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TIMP-1 ACTIVATES A UNIQUE CARDIAC STEM CELL POPULATION, CD63^{+ve}/C-KIT^{+ve}, THEREBY ENHANCING CARDIAC DIFFERENTIATION, AND PROTECTS THE HEART FROM ADVERSE CARDIAC REMODELING FOLLOWING MYOCARDIAL INFARCTION

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
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ABSTRACT

We previously demonstrated that embryonic stem (ES) cells over-expressing tissue inhibitor of metalloproteinase-1 (TIMP-1) have increased potential to engraft and differentiate into cardiac myocytes following transplantation into the infarcted heart. However, the ability of TIMP-1 to activate endogenous stem cells and enhance their differentiation into cardiac regenerative cell types is still unknown. We postulate that TIMP-1 may additionally activate a stem cell population that enhances cardiac cell type differentiation in the infarcted myocardium. To prove this hypothesis, we isolated c-kit⁺ cells from four weeks old C57BL/6 mice and cultured them *in vitro* in presence of ES conditioned media (ESCM), ES-TIMP-1-CM or TIMP-1. Our immunostaining data validate the existence of a novel CD63⁺/c-kit⁺ cells. When treated with TIMP-1, these cells showed significantly ($p < 0.05$) increased proliferation and differentiation into cardiac myocytes, vascular smooth muscle cells, and endothelial cells. Western blot analysis revealed significantly ($p < 0.05$) increased expression of CD63, phosphorylated and total β -catenin proteins. Furthermore, our RT-PCR data showed increased cardiac gene expression (GATA-4, Mef2C, and Nkx-2.5) when compared to ESCM and control cells. Based on the *in vitro* findings, we investigated the effect of intramyocardial delivery of TIMP-1 on endogenous CD63⁺/c-kit⁺ cells following myocardial infarction (MI). C57BL/6 and TIMP-1 KO mice underwent coronary artery ligation followed by intramyocardial delivery of 20 μ l of culture media (CC), ESCM, ES-TIMP-1-CM or TIMP-1. Subsequent immunohistochemistry analysis demonstrated the presence of a CD63⁺/c-kit⁺ cell population within the peri-infarct area and confirmed intramyocardial delivery of ES-TIMP-1-CM or TIMP-1 significantly ($p < 0.05$) enhanced their proliferation.

Percentage of CD63^{+ve}/c-kit^{+ve} cells was significantly ($p < 0.05$) lower in TIMP-1 KO mice compared to C57BL/6 animals. RT-PCR analysis revealed TIMP-1 KO animals expressed significantly less CD63 and TIMP-1 mRNAs compared to C57BL/6 mice. Activated CD63^{+ve}/c-kit^{+ve} cells were also able to differentiate into major cardiac cell types as previously shown *in vitro*. The differentiation potential of these cells was however higher in C57BL/6 mice compared to TIMP-1 KO mice. We also demonstrate that CD63^{+ve}/c-kit^{+ve} cells differentiation is regulated by CD63/ β -catenin pathway *in vivo*. Additionally, we provide evidence that TIMP-1 protects the heart from adverse cardiac remodeling through inhibition of cardiac apoptosis and fibrosis leading to significantly ($p < 0.05$) improved contractile function. Collectively, our data show TIMP-1 plays a dual protective role in the MI heart. It activates a unique stem cell population, CD63^{+ve}/c-kit^{+ve}, which proliferates and differentiates into functional myocytes, smooth muscle cells and endothelial cells mediated through CD63/ β -catenin pathway. TIMP-1 also protects the heart from adverse cardiac remodeling. Increased cardiac regeneration and inhibition of adverse cardiac remodeling consequently lead to restored cardiac function.

To the men of my life, *Karim, Elias* and *Yaseen Bouchoucha*, whose love and support
has empowered me to conquer the most unattainable frontiers.

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“When our insecurities become our identities, unhappiness and discontentment infect us like a festering wound and render us blind to what makes us special. But when our insecurities hold no power and our identities reflect the good rather than the bad in us, that’s when positive growth and success can make their ways into our lives.”

– Asperger Experts –

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AHA	American heart association
AU	Arbitrary units
BMP2	Bone morphogenetic protein-2
BMSCs	Bone marrow stem cells
CC	Cell culture media
cDNA	Complementary deoxyribonucleic acid
Cdk9	Cyclin-dependent kinase-9
CD31	Cluster of differentiation 31
CD63	Cluster of differentiation 63
CHD	Coronary heart disease
CM	Conditioned media
CPC	Cardiac progenitor cell
CSC	Cardiac stem cell
D14	Day 14
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EDV	Left ventricular volume at end diastole

EF	Ejection fraction
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cells
ES	Embryonic stem
ESCM	Embryonic stem conditioned media
ES-TIMP-1-CM	Embryonic stem overexpressing TIMP-1 conditioned media
ESV	Left ventricular volume at end systole
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FGF-4	Fibroblast growth factor-4
FGF-9	Fibroblast growth factor-9
FITC	Fluorescein isothiocyanate
FS	Fractional shortening
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-4	GATA binding protein-4
G418	Geneticin
G-CSF	Granulocyte-colony stimulating factor
GM	Growth media
HGF	Hepatic growth factor
H ₂ O ₂	Hydrogen peroxide
H9c2	Rat cardiomyoblasts
HRP	Horse radish peroxidase

IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IL-6	Interleukin-6
iPS	Induced pluripotent stem
Isl1	Insulin gene enhancer protein 1
LAD	Left anterior descending artery
LIF	Leukemia inhibitory factor
LVIDd	Left ventricular internal dimension-diastole
LVIDs	Left ventricular internal dimension-systole
Mef2C	Myocyte-specific enhancer factor 2C
MHC	Myosin heavy chain
MI	Myocardial infarction
MMP-9	Metalloproteinase-9
MMPs	Metalloproteinases
MSCs	Mesenchymal stem cells
mRNA	Messenger ribonucleic acid
NGS	Normal goat serum
NIH	National institute of health
Nkx2.5	NK2 homeobox 5
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation

RNAs	Ribonucleic acids
RT-PCR	Real-time polymerase chain reaction
SCF	Stem cell factor
SEM	Standard error of the mean
SP cells	Side population cells
TGF- β 2	Transforming growth factor β -2
TIMPs	Tissue inhibitor of metalloproteinases
TIMP-1	Tissue inhibitor of metalloproteinase-1
TIMP-1-ES cells	TIMP-1 overexpressing embryonic stem cells
TNF- α	Tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UCBCs	Umbilical cord blood cells
VA	Vessel area
VF	Vessel fibrosis
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle

CHAPTER ONE: INTRODUCTION

MI Prevalence and Epidemiology

Myocardial infarction (MI) is a major health threat in the United States and other western countries. According to the American Heart Association (AHA) statistics for 2014, more than 620,000 Americans will be hospitalized for an MI or coronary heart disease (CHD) and ~295,000 will have a recurrent attack. In terms of mortality rate, approximately every 34 seconds, 1 American has an MI, and approximately every 2 minutes, an American will die of one (30). The incidence of MI is dependent on predisposing risk factors including: hypertension, diabetes mellitus, tobacco use, hyperlipidemia and family history of CHD (30). Any risk factor, if present, doubles the relative risk of developing atherosclerotic coronary artery disease. Currently used medications have significantly improved patients quality of life; however, these therapies are unable to prevent future episodes of MI, and mortality rate is still at record high (30). Therefore, finding alternative therapies with better short-term and long-term outcomes for MI patients is still at the forefront of medical research.

Myocardial Infarction Pathophysiology

Atherosclerotic plaque rupture in the main arteries of the left ventricle blocks blood and nutrient supply to the myocardium, leading to ischemia and MI (6; 32). Prolonged ischemia results in myocardial remodeling, which includes cardiac cell loss, generation of fibrotic scar tissue, cardiac myocyte hypertrophy and left ventricular dilation (2-4; 6). Necrosis and apoptosis are distinct forms of cell death, both occurring in ischemic heart, although in the first 24 h following infarction, apoptosis is the main form of cell death observed (8; 11). It is a highly orchestrated programmed cell death

characterized by cell cytoskeleton network rupture, leading to cytoplasmic membrane breakage, cell shrinkage, and DNA fragmentation (8; 11). Cardiac myocyte apoptosis is mediated by two distinct pathways; extrinsic and intrinsic (9). Extrinsic pathway is triggered by extracellular death ligands such as FAS, tumor necrosis factor (TNF)- α , and TRAIL, while intrinsic pathway is mediated through multiple Caspases (9).

Cardiac myocyte apoptosis in the infarct and peri-infarct area activates cardiac fibroblasts, which are the main cellular modulators of cardiac fibrosis. They control extra cellular matrix (ECM) turn over through secretion of matrix metalloproteinases (MMPs), otherwise called Matrixins, and their direct inhibitors; tissue inhibitor of metalloproteinases (TIMPs) (19; 22; 33; 38). Imbalance between TIMPs and MMPs expression has been documented in MI and is known to precipitate collagen deposition in the interstitial and vascular spaces, leading to stiffening of the left ventricle, fibrosis and cardiac dysfunction (7; 12; 21). Optimal contractile function relies on the integrity of the syncytial myocardium which contracts rhythmically during each cardiac cycle to pump the blood out of the cardiac chambers. It is then inevitable that death of cardiac cells and accumulation of stiff collagen deposits within the infarct area would dramatically impact contractile function, eventually leading to heart failure (6; 32).

Cell Transplantation in the Infarcted Myocardium

Cell transplantation therapies to replace dead heart cells and to prevent adverse cardiac remodeling following MI have been extensively investigated. Such cells include skeletal myoblasts, neo-natal and adult cardiac myocytes, human umbilical cord blood cells (UCBCs), endothelial progenitor cells (EPCs), embryonic stem cells (ESCs, bone marrow stem cells (BMSCs), mesenchymal stem cells (MSCs), induced pluripotent stem

cells (iPSCs) and cardiac stem cells (CSCs) (1; 5). Unequivocally, transplanted cells showed some regenerative capabilities, however, cell survival rate and differentiation were limited post-transplantation (1; 5; 27; 28). Furthermore, ESCs transplantation involves immune response, teratoma formation and ethical concerns. iPSCs and BMSCs on the other hand have limited engraftment and may induce tumorigenesis (1). Additionally, MSCs were shown to induce bone formation which greatly interferes with the myocardium contractility (1). In light of all these limitations, it is imperative to identify ways to improve existent cell therapies or identify a new cell population for cardiac regeneration.

One very promising characteristic of transplanted cells is their ability to influence injured myocardium through paracrine mechanisms. Indeed, many factors released from these cells have been demonstrated to boost cardiac cell self-renewal [(Insulin Growth Factor (IGF), Hepatic Growth Factor (HGF) and Stem Cell Factor (SCF)], neovascularization [Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor-9 (FGF-9)], and inhibition of adverse cardiac remodeling [Tissue Inhibitor of Metalloproteinases-1 (TIMP-1)] (17; 21; 36; 37). Therefore, direct delivery of factors released by stem cells to repair the damaged myocardium opens a new avenue for cardiac regeneration.

Endogenous Cardiac Stem Cells

The identification of cardiac stem cells (CSCs) greatly challenged the belief that the heart is a post mitotic organ, therefore increasing enthusiasm among scientific community in their quest to repair the damaged heart. CSCs have been localized throughout the myocardium in clusters called niches (29). They were isolated from the

postnatal and adult human hearts and other mammalian species (1; 23; 29; 34). They exhibit capacity to self-renew and differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells in appropriate conditions (1; 23; 29; 34). Different sub-populations of CSCs have been identified based on extra cellular markers, such as side population (SP) cells, cardiac progenitor cells (CPCs), Sca-1^{+ve} cells, cardiosphere cells, Isl1^{+ve} cells, and c-kit^{+ve} cells, which are still the most abundant and most extensively characterized CSCs (1; 5; 29). CSCs are believed to participate in cardiac cell turn over and homeostasis in the healthy and injured organ. However, the reported rate of myocyte regeneration following MI is rather low; suggesting a limited regenerative potential of these resident stem cells (1; 5; 18; 27; 28). Therefore, identifying mechanisms to boost endogenous CSCs activation and participation in cardiac repair following MI appears as the most promising strategy for MI patients.

TIMP-1 in Cardiac Remodeling

Following MI, a maladaptive cardiac remodeling takes place leading to decreased cardiac performance. The gross pathological changes involved in cardiac remodeling include massive cardiac myocyte cell loss through apoptosis, increased infiltration of collagen fibers throughout the infarct zone (fibrosis) and cardiac myocyte hypertrophy leading to deregulated contractile function (2; 3). Cardiac fibroblasts, the main cellular modulators of cardiac fibrosis, lead to overstimulation of MMPs and downregulation of their direct inhibitors TIMPs (19; 22; 33; 38). MMPs are a family of endopeptidases which play a role in aberrant production of ECM proteins leading to adverse ventricular architecture post-MI (31; 38). Tissue inhibitors of metalloproteinases 1 to 4 (TIMPs 1-4) are endogenous protease inhibitors of MMPs (31;

38). TIMPs non-covalently bind the active site of pro-MMP, preventing its activation (31; 38). In the heart, decreased expression of TIMP-1 was reported following MI, correlating with enhanced expression of both MMP-2 and 9 (14; 22; 38). Additionally, up regulation of TIMP-1 in the MI heart by direct delivery of TIMP-1 protein or vector to the peri-infarct area has demonstrated decreased overall infarct size and infiltrated fibrosis (20). TIMP-1 has additionally been shown to be anti-apoptotic in cardiac and non-cardiac cell lines (12; 21; 25; 26). Based on these reported beneficial effects, we generated embryonic stem (ES) cells overexpressing TIMP-1 factor (ES-TIMP-1 cells). When transplanted to the MI heart, these cells demonstrated limited engraftment to the host myocardium; however, collected data showed a significant decrease in adverse cardiac remodeling parameters as well as improved functional capacity of the heart (19). Whether the reported beneficial effects are the result of TIMP-1 released by transplanted cells alone or other factors also released by these cells remains obscure.

TIMP-1 in Cardiac Differentiation

According to previous studies, ~ 10 % of transplanted stem cells survive in the host milieu following transplantation (5; 23; 34). Consequently, limited cardiac myocyte differentiation is reported from these cells (1; 20; 34). Therefore, efficacy of cell transplantation therapy for myocardial regeneration remains a major challenge. Multiple attempts were made to improve aforementioned statistics using various growth factors, such as vascular endothelial growth factor (VEGF), transforming growth factor β -2 (TGF β -2), insulin-like growth factor-1 (IGF-1) and (IGF-2), fibroblast growth factor-2 (FGF-2), FGF-4 and epidermal growth factor (EGF) (35-37). Additional factors examined include bone morphogenetic protein-2 (BMP-2), Interleukin-6 (IL-6) and

Activin-A. Results from these studies described relative improvement in survival plus differentiation rate of transplanted cells, which remains insufficient to restore pre-MI normalcy (1).

Involvement of TIMP-1 in cell proliferation has been demonstrated in various cell types; however, whether TIMP-1 can promote stem cell differentiation is still unknown. In our previously published work, we were able to enhance ES cell engraftment, survival, and differentiation post transplantation in the MI heart by engineering them to over-express TIMP-1 (20). Indeed, transplanted ES-TIMP-1 cells showed significantly increased cardiac myocyte differentiation compared to non-engineered ES cells demonstrating the role of TIMP-1 in stem cell differentiation. Based on this data, we postulated that TIMP-1 released by ES-TIMP-1 cells may additionally activate endogenous cardiac stem cells (CSCs) that express both CSC marker (c-kit) and TIMP-1 receptor (CD63), leading to cardiac cell differentiation and myocardial regeneration post MI injury.

CD63 marker is a cell surface receptor for TIMP-1 and a member of the tetraspanin family (13; 15; 24). It is expressed in multiple cell types including cardiac myocytes, endothelial cells and MSCs. However, no one has previously reported existence of a CSC population which expresses CD63 marker. Furthermore, CD63 activation by TIMP-1 was shown to activate non-canonical β -catenin pathway leading to enhanced cell proliferation in various cell types (25). However, whether activation of CD63/ β -catenin pathway in CD63^{+ve}/c-kit^{+ve} CSCs can trigger cardiac differentiation is completely unknown.

Rationale and Aims

MI is a leading cause of death in the western world (54). The reported mortality rate following MI is caused by the dramatic myocardium loss consecutive to ischemia (5; 6; 30). Additionally, the pool of CSCs discovered in the heart are unable to repopulate the infarcted myocardium with functional cardiac myocytes and vascular cells to compensate for the millions of cells lost during an MI event (1; 5). To date, major efforts have been made to salvage as much myocardium as possible in the first hours following MI; however, the permanent damage caused by cell death eventually leads to recurrent MIs and heart failure. Recently, regenerative medicine via cell transplantation therapy has excited the scientific community as it offers the potential to repopulate the infarcted area with functional cardiac myocytes (2). Somatic, iPS, adult and embryonic stem cells have been utilized to replenish the myocardium with mitigated results (1). Our engineered ES-TIMP-1 cells on the other hand demonstrated improved engraftment and differentiation following transplantation when compared to regular ES cells (20). Based on this data we postulated that TIMP-1 might be utilized to boost dormant endogenous cardiac stem cells (CSCs) that express TIMP-1 receptor (CD63) leading to cardiac cell differentiation and myocardial regeneration post MI injury.

The goal of the first study was to demonstrate existence of a CSC population positive for c-kit (CSC marker) as well as CD63 (TIMP-1 receptor) from isolated murine CSCs. We hypothesized that TIMP-1 released by ES-TIMP-1 cells activates isolated CD63⁺/c-kit⁺ CSCs, enhancing their proliferation, cardiac gene expression, and differentiation into major cardiac cell types. To test our hypothesis, the following aims were proposed:

1. Determine the existence of CD63⁺/c-kit⁺ CSCs in isolated murine CSCs.

2. Assess the impact of ES-TIMP-1-CM and TIMP-1 on CD63^{+ve}/c-kit^{+ve} CSCs proliferation and differentiation in vitro.
3. Identify the mechanism that mediates CD63^{+ve}/c-kit^{+ve} CSCs proliferation and differentiation in vitro.

Within the second study, we aimed at identifying CD63^{+ve}/c-kit^{+ve} CSCs *in vivo* as well as verifying the ability of TIMP-1 to activate these cells following MI. The goal of this study was to validate whether intramyocardial delivery of ES-TIMP-1-CM or TIMP-1 would activate CD63^{+ve}/c-kit^{+ve} CSCs, enhance their proliferation and differentiation into major cardiac cell types and replenish the lost myocardium. We hypothesized that TIMP-1 transplantation into the infarcted myocardium targets CD63^{+ve}/c-kit^{+ve} CSCs, binds to its receptor on their surface and activates β -catenin pathway, leading to enhanced cell proliferation and differentiation. To test our hypothesis, we proposed the following aims:

1. Determine the existence of CD63^{+ve}/c-kit^{+ve} CSCs in C57BL/6 and TIMP-1 KO peri-infarcted hearts and verify the effect of intramyocardial delivery of TIMP-1 on their proliferation.
2. Verify the effect of intramyocardial delivery of TIMP-1 on CD63^{+ve}/c-kit^{+ve} CSCs differentiation following MI.
3. Identify mechanisms by which TIMP-1 mediates CD63^{+ve}/c-kit^{+ve} CSCs proliferation and differentiation in vivo.

Finally, within the third study, we evaluated the impact of TIMP-1 transplantation on adverse cardiac remodeling in the infarcted myocardium of C57BL/6 and TIMP-1 KO mice. The goal of this study was to demonstrate that TIMP-1 is a multifunctional protein

that not only enhances cardiac proliferation and differentiation but also prevents cardiac cell death and fibrosis leading to overall better cardiac function. We hypothesized that TIMP-1 would significantly inhibit cardiac apoptosis, fibrosis and improve cardiac function 14 days post cardiac transplantation compared to ESCM treatment leading to improved contractile function. To test our hypothesis, we proposed to address the following aims:

1. Evaluate the anti-apoptotic effect of TIMP-1 transplanted into the host myocardium following MI.
2. Evaluate the anti-fibrotic effect of TIMP-1 transplanted into the host myocardium following MI.
3. Determine the impact of transplanted TIMP-1 infarct size and cardiac function two weeks post-MI.

The long term goal of our research is to identify a multi-functional protein for cardiac repair and regeneration following MI which targets endogenous CSCs, inducing their proliferation and differentiation into all cardiac cell types, while effectively inhibiting apoptosis and fibrosis. Identification of such factor as well as its targeted CSC population would provide a strong foundation for cardiac regeneration research.

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CHAPTER TWO: A CD63^{+ve}/C-KIT^{+ve} STEM CELL POPULATION ISOLATED FROM THE MOUSE HEART

Abstract

Cardiac cell regeneration from endogenous cardiac stem cells (CSCs) following MI is rather low. Therefore, identifying mechanisms to boost endogenous CSC activation and participation in cardiac repair appears to be the most promising strategy for MI patients. We previously engineered tissue inhibitor of metalloproteinases-1 (TIMP-1) overexpressing embryonic stem (ES-TIMP-1) cells and transplanted them into the infarcted murine heart. Collected data demonstrated that TIMP-1 enhanced transplanted ES cell engraftment, survival and differentiation into cardiac myocytes post transplantation. Therefore, we postulated that there may be a new stem cell population present in the heart that is regulated by extracellular protein TIMP-1. Furthermore, we hypothesized that this cell population has a potential for cell proliferation and differentiation into cardiac cell types. Therefore, we isolated CSCs from four weeks old C57BL/6 mice and cultured them in vitro in presence of ESCM, ES-TIMP-1-CM or TIMP-1. Our immunostaining data demonstrated the existence of a novel CSC sub-population, CD63^{+ve}/c-kit^{+ve}. When treated with TIMP-1, these cells showed significantly ($p < 0.05$) increased proliferation rates compared to control cells, enhanced TIMP-1 receptor (CD63), along with improved expression of phospho and total β -catenin proteins as demonstrated by western blot analysis. Next, we demonstrate significantly ($p < 0.05$) improved cardiac myocyte, vascular smooth muscle cell, and endothelial cell differentiation. Furthermore, our RT-PCR data shows increase in cardiac gene (GATA-4, Mef2C, and Nkx-2.5) expression when compared to ESCM and control cells. Collectively, this data, for the first time, establish the existence of a new CD63^{+ve}/c-kit^{+ve}

CSC sub-population that has a significant potential for proliferation and differentiation into cardiac cell types once stimulated with TIMP-1.

Keywords: Cardiac Stem Cells, TIMP-1, c-kit, CD63, β -catenin, proliferation, differentiation.

Introduction

The identification of cardiac stem cells (CSCs) in the adult heart greatly challenged the belief that the heart is a terminally differentiated organ with no regenerative capacity [1,2]. This information greatly increased enthusiasm among the scientific community in their quest to repair damaged heart tissue following major cardiac cell loss consequent to myocardial infarction (MI). CSCs are localized throughout the myocardium in clusters called niches [2]. They exhibit the capacity, both *in vivo* and *in vitro*, to self-renew and differentiate into three major cardiac cell types including cardiac myocytes, vascular smooth muscle (VSM) cells and endothelial cells (ECs) [2-4]. Different sub-populations of CSCs have been identified based on extracellular markers, such as side population (SP) cells, cardiac progenitor (CP) cells, Sca-1^{+ve} cells, cardiosphere cells, Isl1^{+ve} cells and c-kit^{+ve} cells [2]. CSCs are believed to participate in cardiac cell turn over and homeostasis in the healthy and injured heart [2,3]. However, the reported rate of myocyte regeneration from CSCs following MI is rather low suggesting a limited regenerative potential of these resident stem cells [1,2]. Therefore, identifying mechanisms to boost endogenous CSC activation and their participation in cardiac repair following MI appears to be the most promising strategy for MI patients.

Multiple factors have been shown to promote stem cell recruitment, proliferation and differentiation. Such factors include, but are not limited to, insulin growth factor

(IGF), hepatic growth factor (HGF), granulocyte-colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), fibroblast growth factor-9 (FGF-9), and transforming growth factor beta-2 (TGF- β 2) [5]. Results from these studies described relative improvement in cell proliferation and differentiation. However, their efficacy remains insufficient to restore the post-MI myocardium to its non-pathological architecture and functional capacity [6,7]. Therefore, we hypothesize that there may be additional populations of CSCs present in the heart that could be activated by specific factors, driving their proliferation and differentiation.

In our previously published work, we engineered tissue inhibitor of metalloproteinases-1 (TIMP-1) overexpressing ES cells and transplanted them into the infarcted murine heart [8]. Collected data demonstrated that TIMP-1 enhanced transplanted ES cell engraftment, survival and differentiation into cardiac myocytes post transplantation [8]. In lieu of our previous findings, we postulated that TIMP-1 released by ES-TIMP-1 cells may have additionally activated a subpopulation of endogenous CSCs that express the CSC marker (c-kit) and TIMP-1 receptor (CD63).

To test the hypotheses, we aimed at identifying a subset of CSCs, CD63^{+ve}/c-kit^{+ve}, from isolated murine CSCs. Next, we verified the effect of ES cell released factors, present in their conditioned medium, with or without TIMP-1 (ESCM and ES-TIMP-1-CM) or TIMP-1 alone on CD63^{+ve}/c-kit^{+ve} CSCs proliferation and differentiation rates. Our data will show for the first time that we were able to identify a CD63^{+ve}/c-kit^{+ve} sub-population of CSCs that is susceptible to proliferate, express cardiogenic factors, and differentiate into major cardiac cell types upon treatment with either ES-TIMP-1-CM or TIMP-1 in vitro.

Materials and Methods

Isolation of Cardiac Stem Cells (CSCs)

CSCs were isolated from 4 to 6 weeks old C57BL/6 mice hearts following instructions provided with the Millipore CSCs Isolation kit (Cat# SCR061). In brief, hearts were removed from the thoracic cavity, washed, and minced in CSC isolation buffer. Next, a cell suspension was created through gentle agitation in dissociation buffer for 1 hr and filtered using a self-contained Steriflip® filtration device. The filter allows for the separation of larger cellular material while maintaining a sterile environment. A differential gradient centrifugation was then applied to the cells, allowing for the formation of a clear mid layer containing total CSCs. Isolated CSCs were plated on gelatin-coated plates in CSCs maintenance medium (MM) purchased from Millipore (Catalog# SMC101). During the first 7 to 12 days, cells gradually adhered to the plate and were maintained regularly as per our standard protocol [8].

Preparation of ESCM, ES-TIMP-1-CM, and TIMP-1

ES-TIMP-1 cells were generated in our lab as previously described (8). Next, condition medium (CM) was prepared by treating ES and ES-TIMP-1 cells with Dulbecco's Modified Eagle Medium (DMEM) containing leukemia inhibitory factor (LIF) along with other components as we reported previously (5; 8). LIF was then removed and cells were cultured for 48 hrs on gelatin-coated plates. Following incubation, ESCM and ES-TIMP-1-CM were collected, filtered, and stored at -20°C. Recombinant mouse TIMP-1 was purchased from R&D (cat # 980-MT) as a lyophilizate and reconstituted to a concentration of 100µg/ml following manufacturer instructions and stored at -20°C.

CSCs treatment with ESCM, ES-TIMP-1-CM, and TIMP-1

CSCs were cultured in 6 well plates (80,000 cells/well) pre-coated with 0.1% gelatin in MM until they adhered to the bottom of the plates. CSCs were then divided into 4 groups (n=4 wells/group); Control (CSCs supplemented with 2ml growth media (GM)), ESCM (CSCs supplemented with 2ml of ESCM), ES-TIMP-1-CM (CSCs supplemented with 2ml of ES-TIMP-1-CM) and TIMP-1 (CSCs treated with 50ng TIMP-1 in 2ml GM). Treated cells were incubated for 48 hrs followed by a media exchange back to MM in all groups for an additional 24 hrs.

Plating and Immunostaining Detection of CD63^{+ve}/c-kit^{+ve} CSCs

CSCs were cultured in 8 well plates (15,000 cells/well) pre-coated with 0.1% gelatin. Cells were divided, cultured, and treated as aforementioned. Next, cells were washed and fixed with 10% formalin and Triton X for 10 min each followed by 1 hr with 10% normal goat serum (NGS; Vector Laboratories). To determine whether cells express CD63 and c-kit markers, cells were incubated with anti-c-kit (1:50; Santa Cruz) or anti-MLA-CD63 (1:500; Bios) rabbit monoclonal antibodies diluted in 10% NGS for 1 hr at room temperature or overnight at 4°C. Cell were then washed and incubated for 1 hr with secondary antibodies (goat anti-rabbit Alexa Fluor 568 (c-kit) or 660 (CD63)) at a concentration of 1:20 diluted in 1x PBS. Total nuclei were detected by mounting each section with Antifade Vectashield medium containing 4',6-diamino-2-phenylindole (DAPI; Vector Laboratories). Olympus and confocal fluorescence microscopes were used to visualize and quantify CD63^{+ve}/c-kit^{+ve} cells over total nuclei. Data are presented as % of c-kit^{+ve} cells over DAPI and % CD63^{+ve}/c-kit^{+ve} cells/total c-kit^{+ve} cells.

Immunostaining Determination of CD63⁺/c-kit⁺ CSCs Differentiation into Cardiac Myocytes, VSM Cells, and ECs

To verify whether treatment with ES-TIMP-1-CM or TIMP-1 can trigger CSC differentiation into cardiac myocytes, VSMs, and/or ECs in vitro, CD63⁺/c-kit⁺ cells were stained with either sarcomeric (Src) α -actin, smooth muscle (SM) α -actin or CD31, respectively [9]. After 1 hr incubation in NGS, cells were incubated with rabbit anti-CD31 monoclonal antibody (1:25, Santa Cruz Biotechnologies) prepared in 10% NGS, followed by FITC-conjugated goat anti-rabbit IgG secondary antibody (1:50, Invitrogen) for 60 min. A mouse anti-mouse kit (MOM, Vector Laboratories) was used for SM and Src- α -actin staining. After blocking, cells were incubated with anti-SM and anti-Src α -actin antibodies (1:50 and 1:100 respectively, Sigma) for 30 min followed by an IgG antibody and fluorescein avidin solution for 5 and 10 min each. Stained cells were then mounted with Vectashield antifade medium containing DAPI (Vector Laboratories) for nuclear visualization. Olympus, confocal microscope and Image J software were used to quantify cardiac cell differentiation of CD63⁺/c-kit⁺ cells using the following formulas: % cardiac myocyte differentiation = Cells positive for Src α -actin, c-kit, and CD63 over DAPI x 100. % VSM differentiation = Cells positive for SM α -actin, c-kit, and CD63 cells over DAPI x 100. % ECs differentiation = Cells positive for CD31, c-kit, and CD63 cells over DAPI x 100.

Cell Proliferation Assay

Previously treated cells were trypsinized and re-seeded on a 96 well plate at a density of 10^4 cells/well supplemented with MM for 48 hrs. Next, cell proliferation was measured using a tetrazolium salt cell proliferation assay kit (Cat# 10008883; Chayman

Chemical). In brief, each well received 10µl of reconstituted tetrazolium salt solution and the plate was incubated at 37°C on an orbital shaker for 3 hrs. Finally, absorbance of each sample was read using a microplate reader at a 450nm. Measured absorbance directly correlated with proliferation rate and was plotted as a bar graph.

Western Blot Detection of CD63, phospho (p) and total β-catenin Proteins Expression

To determine CD63, phospho (p) and total β-catenin expression on isolated CSCs as well as the effect of TIMP-1 on their expression, western blot analysis was performed on cell lysates obtained from all four treatments groups as previously described [10]. In brief, an equal amount of protein (100ug) was loaded on a 10% polyacrylamide gel and subjected to electrophoresis at 150V for 1 hr. The gel was then transferred for 45 min to a polyvinylidene fluoride membrane (BioRad) using a semi-dry Immuno-Blot (Bio-Rad) at 15V. The membranes were blocked with 5% skim milk for 1 hr, incubated with primary rabbit antibodies (CD63/MLA1; cat # ABIN687742), (β-catenin (6B3) ; cat # 9582), phospho-β-catenin (Ser33/37/Thr41; cat # 9561, Cell signaling), and β-actin (cat # 4967L from Cell Signaling) for one hr on an orbital shaker at room temperature or at 4°C overnight, and incubated with anti-rabbit IgG horseradish peroxidase secondary antibody (Cat # 7074S; Cell Signaling) for an additional 1 hr. Blots were detected with Pierce® ECL Western Blotting Substrate (Thermo Scientific).

Real-Time PCR for GATA-4, Mef2C and Nkx-2.5

Cells were plated as previously described and total RNA was extracted using the TRI® reagent protocol (Cat # T9424; Sigma Aldrich) (11). In brief, cells were lysed using

1ml TRI reagent in an RNase free environment followed by a separation step in chloroform and RNA precipitation in isopropyl alcohol. cDNA was synthesized from 1µg total RNA using Superscript II reverse transcriptase and random primers (Bio-Rad). Next, real-time quantitative PCR was performed using a CFX96™ Real-Time System (Bio Rad). Supermix 2X (Life Technologies) containing FAM fluorescent dye and dNTPs was added to the reaction mixture containing cDNA template and forward and reverse primers. GATA-4, Mef2C, Nkx-2.5 and GAPDH primer assays were purchased from Life Technologies. The following cycling conditions were used: 95°C for 5 min followed by 35 cycles of amplification (denature at 95°C for 15 seconds and combined annealing /extension at 60°C for 60 seconds). The relative fold expression of GATA-4, Mef2C, and Nkx-2.5 in each group was normalized against GAPDH and plotted.

Statistical Analysis

Data was analyzed using One-way analysis of variance (ANOVA) followed by the Tukey Test. Data is presented as a mean ± SEM and statistical significance assigned when p-value <0.05.

Results

Identification of CD63⁺/c-kit⁺ Population from Isolated Murine CSCs.

CSCs were isolated from adult C57BL/6 mice hearts and cultured in vitro in the presence of ESCM, ES-TIMP-1-CM or TIMP-1. Immunostaining to identify c-kit⁺ and CD63⁺/c-kit⁺ CSCs was performed. Our data demonstrate that about 80% of isolated CSCs were c-kit⁺ (Figure1B) prior to treatment and up to 60% of these cells shared the CD63 marker (Figure 1A and C). Following treatment, our data demonstrate the % of c-

kit⁺ cells significantly increased in the ES-TIMP-1-CM group compared to the control group ($p < 0.05$, ES-TIMP-1-CM: $94\% \pm 1.91\%$ vs. control: $76.5\% \pm 0.5\%$, Figure 1B). However, the percent of double positive CD63⁺/c-kit⁺ CSCs significantly increased with both ES-TIMP-1-CM ($95.5\% \pm 1.19\%$) and TIMP-1 ($88.11\% \pm 4.3\%$) treatments compared to ESCM ($66.8\% \pm 1.06\%$) and control ($60.61\% \pm 5.8\%$) cells ($p < 0.05$, Figure 1C). Moreover, CD63, (p) and total β -catenin expression significantly increased upon CSC treatment with TIMP-1 compared to control cells ($p < 0.05$, Figures 1D-F). This data, for the first time, identify a subset of CSCs that are CD63⁺/c-kit⁺ and TIMP-1 enhances CD63 expression on their surface and β -catenin downstream of CD63 (Figure 1).

TIMP-1 Enhances CD63⁺/c-kit⁺ CSC Proliferation in vitro

The effect of TIMP-1 on CD63⁺/c-kit⁺ CSC proliferation was examined (Figure 2). Our data show enhanced cell proliferation in all treatment groups compared to control cells ($p < 0.05$, Figure 2A and B). Of note, although no significant difference among treatment groups (ES-CM, ES-TIMP-1-CM, and TIMP-1) was achieved, it is worth noting that TIMP-1 alone was able to induce a similar proliferation rate as that in the ES-TIMP-1-CM group (Figure 2B). This data demonstrate that TIMP-1 alone can promote CSC proliferation *in vitro*.

TIMP-1 Enhances Isolated CD63⁺/c-kit⁺ CSC Differentiation into Cardiac Myocytes, VSM Cells, and ECs

Triple immunostaining targeting newly differentiated CD63⁺/c-kit⁺ CSCs expressing either Src α -actin (cardiac myocytes), SM α -actin (VSM cells) or CD31 (ECs)

was performed (Figure 3). Panels A, C, and E demonstrate confocal representative images of CD63⁺/c-kit⁺ CSCs undergoing cardiac myocyte, VSM cell, or EC differentiation, respectively, with c-kit⁺ cells shown in red (a,f and k), CD63⁺ cells shown in purple (b,g, and l), total nuclei shown in blue (c,h, and m), Src α -actin⁺, SM α -actin⁺, or CD31⁺ cells shown in green (d,i, and n, respectively), and merged images of all four colors in e,j, and o. Our quantitative data revealed control and ESCM groups underwent a similar rate of cardiac myocyte differentiation, while ES-TIMP-1-CM and TIMP-1 treatments significantly enhanced this parameter relative to the control and ESCM groups ($p < 0.05$, ES-TIMP-1-CM; 31.24% \pm 1.5% and TIMP-1; 29.39% \pm 1.5% vs. Control; 17.09% \pm 0.6% and ESCM; 18.49% \pm 0.9%, Figure 3B). A similar differentiation pattern was also noted for VSM cells ($p < 0.05$, ES-TIMP-1-CM; 27.94% \pm 0.8% and TIMP-1; 24.31% \pm 1.6%) compared to control and ESCM groups (Control; 13.9% \pm 0.35% and ESCM; 14.93% \pm 1.11%, Figure 3D) and ECs ($p < 0.05$, ES-TIMP-1-CM; 14.7% \pm 0.25% and TIMP-1; 15.9% \pm 0.5%, Figure 3F) compared to control and ESCM groups (Control; 9.01% \pm 0.43% and ESCM; 10.53% \pm 0.32%, Figure 3F). This overall data demonstrate, for the first time, that TIMP-1 can trigger isolated CD63⁺/c-kit⁺ CSC differentiation into the three major cardiac cell types (cardiac myocytes, VSM cells, and ECs).

TIMP-1 Enhances Expression of Cardiac Genes in Isolated CD63⁺/c-kit⁺ CSCs

To verify expression of pro-cardiac markers GATA-4, Mef2C, and Nkx-2.5 following TIMP-1 treatment, RT-PCR analysis was performed (Figure 4). Our data demonstrate no significant change in GATA-4 (Figure 4A), Mef2C (Figure 4B), and Nkx-

2.5 (Figure 4C) mRNA levels in ESCM treated cells compared to control. However, cells treated with ES-TIMP-1-CM and TIMP-1 showed significantly increased expression of the three genes; GATA-4 ($p < 0.05$, ES-TIMP-1CM; 6.81 ± 1.02 and TIMP-1; 7.39 ± 1.01 vs Control; 1 ± 0.71 and ESCM; 1.35 ± 0.23 , Figure 4A), Mef2C ($p < 0.05$, ES-TIMP-1CM; 6.37 ± 0.99 and TIMP-1; 7.34 ± 0.33 vs Control; 1 ± 0.8 and ESCM; 0.58 ± 1.18 , Figure 4B), and Nkx-2.5 ($p < 0.05$, ES-TIMP-1CM; 1.55 ± 0.21 and TIMP-1; 1.4 ± 0.03 vs Control; 1 ± 0.1 and ESCM; 0.86 ± 0.06 , Figure 4C). This data further confirm that TIMP-1 enhances CD63⁺/c-kit⁺ CSC differentiation as demonstrated by increased cardiac gene expression.

Discussion

Several CSC sub-populations have been described in the adult heart. However, the role of each category in myocardial regeneration remains an open question [2,3]. Notably, endogenous cardiac progenitor cells were shown to give rise to differentiated progeny including cardiac myocytes, VSM cells and ECs, *in vitro* as well as *in vivo*. Nonetheless, the extent of cardiac regeneration observed in the post-MI heart by CSCs remains insufficient to regain pre-infarct homeostasis [2,3,12]. Therefore, investigating alternative ways to enhance the differentiation potential of endogenous CSCs remains an important area of cardiac research. In the present study, we identify a novel sub-population of CSCs, CD63⁺/c-kit⁺. When treated with TIMP-1, they showed 1) increased proliferation rates compared to control cells, 2) enhanced CD63, (P) and Total β -catenin expression, 3) significantly improved cardiac myocyte, VSM cell and EC differentiation, and 4), increased cardiac gene (GATA-4, Mef2C and Nkx-2.5)

expression. Taken together, this data, for the first time, establish the existence of a CSC sub-population, which is CD63⁺/c-kit⁺ and is specifically targeted by TIMP-1. Furthermore, in the presence of TIMP-1, these cells undergo proliferation and differentiation into major cardiac cells in vitro. We also postulate that TIMP-1 induced CSC proliferation and differentiation is mediated through activation of CD63/ β -catenin pathway; however, additional experiments are required to confirm this hypothesis.

TIMP-1 is a glycoprotein that is ubiquitously expressed in numerous human cells and tissues [8]. It is expressed in the normal heart to repress matrix metalloproteinases (MMPs), which are responsible for extracellular matrix breakdown [8]. In the post-MI heart, TIMP-1 has been extensively studied for its protective role against aberrant collagen accumulation and fibrosis [8]. Furthermore, its involvement in cell proliferation has been demonstrated in various cell types including human erythroid progenitor cells and cancer cells [13,14]. However, whether TIMP-1 can promote stem cell differentiation is still unknown. In our previously published work, we were able to enhance ES cell differentiation post-transplantation in the post-MI heart by engineering them to over-express TIMP-1[8]. Therefore, we speculated that beyond its MMP-dependent activity, TIMP-1 released by ES-TIMP-1 cells may additionally act as a differentiation and proliferation factor on endogenous CSCs.

To test this hypothesis, we isolated murine CSCs and performed immunostaining analysis to identify CD63⁺/c-kit⁺ cells. Our data revealed the presence of a CD63⁺/c-kit⁺ sub-population, which makes up almost 60% of the whole c-kit⁺ cell population. Moreover, treatment with ES-TIMP-1-CM and TIMP-1 induced an increase in CD63 expression on these cells as well as enhanced their proliferation rate to about

80% of total c-kit⁺ CSCs. CD63 is a transmembrane protein belonging to the tetraspanin family and has been shown to be expressed in multiple cell types including cardiac myocytes, ECs and human mesenchymal stem cells (hMSCs) [14,15]. However, we are first to report its expression by CD63⁺/c-kit⁺ CSCs. This data strongly support our hypothesis and is in accordance with previously published work demonstrating enhanced hMSCs proliferation through TIMP-1/CD63 interaction on the cell surface [14].

Next, we aimed at investigating whether TIMP-1 alone could influence CD63⁺/c-kit⁺ CSC differentiation in vitro. Previously published studies demonstrated that ESCM contains factors such as IGF1/2, bone morphogenetic protein-2 (BMP-2), platelet-derived growth factor (PDGF) and VEGF that can promote cardiac differentiation [5]. However, none of these factors were able to promote significant cardiac differentiation capable of restoring pre-MI capacity [16]. The role of TIMP-1 in cell differentiation, on the other hand, has been the center of debate. In fact, TIMP-1 was reported to promote oligodendrocyte differentiation and enhance central nervous system (CNS) myelination as well as induce differentiation of germinal center B cells [17], while others claim it inhibits hMSCs differentiation and promotes cell proliferation through modulation of Wnt/ β -catenin pathway [14]. Its implication in cardiac differentiation, however, has not yet been delineated. In the present study, we provide evidence that TIMP-1 promotes CD63⁺/c-kit⁺ CSCs differentiation into the three major cardiac cells.

To further establish the involvement of TIMP-1 in CD63⁺/c-kit⁺ CSC differentiation, we measured cardiac gene expression on isolated CSCs. GATA-4, Mef2C and Nkx-2.5 are cardiogenic factors first expressed during embryonic

development to promote cardiac differentiation [18-21]. In the adult organ, these factors are mainly expressed by adult stem cells undergoing cardiac differentiation. More particularly, GATA-4 and Nkx-2.5 are linked to myocyte differentiation while Mef2C is linked to both cardiac myocyte and vascular cell differentiation [20-21]. Based on these previous findings, increased expression of these genes was assumed to be an indicator of CSC differentiation. Interestingly, our data show ES-TIMP-1-CM and TIMP-1 significantly enhanced expression of all three cardiac genes while ESCM treatment did not.

Taken together, our study demonstrates the existence of a CD63⁺/c-kit⁺ CSC population that is susceptible to proliferation and differentiation into cardiac myocytes and vascular cells in vitro upon treatment with TIMP-1. Furthermore, we show that cardiac differentiation is mediated through TIMP-1 binding to its receptor, CD63, leading to activation of β -catenin protein that enhances cardiac gene expression (GATA-4, Mef2C and Nkx-2.5). However, it is still unclear how β -catenin mediates cardiac myocyte or vascular cell differentiation when receiving one unique signal, TIMP-1. Furthermore, the ability of TIMP-1 to enhance CD63⁺/c-kit⁺ CSC differentiation in vivo has yet to be attempted. Therefore, future studies aiming at identifying CD63⁺/c-kit⁺ CSCs in vivo, their localization in respect to the infarct zone following MI, as well as investigating their ability to migrate, proliferate and differentiate upon TIMP-1 transplantation is warranted.

Conflict of Interest

None declared

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Figures

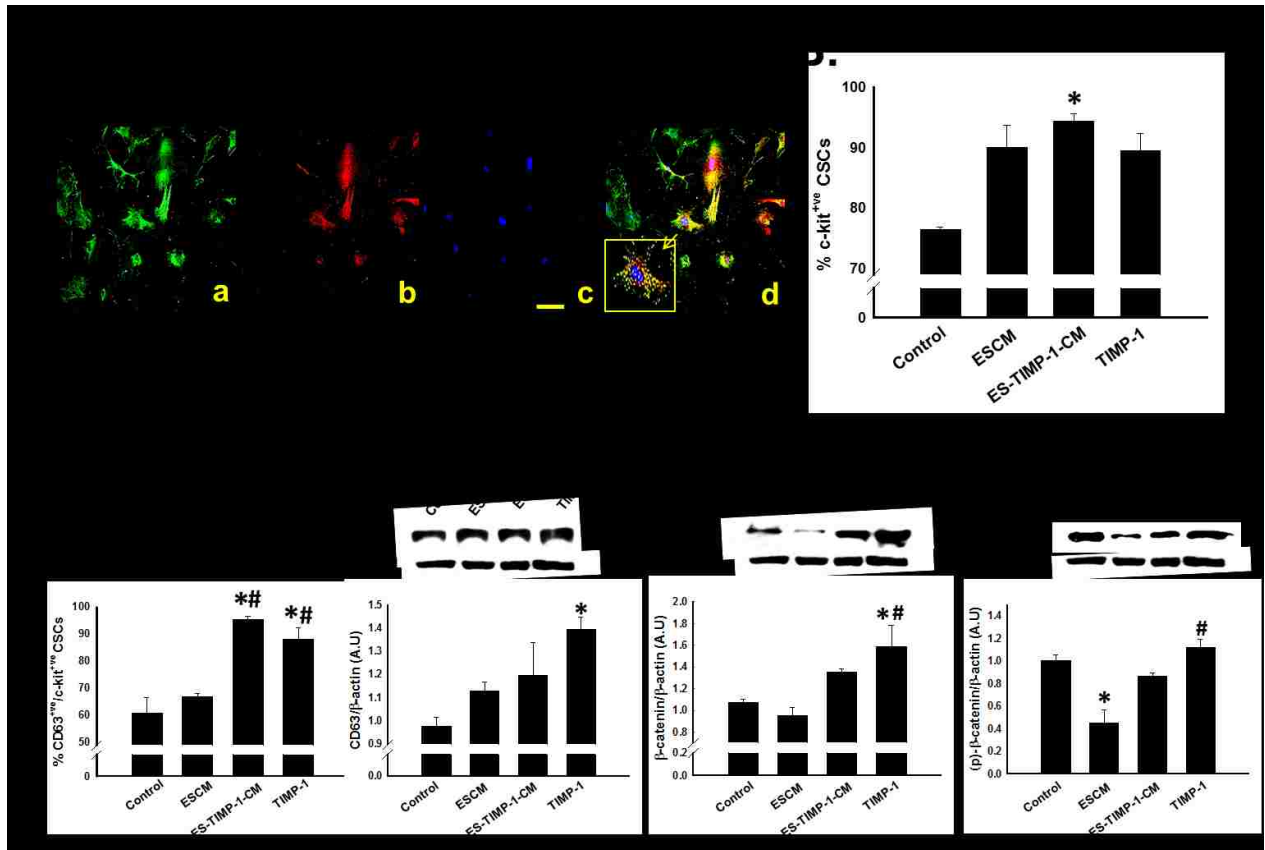


Figure 1. Identification of CD63⁺ve/c-kit⁺ve CSC Population from Isolated CSCs.

Panel A depicts representative confocal images of immunostained CD63⁺ve/c-kit⁺ve CSCs with c-kit⁺ve cells shown in green (a), CD63⁺ve cells shown in red (b), DAPI shown in blue (c) and merged images of all three channels (d). Boxed area with arrow pointing towards it within d depicts an enlarged cell. Scale bar= 300μm. Graph 1B demonstrates percent c-kit⁺ve CSCs/DAPI results. Figure 1C represents quantification analysis of percent CD63⁺ve/c-kit⁺ve CSCs/ c-kit⁺ve cells. Figures 1D-F depict densitometry analysis of CD63, β-catenin and (P)-β-catenin respectively over β-actin via western blot. Data was collected from n=4-5 experiments per group.*p<0.05 vs. Control and #p<0.05 vs. ESCM.

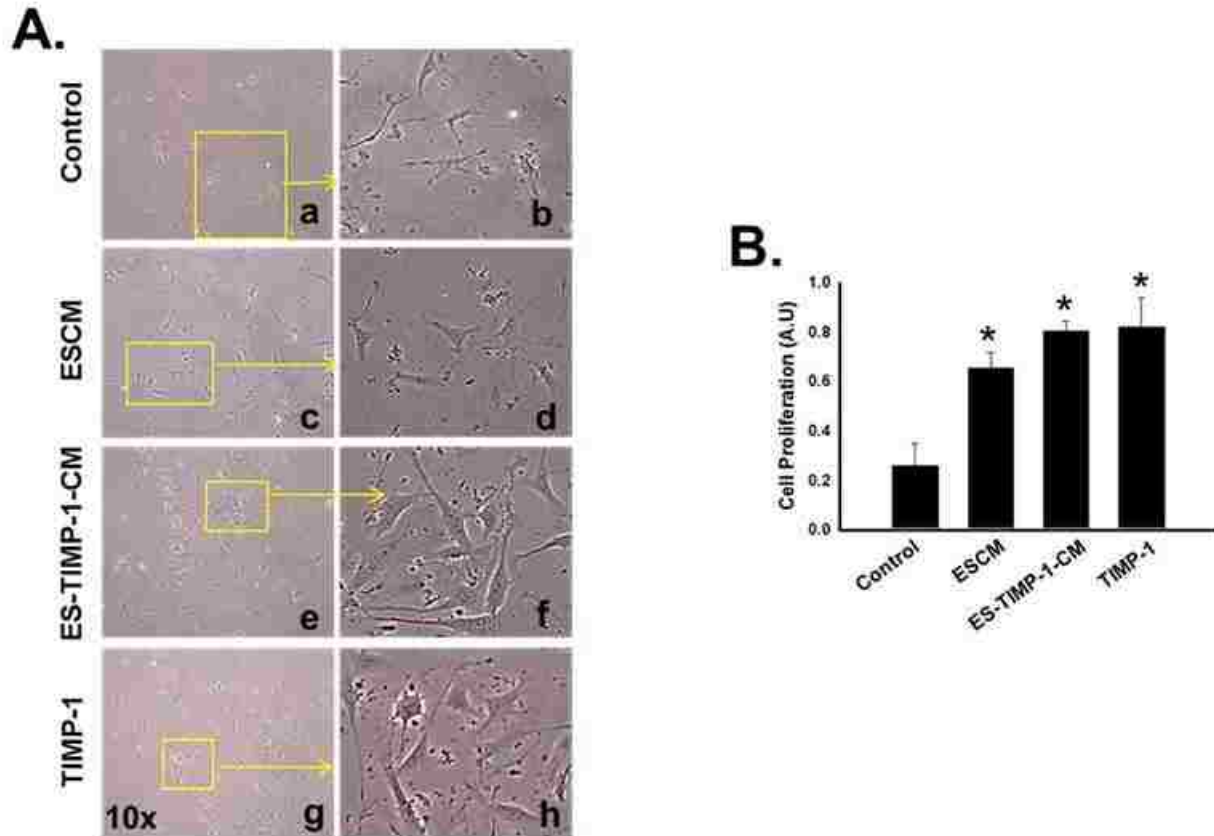


Figure 2. TIMP-1 Enhances CD63⁺/c-kit⁺ CSCs Proliferation. Bright field representative images for all four control and treatment groups are depicted in Figure 2, panel A (a, c, e, and g). Boxed areas from each photomicrograph are enlarged in A (b, d, f, and h). Magnification = 10x. Quantitative data obtained from a proliferation assay are depicted in Figure 2B. A.U= Arbitrary units. Data was collected from n=4-5 experiments per group. *p<0.05 vs. Control and #p<0.05 vs. ESCM.

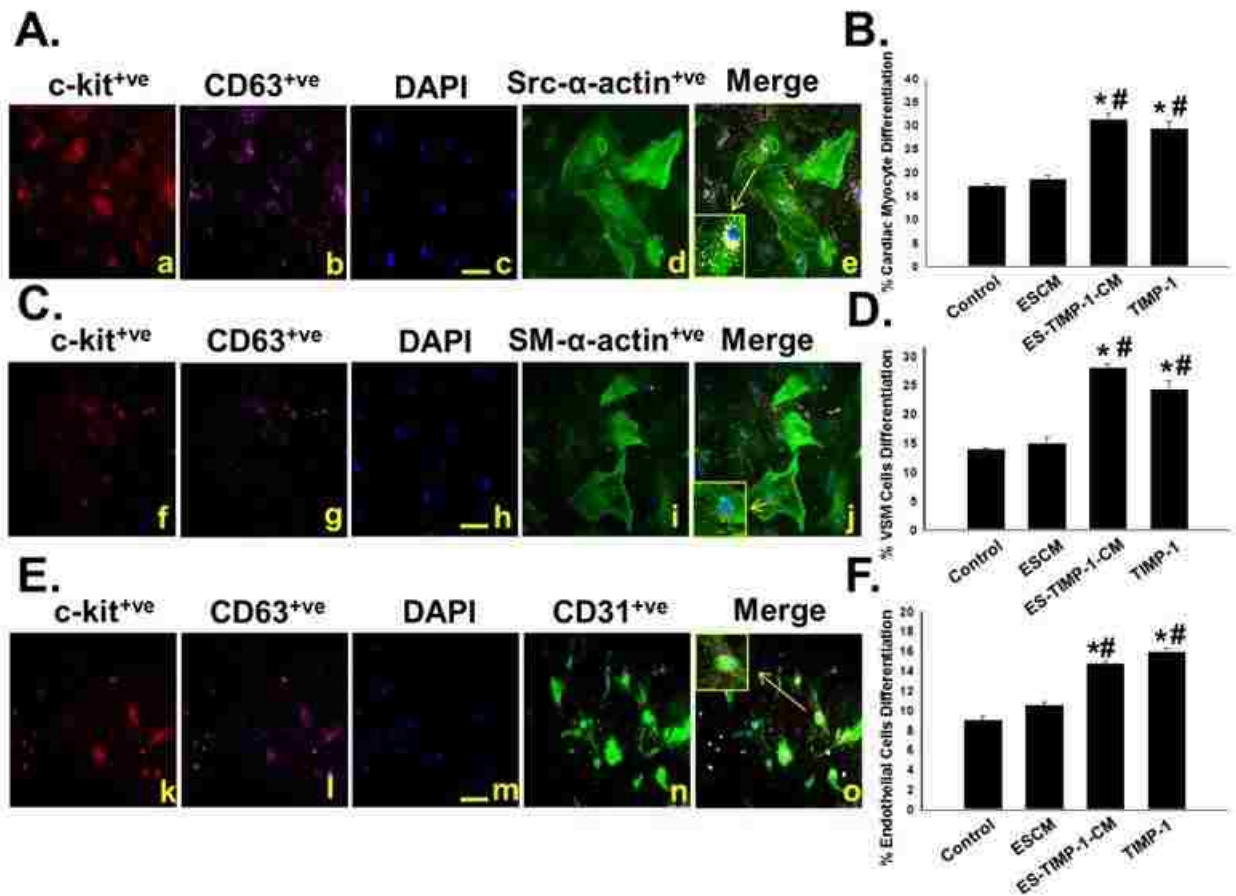


Figure 3. TIMP-1 Enhances CD63⁺/c-kit⁺ CSCs Differentiation into Cardiac Myocytes, VSMs, and ECs. Panels A, C, and E demonstrate confocal representative images of CD63⁺/c-kit⁺ CSCs undergoing cardiac myocyte, VSM cell, and ECs differentiation, respectively, with c-kit⁺ cells shown in red (a, f and k), CD63⁺ cells shown in purple (b, g, and l), total nuclei shown in blue (c, h, and m), Src α -actin⁺, SM α -actin⁺, and CD31⁺ cells shown in green (d, i, and n), and a merged image of all four colors in (e, j, and o). Each merged image contains an enlarged area boxed in the corner. Scale bar = 300 μ m. Figure 3B represents quantitative analysis of percent CD63⁺/c-kit⁺ /Src- α -actin⁺ CSCs over DAPI. Figure 3D represents quantitative analysis of percent CD63⁺/c-kit⁺/SM- α -actin⁺ CSCs over DAPI. Figure 3F represents quantitative analysis of percent CD63⁺/c-kit⁺/CD31⁺ cells over DAPI. Data were collected from n=4-5 experiments per group. *p<0.05 vs. Control and #p<0.05 vs. ESCM.

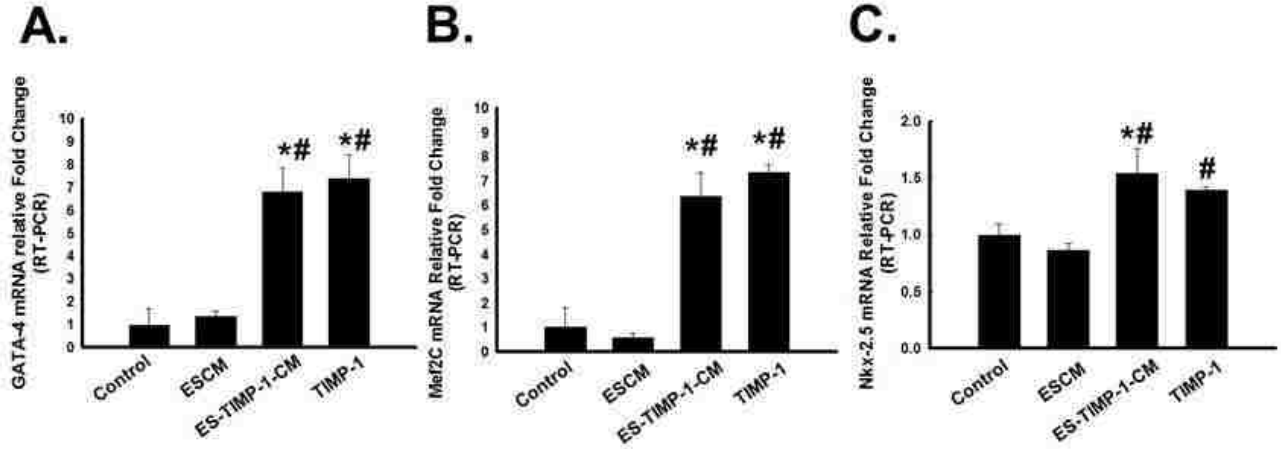


Figure 4. TIMP-1 Enhances Expression of Cardiac Genes in Isolated CD63⁺/c-kit⁺ CSCs. Figure 4 depicts RT-PCR analysis of pro-cardiac markers GATA-4 (A), Mef2C (B), and Nkx-2.5 (C). Data are plotted as mRNA relative fold change vs. control group and normalized against GAPDH. Data were collected from n=4 experiments per group. *p<0.05 vs. Control and #p<0.05 vs. ESCM.

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CHAPTER THREE: TIMP-1 ACTIVATED CD63^{+ve}/C-KIT^{+ve} CARDIAC STEM CELLS DIFFERENTIATE INTO HEART CELL TYPES AND ENHANCE REGENERATION IN THE INFARCTED MYOCARDIUM

Abstract

We previously demonstrated that embryonic stem (ES) cells over-expressing tissue inhibitor of metalloproteinase-1 (TIMP-1) have increased potential to engraft and differentiate into cardiac myocytes following transplantation in the infarcted heart. However, the ability of TIMP-1 to activate endogenous cardiac stem cells (CSCs) and enhance their differentiation potential is still unknown. Based on the observed beneficial effects of TIMP-1 on transplanted ES cells, we postulated that TIMP-1 released by ES-TIMP-1 cells may additionally activate endogenous cardiac stem cells (CSCs) that express both CSC marker (c-kit) and TIMP-1 receptor (CD63) leading to cardiac regeneration in the infarcted myocardium. Accordingly, C57BL/6 and TIMP-1 KO mice underwent coronary artery ligation followed by intramyocardial delivery of 20 μ l of culture media (CC), ES conditioned media (ESCM), ES-TIMP-1-CM or TIMP-1. Immunohistochemistry analysis demonstrated a significant ($p < 0.05$) increase in CD63^{+ve}/c-kit^{+ve} CSCs following ES-TIMP-1-CM and TIMP-1 treatments compared with controls. Interestingly, ratios of CD63^{+ve}/c-kit^{+ve} CSCs were significantly ($p < 0.05$) lower in TIMP-1 KO mice compared to C57BL/6 animals. RT-PCR analysis revealed TIMP-1 KO animals expressed significantly less CD63 and TIMP-1 mRNAs compared to C57BL/6 mice. Additionally, activated CD63^{+ve}/c-kit^{+ve} CSCs were able to differentiate into cardiomyocytes, endothelial and vascular smooth muscle cells. The differentiation rate, however, was more robust in C57BL/6 mice compared to TIMP-1 KO mice. Finally, we demonstrated that CSCs differentiation is regulated by CD63/ β -catenin pathway.

Overall, this study demonstrates that transplantation of TIMP-1 or ES-TIMP-1-CM into the infarcted environment activates CD63^{+ve}/c-kit^{+ve} CSC population enhancing their proliferation and cardiac regenerative potential through the CD63/ β -catenin pathway.

Keywords: Myocardial infarction, TIMP-1, Cardiac stem cells, CD63/ β -catenin pathway.

Introduction

The belief that the adult human heart is a post-mitotic organ incapable of regenerating itself has long been challenged by multiple studies uncovering the existence of small populations of stem cells residing within the myocardium, capable of mitotic activity and differentiation into heart cells (1-3). Despite this, the reported rate of myocyte regeneration is rather low; suggesting a limited regenerative potential of these resident stem cell niches (1; 2; 10; 15; 16). Adult and embryonic stem cell based transplantation therapies have gained much interest in the last decade for their ability to regenerate injured myocardium (4; 7; 14-16; 18; 19; 23). Despite their varied regenerative capabilities, the cell survival rate and differentiation were limited post-transplantation (1; 2; 15; 16). Nonetheless, the heart still benefits from factors released from these cells. These factors were shown to play a role in cell self-renewal [(Insulin Growth Factor (IGF), Hepatic Growth Factor (HGF) and Stem Cell Factor (SCF)], neovascularization [Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor-9 (FGF-9)], and inhibition of adverse cardiac remodeling [Tissue Inhibitor of Metalloproteinases-1 (TIMP-1)] (9; 11; 26-28).

Our published data show that tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits apoptosis and fibrosis in the infarcted heart (11). TIMP-1 Knocked out (KO) mice showed exacerbated cardiac fibrosis two weeks after MI (12, 21). Furthermore, TIMP-1 KO mice treated with exogenous delivery of TIMP-1 or PD-166793, a pharmacological inhibitor of MMPs, was sufficient to reduce cardiac fibrosis (12). Additionally, we previously demonstrated that ES over-expressing TIMP-1 cells have increased potential to engraft and differentiate into cardiac myocytes following transplantation in the infarcted heart (11). However, the ability of TIMP-1 to activate endogenous CSCs and enhance their differentiation potential is still unknown. Based on the observed beneficial effects of TIMP-1 on transplanted ES cells, we postulate that TIMP-1 released by ES-TIMP-1 cells may additionally activate endogenous cardiac stem cells (CSCs) leading to cardiac regeneration (11).

TIMP-1 enhances proliferation of various cell types through regulation of the CD63/ β -catenin signaling pathway (13). CD63 is a cell surface receptor for TIMP-1 and a member of the tetraspanin family. CD63 activation by TIMP-1 induces downstream phosphorylation of β -catenin protein. The activated β -catenin then translocates to the nucleus, interacts with T-cell factor-1 (TCF-1) transcription factor, and causes transcription of target genes Cyclin D1, Survivin and Vascular Endothelial Growth Factor (VEGF). If not activated, β -catenin is ubiquitinated in the cytoplasm and degraded through the activity of the Axin complex (5; 6).

In the previous chapter, we identified a novel cardiac stem cell population, CD63^{+ve}/c-kit^{+ve}, from isolated murine CSCs. These cells were able to proliferate and differentiate into cardiac myocytes, endothelial (ECs) and vascular smooth muscle

(VSM) cells *in vitro* upon TIMP-1 treatment. However, identification of these cells in the MI heart and their ability to promote sufficient cardiac regeneration upon TIMP-1 treatment is yet to be investigated.

The involvement of TIMP-1 and CD63/ β -catenin pathway in cardiac regeneration is not yet understood; therefore, we hypothesized that TIMP-1 enhances cardiac regeneration in the MI heart through targeting a unique CD63^{+ve}/c-kit^{+ve}, CSC population. These cells upon activation undergo cardiac differentiation resulting in overall improved contractile function. Furthermore, we investigated CD63/ β -catenin pathway as the potential downstream mechanism controlling CSCs differentiation in the infarcted heart.

Materials and Methods

Preparation of ESCM and ES-TIMP-1-CM

Embryonic stem (ES) cells overexpressing TIMP-1 factor were generated in our lab as previously described (31). ES and ES-TIMP-1 cells were treated with Dulbecco's Modified Eagle Medium (DMEM) containing leukemia inhibitory factor (LIF) along with other components as we reported. LIF was then removed and cells were cultured for 48 hours on gelatin coated plates. Following incubation, cell media enriched (Conditioned media, CM) with factors released by both ES (ESCM) and ES-TIMP-1 (ES-TIMP-1-CM) cells was collected, filtered, and stored in -20°C (27).

TIMP-1 Preparation

Recombinant mouse tissue inhibitor of metalloproteinases (TIMP-1) was purchased from R&D (cat # 980-MT) as a lyophilizate and reconstituted to a

concentration of 100ug/ml in a mixture of Tris (50mM), CaCl₂ (10mM), NaCl (150mM), and Brij₃₅ (0.05%) dissolved in sterile double distilled water (ddH₂O). Stock solutions were aliquoted into 100µl volumes and stored in -20°C.

Myocardial Infarction and Treatment Administration

C57BL/6 (cat# 000664) and TIMP-1 KO on C57BL/6 background (B6.129S4-*Timp1*^{tm1Pds/J}; Cat# 006243) were purchased from Jackson Laboratories and bred at the University of Central Florida (UCF) transgenic animal facility (TAF). All experiments were in compliance with the UCF Institutional Animal Care and Use Committee (IACUC) guidelines. 8 to 10 weeks old, mice were segregated into five study groups; Sham (n=8), MI + Culture Media (CC; n=8), MI+ESCM (n=8), MI+ESTIMP-1-CM (n=8) and MI+TIMP-1 (n=8). Myocardial infarction (MI) surgery was performed as previously reported (9; 27; 29; 30). In brief, animals were sedated with 4% isoflurane and 2% oxygen through an endotracheal intubation system (Minivent Type 845; Harvard Apparatus). Animals were injected with an intramuscular dose of Buprenorphine (0.5mg/kg) prior to surgery and 2-3 hours post-surgery to aid in pain relief. During the procedure, the chest was shaved and a left thoracotomy was performed. The heart was exposed and visualized under a light microscope to locate the Left Anterior Descending (LAD) coronary artery coming down the left atrium and branching onto the left ventricle. Using a surgical suture needle (Monofilament polypropylene non-absorbable suture, 7/0; cat # 8703P; CP Medical, Portland), a node was made around the LAD and the artery was ligated. Artery ligation was confirmed by visualizing a bleaching area around the ligation site, demonstrating a lack of blood supply to the ventricle. 20µl of CC,

ESCM, ES-TIMP1-CM or TIMP-1 (200µg/kg of body weight) were injected at two sites within the periphery of the bleached area (peri-infarct area) using a 700 Series MICROTITER Syringe supplemented with a gauge floating needle (Hamilton CO., Nevada). Sham operated animals underwent complete surgery; however, the node created around the LAD was left loose. The chest was closed and animals were allowed to recover. Two weeks post-surgery, animals were sacrificed via isoflurane over-exposure and cervical dislocation.

Preparation of Paraffin Sections

Mice hearts were harvested upon sacrifice and subjected to histological evaluation as previously detailed (9; 27; 29; 30). The bottom portion containing both ventricles was fixed in paraformaldehyde (PFA 4%) for 24 to 48 hours, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Resulting blocks were subsequently sectioned using our microtome and 5µm thickness sections were generated and placed on microscope slides (Fisher Scientific). Prior to any staining, sections were incubated for 30 minutes at 60°C to allow extra paraffin to melt. Complete deparaffinization and rehydration was achieved by subsequent dipping in xylene two times for 5 minutes, xylene: absolute alcohol (50:50), absolute alcohol (100%), and 90, 70, 50 and 30% ethyl alcohol solutions for 5 minutes each. Tissues were finally washed in distilled water and phosphate-buffered saline (PBS 1X) for an additional 5 minutes.

Immunostaining Identification of CD63⁺/c-kit⁺ CSCs in the Heart

To determine the existence of a subset CSC population expressing CD63 marker in the murine heart, a double immunostaining for c-kit (CSCs marker) and CD63 (TIMP-

1 receptor) was performed on tissue sections from 7 to 8 animals per group from both C57BL/6 and TIMP-1 KO mice. In brief, sections were incubated with 10% normal goat serum (NGS; Vector Laboratories) for 1 hour to ensure blockage of nonspecific sites followed by one hour at room temperature or overnight at 4°C incubation with anti-c-kit (1:50; Santa Cruz), or anti-MLA-CD63 (1:500; Bios) rabbit monoclonal antibodies diluted in 10% NGS. Sections were then washed and incubated for 1 hour with secondary antibody goat anti-rabbit Alexa Fluor 568 for c-kit and 660 for CD63 at a concentration of 1:20 diluted in 1x PBS. Total nuclei were detected by mounting each section with Antifade Vectashield medium containing 4',6-diamino-2-phenylindole (DAPI; Vector Laboratories). Olympus and confocal fluorescence microscopes were used to visualize and quantify CD63⁺/c-kit⁺ cells over total nuclei. Four representative areas per section were taken and quantified using Image J software. Data is presented as % of CD63⁺/c-kit⁺ cells/total DAPI.

Immunostaining Determination of CD63⁺/c-kit⁺ CSCs Differentiation.

To verify whether ES-TIMP-1CM and TIMP-1 treatments can trigger CSC differentiation into cardiac myocytes (CM), vascular smooth muscle (VSM) and endothelial cells (ECs) in the MI heart, previously double stained CD63⁺/c-kit⁺ cells were triple stained with either sarcomeric (Src) α -actin, smooth muscle (SM) α -actin, or CD31, respectively (11; 29; 30). After 1 hour incubation in NGS, sections were incubated with 1:25 dilution of rabbit anti-CD31 monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) prepared in 10% NGS, followed by FITC-conjugated goat anti-rabbit IgG secondary antibodies (Invitrogen) diluted at 1:50 in PBS for 60

minutes. For both Src and SM α -actin staining, mouse anti-mouse kit (MOM, Vector Laboratories) was used. In brief, sections were incubated for 1 hour in MOM blocking reagent followed by 5 minutes in protein working solution (PWS) provided in the kit. Next, anti-SM and anti-Src α -actin antibodies (1:50 and 1:100 respectively, Sigma) were diluted in PWS and exposed on the top of the sections for 30 min followed by IgG antibody and fluorescein avidin solution for 5 and 10 min each.

Finally, triple stained sections were mounted with Vectashield antifade medium containing DAPI (Vector Laboratories) for nuclear visualization. Four representative images per slide were taken using Olympus and confocal microscopes and Image J software was used to quantify cardiac cell differentiation using these formulas: % CM differentiation = triple Src α -actin^{+ve}/c-kit^{+ve}/CD63^{+ve} cells over total Src α -actin cells x 100. % VSM differentiation = triple SM α -actin^{+ve}/c-kit^{+ve}/CD63^{+ve} cells over total vascular nuclei x 100. % ECs differentiation = triple CD31^{+ve}/c-kit^{+ve}/CD63^{+ve} cells over total vascular nuclei x 100. Of note, representative pictures in Figure 6 were taken from the peri-infarct zone of the heart where we localized CSCs differentiating into cardiac myocytes.

Real-Time PCR for CD63 and TIMP-1 mRNAs

Total RNA was extracted using TRI® reagent protocol (Cat # T9424; Sigma Aldrich). In brief, heart pieces from different study groups were homogenized using 1ml TRI reagent in an RNase free environment followed by a separation step in chloroform and RNA precipitation in isopropyl alcohol. RNA purity and concentration was verified using spectrophotometer analysis from absorbance readings of 260 and 280nm. An

absorbance ratio of A260/A280 ranging between 1.8 to 2.0 was considered pure. cDNA was synthesized from 1 µg total RNA using Superscript II reverse transcriptase and random primers (Bio-Rad). Next, Real-time quantitative PCR was performed using a CFX96™ Real-Time System (Bio Rad). Supermix 2X; Life Technologies containing FAM fluorescent dye and dNTPs in a reaction buffer was added to the reaction mixture containing cDNA template, forward, and reverse primers. CD63, TIMP-1 and GAPDH primer assays were purchased from Life Technologies. The following cycling conditions were used: 95°C for 5 min followed by 35 amplification cycles at 95°C to denature for 15 seconds and 60°C for combined annealing/extension for 60 seconds. The relative fold expression of CD63 and TIMP-1 in each group was normalized against GAPDH and plotted.

Western Blots for CD63, β -catenin and phospho- β -catenin Proteins

To determine the effect of TIMP-1 on CD63/ β -catenin pathway, protein level was measured on heart homogenates taken from all treatment groups and both mouse strains using SDS-PAGE. In brief, an equal amount of protein (100ug) was loaded on a 10% polyacrylamide gel and subjected to electrophoresis at 150V for 1 hour. The gel was then transferred for 45 min to a polyvinylidene fluoride membrane (PVDF; BioRad) presoaked in methanol and transfer buffer using a semi-dry Immuno-Blot (Bio-Rad) at 15V current. The membranes were then blocked with 5% skim milk for 1 hour and incubated with primary rabbit antibodies (CD63/MLA1; cat # ABIN687742, Antibodies), (β -catenin (6B3) ; cat # 9582, and phospho- β -catenin (Ser33/37/Thr41; cat # 9561, Cell signaling) and (β -actin; cat # 4967L from Cell Signaling) for one hour on an orbital

shaker at room temperature or at 4°C overnight. Membranes were then incubated with anti-rabbit IgG horseradish peroxidase secondary antibody (Cat # 7074S; Cell Signaling) for one hour, and blots were detected with Pierce® ECL Western Blotting Substrate (Thermo Scientific) (22).

Statistical Analysis

All data presented in this study were subjected to statistical analysis using SigmaStat 3.5 software. One-way analysis of variance (ANOVA) was run for all experiments followed by Tukey test and statistical results were presented as a mean ± standard error of the mean (SEM). The difference between two study groups was considered statistically significant if the $P < 0.05$.

Results

ES-TIMP-1-CM and TIMP-1 Enhance Endogenous CD63⁺/c-kit⁺ CSCs Proliferation in the Infarcted Myocardium

The effect of transplanted ES-TIMP-1-CM and TIMP-1 on proliferation of endogenous CD63⁺/c-kit⁺ CSCs was examined in the infarcted TIMP-1 KO and C57BL/6 hearts. Our c-kit cell specific staining combined with CD63 confirms that following intramyocardial delivery of ES-TIMP-1-CM and TIMP-1, percent of CD63⁺/c-kit⁺ CSCs significantly increases ($p < 0.05$, ES-TIMP-1-CM: 2.05%±0.33% and TIMP-1: 1.46%±0.14%) compared with (Sham: 0.48%±0.11% and MI+CC: 1.14%±0.12%) in C57BL/6 mice. TIMP-1 KO mice showed similar pattern but with lower ratios ($p < 0.05$, ES-TIMP-1-CM 1.52%±0.24% and TIMP-1: 0.86%±0.1%) compared with (Sham: 0.29%±0.08% and MI+CC: 0.53%±0.05%) (Figure 5).

ES-TIMP-1-CM and TIMP-1 Enhance Endogenous CD63⁺/c-kit⁺ CSCs Differentiation into CMs, VSMs, and ECs in the Infarcted Myocardium

The effect of transplanted ES-TIMP-1-CM and TIMP-1 on endogenous CD63⁺/c-kit⁺ CSCs differentiation into CM, VSM, and ECs was examined in the infarcted TIMP-1 KO and C57BL/6 hearts. Triple immunostaining targeting newly differentiated CMs from CSCs expressing c-kit, CD63 and Src α -actin was performed. Our quantitative data revealed no significant alteration in cardiac myocyte differentiation in MI and MI+ESCM treatment compared to control in both TIMP-1 KO and C57BL/6 mice, while enhanced cardiac myocyte differentiation was observed following ES-TIMP-1-CM and TIMP-1 treatments relative to controls ($p < 0.05$, ES-TIMP-1-CM; 24.72% \pm 1.32% and TIMP-1; 22.91% \pm 1.03%) against (Sham; 6.93% \pm 0.59%, MI+CC; 8.91% \pm 0.75%, and ESCM; 10.18% \pm 0.82%) in C57BL/6 and ($p < 0.05$, ES-TIMP-1-CM; 14.72% \pm 0.71% and TIMP-1; 11.35% \pm 0.86%) against (Sham; 4.35% \pm 0.64%, MI+CC; 4.36% \pm 0.35%, and ESCM; 6.48% \pm 1.65%) in TIMP-1 KO mice (Figure 6).

One of the major cardiac cell types lost during a myocardial event are VSM cells, which make up cardiac vasculature (29). We aimed at investigating the ability of ES-TIMP-1-CM or TIMP-1 to promote their regeneration from CD63⁺/c-kit⁺ CSCs using a triple immunostaining targeting newly differentiating CSCs that express three markers; c-kit, CD63 and SM- α -actin (Figure 7). C57BL/6 data revealed decreased sm- α -actin cells in MI+CC group compared to control animals, although not significant (MI+CC; 5.81% \pm 1.59% vs Sham; 8.78% \pm 1.77%). Conversely, TIMP-1 KO animals showed a slight increase in VSMs differentiation in MI+CC and ESCM against sham animals, with no significance (MI+CC; 3.14% \pm 0.46%, and ESCM; 3.36% \pm 0.21% against Sham;

1.69%±0.22%). ES-TIMP-1-CM and TIMP-1 treatments both significantly ($P<0.05$) increased smooth muscle differentiation compared to MI+CC and Sham groups in both strains ($p<0.05$, ES-TIMP-1-CM; 23.47%±5.30% and TIMP-1; 19.13%±2.66%) in C57BL/6 and ($p<0.05$, ES-TIMP-1-CM; 7.17%±1.34% and TIMP-1; 5.43%±0.28%) in TIMP-1 KO mice against controls (Figure 7C).

Endothelial cells are important components of cardiac vasculature (29). Thus, we aimed at analyzing their regeneration rate from CD63⁺/c-kit⁺ CSCs in the same aforementioned conditions. A triple immunostaining targeting newly differentiating CSCs expressing c-kit, CD63 and CD31 was performed. C57BL/6 data revealed significant depletion of CD63⁺/c-kit⁺ CSCs positive for CD31 marker in MI+CC compared to Sham ($P<0.05$, MI+CC; 4.99%±0.3% vs Sham; 6.91%±0.64%). This decrease was significantly ($p<0.05$) reverted by ESCM, ES-TIMP-1-CM and TIMP-1 treatments ($P<0.05$, ESCM; 7.86%±0.1%, ES-TIMP-1-CM; 11.32%±0.42% and TIMP-1; 10.20%±0.47%). Of note, ES-TIMP-1-CM treatment showed the strongest effect on endothelial cell differentiation when compared to sham, MI+CC and ESCM groups. TIMP-1 KO data demonstrated no significant change in MI+CC and ESCM groups compared to Sham; however, ES-TIMP-1-CM and TIMP-1 significantly increased ratios of CD63⁺/c-kit⁺ CSCs differentiating into ECs compared to MI+CC and Sham groups ($P<0.05$, ES-TIMP-1-CM; 10.78%±0.92% and TIMP-1; 9.03%±0.51% vs MI+CC; 5.65%±0.43% and Sham; 5.55%±0.39%) (Figure 8C). Taken all together, differentiation data demonstrate ratios of CD63⁺/c-kit⁺ cells positive for CM, VSM, and ECs markers are significantly elevated following ES-TIMP-1-CM and TIMP-1 treatments in both C57BL/6 and TIMP-1 KO mice compared to their controls, suggesting TIMP-1 may have

activated endogenous CD63⁺/c-kit⁺ CSCs and enhanced their differentiation into CMs, VSMs and ECs in both C57BL/6 and TIMP-1 KO mice (Figure 6-8).

Representative confocal images demonstrating c-kit/CD63⁺ CSCs differentiation into CMs, VSMs, and ECs are shown in Figure 6, 7 and 8 respectively. In each figure, panel A represents C57BL/6 representative figures and panel B depicts TIMP-1 KO representative images. Each panel demonstrates CD63⁺ cells in purple (a,f,k,p and u), c-kit⁺ in red (b,g,l,q and v), Src- α -actin, SM or CD31⁺ in green (c,h,m,r and w), total nuclei in blue (d,i,n,s and x), and merged images (e,j,o,t, and y). Enlarged images of triple stained cells are depicted at the margin of merged images (Figures 6-8).

Effect of ES-TIMP-1-CM and TIMP-1 on Endogenous TIMP-1 and CD63 mRNAs Expression

To determine effect of ES-TIMP-1-CM and TIMP-1 delivery on endogenous TIMP-1 and CD63 expression, we performed RT-PCR analysis for both mRNAs on heart tissue specimens collected from TIMP-1 KO and C57BL/6 animals. RT-PCR data shows near null expression of TIMP-1 mRNA in TIMP-1 KO mice compared to wild type (Figure 9A). In parallel, levels of CD63 mRNA significantly dropped in the TIMP-1 deficient mice compared to C57BL/6 ($p < 0.05$, TIMP-1 KO; 0.05 ± 0.01 vs C57BL/6; 1 ± 0.0), suggesting a strong correlation between TIMP-1 and CD63 expression in the heart (Figure 9B). Next, we compared expression levels of TIMP-1 and CD63 following MI and exogenous delivery of ESCM, TIMP-1 and ES-TIMP-1-CM treatments. Our data show no effect of any treatments on TIMP-1 mRNA levels in TIMP-1 KO animals. However, C57BL/6 mice showed a noticeable decrease in TIMP-1 expression in MI+CC group followed by 2 to 4 fold increase upon ES-TIMP-1-CM and TIMP-1 treatments,

respectively (Figure 9C). Next, CD63 mRNA data demonstrate a slight decrease in CD63 mRNA expression following MI+CC and ESCM treatment; however, no significance was achieved relative to sham group. ES-TIMP-1-CM and TIMP-1 treated C57BL/6 mice induced 4 and 2.5 fold increase in CD63 expression compared to MI+CC and sham C57BL/6 animals. Ratios of CD63 in TIMP-1 KO animals followed the same trend described in C57BL/6 mice; however, at much lower ratios (TIMP-1 KO; ES-TIMP-1-CM; 0.07 ± 0.03 and TIMP-1; 0.07 ± 0.04) vs (C57BL/6 ES-TIMP-1-CM; 3.98 ± 0.92 and TIMP-1; 2.21 ± 0.6) (Figure 9D).

Mechanism of TIMP-1 Mediated CD63⁺/c-kit⁺ CSCs Regeneration in the Infarcted Myocardium

We compared the effect of TIMP-1 delivery on CD63, phospho (P), and total β -catenin proteins on heart homogenates (Figure 10). Western blot results on TIMP-1 KO mice showed significantly ($p<0.05$) decreased CD63 levels in MI+CC and MI-ESCM groups compared to control. This decrease was drastically ($p<0.05$) reversed in ES-TIMP-1-CM and TIMP-1 treatments (Figure 10D). C57BL/6 mice showed a slight decrease in CD63 levels in MI+CC group against control; however, no significance was achieved. Additionally, ESCM and TIMP-1 treatments slightly increased CD63 expression without reaching significance. Nonetheless, ES-TIMP-1-CM treatment showed a strong increase ($p<0.05$) in CD63 levels (Figure 10A). Next, we measured total β -catenin protein levels (Figure 10B and E). TIMP-1 KO data showed a significant decrease in β -catenin protein in MI+CC and ESCM groups relative to sham, however, upon ES-TIMP-1-CM and TIMP-1 treatments, the decrease in β -catenin significantly improved compared to MI+CC and MI+ESCM animals (Figure 10E). C57BL/6 western

blot analysis revealed no significant change in all study groups except ES-TIMP-1-CM which showed significantly ($p < 0.05$) increased β -catenin protein expression as compared to sham and MI+CC animals (Figure 10B). Finally, we evaluated the ability of exogenous TIMP-1 to activate β -catenin. Densitometry analysis of (P)- β -catenin levels in TIMP-1 KO mice demonstrated a drastic decrease ($p < 0.05$) in (P)- β -catenin levels in MI+CC and ESCM groups compared to sham; however, ES-TIMP-1-CM and TIMP-1 treatments largely increased ($p < 0.05$) (P)- β -catenin levels (Figure 10F). C57BL/6 hearts showed no significant variation in (P)- β -catenin levels in Sham, MI+CC and MI+ESCM animals; however, upon ES-TIMP-1-CM and TIMP-1 treatments, activated (P)- β -catenin levels strongly ($p < 0.05$) increased (Figure 10C). Overall, this data suggest ES-TIMP-1-CM and TIMP-1 delivery activated CD63/ β -catenin pathway following MI.

Discussion

Stem cell transplantation has largely been utilized as a source for cardiac regeneration following MI. These cells include ES, Bone Marrow (BM) derived stem cells, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and umbilical-cord-blood-derived mesenchymal stem cells (UCB-MSCs) (1; 2). The results from these experiments were mitigated by inconsistent cell survival following transplantation and a limited trans-differentiation rate given their non-cardiogenic origin (1; 2; 14-16; 18; 19; 25). CSCs are thus, prime candidates for cardiac transplantation given their cardiac differentiation potential. Published work demonstrated intramyocardial delivery of cardiac progenitor cells (CPCs) to the injured rat myocardium differentiate into cardiac myocytes and vascular cells (24). Activation of endogenous CSCs was additionally

suggested to occur through paracrine factors released by transplanted CPCs; however, the nature of these factors was yet to be determined.

We previously investigated the ability of ES over-expressing TIMP-1 cells to regenerate the lost myocardium in the murine model for MI (11). Data collected from this study demonstrated enhanced cardiac regeneration regardless of the low engraftment rate of transplanted cells. We therefore hypothesized that TIMP-1 released by transplanted ES cells might target a subset of endogenous CSCs which express TIMP-1 receptor (CD63) and trigger their differentiation into the three main cardiac populations; cardiac myocytes, smooth muscle, and endothelial cells.

To address this hypothesis, we performed MI surgery on TIMP-1 KO and C57BL/6 mice followed by intramyocardial delivery of ESCM, ES-TIMP-1-CM or recombinant TIMP-1 protein. ESCM, TIMP-1, and ES-TIMP-1-CM groups were performed to compare the effect of factors released by stem cells, TIMP-1, or both respectively. We stipulated that TIMP-1 alone would be sufficient to activate CD63⁺/c-kit⁺ CSCs and regenerate the damaged myocardium. Our data indeed demonstrated that ESCM performed less than TIMP-1 and ES-TIMP-1-CM did not provide a better outcome when compared to TIMP-1 alone, demonstrating that other factors do not play a role in CD63⁺/c-kit⁺ activation. Our collected data demonstrate *in vivo* delivery of ES-TIMP-1-CM or TIMP-1 equivalently: 1) enhance CD63⁺/c-kit⁺ CSCs proliferation and differentiation into cardiac myocytes, endothelial and vascular smooth muscle cells, 2) promote additional CD63 expression in the heart, 3) up regulate phospho and total β -catenin expression. This study additionally demonstrates that TIMP-1 deletion significantly reduces the number of CD63⁺/c-kit⁺ CSCs population in the heart and

exogenous delivery of TIMP-1 is sufficient to replenish their pool. We believe that endogenous TIMP-1 triggers expression of CD63 receptor on c-kit positive cells as demonstrated by the dramatic decrease in CD63 mRNA expression in TIMP-1 KO hearts. Furthermore, our data shows TIMP-1 enhances CD63⁺/c-kit⁺ CSCs proliferation which explains the decreased number of CD63⁺/c-kit⁺ cells in the heart of TIMP-1 KO vs. C57 control animals. This finding is in accordance with prior study suggesting paracrine factors released by transplanted CPCs activate endogenous CPCs (64), and previously published data suggesting TIMP-1 released by stem cells mediates human mesenchymal stem cell (hMSC) proliferation and differentiation (13).

Next, we provide evidence that CD63⁺/c-kit⁺ CSC activation is mediated through CD63/ β -catenin pathway as suggested by increased CD63, β -catenin and phospho- β -catenin expression in MI hearts treated with ES-TIMP-1-CM and TIMP-1. Enhancement of β -catenin and phospho- β -catenin expression in C57BL/6 infarcted heart mice after ES-TIMP-1-CM and TIMP-1 treatment and/or their rescuing after these same interventions in TIMP-1 KO mice might be explained by activation of CD63 which induces dephosphorylation of Axin complex preventing β -catenin ubiquitination and degradation. Additionally, released β -catenin is subsequently activated by phosphorylation prior to nucleus translocation. We additionally stipulate that endogenous TIMP-1 triggers expression of additional CD63 receptor on c-kit positive cells by activating CD63/ β -catenin pathway in a positive feedback loop. Furthermore, our data show TIMP-1 enhances CD63⁺/c-kit⁺ CSCs proliferation which additionally explains the increased amount of available CD63 mRNA as well as the dramatic decrease in CD63 mRNA expression in TIMP-1 KO hearts.

Activated CD63/ β -catenin pathway and subsequent enhanced cardiac differentiation following TIMP-1 administration contradicts published work attesting TIMP-1 enhances cell proliferation but hinders cardiac differentiation in hMSCs through blockage of the CD63/ β -catenin pathway (8). Nonetheless, our results are in accordance with multiple other studies demonstrating ability of ES cells to differentiate into cardiac cells through activation of β -catenin pathway and subsequent expression of cardiac genes, such as Nkx2.5, β -myosin heavy chain (β -MHC) and cardiac troponin T (cTnT) (17; 20).

In the present study, we choose to identify c-kit^{+ve} CSCs because they represent the most prevalent and extensively characterized subset of CSCs pool. Other CSCs involve side population (SP) cells, cardiac progenitor cells (CPCs), Sca-1^{+ve} cells, cardio sphere cells and Isl1^{+ve} cells, which are less abundant and poorly characterized (2). It is therefore, imperative to identify the relative contribution of CD63^{+ve} CSCs that are not c-kit^{+ve}. CD63 was also shown to be expressed in adult cardiac myocytes and vascular endothelial cells (13). These cells might have differentiated from CD63^{+ve}/c-kit^{+ve} CSC origin and kept CD63 marker expression when fully differentiated. Furthermore, we and others have previously demonstrated that TIMP-1 reduces cardiac apoptosis, fibrosis and enhances cell proliferation following MI; however, not much is known about the molecular mechanism mediating this protective effect and whether ES-TIMP-1-CM or ESCM would provide a better protection (11). In the current paper we stipulate that the anti-apoptotic, anti-fibrotic and proliferative role of TIMP-1 in the heart is most likely mediated through CD63 receptor maintained on these cells. Additional protective

mechanisms mediated by other factors present in the conditioned media are also very likely. Nonetheless, future studies are warranted to support or refute this hypothesis.

In conclusion, our data demonstrate for the first time existence of a unique subset of CSCs in the peri-infarct area, double positive for c-kit and CD63 markers. These cells can be triggered to differentiate into cardiac cells by exogenous delivery of TIMP-1 factor or ES-TIMP-1-CM. Stimulation of CD63⁺/c-kit⁺ CSCs leads to enhanced cardiac regeneration. Additionally, we provide evidence that CD63⁺/c-kit⁺ CSCs activation is mediated through CD63/ β -catenin pathway. This paper demonstrates for the first time that besides the anti- cardiac remodeling effect of TIMP-1, this molecule has a strong regenerative potential. This multifaceted characteristic of TIMP-1 makes it a suitable candidate for MI patients.

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Figures

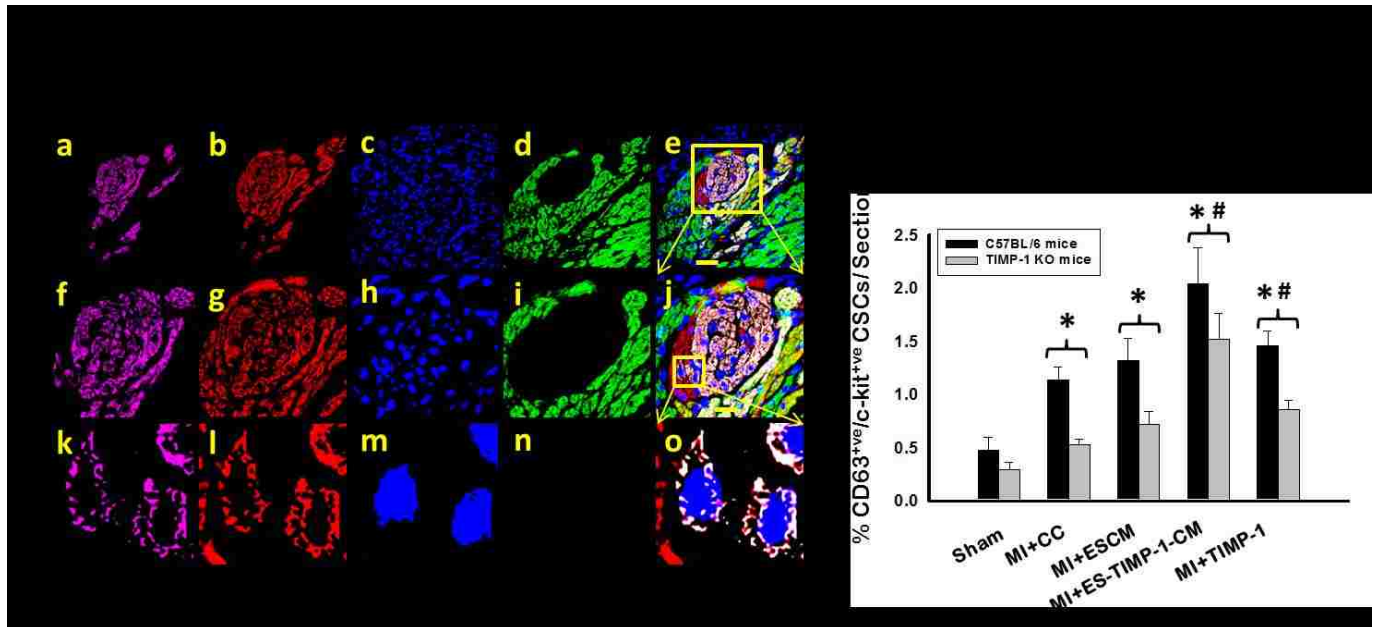


Figure 5. ES-TIMP-1-CM and TIMP-1 Enhance Endogenous CD63^{ve}/c-kit^{ve} CSCs Proliferation in the Infarcted Myocardium. A triple immunostaining targeting CD63^{ve}/c-kit^{ve} CSCs niche and sarcomeric (Src) α -actin (cardiac myocytes marker) was performed and representative confocal images are shown in Figure 5A. CD63^{ve} cells are shown in purple (a,f, and k), c-kit^{ve} in red (b,g and l), Src- α -actin^{ve} in green (d,i and n), total nuclei in blue (c, h and m), and merged images (e,j and o). Scale bar = 50 μ m (a-e) and 25 μ m (f-j). Enlarged images of double stained cells are depicted in (k-o). Ratio of double positive CD63^{ve}/c-kit^{ve} cells over total DAPI was quantified on C57BL/6 and TIMP-1 KO mice and results are plotted in Figure 5B. Data was collected from n=5 animals per group and 1 section per animal. *p<0.05. vs Sham, #p<0.05 vs MI+CC and \$ p<0.05 vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

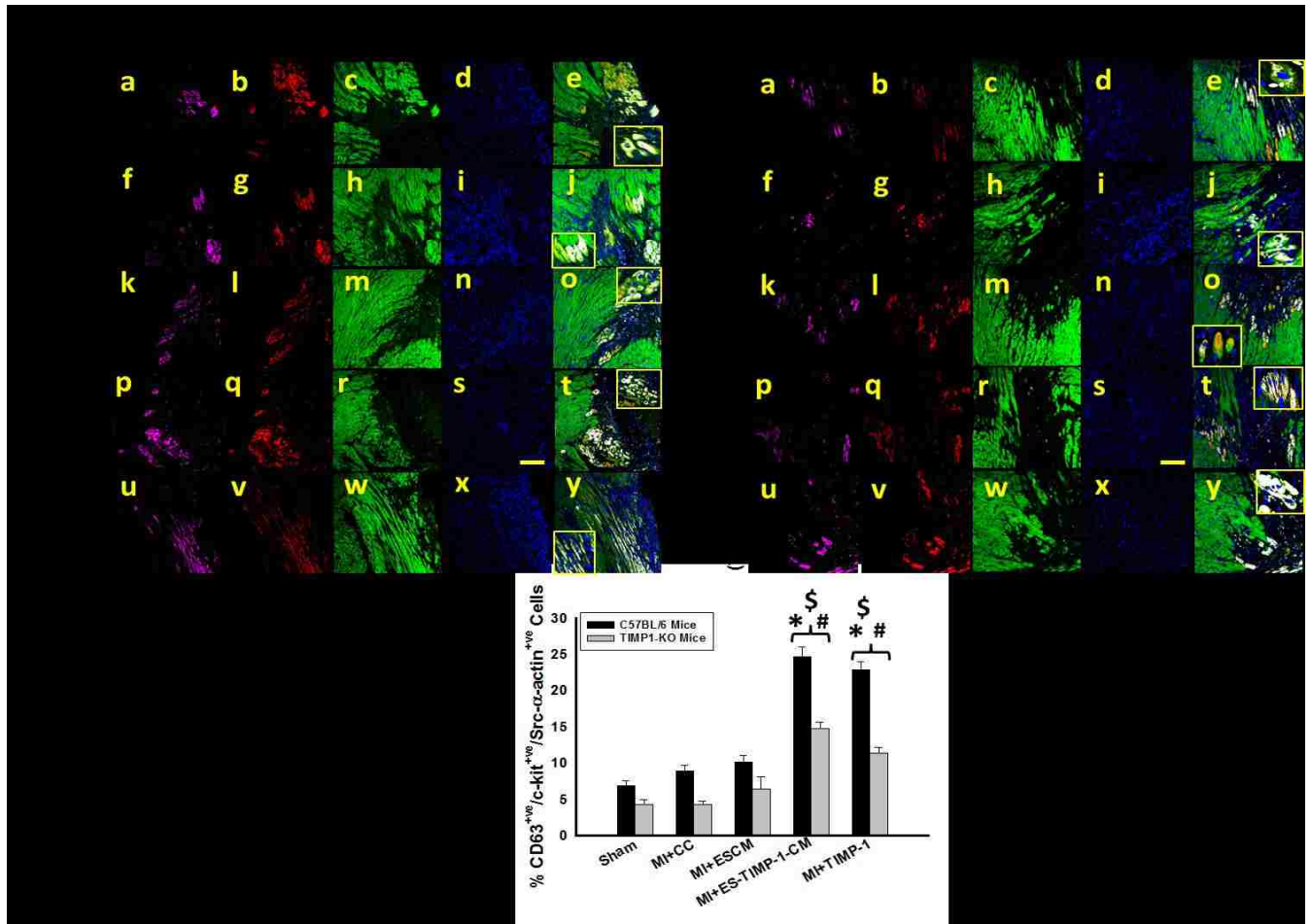


Figure 6. ES-TIMP-1-CM and TIMP-1 Enhance Cardiac Myocyte Differentiation of CD63^{+ve}/c-kit^{+ve} CSCs in the Infarcted Myocardium. A triple immunostaining targeting newly differentiated CSCs expressing the three markers; c-kit, CD63 and sarcomeric (Src) α -actin (cardiac myocytes marker) was performed and representative confocal images are shown in Figure 6A for C57BL/6 and Figure 6B for TIMP-1 KO. CD63+ve cells are shown in purple (a,f,k,p and u), c-kit +ve in red (b,g,l,q and v), Src- α -actin +ve in green (c,h,m,r and w), total nuclei in blue (d,i,n,s and x), and merged images (e,j,o,t, and y). Scale bar = 25 μ m. Enlarged images of triple stained cells are depicted at the margin of merged images. Figure 6C shows % CD63^{+ve}/c-kit^{+ve} CSCs positive for sarcomeric α -actin. Data was collected from n=5 animals per group and 1 section per animal. *p<0.05. vs Sham, #p<0.05 vs MI+CC and \$ p<0.05 vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

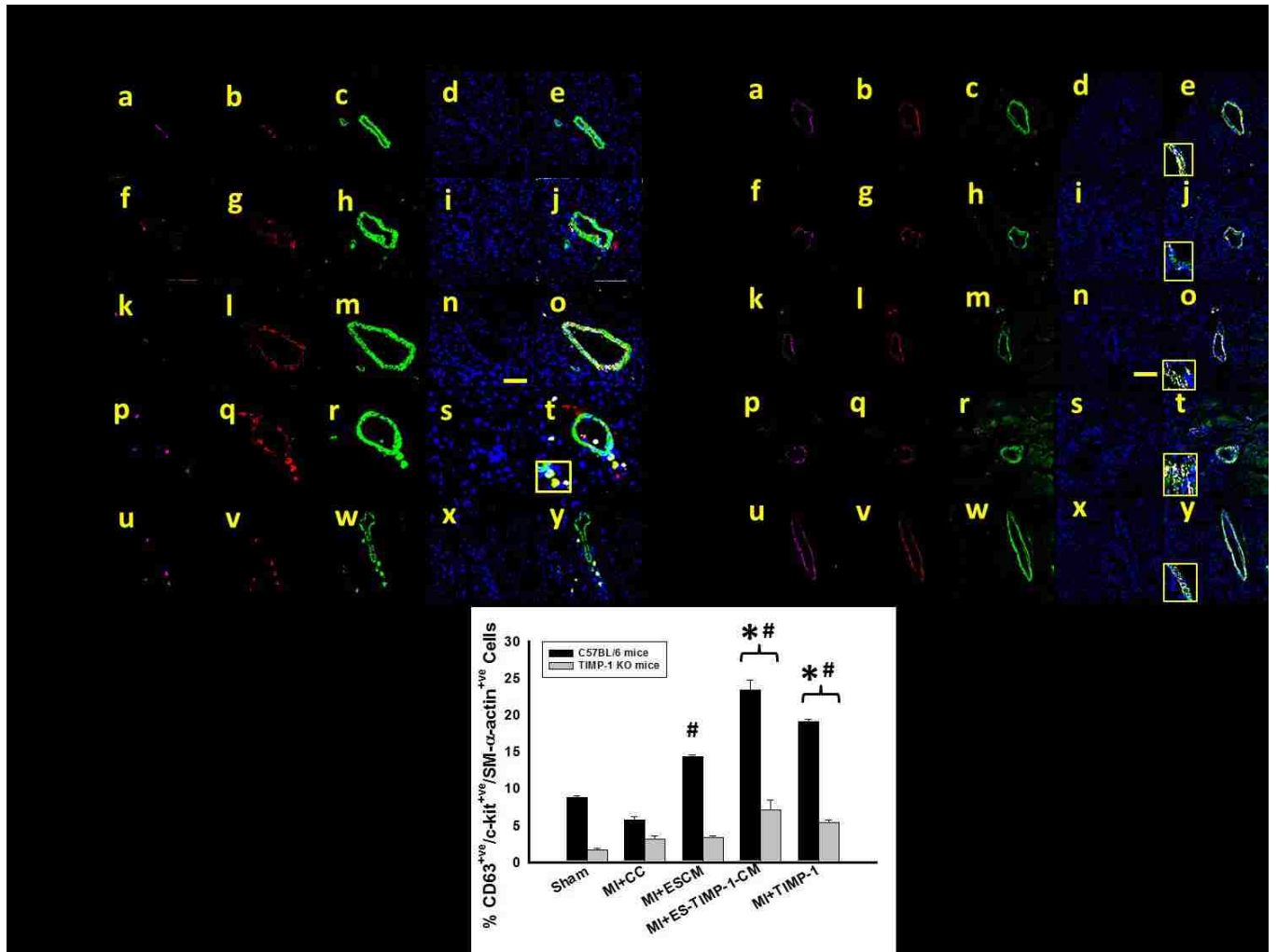


Figure 7. ES-TIMP-1-CM and TIMP-1 Enhance Smooth Muscle Differentiation of CD63⁺/c-kit⁺ CSCs in the Infarcted Myocardium. A triple immunostaining targeting newly differentiated CSCs expressing the three markers, c-kit, CD63 and smooth muscle (SM) α -actin was performed. Representative confocal images are shown in Figure 7A for C57BL/6 and Figure 7B for TIMP-1 KO. CD63⁺ cells are shown in purple (a,f,k,p and u), c-kit⁺ in red (b,g,l,q and v), Sm- α -actin⁺ in green (c,h,m,r and w), total nuclei in blue (d,i,n,s and x), and merged images (e,j,o,t, and y). Scale bar = 25 μ m. Enlarged images of triple stained cells are depicted at the margin of merged images. Histogram 7C shows quantitative data of % CD63⁺/c-kit⁺ also positive for smooth muscle cell marker. Data was collected from n=5 animals per group and 1 to 2 section per animal. *p<0.05. vs Sham, #p<0.05 vs MI+CC and \$ p<0.05 vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

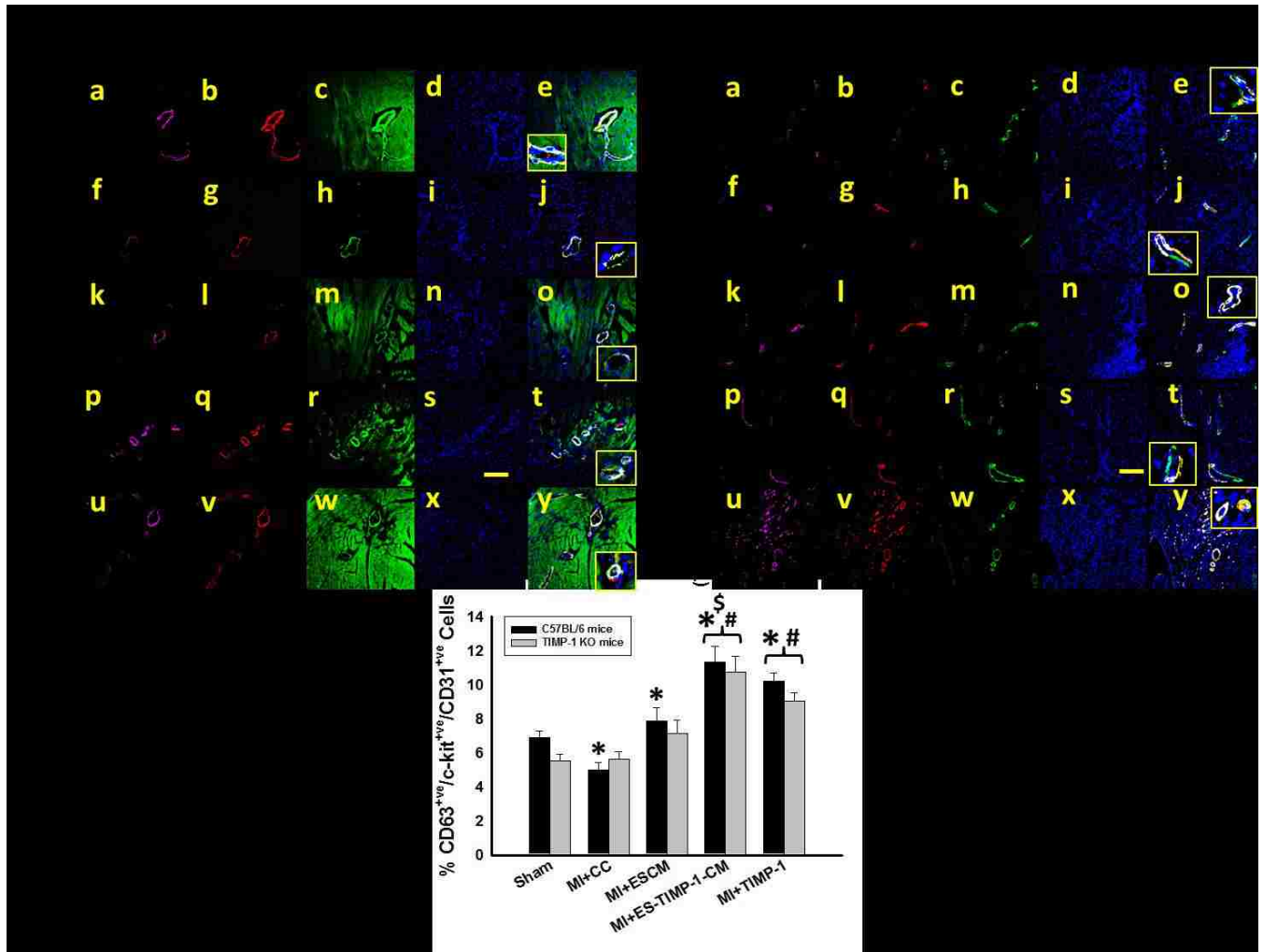


Figure 8. ES-TIMP-1-CM and TIMP-1 Enhance Endothelial Cell Differentiation of CD63⁺/c-kit⁺ CSCs in the Infarcted Myocardium. Figure 8 depicts confocal representative images taken from a triple immunostaining targeting newly differentiated CSCs expressing the three markers: c-kit, CD63 and CD31. Figure 8A represents C57BL/6 experiment and Figure 8B for TIMP-1 KO. CD63⁺ cells are shown in purple (a,f,k,p and u), c-kit⁺ in red (b,g,l,q and v), CD31⁺ in green (c,h,m,r and w), total nuclei in blue (d,i,n,s and x), and merged images (e,j,o,t, and y). Scale bar =25µm. Enlarged images of triple stained cells are depicted at the margin of merged images. Histogram 8C shows quantitative data of % CD63⁺/c-kit⁺ CSCs also positive for endothelial cell marker. Data was collected from n=5 animal per group and 1 to 2 section per animal. *p<0.05. Vs Sham, #p<0.05 vs MI+CC and \$ p<0.05 vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

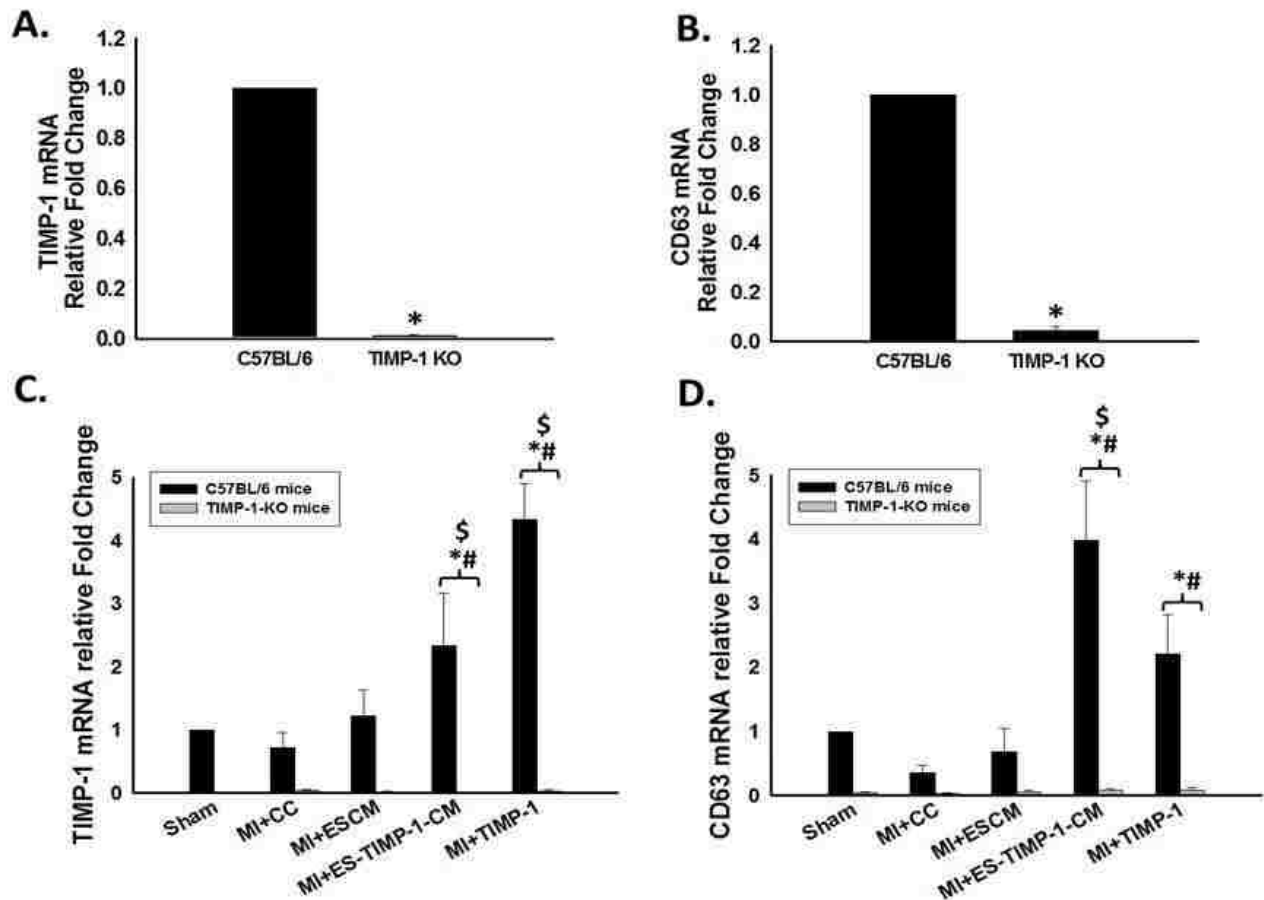


Figure 9. Effect of ES-TIMP-1-CM and TIMP-1 on Endogenous TIMP-1 and CD63 mRNAs Expression in the Infarcted Myocardium. CD63 and TIMP-1 mRNA levels were measured on heart tissue specimens collected from C57BL/6 and TIMP-1 KO control animals. Quantitative RT-PCR relative fold change data for TIMP-1 mRNA is plotted in Figure 9A and CD63 mRNA in Figure 9B. Histograms 9C and 9D demonstrate levels of these two molecules in MI+CC and MI plus exogenous delivery of ESCM, TIMP-1 and ES-TIMP-1-CM treatments in both animal models. Data was collected from n=5 samples per group for TIMP-1 and n=6 to 7 for CD63 measurements. *p<0.05. vs Sham, #p<0.05 vs MI+CC and \$ p<0.05 vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

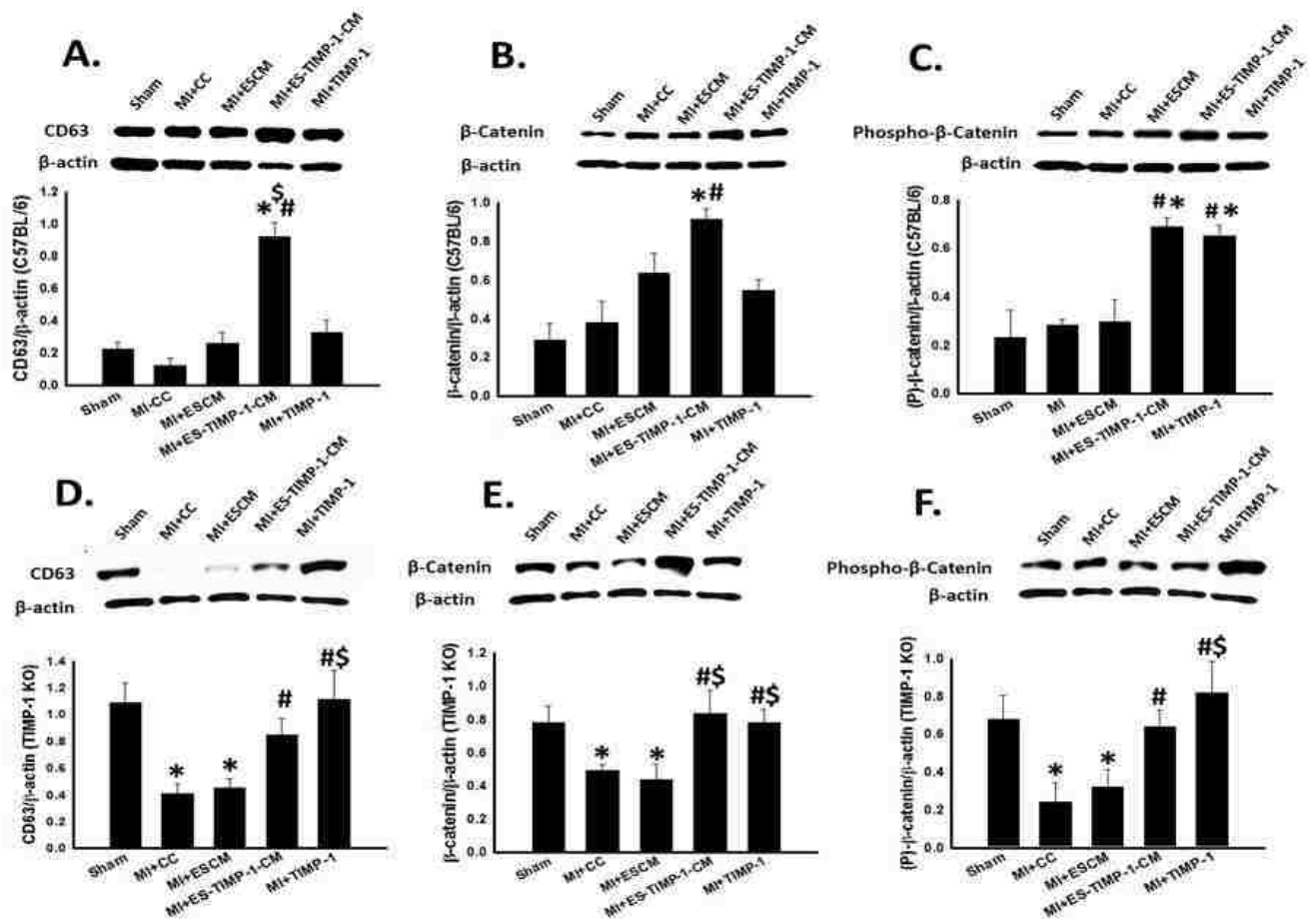


Figure 10. Mechanism of TIMP-1 Mediated CD63^{ve}/c-kit^{ve} CSCs Regeneration in the Infarcted Myocardium. Figure 10 depicts densitometry analysis of western blots for CD63, activated phospho (p) and total β -catenin proteins measured on heart homogenates from both C57BL/6 mice (Figures 10A-C) and TIMP-1 KO mice (Figure 10D-F). Representative blots for each histogram are also included above each corresponding histogram. Data was collected from n=4-6 samples per group. *p<0.05 vs Sham, #p<0.05 vs MI+CC and \$ p<0.05 vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

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CHAPTER FOUR: TIMP-1 AND ES CELLS OVEREXPRESSING TIMP-1 CONDITION MEDIA BLUNT ADVERSE CARDIAC REMODELING FOLLOWING MYOCARDIAL INFARCTION

Abstract

Transplanted mouse ES cells overexpressing tissue inhibitor of metalloproteinase-1 (TIMP-1) in the infarcted myocardium demonstrated inhibited adverse cardiac remodeling and improved cardiac function. However, whether these beneficial effects are provided by transplanted cells or TIMP-1 factor released in the conditioned media (CM) was previously unknown. Therefore, we hypothesized that intramyocardial delivery of ES-TIMP-1-conditioned medium (CM) or TIMP-1, following myocardial infarction (MI), inhibit cardiac apoptosis and fibrosis and resume contractile function. Accordingly, age matched C57BL/6 and TIMP-1 KO mice were subjected to coronary artery ligation followed by intramyocardial delivery of 20 μ l of culture media (CC), ESCM, ES-TIMP-1-CM or TIMP-1. TUNEL staining, Caspase-3 activity ELISA, Masson's Trichrome, and echocardiography analysis were used to assess apoptosis, fibrosis, and heart function respectively. Two weeks post-MI, ES-TIMP-1-CM and TIMP-1 significantly ($p < 0.05$) reduced overall cardiac apoptosis as well as Caspase-3 mediated cardiac myocytes cell death compared with ESCM group in both TIMP-1 KO and control animals. Infarct size, interstitial and vascular fibrosis were significantly ($p < 0.05$) decreased in the ES-TIMP-1-CM and TIMP-1 groups compared to controls. Furthermore, we demonstrated that TIMP-1 factor alone is sufficient to significantly resorb cardiac remodeling in both TIMP-1 KO and control animals regardless of the severity of damage. Finally, echocardiography data showed fractional shortening and ejection fraction were significantly ($p < 0.05$) improved in the ES-TIMP-1-CM and TIMP-1

groups in both strains. Our data suggest that ES-TIMP-1-CM or TIMP-1 alone provide an equivalent protection against adverse cardiac remodeling and shall be considered as potential therapeutic tools for MI patients.

Keywords: Myocardial Infarction, TIMP-1, Apoptosis, Fibrosis, Cardiac Function.

Introduction

Adverse cardiac remodeling is the process by which the myocardium size, conduction and function are altered as a result of myocardial infarction (MI), myocarditis, or idiopathic dilated cardiomyopathy (1). The gross pathological changes involved in cardiac remodeling are; massive cardiac myocyte cell loss through apoptosis, increased infiltration of collagen fibers throughout the infarct zone and cardiac myocyte hypertrophy, leading to deregulated contractile function (1-3). Cardiac myocyte apoptosis following MI activates cardiac fibroblasts, which are the main cellular modulators of cardiac fibrosis. They control extracellular matrix (ECM) turn over through secretion of matrix metalloproteinases (MMPs), otherwise called Matrixins, and their direct inhibitors; tissue inhibitor of metalloproteinases (TIMPs) (3-6). The main function of matrixins is the degradation and removal of ECM molecules as well as stimulation of collagen I, III and IV production from fibroblasts (7; 8). Following MI, upregulation of MMP2 and MMP9 has been correlated with aberrant collagen build up in the infarct area (7; 8). Concurrently, levels of TIMP-1 have been shown to be significantly down-regulated post MI (4). Furthermore, published data demonstrated TIMP-1 downregulation correlates with exacerbated cardiac myocyte apoptosis and increased fibrotic tissue (7). Increased cardiac myocyte death and accumulated collagen

deposition within the infarct zone leads to ventricular wall stiffening resulting in impaired systolic and diastolic functions (1; 4; 7).

Up regulation of TIMP-1 in the MI heart by direct delivery of TIMP-1 protein or vector to the peri-infarct area has demonstrated decreased overall infarct size, infiltrated fibrosis and cardiac myocyte apoptosis which positively impacted contractile function (9). Similarly, pharmacological inhibition of MMPs using PD-166793 compound was able to rescue the MI heart (7). Others have investigated stem cell transplantation as a way to replace apoptotic cardiac myocytes and vasculature in different animal models for MI (4; 8; 10; 11). Stem cells have the potential to regenerate the myocardium and consequently prevent fibrotic tissue accumulation. However, their survival rate in the host myocardium is very timid which greatly limits their differentiation potential in vivo (12; 13). Previously, we have generated embryonic stem (ES) cells overexpressing TIMP-1 factor (ES-TIMP-1 cells). When transplanted to the MI heart, these cells demonstrated limited engraftment to the host myocardium; however, collected data showed a significant decrease in cardiac remodeling parameters as well as improved functional capacity of the heart (4). Therefore we hypothesized that TIMP-1 released by ES-TIMP-1 cells provided the aforementioned protective effect to the heart. Furthermore, ES cells are known to secrete other beneficial factors such as; Insulin Growth Factor (IGF), Hepatic Growth Factor (HGF), Stem Cell Factor (SCF), and Vascular Endothelial Growth Factor (VEGF), all of which regulate cell proliferation and angiogenesis and might be aiding TIMP-1 in blunting left ventricular remodeling (14-16).

In the present study, we compared the anti-cardiac remodeling effect of direct transplantation of TIMP-1 protein, ES condition media (CM), which contains factors

released by ES cells, and ES-TIMP-1-CM in the MI heart of both C57BL/6 and TIMP-1 KO mice. Our study will provide evidence that ES cells released factors combined with TIMP-1 or TIMP-1 alone provide an equivalent protection against adverse cardiac remodeling and shall be considered as potential therapeutic tools for MI patients.

Materials and Methods

Condition Media Preparation from ES and ES-TIMP-1 Cells

Embryonic Stem (CGR8) cells overexpressing TIMP-1 factor (ES-TIMP-1 cells) were generated by transfection of pTurboFP635-C (Homemade) vector encoding red fluorescent protein (RFP), mouse TIMP-1 cDNAs and neomycin resistance gene. Transfection was carried out using Lipofectamine 2000 (Invitrogen). Resulting cells were selected by treatment with G418 antibiotic (Invitrogen) (4). Condition media preparation from both cell lines was carried out as previously detailed (4). Briefly, ES and ES-TIMP1 cells were cultured till confluence in presence of DMEM containing LIF, sodium pyruvate, glutamine, β -mercaptoethanol, penicillin-streptomycin, nonessential amino acids, and 15% ES cell-qualified fetal bovine serum (Invitrogen). LIF was then removed and cells were cultured for 48 hours on gelatin coated plates supplemented with cell culture media (CC). Following the 48 hours incubation, cell media containing factors released from ES and ES-TIMP-1 cells were collected in separate tubes, labeled ESCM and ES-TIMP-1-CM respectively and saved for future use in -20°C .

TIMP-1 Preparation

Recombinant mouse TIMP-1 (R&D; cat # 980-MT) lyophilizate was reconstituted in sterile water containing a mixture of Tris (50mM), CaCl_2 (10mM), NaCl (150mM), and

Brij₃₅ (0.05%) to a concentration of 100ug/ml. aliquots of 100µl volumes were made and stored in -20°C.

Myocardial Infarction and Treatment Administration

All animal procedure detailed hereafter was approved by the University of Central Florida (UCF) Institutional Animal Care and Use Committee (IACUC). C57BL/6 and TIMP-1 KO on C57BL/6 background (B6.129S4-*Timp1*^{tm1Pds}/J; Jackson laboratories; Cat# 006243) were bred at the UCF's Transgenic Animal Facility (TAF) until 8 to 10 weeks old. Next, equal number of males and females from both strains were separated into five study groups; Sham (n=8), MI+Culture Media (CC; n=8), MI+ESCM (n=8), MI+ES-TIMP-1-CM (n=8) and MI+TIMP-1 (n=8), where "n" is the number of animals per study group. Left anterior descending (LAD) coronary artery ligation surgery was performed on the last four groups following previously reported procedure (17-19). In brief, animals were sedated with 4% isoflurane and 2% oxygen provided through an endotracheal intubation system (Minivent Type 845; Harvard Apparatus). Buprenorphine intra-muscular injection (0.5mg/kg) was also given to animals prior to surgery and 2-3 hours post-surgery to aid in pain relief. As part of the pre-operative procedure, the chest was shaved, alcohol sterilized and covered with betadine. A thoracotomy was then performed, the heart was exposed and the LAD ligated using a surgical suture needle (Monofilament polypropylene non-absorbable suture, 7/0; cat # 8703P; CP Medical, Portland). Artery ligation was confirmed when a bleaching area around the ligation site occurs following ligation. 20µl of CC, ESCM, ES-TIMP1-CM or TIMP-1 (200ug/kg of body weight) was injected at two peri-infarct sites using a 700

Series MICROTITER Syringe supplemented with a gauge floating needle (Hamilton CO, Nevada). Sham operated animals were put under the same stress but the ligation was kept loose. Following treatments, thoracotomy was sutured back up and the mice were allowed to recover under strict supervision. Two weeks post-surgery, mice were sedated with Isoflurane overdose and sacrificed by cervical dislocation.

Tissue Embedding and Preparation of Paraffin Sections

Mice hearts were excised two weeks following surgery, washed in 1 X PBS and the bottom portion containing both ventricles was fixed in paraformaldehyde (PFA 4%) (17-19). PFA stored hearts were dehydrated by exposure to serial concentrations of Ethanol and embedded in paraffin. 5 μ m thickness sections were made using a tissue processor than placed on microscope slides (Fisher scientific). The wax remaining on the tissue section was melted down by incubating section in 60°C for 30 minutes. Prior to any staining, deparaffinization and rehydration of the tissue specimens was achieved by sequential dipping in xylene and descending concentrations of alcohol. Tissues were finally washed in distilled water and phosphate-buffered saline (PBS 1x) for an additional 5 minutes.

Determination of Infarct size

A standard Masson's trichrome protocol was performed on tissue sections to detect collagen deposition in the MI area (4). In brief, deparaffinized and rehydrated sections were successively immersed into Weigert iron hematoxylin, acid fuchsin, phosphomolybdic-phosphotungstic acid, and aniline blue. Under bright field Olympus microscope, healthy cells stained in pink, nuclei stained in dark purple and collagen

deposits stained in bright blue. Whole heart micrographs were taken under 2 X objective and Image J software was used to measure the whole heart and the infarct site area. Infarct size was calculated using this formula: LV infarct area/LV area x 100%.

Determination of Infiltrated Interstitial and Vascular Fibrosis

Interstitial fibrosis defined as the amount of collagen infiltrated within the interstitial space was detected on previously Masson's Trichrome stained sections. In brief, four to six photomicrographs were taken under 20X bright field light Olympus microscope objective and the blue area (collagen) was measured in each photo using Image J software. The average of 4 measured areas was taken as representative of the section. Seven to eight hearts per group were used for statistical analysis (4). Vascular fibrosis was averaged from 6 different vessels per sections using this equation: vascular fibrosis/ vessel area x 100% in one to two sections from seven to eight hearts per group.

Determination of Apoptotic Nuclei and Apoptotic Cardiac Myocytes by TUNEL Staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on tissue sections to determine the ratio of apoptotic cells versus total viable cells between the different study groups. Briefly, deparaffinized and rehydrated heart sections were permeabilized with proteinase K (25 ug/ml in 100mM Tris-HCL), then immunostained using TUNEL kit (TMR red; Roche Applied Bio Sciences) which stains apoptotic nuclei in red. For detection of apoptotic cardiac myocytes, previously TUNEL stained section were double stained with mouse anti sarcomeric α -actin (Sigma

Aldrich) following mouse on mouse protocol (MOM, Vector Laboratories). Succinctly, sections were incubated for 1 hour in MOM blocking reagent followed by 5 mins in protein working solution (PWS) provided with the kit. Next, sections were incubated for 30 min with anti sarcomeric α -actin antibody (1:100), followed by 5 mins of IgG antibody and 10 mins of Fluorecin avidin solution. Finally, to detect whether cardiac myocytes apoptosis was mediated by Caspase-3 cascade, a third staining was performed using anti-rabbit polyclonal Caspase-3 primary antibody (1:1000, cell signaling). Hearts were incubated overnight with primary antibody followed by anti-rabbit secondary Alexa 660 (1:50, Invitrogen) for an hour. To detect total nuclei, sections were covered with antifade vectashield mounting medium containing 4',6-diamino-2-phenylindole (DAPI; Vector Laboratories). Olympus fluorescence microscope and LEICA laser scanning confocal microscope were used to identify triple stained sections and 4 images of randomly selected areas per sections were taken under 20X objective. Data per animal was obtained by averaging measurements from all four areas. Percent TUNEL positive nuclei was measured using the following formula: total positive cells/Total DAPI X100%. For Apoptotic cardiac myocytes: double positive cells for Sarcomeric α -actin and TUNEL were quantified and divided by total cardiac myocytes number. 1 to 2 sections per heart were analyzed and 5 to 6 hearts per group were used for statistical analysis (4).

Caspase-3 Activity Assay

To verify cardiac apoptosis is mediated by Caspase-3 machinery, heart homogenates from all study groups and both strains were assayed with the Caspase-3/

CPP32 colorimetric assay kit (K106-200, Biovision). Briefly, 1:10 dilutions were made from homogenates using lysis buffer provided with the kit and protein estimation was measured using Bio-Rad protein assay kit. Caspase-3 assay steps were carried out on a 96 well plate according to manufacturer's protocol until detection of blue color indicative of presence of active form of the protein. A microtiter plate reader was used to measure intensity of the blue color under 400nm to 405nm. Caspase-3 activity graph was plotted as arbitrary units (A.U) (4).

Echocardiography Analysis for Cardiac Function

To evaluate how ES-TIMP-1-CM and TIMP-1 treatments translate at the physiological level, echocardiography analysis was performed on mice hearts at day 14 post surgery using a Phillips Sonos 5500 Ultrasound system in a similar fashion previously reported (17-19). Mice were sedated with 2 to 4% volume of isoflurane and 2% oxygen delivered via nose cone, their chest hair was removed and the sonogram probe was positioned on top of the chest facing the head to record two dimensional (2D) images in a long axis projection with a guided M-mode. Three to four 2D images were obtained and the left ventricular fractional shortening (%FS) and left ventricular ejection fraction (%EF) were recorded. %EF was calculated using this formula [(left ventricular volume at end diastole (EDV) - left ventricular volume at end systole (ESV)/ left ventricular volume at end diastole (EDV)]. EDV and ESV were calculated using *Teichholz formula*:

$$EDV = [7 / (2.4 + LVIDd)] \cdot LVIDd^3.$$

$$ESV = [7 / (2.4 + LVIDs)] \cdot LVIDs^3.$$

Left ventricular interior diameter during diastole (LVIDd) and left ventricular interior diameter during systole (LVIDs) were manually measured on the motion mode screen shots taken for each animal.

Statistical Analysis

Data are reported as Means \pm SEMs (Standard Error of Mean). Significance was determined using one-way analysis of variance (ANOVA) performed on SigmaStat3.5 software followed by Tukey or Bonferroni tests. Difference between two study groups was considered statistically significant if $p < 0.05$.

Results

ES-TIMP-1-CM and TIMP-1 Blunt Apoptosis in the Infarcted Myocardium

To demonstrate the anti-apoptotic effect of ES-TIMP-1-CM and TIMP-1 on the host myocardium following MI, tissue sections were subjected to TUNEL staining which distinguishes apoptotic nuclei with a red color. Quantification of percent apoptotic nuclei against total DAPI revealed apoptosis dramatically increases in MI-CC hearts relative to control ($p < 0.05$, MI-CC; $2.1\% \pm 0.3\%$ vs Sham; $0.3\% \pm 0.05\%$) for C57BL/6 and ($p < 0.05$, MI-CC; $2.86\% \pm 0.4\%$ vs Sham; $0.4\% \pm 0.07\%$) for TIMP-1 KO mice (Figure 11B). Importantly, ESCM, ES-TIMP-1-CM and TIMP-1 transplantation significantly mitigated apoptosis ($p < 0.05$, ESCM; $0.95\% \pm 0.12\%$, ES-TIMP-1-CM; $0.31\% \pm 0.07\%$ and TIMP-1; $0.71\% \pm 0.1\%$) in C57BL/6 mice and ($p < 0.05$, ESCM; $1.59\% \pm 0.2\%$, ES-TIMP-1-CM; $0.45\% \pm 0.17\%$ and TIMP-1; $0.67\% \pm 0.1\%$) in TIMP-1 KO mice relative to their respective MI-CC groups (Figure 11B). Notably, although all treatments significantly decreased

($p < 0.05$) apoptosis compared to MI-CC, only ES-TIMP-1-CM and TIMP-1 groups were able to decrease it up to Sham levels (Figure 11B).

Next, we aimed at evaluating whether ES-TIMP-1-CM and TIMP-1 treatments were able to blunt Caspase-3 mediated cardiac myocytes apoptosis. Therefore, a triple immunostaining for TUNEL, sarcomeric α -actin and Caspase-3 markers was performed on heart sections. Figure 12A depicts confocal representative images obtained from MI-CC animals from C57BL/6 mice (a-f) and TIMP-1 KO (g-l) demonstrating apoptotic nuclei in red (a and g), Caspase-3 positive cells in yellow (b and h), sarcomeric α -actin positive cells in green (c and i), and total nuclei in blue (d and j). A merge of four stainings is depicted in white (e and k) and enlarged areas from e and k are presented in (f) and (l), respectively. Our quantitative data for percent TUNEL positive cardiac myocytes show cardiac myocyte apoptosis significantly elevated following MI ($p < 0.05$, MI-CC; $2.45\% \pm 0.29\%$ vs Sham; $0.36\% \pm 0.06\%$) for C57BL/6 and ($p < 0.05$, MI-CC; $5.42\% \pm 0.68\%$ vs Sham; $0.42\% \pm 0.03\%$) for TIMP-1 KO mice (Figure 12B). However, upon treatment with ESCM, ES-TIMP-1-CM or TIMP-1, these ratios significantly reduced compared with MI-CC hearts and this regardless of the strain ($p < 0.05$, ESCM; $1.07\% \pm 0.11\%$, ES-TIMP-1-CM; $0.84\% \pm 0.06\%$ and TIMP-1; $0.92\% \pm 0.19\%$) in C57BL/6 mice and ($p < 0.05$, ESCM; $2.9\% \pm 0.3\%$, ES-TIMP-1-CM; $1.01\% \pm 0.14\%$ and TIMP-1; $1.22\% \pm 0.17\%$) in TIMP-1 KO mice (Figure 12B). Data additionally show ES-TIMP-1-CM and TIMP-1 provide the greatest improvement against non-treated MI hearts in TIMP-1 KO mice when compared to ESCM treatment, while the three aforementioned treatments had similar effects on cardiac myocyte apoptosis in C57BL/6 mice (Figure 12B). Interestingly, caspase-3 activity measured on heart homogenates collected from

all study groups and both stains revealed similar pattern to cardiac myocyte apoptosis (Figure 12C). In brief, data show increased caspase-3 activity in MI-CC animals for both strains; however, ES-TIMP-CM and TIMP-1 treatments significantly ($p<0.05$) reduced its activity. Of note, even though ESCM treatment decreases Caspase-3 activity, it did not reach significance against MI-CC animals for either strain (Figure 12C).

Reduced Interstitial and Vascular Fibrosis Following ES-TIMP-1-CM and TIMP-1 Transplantation in the Infarcted Myocardium

Masson's trichrome stained heart sections were used to quantify interstitial and vascular fibrosis. Figure 13A depicts interstitial fibrosis (IF) representative images obtained from all study groups per strain. Measured interstitial fibrosis area demonstrate a significant ($p<0.05$) increase in IF area (mm^2) following MI in both C57BL/6 and TIMP-1 KO animals compared to their respective controls ($p<0.05$, MI-CC; 1.04 ± 0.1 vs Sham; 0.03 ± 0.008) for C57BL/6 and ($p<0.05$, MI-CC; 1.43 ± 0.2 vs Sham; 0.03 ± 0.01) for TIMP-1 KO mice (Figure 3B). Our data additionally demonstrate significantly ($p<0.05$) decreased interstitial fibrosis following ESCM, ES-TIMP-1-CM and TIMP-1 treatments compared to MI-CC hearts. Notably, ES-TIMP-1-CM and TIMP-1 showed the greatest decrease in IF measurements regardless of the strain when compared to ESCM treatment ($p<0.05$) (Figure 13B). Next, we quantified accumulated vascular fibrosis (VF) and our data revealed significantly increased ($p<0.05$) collagen deposition around vessels in MI-CC animals (Figure 14B). This increase was however, significantly blunted with ESCM, ES-TIMP-1CM and TIMP-1 treatments ($p<0.05$ vs MI-CC) in both C57BL/6 and TIMP-1 KO animals (Figure 14B). Moreover, ES-TIMP-1-CM and TIMP-1 treatments were able to blunt VF and IF in a similar fashion for both strains

even though TIMP-1 KO animals demonstrated higher IF and VF measurements following MI compared to their C57BL/6 counterparts.

Reduced Infarct Size Following ES-TIMP-1-CM and TIMP-1 Transplantation in the Infarcted Myocardium

To evaluate the effect of ES-TIMP-1-CM and TIMP-1 treatments on the infarct area post MI, heart sections were subjected to Masson's trichrome staining and whole heart images were taken under 2x objective of bright field Olympus microscope. Representative whole-heart images depicting infarct size from both strains and all corresponding treatment groups are represented in Figure 15A. Quantification analysis revealed that the dramatic infarct size induced by MI surgery was significantly blunted following transplantation of ESCM, ES-TIMP-1-CM and TIMP-1 ($p < 0.05$, ESCM; $8.24\% \pm 0.92\%$, ES-TIMP-1-CM; $3.21\% \pm 0.6\%$ and TIMP-1; $4.85\% \pm 1\%$) in C57BL/6 mice and ($p < 0.05$, ESCM; $9.5\% \pm 1.58\%$, ES-TIMP-1-CM; $4.79\% \pm 0.8\%$ and TIMP-1; $4.25\% \pm 0.51\%$) in TIMP-1 KO mice relative to their respective MI-CC groups (C57BL/6, MI-CC; $14.25\% \pm 1.25\%$ and TIMP-1 KO, MI-CC; $17.69\% \pm 0.5\%$) (Figure 15B). ESCM treatment alone provided a significant ($p < 0.05$) improvement in infarct size reduction, however, to a lower degree compared to the later treatments. More importantly, TIMP-1 KO animals showed a drastic increase in infarct size compared to their C57BL/6 counterparts, however, ES-TIMP-1-CM and TIMP-1 treatments were able to significantly ($p < 0.05$) resorb the infarct area in a similar fashion for both strains (Figure 15).

Improved Heart Function Following ES-TIMP-1-CM and TIMP-1 Treatments in the Infarcted Myocardium

Cardiac function was measured 14 days after MI procedure. Our data suggest a significant improvement in cardiac fractional shortening (FS) following ES-TIMP-1-CM and TIMP-1 treatments in C57BL/6 mice ($p < 0.05$, ES-TIMP-1-CM; $40.89\% \pm 1.33\%$ and TIMP-1; $37.64\% \pm 0.92\%$ vs MI+CC; $32.35\% \pm 0.34\%$ and ESCM; $34.55\% \pm 1.46\%$) and TIMP-1 KO mice ($p < 0.05$, ES-TIMP-1-CM; $38.21\% \pm 1.67\%$ and TIMP-1; $36.68\% \pm 1.13\%$ vs MI+CC; $27.85\% \pm 1.28\%$ and ESCM; $31.57\% \pm 1.95\%$) (Figure 16B). However, improved function following either ES-TIMP-1-CM or TIMP-1 transplantation was lower than sham in both TIMP-1 KO ($46.89\% \pm 1.37\%$) and C57BL/6 ($51.83\% \pm 1.34\%$) groups. We also determined that left ventricular ejection fraction (EF) was more significantly impacted in C57BL/6 than TIMP-1 KO hearts following MI. However, its levels significantly ($p < 0.05$) increased in the ES-TIMP-1-CM ($72.59\% \pm 3.29\%$) and TIMP-1 ($69.54\% \pm 0.52\%$) compared with MI+CC ($59.68\% \pm 0.4\%$) and ESCM ($63.45\% \pm 3.77\%$) in TIMP-1 KO mice (Figure 16C). Moreover, significantly ($P < 0.05$) increased ejection fraction was also observed in the ES-TIMP-1-CM ($83.58\% \pm 1.34\%$) and TIMP-1 ($69.04\% \pm 0.56\%$) groups compared with MI+CC ($62.30\% \pm 0.52\%$) and ESCM ($64.1\% \pm 2.1\%$) groups in C57BL/6 mice (Figure 16B). Finally, improved EF ratios following ES-TIMP-1-CM transplantation were significantly ($p < 0.05$) lower than sham in both TIMP-1 KO ($81.37\% \pm 1.31\%$) and C57BL/6 ($83.58\% \pm 1.34\%$). TIMP-1 treatment data for the same parameter showed significance ($p < 0.05$) only in C57BL/6 animals against sham, whereas TIMP-1 KO animals resumed normal ejection fraction.

Discussion

In the current study, we have examined the protective potential of ES-TIMP-1-CM and TIMP-1 against adverse cardiac remodeling consecutive to MI in TIMP-1 KO mice and their controls (C57BL/6 mice). Our cumulative data demonstrate ES-TIMP-1-CM and TIMP-1 significantly: 1) blunt percent cardiac apoptosis, 2) decrease Caspase-3 mediated cardiac myocyte cell death, 3) reduce interstitial and vascular fibrosis, 4) shrink infarct area and most importantly, 5) improve left ventricular contractile function in both C57BL/6 and TIMP-1 KO animals. Finally, we show evidence that TIMP-1 is sufficient to attenuate adverse remodeling to a similar extent between TIMP-1 KO mice and C57BL/6 mice.

Cardiac apoptosis is considered the hallmark of cardiac ischemia. It is mediated by two distinct pathways; the extrinsic and the intrinsic pathways (20; 21). Extrinsic pathway is triggered by extracellular death ligands such as FAS, tumor necrosis factor (TNF)- α , or TRAIL, while intrinsic pathway is mediated through activation of Caspases activity (20; 21). TIMP-1 factor has been shown to blunt apoptosis in different cancer cell lines through modulation of the intrinsic pathway, however, not much is known about its anti-apoptotic role in the heart (9; 22). We have previously demonstrated that TIMP-1 released by ES cells inhibits apoptosis on cultured H9C2 cells (embryonic cardiac myoblasts) previously exposed to hydrogen peroxide (H₂O₂) (23). Furthermore, our next published data demonstrated ES-TIMP-1 cell delivery in the MI heart decreased Caspase-3 mediated apoptosis, however, whether this effect is mediated through TIMP-1 or other factors additionally released by ES-TIMP-1 cells was not clear (4). In the present study, we provide evidence that TIMP-1 alone is sufficient to

decrease overall cardiac apoptosis and specifically blunt Caspase-3 activity in cardiac myocytes following MI (Figures 11 and 12). Furthermore, TIMP-1 KO animals showed significantly higher Caspase-3 activity in cardiac myocytes compared to control mice following MI (Figure 12C). This further demonstrates the anti-apoptotic role of TIMP-1 in cardiac myocytes through inhibition of Caspase-3 activity.

Cardiac myocyte apoptosis in the infarct and peri-infarct area precipitates collagen deposition in the interstitial and vascular spaces leading to stiffening of the left ventricle (9; 17; 22). TIMP-1 ability to blunt cardiac fibrosis through inhibition of MMPs is a well-documented fact (9; 17; 22). Additionally, previously published work has shown decreased fibrosis following ES-TIMP-1 cells transplantation in the MI heart (4). This decrease was associated with inhibition of endogenous MMP-9, a known promoter of collagen secretion and ECM degradation in the heart (4). However, it was unclear whether ES-TIMP-1-CM could provide a better outcome than TIMP-1 alone. Therefore, we measured interstitial and vascular fibrosis on TIMP-1 KO and C57BL/6 infarcted hearts following ESCM, ES-TIMP-1-CM and TIMP-1 treatments. Our data showed that although all treatments were efficient at blunting fibrosis (interstitial and vascular); the last two treatments provided the best outcome regardless of the strain. ES cells have been shown to release other anti-fibrotic factors such as hepatic growth factor (HGF) which has been linked to decreased fibrosis in lung alveolar epithelium (24). This explains, in part, the anti-fibrotic effect of our transplanted ESCM. However, TIMP-1 seems to provide a stronger anti-fibrotic effect as demonstrated by the extent of fibrosis decrease we report following TIMP-1 treatment compared with any other combined factors released by ES cells.

Finally, we aimed at determining whether combined inhibition of apoptosis and fibrosis in the host myocardium contribute to decreased infarct size and improved cardiac function. Infarct size measurements are based on measurement of the infarcted area in tissue sections of the heart (25). Our data is in corroboration with previously published work, which demonstrated larger infarct area in TIMP-1 KO animals compared with their C57BL/6 counterparts two weeks following MI (7; 17; 22). Additionally, ESCM, ES-TIMP-1-CM and TIMP-1 treatments, all significantly reduced infarct size in both mice strains following MI; however, the extent of infarct resorption was more prominent in the last two groups (Figure 15). Reduction of infarct size upon TIMP-1 or MMP inhibitor transplantation in the infarcted myocardium has already been demonstrated (7; 17); however, we are first to report that ES-TIMP-1-CM will provide similar outcome. Furthermore, the infarct size measured in TIMP-1 KO and C57BL/6 hearts were similar post TIMP-1 delivery even though the extent of infarction was bigger in the TIMP-1 depleted mice prior to treatment. The efficiency of infarct resorption in TIMP-1 KO animals treated with TIMP-1 exemplifies its importance in ECM regeneration and balance.

Extent of infarct size area severely impacted left ventricular contractile ability as demonstrated by decreased fractional shortening and ejection fraction parameters measured following MI (Figure 16). Of note, the fractional data collected from TIMP-1 KO animals were expectedly lower than the ones collected from C57BL/6 mice; however, ejection fraction was affected in the same way for both strains (Figure 16). The rather unpredicted EF data is in accordance with multiple previously published works demonstrating exacerbated left ventricular remodeling and decreased FS indices

in TIMP-1 KO mice following MI, with pseudo-normal to minimally affected ejection volumes (26). This was attributed to mitral regurgitation which accompanies acute MI in TIMP-1 KO mice and mitigates volumes measurements (26; 27). Nonetheless, ES-TIMP-1-CM and TIMP-1 delivery significantly improved both FS and EF data in the infarcted heart of both strains.

In conclusion, the present study compared the beneficial effect of direct intramyocardial delivery of TIMP-1 or ES-TIMP-1-CM in the MI setting. Our data revealed that both treatments significantly diminish Caspase-3 mediated cardiac apoptosis and fibrosis (interstitial and vascular) leading to significantly smaller infarct area and improved contractile function. Improved cardiac function is thought to be the result of limited cardiac myocytes loss and less collagen deposition within the host myocardium. Furthermore, we demonstrate that TIMP-1 factor alone is sufficient to significantly resorb cardiac remodeling in both TIMP-1 KO and control animals and resume similar cardiac function in both strains regardless of the extent of starting damage. However, future studies are necessary to identify the mechanisms by which TIMP-1 prevents adverse cardiac remodeling in the infarcted myocardium and how it overpasses any other anti-apoptotic anti-fibrotic mechanism provided by other ES cells released factors.

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Figures

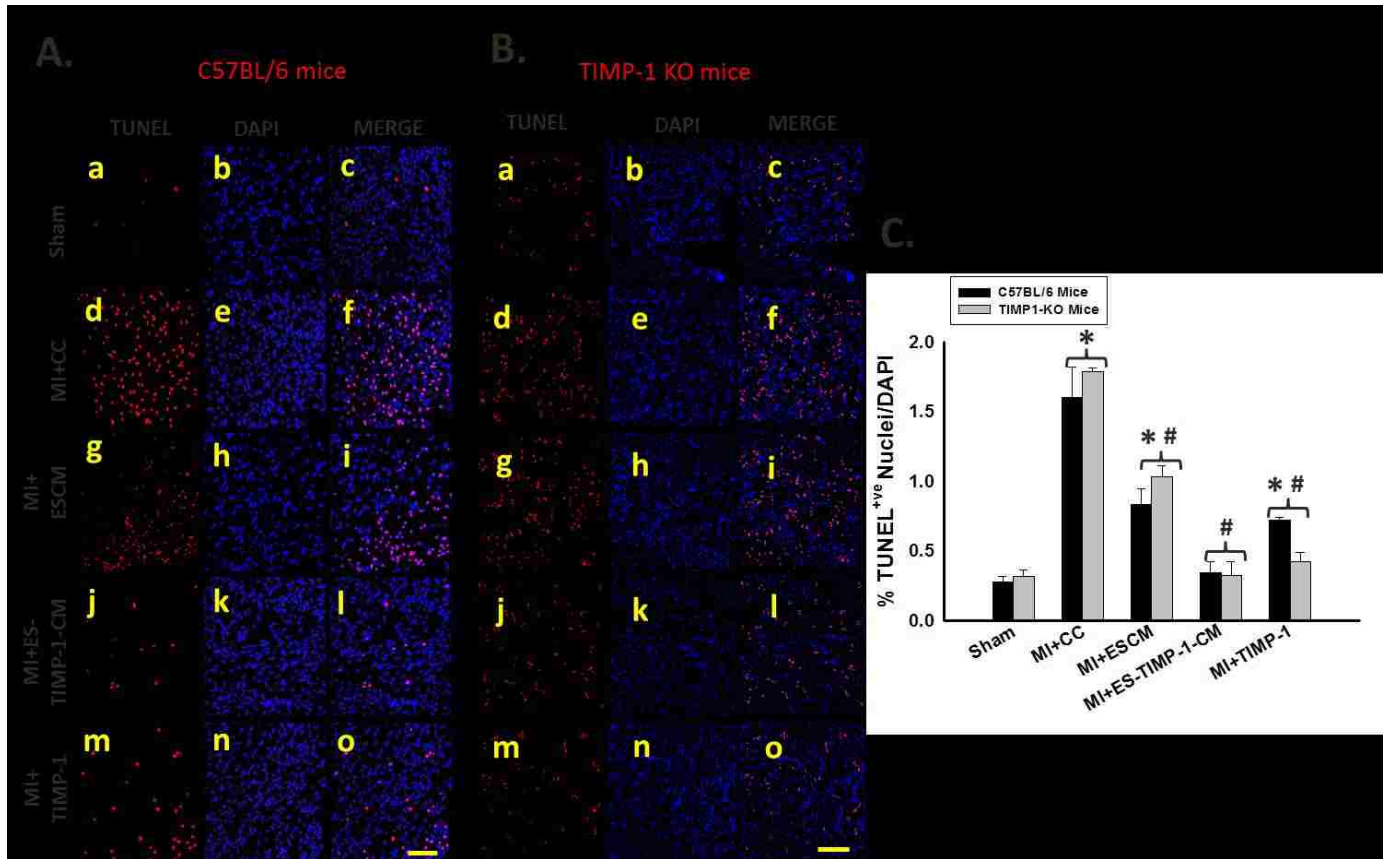


Figure 11. ES-TIMP-1-CM and TIMP-1 blunt apoptosis in the Infarcted Myocardium of C57BL/6 and TIMP-1 KO mice. TUNEL representative confocal photomicrographs demonstrating cardiac apoptosis are detailed in Figure 11A for C57BL/6 and Figure 11B for TIMP-1 KO mice. TUNEL positive nuclei are shown in red (a,d,g,j,and m), total nuclei stained blue with DAPI (b,e,h,k, and n) and merged TUNEL and DAPI images are depicted in (c,f,i,l and o). Histogram 11B depicts quantification of percent apoptotic nuclei against total DAPI for both C57BL/6 and TIMP-1 KO mice. Scale bar= 25 μ m. Data was collected from N=6 per group and 1 to 2 sections per animal.* p <0.05. vs Sham and # p <0.05 vs MI+CC. Brackets over each group means the given significance above it applies to both strains.

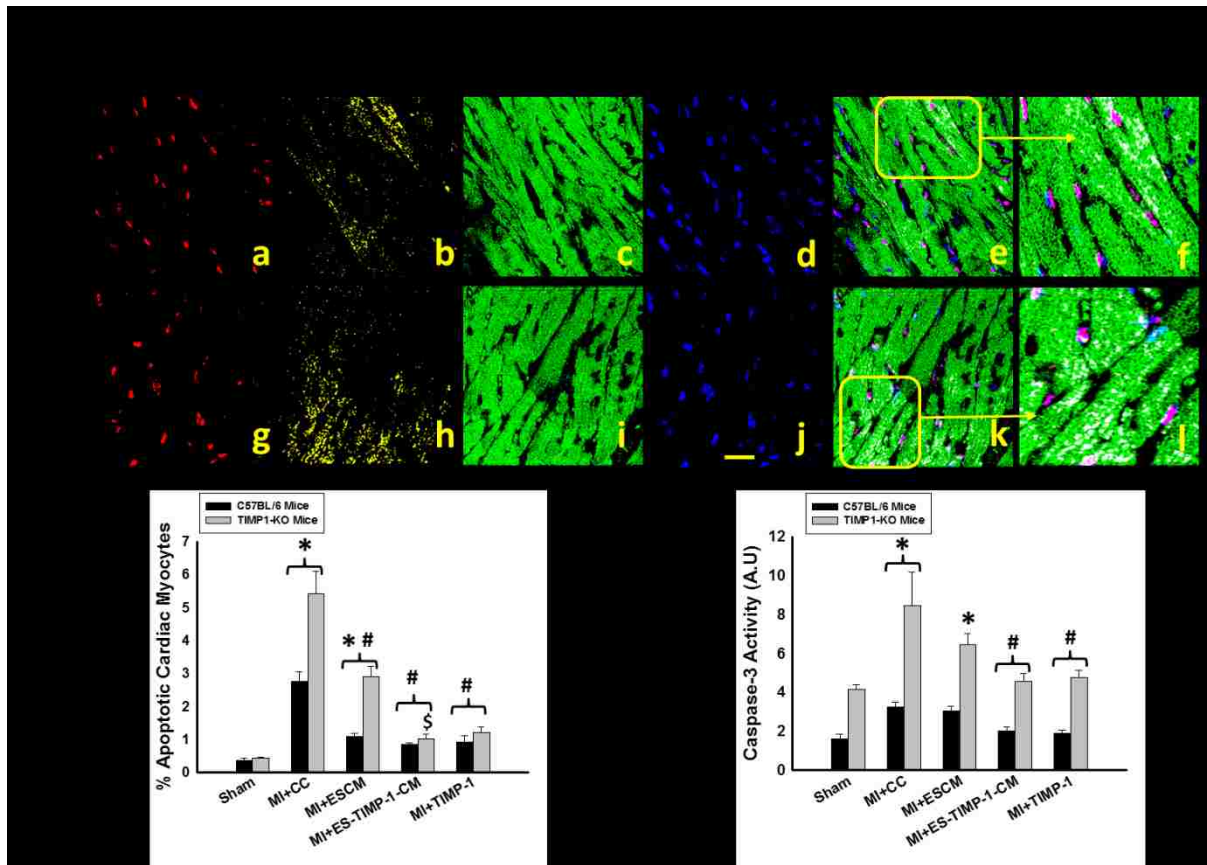


Figure 12. ES-TIMP-1-CM and TIMP-1 Blunt Caspase-3 Mediated Apoptosis in Cardiac Myocytes. Figure 12A depicts confocal representative images obtained from MI-CC animals from C57BL/6 mice (a-f) and TIMP-1 KO (g-l) demonstrating apoptotic nuclei in red (a and g), Caspase-3 positive cells in yellow (b and h), sarcomeric α -actin positive cells in green (c and i), and total nuclei in blue (d and j). A merge of four stainings is depicted in white (e and k) and enlarged areas from e and k images are presented in f and l respectively. Scale bar= 100 μ m. Histogram 12B represents quantitative analysis results of % apoptotic cardiac myocytes over total cardiac myocytes. Histogram 12C demonstrates Caspase-3 activity assay results for both strains and their respective treatments. Data was collected from N=5 per group and 1 to 2 sections per animal. * $p < 0.05$. vs Sham and # $p < 0.05$ vs MI+CC. Brackets over each group means the given significance above it applies to both strains.

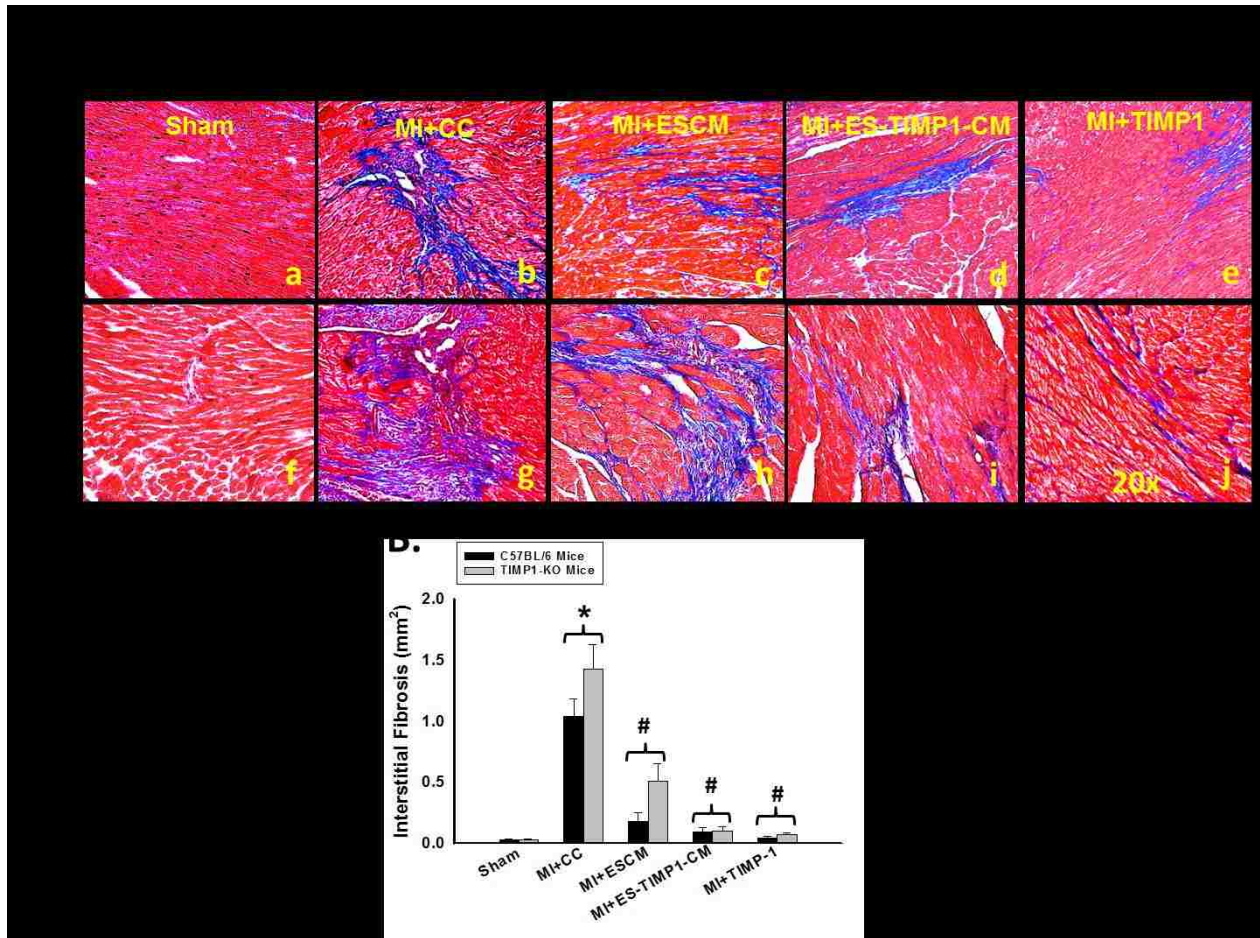


Figure 13. Reduced Interstitial Fibrosis Following ES-TIMP-1-CM and TIMP-1 Transplantation in the Infarcted Myocardium. Figure 13A depicts interstitial fibrosis (IF) representative images obtained from all study groups per strain; C57BL/6 mice (a-e) and TIMP-1 KO animals (f-j). 20x magnification. Histogram 13B shows quantitative analysis of measured interstitial fibrosis area in C57BL/6 and TIMP-1 KO animals. Data was collected from N=7 per group. * $p < 0.05$. vs Sham and # $p < 0.05$ vs MI+CC. Brackets over each group means the given significance above it applies to both strains.

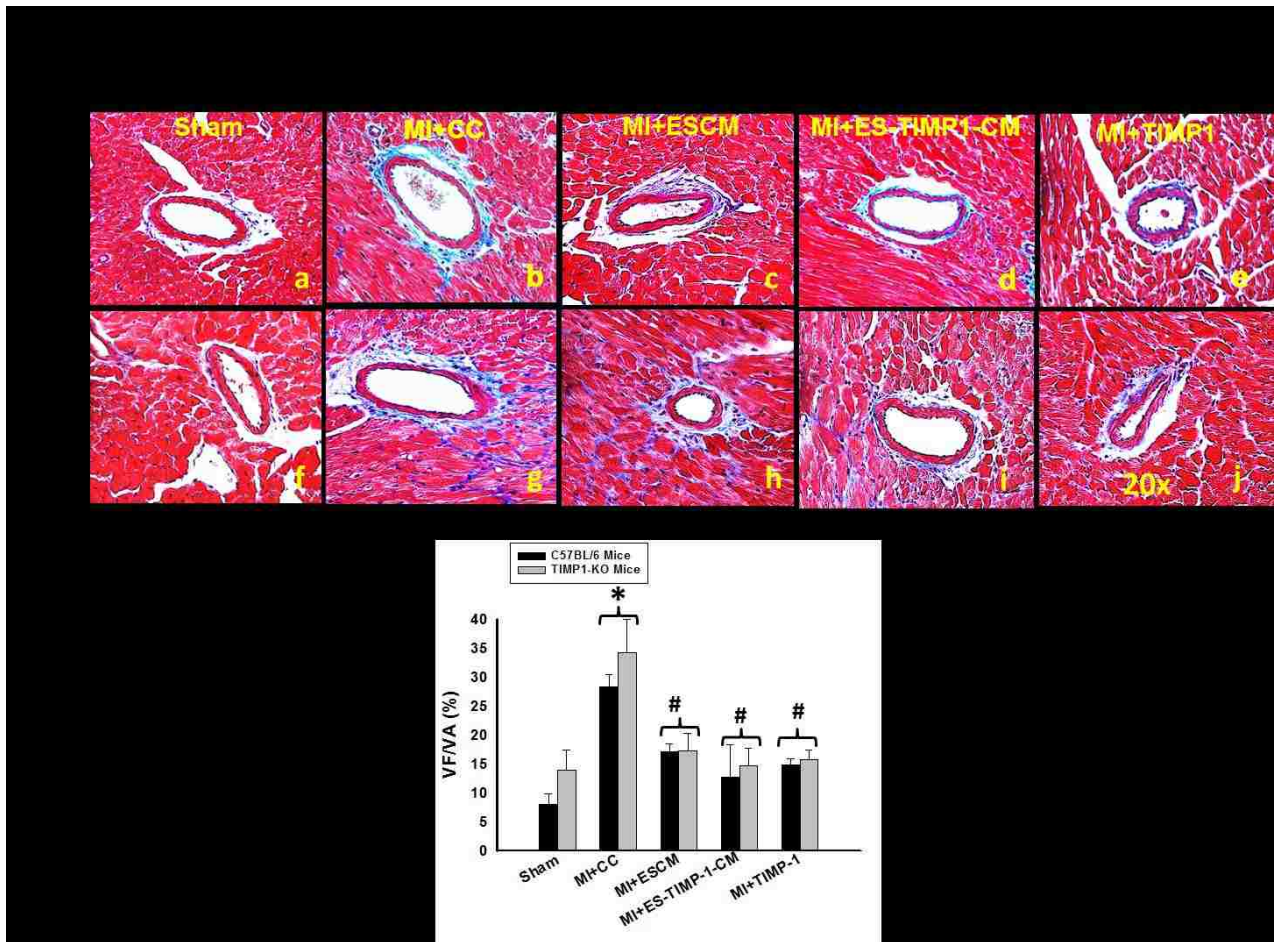


Figure 14. Reduced Vascular Fibrosis Following ES-TIMP-1-CM and TIMP-1 Transplantation in the Infarcted Myocardium. Figure 14A depicts representative photomicrographs of vascular fibrosis obtained under a 20x objective of bright field Olympus microscope. C57BL/6 mice are in (a-e) and TIMP-1 KO animals are in (f-j). Figure 14B shows %vascular fibrosis per vascular area quantified on C57BL/6 and TIMP-1 KO animals. Data was collected from N=7 per group. * $p < 0.05$. vs Sham and # $p < 0.05$ vs MI+CC. Brackets over each group means the given significance above it applies to both strains.

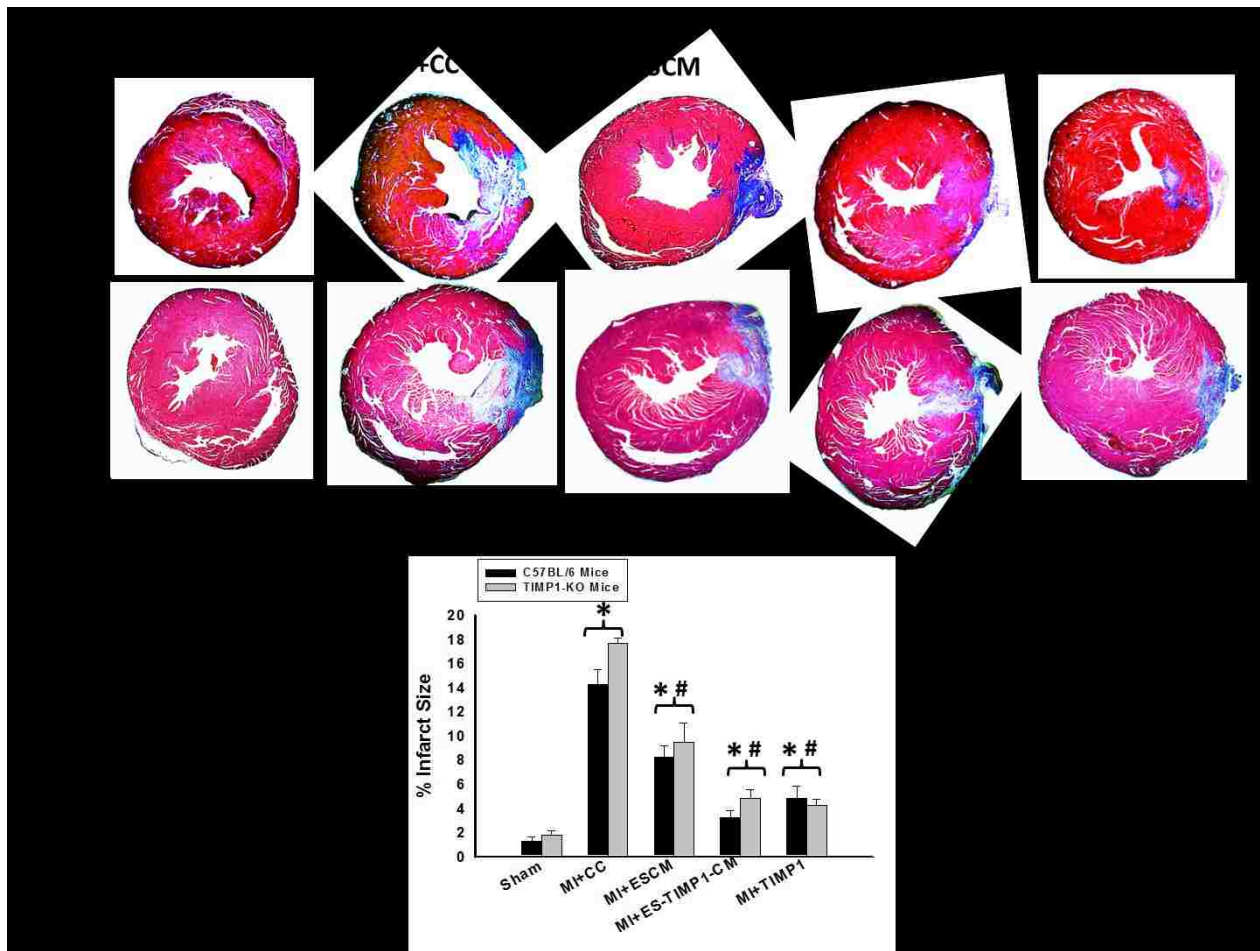


Figure 15. Reduced Infarct Size Following ES-TIMP-1-CM and TIMP-1 Transplantation in the Infarcted Myocardium. Figure 15A shows representative whole-heart images depicting infarct size from both strains and all corresponding treatment groups under 2x magnification. Pink color represents healthy cardiac cells, blue areas represent fibrotic tissue (Infarct area). Histogram 15B shows quantification analysis of % infarct size in both C57BL/6 and TIMP-1 KO mice and their respective treatments. Data was collected from N=7 per group.* $p < 0.05$. vs Sham and # $p < 0.05$ vs MI+CC. Brackets over each group means the given significance above it applies to both strains.

	FS% (C57BL/6)	EF% (C57BL/6)	FS% (TIMP-1KO)	EF% (TIMP-1 KO)
Sham	51.83 ± 1.34	83.58 ± 1.34	46.89 ± 1.37	81.37 ± 1.31
MI+CC	32.35 ± 0.34	62.30 ± 0.52	27.85 ± 1.28	59.68 ± 0.4
MI+ESCM	34.55 ± 1.46	64.1 ± 2.1	31.57 ± 1.95	63.45 ± 3.77
MI+ES-TIMP-1-CM	40.89 ± 1.33	74.1 ± 1.84	38.21 ± 1.67	72.59 ± 3.29
MI+TIMP-1	37.64 ± 0.92	69.04 ± 0.56	36.68 ± 1.13	69.54 ± 0.52

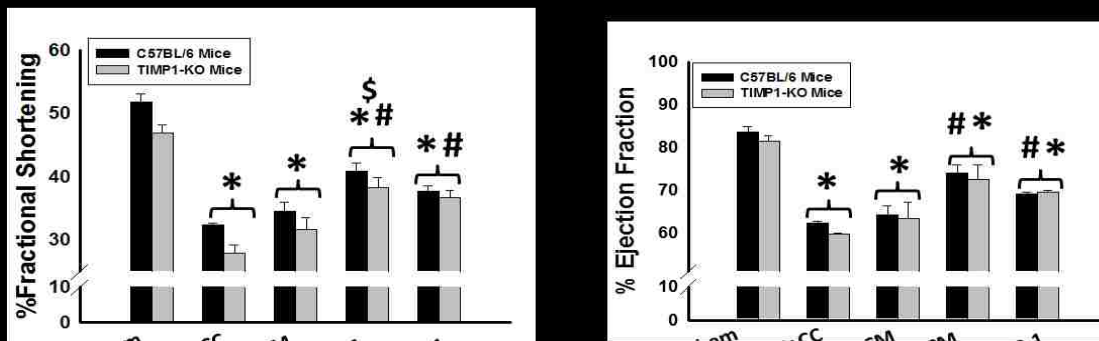


Figure 16. Improved Heart Function Following ES-TIMP-1-CM and TIMP-1 Transplantation in the Infarcted Myocardium. Results of echocardiography analysis D14 post-surgery are presented in Figure 16. Table 16A summarizes quantitative data for % fractional shortening (FS) and ejection fraction (EF) parameters from all groups measured in both C57BL/6 and TIMP-1 KO mice. Histogram 16B represents FS% quantitative data in both stains and histogram 16C depicts %EF values obtained from both stains and all five treatment groups. ($p < 0.05$, Figure 16). Data was collected from $n = 6-8$ animals per group. * $p < 0.05$ vs Sham, # $p < 0.05$ vs MI+CC and \$ $p < 0.05$ vs MI+ESCM. Brackets over each group means the given significance above it applies to both strains.

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CHAPTER FIVE: DISCUSSION

Cell transplantation therapy for cardiac repair following MI has shown timid benefits given the limited survival and plasticity of transplanted cells (1; 2). Therefore, boosting endogenous CSCs populations to promote cardiac regeneration is necessary to promote sufficient myocardium repair. Tremendous efforts have been put in place to identify existent pools of endogenous CSCs (2; 3; 15; 21). Results from these studies demonstrate that following injury, CSCs proliferate and differentiate in an attempt to repair the damaged organ; however, the differentiation rate of these cells remains disproportionate as compared to the extent of cell loss (2; 3; 15; 21; 25). Therefore, identifying factors that can promote sufficient CSCs differentiation into cardiac myocyte, VSM and ECs to replace lost cells and regain pre-MI homeostasis is at the forefront of cardiac regenerative research.

TIMP-1 is a protease inhibitor expressed in the ECM of many tissues including the myocardium to control MMPs-mediated aberrant collagen deposition (4; 5; 14). Additionally, it has been linked to multiple cellular processes including enhanced cell proliferation and inhibition of apoptosis (5; 19; 20; 26). However, the ability of TIMP-1 to promote CSC proliferation and differentiation has not been established. In our previously published work, we engineered ES-TIMP-1 cells and transplanted them into the infarcted murine heart (14). Collected data demonstrated improved contractile function resulting at least in part from TIMP-1 enhancing transplanted ES cell engraftment, survival, and differentiation into cardiac myocytes (14). Therefore, we postulated that TIMP-1 released by ES-TIMP-1 cells may have further activated endogenous cardiac stem cells (CSCs) that express TIMP-1 receptor (CD63) leading to

indigenous cardiac regeneration therefore contributing to the reported improvement in cardiac function post MI injury.

In this regard, our first study (Chapter 2) aimed at identifying a sub-population of CSCs expressing CD63 and c-kit markers from isolated murine CSCs. We verified that TIMP-1 treatment enhances CD63⁺/c-kit⁺ CSCs proliferation and differentiation into cardiac myocytes, VSM cells and ECs *in vitro*. Finally, we provided evidence that TIMP-1 induces CD63⁺/c-kit⁺ CSCs differentiation through enhancing cardiac genes (GATA-4, Mef2C and Nkx-2.5) expression.

Our *in vitro* data established that 60% of isolated c-kit⁺ CSCs were CD63⁺/c-kit⁺ and ES-TIMP-1-CM and TIMP-1 treatments significantly increased their proliferation rate. As previously mentioned in chapter 2, CD63 receptor is expressed in multiple cell types; however, we are first to report its expression by CSCs. Furthermore, enhanced CSCs proliferation mediated through TIMP-1/CD63 axis, demonstrated in this first study, is in accordance with previously published work on hMSCs which showed enhanced stem cell proliferation upon TIMP-1 binding CD63 on the cell surface (12).

Next, we investigated whether TIMP-1 could promote significant CD63⁺/c-kit⁺ CSC differentiation *in vitro*. Multiple factors released in the ES media have been shown to promote cardiac differentiation such as IGF1/2, bone morphogenetic protein-2 (BMP-2), platelet-derived growth factor (PDGF) and VEGF (27; 28). However, the extent of cardiac differentiation is insufficient to restore pre-MI capacity (2; 21; 27; 28). Of note, TIMP-1 role in cell differentiation has been previously reported on germinal center B cells and oligodendrocyte (23). Its involvement in cardiac differentiation, however, has

not yet been established. In the present study, we are first to report that TIMP-1 enhances cardiac differentiation by targeting a unique CSC population, CD63⁺/c-kit⁺.

To further delineate the mechanism mediating CD63⁺/c-kit⁺ CSC differentiation upon TIMP-1 treatment, we measured cardiac gene expression on isolated CSCs. GATA-4, Mef2C and Nkx-2.5 are cardiogenic factors expressed by stem cells undergoing cardiac differentiation (8-11; 31). Particularly, GATA-4 and Nkx-2.5 are indicative of myocyte differentiation while Mef2C is expressed in both cardiac myocyte and vascular differentiating cells (8-11; 31). Our collected data demonstrated that TIMP-1 binding to its receptor expressed on CD63⁺/c-kit⁺ CSCs surface triggers a downstream pathway which leads to enhanced cardiac genes expression resulting in CD63⁺/c-kit⁺ CSCs differentiation. However, the intermediate signaling mechanism determining the differentiation fate of CSCs upon CD63 activation has not been the focus of this study and therefore remains to be elucidated.

Taken together, our first study (Chapter 2) establishes the existence of a CD63⁺/c-kit⁺ CSC population that is susceptible to proliferate and differentiate into cardiac myocytes and vascular cells in vitro upon treatment with TIMP-1. Furthermore, we show that TIMP-1/CD63 interaction leads to enhanced expression of GATA-4, Mef2C and Nkx-2.5 genes leading to enhanced cardiac differentiation.

We conclude our first study (Chapter 2) by highlighting the need for additional experiments aiming at identifying CD63⁺/c-kit⁺ CSCs in vivo, their localization in respect to the infarct zone and their ability to migrate, proliferate and differentiate upon TIMP-1 transplantation. Additionally, identifying the molecular mechanisms promoting

their proliferation and differentiation could provide more insight on how these cells could be best taken advantage of in the clinical set up.

In study 2 (Chapter 3), we aimed at addressing the above highlighted points by performing MI surgery on TIMP-1 KO and C57BL/6 mice followed by intramyocardial delivery of ESCM, ES-TIMP-1-CM or recombinant TIMP-1 protein. These three groups were done to emphasize the effect of TIMP-1 over any other factors released by stem cells present in the CM. We stipulated that TIMP-1 alone would be sufficient to activate CD63⁺/c-kit⁺ CSCs and regenerate the damaged myocardium. Our collected data from study 2 demonstrate existence of CD63⁺/c-kit⁺ CSCs population in the peri infarct heart. Furthermore, we provide evidence that intramyocardial delivery of ES-TIMP-1-CM or TIMP-1 equivalently enhance CD63⁺/c-kit⁺ CSCs proliferation and differentiation into cardiac myocytes, ECs and VSM cells, and promote additional CD63 expression in the heart. Most importantly, this study for the first time demonstrates that endogenous TIMP-1 is necessary for CD63 expression on CD63⁺/c-kit⁺ CSCs. This novel role of TIMP-1 was evidenced by the drastic reduction in CD63 expression resulting in decreased number of CD63⁺/c-kit⁺ CSCs population in TIMP-1 KO animals compared to control mice. Moreover, exogenous delivery of TIMP-1 was sufficient to rebound CD63 expression and replenish CD63⁺/c-kit⁺ CSCs pool in TIMP-1 KO animals.

Next, we aimed at identifying the downstream mechanism governing CD63⁺/c-kit⁺ CSCs proliferation and differentiation upon TIMP-1 treatment. Stem cell differentiation is traditionally mediated through the canonical Wnt/ β -catenin pathway;

however, our data from study 2 demonstrate CD63^{+ve}/c-kit^{+ve} activation bypasses Wnt but requires CD63 and β -catenin intermediates (6; 17; 18). Activation of the non-canonical CD63/ β -catenin pathway is suggested by increased CD63, β -catenin and phospho- β -catenin expression in MI hearts treated with ES-TIMP-1-CM and TIMP-1 when compared to non-treated animals. We therefore postulated that TIMP-1 treatment activates CD63 which induces dephosphorylation of Axin complex, preventing β -catenin ubiquitination and degradation. Released β -catenin then accumulates in the cytoplasm and is phosphorylated prior to nucleus translocation. β -catenin thereafter induces cardiac gene expression and promotes cardiac proliferation and differentiation as previously reported (6; 17; 18). We additionally stipulated that endogenous TIMP-1 activates CD63/ β -catenin on CD63^{+ve}/c-kit^{+ve} CSCs promoting additional CD63 expression. Our results are in accordance with multiple studies demonstrating ES cells differentiate into cardiac cells through activation of β -catenin pathway and subsequent expression of cardiac genes, such as; Nkx2.5, GATA-4, Mef2C, β -myosin heavy chain (β -MHC) and cardiac troponin T (cTnT) (6; 9; 11; 31).

In the present study, we choose to identify c-kit^{+ve} CSCs population because of their prevalence in the heart. However, other CSCs exist such as side population (SP) cells, cardiac progenitor cells (CPCs), Sca-1^{+ve} cells, cardio sphere cells and Isl1^{+ve} cells which are less abundant and less characterized (2; 15; 21; 22; 25). It is therefore imperative to identify the relative contribution of CD63^{+ve} CSCs that are not c-kit^{+ve} in cardiac regeneration following MI.

In conclusion for study 2, we provide evidence for the existence of a CSC population, CD63^{+ve}/c-kit^{+ve}, within the peri-infarct area of the murine heart. We show

that these cells can be triggered to differentiate into cardiac cells by exogenous delivery of TIMP-1 factor or ES-TIMP-1-CM. Finally, we demonstrate that CD63^{+ve}/c-kit^{+ve} CSCs activation is mediated through non-canonical CD63/ β -catenin pathway.

Besides the regenerative role identified in study 1 and 2, TIMP-1 is known to alleviate cardiac remodeling through inhibition of MMPs; however, not much is known about the molecular mechanism mediating this protective effect and whether other factors released in ESCM would provide a better protection (7; 16; 29). In study 3 (Chapter 4), we compared the anti-cardiac remodeling effect of direct transplantation of TIMP-1 protein, ESCM, and ES-TIMP-1-CM in the MI heart of both C57BL/6 and TIMP-1 KO mice. We hypothesized that parallel to enhancing cardiac proliferation and differentiation, TIMP-1 will surpass ESCM in blunting apoptosis and fibrosis in the MI heart.

Our previously published data demonstrated ES-TIMP-1 cell delivery in the MI heart decreased Caspase-3 mediated apoptosis, however, whether this effect is mediated through TIMP-1 or other factors additionally released by ES-TIMP-1 cells was not clear. Within study 3, we provide evidence that TIMP-1 alone is sufficient to decrease overall cardiac apoptosis and specifically blunt Caspase-3 activity (an intrinsic mediator of apoptosis) in cardiac myocytes following MI. Furthermore, TIMP-1 KO animals showed significantly higher Caspase-3 activity in cardiac myocytes compared to control mice following MI which further establishes that TIMP-1 acts as a shield against cardiac myocytes apoptosis through inhibition of Caspase-3 activity.

One of the hallmarks of cardiac remodeling is fibrosis, which results from collagen deposition in the interstitial and vascular spaces leading to stiffening of the left

ventricle, thereafter hindering its contractile capacity (7; 24; 29; 30). MMPs are well documented fibrosis mediators which function is predominantly controlled by TIMP-1(7; 24; 29; 30). Our previously published work showed ES-TIMP-1 cells transplantation in the MI heart decreased overall interstitial and vascular fibrosis . However, it was unclear whether the anti-fibrotic effect was the result of TIMP-1 alone or other factors additionally released in ES-TIMP-1-CM. To address this question, we measured interstitial and vascular fibrosis on TIMP-1 KO and C57BL/6 infarcted hearts following ESCM, ES-TIMP-1-CM and TIMP-1 treatments. Our data showed that although all treatments were efficient at blunting fibrosis (interstitial and vascular); the last two treatments provided the best outcome regardless of the strain. Of note, hepatic growth factor (HGF), an ES cell-released factor present in their media and previously linked to decreased fibrosis in lung alveolar epithelium, might additionally be responsible for the decreased fibrosis reported in our ESCM hearts (13) . However, TIMP-1 seems to provide a stronger anti-fibrotic effect as demonstrated by the extent of fibrosis decrease we report following TIMP-1 treatment compared with any other combined factors released by ES cells.

Finally, as part of study 3, we investigated the impact of TIMP-1 on infarct size measurements and left ventricular contractile function. Expectedly, our results were in accordance with previous work demonstrating larger infarct areas in TIMP-1 KO animals compared with their C57BL/6 counterparts two weeks following MI (7; 16). Moreover, infarct size measurements were significantly lower in ES-TIMP-1-CM and TIMP-1 treatments as compared to either ESCM or non-treated animals. Furthermore, we are first to report that TIMP-1 anti-fibrotic effect is more efficient than any combined effects

from factors released by ES-TIMP-1 cells. Finally, we show that exogenous delivery of TIMP-1 is able to salvage equivalent amount of myocardium in TIMP-1 KO and C57BL/6 hearts even though the extent of infarction was bigger in TIMP-1 depleted mice prior to treatment.

Functional data is greatly impacted by the extent of cardiac cell death and fibrotic tissue accumulation following MI. In fact, the larger the infarct size, the lower the ability of the ventricle to efficiently pump (7; 16). This in part explains why FS percent in TIMP-1 KO animals were lower than C57BL/6 mice. Surprisingly though, EF data which reflect the amount of blood pumped out within every single contraction was similar in both strains. In other words, although infarct measurements and FS data were drastically lower in TIMP-1 KO infarcted hearts compared to C57BL/6 mice, volume measurements between both strains were apparently not affected. To justify this observation, we postulated that mitral regurgitation which occurs in severely affected MI hearts and TIMP-1 KO animals for instance might have mitigated volume measurements in TIMP-1 KO animals leading to pseudo normal EF numbers regardless to their dramatic left ventricular remodeling (7). Nonetheless, ES-TIMP-1-CM and TIMP-1 delivery significantly improved both FS and EF data in the infarcted heart of both strains.

In conclusion, study 3 (Chapter 4) compared the beneficial effect of intramyocardial delivery of TIMP-1 or ES-TIMP-1-CM in the MI setting. Our data revealed that both treatments significantly diminish Caspase-3 mediated cardiac apoptosis, and reduce vascular and interstitial fibrosis leading to significantly smaller infarct sizes and improved contractile function in both control and TIMP-1 KO animals. Furthermore, we demonstrated that exogenous delivery of TIMP-1 factor can sufficiently

reduce cardiac remodeling in both TIMP-1 KO and control animals which resume similar cardiac function. In the current study, we stipulate that the anti-apoptotic and anti-fibrotic role of TIMP-1 in the heart is most likely mediated through CD63 receptor expressed on cardiac cells which most likely differentiated from CD63⁺/c-kit⁺ CSCs previously identified in study 1 and 2. Nonetheless, future studies are warranted to verify this hypothesis.

Overall, study 1, 2 and 3 demonstrate that CD63⁺/c-kit⁺ cells are a novel CSC population present in the peri-infarct area of the heart. When treated with TIMP-1 both in vitro and in vivo, CD63⁺/c-kit⁺ CSCs showed 1) increased proliferation compared to control cells, 2) enhanced CD63 expression, 3) significantly improved cardiac myocyte, VSM cell, and EC differentiation, 4) increased cardiac gene (GATA-4, Mef2C, and Nkx-2.5) expression, 5) CD63⁺/c-kit⁺ differentiation is mediated through CD63/ β -catenin pathway, and 6) TIMP-1 inhibits apoptosis and fibrosis and improves contractile function following myocardial transplantation. Our data provided in the 3 studies demonstrate for the first time the multi-faceted role of TIMP-1. This molecule has a strong regenerative potential through activation of endogenous CD63⁺/c-kit⁺ CSCs while alleviating adverse cardiac remodeling consecutive to MI, therefore opening a new era for cardiac regeneration research.

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