Cardiac Repair Using Stem Cells Conditioned Media Based Therapy In a Rodent Model of Myocardial Infarction and Heart Failure

By
Mohammad Talal Alrefai

Supervisor: Dr. Dominique Shum-Tim
Co-Supervisor: Dr. Adel Schwertani

Department of Surgery
Division of Cardiac Surgery
Division of Experimental Surgery
McGill University, Montreal
Quebec, Canada

JULY 2017

A thesis submitted to McGill University in partial fulfillment of the requirement of the degree of

Master of Science in Experimental Surgery

TABLE OF CONTENTS

ABSTRACT

Objectives:

Myocardial infarction (MI) is the most common cause of congestive heart failure (CHF). Both diseases are associated with significant morbidity and mortality. Cell-based therapy has demonstrated variable degrees of success in the treatment of myocardial infarction and heart failure. Recently, a major advancement in the stem cell field has been made after the introduction of human induced pluripotent stem cells (HiPSCs) as it carried the advantage of non-invasive harvesting methods over the other types of cell based therapy. Here, we investigated the effects of conditioned media (CM) of HiPSCs and mesenchymal stem cells (MSC) on the cardiac function and remodeling of experimental MI and CHF rat model. It was *hypothesized that signaling molecules VEGF and PDGF present in cell conditioning media would have angiogenesis and neovascularization properties to induce improvement in myocardial function and reduction in scar size after MI.*

Methods:

In-vitro preparations: HiPSCs and MSCs were cultured in DMEM containing 5% pluripotent stem cell growth supplement and DMEM alone, respectively. Cells were incubated for 24 hours in the conditioning medium in CO2 chamber at 37C°, thereafter conditioning media was collected and stored in -80C° for further use.

In-vivo preparations: A total of 30 female Lewis rats underwent surgical ligation of the left anterior descending coronary artery through a left thoracotomy. Animals were randomized (n=10/group) to receive one of three treatments injected into the peri-infarct area; normal saline (NS), HiPSC (HiPSC CM) and MSC (MSC CM). Group NS, received (500 μL) of normal saline, group HiPSC and MSC received intramyocardial injection of HiPSCs-CM (500 μL) or MSC-CM, respectively injected into the peri-infarcted zone 15 minutes after the ligation. Left ventricular ejection fraction (LVEF), fractional shortening (FS), histology was evaluated in a blinded fashion, at pre-op day 0 then at 1, 2, 4 and 6 weeks and serum proteomics were evaluated at 2 weeks.

Results:

Echocardiography showed a significant improvement of EF and FS in both group HiPSC and MSC compared to group NS ($P<0.05$) at week 6. There was no significant difference between groups HiPSC and MSC over time. Scar size was significantly smaller in group HiPSC compared to groups NS or MSC ($P<0.05$) at week 6. Group MSC had significantly less scar than group NS ($P<0.05$) at week 6. There was a significant increase in CD31 immunostaining in-groups HiPSC and MSC compared to group NS ($P<0.05$) at week 6. Proteomic analysis which was done at week 2 revealed abundant presence of VEGF in the serum of group HiPSC that was absent in groups NS and MSC.

Conclusion:

The current study demonstrated a significant improvement in cardiac function and remodeling in response to CM from HiPSCs and MSCs compared to that of control. The utilization of culture media may avoid the potential malignant transformation or inadvertent effects of the presence of implanted cells of cell-based therapies.

RÉSUMÉ

Objectif

L'infarctus du myocarde (IM) est la cause la plus fréquente d'insuffisance cardiaque congestive (ICC). Les deux maladies sont associées à une morbidité et à une mortalité significative. La thérapie cellulaire a démontré des degrés de succès variables dans le traitement de l'infarctus du myocarde et de l'insuffisance cardiaque. Récemment, une avancée majeure dans le domaine des cellules souches a été réalisée après l'introduction de cellules souches pluripotentes induites par l'homme (HiPSC) car elle a porté l'avantage des méthodes de récolte non invasives par rapport aux autres types de thérapie à base de cellules. Ici, nous avons étudié les effets des médias conditionnés (CM) des HiPSC et des cellules souches mésenchymateuses (MSC) sur la fonction cardiaque et le remodelage du modèle expérimental d'IM et de rat CHF. On a émis l'hypothèse que les molécules de signalisation VEGF et PDGF présentes dans les milieux de conditionnement cellulaire auraient des propriétés d'angiogenèse et de néovascularisation pour induire une amélioration de la fonction myocardique et une réduction de la taille de la cicatrice après l'IM.

Méthodes

Préparations in vitro : HiPSCs et MSC ont été cultivés dans du DMEM contenant 5% de suppléments de croissance de cellules souches pluripotentes et DMEM seul, respectivement. Les cellules ont été incubées pendant 24 heures dans le milieu de conditionnement dans une chambre de CO2 à 37 ° C, puis on a recueilli les milieux de conditionnement et on les a conservés dans -80 ° C pour une utilisation ultérieure. Préparations in vivo : Au total, 30 rats Lewis ont subi une ligature chirurgicale de l'artère coronaire descendante antérieure par une thoracotomie gauche. Les animaux ont été randomisés (n = 10 / groupe) pour recevoir l'un des trois traitements injectés dans la zone péri-infarctale ; La solution saline normale (NS), HiPSC (HiPSC CM) et MSC (MSC CM). Le groupe NS, reçu (500 µl) de solution saline normale, groupe HiPSC et MSC a reçu une injection intramyocardiale de HiPSCs-CM (500 µL) ou MSC-CM, respectivement injectée dans la zone péri-inférée 15 minutes après la ligature. La fraction d'éjection ventriculaire gauche (FEVI), le raccourcissement fractionnaire (FS),

l'histologie ont été évalués de manière aveugle, au jour pré-op, puis à 1, 2, 4 et 6 semaines et la protéomique sérique a été évaluée à 2 semaines.

Résultats

L'échocardiographie a montré une amélioration significative de l'EF et du FS dans le groupe HiPSC et MSC par rapport au groupe NS (P <0,05) à la semaine 6. Il n'y avait pas de différence significative entre les groupes HiPSC et MSC avec le temps. La taille de la cicatrice était significativement plus faible dans le groupe HiPSC par rapport aux groupes NS ou MSC (P <0,05) à la semaine 6. Le groupe MSC avait nettement moins de cicatrices que le groupe NS (P <0,05) à la semaine 6. Il y avait une augmentation significative de l'immunocoloration CD31 dans -groupes HiPSC et MSC par rapport au groupe NS (P <0,05) à la semaine 6. L'analyse protéomique qui a été effectuée à la semaine 2 a révélé une présence abondante de VEGF dans le sérum du groupe HiPSC qui était absent dans les groupes NS et MSC.

Conclusion

L'étude actuelle a démontré une amélioration significative de la fonction cardiaque et du remodelage en réponse au CM des HiPSC et des MSC par rapport à celui du contrôle. L'utilisation des milieux de culture peut éviter la transformation maligne potentielle ou les effets involontaires de la présence de cellules implantées de thérapies à base de cellules.

ACKNOWLEDGMENT

First and foremost, I would like to express my deepest appreciation to my supervisor Dr. Dominique Shum-Tim for his support and guidance throughout, and for allowing me the opportunity to pursue my goals in advancing my academic career and knowledge in research at this respectable institute.

I would also like to thank my co-supervisor Dr. Adel Schwertani for his kind help and valuable advice that made this experience an easier journey.

My gratitude also extends to the members of my master's committee for their assistance and for taking the time from their busy daily schedules to attend my meetings.

I have had a great experience working at the Cardiac Surgery Research Laboratory at the Montreal General Hospital, mainly due to the assistance from members of my own lab and other labs.

I would like to thank (Mrs. Minh Duong) who has been an invaluable addition to our lab with her great supervision and guidance.

CONTRIBUTION OF AUTHORS

MT ALREFAI[1-2], CL TAROLA[3], R. RAAGAS[1], K. RIDWAN[1], M. SHALAL[1], N LOMIS[4], A PAUL[5], S. PRAKASH[4], A. SCHWERTANI[1], D. SHUM-TIM[1].

[1]Divisions of Cardiac Surgery and Cardiology, McGill University Health Center, Montreal, QC, Canada; [2]King Faisal Specialist Hospital and Research Center, Jeddah, Saudi Arabia; [3]Division of cardiac surgery, University of Western Ontario [4]Division of Biomedical Engineering, McGill University Health Center, Montreal, QC, Canada. [5]Department of Chemical and Petroleum Engineering, School of Engineering, University of Kansas, Lawrence, KS, USA.

Disclosure

This research is in-part supported by FRSQ-The Cell grant for Dr. D Shum-Tim, by a CIHR research grant for Dr. Adel Schwertani and by Saudi ministry of education / the Saudi cultural bureau in Canada / King Faisal specialist hospital and research center for Dr M Alrefai.

DEDICATIONS

I dedicate this work to my loving mother and father Dr. Nahla Alrefai and Prof. Talal Alrefai and to my beloved wife Hibah Altowairqi, my wonderful children Nahla, Tala and Talal and my amazing brothers Moaid, Ahmad, Jameel, Mahmoud and Abdullah.

LIST OF TABLES

Table 1: *Difference in quantity of VEGF and PDGF in the culture medium in each group at 0 (baseline) and 60 minutes.*

	0 min		60 min	
Comparison	**Mean Difference* [SD]**	**P-value**	**Mean Difference* [SD]**	**P-value**
		VEGF		
MSC-CM VS HIPSC-CM	0.001 [0.02]	0.9611	0.173 [0.096]	0.0016**
MSC-CM VS MSC-PRE	0.382 [0.03]	0.0006**		
HIPSC-CM VS HIPSC-PRE	0.388 [0.01]	<0.0001**		
HIPSC-CM VS HIPSC-PRE-R	0.268 [0.01]	<0.0001**		
		PDGF		
MSC-CM VS HIPSC-CM	0.83 [0.03]	0.0562	0.263 [0.157]	0.022**
MSC-CM VS MSC-PRE	0.013 [0.02]	0.6430		
HIPSC-CM VS HIPSC-PRE	0.069 [0.01]	0.0327**		
HIPSC-CM VS HIPSC-PRE-R	0.073 [0.01]	0.0223**		

*mean difference represents mean difference in concentration between two samples (ng/mL)
**Statistically significant

Table 2: *Pre-operative characteristics and post-operative changes in left ventricular ejection fraction and fractional shortening.*

	Pre-operative Ejection Fraction			**Pre-Operative Fractional Shortening**		
Group	**Mean (%)**	**SD**	**P-value**	**Mean (%)**	**SD**	**P-value**
NS	74.6	8.5		39.0	8.1	
HiPSC	73.2	1.7	0.928*	37.1	1.5	0.849*
MSC	73.6	2.1	0.967*	37.5	1.8	0.903*
	Post-operative Ejection Fraction			**Post-Operative Fractional Shortening**		
NS	34.7	3.5	$<0.0001^{+}$	14.02	1.6	$<0.0001^{+}$
HiPSC	35.7	2.7	$<0.0001^{+}$	14.80	1.3	$<0.0001^{+}$
MSC	33.2	4.5	$<0.0001^{+}$	13.51	2.1	$<0.0001^{+}$

**p-value identifies significant difference between treatment groups compared to control.*

$^{+}$p-value identifies significant difference between pre- and post-operative measurements within the same group

LIST OF FIGURES

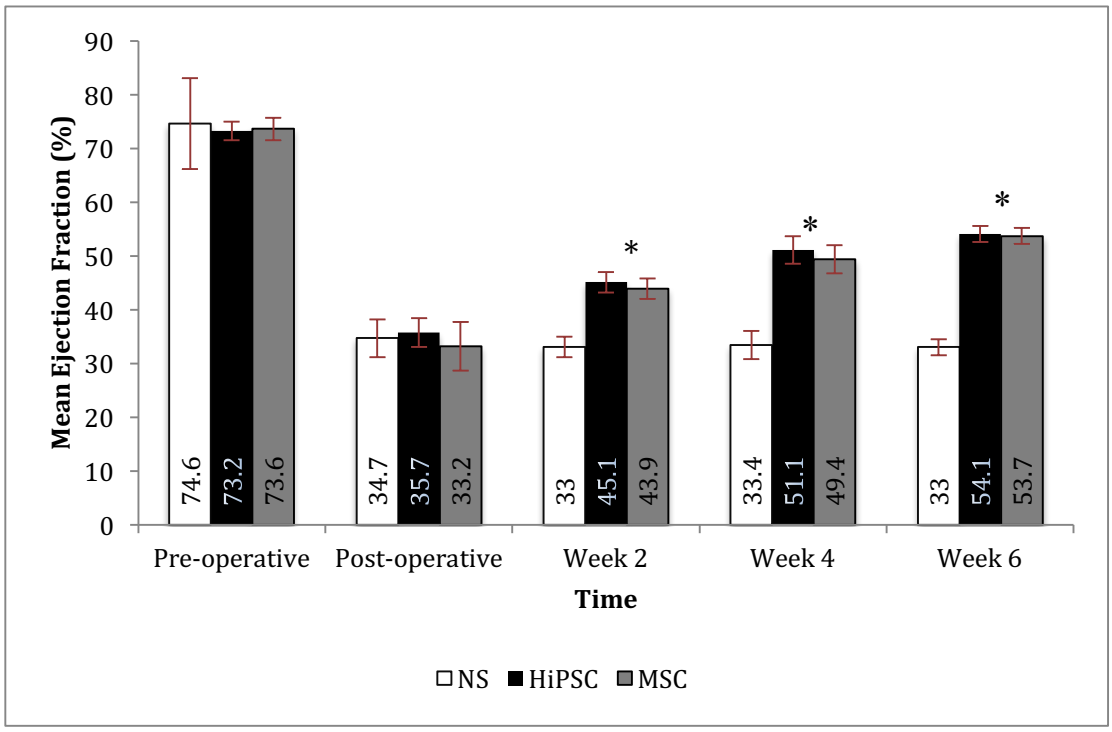


Figure 1: Change in mean ejection fraction over time across treatment groups.
* indicates significant difference between both MSC and HiPSC compared to control.

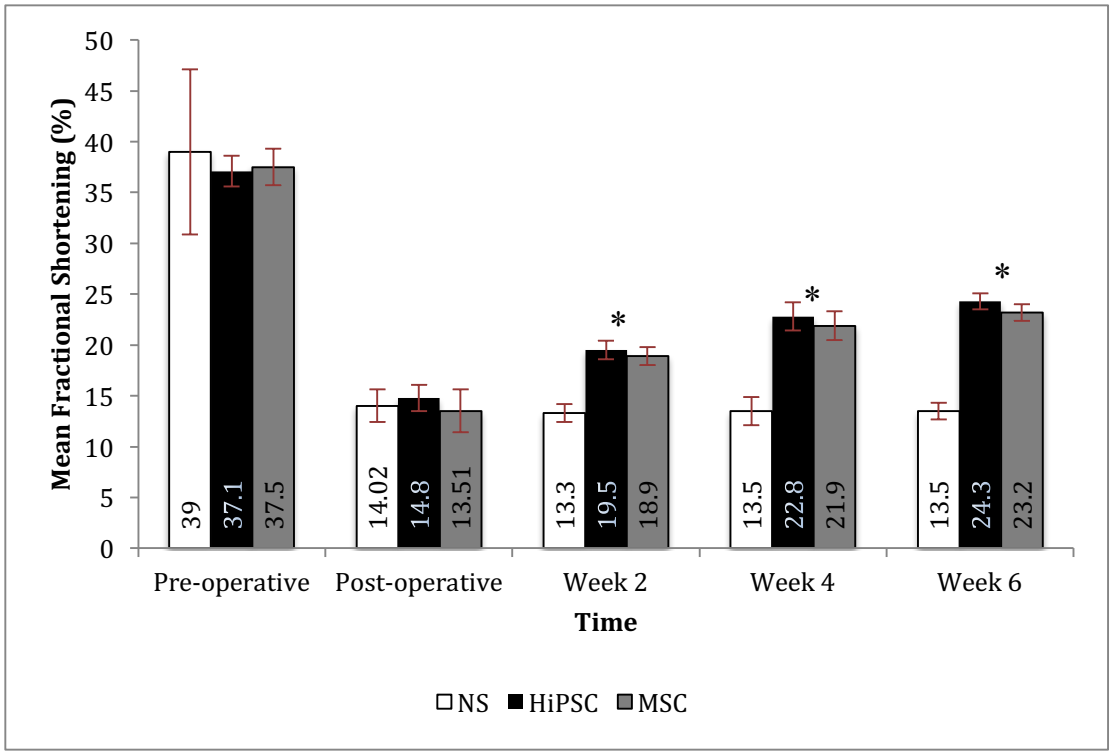


Figure 2: Change in mean fractional shortening over time across treatment groups.
* indicates significant difference between both MSC and HiPSC compared to control.

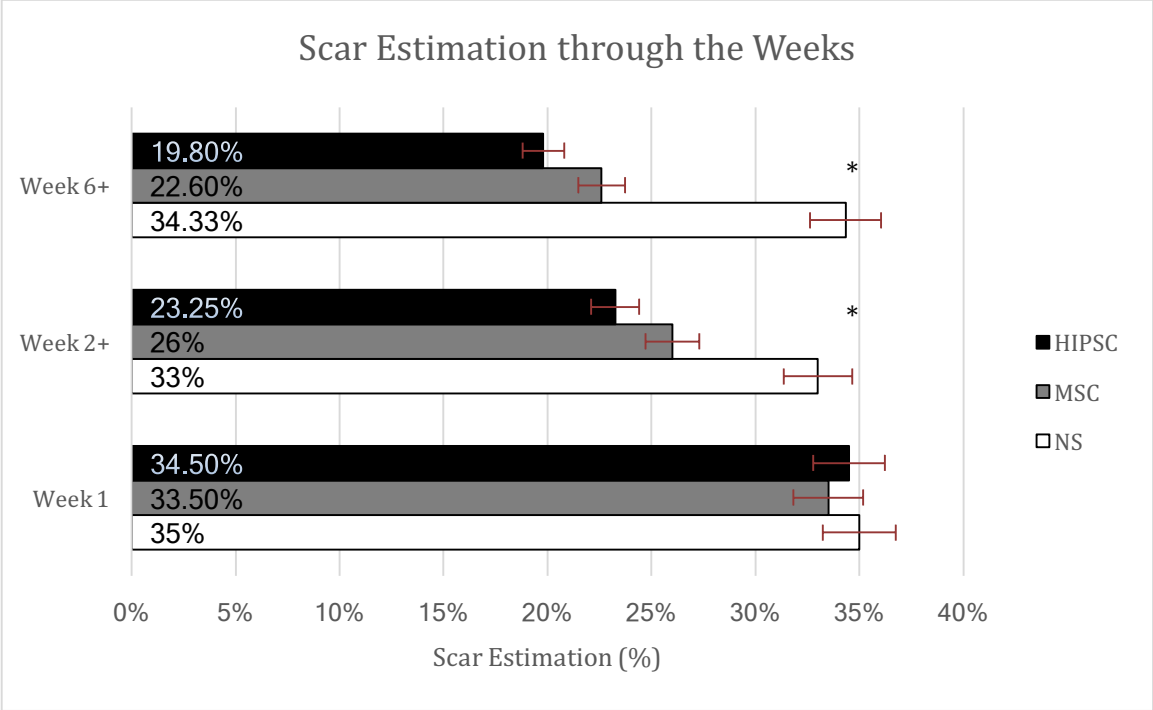


Figure 3: Estimated scar size post-operatively and following treatment at weeks 2+ (2-4) and 6+ (6-8). * indicates significant difference between both MSC and HiPSC compared to control as well between HiPSC and MSC Groups.

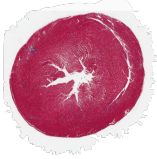
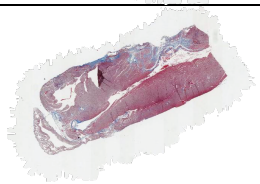
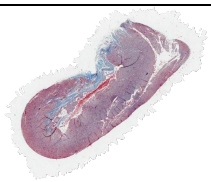
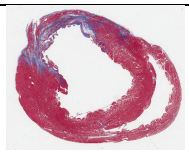
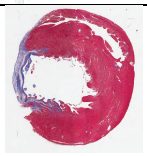
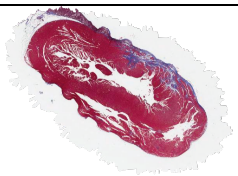
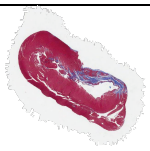
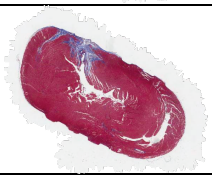
Group	Week 1	Week 2+	Week 6+
Sham			
NS			
HiPSC			
MSC			

Figure 4: Histological scar area post-operatively and after treatment at weeks 2-4 (2+) and 6-8 (6+).

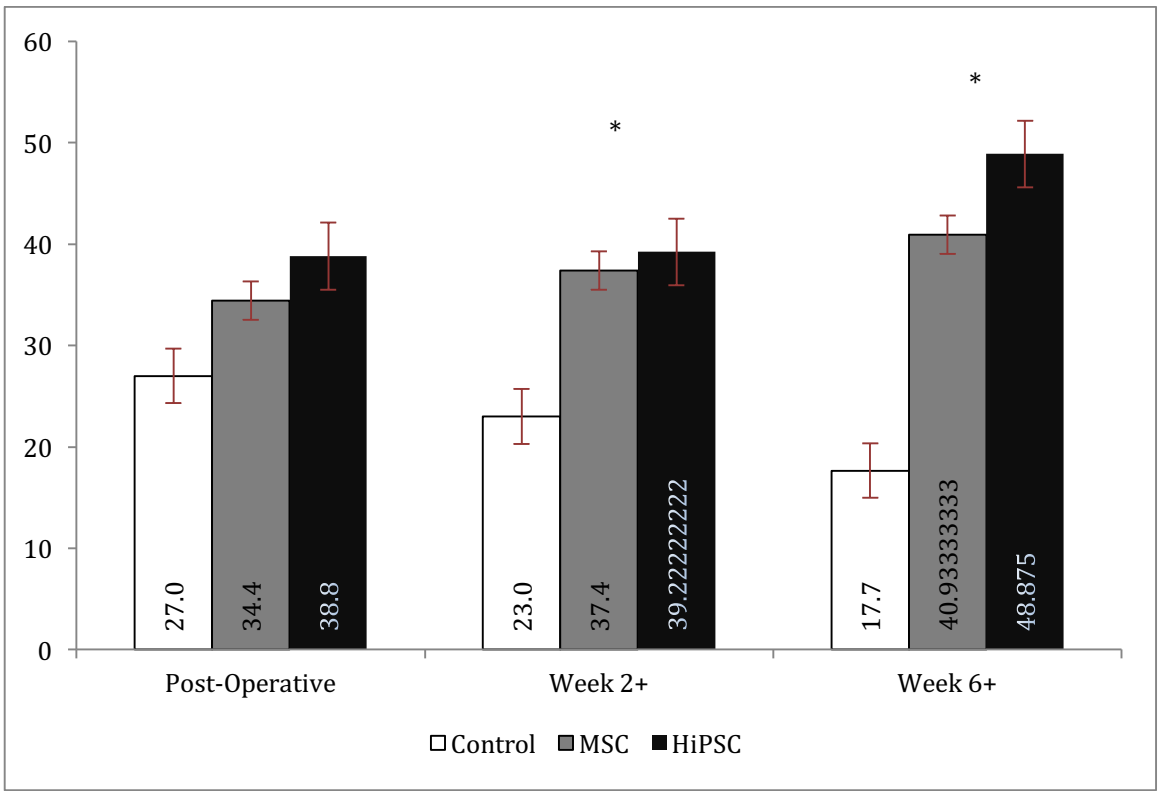
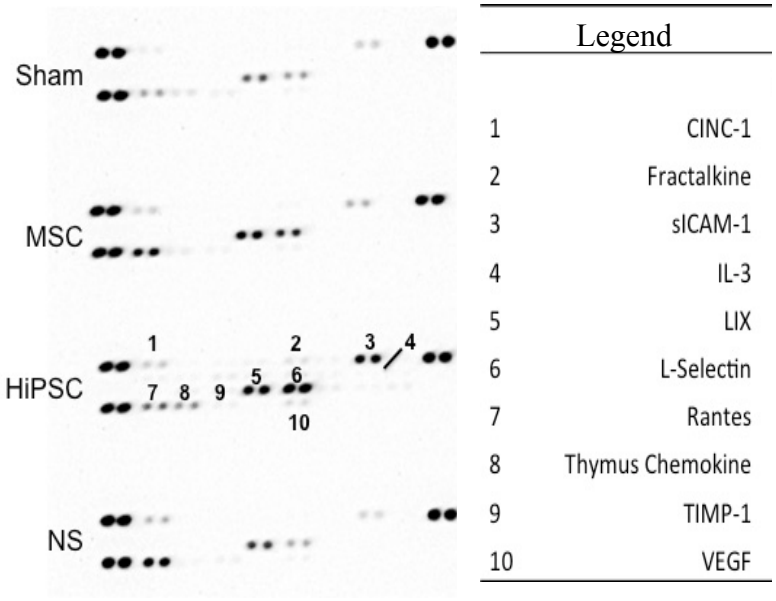


Figure 5: CD31 immunostaining to identify peri-infarct endothelial cell density post-infarct.



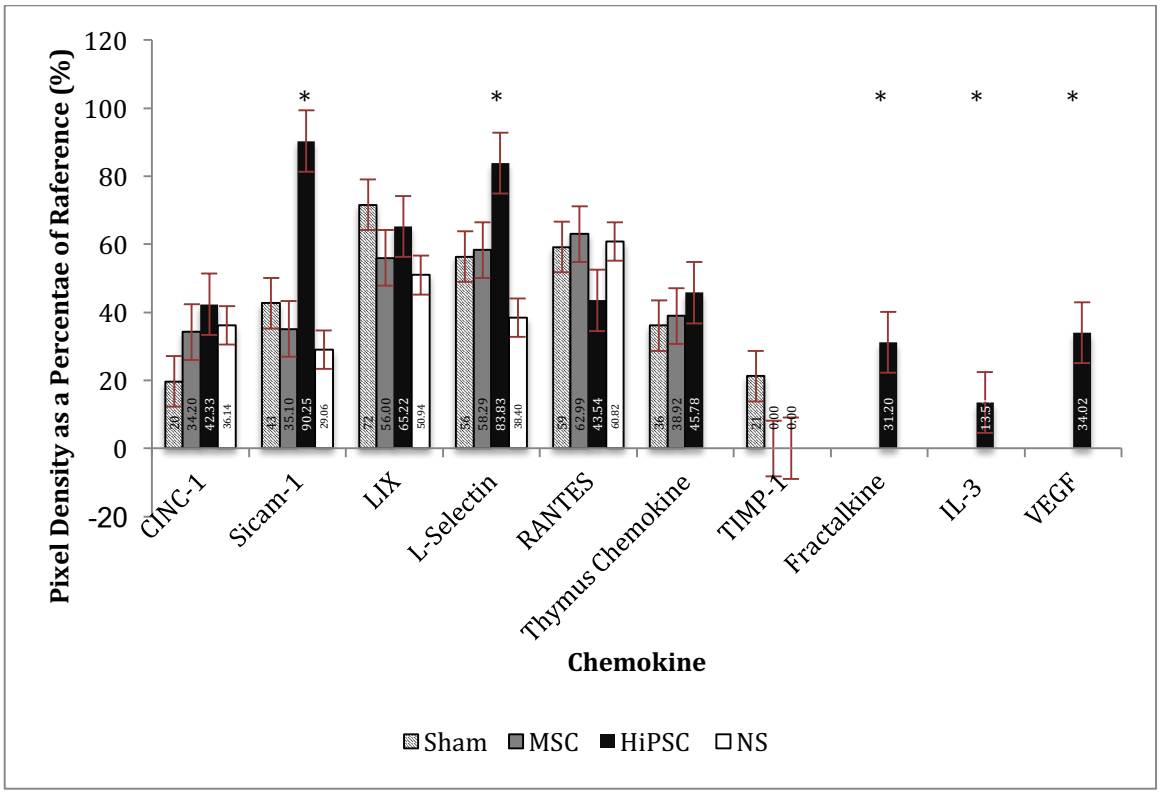


Figure 6: Rat cytokine array for **s**ham, control (NS), and treatment group (MSC, HiPSC) rats. * indicate high concentration and statistical significance of HiPSC in compare to the other groups.

LIST OF ABBREVIATIONS

American Type Culture Collection (ATCC)

Avidin-horseradish peroxidase (HRP)

Congestive heart failure (CHF)

Cytokine induced neutrophil chemo-attractant (CINC-1)

Dulbecco's modified eagle's medium (DMEM)

Fetal bovine serum (FBS)

Human induced pluripotent stem cells (HiPSC)

L-selectin (CD62L)

Left Ventricle end-diastolic diameters (LVEDD)

Left Ventricle end-systolic diameters (LVESD)

Left Ventricular end-diastolic volume (LVEDV)

Left Ventricular end-systolic volume (LVESV)

Lipopolysaccharide-induced CXC *chemokine (LIX)*

Mesenchymal stem cells (MSC)

Myocardial infarction (MI**)**

Phosphate-buffered saline (PBS)

Platelet-derived growth factor (PDGF)

Soluble intercellular adhesion molecule-1 (SICAM-1)

Vascular endothelial cell growth factor (VEGF)

Octamer binding transcription factor-4 (OCT-4)

Sex determining region Y)-box 2 (SOX2)

Kruppel Like Factor-4 (KLF4)

CHAPTER 1: INTRODUCTION

1.1 Ischemic Heart Disease

Ischemic heart disease (IHD) or Myocardial infarction (MI) is among the most common causes of congestive heart failure (CHF), contributing to the significant worldwide morbidity and mortality associated with cardiovascular disease[1]. Following MI, contractile cardiomyocytes became necrotic and are replaced by non-contractile fibroblasts and collagen-rich scar tissue, resulting in thin ventricular walls, decreased ejection fraction, and heart failure. Although some evidence exists demonstrating age-dependent cardiomyocyte annual turnover (between 0.45% - 1%) and limited regeneration post-MI, this response to inflammation is not clinically significant[2-4]. Several investigations, however, have demonstrated cardiomyocyte mitotic indices of 0.015 to 0.08% in heart failure and post-infarct specimens, offering evidence that challenges the notion that the heart is a post-mitotic organ and suggests there may be a myocyte subpopulation that remains subtotally differentiated[4-6].

Recently, there has been interest in the utilization of stem cells to enhance cardiomyocyte regeneration and ventricular remodeling post-MI and in heart failure patients. A number of autologous cell lines, to reduce the risk of allogenic rejection, have been investigated, including bone-marrow derived stem cells, resident cardiac stem cells, skeletal myoblasts, and adipose derived stem cells. These cell lines have been administered transendocardially, transepicardially, and via the coronary arteries in both human and animal models, to varying degrees of success[7-23]. In animal models, these cell-based therapies have demonstrated effectiveness in reducing infarct size and ventricular dilatation, and improving myocardial function[24-26]. Similar results have been demonstrated in human models, however clinical translation is limited[8,13,19]. Primarily, the ideal source of human cardiomyocyte progenitors remains unidentified, though further understanding of the differentiation of embryonic stem cells and human induced pluripotent stem cells (HiPSCs) into cardiomyocytes is curbing this concern[27,28]. Additionally, adverse events including rhythm disturbances and teratoma/teratocarcinoma formation have been identified utilizing stem cell grafting techniques, hampering this

methodology[29-32].

Signaling factors are one of the main elements needed for stem cell function. The paracrine mechanism of function suggests that stem cells secrete multiple complementary cellular pathways, promoting different cellular functions include anti-apoptosis, angiogenesis, and attenuation of fibrosis. Previous investigations have endeavored to exploitate this mechanism using cell lysates/extracts to improve cardiac function post-MI and angiogenesis. Grossman and colleagues injected bone marrow cell extracts into peri-infarct myocardium using a mouse model, and several investigators have isolated microparticles and extracellular vesicles to promote neovascularization[33-35]. This paracrine mechanism represents a new, alternative approach to improve post-infarct cardiac regeneration and function using stem cells.

Multiple clinical trials were done using cell based therapy to address heart failure both in the acute and chronic phase post MI. Depending on the period of presentation, we can divide trials of stem cell injection into treatment during acute presentation of less than 7 days, acute presentation for more than 7 days and chronic presentation for more than a month post MI. For instance, trials experimenting in the acute presentation of less than 7 days, such as the Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial, showed a trend of improvement in regional wall motion in the infarcted zone and reduction of end-systolic LV volume immediately after the injection as well as during the 4-month follow-up[36] and Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) that showed an initial result of increase in LVEF[37], both used bone marrow derived MSC as the choice of stem cells. In regards to the acute presentation for more than 7 days, trials such as CArdiosphere-Derived aUtologous Stem CElls to reverse ventricULar dySfunction (CADUCEUS) showed a significant decrease in the scar size and increase in viable myocardium at 6 and 12 months [17] it mainly used cardiosphere derived stem cells using resident cardiac stem cells. The MYocardial STem cell Administration after acute myocardial infaRction (MYSTAR) trial also showed a trend toward improvement that didn't reach a statistical significance using bone marrow derived MSC.[11] Finally, in regards to chronic presentation heart failure, trials such as

Transplantation of progenitor cells and recovery of left ventricular function in patients with chronic ischemic heart disease (TOPCARE-CHD) also used bone marrow derived MSC found that the treatment was associated with a reduction in natriuretic peptide serum levels (which is a marker for chronic heart failure) and improved the survival of patients with chronic heart failure post-MI[38]. The Percutaneous stem cell injection delivery effects on neomyogenesis Comparison of Allogeneic versus Autologous Bone Marrow-Derived Mesenchymal Stem Cells Delivered by Trans-Endocardial Injection in Patients with Ischemic Cardiomyopathy (POSEIDON) trial also used MSC even though there was immunologic reaction, MSC injection favorably affected patient's quality of life, functional capacity, and ventricular remodeling in one of its groups.[39]

In this study, our hypothesis state *that signaling molecules VEGF and PDGF present in cell conditioning media would have angiogenesis and neovascularization properties to induce improvement in myocardial function and reduction in scar size after MI.*

we investigated the paracrine effects of stem cells on post-MI functional recovery and scar size in a rat model. We utilized 2 stem cell lines, bone marrow derived mesenchymal stem cells (MSC) and subcutaneous tissue reprogrammed cells to produce human induced pluripotent stem cells (HiPSC), and focused on 2 particular growth factors that drive neovascularization and potentially myocardial recovery, platelet-derived growth factor (PDGF) and vascular endothelial cell growth factor (VEGF).

1.2 Mesenchymal Stem Cells (MSCs)

MSCs are stem cells which characterized by its multipotent ability and can be differentiated into cardiomyocytes or endothelial cells[40,41] which made it a potential source of IHD treatment. It does own certain characters such as stability and reproducibility with almost no concerns from the ethical aspect specially if it was harvested from bone marrow, in addition to its versatility and availability as well. It was first discovered back in the mid-sixties in the bone marrow[42] then later on it was found in other different tissues such as adipose tissue, placenta and muscles.[43]

1.3 Human Induced pluroptent stem cells (HiPSC)

1.3.1 Description

HiPSC are autologous mature cells that can be transformed into pluripotent cells. Through specific genetic alterations, mature cells can be reprogrammed to express embryonic genes, allowing them to differentiate into tissues other than their specific lineage. This was introduced by Takahashi and colleagues when they started reprograming the cells using the OCT4, SOX2, KLF4 and MYC gene (which is commonly known as Yamanaka factors). The genetic expression was forced into cells using epsiomal plasmid, retroviral and Sendai viruses.[44,45]

1.3.2 Applications

One very important aspect of HiPSCs is that, they can be aimed to differentiate into a specified lineage which unlocked many opportunities for the research in regenerative medicine, therapeutic for disease modeling and drug discovery.[46-51] It carries the advantage of eliminating the ethical controversy, minimize the immune rejection and personalized approach for drug administration.

1.4 Cytokines role "VEGF and PDGF"

VEGF and PDGF has direct relationship to angiogenesis and neovascularization. VEGF, expressed mainly on endothelial cells, has been previously demonstrated as a potent stimulator of angiogenesis through tyrosine kinase receptor Flk-1 and the fms-like tyrosine kinase receptor Flt-1, and was shown to slightly improve cardiac micro vascular reconstruction and function following intravenous injection[52-55]. PDGF has also been identified as a significant contributor to angiogenesis and cardiac remodeling following MI[56-58]. Notably, both VEGF and PDGF have demonstrated some adverse effects, particularly when given in high intravenous doses. For example, high intravenous doses of VEGF have been associated with tumor pathogenesis and cancer biology, and PDGF has been associated with organ fibrosis[59,60]. However, we have utilized both substrates in a localized fashion, with direct injection into peri-infarct tissue, and avoided intravenous administration.

CHAPTER 2: METHODS

Cell culture: Mesenchymal Stem Cells

Human bone marrow-derived MSC's were donated by Dr. Yen BL and colleagues at the Institute of Cellular and System Medicine (Zhunan, Taiwan). MSC's were isolated using previously described protocols[61]. These cells were cultured in T75 TC flasks in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 ū/ml penicillin (Invitrogen, Carlsbad, CA). Cell culture medium was changed every 24 hours. Cells at 80-90% confluence were recovered or harvested. Regarding cell passage, culture medium was aspirated, the cells were washed twice with 5 mL phosphate-buffered saline (PBS), and 1 mL 0.25% trypsin was utilized to separate cells from the T75 flasks. Cells were incubated for 5-10 minutes at 37ºC. DMEM (6 ml) was subsequently added and the homogenate was split into 2x15 ml tubes and centrifuged for 5 minutes at 1200 rpm. The medium was aspirated and the pellet was diluted in new DMEM. Cells were seeded into new T75 flask and incubated at 37°C in a humidified incubator containing a 5% CO_2 and 21% O_2 atmosphere.

Cell Culture: Human induced Pluripotent Stem Cells

HiPSCs, isolated from fibroblasts within the skin of normal tissue donors and reprogrammed by episomal plasmid retroviral expression of OCT4, SOX2, KLF4 and MYV genes, were obtained from American Type Culture Collection (Manassas, VA). The cells were feeder-free (Pluripotent Stem Cell SFM XF/FF, ATCC no. ASC-3002), (which does not contain any FBS) and a biological matrix (cell matrix basement gel, ATCC no. ACS-3035) was used in place of fibroblast feeders to provide a surface for attachment of the hiPSCs. DMEM:F-12 medium (ATCC no. 30-2006) was utilized as culture medium.

Cells were prepared from the frozen cryovial according to ATCC protocol. Culture media was aspirated and the cells were rinsed twice with 4 mL of Dulbecco's-PBS (D-PBS, ATCC no.30-2200). Stem cell dissociation reagent, 2 mL, (ATCC no. ACS-3010) was added, followed by 10 to 15 minutes of incubation at 37°C in a humidified incubator containing a 5% CO_2 and 21% oxygen atmosphere. The dissociation reagent was

aspirated and 2 mL of DMEM:F-12 with ROCK inhibitor Y27632 (ATCC no. ACS-3030) was added to detach the cells. Suspended cell aggregates were then centrifuged at 200xg for 5 minutes at room temperature to pellet cells. The supernatant was aspirated and discarded, the cells were resuspended in 1 mL of DMEM:F12 + ROCK inhibitor, and re-plated.

Sample Collection

After 10-12 passage cycles, conditioning media from both the MSC's and HiPSCs was collected for subsequent analysis. For both cell types, culture media was collected and filtered using an Amicon® Ultra-15 Centrifugal Filter (MW 10 kDa, Merck Millipore, Billerica, MA). The aspirate was then centrifuged at 14000 rpm for 10 minutes, and stored at -80ºC. We completed this for a subsequent 10-12 consecutive cycles.

Measurement of VEGF and PDGF

An ELISA kit (Peprotech, rocky hill, NJ) was used to estimate the quantities of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) present in each cell line's conditioning media. Elisa microtiter plates were pre-coated with a murine monoclonal antibody against the media cytokine (VEGF or PDGF) being measured. Standards of the culture medium were added in triplicate. We added 100 µL of each sample to appropriate wells of the ELISA strips. The samples were incubated for 2 hours. We then added 100 µL of detection antibody solution to each well, and incubated for a further 2 hours at room temperature. We added 100 µL of avidin-horseradish peroxidase (HRP) solution to all wells, and incubated for 30 minutes at room temperature. Subsequently, 100 µL of substrate solution was added to each well and incubated for 15 minutes at room temperature in the dark. Finally, absorbance was measured at 405 nm with wavelength correction at 650 nm within a period of 60 minutes at 15 minutes intervals. The reaction and standard curves were plotted. All assays were conducted in a blinded fashion.

In-Vivo

We selected female, Lewis rats, 200–250 g (Charles River Laboratories, Senneville, Canada) as our model. All animal studies were performed in accordance with the guidelines set forth by the Canadian Council on Animal Care, and were approved by the institutional ethics committee. Rats were housed in groups of three per cage, at 23° C, fed with Purina rat ration, and were allowed free access to water.

Rats were anesthetized using 5% isoflurane, intubated, and mechanically ventilated at 85 breaths/minute. Via a left thoracotomy through the fourth intercostal space, the left coronary artery was permanently ligated 2 mm from its origin with a 7/0 polypropylene suture (Ethicon Inc, Somerville, NJ). The ischemic myocardial segment rapidly became identifiable through its pallor and akinesia, corresponding to the distribution of the left coronary artery territory. This resulted in infarction of the left ventricle (LV) and subsequently reduced in ventricular function. Fifteen minutes after ligation of the artery, 3 equal peri-infarct intramyocardial injections, total 500 μL, of the previously harvest MSC or HiPSC culture media or normal saline were performed, using a 27-gauge needle. Rats were randomized into three groups: (i) control group NS (n = 10, normal saline), (ii) treatment group HiPSC (n = 10, HiPSC culture media extract), (iii) treatment group MSC (n = 10, MSC culture media extract). All of these groups had coronary ligation and injection of treatment into the peri-infarct area. In addition, there is Group of Sham rats (n=6), which is normal rats didn't get exposed to any surgical intervention, were used for histological comparison as well as proteomics analysis.

Echocardiography

Trans-thoracic echocardiographic examinations were performed under inhaled isoflurane anesthesia (2.5% in oxygen, 500–700 mL/minute) in a blinded fashion. Transthoracic echocardiography was performed for each rat as a baseline before the surgery, immediately after ligation, and at 1 week, 2 weeks, 4 weeks and 6 weeks. Images were obtained using a commercially available system (Micromaxx P04224; SonoSite, Bothell, WA), equipped with a linear probe 7–13 MHz 25 mm footprint turbo transducer (P06519.11; SonoSite).

Briefly, LV end-diastolic diameters (LVEDD) and end-systolic diameters

(LVESD) were measured as the distance between the anterior and posterior LV walls using M-mode tracings in the parasternal short-axis view. Measurements were recorded slightly inferior to the apex of the papillary muscles of the mitral valve. The time of end-diastole was defined as time of maximum diameter of the LV in one heart cycle. Accordingly, end-systole was defined as the minimum diameter. Following the "leading-edge" method as described by the American Society of Echocardiography, two images on average were obtained in each view and averaged over three consecutive cycles.

Left-ventricular fractional shortening (LVFS) was determined according to the following formula:

$$LVFS = [(LVEDD - LVESD)/LVEDD] \times 100$$

The left-ventricular end-diastolic volume (LVEDV) and left-ventricular end-systolic volume (LVESV) were measured using Teichholz formula, as follows:

$$LVEDV = 7.0 \times LVEDD^3/ (2.4 + LVEDD)$$
$$LVESV = 7.0 \times LVESD^3/(2.4 + LVESD)$$

The left ventricular ejection fraction (LVEF) was determined according to the following formula:

$$LVEF = (LVEDV - LVESV) / LVEDV$$

Scar area analysis

To analyze the effect of treatment on scar size, scar area analysis was performed as follows - after cardiac excision, the hearts were immediately immersed in saline to remove excess blood from the ventricles. Samples were then fixed in neutral-buffered 4% formalin and paraffin. Paraffin-embedded samples were sectioned at 5 μm. Masson's trichrome staining (DBS, Pleasanton, CA) was performed to delineate scar tissue (blue color) from the total area of normal myocardium. Masson's trichrome-stained sections were captured as digital images and analyzed using Image-J software (version 1.41;

National Institutes of Health, Bethesda, MD) and ImageScope software (© 2016 Leica Biosystems Imaging). Infarct areas were calculated and expressed as a percentage. Samples were obtained at time points 1, 2, 4, 6, and 8 weeks. We combined results from weeks 2 and 4, and 6 and 8 in order to increase our sample size for analysis.

Assessment of Angiogenesis

Neovascularization was evaluated by analyzing the capillary and arteriole density in the peri-infarct zone. Immunofluorostaining was performed with antibodies against CD31 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) to identify capillary endothelial cells. To measure capillary density, three fields in the peri-infarct area were imaged, and the number of capillaries with diameter of 10μm or greater were counted. The capillary density (mean total CD31-positive microvessels)/mm^2) was quantified using the average of three tissue sections spanning the peri-infarct tissue region of each heart.

Proteomics Analysis

We used a rat cytokines array panel A (R &D system, Inc., Minneapolis, MN) to determine the concentration of soluble cytokines in rat serum from the 3 groups (MSC, HiPSC and NS groups) and Sham group as well. Serum samples were collected as per the protocol. In brief, reagents were prepared at room temperature, and a 4-well multi-dish was used and prepared with buffer and reconstituted detection antibody cocktail. We diluted the Streptavidin-HRP in Array Buffer 6 using the dilution factor on the vial label, and added 2mL of this to each well of the 4-Well Multi-dish. Each membrane was carefully removed from its wash container and excess buffer was drained from the membrane. The membrane was returned to the 4-well multi-dish containing the diluted Streptavidin-HRP and incubated for 30 minutes at room temperature on a rocking platform shaker. We subsequently added 1mL of the prepared Chemi Reagent Mix evenly onto each membrane, and incubated them for 1 minute at room temperature. The membranes were placed on autoradiography film cassette and exposed to X-ray.

Statistical Analysis

Values are expressed as mean value ± SD. Typically, groups were tested and

performed in triplicates and were compared with the Student's t-test, or by using the multivariant analysis of variance (MANOVA) test. Results were considered significant if the *p*-value was < 0.05.

CHAPTER 3: RESULTS

In-vitro Results

Our initial analysis quantified VEGF and PDGF produced by *in*-vitro MSCs and HiPSCs. We created 5 groups, comprising 2 treatments and 3 control groups: "MSC-CM" was the conditioning media after the culture from MSCs, "HiPSC-CM" was the conditioning media after the culture from HiPSCs, "MSC-pre" was the MSC conditioning media before the culture (DMEM), "HiPSC-pre" was HiPSC culture media before culture (DMEM:F12-no rock), "HiPSC-pre-r" was HiPSC conditioning media before culture with *rock inhibitor* (DMEM:F12-with rock). There was a significant increase in VEGF and PDGF release between each cell line and its control at 0 through 60 minutes, as shown in Table 1. Additionally, there was a significant increase in the release of both growth factors in the HiPSC culture media compared to the MSC media at 60 minutes (PDGF: P= 0.022; VEGF: P= 0.0016).

In-vivo results

Echocardiography Results

We formed 3 Groups: Group NS, Group HiPSC and Group MSC. Pre-operative echocardiography demonstrated no significant difference in left ventricular ejection fraction (LVEF) or fractional shortening (FS) between the three groups (Table 2). Additionally, there was no significant difference between pre-operative LVEF or FS between the two treatment groups, where LVEF for Group HiPSC and Group MSC (73± 2.4) P=0.928 vs. (73± 2.7) P= 0.967, respectively and FS for group HiPSC and Group MSC (37.3±2) P=0.849 vs. (37.4 ± 2) P=0.903, respectively. Following surgery, there was a rapid deterioration in mean LVEF and mean FS in all groups when compared to the pre-operative measurements (Table 2). There was no significant difference in mean LVEF or FS between groups immediately following surgery (*LVEF*, NS vs. HiPSC:

(34.7± 3.4) vs. (35.7 ± 2.6) p = 0.913, NS vs. MSC: (34.7± 3.4) vs. (33.1± 4.4) p = 0.824, MSC vs. HiPSC: (35.7 ± 2.6) vs. (33.1± 4.4) p = 0.501; *FS*, NS vs. HiPSC: (14±1.6) vs. (14.8 ± 1.3) p = 0.799, NS vs. MSC: (14 ±1.6) vs. (13.5 ± 2) p = 0.907, MSC vs. HiPSC: (13.5± 2) vs. (14.8±1.3) p = 0.438). In contrast, there was a significant difference in LVEF between treatment and control groups as early as week 2 (42.7± 8.1, P< .00001) (Figure 1). Similarly, there was a significant difference in FS at weeks 2, 4, and 6 compared to baseline in both the HiPSC and MSC groups (Figure 2). There was no significant difference in mean LVEF (2 weeks: P=0.169; 4 weeks: P=0.864; 6 weeks: P=0.942) or mean FS (2 weeks: P=0.166; 4 weeks: P=0.907; 6 weeks: P=0.893) between the two treatment groups at any pre-determined time point.

Scar Area Analysis

In the control, MSC, and HiPSC groups, hearts were harvested at time points 1, 2, 4, 6, and 8 weeks post surgery. We quantified scar tissue area at weeks 1, 2+ (2-4 grouped), and 6+ (6-8 grouped). There was no statistically significant difference between all groups at week 1 (34.16 ± .75, P=.385). There was a statistically significant decrease in scar area in MSC and HiPSC groups compared to the control group NS (Figure 3) at weeks 2-4 (NS vs. MSC: (33±0.01) vs. (26 ±0.01) P=0.001; NS vs. HiPSC: (33±0.01) vs. (23.25±0.009) P< 0.0001) and 6-8 (NS vs. MSC: (34.3 ±0.011) vs. (22.6 ± 0.016) P<0.0001; NS vs. HiPSC: (34.3 ±0.011) vs. (19.8 ±0.017) P<0.0001). Furthermore, at 2-4 weeks post-surgery, there was significant reduction in scar area in the MSC group compared to the HiPSC group, with mean difference of 24.42 ±1.7, P=0.014, this was evident at weeks 6-8, with mean difference of (21±2.1, P=0.024 (Figure 3). Histological demonstration of the reduction in scar size in response to treatment is shown in Figure 4.

Neovascularization and CD31 Immunostaining

We utilized immunofluorescent staining to assess CD31 staining in endothelial cells at the peri-infarct zone. Each group was again divided into three time frames, week 1 (post-operative), week 2+ (weeks 2-4) and week 6+ (weeks 6-8). We identified a

significant increase in peri-infarct angiogenesis in the HiPSC and MSC groups compared to controls at weeks 2+ MSC vs NS: (37.4±6.9) vs. (23± 3.7) with mean difference of (29.5 ± 9), P=0.003, HiPSC vs. NS: (39.2 ± 11.5) vs. (23± 3.7) with mean difference of (32.7±12), P=0.006) and weeks 6+ (MSC vs NS: (40.9 ± 14) vs. (17.6 ± 8), with mean difference of (32± 16), P<0.0001, HiPSC vs. NS: (48.8±11.4) vs. (17.6 ± 8) with mean difference of (37.6± 18), P<0.0001). There was no significant difference in angiogenesis between the HiPSC and MSC groups at weeks 2+ (39.2 ± 11.5) vs. (37.4±6.9), (38.5 ±9.8), P=0.72 or 6+ (48.8±11.4) vs. (40.9 ± 14), (45± 13), P=0.097.

Proteomics Analysis

The proteomics analysis (Figure 6) revealed abundant presence of VEGF, fractalkine and Interleukin-3 in the serum of group HiPSC that was absent in the NS and MSC groups. Among the other factors detected at HiPSC group, we noticed a presence of a higher concentration of soluble intercellular adhesion molecule-1 (SICAM-1), Fractalkine, IL-3, VEGF *and L-selectin (CD62L) which reach a statistical significance in compare to the other Groups (MSC, NS and Sham).*

CHAPTER 4: DISCUSSION

Although the use of stem cell-based therapy has shown a promise in the treatment of myocardial infarction and congestive heart failure, the benefits remains limited and associated with significant inadvertent side effects. In the present study, we assessed 2 main questions, first whether there is a potential role for cytokines to initiate myocardial repair in infarcted myocardial tissue, and second, to identify a cell lineage capable of producing these cytokines, in this case VEGF and PDGF. We had several key findings: i) both HiPSCs and MSCs are capable of producing VEGF and PDGF in an *in-vitro* environment; ii) injection of the culture medium from both cell types containing these cytokines into peri-infarcted tissue resulted in improved LVEF and FS, post MI in the LAD territory; iii) Stem cells secrete a number of other growth factors and chemokines that may improve cardiac function post-MI.

Our initial hypothesis was built upon the paracrine effect of stem cells, under the assumption that stem cells produce growth factors, which facilitate the growth and differentiation of the cell itself and enhances growth of the cells in the surrounding environment. We focused on VEGF and PDGF given their direct relationship to angiogenesis and neovascularization.

We selected HiPSCs and bone marrow derived MSCs given their previously demonstrated efficacy in improving post-MI cardiac function after stem cell grafting. HiPSCs are autologous adult cells that, through specific alteration, can be reprogrammed to express embryonic genes allowing them to differentiate into different tissue types. In 2006-2007, Yamanaka and colleagues reported the generation if iPSCs in a murine model, and later in a human model[44,62]. HiPSCs have since been trialed in murine and porcine models, and demonstrated improved cardiac function after grafting into infarcted myocardium[30,63]. MSCs have been utilized in a similar fashion, and have demonstrated favorable results[39,64]. Here, we hypothesized that these cells release growth factors such as VEGF and PDGF in the culture medium which can be injected directly into the peri-infarct zone. Indeed, we were able to demonstrate a measurable amount of VEGF and PDGF in the culture media collected from both HiPSCs and MSCs, which upon injection ameliorated the myocardial remodeling properties associated with experimental models of myocardial infarction.

We elected to inject the growth-factor containing cell passage media from both stem cell lines into the peri-infarct area, rather than graft the stem cells for several reasons. Previous investigations have demonstrated teratogenicity and arrhythmia disturbances with stem cell transplantation[29-32]. Hentze and colleagues demonstrated that grafting of human embryonic stem cells containing as little as 0.025% undifferentiated cells could result in teratomas formation[65]. This has resulted in further investigation to identify means of cell delivery to reduce these adverse effects, or increase the purity of stem cell grafts. Hence, injection of the culture media, however, avoids the grafting of cells, yet still exploits the growth factors produced by cell lines that have been demonstrated to be effective in modifying cardiac function post-infarct or in chronic ischemic heart failure. We achieved a reduction in scar area, increase in peri-infarct angiogenesis, and improved left ventricular ejection fraction and fractional shortening using this technique.

Our results are comparable to investigations utilizing stem cell grafting techniques which showed improved cardiac function following MI or in ischemic cardiomyopathy. We demonstrated a 51.5% and 61.7% increase in ejection fraction in rats treated with HiPSC or MSC culture media from baseline to 6 weeks, respectively. Compared to rats treated with NS, mean ejection fraction improved by roughly 60% in both cell lines. Kawamura and colleagues utilized HiPSCs in a porcine model, and demonstrated a roughly 25% increase from baseline among pigs treated with HiPSCs, and a 43% increase in ejection fraction compared to the sham group[30]. Ong and colleagues similarly demonstrated a roughly 60% increase in ejection fraction at 35 days post-infarct using grafted HiPSCs in a murine model[66]. Cell-free therapies have also demonstrated similar results, as described by Barile and colleagues, and Yeghiazarians and colleagues, who, respectively, demonstrated improved cardiac function utilizing microvesicles from HiPSCs and extract from fractionated bone marrow stem cells in murine models[35,61]. Therefore, our data suggests that a paracrine mechanism may play a significant role in cardiac remodeling and improvement in cardiac function in ischemic cardiomyopathy.

Even though the MSC Group received CM which contain FBS, that might contribute to the improvement of the ejection fraction for that group, the HiPSC Group has no FBS as we used feeder free medium which make it less likely to be the most

effective contributor in the improvement of cardiac function and neovascularization process.

To further explore this possibility, we utilized CD-31 immunostaining of the endothelium of blood vessels to investigate vascularity following treatment. Our results demonstrate that there was significantly more peri-infarct vascularization in rats treated with either MSC or HiPSC culture media compared to those treated with NS. In corroboration with our echocardiographic results, our findings are consistent with those of Schuleri and colleagues, who identified an increase in myocardial blood flow in infarcted tissue in post-infarct porcine hearts grafted with MSCs and suggested that early post-insult increases in myocardial tissue perfusion may reduce apoptosis and improve cardiac function[67]. This may, in turn, result in the small scar size that we identified in rat hearts treated with MSC and HiPSC culture medium at 6-8 weeks post-infarct. However, the exact mechanism of cardiac functional benefit utilizing this technique is unknown, though several explanations are possible. First, through improved blood supply and function of peri-infarct myocardium. Second, through recruitment of cardiomyocyte progenitor cells.

Our proteomics analysis demonstrated an increased presence of number of cytokines and growth factors involved in cardiac repair and remodeling in treated rats. We identified a high presence of VEGF, interleukin-3 (or multi-colony stimulating factor) and fractalkine in rats treated with HiPSCs compared to those that received intramyocardial NS or MSC culture media. Several investigations have demonstrated the effect of these cytokines on cellular proliferation, differentiation, maturation, angiogenesis, and cardiac remodeling[68-77]. Interleukin-3 has demonstrated improvement in left ventricular function and survival in animal models with acute MI or ischemic cardiomyopathy, which might be explained by promotion of angiogenesis, inhibition of apoptosis or differentiation of cardiomyocytes[69,71,72,75]. Fractalkine has demonstrated a role in delaying the enlargement of ventricular chambers following myocardial infarction[77]. We also identified higher concentrations of CINC-1, SICAM-1, and L-selectin in HiPSC culture media treated rats compared to the NS group, CINC-1 acts as a chemoattractant for PMNs to peri-infarct areas by interacting with cell surface chemokine

receptors[70,73]. Finally, SICAM-1 and L-selectin have demonstrated roles as chemo-attractants and neutrophil function[68,76,78].

Since heart failure due to ischemic cardiomyopathy continues to remain a prominent cause of morbidity and mortality worldwide, investigation utilizing stem cell therapy to improve cardiac function will continue. We have developed an approach to stem-cell based therapy which exploits the paracrine effect of stem cells to promote cardiac angiogenesis, reduce scar area, and improve cardiac function through a means that minimizes some of the adverse effects associated with stem cell grafting. Translated to the clinical setting, our findings also suggest that conditioned media from MSC and HiPSC can be prepared and made available on the shelf and potentially used in patients presenting with acute MI and or ischemic cardiomyopathy. Therefore, it eliminates the logistics of highly specialized institute and complex harvesting, proliferating and implantation process while offering the benefits of stem cell-based therapy to the generalized populations. Future studies aimed at characterizing the secretory profile of cell-based therapy may provide target specific treatment for patient with myocardial infarction and congestive heart failure.

REFERENCES

1. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012;380(9859):2095-2128.
2. Bergmann O. Evidence for cardiomyocyte renewal in humans. . *Science.* 2009;324:98–102.
3. Bergmann O, Bhardwaj RD, Bernard S, et al. Evidence for cardiomyocyte renewal in humans. *Science.* 2009;324(5923):98-102.
4. Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med.* 2001;344(23):1750-1757.
5. Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P. Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl Acad Sci U S A.* 1998;95(15):8801-8805.
6. Meckert PC, Rivello HG, Vigliano C, Gonzalez P, Favaloro R, Laguens R. Endomitosis and polyploidization of myocardial cells in the periphery of human acute myocardial infarction. *Cardiovasc Res.* 2005;67(1):116-123.
7. Ang KL, Chin D, Leyva F. Randomized, controlled trial of intramuscular or intracoron- ary injection of autologous bone marrow cells into scarred myocardium during CABG versus CABG alone. *Nat Clin Pract Cardiovasc Med* 2008;5:663–670.
8. Bartunek J, Behfar A, Dolatabadi D, et al. Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem Cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics. *J Am Coll Cardiol.* 2013;61(23):2329-2338.
9. Dib N, Dinsmore J, Lababidi Z. One-year follow-up of feasibility and safety of the first U.S., randomized, controlled study using 3-dimensional guided catheter-based delivery of autologous skeletal myoblasts for ischemic cardiomyopathy (CAuSMIC study). . *JACC Cardiovasc Interv.* 2009;2:9-16.
10. Duckers HJ, Houtgraaf J, Hehrlein C, et al. Final results of a phase IIa, randomised, open-label trial to evaluate the percutaneous intramyocardial transplantation of autologous skeletal myoblasts in congestive heart failure patients: the SEISMIC trial. *EuroIntervention : journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology.* 2011;6(7):805-812.
11. Gyongyosi M, Lang I, Dettke M. Combined delivery approach of bone marrow mononuclear stem cells early and late after myocardial infarction: the MYSTAR prospective, randomized study. *Nat Clin Pract Cardiovasc Med.* 2009;6:70–81.
12. Hirsch A, Nijveldt R, van der Vleuten PA, et al. Intracoronary infusion of mononuclear cells from bone marrow or peripheral blood compared with standard therapy in patients after acute myocardial infarction treated by

primary percutaneous coronary intervention: results of the randomized controlled HEBE trial. *Eur Heart J.* 2011;32(14):1736-1747.

13. Huikuri HV, Kervinen K, Niemela¨ M. Effects of intracoronary injection of mononuclear bone marrow cells on left ventricular function, arrhythmia risk profile, and restenosis after thrombolytic therapy of acute myocardial infarction. . *Eur Heart J.* 2008;29:2723–2732.
14. Janssens S, Dubois C, Bogaert J. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised con- trolled trial. *Lancet.* 2006;367:113–121.
15. Losordo DW, Henry TD, Davidson C, et al. Intramyocardial, autologous CD34+ cell therapy for refractory angina. *Circulation research.* 2011;109(4):428-436.
16. Lunde K, Solheim S, Aakhus S. Intracoronary injection of mono- nuclear bone marrow cells in acute myocardial infarction. *N Engl J Med.* 2006;355:1199–1209.
17. Makkar RR, Smith RR, Cheng K, et al. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *The Lancet.* 2012;379(9819):895-904.
18. Perin EC, Willerson JT, Pepine CJ, et al. Effect of transendocardial delivery of autologous bone marrow mononuclear cells on functional capacity, left ventricular function, and perfusion in chronic heart failure: the FOCUS-CCTRN trial. *Jama.* 2012;307(16):1717-1726.
19. Schachinger V, Erbs S, Elsasser A, et al. Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J.* 2006;27(23):2775-2783.
20. Surder D, Manka R, Lo Cicero V, et al. Intracoronary injection of bone marrow-derived mononuclear cells early or late after acute myocardial infarction: effects on global left ventricular function. *Circulation.* 2013;127(19):1968-1979.
21. Tendera M, Wojciech Wojakowski, Witold R, et al. Intracoronary infusion of bone marrow-derived selected CD341CXCR41 cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial. *European heart journal.* 2009;30:1313–1321.
22. Traverse JH., Henry T. D., Pepine C. J., et al. Effect of the use and timing of bone marrow mononuclear cell delivery on left ventricular function after acute myocardial infarction: the TIME randomized trial. *Jama.* 2012;308(22):2380-2389.
23. Traverse JH, Henry TD, Vaughan DE, et al. LateTIME: a phase-II, randomized, double-blinded, placebo-controlled, pilot trial evaluating the safety and effect of administration of bone marrow mononuclear cells 2 to 3 weeks after acute myocardial infarction. *Tex Heart Inst J.* 2010;37(4):412-420.

24. Etzion S, Battler A, Barbash IM, et al. Influence of embryonic cardiomyocyte transplantation on the progression of heart failure in a rat model of extensive myocardial infarction. *J Mol Cell Cardiol.* 2001;33(7):1321-1330.
25. Menard C, Hagege AA, Agbulut O, et al. Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. *Lancet.* 2005;366(9490):1005-1012.
26. Muller-Ehmsen J, Peterson KL, Kedes L, et al. Rebuilding a damaged heart: long-term survival of transplanted neonatal rat cardiomyocytes after myocardial infarction and effect on cardiac function. *Circulation.* 2002;105(14):1720-1726.
27. Caspi O, Huber I, Kehat I, et al. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol.* 2007;50(19):1884-1893.
28. Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. . *Nat Biotechnol* 2007;25:1015–1024.
29. Cai J, Yi FF, Yang XC, et al. Transplantation of embryonic stem cell-derived cardiomyocytes improves cardiac function in infarcted rat hearts. *Cytotherapy.* 2007;9(3):283-291.
30. Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T. Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation.* 2013;126(11 Suppl 1):S29–37.
31. Lee AS, Tang C, Cao F, et al. Effects of cell number on teratoma formation by human embryonic stem cells. *Cell cycle.* 2009;8(16):2608-2612.
32. Menasche P, Alfieri O, al. JSe. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast trans- plantation. *Circulation* 2008;117:1189–1200.
33. Barile L, Lionetti V, Cervio E, et al. Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. *Cardiovasc Res.* 2014;103(4):530-541.
34. Leroyer AS, Ebrahimian TG, Cochain C, et al. Microparticles from ischemic muscle promotes postnatal vasculogenesis. *Circulation.* 2009;119(21):2808-2817.
35. Yeghiazarians Y, Zhang Y, Prasad M, et al. Injection of bone marrow cell extract into infarcted hearts results in functional improvement comparable to intact cell therapy. *Mol Ther.* 2009;17(7):1250-1256.
36. Assmus B, Schächinger V, Teupe C. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation.* 2002;106:3009–3017.
37. Schachinger V, Erbs S, Seeger FH. Improved clinical outcome after intra-coronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J.* 2006;27:2775–2783.
38. Assmus B, Fischer-Rasokat U, Honold J, et al. Transcoronary transplantation of functionally competent BMCs is associated with a decrease in natriuretic

peptide serum levels and improved survival of patients with chronic postinfarction heart failure: results of the TOPCARE-CHD Registry. *Circulation research.* 2007;100(8):1234-1241.

39. Hare JM, Fishman JE, Gerstenblith G, et al. Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *Jama.* 2012;308(22):2369-2379.
40. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation.* 2002;105(1):93-98.
41. Silva GV, Litovsky S, Assad JA, et al. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation.* 2005;111(2):150-156.
42. Friedenstein AJ, Piatetzky S, II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol.* 1966;16(3):381-390.
43. Fukuchi Y, Nakajima H, Sugiyama D, Hirose I, Kitamura T, Tsuji K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells.* 2004;22(5):649-658.
44. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126(4):663-676.
45. Okita K, Yamanaka S. Induced pluripotent stem cells: opportunities and challenges. *Philos Trans R Soc Lond B Biol Sci.* 2011;366(1575):2198-2207.
46. Lee G, Papapetrou EP, Kim H, et al. Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature.* 2009;461(7262):402-406.
47. Briggs JA, Mason EA, Ovchinnikov DA, Wells CA, Wolvetang EJ. Concise review: new paradigms for Down syndrome research using induced pluripotent stem cells: tackling complex human genetic disease. *Stem Cells Transl Med.* 2013;2(3):175-184.
48. Tan Q, Lui PP, Rui YF, Wong YM. Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. *Tissue engineering Part A.* 2012;18(7-8):840-851.
49. Nori S, Okada Y, Yasuda A, et al. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108(40):16825-16830.
50. Liu H, Kim Y, Sharkis S, Marchionni L, Jang YY. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. *Science translational medicine.* 2011;3(82):82ra39.
51. Desnuelle C, Dib M, Garrel C, Favier A. A double-blind, placebo-controlled randomized clinical trial of alpha-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis. ALS riluzole-tocopherol Study Group. *Amyotrophic lateral sclerosis and other motor neuron disorders : official*

publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases. 2001;2(1):9-18.
52. Crafts TD, Jensen AR, Blocher-Smith EC, Markel TA. Vascular endothelial growth factor: therapeutic possibilities and challenges for the treatment of ischemia. *Cytokine.* 2015;71(2):385-393.
53. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science.* 1992;255(5047):989-991.
54. Li C, Liu B, Dai Z, Tao Y. Knockdown of VEGF receptor-1 (VEGFR-1) impairs macrophage infiltration, angiogenesis and growth of clear cell renal cell carcinoma (CRCC). *Cancer Biol Ther.* 2011;12(10):872-880.
55. Liu G, Li L, Huo D, et al. A VEGF delivery system targeting MI improves angiogenesis and cardiac function based on the tropism of MSCs and layer-by-layer self-assembly. *Biomaterials.* 2017;127:117-131.
56. Li H, Fredriksson L, Li X, Eriksson U. PDGF-D is a potent transforming and angiogenic growth factor. *Oncogene.* 2003;22(10):1501-1510.
57. Wang Z, Kong D, Banerjee S, et al. Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling. *Cancer Res.* 2007;67(23):11377-11385.
58. Zhao W, Zhao T, Huang V, Chen Y, Ahokas RA, Sun Y. Platelet-derived growth factor involvement in myocardial remodeling following infarction. *J Mol Cell Cardiol.* 2011;51(5):830-838.
59. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev.* 2004;15(4):255-273.
60. Epstein SE, Kornowski R, Fuchs S, Dvorak HF. Angiogenesis therapy: amidst the hype, the neglected potential for serious side effects. *Circulation.* 2001;104(1):115-119.
61. Yen BL, Huang HI, Chien CC, et al. Isolation of multipotent cells from human term placenta. *Stem Cells.* 2005;23(1):3-9.
62. Takahashi K., Tanabe K., Ohnuki M., et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861-872.
63. Dai B, Huang W, Xu M, et al. Reduced collagen deposition in infarcted myocardium facilitates induced pluripotent stem cell engraftment and angiomyogenesis for improvement of left ventricular function. *J Am Coll Cardiol.* 2011;58(20):2118-2127.
64. Heldman AW, DiFede DL, Fishman JE, et al. Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy: the TAC-HFT randomized trial. *JAMA.* 2014;311(1):62-73.
65. Hentze H, Soong PL, Wang ST, Phillips BW, Putti TC, Dunn NR. Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. *Stem Cell Res.* 2009;2(3):198-210.
66. Ong SG, Huber BC, Lee WH, et al. Microfluidic Single-Cell Analysis of Transplanted Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes After Acute Myocardial Infarction. *Circulation.* 2015;132(8):762-771.

67. Schuleri KH, Amado LC, Boyle AJ, et al. Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. *Am J Physiol Heart Circ Physiol.* 2008;294(5):H2002-2011.
68. Chandrasekar B, Smith JB, Freeman GL. Ischemia-reperfusion of rat myocardium activates nuclear factor-KappaB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. *Circulation.* 2001;103(18):2296-2302.
69. Deindl E, Zaruba MM, Brunner S, et al. G-CSF administration after myocardial infarction in mice attenuates late ischemic cardiomyopathy by enhanced arteriogenesis. *FASEB J.* 2006;20(7):956-958.
70. Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. *Circ Res.* 2012;110(1):159-173.
71. Fukuhara S, Tomita S, Nakatani T, et al. G-CSF promotes bone marrow cells to migrate into infarcted mice heart, and differentiate into cardiomyocytes. *Cell Transplant.* 2004;13(7-8):741-748.
72. Harada M, Qin Y, Takano H, et al. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. *Nat Med.* 2005;11(3):305-311.
73. Kim D, Haynes CL. Neutrophil chemotaxis within a competing gradient of chemoattractants. *Anal Chem.* 2012;84(14):6070-6078.
74. Kobayashi M, Van Leeuwen BH, Elsbury S, Martinson ME, Young IG, Hapel AJ. Interleukin-3 is significantly more effective than other colony-stimulating factors in long-term maintenance of human bone marrow-derived colony-forming cells in vitro. *Blood.* 1989;73(7):1836-1841.
75. Sanganalmath SK, Abdel-Latif A, Bolli R, Xuan YT, Dawn B. Hematopoietic cytokines for cardiac repair: mobilization of bone marrow cells and beyond. *Basic Res Cardiol.* 2011;106(5):709-733.
76. Sellak H, Franzini E, Hakim J, Pasquier C. Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood.* 1994;83(9):2669-2677.
77. Xuan W, Liao Y, Chen B, et al. Detrimental effect of fractalkine on myocardial ischaemia and heart failure. *Cardiovasc Res.* 2011;92(3):385-393.
78. Hafezi-Moghadam A, Thomas KL, Prorock AJ, Huo Y, Ley K. L-selectin shedding regulates leukocyte recruitment. *J Exp Med.* 2001;193(7):863-872.