) Role of integrin signaling through integrin-linked kinase in skin physiology and pathology. Exp Dermatol 23(7):453-456

397. Hara M, Yaar M, Tang A, et al (1994) Role of integrins in melanocyte attachment and dendricity. J Cell Sci 107(pt 10):2739-2748

398. Banito A, Rashid ST, Acosta JC, et al (2009) Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 23:2134-2139

399. Esteban MA, Pei DQ (2012) Vitamin c improves the quality of somatic cell reprogramming. Nat Genet 44:366-367

400. Gore A, Li Z, Fung HL, et al (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature 471:63-67

401. Mayshar Y, Ben-David U, Lavon N, et al (2010) Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell 7:521-531

402. Cheng LZ, Hansen NF, Zhao L, et al (2012) Low incidence of DNA sequence variation in human induced pluripotent stem cells generated by nonintegrating plasmid expression. Cell Stem Cell 10:337-344

403. Hussein SM, Batada NN, Vuoristo S, et al (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471: 58-62

404. Laurent LC, Ulitsky I, Slavin I, et al (2011) Dynamic changes in the copy number of pluripotency and cell proliferation genes in human escs and ipscs during reprogramming and time in culture. Cell Stem Cell 8:106-118

405. Yoshishara M, Hayashizaki Y, Murakawa Y (2017) Genetic instability of ipscs: Challenges towards their clinical applications. Stem Cell Rev 13:7-16

406. Medvedev SP, Shevchenko AI, Zakian SM (2010) Induced pluripotent stem cells: problems and advantages when applying them in regenerative medicine. Acta Naturae 2(5):18-27

407. Yoshida Y, Takahashi K, Okita K, et al (2009) Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 5:237-241

408. Mathieu J, Zhou WY, Xing YL, et al (2014) Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. Cell Stem Cell 14:592-605

409. Rodesch F, Simon P, Donner C, et al (1992) Oxygen measurements in endometrial and trophoblastic tissues during early-pregnancy. Obstet Gynecol 80:283-285

410. Wang PY, Thissen H, Kingshott P (2016) Modulation of human multipotent and pluripotent stem cells using surface nanotopographies and surface-immobilised bioactive signals: A review. Acta Biomaterialia 45:31-59

411. Chen AKL, Reuveny S, Oh SKW (2013) Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction. Biotechnol Adv 31:1032-1046

412. Guo G, von Meyenn F, Santos F, et al (2016) Naïve pluripotent cells derived directly from isolated cells of human inner cell mass. Stem Cell Rep 6:437-446

413. Pan F, Zhang M, Wu G, et al (2013) Topographic effect on human induced pluripotent stem cells differentiation towards neuronal lineage. Biomaterials 34:8131-8139

414. Chan LY, Birch WR, Yim EK, et al (2013) Temporal application of topography to increase the rate of neuronal differentiation from human pluripotent stem cells. Biomaterials 34:382-392

415. Mosqueira D, Pagliari S, Uto K, et al (2014) Hippo pathway effectors control cardiac progenitor cell fate by acting as dynamic sensors of substrate mechanics and nanostructure. ACS Nano 8:2033-2047

416. Wan CR, Frohlich EM, Charest JL, et al (2011) Effect of surface patterning and presence of collagen I on the phenotypic changes of embryonic stem cell derived cardiomyocytes. Cell Mol Bioeng 4:56-66

417. Kim DH, Kshitiz RR, Smith P, et al (2012) Nanopatterned cardiac cell patches promote stem cell niche formation and myocardial regeneration. Integr Biol 4:1019-1033

418. Reimer A, Vasilevich A, Hulshof F, et al (2016) Scalable topographies to support proliferation and Oct4 expression by human induced pluripotent stem cells. Sci Rep 6:18948.

419. Chou YF, Chen HH, Eijpe M, et al (2008) The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. Cell 135:449–461

420. Aasen T, Raya A, Barrero MJ, et al (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26:1276–1284

421. Redmer T, Diecke S, Grigoryan T,et al (2011) E-cadherin is crucial for embryonic stem cell pluripotency and can replace OCT4 during somatic cell reprogramming. EMBO Rep 12:720–726

422. Rowland TJ, Miller LM, Blaschke AJ, et al (2010) Roles of integrins in human induced pluripotent stem cell growth on Matrigel and vitronectin. Stem Cells Dev 19:1231–1240

423. Magli A, Incitti T, Kiley J, et al (2017) PAX7 targets, CD54, Integrin α9β1, and SDC2, allow isolation of human ESC/iPSC-derived myogenic progenitors. Cell Rep 19(13):2867-2877

424. Ozeki N, Hase N, Higuchi N, et al (2017) Gelatin scaffold combined with bone morphogenetic protein-4 induces odontoblast-like cell differentiation involving integrin profile changes, autophagy-related gene 10, and Wnt5 sequentially in human induced pluripotent stem cells. Differentiation 93:1-14

425. Shah N, Morsi Y, Manasseh R (2014) From mechanical stimulation to biological pathways in the regulation of stem cell fate. Cell Biochem Funct 32(4):309-325

426. Cho M, Titushkin I, Sun S, et al (2010) Physicochemical control of adult stem cell differentiation: shedding light on potential molecular mechanisms. J Biomed Biotechnol 2010:743476

427. Huang Y, Fan Y (2013) Effect of intermittent fluid shear stress on cardiomyogenic differentiation of rat bone marrow mesenchymal stem cells. IFMB 39:1953-1956

428. Pavalko FM, Chen NX, Turner CH, et al (1998) Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. Am J Physiol 275:C1591-C1601

429. Mathieu PS, Loboa EG (2012) Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. Tissue Eng Part B Rev 18(6):436-444

430. Kearney EM, Farrell E, Prendergast PJ (2008) Involvement of stretch-activated ion channels in strain-regulated glycosaminoglycan synthesis in mesenchymal stem cell-seeded 3D scaffolds. J Biomech 41(9):2055-2059

431. Leong W, Wu S, Pal M, et al (2012) Cyclic tensile loading regulates human mesenchymal stem cell differentiation into neuron-like phenotype. J Tissue Eng Regen Med 6(Suppl 3):s68-s79

432. Huang Y, Zheng L, Gong X, et al (2012) Effect of cyclic strain on cardiomyogenic differentiation of rat bone marrow derived mesenchymal stem cells. PLoS One 7(4):e34960

433. Shin JW, Jang JY, Lee SW, et al (2011) Combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagents. J Biomed Biotechnol 2011: 860652

434. Li J, Wang J, Zou Y, et al (2012) The influence of delayed compressive stress on TGF-β1-induced chondrogenic differentiation of rat BMPCs through Smad-dependent and Smad-independent pathways. Biomaterials 33(33):8395-8405

435. Li J, Zhao Z, Yang J, et al (2009) p38 MAPK mediate in compressive stress-induced chrondrogenesis of rat bone marrow MPCs in 3D alginate scaffolds. J Cell Physiol 221(3):609-617

436. Park JS, Chu JS, Tsou AD, et al (2011) The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta. Biomaterials 32(16):3921-3930

437. Kim DH, Heo SJ, Kim SH, et al (2011) Shear stress magnitude is critical in regulating the differention of mesenchymal stem cells even with endothelial growth medium. Biotechnol Lett 33(12):2351-2359

438. Huebsch N, Arany PR, Mao AS, et al (2010) Harnessing traction-mediated manipulation of the cell-matrix interface to control stem-cell fate. Nat Mater 9(6):518-526

439. Parekh SH, Chatterjee K, Lin-Gibson S, et al (2011) Modulus-driven differentiation of marrow stromal cells in 3D scaffolds that is independent of myosin-based cytoskeletal tension. Biomaterials 32:2256-2264

440. Shih Y-RV, Tseng K-F, Lai H-Y, et al (2010) Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. J Bone Miner Res 26(4):730.

441. Dahl KN, Ribeiro AJ, Lammerding J (2008) Nuclear shape, mechanics, and mechanotransduction. Circ Res 102(11):1307-1318

442. Meaburn KJ, Misteli T (2007) Cell biology: chromosome territories. Nature 445(7126): 379-381

443. Parnaik VK, Manju K (2006) Laminopathies: multiple disorders arising from defects in nuclear architecture. J Biosci 31:405-421

444. Yi S , Ding F, Gong L, et al (2017) Extracellular matrix scaffolds for tissue engineering and regenerative medicine. Curr Stem Cell Res Ther 12(3):233-246

445. Faulk DM, Johnson SA, Zhang L, et al (2014) Role of the extracellular matrix in whole organ engineering. J Cell Physiol 229(8):984-989

446. Hubbell JA (1995) Biomaterials in tissue engineering. Biotechnology (NY) 13(6):565-576

447. Gunatillake PA, Adhikari R (2003) Biodegradable synthetic polymers for tissue engineering. Eur Cell Mater 5:1-16

448. Di Lullo GA, Sweeney SM, Korkko J, et al (2002) Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 277(6):4223-4231

449. Khoshnoodi J, Cartailler JP, Alvares K, et al (2006) Molecular recognition in the assembly of collagens: terminal noncollagenous domains are key recognition modules in the formation of triple helical protomers. J Biol Chem 281(50):38117-38121.

450. Badylak SF, Freytes DO, Gilbert TW (2009) Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater 5(1):1-13

451. Wolf K, Friedl P (2011) Extracellular matrix determinants of proteolytic and non-proteolytic cell migration. Trends Cell Biol 21(12):736-744

452. Paranteau-Bareil R, Gauvin R, Berthod F (2010) Collagen-based biomaterials for tissue engineering applications. Materials 3(3):1863-1887

453. Glowacki J, Mizuno S (2008) Collagen scaffolds for tissue engineering. Biopolymers 89(5):338-344

454. Loo Y, Lakshmanan A, Ni M, et al (2015) Peptide bioink: self-assembling nanofibrous scaffolds for three-dimensional organotypic cultures. Nano Lett 15(10): 6919-6925

455. Helary C, Bataille I, Abed A, et al (2010) Concentrated collagen hydrogels as dermal substitutes. Biomaterials 31(3):481-490

456. Nöth U, Rackwitz L, Heymer A, et al (2007) Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels. J Biomed Mater Res Part A 83A(3):626-635

457. Liu CZ, Xia ZD, Han ZW, et al (2008) Novel 3D collagen scaffolds fabricated by indirect printing technique for tissue engineering. J Biomed Mater Res B Appl Biomater 85(2):519-528

458. Park JY, Choi JC, Shim JH, et al (2014) A comparative study on collagen type I and hyaluronic acid dependent cell behavior for osteochondral tissue bioprinting. Biofabrication 6(3):035004

459. Lee W, Debasitis JC, Lee VK, et al (2009) Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. Biomaterials 30(8):1587-1595

460. Lee W, Pinckney J, Lee V, et al (2009) Three-dimensional bioprinting of rat embryonic neural cells. Neuroreport 20(8):798-803

461. Lee V, Singh G, Trasatti JP, et al (2014) Design and fabrication of human skin by three-dimensional bioprinting. Tissue Eng Part C Methods 20(6):473-484

462. Wu Z, Su X, Xu B, et al (2016) Bioprinting three-dimensional cell-laden tissue constructs with controllable degradation. Sci Rep 6:24474

463. Inzana JA, Olvera D, Fuller SM, et al (2014) 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. Biomaterials 35(13):4026-4034

464. Xu T, Gregory CA, Molnar P, et al (2006) Viability and electrophysiology of neural cell structure generated by the inkjet printing method. Biomaterials 27(19):3580-3588

465. Roth EA, Xu T, Das M, et al (2004) Inkjet printing for high-throughput cell patterning. Biomaterials 25(17):3707-3715

466. Xu T, Jin J, Gregory C, et al (2005) Inkjet printing of viable mammalian cells. Biomaterials 26(1):93-99

467. Lee W, Lee V, Polio S, et al (2010) On-demand three-dimensional freeform fabrication of multi-layered hydrogel scaffold with fluidic channels. Biotechnol Bioeng 105(6):1178-1186

468. Zhao L, Lee VK, Yoo S-S, et al (2012) The integration of 3-D cell-printing and mesoscopic fluorescence molecular tomography of vascular constructs with thick hydrogel substrates. Biomaterials 33(21): 5325-5332

469. Duarte Campos DF, Blaeser A, Korsten A, et al (2014) The stiffness and structure of three-dimensional printed hydrogels direct the differentiation of mesenchymal stromal cells toward adipogenic and osteogenic lineages. Tissue Eng Part A 21(3-4):740-756

470. Schense JC, Hubbell JA (1999) Cross-linking exogenous bifunctional properties into fibrin gels with factor XIIIa. Bioconjugate Chem 10(1):75-81

471. Rowe SL, Lee S, Stegemann JP (2007) Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels. Acta Biomater 3(1):59-67

472. Wlodarczyk-Biegun MK, Del Campo A (2017) 3D bioprinting of structural proteins. Biomaterials 134:180-201

473. Murphy SV, Skardal A, Atala A (2013) Evaluation of hydrogels for bio-printing applications. J Biomed Mater Res A 101(1):272-284

474. Bensaid W, Triffitt JT, Blanchat C, et al (2003) A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials 24(14):2497-2502

475. Skardal A, Mack D, Kapetanovic E, et al (2012) Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. Stem Cells Transl Med 1(11):792-802

476. Xu T, Binder KW, Albanna MZ, et al (2013) Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications. Biofabrication 5(1):015001

477. Grimm C, Polterauer S, Helmy S, et al (2014) A collagen-fibrin patch (Tachosil®) for the prevention of symptomatic lymphoceles after pelvic lymphadenectomy in women with gynecologic malignancies: a randomized control trial. BMC Cancer 14:635

478. Filosso PL, Ruffini E, Sandri A, et al (2013) Efficacy and safety of human fibrinogen-thrombin patch (TachoSil(®)) in the treatment of postoperative air leakage in patients submitted to redo surgery for lung malignancies: a randomized trial. Interact Cardiovasc Thorac Surg 16(5):661-666

479. Hüttner FJ, Mihaljevic AL, Hackert T, et al (2016) Effectiveness of Tachosil (®) in the prevention of postoperative pancreatic fistula after distal pancreatectomy: a systematic review and meta-analysis. Langenbecks Arch Surg 401(2):151-159

480. Erdas E, Medas F, Podda F, et al (2015) The use of a biologic topical haemostatic agent (TachoSil(®)) for the prevention of postoperative bleeding in patients on antithrombotic therapy undergoing thyroid surgery: a randomized controlled pilot trial. Int J Surg 20:95-100

481. Snyder JE, Hamid Q, Wang C, et al (2011) Bioprinting cell-laden matrigel for radioprotection study of liver by pro-drug conversion in a dual-tissue microfluidic chip. Biofabrication 3(3):034112

482. Hughes CS, Postovit LM, Lajoie GA (2010) Matrigel: a complex protein mixture required for optimal growth for cell culture. Proteomics 10(9): 1886-1890

483. Carey DJ, Todd MS, Rafferty CM (1986) Schwann cell myelination: induction by exogenous basement membrane-like extracellular matrix. J Cell Biol 102:2254-2263

484. Benton G, Amaoutova I, George J, et al (2014) Matrigel: From discovery and ECM mimicry to assays and models for cancer research. Advanced Drug Delivery Reviews 79-80:3-18

485. Mettouchi A (2012) The role of extracellular matrix in vascular branching morphogenesis. Cell Adhes Migr 6(6):528-534

486. Marcelo KL, Goldie LC, Hirschi KK (2013) Regulation of endothelial cell differentiation and specification. Circ Res 112(9):1272-1287

487. Arnaoutova I, Kleinman HK (2010) *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 5(4):628-635

488. Barna J, Princz A, Kosztelnik M, et al (2012) Heat shock factor-1 intertwines insulin/IGF-1, TGF-β, and cGMP signaling to control development and aging. BMC Dev Biol 12:32

489. Shi Z, Neoh KG, Kang ET, et al (2014) Enhanced endothelial differentiation of adipose-derived stem cells by substrate nanotopography. J Tissue Eng Regen Med 8(1):50-58

490. Albini A, Noonan DM (2010) The “chemoinvasion” assay, 25 years and still going strong: the use of reconstituted basement membranes to study cell invasion and angiogenesis. Curr Opin Cell Biol 22(5):677-689

491. Albini A, Iwamoto Y, Kleinman HK, et al (1987) A rapid *in vitro* assay for quantitating the invasion potential of tumor cells. Cancer Res 47(12):3239-3245

492. Benton G, George J, Kleinman HK, et al (2009) Advancing science and technology via 3D culture on basement membrane matrix. J Cell Physiol 221(1):18-25

493. Barkan D, Green JE, Chambers AF (2010) Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. Eur J Cancer 46(7):1181-1188

494. Barkan D, Kleinman H, Simmons JL, et al (2008) Inhibition of metastatic growth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res 68(15):6241-6250

495. Lo AT, Mori H, Mott J, et al (2012) Constructing three-dimensional models to study mammary gland branching morphogenesis and functional differentiation. J Mammary Gland Biol Neoplasia 17(2):103-110

496. Kenny PA, Lee GY, Myers CA, et al (2007) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 1(1):84-96

497. Mehlhorn AT, Niemeyer P, Kaiser S, et al (2006) Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. Tissue Eng 12(10):2853-2862

498. Rao Pattabhi S, Martinez JS, Keller TC 3rd (2014) Decellularized ECM effects on human mesenchymal stem cell stemness and differentiation. Differentiation 88(4-5):131-143

499. Hoshiba T, Kawazoa N, Tateishi T, et al (2010) Development of extracellular matrices mimicking stepwise adipogenesis of mesenchymal stem cells. Adv Mater 22(28):3042-3047

500. Chen XD, Dusevich V, Feng JQ, et al (2007) Extracellular matrix made by bone marrow cells facilitates expansion of marrow-derived mesenchymal progenitor cells and prevents their differentiation into osteoblasts. J Bone Miner Res 22(12):1943-1956

501. Harvey A, Yen TY, Aizman I, et al (2013) Proteomic analysis of the extracellular matrix produced by mesenchymal stromal cells: implications for cell therapy mechanism. PLoS One 8(11):e79283

502. Lai Y, Sun Y, Skinner CM, et al (2010) Reconstitution of marrow-derived extracellular matrix *ex vivo*: a robust culture system for expanding large-scale highly functional human mesenchymal stem cells. Stem Cells Dev 19(7):1095-1107

503. Hoch AI, Mittal V, Mitra D, et al (2016) Cell-secreted matrices perpetuate the bone-forming phenotype of differentiated mesenchymal stem cells. Biomaterials 74:178-187

504. Choi DH, Suhaeri M, Hwang MP, et al (2014) Multi-lineage differentiation of human mesenchymal stromal cells on the biophysical microenvironment of cell-derived matrix. Cell Tissue Res 357(3):781-792

505. Liu X, Zhou L, Chen X, et al (2016) Culturing on decellularized extracellular matrix enhances antioxidant properties of human umbilical cord-derived mesenchymal stem cells. Mater Sci Eng C Mater Biol Appl 61:437-448

506. Prewitz MC, Stiβel A, Friedrichs J, et al (2015) Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding. Biomaterials 73:60-69

507. Chen RN, Ho HO, Tsai YT, et al (2004) Process development of an acellular dermal matrix (ADM) for biomedical applications. Biomaterials 25(14):2679-2686

508. Hülsmann J, Grün K, El Amouri S, et al (2012) Transplantation material bovine pericardium: biomechanical and immunogenic characteristics after decellularization vs glutaraldehyde-fixing. Xenotransplantation 19(5):286-297

509. Morticelli L, Thomas D, Roberts N, et al (2013) Investigation of the suitability of decellularized porcine pericardium in mitral valve reconstruction. J Heart Valve Dis 22(3):340-353

510. Chen F, Yoo JJ, Atala A (1999) Acellular collagen matrix makes a possible "off the shelf" biomaterial for urethral repair. Urology 54:407-410

511. Freytes DO, Badylak SF, Webster TJ, et al (2004) Biaxial strength of multilaminated extracellular matrix scaffolds. Biomaterials 25(12):2353-2361

512. Flynn LE (2010) The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. Biomaterials 31:4715-4724

513. Grandi C, Baiguera S, Martorina F, et al (2011) Decellularized bovine reinforced vessels for small-diameter tissue-engineered vascular grafts. Int J Mol Med 28(3):315-325

514. Cartmell JS, Dunn MG (2000) Effect of chemical treatments on tendon cellularity and mechanical properties. J Biomed Mater Res 49:134-140

515. Woods T, Gratzer PF (2005) Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. Biomaterials 26:7339-7349

516. Parmaksiz M, Dogan A, Odabas S, et al (2016) Clinical applications of decellularized extracellular matrices for tissue engineering and regenerative medicine. Biomed Mater 11(2): 022003

517. Levenda AC, Sanders NR (2015) A simplified approach for arthroscopic repair of rotator cuff tear with dermal patch augmentation. Adv Orthop Surg 2015:423949

518. Longo UG, Lamberti A, Maffuli N, et al (2010) Tendon augmentation grafts: a systematic review. Br Med Bull 94:165-188

519. Mostow EN, Haraway GD, Dalsing M, et al (2005) Effectiveness of an extracellular matrix graft (OASIS Wound Matrix) in the treatment of chronic leg ulcers: a randomized clinical trial. J Vasc Surg 41(5):837-843

520. Lam MT, Wu JC (2012) Biomaterial applications in cardiovascular tissue repair and regeneration. Expert Rev Cardiovasc Ther 10:1039-1049

521. Yanagawa B, Rao V, Yau TM, et al (2013) Initial experience with intraventricular repair using CorMatrix extracellular matrix. Innovations 8(5):348-352

522. Sündermann SH, Müggler O, Caliskan E, et al (2015) Extracellular matrix for reconstruction of cardiac structures after tumour resections. Interact Cardiovasc Thorac Surg 20(1):10-14

523. Badylak SF, Weiss DJ, Caplan A, et al (2012) Engineered whole organs and complex tissue. Lancet 379(9819):943-952

524. Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. Biomaterials 27:3675-3683

525. Parmaksiz M, Elcin AE, Elcin YM (2017) Decellularization of bovine small intestinal submucosa and its use for the healing of a critical-size full-thickness skin defect alone and in combination with stem cells in a small rodent model. J Tissue Eng Regen Med 11(6):1754-1765

526. Piccoli M, D'Angelo E, Crotti S, et al (2018) Decellularized colorectal cancer matrix as bioactive microenvironment for in vitro 3D cancer research. J Cell Physiol 233: 5937-5948

527. Parker MW, Rossi D, Peterson M, et al (2014) Fibrotic extracellular matrix activates a profibrotic positive feedback loop. J Clin Invest 124: 1622-1635

528. Hedström U, Hallgren O, Öberg L, et al (2018) Bronchial extracellular matrix from COPD patients induces altered gene expression in repopulated human bronchiol epithelial cells. Sci Rep 8: 3502

529. Friedernstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-274

530. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641-650

531. Mills EJ, Wu P, Chong G (2011) Efficacy and safety of statin treatment for cardiovascular disease: a network meta-analysis of 170 255 patients from 76 randomized trials. Q J Med 104:109-124

532. Liapis CD, Bell PR, Mikhailidis D et al (2009) ESVS guidelines. Invasive treatment for carotid stenosis: indications, techniques. Eur J Vasc Endovasc Surg 37:1-19

533. Liapis CD, Bell PR, Mikhailidis D et al (2010) ESVS guidelines: Section A – prevention in patients with carotid stenosis. Curr Vasc Pharmacol 8:673-681

534. Tahara N, Kai H, Ishibashi M, Nakuara H, Kaida H, Baba K, Hayabuchi N, Imaizumi T (2006) Simvastatin attenuates plaque inflammation: evaluation by fluorodeoxyglucose positron emission topography. J Am Coll Cardiol 48:1825-1831

535. Sillesen H (2009) Statins and their use in preventing carotid disease. Curr Atherscler Rep 11:309-314

536. Verzini F, De RP, Parlani G, Giordano G, Caso V, Cieri E, Isernia G, Cao P (2011) Effects of statins on early and late results of coronary stenting. J Vasc Surg 53:71-79

537. Kertai MD, Boersma E, Westerhout CM, van Domburg R, Klein J, Bax JJ, van Urk H, Poldermans D (2004) Association between long-term statin use and mortality after successful abdominal aortic aneurysm surgery. Am J Med 116:96-103

538. Kalyanasundaram A, Elmore JR, Manazer JR, Golden A, Franklin DP, Galt SW, Zakhary EM, Carey DJ (2006) Simvastatin suppresses experimental aortic aneurysm expansion. J Vasc Surg 43:117-124

539. Schouten O, van Laanen JH, Boersma E, Vidakovic R, Feringa HH, Dunkelgrün M, Bax JJ, Koning J, van Urk H, Poldermans D (2006) Statins are associated with a reduced infrarenal abdominal aortic aneurysm growth. Eur J Vasc Endovasc Surg 32:21-26

540. Giri J, McDermott MM, Greenland P, Guralnik JM, Criqui MH, Liu K, Ferrucci L, Green D, Schneider JR, Tian L (2006) Statin use and functional decline in patients with and without peripheral artery disease. J Am Coll Cardiol 47:998-1004

541. Heart Protection Study Group (2007) Randomized trial of the effects of cholesterol-lowering with simvastatin on peripheral vascular and other major vascular outcomes in 20 536 people with peripheral arterial disease and other high-risk conditions. J Vasc Surg 45:645-654

542. Kobashigawa JA, Katznelson S, Laks H et al (1995) Effect of pravastatin on outcomes after cardiac transplantation. N Engl J Med 333:621-627

543. Nissen SE, Nicholls SJ, Sipahi I et al (2006) Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. JAMA 295:1556-1565

544. Mehra MR, Uber PA, Vivekananthan K, Solis S, Scott RL, Park MH, Milani RV, Lavie CJ (2002) Comparative beneficial effects of simvastatin and pravastatin on cardiac allograft rejection and survival. J Am Coll Cardiol 40:1609-1614

545. Kobashigawa JA, Moriguchi JD, Laks H, Wener L, Hage A, Hamilton MA, Cogert G, Marquez A, Vassilakis ME, Patel J, Yeatman L (2005) Ten-year follow-up of a randomized trial of pravastatin in heart transplant patients. J Heart Lung Transplant 24:1736-1740

546. Schachter M (2005) Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. Fundam Clin Pharmacol 19:117-125

547. Bullano MF,Wertz DA, Yang GW, Kamat S, Borok GM, Gandhi S, McDonough KL, Willey VJ (2006) Effect of rosuvastatin compared with other statins on lipid levels and national cholesterol education program goal attainment for low-density lipoprotein cholesterol in a usual care setting. Pharmacotherapy 26:469-478

548. Lennernas H, Fager G (1997) Pharmacodynamis and pharmacokinetics of the HMG-CoA reductase inhibitors: similarities and differences. Clin Pharmacokinet 32:403-425

549. Singhvi SM, Pan HW, Morrison RA, Willard DA (1990) Disposition of pravastatin sodium, a tissue-selective HMG-CoA reductase inhibitor, in healthy subjects. Br J Clin Pharmacol 20:239-243

550. Zhou Z, Rahme E, Pilote L (2006) Are statins created equal? Evidence from randomized trials of pravastatin, simvastatin, and atorvastatin for cardiovascular disease prevention. Am Heart J 151:273-281

551. van Amerongen MJ, Bou-Gharios G, Popa E, van Ark J, Petersen AH, van Dam GM, van Luyn MJ, Harmsen MC (2008) Bone marrow-derived myofibroblasts contribute functionally to scar formation after myocardial infarction. J Pathol 214:377-386

552. Möllmann H, Nef HM, Kostin S, von KC, Pilz I, Weber M, Schaper J, Hamm CW, Elsässer A (2006) Bone marrow-derived cells contribute to infarct remodelling. Cardiovasc Res 71:661-671

553. Salama M, Andrukhova O, Roedler S, Zuckermann A, Laufer G, Aharinejad S (2011) Association of CD14+ monocyte-derived progenitor cells with cardiac allograft vasculopathy. J Thorac Cardiovasc Surg 142:1246-1253

554. Carlson S, Trial J, Soeller C, Entman ML (2011) Cardiac mesenchymal stem cells contribute to scar formation after myocardial infarction. Cardiovasc Res 91:99-107

555. van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J (2010) Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 7:30-37

556. Hombach-Klonisch S, Panigrahi S, Rashedi I, Seifert A, Alberti E, Pocar P, Kurpisz M, Schulze-Osthof K, Mackiewicz A, Los M (2008) Adult stem cells and their trans-differentiation potential-perspectives and therapeutic applications. J Mol Med 86:1301-1314

557. Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells 25:2739-2749

558. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 938:221-229

559. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701-705

560. Bittira B, Kuang JQ, Al-Khaldi A, Shum-Tim D, Chiu RC (2002) In vitro preprogramming of marrow stromal cells for myocardial regeneration. Ann Thorac Surg 74:1154-1159

561. Chedrawy EG, Wang JS, Nguyen DM, Shum-Tim D, Chiu RC (2002) Incorporation and integration of implanted myogenic and stem cells into native myocardial fibers: anatomic basis for functional improvements. J Thorac Cardiovasc Surg 124:584-590

562. Bellini A, Mattoli S (2007) The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest 87:858-870

563. Cox N, Pilling D, Gomer RH (2012) NaCl potentiates human fibrocyte differentiation. PLoS One 7:e45674

564. Buhaescu I, Izzedine H (2007) Mevalonate pathway: a review of clinical and therapeutical implications. Clin Biochem 40:575-584

565. Hilgendorff A, Muth H, Parviz B, Staubitz A, Haberbosch W, Tillmanns H, Hölschermann H (2003) Statins differ in their ability to block NF-kappaB activation in human blood monocytes. Int J Clin Pharmacol Ther 41:397-401

566. Dhingra R, Gang H, Wang Y, Biala AK, Aviv Y, Margulets V, Tee A, Kirshenbaum LA (2013) Bidirectional regulation of nuclear factor- κB and mammalian target of rapamycin signaling functionally links Bnip3 gene repression and cell survival of ventricular myocytes. Circ Heart Fail 6:335-343

567. Sciarretta S, Hariharan N, Monden Y, Zablocki D, Sadoshima J (2011) Is autophagy in response to ischemia and reperfusion protective or detrimental for the heart? Pediatr Cardiol 32:275-281

568.Wang EY, Gang H, Aviv Y, Dhingra R, Margulets V, Kirshenbaum LA (2013) p53 mediates autophagy and cell death by a mechanism contingent on Bnip3. Hypertension 62:70-77

569. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, Yang R, Chen W, Wang S, Shi S (2011) Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-γ and TNF-α. Nat Med 17:1594-1601

570. Peng CF, Han YL, Jie-Deng, Yan CH, Jian-Kang, Bo-Luan, Jie-Li (2011) Overexpression of cellular repressor of E1A-stimulated genes inhibits TNF-α-induced apoptosis via NFκB in mesenchymal stem cells. Biochem Biophys Res Commun 406:601-607

571. Kim JM, Cho HH, Lee SY, Hong CP, yang JW, Kim YS, Suh KT, Jung JS (2012) Role of IRAK1 on TNF-induced proliferation and NFκB activation in human bone marrow mesenchymal stem cells. Cell Physiol Biochem 30:46-60

572. Gauthaman K, Fong CY, Bongso A (2009) Statins, stem cells, and cancer. J Cell Biochem 106:975-983

573. Patel S, Mason RM, Suzuki J, Imaizumi A, Kamimura T, Zhang Z (2006) Inhibitory effect of statins on renal epithelial-to-mesenchymal transition. Am J Nephrol 26:381-387

574. Piotrowski PC, Kwintkiewicz J, Rzepczynska IJ, Seval Y, Cakmak H, Arici A, Duleba AJ (2006) Statins inhibit growth of human endometrial stromal cells independently of cholesterol availability. Biol Reprod 75:107-111

575. Shao H, Tan Y, Eton D, Yang Z, Uberti MG, Li S, Schulick A, Yu H (2008) Statin and stromal cell-derived factor-1 additively promote angiogenesis by enhancement of progenitor cells incorporation into new vessels. Stem Cells 26:1376–1384

576. Assmus B, Urbich C, Aicher A, Hofmann WK, Haendeler J, Rössig L, Spyridopoulos I, Zeiher AM, Dimmeler S (2003) HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. Circ Res 92:1049–1055

577. Satoh M, Minami Y, Takahashi Y, Tabuchi T, Itoh T, Nakamura M (2009) Effect of intensive lipid-lowering therapy on telomere erosion in endothelial progenitor cells obtained from patients with coronary artery disease. Clin Sci (Lond) 116:827–835

578. Zhang Y, Zhang R, Li Y, He G, Zhang D, Zhang F (2012) Simvastatin augments the efficacy of therapeutic angiogenesis induced by bone-marrow-derived mesenchymal stem cells in a murine model of hindlimb ischemia. Mol Biol Rep 39:285-293

579. Xu H, Yang YJ, Qian HY, Tang YD, Wang H, Zhang Q (2011) Rosuvastatin treatment activates JAK-STAT pathway and increases efficacy of allogenic mesenchymal stem cell transplantation in infracted hearts. Circ J 75:1476-1485

580. Zhang Q, Yang YJ, Wang H, Dong QT, Wang TJ, Qian HY, Xu H (2012) Autophagy activation: a novel mechanism of atrovastatin to protect mesenchymal stem cell from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway. Stem Cells Dev 21:1321-1332

581. Krenning G, Zeisberg EM, Kalluri R (2010) The origin of fibroblasts and the mechanism of cardiac fibrosis. J Cell Physiol 225: 631-637.

582. Kassem M, Abdallah BM. Human bone-marrow-derived mesenchymal stem cells: Biological characteristics and potential role in therapy of degenerative diseases (2008) Cell and Tissue Research 331: 157-163.

583. Hare JM, Fishman JE, Gerstenblith G, et al (2012) Comparison of allogenic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. JAMA 308: 2369-2379.

584. Yang Z, Xhang F, Ma W, et al (2010) A novel approach to transplanting bone marrow stem cells to repair human myocardial infarction: delivery via a noninfarct-relative artery. Cardiovasc Ther28: 380-385.

585. Perin EC, Dohmann HF, Borojevic R, et al (2003) Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 107: 2994-2302

586. Hare JM, Traverse JH, Henry TD, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am CollCardiol 2009; 54: 2277-2286

587. Reyes M, Lund T, Lenvik T, et al (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98: 2615-2625

588. Niemeyer P, Krause U, Fellenberg J, et al (2004) Evaluation of mineralized collagen and alpha-tricalcium phosphate as scaffolds for tissue engineering of bone using human mesenchymal stem cells. Cells Tissues Organs 177: 68-78

589. Yuge L, Kajiume T, Tahara H, et al (2006) Microgravity potentiates stem cell proliferation while sustaining the capability of differentiation. Stem Cells Dev 15: 921-929

590. Amiel J, de Pontual L, Henrion-Caude A (2012) miRNA, development and disease. Adv Genet 80: 1-36

591. Hobert O (2008) Gene regulation by transcription factors and microRNAs. Science 319: 1785-1786

592. Inose H, Ochi H, Kimura A, et al (2009) A microRNA regulatory mechanism of osteoblast differentiation. PNAS 106: 20794-20799

593. Jiang J, Gusev Y, Aderca I, et al (2008) Association of microRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. Clin Can Res 14; 419-427

594. Mycko MP, Cichalewska M, Machlanska A, et al (2012) MicroRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. PNAS 109: E1248-E1257

595. Tomasek JJ, Gabbiani G, Hinz B, et al (2002) Myofibroblasts and mechano-regulation of connective tissue remodeling. Nature Reviews 3: 349-363

596. Hinz B, Gabbiani G (2003) Mechanisms of force generation and transmission by myofibroblasts. Curr Opin Biotech 14: 538-546

597. Mollmann H, Nef HM, Kostin S, et al (2006) Bone marrow-derived cells contribute to infarct remodeling. Cardiovasc Res 71: 661-671

598. Hanisch A, Sillje HH, Nigg EA (2005) Timely anaphase onset requires a novel spindle and kinetochore complex comprising Ska1 and Ska2. EMBO J 25: 5504-5515

599. Chen Z, Chen LY, Dai HY, et al (2012) miR-301a promotes pancreatic cancer cell proliferation by directly inhibiting bim expression. J of Cell Biochem 113: 3229-3235

600. Lu Z, Li Y, Takwi A, et al (2011) miR-301a as an NF-kappaB activator in pancreatic cancer cells. EMBO J 30; 57-67

601. Zhou P, Jiang W, Wu L, et al (2012) miR-301a is a candidate oncogene that targets the homeobox gene gax in human hepatocellular carcinoma. Big Diseases Sciences 57: 1171-1180

602. Pittenger MF, Mackay AM, Beck SC, et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147

603. Sekiya I, Larson BL, Smith JR, et al (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20: 530-541

604. Lin CS, Xin ZC, Dai J, et al (2013) Commonly used mesenchymal stem cell markers and tracking labels: limitations and challenges. Histol Histopathol 28: 1109-1116

605. Battula VL, Treml S, Bareiss PM, et al 2009) Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. Haematological 94: 173-184

606. Larsson O, Diebold D, Fan D, et al (2008) Fibrotic myofibroblasts manifest genome-wide derangements of translational control. PLoS One 3: e3220

607. Kalluri R, Neilson EG (2003) Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 112: 1776-1784

608. Wang J, Chen H, Seth A, et al (2003) Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. Am J Heart Physiol 285: H1871-H1881

609. Cordes KR, Sheehy NT, White MP, et al (2009) miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 460: 705-710

610. Gong C, Nie Y, Qu S, et al (2014) miR-21 induces myofibroblast differentiation and promotes the malignant progression of breast phyllodes tumors. Cancer Res 74: 4341-4352

611. Digirolamo CM, Stokes D, Colter D, et al (1999) Propagation and senescence of human marrow stromal cells in culture: A simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Brit J Haem 107: 275-281

612. Talele NP, Fradette J, Davies JE, et al (2015) Expression of α-smooth muscle actin determines fate of mesenchymal stromal cells. Stem Cell Reports 4(6): 1016-1030

613. Li CX, Talele NP, Boo S, et al (2017) MicroRNA-21 preserves the fibrotic memory of mesenchymal stem cells. Nat Mater 16(3): 279-289

614. Ryan JA (2008) Evolution of cell culture surfaces. BioFiles 3: 21

615. Dominici M, Le Blanc K, Mueller I, et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy Position Statement. Cytotherapy 8: 315-317

616. Bernstein E, Kim SY, Carmell MA, et al (2003) Dicer is essential for mouse development. Nat Genet 35: 215-217

617. Möllman H, Nef HM, Kostin S, et al (2006) Bone marrow-derived cells contribute to infarct remodeling. Cardiovasc Res 71: 661-671

618. Chen X, Talele NP, Boo S, et al (2017) MicroRNA-21 preserves the fibrotic mechanical memory of mesenchymal stem cells. Nature Materials 16: 379-389

619. The stressful life of cardiac myofibroblasts. Cardiac Fibrosis and Heart Failure: Cause or Effect? Eds Dixon IMC, and Wigle, JT. Springer International Publishing Switzerland.

620. Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326: 1216-1219

621. Kania G, Blyszczuk P, Valaperti A, et al (2008) Prominin-1+/CD133+ bone marrow-derived heart-resident cells suppress experimental autoimmune myocarditis. Cardiovasc Res 80: 236-245

622. Wienholds E, Koudijs MJ, van Eeden EJ, et al (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet 35: 217-218

623. Feng Y, Zhang X, Graves P, and Zeng Y (2012) A comprehensive analysis of precursor microRNA cleavage by human Dicer. RNA 18: 2083-2092

624. Stappert L, Borghese L, Roese-Koerner B, et al (2013) MicroRNA-based promotion of human neuronal differentiation and subtype specification. PLoS One 8: e59011

625. Dalmosso G, Nguyen HT, Yan Y, et al (2010) MicroRNAs determine human intestinal epithelial cell fate. Differentiation 80: 147-154

626. Ribeiro AO, Schoof CRG, Izzotti A, et al (2014) MicroRNAs: modulators of cell identity, and their applications in tissue engineering. Microrna 3: 45-53

627. Marson A, Levine SS, Cole MF, et al (2008) Connecting miRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 134: 521-533

628. Eulalio A, Mano M, Dal Ferro M, et al (2012) Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492: 376-381

629. Suh MR, Lee Y, Kim JY, et al (2004) Human embryonic stem cells express a unique set of microRNAs. Dev Biol 270: 488-498

630. Kitahara H, Yagi H, Tajima K, et al (2016) Heterotropic transplantation of a decellularized and recellularized whole porcine heart. Interact Cardiovasc Thorac Surg 22: 571-579

631. Emerman JT, Burwen SJ, and Pitelka DR (1979) Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue Cell 11:109–119

632. Dixon IM (2010) The soluble interleukin-6 receptor takes its place in the pantheon of interleukin-6 signaling proteins: phenoconversion of cardiac fibroblasts to myofibroblasts. Hypertension 56: 193-195

633. Porter KE, Turner NA, O'Regan DJ, et al (2004) Tumor necrosis factor alpha induces human atrial myofibroblast proliferation, invasion, and MMP-9 secretion: inhibition by simvastatin. Cardiovasc Res 64: 507-515

634. Salamone M, Carfi Pavia F, Ghersi G (2016) Proteolytic enzymes clustered in specialized plasma-membrane domains drive endothelial cells' migration. PLoS One 11: e0154709

635. Dunlevy JR, Couchman JR (1993) Controlled induction of focal adhesion disassembly and migration in primary fibroblasts. J Cell Sci 105: 489-500

636. Duval K, Grover H, Han L-H, et al (2017) Modeling physiological events in 2D vs 3D cell culture. Physiology (Bethesda) 32: 266-277

637. Andrée B, Bär A, Haverich A, et al (2013) Small intestinal submucosa segments as matrix for tissue engineering: review. Tissue Engin Part B: Reviews 19: 279-291

638. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674

639. Bourin P, Gadelorge M, Peyrafitte JA, et al (2008) Mesenchymal progenitor cells: tissue origin, isolation, and culture. Transfus Med Hemother 35: 160-167