TNF-α and Hypoxia-Induced Paracrine Secretion of Rat Bone Marrow-Mesenchymal Stem Cells for Cardiac Repair in Lewis Rats Post-MI

written by

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ABSTRACT

Background: Due to the limited regenerative capacity of mammalian cardiomyocytes, following a myocardial infarction (MI) the chronic loss of functional contractile myocardium and continuous inflammation degrade the structure and function of the heart. Although bone-marrow mesenchymal stem cell (BM-MSC) strategies have been suggested to play a crucial role in promoting the repairing process in ischemic hearts, poor viability and retention of transplanted cells in the harsh microenvironment remains as an obstacle. The paracrine hypothesis suggests that BM-MSCs secrete paracrine factors consisting of biologically active molecules that may work together to manipulate the microenvironment and trigger angiogenesis, cardioprotective, and homing properties at the site of infarct. Tumour Necrosis Factor-α (TNF-α) is a pleiotropic pro-inflammatory cytokine present in ischemic cardiac regions, but the specific roles it plays in cardioprotection and its interaction with BM-MSCs have yet to be established. The current study aims to understand the regulatory effects of TNF-α and hypoxia on rat BM-MSCs (rBM-MSC) and the angiogenic, migratory, cardioprotective, and reparative effects of its secretions which may, initiate and sustain the process of cardiac repair following MI.

Methods: Secretome from rBM-MSC cultures treated/untreated with either conditioned rat cardiomyocyte medium (rCCM), TNF-α, and/or normoxia/hypoxia in various combinations were collected. Immunocytochemistry, western blot analyses, trans-well cell migration, and Annexin V apoptosis assays in conjunction with fluorescence-activated cell sorting were conducted. *In vivo*, echocardiography was performed on induced infarcted rats at three weeks following their treatment with a control (rCCM and hypoxia) or TNF-α Hypoxia-Induced secretome. Histological analyses including Masson's Trichrome staining and immunohistochemistry (IHC) for the CD31 and Ki-67 markers were further conducted. Image J and Prism were used for graphing and statistical analysis.

Results: The immunocytochemistry and western blot analyses confirmed the presence of the TNF-Receptors 1 and 2 (TNFR1/TNFR2) on the surface of rBM-MSCs. Western blot analyses on rBM-MSC lysates treated with rCCM, TNF-α and hypoxia showed increased expression of TGF-β, FGF-2, VEGF-1, Myogenin, and Ang-2, while decreased expression of Ang-1 and FGF-7. The trans-well migration assay showed that TNF-α Hypoxia-Induced secretome exhibits chemotactic properties. The Annexin V apoptosis assay revealed that the TNF-α Hypoxia-Induced secretome treatment on rCMs under hypoxia does not show a statistically significant difference between the

values for the control cells (rCMS with hypoxia only) and itself. In vivo, the rats treated with TNF-α Hypoxia induced secretome had a higher left ventricle fractional shortening (LVFS) by 9.584% $\pm$ 2.162 % (p<0.01) than the control secretome treated, while trichrome staining revealed a decrease in the size of infarct. IHC revealed increased expression of CD31 (marker of angiogenesis) and Ki67 (marker of proliferation) near the area of infarct in the TNF-α Hypoxia-Induced secretome treated rats compared to the control secretome treated rats.

Discussion: The presence of TNFR1 and TNFR2 on rBM-MSCs indicates that TNF-α is able to bind to rBM-MSCs and initiate cell survival pathways through the established TNFR2 pathway. In hypoxic conditions, TNF-α stimulates rBM-MSCs to secrete proteins such as TGF-β, FGF-2, Ang 2, VEGF-1, and Myogenin that contribute to neovascularization, MSC/endothelial cell (EC) proliferation and migration, decreasing acute pro-inflammatory responses, and/or inducing MSC/EC differentiation. The decreased expression of Ang-1 in TNF-α and hypoxia treated rBM-MSCs may be a result of the increased expression of Ang-2. The decreased expression of FGF-7 in TNF-α and hypoxia treated rBM-MSCs may indicate that VEGF-1 is secreted through another mechanism which is not dependent on FGF-7. Furthermore, the TNF-α hypoxia-induced secretome plays a role in the migration of BM-MSCs to the site of infarct. Additionally, there may be no significant difference in the number of apoptotic rCMs between the hypoxia induced control and TNF-α hypoxia-induced secretome treatments, as the presence of the TNF-α cytokine might reduce the pro-survival effect of the secreted secretome. A higher fractional shortening and the decrease in the size of infarct in the TNF-α hypoxia-induced secretome treated Lewis rats shows that myocardial preservation maybe occurring. The CD31 marker indicates the occurrence of angiogenesis and thus, an increase in vessel formation at the site of infarct. Increased expression of the Ki-67 marker shows that proliferation is occurring at the site of infarct in TNF-α hypoxia-induced secretome treated rats. These findings suggest that the paracrine secretions of TNF-α stimulated and hypoxia preconditioned BM-MSCs may play a therapeutic role in initiating neovascularization, mobilization of BM-MSCs, anti-inflammatory mechanisms, and cell survival mechanism in the myocardium post-MI. These results may shed light on developing a potential cell free secretome therapy for cardiac repair in ischemia induced MI patients.

RÉSUMÉ

Background : En raison de la capacité de régénération limitée des cardiomyocytes chez les mammifères, à la suite d'un infarctus du myocarde (MI), la perte chronique du myocarde contractile fonctionnel et l'inflammation continue dégrade la structure et la fonction du cœur. Bien que les stratégies de l'utilisation des cellules souches mésenchymateuses (BM-MSCs) de la moelle osseuse ont été suggérées pour jouer un rôle crucial dans la promotion de guérison descœurs ischémiques, la faible viabilité et la rétention des cellules transplantées dans un microenvironnement dur demeure toutefois un obstacle. L'hypothèse paracrine suggère que les BM-MSCs sécrètent des facteurs paracrines consistant en des molécules biologiquement actives qui peuvent travailler ensemble pour manipuler le microenvironnement et déclencher uneangiogenèse, et des propriétés d'attraction et cardioprotectrice sur le site de l'infarctus.

Le Facteur de Nécrose Tumorale-α (TNF-α) est une cytokine pro-inflammatoire pléiotropie présente dans les régions cardiaques ischémiques, mais son rôle spécifique en cardioprotection et son interaction avec les BM-MSCs doivent encore être établi. L'actuelle étude vise à comprendre les effets régulateurs du TNF-α et de l'hypoxie chez des BM-MSCs de rat (rBM-MSC) ainsi que dans l'angiogénique, la migration, la cardioprotection, et les effets réparateurs de ses sécrétions pouvant initier et soutenir le processus de réparation cardiaque suite à un MI.

Méthodes : Plusieurs données ont été recueillis des sécrétome de culture de rBM-MSC traitées ou non avec un milieu conditionné provenant des cellules cardiaques de rat (rCCM) et par traitement au TNF-α, et/ou normoxie/hypoxie. L'immunocytochimie, l'immunobuvardage par western, la migration cellulaire trans-puits, et le dosage de l'apoptose Annexin V jumelé avec le Tri de Cellules Activées par Fluorescence (TCAF) ont été menés dans cette étude. In vivo, l'échocardiographie a été effectuée sur les cœurs de rats infarcis trois semaines suivant l'injection d'un traitement contrôle (rCCM +hypoxie) ou d'un sécrétome d'hypoxie induite par le TNF-α. Des analyses histologiques incluant la coloration du Trichrome de Masson et l'immunohistochimie (IHC) pour les marqueurs CD31 et Ki-67 ont été également menées. L'image J et Prism ont été utilisés pour l'analyse graphique et statistique des données.

Résultats : L'immunocytochimie et l'immunobuvardage par western blot des analyses confirment la présence des récepteurs TNF 1 et 2 (TNFR1/TNFR2) sur la surface des rBM-MSCs. L'immunobuvardage par western blot sur les lysats des rBM-MSC traités avec rCCM, TNF-α, et l'hypoxie ont montré une augmentation dans l'expression du TGF-β, FGF-2, VEGF-1, la

myogénine et dans l'Ang-2, tandis que l'expression de l'Ang-1 et FGF-7 ont diminué. Le test de migration trans-puits a montré que le sécrétome de TNF- α et d'hypoxie présente des propriétés chimiotactiques. Le traitement par l'apoptose Annexin V a révélé quant à lui que le traitement du sécrétome de TNF- α et d'hypoxie sur les rCMs cultures induite par l'hypoxie ne montre pas de différence statistiquement significative entre les valeurs pour les cellules de contrôle (rCMs avec hypoxie uniquement) et elle-même. In vivo, les rats traités par le sécrétome de TNF- α et d'hypoxie ont eu un raccourcissement fractionnaire supérieur du ventricule gauche (LVFS) avec 9.584 % + 2,162 % ($P < 0,01$) plus que le sécrétome de contrôle traité, tandis que la coloration au trichrome révèle une diminution de la taille de l'infarctus. L'IHC a révélé une augmentation dans l'expression de CD31, marqueur de l'angiogenèse, et du Ki67, un marqueur de prolifération, près de la zone de l'infarctus dans le groupe de rats traités par le sécrétome de TNF- α et d'hypoxie par rapport aux rats du sécrétome control.

Discussion : La présence de TNFR1 et TNFR2 sur les rBM-MSCs indiquent que le TNF-α est capable de se lier aux rBM-MSCs et d'initier les voies de la survie cellulaire par la voie de TNFR2 déjà établie. Le TNF-α en conditions d'hypoxie stimulent les rBM-MSCs à sécréter des protéines telles que le TGF-β, FGF-2, Ang-2, VEGF-1, et la myogénine dans les rBM-MSCs qui contribue soit à une néovascularisation, une prolifération et migration des MSC/cellules endothéliales (EC), une diminution des réactions inflammatoires, et/ou une induction à la différentiation des MSC/EC. La diminution de l'expression des Ang-1 en rBM-MSCs traitées par TNF-α et l'hypoxie peuvent être le résultat de l'augmentation de l'expression de l'Ang-2. La diminution de l'expression des FGF-7 en rBM-MSCs traitées par TNF-α et l'hypoxie peuvent indiquer que le VEGF-1 est sécrétée par un autre mécanisme autre que par la voie FGF-7. En outre, le sécrétome de TNF- α et d'hypoxie joue un rôle dans la migration de rBM-MSCs vers le site de l'infarctus. En plus, il peut y avoir une différence non significative dans le nombre d'apoptotic rCMs entre l'hypoxie induite par le contrôle et le traitement du sécrétome de TNF- α et d'hypoxie, comme la présence de la cytokine TNF-α qui peut réduire l'effet apoptotiques du sécrétome sécrété. Le raccourcissement fractionnaire supérieur et la diminution de la taille de l'infarctus dans le sécrétome de TNF- α et d'hypoxie dans le traitement des rats Lewis montre que la conservation du myocarde peut survenir. Le marqueur CD31 indique l'apparition de l'angiogenèse et par conséquent à une augmentation de la formation de vaisseaux sur le site de l'infarctus. L'augmentation de l'expression du marqueur de Ki-67 montre que la prolifération se produit sur le site d'infarctus chez les rats du sécrétome de

TNF- α et d'hypoxie. Ces résultats suggèrent que les sécrétions paracrines des BM-MSCs préconditionnées par TNF-α et l'hypoxie peuvent jouer un rôle thérapeutique bénéfique à l'initiation de la néovascularisation, la mobilisation des BM-MSCs, les mécanismes anti-inflammatoires, et le mécanisme de survie cellulaire dans le myocarde post-MI. Ces résultats pourraient faire la lumière sur le développement d'une thérapie potentielle d'un sécrétome libre de cellules pour la réparation cardiaque chez les patients ischémiques induite par MI.

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With the deepest gratitude, I would like to dedicate this thesis to the almighty god, who even through the toughest times has guided me towards achieving my goals, while instilling faith in myself and my dreams;

My role model, hero, and loving selfless father Mr. Selvasandran Sinnathurai, who supports me with unconditional love and taught me to be fearless with a lion heart in the face of adversity to successfully pursue my goals;

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। । सत्त्वानुरूपा सर्वस्य । श्रद्धा भवति भारत । श्रद्धा-मयो यम् पुरुषो । यो यच्-छ्रद्धः स ऐव सः । ।

~ Man is made by his belief. As he believes, so he is. ~ (Chapter 17.3, Bhagavat Gita)

LIST OF ABBREVIATIONS

ACCF American College of Cardiology Foundation

ACE Angiotensin-Converting Enzyme

AMA American Heart Association

AMI Acute Myocardial Infarction

Ang 1 Angiopoitein 1

Ang 2 Angiopoitein 2

Akt Phosphorylated Protein Kinase B

ARB Angiotensin Receptor Blocker

ATCC American Type Culture Collection

BMC Unfractionated Bone Marrow Cells

BM-MNCS Bone Marrow-Derived Mononuclear Cells

BSA Bovine Serum Albumin

CABG Coronary Artery Bypass Grafting

CD31 Platelet Endothelial Cell Adhesion Molecule

CHD Coronary Heart Disease

CM Cardiomyocytes

CO_2 Carbon Dioxide

CPC Cardiac Progenitor Stem Cells

CSC Cardiac Resident Stem Cells

cTn Cardiac Troponin

CVD Cardiovascular Disease

DAMP Damage-Associated Molecular Patterns

DAPI 4',6-Diamidino-2-Phenylindole

DLLI Diabetic Lower Limb Ischemia

DMEM Dulbecco's Modified Eagle Medium

EC Endothelial Cells

ECG Electrocardiography

ECM Extra Cellular Matrix

EDTA Ethylenediaminetetraacetic acid

EPC Endothelial Progenitor Cells

ESCs Embryonic Stem Cells

ESC European Society of Cardiology

FACS Fluorescence-Activated Cell Sorting

FGF-2 Fibroblast Growth Factor - 2

FGF-7 Fibroblast Growth Factor - 7

FBS Fetal Bovine Serum

GAPDH Glyceraldehyde 3-Phosphate Dehydrogenase

hASC Human Adipose Tissue-Derived Mesenchymal Stem Cell

HGF Hepatocyte Growth Factor

HIF-1a Hypoxia Inducible Factor-1a

HRP Horseradish Peroxidase

IHD Ischemic Heart Disease

IL Interleukin

IPSC Induced Pluripotent Stem Cells

LAD Left Anterior Descending

LCA Left Circumflex Artery

LDL Low-Density Lipoprotein

LV Left Ventricle

LVFS Left Ventricular Fractional Shortening

MI Myocardial Infarction

MMP Matrix Metalloproteinase

MSCs Mesenchymal Stem Cells

MUHC McGill University Health Centre

NF-κB Nuclear Factor Kappa- B

O_2 Oxygen

PBS Phosphate Buffered Saline

PCI Percutaneous Coronary Intervention

PDGF Platelet-Derived Growth Factor

PFA Paraformaldehyde

PI Propidium Iodide

PMI Perioperative Myocardial Infarction

PMT Pharmacological Management Therapies

PS Penicillin & Streptomycin

PVDF Polyvinylidene Fluoride Membrane

RA Retinoic Acid

rBM-MSC Rat Bone-Marrow Mesenchymal Stem Cells

RCA Right Coronary Artery

rCB H9C2 Rat Cardiomyoblasts

rCM H9C2 Rat Cardiomyocytes

rCCM Rat Cardiomyocyte Conditioned Medium

RIPA Radio immunoprecipitation

SDF-1 Stromal Derived Growth Factor

TBST Tris-Buffered Saline and Tween 20

TGF-B Transforming Growth Factor-Beta

TLR Toll-Like receptors

TNF- Tumour Necrosis Factor Alpha

TNFR1 Tumor Necrosis Factor Receptor 1

TNFR2 Tumor Necrosis Factor Receptor 2

UBC Umbilical Cord Blood

VADs Ventricular Assistive Devices

VSMC Vascular Smooth Muscle Cells

VEGF Vascular Endothelial Growth Factor-1

WB Western Blots

WHF World Heart Federation

WHO World Health Organization

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Chapter 1 - Background

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CHAPTER 1: BACKGROUND

1.1 Cardiovascular Disease

Cardiovascular Disease (CVD) is the number one leading cause of death worldwide. In 2012, CVDs had caused approximately 17.5 million deaths globally (WHO, 2012). Studies show that the major populations that are diagnosed with CVDs are individuals from low and middle income countries with unhealthy behaviours and lifestyles. This may include alarmingly high rates of tobacco and alcohol use, unhealthy diets leading to obesity, physical inactivity, especially in individuals who are co-diagnosed with diseases like diabetes and hypertension. Other risks include: family history, age, and ethnicity (Mendis, 2011).

CVD encompasses a subset of disorders associated with the heart and blood vessels which lead to the brain and lie throughout the body. It is mainly known to be characterized by the following diseases: coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism (WHO, 2014).

1.1.1 Coronary Heart Disease

Coronary Heart Disease (CHD), also known as Ischemic Heart Disease (IHD), is one of many diseases that fall within CVD. In 2012, CHD caused 7.4 million deaths of the total 17.5 million deaths which resulted from CVD. CHD is multifactorial disease, and the underlying pathology of CHD is a consequence of a buildup of plaque within the walls of arteries that lead to the heart (WHO, 2014). Atherosclerosis refers to the narrowing, thickening, and hardening of blood vessels, which usually occurs when the endothelium lining the inner walls of the arteries are damaged and become chronically inflamed, allowing the buildup of plaque to occur. Plaque is constituted of triglycerides, low-density lipoprotein (LDL), cell debris, calcium, and fibrin. Over time, the progressive build-up and rupturing of plaque can result in stenosis or thrombosis and ultimately, hinder the enriched supply of oxygen-rich blood flow through the artery to a portion of the heart (William Insull, 2009). The blocked artery contributes to the depletion of oxygen and nutrients to the heart, and creates an imbalance in the supply and demand of oxygen that is needed for the survival of the heart tissue. This ischemia induced disorder usually results in a myocardial infarction which causes the necrosis of cardiomyocytes in the section of the heart muscle that is

starved of oxygen and nutrients. Although most cases are plaque-induced, vasospasms have been shown to cause a few cases of MI. There are five critical manifestations of CHD: Stable angina (chest pain) pectoris, unstable angina pectoris, MI, heart failure, and sudden death (Mendis et al.,2011).

1.2 Myocardial Infarction

A Myocardial Infarction (MI), conventionally known as a heart attack, results from the occlusion of a coronary artery, which decreases blood flow to the heart tissues and deprives the myocardium of oxygen and essential nutrients (Figure 1). The lack of oxygen and nutrients ultimately, causes the death of resident cardiomyocytes and results in irreversible tissue damage. There are two main coronary arteries that supply blood to the heart: 1) The Right Coronary artery- which branches into the Right Marginal artery and Posterior Descending artery and 2) The Left Coronary artery- which branches into the Circumflex artery and Left Anterior Descending artery (LAD). Blockage of any of the previously listed arteries can result in an ischemia induced MI (Figure 2).

Figure 1. A Visual Representation of a Myocardial Infarction

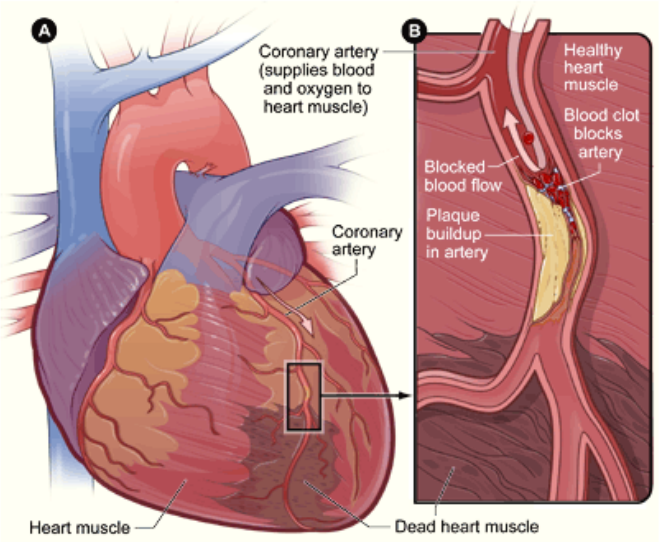


Fig 1. Illustrates the mechanism by which blockage of a coronary artery occurs and results in MI. (Adapted from http://www.nhlbi.nih.gov/health/health-topics/topics/cad/signs)

Table 1. Myocaridal Infarct Sites and Responsible Coronary Arteries

Infarct Site	**Affected Coronary Artery**
Anterior Septum	Proximal LAD
Apex	LAD, RCA, or LCX
High Lateral wall	First diagonal branch of LAD
Anterolateral wall	Proximal LAD including diagonal branch
Extensive Anterior wall	Proximal LAD
Anteroinferior wall	LAD going around apex extensively
Lateral wall	Diagonal branch of LAD, extending to apex
High Posterior wall	LCX
Posterolateral wall	LCX – including obtuse marginal branch
Posteroinferior wall	LCX or large RCA
Inferior wall Apex	RCA or LCX
Inferior wall/ Right ventricle	LCX or large RCA

Table 1. Sites which are highly susceptible to MI and their respective coronary arteries. Left Circumflex Artery (LCA), Right Coronary Artery (RCA), Left Circumflex Artery(LCA). (Adapted from Anzai, T et al., 2001.)

Figure 2. The Coronary Arteries of the Heart

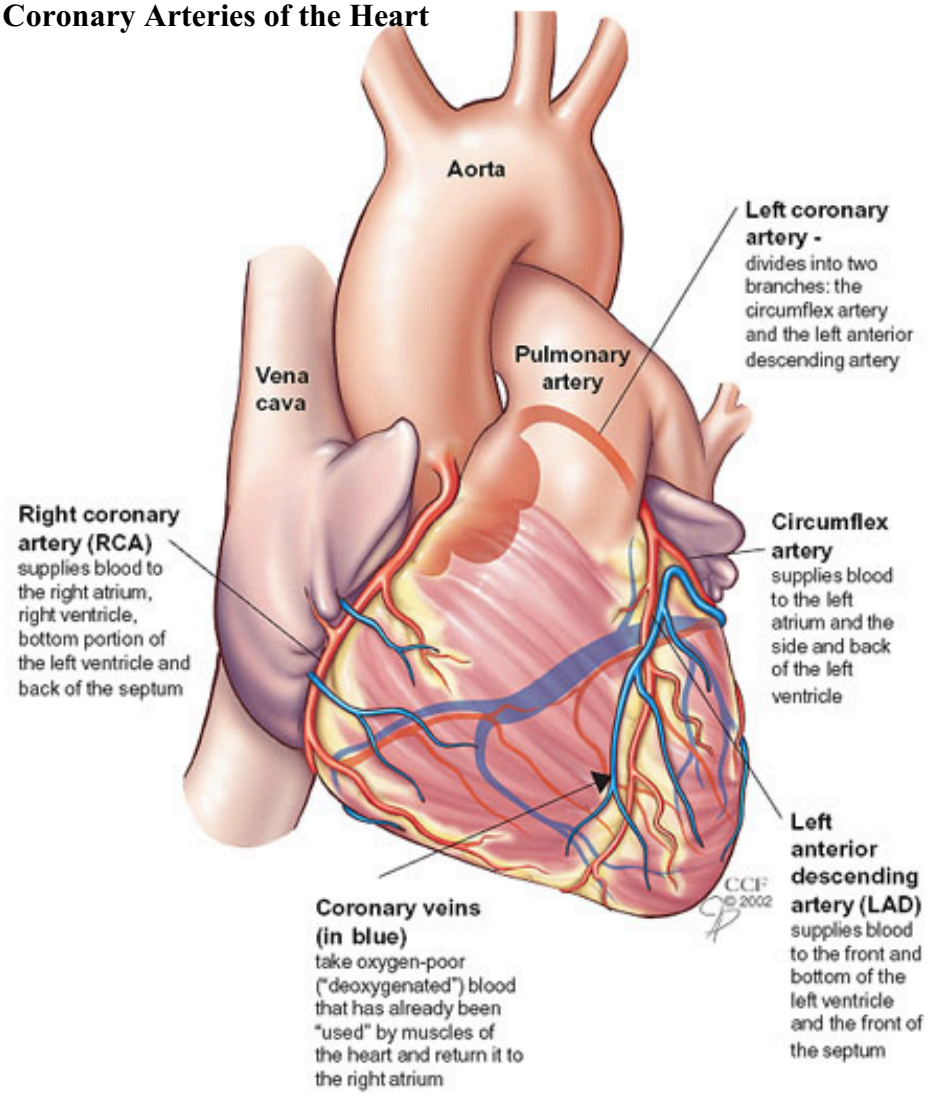


Fig 2. A visual depiction of the coronary arteries (red) and coronary veins (blue)of the heart. (Adapted from http://my.clevelandclinic.org/services/heart/heart-blood-vessels/coronary-arteries)

MI can be distinguished as an Acute Myocardial Infarction (AMI) or a Perioperative Myocardial Infarction (PMI). Confirmation of an AMI occurs upon evidence that suggests the death of cardiomyocytes in a clinical setting consistent with acute myocardial ischemia. "Under the stated conditions, any one of the following criteria [listed in Table 2] meets the diagnosis for myocardial infarction" (Thygesen et al, 2012).

Table 2. Definition of Myocardial Infarction - *Criteria for Acute Myocardial Infarction*

- "Detection of a rise and/or fall of cardiac biomarker values [preferably cardiac troponin (cTn)] with at least one value above the 99th percentile upper reference limit (URL) and with at least one of the following:
  - Symptoms of ischemia.
  - New or presumed new significant ST-segment–T wave (ST–T) changes or new left bundle branch block (LBBB).
  - Development of pathological Q waves in the ECG.
  - Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.
  - Identification of an intracoronary thrombus by angiography or autopsy.

- Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes or new LBBB, but death occurred before cardiac biomarkers were obtained, or before cardiac biomarker values would be increased.

- Percutaneous coronary intervention (PCI) related MI is arbitrarily defined by elevation of cTn values (>5x99th percentile URL) in patients with normal baseline values (≤99th percentile URL) or a rise of cTn values >20% if the baseline values are elevated and are stable or falling. In addition, either (i) symptoms suggestive of myocardial ischemia or (ii) new ischemic ECG changes or (iii) angiographic findings consistent with a procedural complication or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required.

- Stent thrombosis associated with MI when detected by coronary angiography or autopsy in the setting of myocardial ischemia and with a rise and/or fall of cardiac biomarker values with at least one value above the 99th percentile URL.

- Coronary artery bypass grafting (CABG) related MI is arbitrarily defined by elevation of cardiac biomarker values (>10x99th percentile URL) in patients with normal baseline cTn values (≤99th percentile URL). In addition, either (i) new pathological Q waves or new LBBB, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality" (Thygesen et al, 2012).

"Criteria for prior myocardial infarction

Any one of the following criteria meets the diagnosis for prior MI:

● Pathological Q waves with or without symptoms in the absence of non-ischemic causes.

● Imaging evidence of a region of loss of viable myocardium that is thinned and fails to contract, in the absence of a non-ischemic cause.

● Pathological findings of a prior MI" (Thygesen et al, 2012).

Table 2. The Criterion needed to meet the diagnosis for AMI. ("Content adapted from Circulation, 126 /16, Thygesen, Alper, Jaffe, Simoons, Chaitman, & White. Third universal definition of myocardial infarction, 2020-2035., (2012).

PMI occur in high risk cardiac patients who undergo any type of major surgeries. PMIs start within one to two days of surgery during the greatest postoperative stress. “Two distinct mechanisms may lead to PMI: acute coronary syndrome and prolonged myocardial oxygen supply-demand imbalance in the presence of stable coronary artery disease designated type 1 and type 2 by the universal definition of MI” (Landesberg, 2009). In this particular study, we focus on AMI.

Although most cases of MI are triggered by the narrowing of the arteries or by spontaneous rupturing of plaques, few cases have been reported to be caused by obstructing mechanisms such as coronary vasospasms (Thygesen et al, 2012). Depending on the size of the MI, individuals can be clinically asymptomatic, symptomatic, or experience sudden death. An ischemia induced MI can be pathologically subcategorized into being one of two: transmural or nontransmural. The initial is characterized by cell death that extends from the epicardium through the myocardium to the endocardium. This covers the whole thickness of a portion of the heart muscle. The latter describes ischemic necrosis which extends only throughout the endocardium and/or myocardium. A nontransmural MI, does not affect the full thickness of the heart muscle (Bolooki & Askari, 2010).

1.2.1 Universal Classifications of Myocardial Infarction

In 2012, the European Society of Cardiology (ESC), American College of Cardiology Foundation (ACCF), American Heart Association (AMA), and the World Heart Federation (WHF) updated the universal definition and classifications of MI, which are now adopted by the World Health Organization (WHO) and the medical community. This definition allows MIs to be recognized by clinical features (through echocardiographic findings-(ECGs)), elevated values of biochemical markers released by necrotic cardiomyocytes, by imaging (echocardiography, radionuclide imaging, magnetic resonance imaging, and X-Ray computed tomography), or by pathological analysis (Thygesen et al, 2012) (Table 3).

Clinical Features and Classifications of MI

During a clinical classification of MI, patient history and electrocardiographic (ECG) findings are highly assessed and analysed. Patients can be asymptomatic or symptomatic. Atypical symptoms may include: palpitations or cardiac arrest (Thygesen et al, 2012). Usually patients can experience angina, epigastric discomfort, diaphoresis, and fatigue which usually lasts up to or

grater than 20 minutes (Bolooki & Askari, 2010). The discomfort is often diffused and does not remain localized. The ECG is a primary diagnostic tool, when combined with other tools, allows one to detect a suspecting MI. An ECG records the electrical activity of the heart. "The acute or evolving changes in the ST-T waveforms and the Q-waves when present potentially allow the clinician to date the event, to suggest the infarct-related artery, and to estimate the amount of myocardium at risk" (Thygesen et al., 2008). The PQRST & U waveforms in ECGs can be examined in order to determine if there is a ST- elevated MI (STEMI) or non-ST-elevated MI (NSTEMI) which reflects the difference in the acute myocardial infarction, the extent to which an artery is occluded, and the resulting size of necrosis (Bode & Zirlik, 2007) (Figure 3). Furthermore, MI can be clinically classified into five subgroups (Table 3): 1) Spontaneous MI (MI type 1), MI secondary to an ischemic imbalance (MI type 2), 3) MI resulting in death when biomarker values are unavailable (MI type 3), 4) MI related to percutaneous coronary intervention (PCI) (MI type 4a) and MI related to stent thrombosis (MI type 4b), and 5) MI related to coronary artery bypass grafting (MI type 5) (Thygesen et al, 2012).

Figure 3. Basic Electrical Activity of the Heart on a ECG

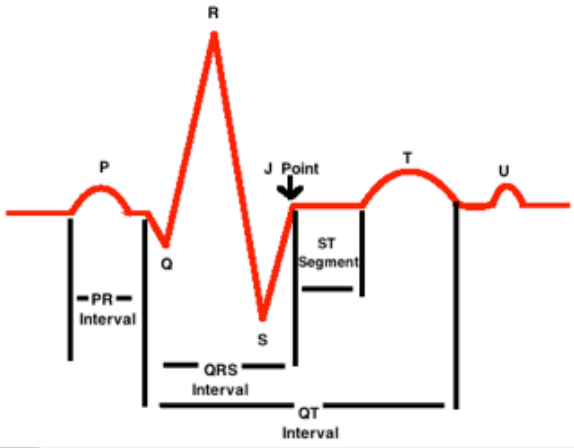


Fig 3. "The ***P wave*** represents **atrial depolarization**. The PR interval is the time between the first deflection of the P wave and the first deflection of the QRS complex. The three waves of the ***QRS complex*** represent **ventricular depolarization**. The ***ST segment/ST interval***, is the time between the end of the QRS complex and the start of the T wave. It reflects the period of **zero potential between ventricular depolarization and repolarization**. ***T waves*** represent **ventricular repolarization** (atrial repolarization is obscured by the large QRS complex" (Ashley & Niebauer, 2004).

Classification of MI using Biomarkers

As stated by the European Society of Cardiology (ESC), the American College of Cardiology Foundation (ACCF), the American Heart Association (AHA), and the World Heart Federation (WHF), the most preferred biomarkers that are used to diagnose an AMI is Cardiac Troponin (cTn) I or T. cTn is a component found in all contractile myocardial cells, and is highly tissue specific, found exclusively in the heart. Elevated levels of cTn in the blood can be a marker of myocardial necrosis, however, the mechanism underlying the cause of the damage cannot be determined. The cycle of rising and/or falling levels of cTn is crucial in diagnosing an acute MI, although it may vary from case to case. Levels of cTn are assessed every 3-6 hours following the initial event and test. "The cTn concentration is defined as a value exceeding the 99th percentile of a normal reference population (upper reference limit)" (Thygesen et al.,2012), and must occur at least once in the cycle of tests that occur depicting a rising and/or fall pattern in cTn levels to be classified as a MI. MB fraction of creatine kinase (CKMB) levels measured by mass assay maybe used to diagnose an MI, with other classification methods in the event that cTn assays are unavailable (Thygesen et al, 2012).

Pathological Characteristics of MI

The pathological analysis of MI induced by ischemia reveals necrosis of cardiomyocytes. Although cell death is a major marker in profiling a MI, post ischemia, it does not occur immediately and takes approximately 20 minutes or less in some animal models to manifest. Furthermore, necrosis can only be identified both microscopically and macroscopically after several hours following the infarction post-mortem (Thygesen et al, 2012).

1.2.2 Current Management Therapies & Surgical Interventions

Patients whom experience an ischemia induced AMI are mainly given symptomatic treatments, while they are further examined to see if they need any surgical interventions in order to unblock the obstructed coronary artery. The primary goals of the therapies and procedures administered to the patient are restoring the normal coronary blood flow to the heart tissue and salvaging any remaining myocardium. The management and treatment plan for a patient with a MI is highly time dependent and definitive therapies differ between a STEMI or NSTEMI.

Table 3. Universal Classification of Myocardial Infarction

Type 1: Spontaneous myocardial infarction	Spontaneous myocardial infarction related to atherosclerotic plaque rupture, ulceration, Assuring, erosion, or dissection with resulting intraluminal thrombus in one or more of the coronary arteries leading to decreased myocardial blood flow or distal platelet emboli with ensuing myocyte necrosis. The patient may have underlying severe CAD but on occasion non-obstructive or no CAD.
Type 2: Myocardial infarction secondary to an ischemic imbalance	In instances of myocardial injury with necrosis where a condition other than CAD contributes to an imbalance between myocardial oxygen supply and/or demand, e.g. coronary endothelial dysfunction, coronary artery spasm, coronary embolism, tachy-/brady-arrhythmias, anemia, respiratory failure, hypotension, and hypertension with or without LVH.
Type 3: Myocardial infarction resulting in death when biomarker values are unavailable	Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes or new LBBB, but death occurring before blood samples could be obtained, before cardiac biomarker could rise, or in rare cases cardiac biomarkers were not collected.
Type 4a: Myocardial infarction related to percutaneous coronary intervention (PCI)	Myocardial infarction associated with PCI is arbitrarily defined by elevation of cTn values > 5 x 99th percentile URL in patients with normal baseline values (<99th percentile URL) or a rise of cTn values >20% if the baseline values are elevated and are stable or falling. In addition, either (i) symptoms suggestive of myocardial ischemia, or (ii) new ischemic ECG changes or new LBBB, or (iii) angiographic loss of patency of a major coronary artery or a side branch or persistent slow- or no-flow or embolization, or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required.
Type 4b: Myocardial infarction related to stent thrombosis	Myocardial infarction associated with stent thrombosis is detected by coronary angiography or autopsy in the setting of myocardial ischemia and with a rise and/ or fall of cardiac biomarkers values with at least one value above the 99th percentile URL
Type 5: Myocardial infarction related to coronary artery bypass grafting (CABG)	Myocardial infarction associated with CABG is arbitrarily defined by elevation of cardiac biomarker values >10 x 99th percentile URL in patients with normal baseline cTn values (<99th percentile URL). In addition, either (i) new pathological Q waves or new LBBB, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

Table 3. Classifies MI based on clinical and molecular manifestations. ("Content adapted from Circulation, 126 /16, Thygesen, Alper, Jaffe, Simoons, Chaitman, & White. Third universal definition of myocardial infarction, 2020-2035., (2012). © 2016 American Heart Association, Inc. All rights reserved. Unauthorized use prohibited).

Contingent upon the type and severity of MI patients experience, patients' treatment options may be comprised of pharmacological treatments which help manage symptoms and/or an

immediate reperfusion therapy through procedural or surgical intervention (Table 4). Pharmacological treatments include the administration of: 1) anti-platelets and thrombolytics, 2) nitrates, 3) aldosterone antagonists, 4) beta blockers, 5) fibrinolytics, 6) angiotensin-converting enzyme inhibitors (ACE inhibitors) and angiotensin receptor blockers (ARB), and 7) statin therapy. Following a coronary angiography, reperfusion therapeutic option may include: 1) percutaneous coronary intervention (PCI) and 2) surgical revascularization through coronary artery bypass surgery (CABAG) (Bolooki & Askari, 2010; Priebe, 2005). During end stage heart failure, the only surgical options that would be available to a patient are: a heart transplant or ventricular assistive devices (VADs) (Friedrich & Böhm, 2007). Reduction of morbidity and mortality from MI is dependent on patients and bystanders immediately recognizing MI symptoms and attaining pharmacological adjunctive therapies.

Table 4. List of Management Therapies and Surgical Interventions Post-MI

Pharmacological Management Therapies (PMT)	***Function of PMT***	***Examples of PMT***	***Procedural and Surgical Interventions***	***Final Surgical Interventions***
Anti-Platelets and Thrombolytics	- Prevents platelet adhesions and cohesion -Dissolves blood clots	-Aspirin -Clopidogrel -Heparin	*PCI*	*Heart Transplant*
Nitrates	- Vasodilators	- Nitroglycerin	*CABG*	*VADs*
Aldosterone Antagonists	- Diuretics	- Spironolactone		
Beta-Blockers	-Reduces myocardial oxygen consumption -Lowers heart rate/blood pressure	- Carvedilol -Bisoprolol - Metoprolol Succinate		
Fibrinolytics	-Dissolves fibrin in blood clots	- Tenecteplase -Streptokinase -Alteplase		
ACE Inhibitors & ARB	-Relaxes blood vessels	- Captopril - Candesartan		
Statin Therapy	- Lowers Cholesterol	- Atorvastatin		

Table 4. Lists pharmacological management therapies, surgical interventions, and final stage surgical interventions that may be selected as a method of treatment for patients who have suffered MI. The combination of therapies administered to a patient is patient specific (Boersma et al.,2003; Hilleman et al.,2007; Friedrich & Böhm, 2007).

1.3 The Microenvironment Post-Myocardial Infarction

Subsequent to an ischemic injury to the myocardium, an acute innate and adaptive inflammatory response is activated in attempt to repair and heal the myocardium, reestablish homeostasis, and initiate the scar formation processes. As the adult mammalian heart has a limited intrinsic regenerative capacity, irreversible myocardial damage may cause ventricular dysfunction and lead to the development of heart failure. Excessive inflammation and their corresponding physiological responses, stimulate a cascade of reactions which leads to increased death of peripheral cardiomyocytes, the degradation of the extracellular matrix (ECM), the regeneration of fibroblasts into ineffective scar tissue, the reorganization of myocyte bundles, and adverse left ventricular remodeling (Venugopal et al., 2012). The progressive ventricular remodeling and dilation, depending on the size of infarct and incurred damages, can ultimately lead to permanent impairment of the myocardium eventually, resulting in heart failure.

1.3.1. Acute Inflammation Post MI

Inflammation is a fundamental biological response that is essential in triggering the healing process following an ischemic event in the myocardium. Post MI, an acute inflammatory response is initiated through endogenous alarm signals which are released by necrotic and damaged cardiomyocytes. These signals are commonly referred to as damage-associated molecular patterns (DAMPs). DAMPs then continue to "activate the complement cascade and stimulates toll-like receptors (TLRs)/interleukin-1 (IL-1) signaling" (Fang et al.,2015). The activation of the TLRs and production of IL-1 actively triggers the NF-κB (Nuclear Factor Kappa- B) complex, which allows for the production of various adhesion molecules, chemokines, and cytokines. These primary biological signaling molecules play a major role in enhancing the recruitment of inflammatory cells (leukocytes and mononuclear cells) to the infarcted region of the myocardium in an attempt to clean and repair the wounded myocardium (Frangogiannis, 2002). Simultaneously, reactive oxygen species (ROS) induce the production of proinflammatory cytokines, which can then induce the production of more ROS (Nian et al.,2004). Adhesion molecules promote and allow leukocytes, mainly neutrophils, to transmigrate across the endothelial surface of vessels and infarcted myocardium to mediate tissue damage through releasing matrix degrading proteins called matrix metalloproteinase (MMP). Monocytes and lymphocytes are further recruited and produce additional proinflammatory cytokines and growth factors while playing a critical role in degrading

damaged myocardium and mediating wound healing (Zuylen, 2015) Monocytes are known to transform into one of two types of macrophages, M1 – related to proinflammatory processes and M2- involved in repair (Hansson, 2001). Transmigration of macrophages and neutrophils throughout the cycle of inflammation causes a cytokine amplification process, which contributes to the various innate mechanisms that are involved in chronic inflammation, the absorbance of necrotic tissue, hypertrophy, degradation of matrix (collagen and integrins), and scar formation (Bodi et., 2008). Figure 4 illustrates the initiation mechanisms of an acute inflammatory response.

Figure 4. Initiation of the Inflammatory Response following an Ischemia Induced MI

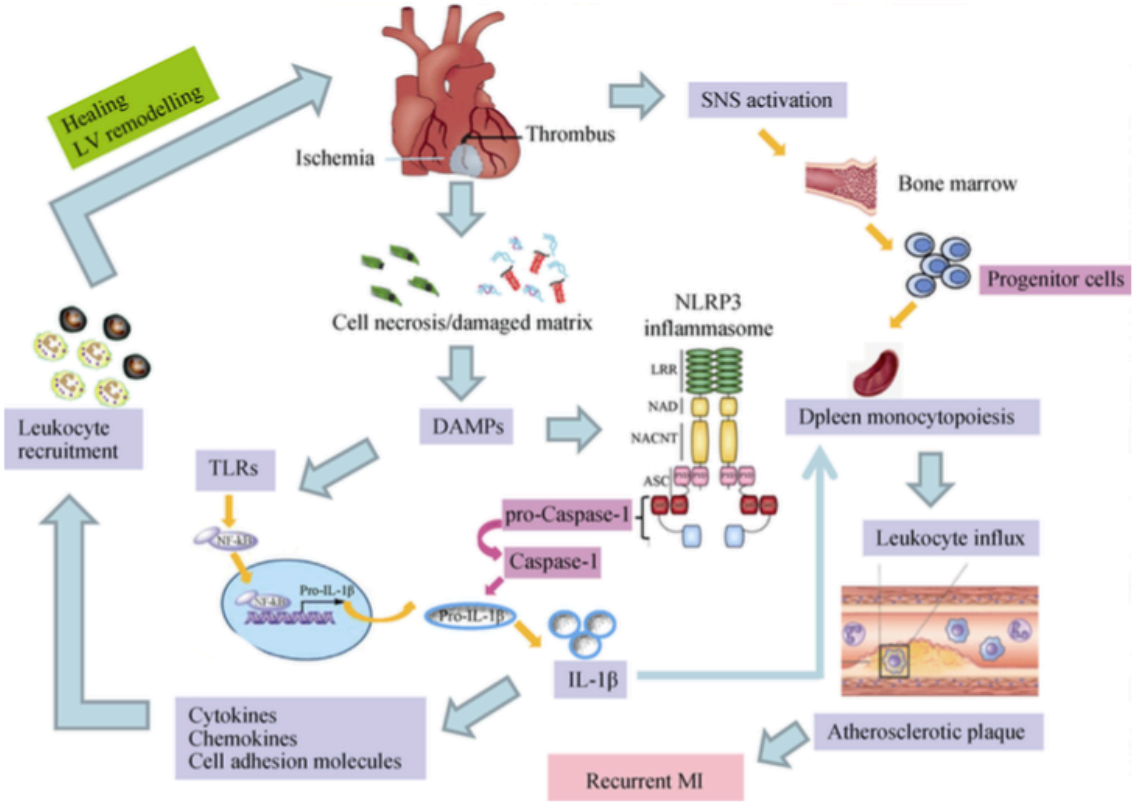


Fig 4. "AMI triggers an intense inflammatory response including elevation of inflammatory mediators, and recruitment of inflammatory cells via DAMPs/TLR/IL-1signaling. Inflammasomes also recognize danger signals and activate caspase-1, and release active IL-1β. Inflammatory response serves to repair the heart, but excessive inflammation leads to adverse LV remodeling and heart failure. AMI is also associated with bone marrow activation via SNS activation and spleen monocytopoiesis, resulting in increased leukocyte influx which aggravates atherosclerosis and contributes to recurrent MI. Spleen monocytopoiesis is also regulated by IL-1β. AMI: acute myocardial infarciton; DAMPs: damage-associated molecular patterns; IL-1: interleukin-1; LV:

left ventricular; TLR: toll-like receptor; SNS: sympathetic nervous system" (Fang et al.,2015). ("Image adapted from Journal of Geriatric Cardiology, 12 /3, Fang, Moore, Dart, & Wang, Systemic inflammatory response following acute myocardial infarction, 306., (2015), with permission from the Open Access Journal. j.issn.1671-5411.2015.03.020").

1.3.2. Chronic Inflammation Post MI

As acute inflammation continues to persist, chronic inflammatory mechanisms of action are thenceforth triggered. Acute inflammation may become chronic as a result of the size of infarct or lack of treatment. The sustained release of proinflammatory cytokines continue to further recruit inflammatory cells, amplify cytokine production, and induce wound healing and repair to occur. Therefore, as the necrosis of myocytes and degradation of the matrix continues to occur, remodelling of the ventricles and hypertrophy occur in an attempt to increase the efficiency of the failing ventricle. Furthermore, inflammation begins to extend into the non-infarcted regions of the myocardium and causes the deterioration of the myocardium. Thus, local collagen compositions and integrin constitutions begin to change, and an increase in fibroblasts recruitment is also witnessed for remodelling to occur (Zuylen, 2015). In addition to the recruitment of fibroblast, mast cells may release various growth factors and histamines in order to promote vascularization (Bovini et al., 2007). However, remodelling is a short-term solution to the problem at hand as the fibroblasts that ultimately replace necrotic cardiomyocytes are non-contractile (Zuylen, 2015). Therefore, although healing is occurring, the functionality of the heart becomes compromised and this can eventually lead to heart failure. Figure 5, visually depicts the difference between the acute and chronic inflammatory response.

Figure 5. Acute and Chronic Inflammation Post MI

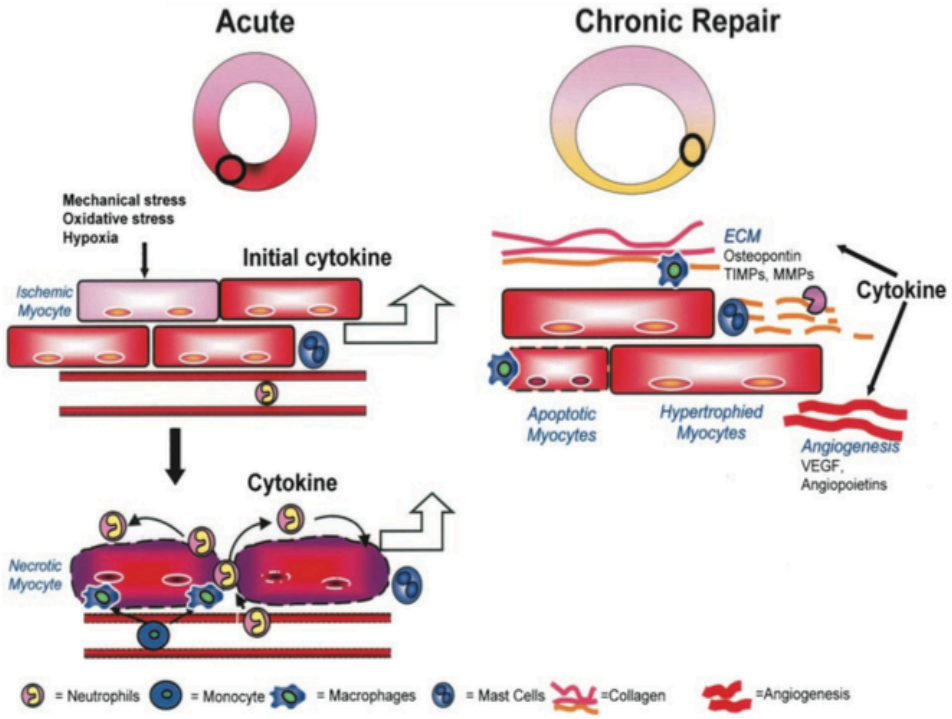


Fig 5. "The induction of inflammatory cytokines after myocardial ischemia and their effect on acute and chronic cardiac remodeling post infarction. Direct myocardial stress, such as mechanical stretch, oxidative stress, and hypoxia in the setting of ischemia, will rapidly induce cytokines such as tumor necrosis factor alpha (TNF-□) or interleukin-6 (IL-6). This can either enhance survival or accelerate myocyte necrosis and apoptosis and decrease contractility. This is also followed by cytokine amplification through transmigration of macrophages and neutrophils. During the chronic phase post infarction, the activation of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) contributes to the laying down of collagen and wound repair. The elaboration of angiogenic and progenitor cell mobilization factors further contributes to the healing of the wound. ECM indicates extracellular matrix; VEGF, vascular endothelial growth factor" (Nian et al.,2004).

"Reprinted from Circulation Research, 94 /12, Nian, Lee, Khaper, Liu, Inflammatory Cytokines and Postmyocardial Infarction Remodeling, 1544., (2014), with permission from Wolters Kluwer Health, Inc. Wolters Kluwer Health, Inc. http://dx.doi.org/10.1161/01.RES.0000130526.20854.fa "

1.4 Research in Stem Cell Therapies

Apart from the existing traditional drug and management therapies and surgical interventions used to treat individuals diagnosed with CHD, efficient treatments that allow for the repair of non-

functional myocardium have still yet to be developed. Thus, an upsurge in research interests associated with investigating models for myocardial repair and regeneration is arising. The limited regenerative capacity of mammalian cardiomyocytes has been a crucial obstacle to developing a treatment that may allow native myocardium to repair and regenerate following an infarction. Current research models encompass novel stem cell treatment designs which in theory would function to restore or regenerate the damaged myocardium. To mediate myocardial repair, stem cells must differentiate into cardiac cell lineages and/or chemically influence the cellular and molecular mechanisms governing repair in the harsh microenvironment-post MI, to replace the injured heart tissue (Mirotsou et al, 2011). Many of these models believe that Mesenchymal Stem Cells (MSCs) may be the key biological tool that maybe required for potentially repairing and regenerating myocardial tissue post-MI (Kern et al.,2006). Two of the countless existing repair and regenerative experimental models specifically focus on tissue engineering approaches through the use of biological scaffolds and administering stem cell-based therapies for cellular cardiomyoplasty (William et al.,2007; Venougopal et al.,2011). However, major limitations to these models include: the poor retention rate and viability of cells injected into the hostile myocardium post-MI, the lack of development of a biological scaffold that mimics the native extracellular matrix of the heart, and the difficulty of inducing the differentiation of stem cells into functional cardiomyocytes in vivo. Despite these limitations, preclinical research results lead scientists to remain optimistic in developing a cellular therapy for myocardial repair. In this current study, we focus on developing a cell-free cytokine dependent therapy for salvaging the healthy myocardium following an ischemia-induced MI.

1.4.1 Mesenchymal Stem Cells

The primary challenge in developing a cellular therapy for cardiac regeneration is selecting the ideal source of stems cells. The endogenous properties of each cell may be critical in deciding the suitable cell type for the job of repairing the damaged myocardium. Reservoirs of stem cells which reside in adult tissues are characterized as being reparative cells. They are known to mobilize to the site of injury and differentiate in response to wound signals that are released within the injured microenvironment (Barry & Murphy, 2004). Several types of adult stem cells exhibit a wide range of therapeutic potentials and some, can be categorized into one of three groups: bone marrow-derived, circulating, and resident progenitor stem cells (Mirotsou et al., 2011). Types of

stem cells that have been examined on a molecular or clinical level in an effort to create a stem cell therapy include: bone Marrow-derived mononuclear cells (BM-MNCs), unfractionated BM cells (BMCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), endothelial progenitor cells (EPCs), cardiac progenitor stem cells (CPCs), cardiac resident stem cells (CSCs), and induced pluripotent stem cells (IPSCs) (Gnecchi et al.,2008; Mirotsou et al.,2011). Although numerous types of stem cells have been examined in developing a therapy to successfully heal the damaged heart tissue following an infarction, the most widely accepted, researched, and promising cell type to show improved heart function in animal and human clinical trials are Mesenchymal Stem Cells (MSCs) (Collins & Russell, 2009).

Within the 1960's to 1970's, Friedenstein and colleagues were the first to run a series of pioneering studies on isolating and culturing MSCs from a subpopulation of mouse bone marrow cells, while examining its differentiation potential and colonization capacity in vitro and in vivo in various models (Friedenstein et al.,1966;1970;1976;1987). Over the last few decades, mesenchymal stems cells, also known as Mesenchymal Stromal Cells, have been extensively studied and have been established as having prospective therapeutic potentials that may revolutionize the field of regenerative medicine and tissue engineering in cardiology.

MSCs are nonhematopoietic undifferentiated spindle shaped stem cells that can be found in numerous adult tissues (Chamberlain et al., 2007). The major source of MSCs is bone marrow, however, more recently MSCs have also been known to exist in adipose tissues, skin, muscle, placenta, peripheral blood, and umbilical cord blood (UCB) (Kern et al.,2006; Boyle, Schulman, & Hare, 2006; Gnecchi et al.,2012). MSCs can be characterized as being self-renewing and multipotent, and therefore capable of multilineage differentiation into various tissues of mesodermal/non-mesodermal origin (Gallina et al.,2015; William & Hare, 2011). Not only can they differentiate into adipocytes, chondrocytes, osteocytes, and myotubules, in vitro and in vivo studies also indicate that MSCs can differentiate into "muscle, neural precursors, cardiomyocytes, [endothelial cells] and possibly other cell types" (Aggarwal & Pittenberg, 2005; Chamberlain et al.,2007). Furthermore, upon isolation of MSCs, they are highly expandable in vitro, plastic adherent, and develop as fibroblast colony-forming- units. MSCs can be easily amplified and are immunologically privileged. Thus, MSCs can be an ideal source of cells for allogeneic

transplantation or for potential immunomodulatory therapy across a diverse patient population as MSCs suppress the proliferation of T-cells at the site of injury (Chamberlain et al.,2007).

The defining properties of MSCs remain inconsistent among scientific investigators. In 2006, the International Society for Cell Therapy proposed that the minimum criteria to characterize MSCs are based on: "1) adherence to plastic, 2) specific surface antigen (Ag) expression, and 3) multipotent differentiation potential" (Dominici et al.,2006; William & Hare, 2011). Thus, the proposed criteria to characterize MSCs are: "(1) adherence to plastic in standard culture conditions; (2) expression of the surface molecules CD73, CD90, and CD105 (3) the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 and (4) a capacity for differentiation to osteoblasts, adipocytes, and chondroblasts in vitro (Figure 6). The criterion was established to standardize human MSC isolation, but may not apply uniformly to other species" (Dominici et al., 2006). MSCs from species differing from humans may express different markers (William & Hare, 2011). MSCs are also known to migrate to the site of injury in response to chemotactic signalling in attempt to supress inflammation and initiate wound healing processes (Chamberlain et al.,2007). This may allow for the systematic delivery of these cells for treatment purposes.

The primary roles of MSCs are still highly disputed, however they are known to maintain the hematopoietic niche in bone marrow and participate in "organ homeostasis, and wound healing" (William & Hare, 2011).

As MSCs are multipotent and possess the ability to differentiate into cardiomyocytes and endothelial cells, they are a highly attractive cell type that can be used in developing a therapy to regenerate myocardium following an infarction. In a 1995 study by Wakitani and colleagues, it was reported that when MSCs are treated with "5-aza-cytidine and amphotericin B, MSCs differentiate into myoblasts that fuse into rhythmically beating myotubes" (Chamberlain et al.,2007). Furthermore, Gojo and coworkers examined and successfully identified the in vivo differentiation potential of MSCs into cardiomyocytes and endothelial cells (Gojo et al.,2003; Barry & Murphy, 2004). "Another advantageous characteristic of MSC is that they are easy to modify ex vivo using viral vectors" and thus can be genetically modified to express pro-survival genes necessary for up regulating survival factors (Gnecchi et al.,2012; Myers et al.,2010). Thus, till recent date, MSCs still remain as one of the leading candidates for cellular cardiomyoplasty (Williams & Hare, 2011).

Figure 6. Characterizing Mesenchymal Stem Cells

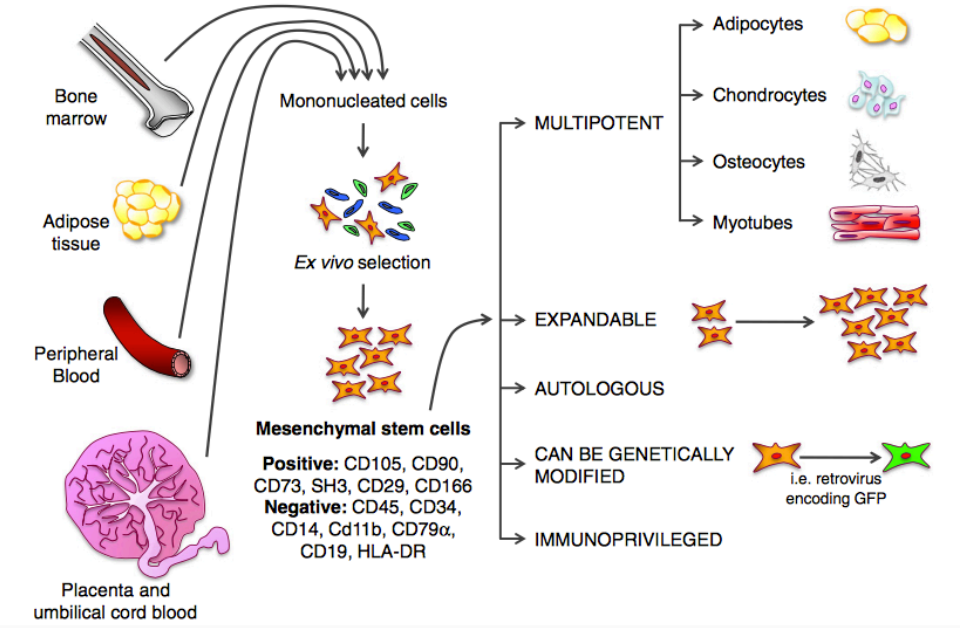


Fig 6. "MSC can be isolated from the bone marrow, adipose tissue, placenta and umbilical cord blood. Some investigators also described MSC in the peripheral blood. MSC can be expanded ex vivo, are multipotent and possess other favorable characteristics that make them suitable for cell therapy and myocardial repair" (Gnecchi et al., 2012). " Reprinted from Vascular Pharmacology, 57/1, Gnecchi, Danieli, Cervio, Mesenchymal stem cell therapy for heart disease, 48-55., (2012), with permission from Elsevier. http://dx.doi.org/10.1016/j.vph.2012.04.002."

1.4.2 Bone-Marrow Mesenchymal Stem Cells

The most extensively studied and prevalently used source of stem cells in clinical cardiac repair studies is the bone marrow. Bone-marrow stroma is composed of a "heterogeneous population of cells [which include] fibroblasts, adherent stromal cells, adipocytes, endothelial cells, and osteogenic cells" (Gnecchi et al.,2012; Caplan et al., 1991). Bone Marrow Mesenchymal Stem cells (BM-MSCs) are a relatively scarce source of stem cells as they only constitute 0.001%–0.01% of bone marrow cells (Gallina et al.,2015). Although they are limited in number, they can be rapidly cultured to produce billions of MSCs in vitro and expanded ex-vivo (Pittenger and Martin, 2004; Myers et al.,2010). Throughout the last few years, BM-MSCs have been widely used in in vitro, in vivo, and clinical studies which solely aim to repair and regenerate damaged myocardial tissue (William & Hare, 2011). The principal reasons of developing a cellular therapy using the administration of exogenous BM-MSCs are: to prevent inefficient and ineffective remodelling of the damaged heart, to salvage the remaining healthy myocardium, to supress

damaging inflammatory responses that may lead to the deterioration of the myocardium and the extra-cellular matrix, and to re-establish the vascular ground work in an attempt to induce neovascularization and BM-MSC differentiation into cardiomyocytes (Myers et al., 2010; William & Hare, 2011). BM-MSCs can actively fulfill the mentioned usages by transdifferentiating into cardiomyocytes, fusing with resident cardiac progenitor cells and instigating proliferation, and/or secreting endogenous trophic factors that may respond to and manipulate the injured microenvironment (Russo et al., 2014).

"Three mechanisms of action have been proposed for adult BM-MSC in heart repair: 1) CMC regeneration, 2) vasculogenesis, and 3) paracrine effects (Fig.11). Regardless of the mechanism of action, there is a general agreement that BM-MSC transplantation is safe and has beneficial effects on infarcted hearts" (Gnecchi et al.,2012; Wu et al.,2007). Overall, although the mechanism of action of BM-MSCs have yet to be established, the use of BM-MSCs in cardiac repair post MI is promising as the administration of BM-MSCs for therapeutic purposes in various studies involving in vivo models have shown either neovascularization, less severe ventricular remodelling, or improved cardiac ejection fractions (Myers et al,2010; William & Hare, 2011).

1.4.3 Homing, Hypoxia & The Suppression of Inflammation

One of the most interesting roles of BM-MSCs is their ability to migrate to the site of myocardial injury or inflammation in an attempt to participate in wound repair. When BM-MSCs migrate to the area of infarction post-MI, the act is known as "homing". The definite mechanism by which BM-MSCs migrate to the damaged tissues is still unclear, but it's suggested that the injured and inflamed tissues likely express specific surface markers that "facilitates trafficking, adhesion, and infiltration of MSCs to the site of injury" (Chamberlain et al.,2007). More recently, it has been determined that BM-MSCs express chemokine receptors, ligands, and adhesion molecules that aid in the process of regulating homing and transendothelial migration. Gradients of chemotactic factors become upregulated in damaged tissue in order to induce the migration of BM-MSCs. BM-MSCs express a variety of surface receptors that play a major role in recognizing these cytokines, growth factors, and integrins that act as potent mobilization factors (stromal derived factor-1 [SDF-1], transforming growth factor-□1 [TGF-□1], and tumor necrosis factor-alpha [TNF-□]), which tend to be upregulated in inflamed tissue. In specific, "signaling through [stromal derived growth factor] - SDF-1/CXCR4, [CX3XR1], and [hepatocyte growth factor

receptor]- HGF/c- MET has been identified as being of importance in stimulating the migration of reparative cells to the ischemic heart" (Russo et al.,2014; Das et al.,2010; Ponte et al.,2007) Other proinflammatory cytokines and proangiogenic growth factors that also contribute to the mobilization of BM-MSCs and endothelial progenitor cells post-MI are: IL-8, vascular endothelial growth factor (VEGF-1), platelet-derived growth factor receptor (PDGF) and fibroblast growth factors (FGF) (Ponte et al.,2007).

Hypoxia is a critical environmental cue that instigates the upregulation of integrins and production of chemokines, and growth factors along with paracrine secretions of BM-MSCs in an attempt to induce homing of BM-MSCs post-ischemic injury. Hypoxia is the lack of oxygen in a particular environment. Physiological levels of O_2 are lower than atmospheric oxygen tension (21%) however, BM-MSCs respond positively to decreased oxygen levels through most notably the transcription factor, hypoxia inducible factor-1a (HIF-1a). HIF-1a then activates several downstream genes that allow for the production of vascular endothelial growth factor (VEGF), TNF- α, other proinflammatory cytokines and chemokines that may aid in the homing and recruitment of BM-MSCs, while simultaneously activating the transcription factor nuclear factor κB that maybe required to initiate pro-survival genes for cell survival in cardiomyocytes (Das et al.,2010). In a study by Rochefort et al, it was concluded that under hypoxic conditions in vivo (rat model), there is a 15-fold increase in MSC mobilization in the pool of circulating MSCs. Hypoxia plays a major role in enhancing BM-MSCs proliferation, differentiation, paracrine activity, migration, and can greatly contribute to understanding the therapeutic benefits of BM-MSCs in myocardial sustainability and repair.

MSCs are immune privileged and evasive, and are known to play numerous roles in suppressing inflammation post myocardial injury, in attempt to heal the wound (Ankrum et al, 2014). The balance between the pro-inflammatory responses and the anti-inflammatory repair mechanisms is key in developing an efficient and effective therapy that may ultimately give rise to a functional myocardium. In a physiological setting, this same balance is fundamental in triggering fibrosis. Fibrosis and scarring are the body's short-term treatment for an ischemic injury. Figure 7 examines the interaction between the MSCs and the inflammatory cells while depicting that MSCs interact with both the innate and adaptive immune cells. MSCs suppress the innate inflammatory cells that defend against non-specific infections. MSCs release IL-6 to reduce the effects of ROS, while inhibiting the differentiation of immature monocytes into dendritic cells. In

addition they also decrease the production of TNF-α and the natural killer cells' production of interferon (IFN-γ) (Wiliam & Hare, 2011). T-cells, that make up the adaptive immune responses, are inhibited by MSC secretions (HGF, IL-10, and TGF-β1) thereby, decreasing the death of targeted cells and further reducing the proliferation and maturation of other T-cells (Aggarwal & Pittenger, 2005). They also alter naïve T-cells to an anti-inflammatory state, which increases the production of IL-4 (anti-inflammatory cytokine) (William & Hare, 2011). In order to understand the therapeutic benefits of MSCs, it is essential to identify and understand the natural immunomodulatory secretions and their effects in response to tissue injury. These paracrine factors may become the crucial component in understanding how MSCs contribute to cardiac repair. Ultimately, BM-MSCs are a safe and feasible source of cells that holds great potential in myocardial repair.

Figure 7. Mesenchymal Stem Cells' Interactions with Immune Cells

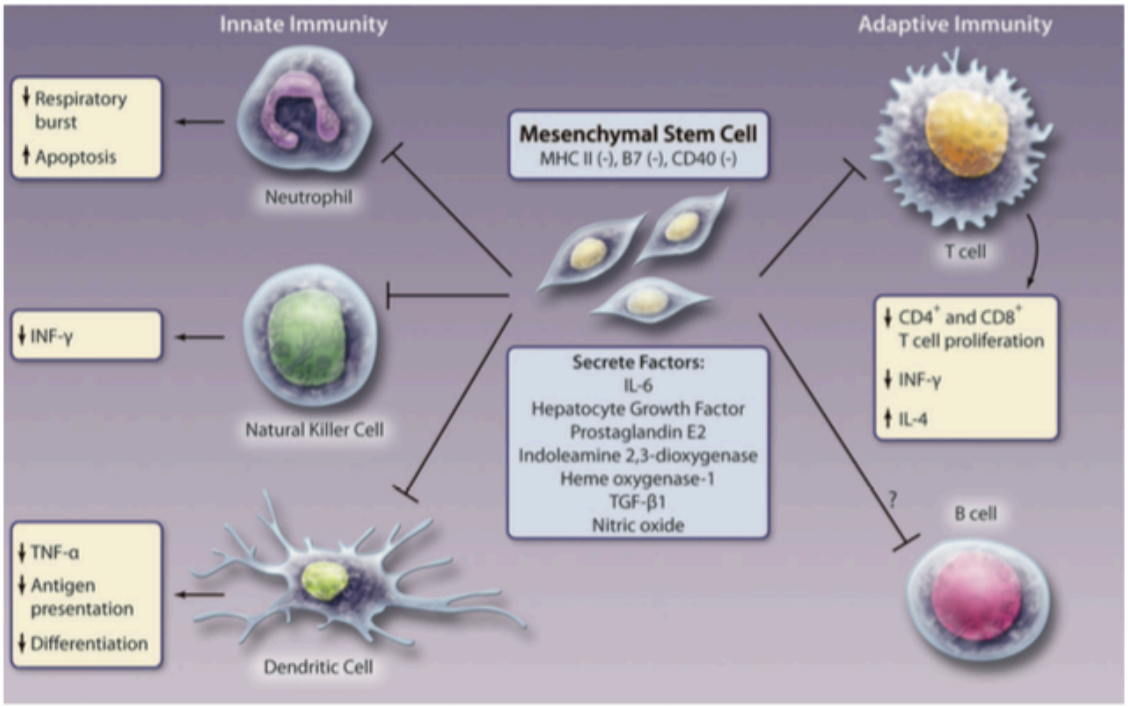


Fig 7. "MSCs are immunoprivileged cells that inhibit both innate (neutrophils, dendritic cells, natural killer cells) and adaptive (T cells and B cells) immune cells" (William & Hare, 2011)

"Reprinted from Circulation Research, 109/8, William & Hare, Mesenchymal Stem Cells: Biology, Pathophysiology, Translational Findings, and Therapeutic Implications for Cardiac Disease, 924-926., (2011), with permission from Wolters Kluwer Health, Inc. http://dx.doi.org/10.1161/CIRCRESAHA.111.243147."

1.4.4 Current Research in Stem Cell Therapy Models

Following an ischemic injury, an apparent lack of an effective intrinsic mechanism for myocardial repair limits cell turnover of cardiomyocytes. Thus, investigative efforts have focused on replacing the lost cells and/ or providing a vehicle that promotes repair mechanisms through paracrine secretions with exogenous administration of MSCs. The ease of isolation, high expansion potential, reproducibility, genetic stability, multipotency, and compatibility with tissue engineering practices make MSCs a prospective source for stem cell therapy models (Pittengar & Martin, 2004) Cellular cardiomyoplasty and tissue engineering using biomaterials and stem cells have become extensively researched and popular among clinical trials. The therapeutic abilities of the cells and method of delivery are highly dependent on the cells ability to differentiate into cardiomyocytes and/or secrete paracrine factors that enable cardiac repair and regeneration to occur. "Experimental studies have shown that bone marrow- derived cells are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis, which leads in turn to amelioration of cardiac function" (Venougopal et al.,2012). In 1999, the first use of BM-MSCS for cardiomyoplasty was reported in the laboratories of Weisel and Lee where "autologous bone marrow cells were implanted in the left ventricle (LV) of rats by direct injection 3 weeks after cryoinjury." Transplanted cells expressed muscle-specific proteins which were absent before implantation (Pittengar & Martin, 2004). Figure 8 looks at the therapeutic models which have been examined till date. They include the administration of MSCS (in specific BM-MSCs) through intracoronary infusion, intravenous infusion, intramyocardial injection; through the uses of biological scaffolds such as patches, hydrogels; and 3D printed scaffolds and organs (Williams & Hare, 2011; Venugopal et al.,2012; Mironov et al.,2003). The process of decellularizing and recellularizing in prospect of creating a functional heart is another approach to stem cell therapy (Maher, 2013). This current study focuses on developing a cell-free therapy that is exclusively focused on administering paracrine secretions secreted by BM-MSCs in response to a proinflammatory cytokine and hypoxia. The goal of this therapy is to aid in BM-MSCs recruitment to the site of infarct and to reduce the size of fibrosis and scarring while improving the survival of cardiomyocytes mediated through vasculogenesis.

1.4.5 The Limitations to Stem Cell Therapy Models

"The efficacy of cell therapy is [highly dependent upon the cell type and] the ability of the delivery mechanism to provide sufficient localization, survival, and retention of the donor cells in

the affected tissues" (Russo et al.,2014). The major obstacles that are faced in the clinical applications of BM-MSC-based therapy is the poor viability and survival of transplanted cells as they tend to be lost in the pulmonary circulation thus, limiting their reparative abilities (Venougopal et al.,2012).

Figure 8. Delivery of MSCs in an Attempt to Repair the Damaged Myocardium

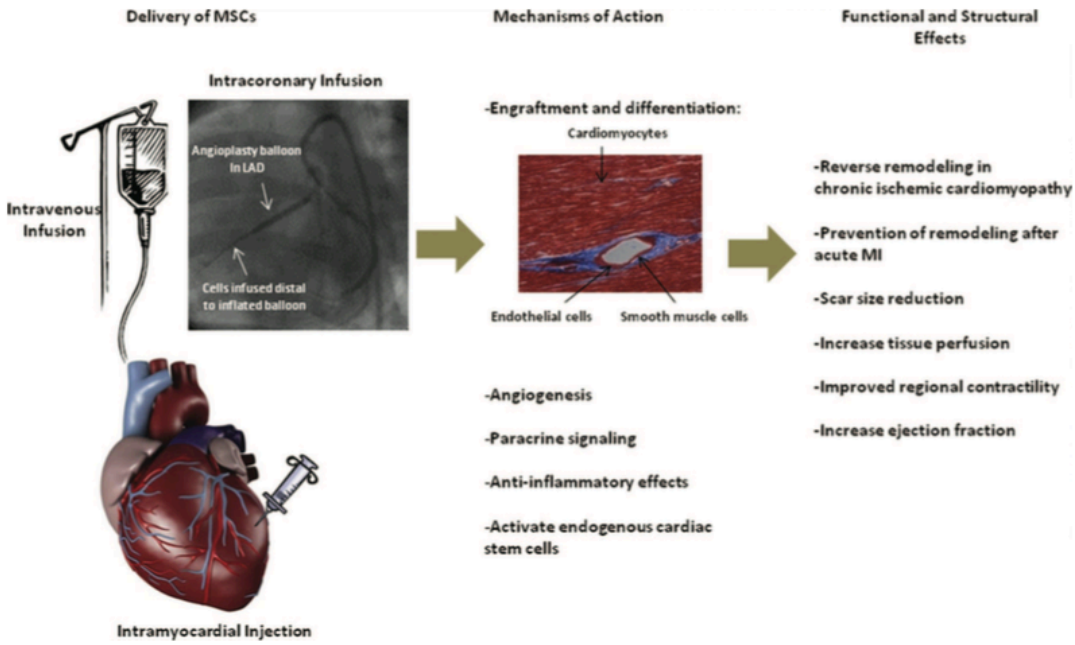


Fig 8. "Delivery and potential effects of MSC therapy in cardiac disease" (William& Hare, 2011)
"Reprinted from Circulation Research, 109/8, William & Hare, Mesenchymal Stem Cells: Biology, Pathophysiology, Translational Findings, and Therapeutic Implications for Cardiac Disease, 924-926., (2011), with permission from Wolters Kluwer Health, Inc. http://dx.doi.org/10.1161/CIRCRESAHA.111.243147."

Only around 5% of the transplanted cells are retained in the myocardium and can survive up to 14 days in an infarcted porcine heart model, while showing only a transient improvement of the left ejection fraction (Kania, 2011). Furthermore, the ischemic host microenvironment also prevents adverse challenges to these engrafted BM-MSCs. Inflammation, lack of survival signals, deprivation of nutrients and oxygen, and the lack of an extracellular matrix can cause exogenous BM-MSCs to become apoptotic. The regenerative and differentiation potential of cells may also become negatively affected (Song et al.,2010). Various biomaterials (Matrigel, Collagen, Chitosan) that can be used to address the engraftment and retention issues must be non-immunogenic and retain the mechanical properties of the native extracellular matrix of the heart in order for the effective transplantation of BM-MSCs (Segers & Lee, 2008). Paracrine secretions of the BM-MSCs have more recently stepped into the limelight as a major contributor that directs

restorative processes including myocardial protection, initiating the process of angiogenesis and differentiation, and supressing inflammation (Mirotsou et al.,2011; Rodrigues et al.,2010). The focus on paracrine secretions may hopefully aid moving towards developing a more cell-free therapy that may take into account the synergistic interactions of proteins at a molecular level.

1.4.6 The Roles of Bone-Marrow Mesenchymal Stem Cells in Myocardial Repair

Currently, the active role of BM-MSCs is widely debated as a consensus on the mechanism of action of BM-MSCs has not been reached yet. Myocardial and vascular regeneration have been proposed as the central function of stem cells in cardiac repair (Gnecchi et al.,2016). Three main hypotheses exist when it comes to the mechanism of action of BM-MSCs in cardiac repair. The initial and primary hypothesis is that the BM-MSCs transdifferentiate into functional cardiomyocytes and endothelial cells at the site of infarct (Pittenger &Martin, 2004). The second hypothesis states that BM-MSCs fuse together with existing cardiac progenitor cells in order to differentiate and support the act of proliferation. The third and most promising hypothesis is that BM-MSCs secrete soluble paracrine factors that synergistically interact with the harsh microenvironment, while protecting the heart from progressive ischemia induced damage, activating neovascularization, attenuating fibrosis, improving myocardial contractility, and supressing inflammation (Mirotsou et al.,2011). Due to the limited engraftment of BM-MSCs at the site of infarct, the first two hypothesis seem highly unlikely to contribute to the transient but significant functional improvement of the heart witnessed in many preclinical studies (Rodrigues et al.,2010). Furthermore, these BM-MSCs released factors have been also shown to exert an autocrine effect on themselves, which may ultimately influence the stem cell biology and future. Therefore, this study currently focuses on examining the effects of the cytokines and growth factors that are secreted by BM-MSCs in response to hypoxia and TNF- α, that then work to induce neovascularization and myocardial preservation.

1.4.6.1 Neovascularization

An important biological process that is greatly influenced by paracrine activity is neovascularization. In vitro, MSCs stimulated with specific cytokines and or growth factors differentiate and acquire an endothelial cell phenotype and further continue to secrete proangiogenic factors. Molecular effectors that induce neovascularization include: nitric oxide,

VEGF, HGF and more (Gnecchi et al.,2008). Vessel enlargement, maturation, and the synthesis of the extracellular matrix are highly induced by these factors. BM-MSC secreted proangiogenic factors may play a more important role in collateral remodeling and in increasing the capillary density in ischemic tissue. An upregulation in angiogenic factors (VEGF, FGF, Angiopoietin 1 (ANG-1), HGF, IGF-1, PDGF) is noticed in hypoxia-induced BM-MSCs (Choi, Kurtz, & Stamm, 2011). These factors may aid in the recruitment of endothelial progenitor cells, while pretreatment with hypoxia conditioned medium promoted proliferation and migration of endothelial cells and vascular smooth muscle cells to the site of cardiac injury (Choi, Kurtz, & Stamm, 2011). Figure 9 depicts the three mechanisms that adult stem cells are involved in to promote postnatal neovascularization. "The first is referred to as postnatal vasculogenesis, which consists in the assembly of new blood vessels by fusion and differentiation of endothelial precursor cells originating from the bone marrow. The second mechanism is angiogenesis and consists of the sprouting of new vessels from pre-existing vessels. The third mechanism is collateral enlargement and muscularization, namely arteriogenesis. The release of proangiogenic and proarteriogenic factors by transplanted stem cells positively influences neovascularization in a paracrine fashion" (Gnecchi et al, 2008;Timmers et al., 2011) These mechanisms play a critical role in establishing the vasculature that maybe required to initiate cardiac repair.

Figure 9. The Stem Cell Derived Mechanisms that Drive Neovascularization

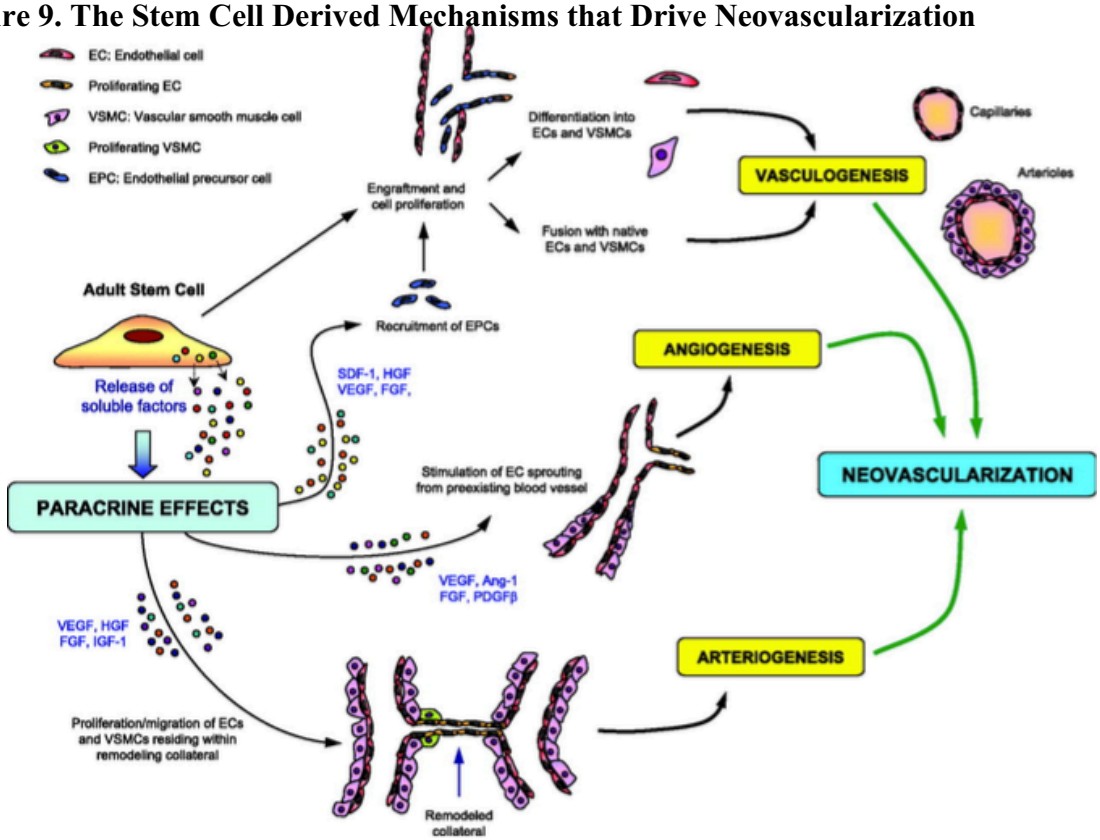


Fig 9. Illustrates the three mechanisms of neovascularization: 1) vasculogenesis, 2) angiogenesis, and 3) arteriogenesis (Gnecchi et al, 2008). "Reprinted from Circulation Research, 103/11, Gnecchi, Zhang, Ni, Dzau, Paracrine Mechanisms in Adult Stem Cell Signaling and Therapy, 1210-1212., (2008), with permission from Wolters Kluwer Health, Inc. http://dx.doi.org/10.1161/CIRCRESAHA.108.176826."

1.4.6.2 The Regeneration of Cardiomyocytes

Adult stem cells such as BM-MSCs, are hypothesized to play a major role in driving cardiac regeneration, but the physical mechanism by which BM-MSCs maybe involved in cardiac repair still remains unclear. Adult stem cells injected into the myocardium may potentially lead to cardiac regeneration through transdifferentiation into novel cardiomyocytes. “Cell fusion of stem cells with native cardiomyocytes represents a second possibility but the biological meaning of this event remains unclear. Finally, soluble paracrine factors released by the stem cells may induce activation, migration and differentiation of CSCs and/or enhance proliferation of resident cardiomyocytes” (Gnecchi et al, 2008) (Figure 10). Conversely, transdifferentiation or cell fusion modes of action are still relatively inferior modes of action when justifying the recovery of injured hearts in various studies where intramyocardial BM-MSCs administration was conducted. “The presence and the role of endogenous cardiac regeneration requires further in depth investigation” (Gnecchi et al.,2008; 2012).

Figure 10. The Stem Cell Derived Mechanisms that Drive Cardiac Regeneration

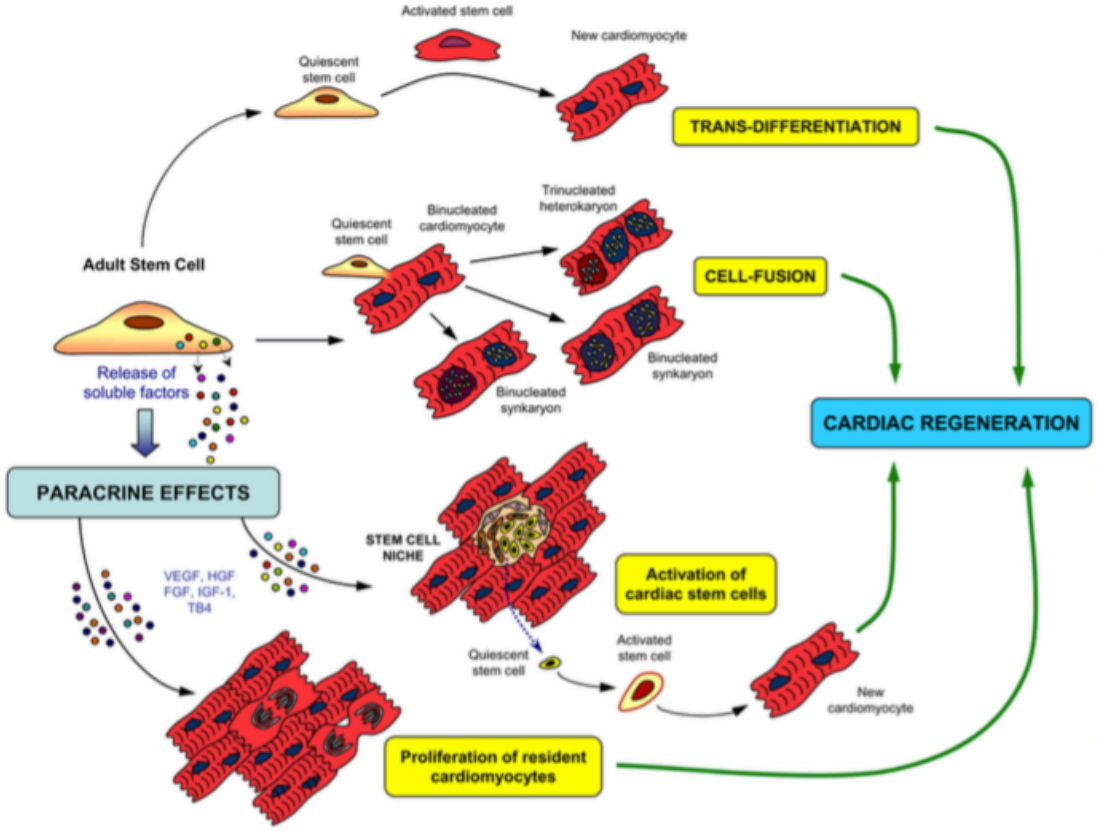


Fig 10. Illustrates the three mechanisms of cardiac regeneration: 1) Transdifferentiation, 2) Cell-Fusion, 3) Paracrine activation of CSC/proliferation of resident CM (Gnecchi et al, 2008). "Reprinted from Circulation Research, 103/11, Gnecchi, Zhang, Ni, Dzau, Paracrine Mechanisms in Adult Stem Cell Signaling and Therapy, 1210-1212., (2008), with permission from Wolters Kluwer Health, Inc. http://dx.doi.org/10.1161/CIRCRESAHA.108.176826."

1.4.7 The Paracrine Effect

Research in the field of regenerative cardiac medicine is rapidly growing, and one of the prime research interests of the field is the paracrine hypothesis of BM-MSC function. The paracrine hypothesis states that paracrine mechanisms facilitated by trophic factors released by BM-MSCs, play a critical role in initiating and mediating cardiac repair mechanisms following stem cell mobilization or exogenous administration of stem cells into the infarcted myocardium (Yao et al, 2015; Gnecchi et al.,2016; Wu et al.,2007) (Figure 11). There is a dynamic and synergistic regulation of BM-MSC secreted cytokines and growth factors that influence vascularization, differentiation (of BM-MSCs & EPCs), mobilization, and more importantly anti-apoptotic cell survival mechanisms. Additionally, paracrine factors secreted by BM-MSCs in response to a myocardial infarction may further influence adjacent cardiomyocytes near the periinfarct region in an attempt to salvage any remaining healthy myocardium. The BM-MSC secreted regulatory paracrine factors are as whole referred to as "MSC Secretome." The secretome is composed of growth factors, cytokines, and chemokines (Rangnath et al, 2012). The composition of a secretome can be greatly influenced by manipulating physiological conditions (normoxia or hypoxia), pre-conditioning BM-MSCs with cytokines or growth factors, or genetically modifying BM-MSCs. "In vitro studies have shown that conditioned medium from hypoxic genetically modified MSCs overexpressing [phosphorylated protein kinase B] -Akt (Akt-MSCs) inhibits apoptosis and can trigger spontaneous contractions of rat cardiomyocytes" (William & Hare, 2011; Gnecchi et al.,2016). Moreover, "when this conditioned medium from Akt-MSCs were injected into post-MI rat hearts, infarct size was reduced and LV function improved" (William & Hare, 2011; Gnecchi et al., 2005;2006). The upregulation of various cytoprotective and proangiogenic growth factors such as VEGF, FGF, HGF, IGF-1 was also witnessed, thus indicating that the genetically modified BM-MSCs exert protection via secretome. In addition, these BM-MSC released factors may have an autocrine response on the biology of the BM-MSCs itself. Thus, the paracrine hypothesis examines the influence of BM-MSC released factors on the microenvironment thereby, modulating stem cell biology, tissue response, wound healing, and neovascularization post MI (Rodrigues et al.,2010). The road to manufacturing a

novel protein therapy with the intent of myocardial protection and neovascularization may be long, but is promising. Harnessing the paracrine effect and developing a therapy that may use a secretome cocktail of essential proteins required for neovascularization, myocardial protection, extracellular remodeling, and cell survival may help the field of cardiac regenerative medicine become dependent upon cell-free therapies to treat myocardial ischemic injuries. BM-MSCs preconditioned with various growth factor and cytokines in physiological conditions that may upregulate beneficial proteins may act synergistically to achieve a therapeutic effect (Ranganath et al, 2012). The administration of a secretome at various time points to repair a myocardial injury instead of stem cells maybe easily translated into clinical research as it bypasses many hurdles associated with prospective cell-based therapies (immune compatibility, tumorigenicity, unspecific differentiation) (Gnecchi et al.,2012). This cell-free secretome approach may aid in pioneering novel "off -the-shelf" stem cell-derived products that treat the effects of ischemia induced myocardial infarctions (Gnecchi et al., 2016).

Figure 11. The MSC Paracrine Effect Triggering Cardiac Repair Mechanisms

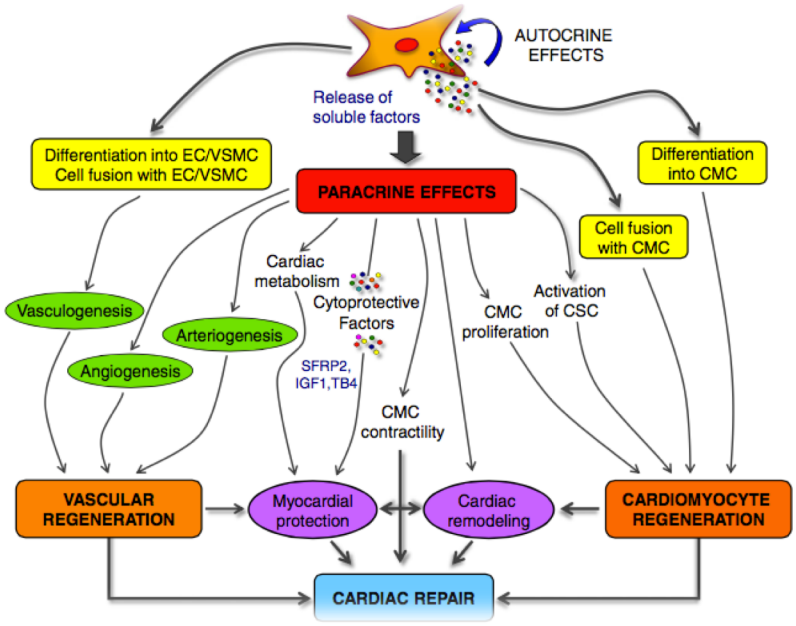


Fig 11. "Mechanisms of mesenchymal stem cell (MSC) action in cardiac regeneration and repair. Cardiomyocyte and vascular regeneration represent the two mechanisms of action originally proposed to explain the reparative effects observed after MSC therapy in ischemic heart disease models. More recently, it has been demonstrated that soluble factors produced and released by

MSC determine beneficial paracrine effects, resulting in myocardial repair" (Gnecchi et al.,2012).

1.5 The Proinflammatory Cytokine Tumour Necrosis Factor □

1.5.1 The Cytokine as a Signaling Molecule Post Myocardial Infarction

Tumour Necrosis Factor-□ (TNF-□), is a proinflammatory intercellular cytokine that is biologically known to be pleiotropic. It is a trimeric polypeptide which has a molecular weight of either 26 kDa or 17-kDa depending on the form. It is highly upregulated in the hostile microenvironment that is created following a MI. TNF-□ exists in one of two forms, as an integral transmembrane precursor protein (Type II) or in a soluble secreted form (Type I) that exists in the cleaved form of the previously mentioned (Ferrari, 1999). TNF-□ is produced within a few minutes by cardiomyocytes, activated monocytes, macrophages, B-cells, T-cells, and fibroblasts as an intrinsic cardiac response to myocardial injury. The cytokine is highly implicated in ischemia induced myocardial damages, and is known to robustly express the soluble form of TNF-□ when ultimately leading to heart failure (Papathanasiou et al.,2015). However, growing evidence suggests that TNF-□ may play an important role in myocardial homeostasis aside from regulating local inflammatory responses (IL-6 & IL-1)(Irwin et al,1999; Feldmen et al., 2000). It is known that time and dosage of TNF-□ is extremely important in influencing the effect it has on the myocardium. Short term, low doses of TNF-□ protect the myocardium, while high doses have been shown to have toxic effects (Tian et al.,2015). Therefore, the net effect of TNF-□ may contribute crucially to both the adverse and beneficial physiological effects in individuals with MI.

1.5.2 Tumour Necrosis Factor Receptors 1 & 2

The biological activities of TNF-a are physiologically mediated through two distinct cell surface receptors, Tumour Necrosis Factor Receptor 1 (TNFR1) also known as p55 and Tumour Necrosis Factor Receptor 2 (TNFR2) also known as (P75) (Kubota et al.,2001; Irwin et al.,1999) (Figure 12). TNFR1 contains a death domain in the cytoplasmic tail, and is known to initiate the caspase cascade which causes cell death via apoptosis (Choisi et al., 2007). Its complex is mainly involved in inflammatory reactions and ventricular remodeling (Tian et al.,2015). TNFR2 is the receptor that is known to be associated with cell survival and differentiation. TNFR2 is most often

present within the cardiomyocytes that are located within the myocardial infarct zone and play a role in inhibiting inflammatory reactions and ventricular remodeling. They also promote cardioprotective and angiogenic mechanisms (Tian et al.,2015). Thus, the interaction between TNF-α and TNFR2 increase the production of angiogenic growth factors that accelerate neovascularization. Furthermore, "downregulated TNFR2s can cause enhancement of TNFR1" (Tian et al.,2015). Lacerda and colleagues found that the secretion of Leptin could be inhibited by soluble TNF-α in vivo thus, reducing the damage caused by MI (Lacerda et al.,2010). Furthermore, "studies found that MSCs expressing TNFR2 could reduce inflammatory reactions and improve cardiac functions in individuals post-MI" (Tian et al.,2015; Bao et al.,2008). Although the pathophysiological effects of TNF-α have been extensively researched, the downstream signalling mechanisms have not been elucidated (Higuchi et al, 2006).

Figure 12. The TNFR1 and TNFR2 Signalling Cascade

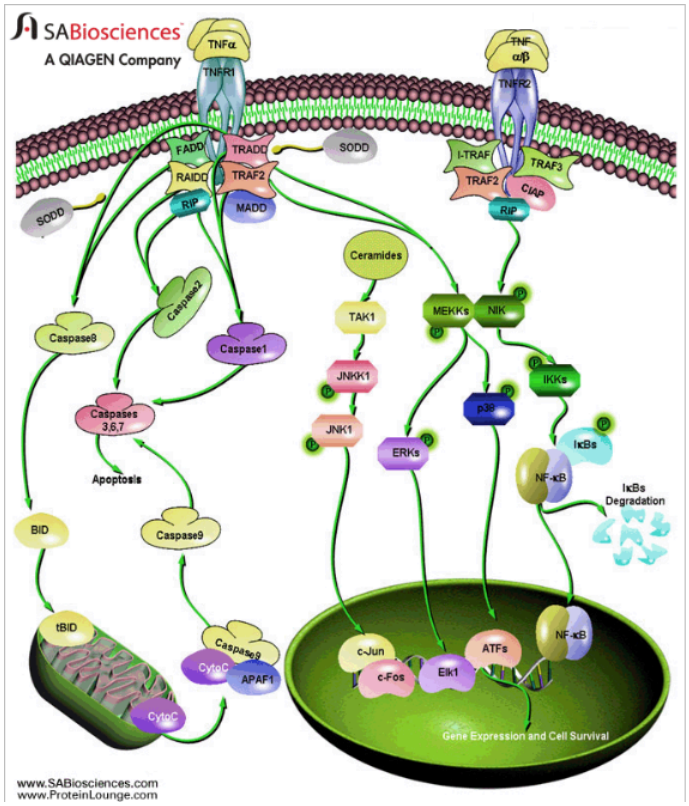


Fig 12. TNF Signaling (Adpated from http://www.sabiosciences.com/pathway.php?sn=TNF_Signaling) with the permission of QIAGEN, Germany copyright).

1.5.3 Cell Survival Pathways

In a regular healthy heart, TNF-α is rarely secreted, and the concentration of the cytokine in the heart is low (Schulz&Heusch, 2009). Conversely, in ischemia induced infarcted hearts, TNF-

 is known to be expressed up to 50 folds more in the infarcted tissue regions while up to 15 folds in the non-infarcted areas in rodent models (Nian et al.,2004). Furthermore, preconditioning the myocardium with TNF-, has been shown to reduce infarct size. However, the mechanism of action remains unclear (Schulz & Heusch, 2009). One pathway that is highly associated with TNF- signaling is the ubiquitous nuclear factor-κB transcription factor. This transcription factor is well known in initiating immune and inflammatory responses, cell survival mechanisms, and programmed cell-death (Higuchi et al.,2006). Another signaling pathway that has been associated with TNF- and it's contribution to cell survival by inhibiting apoptosis, is the Protein Kinase B pathway (Akt) (Higuchi et al.,2006). Although these two signaling pathways have been proposed as the main routes that TNF- actively participates through, extensive research is still needed to unveil the primary mechanism. Figure 13 looks at the proposed signaling mechanisms of TNF- and their ultimate roles.

Figure 13. The Proposed Mechanism of TNF--TNFR1/TNFR2 Signaling Pathways

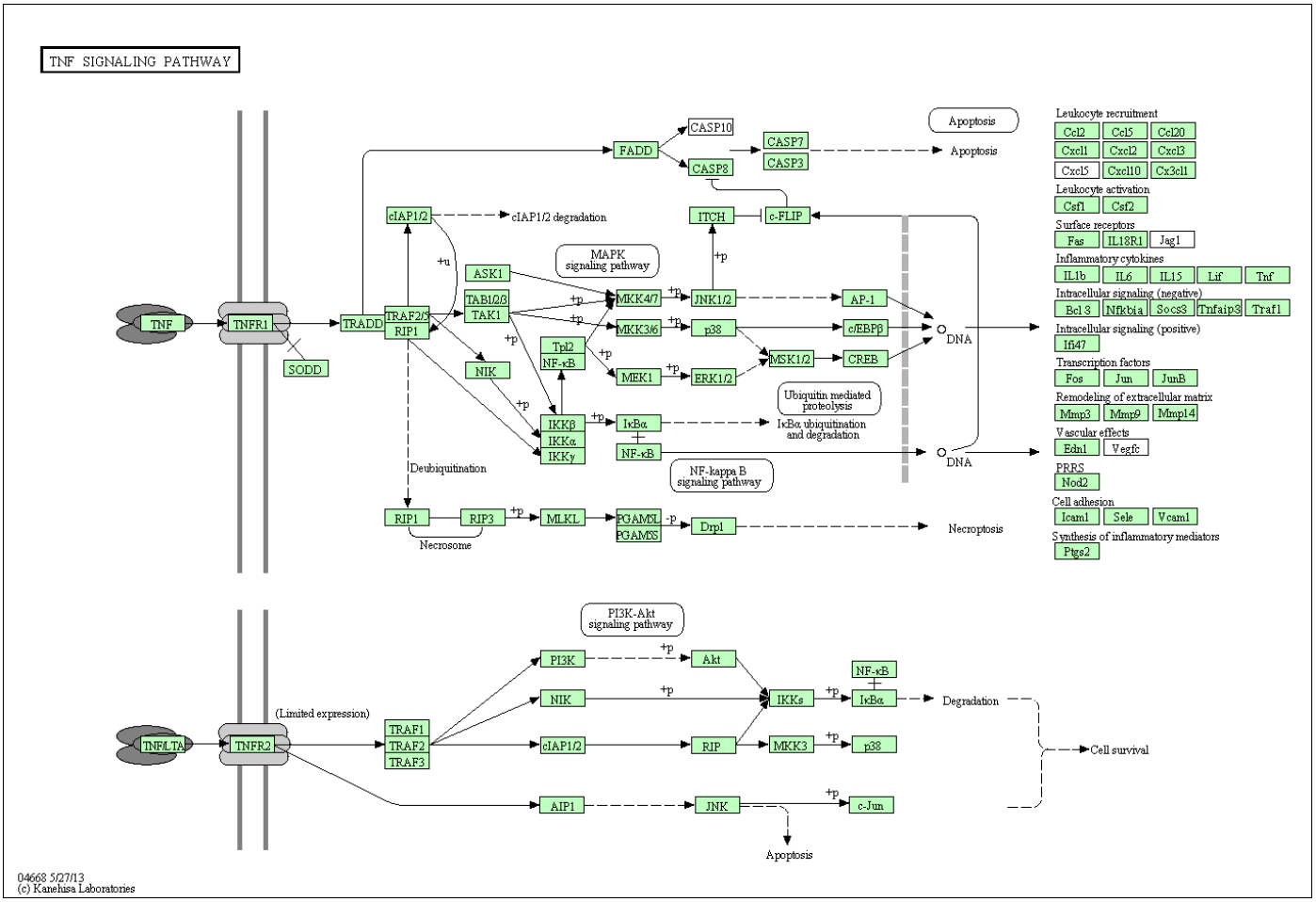


Fig 13. Depicts the TNF signaling pathway in Rattus norvegicus (rat) (Adapted from http://www.genome.jp/kegg-bin/show_pathway?org_name=rno&mapno=04668&mapscale=&show_description=hide)

1.5.4 TNF-α induced Protein Network

TNF-α is a primary proinflammatory cytokine that plays a major role in triggering the production of supplementary proinflammatory cytokines along with growth factors and transcription factors that are critical in starting the cardiac repair process post-MI. As TNF-α is secreted at the early stages of an ischemic infarction, it works in multiple ways to not only activate an immune response, but also prompts the cytokine amplification process in attempt to clean up and repair the damaged area. Thus, this leads to the secretion of both beneficial and unfavourable proteins by MSCs that are required for cardiac healing and repair to occur. Numerous proteins can be found within the secretome network of MSCs stimulated with TNF-α using the online protein association network analysis software called STRING (http://string-db.org). A selective biased approach was undertaken in order to narrow down on the most immediate proteins that were secreted by MSCs when stimulated by TNF-α. Figure 14 looks at the protein network that is closely associated with TNF-α in the norvegicus rattus species. Table 5 lists the key signature molecules that are secreted by MSCs when stimulated with TNF-α and how they affect the activity of MSCs at the site of infarct. Key signature molecules that are closely examined in this current study include: VEGF-1, FGF-2, FGF-7, Ang-1, Ang-2, TGF-B1, and Myogenin. Activities of each protein ranges from regulating migration to cardiac muscle differentiation. Deciphering the protein network that comprises the TNF-α and hypoxia stimulated rBM-MSCs secretion network can not only aid in unveiling the the paracrine effect of TNF-α in cardiac repair, but may also aid in understanding and developing the most optimal cocktail of proteins which can work as a cytokine-dependent secretome therapy for prospective myocardial preservation.

Figure 14. TNF-α Protein Network That Regulates Essential Factors for Cardiac Repair

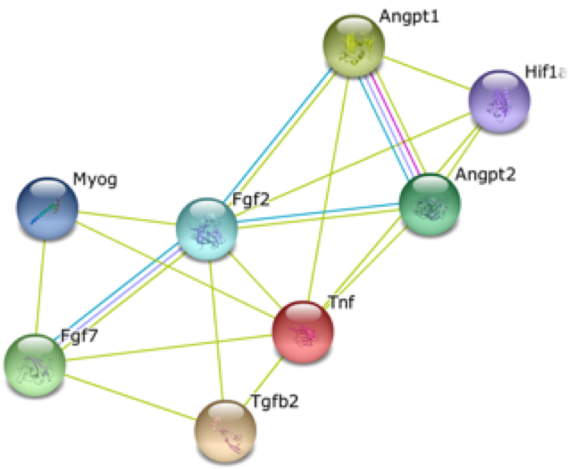


Fig 14. This functional protein network analysis was conducted at string-db.org on TNF-α in order to determine the most closely linked and functional proteins in the TNF network. A selective biased approach was taken when determining what proteins to examine.

Table 5. Expected Roles of MSCs and their Protein Secretions Under the Paracrine Effect

Expected Roles of MSCs Under the Paracrine Effect	**Key Signature Molecule**	**Reference**
MSC & EC migration, self-renewal, & adhesion	FGF-2, VEGF-1	(Eiselleova, et al.,2009) (Zisa et al.,2009)
Neovascularization/ angiogenesis (Blood vessel stabilization)	VEGF-1, FGF-2, FGF-7, Ang-1, Ang-2	(Hausenloy & Yellon, 2009) (Sandhu et al.,2004) (Gnecchi et al.,2008)
Cardiac remodeling (Cardiac hypertrophy)	Ang-2	(Thurston & Daly et al.,2012)
Cell proliferation (Survival)	FGF-7, FGF-2, VEGF-1	(Eiselleova, et al.,2009) (Zisa et al.,2009)
Cardiac hypertrophy, EC proliferation, & anti-inflammatory role	TGF Beta 1	(Hausenloy & Yellon, 2009). (Dobaczewski et al.,2011)
Cardiac muscle cell differentiation & formation	Myogenin	(Gnecchi et al.,2008)

Table 5. Listed above are the key signature molecules which are secreted by BM-MSCs in response to a MI when stimulated with TNF-α. The roles of each protein and how they affect BM-MSCs in the process of cardiac repair have also been indicated.

OBJECTIVES

IHD and heart failure remain as leading causes of morbidity and mortality worldwide. Due to the limited intrinsic regenerative capacity of mammalian cardiomyocytes, "the myocardial loss after a myocardial infarct is irreversible and is subsequently replaced by non-contractile scar tissue, often initiating heart failure" (Song et al.,2010). Unfortunately, the conventional therapies that are available focus solely on treating the symptoms that accompany MI and on re-establishing blood flow to the deprived tissues of the heart. Although pharmacological therapies are the major approaches of treatment, the discovery of BM-MSCs' potential to improve cardiac function has lead to the up rise in research which aims at understanding the mechanism by which stem cell mediated cardiac repair occurs (William & Hare, 2011). Increasing evidence suggests that paracrine factors such as cytokines, growth factors, and chemokines are secreted by BM-MSCs into the injured tissues. These paracrine factors may play a crucial role in providing myocardial protection and repair by directly modifying the healing process in the heart (Rodrigues et al.,2010). TNF-α is a proinflammatory cytokine that is produced in response to an ischemia induced infarction and is known to play an active role in triggering a cascade of molecular responses essential for inflammation and cardiac wound healing. Nonetheless, the definitive role it plays in cardiac repair and protection has not been elucidated yet. Preconditioning with TNF-α and hypoxia may beneficially impact, change, and enhance the composition of the native BM-MSCs secreted factors and its' therapeutic effects, while modifying the composition towards exclusively treating ischemic myocardial injuries (Schulz & Heusch, 2009). Furthermore, it may also interact with the BM-MSCs and alter its morphology. Neovascularization, mobilization of BM-MSCs/progenitor cells, cell survival, differentiation, suppression of inflammation, and fibrotic processes at the area of infarct may be potentially driven and upregulated by these preconditioned BM-MSCs secreted paracrine factors (Burchfield & Dimmeler, 2008).

Therefore, the primary objective of the current study is to examine and understand the role of TNF-α and hypoxia on rBM-MSCs and its' secretions in vitro, while investigating the potential angiogenic, migratory, cardioprotective, and reparative effects of the TNF-α Hypoxia-induced secretome following an ischemia induced MI. We further sought to supplement the in vitro study with an in vivo rodent model study which examined if the TNF-α Hypoxia - induced secretome prompted cardiac repair post-MI by preventing adverse damage in cardiomyocytes adjacent to the infarcted region, while promoting angiogenesis.

CHAPTER 2: MATERIALS & METHODS

2.1 Cells and Reagents

H9C2 cardiomyoblasts (rCB) from the Rattus norvegicus species, were commercially obtained from the ATCC (ATCC, CRL-1446). Rat Bone Marrow Mesenchymal Stem Cells (rBM-MSCs) were commercially purchased from LONZA (Lonza, PT-2505). rBM-MSCs were obtained from female Fischer 344 rats. The rBM-MSCs were withdrawn from the femur and tibia of these rats. Primary antibodies that were used throughout the experiments include: Beta-Actin - mouse monoclonal (Santa Cruz, SC-4778), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) - goat polyclonal (Santa Cruz, 20357), Ang-1- goat polyclonal (Santa Cruz, SC-6319), Ang-2- goat polyclonal (Santa Cruz, SC-7017), FGF-2- goat polyclonal (Santa Cruz, SC-1360), FGF-7- rabbit polyclonal (Santa Cruz, SC-7882), Myogenin-rabbit polyclonal (Santa Cruz, SC-576), TGF-B 1,2,3- rabbit polyclonal (Santa Cruz, SC-7892), VEGF-1- rabbit polyclonal, (Santa Cruz, SC-507), Anti-TNFR1- rabbit polyclonal (Abcam,ab19139) , and Anti-TNFR2- rabbit polyclonal (Abacam, ab15563). Secondary antibodies that were used include: Alexa Fluor 488 - donkey-anti-rabbit (Santa Cruz, SC-2314), donkey-anti-goat (Santa Cruz, SC-2020), and goat-anti-rabbit (Santa Cruz, SC-2004).

2.2 Cell Culture

rCBs were initially cultured in T75 flasks (Greiner bio-one, 82050-856) with Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% antibiotics - penicillin and streptomycin (PS). The medium was changed every 48 hours and the cells were split when the flask reached 80% confluence. The same cell culture method was used for rBM-MSCs. Throughout the experiments, rCBs with passage numbers between three and eight were used, while rBM-MSCs with passage numbers between three and seven were used. Cell culture flasks were kept in a humidified incubator with 5% CO_2 and 21% O_2 at 37°C.

2.2.1 Differentiating Rat Cardiomyoblasts to Rat Cardiomyocyte like Cells

Confluent rCB flasks were washed once with phosphate buffered saline (PBS) and were trypsinized with 0.25% trypsin/EDTA to detach the cells from the surface of the flask. The non-adherent cells were then observed under bright field microscopy (Olympus CKX41). Trypsin was inactivated using 10% FBS 1% PS DMEM. The medium was then collected in 15 ml tubes

(Sarstedt, 62.554.205) centrifuged at 1200 rpm for 5 minutes and was then resuspended in 1ml of 10% FBS 1% PS DMEM. 0.4% Tryphan Blue (1:1) was added to 100ul of cells and then were counted using a hemocytometer (PlanoPtik, Neubauer Improved) under a bright field microscope. Two-six well plates with 150 000 rCBs/well were then plated and kept in the humidified incubator with 5% CO_2 and 21% O_2 at 37°C for 24 hours. They were further differentiated into cardiomyocyte like cells (rCM) with four treatments of 10nM of retinoic acid (RA) (Sigma, R2625), previously diluted in dimethyl sulfoxide (DMSO), on alternate days over the duration of a week in 1% FBS 1% PS DMEM.

Figure 15. Differentiating rCBs to rCMs using Retinoic Acid

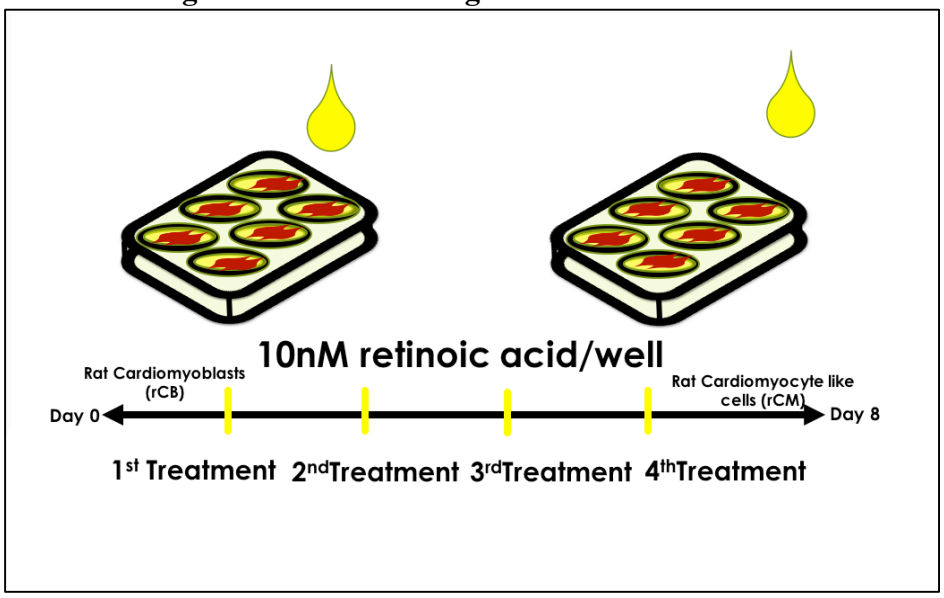


Fig 15. rCBs were treated with 10nM of RA every other day across a span of 8 days.

2.2.2 Rat Cardiomyocyte Conditioned Medium - The Microenvironment of a MI

Following the four RA treatments, differentiated rCMs were then induced to hypoxic conditions in a hypoxic chamber (Mitsubishi Gas Chemical CO., INC., 50-25; AnaeroPack System 10-01) for 16 hours overnight in serum deprived 1% PS DMEM. The next day, the remaining supernatant/rat cardiomyocyte conditioned medium (rCCM) were collected (Branco, 2015).

2.2.3 Rat Bone Marrow Mesenchymal Stem Cell Culture

The rBM-MSCs were trypsinized and counted in the same manner as the rCM. Subsequently, six-six well plates with 100,000 rBM-MSCs/well were plated and left for 24 hours in the incubator with 5% CO_2 and 21% O_2 at 37°C. The plating of the rBM-MSCs was done one day prior to acquiring the rCCM.

2.2.4 Deriving the Secretome

Following the 24-hour incubation period, which allowed the cells to adhere to the plate, the rBM-MSCs were treated with rCCM, normoxia (5% CO_2, 21% O_2, 37 °C), hypoxia (0-0.1% O_2), and/or TNF-α in serum free 1% PS DMEM in various combinations. There were a total of eight treatment conditions. Every three wells of the six-six well plates were allocated to each experimental condition. The experimental groups included four treatments in both normoxic or hypoxic conditions. Within normoxia or hypoxia, the negative controls were the untreated rBM-MSCs in serum free 1% PS DMEM. The experimental controls were rBM-MSCs treated with 2ml of rCCM and rBM-MSCs treated with 100ng/well of TNF-α (Peprotech, 400-14) in serum free 1% PS DMEM. The experimental conditions of interest were rBM-MSCs induced with 100ng/well of TNF-α and 2ml of rCCM. Three of the six well plates with four different treatments were placed in normoxia, while the other three plates were placed in a hypoxic chamber and placed back into the incubator for 16 hours. After 16 hours, the resulting secretomes were collected (6ml per treatment) and stored at -80°C. Subsequently, they were than lyophilized (Labconco, Freezone 2.5) at the Analytical Chemistry Laboratory in the Department of Chemical Engineering at McGill University. Once lyophilized, each sample was reconstituted with 500 ul of serum free 1% PS DMEM (1/12 of the initial secretome volume) and stored at -20°C until use.

2.3 TNFR1 & TNFR2 Receptor Expression

2.3.1 Immunofluorescence

rBM-MSCs ($2x10^5$ cells) were seeded onto sterile 24mm X 40mm microscopic glass cover slips (Fisher Scientific) coated with polylysine (Sigma, P-6407) in 6 cm cell culture dishes. The negative control had cells treated with no serum 1% PS DMEM and was kept in normoxia. The experimental control cells were treated with rCCM with no serum 1% PS DMEM while the treatment condition had cells treated with both rCCM with TNF-α. These two conditions were kept in a hypoxia environment. Cells were left for 16 hours in their respective conditions. After removing the supernatant and washing the cover slips twice with PBS, the cells were fixed with 3.7% paraformaldehyde (PFA) for 3-4 minutes. The cover slips were then rewashed thrice with PBS and blocked with 3% bovine serum albumin (BSA) at room temperature for 1 hour. After blocking, the BSA was removed and the slides were incubated overnight with either the anti-rabbit TNFR1 or TNFR2 primary antibody (1:1000 and 1:200) at 4°C. The slips were next incubated

with the donkey-anti-rabbit labelled secondary antibody Alexa Fluor 488 green (1:250) for 2 hours at room temperature and then were washed five times with PBS. Nuclei of the cells were stained using 4',6-diamidino-2-phenylindole (DAPI) for 4 minutes. Cover slips were then observed under the fluorescence microscope (Biorad, ZOE Fluorescent Cell Imager 1450031) at a 20X magnification.

Figure 16. Deriving the Secretome In Vitro

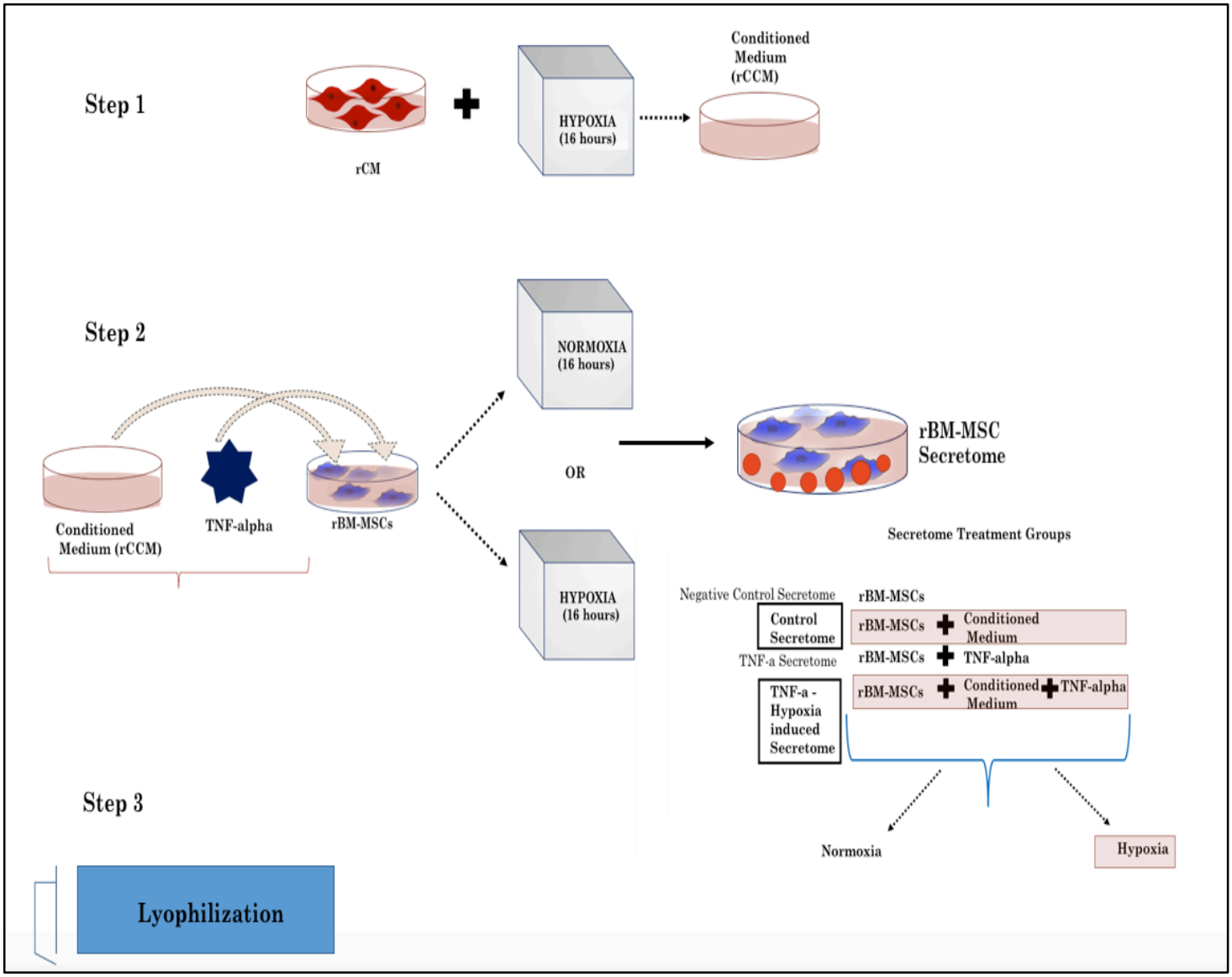


Fig 16. Illustrates the process by which BM-MSC secretome is derived. Step 1 shows how the rCCM is made. Step 2 looks at the various combinations (rCCM, hypoxia/normoxia, TNF-□) of

treatments required to make the respective treatment and control secretomes. Step 3 shows that the secretome is lyophilized.

2.3.2. Receptor Protein Western Blots

rBM-MSCs (150 000 cells/well) treated with various combinations of hypoxia, normoxia, rCCM, and/or TNF-α for 16 hours were first lysed using the radio immunoprecipitation (RIPA) buffer (Tris-HCl pH 7.4, 50 mM, Triton X-100 1%, Sodium deoxycholate 1%, SDS 0.1%, EDTA, 1 mM, NaCl 150 mM at pH 7.0) with NaF and protease inhibitors (Sigma, P8340) for 30 minutes on ice on a shaker. The lysates were then scrapped off into ependorff tubes and centrifuged at 13 000rpm for 5 minutes. The lysate was then denatured using the Laemmli 2X loading buffer (Biorad, 161-0737) and was boiled at 100°C for 4 minutes.

The cell lysate supernatants (30ul per well) and Colour Burst protein ladder - 10ul/well (Sigma, C1992) were loaded onto and run on 12% SDS-polyacrylamide gels. The proteins on the SDS-gels were then transferred onto Immunoblot PVDF membranes (Bio-Rad, 162-0177), visualized with ponceau staining for assurance of effective transfer (Sigma, P7170-1L), and blocked with 5% skimmed milk in TBST (Carnations) for 1 hour at room temperature. All antibody solutions were made with 2.5% BSA and primary antibodies had 2% sodium azide.

After blocking, the membranes were then incubated in the TNFR1 or TNFR2 primary antibody (1:1000 and 1:200) overnight at 4°C on a shaker. The membranes were then washed five times with TBS Tween solution (TBST) and again incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:3000) for 1 hour at room temperature. They were rewashed five times for 5 minutes with TBST and were incubated with the chemiluminescent reagents (Western Lightening Plus-ECL, Perkin Elmer Inc, NEL10001EA). The membranes were finally developed and protein expression was visualized using the Image Quantum LAS 4000 (GE Healthcare Life Sciences, 28-9558-10). As housekeeping proteins, both Beta-Actin and GAPDH were used to assess the quality of the expression of the examined protein on each membrane and to standardize the protein expression to the housekeeping proteins. For TNFR1, the positive control that was used was the A549 cell lysate (Abcam, ab7910) and for TNFR2 the positive control was Jurkat cell lysate (Abcam, ab7899).

2.4 Rat Bone Marrow Mesenchymal Stem Cell Protein Western Blots

rBM-MSCs (150 000 cells/well) treated with various combinations of hypoxia, normoxia, rCCM, and/or TNF-□ for 16 hours were initially lysed using the radio immunoprecipitation (RIPA) buffer with NaF and protease inhibitors for 30 minutes on ice on a shaker. The lysates were then scrapped off into ependorff tubes and centrifuged at 13 000rpm for 5 minutes. The lysate was then denatured using the laemmli 2X buffer and was boiled at 100°C for 4 minutes.

The cell lysate supernatants (30ul per well) and protein ladder (10ul/well) were loaded onto and run on 12% SDS-polyacrylamide gels. The proteins on the SDS-gels were then transferred onto Immunoblot PVDF membranes, visualized with ponceau staining for assurance of effective transfer, and blocked with 5% skimmed milk in TBST for 1 hour at room temperature. All antibody solutions were made with 2.5% BSA and primary antibodies had 2% sodium azide.

After blocking, the membranes were then incubated overnight at 4°C on a shaker with a series of primary antibodies (1:200) that were used to test the presence of various proteins that are involved in cardiac repair, cell differentiation, and vasculogenesis. The membranes were then washed five times with TBST and again incubated with HRP-conjugated anti-mouse, anti-goat, or anti-rabbit secondary antibodies (1:3000) for 1 hour at room temperature. Five 5 minute washes with TBST were conducted followed by the incubation with the chemiluminescent reagents. The membranes were then developed and imaged using the Image Quantum LAS 4000. As housekeeping proteins Beta-Actin was used to assess the quality of the expression of the examined protein on each membrane and to standardize the protein expression to the housekeeping proteins. Numerous western blots were run and expression of: Ang-1, Ang-2,FGF-2, FGF-7, Myogenin, TGF-β, and VEGF-1 were examined.

2.5 Characterizing the Secretome

2.5.1 Cell Trans-Well Migration Assay

500 ul of 10% FBS 1% PS DMEM was added to 15 wells in a 24 well plate. 8.0 um pore size PET track-etched membrane cell culture filter inserts were placed in each of the 15 wells (Becton Dickinson, 353097). 25 000 rBM-MSCs were seeded onto each filter. 200ul of 10% FBS 1% PS DMEM was added to the filters on top after seeding the cells. The 24 well plate was then placed in the incubator with 5% CO_2 21% O_2 at 37°C for 6 hours for the cells to proliferate and attach to the filters.

There were five treatment groups and the experiment was conducted in triplicates. The negative control was the serum free 1%PS DMEM, the positive control was SDF-1 (15ng/ml) (Abcam, ab9798), the experimental controls included 1) serum free 1%PS DMEM and TNF-α (100ng/well) and 2) the control secretome, while the treatment of interest had serum free 1%PS DMEM with the TNF-α hypoxia induced secretome.

A secretome solution was created for both the control and TNF-α hypoxia induced secretomes. 84 ul of the selected secretome was added to 216ul of serum free 1%PS DMEM to get a total of 300ul. 400 ul of serum free 1%PS DMEM was added to 12 wells and 500 ul of serum free 1%PS DMEM was added to 3 wells as a negative control in a new 24 well plate. 100 ul of the each secretome solution was then added to each of the 3 respective treatment wells accordingly (3 wells/secretome treatment). 100ng/well of TNF-α was added to three wells, and 15ng/ml of SDF-1 was added to the last three wells containing serum free DMEM. Following the adhesion of the cells on the filter from the old 24 well plate, the medium in the filters were removed and the filters were then transferred to the new 24 well plate with the treatments. 200 ul of 1%PS DMEM with no serum was then added on top of the filters containing the treatments. The 24 plate was placed in a hypoxic chamber for 16 hours overnight in a 5% $C0_2$ 21% O_2 37 °C humidified incubator.

The following day, the plate was removed from the hypoxic chamber and each of the filters were then emptied and washed with a two-step 1X PBS wash. The cells were then fixed on the filter with 600 ul (400 ul below filter and 200 ul above the filter) of 3.7% formaldehyde for two minutes. A two-step PBS wash was then repeated. Next, 600ul (400 ul below filter and 200 ul above the filter) of 100% methanol was added to each well for 20 minutes to permeabilize the cells. A two-step PBS wash was then repeated. The nuclei were then stained using DAPI. A two-step PBS wash was once again repeated. A cotton swab was then used to disrupt the cells on the filters. The filters were then cut and placed on a slide making sure the bottom of the filter was facing up. Mounting solution was used to mount the filter on the slide. Fluorescence microscopy was then performed to count the number of cells that had migrated onto the filter in response to the secretome (20X magnification). Cells were counted manually and using Image J.

2.5.2 Cell Survival - Annexin V Apoptosis Assay

Six-six well plates were seeded with 150 000 rCMs/well. The following day, various combinations of treatments were administered to the rCMs in triplicates. Treatments included: 1)

A calibration control for the fluorescent-activated cells sorting (FACS) machine where the cells were treated with fresh serum-free 1%PS DMEM, 2) a negative control with serum-free 1%PS DMEM treated in hypoxia for 32 hours, 3) a cytokine treatment control – cells treated with 100ng/well of the TNF-α cytokine in fresh serum-free 1%PS DMEM, 4) an experimental control – cells treated with the control secretome (1X), and 5) an experimental treatment group – cells treated with the TNF-α hypoxia induced secretome (1X). All treatment conditions were then incubated in normoxia or hypoxia separately for 16 hours overnight in a 5% $C0_2$ 21% O_2 37 °C humidified incubator. In this experiment, apoptosis was induced by 16 hours of hypoxia. After the incubation period, the cells were washed with 1X PBS, harvested, and once again rewashed with cold 1X PBS. The washed cells were then centrifuged at 500xg for five minutes (MBI Lab Equipment, Sigma 2-16P) and the supernatants were discarded. The Annexin V Live Dead Apoptosis Kit was used to conduct the lived/dead assay (Thermo Fisher Scientific, V13245). The cells were then resuspended in 106 ul of a 1X annexin-binding buffer, alexafluor 488, and propidum iodide (PI) master mix and transferred into 5ml polystyrene round-bottom FACS tubes (BD Falcon, 352235). Cells from the FACS machine calibration control group were either left unstained, stained with alexa fluor 488 only, stained with PI only, or were stained with alexa fluor 488 and PI. The stained and unstained cells were then left to incubate for 15 minutes at room temperature. Following the incubation period, 200 ul of the 1X annexin binding buffer was added to each of the groups, mixed gently, and placed on ice. The cells were then analyzed using flow cytometry measuring the fluorescence emission at 530 nm and 575nm using 488 nm excitation. FACS was conducted at the Immunophenotyping Platform located at the Research Institute of MUHC - Glen Site using the BD FACSCanto II (Becton Dickinson). Results were analyzed using the FlowJO software.

2.6 LAD Ligations in the Lewis Rat Model

Three of the four sets (three per set) of Lewis rats (Charles River Laboratories, Canada) underwent a left antero-lateral thoracotomy and were surgically induced with an ischemic myocardial infarction using the left anterior descending artery (LAD) ligation procedure with a 7.0 polypropylene suture (Ethicon, 8703H). Group 1 did not undergo a myocardial infarction, and remained as the SHAM baseline group for the study. All other rats were anesthetized (Anesthesia ventilation adaptation kit, 341-389) using 5% isoflurane (PPC Inc. Canada) before the surgical

procedure, intubated, and were ventilated at 80–90 breaths/min using the rodent ventilator (Harvard Apparatus Co) pre-op. Group 2 had induced infarcted rats which were not treated with any treatment and remained as the negative control for the study. The ischemia induced myocardial infraction was visually identified by the transparency of the tissue. A 28 gauge, 1/2 –inch insulin syringe (Becton Dickinson, 329461) was used to administer 100µL (28ul of secretome +72ul of serum free DMEM) of a concentrated supernatant, in both Group 3 - control secretome or Group 4 - TNF- α hypoxia induced secretome, at five distinct locations (20µL per site) at the peri-infarct zone of the infarction. Rats were closed up with a 3-0 Vicryl coated suture (Ethicon, J423H) and post-operatively they were administered carprofen (5 mg/kg) subcutaneously and were left to recover safely in their respective chambers. Protocol number FACC2015-7692 was closely followed and all MUHC Animal Facility policies, Canadian Council on Animal Care regulations, and McGill's ethics committee's mandates were respected during the surgical procedures and caretaking period of time. All surgeries were performed under sterile conditions.

2.7 Echocardiography

Echocardiograms were conducted at three week intervals post-op to assess cardiac function using the MicroMaxx ultrasound system (SonoSite Inc., P05324-01) which was attached to a linear probe 13-6 MHz transducer (Sonosite, HFL38xp). Echoes were taken using the parasternal short-axis view (M-mode tracing) which, allowed us to view the area between the anterior and posterior walls of the left ventricle at the level of the papillary muscles of the mitral valve. We then assessed the diameters between the anterior and posterior left ventricular walls at end systole and end diastole (LVESD and LVEDD). Rats were under 3%-5% isoflurane during the examination. Left Ventricular Fractional Shortening was calculated using the formula (LVFS%)= (LVEDD-LVESD)/ LVEDD X 100.

Figure 17. In Vivo Experimental Design

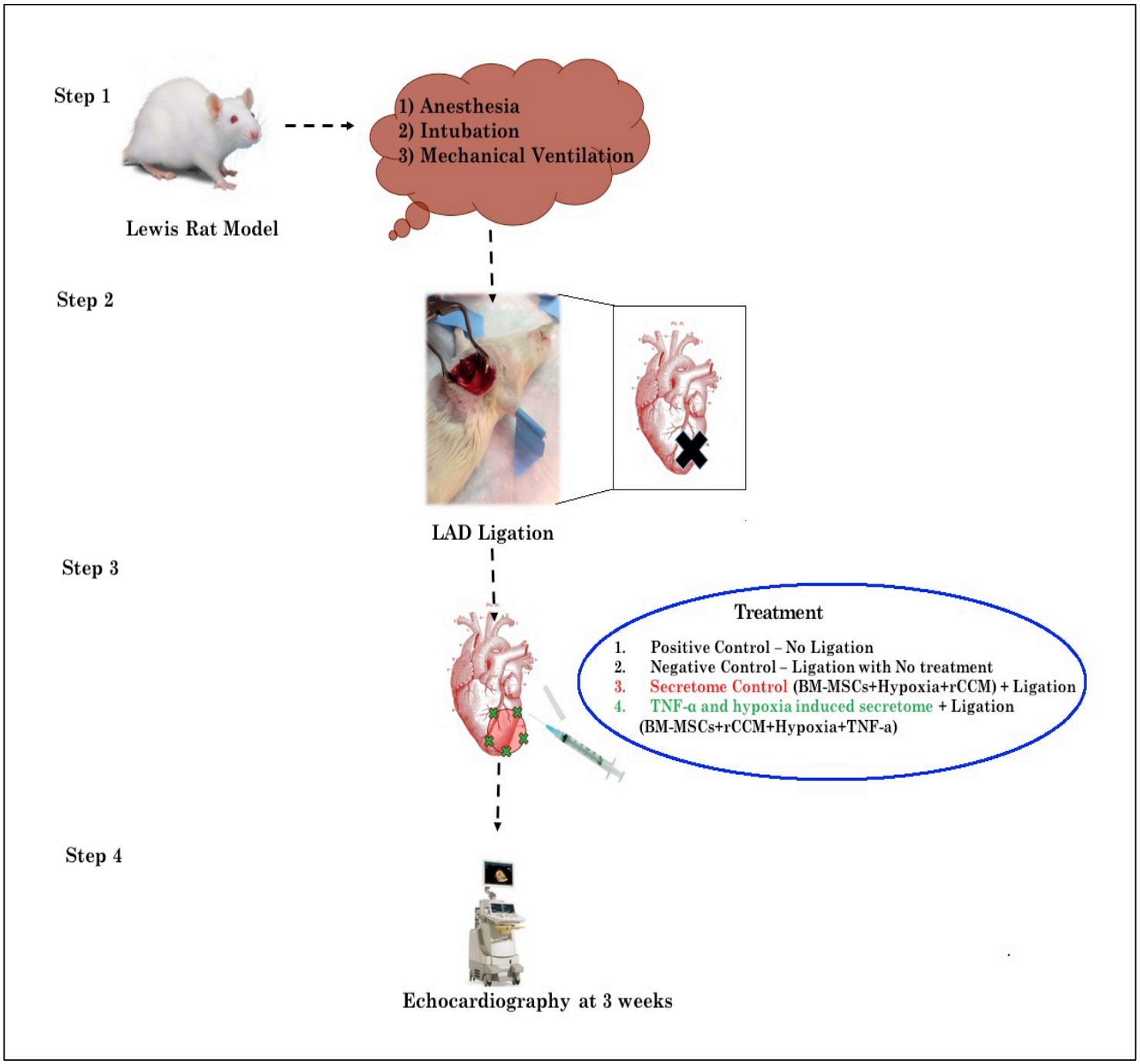


Fig.17 Visually depicts the steps required to conduct the in-vivo LAD ligation (in Lewis rat Models) essential to induce MI. Subsequent to the MI, no treatment or treatment secretomes are administered and are followed up with an echocardiography at 3 weeks' post-treatment/infarction.

2.8 Masson's Trichrome Staining

Following a three-week interval, the Lewis rats were sacrificed and the infarcted hearts were extracted in accordance to MUHC Animal Facility policies. Sagittal and transversally cut samples of the infarcted region of the heart were fixed in 10% buffered formalin phosphate. They were embedded in wax, cut at a thickness of 6um/section by a microtome (Spencer, 820), placed on a 24mm X 50mm microscope slide, and left overnight to dry in the incubator. Citrate solvent clearing agent was then used to remove the paraffin wax. Slides were then rehydrated using a series of decreasing concentrations of ethanol starting from 100%, 95%, 80%, to 75%. Tissue samples were then fixed overnight at room temperature in Boulin's fixative (Ricca Chemical Company,1120-32). The following day, slides were run under tap water in order to remove any residues. Slides were then immersed in Weigert's hemotoxylin and washed down with distilled water. Samples were stained with Biebrich Scarlet-Acid Fuchsin Solution (Sigma, HT-151), washed down with distilled water, immersed in phosphomolybidic-phosphotungstic acid (Sigma, HT-153-250 & HT-152-250), stained again with Aniline Blue (Sigma, B8563), and introduced to 1% acetic acid. Finally, the slides were washed again with distilled water, submerged in increasingly concentrated ethanol solutions ranging from 95% to 100%, and cleaned using citrate solution. The slides were dried and mounted with permount. All trichrome stained slides were scanned using the hp scannerjet (Hewlett-Packard, 3750c) at 1200 dpi. The Masson's Trichrome Staining protocol was attained from Montreal Children's' Hospital- Pathology. The size of infarct was measured using Image J and was calculated using the formula: % Infarction Size = {Total area of tissue section/Area of infarction} x 100 (Takagawa et al., 2007).

2.9 Immunohistochemistry

Further examination of the tissue samples were conducted via immunohistochemistry (IHC) using the Ki67 (Abcam, ab16667) and CD31 (Novus Bio, NB100-2284) markers. IHC was performed by the Histology Core Facility at the Goodman Cancer Research Centre. Images were taken at 4X, 10X, 20X, and 40X at the sites of infarct, transition areas, and area of infarctions, in both control secretome treated rats and TNF-□ hypoxia induced secretome treated rats using the Olympus BX40 bright field microscope.

2.10 Statistical Analysis

All quantitative measurements and statistical analysis were conducted using the Image J and GraphPad Prism 6.0 software. All experiments were performed in triplicates with samples run in triplicates. Raw results were statistically analyzed using either the Student's unpaired t-test (t-test) or a One-way analysis of variance (ANOVA) test, where values are expressed as means ± standard deviation (SD) were compared against each other (Tuckey Test) to determine if there was a significant difference. P-values considered $p<0.05$ were considered to be statistically different and significant. Throughout the results and graphs, * visually denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$. Error bars on graphs represent the standard deviation.

CHAPTER 3: RESULTS

3.1 Expression of TNFR1 & TNFR2 Receptors in rBM-MSCs

Immunofluorescence and western blot analysis were conducted in order to determine the presence and expression of the TNFR1(52 kDa) and TNFR2 (55 kDa) receptors on the surface of rBM-MSCs. Both immunofluorescence and western blots confirmed the presence of the TNFR1 and TNFR2 receptors on the surface of rBM-MSCs at the protein level. As shown in Figure 18A, immunofluorescence confirmed the expression of the TNFR1 and TNFR2 receptors in three different conditions which included, rBM-MSCs cultured in normoxic conditions for 16 hours, rBM-MSCs treated with rCCM in a hypoxic environment for 16 hours, and lastly rBM-MSCs treated with rCCM and TNF-α in hypoxia for 16 hours. All groups were supplemented with no serum 1% PS DMEM in addition to their respective treatments. The expression of the cell surface receptors is represented in green (Alexa Fluor 488), while the blue (DAPI) represents the counterstained nuclei of the cells. Figure 18B depicts the western blot protein analysis of the expression of the TNFR1 or TNFR2 proteins in rBM- MSC lysates previously treated in various conditions which include: no treatment (no serum 1% PS DMEM) in normoxia, treatments with rCCM in normoxia, TNF-α in normoxia, rCCM and TNF-α in normoxia, rCCM in hypoxia, and rCCM and TNF-α in hypoxia. Although the expression of TNFR2 on rBM-MSCs was reconfirmed in the western blot analysis within all treatment groups including the positive control for TNFR2 (Jurkat cells), the expression of TNFR1 was not detectable in the western blots in any examined conditions. However, the positive control – A549 cells- for TNFR1 had mildly expressed the TNFR1 protein. Beta-Actin and GAPDH proteins were used as loading controls.

A

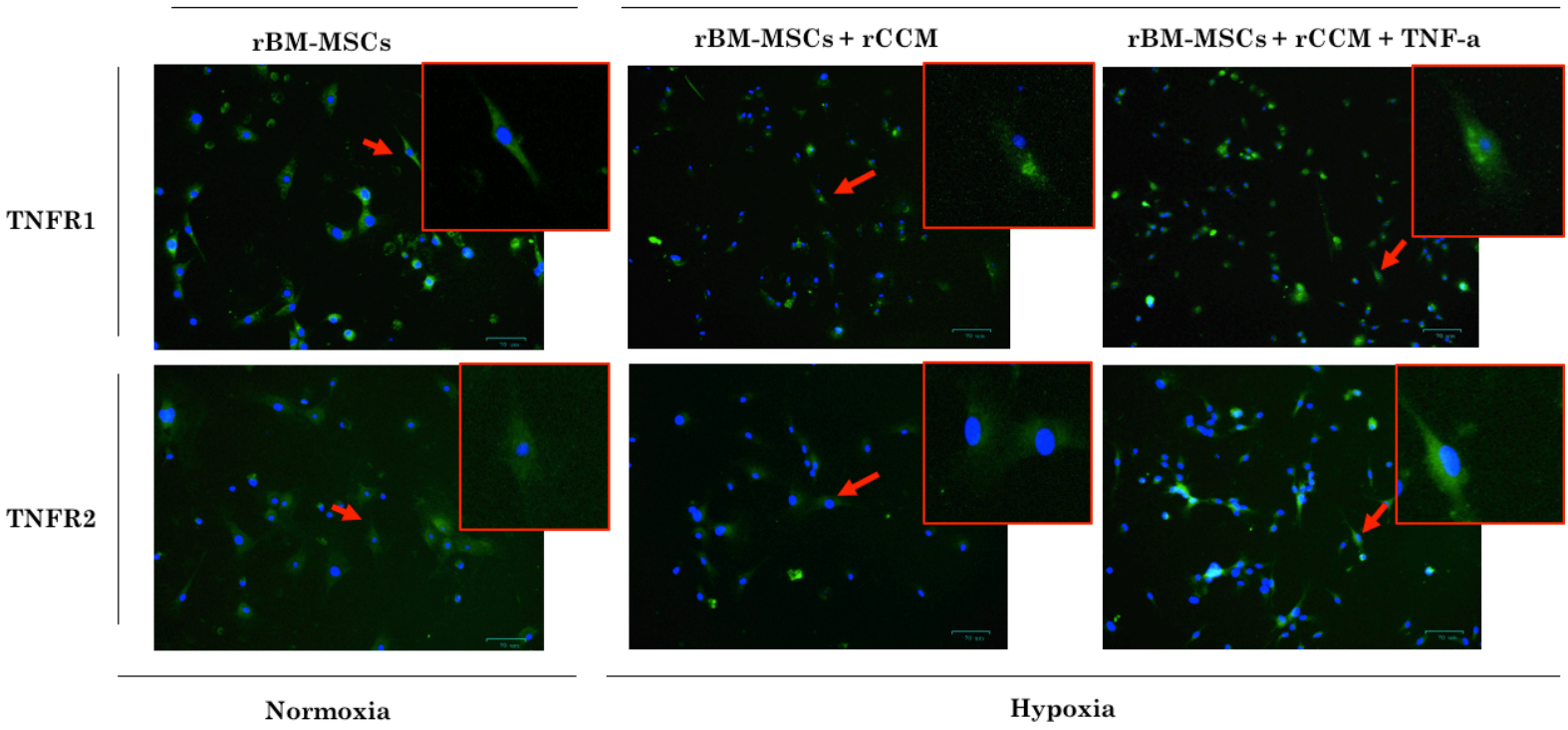


Fig 18A. Immunofluorescence was used to identify the Tumor Necrosis Factor Receptors - TNFR1 & TNFR2. Both receptors are stained with Alexa Fluor 488 and are expressed in green on the surface of Rat Bone Marrow Mesenchymal Stem Cells (rBMSCs) in the mentioned conditions. The nuclei are stained with DAPI and is visualized in blue. The scale bar is 70 μm and the images were taken using the 20X objective.

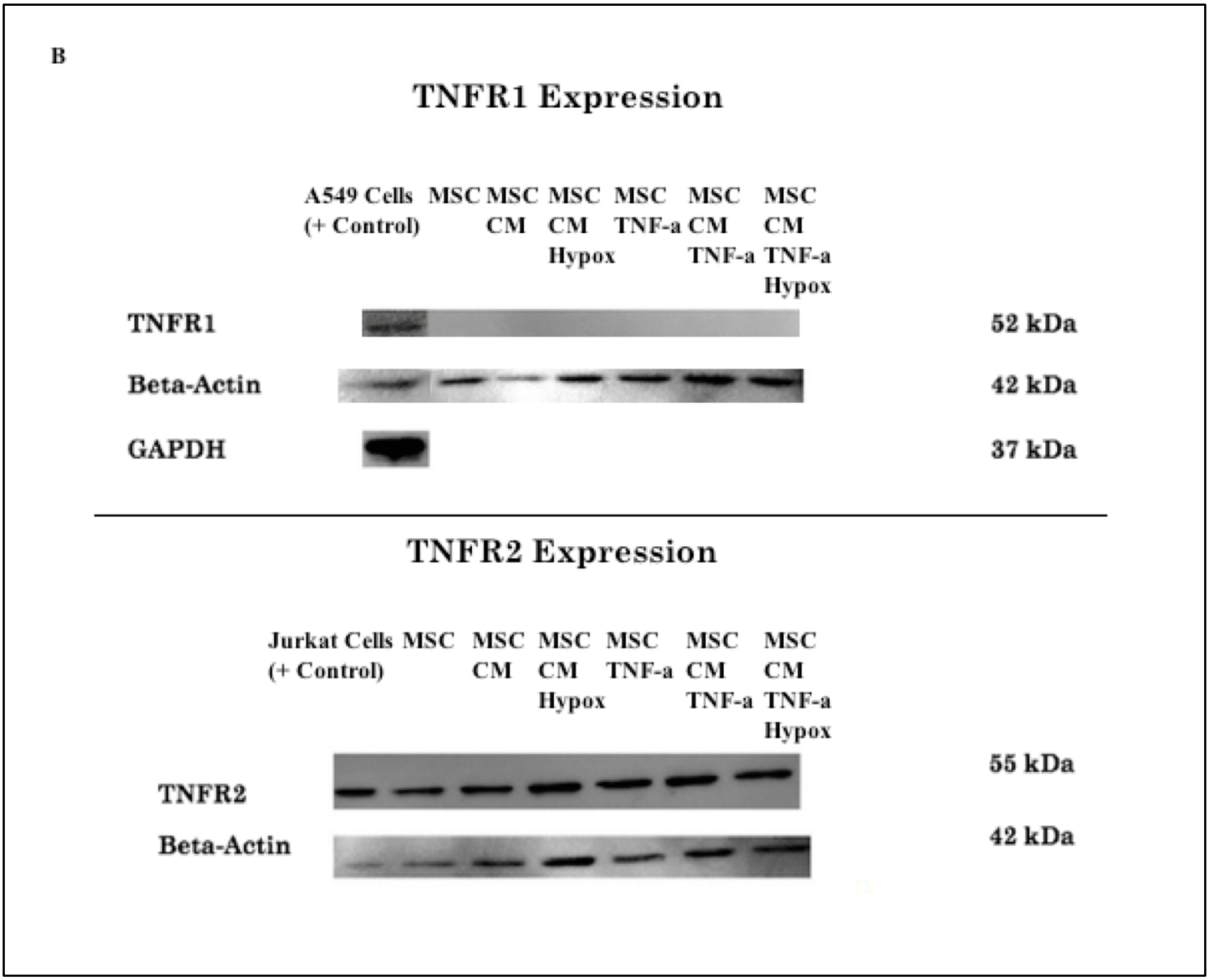
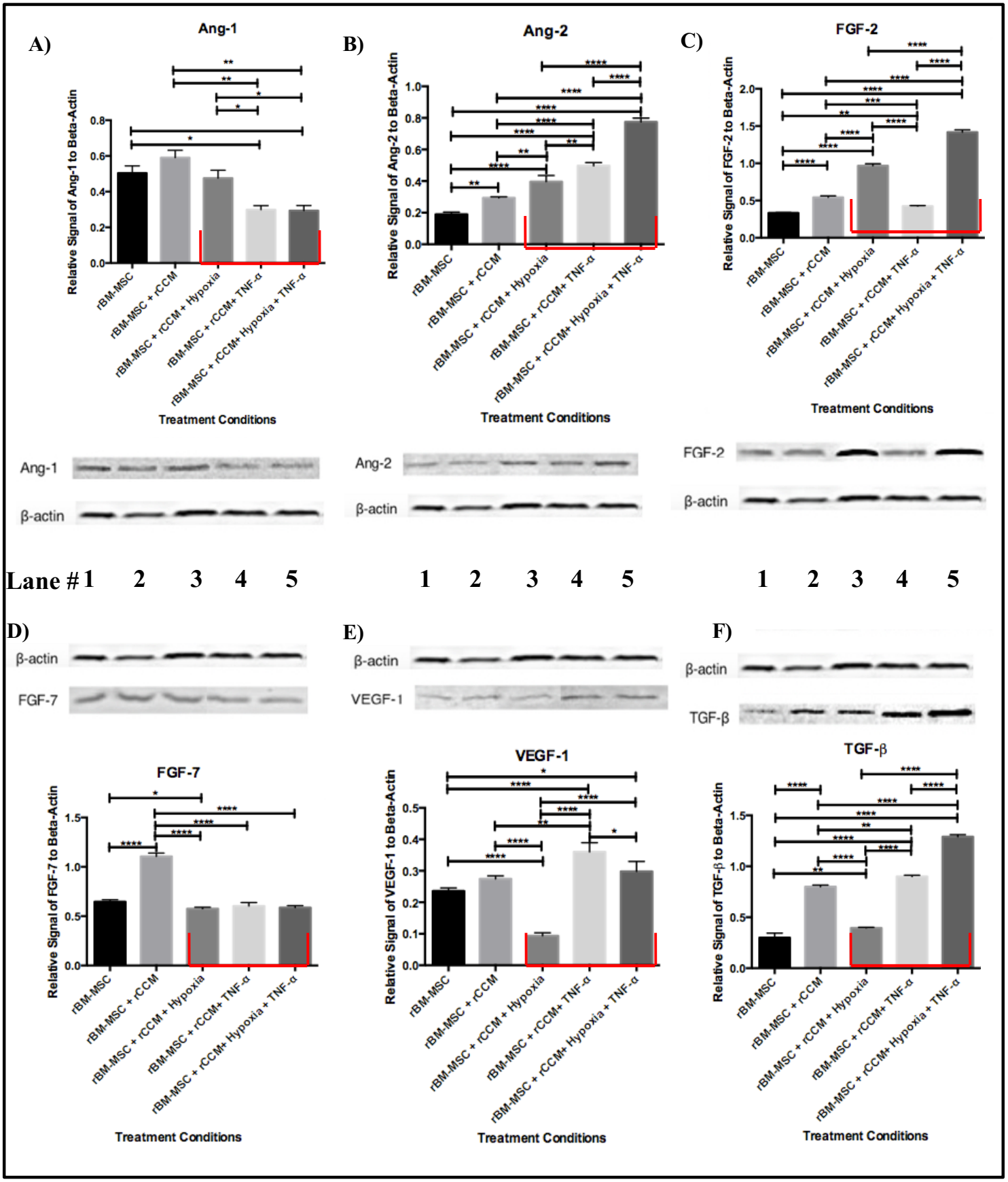


Fig 18B. Western Blot Expression of TNFR1 (top) was not detected in any of the examined treated/untreated rBM-MSCs groups, however TNFR1 was detected in the A549 cells, which acted as the positive control. Both GAPDH and Beta Actin were used as loading controls for TNFR1. TNFR2 (bottom) in rBM-MSCs were expressed at high levels throughout all treated/untreated groups. TNFR2 was also expressed in Jurkat cells (positive control). Beta-actin was used as the loading control for TNFR2. Equal amounts of lysate were run in each condition. *MSC= rBM-MSC, *CM=rCCM & *Hypox=Hypoxia.

3.2 rBM-MSCs Express Markers of Neovascularization, Migration, & Cell-Survival

Western blot analysis on rBM-MSCs treated under five different conditions were performed in order to determine the role of rBM-MSCs in cardiac repair and to identify the secreted proteins that may aid in initiating processes such as neovascularization, suppression of inflammation, mobilization of EPCs and BM-MSCs, and pro-survival mechanisms. Using a biased approach following the string analysis on TNF-α, several protein factors including Ang-1, Ang-2, FGF-2, FGF-7, VEGF-1, TGF-B, and myogenin were selected and examined for within rBM-MSCs lysates. rBM-MSCs were initially treated with one of five conditions for 16 hours: no serum 1% PS DMEM (negative control); rCCM only; rCCM and hypoxia; rCCM and TNF-α (experimental controls); or rCCM, hypoxia, and TNF-α. All signals of protein expression were standardized to beta-actin, which acted as the loading control. In Figure 19A, the expression of Ang-1 was decreased by 1.62 folds (p<0.05) in the rCCM, hypoxia, and TNF-α group and by 1.59 folds (p<0.05) in the rCCM and TNF-α group when compared to the control group treated with only the rCCM and hypoxia. In Figure 19B, the expression of Ang-2 increases significantly amongst the groups. rBM-MSCs treated with rCCM, hypoxia, and TNF-α have an additive effect on the increased expression of Ang-2 (1.95 and 1.56 fold increase - p<0.0001) in comparison to the rCCM and hypoxia group or rCCM and TNF-α group. Figure 19C indicates that treatment with rCCM and hypoxia increases FGF-2 expression in rBM-MSCs by 1.79 folds (p<0.0001) in comparison to treatment with only rCCM. There is a 1.47 fold (p<0.0001) increase in FGF-2 in the cells treated with rCCM, hypoxia, and TNF-α compared to the control with just rCCM and hypoxia. Furthermore, the addition of TNF-α to the rCCM decreases the expression of FGF-2 by 1.28 folds (p<0.001) when compared to just the rCCM. Figure 19D shows that the addition of hypoxia alone, TNF-α alone, or the combination of both to the cells with rCCM, decreases the relative signal of FGF-7 by 1.92 folds, 1.83 folds, and 1.88 folds (p<0.0001). Figure 19E looks at the expression of VEGF-1 which shows that there is a 3.18 fold increase (p<0.0001) in VEGF-1 in rCCM, hypoxia, and TNF-α treated cells, while there is a 3.85 fold increase (p<0.0001) in the rCCM and TNF-α treated group compared to the rCCM and hypoxia group. Figure 19F indicates that there is a 3.27 fold (p<0.0001) increase in expression of TGF-β in rBM-MSCs treated with rCCM, hypoxia, and TNF-α in contrast to the rCCM and hypoxia group. There is also a 1.13 (p<0.01) fold increase in TGF-β in the rCCM and TNF-α group when compared to just the rCCM

treated cells. When treated with both hypoxia and TNF-α, in the presence of hypoxia, there is a 1.43 fold increase (p<0.0010) in the expression of TFG-β compared to the rCCM and TNF-α treated cell expression. Finally, Figure 19G indicates that myogenin expression is decreased by 1.46 folds (p<0.0001) and increased by 1.69 folds (p<0.0001) in cells treated with rCCM and hypoxia or rCCM and TNF-α when compared to cells with only rCCM treated cells. Additionally, there is a 4.55 fold (p<0.0001) and 1.84 fold (p<0.0001) increase in myogenin in rCCM, hypoxia, and TNF-α treated cells when compared to rCCM and hypoxia or rCCM and TNF-α treated cells. Thus, a critical trend that can be seen in Figure 19 is that Ang-2, FGF-2, VEGF-1, TGF- β, and myogenin expression all show an increase in protein expression (by ~ $\geq$1.5 folds) in rBM-MSCs treated with rCCM, hypoxia, and TNF-α when compared to rBM-MSCs treated with just rCCM and hypoxia (control). However, there is a decrease in Ang-2 expression and no change in FGF-7 expression between the rBM-MSCs treated with rCCM, hypoxia, and TNF-α and the rBM-MSCs treated with just rCCM and hypoxia (control). Overall, the expression of these crucial proteins may play a significant role in inducing cardiac repair through numerous beneficial mechanisms of action.


A)
Ang-1
Relative Signal of Ang-1 to Beta-Actin
rBM-MSC
rBM-MSC + rCCM
rBM-MSC + rCCM + Hypoxia
rBM-MSC + rCCM+ TNF-α
rBM-MSC + rCCM+ Hypoxia + TNF-α
Treatment Conditions
B)
Ang-2
Relative Signal of Ang-2 to Beta-Actin
Treatment Conditions
C)
FGF-2
Relative Signal of FGF-2 to Beta-Actin
Treatment Conditions
Ang-1
β-actin
Ang-2
β-actin
FGF-2
β-actin
Lane # 1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
D)
β-actin
FGF-7
FGF-7
Relative Signal of FGF-7 to Beta-Actin
Treatment Conditions
E)
β-actin
VEGF-1
VEGF-1
Relative Signal of VEGF-1 to Beta-Actin
Treatment Conditions
F)
β-actin
TGF-β
TGF-β
Relative Signal of TGF-β to Beta-Actin
Treatment Conditions

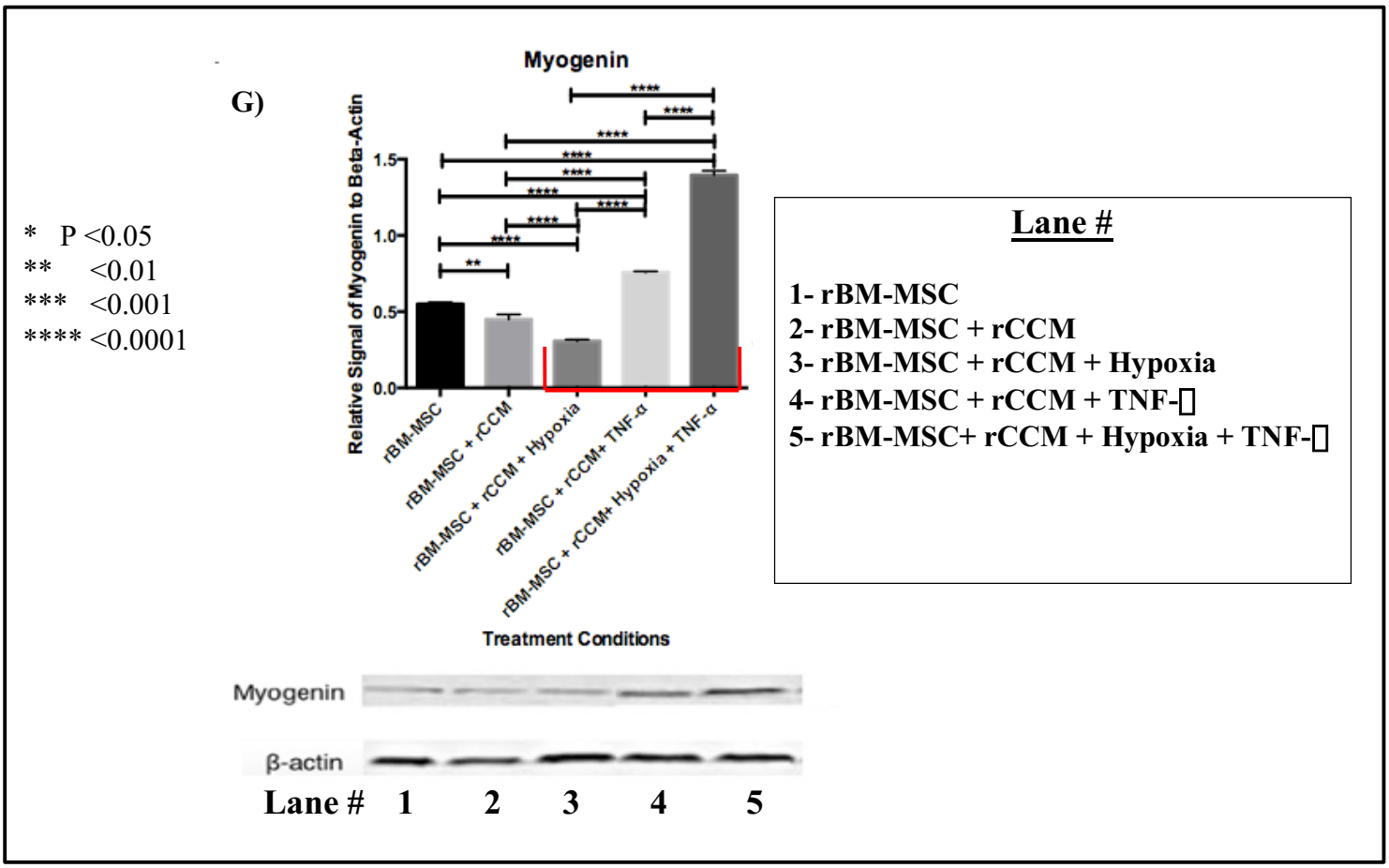


Fig 19. *rBM-MSCs Express Markers of Neovascularization, Migration, & Cell-Survival.* Western blot analysis on rBM-MSCs were performed to determine the role of rBM-MSCs in cardiac repair and to identify the secreted proteins that aid in initiating processes such as neovascularization, suppression of inflammation, mobilization of EPCs and BM-MSCs, and pro-survival mechanisms. Ang-1, Ang-2, FGF-2, FGF-7, VEGF-1, TGF-β, and myogenin were selected and examined for within rBM-MSCs lysates. rBM-MSCs were initially treated with one of five conditions for 16 hours: no serum 1% PS DMEM (negative control); rCCM only; rCCM and hypoxia; rCCM and TNF-α (experimental controls); or rCCM, hypoxia, and TNF-α. Relative signals of protein to beta actin were examined between the control and treatment groups. Beta-actin, which acted as the loading control. *We specifically focus on the groups treated with rCCM and hypoxia (control) or rCCM, hypoxia, and TNF-α for the expression of the proteins to determine the effects of TNF-α.* All signals of protein expression were standardized to beta-actin, which acted as the loading control. rBM-MSCs with no treatment acted as the negative control. A one-way analysis of

variance (ANOVA) test was conducted, where protein expression values from each group were expressed as means ± SD and compared against each other (Tuckey Test), to determine if there was a significant difference between the expression of the tested proteins between the groups. A critical trend that can be seen in Figure 19 is that Ang-2, FGF-2, VEGF-1, TGF- β, and myogenin expression all show an increase in protein expression (by ~ $\geq$1.5 folds) in rBM-MSCs treated with rCCM, hypoxia, and TNF-α when compared to rBM-MSCs treated with just rCCM and hypoxia (control). Values considered $p<0.05$ were considered to be statistically different and significant. Throughout the graphs, * visually denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$. Error bars on graphs represent the standard deviation.

3.3 The TNF-α Hypoxia-Induced Secretome Induces Migration of rBM-MSCs

Secretome have many potential beneficial roles that may contribute to cardiac repair, but the mechanisms by which they do remain a mystery. The trans-well cell migration assay was conducted in order to determine if the TNF-α Hypoxia-Induced secretome is a chemo-attractant, which can aid in the mobilization of rBM-MSCs. Stromal Derived Factor-1 (SDF-1) is an established potent mobilization factor and was used as a positive control in this experiment. Aside from the positive control, two control groups and two experimental secretome groups were assessed. No serum 1%PS DMEM was used across all groups. Control and treatment groups that were assessed included, **1)** DMEM in hypoxia (negative control), **2)** 50ng/ml of the TNF-α cytokine (experimental control), **3)** control secretome, and **4)** TNF-α Hypoxia-Induced secretome. The treated rBM-MSCs were kept under hypoxia for 16 hours before inspection. Figure 20A shows the blue DAPI stained nuclei of the rBM-MSCs that migrated across the filter per group through fluorescent imaging. Results suggest that the TNF-α Hypoxia-Induced secretome induces rBM-MSCs mobilization. Figure 20B is a graph that quantifies the cell migration per mm^2 per treatment group. It shows that rBM-MSCs do not migrate towards no serum 1% PS DMEM, and that only a few cells migrate towards the TNF-α cytokine alone (~108.7 cells). It further indicates that the control secretome induces around ~ 459.3 cells to migrate, whereas the TNF-α Hypoxia-Induced secretome induces ~ 888.7 rBM-MSCs to migrate per mm^2. Ultimately, approximately 429.3$\pm$103.9 ($p<0.05$) more rBM-MSCs migrated towards the TNF-α Hypoxia-Induced secretome in contrast to the control secretome. The number of cells that migrated towards the positive control (~1182 cells) was similar to the TNF-α Hypoxia-Induced secretome. Furthermore, the TNF-α

Hypoxia-Induced secretome increased the rBM-MSC mobilization by ~ 780±103.9 rBM-MSCs (p<0.001) when compared to the treatment group with the TNF-α cytokine only.

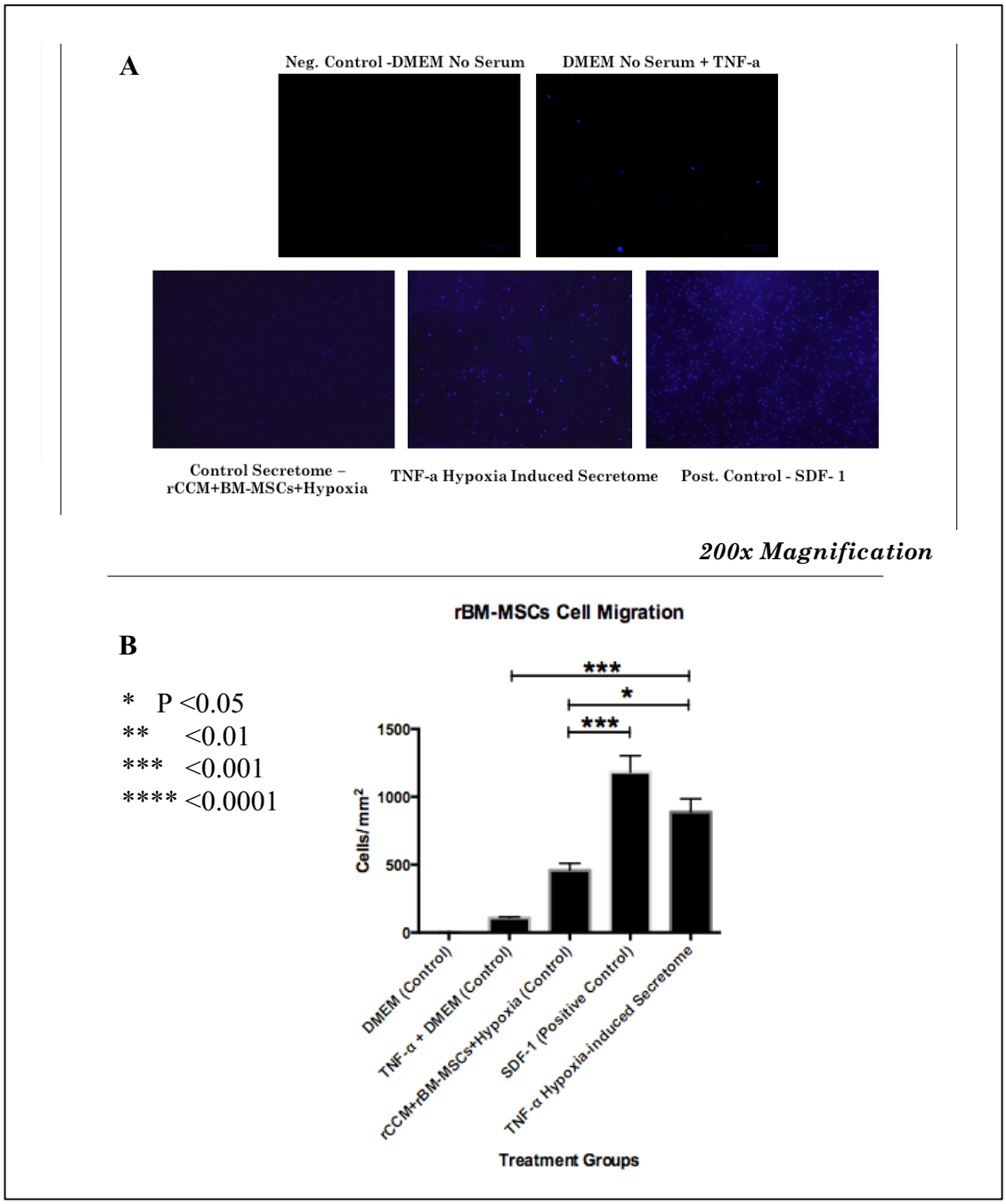
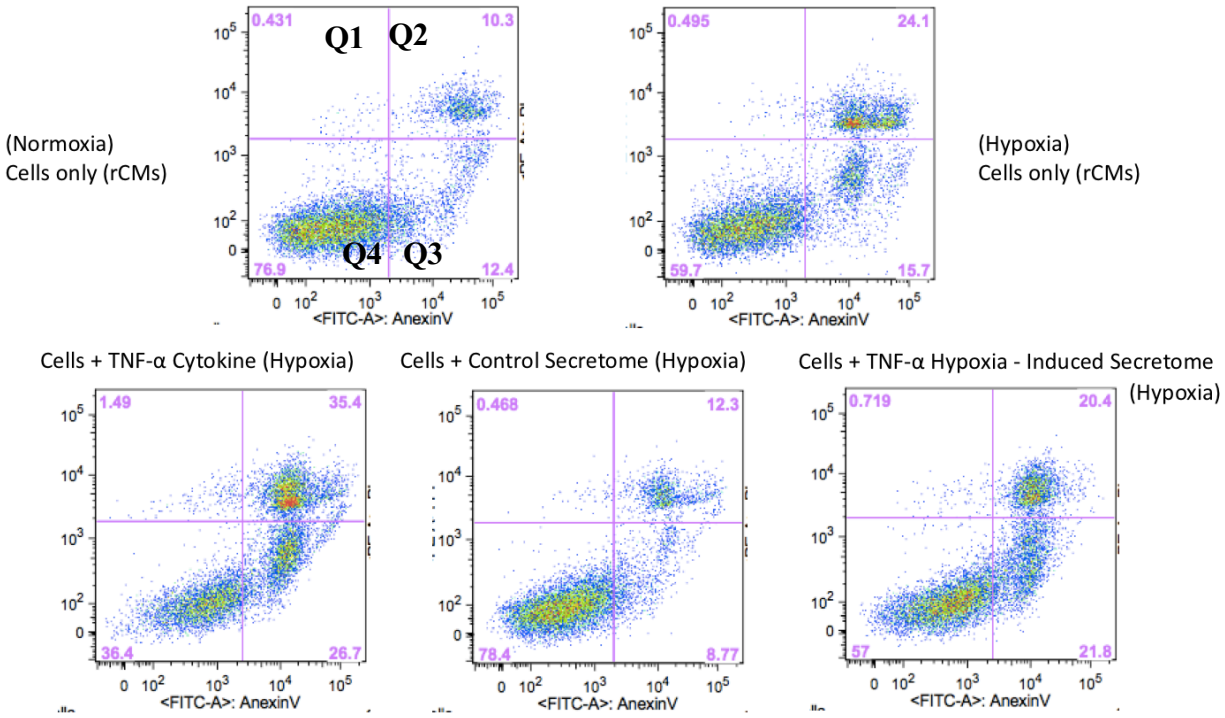


Fig 20. *The TNF-a Hypoxia Induced Secretome Induces Migration of rBM-MSCs.* (A) At the 20X objective, rBM-MSC migration was assessed through fluorescent imaging towards four different conditions. SDF-1 was the positive control. DAPI (blue) shows the nuclei of the cells. (B) graphically represents the number of cells that migrated per mm^2 for each group.

3.4 The TNF-α Hypoxia-Induced Secretome May Downregulate Apoptosis Under Hypoxia

An Annexin V apoptosis assay was conducted in order to determine if the TNF-α Hypoxia-Induced secretome prevents hypoxia induced rCMs from undergoing apoptosis through possibly initiating cell survival mechanisms. 16 hours of hypoxia was used to induce apoptosis in this assay before treating the rCMs with the secretome to understand the anti-apoptotic effect of the secretome. rCMs were treated wth eight different conditions including, **1)** no serum 1%PS DMEM in normoxia (negative control), **2)** no serum 1%PS DMEM in hypoxia, **3)** no serum 1%PS DMEM and 50ng/ml of the TNF-α cytokine of the in hypoxia, **4)** control secretome in hypoxia **5)** TNF-α Hypoxia-Induced secretome in hypoxia, and the last three control groups are similar to groups 3-5 except they were kept in normoxia (Appendix, Table 7). We strictly focus on the first five groups which were all conducted in triplicates. Cells that were apoptotic expressed annexin V and were stained with alexa fluor 488 (green), while necrotic cells were stained with PI (red). In Figure 21A, FACS analysis visually depicts the percentage of rCMs that were necrotic in Q1, in late apoptosis in Q2, in early apoptosis in Q3, and alive in Q4 throughout all five experimental groups. Figure 22B graphically depicts the FACS results which show that the percentage of live cells treated with only hypoxia decreased by 17.05% $\pm$ 1.282% (p<0.0001) to 60.56% in comparison to the normoxia treated 77.61% rCMs. Furthermore, the percentage of live cells treated with the TNF-α cytokine decreases by 41.02% $\pm$ 1.282% (p<0.0001) to 36.59%, while TNF-α Hypoxia-Induced secretome decreases live cells by 21.90% $\pm$ 1.282% (p<0.0001) to 55.72% in contrast to the normoxia treated cells. rCMS treated with the TNF-α cytokine or TNF-α Hypoxia-Induced secretome experience a decrease in live cells by 23.97%$\pm$ 1.282% (p<0.0001) or 4.846%$\pm$ 1.282% (p<0.05), while rCMs treated with the control secretome experience an increase in live cells by 18.87% $\pm$ 1.282% (p<0.0001) to 79.44% in contrast to cells treated with only hypoxia. In addition, rCMs treated with the TNF-α cytokine show a decrease in live cells by 19.12%$\pm$1.282% (p<0.0001) when compared to the TNF-α Hypoxia-Induced secretome treated cells. More importantly, there is a 23.72%$\pm$1.282% (p<0.0001) decrease in live rCMs in TNF-α Hypoxia-Induced secretome treated cells, when compared to the control secretome treated cells. Therefore, hypoxia does induce apoptosis in rCMs, while the control secretome and TNF-α Hypoxia-Induced secretome protect the cells from the hypoxia induced damage. The TNF-α cytokine alone further induces apoptosis in rCMs. The TNF-α Hypoxia-Induced secretome has no effect on the hypoxia induced cells.

A)


(Normoxia)
Cells only (rCMs)
Q1
Q2
Q4
Q3
0.431
10.3
76.9
12.4
<FITC-A>: AnexinV
(Hypoxia)
Cells only (rCMs)
0.495
24.1
59.7
15.7
Cells + TNF-α Cytokine (Hypoxia)
1.49
35.4
36.4
26.7
Cells + Control Secretome (Hypoxia)
0.468
12.3
78.4
8.77
Cells + TNF-α Hypoxia - Induced Secretome (Hypoxia)
0.719
20.4
57
21.8

B)

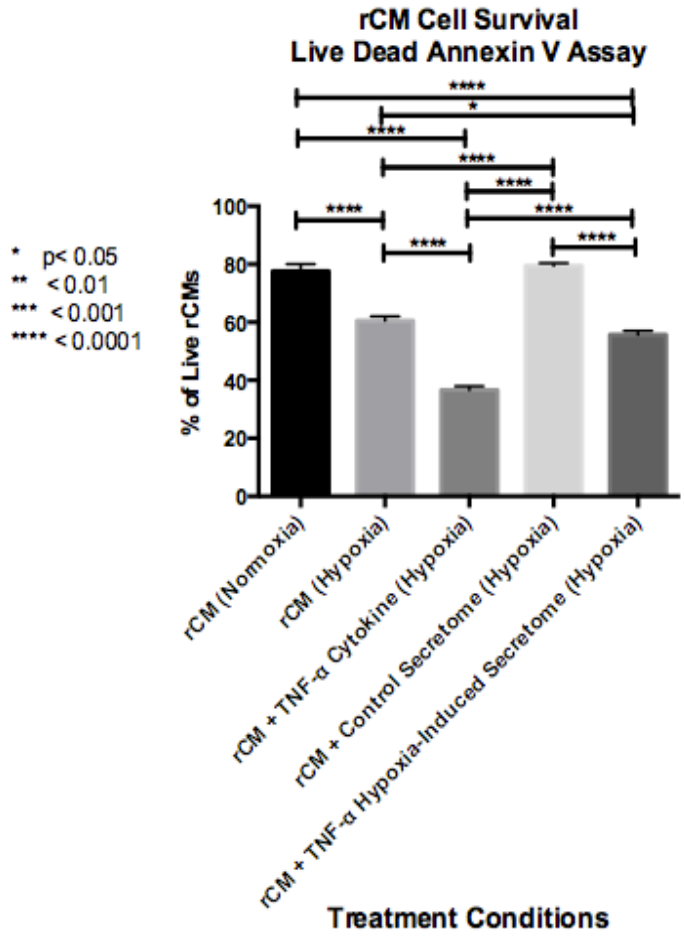

rCM Cell Survival
Live Dead Annexin V Assay
* p< 0.05
** < 0.01
*** < 0.001
**** < 0.0001
% of Live rCMs
rCM (Nomoxia)
rCM (Hypoxia)
rCM + TNF-α Cytokine (Hypoxia)
rCM + Control Secretome (Hypoxia)
rCM + TNF-α Hypoxia-Induced Secretome (Hypoxia)
Treatment Conditions

Fig 21. *The TNF-α Hypoxia-Induced Secretome may Downregulate Apoptosis Under Hypoxia.* FACS analysis of the Cell Survival- Annexin V Apoptosis Assay was conducted on rCMs treated in one of five different conditions under normoxia or hypoxia (16 hours). They were analyzed to see if the treatments initiated cell survival mechanisms and prevented apoptosis. The average percentage of live cells were calculated and compared between the groups to determine the percentage of cells being rescued by the treatments. Unstained samples were used to calibrate the FACS machine, while samples stained with only annexin V or PI were set as negative controls in order to deduct the background signals. (A) FACS analysis visually depicts the percentage of rCMs that were necrotic in Q1, in late apoptosis in Q2, in early apoptosis in Q3, and alive in Q4 throughout all five experimental groups. (B) graphically depicts the FACS results indicating the % of live cells in each group.

3.5 The TNF-α Hypoxia-Induced Secretome Treated Rats have Higher LVFS

Echocardiography was conducted at three weeks on four groups of Lewis rats which included, 1) baseline rats with no infarction (LVFS = 42.55%), 2) infarcted rats with no treatment(LVFS = 21.57%), 3) infarcted rats treated with the control secretome (LVFS = 28.78%), and 4) infarcted rats treated with the TNF-α Hypoxia-Induced secretome (LVFS = 38.36%). The left ventricular fractional shortening (LVFS) describes the contractility of the left ventricle, thereby determining the efficiency and functionality of the heart. The echoes were conducted using the parasternal short-axis view at the level of the papillary muscles. Figure 22B displays the M-mode short axis views of the left ventricle in both control secretome (***left***) and TNF-α Hypoxia-Induced secretome (***right***) treated rats. Points A & C on the echoes represent the end diastole diameter (EDD), while B&D represent the systole diameter (ESD). (EDD-ESD)/EDD X 100 is the formula used to calculate the LVFS. Figure 22A depicts the calculated average left ventricle shortening between four the groups. There is a 20.98 % $\pm$ 2.162% decrease ($p<0.0001$) in the LVFS in infarcted rats with no treatment and 13.77% $\pm$ 2.162% decrease ($p<0.001$) in the control secretome treated rats in contrast to the baseline group. Between the infarcted rats with no treatment and the control secretome treated rats, there is a 7.204% $\pm$ 2.162% increase ($p<0.05$) in the LVFS of the control secretome. The TNF-α Hypoxia-Induced secretome treated rats have an increase in LVFS by 16.79% $\pm$ 2.162% ($p<0.001$) when compared to the infarcted rats with no treatment. More importantly, there is 9.584% $\pm$ 2.162 % increase ($p<0.01$) in LVFS in the TNF-α Hypoxia-Induced

secretome treated rats in contrast to the control secretome treated rats. There is no statistically significant difference between the baseline and TNF-α Hypoxia-Induced secretome.

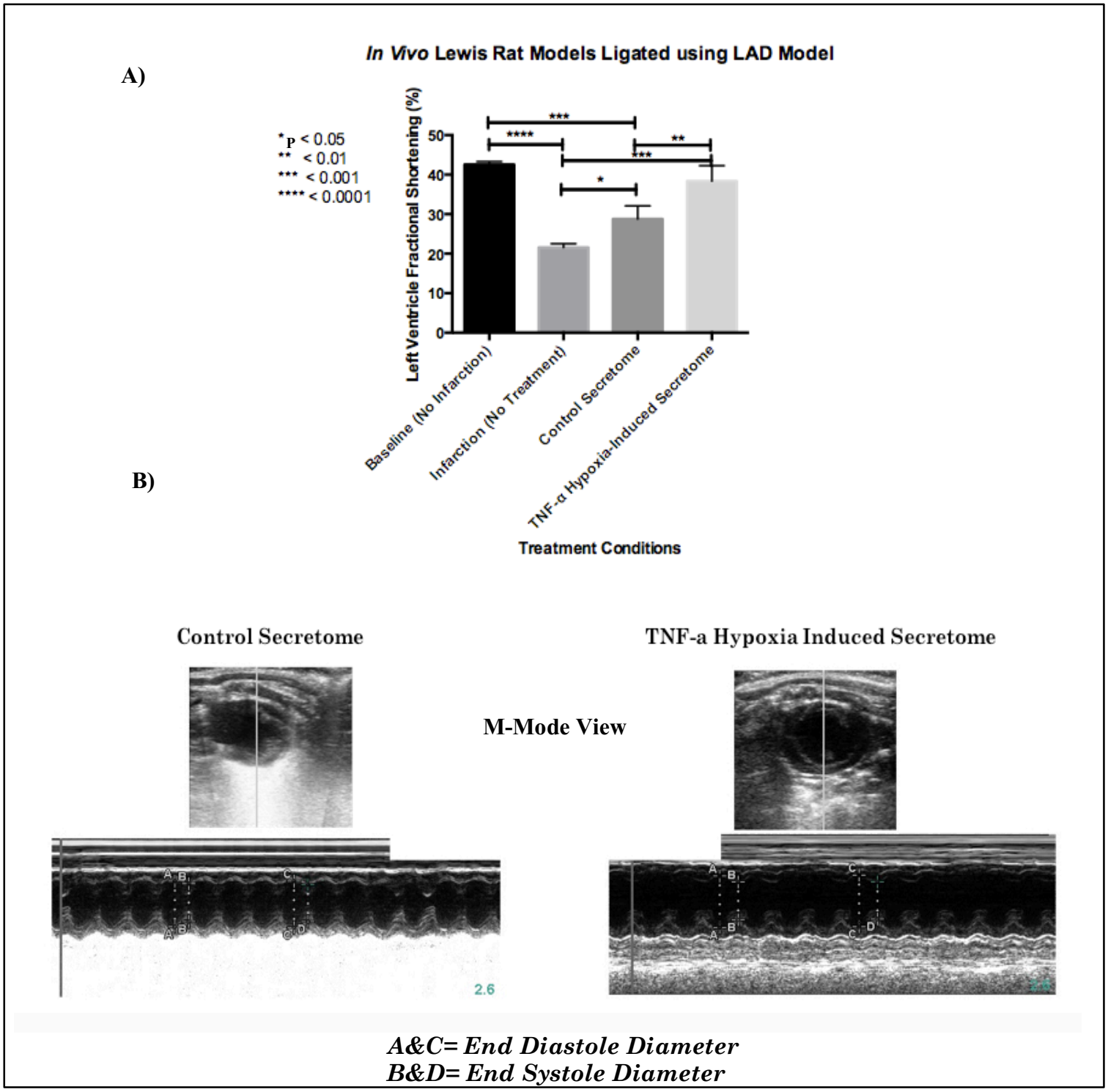


Fig 22. *The TNF-α Hypoxia-Induced Secretome Treated Rats have Higher LVFS than the control.* (EDD-ESD)/EDD X 100 was used to calculate the LVFS. (A) Graph depicts the difference in

LVFS between the baseline, infarcted rats with no treatment, the control secretome treated groups, and the TNF-α Hypoxia-Induced secretome treated rats. (B) shows the parasternal short-axis view (m-mode) at the level of the papillary muscles in both control and TNF-α Hypoxia-Induced secretome treated rats.

3.6 The TNF-α Hypoxia Induced Secretome may Prevent Cell Death at the Peri-infarct

Initially the paraffin wax embedded rat hearts were cut transversally from top to bottom into sections with a thickness of 6μm. Masson's Trichrome staining was conducted on a series of transverse cut tissue sections which reflected the entire infarcted region (top to apex) of the Control (***Top***) and TNF-α Hypoxia-Induced secretome (***Bottom***) treated rat hearts post-MI (Figure 23A). **Red** represents the viable myocardial tissue, while **blue** represents the collagen in the fibrotic region. Figure 23A visually represents the difference in the size of infarct between the control and TNF-α Hypoxia-Induced secretome treated rat heart sections, as the size of the infarct, represented in blue, is much smaller in the TNF-α Hypoxia-Induced secretome treated rat hearts than in the control secretome treated rat hearts. Figure 23B graphically represents the average infarct size (%) from the Lewis rats treated with the Control or TNF-α Hypoxia-Induced secretome post-MI. Image J was use to calculate the area of each infarct versus the area of the full section in order to calculate the average % infarct size. The average infarct size for the control secretome treated rats was 16.80% $\pm$ 0.6391%, and the average infarct size for the TNF-α Hypoxia-Induced secretome treated rats post-MI was 2.346% $\pm$ 0.4215% (p<0.0001). There is approximately a 14.45% $\pm$ 0.7655% decrease in the average size of infarct when the induced infarcted rats are treated with the TNF-α Hypoxia-Induced secretome when compared to the control secretome. The decrease in infarct size when treated with the TNF-α Hypoxia-Induced secretome indicates that myocardial preservation maybe occurring.

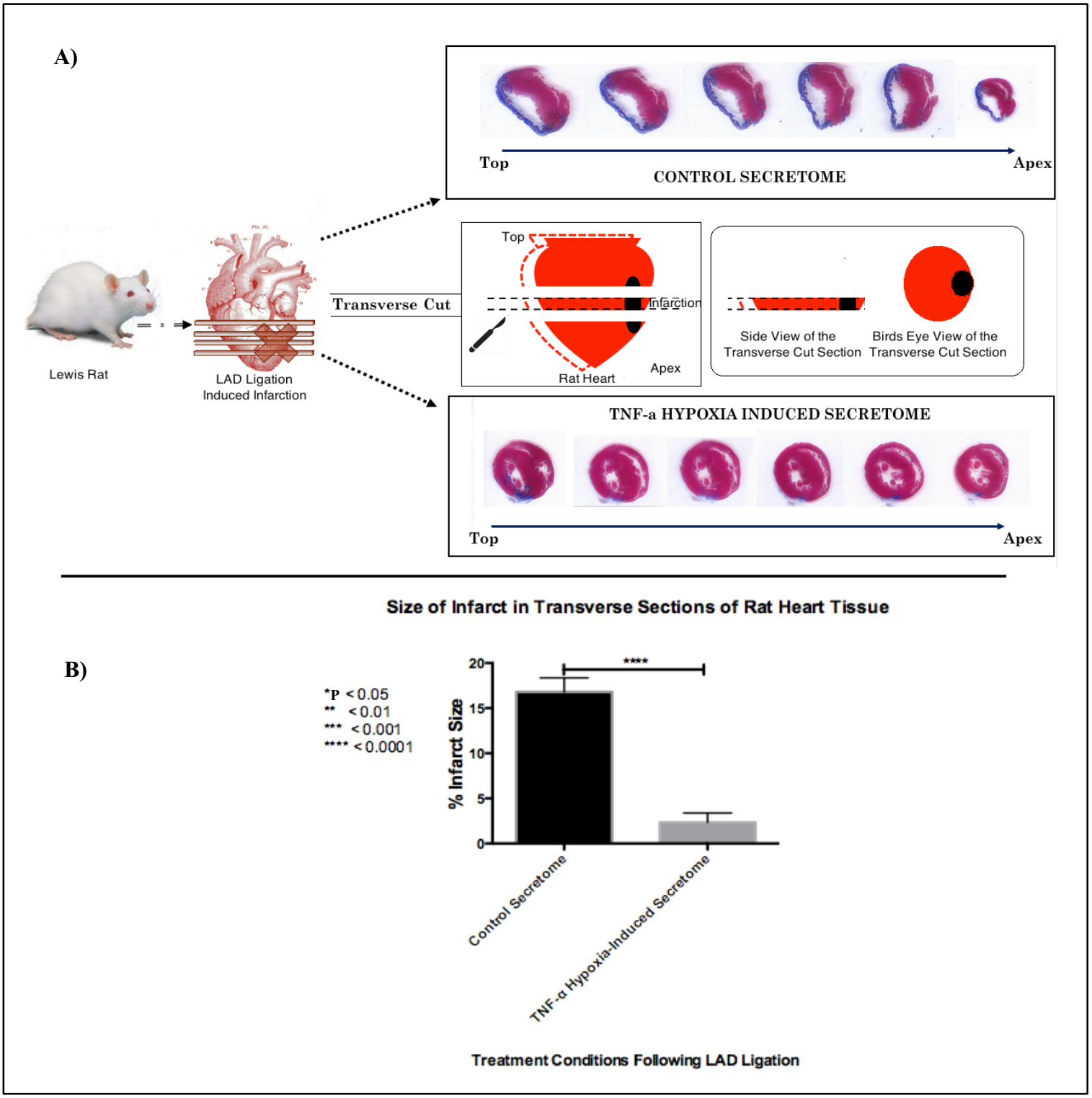


Fig 23. *Masson's Trichrome staining and average infarct size reveals that TNF-α Hypoxia-Induced secretome may prevent cell death at the peri-infarct.* Figure 23A looks at the Masson's Trichrome stained tissue sections of the Control (Top) and TNF-α Hypoxia-Induced secretome

(B) treated rat hearts following an LAD ligation induced MI. Each of the six transverse cut tissue sections in each treatment group lie throughout the infarcted zone (top through apex of the heart). **Red** represents the myocardial tissue, while **Blue** represents the collagen in the fibrotic region. Figure 23B graphically represents the average infarct sizes (%) from the Lewis rats treated with the Control or TNF-α Hypoxia-Induced secretome post-MI. There is approximately a 14.45% ± 0.7655% decrease in the average size of infarct when the induced infarcted rats are treated with the TNF-α Hypoxia-Induced secretome compared to control secretome.

3.7 The Vascular Regenerative Potential of the TNF-α Hypoxia Induced Secretome in Lewis Rats

CD31, also known as the Platelet/endothelial cell adhesion molecule-1, is a protein marker that can characterize angiogenesis. Immunohistochemistry for the CD31 marker was performed on Lewis rat heart tissue sections that were of 6μm thickness. IHC was conducted to determine if there was an increase in vessel formation at the site of infarct in the TNF-α Hypoxia-Induced secretome (***top***) treated rat hearts compared to the control secretome (***bottom***) treated rat hearts. As shown in Figures 24 (**A-F**), the secretome treated rat heart tissues were examined in order of healthy myocardium (***left***), transition zone (***middle***), and infarcted region (***right***) for each transverse cut tissue section. Figures 24 (**A-C**) looks at the control secretome treated rat heart tissue post-MI, while Figures 24 (**D-F**) look at the heart tissue treated with the TNF-α Hypoxia-Induced secretome. The staining was done in triplicates for each treatment. The purple dots indicate the nuclei of the cells, while the light brown circles indicate the expression of the CD31 marker on the cell surface of endothelial cells. The number of vessels in each region for each treatment was counted by a blinded individual. Figure 24G graphically represents the average number of vessels per optical field in each tissue region for each treatment group. In the control secretome treated rat tissue sections, among the healthy and infarcted region, the formation of vessels is low with no significant differences between the two groups. In the TNF-α Hypoxia-Induced secretome treated tissue sections, there is a 8.3 ± 1.15 increase in the average number of vessels formed in the the infarcted region versus the healthy region per optical field (p<0.001). More significantly, there is a 8.6 ± 1.15 increase in the average number of vessels formed (p<0.001) at the site of infarct in the TNF-α Hypoxia-Induced secretome treated rats than in the control secretome treated rats (Figure 24 (**C & F)**).

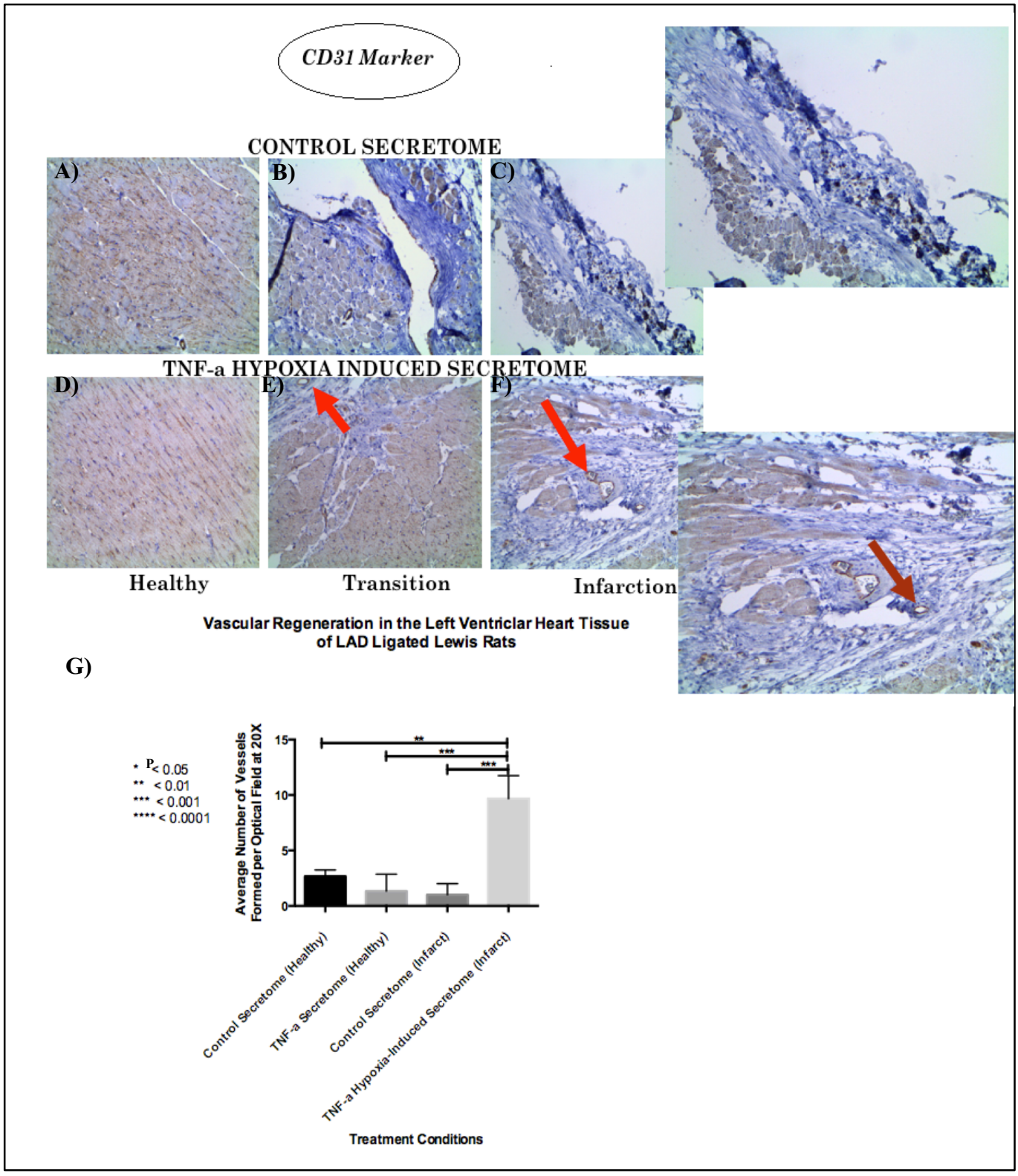


Fig 24. *Potential Vascular Regeneration in TNF-α Hypoxia Induced Secretome Treated Rats through the expression of CD31(**F**).* Rat heart tissue sections (6μm) were stained for the CD31

protein in two different treatment groups, which included the control secretome **(*top*)** and TNF-α Hypoxia-Induced secretome (***bottom***). The marker was examined for in each tissue section in healthy (**A & D**), transition (**B & E**), and infarcted sites (**C & F**). The purple dots indicate the nuclei of the cells, while the light brown circles indicate the CD31 marker. The light brown circles represent vessels that are outlined by the CD31 marker. The increase in CD31 expression is represents the increasing number of vessels present per optical field in the infarcted region of the TNF-α Hypoxia-Induced secretome treated tissues over the control treatment ***(F)*** The images were taken using the 20X objective. Haematoxylin was used to stain the nuclei. (**G**) shows that there is a 8.6 ± 1.15 increase in the average number of vessels formed ($p<0.001$) at the site of infarct in the TNF-α Hypoxia-Induced secretome treated rats than in the control secretome treated rats.

3.8 The Increased Occurrence of Proliferation at the Site of Infarct in TNF-α Hypoxia-Induced Secretome Treated Lewis Rats

Ki-67 is a protein marker that is strictly associated with cell proliferation and is found in the nucleus of cells (Scholzen & Gerdes, J, 2000). IHC for the Ki-67 marker was performed on Lewis rat heart tissue sections that were of 6μm thickness. IHC was conducted to determine if the proliferation of cells were occurring and upregulated at the site of infarct in the TNF-α Hypoxia-Induced secretome (***top***) treated rat hearts compared to the control secretome (***bottom***) treated rat hearts. As shown in Figures 25 (**A-F**), the secretome treated rat heart tissues were examined in order of healthy myocardium (***left***), transition zone (***middle***), and infarcted region (***right***) for each tissue section. Figures 25 (**A-C**) represents rat heart tissues post-MI treated with control secretome, while Figures 25 (**D-F**) represent heart tissues treated with the TNF-α Hypoxia-Induced secretome. The staining was done in triplicates for each treatment. The purple dots indicate the nuclei of the cells, while the dark brown spots indicate the Ki-67 marker. In the control secretome treated rat tissue sections, among the healthy, transition zone, and infarcted region the expression of the Ki-67 protein is low and remains consistent. Conversely, in the TNF-α Hypoxia-Induced secretome treated rat heart tissue samples, the expression of Ki-67 varies. The healthy region of the tissue, is similar to the healthy myocardium in the control secretome treated rats. However, the transition zone and specifically the infarcted region has increased levels of expression of Ki-67. More importantly, when comparing the two infarcted regions between the secretome treatment

groups, the TNF-α Hypoxia-Induced secretome treated heart sections show an upregulation in the expression of the Ki-67 protein marker (Figure 25 (**C & F)**).

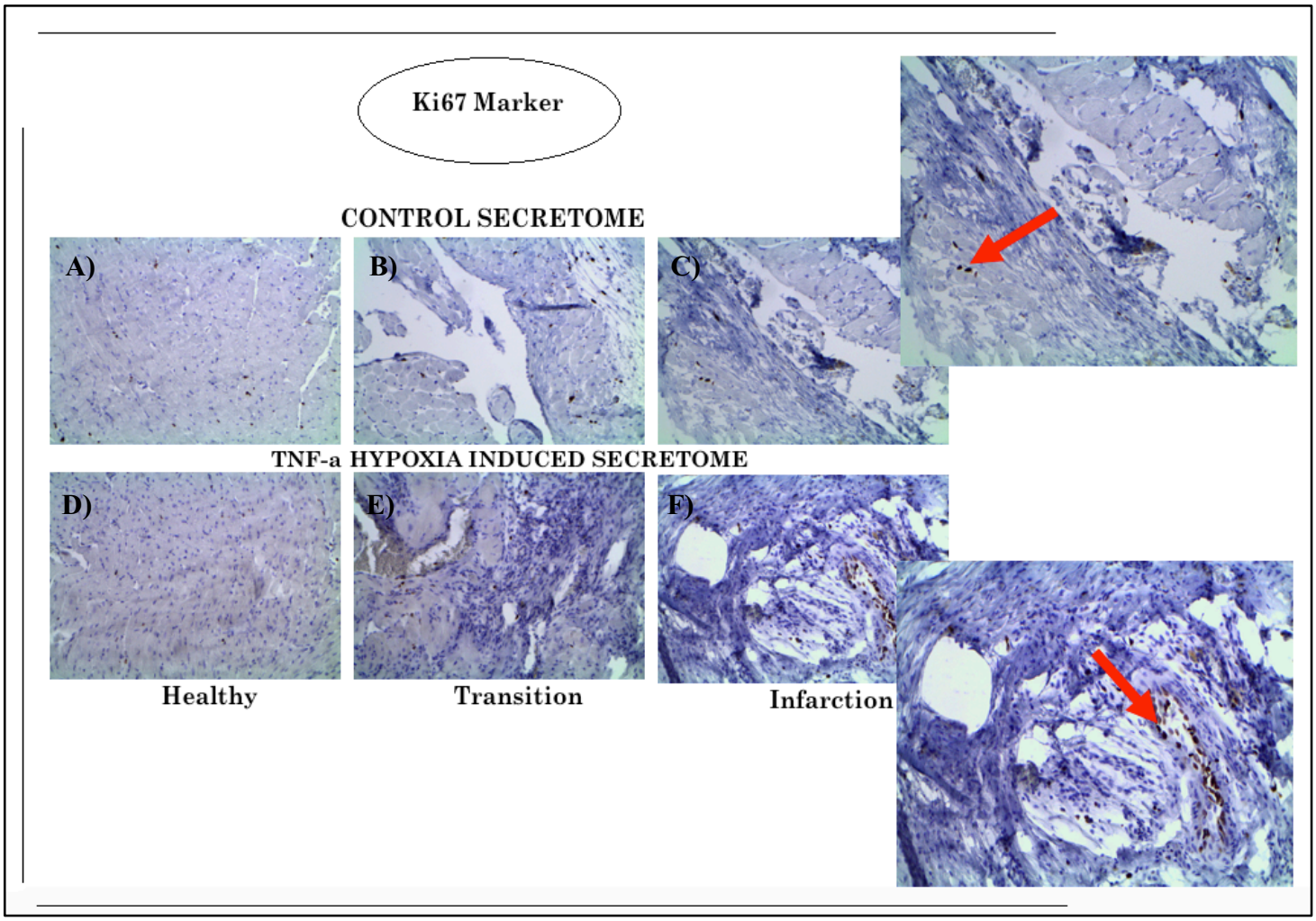


Fig 25. *The Increased Occurrence of Proliferation at the Site of Infarct in TNF-α Hypoxia-Induced Secretome Treated Rats through the expression of Ki67 (**F**).* Rat heart tissue sections (6μm) were stained for the Ki-67 protein in two different treatment groups, which included the control secretome **(*top*)** and TNF-α Hypoxia-Induced secretome (***bottom***). The marker was examined for in each tissue section in healthy (**A & D**), transition (**B & E**), and infarcted sites (**C & F**). Increased expression of Ki-67 can be seen in the infarcted region of the *TNF-α Hypoxia-Induced secretome treated tissues (**F**).* The purple dots indicate the nuclei of the cells, while the dark brown spots indicate the Ki-67 marker. The images were taken using the 20X objective. Haematoxylin was used to stain the nuclei.

CHAPTER 4: DISCUSSION

AMIs mainly occur as a result of the occlusion of a coronary artery which deprives the heart tissue of essential oxygen and nutrients needed for CMs to survive. Chronic inflammation and progressive remodelling may cause irreversible damage to the myocardium, which can ultimately lead to end-stage heart failure (Song et al.,2010). Currently, the existing treatment methods are not sufficient enough to repair and regenerate the damaged myocardium in order to re-establish the hearts' function, as they mainly focus on treating the cause and the symptoms presented as a result of the occlusion. BM-MSCs have been extensively studied and have been shown to be encouraging in developing a therapy for cardiac repair following MI (William & Hare, 2011). Paracrine factors secreted by the BM-MSCs at the site of injury have recently been shown in many studies to have beneficial effects on repairing the damaged myocardium, however the mechanisms by which it promotes repair remain unknown (Yao et al.,2015). In addition, the idea of preconditioning BM-MSCs with hypoxia and cytokines have been proposed to upregulate the expression of secreted beneficial proteins which have reparative effects in response to the ischemic microenvironment (Burchfield & Dimmeler, 2008). TNF-, is a pleiotropic proinflammatory cytokine that is secreted by CMs and immune cells immediately following myocardial injury and is known to be upregulated in hypoxia (Papathanasiou et al.,2015; Rochefort et al, 2006). In this study we concentrate on shifting the focus from stem cell therapeutic models to developing a cell-free secretome therapy for cardiac repair. Thus, we hypothesized that TNF-α and hypoxia treated rBM-MSCs enhance the homing and recruitment of rBM-MSCs, while simultaneously altering their paracrine responses to promote angiogenesis, cell survival, and anti-inflammatory responses in a hostile infarcted environment.

4.1 rBM-MSCs are Responsive to TNF-α and Promote Mobilization of rBM-MSCs via TNF-α Hypoxia-Induced Secretome In Vitro

TNF-α is secreted by damaged CMs and immune cells and is known to initiate inflammatory, apoptotic, and possible reparative processes by binding to cell surface receptors TNFR1 and TNFR2 (Papathanasiou et al.,2015). Our immunofluorescence and western blot data confirm the presence of TNFR1 and TNFR2 on the surface of rBM-MSCs in both normoxic or hypoxic conditions treated or untreated with various combinations of rCCM and/or TNF-α. Although both receptors are visualized through immunofluorescence in Figure 18A, western blot

analysis shows the presence of only TNFR2 (Figure 18B). This maybe a result of the low receptor concentration (expression) of TNFR1 on rBM-MSCs in their respective treatment conditions, as the conditions may not have induced the optimal stress needed for overexpression of the pro-apoptotic receptor TNFR1. Therefore, the western blot technique may not be as sensitive to detect the extremely low concentration of TNFR1. Given its higher expression, our results seem to indicate that TNFR2 is more relevant to stress-induced TNF-α action on rBM-MSCs. Ultimately, these results indicate that TNF-α is able to bind to rBM-MSCs in order to initiate possible cell survival pathways through the stress-induced TNFR2 mechanism.

Endogenous homing and recruitment mechanisms of BM-MSCs to the site of injury are mediated by hypoxia and cytokines via paracrine effects, and this homing mechanism is essential for the suppression of inflammation and the initiation of cardiac repair (Russo et al.,2014). However, this mechanism may not be effective enough to induce mass migration of BM-MSCs. Although molecules such as SDF-1 and HGF play a significant role in mobilization, preconditioning BM-MSCs may upregulate the production of these factors and increase recruitment rates. A 2009 study conducted by Tang et al revealed that preconditioning cardiosphere-derived, Lin−/c-kit+ (CLK) progenitor cells with hypoxia significantly increased the expression of CXCR4, there by enhancing CLK-cell migration in vitro and recruitment in vivo in the infarcted myocardium. Thus, by preconditioning BM-MSCs with hypoxia and possible migration inducing factors, one may be able to enhance the BM-MSCs migratory properties to the site of infarct. Our migration assay results demonstrate that the rBM-MSCs' TNF-α and Hypoxia-Induced secretome provokes approximately twice the amount of rBM-MSCs ($p<0.05$) to migrate compared to the control secretome in vitro. Additionally, in an effort to assess and remove the chemotactic effects of the TNF-α cytokine alone, we examined and found that the cytokine alone itself does not significantly impact the mobilization of rBM-MSCs. Therefore, TNF-α and hypoxia play a crucial role in stimulating rBM-MSCs, while enhancing the secretion of potent mobilization factors amongst other factors, which may further promote and amplify the homing and recruitment of rBM-MSCs to the site of infarct. The TNF-α and Hypoxia-Induced secretome maybe enhanced with high levels of SDF-1, HGF, platelet-derived growth factor (PDGF), and other mobilization factors that aid in BM-MSC recruitment. A study by Yu et al in 2013 tested the potential of hypoxia treated "MSC transplantations to enhance the efficacy of cell therapy on acute kidney injury." The study confirmed the up-regulation of CXCR4/SDF-1 in vitro after preconditioning

with hypoxia. Furthermore, the upregulation of the mechanism increased the MSCs' migration potential in vitro. Therefore, the TNF-α Hypoxia-Induced secretome is a chemoattractant that maybe overexpressing various mobilization factors and thus, the secretome may play a crucial role in the migration of BM-MSCs to the site of infarct.

4.2 TNF-α and Hypoxia-Induced Secretome Triggers Neovascularization for Cardiac Repair In Vitro and In Vivo

"Neovascularization is an important biological process positively influenced by MSCs in cardiac repair post-MI" (Wen et al.,2011). Several mechanisms of neovascularization which underlie cardiac repair have been proposed, however our results reinforce the paracrine hypothesis. In this study, a string analysis was conducted to analyze the protein network that is closely associated with TNF- in the norvegicus rattus species. Using a biased approach, the expression of angiogenic proteins such as VEGF-1, FGF-2, FGF-7, Ang-1 and Ang-2 were examined for within rBM-MSCs that were treated with various combinations of rCCM, hypoxia/normoxia, and or TNF-α in vitro. In this study, we mainly focus on the effects of TNF-α and hypoxia on rBM-MSCs' protein expression, keeping in mind that the rCCM and hypoxia treated rBM-MSCs are simulating an in vitro model of the endogenous physiological BM-MSCs' response to an in vivo ischemic MI, thus acting only as a control. Our data shows that there is an increase in the expression of VEGF-1 by 3.18 folds (p<0.0001), FGF-2 by 1.47 folds (p<0.0001), and Ang-2 by 1.95 folds (p<0.0001) in rCCM, TNF-α and hypoxia treated rBM-MSCs as opposed to the rCCM and hypoxia treated cells. This suggests that TNF-α and hypoxia may work together in the hostile environment post-MI to increase the expression of VEGF-1, FGF-2, and Ang-2. VEGF-1 may induce neovascularization through the proliferation of ECs and vascular smooth muscle cells (VSMCs), while stimulating EC sprouting from pre-existing blood vessels. More importantly, it is known to mobilize bone marrow progenitor cells and promotes differentiation to ECs (Zisa et al.,2009). In a 2007 study by Wang et al, it was determined that an increase in VEGF was inherent succeeding the preconditioning of mouse BM-MSCs with hypoxia or TNF-α, which was mediated through the STAT3 and p38 MAPK mechanisms. In our current study, we see that the additive effect of hypoxia and TNF- α increase the expression of VEGF by approximately 3-folds and thus, it can be hypothesized that the STAT3 and p38 MAPK mechanisms are working in order to initiate and sustain the process of angiogenesis through BM-MSC secretion in the infarcted

microenvironment. FGF-2 may aid in the process of neovascularization by allowing endothelial progenitor cell maturation and migration (Eiselleova, et al.,2009). Liu et al., investigated the preconditioning of rBM-MSCs with hypoxia (48h) to facilitate revascularization in diabetic lower limb ischemia (DLLI). Angiography and immunohistochemistry demonstrated accelerated vascular reconstruction in the in vivo rat models with DLLI when hypoxia preconditioned rBM-MSCs were administered. Western blots results suggested increased expression of VEGF, bFGF, Ang, and other proteins which played a major role in increasing microvessel density within the DLLI model that was treated with the hypoxia preconditioned rBM-MSCs (Liu et al.,2015). However, in our study, Ang-1 was downregulated in rBM-MSCs treated with rCCM, TNF- α, and hypoxia when compared to rBM-MSCs treated with just hypoxia and rCCM. Ang-2 not only triggers hypertrophy, but also regulates angiogenesis through regulating weak productions of Ang-1 and through triggering VEGF-1 expression. Ang-2 functions are context-dependent (Sandhu et al.,2004). In this study, our data shows that Ang-1 is downregulated by 1.62 folds ($p<0.05$) in rCCM, TNF-α, and hypoxia treated rBM-MSCs compared to the rCCM and hypoxia treated cells, which means that Ang-2 maybe upregulated in an effort to increase Ang-1 expression. This in turn could lead to the proliferation and/or survival of endothelial cells. Conversely, the expression of FGF-7 in rBM-MSCs remained unchanged between cells untreated or treated with rCCM and hypoxia or rCCM, hypoxia, and TNF-α. Although FGF-7 is known to upregulate the expression of VEGF-1, in this case VEGF-1 in the myocardium maybe activated through FGF-2 and other protein mechanisms (Ferrara & Davis-Smyth, 1997; Eiselleova, et al.,2009).

Furthermore, in vivo IHC studies revealed the presence of CD31, a marker of angiogenesis, at the site of infarct, in infarcted models treated with the TNF-α Hypoxia-Induced secretome. CD31 aids in forming new endothelial cell-cell interactions (DeLisser et al, 1997). CD31 staining revealed that there is a greater number of vessels formed in TNF-α Hypoxia-Induced secretome treated rats in contrast to the control secretome following MI. There was a 8.6 ± 1.15 increase in the average number of vessels formed ($p<0.001$) at the site of infarct in the TNF- Hypoxia-Induced secretome treated rats than in the control secretome treated rats. Accordingly, Wu et al., conducted a study using an excisional wound splinting model, to show that green fluorescence labelled allogeneic mouse BM-MSCs injected around and on the wound "significantly enhanced wound healing in normal and diabetic mice compared with that of allogeneic neonatal dermal fibroblasts or vehicle control medium" (Wu et al, 2007). Proangiogenic factors secreted by the

BM-MSCs played a role increasing capillary densities in wounds at 14 days. These were assessed morphometrically through IHC staining for CD31. Similarly, we can conclude that angiogenesis is occurring at the site of infarct following the treatment of the TNF-α Hypoxia-Induced secretome. Hence, TNF-α and hypoxia may play a critical role in inducing neovascularization in an effort to initiate cardiac repair processes.

4.3 rCM Survival and Myocardial Preservation is Initiated by the TNF-α and Hypoxia-Induced Secretome in a Hypoxic Microenvironment in the Absence of Nutrients

Post-MI, the resulting damage caused by the death of CMs is close to irreversible as the endogenous regenerative capacity of CMs in the myocardium is extremely limited. Pro-survival and anti-apoptotic properties may be triggered through the TNFR2 receptors in rCMs at the peri-infarct in an attempt to salvage healthy myocardium, but the mechanism of action still remains unknown (Tian et al.,2015). In a 2010 study by Kelly et al., it was reported that 1) MSCs derived from TNFR1 knockout mice can further improve myocardial function when administered pre-ischemia and 2) TNFR1 and TNFR2 knockouts eliminate MSCs protective function in the heart post MI. The study concluded that the "TNFR1 signaling may damage MSC paracrine effects and decrease MSC- mediated cardioprotection, whereas TNFR2 likely mediates beneficial effects in MSCs" (Kelly et al.,2010). Appropriately, in our receptor expression study of TNFR1 and 2, TNFR2 was prevalent in the rBM-MSCs over TNFR1 expression in stress induced conditions.

In 2010, Lee et al., examined the composition and effects of the TNF-α induced secretome of human adipose tissue-derived mesenchymal stem cells (hASCs) with a purpose of regenerating or repairing inflamed/injured tissue. The study identified that 118 of the 187 proteins that were secreted by hASC in the conditioned media were upregulated when stimulated with TNF-α. The TNF-α induced conditioned media derived from hASCs was analyzed through liquid chromatography coupled with tandem mass spectrometry. Results from the study exhibited that the TNF-α induced hASC secretome stimulated human monocyte migration, while secreting several cytokines (IL-6 & Il-8) which may play an important role in inflammation. Additionally, TNF-α treatments increased the secreted levels of cathepsin L and pentraxin 3 proteins which are involved in the "regulation of ECM degradation and remodeling, motility, angiogenesis, cell signaling," and inflammation in a time and concentration dependent manner (Lee et al.,2010). Our Annexin V assay results suggest that when rCMs are stressed under hypoxia for 16 hours,

hypoxia induces apoptosis and allows only 60.56% of rCMs to remain viable when compared to the normoxia treated cells (77.61%). The effect of the TNF-α cytokine on rCMs in hypoxia further reduces the viability of the cells through increased cell apoptosis and necrosis to 36.59%. Furthermore, the control secretome protects the rCMs against hypoxia-induced apoptosis which allows the survival of rCMs to increase to 79.44%, while the TNF-α Hypoxia-Induced secretome decreases the percentage of cells to 55.72%. Remarkably, the TNF-α Hypoxia-Induced secretome treated rCMs show no statistical difference between the hypoxia treated group and itself, while the control secretome group shows a statistically significant increase in the number of cells that are protected from apoptosis. Thus, regular paracrine secretions released in response to the infarction of BM-MSCs seem to protect rCMs from apoptosis. However, the TNF-α cytokine alone is detrimental to the rCMs in hypoxia. Interestingly, the TNF-α Hypoxia stimulated BM-MSC secretome results show that they neither protect nor induce further apoptosis and necrosis of rCMs when compared to the hypoxia treated cells. This may be caused by the presence of the unused soluble TNF-α cytokine within the TNF-α Hypoxia-Induced rBM-MSC secretome itself. This may counter the anti-apoptotic properties of the secretome itself, thereby, neutralizing the beneficial effects of the TNF-α Hypoxia-Induced secretome. Moreover, as the effect of TNF-α is time dependent, increasing or decreasing the incubation length of rBM-MSCs treated with TNF-α when creating the TNF-α and hypoxia induced rBM-MSCs secretome may have varying effects. Thus, the TNF-α Hypoxia-Induced secretome may indeed have anti-apoptotic properties that promote the survival of rCMs. Furthermore, timing of the administration of TNF-α to the rBM-MSCs and the duration and concentration of TNF-α that is present within the cellular microenvironment can play a major role in deciding if TNF-α will have beneficial or detrimental roles in cardiac tissue preservation.

In vivo, LAD ligated infarcted Lewis rats were used to assess if the TNF-α Hypoxia - Induced secretome initiated cardiac repair and myocardial preservation by preventing extensive damage in cardiomyocytes adjacent to the infarcted region, while promoting angiogenesis. Echocardiography was conducted two weeks post-operative and results showed that the TNF-α Hypoxia-Induced secretome treated rats showed an increased LVFS of 38.36% in contrast to the infarcted rats that were untreated (LVFS=21.75%) (p<0.001). The TNF-α Hypoxia-Induced secretome treated rats also had a higher LVFS when compared to the control secretome treated

rats (LVFS= 28.78%) (p<0.01). Baseline rats with no infarction had a LVFS of 42.55%. The higher LVFS of the TNF-α Hypoxia-Induced secretome treated rats indicate an increase in the contractility of the heart as a result of the treatment. This may be caused by increased vessel densities and through the prevention of extensive inefficient remodeling. Therefore, this indicates that a moderate recovery in the cardiac mechanical function had occurred in TNF-α Hypoxia-Induced secretome treated rats. Furthermore, there was no statistical difference between the LVFS in the baseline versus TNF-α Hypoxia-Induced secretome treated rats.

Further investigation on the differences between the infarct sizes in the groups was also conducted via masson's trichrome staining (Figure23A). Results revealed that the average infarct size within the TNF-α Hypoxia-Induced secretome treated rats was smaller by 14.45%± 0.7655% (p<0.0001) than in the control secretome rats. This indicates that preservation of the myocardium is occurring most likely near the peri-infarct, thus not allowing the cells adjacent to the infarct zone to become apoptotic or necrotic. It further shows that the amplification of the innate signaling factors of repair, may not be as effective in preserving the damaged myocardium as much as the TNF-α Hypoxia-Induced secretome.

The Ki-67 protein marker is mainly present during all active phases of the cell cycle including G1, S, G2, and mitosis, but is absent from resting cells (Scholzen & Gerdes, J, 2000). Thus, when IHC for the Ki-67 marker was conducted on the heart sections from both treatments, it was determined that cell proliferation at the site of infarct was qualitatively increased in the TNF-α Hypoxia-Induced secretome than in the control secretome heart sections. Although we cannot state that CPC or BM-MSCs are the cells that are proliferating at the site of infarct, we can state that proliferation at the site of infarct is heavily influenced by the TNF-α Hypoxia-Induced secretome post-MI. The secretome may ultimately affect a whole group of cells which include, CPCs, CSCs, BM-MSCs, fibroblasts, and immune cells.

Proliferation and differentiation of BM-MSCs may be affected by the secretome as, western blot analysis in the rBM-MSCs treated with rCCM, TNF-α, and hypoxia revealed that VEGF-1, FGF-2, myogenin, and TGF-B were highly expressed compared to the rCCM and hypoxia control group. These results show that VEGF-1 may play a role in mobilizing bone-marrow progenitor cells and promoting differentiation into CMs or ECs (Zisa et al.,2009). The increased levels of FGF-2 expression in rBM-MSCs may promote BM-MSCs migration, self renewal, adhesion properties (Eiselleova, et al.,2009). The 3.27 fold (p<0.0001) increase in

expression of TGF-B in rBM-MScs treated with rCCM, hypoxia, and TNF-α compared to the rCCM and hypoxia control may play a role in differentiating MSCs to smooth muscle cells. This may promote neovascularization while TGF-B also acts as an anti-inflammatory molecule that has a role in cardioprotection by promoting anti-apoptotic properties in CMs (Hausenloy & Yellon, 2009). Myogenin is a transcription factor that is implicated in cardiac muscle cell differentiation, muscle formation, and myocardial repair (Gnecchi et al.,2008). Thus, the increased protein expression of myogenin in rBM-MSCs treated with rCCM, TNF-α, and hypoxia may contribute to and be involved in the healing and reparative process. A significant variable in this experiment is the effect of hypoxia on stem cells. This is because the "effect of hypoxia can vary widely with differences in the oxygen concentration and duration of exposure" to hypoxia (Yu et al.,2013).

Overall, myocardial preservation following an MI through the promotion of anti-apoptotic properties in rCMs can indeed be induced by the TNF-α Hypoxia-Induced secretome. Thus, the TNF-α Hypoxia-Induced secretome induces anti-apoptotic, migratory, anti-inflammatory, and neovascularization properties and is a viable treatment option for myocardial infarction.

4.4 Limitations

Study limitations include the use of a selective biased approach when considering which critical proteins to assess in the rBM-MSCs, using a small sample size in the in-vivo model to understand the initial effects of the secretomes, and having limited control over the size of infarcts that were induced through the LAD ligation of Lewis rats. Variability in the later is difficult to control, however increasing the size of the samples may minimize the variability of the infarct size within and between each group. Furthermore, a series of transverse cut sections were accounted for when calculating the infarct size, as the average is a more suitable representation of the infarct. The limitation to developing a cell-free therapy for cardiac repair is that numerous mechanisms work synergistically through the paracrine effect to repair injured tissue, and each secreted protein may be involved in numerous tasks. Identifying the proteins that make up a specific secretome and the signaling pathways they trigger is key to understanding the entire paracrine biology mediating the therapeutic benefits of the secretome, however this is a work in progress. Therefore, in this study we focused on one pivotal cytokine and the reparative effects it induces through rBM-MSCs. However, for future work an integrative approach should be used to examine the multiple

interactions between the various cytokines and effectors that make up the secretome in order to better elucidate their overall net effects.

CONCLUSION

Initially, we set out to determine the role of TNF-α and hypoxia on rBM-MSCs and its' secretome in vitro, while investigating the potential angiogenic, migratory, cardioprotective, and reparative effects of the TNF-α Hypoxia-Induced secretome following MI. In this study, we determined the increased expression of beneficial proteins in rBM-MSCs that aid in neovascularization, immune suppression, mobilization, and differentiation when stimulated with TNF-α and hypoxia. Furthermore, we discovered that the TNF-α Hypoxia-Induced secretome is a chemo-attractant which may enhance rBM-MSC recruitment to the site of infarct, while displaying anti-apoptotic properties that may aid in the survival of the rCMs. In vivo studies continued to show a greater LVFS and a possible reduction in infarct size, while histological analyses indicated an increase in cell proliferation and number of vessels formed in the infarcted regions of the rat hearts treated with the TNF-α Hypoxia-Induced secretome. Overall, our findings are promising in terms of developing a therapy that preserves the myocardium while promoting mechanisms essential for cardiac repair post-MI. This research is significant in helping us understand the effects of preconditioning rBM-MSCS to alter the paracrine responses in attempt to initiate, sustain, and enhance endogenous cardiac repair mechanisms. It is a major stepping-stone in moving towards creating a potential cell free cytokine-dependent therapy that may be minimally invasive in the future. Ultimately, it may one day exclusively focus on the administration of secretome injections to ischemic patients in an attempt to enhance innate cardiac repair mechanisms following an MI.

REFERENCES

World Health Organization. (2014). Global status report on noncommunicable diseases 2014. World Health Organization, 1-298.

Insull, W. (2009). The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. The American journal of medicine, 122(1), S3-S14.

Mendis, S., Thygesen, K., Kuulasmaa, K., Giampaoli, S., Mähönen, M., Blackett, K. N., & Lisheng, L. (2011). World Health Organization definition of myocardial infarction: 2008–09 revision. International journal of epidemiology, 40(1), 139-146.

Bolooki, H. M., & Askari, A. (2010). Acute myocardial infarction. Disease Manag Proj.

Thygesen, K., Alpert, J. S., & White, H. D. (2008). Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction. G Ital Cardiol (rome).

Jennings, R. B., & Ganote, C. E. (1974). Structural changes in myocardium during acute ischemia. Circulation Research, 35, 156.

Jaffe, A. S., Babuin, L., & Apple, F. S. (2006). Biomarkers in acute cardiac disease: the present and the future. Journal of the American College of Cardiology, 48(1), 1-11.

AL-Ziarjawey, H. A. J., & Çankaya, I. (2015). Heart Rate Monitoring and PQRST Detection Based on Graphical User Interface with Matlab. International Journal of Information and Electronics Engineering, 5(4), 311.

Bode, C., & Zirlik, A. (2007). STEMI and NSTEMI: the dangerous brothers. European heart journal, 28(12), 1403-1404.

Ashley EA, Niebauer J. Cardiology Explained. London: Remedica; (2004). Chapter 3, Conquering the ECG. Available from: http://www.ncbi.nlm.nih.gov/books/NBK2214/

Dybdahl, B., Slørdahl, S. A., Waage, A., Kierulf, P., Espevik, T., & Sundan, A. (2005). Myocardial ischaemia and the inflammatory response: release of heat shock protein 70 after myocardial infarction. Heart, 91(3), 299-304.

Bodi, V., Sanchis, J., Nunez, J., Mainar, L., Minana, G., Benet, I., ... & Llacer, A. (2008). Uncontrolled immune response in acute myocardial infarction: unraveling the thread. American heart journal, 156(6), 1065-1073.

Hansson, G. K., & Libby, P. (2006). The immune response in atherosclerosis: a double-edged sword. Nature Reviews Immunology, 6(7), 508-519.

Neumann, F. J., Ott, I., Gawaz, M., Richardt, G., Holzapfel, H., Jochum, M., & Schömig, A. (1995). Cardiac release of cytokines and inflammatory responses in acute myocardial infarction. Circulation, 92(4), 748-755.

Frangogiannis, N. G., Smith, C. W., & Entman, M. L. (2002). The inflammatory response in myocardial infarction. Cardiovascular research, 53(1), 31-47.

Priebe, H. J. (2005). Perioperative myocardial infarction—aetiology and prevention. British journal of anaesthesia, 95(1), 3-19.

Smith, S. C., Benjamin, E. J., Bonow, R. O., Braun, L. T., Creager, M. A., Franklin, B. A., ... & Lloyd-Jones, D. M. (2011). AHA/ACCF secondary prevention and risk reduction therapy for patients with coronary and other atherosclerotic vascular disease: 2011 update: a guideline from the American Heart Association and American College of Cardiology Foundation endorsed by the World Heart Federation and the Preventive Cardiovascular Nurses Association. Journal of the American college of cardiology, 58(23), 2432-2446.

Anzai, T., Ogawa, S., Motomiya, T., Nonogi, H., Yasue, H., Ogawa, N., ... & Katada, K. (2001). Acute myocardial infarction, 45(4), 135-142.

Thygesen, K., Alpert, J. S., Jaffe, A. S., Simoons, M. L., Chaitman, B. R., & White, H. D. (2012). Third universal definition of myocardial infarction. Circulation, 126(16), 2020-2035.

White, H. D., & Chew, D. P. (2008). Acute myocardial infarction. The Lancet, 372(9638), 570-584.

Friedrich, E. B., & Böhm, M. (2007). Management of end stage heart failure. Heart, 93(5), 626-631.

Boersma, E., Mercado, N., Poldermans, D., Gardien, M., Vos, J., & Simoons, M. L. (2003). Acute myocardial infarction. The Lancet, 361(9360), 847-858.

Hilleman, D. E., Tsikouris, J. P., Seals, A. A., & Marmur, J. D. (2007). Fibrinolytic Agents for the Management of ST-Segment Elevation Myocardial Infarction. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 27(11), 1558-1570.

Venugopal, J. R., Prabhakaran, M. P., Mukherjee, S., Ravichandran, R., Dan, K., & Ramakrishna, S. (2012). Biomaterial strategies for alleviation of myocardial infarction. Journal of The Royal Society Interface, 9(66), 1-19.

Bonvini, R. F., Hendiri, T., & Camenzind, E. (2005). Inflammatory response post-myocardial infarction and reperfusion: a new therapeutic target? European heart journal supplements, 7(I), l27-l36.

Fang, L., Moore, X. L., Dart, A. M., & Wang, L. M. (2015). Systemic inflammatory response following acute myocardial infarction. Journal of geriatric cardiology: JGC, 12(3), 305.

Nian, M., Lee, P., Khaper, N., & Liu, P. (2004). Inflammatory cytokines and postmyocardial infarction remodeling. Circulation research, 94(12), 1543-1553.

V.V., Zuylen,den Haan, M. C., Geutskens, S. B., Roelofs, H., Fibbe, W. E., Schalij, M. J., & Atsma, D. E. (2015). Post-myocardial Infarct Inflammation and the Potential Role of Cell Therapy. Cardiovascular Drugs and Therapy, 29(1), 59-73.

Mirotsou, M., Jayawardena, T. M., Schmeckpeper, J., Gnecchi, M., & Dzau, V. J. (2011). Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. Journal of molecular and cellular cardiology, 50(2), 280-289.

Friedenstein, A. J., Piatetzky-Shapiro, I. I., & Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. Development, 16(3), 381-390.

Friedenstein, A. J., Gorskaja, J. F., & Kulagina, N. N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Experimental hematology, 4(5), 267-274.

Friedenstein, A. J., Chailakhyan, R. K., & Gerasimov, U. V. (1987). Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell proliferation, 20(3), 263-272.

Friedenstein, A. J., Chailakhjan, R. K., & Lalykina, K. S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Proliferation, 3(4), 393-403.

Bianco, P., Robey, P. G., & Simmons, P. J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. Cell stem cell, 2(4), 313-319.

Kern, S., Eichler, H., Stoeve, J., Klüter, H., & Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem cells, 24(5), 1294-1301.

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(11), 2739-2749.

Barry, F. P., & Murphy, J. M. (2004). Mesenchymal stem cells: clinical applications and biological characterization. The international journal of biochemistry & cell biology, 36(4), 568-584.

Aggarwal, S., & Pittenger, M. F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood, 105(4), 1815-1822.

Caplan, A. I. (1991). Mesenchymal stem cells. Journal of orthopaedic research, 9(5), 641-650.

Pittenger, M. F., & Martin, B. J. (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. Circulation research, 95(1), 9-20.

Williams, A. R., & Hare, J. M. (2011). Mesenchymal stem cells biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circulation research, 109(8), 923-940.

Dominici, M. L. B. K., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., ... & Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8(4), 315-317.

Ankrum, J. A., Ong, J. F., & Karp, J. M. (2014). Mesenchymal stem cells: immune evasive, not immune privileged. Nature biotechnology, 32(3), 252-260.

Choi, Y. H., Kurtz, A., & Stamm, C. (2010). Mesenchymal stem cells for cardiac cell therapy. Human gene therapy, 22(1), 3-17.
Timmers, L., Lim, S. K., Hoefer, I. E., Arslan, F., Lai, R. C., van Oorschot, A. A., ... & Piek, J. J. (2011). Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. Stem cell research, 6(3), 206-214.

Gallina, C., Turinetto, V., & Giachino, C. (2015). A new paradigm in cardiac regeneration: the mesenchymal stem cell secretome. Stem cells international, 2015, 1-10.
Branco, A. F., Pereira, S. P., Gonzalez, S., Gusev, O., Rizvanov, A. A., & Oliveira, P. J. (2015). Gene Expression Profiling of H9c2 Myoblast Differentiation towards a Cardiac-Like Phenotype. PloS one, 10(6), 1-18.

Takagawa, J., Zhang, Y., Wong, M. L., Sievers, R. E., Kapasi, N. K., Wang, Y., ... & Springer, M. L. (2007). Myocardial infarct size measurement in the mouse chronic infarction model: comparison of area-and length-based approaches. Journal of Applied Physiology, 102(6), 2104-2111.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., ... & Leri, A. (2001). Bone marrow cells regenerate infarcted myocardium. Nature, 410(6829), 701-705.

Orlic, D., Kajstura, J., Chimenti, S., Bodine, D. M., Leri, A., & Anversa, P. (2003). Bone marrow stem cells regenerate infarcted myocardium. Pediatric transplantation, 7(s3), 86-88.

Kania, G. (2011). Do stem cells contribute to heart regeneration or rather to pathological remodelling in inflammatory cardiomyopathy. Cardiovasc. Med., 14, 46-52.

Rodrigues, M., Griffith, L. G., & Wells, A. (2010). Growth factor regulation of proliferation and survival of multipotential stromal cells. Stem cell research & therapy, 1(4), 1-12.

Gnecchi, M., Zhang, Z., Ni, A., & Dzau, V. J. (2008). Paracrine mechanisms in adult stem cell signaling and therapy. Circulation research, 103(11), 1204-1219.

Gnecchi, M., Danieli, P., & Cervio, E. (2012). Mesenchymal stem cell therapy for heart disease. Vascular pharmacology, 57(1), 48-55.

Gnecchi, M., Danieli, P., Malpasso, G., & Ciuffreda, M. C. (2016). Paracrine Mechanisms of Mesenchymal Stem Cells in Tissue Repair. Mesenchymal Stem Cells: Methods and Protocols, 123-146.

Yao, Y., Huang, J., Geng, Y., Qian, H., Wang, F., Liu, X., ... & Dong, J. (2015). Paracrine action of mesenchymal stem cells revealed by single cell gene profiling in infarcted murine hearts. PloS one, 10(6), e0129164.

Burchfield, J. S., & Dimmeler, S. (2008). Role of paracrine factors in stem and progenitor cell mediated cardiac repair and tissue fibrosis. Fibrogenesis & tissue repair, 1(1), 1.

Boyle, A. J., Schulman, S. P., & Hare, J. M. (2006). Stem cell therapy for cardiac repair ready for the next step. Circulation, 114(4), 339-352.

Collins, J. M., & Russell, B. (2009). Stem cell therapy for cardiac repair. The Journal of cardiovascular nursing, 24(2), 93.

Wakitani, S., Saito, T., & Caplan, A. I. (1995). Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle & nerve, 18(12), 1417-1426.

Gojo, S., Gojo, N., Takeda, Y., Mori, T., Abe, H., Kyo, S., ... & Umezawa, A. (2003). In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Experimental cell research, 288(1), 51-59.

Myers, T. J., Granero-Molto, F., Longobardi, L., Li, T., Yan, Y., & Spagnoli, A. (2010). Mesenchymal stem cells at the intersection of cell and gene therapy. Expert opinion on biological therapy, 10(12), 1663-1679.

Russo, V., Young, S., Hamilton, A., Amsden, B. G., & Flynn, L. E. (2014). Mesenchymal stem cell delivery strategies to promote cardiac regeneration following ischemic injury. Biomaterials, 35(13), 3956-3974.

Wu, Y., Chen, L., Scott, P.G., & Tredget, E.E. (2007). Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells, 25(10), 2648-2659.

Das, R., Jahr, H., van Osch, G. J., & Farrell, E. (2009). The role of hypoxia in bone marrow–derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Engineering Part B: Reviews, 16(2), 159-168.

Rochefort, G. Y., Delorme, B., Lopez, A., Herault, O., Bonnet, P., Charbord, P., ... & Domenech, J. (2006). Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem cells, 24(10), 2202-2208.

Ponte, A. L., Marais, E., Gallay, N., Langonne, A., Delorme, B., Herault, O., ... & Domenech, J. (2007). The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem cells, 25(7), 1737-1745.

Daley, G. Q., & Scadden, D. T. (2008). Prospects for stem cell-based therapy. Cell, 132(4), 544-548.

Maher, B. (2013). How to build a heart. Nature, 499(7456), 20-22.

Mironov, V., Boland, T., Trusk, T., Forgacs, G., & Markwald, R. R. (2003). Organ printing: computer-aided jet-based 3D tissue engineering. TRENDS in Biotechnology, 21(4), 157-161.
Segers, V. F., & Lee, R. T. (2008). Stem-cell therapy for cardiac disease. Nature, 451(7181), 937-942.

Song, H., Song, B. W., Cha, M. J., Choi, I. G., & Hwang, K. C. (2010). Modification of mesenchymal stem cells for cardiac regeneration. Expert opinion on biological therapy, 10(3), 309-319.

Gnecchi, M., He, H., Noiseux, N., Liang, O. D., Zhang, L., Morello, F., ... & Dzau, V. J. (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. The FASEB Journal, 20(6), 661-669.

Schulz, R., & Heusch, G. (2009). Tumor Necrosis Factor-α and Its Receptors 1 and 2 Yin and Yang in Myocardial Infarction?. Circulation, 119(10), 1355-1357.

Kubota, T., Miyagishima, M., Frye, C. S., Alber, S. M., Bounoutas, G. S., Kadokami, T., ... & Feldman, A. M. (2001). Overexpression of tumor necrosis factor-α activates both anti-and pro-apoptotic pathways in the myocardium. Journal of molecular and cellular cardiology, 33(7), 1331-1344.

Al-Lamki, R. S., Lu, W., Wang, J., Yang, J., Sargeant, T. J., Wells, R., ... & Lebastchi, A. H. (2013). TNF, acting through inducibly expressed TNFR2, drives activation and cell cycle entry of c-Kit+ cardiac stem cells in ischemic heart disease. Stem Cells, 31(9), 1881-1892.

Tian, M., Yuan, Y. C., Li, J. Y., Gionfriddo, M. R., & Huang, R. C. (2015). Tumor necrosis factor-α and its role as a mediator in myocardial infarction: A brief review. Chronic Diseases and Translational Medicine, 1(1), 18-26.

Papathanasiou, S., Rickelt, S., Soriano, M. E., Schips, T. G., Maier, H. J., Davos, C. H., ... & Capetanaki, Y. (2015). Tumor necrosis factor-[alpha] confers cardioprotection through ectopic expression of keratins K8 and K18. Nature medicine, 21(9), 1076–1084.

Higuchi, Y., Chan, T. O., Brown, M. A., Zhang, J., DeGeorge, B. R., Funakoshi, H., ... & Feldman, A. M. (2006). Cardioprotection afforded by NF-κB ablation is associated with

activation of Akt in mice overexpressing TNF-α. American Journal of Physiology-Heart and Circulatory Physiology, 290(2), H590-H598.

Sharma, H. S., & Das, D. K. (1997). Role of cytokines in myocardial ischemia and reperfusion. Mediators of inflammation, 6(3), 175-183.

Feldman, A. M., Combes, A., Wagner, D., Kadakomi, T., Kubota, T., Li, Y. Y., & McTiernan, C. (2000). The role of tumor necrosis factor in the pathophysiology of heart failure. Journal of the American College of Cardiology, 35(3), 537-544.

Chiosi, E., Spina, A., Sorrentino, A., Romano, M., Sorvillo, L., Senatore, G., ... & Illiano, G. (2007). Change in TNF-α receptor expression is a relevant event in doxorubicin-induced H9c2 cardiomyocyte cell death. Journal of Interferon & Cytokine Research, 27(7), 589-598.

Lacerda, L., McCarthy, J., Mungly, S. F., Lynn, E. G., Sack, M. N., Opie, L. H., & Lecour, S. (2010). TNFα protects cardiac mitochondria independently of its cell surface receptors. Basic research in cardiology, 105(6), 751-762.

Bao, C., Guo, J., Lin, G., Hu, M., & Hu, Z. (2008). TNFR gene-modified mesenchymal stem cells attenuate inflammation and cardiac dysfunction following MI. Scandinavian Cardiovascular Journal, 42(1), 56-62.

Dobaczewski, M., Chen, W., & Frangogiannis, N. G. (2011). Transforming growth factor (TGF)-β signaling in cardiac remodeling. Journal of molecular and cellular cardiology, 51(4), 600-606.

Eiselleova, L., Matulka, K., Kriz, V., Kunova, M., Schmidtova, Z., Neradil, J., ... & Dvorak, P. (2009). A Complex Role for FGF-2 in Self-Renewal, Survival, and Adhesion of Human Embryonic Stem Cells. Stem Cells, 27(8), 1847-1857.

Zisa, D., Shabbir, A., Suzuki, G., & Lee, T. (2009). Vascular endothelial growth factor (VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal stem cell-mediated cardiac repair. Biochemical and biophysical research communications, 390(3), 834-838.

Zisa, D., Shabbir, A., Mastri, M., Suzuki, G., & Lee, T. (2009). Intramuscular VEGF repairs the failing heart: role of host-derived growth factors and mobilization of progenitor cells. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 297(5), R1503-R1515.

Hausenloy, D. J., & Yellon, D. M. (2009). Cardioprotective growth factors. Cardiovascular research, 83 (2), 174-194.

Sandhu, R., Teichert-Kuliszewska, K., Nag, S., Proteau, G., Robb, M. J., Campbell, A. I., ... & Stewart, D. J. (2004). Reciprocal regulation of angiopoietin-1 and angiopoietin-2 following myocardial infarction in the rat. Cardiovascular Research, 64(1), 115-124.

Thurston, G., & Daly, C. (2012). The complex role of angiopoietin-2 in the angiopoietin–tie signaling pathway. Cold Spring Harbor perspectives in medicine, 2(9), a006650.

Daly, C., Pasnikowski, E., Burova, E., Wong, V., Aldrich, T. H., Griffiths, J., ... & McDonald, D. M. (2006). Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proceedings of the National Academy of Sciences, 103(42), 15491-15496.

Fiedler, U., & Augustin, H. G. (2006). Angiopoietins: a link between angiogenesis and inflammation. Trends in immunology, 27(12), 552-558.

DeLisser, H. M., Christofidou-Solomidou, M., Strieter, R. M., Burdick, M. D., Robinson, C. S., Wexler, R. S., ... & Albelda, S. M. (1997). Involvement of endothelial PECAM-1/CD31 in angiogenesis. The American journal of pathology, 151(3), 671.

Lertkiatmongkol, P., Liao, D., Mei, H., Hu, Y., & Newman, P. J. (2016). Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). Current opinion in hematology, 23(3), 253-259.

Scholzen, T., & Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. Journal of cellular physiology, 182(3), 311-322.

Tang, Y. L., Zhu, W., Cheng, M., Chen, L., Zhang, J., Sun, T., ... & Qin, G. (2009). Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circulation research, 104(10), 1209-1216.

Yu, X., Lu, C., Liu, H., Rao, S., Cai, J., Liu, S., ... & Ding, X. (2013). Hypoxic preconditioning with cobalt of bone marrow mesenchymal stem cells improves cell migration and enhances therapy for treatment of ischemic acute kidney injury. PloS one, 8(5), 1-12.

Wang, M., Zhang, W., Crisostomo, P., Markel, T., Meldrum, K. K., Fu, X. Y., & Meldrum, D. R. (2007). STAT3 mediates bone marrow mesenchymal stem cell VEGF production. Journal of molecular and cellular cardiology, 42(6), 1009-1015.

Liu, J., Hao, H., Xia, L., Ti, D., Huang, H., Dong, L., ... & Fu, X. (2015). Hypoxia pretreatment of bone marrow mesenchymal stem cells facilitates angiogenesis by improving the function of endothelial cells in diabetic rats with lower ischemia. PloS one, 10(5), 1-18.

Kelly, M. L., Wang, M., Crisostomo, P. R., Abarbanell, A. M., Herrmann, J. L., Weil, B. R., & Meldrum, D. R. (2010). TNF receptor 2, not TNF receptor 1 enhances mesenchymal stem cell-mediated cardiac protection following acute ischemia. Shock (Augusta, Ga.), 33(6), 602.

Lee, M. J., Kim, J., Kim, M. Y., Bae, Y. S., Ryu, S. H., Lee, T. G., & Kim, J. H. (2010). Proteomic analysis of tumor necrosis factor-α-induced secretome of human adipose tissue-derived mesenchymal stem cells. Journal of proteome research, 9(4), 1754-1762.

Wen, Z., Zheng, S., Zhou, C., Wang, J., & Wang, T. (2011). Repair mechanisms of bone marrow mesenchymal stem cells in myocardial infarction. Journal of cellular and molecular medicine, 15(5), 1032-1043.

Ferrara, N., & Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. Endocrine reviews, 18(1), 4-25.

APPENDIX

Table 6. Number of Migrated rBM-MSCs per Treatment/Well

Transwell Migration Assay																
Treatments																
		Controls												TNF-a & Hypoxia-induced Secretome		
		DMEM No Serum (Negative Control)			SDF-1 (Positive Control)			Sample Controls								
								DMEM No Serum +TNF-a			Secretome Control					
	Slide #	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Number of Cells		0	2	1	1046	1424	1075	96	110	120	410	405	563	881	1061	724
Average Number of Cells that Migrated		1			1181.666667			108.6666667			459.3333333			888.6666667		

Table 6. The raw data acquired from the trans-well cell migration assay. The average number of rBM-MSCs that migrated in response to the secretome treatment or control treatments have been calculated. Each treatment group was conducted in triplicates. Images were taken using 20X objective.

Table 7. Live Dead Annexin V Raw Data – FACS ANALYSIS - % of Apoptotic Cells

Cell Survival - Live Dead Annexin V Assay Raw Data -FACS Results					
	rCM (Normoxia)	rCM (Hypoxia)	rCM + TNF-a Cytokine (Hypoxia)	rCM + Control Secretome (Hypoxia)	rCM+ TNF- a Hypoxia-Induced Secretome (Hypoxia)
	24.22	39.8	61.6	21.07	44.8
	22.7	37.1	62.1	20.11	42.2
	19.33	39.8	64.6	18.8	43.2
Average % of Apoptotic Cells	22.08333333	38.9	62.76666667	19.99333333	43.4
	rCM (Normoxia)	rCM (Hypoxia)	rCM + TNF-a Cytokine (Hypoxia)	rCM + Control Secretome (Hypoxia)	rCM + TNF- a Hypoxia-Induced Secretome (Hypoxia)
	0.111	0.449	0.73	0.468	0.796
	0.431	0.674	0.9	0.421	0.719
	0.372	0.495	0.29	0.825	1.14
Average % of Necrotic Cells	0.304666667	0.539333333	0.64	0.571333333	0.885
Average % of Live Cells	77.612	60.56066667	36.59333333	79.43533333	55.715
	rCM(Normoxia)	rCM (Hypoxia)	rCM + TNF-a Cytokine (Normoxia)	rCM+ Control Secretome (Normoxia)	rCM + TNF- a Hypoxia-Induced Secretome (Normoxia)
	24.22	39.8	46.7	17.54	33.1
	22.7	37.1	42.9	18.83	34.6
	19.33	39.8	52.8	18.12	34.9
Average % of Apoptotic Cells	22.08333333	38.9	47.46666667	18.16333333	34.2
	rCM (Normoxia)	rCM (Hypoxia)	rCM + TNF-a Cytokine (Normoxia)	rCM + Control Secretome (Normoxia)	rCM + TNF- a Hypoxia-Induced Secretome (Normoxia)
	0.111	0.449	0.73	0.064	4.26
	0.431	0.674	0.9	0.0745	0.64
	0.372	0.495	0.29	0.225	0.621
Average % of Necrotic Cells	0.304666667	0.539333333	0.64	0.121166667	1.840333333
Average % of Live Cells	77.612	60.56066667	51.89333333	81.7155	63.95966667

Table 7. The raw data acquired from the FACS analysis of the Cell Survival- Annexin V Apoptosis Assay. rCM treated in one of eight different conditions under hypoxia (16 hours) were analyzed to see if the treatments initiated cell survival mechanisms and prevented apoptosis. The groups were examined in triplicates and the average percentage of apoptotic cells were calculated and compared between the groups to determine the percentage of cells being rescued by the treatments. The average percentage of necrotic and live cells in each group were also calculated.

Table 8. Echocardiography Raw Data – LVFS (%) of the Lewis Rats

		Echocardiography Raw Data - LVFS % in Lewis Rats		
	Baseline (No Infarction)	Infarction (No Treatment)	Control Secretome	TNF-a Hypoxia-Induced Secretome
	41.73	22.37	32.05128	42.76
	42.65	20.55	25.37	35.22
	43.27	21.8	28.91	37.10342
Average LVFS(%)	42.55	21.57333333	28.77709333	38.36114

Table 8. The raw LVFS data attained from four groups of Lewis rats at baseline and from induced infarcted rats not treated or treated with secretome. Each group was done in triplicates.

Table 9. Average Infarct Size (%)

		Average Area of Infarction for each Transverse Cut Masson's Trichome Stained Lewis Rat Heart Section				
Section #	Average Area of each Cut Section	Average Infarct Area	Average Epicardial Area	Final Area= Section Full Area-Epicardial Area		Infarct Area/Final Area *100
TNF-a Hypoxia-Induced Secretome Treated						
1	3.6415	0.1515	0.1045		3.537	4.283290925
2	3.845	0.054	0.2895		3.5555	1.518773731
3	3.8175	0.0635	0.242		3.5755	1.775975388
4	3.653333333	0.084	0.175666667		3.477666667	2.415412633
5	3.708	0.086	0.2345		3.4735	2.475888873
6	3.6055	0.0525	0.339		3.2665	1.607224858
					AVERAGE Infarct Size (%)	2.346094401
Control Secretome Treated						
1	1.6295	0.232	0.245		1.3845	16.75695197
2	1.67825	0.285	0.24125		1.437	19.83298539
3	1.8355	0.25	0.2545		1.581	15.81277672
4	1.648	0.2215	0.2565		1.3915	15.91807402
5	1.756	0.245666667	0.295		1.461	16.81496692
6	1.850333333	0.249	0.260333333		1.59	15.66037736
					AVERAGE Infarct Size (%)	16.7993554

Table 9. The calculation of the infarct sizes from 12 transverse cut heart sections from Lewis rats treated with the Control or TNF-a Hypoxia-Induced secretome post-MI is listed above. Image J was use to calculate the area of each infarct versus the area of the full section in

order to calculate the % infarct Size. Each of the six sections lie throughout the infarct zone (top to apex).

Table 10. Raw Data of the Number of Vessels Formed Per Optical Field Per Treatment

Immunohistochemistry	20X objective					
Antibody		Secretome Treatment	Area of tissue	Slide Number	Number of Vessels Per Optical Field	Number of existing Vessls
CD31						
		Control	Healthy	1	3	5
		Control	Healthy	2	3	1
		Control	Healthy	3	2	3
	Average # of Vessels Per Optical Field			2	2.666666667	3
		Control	Infarct	1	0	0
		Control	Infarct	2	2	3
		Control	Infarct	3	1	4
	Average # of Vessels Per Optical Field			2	1	2.333333333
		TNF-a + Hypoxia Induced	Healthy	1	1	3
		TNF-a + Hypoxia Induced	Healthy	2	0	3
		TNF-a + Hypoxia Induced	Healthy	3	3	5
	Average # of Vessels Per Optical Field			2	1.333333333	3.666666667
		TNF-a + Hypoxia Induced	Infarct	1	9	3
		TNF-a + Hypoxia Induced	Infarct	2	8	1
		TNF-a + Hypoxia Induced	Infarct	3	12	6
	Average # of Vessels Per Optical Field			2	9.666666667	3.333333333

Table 10. The raw data acquired from counting the number of vessels per optical field between the Control and the TNF-a Hypoxia-Induced secretome treated rats post-MI. The sections were transverse cut and stained with CD31 to identify vessels. Vessels were counted in healthy, transition, and infarcted regions of each tissue section at a 20X objective. Each treatment was done in triplicates. Vessels were counted blindly.