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TRANSPLANTATION OF PLURIPOTENT STEM CELLS CONFERS CARDIAC
PROTECTION IN DOX-INDUCED HEART FAILURE THROUGH NOTCH-1 PATHWAY

by

HILDA MERINO-CHAVEZ
M.D. University of Central Ecuador, 2007

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
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Major Professor: Dinender K. Singla

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ABSTRACT

Doxorubicin (DOX) is the antineoplastic drug of preference used to treat a wide variety of malignancies, with high survival rates among treated patients. However, the benefits of this drug have become less appealing due to the side effects that occur such as DOX-induced cardiomyopathy (DIC) and an increased risk of myocardial infarction (MI). Therefore, there is an urgent need to explore the therapeutic options to treat DIC. In this context, adult stem cells have been used as a source to reduce cardiomyocyte apoptosis in DIC; however, the effects of transplanted embryonic stem (ES) cells and induced pluripotent stem (iPS) cells in DIC post MI are unknown. As a result, we wanted to understand how transplanted ES and iPS cells and the factors released by them inhibit apoptosis and improve cardiac function in DIC post MI. C57BL/6 mice were divided into five groups: Sham, DOX-MI, DOX-MI+cell culture (CC) media, DOX-MI+ES cells, and DOX-MI+iPS cells. Mice were treated with DOX (12 mg/kg, cumulative dose) followed by left coronary artery ligation to induce MI. ES or iPS cells (5×10^4) were delivered into the peri-infarct region. At day 14 post-MI, echocardiography was performed, mice sacrificed, and hearts harvested for further analyses. To investigate if protective effects are provided by factors released from ES and iPS cells in DIC, we performed in vitro studies using condition media (CM) obtained from ES or iPS cells to treat DOX-induced cardiotoxicity in H9c2 cells. Our data reveal that apoptosis was significantly inhibited in the ES and iPS cell transplanted hearts as well as ESCM and iPSCM treated cells compared with the untreated controls. Furthermore, a significant increase in levels of Notch-1, Hes1, and pAkt survival protein were observed. Decreased levels of PTEN, a negative regulator of Akt pathway, along with improved

heart function were also observed in the stem cell transplanted groups. In conclusion, our data show that transplantation of ES and iPS cells blunt DOX-induced apoptosis in vivo, which is associated with improved cardiac function. Moreover, decreased apoptosis in both in vitro and in vivo models is mediated by the Notch pathway.

*"Nothing can stop who firmly believes that the wind in his sails comes directly from
God." - Rex Rouis*

For my loving husband whose love has been the engine of my dreams and whose
support was always there when I needed it.
For my parents and my sister who taught me that my dreams can come true with love,
dedication, and hard work.

*Esta tesis esta dedicada para mi amado esposo cuyo amor es la máquina de mis
sueños y quien estuvo siempre a mi lado cuando lo necesité.*

*Para mis padres y hermana quien con su ejemplo me enseñaron que mis sueños
pueden hacerse realidad si lo hago con amor, dedicación y trabajo duro.*

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"It matters not how strait the gate, How charged with punishments the scroll.

I am the master of my fate: I am the captain of my soul."

-William Ernest Henley

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I like to express my sincere gratitude to Reetu Singla for her support and encouragement during my professional journey throughout these years and to all my fellow lab members present or not who shared along this journey with me.

"Don't walk in front of me, I may not follow. Don't walk behind me, I may not lead.

Walk beside me and be my friend."

I would like to thank Latifa Abdelli, Princess Urbina, and Crystal Rocher, my fellow graduate students and friends who shared with me their dreams and their good and bad days, who help me and encourage me every day during this journey and who I will keep always in my heart.

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LIST OF ABBREVIATIONS

2D	Two dimensional
Akt	Adenosine triphosphate-dependent tyrosine kinase
ANOVA	Analysis of variance
AU	Arbitrary units
bFGF	Basic fibroblast growth factor
BM	Bone Marrow
BMSCs	Bone marrow mesenchymal stem cells
BMCs	Bone marrow-derived stem cells
C57BL/6	C57 Black/6 mice
CC	Cell culture media
CM	Conditioned media
CPCs	Cardiac Progenitor Cells
D14	Day 14
DAPI	4',6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine- butyl ester
DEVD	Amino acid sequence Asp-Glu-Val-Asp
DIC	Doxorubicin induced cardiomyopathy
DMEM	Dulbecco's modified eagle medium
DOX	Doxorubicin
ECL	Enhanced chemiluminescent substrate
EDV	Left ventricular volume at end diastole

EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay
ES	Embryonic stem
ESCM	Embryonic stem cells Condition Media
ESV	left ventricular volume at end systole
FBS	Fetal bovine serum
FS	Fractional shortening
GFP	Green fluorescent protein
H ₂ O ₂	Hydrogen peroxide
H9c2	Rat cardiomyoblasts
Hes1	Hairy and enhancer of split-1
H&E	Hematoxylin and eosin
HRP	Horse radish peroxidase
hUCB	Transplanted human umbilical cord blood derived stem cells
IP	Intraperitoneal
IP3	Inositol trisphosphate
iPS	Induced pluripotent stem
iPSCM	Induced pluripotent stem cells Condition Media
LAD	Left anterior descending artery
LIF	Leukemia inhibitory factor
LV	Left ventricle
LVIDd	Left ventricular internal dimension-diastole

LVIDs	Left ventricular internal dimension-systole
MI	Myocardial infarction
MOM	Mouse on Mouse
NIH	National institute of health
Notch-1	Notch homolog 1, translocation-associated
p-Akt	Phosphorylated Akt
p-PTEN	Phosphorylated phosphatase and tensin homolog
PFA	Paraformaldehyde
PI3K	Phosphoinositide-3-kinase
PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SEM	Standard error of the mean
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

CHAPTER 1: INTRODUCTION

Doxorubicin

Doxorubicin (DOX) is the antineoplastic drug of preference used to treat a wide variety of malignancies such as acute leukemia, lymphoma, and breast cancer, producing high survival rates among treated patients (Hershman et al., 2008; De et al., 2010). The five-year survival rate of children with leukemia has increased from 30% to 80% due to use of DOX as a chemotherapeutic treatment (Geisberg and Sawyer, 2010). However, DOX's induced cardiotoxicity as a side effect make this drug less appealing (Singal and Iiskovic, 1998; Miranda et al., 2003). This cardiac dysfunction is dose- and time-dependent (Deng et al., 2009; Huang et al., 2010). In fact, DOX-induced cardiomyopathy (DIC) can develop as early as one year after its use or may take up to 20 years (Minotti et al., 2004; Geisberg and Sawyer, 2010; Psaltis et al., 2011). Patients having received DOX had an increased risk of myocardial infarction (MI) that persisted up to 25 years after treatment. Five percent of Hodgkin's lymphoma patients who received DOX as part of their chemotherapy treatment developed MI at about 11.5 years after complete remission (Swerdlow et al., 2007).

DOX-induced cardiomyopathy mechanism

Studies have proposed that DOX induces oxidative stress in the heart leading to heart dysfunction (Horenstein et al., 2000; Kumar et al., 2002; Ludke et al., 2009; Menna et al., 2010). The chemical structure of DOX produces one electron reduction to the drug molecule resulting in formation of a semiquinone, which under aerobic conditions, forms reactive oxygen species (ROS) such as superoxide radicals, hydroxy

radicals, and hydrogen peroxide (Ludke et al., 2009). In addition to the induction of high levels of ROS, DOX decreases the levels of endogenous antioxidants that scavenge the free radicals. The resulting increase in free radicals and the decrease of antioxidants results in heart failure (Singal et al., 1997; Minotti et al., 2004; Geisberg and Sawyer, 2010; Psaltis et al., 2011). Furthermore, DOX ROS induction has also been associated with lipid peroxidation. ROS harms the cell membrane and the proteins that are integrated to its phospholipid bilayer such as enzymes, receptors, and transporters, impairing their function to maintain metabolic equilibrium in the cell (Singal et al. 1998).

Stem cells as adjuvant therapy for DIC

Adjuvant therapies have been proposed to decrease cardiotoxic effects induced by DOX. For instance, the use of antioxidants such as probucol, taurine, and fenofibrate have been shown to suppress DOX-induced oxidative stress and decrease cardiomyocyte apoptosis (Li and Singal, 2000; Ichihara et al., 2007; Das et al., 2011). However, these approaches have been used to inhibit cell death, so a new approach is needed to replace cell loss. Since cardiomyocytes have limited self-renewal, stem cells have gained significant consideration as an alternative method to repair and regenerate cardiac cells in DIC (Gopinath et al., 2010). Adult stem cells, such as bone marrow-derived and human umbilical cord blood-derived stem cells have been used as a source for cell replacement after cardiomyocyte apoptosis in DIC. Previous studies have shown that transplantation of bone marrow mesenchymal stem cells (BMSCs) improved left ventricular cardiac dysfunction in a DIC animal model. However, BMSCs were not able to differentiate to cardiomyocytes and did not express all muscle markers required to be

considered cardiac cells (Chen et al., 2010). Furthermore, bone marrow-derived stem cells (BMCs) similarly attenuate the impaired cardiac function in DIC and increase capillary density in remote areas of the heart. However, differentiation of BMCs to cardiomyocytes was not demonstrated and the benefits seen after transplantation were attributed to paracrine secretion of transplanted stem cells (Garbade et al., 2009). Finally, human umbilical cord blood (hUCB)-derived stem cells when transplanted in DIC differentiated towards cardiomyocytes, decreased the expression of hypertrophic markers, and decreased fibrosis, resulting in improved cardiac function (Gopinath et al., 2010).

Embryonic and induced pluripotent stem cells as valuable sources to restore DIC post-MI

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells can differentiate into any tissue in the human body (pluripotency), including cardiomyocytes, and are able to self-renew, converting them in a noble option to treat ischemic or toxic cardiomyopathies (Singla and Sobel, 2005; Singla et al., 2007; Singla et al., 2011). For instance, ES cells and their condition media (CM) have been shown to inhibit apoptosis and improve cardiac function when transplanted in a DIC animal model (Singla et al., 2012). Studies have shown that delivery of ES and iPS cells and the factors they secrete improve cardiac function and decrease apoptosis after an MI (Singla et al., 2007; Nelson et al., 2009; Singla et al., 2011). Evidence has suggested that transplanted ES cells inhibit cardiac remodeling and apoptosis, thus decreasing oxidative stress generated during MI (Singla et al., 2007). Rat cardiomyoblasts (H9c2) exposed to H₂O₂ and treated with ES CM resulted in decreased apoptosis and activated

Akt pro-survival protein. This suggests that ES cell-secreted factors are involved in the IP3/Akt survival pathway, resulting in regeneration of cardiomyocytes after an MI (Singla et al., 2007; Singla et al., 2008). Induced pluripotent stem (iPS) cells generated from H9c2 cells, when transplanted after MI, were shown to differentiate into cardiomyocytes and inhibited apoptosis, cardiac fibrosis, and improved cardiac function (Singla et al., 2011). All these characteristics make ES and iPS cells valuable sources to restore the cardiomyocyte population lost in DIC. However, the mechanisms by which stem cells are recruited to the heart and exert their benefits are not well understood.

Stem cells and Notch-1

Notch-1 receptors are regulators of embryonic development through cell-cell interactions, and these receptors mediate self-renewal, survival, and differentiation of stem cells (Li et al., 2011). In the heart, Notch-1 regulates the fate of cardiac progenitor cells (CPCs) and stimulates proliferation of cardiomyocytes mediated by c-Met and Akt survival proteins. Studies have shown that Notch-1 plays a protective role of the adult heart in MI. Levels of Notch-1 and its downstream target, Hes1, increase as a response to ischemic injury. Additionally, the overexpression of Notch-1 has been shown to improve cardiac function in murine models of MI (Gude et al., 2008; Li et al., 2011). Notch-1 has also been implicated in repair of the infarcted heart through transplantation of bone marrow (BM)-derived stem cells (Li et al., 2011). The protective role of Notch-1, however, may not be present after DOX treatment. A previous study showed that Notch-1 receptor expression levels in CPCs were down-regulated compared to Notch-1 ligands, delta-like 3 and jagged, after DOX treatment (De et al., 2010). Since Notch-1

receptor regulates transition of CPCs into myocytes after ischemic damage, it is important to maintain integrity of the receptor after DOX treatment to decrease the cardiotoxic effects while preserving antineoplastic functions of the drug. Evidence shows that there is a high necessity and priority to find an effective adjuvant treatment that can be used in conjunction with DOX to reduce its cardiotoxic effects without reducing its efficacy as a cancer treatment.

Hypothesis

In the present study, we hypothesize that:

- I. Transplanted ES and iPS cells inhibit cardiomyocyte apoptosis and improve cardiac function in DIC following MI
- II. Decrease in cardiomyocyte apoptosis is mediated by the activation of the Notch-1 pathway

Aims

Aim 1. Determine the effects of transplanted ES and iPS cells on apoptosis and cardiac function in DIC post-MI.

Aim 2. Determine that activation of Notch-1 pathway is the mechanism by which transplanted ES and iPS cells decrease apoptosis in DIC-post MI. Furthermore, confirm this mechanism in an in-vitro model of DOX-induced cardiotoxicity.

CHAPTER 2: MATERIALS AND METHODS

Cell culture and drug treatment

H9c2 cell line: H9c2 cells were grown on 100mm non-coated tissue culture plates for 48-72 hours, or until about 80 to 90% confluent, and were maintained in Dulbecco's modified eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum, penicillin/streptomycin, glutamine, sodium pyruvate, and non-essential amino acids. Plates were divided into six groups: CONTROL, DOX, DOX+ESCM, DOX+iPSCM, DOX+DAPT+ESCM, and DOX+DAPT+iPSCM. DOX at a concentration of 2 μ M (Fisher, BP2516) was added to all plates except the control group for 24 hours. The next day, the DAPT groups were pre-exposed to 30 μ M of DAPT (Sigma, D5942) for another four hours. Following DAPT pre-exposure, medium was replaced with fresh medium, conditioned medium was derived from ES and iPS cells, and ES and IPS CM was supplemented with or without DAPT for another 24 hours. Finally, cells were washed and detached from the plates using trypsin/EDTA and collected in RIPA buffer-containing protease inhibitors for 30 minutes on ice. The supernatant containing the proteins was collected after centrifugation.

ES cells: Were maintained in DMEM as previously reported (Singla et al., 2007). Cells were trypsinized and used the same day to be transplanted during MI surgery at a concentration of 2.5 million cells/mL.

iPS cells: Maintained in Dulbecco's DMEM (Invitrogen, USA) on a 60mm gelatin-coated tissue culture plate as reported previously (Singla et al., 2011). Cells were trypsinized and given in a concentration of 2.5 million cells/mL. ES and iPS cells were

previously tagged with red fluorescent protein (RFP) for identification once transplanted in the heart.

ESCM and iPSCM: Were obtained and prepared as previously published (Singla et al., 2012).

In vivo study groups and induction of DIC

All animal protocols were approved by the University of Central Florida animal care committee. C57BL/6 mice male and females of 8-10 weeks of age were divided into five groups: Sham-operated control, DOX-MI, DOX-MI+cell culture (CC) media, DOX-MI+ES cells, and DOX-MI+iPS cells with an n=6-8 in each group. Mice were treated with DOX as previously reported (Singla et al., 2012). In brief, mice were injected one time every other day (Monday, Wednesday, and Friday) for three days with DOX to obtain a cumulative dose of 12 mg/kg via intraperitoneal injection (IP). Two weeks after the last dose of DOX, MI was induced by coronary artery ligation.

Coronary artery ligation and stem cell transplantation

A coronary artery ligation procedure was performed as reported previously (Kumar et al., 2005; Singla et al., 2006) under isoflurane inhalatory anesthesia administered via an endo-tracheal tube. In brief, a left thoracotomy was performed; the left anterior descending (LAD) coronary artery was visualized using a dissecting microscope and subsequently ligated using a 7.0 polypropylene suture. Sham-operated mice were subject to the same procedure, however, the suture was not tied off. Following ligation, 5×10^4 ES or iPS cells were delivered into two different sites in the

peri-infarct region, identified as the white area around the tight LAD region where the suture was placed, using a 29-gauge floating needle.

Echocardiographic analysis

At day (D) 14 post-MI, two-dimensional (2D) echocardiography was performed under 2% isoflurane via nose cone to analyze cardiac function as previously described (Singla et al., 2012). In brief, M-mode images of the left ventricle were documented. Left ventricular (LV) dimensions such as LV fractional shortening (FS), left ventricular end-diastolic internal diameter (LVIDd), left ventricular internal diameter in systole (LVIDs), as well as end diastolic volume (EDV), end systolic volume (ESV), and LV ejection fraction (EF) were measured. After echocardiography analysis was performed, animals were sacrificed using pentobarbital (80mg/Kg) and cervical dislocation. The hearts were harvested, rinsed with PBS and sectioned transversally with the top portion kept in RNA later and the bottom portion in 10% paraformaldehyde (PFA).

Notch-1 and α -sarcomeric actin double staining

The bottom part of the heart was embedded in different ethanol dilutions (75%, 80%, 90%, and 100%), and paraffin blocks were formed containing the heart tissue. Five micron sections were obtained and placed on microscope slides. A double immunofluorescent staining protocol was applied as reported previously (Boni et al., 2008). Sections from five to six different hearts in each group were deparaffinized, rehydrated, and then blocked with a mouse antigen blocking reagent (MOM kit, Vector Laboratories, Cat. #FMK-2201) for one hour and then incubated with mouse Notch-1 antibody (1:20 dilution, abcam Cat. #44986) and a monoclonal anti- α -sarcomeric actin

antibody (1:30 dilution; Sigma-Aldrich Cat. #A2172). Biotinylated Anti-Mouse IgG reagent (MOM kit, Vector Laboratories, Cat. #FMK-2201) was used as a secondary antibody. Finally, sections were incubated for five minutes with fluorescein Avidin DCS (16ul/ml) and Texas Red Avidin DCS (15ug/ml), respectively, then washed and covered with mounting media containing 4',6-diamino-2-phenylindole (DAPI). Sections were analyzed and representative photomicrographs were taken under fluorescent (Olympus 1X70 and Nikon TE 2000-E) and confocal (LEICA laser scanning) microscopes. Proper control slides were also performed.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

In-vitro: H9c2 cells were fixed with 0.4% formalin for 10 minutes and then washed with 1xPBS. Apoptotic positive nuclei was determined by TUNEL staining using an in situ cell death detection kit (Roche, Cat# 12156792910) according to manufacturer's instructions. In brief, cells were incubated with a TUNEL reaction mixture (enzyme and label solutions 1:9) for one hour, and then washed with 1x PBS three times. Finally, mounting media with DAPI was used to cover the slides. Each slide was analyzed under a fluorescent microscope. Photomicrographs were taken under 20X and apoptotic nuclei (red) were counted as well as the total number of nuclei (blue). The percentage of TUNEL-positive nuclei was obtained dividing the total number of merged TUNEL-positive nuclei to the total number of nuclei multiplied by 100. The in vitro experiment was repeated three times.

In-vivo: Heart sections were deparafinized, rehydrated, and then incubated with proteinase K (Sigma, Cat. #P6556-5mg) at a dose of 25 ug/ml in 100 mM Tris-HCL for

15 minutes. Apoptotic positive nuclei was determined by TUNEL staining using the same method described in the in-vitro experiment according to manufacturer's instructions. In brief, sections were incubated with a TUNEL reaction mixture for one hour, and then washed with 1xPBS three times. Finally, mounting media with DAPI was used to cover the slides. Each slide was analyzed under an Olympus fluorescent microscope. Photomicrographs were taken under 20X and the percentage of apoptotic nuclei (red) was determined by counting the total number of red positive cells and the total number of nuclei (blue). Red cells that merge with DAPI in blue were considered positive. The following formula was applied to get the percentage: $(\text{red+nuclei}/\text{total blue nuclei}) \times 100$. One to two sections of 6-8 animals per group were analyzed.

Caspase-3 activity assay

In vitro: H9c2 cells were washed with 1xPBS, dissociated using trypsin/EDTA, and collected in modified RIPA buffer. Cell lysates were then used for protein quantification using a Bradford assay (Bio-Rad, Cat. #500-0006). Caspase-3 colorimetric activity assay (Bio Vision, Cat. #K106-25) was performed according to manufacturer's instructions. In brief, 70ul of 2X reaction buffer having 10 mM of DTT was added to 30ul of protein lysates. Additionally, 5 ul of 4mM DEVD-pNA was added to each sample and incubated for 1-2 hours at 37°C. The colorimetric reaction was then measured at 405 nm in a 96 well micro plate reader (Biorad Model 680). Readings were plot and expressed in arbitrary Units (A.U.).

In vivo: The top part of the heart was homogenized in modified RIPA buffer and the supernatant was collected and then used for protein quantification using a Bradford

assay. Caspase-3 colorimetric activity assay (Bio Vision, Cat. #K106-25) was performed according to manufacturer's instructions. In brief, 70ul of 2X reaction buffer having 10 mM of DTT was added to 30ul of heart homogenates. Additionally, 5 ul of 4mM DEVD-pNA was added to each sample and incubated for 1-2 hours at 37°C. The colorimetric reaction was then measured at 405 nm in a 96 well micro plate reader (Biorad Model 680). Readings were plot and expressed in arbitrary Units (A.U.).

Western blot

Protein lysates from H9c2 cells and tissue homogenates were obtained as stated before and used for protein quantification with Bradford assay and using a microplate reader (Biorad, Model 680) and measured at 590nm. 100 to 150ug of protein were loaded in an 8% or 10% polyacrylamide gel and run at 150V for one hour. Next, proteins were transferred to a PVDF membrane (BioRad, #162-0177) using a Trans-Blot Semi-dry transfer Cell for 35 to 60 minutes. Membranes were blocked with 5% skim milk in TBST for one hour and then incubated with Notch-1 (Cell signaling, Cat. #3268S), c-Notch-1 (Cell signaling, Cat. #4147S), pPTEN (Cell Signaling Cat. #95595), pAKT (Cell Signaling, Cat. #4058S), Hes1 (Abcam, Cat. #ab49170) and β -actin (Cell signaling, Cat. #4967L) primary antibodies at appropriate dilutions for one hour at room temperature or overnight at 4°C. Following the incubation of primary antibodies, the proper secondary antibody was used and membranes were incubated for one hour. Finally, membranes were treated with an enhanced chemiluminescent substrate (ECL, Thermo Scientific, and Cat # 32106) for 1-2 minutes and then exposed at different exposure times.

pAkt activity assay

Cell treatment, heart homogenization, and protein quantification were performed as stated before. Phosphorylated Akt (pAkt) activity assay (Exalpha Biological, Cat. #X1844K) was performed following manufacturer's instructions. In brief, 50ul of each sample was pipetted into microtiter wells that have capture antibodies, provided by the manufacturer, and incubated for two hours. Next any unbound material was washed and a biotin conjugated anti-pAkt1 antibody was added to each well and incubated for two hours. Excess antibody was washed and a HRP-conjugated streptavidin was added to each well for 30 minutes, producing a colorimetric reaction that was measured at 450nm in a microplate reader. Values were recorder and expressed in arbitrary units (A.U.).

Statistical analysis

One-way analysis of variance (ANOVA) was performed followed by Tukey test for all samples. All values were presented as a mean \pm SEM. $P < 0.05$ was considered to be statistically significant between the values.

CHAPTER 3: RESULTS

Effects of transplanted ES and iPS cells on apoptosis in DIC post-MI

Previous studies have shown that DOX induces apoptosis in cardiomyocytes (Minotti et al., 2004; Geisberg and Sawyer, 2010; Psaltis et al., 2011; Singla et al., 2012). To determine if ES and iPS cell transplantation has an effect on cardiomyocyte apoptosis in DIC post-MI, TUNEL staining was performed. At D14 post-MI, a significant number of apoptotic nuclei were observed in heart sections of DOX and DOX-MI+CC groups when compared with Sham (Figure 1, D-I). However, quantitative analysis of TUNEL staining was significantly reduced in DOX-MI+ES and DOX-MI+iPS cell transplanted hearts (Figure 1, J-O and P). The number of apoptotic nuclei was not significant between the ES and iPS cell treated groups. Moreover, a caspase 3 activity assay was performed. Quantitative analysis of caspase 3 activity was significantly reduced ($P < 0.05$) in DOX-MI+ES and DOX-MI+iPS cell transplanted hearts compared with the stem cell non-treated hearts, DOX-MI, and DOX-MI+CC (Figure 1, Q). Caspase 3 activity was not significant between ES and iPS cell groups.

Transplantation of ES and iPS cells contributes to cardiac repair in DIC post-MI through Notch-1

Notch-1 regulates the fate of cardiac progenitor cells (CPCs) and stimulates proliferation of cardiomyocytes (Gude et al., 2008; Li et al., 2011). Previous studies have shown that after treatment with DOX, Notch-1 receptor expression levels in CPCs are low compared with its ligands, delta-like 3 and jagged (De et al., 2010). Therefore, one to two heart sections from each group were stained for the presence of Notch-1^{+ve} cells and co-labeled with α -sarcomeric actin (Figure 2 A-T).

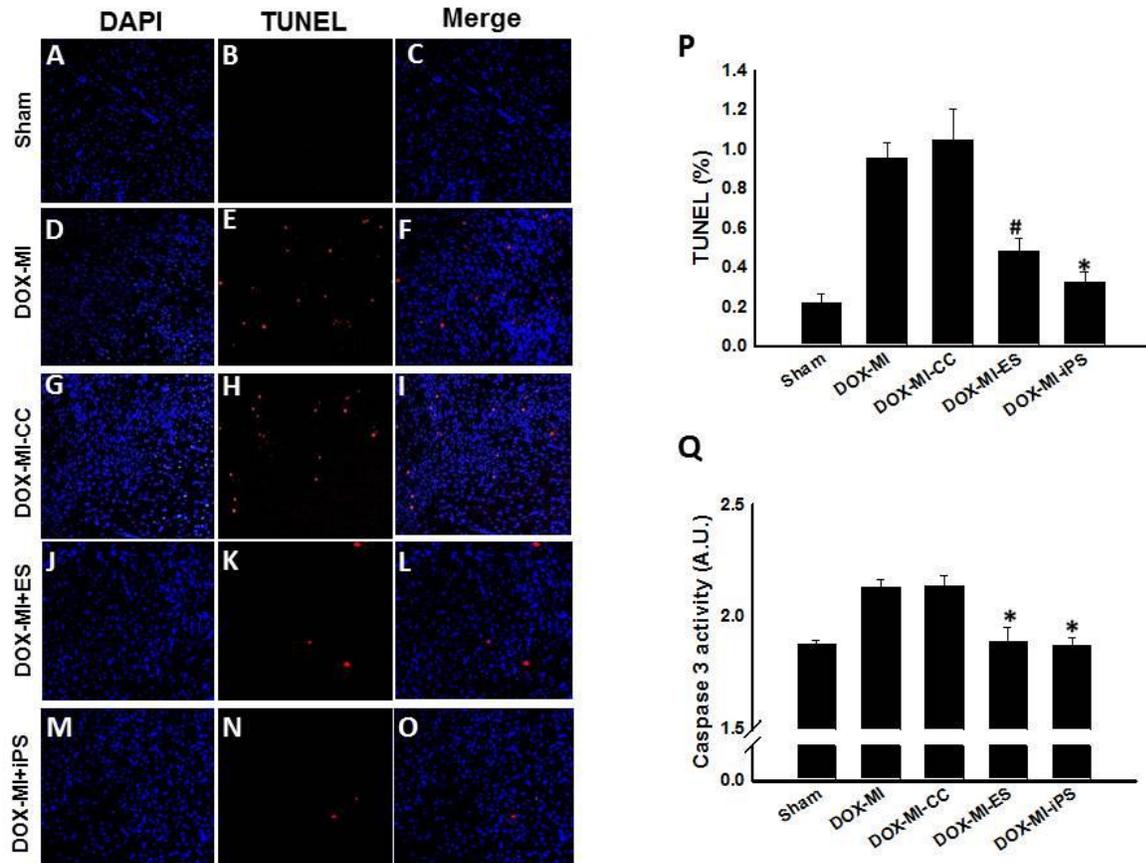


Figure 1: Effects of transplanted ES and iPS cells on cardiomyocyte apoptosis using TUNEL staining. To determine if ES and iPS cell transplantation has an anti-apoptotic effect, TUNEL staining was performed. Representative photomicrographs are shown on the left with DAPI in blue (A, D, G, J, and M), TUNEL in red (B, E, H, K, and N) and merged images (C, F, I, L, and O). Top right histogram (P) shows the percentage of total apoptotic nuclei at two weeks post-MI in ES and iPS cell treated groups (* $P < 0.05$) vs. DOX-MI and DOX-MI+CC groups with an $n = 7-9$ in each of the study groups. Bottom right histogram (Q) shows quantitative analysis of caspase 3 activity assay in arbitrary units (* $P < 0.05$ vs. DOX-MI and DOX-MI+CC).

Quantitative analysis of Notch-1^{+ve} cardiomyocytes show a significant decrease in DOX-MI and DOX-MI+CC groups compared with Sham. However, the number of Notch-1^{+ve} cardiomyocytes significantly increased ($P < 0.05$) in DOX-MI+ES and DOX-MI+iPS cell transplanted groups when compared with the stem cell untreated groups (Figure 2, U). Furthermore, western blot (WB) analyses were performed to confirm the

results obtained during immunohistochemistry. Densitometric analysis confirms that levels of Notch-1 in hearts treated with ES and iPS cells significantly increased compared with the untreated groups, DOX-MI and DOX-MI+CC (Figure 2, V); however, the levels of Notch-1 between DOX-MI+ES and DOX-MI+iPS were not significant.

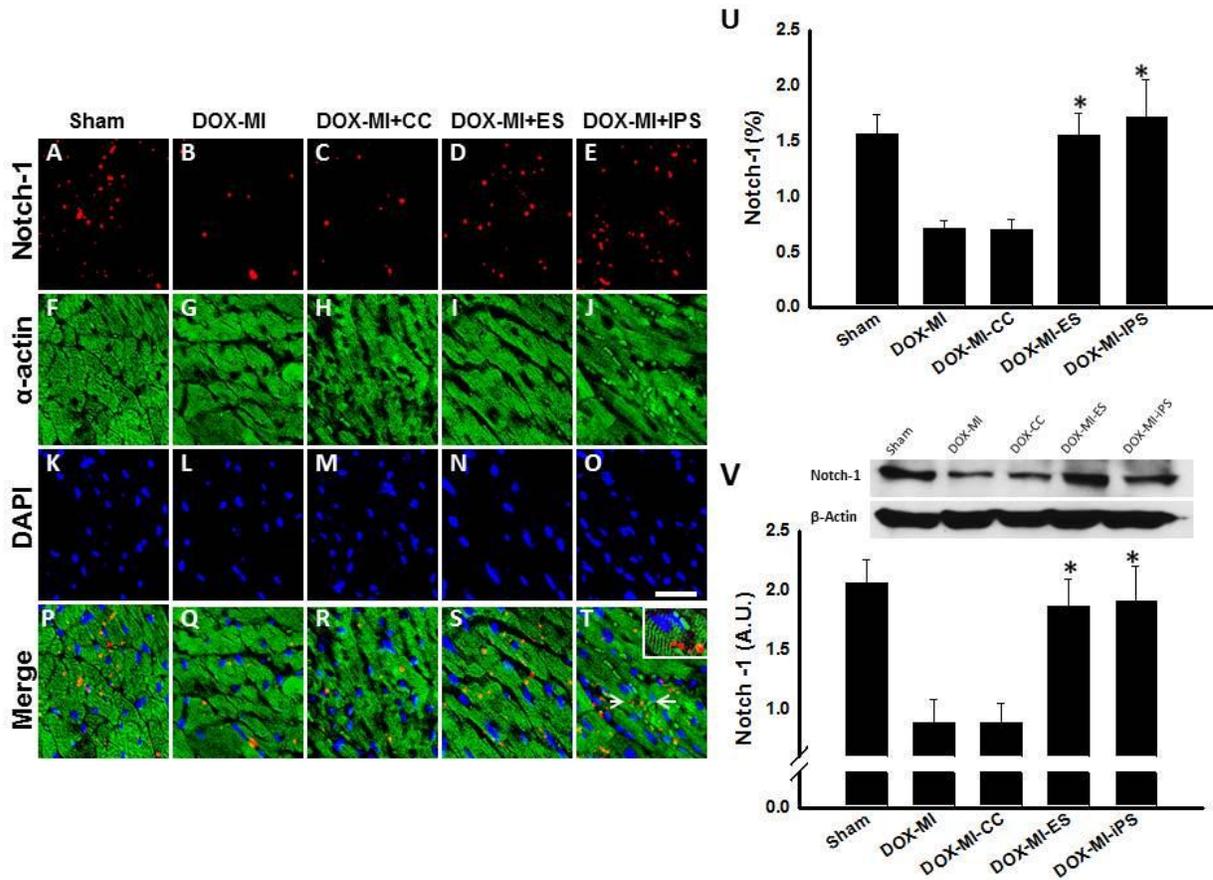


Figure 2: Effects of transplanted ES and iPS cells on Notch-1 expression. To study the expression of Notch-1 in DIC post-MI, a double staining with α -sarcomeric actin was performed. Representative photomicrographs are shown on the left with Notch-1 in red (A-E), α -sarcomeric actin in green (F-J), DAPI in blue (K-O), and merged images (P-T). The box in T demonstrates colocalization of Notch-1 with α sarcomeric actin. Scale bar=20 μ m. Top right histogram (U) shows a significant decrease of the percentage of Notch-1^{+ve} cells at two weeks post-MI in DOX-MI and DOX-MI+CC groups (*P<0.05). However, the percentage of Notch-1^{+ve} cells was significantly higher in the stem cell treated groups DOX-MI+ES and DOX-MI+iPS with an n=5-6 in each study group. Bottom right panel and histogram (V) show representative WB bands of Notch-1 receptor and control loading β -actin with densitometric analysis that show a significant decrease in expression of Notch-1 in DOX-MI and DOX-MI+CC groups and a significant increase in levels of Notch-1 in ES and iPS cell treated groups (*P<0.05) with an n=6 in each study group.

Transplanted ES and iPS cells regulate activation of PTEN in DIC post MI through Hes1

Phosphatase and tensin homolog (PTEN) is a cell cycle regulator and a tumor suppressor gene. Previous studies have shown that during MI, levels of PTEN protein increase (Keyes et al., 2010; Glass and Singla, 2011). In addition, overexpression of PTEN in Ishikawa cells has shown to enhance their chemosensitivity to DOX inducing apoptosis (Wan et al., 2007). Notch-1 has an inhibitory effect on PTEN through Hes1 in T-cell lymphoblastic leukemia (T-ALL) cells (Gutierrez and Look, 2007; Gutierrez et al., 2009). Therefore, to further characterize the effects of Notch-1 in cardiac repair in DIC post-MI treated with ES and iPS cells, Notch-1 downstream effector, Hes1, was studied.

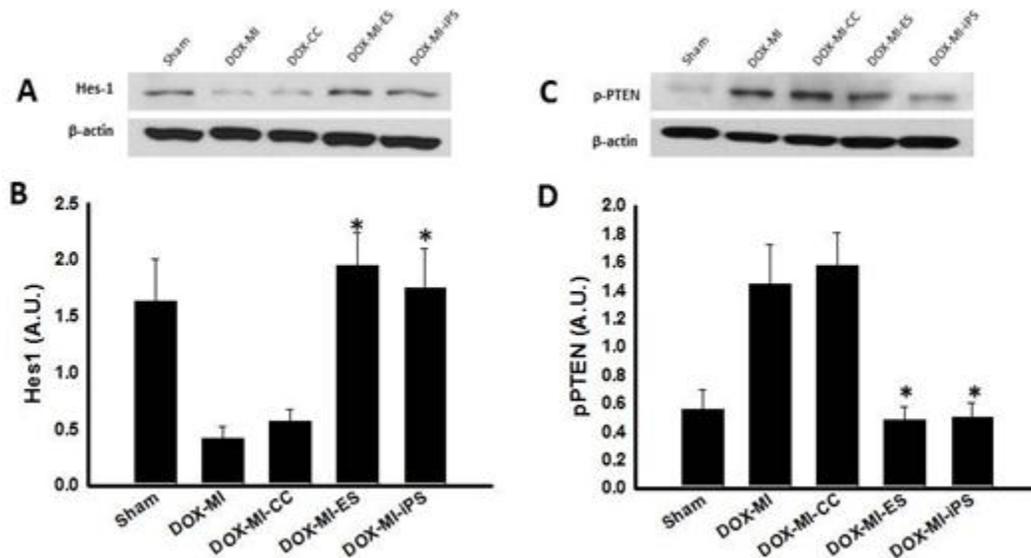


Figure 3: Hes1 regulates expression of PTEN in DIC post MI. **(A)** Top left panel displays representative photomicrographs of WB bands for Hes1. **(B)** Bottom left histogram shows densitometric analysis of WB bands with a significant increase in levels of Hes1 in groups treated with ES and iPS cells ($*P < 0.05$) vs. DOX-MI and DOX-MI+CC groups ($n=4-5$). Top right upper panel **(C)** shows representative WB bands of pPTEN and β -actin. Right bottom histogram **(D)** shows densitometry analysis of WB bands with a significant decrease in levels of pPTEN in hearts transplanted with ES and iPS cells. $*P < 0.05$ vs. DOX-MI and DOX-MI+CC ($n=4-6$).

As observed in Figure 3 A-B, densitometric analysis of Hes1 WB bands showed a significant increase in expression of Hes1 in the ES and iPS cell transplanted hearts ($P<0.05$) compared with a reduced expression of Hes1 in DOX-MI and DOX-MI+CC groups. However, Hes1 protein levels between ES and iPS cell transplanted groups were not significant. WB analyses were performed to study protein levels of PTEN. The densitometric analysis of WB bands of activated PTEN revealed a significant decrease ($P<0.05$) in levels of PTEN in hearts transplanted with ES and iPS cells compared with DOX-MI and DOX-MI+CC groups (Figure 3, C-D).

Akt protein levels are regulated by ES and iPS cells transplanted in DIC post-MI

Notch-1 crosstalk occurs with Akt in the border zone of an MI working together as a protective mechanism in the ischemic heart (Li et al., 2011). However, it has been shown that after treatment with DOX, levels of pAkt decrease in the heart. Therefore, we analyzed the pAkt activity in DIC post-MI. pAkt activity was significantly decreased in DOX-MI and DOX-MI+CC, but the decrease in pAkt was reverted in hearts transplanted with ES and iPS cells, showing a significant increase ($P<0.05$) in the activity of this survival protein (Figure 4, A). Moreover, WB densitometric analysis of pAkt bands confirm a significant increase ($P<0.05$) of activated Akt in the ES and iPS cell treated groups when compared with DOX-MI and DOX-MI+CC groups (Figure 4, A-B). There was not a significant difference between DOX-MI+ES and DOX-MI+iPS groups in pAkt activity.

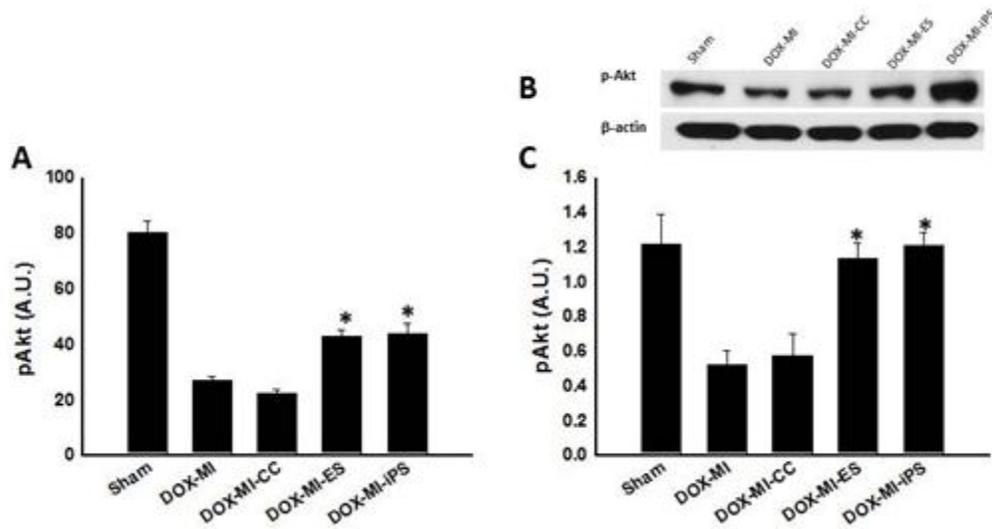


Figure 4: Effects of transplanted ES and iPS cells on Akt pathway in DIC post MI. Left histogram **(A)**, reveals a significant increase in pAkt activity in hearts transplanted with ES and iPS cells. * $P < 0.05$ vs. DOX-MI and DOX-MI+CC with an $n = 4-5$. WB analysis was performed using heart homogenates from each group and an antibody specific to pAkt. **(B)** Top right panel displays representative photomicrographs of WB bands for pAkt with its appropriate loading control. Bottom right histogram **(C)** shows densitometric analysis of WB bands with a significant increase in levels of pAkt levels in DOX-MI+ES and DOX-MI+iPS cell treated hearts (* $P < 0.05$, $n = 4-5$).

ES and iPS cell delivery in DIC post-MI improves cardiac function

Two weeks following MI surgery, cardiac function was analyzed in each of the study groups. A significant deficiency in the contraction function of the left ventricle (LV) in DOX-MI and DOX-MI+CC hearts was observed when compared with Sham group ($P < 0.001$) (Figure 5, A-G). However, a significant ($P < 0.05$) improvement in cardiac function expressed in the average percentage of fractional shortening (FS %) and ejection fraction (EF%) at two weeks post MI in DOX-MI+ES and DOX-MI+iPS cell treated hearts was observed (Figure 5, C and F). There was also a significant ($P < 0.05$) improvement in the internal diameter of the LV during systole (LVIDs) and in the end-systolic volume (ESV) values in the ES and iPS cell treated hearts (Figure 5, B and E).

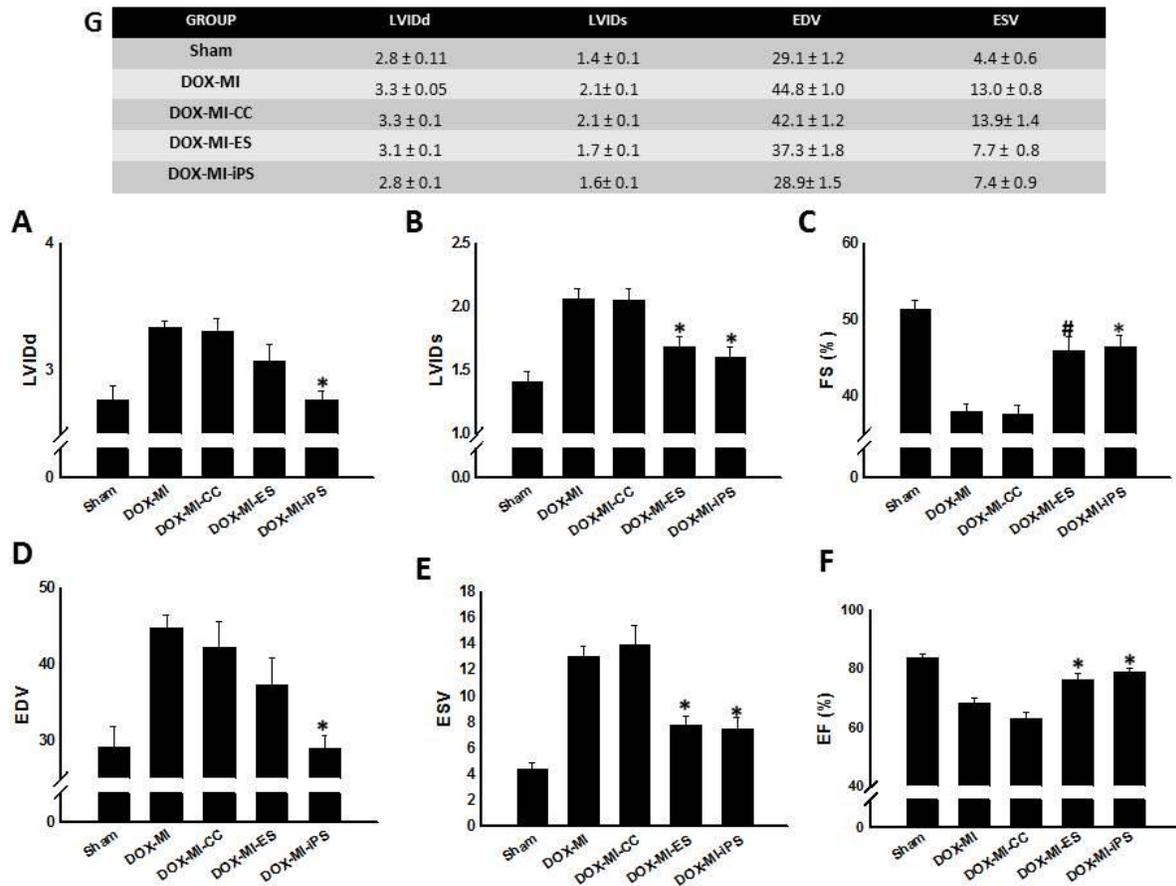


Figure 5: Effects of transplanted ES and iPS cells on cardiac function. To determine the effect of transplanted ES and iPS cells on cardiac function, echocardiography analysis was performed at D 14 post-MI in the DIC animal model. Left panels **A and B** show LVIDd and LVIDs values in each of the study groups. Panels **D and E** show the end-diastolic volume (EDV) and end-systolic volume (ESV) values of the left ventricle. Right histograms **C and F** show that average left ventricular fractional shortening percentage (FS) and average ejection fraction percentage (EF) significantly improved two weeks post-MI in the ES and iPS cell treated groups (* $P < 0.05$) compared with Sham, DOX-MI and DOX-MI+CC groups ($n=5-8$). Upper panel (**G**) shows the mean values and SEM of each of the study groups of LVIDd, LVIDs, EDV, and ESV.

The internal diameter of the LV during diastole (LVIDd) and end-diastolic volume (EDV) was improved in the iPS cell transplanted hearts compared with DOX-MI and DOX-MI+CC. We did not observe a significant improvement of LVIDd and EDV in the DOX-MI+ES group compared with DOX and DOX-MI+CC.

ESCM and iPSCM decrease apoptosis in H9c2 cells treated with DOX

To further investigate if protective effects seen in vivo are due to factors released from ES and iPS cells, in vitro studies were performed using condition media (CM) obtained from ES or iPS cells to treat DOX-induced cardiotoxicity in H9c2 cells. H9c2 cells were treated with DOX, DAPT (Notch-1 inhibitor), ESCM, and/or iPSCM. TUNEL staining was significantly reduced ($P<0.05$) in the ESCM and iPSCM treated groups compared with DOX and DOX+DAPT treated groups (Figure 6A). In addition to TUNEL staining, a caspase 3 activity assay was performed. As shown in Figure 6B, a significant increase ($P<0.01$) in caspase 3 activity was observed in DOX, DOX+DAPT+ESCM, and DOX+DAPT+iPSCM, compared with the CONTROL group. However caspase 3 activity was significantly attenuated ($P<0.05$) in the ESCM and iPSCM treated groups when compared with DOX and DOX+DAPT+ESCM but not with DOX+DAPT+iPSCM.

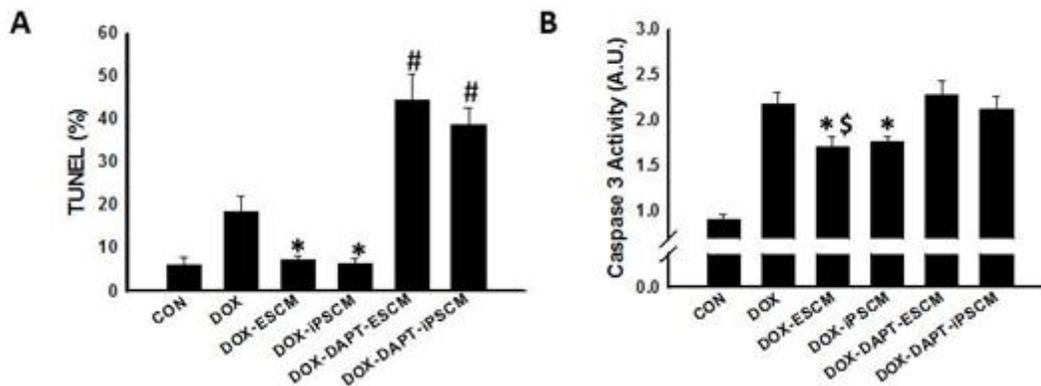


Figure 6: Effects of ESCM and iPSCM on DOX-induced apoptosis in H9c2 cells. To further investigate the effect of ES and iPS cells on DOX-induced apoptosis, H9c2 cells were treated with DOX, DAPT, and/or ESCM and iPSCM. Left histogram **(A)** shows quantitative analysis of the percentage of apoptotic H9c2 cells with a significant decrease in levels of apoptosis in ESCM and iPSCM treated groups ($*P<0.05$) compared with the DOX, DOX+DAPT+ESCM, and DOX+DAPT+iPSCM groups ($\#P<0.001$) ($n=5-8$). **(B)** Right histogram shows quantitative analysis of caspase 3 activity in arbitrary units with a significant decrease ($*P<0.05$) in ESCM and iPSCM treated groups compared with DOX and DOX+DAPT+ESCM groups ($n=5-10$).

ESCM and iPSCM regulate Notch-1 expression in H9c2 cells treated with DOX

As observed in the previous in-vivo results, transplantation of ES and iPS cells in DIC post-MI regulates expression of Notch-1. Therefore, to investigate if the effects on Notch-1 are also affected by factors released from ES and iPS cells, H9c2 cells were treated with DOX, DAPT, and/or ESCM and iPSCM. Western blot analysis of Notch-1 and cleaved Notch-1 showed a significant increase ($P<0.05$) in the group treated with ES CM compared with DOX and DAPT treated groups respectively (Figure 7, A-D). The same tendency was observed in the iPSCM group (Figure 7, E-G).

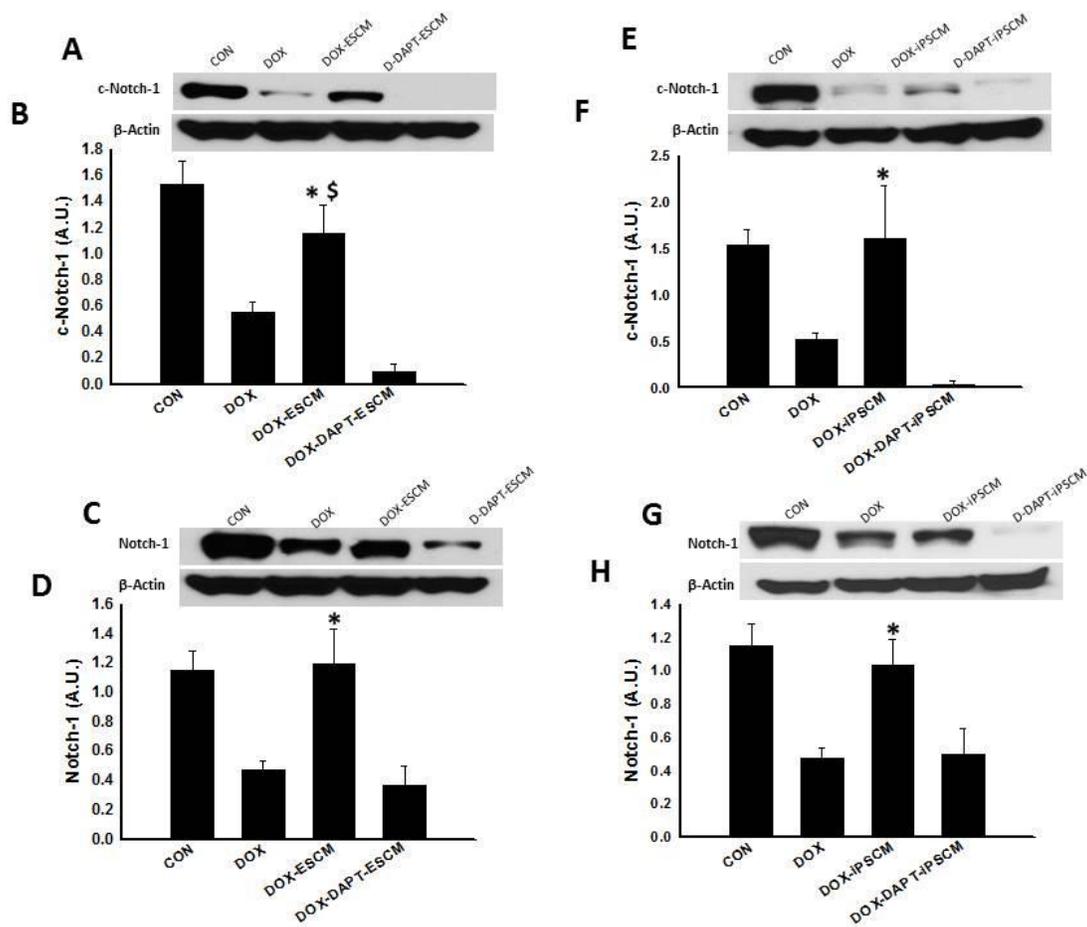


Figure 7: Effects of ESCM and iPSCM on Notch-1 in DOX induced cardiotoxicity in H9c2 cells. To study the effects of ESCM and iPSCM on Notch-1 in DIC, a gamma secretase inhibitor was used (DAPT) to inhibit the Notch-1 intracellular domain (c-Notch-1). **(A)** Upper left panel shows representative WB bands of c-Notch-1. **(B)** Upper left histogram shows densitometric analysis of c-Notch-1 WB bands with a significant increase in levels of c-Notch-1 ($*P<0.05$) in the group treated with ESCM compared with DOX and ($\$P<0.001$) DOX+DAPT+ESCM with an $n=7-10$ in each group. **(C)** Bottom left panel shows representative WB bands of Notch-1 with its respective loading control. **(D)** Bottom left histogram shows densitometric analysis of Notch-1 WB bands with a significant increase in levels of Notch-1 ($*P<0.05$) in the group treated with ESCM compared with DOX and DOX+DAPT+ESCM with an $n=6-7$ in each group. **(E)** Right upper panel shows representative WB bands of c-Notch-1 and β -actin. **(F)** Upper right histogram shows densitometric analysis of c-Notch-1 WB bands with a significant increase in levels of c-Notch-1 ($*P<0.05$) in the group treated with iPSCM compared with DOX and DOX+DAPT+iPSCM with an $n=6-9$ in each group. **(G)** Bottom right panel shows representative WB bands of Notch-1 receptor. **(H)** Bottom right histogram shows densitometric analysis of Notch-1 WB bands with a significant increase in levels of Notch-1 ($*P<0.05$) in the group treated with iPSCM compared with DOX and DOX+DAPT+iPSCM with an $n=8-6$ in each group.

Hes1 regulates expression of Akt in H9c2 cells treated with DOX

Densitometric analysis of WB bands of Hes1 showed a significant increase ($P < 0.05$) in levels of Hes1 in the ESCM and iPSCM treated groups compared with DOX and DOX+DAPT treated groups (Figure 8, A-B). To assess the effect of Hes1 on Akt activity in H9c2 cells treated with DOX, a pAkt activity assay was performed. Figure 8C shows a significant increase ($P < 0.05$) in pAkt activity in DOX+ESCM and DOX+iPSCM treated cells compared with DOX and DOX+DAPT+iPSCM but not when compared with DOX+DAPT+ESCM. Akt activity was not significant between ESCM and iPSCM treated groups.

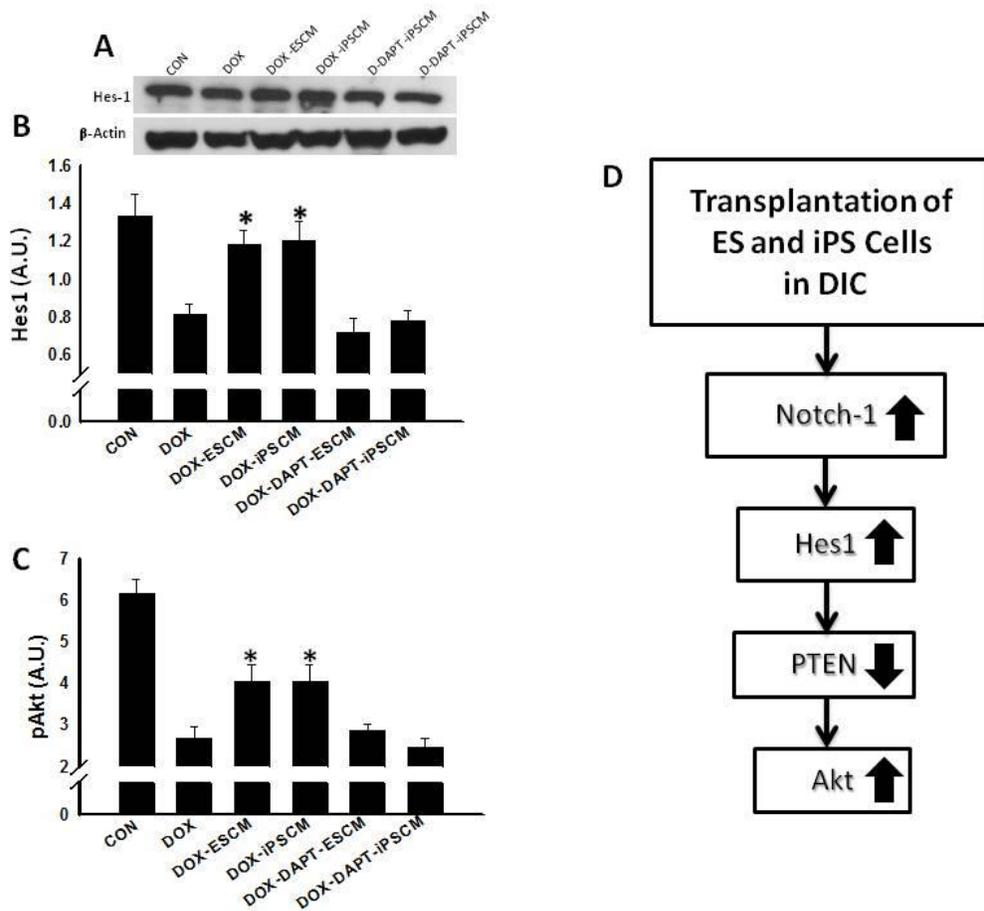


Figure 8: Hes1 regulates expression of Akt in H9c2 cells exposed to DOX. **(A)** Upper panel shows representative pictures of WB bands for Hes1 and its β -actin control. **(B)** Bottom panel shows densitometric analysis of WB for Hes1 that shows a significant increase (* $P < 0.05$) in Hes1 levels compared with DOX, DOX+DAPT+ESCM, and DOX+DAPT+iPSCM groups ($n = 5-9$). **(C)** Right histogram reveals a significant increase in pAkt activity in H9c2 cells exposed to DOX and treated with ESCM and iPSCM. * $P < 0.05$ vs. DOX and DOX+DAPT+iPSCM with an $n = 4-6$. **(D)** Diagram shows that transplantation of ES and iPS cells in DIC increase expression of Notch-1 and its downstream effector Hes1, therefore decreasing the levels of PTEN protein and increasing the levels of pro-survival Akt protein.

CHAPTER 4: DISCUSSION

The benefits of DOX as an antineoplastic drug have become less appealing due to the cardiotoxicity that this drug induces. In a study of 115 leukemia survivors who received DOX during their childhood, 57% developed cardiac dysfunction (Lipshultz et al., 2005). Furthermore, patients that received DOX as a cancer treatment at a young age were at high risk of experiencing an MI in their adulthood (Swerdlow et al., 2007; Ng and Mauch, 2009). The cardiac dysfunction induced by DOX is dose dependent and can develop months or years after its use (Steinherz et al., 1991; Horenstein et al., 2000; Huang et al., 2010). The cardiotoxic effects of DOX occur through modification of the function and architecture of the heart. DOX treatment results in cardiomyocyte apoptosis and inevitably leads to dilated cardiomyopathy and impaired ejection fraction (Minotti et al., 2004; Geisberg and Sawyer, 2010; Psaltis et al., 2011).

The limited self-renewal of cardiomyocytes makes stem cells an attractive option for replenishing cardiac cell loss resulting from toxic or ischemic injury (Gopinath et al., 2010). Previous studies have used stem cells in DIC and have attributed the benefits to the paracrine secretion of stem cells (Agbulut et al., 2003; Garbade et al., 2009; Gopinath et al., 2010). Studies suggest that treatment of DIC with ES cells or ES CM leads to inhibited cardiomyocyte apoptosis, attenuated cardiac fibrosis, and improved cardiac function (Singla et al., 2012). Furthermore, evidence has indicated that factors secreted from ES and iPS cells improve cardiac function and decreased apoptosis after MI (Singla et al., 2007; Singla et al., 2011). However, to the best of our knowledge this is the first study in which ES and iPS cells were transplanted in a mouse model of DIC with myocardial infarction. The mechanisms by which stem cells decrease

cardiomyocyte apoptosis and improve cardiac function in DIC are not well understood. In the present study, ES and iPS cells decreased cardiomyocyte apoptosis and improved cardiac function in vivo and in-vitro in DOX-induced heart failure. Moreover, data presented in this study reveal that after treatment with ES and iPS cells or ESCM and iPSCM, there is an up-regulation of Akt activity. A decrease in the levels of PTEN protein, a negative inhibitor of Akt in hearts treated with ES and iPS cells, was also observed. Furthermore, the expression of Notch-1 and Hes1 were shown to decrease significantly in DIC post-MI and in DOX-induced cardiotoxicity in H9c2; however, ES and iPS cells and their CM significantly increased the levels of these proteins. This strongly implicates Notch-1 as having an important role in DOX-induced heart failure.

Previous studies have shown that DOX induces cardiac dysfunction and that cardiomyocyte apoptosis is one of the primary elements that leads to heart failure (Singal et al., 1997; Singal et al., 2000). During an MI, cardiomyocyte apoptosis also occurs (Singla and Sobel, 2005; Singla et al., 2007; Singla et al., 2011). In this study, TUNEL staining and a caspase-3 activity assay show significantly reduced cardiomyocyte apoptosis in the ES and iPS cell-treated hearts as compared to the DOX-MI and DOX-MI+CC groups. To further examine the effects of ES and iPS cells and the factors secreted by these cells on DOX-induced cardiotoxicity, H9c2 cells were treated with DOX. The present data demonstrate that DOX-exposed H9c2 cells treated with ESCM and iPSCM had significantly decreased the levels of apoptosis and caspase-3 activity. This data is similar to that of previous studies suggesting that transplanted ES and iPS cells decrease cardiomyocyte apoptosis in DIC and in MI

respectively (Singla and Sobel, 2005; Singla et al., 2007; Singla et al., 2011; Singla et al., 2012).

The mechanisms by which ES and iPS cells decrease apoptosis when transplanted in the heart are not well understood. Previous studies have shown that levels of the pro-survival protein, Akt, decreases in the heart after DOX treatment and MI (Singla et al., 2012; Glass and Singla, 2011), while PTEN protein, a negative regulator of Akt, is up-regulated after MI (Keyes et al., 2010; Glass and Singla, 2011). In the present study, our data suggest that after experiencing an MI, DIC hearts had significant down-regulation of Akt and significantly increased levels of PTEN as compared to the Sham group. However, hearts treated with ES and iPS cells had increased levels of Akt and significantly decreased levels of PTEN as compared to the DOX-MI and DOX-MI+CC groups. In the in vitro experiments, the same trend in levels of Akt was observed in the groups treated with ESCM and iPSCM. These data suggest that transplanted ES and iPS cells regulate Akt and PTEN and that these proteins may play a role in DIC post-MI.

Previous studies have shown that Notch-1 mediates cardiac repair after MI (Li et al., 2011); however, DOX treatment decreases the levels of Notch-1 expression in cardiac progenitor cells (CPCs) (De et al., 2010). Therefore, the expression of Notch-1 was analyzed in DIC post MI. Our immunohistochemistry and WB analyses show that the expression of Notch-1 decreases significantly in DIC post-MI as compared to the Sham group. Moreover, levels of Notch-1 significantly increase in the ES and iPS cell-treated hearts. In addition to our in-vivo studies, in-vitro studies were conducted to analyze the effects of factors released from ES and iPS cells. H9c2 cells were treated

with DOX and also exposed to a gamma secretase inhibitor (DAPT). DAPT inhibits the activation of Notch-1 by preventing its cleavage and the release of its intracellular domain (NICD). Following DOX and DAPT exposure, cells were treated with ESCM or iPSCM. H9c2 cells treated with DOX showed a significant decrease in the expression of Notch-1 and c-Notch-1; however, when treated with ESCM or iPSCM, the levels of Notch-1 significantly increased. These results are in accordance with previous studies, which show a decrease in the levels of Notch-1 in CPCs after DOX treatment (De et al., 2010). Nevertheless, to the best of our knowledge this is the first study to show that expression of Notch-1 increases following transplantation of ES and iPS cells in DIC post-MI.

To further characterize the role of Notch-1 on cardiac repair in DOX-induced heart failure, a Notch-1 downstream effector, Hes1, was also studied. It has been shown that Notch-1 crosstalk occurs with the PI3K/Akt pathway along the border of an MI, thus protecting the heart from an acute pathological injury (Li et al., 2011). Recent studies have shown that in certain types of cells, such as T-cell lymphoblastic leukemia (T-ALL) cells, Notch-1 has an inhibitory effect on PTEN and that its negative regulation signals through Hes1 (Gutierrez and Look, 2007; Gutierrez et al., 2009). The western blot data presented in this study shows that after MI was induced in a DIC animal model, the levels of Hes1 significantly attenuated. However, following the transplantation of ES and iPS cells, the levels of Hes1 increased significantly as compared to the non-transplanted DOX-MI and DOX-MI+CC hearts. The same tendency in Hes1 levels was observed in DOX-induced cardiotoxicity in H9C2 cells treated with ESCM and iPSCM.

Lastly, we studied the effects of transplanted ES and iPS cells on cardiac function in DIC post-MI. Our data show that 14 days post-MI, animals treated with ES and iPS cells had significantly improved EF and FS percentages as compared to the untreated DOX and DOX-MI+CC groups.

In conclusion, the data obtained during this study suggest that transplantation of ES and iPS cells in DOX-induced heart failure decrease apoptosis and improved heart function. Furthermore, the decrease in apoptosis observed in the in vitro and in vivo data is mediated by the activation of the Notch-1 pathway

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