STANDARDIZATION OF NICHE FOR *IN VITRO* DIFFERENTIATION OF ADIPOSE DERIVED MESENCHYMAL STEM CELLS INTO CARDIOMYOCYTES AND CO-CULTURE WITH ENDOTHELIAL PROGENITORS

A THESIS PRESENTED BY

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TO

SREE CHITRA TIRUNAL INSTITUTE
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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

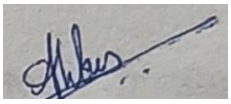
FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2020

CERTIFICATE

I, **Subha S**, hereby certify that I had personally carried out the work depicted in the thesis entitled, ***"Standardization of niche for in vitro differentiation of adipose derived mesenchymal stem cells into cardiomyocytes and co-culture with endothelial progenitors",*** except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.



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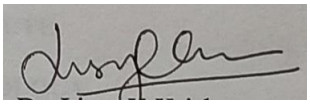

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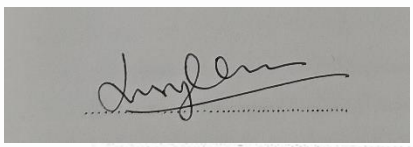

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The Thesis is dedicated to……

My ever loving Family and Teachers ……

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ABBREVIATIONS

ABAM	Antibiotic Antimycotic
ACD	: Acid Citrate Dextrose
ADMSC	: Adipose Derived Mesenchymal Stem Cell
ADP	: Adenosine diphosphate
AHA	: American Heart Association
AHF	: Acute Heart Failure
ATF	: Activating Transcription Factor
ATP	: Adenosine Triphosphate
AV	: Atrioventricular
AVC	: Atrioventricular Canal
BMMNC	: Bone Marrow Mononulcear Cell
BMMSC	: Bone marrow derived Mesenchymal Stem Cell
BMP	: Bone Morphogenetic Protein
BSA	: Bovine Serum Albumin
Ca^{2+}	: Calcium
CABG	: Coronary Artery Bypass Grafting
CD	: Cluster of Differentiation
cDNA	: Complimentary Deoxy ribo Nucleic Acid
CFB	: Cardiac Fibroblasts
CFP	: Cardiac Fibroblast Progenitor Cell
CHD	: Coronary Heart Disease
CHF	: Congestive Heart Failure
CM	: Cardiomyocyte
CMP	: Cardiomyocyte Progenitor Cell
CPC	: Cardiac Progenitor Cell
CSC	: Cardiac Stem Cell
CVD	: Cardiovascular disease
CVS	: Cardiovascular system
Cx 43	: Connexin 43

DAPI	: 4', 6-diamidino-2-phenylindole
DDR 2	: Discoidin Domain Receptor 2
DNA	: Deoxyribonucleic acid
dNTP	: deoxy Nucleotide Tri Phosphate
DPX	: Dibutylphthalate Polystyrene Xylene
EB	: Embryoid Body
EC	: Endothelial Cell
ECM	: Extracellular matrix
EDTA	: Ethylene Diamine Tetra acetic Acid
EMT	: Epithelial to Mesenchymal Transition
EPC	: Endothelial Progenitor Cell
ESC	: Embryonic Stem Cell
eSec	: Engineered VEGF Over-expressed hADMSC secretome
FACS	: Fluorescence Activated Cell Sorter
FBS	: Fetal Bovine Serum
FGF	: Fibroblast Growth Factor
Fib	: Fibrin
GAPDH	: Glyceraldehyde 3- Phosphate Dehydrogenase
GF	: Growth Factor
GJA	: Connexin 43
H & E	: Hematoxylin and Eosin
HAND	: Heart and Neural crest Derivatives
HBSS	: Hank's Balanced Salt Solution
HGF	: Hepatocyte Growth Factor
HLA	: Human Leukocyte Antigen
HSC	: Hematopoietic Stem Cell
IAEC	: Institutional Animal Ethics Committee
ICD	: Implantable Cardioverter- Defibrillator
IEC	: Institutional Ethics Committee
IGF-1	: Insulin-like growth factor 1
IL	: Interleukin

IP	: Induction Protocol
iPSC	: Induced Pluripotent Stem Cell
Irx	: Iroquious Homeobox Protein
ISCT	: International Society for Cellular Therapy
LAD	: Left Anterior Descending Coronary Artery
LDL	: Low Density Lipoprotein
LV	: Left Ventricle/Ventricular
LVAD	: Left Ventricular Assist Device
LVEF	: Left Ventricular Ejection Fraction
MCP	: Monocyte Chemo attractant Protein
MDR	: Multi Drug Resistance
MEF	: Myocyte Enhancer Factor
MHC	: Myosin Heavy Chain
MI	: Myocardial Infarction
MLC	: Myosin Light Chain
MMP	: Matrix Metalloproteinase
MSC	: Mesenchymal Stem Cell
NK	: Natural Killer
Nkx	: NK2 transcription – factor related
nSec	: Non-engineered hADMSC secretome
OFT	: Outflow Tract
PBS	: Phosphate Buffered Saline
PCI	: Percutaneous Coronary Intervention
PDGF	: Platelet derived growth factor
PGF	: Platelet Growth Factor
PRP	: Platelet Rich Plasma
PTCA	: Percutaneous Transluminal Coronary Angioplasty
qRT-PCR	: Quantitative Real Time Polymerase Chain Reaction
RA	: Retinoic acid
RNA	: Ribo Nucleic Acid
ROS	: Reactive Oxygen Species

SA	: Sinoatrial
SP	: Side Population
TCPS	: Tissue Culture Polystyrene
TGF	: Transforming Growth Factor
TIMP	: Tissue Inhibitor of Metalloproteinase
TNF	: Tumor Necrosis Factor
TNNI	: Cardiac Troponin I
TNNT	: Cardiac Troponin T
VAD	: Ventricular Assist Devices
VCAM	: Vascular Cell Adhesion Molecule
VEGF	: Vascular endothelial growth factor
VSMC	: Vascular Smooth Muscle Cell
vWF	: von Willebrand Factor
Wnt	: Wingless

ANNOTATIONS

%	: Percentage
<	: Less than
>	: Greater than
µg	: Microgram
µl	: Microlitre
µm	: Micromolar
d	: Days
h	: hours
IU	: International Unit
mA	: Milli Ampere
min	: Minutes
mM	: Milli Molar
ng	: Nanogram
nm	: Nanometers
pmol	: Picomol

SYNOPSIS

Cardiovascular diseases (CVDs) are a major cause of death and disability, accounting for 17.5 million deaths globally each year; and the CVD death rate in India is higher than the global average. Myocardial infarction (MI) results from an abrupt occlusion of one or more coronary arteries that supply blood to the heart muscles. Consequently, the reduction of oxygen and nutrients to the heart muscle leads to irreparable damage to the myocardial tissue, thus compromising the cardiac function. The current treatment modalities remain ineffective for the regeneration of damaged tissue.

Several cell-based therapies using embryonic stem cells (ESC) and adult stem cells (ASC) have indicated improvement in cardiac regeneration following MI; hence, gaining optimism for the repair of damaged myocardium. Irrespective of the cell type used, issues like poor proliferation and differentiation ability to replace the damaged myocardium, poor survival of transplanted cells, and lack of cell-cell connections remain to be addressed. In preclinical trials and some clinical trials, bone marrow-derived mesenchymal stem cells (BMMSCs) have shown promising results in cardiac regeneration, but the hurdles in isolation procedures limit its clinical application.

In this context, the adipose tissue-derived mesenchymal stem cell population (ADMSCs) has gained attention among the different cell-based therapies tried so far for cardiac regeneration due to its ease of isolation, high proliferation potential, and multipotency. Some studies have reported the use of ADMSCs for cardiac regeneration as well. The survival of stem cells in the inherent tissue and the transplanted site is considered to be influenced by the niche which elicits signals for proliferation and/or differentiation. Since the ADMSCs are multipotent, lineage commitment to the target cells may reduce undesirable cell fate, post-transplantation. The differentiation of stem cells *in vitro* is known to be influenced and controlled by the growth factors (GF) and the extracellular matrix (ECM) combination in the culture environment. Also, the post-transplant survival, proliferation, differentiation, and integration to the native tissue require a niche that produces optimum signals. In the cardiac tissue, there are supporting cells such as cardiac fibroblasts to supply ECM molecules, apart from the vascular environment that provides oxygen/nutrient supply. Therefore, it is anticipated

that regenerative therapies may also attempt to provide all important cells and biomimetic ECM of cardiac tissue in a combination.

In this context, this study hypothesized that from the same donor hADMSC, important progenitor cells of cardiac tissue may be derived and co-cultured to exploit transplantation efficiency, post-MI. Therefore, the study focused on the development of cardiomyocyte progenitors (CMPs), cardiac fibroblast progenitors (CFPs), and endothelial progenitor cells (EPCs), and their co-culture in biomimetic ECM environment and further for studying transplantation outcome. The goal of the study was to standardize niche conditions for the co-existence of CMPs, CFPs, and EPCs using the biomimetic components of ECM, composed of artificially clotted fibrin and over-expressed ADMSC-derived growth factors. The study considered the translational potential of cell-based combination therapy in the near future.

To test the study hypothesis, the following objectives were proposed:

1) To establish stem-ness of hADMSCs isolated from patients undergoing cardiac by-pass surgery.
2) To study the role of fibrin as a niche component in inducing differentiation of hADMSCs to CMPs *in vitro.*
3) To standardize culture conditions and establish differentiation of hADMSCs to CMPs, CFPs, and EPCs.
4) To co-culture CMPs, CFPs and EPCs and study the influence of each cell type on the expression of cell-specific markers, if any.
5) To identify if hADMSC-derived secretome influences the cell-specific markers of CMPs and CFPs.
6) To study the influence of fibrin matrix for post-transplantation survival of rat CMPs in animal model of MI.

The thesis is divided into 6 chapters:

In **Chapter 1**, the research problem is introduced and defined. Information regarding the early development of the heart, adult heart structure, function, functions of different cell types and the composition of cardiac ECM, the interactions between different cell types and the ECM in the cardiac tissue, the homeostasis process of

cardiac tissue, and the pathology of MI are briefly described to state the problem. The limitations of conventional therapy and the promise of cell-based therapy are described to justify the rationale of the study. The transplantation failures of previous studies related to using of (i) undifferentiated stem cells, (ii) differentiated cardiomyocytes (CM); (iii) lack of a program for stable CM lineage commitment of hADMSC *in vitro* and (iv) lack of proper niche for cell delivery and homing were identified as gap area to be addressed in this study. With more reading, major lacunae identified in the current strategies with cell-based therapy is that the influence of supporting cell types for *in vitro* and *in vivo* cell survival and differentiation has not been explored by other investigators. Therefore, the importance of co-culture of cardiac fibroblasts and endothelial cells with cardiac myocytes was identified as a prospective research problem. The significance of ECM including adhesive proteins and growth factors in regulating hADMSC differentiation and survival *in vitro* and *in vivo* has been described in this chapter. Considering all these points, a hypothesis is developed and the study objectives are defined.

In **Chapter 2**, the literature in the field of the identified research problem is elaborately reviewed. This section describes, i) various stages and signaling pathways in the mammalian heart development, iii) the role of various cardiac markers, ECM and the supporting cell populations of the heart, iv) mechanisms of cell death in acute MI and the role of GFs in cardiac regeneration, vi) reparative strategies of the damaged myocardium using various cell-based therapies for cardiac regeneration and the major hurdles faced in different trials, and vii) progression and effect of cell transplantation in animal models of MI.

In **Chapter 3**, the methods used for the standardization of *in vitro* differentiation of ADMSCs into CMPs and CFPs are described. This chapter then describes the development of MI in the rat model and the cell transplantation strategy into the infarcted myocardium. The detailed procedures include both *in vitro* and *in vivo* experiments starting from cell culture and subculture techniques, qualitative and quantitative immunocytochemistry, conventional reverse transcriptase-polymerase chain reaction (RT-PCR), quantitative real-time PCR analysis, bioengineering to over-express GFs in hADMSC and collection of secretome, standardization of cell

transplantation in rat MI model, labeling rat cells, tracking of the transplanted cell by tissue imaging, conventional tissue sectioning, cryosectioning of explanted tissues, histological and immune staining of tissue sections and image analysis to quantify the results in stained sections. The statistical methods used for the analysis of quantitative data is described.

Chapter 4 highlights the results obtained in the study and is divided into 3 phases and presented with illustrations for each experiment. The first phase of the study focused on isolation, expansion, and characterization of hADMSCs to establish stem-ness and standardization of niche for CMP induction. The role of fibrin in inducing the hADMSCs to CMPs *in vitro* was proven by the up-regulated expression of early cardiac transcription factor GATA-4. By modifying the niche constituents and compositions in different experiments, the most suitable one for obtaining CMPs with specific markers was identified. Balanced growth and maintenance of both CMP and CFP were noted in the selected protocol, confirmed by the specific markers using RT-PCR. An angiogenic response was induced by seeding EPC to the CMP-CFP population and further culture of all 3 cell types. The co-survival with the expression of markers for all 3 types of cells was observed. The addition of non-engineered or vascular endothelial growth factor overexpressing hADMSC-derived secretome/GFs to CMP-CFP population also promoted angiogenic response, probably by acting on undifferentiated ADMSC in the culture, without affect CMP marker expression. In the third phase, the effect of fibrin as a delivery vehicle for the cell transplantation into infarcted myocardium was studied using animal models of MI. Rat ADMSCs, induced to CMPs *in vitro* survived in the fibrin niche post-transplantation for 28d and showed signs of differentiation. The cells tracked by PKH26 labeling also expressed connexin 43 and troponin T in the MI tissue.

Chapter 5 discusses the results in the light of current concepts and speculations published in the literature highlighting the study outcome. This study seems to be the first to report the differentiation of hADMSCs to CMPs and CFPs simultaneously in the same culture. The influence of ADMSC-derived EPCs in the initiation of angiogenic activity in the co-culture was evident. Also, the engineered ADMSC-derived secretome seemed to act on undifferentiated ADMSC to develop an

angiogenic response. The study also demonstrated the advantage of injectable fibrin as a delivery vehicle for transplantation of cells in infarcted myocardium.

Chapter 6 summarizes the outcome of various experiments. Derivation of CMP and tissue-specific supporting cells-CFP and EPC using fibrin-based niche and the secretome from the engineered hADMSC seemed to have an additive effect for CMP survival and for inducing angiogenic activity in co-culture. Combinatorial application of the CMP-CFP-EPC population is likely to regenerate vascularized cardiac tissue. The feasibility of such a combinational approach is established in this study. The importance of fibrin-based niche for differentiation and survival of ADMSC-derived cardiac tissue-specific cells has been established in both *in vitro* and *in vivo*. The study has certain limitations that have been highlighted in this chapter. Transplantation of an increased cell number is an important aspect to produce better regenerative outcomes; in the current study, dose-response was not attempted.

The bibliography section provides a list of references considered for defining the problem, designing the study, and discussing the results. Finally, the conferences attended and the appendix is included.

CHAPTER 1

1. INTRODUCTION

The heart is a highly modified muscular pump that regulates the cardiovascular system (CVS) which includes the systemic and pulmonary circuits. It is one of the first organs to be formed in the mammalian embryo and a continuous pump that works right from fetal life. Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and are the leading cause of death globally. It is estimated that almost 17.5 million people die each year from CVDs, which accounts for 31% of all deaths worldwide. According to the American Heart Association (AHA) report, 23.6 million people will die from CVDs by 2030 (Mozaffarian et al., 2015). Among the CVDs, myocardial infarction (MI), acute heart failure (AHF), and acute cerebral failure (Stroke) account for 80% of death globally. The Global Burden of Disease study estimates an age-standardized CVD death rate of 272 per 100000 population in India, which is higher than the global average rate (Prabhakaran et al., 2016). MI results from the abrupt occlusion of one or more coronary arteries that supply blood to the heart. The occlusion results in thrombosis within the vessels thus reducing the supply of oxygen and nutrients to the myocardium, the heart muscle. Failure to restore blood supply rapidly results in irreversible death of myocardial tissue impairing cardiac performance. An acute heart attack event is followed by the formation of non-contractile fibrotic scar tissue resulting in subsequent heart failure that impedes the quality of life, reduces life expectancy, and increases the financial burden of patients.

The mammalian heart has a limited capacity for self-repair and regenerative potential following an injury resulting in irreversible loss of heart muscle. Replacement of the injured area by fibrotic scar tissue poses an extra burden on the weakened heart muscle resulting in reduced contractile efficiency and reduced cardiac output.

Even though medications like the use of aspirin, statins, and beta-blockers are commonly used to slow down progressive heart failure, these therapies are ineffective in reversing the progression of failing heart. Heart transplantation is the gold standard strategy to overcome MI followed by heart failure, but the ever-increasing incidence of MI and decreased donor supply limits the effectiveness of heart transplantation. Although other strategies like coronary artery bypass graft (CABG), use of ventricular assist devices (VADs), and biventricular pacemakers that prevent ventricular dilatation are being widely used, these poses certain side effects and financial burdens for patients. This necessitates alternative treatment modalities to be explored with the potential option to treat the infarcted heart.

1.1 Heart Anatomy and Physiology

The heart is a complex muscular pump that is located in the chest between the lungs behind the sternum and above the diaphragm. It pumps blood through the three divisions of the circulatory system – the coronary (vessels supplying blood to the heart muscles), pulmonary (heart and lungs), and systemic (various systems of the body). It is composed of a special type of muscle tissue, the cardiac muscle, or myocardium that maintains the contractile ability of the heart thus creating a regular pumping rhythm. The heart has mainly three major components – the chambers, valves, and the electric nodes. The mammalian heart has four chambers – the two upper atria and the two lower ventricles which are separated by an atrioventricular septum. The right atrium receives the deoxygenated blood from the upper arms and organs through superior vena cava, from lower organs and legs through inferior and from coronary sinus that drains deoxygenated blood from the heart itself. The deoxygenated blood is then pumped through the tricuspid valve to the right ventricle. Deoxygenated blood is carried to the lungs for re-oxygenation by pulmonary arteries. Oxygenated blood then traverses through the pulmonary vein to the left atrium. The bicuspid valve or mitral valve passes the oxygenated blood to the left ventricle which then pumps the same through the aorta, the largest artery to various parts of the body thus completing the circuit.

Two major electrical nodes that are composed of groups of specialized cells are responsible for the autorhythmicity of the heart – the sinoatrial (SA) node and the

atrioventricular (AV) node. SA node acts as the primary pacemaker of the heart and is located in the wall of the right atrium. The rhythmic excitation experienced by the SA node is responsible for the rapid impulse transmission to atria causing muscular contraction and pumping of blood from atria to ventricles. The excitation wave then passes through the AV node and Purkinje fibers, which runs down the interventricular septum before spreading out to the walls of the ventricles. The small pause of the excitation wave in the AV node allows the ventricles to be filled with blood. The contraction phase (systole) and the relaxation phase (diastole) thus constitute the cardiac cycle. The cycle of contraction of heart muscle thus generated is the heart beat. Therefore, coordinated contraction of heart muscle cells and involvement of extracellular matrix (ECM) proteins are crucial for the maintenance of the rhythm of heart function.

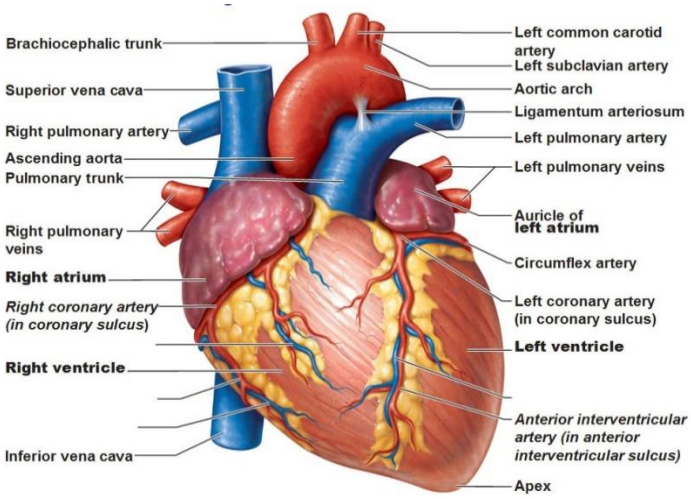


Figure 1 – Structure of the heart

1.1.1 Layers of heart tissue

The heart is surrounded by a membranous layered structure, the pericardium that protects the heart and provides room for vigorous pumping. The heart is composed of three layers – the epicardium, the myocardium, and the endocardium. The endocardium lines the inner wall of the heart. The myocardium, the middle wall makes up the bulk of the heart wall and it is composed of specialized heart muscle cells called cardiomyocytes (CMs). The outer layer of the heart is the epicardium or visceral pericardium, which is the inner layer of the pericardium.

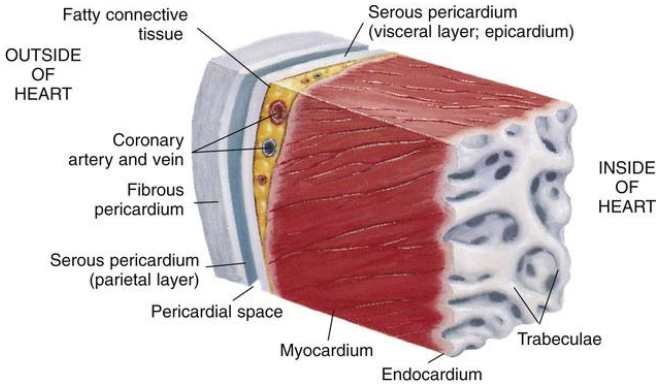


Figure 2 – Layers of the heart muscle

The endocardium is mainly composed of endothelial cells that form the inner lining of blood vessels and cardiac valves. Smooth muscle cells are found in the coronary arteries and the inflow and outflow vasculature. Epicardium contributes to the precursors of coronary vasculature and cardiac fibroblasts and is a part of the inner layer of the pericardium. The myocardium is mainly composed of a three-dimensional arrangement of high- dense cylindrical CMs (atrial and ventricular) and fibroblasts with an abundant vascular network and collagen-rich extracellular matrix. Each CM has a bundle of myofibrils divided into contractile units, the sarcomeres. Pacemaker cells and Purkinje fibers are specialized CMs that are involved in the conduction system of the heart, that generate and conduct electrical impulses. CMs pulsate via sodium and calcium ion transient through the cell membrane. Myocardial cells are electromechanically coupled through connexins and cadherins. Even though the myocardial cells appear to be structurally connected, the heart is a functional syncytium.

1.1.2 Cellular composition of heart

The adult mammalian heart is composed of many cell types, including cardiomyocytes (CMs), cardiac fibroblasts (CFBs), endothelial cells (ECs), and perivascular cells. CMs occupy 70-85% of the volume of the mammalian heart. It is estimated that the myocardium contains approximately 20 million cardiomyocytes per gram of tissue. Non-myocytes occupy a relatively small volume

fraction and are essential for the normal heart homeostasis, providing extracellular matrix (ECM), intercellular communication, and vascular supply needed for efficient CM contraction and long-term survival. The ECs play a major role in cardiac morphogenesis and function during heart development (Zhou and Pu, 2016). The cardiac fibroblasts are primarily called the 'Renaissance cells' of the heart that have a mesenchymal origin and provide a structural framework in the form of ECM and play central roles in cardiac fibrosis, adverse remodeling, and arrhythmogenesis. These cells have numerous functions including synthesis and deposition of ECM, cell-cell communication with myocytes, cell-cell signaling with other fibroblasts, and with endothelial cells. In normal myocardium, fibroblasts are involved in metabolite regulation, waste removal, and biochemical signaling by forming a highly coupled network with other fibroblasts as well as with the CMs. The other transient cell populations in the heart include lymphocytes, mast cells, and macrophages. During normal cardiac functioning, cellular components of the heart interact dynamically to respond to changes in developmental, homeostatic, and pathologic stimuli (Souders et al., 2009). The mammalian heart also consists of a pool of Cardiac stem cells (CSCs) that are distributed throughout the myocardium. It is estimated that 1 in 30,000 to 40,000 cells of the myocardium is a CSC that is capable of self-renewal, clonogenic, and multipotent that can differentiate into CMs, ECs, or vascular smooth muscle cells (VSMCs) *in vivo* and *in vitro*. The CSC clusters are involved in maintaining the myocardial cell homeostasis throughout life and participate in remodeling in cardiac pathology. Four major classes of CSCs have been identified in the mammalian heart based on the expression of surface markers – c-Kit$^+$ cells, Sca-1$^+$ cells, Side Population cells, and Islet – 1$^+$ cardiac stem cells. These CSCs are maintained in a microenvironment within which they remain undifferentiated and myocytes and fibroblasts act as the supporting cells in the niche surrounding the CSCs. Following a cardiac injury, these CSCs are mobilized from their niche where they undergo asymmetric division to differentiate into CMs, ECs, or VSMCs (Barile et al., 2007).

1.1.3 Extracellular matrix of the heart

The ECM consists of the acellular components of the heart and it provides a 3-dimensional network for myocytes and other cells of the heart to maintain proper cardiac function and structure. ECM is mainly synthesized and degraded by the cardiac fibroblasts. The major components of cardiac ECM include interstitial collagens that make up to 1-2% of the volume of the heart, proteoglycans, glycoproteins, cytokines, growth factors, matrikines, and proteases. The ECM also plays an important role in distributing the mechanical forces throughout the myocardium, by conveying mechanical signals to individual cells via cell surface ECM receptors (Souders et al., 2009). ECM plays an important role in post-natal myocardial growth and pathological myocardial hypertrophy like in hypertension and hemodynamic overload. Changes in the ECM structure and composition alter the morphogenesis, cell function, proliferation, and migration processes in the heart (Corda et al., 2000).

1.1.4 Blood vessels

There are three main types of blood vessels associated with the CVS – arteries, veins, and capillaries. Arteries are thick-walled blood vessels that carry blood away from the heart. The capillaries are a network of tiny vessels that are having extremely thin walls that permit the exchange of gases, nutrients, and fluids between blood, body tissues, and alveoli of the lungs. The capillaries merge to form the veins that return the blood to the heart. The heart has its blood vessels that supply the heart muscle with oxygen and nutrients. The coronary arteries supply oxygenated blood to heart tissue which is distributed to the cells via capillaries. The coronary veins formed by the convergence of capillaries drain out the deoxygenated blood to the right atrium.

1.2 Cardiovascular diseases (CVDs)

CVDs include a wide range of diseases like Coronary heart disease (CHD) leading to heart attacks, cerebrovascular diseases leading to stroke, rheumatic heart disease, congenital heart disease, peripheral artery disease, cardiomyopathies, and cardiac

arrhythmias. Among all the CVDs, CHD is the major culprit of mortality which accounts for around 7 million deaths per year.

1.2.1 Myocardial Infarction (MI)

One of the main underlying pathological processes that cause MI is atherosclerosis. Atherosclerosis is an inflammatory process affecting medium and large-sized blood vessels of the CVS. Atherosclerosis develops through a series of reactions, resulting in the accumulation of low-density lipoprotein (LDL) cholesterol, LDL-cholesterol engulfed macrophages or foam cells, smooth muscle cells, and collagen resulting in the formation of an atheromatous plaque covered by a fibrous cap. Rupture of the plaque results in the release of cell debris and lipid fragments to the vessel lumen which gets exposed to the thrombogenic agents on the endothelial surface of blood vessels resulting in the formation of occlusion or thrombus. The thrombus formation in coronary arteries results in severe ischemia or lack of oxygen supply to the heart muscle tissue leading to MI.

1.2.2 Pathophysiology of MI

MI caused by coronary artery occlusion begins to develop after 15-20 minutes of severe ischemia. The human left ventricle has 2-4 billion CM and an infarction wipes out almost 25% of the cells in a few hours after the onset of ischemia (Laflamme and Murry, 2011). After a myocardial injury, a normal healing response is initiated through a series of complex events. Four phases can be distinguished during cardiac wound healing (Cleutjens et al., 1999). Phase 1 is characterized by the death of CMs. The ischemic environment causes CM apoptosis and necrosis. The cell death after injury extends up to 4 days after infarction and evokes an early inflammatory response which forms Phase 2, which gets further aggravated by reoxygenation following reperfusion. This causes the accumulation of reactive oxygen species and toxic agents in the injured area. In response, the cells in and around the infarcted area secrete various chemokines and cytokines like tumor necrosis factor – α (TNF- α), monocyte chemoattractant protein 1 (MCP-1), interleukin (Il) -1β, Il -6 or Il-8 which causes infiltration of circulating leukocytes.

The up-regulation of cell adhesion molecules by endothelial cells further enhances the infiltration of pro-inflammatory immune cells into the injured area. Later monocytes home into the damaged area and mature into macrophages that clear cellular debris and matrix degradation products at the injury site. Two to three days after the infarction new ECM proteins are being deposited, initially in the border zone between the infarcted and non-infarcted tissue and later in the central area of the infarct. This marks Phase 3 of cardiac wound healing. The formation of the granulation tissue increases the tensile strength of the infarct and prevents cardiac rupture. The infarcted area is first deposited with fibrin, followed by the deposition of other ECM proteins. Few days following the injury, the infarcted area is occupied by myofibroblasts and these cells contribute to the formation of granulation tissue which is mainly composed of myofibroblasts, blood vessels, macrophages and collagen, and other extracellular matrix proteins. The granulation tissue then gets developed into a dense scar rich in collagen deposits, which is non-contractile because of the lack of cardiomyocyte regeneration. The series of the inflammatory response and the fibrotic scar poses an extra mechanical burden to the viable myocardial tissue surrounding the infarct region thus resulting in ventricular dysfunction. The disruption of the electromechanical continuum of the ventricular muscle tissue thus compromises the pumping efficiency of the heart leading to congestive heart failure (CHF) (Konstantinos D. Boudoulas and Hatzopoulos, 2009).

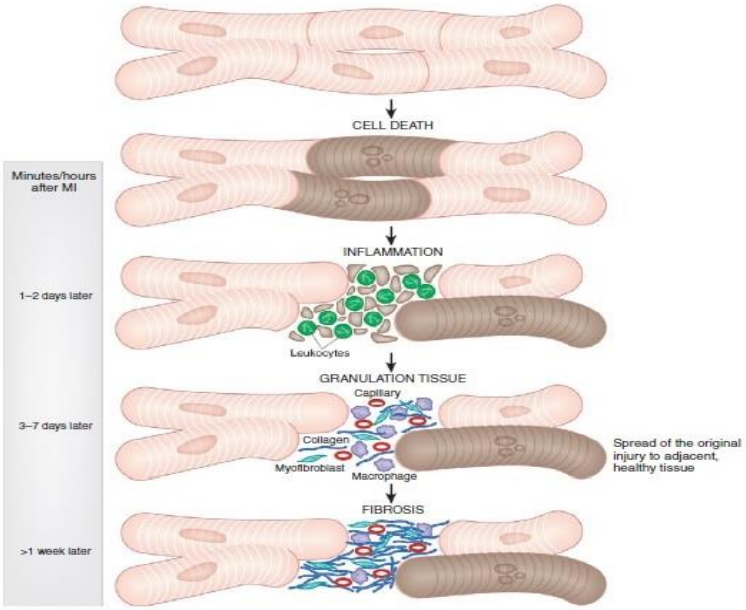


Figure 4 – Stages of events in MI

(Picture Courtesy: Boudoulas and Hatzopoulos, 2009)

1.2.3 Alterations in the non-infarcted myocardium

Initially, the events in the non-infarcted myocardium are not as dramatic as the changes in the infarct. However, following an MI, many constituents of the non-infarcted myocardium, including CMs, ECs, and the ECM undergo changes following MI. Changes in the non-infarcted myocardium are marked by CM hypertrophy within few days after the onset of an MI. DNA synthesis in CMs is a limited process and a large number of CMs in the non-infarcted region undergoes apoptosis contributing to the remodeling process after infarction. DNA (deoxyribonucleic acid) synthesis is more prominent in the ECs lining the capillaries. But EC proliferation is not high enough to fully compensate for the amount of cardiac hypertrophy resulting in a decrease in the capillary to myocyte fiber ratio, thereby increasing the oxygen diffusion distance (Cleutjens et al., 1999). Though the pool of endogenous CSCs is remarkably increased following a cardiac injury, the poor oxygen diffusion results in the death of the majority of CSCs by apoptosis. Thus, the intrinsic repair mechanism initiated by the pool of

stem cells present in the adult heart is limited due to potential barriers like ischemia, inflammation, and fibrosis that constitute the hostile microenvironment of injured myocardium (Torella et al., 2006).

The primary therapy of MI is aimed to restore the blood flow to the region of the myocardium, affected by the thrombotic occlusion of the coronary artery. Current therapeutic regimens to treat MI followed by heart failure include pharmacological interventions like the use of thrombolytic agents like the use of aspirin, clopidogrel that inhibits platelet aggregation by inhibiting adenosine diphosphate (ADP) action on platelet receptors, beta-blockers, and calcium channel blockers. Beta-blockers are reported to reduce the myocardial infarct size, lower the reinfarction incidence, and decrease mortality whereas, Calcium channel blockers have vasodilatory effects, decreasing the blood pressure. But several contraindications to thrombolytic therapy have been reported including hemorrhagic stroke, gastrointestinal bleeding, etc. Interventional therapies for MI include percutaneous transluminal coronary angioplasty (PTCA), implantable cardioverter-defibrillator (ICD), and cardiac resynchronization therapy. Primary percutaneous intervention (PCI) with or without stenting promises a better outcome than pharmacological therapy with fibrinolytic agents (Reddy et al., 2015). Though heart transplantation remains to be the gold standard therapy for MI followed by heart failure, patients receiving heart transplantation require lifelong immunosuppression and face chances of severe post-operative complications including primary graft failure and transplant vasculopathy. The success of heart transplantation for end-stage heart failure treatment is also limited due to the increasing gap between the supply and demand. Use of left ventricular assist device (LVADs) have shown improvements in symptoms and quality of life in patients, it also faces the problems like infections, bleeding, and device failure (Hsiao, 2013).

Even though these treatment modalities have alleviated the symptoms, improving the quality of patient care, and reduced mortality rates in patients with CVDs, a definitive therapy that can reverse the progression to end-stage heart failure and regenerate the lost functional myocardium is still lacking. So this scenario has

prompted the search for alternative therapeutic strategies that can repair and regenerate the damaged myocardium.

1.3 Current Strategies for myocardial regeneration

Cellular therapy and tissue engineering are emerging as promising alternative approaches to treat CVDs. Cellular therapy focuses on isolating cells and delivering the cells to the site of cardiac injury to restore blood flow and contractility to the scarred, dysfunctional heart. Myocardial tissue engineering approaches involve engineering heart tissue by seeding cells in three – dimensional matrices or scaffolds, either natural or synthetic polymers, that act as mechanical and biological support for cell growth and differentiation. The difficulty in regenerating the damaged myocardial tissue has led researchers to explore various cellular repair strategies to repair the infarcted heart. Cellular therapies used for myocardial repair focus to restore cardiac function by replacing the injured myocardium with functional CM. The major cellular repair strategies include the following:

a) Therapies that prompt the heart to regenerate.
b) Direct transplantation of cells into damaged myocardium
c) Tissue engineering techniques

In situ regeneration of injured myocardium was reported by several groups by direct delivery of cytokines and growth factors that potentially stimulate the healing and repair of the injured myocardium. The major mechanism of action of the direct delivery of such growth factors and cytokines may be attributed to their role in mobilizing endogenous CSCs and bone marrow-derived progenitor cells to the infarct site, where they undergo differentiation into ECs and improve angiogenesis or promote myocardial regeneration by their anti-apoptotic and anti-inflammatory effects (Leor et al., 2005). Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are key regulatory growth factors of angiogenesis and studies have reported that dual delivery of these growth factors using a single structural polymeric scaffold resulted in the rapid formation of a mature vascular network (Richardson et al., 2001). Similarly, Li and colleagues have reported that

overexpression of Insulin-like growth factor 1 (IGF-1) prevented cell death in viable myocardium after infarction, reduced ventricular dilation and cardiac hypertrophy (Li et al., 1997).

Direct transplantation strategy aims to repopulate the injured myocardium with the transplantation of healthy cells. Several cell types like fetal CM and skeletal myoblasts were found to improve cardiac function after transplanting to the injured myocardium. But issues like the inability of the transplanted cells to produce myocardial fibers in clinically relevant numbers and the inability of skeletal myoblasts to differentiate and integrate within the host myocardium limits its beneficiary effects.

1.3.1 Cell sources explored for Cardiac regeneration

An optimal cell source to create a myocardial patch should be easy to harvest, proliferative, non-immunogenic, and most importantly be able to differentiate into mature, functional CM. A variety of cell sources have been used for the treatment of MI including fetal CM, skeletal myoblasts, endothelial progenitor cells (EPCs), embryonic stem cells (ESCs), endogenous cardiac stem cells, bone marrow stromal cells, smooth muscle cells, mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) with each cell type having certain advantages and disadvantages.

CMs serves as the ideal source for the tissue engineering approach since these cells can maintain the excitation-contraction coupling with the native myocardium following transplantation. But lack of availability of enough adult CMs, their poor *in vitro* proliferative and survival capacity forced the use of fetal CM. The use of human fetal CMs for the development of cardiac patches is limited due to the limited availability of aborted embryos and also due to various ethical and political issues.

Embryonic stem cells (ESCs) that are derived from the inner cell mass of the blastocyst have the totipotent ability and have been used for differentiation into cardiac lineage *in vitro* under specific culture conditions. Transplantation of ESCs

into animal models of MI was found to exert positive effects on the left ventricle that was analyzed by measuring various hemodynamic parameters like regional wall motion and diastolic dimensions of the expanded left ventricle. However, only a small proportion of ESCs differentiate into CMs under specific culture conditions *in vitro* and the exact mechanism by which these cells differentiate into CMs has not been elucidated yet. ESCs also pose the risk of teratoma formation and immune rejection response at the site of transplantation which limits its use in clinical trials as the ideal cell source for cardiac regeneration.

Induced pluripotent stem cells (iPSCs) are stem cells derived from mature somatic cells using reverse transcription factors Oct3/4, Sox2, Klf4, and c-Myc and have the ability to differentiate into a variety of cell lineages including CM. But iPSCs share the issues like poor efficiency of CM differentiation, the formation of a heterogeneous cell population, risk of teratoma formation, and immune rejection response similar to that of ESCs which limits its use for cardiac regeneration.

The disadvantages of ESCs and iPSCs have led most of the researchers to focus on various adult stem cell sources as potential candidates for cardiac regeneration. Skeletal myoblasts have been used in certain clinical trials and were found to improve left ventricular ejection fraction, but the follow-up studies proved that they posed the risk of developing severe arrhythmias due to lack of electromechanical coupling with the native myocardium since they cannot transdifferentiate into functional CMs. EPCs have been used in certain animal models of MI and were found to improve cardiac function post-MI. However, such an improvement in cardiac function was due to the role of EPCs in promoting angiogenesis and paracrine effects to maintain the survival and division of CMs. But the insufficient characterization of EPC-derived cells limits its use as a cell source for cardiac regeneration.

Several research groups have identified the presence of various endogenous cardiac stem cell (CSCs) populations like Stem cell antigen 1^+ (Sca-1^+) cells, c-Kit^+ cells, Isl-1^+ cells, and cardiac side population (SP) cells in the adult mammalian heart, that can differentiate into CMs, endothelial cells, and smooth muscle cells. These

endogenous cardiac stem cells reside in a niche within the healthy myocardium in an undifferentiated state. Following a myocardial injury, most of the human cardiac stem cell pools acquire a senescent phenotype that fails to differentiate into functional CM or vascular cells. Such senescent CSCs become non-cycling and non-differentiating thus resulting in regenerative cell deficit which later leads to the development of end-stage heart failure. The use of CSCs for the regeneration of damaged heart is limited due to its disadvantages like functional impairment when isolated from biopsy tissue of older patients.

One of the most widely used cell sources for cardiac regeneration is mesenchymal stem cells (MSCs). MSCs are multipotent cells that can differentiate into a variety of lineages including chondrocytes, osteoblasts, adipocytes, skeletal myoblasts, and CMs, and are present in a variety of tissues like bone marrow, adipose tissue, umbilical cord blood, synovium, dermis, and deciduous teeth. They are also present in various organs like the liver, spleen, and lungs. The MSCs can be easily isolated, expanded *in vitro* in large numbers, and can be genetically modified which makes them a promising candidate for cardiac regeneration. Moreover, MSCs are hypo immunogenic, since they lack HLA (human leukocyte antigen) class II and co-stimulatory molecules and have immunosuppressive properties. Bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-derived mesenchymal stem cells (ADMSCs) have been used in a variety of animal studies and was found to improve heart function by reducing infarct size and increased left ventricular ejection fraction. Based on the success of preclinical trials BM-MSCs were used in clinical trials since 2002 and an improvement in cardiac output of patients treated with BM-MSCs was reported by various research groups. Three major mechanisms of action have been proposed for MSC in heart repair.

a) CM regeneration
b) Vasculogenesis
c) Paracrine effects

Makino et al, in 1999 reported CM differentiation of mouse BM-MSC *in vitro* using 5- azacytidine induction. Similar studies were conducted using human MSCs in

animal models of MI and the cells were engrafted to the infarct border zone and were found to differentiate into cardiac-like cells expressing cardiac markers. MSCs were also found to induce vasculogenesis by turning into pericytes thus stabilizing and favoring the maturation of new blood vessels (Segers and Lee, 2008). MSCs secrete a variety of growth factors and cytokines. These cytokines and growth factors were found to favor neovascularization, cytoprotection, and activation of resident cardiac stem cells.

Adult bone marrow has been considered as the main source for the isolation of mesenchymal stem cells. But harvesting MSCs from bone marrow is a highly invasive procedure. Stem cells constitute about 0.001% to 0.01% per milliliter of bone marrow aspirate while the number of stem cells present per gram of adipose tissue is around 1% - 10%, mostly depending upon the donor and tissue harvesting site. The isolation procedure of MSCs from adipose tissue is minimally invasive as compared to that of bone marrow, more practical, and cost-effective. Furthermore, the feasibility of using allogenous human ADMSCs (hADMSCs) is considered better as it faces fewer ethical concerns as compared to that of BM-MSCs since the fat tissue is discarded as medical waste. Also, it is feasible to be collected from the patient's fat for autologous patient-specific cell therapy. hADMSCs, like BM-MSCs, are capable of self-renewal and exhibit multipotency, and have the potential to differentiate into cardiovascular lineages, CMs, ECs, and Vascular Smooth Muscle Cells (VSMCs) (Fraser et al., 2006).

1.4 Status of cell-based therapy in MI regeneration

In preliminary clinical studies, researchers have used several approaches to deliver the stem cells to the injury site. Common routes of delivery include intravenous injection and direct infusion into coronary arteries (intracoronary) and intramyocardial routes, during open-chest surgery. Retention of cells immediately after delivery is highly dependent on the delivery strategy; among these methods, intracoronary infusion offers the advantage of directed local delivery (Goldthwaite Jr, 2007), whereas intramyocardial delivery route results in loss of many cells through the vasculature. Survival in the inflammatory environment of infarcted

myocardium is a challenge to all types of transplanted cells, as almost 90% of the cells die within a week. Persistent ischemia in the infarcted myocardium also limits cell survival and revascularization. Proper structural and functional electromechanical integration of transplanted cells with the host myocardium is also necessary for maintaining the electromechanical continuum of the heart to prevent any arrhythmic event followed by stem cell transplantation to the injured myocardium (Segers and Lee, 2008).

The therapeutic efficacy of transplanted stem cells for regeneration of infarcted myocardium depends on the ability of these cells to home, proliferate, differentiate and survive in the host tissue and this is very much influenced by the microenvironment within which the cells are grown. The ECM composition and the growth factor combination is critical for the regulation of stem cell properties and its differentiation during *in vitro* culture and for proper integration *in vivo* upon transplantation (Chavakis et al., 2010). Hence, the constitution of a biomimetic niche composed of insoluble and soluble ECM components and growth factors may be a suitable approach for inducing *in vitro* differentiation of hADMSCs into cardiomyocytes and cardiac fibroblasts. It has been reported that peripheral blood mononuclear cells were induced to differentiate into endothelial cells and smooth muscle cells *in vitro* in a natural scaffold, fibrin, along with fibronectin, gelatin, and appropriate growth factor cocktail (Sreerekha et al., 2006). The effect of fibrin for the development of cardiac cell sheets using neonatal rat ventricular cardiomyocytes that exhibited strong spontaneous beating and well-differentiated striations was reported earlier by Itabashi and colleagues (Itabashi et al., 2005).

Even though hADMSCs are reported to be a promising candidate for cardiac regeneration, the *in vivo* differentiation potential of hADMSCs into CMs is not well established. Direct transplantation of hADMSCs to the infarcted heart also failed to produce any remarkable improvement in heart function, due to its multipotent nature. The hostile microenvironment persisting in the infarcted heart further reduced the chances of cell retention and its differentiation. This necessitates the need for *in vitro* manipulation of hADMSCs before transplantation to improve the cardiac function following MI.

1.5 Definition of the problem

Although a plethora of cell types have been used for regeneration of the heart tissue following a MI, an ideal cell source that can be used for myocardial tissue engineering is still unknown. Recent developments in stem cell therapy have effectively explored the potential of adult stem cells for cardiac regeneration. Even though hADMSCs were found to be a promising candidate for heart regeneration, it poses several challenges to address.

a) The *in vivo* differentiation potential of hADMSCs into CM is not well established.
b) Upon transplantation of hADMSCs in their multipotent stem cell state into an infarcted heart may result in undesirable cell fate and outcome.
c) Poorly defined conditions for *in vitro* lineage commitment of hADMSCs into CM progenitor cells (CMPs) for transplantation purposes.
d) *In vitro* methods yielding low percentage hADMSC differentiation to CMPs due to the absence of an appropriate niche
e) Poor survival of transplanted ADMSC or lineage-committed ADMSC when transferred to hostile and infarct microenvironment.

In the above context, the gaps identified for the current study are limited as follows.

- Association of angiogenic responses for the organization of cardiac tissue upon cell-based therapy has not been studied
- Both cell-based and protein-based niche may be important for the survival of progenitors *in vitro* and *in vivo;* this concept has not been addressed
- The influence of the cell delivery matrix on the survival of transferred cells has not been studied.
- Tissue level analysis for determining the survival and differentiation of transplanted cells in the cardiac tissue is not seen.

To address the identified gaps, in the field of stem cell therapy for cardiac tissue regeneration, a study hypothesis has been developed.

1.6 Hypothesis

The concept of autologous hADMSCs as a favored source has been proposed by many investigators. Also, several reports suggest that the hADMSC from CVD patients may not show true stem cell properties such as proliferation potential and multi-potency. Therefore, to plan and implement patient-specific cell-based therapy, the first step is to prove that ADMSCs may be isolated from patients and get them pre-differentiated to cardiac lineage. Also, studies have proven that cardiomyocytes are difficult to survive in culture. So the major focus of this study is to explore the designing of a biomimetic matrix along with supporting cells for improving the differentiation of hADMSCs to CMPs. It was also recognized that obtaining proliferating progenitor stage CMPs may be an important aspect of implementing effective cell-based therapy. Also, because MI damages the tissue architecture, the study envisaged that an injectable matrix with *in situ* gelling property may be a suitable strategy to retain the transplanted cells at the site of injury. Therefore, it is proposed that:

A biomimetic niche that could support the induction of hADMSCs to cardiomyocyte progenitors (CMPs) and cardiac fibroblast progenitors (CFPs) may be designed for co-survival with endothelial progenitor cells (EPCs) to induce angiogenic responses in the co-culture in vitro.

The study planned: (i) Isolation of hADMSC from CVD patients for the derivation of CMPs, CFPs and EPCs to establish feasibility of using autologous cells for transplantation, (ii) Standardization of niche conditions for differentiation of hADMSCs to CMPs, CFPs, using the biomimetic components of ECM and growth factors, (iii) understanding the influence of seeding hADMSC derived EPCs to the CMPs-CFPs population for maintaining differentiation status of each cell type and for induction of angiogenic response, and (iv) conducting a preliminary study to establish the feasibility of using injectable fibrin matrix as a delivery medium to achieve the survival of progenitors transferred to MI in an animal model.

1.7 Objectives of the study

- To establish stem-ness of hADMSCs isolated from patients undergoing cardiac bypass surgery.
- To study the role of the fibrin-based niche in inducing differentiation of hADMSCs to CMPs *in vitro*.
- To standardize culture conditions and establish differentiation of hADMSCs to CMPs, CFPs, and EPCs.
- To co-culture CMPs, CFPs and EPCs and study the influence of each cell type on the expression of cell-specific markers, if any.
- To identify if hADMSC-derived secretome influences the cell-specific markers of CMPs and CFPs.
- To study the influence of fibrin matrix for post-transplantation survival of rat CMPs in an animal model of MI.

1.8 Rationale of the study

This study aims to utilize human ADMSCs as a promising candidate for cardiac regeneration following a MI. The study goal is pre differentiating the stem cells into cells that may be used for the treatment of MI. The major goal of this study is attempting to establish that hADMSC derived from CVD patients could be used for generating 3 different functions upon cell transplantations, such as (i) produce CMPs, (ii) provide supporting fibroblast cell to produce ECM niche through the derivation of CFPs from hADMSCs (iii) produce angiogenic activity in cell cultures by providing ADMSC-derived EPCs and vascular endothelial growth factor (VEGF) transfection of hADMSCs. Taken together, the standardized methods could probably be a suitable system for cell-based therapy. Ultimately the survival of CMPs upon transplantation to the MI is the initial concern to predict the outcome of the therapy. Therefore, the transfer of progenitors and niche components to the MI in the animal model was planned to establish that CMPs survived in the region of injury.

CHAPTER 2

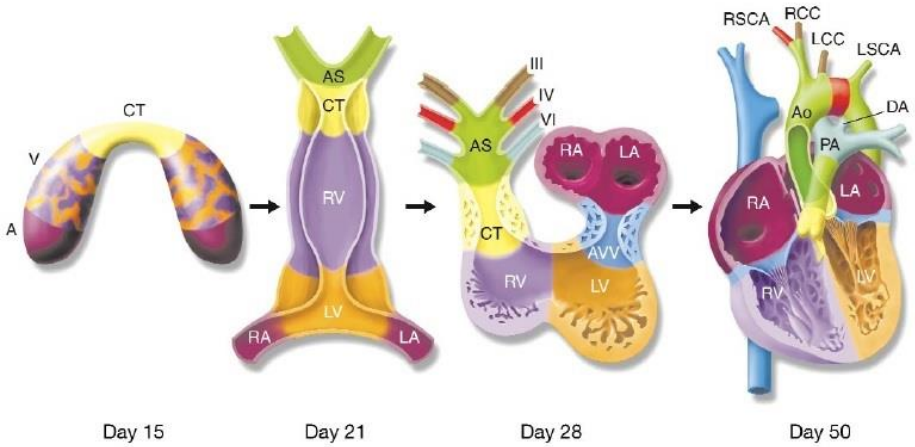
2. LITERATURE REVIEW

This section summarizes the recent advancements in the field of myocardial tissue regeneration based on published literature. The embryonic development of the heart and the role of various signaling mechanisms that play a pivotal role in the development of the adult mammalian heart are reviewed in detail in this section. The hurdles in cardiac regeneration are briefly mentioned in this section. The need for *in vitro* pre-differentiation of adult stem cells for cardiac regeneration using extracellular matrix components and various growth factors for transplantation purposes is also reviewed in this section.

2.1 Mammalian heart development

The mammalian heart is the first organ to form during progressive embryonic development and it involves precisely orchestrated molecular and morphogenetic events. Cardiogenesis is an active and complex process which includes co-ordinated phases of pattern formation, morphogenesis, and gene expression regulation. The heart develops from the mesodermal germ layer of the embryo (Franco et al., 1998). Heart tissue is derived from the anterior splanchnic mesoderm and the embryonic development of the heart is mainly divided into four stages – a) cardiac crescent formation, b) formation of the linear heart tube, c) tube looping and segmentation, and d) chamber formation and maturation (Harvey, 2002). During gastrulation, the bilaterally arranged cardiac progenitor cells migrate through the node and primitive streak and move to the anterior and lateral aspects of the embryo to form the paired heart fields. Later, as the embryonic disc folds, these cells form the 'cardiac crescent' around day 15 of human embryonic development. The cells from the primary heart field contribute to a major portion of the left ventricular myocardium and the cells from the second heart field contribute to the formation of the right ventricular myocardium, outflow tract, endocardium, and smooth muscles of great vessels. As the embryonic development progresses, the lateral folding of the embryonic disc causes

the fusion of the heart progenitor cells in the median of the embryo resulting in the formation of the linear heart tube or the primitive heart tube. The primitive heart tube begins to beat as early as embryonic day 22 in humans and the circulation begins by day 27-29 (Abdulla et al., 2004). The primitive heart tube develops many constrictions that outline future structures. The cranial-most area is the bulbus cordis and extends cranially into the truncus arteriosus. The primitive ventricle is caudal to bulbus cordis and the primitive atrium forms the caudal-most structure of the tubular heart. Looping of the primitive heart begins around day 23 of development and it is genetic property of the myocardium. The cephalic end of the heart tube bends ventrally, caudally and to the right. The looping of the heart results in the formation of a U- shaped structure with the bulbus cordis forming the right arm of the U-shaped heart tube and the primitive ventricle forming the left arm. The final stage of heart development is marked by chamber formation and maturation during which period the growth of the heart from thin-walled structure to fully functional chambers happen. The formation of atrioventricular and semilunar valves occurs during the period of embryonic day 50 till birth. The formation of the early cardiac conduction system occurs during the final stages of heart development in human embryos.



A- atrium, Ao- aorta, AS - aortic sac, AVV- atrioventricular valve, CT - conotruncal, DA - ductus arteriosus, LA - left atrium, LCC - left common carotid, LSCA - left subclavian artery, LV - left ventricle, PA - pulmonary artery, RA - right atrium, RCC - right common carotid, RSCA - right subclavian artery, RV - right ventricle, V - ventricle

Figure 4 – Stages of mammalian heart development.

Picture courtesy: (Srivastava and Olson, 2000)

2.2 Signalling in heart development

The heart develops from the mesodermal germ layer of the embryo. The development of the heart from a linear heart tube into a four-chambered structure involves complex and well-coordinated morphogenetic processes that involve a variety of cell and tissue interactions mediated by various signal transduction pathways (Wagner and Siddiqui, 2007). Heart formation begins with the migration of mesodermal cells from the embryonic midline cranially to form the cardiac crescent or the primary heart field. Mesoderm in mammals is divided into dorsal somatic mesoderm and ventral splanchnic mesoderm. The cell layers neighboring the splanchnic mesoderm – the anterior endoderm, neuroectoderm, and the node produce positive and negative signals for the induction of cardiogenesis. The positive signals mainly emerging from the endoderm induce mesoderm to cardiogenic lineage by inducing cardiac-specific transcription factor expression, while the negative signals play to delimit the cardiogenic induction to a specific population of mesodermal cells. The concerted action of positive and negative signals from the neighboring tissues drive the determination and differentiation of the primary and secondary heart field cells (Harvey, 2002). The main positive signals include Bone morphogenetic proteins (BMPs), Fibroblast growth factors (FGFs), Crescent, and mesodermally derived Wnt 11. The major inhibitory signals that are involved in cardiogenesis include Chordin, Noggin, and Wnts 3a, and 8 (Wagner and Siddiqui, 2007).

2.2.1 BMP signaling

BMPs belong to the transforming growth factor –β (TGF- β) superfamily and are disulfide-linked dimeric proteins. Among the members of the BMPs, BMP-2 and BMP-4 growth factors induce the formation of cardiogenic cells in non-precardiac mesoderm *in vitro*. The BMP signal transduction pathway acts via the Smad pathway and the TAK-MKK3/6–p38/JNK pathway. Both pathways activate the nuclear transcription factor Activating transcription factor 2 (ATF-2) having the histone acetyltransferase activity thus resulting in the activation of subordinate genes which include early cardiac transcription factor genes *Nkx 2.5*, *GATA -4, Tbx 2,3,* and *MHC*.

Thus BMP signaling plays an important role in the early steps of cardiogenic induction by inducing mesodermal cells into cardiogenic lineage (Schultheiss et al., 1997).

2.2.2 FGF signaling

Fibroblast growth factors (FGFs) comprise a large family of polypeptide growth factors that are involved in a variety of cellular processes including cell migration, chemotaxis, proliferation, angiogenesis, differentiation, cell survival, and apoptosis. FGFs induce cardiogenic induction synergistically with BMPs (Wagner and Siddiqui, 2007). FGF-8 mediates cardiac induction in the secondary heart field by inducing the expression of cardiogenic genes *Nkx 2.5* and *MEF 2c*. FGF signals are transmitted via 3 pathways - the Ras/MAPK pathway, phospholipase C-γ/ Ca^{2+} pathway, and the phosphatidylinositol 3 (PI3) – kinase/Akt pathway. Thus FGF signals play a major role in the proliferation and survival of differentiated cardiomyocytes in synergy with the BMP signaling during heart development (Solloway and Harvey, 2003).

2.2.3 Wnt signaling

Wnt proteins are a family of secreted glycoproteins that play a pivotal role in various developmental processes like cell fate determination, proliferation, differentiation, migration, and establishment of cell polarity of various cell types. The Wnt proteins play a dual role in cardiogenesis based on the signal transduction pathway through which it acts on the cells. Wnt 11 activates the Wnt/polarity pathway that positively induces cardiogenesis while Wnt 3a and Wnt 8, expressed in the cardiogenic mesoderm activate the Wnt/β- catenin pathway thus inhibiting Cardiogenesis (Solloway and Harvey, 2003).

2.3 Formation of Left-right asymmetry (L/R asymmetry) and Chamber septation

The conversion of anterior/posterior organization of the linear heart tube to a primitive heart with two atrial and ventricular chambers in the left-right identity is critical to normal heart chamber formation. The formation of left-right asymmetry begins with the rightward looping out of the heart tube and is driven by the presence of a molecular

asymmetry within the heart tube. The process of left-right asymmetry development has four main stages - (1) initial breaking of L/R identity in/near Node, (2) transfer of L/R biased signals from node to lateral plate mesoderm, (3) L/R asymmetric expression of signaling molecules and (4) L/R asymmetric morphogenesis (Hamada et al., 2002). The major signaling molecules that are involved in the L/R asymmetry generation include Nodal, Lefty 1, Lefty 2, Pitx2, and Hedgehog.

Following the rightward looping of the heart tube, the looped heart gets segmented into the atrium, atrioventricular canal (AVC), ventricles, and the outflow tract (OFT). The looped heart has an inner endocardial lining and an outer myocardial layer. Local tissue swellings or endocardial cushions are formed in the lumen of AVC and proximal OFT by the accumulation of abundant extracellular matrix, which is also termed as the 'cardiac jelly' in between the endocardium and the myocardium (Lockhart et al., 2011). Mesenchymal cells originating from the endocardium later occupy these spaces. In the distal OFT, mesenchymal cells originating from the neural crest later populate the local tissue swellings. The outermost epicardial layer of the heart is then formed by a sheath of cells originating from the proepicardial organ, which grows over the myocardium. The atria and ventricle then undergo septation to from the four-chambered heart. AVC also gets separated into left (mitral) and right (tricuspid) outlets that connect the respective ventricle to the atrium. OFT divides into left and right ventricular outlets that connect the left ventricle to the aorta and right ventricle to the pulmonary trunk respectively. Several signaling pathways play a pivotal role in AVC and OFT cushion formation and septation and the major signaling pathways involved in the process include, Notch signaling, Wnt signaling, VEGF and TGF/BMP signaling, and the Sonic hedgehog signaling pathways (Lin et al., 2012).

2.4 Cardiac transcription factors – role in vertebrate heart development

The myogenic and morphogenic programs are regulated by a network of transcription factors expressed in time and tissue-specific manner (Olson, 2006). Some of the major transcription factors that play a central role in all muscle lineages include *Nkx2-5* (NK2 transcription- factor related, locus 5), *GATA 4/5/6*, myocyte enhancer factor (*Mef2b/Mef2c*), *Hand1/Hand2* (Heart and neural crest derivatives expressed transcript

1 or 2) and T – box 5/20 (*Tbx5/Tbx20*) (Solloway and Harvey, 2003). Nkx2-5 is considered as one of the earliest molecular markers of cardiac lineage and is crucial for the differentiation process and maintenance of ventricular gene expression program and looping morphogenesis (Schwartz and Olson, 1999). *Mesp 1* and *Mesp 2* are basic helix-loop-helix transcription factors required for the migration of cardiac precursors. *GATA* family of transcription factors is a family of zinc-finger containing transcription factors that contribute to the activation of a cardiac-specific gene program. *GATA 4, 5,* and *6* genes are expressed in the developing heart and can interact with other transcription factors like *Nkx2.5, MEF-2, dHand,* etc. and plays a pivotal role in cardiac precursor cell migration from primitive streak and formation of the linear heart tube. The major factors that contribute to patterning and chamber formation in the developing heart include Retinoic acid (RA), Iroquois homeobox protein 4 (*Irx 4*), T-box genes, and *GATA*. RA is essential for the regulation of asymmetry inducing genes. *Irx 4* is involved in the chamber-specific gene expression and the development of ventricular chambers. It also regulates the expression of ventricular myosin heavy chain (*VMHC*) (Solloway and Harvey, 2003). The T-box family of genes is involved in the formation of posterior heart structures and *Tbx* deficiency is found to downregulate *Nkx2.5, GATA, Irx 4,* and myosin light chain (*MLC 2V*) expression. *Tbx 5* is involved in the ventricular differentiation and its misexpression in embryos resulted in a lack of ventricular septum formation and the development and maturation of the cardiac conduction system. *Mef 2* families of transcription factors are involved in the development of secondary heart field structures, ie. Right ventricle and outflow tract. It also binds to the promoter regions of MLC and α – myosin heavy chain and regulates their expression pattern. The Hand factors (*Hand 2/dHand and Hand1/eHand*) play an essential role in cardiac morphogenesis, cardiomyocyte differentiation, and cardiac-specific transcription (Olson, 2006).

2.5 Markers of cardiac differentiation

The heart is an organ that acts as a functional syncytium and cardiac cells are connected by the formation of intercalated discs that facilitate electrical conduction. Another unique feature of the heart is that it spontaneously generates its electrical impulses. Three main types of proteins are found in the mature myocardium of the heart –

myofibrillar proteins, metabolic proteins, and extracellular proteins (Forough et al., 2011). Myofibrillar proteins transform the chemical energy of ATP (adenosine triphosphate) to the mechanical work of the heart and are only expressed by the heart, thus serving as excellent biomarkers for CMs. The two most important among them are the Troponin proteins – cardiac Troponin T (TNNT) and cardiac Troponin I (TNNI). They form an integral part of the contractile machinery of the sarcomeres, the structural and functional unit of muscle fiber. The other major contractile proteins include myosin and actin. Myosins are thick filament proteins with a molecular weight of 460kDa and consists of two heavy chains and two light chains (Tang et al., 2017). Two myosin heavy chain (MHC) isoforms are expressed during development, α- MHC and β – MHC, and the patterns of expression of this protein varies during different stages of development and adulthood. The human heart predominantly expresses β – MHC in the ventricles that are encoded by the *MYH7* gene. Three isoforms of regulatory myosin light chain (MLC) is reported in striated muscles, with MLC2a confined to atrial myocardium and MLC2v expression in ventricular myocardium and MLC2f expression in the embryonic heart (Franco et al., 1998). Alpha actinin is a major structural protein that helps in maintaining the myofibrillar structure (Adamcová et al., 2006). Connexin 43 is an important cardiac surface protein for forming hemichannels and gap junctions within the heart and is involved in the formation of intercalated discs. The major extracellular proteins in the heart include collagens, glycoproteins, glycosaminoglycans, and elastins. Among the 24 different varieties of collagens found in the heart ECM, Collagen type I and III are the predominant interstitial collagens that constitute about 1-2% of the total volume of the heart. The other major proteins found in the ECM of the heart include laminins, fibronectin, thrombospondin, fibrillin 1 and tenascin, among which fibrillin -1 forms the second abundant protein in the adult heart ECM (Jourdan-Lesaux et al., 2010) (Williams et al., 2014). Fibrillin 1, a 350kDa protein act as the major structural component of the heart ECM microfibrils and is located in the periphery of elastic fibres. Cardiac fibroblasts, that are primarily involved in the ECM production of the heart is characterized by the presence of Discoidin Domain Receptor 2 (DDR2), a receptor tyrosine kinase that uses triple helix Collagen I and III as a ligand. Studies have shown that mice lacking DDR2 gene are small and have abnormal cardiac structure and

function. Activation of DDR2 mediated signalling increased transcription of multiple genes including matrix metalloproteinases and are responsible for LV (left ventricular) remodeling followed by MI (Blankesteijn, 2015).

2.6 Extracellular matrix in heart development and disease

The extracellular matrix (ECM) of the heart is a network structure that provides structural and functional integrity to the heart. It forms a highly dynamic milieu that plays an active and crucial role in the regulation of a wide variety of cellular events like cell-cell signaling, regulation of cell proliferation, cell differentiation, and cell migration. The ECM plays an essential role in embryogenesis by mediating the spatiotemporal regulation of cell migration, reorganization, and differentiation of individual heart structures. Cardiac ECM maintains the mechanical connection among the cardiomyocytes, cardiac fibroblasts, and blood vessels within the myocardium and also transmits extracellular mechanical signals to cardiomyocytes. Cardiac fibroblasts are the primary source of all major ECM proteins. Cardiac ECM also comprises ECM – regulatory proteins called Matrix metalloproteinases (MMPs) that degrade ECM proteins, and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Homeostasis of cardiac ECM is maintained by a well-controlled balance between the functions of MMPs and TIMPs (Fan et al., 2012). ECM is particularly abundant in the developing mesenchymal structures of the heart that plays a major role in the valvuloseptal morphogenesis of the heart. Four major mesenchymal tissues in the developing heart include atrioventricular cushions, outflow tract cushions, dorsal mesenchymal protrusion, and the epicardium. A wide variety of ECM macromolecules play an important role in the developing cardiac tissues and these include collagens, fibrillins, hyaluronans, proteoglycans, fibronectin, laminin, elastin, and tenascin (Lockhart et al., 2011). In the myocardium, ECM is linked to the cellular cytoskeleton by integrins and is mainly involved in the bi-directional signal transduction, mediated through integrin receptors that are transmembrane heterodimers. The composition of ECM molecules and integrin receptors vary during different developmental stages like fetal, neonatal and adult stages with fetal and neonatal heart having higher expression of fibronectin and periostin as the major ECM molecules while adult heart having a relative abundance of Collagen I and Fibrillin 1 (Williams et al., 2014).

The predominant molecules present in the ventricular ECM network of a healthy myocardium are Collagen I and Collagen III, among other structural proteins. In the normal healthy myocardium, Collagen I is the major collagen type, and upon an injury, Collagen III is produced. Cardiac remodeling in heart disease is marked by a change in mass, volume, and shape of the ventricles. Chronic heart failure is characterized by an overall imbalance of ECM turnover with myocardial collagen accumulation, collagen fibril disruption, myocyte loss, and alterations in the spatial orientation of cells and intracellular components. Though there is no significant reduction in the total collagen content in the heart post-MI, altered arrangement and poor cross-linking between the collagen fibrils are the major reasons that compromise the structural integrity of the heart leading to Left Ventricular (LV) dilation (Kassiri and Khokha, 2005). During left ventricular remodeling post-MI, the following ECM components are upregulated – laminins, fibronectins, thrombospondins, and tenascins. Laminin helps to maintain tissue organization by crosslinking other ECM components and act as a molecular sieve. Fibronectin plays a pivotal role by crosslinking platelets and facilitating blood clotting and is involved in mechanotransduction. Biglycans, decorins, and perlecans are the major proteoglycans that are upregulated post-MI (Jourdan-Lesaux et al., 2010).

2.7 Cardiac fibroblasts

The myocardium is composed of cell types like cardiomyocytes, cardiac fibroblasts, endothelial cells, and smooth muscle cells (Fan et al., 2012). Though two-thirds of myocardial tissue volume is occupied by cardiomyocytes, cardiac fibroblasts (CFBs) account for the most abundant cell type in the mammalian heart. CFBs are of mesenchymal origin and they lack a basement membrane that makes it a unique cell type. Though CFBs have diverse origins, the majority of embryonic CFBs are derived from the proepicardial organ. Some of the cells of the proepicardial organ undergo epithelial - to - mesenchymal transition (EMT) that later invades the atrial and ventricular walls and differentiate into CFBs. This EMT is tightly regulated by various signaling factors including Ets factors, Fibroblast growth factors (FGFs), Platelet-derived growth factor β (PDGF β), Tbx5, Thymosin β4, and TGFs. The other sources of CFB are endocardium, bone- marrow-derived precursors, fibrocytes, and

monocytes (Lajiness and Conway, 2014). CFBs are involved in a variety of aspects of cardiac function including homeostasis and remodeling of cardiac ECM, cell-cell communication with cardiomyocytes, electrical activity, production of growth factors and cytokines, and intercellular signaling with other CFBs thus maintaining the normal structure, function, biochemical and electrical features of the heart (Fan et al., 2012). CFBs form a dynamic cell population in the heart and have an extensive and balanced communication with the cardiomyocytes via several signaling molecules. Following an ischemic injury to the heart, CFBs differentiate into highly proliferative and migratory cell type called the "activated" cardiac fibroblasts or myofibroblasts. These myofibroblasts are mainly responsible for the remodelling of cardiac ECM following an insult to the heart muscle (Lajiness and Conway, 2014).

2.8 Acute myocardial infarction – mechanisms of cell death

The heart is an organ with a limited capacity for regeneration and repair. Cell death, either progressive or acute, is considered a hallmark characteristic of cardiac diseases including MI, heart failure, and ischemia/reperfusion. In the infarcted myocardium, an area of total ischemia has no flow and diffusion is very slow from the center of the area of ischemia. The extent of myocardial damage may vary from small regional injury to a subendocardial lesion or transmural damage to the myocardial structures. Major mechanisms of cardiac injury include oxygen deprivation and depletion of high energy phosphates, osmotic cell stress, and water accumulation leading to disruption of the sarcolemma, activation of lysosomes that hydrolyze the myocyte cell membrane and cell content. Severe ischemia increases the probability of sarcoplasmic Ca^{2+} channels to remain open leading to damage of membrane phospholipids and ion channels thus lowering ATP production and accelerating myocyte necrosis. MI also induces the activation of the complement system by the infiltration of various pro-inflammatory cytokines in the injured area. Major cell death pathways following an ischemic event include apoptosis, autophagy, and necrosis (Konstantinidis et al., 2012). Apoptosis is characterized by cell shrinkage, fragmentation into membrane-enclosed apoptotic bodies, and phagocytosis of the debris by macrophages. Myocyte apoptosis begins in 2-4 hours following a MI and a peak of myocyte apoptosis is reported after 6-8 hours after the onset of infarction and is the main source of

cardiomyocyte loss after infarction. Myocyte loss by necrosis occurs from 12 hours till the 4th day after the onset of an infarct event and is characterized by loss of plasma membrane integrity, cellular and sub-cellular swelling followed by inflammation (Haunstetter and Izumo, 1998). Autophagy is a physiological process that is involved in the degradation of long-lived proteins and removing excess or damaged organelles. The rate of decline of ATP plays an important role in determining whether myocytes enter apoptosis or necrosis in response to ischemia or other stimuli. Studies have reported that the number of autophagosomes increases in the heart both during ischemia and reperfusion. In fetal mouse hearts, a period of hypoxia combined with glucose deprivation followed by reperfusion enhanced autophagy. Signals that induce autophagy in the heart include ATP levels, hypoxia, reactive oxygen species, and reactive nitrogen species (Chiong et al., 2011).

2.9 Cardiac regeneration – the role of cardiac stem cells

For more than 50 years, the heart was considered to be a terminally differentiated organ and cardiac hypertrophy of existing myocytes was considered as the only mechanism in response to increased workload. When myocardial hypertrophy gets exhausted, ventricular dysfunction supervenes resulting in cardiac failure. The presence of a multipotent progenitor cell population in the heart was identified after the sex-mismatched cardiac transplants in humans. In such studies, the female heart transplanted into a male host was documented to have a significant number of Y-chromosome-positive myocytes and coronary vessels that originated from the host and colonized the transplanted heart and subsequently differentiated. These cells were negative for all markers of hematopoietic lineages (Lin-) and expressed stem cell marker epitopes c-kit, Sca-1- like and multidrug resistance 1 (MDR1) which was also expressed in normal hearts (Quaini et al., 2002). These undifferentiated cells together with early committed cells were considered as the Cardiac stem cell (CSC) population and play an important role in maintaining the homeostasis of normal and stressed myocardium. The CSCs are self-renewing, clonogenic and multipotent cells – having the ability to differentiate into CMs, ECs, and VSMCs. They reside in a microenvironment within which the cells are maintained in their undifferentiated state. The CSCs act as a ‘cardiac cellular reserve’ throughout the lifespan of an individual

that helps in the myocyte renewal resulting from wear and tear (Beltrami et al., 2003). The CSCs are the primitive and developmentally multipotent cells that undergo differentiation into cardiac progenitor cells (CPCs) characterized by its immature and committed nature. The CPCs then differentiate into precursor cells that can develop into any of the three main cardiac lineage cells and they develop into transit-amplifying cells that actively divide to terminally differentiate into myocyte, smooth muscle, or endothelial cell type.

Following an ischemic injury, myocardial regeneration occurs, but myocyte proliferation is restricted to the viable myocardium adjacent to and remote from the infarct. The endogenous regenerative potential of resident CSCs is limited mainly due to the following factors – 1) after an ischemic injury, stem cells within the infarct die, similar to other cells due to oxygen deprivation, and 2) resident CSCs cannot migrate to the damaged area from the viable tissue due to the hostile microenvironment marked mainly by inflammation, fibrosis, and poor angiogenesis. The presence of a sharp boundary that separates the viable myocardium of the border zone from the dead tissue of the infarct thus hampers the regeneration of the dead tissue and the recovery of function (Barile et al., 2007). Even though a remarkable increase in the number of human CSCs is reported in acute and chronic infarcted hearts, a large proportion of the human CSCs acquire a senescent phenotype. The senescent CSCs are non-cycling and non-differentiating thus creating a 'regenerative cell deficit' that fails to address the acute and chronic loss of cardiomyocytes and vascular structures following an MI which later leads to end-stage heart failure (Torella et al., 2006). Aging is also associated with a progressive decrease in the human CSC pool and the loss of CSC function with aging is partly mediated by an imbalance between factors promoting growth and survival and factors that enhance oxidative stress, telomere attrition, and apoptosis (Terzic and Behfar, 2016).

2.10 Cardiac regeneration using growth factors

Growth factors and cytokines are cell-secreted molecules that regulate a variety of cellular functions like cell proliferation, migration, differentiation, and apoptosis. The major mechanisms of action by which the growth factors exert cardiac regeneration

include its role in the positive remodeling of the ECM, promoting the proliferation of adult CMs, recruiting or homing CSCs to the injury site, anti-apoptotic effects, and angiogenic effects (Li et al., 2014). The endogenous regenerative capacity of the heart is limited due to various factors like inflammation, fibrosis, and lack of angiogenesis. Mitigation of the hostile microenvironment by local delivery of growth factors may reduce the extent of fibrosis, inflammation, and apoptosis. Angiogenesis is one of the major growth factor-induced reparative mechanisms most often investigated. Nakamura and colleagues have reported a reduction in CM apoptosis and infarct size in rats undergoing ischemia-reperfusion upon intravenous administration of Hepatocyte growth factor (HGF) (Nakamura et al., 2000). Similarly, delivery of Platelet-derived growth factor (PDGF) – BB delivery using injectable self-assembling nanofibres in rats post coronary artery ligation, enabled protection of CMs, reduced infarct size, and preserved systolic function 14 days post-injection (Hsieh et al., 2006). But the therapeutic potential of growth factors is limited by their short biological half-life, low plasma stability, and poor specificity to the target organ and aspects related to dosage and route of administration (Rebouças et al., 2016).

2.11 Cell-based strategies of myocardial repair

Cellular therapies using various sources of cells has gained focus to regenerate the damaged myocardium. The cellular repair strategies can include – 1) therapies that modulate the heart to regenerate the damaged tissues, 2) direct transplantation of cells into the damaged environment and 3) tissue engineering techniques (Leor et al., 2005). Cellular therapy can be attained in one of the following ways – 1) by transplanting cells through transepicardial, transendocardial, or intracoronary routes or 2) by mobilizing resident stem cells to the injury site using cytokines (Wu et al., 2006). The possible mechanisms of myocardial tissue regeneration by the administration of cells include secretion of several cytokines by the transplanted cells which promote neovascularization, the prohibition of fibrosis, a decrease of cell death and recruitment of stem cells to the injured area, and transdifferentiation of transplanted cells to CMs to improve the heart function. The major cell sources used for cell-based regenerative therapy of damaged myocardium include skeletal myoblasts, endothelial progenitor cells, fetal cardiomyocytes, embryonic stem cells,

induced pluripotent stem cells, bone marrow-derived stem cells, hematopoietic stem cells, umbilical cord-derived stem cells, mesenchymal stem cells, fibroblasts, and cardiac stem cells (Venugopal et al., 2012).

2.11.1 Direct transplantation of cells to the damaged heart

Direct transplantation strategy for cardiac regeneration aims at repopulating the injured myocardium by transplantation of healthy cells. The following cell types have been used for replacing the necrotic tissue and minimize scarring.

Skeletal myoblasts

One of the initial few cell types first considered for cardiac regeneration is skeletal myoblasts (Marelli et al., 1992). Skeletal myoblasts or the satellite cells are the precursor cells that lie in a quiescent state under the basal membrane of muscle fibers. Following a muscle injury, these cells are rapidly mobilized to the injury site where they proliferate and fuse to regenerate the damaged fibers. The myoblasts also have several advantages including high proliferative potential under appropriate *in vitro* culture conditions, its restricted potential to differentiate into a myogenic lineage that eliminates the risk of tumorigenicity, and high resistance to ischemic conditions, and the ability to contract (Wu et al., 2006). Skeletal myoblasts were injected via epicardium for patients undergoing CABG by Menasche and colleagues and were found to improve the pumping efficiency and cardiac function 5 months post-infarction (Menasché et al., 2003). Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) was the first randomized study of myoblast transplantation but it did not demonstrate an incremental improvement in left ventricular function over the CABG procedure (Dixit and Katare, 2015). The major disadvantage of skeletal myoblasts is their inability to differentiate into cardiomyocytes. Skeletal myoblasts are unable to establish electromechanical connections with the host myocardium due to the absence of Connexin 43 and N-cadherin and form a functional syncytium with the viable myocardium. Failure to electrically couple these cells with the viable myocardium and integration into the host tissue resulted in the occurrence of ventricular arrhythmias following transplantation in patients (Fernandes et al., 2009).

Endothelial progenitor cells (EPCs)

Endothelial progenitor cells (EPCs) are functionally and phenotypically distinct from the mature endothelial cells (ECs) that are present in the circulating human peripheral blood and can differentiate into mature ECs. EPCs are bone-marrow-derived stem cells that are recruited to the peripheral blood in response to tissue ischemia. EPCs home in the ischemic areas and differentiate into new blood vessels. Studies have shown that intravenously injected EPCs home to the damaged region of the heart within 48 hours (Kocher et al., 2001). The new vascularization induced by the transplanted EPCs prevented cardiomyocyte apoptosis and left ventricular remodeling thus preserving the ventricular function (Schuster et al., 2004). The major mechanism of action of transplanted EPCs for improvement of cardiac function is mainly mediated by its paracrine factors that promote angiogenesis and not by transdifferentiation into cardiomyocytes.

Bone marrow-derived stem cells

One of the preliminary research in the field of stem cells was conducted on bone marrow as early as 1950, which contained at least two different populations of stem cells – hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Orlic and colleagues in 2001 were the first to report the regeneration of infarcted myocardium with bone marrow-derived cells, where the transplantation of c- Kit^+ (a surface marker protein) bone marrow cells to a murine model of MI, exhibited the ability to transdifferentiate into mature CMs, VSMCs, and ECs. Bone marrow mononuclear cells (BMMNCs) include a mixed population of blood and stem/progenitor cells and intravenous injection of human bone marrow donor cells into infarcted myocardium of rats resulted in a significant increase in neovascularization, attenuation of cardiomyocyte apoptosis, and left ventricular remodeling (Kocher et al., 2001). Most clinical studies have used BMMNCs and have shown mixed results. Intracoronary infusion of BMMNCs following a heart attack had shown significant improvement in the left ventricular ejection fraction in several clinical trials including Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) and the Transplantation of Progenitor Cells and

Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE- AMI). However, other studies indicated no improvement in left ventricular ejection fraction as compared to that of the control group (Goldthwaite Jr, 2007). The exact mechanism of action that improves heart function following bone marrow cell transplantation remains unclear, but the paracrine effects of these transplanted cells are proposed as the major mechanism than differentiation of these cells into functional CMs (Segers and Lee, 2008). Though Bone marrow cells are the most widely investigated cell type for myocardial repair, these cell types also pose the risk of differentiating into undesired cell types – like osteoblasts in the transplanted heart (Breitbach et al., 2007).

Cardiac stem cells (CSCs)

CSCs comprise less than 1% of the cells in the heart and several subpopulations of CSCs have been reported based on their expression of surface markers transcription factors like c-Kit$^+$, Sca-1$^+$, MDR-1$^{+,}$ and Isl-1$^+$ cells. Beltrami and colleagues have reported the presence of a population of c-Kit$^+$ CSCs in rat hearts and upon transplantation of these cells into infarcted rat hearts 5 hours post-MI. A 20-day follow-up study showed that c-Kit$^+$ CSCs improved left ventricular ejection fraction (LVEF) by 11% compared to the control rats that did not receive cell transplantation (Beltrami et al., 2003). Transplantation studies using Sca-1$^+$ CSCs in infarcted mice hearts also showed an improvement in cardiac function and enhanced neovascularization possibly due to the paracrine effects of transplanted cells. Similarly, Messina and colleagues have demonstrated the formation of cardiospheres in suspension from mouse and human heart biopsies that have the potential to differentiate into CMs, ECs, and VSMCs. Transplantation studies using cardiospheres reported an improvement in ventricular function in mice and swine models (K. D. Boudoulas and Hatzopoulos, 2009). Increased LVEF and decreased infarct size 12 months post-transplantation was reported in phase 1 clinical study, stem cell infusion in patients with ischemic cardiomyopathy (SCIPIO), in which c-Kit$^+$ CSCs were administered by intracoronary route. Similarly, phase 1 clinical trial Cardiosphere- derived autologous stem cells to reverse ventricular dysfunction study (CADUCEUS), which explored the potential of cardiosphere derived cells for

cardiac regeneration showed reduction in scar mass without any differences in LVEF. But despite the positive observations made in the clinical trials, improvement in cardiac function was minimal due to the low retention of transplanted cells in the infarcted myocardium (Leong et al., 2017).

2.11.2 Stem cell-based differentiation strategies

Ever since the isolation of human embryonic stem cells (ESCs), that can undergo differentiation *in vitro* to generate derivatives of three primary germ layers, and almost all cell types in the body, a variety of stem cell types, both embryonic and adult, have been used for cardiac regeneration. The *in vitro* differentiation strategies recapitulates the stepwise stages of embryological development for any cell type of interest (Mummery et al., 2012). The following section summarizes various stem cell types and strategies used for cardiac repair and regeneration.

Embryonic stem cells (ESCs)

Embryonic stem cells are derived from the inner cell mass of the blastocyst that can differentiate into any cell present in the adult organism. Studies using mouse ESCs demonstrated that upon dissociation of ESCs into single cells followed by its aggregation in hanging drops resulted in the development of spheroids that can differentiate into derivatives of three primary germ layers and this spheroid- like structures were called “Embryoid bodies” (EBs) (Mummery et al., 2012). The first EB- mediated differentiation of human ESCs to CMs was performed by Kehat and colleagues using the H9 human ESC cell line and the EBs formed in this study were small clusters of 3- 20 cells grown in suspension. Such EBs exhibited spontaneous beating 4 days post-plating onto 0.1% gelatin-coated culture plates. Kehat and colleagues have also demonstrated that the *in vitro* spontaneously contracting EBs stained positive for cardiac markers like cardiac MHC, α - actinin, desmin, and TNNI. The *in vitro* differentiated cells were also shown to have myofibrillar organization and exhibited intracellular calcium transients (Kehat et al., 2001). Embryonic development of the heart has revealed the role of the primitive streak and visceral endoderm in inducing the mesodermal germ layer to differentiate into cardiogenic precursors *in vivo*. Mummery and colleagues have demonstrated the

importance of a feeder cell layer for cardiomyogenic differentiation of EBs. In this study, the co-culture of mitotically inactivated mouse visceral endoderm-like cell line (END-2) with hES2 human ESCs induced the differentiation of the latter cell type into immature CMs, and areas of beating cells were observed within one week of co-culture (Mummery et al., 2002). But both EB based culture and feeder cell-based culture had poor reproducibility due to diffusional barriers and heterogeneity in the CM population generated, respectively (Mummery et al., 2012). The presence of serum in the culture medium and insulin were also reported to be detrimental factors for CM differentiation of EBs.

Numerous studies have explored the use of various growth factors that play a pivotal role in embryonic development of the heart to guide *in vitro* differentiation of ESCs to CMs. Growth factor-based induction protocols have tested varying concentrations and combinations of growth factors like BMP-4, Activin A, FGF2, Wnt agonists and antagonists, and VEGF that play a major role in various stages of cardiogenesis *in vivo*. Laflamme et al., have demonstrated the guided differentiation of human ESCs into CMs, in which H7 human ESCs were plated as monolayers in the presence of mouse embryonic fibroblast conditioned media on Matrigel, a commercially available ECM. Cardiac differentiation was achieved by treating the cells sequentially using growth factors Activin A for 24 hours followed by BMP-4 induction in serum-free RPMI medium and resulted in > 30% CMs (Laflamme et al., 2007). Similar studies have been conducted by various research groups, where growth factors like TGF-β, ascorbic acid, retinoic acid, etc. have been used to induce CM differentiation from ESCs (Becker et al., 2011). Though ESCs have shown maximum efficiency of cardiac differentiation as compared to other cell types, the clinical use of this cell type is limited due to various factors like its genetic instability, chances of immunological rejection, risk of tumor formation upon transplantation, and ethical considerations related to the origin of these cell types (Dixit and Katare, 2015).

Induced Pluripotent Stem Cells (iPSCs)

Initially adult somatic cells were considered to be terminally differentiated cells and the cellular differentiation process was considered to be irreversible. But in 2006, Takahashi and Yamanaka induced pluripotency in somatic cells through retroviral transduction of several transcription factors involved in the self-renewal of ESCs. A combination of transcriptional factors commonly used for cellular reprogramming includes Krüppel-like factor 4 (Klf-4), sex-determining region Y-box 2 (Sox-2), c-Myc or octamer-binding transcription factor 4 (Oct3/4), Nanog, and Lin-28 (Takahashi and Yamanaka, 2006). iPSCs have been shown to differentiate into CMs, ECs, and VSMCs *in vitro*. Lei Ye and colleagues have demonstrated effective cardiomyocyte differentiation using four different iPSC cell lines derived from human cord mononuclear blood cells, human bone marrow $CD34^+$ cells and the other two from two neonatal human dermal fibroblasts derived iPSCs, in which the iPSC cell lines were treated with a combination of growth factors Activin A, BMP-4, and VEGF. The induced cells were found to have the gene expression profile of CMs and expressed TNNT, alpha sarcomeric actin, and MLC2v by immunocytochemistry and also exhibited cardiac myocyte –like action potential (Ye et al., 2013). Though iPSCs are being explored for its ability to differentiate into CMs and its use in myocardial regeneration, they also pose a risk of producing a heterogeneous population of cells including non-cardiomyocytes upon transplantation and teratoma formation. Hence the *in vivo* safety and functionality of these cells need to be further studied before their use in clinical studies (Dixit and Katare, 2015).

Cardiac fibroblasts (CFBs) play pivotal roles in various stages of heart development, homeostasis, and disease. The studies investigating the biology and applications of CFBs are limited due to the minimal availability of human CFBs from the native heart. Though several studies have been conducted by various research groups that reported directed differentiation of ESCs or iPSCs into CMs, ECs, and VSMCs, efficient *in vitro* differentiation of these stem cells to CFBs is less explored. Zhang and colleagues have reported directed differentiation of human pluripotent stem cells to CFBs via second heart field progenitors by sequential modulation of Wnt and FGF signaling. CFBs generated through this method resembled native heart CFBs in

overall gene expression and also produced abundant ECM composed of collagen and fibronectin upon culturing for 10 days at high seeding density (Zhang et al., 2017).

Since ECM proteins play a major role in the developmental pathway of the heart, the use of ECM proteins along with cytokines have been studied for monolayer based ESC and iPSC differentiation towards cardiac lineage. Zhang and colleagues have demonstrated a matrix sandwich method in which, pluripotent stem cells were cultured as monolayers on matrigel and subsequently over layered with matrigel to promote epithelial-to-mesenchymal transition that generated N-cadherin^{+} mesenchymal cells. These cells were further guided to cardiac lineage with the sequential application of growth factors Activin A, BMP-4, and FGF-2 that generated high purity CMs (Zhang et al., 2012). Reports from earlier studies indicated that ECM identity is important for CM differentiation. Comparative study of the effect of decellularized heart and liver ECM on cardiomyogenic differentiation of murine ESCs *in vitro* showed that the cells cultured in decellularized heart ECM expressed higher levels of cardiac Myosin heavy chain (MHC) and cTnI two weeks after culture (Higuchi et al., 2013).

Umbilical cord-derived stem cells

Umbilical cord-derived stem cells are multipotent, fibroblast-like cells that can differentiate into osteogenic, adipogenic, cardiomyogenic, and endothelial lineages under appropriate conditions. They have high plasticity and can be easily extracted and cryopreserved as compared to that of BMMNCs (Wu et al., 2006). Studies have demonstrated the differentiation of human umbilical cord-derived stem cells into cardiomyocytes using Sphingosine-1-phosphate and the development of an engineered cell sheet with the umbilical cord-derived cardiomyocytes using a temperature-responsive polymer, poly (N- isopropyl acrylamide) or PIPAAm (Zhao et al., 2011). In animal studies, human umbilical cord mononuclear cells have significantly reduced the infarct size by as much as 50% and reduced left ventricular remodeling by preserving the left ventricular ejection fraction without the requirements for host immune suppression (Henning, 2011).

Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells are a subset of adult stem cells that were preliminarily identified from bone marrow by Friedenstein et al. MSCs are the precursors of non-hematopoietic tissues like bone, muscle, tendons, ligaments, adipose tissue, and fibroblasts (Wu et al., 2006). They are present in almost all tissues of the body and are located mainly in the perivascular niche and they have been isolated from a wide variety of tissue including bone marrow, peripheral blood, fetal lung, fetal liver, in neonatal tissue like amnion, placenta, cord blood and adipose tissue (Hass et al., 2011). MSCs are multipotent stem cells capable of differentiating into cell types of mesodermal lineage and are characterized by their plastic adherent nature, expression of surface markers like CD 73, CD 105, and CD 90, and lack of other surface markers typically present in HSCs like CD 45, CD 14, CD 34, CD 11b, CD 19 and their ability to differentiate into adipocytes, chondrocytes and osteoblasts *in vitro* (Dominici M et al., 2006). Since the MSCs lack HLA- DR surface molecules, they are reported to have low immunogenicity and have shown to possess immunomodulatory effects by their inhibitory effect towards both B-cell, T-cell, dendritic cell, and Natural Killer (NK) cell proliferation to promote allograft survival. Bone marrow represents the major source of MSCs in the body and bone marrow-derived mesenchymal stem cells (BMMSCs) have shown promising potential in cardiac repair due to its powerful proliferative capacity, its ability to reduce infarct size. Werong Xu and colleagues have demonstrated that human MSCs isolated from bone marrow using Ficoll density gradient centrifugation was shown to get differentiated into CMs upon induction with 5- Azacytidine and basic FGF and these differentiated cells were characterized by immunocytochemistry for the cardiac markers MHC, desmin, and alpha cardiac actin and by transmission electron microscopy for the presence of myofilament – like structures (Xu et al., 2004). In another study, Atta Behfar and colleagues have demonstrated that human BMMSCs were differentiated into cardiac phenotype *in vitro* using a cocktail of growth factors and cytokines and injected intramyocardially into a mouse model of chronic MI. A one-year follow-up study revealed that the cardiopoietic MSC treated group showed improved ejection fraction over patient-matched naïve human MSCs 1 and 2 months post-transplantation. Pathological

evaluation of heart tissues also revealed that cardiopoietic MSC treated groups showed a reduction in myocardial fibrosis and scar size over 3 to 20 months post-transplantation. The study also revealed the presence of human-specific troponin positive cardiomyocytes in the anterior wall of the cardiopoietic MSC treated group as compared to that of the naïve MSC treated group (Behfar et al., 2010). In another study, infarcted rat hearts were treated with a combination of bone marrow MSC/silk fibroin/hyaluronic acid (BMSC/SH) and was found to improve the thickness of the left ventricular wall, reduce apoptosis, stimulate the secretion of angiogenic growth factors like VEGF thereby promoting neovascularization (Chi et al., 2012).

Though BMMSCs are one of the most extensively studied cell types for the regeneration of injured myocardium, it poses certain risk factors. Harvesting of BMMSCs is invasive and immensely painful with potential morbidity due to infections. Bone marrow is also not a good choice for the isolation of MSCs due to its low yield (0.001% to 0.01%), the potential for high- grade viral infection, and a substantial reduction in the proliferative capacity of cells with age (Fraser et al., 2006).

Adipose tissue is derived from the embryonic mesenchyme and contains a stroma that can be easily isolated. The presence of mesenchymal stem cells in processed lipoaspirate samples was first discovered and defined by Zuk and colleagues in 2001 (Zuk et al., 2001). Adipose tissue was also found to have a significantly higher stem cell density than bone marrow (5% versus 0.01%). Adipose tissue-derived mesenchymal stem cells (ADMSCs) can be harvested in larger amounts and using a simple non-invasive procedure as compared to BMMSCs (Fraser et al., 2006). One of the earliest studies in which ADMSCs were used for cardiac regeneration was reported by Rangappa and colleagues. In their study, Rabbit ADMSCs were induced to differentiate *in vitro* using 5- Azacytidine, a nucleoside analog, and after one week of induction, 20- 30% of cells showed binucleation and extended cytoplasmic processes with adjacent cells. At 3 weeks, the cells began to beat spontaneously in culture and expressed cardiac-specific markers alpha-actinin and troponin I (Rangappa et al., 2003). Similarly in another study, ADMSCs were induced to differentiate into cardiomyocytes with Angiotensin II and the differentiated cells

were characterized by Connexin 43, Troponin I, and MHC expression 4 weeks after induction (Song et al., 2013). ADMSCs also exhibit the property of differentiating into endothelial cells and smooth muscle cells *in vitro*. In one study, a subset of ADMSCs was induced to differentiate into ECs *in vitro* using endothelial growth medium supplemented with Insulin-like growth factor (IGF) and VEGF, and the differentiated cells were characterized by increased expression of CD 31 and von Willebrand factor (vWF) (Miranville et al., 2004). Vascular smooth muscle cells (VSMCs) are the other major cell type that maintains the physiological functioning of the blood vessel wall. ADMSCs were induced into VSMCs *in vitro* by culturing them in an inductive medium consisting of MCDB 131 and heparin for 6 weeks (Rodríguez et al., 2006).

The first clinical trial using ADMSCs, the APPOLO trial evaluated the safety and feasibility of ADMSCs delivered via intracoronary injections in a dose of 20-40 million cells by magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and echocardiography. A 6- month follow-up study showed an improvement in revascularization, improved cardiac function, and reduced scar formation in the heart. In another study, ATHENA, ADMSCs were delivered via the intramyocardial route and the results highlighted an improvement in the occluded blood perfusion (Ma et al., 2017).

2.11.3 Mechanism of action of MSCs

The major mechanism of action of MSCs for cardiac regeneration is proposed to be the following ones – 1) CM regeneration, 2) Vasculogenesis and 3) Paracrine effects. In a non- infarcted myocardium, MSCs are found to be present in low numbers. Upon a cardiac injury, the apoptotic cardiomyocytes express Hepatocyte growth factor (HGF) and in response to this signal, MSCs get recruited to the apoptotic cell death site attributed by the presence of HGF receptor on its surface. Similarly, following a cardiac insult, levels of SDF- 1α get upregulated and it mediates the recruitment of endogenous MSCs by the interaction of chemokine receptor CXCR4 with SDF- 1α. MSCs secrete a range of different growth factors like VEGF, IGF-1, HGF, and a variety of micro RNAs that promote angiogenesis (Singh et al., 2016). MSCs are

reported to participate in neovascularization mediated by the secretion of placental growth factor. It also promoted the polarization of macrophages that further secreted placental growth factors thereby inducing local neovascularization and enhanced cardiac repair post-injury (Zhang et al., 2015). The majority of the beneficial effects of MSC transplantation for cardiac regeneration is attributed to its paracrine effects rather than direct differentiation into CMs. MSCs secrete a plethora of cytokines and growth factors depending on the microenvironment in which they reside. This has attracted much attention to the potential of MSC secretome for its potential use in tissue repair and regeneration. The secretome is defined as the set of factors or molecules secreted by a cell to the extracellular space and these factors include soluble proteins, free nucleic acids, lipids, and extracellular vesicles. MSC – sourced secretome is a major replacement for cell-free therapies due to the following reasons: application of secretome resolves many safety considerations associated with the transplantation of living cells like immune compatibility, tumorigenicity, emboli formation, and transmission of infections. MSC sourced secretome, like the use of the conditioned medium is more economical and more practical for clinical applications and the time, cost of expansion and maintenance of cultured stem cells can be reduced to a large extend for treatment of acute conditions like cerebral ischemia and MI (Vizoso et al., 2017).

2.12 Challenges in the use of MSCs for cardiac regeneration

Though MSCs have been attributed with several advantages that make them a suitable cell type for cardiac regeneration, beneficial effects of direct transplantation of unmodified MSCs into the infarcted heart is much limited. Even though MSCs are said to be immune-privileged, the formation of malignant tumors upon transplantation of unmodified BMMSCs in the peri-infarct area of the mouse model of MI has been reported (Jeong et al., 2011). In another study, MSC injection was found to result in the formation of calcified or ossified encapsulated structures in the infarcted heart. Similarly, *in vivo* spontaneous transformation of BMMSCs to osteosarcoma genesis in patients was reported. Though MSCs can be generated in higher numbers *in vitro*, the delivery methods for MSC transplantation to a specific injury site has not been established yet. Also, most of the grafted MSCs undergo

Anoikis, a programmed cell death induced by loss of matrix attachments. The transplanted MSCs injected into the infarcted region encounter harsh conditions like loss of survival signals because of inadequate interaction between cells and matrix, due to deprivation of oxygen, nutrients, and predominant inflammation in the infarct area. Adhesion of cells to the matrix via integrin molecules is necessary for the survival of differentiated adherent cells in the cardiovascular system, including CMs, ECs, VSMCs, and CFBs. This poor cell retention in the hostile microenvironment of the infarcted heart is further aggravated by the reactive oxygen species (ROS). *Ex vivo* manipulation of MSCs has been used to overcome some of these issues, and the various strategies are categorized as follows: 1) pre-treatment with growth factors or cytokines, 2) preconditioning such as hypoxia and 3) genetic modifications of MSCs to enhance survival, proliferative capacity, metabolic characteristics and directed differentiation (Heesang Song et al., 2010).

Mangi and colleagues have reported genetic modifications of MSCs by over-expressing the *Akt* gene and observed that *Akt* over-expressing MSCs were more resistant to apoptosis *in vitro* and *in vivo* (Mangi et al., 2003). They also demonstrated a drastic improvement in the cardiac MI rodent model. Similarly, the transduction of Heme oxygenase -1 *(HO-1)* to MSCs using adenoviral vector showed an improvement in angiogenic effects, anti-oxidative and anti-apoptotic capacities, that improved cardiac function post –MI. Transplantation of glycogen synthase kinase GSK) -3β overexpressing MSCs into coronary ligated heart resulted in improved mortality, reduced infarct size, LV remodeling, and improved CM differentiation rate. It also upregulated the paracrine factor VEGF-A resulting in an increase in capillary density and survival of MSCs in the infarcted myocardium (Singh et al., 2016). But *in vitro* transdifferentiation of MSCs into CMs is minimal probably due to the absence of supporting cells and the ECM that are essential for the proliferation and survival of CMs *in vitro*. This issue needs to be well addressed to use ADMSCs as a suitable cell source for cardiac regeneration and repair following MI.

2.13 Animal models of MI

The biological complexity of the cardiovascular system necessitates the need for preclinical models to evaluate various aspects of the disease diagnosis and assessment of various pharmacological therapies. An ideal model system should be inexpensive, easily manipulated, reproducible, and most importantly, should present the cardinal signs and pathology that resemble the human disease and ethically sound (Kumar et al., 2016). The small animal models commonly used for a range of CVDs include mice and rats. The main advantages of small animal models include its low maintenance cost, easy handling, and short lifespan. Rat models dominate in the field of CVDs mainly due to its larger size that facilitates surgical and post-surgical procedures. Myocardial damage in rats can be induced by three procedures – surgical, pharmacological, or electrical. The most commonly used surgical procedure to create MI is ligating the left coronary artery. This method was introduced by Pfeffer and colleagues in which the anesthetized rats are subjected to left thoracotomy and the heart is exteriorized by gentle pressure. The left coronary artery is then ligated or heat cauterized between the pulmonary artery outflow tract and left atrium. Following ligation, the heart is returned to its normal position and the thorax is closed (Pfeffer et al., 1979). Modifications like temporary occlusion of the coronary artery followed by reperfusion are also done to evaluate the diverse parameters resulting from ischemia/reperfusion. Bagdon and colleagues introduced the pharmacological induction of heart damage by treatment with beta-one adrenergic receptor agonist isoproterenol. The electrical method consists of generating overlapping burns in exposed rat hearts by applying 2-mm – tipped soldering iron to the epicardium of the LV. Cryo-injuries to the epicardium is also a method to induce MI in mice and rat models of MI.

Though rodent models have provided significant insight into human cardiac pathophysiology, rodent hearts differ from that of the human heart in terms of architecture, heart rates, oxygen consumption, contractility, protein expression. The most commonly studied large animal models of CVDs include rabbits, dogs, pigs, sheep, and non-human primates. Swine models are much preferred among the large animal models due to their similarity in the collateral coronary circulation and arterial

anatomy with that of the humans and the accurate prediction of infarct size. The most common methods of inducing heart damage in large animal models include left anterior descending coronary artery (LAD) ligation and balloon catheter procedures (Zaragoza et al., 2011).

2.14 Cell transplantation outcome in animal models: Tissue regeneration

The function of a ventricle with a healed MI can be assessed by a variety of parameters to study the recovery after injury. The most widely used method to assess infarct size is the histological examination of the damaged tissue. The area at risk (AAR) represents the entire myocardial perfusion bed distal to the occluded coronary artery area. Conventional histology for infarct size assessment uses paraffin-embedded formalin-fixed heart sections about 1mm thick following euthanization of the animal. The tissue slices can be stained using Masson's trichrome stain to determine the extent of fibrotic tissue in the sections and the infarct size is calculated as the percentage of entire LV. Another method for post-mortem determination of the infarct size is by using 2, 3, 5- triphenyl tetrazolium (TTC). In TTC staining, the heart tissue sections are immersed in the TTC solution for 15 to 20 minutes. The active mitochondrial dehydrogenases convert the water-soluble TTC into an insoluble red precipitate. This enables the differentiation of viable tissue from non-viable tissue. The AAR assessment is made by injecting a dye into the circulation of the animal, either *in vivo* or post-mortem through retrograde perfusion. Evans Blue staining is generally used to measure AAR (Redfors et al., 2012).

2.15 Use of delivery vehicles in cell-based therapy

A major translational challenge to implement cell-based therapy for tissue regeneration is to determine suitable delivery protocols that ensure accuracy, improved cell survival, and reproducibility in administering cells for therapeutic efficacy (Amer et al., 2017). Several delivery systems like the use of biomaterials and engineered tissue constructs have been tried by various groups to improve transplanted cell retention at the injury site (Singh et al., 2016). Though many transplantation studies have employed saline-based delivery vehicles, recent trials

have developed alternative cell carriers like hydrogels for injection-based cell therapy. Different synthetic or natural hydrogel matrices have been used as carriers for either cell delivery/growth factor delivery. The commonly used natural hydrogel matrices include collagen, fibrin, gelatin, hyaluronan, alginate, and chitosan while the synthetic hydrogels include poly (lactic-co-glycolic acid) (PLGA) and polyethylene glycol (PEG) (Fakoya, 2017). In one study, the effects of fibrin glue as an injectable polymer for cardiac regeneration was studied by Christman and colleagues. In their study, MI was induced in female Sprague-Dawley rats by LAD ligation, and following one week after MI, the infarcted hearts were injected with either BSA in PBS, fibrin glue alone, skeletal myoblasts alone, or skeletal myoblasts along with fibrin glue. A six-week follow-up study was performed and histological examinations and immunohistochemistry analysis revealed that the treatment group with fibrin glue alone and cell-fibrin gel mixture attenuated the decrease in thickness of infarct wall size and preserved cardiac function (Christman et al., 2004). Similarly, Ryu and colleagues reported an improvement in neovascularization following MI in rats after injection of a mixture of bone marrow mononuclear cells and fibrin gel into the infarcted myocardium. The BMMNCs were injected 3 weeks following MI, either in medium or with fibrin and the neovascularization was assessed by Hematoxylin and eosin staining for measuring the microvessel density and internal diameter 8 weeks after treatment. The results indicated that the microvessel density in the infarction site was significantly higher when BMMNCs were implanted with fibrin matrix compared to cell implantation without matrix (Ryu et al., 2005). Also, immunohistochemistry of tissue sections using α – smooth muscle actin (α –SMA) antibody showed that a larger number of microvessels were positively stained for α –SMA in BMMNCs delivered with fibrin gel treatment group. In another study, the efficacy of autologous ADMSCs embedded in platelet-rich fibrin (PRF) to promote myocardial regeneration and repair following acute MI in rodent models was evaluated and the results indicated an enhancement of angiogenesis, preservation of LV function, and suppression of LV remodeling (Chen et al., 2015). The protein expressions of CXCR4, SDF-1α, and VEGF, which are the biomarkers of angiogenesis, were highest in ADMSCs embedded in the PRF treatment group as

compared to that of the other control groups indicating that the treatment improved endothelial cell function following MI.

2.16 Imaging and histology analysis for tracking cells

Current regenerative modalities to repair the infarcted myocardium employs the delivery of stem cells/ pre-differentiated cells through different delivery routes. Imaging and monitoring the biodistribution, viability, and the differentiation status of the implanted stem cells in the infarcted myocardium via non – invasive means *in vivo* offers massive clinical and research benefits (Pei et al., 2017). Labeling of cells for transplantation can be performed using a variety of paradigms and some of them include the use of superparamagnetic iron oxide (SPIO) nanoparticle labeling of cells which allows the detection of transplanted cells through magnetic resonance imaging. But reports from a few studies indicated that these nanoparticles decreased the migration of stem cells and negatively affected the cell functions. Radionuclide imaging of transplanted cells mainly by positron emission tomography has been employed by researchers to track the transplanted cells *in vivo*. But this technique is limited since the only immediate fate of the transplanted cells can be tracked. Bioluminescence (BLI) imaging is commonly used for tracking the stem cells transplanted into the injured myocardium by transducing the stem cells with the luciferase gene. BLI imaging is a sensitive, quantitative and inexpensive technique but the major limitation of this technique to label and track the cells is due to its poor tissue penetration that hampers evaluating the exact location of the transplanted cells (Ruggiero et al., 2012). Direct labeling of stem cells/ pre-differentiated cells with fluorescent probes enables visualization of transplanted cells both *in vitro* and *in vivo*. PKH26 is a red – fluorescent lipophilic dye that binds to the lipid bilayer of cell membranes. The fluorescent nature of this dye allows for tracking of cell proliferation *in vitro* and for long-term tracking of transplanted cells *in vivo*.

Histological analysis of heart tissue sections by routine staining techniques like Hematoxylin and eosin (H & E) staining allows us to study the changes in tissue architecture following an injury to the myocardium. The presence of viable cardiomyocytes in healthy tissue sections can be tracked by the appearance of pale

eosinophilic cytoplasm and cross striations with intact nuclei, whereas, absence of cross striations indicates the dead myocardium upon H & E staining of tissue sections. Degenerative changes in tissues can be tracked by immunohistological imaging of tissue sections for cardiomyocyte-specific markers like alpha-actinin, cardiac troponins, and gap junctional proteins (Pieperhoff et al., 2014).

CHAPTER 3

3. MATERIALS AND METHODS

In this study, the major focus was made to standardize the *in vitro* differentiation ability of ADMSCs to cardiac lineage cells, CMPs, and CFPs. Three different growth factor induction protocols were carried out for proving the differentiation of ADMSCs to CMPs and CFPs on a fibrin-based matrix. Among the three induction protocols tried for differentiation, one protocol was selected based on its ability to support the co-survival of both cardiac populations CMPs and CFPs *in vitro*. The materials used for conducting the experiments and the details of the standardized methods are described in this section.

3.1 Preparation of Platelet growth factor (PGF)

Platelet growth factor was prepared from platelet-rich plasma (PRP) based on a previously published protocol (Resmi and Krishnan, 2002). Fresh PRP bags were collected from the blood bank and was washed with 10% excess acid citrate dextrose (ACD) solution and centrifuged at 750g. Following centrifugation, cells were washed thrice with ACD-Tyrode's buffer. Calcium chloride ($CaCl_2$) was added to get 2mM final concentration, and challenged with 1IU/ml thrombin for 5 min. EDTA at a concentration of 5mM was used to stop further activation of platelets. Activated platelets were then centrifuged at 36000g for 1h at 4°C and the released proteins were collected in the supernatant and dialyzed against Ca^{2+} - Mg^{2+} - free Hank's Balanced Salt Solution (HBSS) overnight. The dialyzed sample was then sterile filtered using a 0.22µm syringe filter (Merck Millipore; USA) and aliquoted and stored at -20°C until use. The concentration of the PGF cocktail was determined by Lowry's assay and the concentration in the medium was adjusted as per the requirement.

3.2 Isolation and Characterization of ADMSCs

Human adipose tissue was collected with the approval of the Institute Ethics Committee (IEC- SCTIMST) approval (SCT/IEC/1231/JUNE-2018, dated 17.07.2018). Tissue samples were collected from patients of the age group 30-65 during coronary artery bypass grafting. ADMSC isolation protocol was carried out as described by Zhu et al., (2008). Briefly, ~ 5g to 10g of tissue was thoroughly washed with sterile Hank's Balanced Salt Solution (HBSS) to remove the blood and was finely minced into small pieces. Cell dissociation was achieved by treating the minced pieces with 0.15PZ U/ml of Collagenase NB 4 Standard Grade enzyme (Serva Electrophoresis, Germany) for a period of 45- 60 min at 37°C with continuous shaking. The digested cell suspension was then mixed with an equal volume of serum-containing medium and passed through a sterile 70µm cell strainer (BD Falcon, USA). Cells were washed by centrifugation at 400g and the cell pellet obtained was resuspended in low glucose Dulbecco's modified Eagle's medium (DMEM- LG; Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA) and Antibiotic- antimycotic solution (ABAM; Invitrogen, USA). The cells were seeded to a 25cm^2 tissue-culture polystyrene flask (TCPS; Nunc, Denmark) and incubated at 37°C under 5% CO_2. Medium change was given at 3- day intervals. Upon reaching ~ 80% confluence, cells were passaged by a standard trypsinization protocol using 0.25% Trypsin- EDTA (Invitrogen, USA) for the expansion of cell numbers.

ADMSCs from passage 2-4 were used for characterization using surface markers by flow cytometry as listed in table 1, and its multipotent nature was confirmed by tri-lineage differentiation and specific staining. Characterization of ADMSCs was done by the analysis of cell surface markers using flow cytometry-based on the International Society for Cellular Therapy (ISCT) norms. Briefly, cells at passage 2- 4 were trypsinized using a standard protocol and fixed in 3.7% formaldehyde (Merck, Germany) for 20 min at room temperature. The cells were washed by centrifugation at 400g for 5 min following which the supernatant was discarded and the cell pellet was dissolved in 0.3% bovine serum albumin/phosphate-buffered saline (BSA/PBS) and incubated for 30 min. The cells were then incubated with the primary antibodies against CD 105 (1:100), CD 90 (1:100) CD 73 (4µl/assay), CD 14 (1:100) and CD 45

(1:100) for 1h. Following primary antibody incubation, the cells were washed by centrifugation and were analyzed by flow cytometry using BD FACS Aria I. The source of antibodies used for ADMSC characterization is listed in Table 1. The unstained control was used to acquire a histogram of fluorescence against side scatter. The percentage of cells expressing the specific markers was estimated using FlowJo software (Tree Star Inc., USA).

Adipogenic, osteogenic, and chondrogenic differentiation of ADMSCs was done to prove the property of the multipotency of ADMSCs using Stem Pro medium (Life Technologies, USA), as per the standard procedure. For adipogenic induction 1×10^4 cells/cm^2 and osteogenic induction 5×10^3 cells/cm^2 respectively, were seeded and cultured in respective Stem Pro induction medium for 21 days with the medium change in 2-3 days. For chondrogenic differentiation, cells were seeded at high density as microdroplets and grown for 14 days in the respective medium. Differentiation to adipogenic, osteogenic, and chondrogenic lineages was confirmed by staining with Oil Red O, Alizarin Red S, and Toluidine blue stains respectively, as per the standard protocols.

Table 1 – Source of antibodies used for ADMSC characterization

Name	Isotype	Source	Catalog No.
CD 105 PE	Mouse IgG	Santa Cruz	sc-18838
CD 73 PE	Mouse IgG	Biolegend	344003
CD 90 PE	Mouse IgG	BD Pharmingen	555596
CD 14 FITC	Mouse IgG	Millipore	CBL453F
CD 45 FITC	Mouse IgG	Beckman Coulter	A07782

3.3 ADMSCs induction to Cardiomyocyte lineage

For induction of differentiation, tissue culture polystyrene (TCPS) dishes coated with fibrin matrix as described earlier was used (Prasad Chennazhy and Krishnan, 2005). Briefly, the pharmacopeia grade fibrin sealant (Drug controller approved) was

reconstituted. Diluted (5IU) thrombin treated surface was layered with a thin layer of (12.5 ul per cm^2) diluted (2mg/ml) fibrinogen containing 0.2 mg fibronectin and exogenous gelatin (0.2%; Sigma Chemicals, USA), and was allowed to clot and the clot formed was stabilized by incubating the culture dishes at 37°C for 30 min, lyophilized (Modulyo 4K, Edwards, UK) in a sterile atmosphere, and stored at 4°C until used.

In preliminary experiments, a comparison between seeding density of 5000 cells/cm^2 and 10000 cells/cm^2 showed better expression of connexin 43 in RPMI1640. So for all subsequent experiments, 10000 cells/cm^2 was considered the best seeding density.

Mainly three different growth factor induction protocols (IP) were compared for the *in vitro* pre-differentiation of ADMSCs to CMPs and CFPs. The fibrin-based matrix and the culture medium supplemented with growth factors together constituted the biomimetic niche. ADMSCs cultured using DMEM-LG medium with 10% FBS and ABAM on bare TCPS dish was used as the control.

In IP1, cells were seeded and cultured in low glucose DMEM for 24h under standard culture conditions. After 24h culture, cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% FBS, ABAM, and 5ng/ml of bone morphogenetic protein -4 (BMP-4; R & D systems, USA). After 48h, BMP-4 was withdrawn and cells were allowed to grow in RPMI 1640 supplemented with 10% FBS, ABAM, and 5ng/ml of Vascular Endothelial growth factor (VEGF; Cell Signaling Technology, USA) for 72h. Later, the culture was maintained in 10% RPMI medium without any growth factors for 16 days with medium change on alternate days under standard culture conditions.

In IP2, cells were seeded and cultured in DMEM-LG medium for 24h under standard culture conditions. After 24h, cells were supplemented with a combination of 5ng/ml of Activin A (Invitrogen, USA) and 5ng/ml of BMP-4 for 48h. Following Activin A and BMP-4 withdrawal, the cells were supplemented with 5ng/ml VEGF-165 for 72h. The culture was maintained in 10% RPMI medium without any growth factors for the remaining period of culture with medium change on alternate days till 16 days under standard culture conditions.

In IP3, cells were cultured in DMEM/F12 medium (Gibco, USA) supplemented with 10% FBS, ABAM, 1.2µg/ml Platelet growth factor (PGF, in-house prepared), 50ng/ml of Insulin-like growth factor -1 (IGF-1, Cell Signaling Technology, USA) and 50 µg/ml of L-ascorbic acid (Sigma, USA) for a period of 48h followed by withdrawal of PGF and IGF-1. The cells were then maintained in DMEM/F12 medium with 10% FBS, ABAM, and 50 µg/ml L-ascorbic acid for 16 days with medium change on alternate days.

3.4 Analysis of differentiation

Following ADMSC induction into CMPs and CFPs using different growth factor induction/withdrawal protocols on the fibrin-based biomimetic niche for specified periods, the cells were characterized. Morphological analysis using phase-contrast microscopy (DMIRB, Leica Microsystems, Wetzlar, Germany) was used as the first level of evidence for the transformation of cells. The morphology was continuously monitored during the culture period and was documented before further characterization.

3.4.1 Quantitative Real-Time polymerase chain reaction

The cells induced to differentiate into CMP lineage under three different culture conditions were analyzed for the expression of differentiation markers on days 8, 16 of differentiation. RNA was isolated from the cells at respective periods using TRIZOL reagent (Invitrogen, USA) based on the manufacturer's protocol. RNA quantification was done spectrophotometrically by using Nanodrop (ND 2000; Thermo Scientific, USA). 200ng of total RNA was converted to cDNA using the OrionX cDNA kit (Origin, India) in a thermal cycler (Master cycler; Eppendorf). Amplification of genes using specific Intron-spanning primers for CMP markers and CFP specific markers were analyzed by qRT-PCR. Real-time PCR was carried out in a total volume of 15µl containing 20ng cDNA, 100 pmol each of respective forward and reverse primers, and 7.5 µl of OrionX 2X Real-time PCR master mix (Origin, India). Forty cycles of reaction were performed using the Bio-Rad iQ5 Real-time PCR detection system (Bio-Rad, USA) under the following conditions: Enzyme activation,

95°C for 15 minutes; denaturation, 95°C for 30 seconds; annealing, 50°C for 20 seconds; and extension, 72°C for 20 seconds. GAPDH and β- actin were used as house-keeping genes. Melt curve analysis was performed for each gene to confirm the specificity of each reaction. Products were analyzed by agarose gel electrophoresis for correct amplicon size. Fold change in expression was calculated after normalization with GAPDH expression on each day of analysis using the formula $2^{-\Delta\Delta Ct}$. A list of primers used for the study is given in table 2.

Table 2 – Primer sequences used for qRT PCR analysis

Genes	Amplicon Size	Primer sequence (5'-3')
GAPDH	120bp	FP - GAA ATC CCA TCA CCA TCT TCC AGG RP - GAG CCC CAG CCT TCT CCA TG
β Actin	434bp	FP - TGT GCC CAT CTA CGA GGG GTA TGC RP - GGT ACA TGG TGG TGC CGC CAG ACA
TNNT 2	114bp	FP - GTT ACA TCC AGA AGA CAG AG RP - CTT CAT TCA GGT GGT CAA T
MYL 2	120bp	FP - ATT CTC AAC GCA TTC AAA GT RP - CAT CTG GTC AAC CTC CTC
MYH 6	94bp	FP - GAC CGA GAA TGG AGA GTT RP - CAT TTG CTG GGT ATA AGA GAG
GJA 1	97bp	FP - GTC TGA GTG CCT GAA CTT RP - GCA CCA CTC TTT TGC TTA A
DDR 2	167bp	FP - AGT GCC ATC AAG TGT CAA TA RP - CGA GTG TTG CTG TCA TCA
Fibrillin 1	206bp	FP - TGA TGG CTC CTA CAG ATG TGA ATG C RP - GAC ACG GCT GGC AAG GTT CC
Collagen 1	120bp	FP - CCA AGG GTA ACA GCG GTG A RP - GCT TTC CTT CCT CTC CAG CA
CD 31	118bp	FP - CAG TCA TTA CGG TCA CAA T RP - CTG AGG ACA CTT GAA CTT C
VCAM-1	90bp	FP - CCTCCTTAATAATACCTGCCATTG RP - TCTGTGCTTCTACAAGACTATATGA
MCP-1	120bp	FP - CCGAGAGGCTGAGACTAAC RP - ATGAAGGTGGCTGCTATGA
TGF-β I	152bp	FP - AGTTGTGCGGCAAGTGGTTGA RP - GCCATGAATGGTGGCCAGGT
PDGFR α	146bp	FP - AGGTTGAGAGGAGGACTT RP - CCACTGAGATGCTACTGAG

3.4.2 Immunostaining

The lineage-specific marker expressions at the translational level for CMP and CFP markers were analyzed by immunocytochemistry of induced cells after 16 days of culture. The cells cultured for a specific period was fixed with 3.7% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature, permeated using 0.2% Triton X 100 (Sigma Aldrich, USA) for 5 minutes, and blocked with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes. The cells were then incubated with primary antibodies overnight at 4°C, secondary antibodies at 37°C for one hour at room temperature, and the cells were then stained for nucleus using Hoechst 33342 (Life technologies, USA). The source of antibodies used for the immunostaining is listed in Table 3.

The percentage of cardiac Troponin T positive cells was estimated semi-quantitatively by Image J analysis. The number of nuclei per field was counted from 4 different fields from different donors and the distribution of cardiac Troponin T positive cells per field was counted. The percentage of Troponin T +ve cells was estimated based on the total number of cells in 4 fields and total TNNT 2 cells in the corresponding fields. The average and standard deviation were also calculated.

Table 3 – Sources of antibodies used for Immunostaining studies

Name	**Isotype**	**Source**	**Catalog No.**
Cardiac Troponin T	Mouse IgG	Abcam	ab8295
Cardiac Troponin I	Mouse IgG	Thermo Scientific	MA1-20113
Connexin 43	Rabbit polyclonal	Thermo Scientific	PA5-11632
Alpha actinin	Mouse IgG	Abcam	ab9465
Fibrillin 1	Mouse IgG	Novus Biologicals	NB110-8146
Collagen I	Mouse IgG	Abcam	ab6308
CD 31	Mouse IgG	BioLegend	102401
Anti-mouse AF 488	Goat IgG	Abcam	ab150113
Anti-rabbit AF 488	Goat IgG	Abcam	ab150077

3.4.3 Characterization of deposited ECM

To determine ECM deposition by cells during the culture period, cultures were subjected to decellularization, as standardized by Divya et al. (2007), stained using antibodies. Briefly, after specific periods of culture, the surfaces were treated with a solution containing 0.2% NaOH (Merck, Germany), and 1% Triton X – 100 (Sigma Aldrich, USA) in double-distilled water for 30 min. The insoluble matrix recovered was fixed with 3.7% formaldehyde (Merck, Germany) for 20 min and washed. The culture dishes were incubated with primary antibody against Collagen I (Abcam, UK) for 2h at room temperature. Following primary antibody incubation, the culture dishes were stained with secondary antibody Alexa Fluor 488 anti-mouse IgG (Abcam, UK). Fluorescent images of immunostained Collagen I positive areas were imaged using a fluorescent microscope (Leica, Germany).

3.5 Co-culture of EPCs with CMP-CFPs to assess the initiation of the angiogenic response

hADMSC derived EPCs was a kind gift by Ms. Renu. Briefly, to assess the effect of co-culture of EPCs with CMP-CFP culture in the initiation of angiogenic response, hADMSCs induced to CMP-CFPs using IP3 protocol were co-cultured with EPCs derived from the same donor hADMSC in a 2:1 ratio. The EPCs were trypsinized and a seeding density of 5000cells/cm^2 was seeded to CMP-CFPs on the 5^{th} day of induction *in vitro*. The co-culture was maintained in a 1:1 ratio of DMEM/F12 medium supplemented with L-ascorbic acid and MCDB 131 medium supplemented with L-glutamine, L-ascorbic acid, and heparin sulfate for 16 days.

3.5.1 Assessment of stability of CMPs, CFPs, and EPCs in co-culture

Replicate cultures described in section 3.5 were terminated after 16d for quantification of genetic markers for CMP, CFP, and EPC. The protocol is as described in section 3.4.1 and primers used are listed in Table 2.

The lineage-specific marker expressions at the translational level for CMP, CFP, and EPC markers was analyzed by immunocytochemistry of induced cells after 8 and 16 days of culture. The cells cultured for a specific period was fixed with 3.7% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature, permeated using 0.2% Triton X 100 (Sigma Aldrich, USA) for 5 minutes, and blocked with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes. The cells were then incubated with primary antibodies overnight at 4°C, secondary antibodies at 37°C for one hour at room temperature, and the cells were then stained for nucleus using Hoechst 33342 (Life technologies, USA). The source of antibodies used for the immunostaining is listed in Table 3.

3.6 Effect of hADMSC secretome in CMP-CFP culture in the initiation of angiogenic potential

Secretome containing overexpressed VEGF released into the culture medium was a kind gift by Ms, Amita Ajit. Briefly, secretome from hADMSCs transfected with VEGF-A was obtained by collecting culture medium in which engineered cells were grown; after estimating VEGF –A content using ELISA kit. Secretome obtained from normal ADMSC (non-engineered) was used as negative control and recombinant VEGF-A was used as the positive control. The total protein content of both non-engineered hADMSC secretome (nSec) and engineered VEGF – over-expressed hADMSC secretome (eSec) was normalized by Lowry's estimation method and added equal concentrations of eSec secretome and nSec secretome to the IP3 induced hADMSCs culture. To assess the effect of engineered and non-engineered ADMSC secretome on cells induced with IP3, CMP proliferation was analyzed by tritiated thymidine uptake assay and the effect of engineered and non-engineered ADMSC secretome on the growth of CMPs was evaluated by gene expression studies by qRT-PCR using cardiac-specific markers, EC markers, CFP marker, and ECM markers. The cells cultured using IP3 induction protocol on fibrin matrix was used as the experimental control and the cells cultured using IP3 protocol along with the addition of recombinant VEGF (VEGF-165; Cell Signaling Technology, MA, USA) was used as the positive control.

3.6.1 Tritiated thymidine uptake assay

To quantify the initial cell attachment, ADMSCs in the log phase were labeled with tritiated thymidine (^{3}H – thymidine) (American Radiolabelled Chemicals, USA) by adding 20μCi ^{3}H – thymidine/25cm^2 flask for 96h at 37°C (Badylak et al., 1999). The cells were harvested and seeded onto fibrin coated culture dishes at a seeding density of 10,000cells/cm^2. Cell numbers were calculated based on radioactivity of the known number of cells and an increase in cell number was calculated based on the number of cells attached on 24h post-seeding.

3.6.2 Analysis of Differentiation

Replicate cultures described in section 3.6 were terminated after 16d for quantification of genetic markers for CMP, CFP, and EPC. The protocol is as described in section 3.4.1 and primers are as listed in Table 2.

The lineage-specific marker expressions at the translational level for CMP markers were analyzed by immunocytochemistry of induced cells after 8 and 16 days culture. The cells cultured for a specific period was fixed with 3.7% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature, permeated using 0.2% Triton X 100 (Sigma Aldrich, USA) for 5 minutes, and blocked with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes. The cells were then incubated with primary antibodies overnight at 4°C, secondary antibodies at 37°C for one hour at room temperature, and the cells were then stained for nucleus using Hoechst 33342 (Life technologies, USA). The source of antibodies used for the immunostaining is listed in Table 3.

3.7 *In vivo* evaluation

3.7.1 Isolation of rat ADMSCs and induction to CMP lineage

Rat adipose tissue was collected during tracheostomy, with the approval of the Institutional Animal Ethics Committee (SCT/IAEC-266/FEBRUARY/2018/95) from male Sprague –Dawley rats above 250g bodyweight. Rat ADMSCs (rADMSCs) were isolated as prescribed in section 3.2 and passage 3 rADMSCs were induced into

cardiac lineage *in vitro* as per the IP3 induction protocol used for induction of human ADMSCs to cardiac lineage (described in section 3.3). The induced cells (rCMPs) were characterized for the expression of CMP lineage-specific and EPC lineage-specific markers by qRT-PCR (described in section 3.4.1) and the characterized cells were used for transplantation into animal models of MI. The Primer sequences used for characterization are listed in Table 4.

Table 4 – List of primers used for rCMP characterization

Gene	Gene Bank No.	Primer sequence (5'-3')	Amplicon size
TNNT 2	NM_012676	Forward - GATGCTGAAGATGGTCCA Reverse - TCATCAAAGTCCACTCTCTC	110bp
GJA 1	NM_012567	Forward - TTGACTTCAGCCTCCAAG Reverse - GGCACCTCTCTTTCACTTA	75bp
CD 31	NM_031591.1	Forward - TTGCCGCCTTGATAGTTG Reverse - CCTTCTCACTGTTGGAGTTC	117bp
Flk-1	NM_013062.1	Forward - AGCATCAGCATAAGAAGATTGTA Reverse - GTCACACTGTCTATGGTCAAG	97bp
GAPDH	NM_017008.4	Forward - GGCACAGTCAAGGCTGAGAATG Reverse - ATGGTGGTGAAGACGCCAGTA	143bp

3.7.2 Allocation of animals

All experiments obeyed the guidelines issued by the CPCSEA committee and approved by the Institutional Animal Ethics Committee (IAEC). Forty-two adult male inbred strains of Sprague-Dawley rats (body weight: 250-400g each) obtained from Division of Laboratory Animal Sciences, SCTIMST, India were used for the experiment. The animals were kept under standardized animal care conditions and were given free access to food and water throughout the study. Experimental animals were randomly assigned to one of the following seven groups before surgery with at least 6 animals included in each group. The distribution of animals in different groups is indicated in table 5.

Table 5 – Allocation of animals for *in vivo* studies

Group I	Medium injected control (**Med-**)
Group II	Fibrin injected control (**Fib-**)
Group III	rADMSCs transplanted in fibrin as matrix (**rADMSC-Fib-**)
Group IV	rADMSCs transplanted in medium as matrix (**rADMSC-Med-**)
Group V	rCMPs transplanted in fibrin (**rCMP-Fib-**)
Group VI	rCMPs transplanted in medium (**rCMP-Med-**)
Group VII	Human s-e-ADMSC secretome transplanted in fibrin (**Fib-eSec-**)

3.7.3 Preparation and labeling of cells for transplantation

Around 10^6 cells/animal were used for the experiment. Before transplantation, rat ADMSCs from passage 3 *in vitro* culture on bare TCPS and rat CMPs on 6^{th} to 8^{th} day of *in vitro* culture on fibrin matrix were labeled with fluorescent membrane intercalating dye PKH26 (red fluorescence, Sigma, USA) according to the manufacturer's instructions. Briefly, the cells were harvested by trypsinization and were washed in a serum-free medium. The cells were then suspended in the given diluent (diluent C, 0.5ml), mixed with the dye solution in diluent (0.5ml), and incubated for 2.5min at room temperature with periodic mixing in dark. The reaction with dye was stopped by adding an equal volume of FBS to the cell suspension with dye for 1min, to bind with the excess dye and the suspension was centrifuged at 400g for 10min at 22℃. The cells were then washed with a 10% serum-containing medium to remove the unbound dye and were used for transplantation.

3.7.4 Creation of MI model and transplantation

The rats were anesthetized with ketamine (80mg/kg body weight) and xylazine (10mg/kg body weight) and were clipped and sterilized with antiseptic betadine. Mechanical ventilation was be achieved by tracheostomy by connecting the endotracheal tube to a ventilator with cycles of 40-60 breaths per minute and a tidal

volume of 1.2ml per 100-gram body weight. Animals were then maintained under general anesthesia with 1.5% isofluorane. A left thoracotomy was performed between the 4^{th} and 5^{th} intercostal spaces and after retraction of the ribs, the heart was exteriorized. The left coronary artery was transiently ligated with 6-0- prolene suture approximately 2mm from its origin between the left atrium border and the pulmonary artery sulcus. The development of regional myocardial ischemia was observed by the blanching of the tissue over the anterior surface of the LV and the rapid development of akinesia.

Cells were transplanted at the injury site 15 - 20 min after the MI using a 1ml syringe. 10^6 cells/animal suspended in fibrinogen were injected along with thrombin at the site of injury for test animals. Fibrinogen at a concentration of 20mg/ml and thrombin at a concentration of 1IU/ml was used to form a clot. In Group I, 200ul of the serum-free medium was injected whereas in Group II, fibrin alone was injected and in Group III, 10^6 ADMSCs suspended in serum-free media were applied. Soon after transplantation/injection, the chest wall was closed after maintaining negative pressure inside the thoracic cavity by gentle suction. Postoperative care was given to the animals by the administration of antibiotics and painkillers for 5 days after the development of MI and cell transplantation.

3.7.5 Tracking of transplanted cells

The infarcted heart was collected 28 days of post-infarct induction by washing the heart tissue section in PBS. The tissue sections were then used for imaging (IVIS Spectrum Preclinical *in vivo* imaging system, Perkin Elmer, USA). The excitation wavelength used was 500nm and the emission was measured at 600nm. The retention of ADMSC and CMP in the transplanted site was assessed by fluorescent imaging of the explanted tissue area.

3.7.6 Immunohistochemical tracking of the transplanted cells

Following fluorescent imaging, the heart tissue was cryoprotected by dipping it in iso-Pentane, which is cooled to a temperature of around -150°C by placing it in a liquid nitrogen container. The frozen heart tissue was then embedded in tissue – freezing media (Jung, Lecia Microsystems, Germany) and cut into 14um sections using a

cryostat (Leica, Germany). The sections were then washed in PBS and permeated with 0.2% Triton-X 100 for 5min. Following permeation, the sections were blocked with 3% bovine serum albumin (BSA; Sigma, USA) for 30 minutes. The cells were then incubated with primary antibodies against cardiomyocyte marker Troponin T (Abcam, UK), gap junctional protein Connexin 43 (Thermo Scientific), and pro-inflammatory marker CD68 (Abcam, UK) overnight at 4°C. The corresponding secondary antibody conjugated with Alexa Fluor 488 from Abcam was incubated for one hour at room temperature to develop the antigens. Nuclear staining was done using 4, 6, diamidino-2-phenylindole (DAPI, Invitrogen, USA). To identify the presence of lineage-committed transplanted CMPs, dual-labeled cells (PKH26 and cardiac marker) were analyzed.

3.7.7 Hematoxylin and Eosin staining for assessing the injury site

Formalin-fixed, paraffin-embedded tissue sections of rat hearts were stained for analysis of the injury site formed by ischemia of the heart wall. For H & E staining, formaldehyde-fixed sections were processed in 70% alcohol overnight, followed by three changes of acetone (20min each), two changes of xylene (10 and 15min respectively), and two changes of paraffin wax (60min each). Then the tissues were embedded in paraffin wax. Transverse tissue sections were obtained using a microtome (Leica RM 2255, Germany). The sections were deparaffinized in xylene (3 changes, 15min each), rehydrated in descending grades of isopropanol (100% for 3min, 95% for 3 min, 80% for 3min and 70% for 3min), and washed in tap water. The rehydrated sections were stained using Harris's hematoxylin and eosin (H & E). The tissue sections were submerged in Harris's hematoxylin for 15min, washed in tap water for 5min, differentiated in 1% acid-alcohol (1-2 fast dip), and blued with 0.2% ammonia water for 1min. The slides were washed with tap water for 5min, counter-stained with 1% eosin Y for 5min. Tissue sections were dehydrated in isopropanol (95% for 5min, 2 changes in 100% ethanol for 5min each), cleared in xylene (2 changes, 10min each), mounted in DPX, and viewed under a microscope.

3.7.8 Identification of Cell fate

Identification of Cell Fate Immunohistochemical staining was done by standard protocol. Briefly, 4μm formalin-fixed paraffin-embedded sections were de-paraffinized for 30 minutes in the oven at 70°C and in xylene for 10 minutes, followed by dehydration in graded isopropanol. The antigen retrieval was performed using Tris EDTA buffer (Catalog # PS009, Pathn Situ Biotechnologies, India) in a microwave oven at 100°C for 20 minutes. The endogenous peroxidase activity was blocked using 3% hydrogen peroxide followed by washing with Immuno wash buffer (Catalog #PS006, Pathn Situ Biotechnologies) for 5 minutes twice and non-specific background staining was blocked using protein block (Catalog # X0909, Agilent Dako, Santa Clara, CA, USA). The slides were incubated with mouse monoclonal anti-human CD31 primary antibody (Clone JC70A, ready-to-use, Code IR610, Agilent Dako) for one hour and washed with Immuno wash buffer for 5 minutes twice. The sections were visualized using horseradish peroxidase-labeled anti-Rabbit secondary antibody system (Dako REAL EnVision, Catalog # K5007, Agilent Dako) for 30 minutes, washed with Immuno wash buffer for 5 minutes twice followed by incubation with DAB chromogen system (Dako REAL EnVision, Catalog # K5007) for 10 minutes. The sections were finally washed with distilled water, counterstained with hematoxylin, dried, and mounted using DPX (Dibutyl phthalate Polystyrene Xylene). Masson's trichrome staining was carried out in formalin-fixed tissue sections to assess the area of fibrosis. The integrated area of fibrosis was calculated using magnified images of the trichrome stained sections using Image J software (NIH, Maryland, USA).

3.8 Statistical Analysis

Statistical Analysis was calculated by Student's t-test for all quantitative data to determine the significance in variation between the control and the test. Mean values and standard error was calculated for all parameters and are represented in the graphical form. Significance is labelled in the graph wherever applicable.

CHAPTER 4

4. RESULTS

The study results are illustrated and discussed in this chapter in 3 subsections. The first section focuses on human ADMSC isolation, culture expansion *in vitro*, characterization, and standardization of niche for cardiac lineage induction. Based on the preliminary results presented in part I, the most suitable niche for obtaining CMP was selected. The second part focuses on the evaluation of the modified niche that resulted in the co-differentiation of CMPs and CFPs *in vitro*. The evaluation of niche primarily focused on the characterization of differentiated CMPs and CFPs carried out at transcriptional and translational levels. Out of 3 different niche based induction protocols, the most suitable one which resulted in the co-differentiation of CMP and CFP was further selected for detailed study. The initiation of angiogenic response in CMP-CFP culture was studied by either co-culture of ADMSC-derived EPC with CMP-CFP culture or by the addition of VEGF overexpressing bioengineered hADMSC secretome to CMP-CFP culture. The third part evaluates the difference between ADMSC and CMP transplantation in rat MI models induced by LAD ligation. The effect of fibrin as a delivery vehicle for the transplantation success in terms of cell survival in the infarcted region by tracking the presence of transplanted cells in the heart tissue was also evaluated.

4.1 Human ADMSC isolation and characterization

The primary requirement of ADMSC differentiation to cardiac lineage is to obtain a substantial number of a homogenous population of ADMSCs, before inducing them to pre-differentiate into cardiac lineage. The collagenase treated adipose tissue sample contains a heterogeneous population of cells and the initial period for the cells to become confluent was around 8-9 days in culture. Further trypsinization and passaging were carried out in every 4 days and the cells were grown as a monolayer culture (**Figure 1A e, f**).

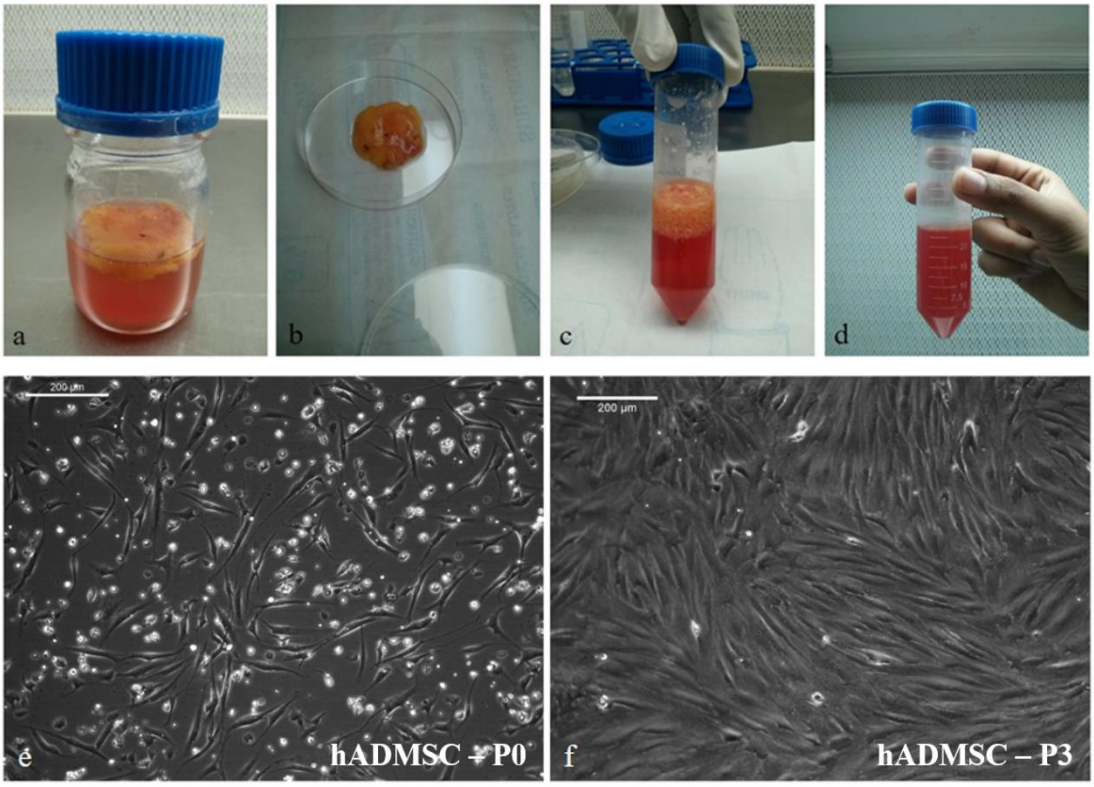


Figure 1A. Images representing the isolation and culture expansion of hADMSCs (a) Adipose tissue collected in sterile HBSS medium, (b) tissue chunk after removal of visible blood clots and blood vessels, (c) minced tissue re-suspended in collagenase enzyme, (d) digested tissue, (e) Passage 0 ADMSC at day 5 of culture, (f) Passage 3 ADMSCs (*Magnification – 10x*).

Surface markers of ADMSC analyzed by flow cytometry at passage 3 indicated that the MSC markers CD105, CD73, and CD90 were around 90% positive with only < 2% expressing hematopoietic stem cell (HSC) markers CD14 and CD34 (**Figure 1B**). The multipotent nature of cultured ADMSCs was confirmed by its ability to differentiate into adipogenic, osteogenic, and chondrogenic lineages upon culturing them in respective induction medium. The Adipogenic and osteogenic differentiation of the cells cultured for 21 days was confirmed by Oil Red O staining (**Figure 1C.a**) and Alizarin Red S (**Figure 1C.b**) staining respectively, whereas, chondrogenic differentiation was confirmed by Toluidine Blue staining of cells cultured for 14 days (**Figure 1C.c**).

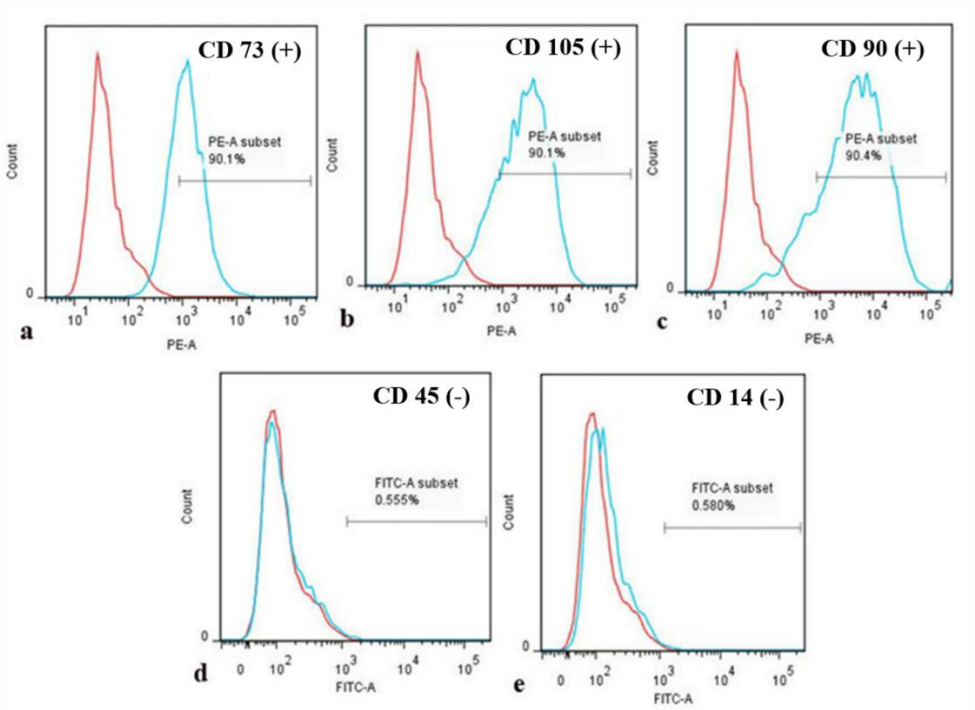


Figure 1B. Histograms for quantitative analysis of surface markers. Harvested cells of P3 ADMSC were used for flow cytometric analysis. a)CD73, b)CD105, c)CD90, d)CD45 and e)CD14 (blue line) as compared to unstained control (red line) indicating > 90% positivity for CD 73,CD105 and CD90 and < 2% positivity for CD45 and CD14.

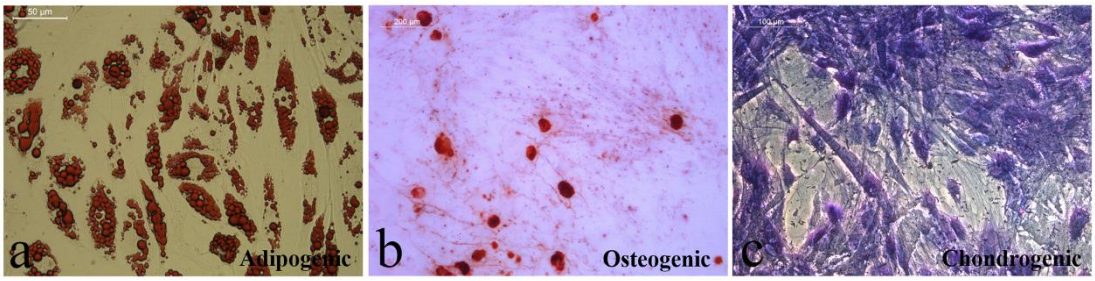


Figure 1C. Representative images demonstrating multi-potency of ADMSCs. a) ADMSC differentiated to Adipogenic lineage confirmed by Oil Red O staining (*magnification -40X*), b) ADMSC differentiated to Osteogenic lineage confirmed by Alizarin Red S (*magnification- 10x*) and into c) Chondrogenic lineage confirmed by Toluidine Blue staining (*magnification - 20X*).

4.2 Confirming advantage of fibrin niche for CMP differentiation

The initial part of the differentiation experiment was to identify if the fibrin matrix influences the stimulation of hADMSCs to cardiac lineage cells. Therefore, fibrin matrix coated (Fib)-grown hADMSCs were compared against the TCPS grown cells. The initial trials to induce hADMSCs to CMPs attempted the use of two different culture medium – DMEM LG and RPMI 1640 medium to identify if there is a medium-specific response to induction. The GFs that induced hADMSCs to CMP lineage was BMP-4 and VEGF-165. Morphological features like the elongation of cells, appearance of cell fusion, and formation of the tube-like structure were more prominent in cells cultured on fibrin matrix with RPMI medium and GFs as compared with those cultured on fibrin matrix with DMEM-LG medium and GFs (**Figure 2A and 2B**).

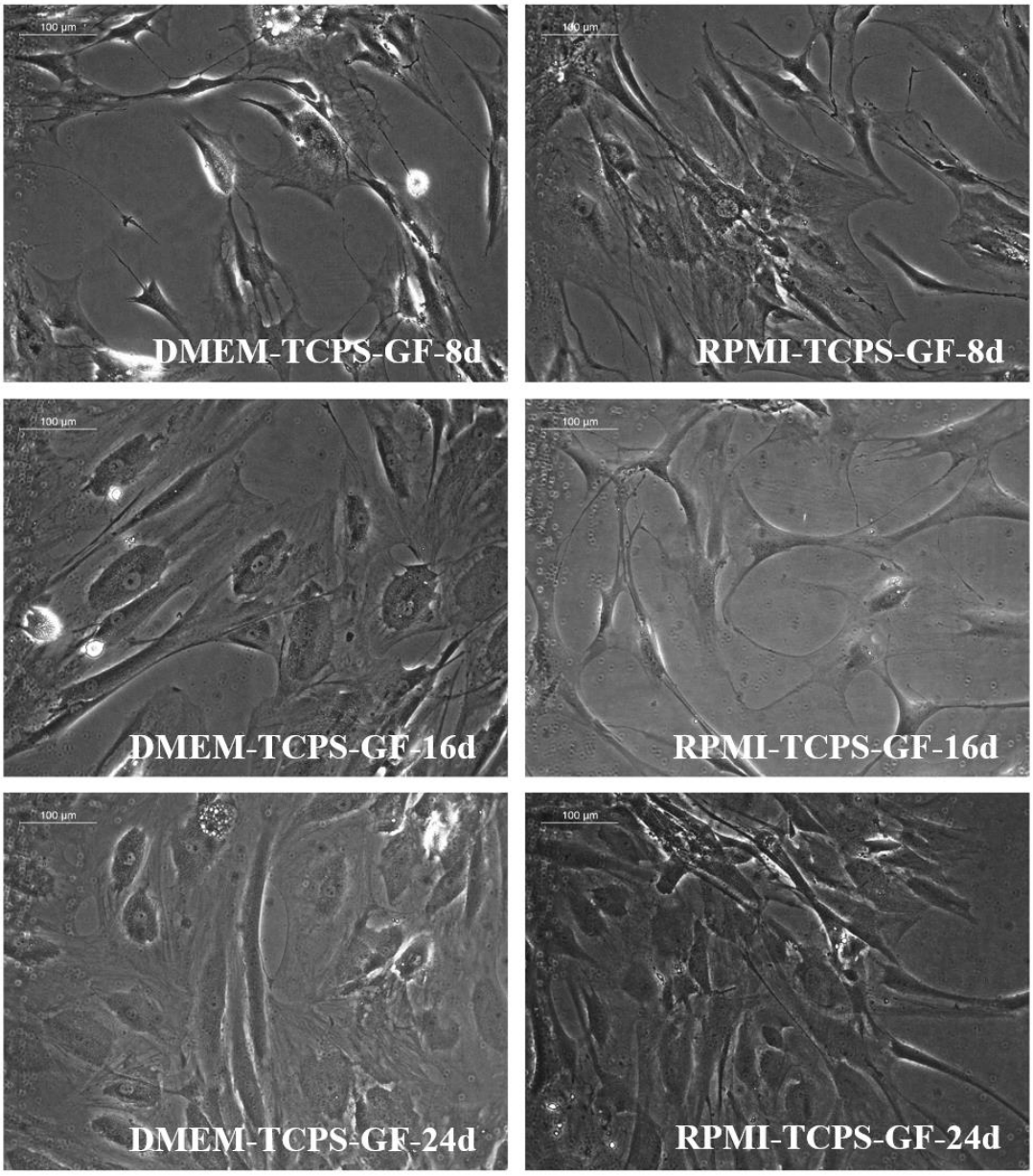


Figure 2A. Representative phase-contrast images of hADMSCs cultured on bare TCPS. Distinct morphological features are seen depending on the culture medium and period of culture. The left panel indicates cells cultured in DMEM-LG- GFs and the right panel indicates cells cultured in RPMI- GFs. 8d, 16d, and 24d indicate cells cultured for 8 days, 16 days, and 24 days *in vitro* respectively.

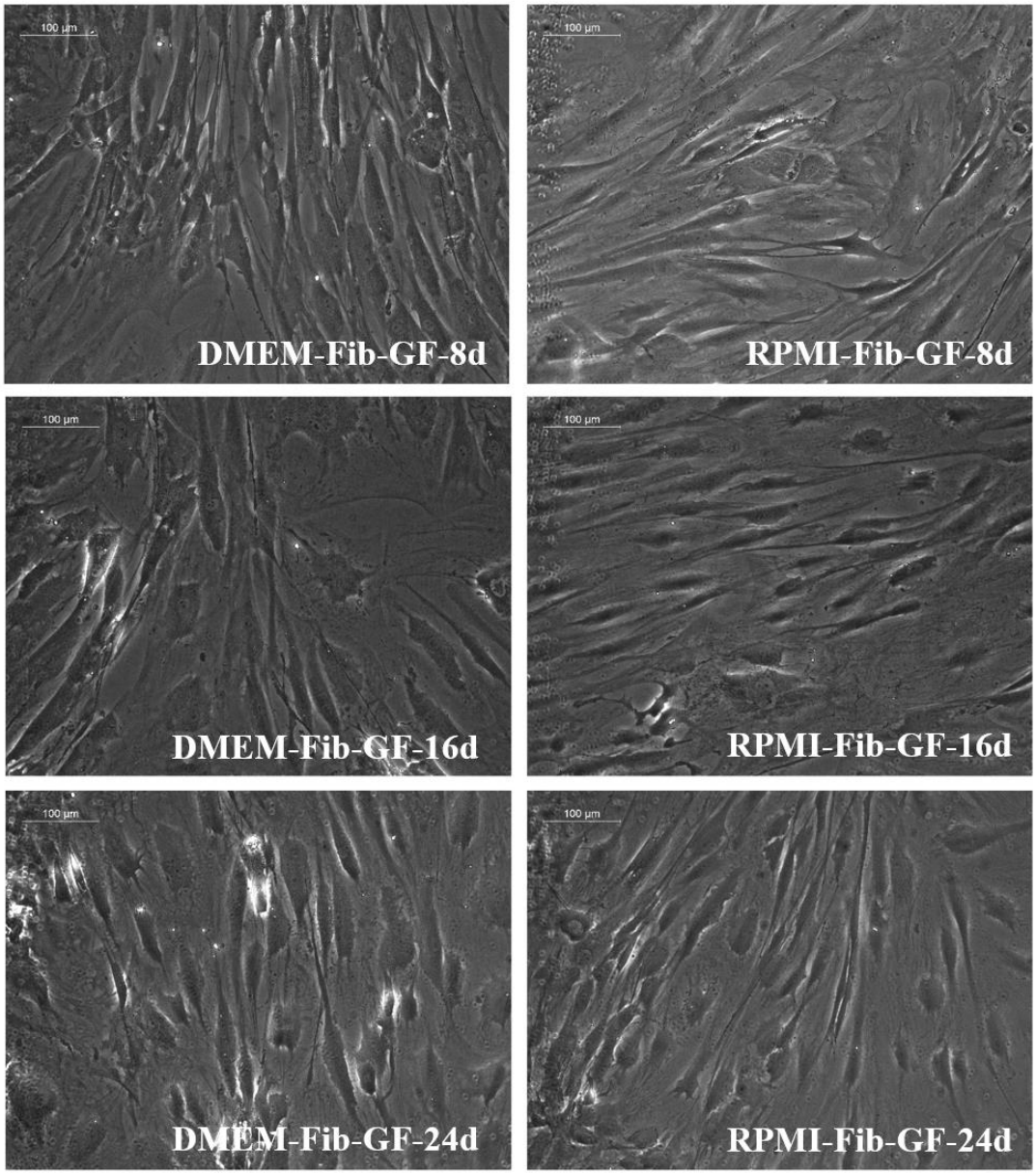


Figure 2B. Representative phase-contrast images of hADMSCs cultured on fibrin coated TCPS. Distinct morphological features are seen depending on the culture medium and period of culture. The left panel indicates cells cultured on fibrin matrix with DMEM-LG and GFs and the right panel indicates cells cultured fibrin matrix with RPMI and GFs. 8d, 16d, and 24d indicate cells cultured for 8 days, 16 days, and 24 days *in vitro* respectively.

4.2.1 Expression of CMP lineage markers

The ADMSCs grown on fibrin matrix, upon induction with GFs expressed mRNA of cardiac transcription factor GATA-4 and cardiomyocyte structural protein Troponin T at different stages of culture. The expression of GATA-4 was higher in the cells cultured on the fibrin matrix with RPMI medium and GFs than the cells cultured on bare TCPS at 8d and 24d. The expression of GATA-4 and Troponin T was absent in the cells cultured using DMEM LG medium, with GFs, on bare TCPS as well as on fibrin matrix (**Figure 3**).

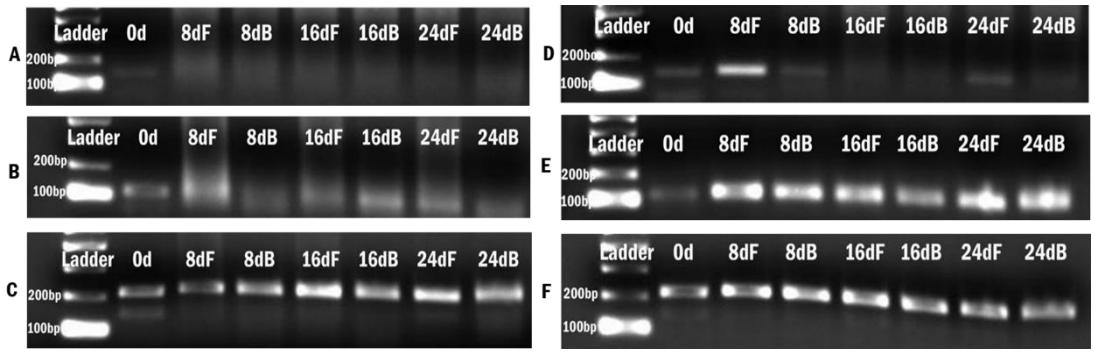


Figure 3. Images showing RT-PCR products of markers expressed upon induction to cardiac cells. A) and D) expression of GATA-4 by cells cultured in DMEM-LG medium and RPMI 1640 medium with GFs respectively. B) and E) expression of TNNT-2 by cells cultured in DMEM-LG medium and RPMI 1640 medium with GFs respectively and C) and F) expression of GAPDH by cells cultured in DMEM-LG medium and RPMI 1640 medium with GFs respectively. 0d indicates day 0; 8dF, 16dF, and 24dF indicate gene expression of cells cultured till day 8, day 16, and day 24 on fibrin matrix, and 8dB, 16dB, and 24dB indicates cells cultured till day 8, day 16, and day 24 on bare TCPS.

Further, the cardiac-specific gene expression was studied by quantitative real-time PCR (qRT-PCR), and the results obtained indicated that GATA-4, MYL2, and MYH6 expression were up-regulated between 8 to 16 days of induction whereas TNNT2 expression was quiescent through all periods of culture. But by 24d, all the cardiac-specific genes were found to get down-regulated indicating instability of CMPs in culture by 24d (**Figure 4**).

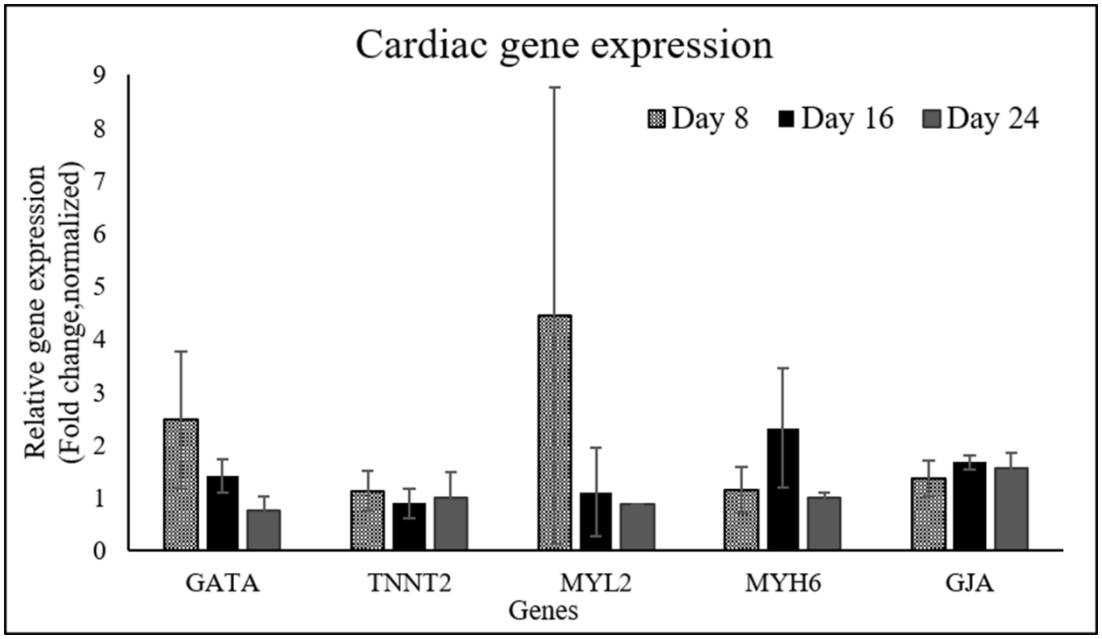


Figure 4. Analysis data of CMP marker expression by qRT-PCR in induced hADMSCs. The expression pattern of CMP specific genes at 8d, 16d, and 24d of culture *in vitro* was represented graphically. Gene expression of each period was normalized with control ADMSCs at respective periods.

4.2.2 Tracking of differentiation by immunostaining

The expression of the cardiac marker at the translational level was confirmed by immunostaining for Troponin I. By 16d of culture, cells induced in RPMI medium with GFs expressed cardiac Troponin I. But the number of cells expressing Troponin I was consistently lower (**Figure 5**). The expression of gap junction protein Connexin 43 was also confirmed by immunostaining and the cells at 16d of induction indicated the presence of Connexin 43 (**Figure 6**).

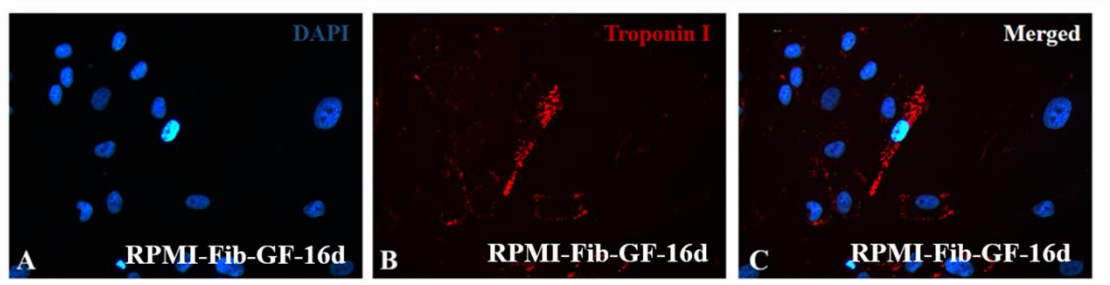


Figure 5. **Expression of the structural cardiac marker.** Immunofluorescent micrograph of ADMSCs induced with RPMI and GFs on fibrin matrix expressing Troponin I. A) Nuclear stain DAPI, B) cardiac troponin I and C) merged image *(magnification 40X).*

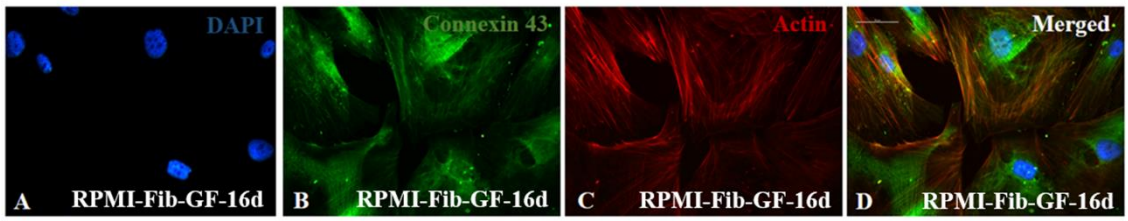


Figure 6. Expression of gap junctional protein. Immunofluorescent micrograph of ADMSCs induced with RPMI and GFs on fibrin matrix expressing Connexin 43. A) Nuclear stain DAPI, B) connexin 43, C) Actin and D) merged image *(magnification 40X).*

4.3 Modified protocols: effect co-differentiation of CMPs & CFPs

The successful proliferation and differentiation of cardiomyocyte progenitors (CMPs) both *in vitro* and *in vivo* may depend on the presence of the supporting cardiac fibroblasts (CFBs). The cardiac fibroblast progenitors (CFPs) can provide structural and functional integrity to the CMPs similar to the *in vivo* microenvironment present in the native heart tissue. This part of the study focused on standardizing a GF- based induction protocol that promoted the co-survival and differentiation of CMPs and CFPs. The Co-survival of both cell types was confirmed by transcriptional and translational level analysis of CMP and CFP markers. The modified niche comprised fibrin matrix in all cases and 10,000cells/cm^2 was the seeding density used. The major difference was variations in the addition/deprivation of various combinations of GFs.

The morphology of cells appeared to be different with all three induction protocols. Features like the elongation of cells, the appearance of branching, and formation of tube-like structures were observed in the cells induced using all three induction protocols from 8d of differentiation. The presence of fibroblastic morphology was also observed frequently along with few phase-bright cells upon induction using IP1, IP2, and IP3 after 8d and 16d of initiation of differentiation *in vitro* (**Figure 7A-D**).

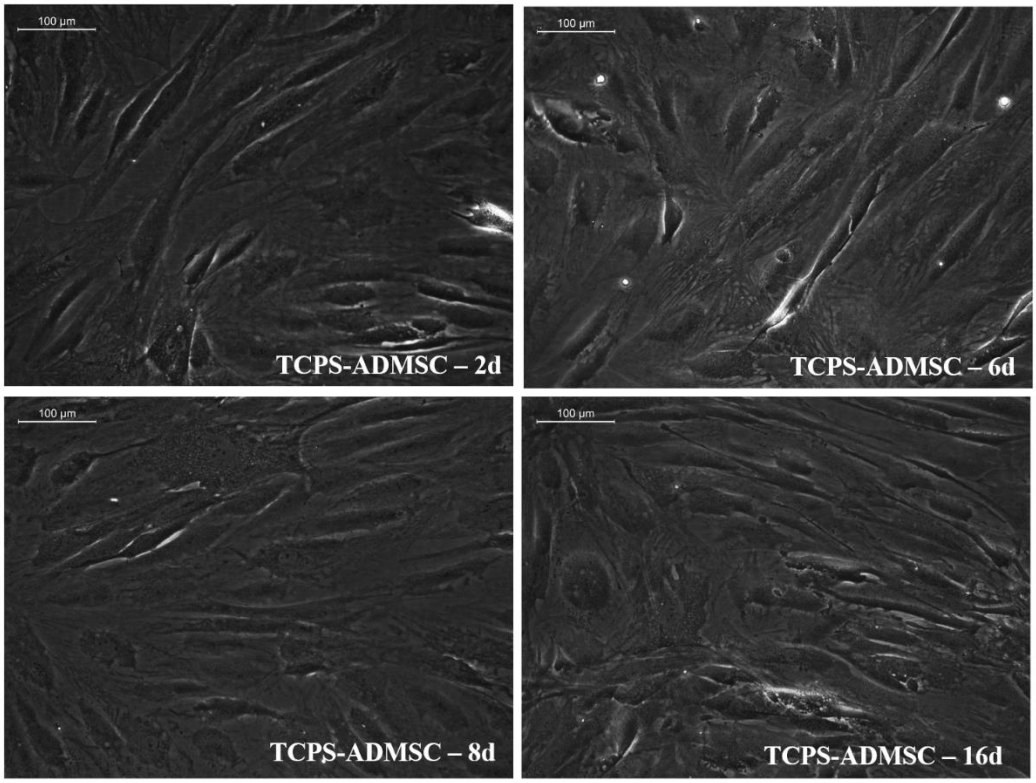


Figure 7A. Representative images showing the morphological stability of hADMSCs. The cells were cultured on bare TCPS for different periods of culture (*magnification -20X).*

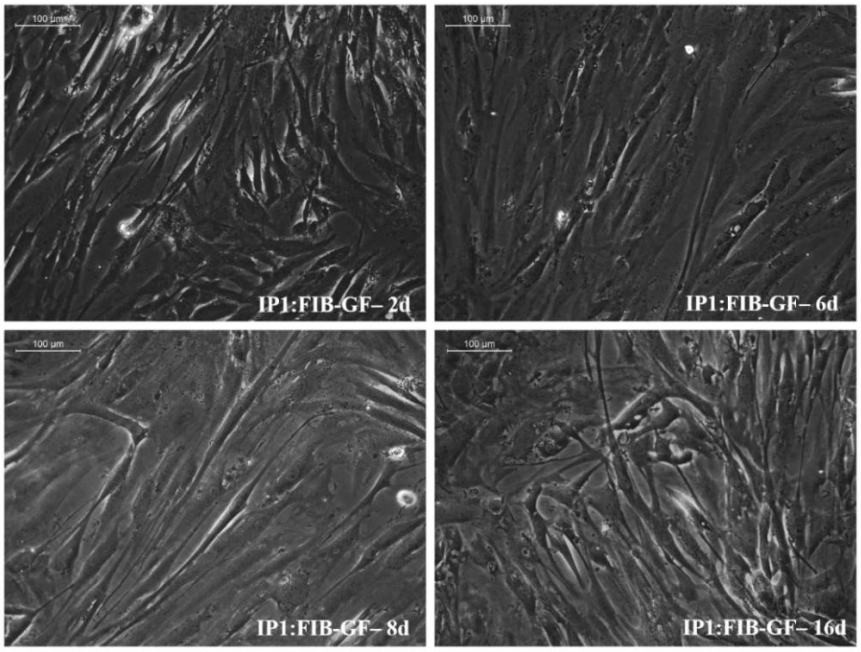


Figure 7B. Representative images of hADMSCs subjected to IP1. ADMSCs induced to CMPs and CFPs on fibrin matrix using different growth factor composition seen at different periods of culture *(magnification -20X).*

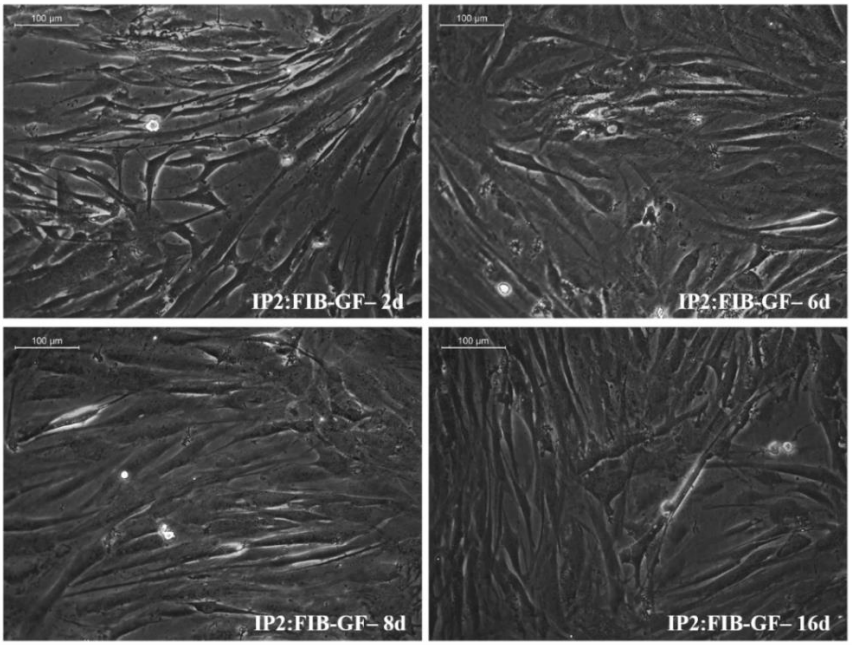


Figure 7C. Representative images of hADMSCs subjected to IP2. ADMSCs induced to CMPs and CFPs on fibrin matrix on different periods of culture *(magnification -20X).*

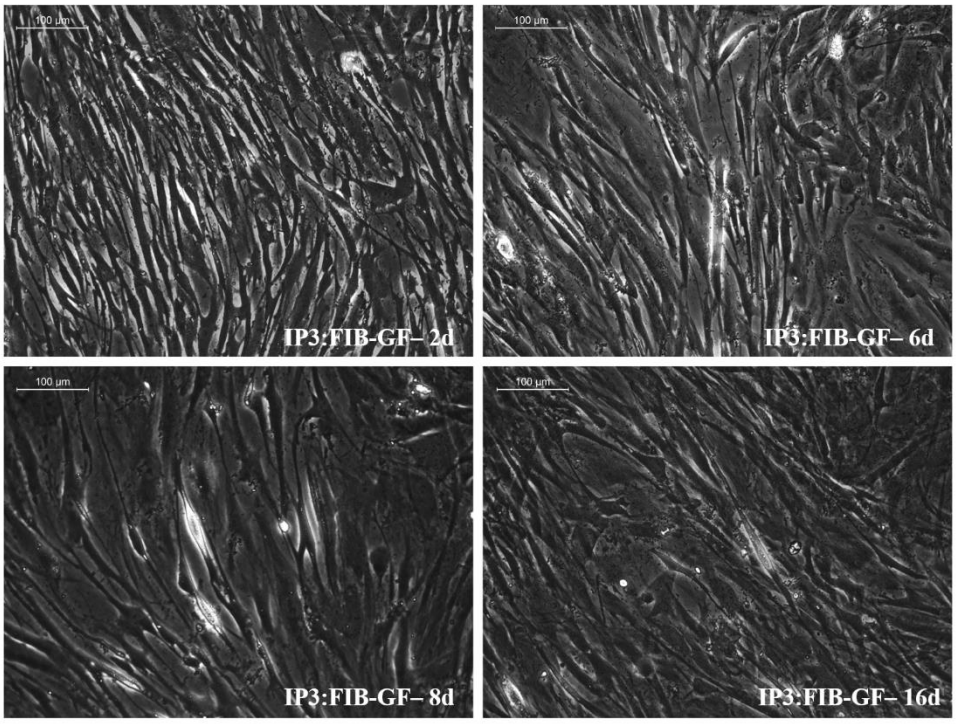


Figure 7D. Representative images of hADMSCs subjected to IP3. ADMSCs induced to CMPs and CFPs on fibrin matrix at different periods of culture *(magnification -20X).*

Since the morphological appearance indicated differentiation with all 3 protocols, a further molecular level analysis was carried out initially at the transcriptional level and later at the translational level.

4.3.1 Identification of CMPs and CFPs by qRT-PCR

To identify the presence of CMPs and CFPs, the cells that were cultured using all three induction protocols were analyzed. Parameters analyzed were based on selected CMP specific and CFP specific markers after 8d and 16d of culture by qRT-PCR. Relative gene expression was calculated using normal hADMSC grown on TCPS for respective periods as the control in all cases; for identifying the effect of induction protocol and period. It was observed that the expression of CMP specific markers TNNT2 (cardiac Troponin T), GJA1 (connexin 43), and MYL2 (myosin light chain 2) was up-regulated in cells induced by IP3 on 8d, whereas MYH6 (myosin heavy chain 6) expression pattern was similar in cells induced by all 3 induction protocols on 8d. The cardiac

fibroblastic marker DDR2 (discoidin domain receptor 2) and fibrillin-1 expression were up-regulated at 16d of induction using the IP1 strategy. The TNNT2 expression in the cells induced using IP2 seemed to get down-regulated at 16d of culture, as compared to the TNNT2 expression at 8d. DDR2 expression and fibrillin-1 expression were up-regulated in these cultures at 16d of induction indicating an increase in CFP differentiation. The expression of the Collagen I gene was found to be down-regulated at 8d and 16d upon induction using IP2 protocol. But later, the melt curve analysis of various genes indicated the presence of non-specific products and therefore, the IP2 protocol was eliminated from further studies. The expression of gap junction protein GJA1 in cells induced using IP3 was up-regulated at 8d of induction and its expression was found quiescent by16d. A 1.5- fold up-regulation of TNNT2 was observed in the cells induced using IP3 at 16d of culture compared to the control hADMSCs indicating continued differentiation of CMPs upon induction by IP3. The CFP markers DDR2 and fibrillin-1 were observed to be nominally up-regulated in cells induced by IP3 by 16d as compared to 8d. The expression of Collagen I, the major cardiac ECM specific gene was found to be 2- fold up-regulated at 8d and 0.5 fold higher by 16d of IP3 induction as compared to control hADMSCs at respective periods **(Figure 8A & 8B)**. The expression of Transforming growth factor β I (TGF β I) and platelet derived growth factor receptor α (PDGFRα) were also analyzed and in all three IPs; TGF β I expression was low on the 8^{th} day of culture as compared to ADMSC cultured for 8 additional days, whereas its expression was up-regulated by the 16^{th} day **(Figure 8C)**. On the other hand, the expression of PDGFRα was upregulated by the 8^{th} day of induction while its expression was found to get reduced by the 16^{th} day **(Figure 8D)**.

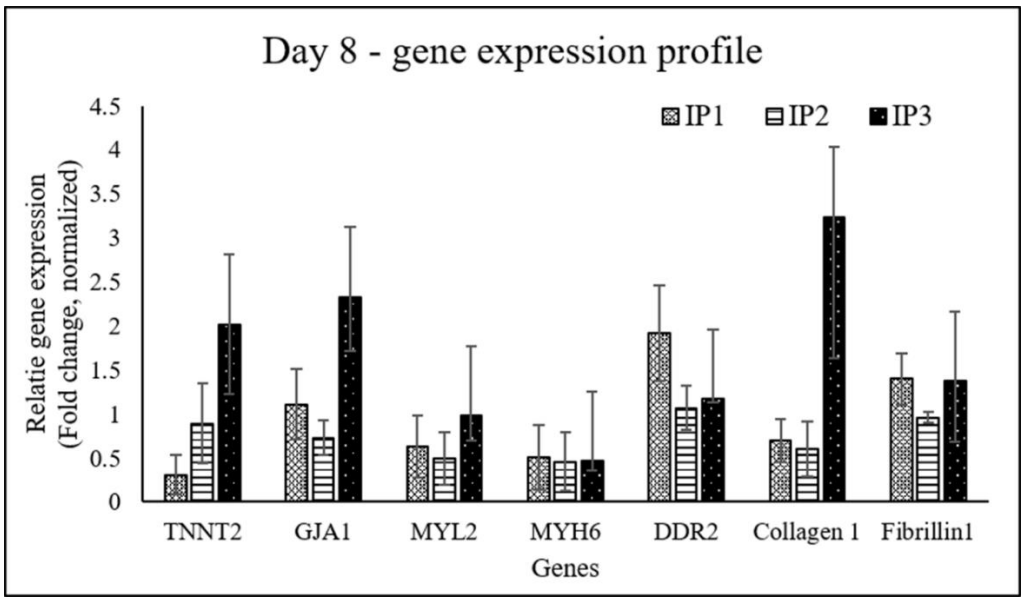


Figure 8A. Gene expression of CMP and CFP genes on 8d. The expression pattern of CMP and CFP specific genes upon induction of cells using IP1, IP2, and IP3 at 8d of culture *in vitro* was represented graphically. Gene expression was normalized to control ADMSC.

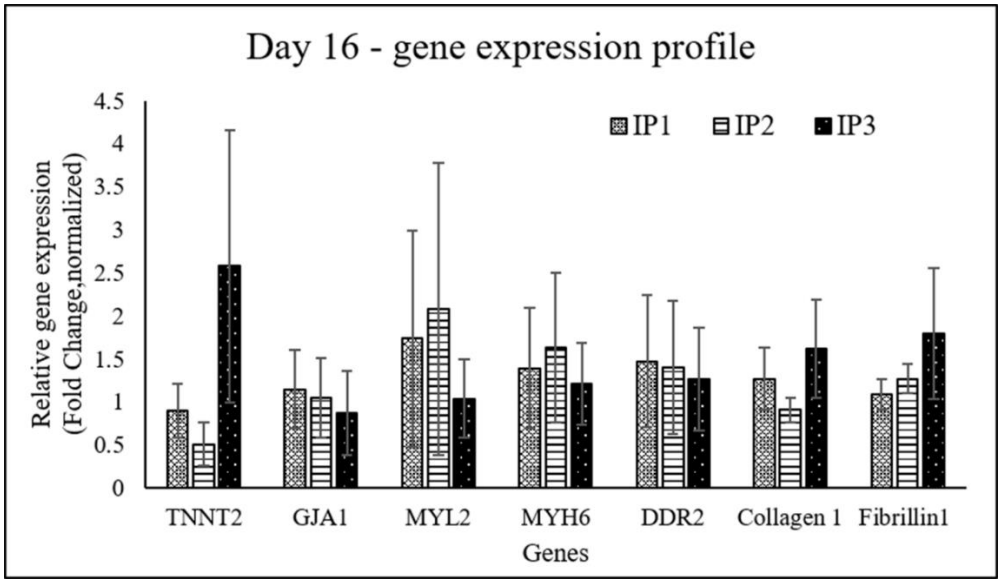


Figure 8B. Gene expression of CMP and CFP genes on 16d. The expression pattern of CMP and CFP specific genes upon induction of cells using IP1, IP2, and IP3 at 16d of culture as compared to the ADMSC control on respective periods was represented graphically.

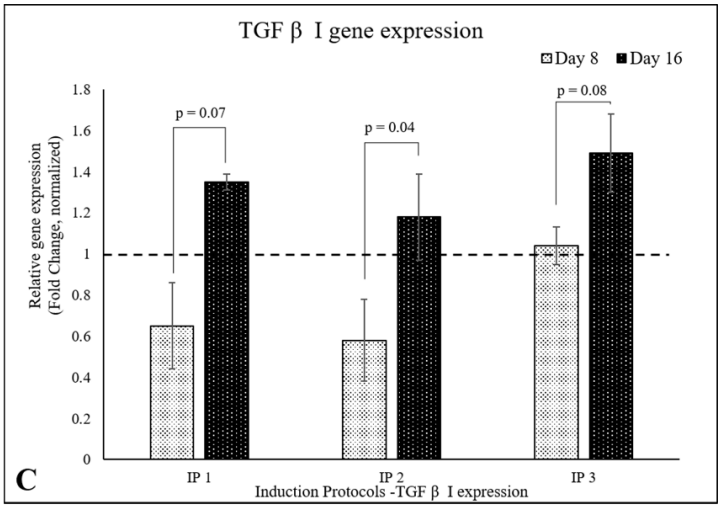


Figure 8C. Graphical representation of the relative gene expression pattern of TGF β I. The expression pattern of TGF β I gene upon induction of cells using IP1, IP2, and IP3 at 8d and 16d of culture as compared to the ADMSC control on respective periods was represented graphically. Error bars represent mean ± Standard error. Statistical analysis using Student's t-test and the respective p values obtained is represented in the graph (n=3).

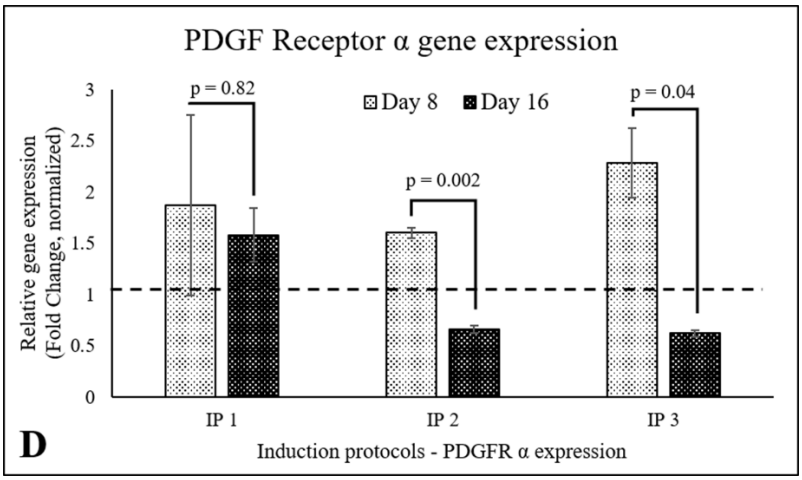


Figure 8D. Graphical representation of the relative gene expression pattern of PDGFRα. The expression pattern of PDGFRα gene upon induction of cells using IP1, IP2, and IP3 at 8d and 16d of culture as compared to the ADMSC control on respective periods was represented graphically. Error bars represent mean ± Standard error. Statistical analysis using Student's t-test and the respective p values obtained is represented in the graph (n=3).

4.3.2 Immunocytochemical characterization

The expression of CMP markers and CFP markers at the translational level was confirmed by immunocytochemistry at 16d of induction. Cells that were induced using IP3 expressed cardiomyocyte-specific marker Troponin T and gap junctional protein Connexin 43 at 16d of induction. For both proteins, high expression is evident in IP3 (Fig 9: IP3/TNNT^{High} and IP3/Cx43^{High}) as compared to that in IP1. The TNNT2 stained cells in IP3 showed typical cardiomyocyte morphology. The gap junctions stained specifically on the cell membrane also confirm the specificity of the cell. Very few cells expressed Troponin T and Connexin 43 at 16d after induction using IP1 (**Figure 9**). The expression of CFP marker Fibrillin 1 was also observed in the cells induced using IP1 and IP3 at 16d of differentiation (**Figure 10**). Upon estimation of cardiac Troponin T expressing cells, around 61.24 ± 19.95% cells in culture were differentiated to CMLs in IP3 **(Table 6)**; whereas, in IP1, around 16.62 ± 5.61% only were transformed into CMLs **(Table 7; p = 0.02)**.

Table 6 - The yield of cardiac Troponin T expressing cells in IP1 culture

IP1 Induction	No. of nucleus/field	No. of TNNT 2 stained cells/field	Percentage of TNNT 2 stained cells/field	Average	S.D
Sample 1	51	10	19.60	16.62	5.61
Sample 2	59	6	10.16		
Sample 3	44	10	22.72		
Sample 4	57	8	14.03		

Table 7 - The yield of cardiac Troponin T expressing cells in IP3 culture

IP3 Induction	No. of nucleus/field	No. of TNNT 2 stained cells/field	Percentage of TNNT 2 stained cells/field	Average	S.D
Sample 1	42	17	40.47	61.24	19.95
Sample 2	46	35	76.08		
Sample 3	46	37	80.43		
Sample 4	50	24	48		

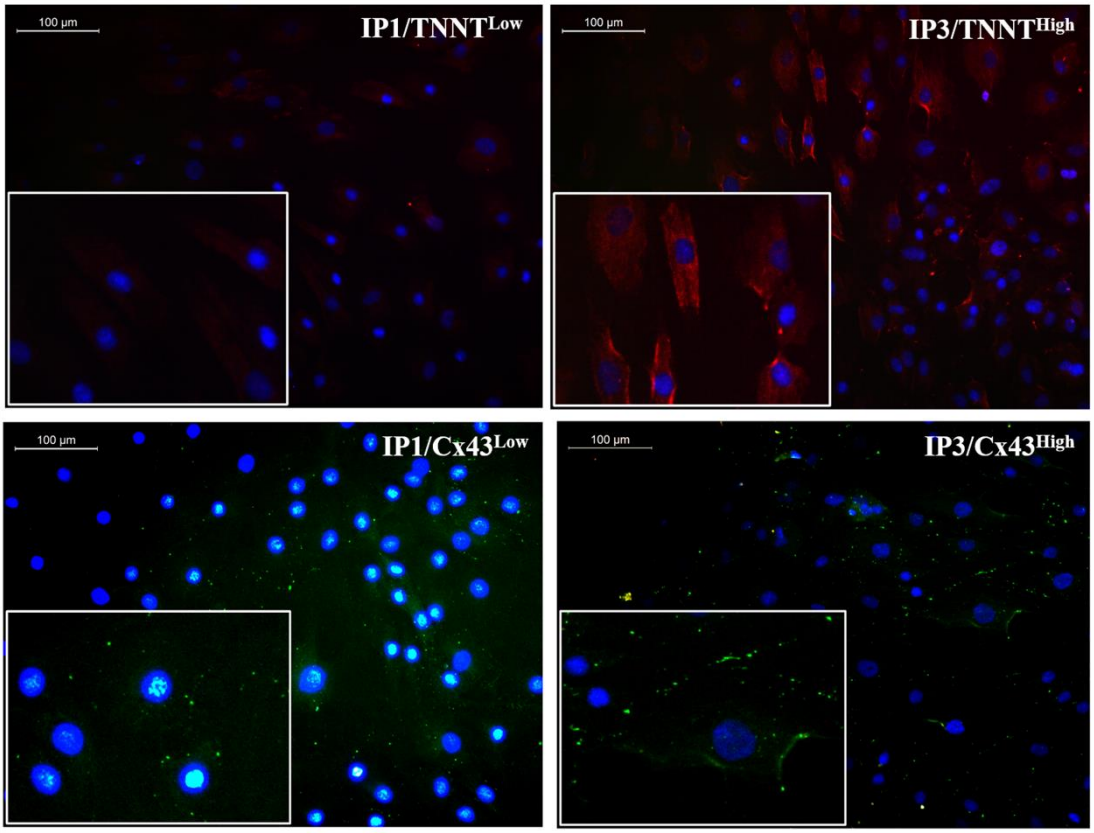


Figure 9. Representative images of IP1 & IP3 cells stained with Troponin T and Connexin 43. Immunocytochemical expression of cardiac Troponin T and connexin 43 at 16d of induction by IP1 and IP3. Low TNNT and Cx43 expression were observed in IP1 while TNNT and Cx43 expressing cells were observed more frequently in IP3 induction. *(Magnification – 40x).*

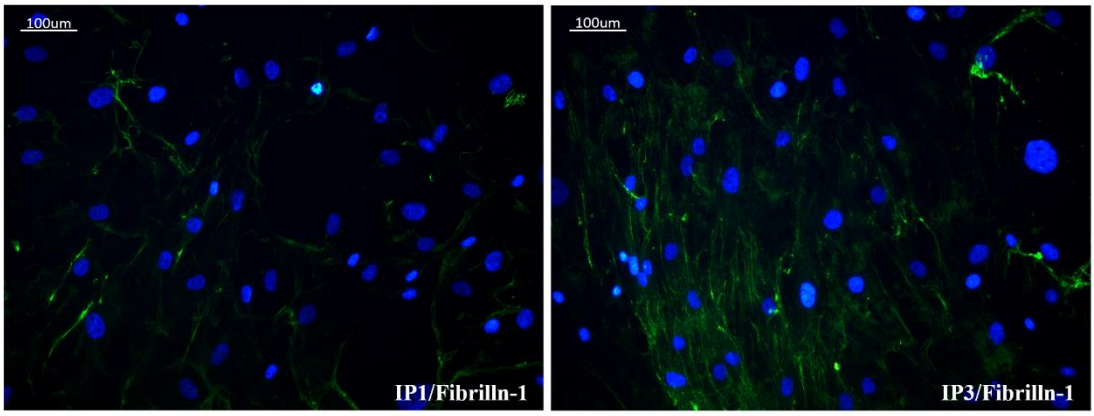


Figure 10. Representative image demonstrating the induction of hADMSC to CFP. Immunocytochemical staining of fibrillin-1 on 16d of induction in cells induced by IP1 and IP3 indicating the presence of cardiac fibroblasts *(magnification – 20x).*

4.3.3 Expression of extracellular matrix protein

The cells induced to differentiate to CMP and CFPs were examined for the presence of ECM protein Collagen I, the most prominent ECM marker of native heart tissue, upon decellularizing the cultures that were induced for a period of 16d and the immunocytochemical evaluation indicated specifically Collagen I deposition by the cells induced by IP3 (**Figure 11**). Cells in IP1 also deposited collagen, but comparatively less quantity.

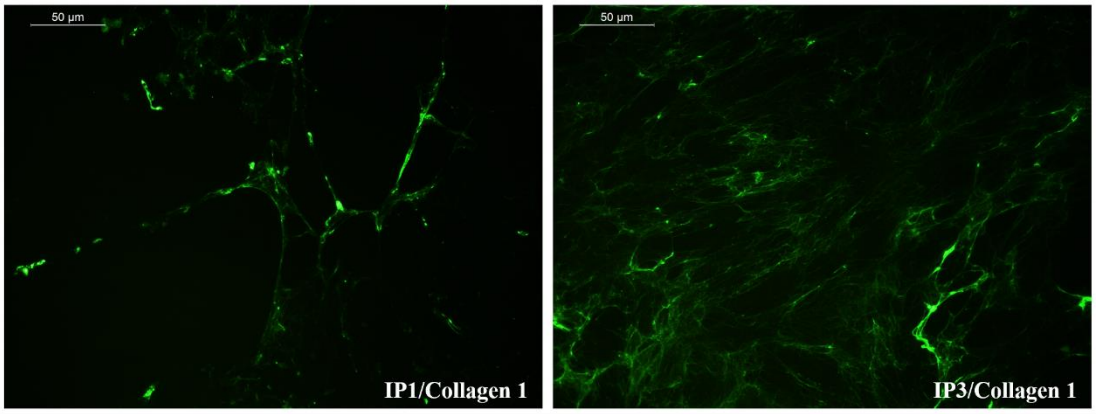


Figure 11. Representative immunostained images of decellularized culture matrix for Collagen I. Expression indicated by immunocytochemistry of IP1 induced cells and IP3 induced cells at 16d of differentiation *(magnification – 40x).*

Thus, the transcriptional level and translational level expression of CMP and CFP specific markers indicated that IP3 protocol induced differentiation of hADMSCs into CMP and CFP lineages in the same culture *in vitro,* markedly.

4.4 Induction of angiogenic activity in IP3 by seeding exogenous EPCs

In addition to the cardiac fibroblast population present in the myocardium, the other major non-myocyte population present within the myocardium is the endothelial cells that provide blood supply to the cardiomyocytes. This section focused on the co-culture of hADMSC-derived EPCs with the CMP-CFP population. Upon seeding EPCs, the microenvironment can change and influence the differentiation in different ways. This is mainly because EPCs may release GFs. So it is important to establish the stability of the CMP and CFP population, in addition to the phenotype maintenance of seeded EPC. Therefore analysis at the transcriptional and translational level was done in the co-culture for markers specific for CMP, CFP, and EPC and compared to IP3 alone and EPC alone in culture. ADMSCs were induced into EPCs by a combination of GFs followed by hypoxia induction for 16h. The hypoxia-induced cells were trypsinized and seeded over IP3 cells (CMP-CFP) at a density of 5000 cells/cm^2. A combination of DMEM/F12 and MCDB 131 at a 1:1 ratio was used for the maintenance of co-culture and the medium composition did not negatively influence cell survival. The EPCs attached to the CMP-CFP layer without causing prominent cell death/detachment (**Figure 12**). Count of total cell in control ADMSCs culture and EPCs alone culture showed an increase by 16d as compared to 8d, indicating that both these cells are in proliferative phenotype whereas, the cell count in CMP-CFP co-culture and CMP-CFP-EPC co-cultures decreased by 16d from that of day 8, indicating the proliferative arrest of a particular cell population.

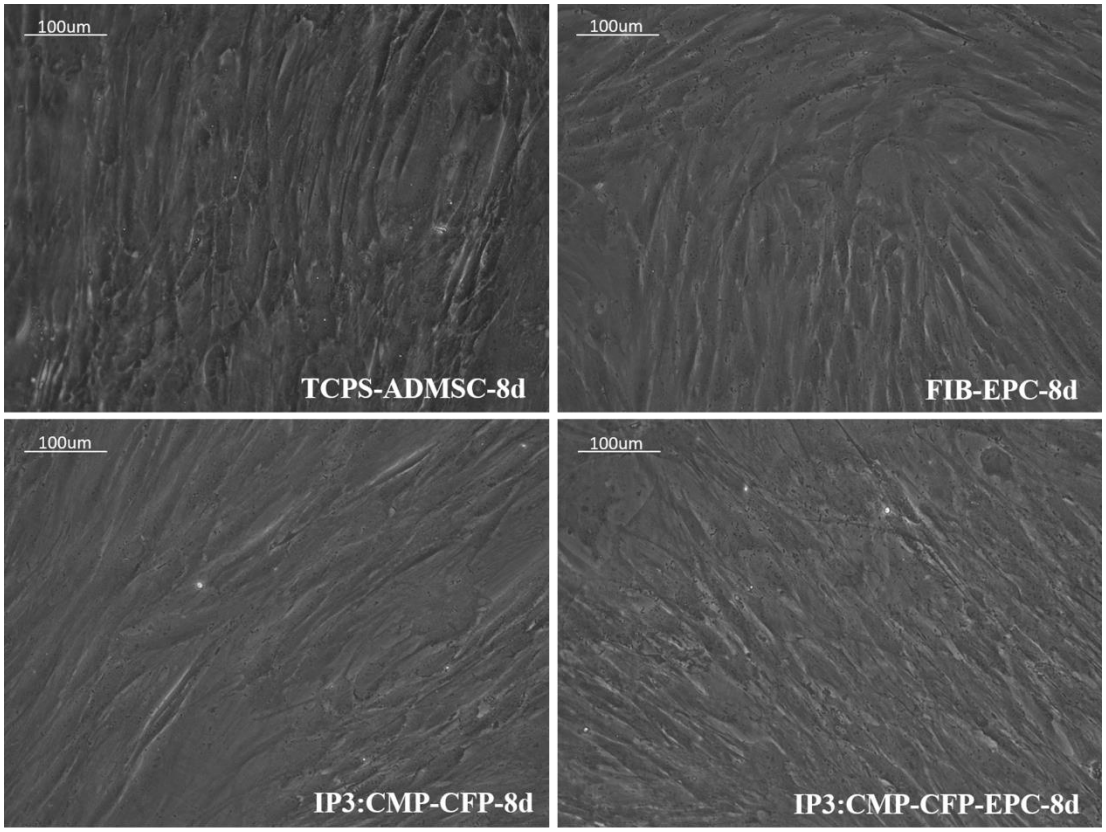


Figure 12. Representative images of co-culture of CMP, CFP & EPC. CMP-CFPs and CMP-CFP-EPCs cultured on fibrin matrix at 8d of differentiation. ADMSCs cultured on bare TCPS was used as the control (*Magnification – 20x*).

4.4.1 Phenotype characterization of cells in co-cultures

The cell-specific markers of all three subpopulations, that is CMPs, CFPs, and EPCs in co-culture was analyzed by qRT-PCR at 8d and 16d of induction. The gene expression pattern indicated a stable and comparable expression profile for the CMP specific genes - TNNT2, GJA1, and MYL2, even after exogenous addition of EPCs into the IP3 induced cells **(Figure 13A)**. Though the endothelial-specific genes – CD31, VCAM-1, eNOS, and MCP-1 were not analyzed in IP3 protocol, after 8d of induction these genes were seen to be expressed, indicating that even without seeding exogenously developed EPCs, the induction protocol derived EPCs along with CMP and CFP (**Figure 13B**). On 16d of induction, all CMP specific genes and endothelial-specific gene expression was observed to be stable and further up-regulated as compared to 8d expression. Only the expression profile of GJA1 and MYL2 was lower

on 16d than that on 8d, but it was expressed to a similar level. The higher expression levels of TNNT2 and other endothelial-specific markers by 16d indicates that the presence of EPC and probably the factors contributed by the cell favored CMP differentiation as well. But the fibroblast specific genes were found to be up-regulated in CMP-CFP culture without EPCs on 16d of induction. This observation points to lower numbers of fibroblasts in the CMP-CFPs-EPCs co-culture which may be considered as favorable maintenance of the fibroblast population **(Figure 13C)**. The gene expression of TGF β I was low on the 8th day while its expression was up-regulated by the 16th day in co- culture. PDGFRα gene expression was up-regulated by the 8th day while its expression pattern was reduced by the 16th day of co-culture **(Figure 13D)**. If the fibroblast population exceeds CMPs and EPCs, it can cause excessive collagen deposition and can hamper the regenerated tissues. Thus a striking observation in this experiment was that EPCs are also generated in the IP3 induction culture. Both on 8d and 16d, IP3 culture showed up-regulation of EC-specific gene; with a marked increase by 16d as compared to 8d.

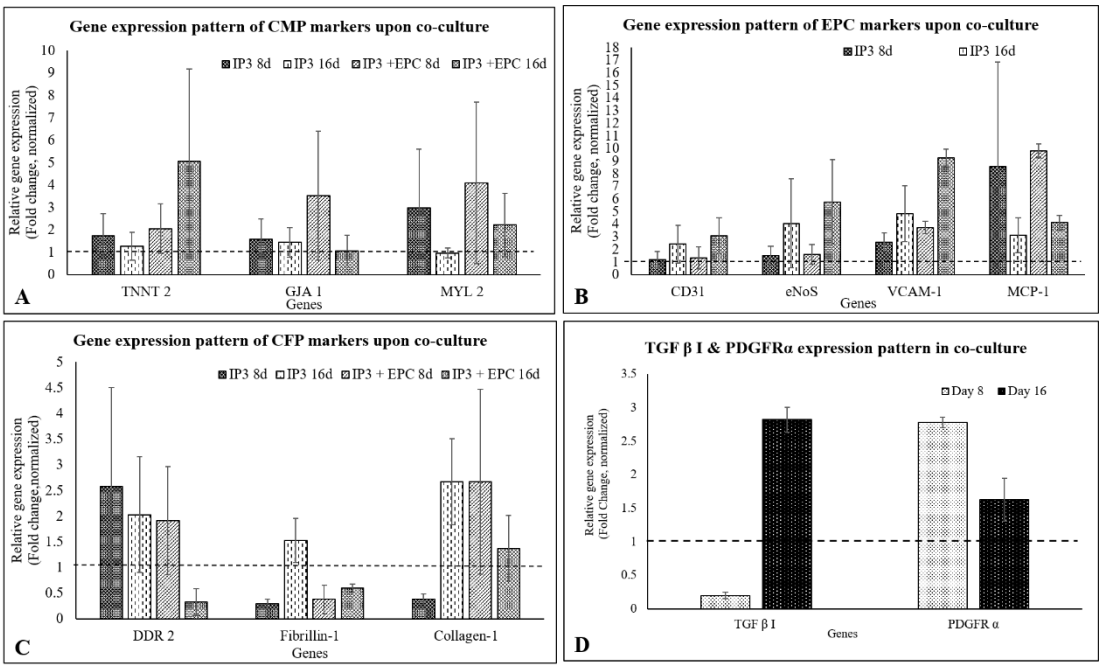


Figure 13. Gene expression pattern of cells upon co-culture. The relative gene expression of (A) CMP-specific, (B) EPC-specific, and (C) CFP-specific markers in

co-culture and (D) the expression of TGF β I and PDGFRα gene expression upon co-culture, normalized to the control ADMSCs is represented graphically.

Similar to the observation at the transcriptional level, at the translational level also, the development of EPC in IP3 is indicated by the immunofluorescence images with VCAM-1 positive cells in IP3 culture (Fig 14, left column). Also, Troponin T positive and fibrillin positive cells were seen in IP3 (CMP-CFP) culture at 8d of induction. But when exogenous EPC was added to IP3 more numbers of VCAM-1 positive cells are evident in CMP-CFP-EPC co-culture at 8d of induction. The fibrillin-1 expression was evident in CMP-CFP culture and the co-cultures of CMP-CFPs with EPCs indicating maintenance of fibroblast population in co-cultures. The expression of endothelial-specific marker VCAM-1 was observed in ADMSCs-derived EPCs and its expression was also evident in CMP-CFP-EPC co-culture at 8d (**Figure 14**).

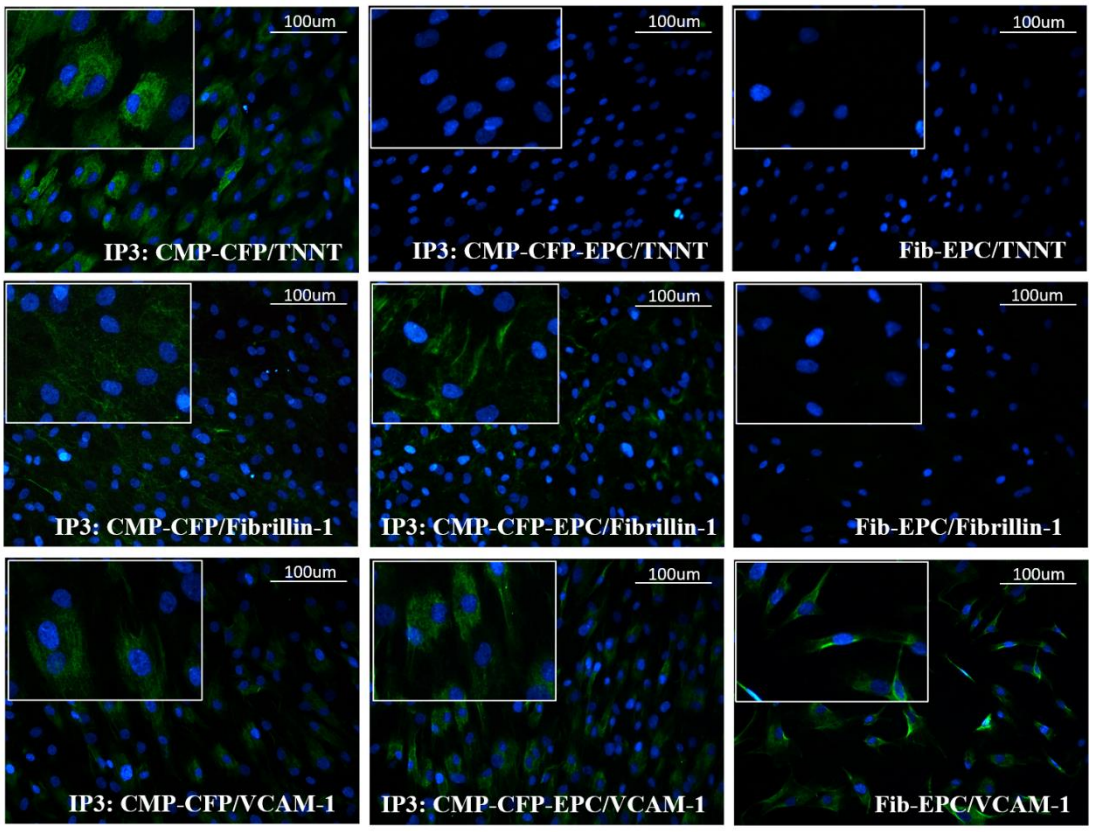


Figure 14. Immuno fluorescent images of CMP CFP and EPC markers upon co-culture. Cardiac troponin T stained images of CMP-CFPs, CMP-CFP-EPCs, and EPC cultures on 8d shown in the top panel mark presence of CMP in co-culture but not in

EPC. Fibrillin-1 in CMP-CFPs, CMP-CFP-EPCs, and EPC in the middle panel marks the presence of CFP only in co-cultures and not in EPC.VCAM-1 immunofluorescence is seen in both co-cultures i.e. CMP-CFPs, CMP-CFP-EPCs, but more prominently in the latter and EPC alone culture on 8d marking the EC lineage cells in IP3 protocol (*Magnification -20x*).

4.5 Effect of addition of hADMSC secretome to CMP-CFP culture in the initiation of angiogenic potential

Mesenchymal stem cells are well known to release a variety of trophic factors that are mainly responsible for the paracrine reparative mechanism of this cell type in various tissue injuries. This part of the study focused on exploiting the effect of the addition of bioengineered hADMSC secretome overexpressed with VEGF or non-engineered hADMSC secretome in combination with IP3 induction to hADMSC cultures, to initiate an angiogenic response by the secreted proteins on the undifferentiated population of hADMSCs present in the CMP-CFP cultures.

The successful incorporation of mammalian expression plasmid encoding human VEGF-A transcript variant 4 gene into ADMSCs by electroporation was demonstrated by the expression of green fluorescent protein (GFP) spark tag in the cytoplasm of ADMSCs (**Figure 15A**).

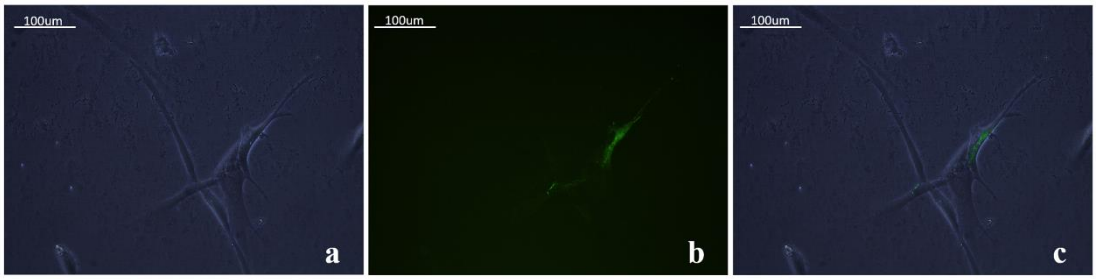


Figure 15A. Electroporated hADMSCs. (a) Phase-contrast micrograph of hADMSC electroporated with VEGF overexpressing vector, (b) fluorescent micrograph of the electroporated cell indicated by GFP expression, and (c) merged image of phase contrast and fluorescent field (*magnification – 20x*).

It was observed that the addition of VEGF overexpressed bioengineered hADMSC secretome (eSec) as well as non-engineered hADMSC secretome (nSec) to the CMP-CFP cultures induced elongation of cells in cultures at 8d of induction (**Figure 15B**).

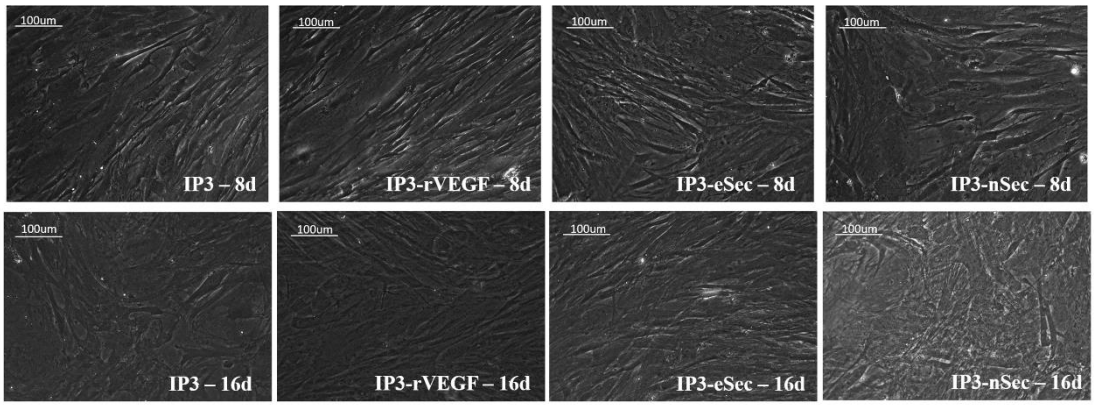


Figure 15B. Morphological features of secretome added CMP-CFPs. Phase-contrast micrograph of CMP-CFPs induced by IP3 alone; CMP-CFPs induced by IP3-rVEGF; CMP-CFPs induced by IP3 - eSec and CMP-CFPs induced by IP3 - nSec on 8d and 16d of induction (*magnification – 20x*).

The CMP-CFP co-culture seemed to undergo good cell proliferation. When eSec was added to the cultures, the proliferation potential was reduced which was further decreased in cultures in which recombinant exogenous VEGF or normal ADMSC secretome was added (**Figure 16**). Since differentiation and proliferation are inversely proportional, further differentiation of the heterogeneous population of cells in IP3 culture into different lineage is indicated by the observed reduction in proliferation. Since it is a mixture of different lineage cells, which cells are proliferating or not proliferating is not clear from this experiment.

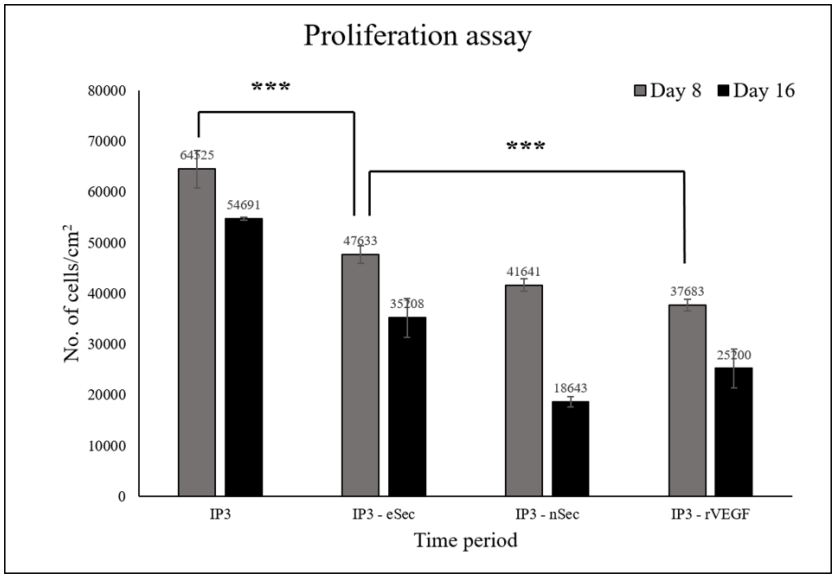


Figure 16. The proliferation of cells by tritiated thymidine uptake assay. Significant proliferation was observed in cells induced by IP3-upon adding eSec (engineered secretome) compared to the cells induced by IP3-rVEGF cultures at 8d of induction. Significance is indicated as '***' ($P < 0.001$).

Upon gene expression analysis by qRT- PCR, results indicated that the expression of endothelial-specific genes VCAM-1, e-NOS, and MCP-1 was up-regulated at 16d of induction in CMP-CFP cultures added with either eSec or nSec as compared to the cells induced by IP3 alone (**Figure 17**). Also, the IP3 culture, without any other added ADMSC secretome, comprises lineage-committed EPCs that expressed all EC markers by 16d, other than CMP and CFP.

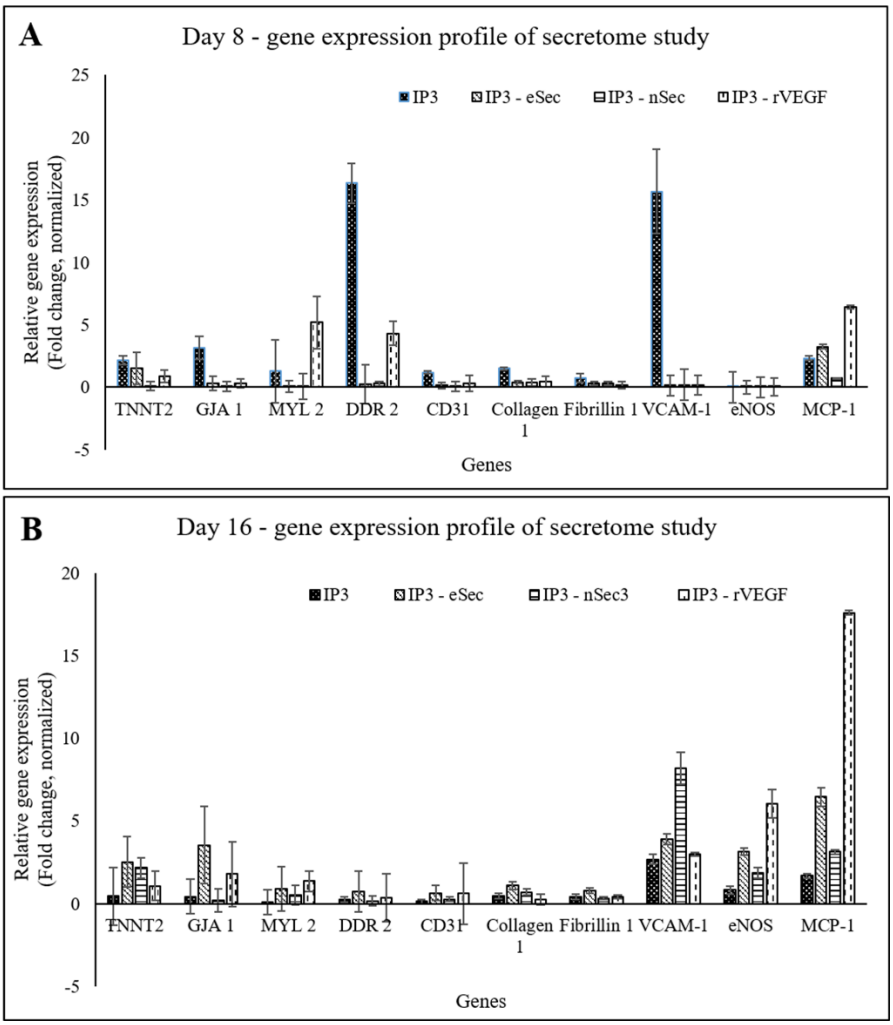


Figure 17. Effect gene expression upon the addition of hADMSC secretome after IP3 induction. The relative gene expression of CMP specific, CFP specific, and EPC specific markers upon addition of eSec/nSec/rVEGF in combination with IP3 on (A) 8d of induction and (B) 16d of induction normalized to the control ADMSCs is represented graphically.

The effect of the addition of VEGF overexpressed bioengineered hADMSC secretome or non-engineered hADMSC secretome into IP3 induced cells was analyzed by the expression of Troponin T positive cells after 16 days of differentiation. The immunostaining for cardiac troponin T in cells indicated more frequent Troponin T positive cells *in vitro* upon addition of either eSec or nSec into IP3 induced cells as compared to control IP3 alone or those treated with rVEGF. The results indicate

improved maintenance of CMP phenotype upon the addition of either eSec or nSec (**Figure 18**).

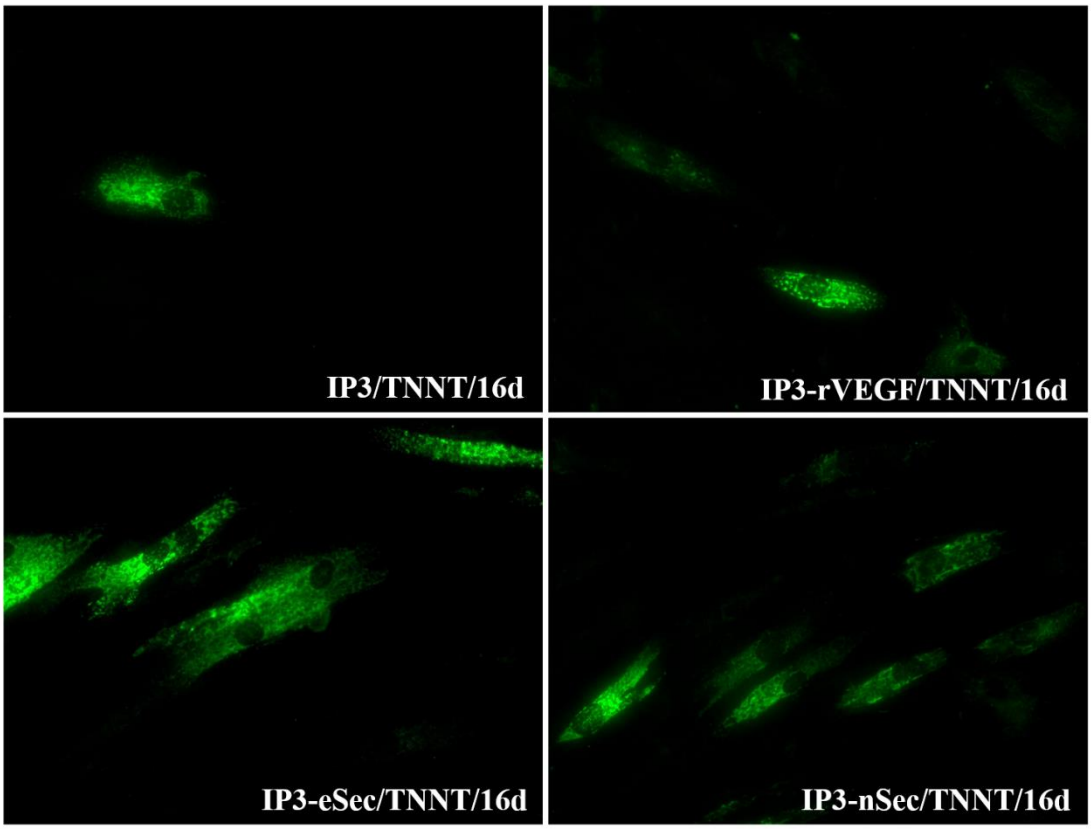


Figure 18. Representative images of TNNT immunofluorescence in IP3 upon adding eSec and nSec. Fluorescent micrograph images of cardiac Troponin T positive cells induced by IP3 alone, IP3 - rVEGF, IP3 – eSec, and IP3 – nSec on 16d of induction. The frequency of TNNT positive cells was higher in IP3 – eSec and IP3 – nSec groups (*Magnification – 20x*).

Clearly, added secretome promotes the derivation of more CMP in culture, irrespective of whether it is engineered to overexpress VEGF or not. The results suggest the paracrine effect of exogenously collected and added ADMSC secretome in IP3 culture. The addition of rVEGF is not enough to produce such an effect for increased differentiation of hADMSCs to CMPs under the conditions of induction protocol IP3, standardized in this study.

4.6 Cell transplantation and evaluation in animal model of MI

This final phase of the study illustrates rat ADMSC isolation, induction to CMP-CFP population, *in vitro* cell labeling before transplantation to track cell survival in the transplanted site of rat MI model.

4.6.1 Rat ADMSC isolation and induction to rCMP lineage

Rat ADMSC was isolated by the collagenase enzyme treatment method standardized for hADMSC isolation and the cells were culture expanded and passaged by standard trypsinization procedure. The passage 3 rADMSCs were then induced into cardiomyocyte progenitors (CMPs) using the niche and induction protocol (IP3) standardized for human cells. Distinct morphological features were observed in induced cells as compared to the control rat ADMSCs from day 5 of induction. The elongation of cells and formation of tube-like structures was observed from the 5^{th} day of induction and the elongated morphology of induced cells was maintained till 16 days of culture. The appearance of phase-bright cells was also evident from the 5^{th} day of induction *in vitro* (**Figure 19A**). The qRT-PCR results indicated a 0.25-fold up-regulation of cardiac-specific markers Troponin T (TNNT2) by 8d and the expression of TNNT2 was almost consistent up to 16d (0.55-fold) of culture *in vitro*. The expression of Connexin 43 (GJA 1) mRNA, a predominant gap junctional protein present in the myocardium was also found to be expressed in primed rCMPs till 16d of culture in vitro. A 0.34-fold up-regulation of Flk-1, the major vascular endothelial growth factor (VEGF) receptor, was observed in the *in vitro* primed cells by 8d and was further up-regulated to 2-fold by 16d as compared to the un-differentiated rADMSCs. However, CD31 mRNA expression was not observed in the *in vitro* primed rADMSCs on the 8^{th} day of culture, but by the 16^{th} day of *in vitro* priming, its expression (0.64-fold) was observed in the rCMPs **(Figure 19B).**

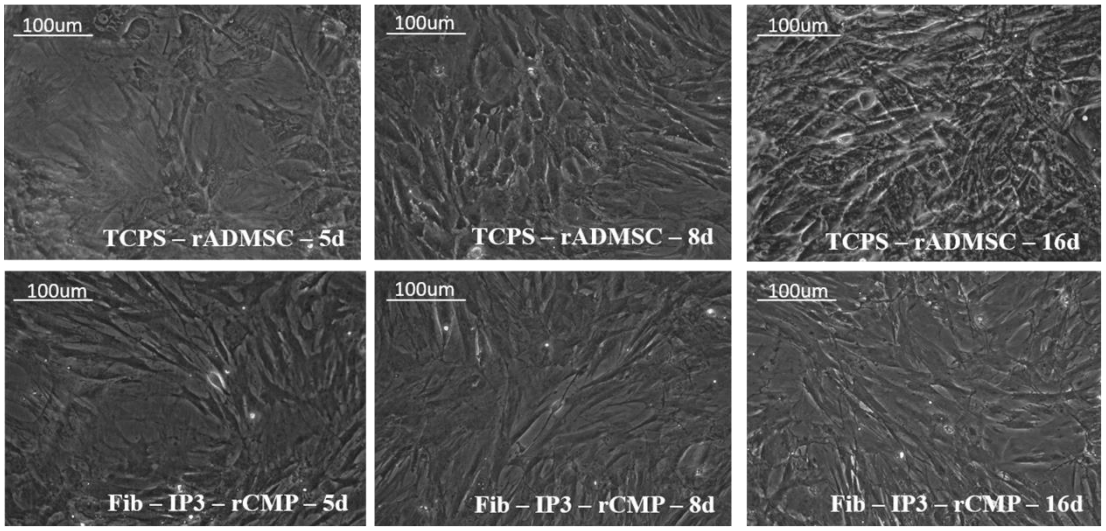


Figure 19A. Morphology of rADMSCs induced to rCMPs *in vitro*. Phase-contrast images of rADMSCs cultured on bare TCPS on days 5, 8, and 16 and phase-contrast images of rCMPs induced by IP3 on fibrin matrix on days 5, 8, and 16 of culture *in vitro (magnification -20x).*

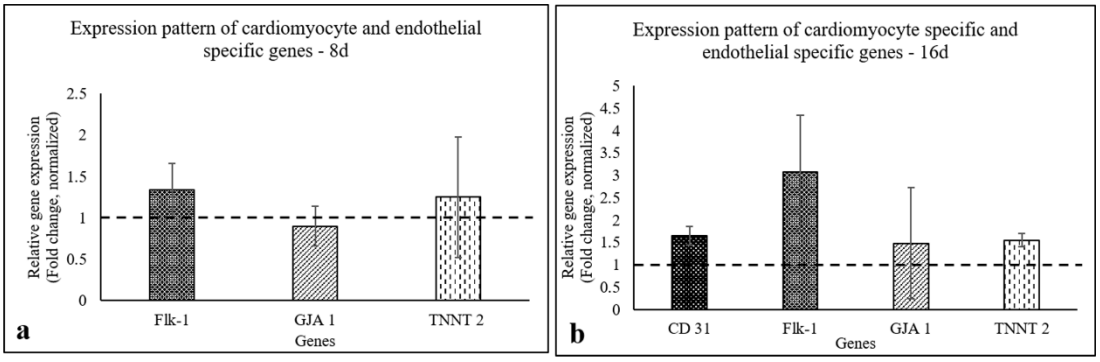


Figure 19B. Graphical representation of relative gene expression pattern of *in vitro* primed rCMPs for the markers: a) Flk-1, GJA1, and TNNT 2 on 8d and b) the relative gene expression pattern of rCMPs for the markers Flk-1, CD31, GJA1 and TNNT 2 at 16d of culture *in vitro* by qRT-PCR. Fold change is indicated relative to GAPDH expression on each day of analysis using the $2^{-\Delta\Delta Ct}$ method, upon normalization with results obtained from the un-differentiated rADMSCs cultured on bare TCPS. The dotted lines represent the basal level of gene expression in un-differentiated rADMSC cultures of the respective time periods. Error bars represent standard error (n=3).

4.6.2 Creation of MI model and cell transplantation

Soon after the animals were anesthetized, a tracheostomy was performed and the animals were ventilated with a small-animal ventilator through a tracheostomy tube followed by anesthesia maintenance with 1.5% isofluorane. MI was induced surgically by left anterior descending artery (LAD) ligation (**Figure 20B**) through a parasternal thoracotomy (**Figure 20A**) and the development of MI was confirmed visually by observing the blanching of the myocardium distal to the ligation (**Figure 20C**).

The MI animals were grouped into 7 groups:-

Gr. I - Medium injected controls (to study the effect of MI and to eliminate auto-fluorescence) – **(Med-)**

Gr.II - Fibrin injected controls (to study the effect of fibrin on injury as compared to Gr.I) – **(Fib-)**

Gr.III - rADMSC mixed in fibrin and transplanted (to study cell retention due to matrix support) – **(rADMSC-Fib-)**

Gr.IV - rADMSC mixed in medium and transplanted (to study cell retention without support) – **(rADMSC-Med-)**

Gr.V- rCMP mixed in fibrin and transplanted (to study the effect of matrix on cell survival and differentiation) – **(rCMP-Fib-)**

Gr.VI - rCMP mixed in medium and transplanted (to study the effect of cell survival and differentiation without matrix) – **(rCMP-Med-)**

Gr.VII - h-s-e-ADMSC mixed in fibrin and transplanted (to study the effect of matrix and GFs on cell survival and differentiation) – **(Fib-eSec-)**

Following development of ischemic area in the myocardium, **Med-, Fib-, rADMSC-Fib-, rADMSC-Med-, rCMP-Fib-, rCMP-Med-, Fib-eSec-** were injected to the peri-infarct zone (**Figure 20D**). Following transplantation, air from the thoracic cavity was removed by suction and the chest wall was closed (**Figure 20E**). Animals were

maintained in the ventilator (**Figure 20F**) and weaned after the restoration of spontaneous breathing.

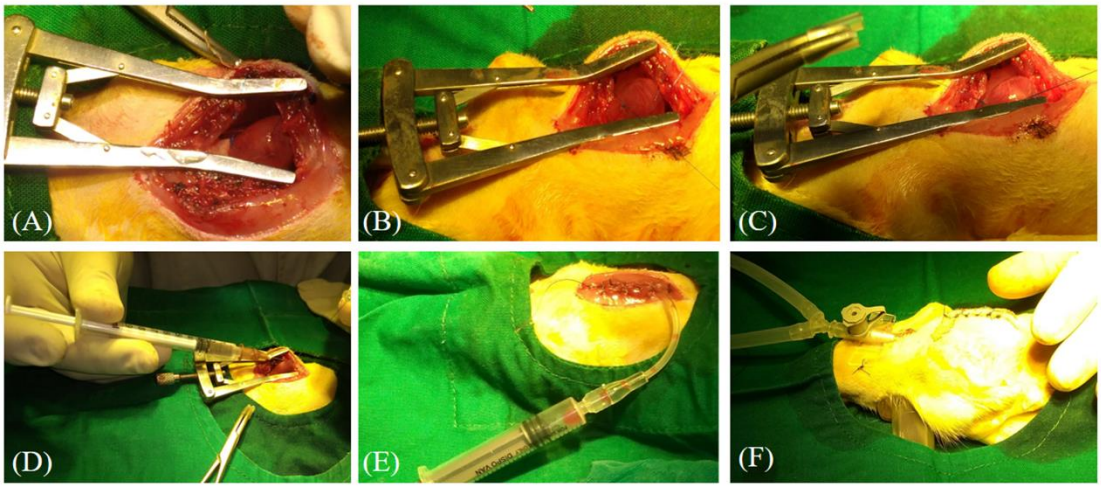


Figure 20. Representative images depicting the MI model and transplantation experiments. (A) Parasternal thoracotomy was done followed by tracheostomy. (B) Surgical creation of MI by ligation of LAD by 6-0 prolene suture. (C) The development of MI was observed by the blanching of the tissue distal to ligation. (D) Transplantation was performed in the peri-infarct zone, (E) air from the thoracic cavity was removed by suction using a syringe and (F) chest wall was closed in layers and animals were ventilated till restoration of spontaneous breathing and allowed to survive for 28 days.

4.6.3 IVIS imaging to track the survival of transplanted cells

The red fluorescence of mixed progenitors harvested from 6-8 d induction cultures and labeled with PKH26- lipophilic dye was confirmed before transplantation. 28 days post-transplantation, the IVIS images of the isolated heart showed distinctly different fluorescence based on the experiment. The induced rCMPs delivered in fibrin showed more red fluorescence in the transplanted site than the heart tissues with rCMPs transplanted in the medium. This indicated that though an equal number of cells was transplanted in all animal groups, the group in which fibrin was used as a cell delivery vehicle retained more cells in the site of injury. There is no autofluorescence from heart tissue as seen in the control IVIS images of the heart (**Figure 21**). Both cell types-

rADMSC (Fig21d) and rCMP (Fig21f) delivered in the medium also retained in the heart tissue. But the intensity of fluorescence was less in both as compared to the rADMSC (Fig21e) and rCMP (Fig21g) delivered in fibrin. So the results confirm both rADMSCs and rCMPs can home better if fibrin is used for cell delivery. Both cells – rADMSCs and rCMPs seemed to have migrated from the site of injury to other areas, irrespective of the cell type upon delivery with the medium.

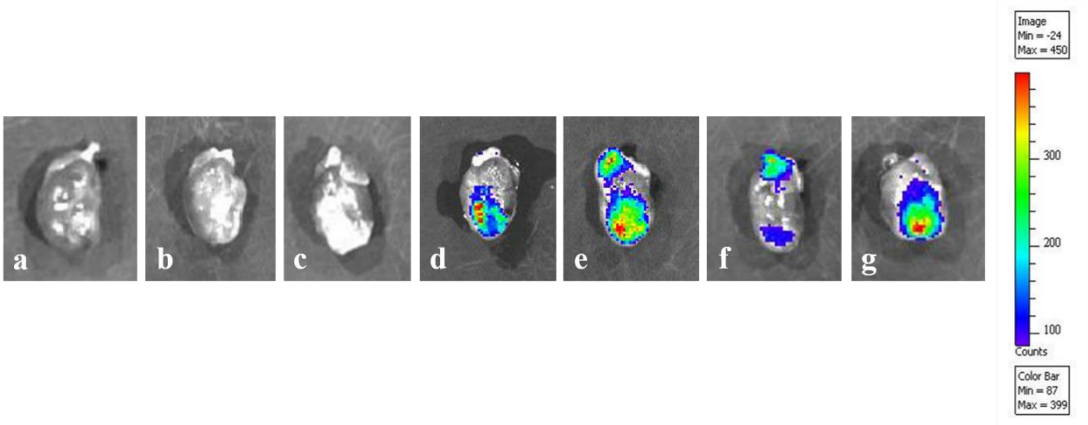


Figure 21. IVIS imaging of whole heart tissue. Absence of red fluorescence in (a) Med-, (b) Fib-, (c) Fib-eSec- indicating absence of auto-fluorescence. Intensity of red fluorescence was higher in (e) rADMSC-Fib- and (g) rCMP-Fib- as compared to (d) rADMSC-Med- and (f) rCMP-Med- 28d post- transplantation.

4.6.4 Identification of transplanted cells in tissue sections

Heart tissue cryo-sections from each group were stained with DAPI to identify the nucleus and thus the presence of cells. The fluorescence microscopy revealed the presence of $PKH26^{+}$ cells co-localized with DAPI in the animal groups transplanted with either cell in medium or cells in fibrin (**Figure 22**) and the results are consistent with the IVIS imaging of whole heart tissue. Analysis of the controls: Med-, Fib- or Fib-eSec- injected tissue sections did not reveal any red fluorescence, confirming that transplanted cells only are responsible for the observation in the cell transplanted section. Upon viewing different fields of sections, more frequent and intense fluorescence was emitted from the tissue section when fibrin was used as a delivery medium for cell transplantation.

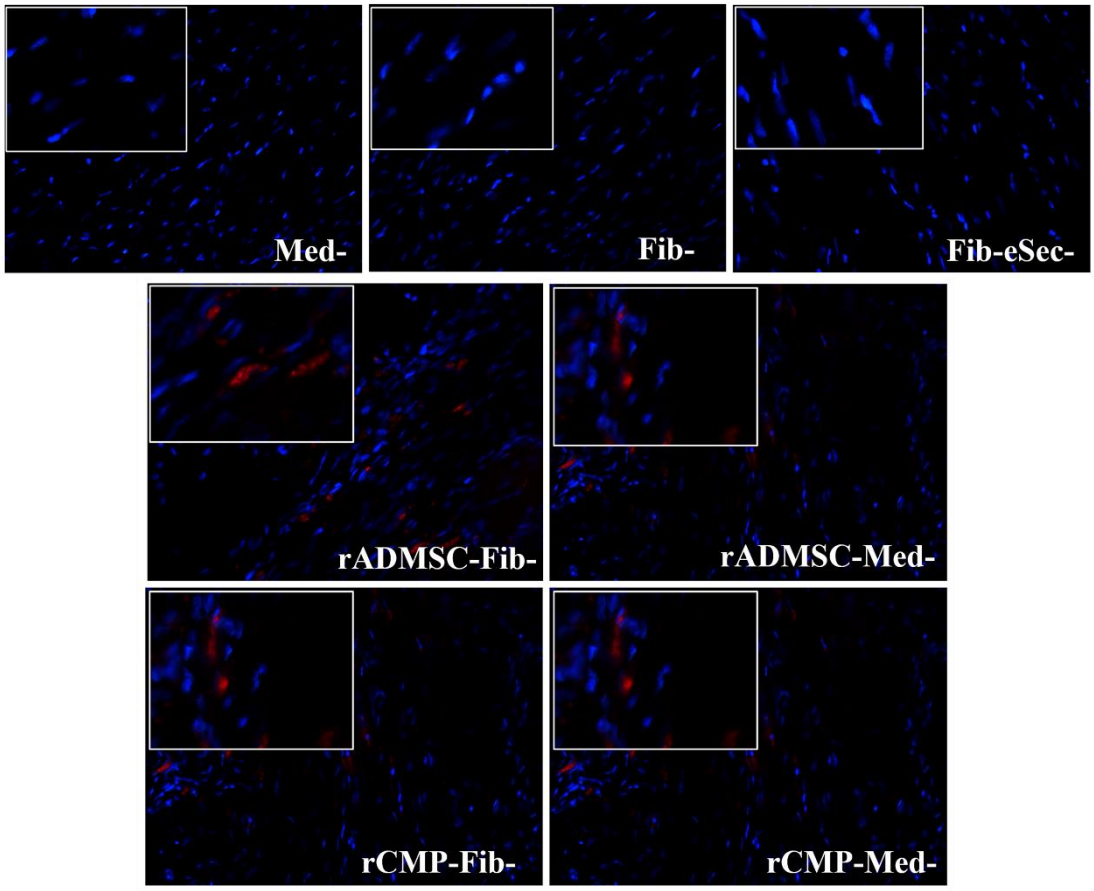


Figure 22. Fluorescent images of tissue cryosections stained with DAPI. Absence of red fluorescence of PKH26 in Med-, Fib-, and Fib-eSec- stained for DAPI. Presence of red fluorescence co-localized with the nuclear stain DAPI indicating the presence of transplanted cells in tissue cryo-sections of animal hearts in the groups – rADMSC-Fib-, rADMSC-Med-, rCMP-Fib- and rCMP-Med-. (*Magnification – 40x*).

4.6.5 Fate of transplanted cells *in vivo*

Tissue cryo-sections of all animal groups were stained for cardiac markers Connexin 43 and Troponin T and distinct differences in tissue integrity was observed between the native heart tissue site and the infarcted heart tissue site. The results indicated that $PKH26^{+}$- Connexin 43 dual-positive cells (**Figure 23**) and $PKH26^{+}$ -Troponin T dual-positive cells (**Figure 24**) were observed in the tissue sections of the MI region with transplanted rCMP-Fib-.The results indicate homing and differentiation of the transplanted rCMPs in the infarcted heart when fibrin was used as the delivery vehicle.

Co-localization of cardiac-specific markers and PKH26 was absent in other groups, with or without cells.

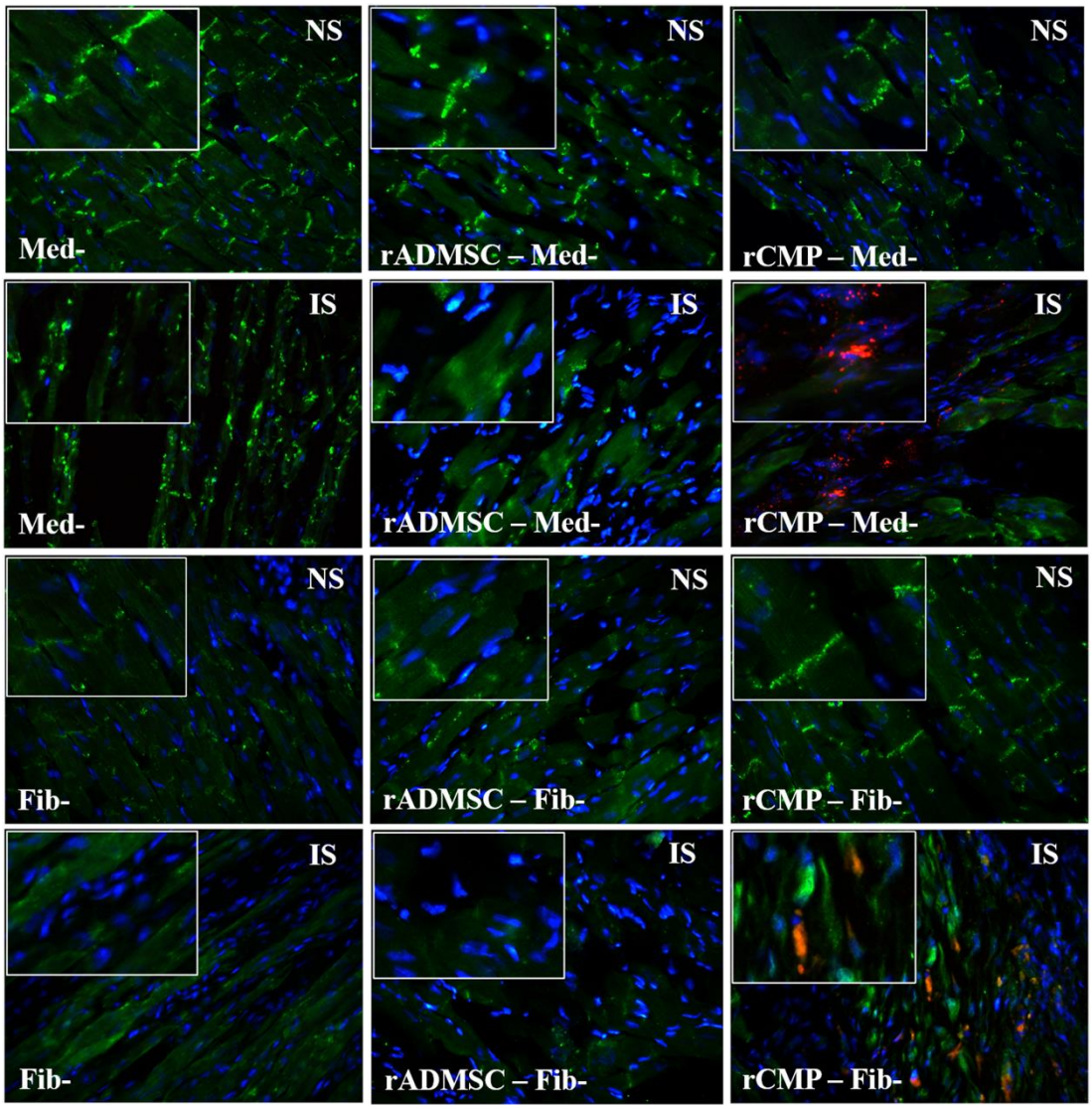


Figure 23. Co-localization of PKH26 and Connexin 43 in tissue sections. Merged images of native tissue site (NS) and injured tissue site (IS) of Groups I to VI stained for Connexin 43. The nuclear stain is indicated by DAPI and Connexin 43 is stained green and PHK26 is stained red. Co-localized area of both red and green showing orange color is magnified and represented as an inset in the group rCMP-Fib-(IS). (*Magnification – 40x*).

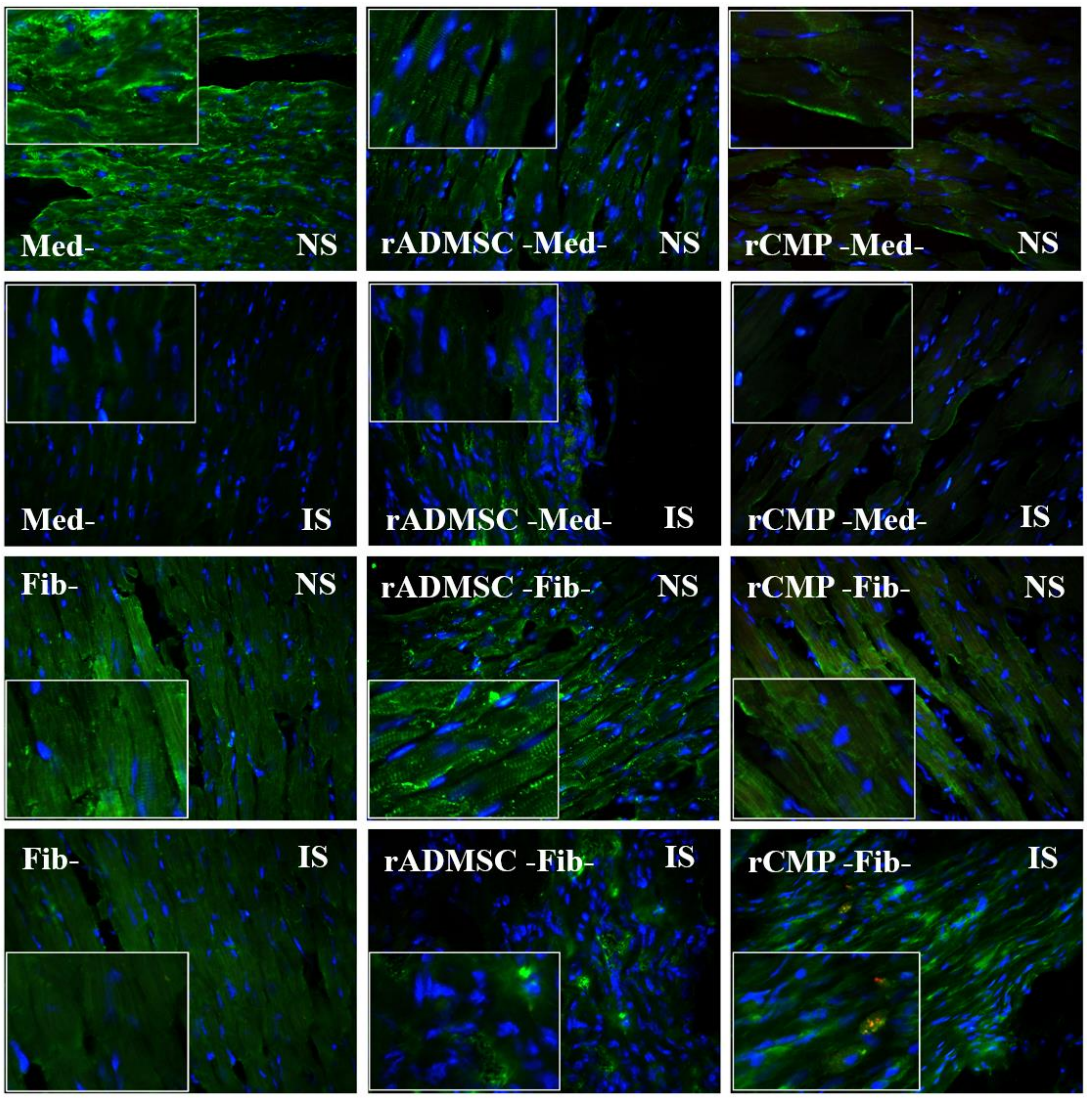


Figure 24. Co-localization of PKH26 and cardiac Troponin T in tissue sections. Merged images of native tissue site (NS) and injured tissue site (IS) of Groups I to VI stained for cardiac Troponin T. Nuclear stain is indicated by DAPI and Troponin T is stained green and PHK26 is stained red. Co-localized area of both red and green showing orange color is magnified and represented as an inset in the group rCMP-Fib- (IS). (*Magnification – 40x*).

4.6.6 Immunostaining for proinflammatory marker

Immunostaining for pro-inflammatory marker CD68 in tissue cryo-sections indicated that the animal groups injected with rCMP-Med- and rCMP-Fib- did not express CD68, 28 days post-transplantation while, tissue sections immunostained from other

treated animal groups; injected with Med-, Fib-, Fib-eSec and rADMSC-Med- expressed CD68 positive cells indicating an inflammatory response in the heart tissues 28 days post-transplantation (**Figure 25**). The results indicate that transplantation of rCMP-Fib- is probably immunomodulatory.

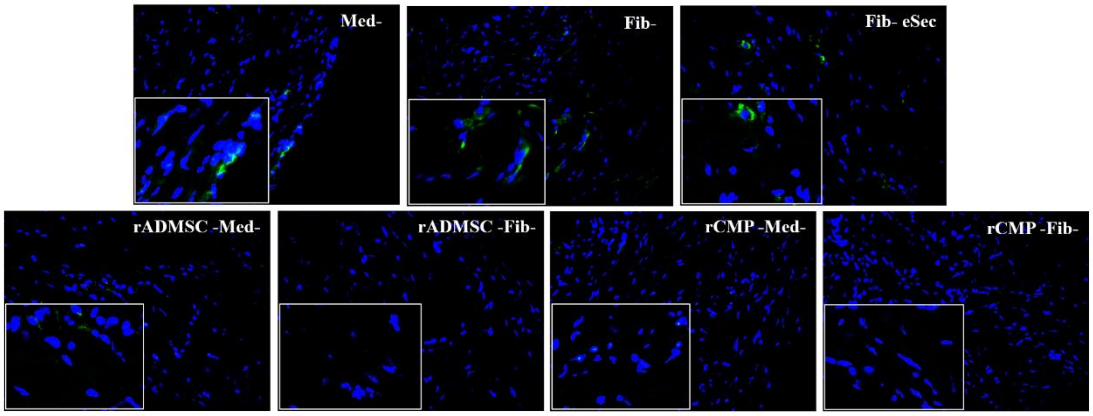


Figure 25. Immunostaining of Inflammatory marker CD68. Fluorescent micrographs of tissue sections of Med-, Fib-, Fib- eSec, and rADMSC-Med- injected groups showed CD68 positive cells represented by green fluorescence. CD68 positive cells were absent in tissue sections of rADMSC-Fib-, rCMP-Med- and rCMP-Fib- injected groups indicated by the absence of green fluorescence. The nucleus is stained with DAPI (*magnification – 40x*).

4.6.7 Histological staining of tissue sections

Tissue sections of infarcted rat hearts stained with hematoxylin and eosin staining confirmed the development of MI as evidenced by the thinning of the LV wall **(Figure 26)**. The formation of blood vessels in the infarcted regions was observed in tissue sections 28 days post-transplantation. Formation of blood vessels was more prominent in the animals transplanted with rADMSC-Fib- and rCMP-Fib-, where the cells were delivered with fibrin as the delivery matrix as compared to the groups rADSMC-Med- and rCMP-Med-, in which medium was used as the delivery vehicle. **(Figure 27A)**. The LV wall thinning and formation of blood vessles was also evident in Fib-eSec- animal groups as indicated in the **Figure 27B**.

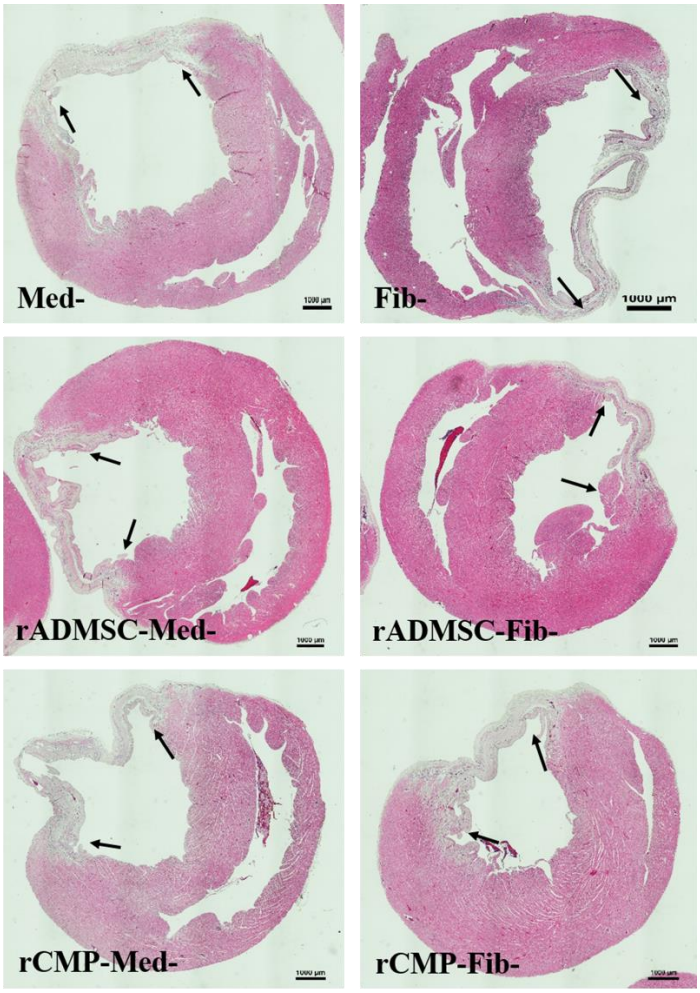


Figure 26. H and E staining of tissue sections. LV wall thinning was evident from H & E staining of tissue sections in animal groups (*magnification – 10x*). The arrows indicate left ventricular wall thinning in each animal groups.

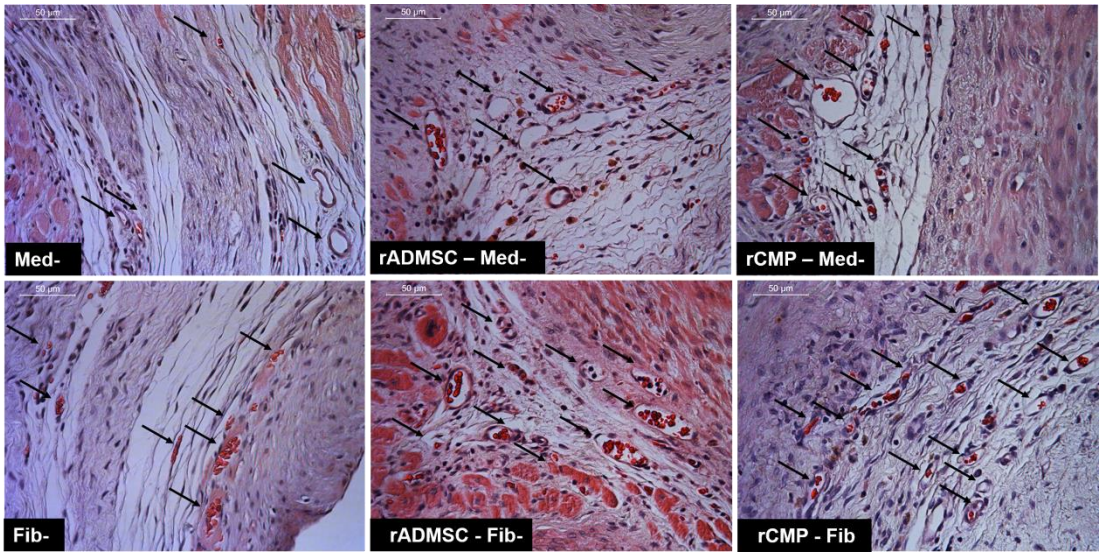


Figure 27A. Blood vessels in tissue sections. H & E staining indicating the formation of blood vessels in tissue sections in all animal groups. The presence of large blood vessels is evident in the groups – rADMSC-Fib and rCMP-Fib. Fewer blood vessels were present in Med-, Fib-, rADMSC- Med, and rCMP-Med- injected groups. (*Magnification – 40x*).

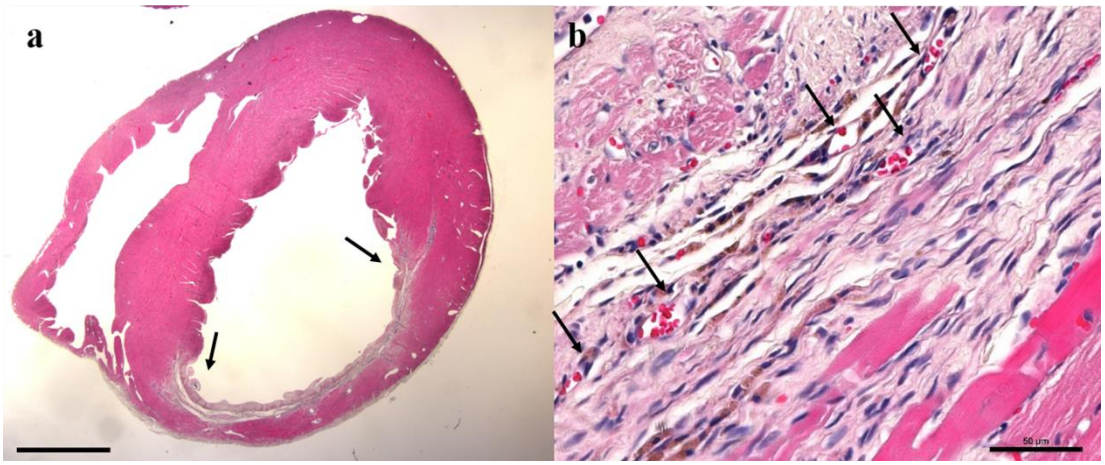


Figure 27B. H and E staining and Blood vessels in Fib-eSec- tissue sections. a) arrows indicate left ventricular wall thinning *(10x)* and b) arrows indicating the formation of blood vessels in tissue sections of Fib-eSec- animal groups *(40x)*.

4.6.8 Evidence for Angiogenic response in MI tissue by 28th day

The immunohistochemistry of tissue sections with CD31 confirmed the observation of H&E stained sections. In Med- injected tissue (Figure 28a) there was no CD31 stained blood vessel seen; whereas, in Fib- delivered tissues (Figure 28d) presence of blood vessels was evident. Peri-infarct zones with CD31 stained blood vessels were seen in rADMSC-Med- (Figure 28b), rADMSCs-Fib- (Figure 28e) and rCMP-Med- (Figure 28c) injected tissues. Distribution of large capillary-like structures was remarkable in rCMP-Fib- (Figure 28f) injected tissues, without discriminating the MI zone.Thus clearly, both fibin and rADMSCs are effective for inducing angiogenesis; but rCMPs and fibrin together makes a major difference in terms of granulating the infarct tissue along with angiogenesis.

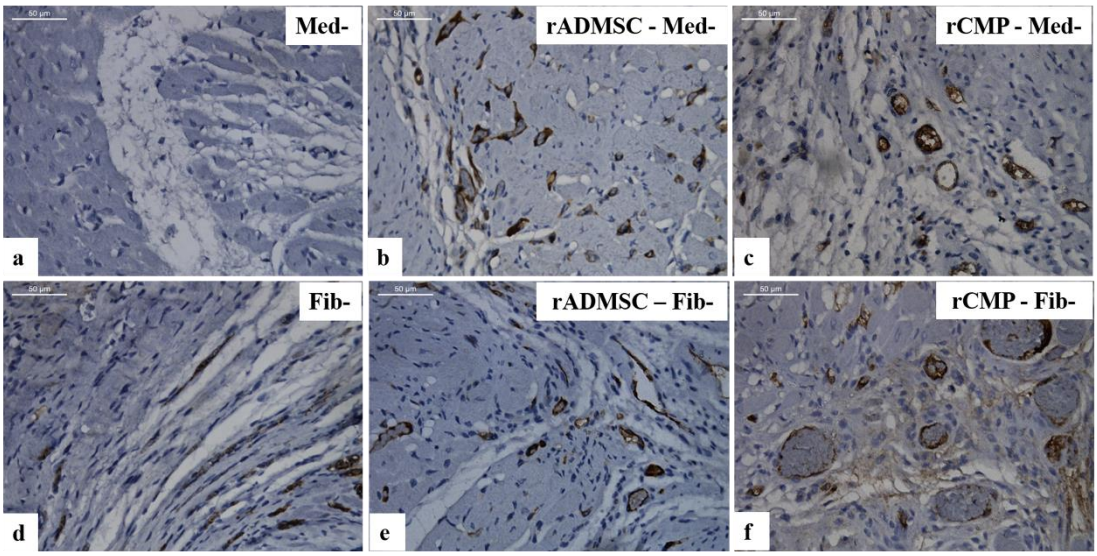


Figure 28. Representative bright-field images of CD 31 immunostaining. Prominent DAB stained large capillary-like structures were visible in rCMP-Fib- and rCMP-Med- groups compared to rADMSC-Med- and rADMSC-Fib- groups in the border areas of infarct zones indicating angiogenesis. CD 31 staining was not prominent in Med- transplanted groups and Fib- alone transplanted groups (magnification – 40x).

4.6.9 The reduced fibrotic response of MI

The reduced fibrotic response of MI Masson's trichrome staining revealed that the fibrotic area was wider in Med- (Figure 29a) tissues and rADMSC-Med- injected tissues (Figure 29b). Upon quantification (Figure 29g) the average area was less in rADMSC-Med- injected tissues as compared to the Med- injected tissues; however, the difference was not significant ($p=0.06$) (Figure 29g). In rCMP-Med- injected tissues also, even though the quantification showed $p=0.05$ (Figure 29g), visually the collagen was distributed similar to that in rADMSC-SFM- tissues (Figure 29b). The quantified reduction from Med- alone transplanted animals indicate that some of the transplanted rADMSCs/rCMPs may have some effects in the injected area; however, since most of the cells migrated away from the injection site (Figure 21d & 21f) the paracrine effect of injected cell was less significant. A significant reduction in the percentage of the fibrotic area was observed in all other tissues, including the fibrin alone injected tissues (Figure 29 d-f), when compared to Med- injected sham control. Overall, it is established that more than rADMSC/rCMP, fibrin is responsible reducing the fibrotic tissue.

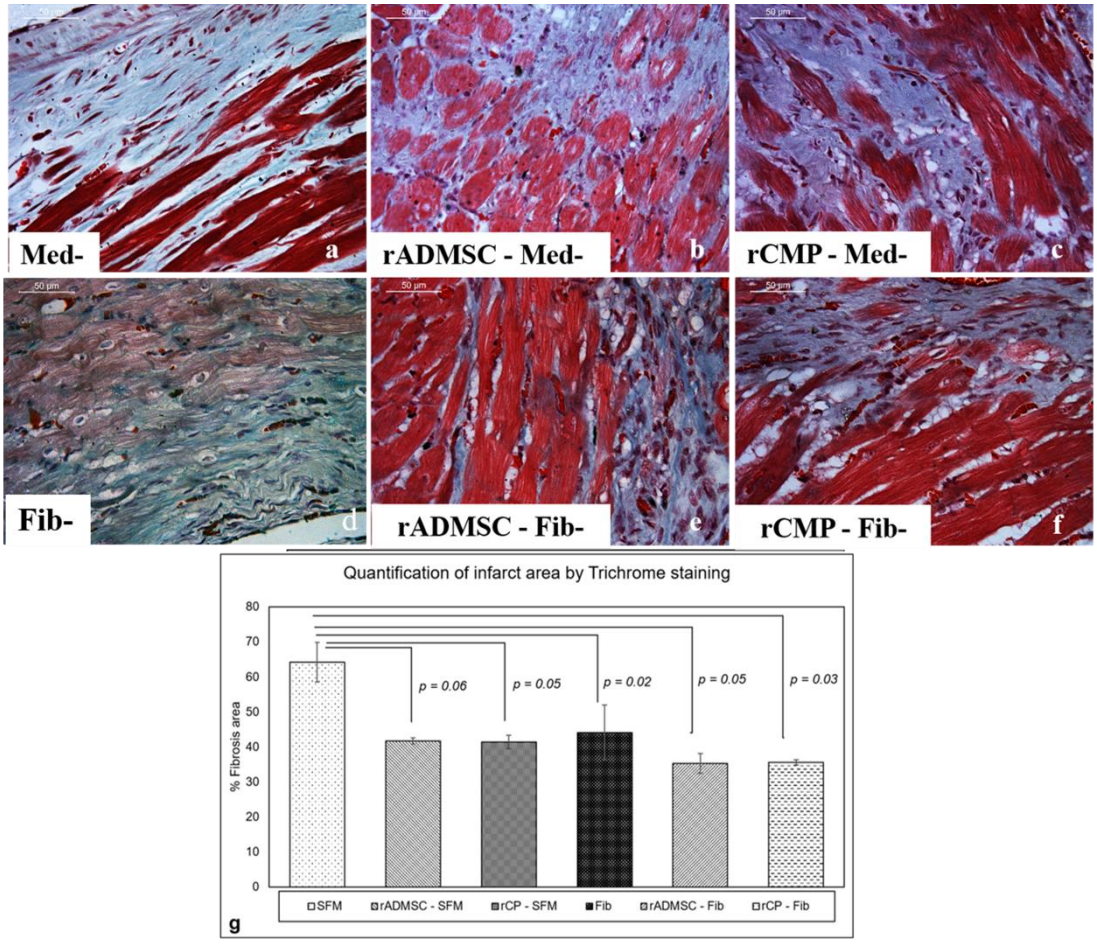


Figure 29. Representative Micrographs of Masson's trichrome staining of border zones of the infarct. (a-f), Masson's Trichrome staining for identifying the fibrotic area in the border zones of LV myocardium among six groups. (Magnification – 40x). (g), Quantification of collagen deposition represented as the percentage of fibrosis area in the border zones of MI was done using Image J software using the high power field of the border zones of all six groups. Error bars represent mean ± Standard error. Statistical analysis using Student's t-test and the respective p values obtained is represented in the graph (n=3).

The results of the animal experiment thus indicate that rCMP transferred from induced cultures have supported cell survival and differentiation into cardiomyocyte and endothelial cells, when fibrin is used as a cell delivery vehicle. Transfer of native rADMSCs did not result in the formation of cardiomyocytes, but it appeared to

promote angiogenesis. The induced rCMP culture may contain un-induced ADMSCs, EPCs other than rCMPs, which can promote angiogenesis.

CHAPTER 5

5. DISCUSSION

The major observations of the study that are illustrated in chapter 4 are discussed in this section, based on the defined objectives and current literature in the relevant field. The limitations of the present study are also mentioned in this chapter.

5.1 Standardization of fibrin-based niche for ADMSC induction to CMPs

The mammalian heart is no longer considered to be a post-mitotic organ and several subpopulations of cardiac stem cells (CSCs) have been identified in the adult mammalian heart. However, an acute MI event results in senescence of the hCSC pool resulting in a 'regenerative cell deficit'. Therefore, the regeneration of the damaged myocardial wall following an acute MI event is a major challenge and remains unaddressed (Torella et al., 2006). Recent studies have focused on the use of multipotent stem cell-based therapies to repair and replenish the injured myocardium with healthy cells. Among the various adult stem cell sources, mesenchymal stem cells have been found to promote cardiac regeneration and angiogenesis (Singh et al., 2016). The possibility of using autologous hADMSCs from CVD patients to derive CMPs and the major non-myocyte population of the mammalian heart *in vitro* is highlighted in this study. An important characteristic of hADMSCs is multilineage differentiation potential. Obtaining hADMSCs isolated from CVD patients with multipotent differentiation potential is essential to proceed with autologous therapeutic approaches. This study demonstrated the trilineage differentiation of ADMSCs from CVD patients into adipogenic, osteogenic, and chondrogenic lineages *in vitro*. The stem-ness of culture-expanded hADMSCs was proven by the expression of surface markers using flow cytometry and the passage 3 cells were found to be homogenous cell population expressing > 90% positivity for the MSC specific 'Cluster of differentiation' (CD) markers – CD105, CD 90 and CD73 and < 5% of these cells expressed hematopoietic stem cell markers. The reparative property of mesenchymal stem cells upon transplantation into the injured tissue site is mainly influenced by the microenvironment or niche within which they reside. Differentiation of MSCs into

undesired tissue types has been reported upon transplantation of unfractionated bone marrow cells into the infarcted heart. Therefore, the pre-differentiation of MSCs either *ex vivo* or *in situ* with various factors/genes may enhance the therapeutic potential of MSCs upon transplantation into the injured heart (Ko and Kim, 2008). An optimized niche derivation for *in vitro* differentiation of hADMSCs into CMPs was the main objective of this study.

Fibrin, used for the constitution of the biomimetic matrix in this study, has been widely used in various clinical and bioengineering applications. It is used clinically as a hemostatic glue and as a soft tissue adhesive. Fibrin acts as a natural scaffold for effective cell attachment, growth, migration, proliferation, and differentiation in wound sites accelerating wound healing and homing of adult stem cells effectively and their differentiation *in vitro* into vascular lineages has been reported previously (Sreerekha et al., 2006). It has been widely used as a natural hydrogel for the development of small-diameter vessels and regeneration of bone, skin, cartilage, nervous tissue, and cardiac tissue (Barsotti et al., 2011; Christman et al., 2004). The potential of fibrin as a provisional ECM to promote *in vitro* differentiation of hADMSCs to CMPs was attempted in this study in combination with growth factors. A time-dependent induction and withdrawal of growth factors was the initial induction strategy in this study to derive CMPs. Similar induction of iPSCs and ESCs to cardiomyocytes was reported in which BMP-4 and VEGF were used for the differentiation of stem cells (Ye et al., 2013).

The present study aimed to improve the differentiation of ADMSCs into cardiomyocyte progenitors using a fibrin-based matrix that was formulated by using fibrinogen and thrombin isolated from human blood plasma. This approach was based on the knowledge that *in vitro* culture survival of cardiomyocyte is poor on bare tissue culture polystyrene. The percentage of hADMSC getting differentiated has been an identified limitation of many such attempts. Therefore, this study hypothesized that Fibrin may support better differentiation and survival. As a biopolymer, it possesses excellent biocompatibility and the potential to incorporate cells/growth factors (Barsotti et al., 2011). It was observed during the niche optimization that GFs alone were not effective to induce hADMSCs to CMPs in a bare tissue culture polystyrene

dish. Whereas, induction of hADMSCs in fibrin – coated niche promoted expression of early cardiac transcription factor GATA-4. Thus, the favorable role of the fibrin-based niche in inducing hADMSCs into cardiac lineage was an important observation. The expression of early cardiac transcription factor GATA-4 and cardiomyocyte-specific markers TNNT2, MYL2, and MYH6 was down-regulated by 24d in culture. Whereas some of the markers were very significantly expressed at early periods such as 8d and 16d. Therefore, it was assumed that once lineage-committed CMPs progress into more mature cardiomyocytes, these cells do not survive *in vitro*. It is well established that cardiomyocytes fail to re-enter the cell cycle to proliferate, once they attain a terminally differentiated phenotype (Foglia and Poss, 2016).

5.2 Promoting co-survival of CMPs with CFPs

In the mammalian heart, CMs co-exist with the major non-myocyte cell populations' viz. cardiac fibroblasts and endothelial cells that constitute more than 50% of the total cell population of the myocardium. Cardiac fibroblasts play a major role in maintaining the electromechanical continuum of the heart and provides a structural backbone for the CMs by maintaining a balance between the synthesis and turnover of cardiac ECM. The cardiac fibroblasts together with ECs play a pivotal role in maintaining a proper 3D architecture of the heart via cell-cell interactions and autocrine and paracrine action of secreted molecules (Souders et al., 2009; Segers et al., 2018). In some initial induction protocols that were attempted, an interesting observation was the presence of fibroblast-like cells along with tube-like cardiomyocyte lineage cells, under the influence of a combination of growth factors, when hADMSCs were grown on a fibrin-based biomimetic matrix. Such heterogeneity in morphology prompted the characterization of cell phenotypes using specific markers. Initial attempts revealed the presence of CMPs and CFPs simultaneously. Therefore, the original plan of getting hADMSC differentiated into fibroblast to co-culture with CMPs was surrendered and attempted to improve the co-differentiation of CMPs and CFPs. The growth factor IGF-1 plays a prominent role in the physiological and pathological remodeling of heart tissue and has been reported to promote cardiac protection following injury (Dai and Kloner, 2011). Similarly, delivery of platelet growth factors has been reported to promote myocardial protection (Hsieh et al., 2006).

Pretreatment of MSCs with a combination of growth factors was found to induce guided cardiopoiesis upon inducing these cells with a cocktail of growth factors consisting of IL-6, FGF-2, thrombin, BMP-4, TGF-β1, retinoic acid, activin A, and IGF-1 (Behfar et al., 2010). Simultaneous derivation of CFs and CMs from MSCs has not been addressed in earlier studies. The results obtained in this study demonstrated that the multipotent nature of hADMSCs may be exploited to derive the major cell populations present in the myocardium simultaneously, using the same culture conditions. Among the three different growth factor induction protocols used for differentiation of hADMSCs into cardiac lineage cells, the IP3 induction strategy using IGF-1, PGF, and L-ascorbic acid was effective in driving co-differentiation. The composition supported the differentiation and co-survival of pADMSCs into CMPs and CFPs lineages *in vitro*. The co-existence of CMPs and CFPs in the same culture is indicated by the expression of cardiac markers TNNT2, GJA1, MYL2, and MYH6 and the cardiac fibroblast markers DDR2 and fibrillin-1 at the transcriptional level. This observation at the genetic level was further confirmed by the cardiac Troponin T and fibrillin-1 expression by immunocytochemistry 16d post- induction *in vitro*. Addition of L-ascorbic acid to the cultures would have promoted the production of collagen I that acts as the primary ECM molecule essential for the differentiation and survival of CMPs *in vitro*. The TGF βI was also observed to get upregulated in the IP3 induction protocol by 16^{th} day, even without the addition of any TGF β superfamily members, indicating that it plays an important role in cardiac differentiation. This observation is in line with previous studies (Gwak et al., 2009; Mohanty et al., 2013; Umbarkar et al., 2019). Another observation was the reduced expression of PDGFRα in IP3 by the 16^{th} day of induction. PDGFRα is a well-characterized marker for cardiac progenitor cells and its expression is reported to be minimal in differentiated cardiomyocytes and endothelial cells (Chong et al., 2013). The results obtained in our study may thus indicate that the IP3 induction protocol resulted in a progressive differentiation of pADMSCs from a progenitor stage towards a more advanced cardiac lineage-committed phenotype. Though various studies have reported the induction of MSCs using 5- azacytidine, only a minor fraction of ADMSCs was differentiated to cardiomyocytes (Martin-Rendon et al., 2008). Induction of MSCs with either Angiotensin II or 5-azacytidine or a combination of both has been reported to derive

cardiomyocyte-like cells upon culturing the MSCs for 3 weeks. However, the stability of derived cardiac progenitors is debated. Exposure to both inducers simultaneously resulted in the death of adherent cells without any favorable additive or synergistic effect on cardiomyocyte differentiation. The estimated yield of cardiomyocytes were; with azacytidine ~ 24.0%, with Angiotensin II ~25.0%, and with a combination of both ~31% CMLs after 21 days of induced MSC induction (Xing et al., 2012). Similarly, a lower frequency of CMLs (20.5 ± 1.9%) was reported to be generated *in vitro* upon treatment of bone marrow-derived MSCs with TGF-β1 (Mohanty et al., 2013). In this study, the IP3 was found to derive >60% cardiac Troponin T positive CMPs in 16 days period *in vitro*. This may be probably due to the co-existence of CFPs in the culture. Also, prominent collagen 1 expression in IP3 indicates that by the time fibrin is degraded, collagen-based ECM could form to support cell attachment, growth, and survival. After the grown progenitors were lysed off, uniform distribution of deposited collagen in the culture dish is demonstrated using immunostaining. By this time the adherence of cell to the culture surface was intense which may be due to the collagen deposited by CFPs. As a result, single cell suspension, suitable for flow cytometry analysis could not be obtained by standard trypsinization; manual counting was done from multiple fields to estimate % CMPs cells in the IP3 induced culture. As compared to other reports, the yield of CMPs in this study is remarkable and the remaining cells could be CFPs, which is responsible for collagen deposition and for stabilizing CMPs in the culture.

5.3 Co-culture of EPCs with CMP – CFP culture to initiate angiogenesis

Revascularization of the damaged myocardium is a key factor that determines the extent of myocardial regeneration. The myocardium has a much higher capillary density of 3000 - 4000/mm^2 and the intricate network of capillaries is lined by the endothelial cells that provide oxygen and nutrients to the working CMs (Segers et al., 2018). In the native myocardium, ECs outnumber CMs by a ratio of 3:1, and studies at the early stages of cardiac tissue development have revealed that the endocardial endothelium plays a crucial role in the myocardial cell maturation and function. Co-culture studies of ECs with neonatal rat CMs in a three- dimensional culture using hydrogel scaffolds have shown that the ECs affect CM reorganization and that the CMs

migrate to or survive near ECs and their survival was improved upon co-culture with ECs (Narmoneva et al., 2004). The results obtained from co-cultures of hADMSC derived EPCs with hADMSC derived CMP-CFPs in this study demonstrate that by manipulating the microenvironment of ADMSCs, these cells can be differentiated to myocytes as well as the major non-myocyte populations present in the mammalian heart. Two different controls used were, IP3 as one control and EPC grown on fibrin as another control. The test was IP3 + exogenously developed EPC. Seeding of EPCs was found to initiate the expression of angiogenic markers CD31, VCAM-1, MCP-1, and eNOS and it also maintained stable expression of cardiac-specific markers TNNT2 and MYL2 till 16d *in vitro*. The addition of EPCs was also found to regulate the CFP differentiation which was reflected in the gene expression pattern of DDR2 at 8d and 16d of co-culture *in vitro* as compared to the IP3 culture. An interesting observation is the development of EPC in IP3, even without co-culture with EPC. Here IP3 was analyzed as control for IP3+EPC culture. However, the control IP3 showed up-regulation of EC markers, more significantly by 16d. Added effect of exogenous EPC was more interesting because TNNT2 expression at gene level and protein level was significantly increased by the presence of exogenous EPCs. The results obtained from this co-culture experiments indicate that hADMSC derived EPCs with hADMSC derived CMPs and CFPs opens new prospects for regenerating the damaged myocardium with autologous cells obtained from the CVD patients.

5.4 Effect of hADMSC secretome in IP3 culture

Recent regenerative strategies have focused on cell-free therapies to overcome the issues of poor cell engraftment and retention in the injured site and issues related to immune reactions. MSCs are known to secrete an array of bioactive molecules, collectively named the ‘secretome’, to the extracellular space. The secretome of MSCs includes a variety of molecules like growth factors, cytokines, free nucleic acids, lipids, and extracellular vesicles (Vizoso et al., 2017). A multitude of growth factors having angiogenic potential like VEGF, placental growth factor (PLGF), hepatocyte growth factor (HGF) and cytokines like interleukin - 1 (IL1) and -6 (IL6) and stem – cell-derived factor (SDF-1) is reported to be present in the MSC secretome (Ward et al., 2018). Among the angiogenic factors, vascular endothelial growth factor (VEGF)

and its receptors play a crucial role in the regulation of growth, development, and differentiation of the vascular bed of the heart and the blood vessels. The transplantation of infarcted rat hearts with VEGF – expressing MSC conditioned medium was found to mobilize homing of bone marrow stem cells and CSCs to the infarct site mediated by SDF-1/CXCR4 pathway (Tang et al., 2011). Moreover, VEGF expressing MSC transplantation to animal models of MI resulted in increased vascular density in the infarct area, contributed by a transient high level of VEGF expression, mainly mediated by the transplantation of VEGF expressing MSCs in the infarct site thereby reducing the infarct size and improved cardiac function (Matsumoto et al., 2005). The addition of VEGF over-expressed bioengineered hADMSC secretome and the non-engineered hADMSC secretome in combination with IP3 induction was found to initiate the expression of angiogenic markers in the CMP- CFP population *in vitro*. This angiogenic response may be attributed to either the EPC developed upon induction or the undifferentiated hADMSC pool present in the CMP – CFP population upon induction with IP3. The addition of either eSec secretome or nSec secretome to the IP3 strategy was also observed to be beneficial in maintaining the CMP population which was indicated by the presence of more frequent cardiac Troponin T positive cells in the culture 16d after induction. The results obtained in this study indicated the advantages of using combinatorial therapy using MSC-derived cardiac precursors and the secretome of MSCs in inducing angiogenesis thereby promoting a cardioprotective effect upon transplantation into the infarcted myocardium.

5.5 Homing of cells transplanted to infarcted myocardium

The commonly available treatment options to restore damaged cardiac tissue include the use of pharmacological agents, interventional therapies using medical devices, and heart transplantation. These treatment modalities alleviate the pain and reduce the workload of the remaining viable myocardial tissue following an MI. Cell transplantation therapies are emerging as a promising strategy to regenerate/repair the damaged myocardium with functional tissue and several cell types have been tried by researchers to regenerate the myocardium. An ideal regenerative cell source may be critical for effective therapy (Wang et al., 2010). The poor retention of transplanted cells in the infarcted heart is a major concern that limits the improvement in cardiac

function following cell transplantation. Several different natural and synthetic polymers have been widely used for guiding the organization, growth, and differentiation of transplanted cells in the injured site. Bioactive materials with appropriate physical strength and degradation kinetics that promote cell-matrix interactions may be an ideal cell delivery source for the treatment of MI (Leor et al., 2005). The primary focus of this study was to develop an injectable cell delivery matrix that may promote retention and homing of transplanted cells in the injured tissue site. Identification of a cell source and their *in vitro* differentiation into cardiomyocyte progenitors suitable for transplantation is also an important step in developing a cell-based therapy for regenerating the injured myocardium.

The major steps in the experimental design involved the isolation of ADMSCs from rat and their culture expansion *in vitro*. The rADMSCs after culture expansion were induced to differentiate into cardiomyocyte progenitors. Rat MI model was developed by ligation of LAD using a 6-0 prolene suture and the successful development of MI was observed by the blanching of the myocardial tissue distal to the ligated site. Cells were labeled *in vitro* before transplantation and the imaging of the whole heart tissue 28 days post-MI development revealed better cell retention in the animal groups that were administered with either undifferentiated cells or induced cells in injectable fibrin matrix as the delivery vehicle.

Fibrin gels are reported to exhibit excellent biocompatibility, promote cell attachment, and have a controlled biodegradable nature. It also acts as a protective cell carrier safeguarding the cells from the forces during application and cell delivery processes. It is also reported to enhance cell viability and tissue regeneration (Li et al., 2015). Improvement in wall thickness was observed in the infarcted rat hearts upon delivery of a mixture of fibrin with myoblasts five weeks post-implantation (Christman et al., 2004). Studies on animal models of MI has revealed that autologous ADMSCs upon transplantation into infarcted hearts with platelet-rich fibrin significantly promoted myocardial regeneration by preserving left ventricular (LV) function and limiting LV remodeling after acute MI (Chen et al., 2015). Ryu and colleagues have reported that the injection of bone marrow mononuclear cells and fibrin gel into the infarcted myocardium enhanced neovascularization by improving the microvessel density and

delivery of cells with fibrin gel exhibited more viable cells and less fibrous tissue compared to the cell-alone injection groups (Ryu et al., 2005).

A major observation in this study is that the cell transplantation to animal models of MI through the intramyocardial route of delivery was successful in terms of homing and retention of the transplanted cells in the injured site which was evident from the cryosections of heart tissues explanted 28 days post-procedure. Evaluation of tissue sections for the expression of cardiac-specific markers revealed that the transplanted rCMPs injected intramyocardially with fibrin as a delivery vehicle expressed cardiac-specific markers Connexin 43 and Troponin T 28 days post-transplantation, which was confirmed by its co-localization with the cell tracker dye PKH26. This co-localization of cardiac-specific markers with PKH26 was absent in the animal groups in which the cell culture medium was used as the delivery matrix. The results obtained from this proof-of-concept study indicated that injectable fibrin may provide a suitable niche for the maintenance of lineage-committed cardiac progenitor cells to survive within the hostile microenvironment of the infarcted myocardium and that it promoted differentiation of these transplanted cells in the injured site. Evaluation of inflammatory marker in tissue sections revealed that rADMSCs in combination with fibrin did not elicit the expression of CD68, a pro-inflammatory macrophage marker, 28 days post-transplantation indicating that the progression of MI to the viable myocardium was minimal. This pattern was also observed in the rCMPs transplanted either with medium or with fibrin as the delivery vehicle indicating that the ADMSCs even upon lineage commitment did not elicit an immune response in the host tissue post-transplantation. Further evaluation is needed to analyze the ratio between the pro-inflammatory and anti-inflammatory macrophage markers to get a better picture of the extent of progression of MI to the viable myocardium and the LV remodeling. Improved angiogenesis was observed when rCMPs delivered with fibrin and a reduction in the extend of fibrosis was also observed in all the animal groups as compared to that of the Medium alone injected animal group.

5.6 Limitations of the study

This study mainly explored the feasibility of differentiating autologous ADMSCs derived from CVD patients into CMPs, CFPs, and EPCs, and hence, the transplantation efficiency of this study from a clinical perspective is limited. Though the induction protocol optimized for differentiation of ADMSCs to CMPs resulted in the expression of cardiac markers at the transcriptional level and translational level, the functionality of differentiated cells in terms of its electrical activity was not evaluated. The effect of EPC transplantation on angiogenic potential was not evaluated in animal models. Though the immunostaining analysis of tissue cryosections revealed the presence of transplanted cells expressing cardiac-specific markers co-localized with the tracker dye, indicating its homing and differentiation in the injured myocardium, the functional evaluation of the hemodynamic parameters could not be carried out (Echo studies, pre and post-surgery). Hence the long-term outcome of cell transplantation in terms of improvement in cardiac function was not derived from this study.

CHAPTER 6

6. SUMMARY AND CONCLUSION

The goal of this study was to develop cell-based therapy for regeneration of myocardial infarct using autologous stem cells. The stem cells targeted was adipose-derived mesenchymal cells from CVD patients. The ADMSCs have several advantages for considering it as a stem cell source. Autologous cells can reduce the chances of immune rejection. Other major advantages are ease of tissue collection, the available procedure for cell isolation, proliferation potential, and multipotency. The potency of stem cells to proliferate and differentiate is dependent on the age and health status of the donor. When CVD patients are considered, they may have different disease conditions like diabetes, hyperlipidemia, hypertension, etc. Therefore, isolation of cells can be difficult, and once isolated, cells may not proliferate well, may drift characteristics, and may not possess differentiation potential. In this study, ADMSC isolation from randomly selected patients was achieved successfully, cells proliferated well and tri-lineage differentiation was achieved easily. Thus the feasibility of collecting autologous ADMSC for the regeneration of MI is established.

The multipotency of hADMSC is a double-edged property. It is an advantage that the cells can differentiate into different lineages, at the same time it is a disadvantage that it might differentiate into undesired lineage upon transplantation. Therefore, pre-differentiation to required lineage is considered to be a prerequisite for successful transplantation for the homing, differentiation, and regeneration of the injured myocardial tissue. Because fibrin is a natural scaffold that promotes wound regeneration in many contexts, this study focused on the use of fibrin coated surface as a matrix for composing a niche to promote lineage commitment of ADMSC to cardiac tissue-specific cells. The study proved the efficacy of a fibrin-based matrix for promoting differentiation into CMPs, CFPs, and EPCs. This a notable achievement of the study to have standardized a protocol to develop 3 different cell types simultaneously using the same ADMSC in a single induction protocol.

Another aspect of the study was to promote angiogenic activity in the culture by seeding exogenously produced hADMSC-derived EPC to the cardiac cells generated by the induction protocol. Growing EPC with cells developed by the induction protocol has produced increased angiogenic response with up-regulated endothelial markers. Other than causing the angiogenic response, the progression of CMP differentiation was also demonstrated using TNNT2 expression at the transcriptional level. Angiogenic response was also up-regulated by adding secretome from normal hADMSCs collected in culture medium and the hADMSCs engineered to secrete more VEGF. The addition of eSec or nSec to the induction protocol also favored the CMP maintenance *in vitro* as indicated by the TNNT2 immunostaining. The increased VEGF concentration in the medium has modulated the presence of cardiac fibroblasts.

Another aspect of the study was to establish the use of injectable fibrin for transplantation of the pre-differentiated CMP-CFP-EPC population for *in vivo* cardiac regeneration. Not only the transferred cells were retained in fibrin upon transplantation, but also, the differentiation of transplanted cells to cardiomyocyte-like cells was demonstrated.

The defined objective of this study was i) isolation and culture expansion of ADMSCs derived from CVD patients and proving their ability of multipotency *in vitro* upon culture expansion, ii) assessing the role of fibrin in promoting the differentiation of hADMSCs to CMPs *in vitro*, iii) optimization of a suitable biomimetic niche composed of growth factors and extracellular matrix components that aided the simultaneous differentiation of hADMSCs into CMPs and CFPs, iv) assessing the effect of co-culture of hADMSC derived EPCs with CMP/CFP population in initiating angiogenic response, v) studying the influence of bioengineered/ non-engineered hADMSC secretome in combination with the optimized induction protocol in promoting an angiogenic effect and maintenance of CMP phenotype *in vitro*. The pilot studies on animal models of MI indicated that cell transplantation was successfully carried out through the intra-myocardial delivery route. The rat ADMSCs were also induced to CMPs using the induction protocol optimized for hADMSC differentiation to CMPs. The results obtained from cryosections of injured tissue indicated successful homing and differentiation of the transplanted CMPs in the infarcted myocardium

upon transplantation of the CMPs with injectable fibrin matrix as a delivery vehicle. The fibrin matrix-assisted as a protective anti-inflammatory cell carrier for the transplanted cells within the hostile microenvironment in the ischemic heart tissue.

6.1 Summary

Human ADMSCs from CVD patients were successfully isolated and culture expanded *in vitro* and their potential to differentiate into cardiac-specific lineage cells was proven. The multipotency of hADMSCs was exploited in this study to derive the major cell populations present in the mammalian myocardial tissue. An appropriate biomimetic niche composed of fibrin and growth factors was designed upon the comparison of hADMSC cultures in bare tissue culture polystyrene dishes and fibrin coated dishes. The expression of early cardiac transcription factor GATA-4 in cells induced on a fibrin matrix confirmed that fibrin may act as a suitable niche in promoting hADMSC differentiation to cardiac lineage cells. Optimum growth factor combinations were derived that promoted the co-differentiation of hADMSCs to CMPs CFPs and EPCs *in vitro* to promote the survival of CMPs *in vitro*. The optimized protocol was found to support the CMP phenotype for 16 days in culture conditions indicated by the expression of CMP specific, CFP specific, and EPC-specific marker expressions at the transcriptional level and translational level. The addition of EPCs derived from the hADMSCs to CMP/CFP cultures was observed to increase angiogenic response in cultures that might promise potential improvement in cardiac function from a clinical perspective. Also, another aspect of inducing angiogenic response was carried out in this study by exploring the effect of the addition of bioengineered/non-engineered hADMSC secretome that is considered as a reservoir of various growth factors and cytokines promoting angiogenesis, along with the optimized induction protocol. The results obtained in the study opens the scope of using both cell-based therapy in combination with the cell-free secretome to promote better outcomes in terms of cardiac regeneration.

The niche conditions that induced hADMSC differentiation to CMPs were used to derive CMPs from rADMSCs. The MI was successfully developed in rat models by ligation of the LAD coronary artery and the transplantation of cells via the intra-

myocardial delivery route was performed. 28 days post-MI, the animals were euthanized and the whole heart tissues were checked for the presence of transplanted cells in the injury site. Fibrin, when used as an injectable cell delivery matrix was found to favor the retention of transplanted cells in the injury site and the immunostaining of tissue sections indicated differentiation of transplanted cells in the injury site.

6.2 Conclusion

1. The feasibility of sourcing autologous hADMSCs for cell-based therapy for cardiac regeneration is proven.
2. The potential of the fibrin-based niche for pre-differentiation of hADMSC to cardiac progenitor cells-CMP, CFP, and EPC, using the same protocol in the same culture well simultaneously has been demonstrated.
3. An added effect of exogenous, hADMSC-derived EPC for promoting angiogenic activity in induced mixed population cell culture has been demonstrated
4. An added effect of exogenous, hADMSC derived EPC for progression of CMP differentiation to cardiomyocyte-like cells with specific marker expression and cell morphology, in induced mixed population cell has been demonstrated.
5. An added effect of exogenous, hADMSC-derived EPC for regulating cardiac fibroblast differentiation in the induced mixed cell population is also demonstrated.
6. The effect of exogenous hADMSC-secretome on promoting angiogenic activity in the heterogeneous population of CMPs, CFPs, and EPCs in induced culture has been demonstrated.
7. The effect of exogenous hADMSC-secretome, over-expressing VEGF, on promoting angiogenic activity in the induced mixed cell population is also demonstrated.
8. The advantage of injectable fibrin as a delivery vehicle for the transfer of rCMPs to MI in the rat model for improving retention and differentiation has been demonstrated.

9. The advantage of engineered ADMSC secretome upon mixing with injectable fibrin as a delivery vehicle for improving retention and differentiation has been demonstrated.

On the whole, preparation of a mixed population of cardiac progenitor cells suitable for effective transplantation using injectable fibrin as a delivery vehicle has been achieved in this study for potential application in the regeneration of ischemic cardiac injury.

6.3 Future perspectives

1. The future studies may focus on assessing the dose-response of cells in the *in vivo* animal study; by transferring higher numbers of cells from culture
2. The effect of cell transplantation after a longer duration such as 3 months or 6 months could be studied for establishing the outcome upon cardiac progenitor transplantation
3. The effect of multiple-dose of cell transplantation through the intracoronary route could be studied.
4. Finally, the outcome in terms of electrical conductivity and cardiac output could be studied to establish regeneration and native tissue integration.

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LIST OF PUBLICATIONS

- **Subha S**, Sachin J Shenoy, Arya Anil, Sabareeswaran A, Deepthi AN, Lissy K Krishnan. Fibrin Hydrogel Aided Cardiac Progenitor Cell Delivery Enhances Regenerative Tendency in Myocardial Infarct Model. *Archives of Clinical and Biomedical Research 4 (2020): 513-541. (IF – 3.1)*

- **Subha S**, Renu Ramesh, K. Jayakumar, Lissy K Krishnan. *In Vitro* Differentiation of Adipose-Derived Mesenchymal Stem cells into Cardiac Tissue-Linked Progenitor Cell Cluster Using Fibrin Matrix Based Niche. *Cardiology and Cardiovascular Medicine 4 (2020): 646-678. (IF – 3.5)*

Presentations and Conference Proceedings

1. **Subha S** & Lissy K Krishnan. Biomimetic niche design for growth factor mediated differentiation of human adipose derived mesenchymal stem cells to Cardiac lineage, 2013. 11th Annual meeting of ISSCR International Conference, Boston, MA, USA (Poster)
2. **Subha S**, K Jayaykumar & Lissy K Krishnan. Derivation of Cardiomyocyte Progenitor cell from adipose derived mesenchymal stem cells, Science Fete 2015. Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India (Oral- Best Paper Award)
3. **Subha S**, Renu Ramesh, K Jayakumar & Lissy K Krishnan. *In vitro* differentiation of adipose derived mesenchymal stem cells into cardiac lineage and to fibroblastic lineage and effect of co-culture in expression of cardiac-specific markers, 2017. National Seminar of Frontiers in Biotechnology, Inter University centre for Genomics and Gene Technology, University of Kerala, Thiruvananthapuram, India (Oral)

APPENDIX

PBS (1000ml) pH 7.4

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g

Added distilled water to 1000ml, filtered and stored at room temperature. PBST was prepared by adding 0.1% Tween-20 to PBS

HBSS (1000ml) pH 7.4

KCl	0.4g
KH_2PO_4	0.06g
NaCl	8g
Na_2PO_4	0.0482g

Added distilled water to 1000ml, filtered, autoclaved and stored at 4℃

SFM (50ml)

DMEM-LG/DMEM:F12	50ml
Antibiotics (100X)	500µl

Filtered and stored at 4℃

DNA Gel Loading dye

Glycerol	6ml
0.5M EDTA	1.2ml
Sterile water	2.8ml
Bromophenol Blue	2mg
Xylene Cyanol	2mg

Mixed all components. Store at room temperature.

URKUND

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Sources included in the report

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High School:	Govt. Higher Secondary School for Girls, Ernakulam, affiliated to Kerala State Board, Passed SSLC with 91.6% in 2004 (2003-2004).

ADDITIONAL QUALIFICATION:

- Has Cleared JRF UGC NET, conducted by CSIR in December 2011 in Life Sciences.

PUBLICATIONS:

- Subha S, Sachin J Shenoy, Arya Anil, Sabareeswaran A, Deepthi AN, Lissy K Krishnan. Fibrin Hydrogel Aided Cardiac Progenitor Cell Delivery Enhances Regenerative Tendency in Myocardial Infarct Model. Archives of Clinical and Biomedical Research 4 (2020): 513-541. (IF – 3.1)
- Subha S, Renu Ramesh, K. Jayakumar, Lissy K Krishnan. In Vitro Differentiation of Adipose-Derived Mesenchymal Stem cells into Cardiac Tissue-Linked Progenitor Cell Cluster Using Fibrin Matrix Based Niche. Cardiology and Cardiovascular Medicine 4 (2020): 646-678. (IF – 3.5)

CONFERENCES AND WORKSHOPS PARTICIPATED:

- Have participated in ISSCR 11th annual meeting held at Boston, MA, USA from 12th-15th June 2013 and delivered a poster presentation entitled "Biomimetic niche design for growth factor mediated

differentiation of human adipose derived mesenchymal stem cells to cardiac lineage."

- Have participated in a National seminar on "Frontiers in Biotechnology" in 2017, organized by Inter-University Centre for Genomics and Gene Technology, University of Kerala, Thiruvananthapuram, India and an Oral presentation was delivered on the paper entitled "*In vitro* differentiation of adipose derived mesenchymal stem cells into cardiac lineage and to fibroblastic lineage and effect of co-culture in expression of cardiac specific markers".

- Have participated in the Science Fete 2015, organized by Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India and an oral presentation was delivered on the paper entitled "Derivation of Cardiomyocyte Progenitor cell from adipose derived mesenchymal stem cells".

- Have participated in World Brain Awareness Week organized by Centre for Neuroscience, Department of Biotechnology, Cochin University of Science and Technology, Cochin on March 19, 2010.

- Have participated in International Conference on Neuroscience Updates [ICN] and annual Meeting of Society for Neurochemistry (India) held at CUSAT during December 12-14, 2009, organized by Centre for Neuroscience, Department of Biotechnology, Cochin University of Science and Technology , Cochin, Kerala.

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Rebuttal to thesis comments – Examiner 1

1. **The candidate has not commented about the cell contractibility efficiency using these protocols.**

   Response: Thank you for the valuable comment. In our study, we aimed to induce differentiation of human adipose derived mesenchymal stem cells (hADMSCs) into cardiomyocyte progenitors (CMPs) and cardiac fibroblast progenitors (CFPs) on a fibrin based biomimetic niche. So our culture was primarily a 2D culture in which the tissue culture polystyrene dish coated with fibrin was gradually replaced by the extracellular matrix deposited by the lineage-committed/induced cells. Hence, measuring the contractility/traction forces imparted by individual cells may change significantly over the course of measurement as mentioned in the following reference. Henceforth, we did not include contractility studies in this work and is mentioned in the Discussion part of the modified thesis.
   Reference: Polacheck, W.J., Chen, C.S., 2016. Measuring cell-generated forces: a guide to the available tools. Nature Methods 13, 415–423. https://doi.org/10.1038/nmeth.3834

2. **The study lacks functional assessment studies like calcium ion imaging which is a must for any cardiac differentiation protocol efficiency.**

   Response: Thank you for the valuable comment. We understand that since we didn't perform calcium ion imaging, our differentiation protocol does not justify the functionality of the induced cells in culture and this has been mentioned in the limitations of the study in the thesis (Section 5.6, page number 118). However, we have tried to demonstrate the presence of lineage-specific markers like cardiac Troponin T and connexin 43 for CMPs and collagen Type I and fibrillin-1 for CFPs, in the induced cultures that was absent in the respective control cultures.

3. **The bright field images are not clear.**

   Response: Thank you for the suggestion. The issue is resolved.

4. **With respect to animal studies, the candidate has not mentioned detailed protocol followed. The study has several lacunae like No control mice relevant data provided, few important parameters required for evaluation of the MI model pre an post-surgery like blood parameters, Echo, etc are missing.**

Response: Thank you for the suggestion. We have mentioned the details of animal experiments conducted in our study in Chapter 3, Materials and Methods under the section subheading, *In vivo evaluation* (section 3.7, page no. 60-64). In our animal studies, intramyocardial injection of cells was performed in MI induced rats and hence, the group I and group II rats, which included the medium injected control rats, and the fibrin injected control rats respectively, served as the control groups (kindly refer to Table 5, page number 62 for the allocation of animals for *in vivo* studies). This was to evaluate if the intramyocardial injections of the respective cell delivery vehicles caused any adverse action on the infarcted rat hearts.

We understand that few important parameters like Echo studies, pre and post-surgery are missing in our study. Unfortunately, we did not have the probe for measuring the Echo parameters of rodents with us and hence we were unable to include that valuable information in our studies. This has been mentioned in the section 5.6, Limitations of the study, page number 118 of the thesis.

5. **The histopathological studies are no clear. None of the features are highlighted. \ with double arrow head in the images thus it is difficult to analyse them.**

Response: Thank you for the valuable suggestion. We have tried to include better images of H & E staining of infarcted rat hearts. Kindly refer to the modified figures 26 and 27 on page number 103. Also we have highlighted the blood vessel formation in the tissue sections with arrows for the ease of analysis.

6. **The candidate has shown IVIS images for cell tracking in heart at 28 days. The candidate could have shown the same after 24hrs and a follow-up studies.**

Response: Thank you for the valuable suggestion. The primary aim of our animal study was to evaluate the retention and differentiation of transplanted cells in MI induced rat hearts upon intramyocardial delivery of cells using either Serum-free medium or fibrin as the cell delivery vehicle. The IVIS images of whole heart tissue was analyzed after euthanasia of animal at 28 days post-MI and cell transplantation, to track the retention of transplanted cells. Hence, tracking the retention of cells 24hrs post cell-transplantation was not included in this study.

Rebuttal to thesis comments – Examiner 2

1. **Phase contrast image (fig 7A, 7B, 7C, 7D, 12, 15B, 19) are not clear and morphological difference is not visible, may be due to printing issue.**

   Response: Thank you for the suggestion and apologies for the lack of clarity. The issue has been resolved.

2. **Confocal image (Fig: 5A): the cell expressing troponin –I has differently stained for DAPI than other cells in the field.**

   Response: Thank you for the comment. It has been reported by Cummings et al., that the color of DAPI changes according to the health status of the cell. We assume that the color of DAPI in Troponin-I stained cell was different from the others due to the nuclear condensation.

   Reference: Cummings, B.S., Wills, L.P., Schnellmann, R.G., 2004. Measurement of Cell Death in Mammalian Cells. Curr Protoc Pharmacol 0 12. https://doi.org/10.1002/0471141755.ph1208s25

3. **Graphs (fig: 8A, 8B, 13, 17) there is lot of standard deviation and there has been no numerical representation of the graph.**

   Response: Thank you for the comment. The data represented in the graphs are from 3 different donor cells. A 100% differentiation is not achieved in any of the induction protocols used in this study and hence a pool of undifferentiated hADMSCs is expected to exist in the cultures along with the differentiated or lineage- committed cells in the culture. This proportion of undifferentiated cells may differ in different donor cells and this variation might have contributed to the high standard deviation observed in the gene expression studies. The numerical representations are included in the modified graphs and are incorporated in the modified thesis.

4. **In fig: 3 the loading control (GAPDH) is not proper.**

   Response: Thank you for the suggestion. We assume that the reuse of TAE buffer to run the PCR products on agarose gel or the voltage fluctuation would have resulted in the lane anomaly of the loading control.

5. **For graphs (gene expression profile) it is advisable to do statistical analysis as performed for proliferation assay.**

Response: Thank you for your valuable suggestion. We have done statistical analysis for gene expression studies, but since we did not obtain any significance, we have not included the p value obtained in the graphs.

6. **For *in vivo* studies why there is no control for Group VII (only medium as matrix) kindly explain.**

   Response: Thank you for the suggestion. We have included the animal group where Serum-free medium alone was used as the matrix (Group I – Medium injected control, Med-). Kindly refer to Table 5 (Allocation of animals for *in vivo* studies) on page number 62. The animal group, Medium with human s-e-ADMSC secretome was not included in our study due to an oversight.

7. **The claim made by the student in the hypothesis of using fibrin as matrix being a novel for her studies, however seems to be not true (Fibrin as a scaffold for cardiac tissue engineering by Maria Chiara Barsotti BSc, PhD Francesca Felice Alberto Balbarini Rosella Di Stefano first published: 05 October 2011). There has been previous attempt where fibrin has been used for stem cells delivery.**

   Response: Thank you for the suggestion. The use of fibrin as a scaffold for cardiac tissue engineering is well established. The reference is also mentioned in the Chapter 5, Discussion part of the thesis (page number 110). In our study, we have transplanted pre-differentiated cardiomyocyte-progenitor cells (CMPs), into the MI model using fibrin as a delivery vehicle. The aim of the study was to evaluate the survival and retention of transplanted progenitors in the infarcted heart upon delivery using fibrin.