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Transplantation Of Ips Cells Reduces Apoptosis And Fibrosis And Improves Cardiac Function In Streptozotocin-induced Diabetic Rats

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TRANSPLANTATION OF IPS CELLS REDUCES APOPTOSIS AND FIBROSIS AND IMPROVES CARDIAC FUNCTION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

by

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A thesis submitted in partial fulfillment of the requirements
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ABSTRACT

Background: Streptozotocin (STZ) induced diabetes leads to various complications including cardiomyopathy. Recent data suggests transplanted bone marrow stem cells improve cardiac function in diabetic cardiomyopathy. However, whether modified ES, iPS cells, or factors released from these cells can inhibit apoptosis and fibrosis remains completely unknown. The present study was designed to determine the effects of transplanted ES cells overexpressing pancreatic transcription factor 1 a (Ptf1a), a pro-pancreatic endodermal transcription factor, iPS cells, or their respective conditioned media (CM) on diabetic cardiomyopathy. **Methods**: Experimental diabetes was induced in male Sprague Dawley rats (8-10 weeks old) by intraperitoneal STZ injections (65 mg/kg body weight for 2 consecutive days). Animals were divided into six experimental groups including control, treated with sodium citrate buffer IP, STZ, STZ + ES-Ptf1a cells, STZ + iPS cells, STZ + ES-Ptf1a CM and STZ + iPS CM. Following STZ injections, appropriate cells (1 X 106/mL/injection/day) or CM (2 mL injection/day) were given intravenously for 3 consecutive days. Animals were sacrificed and hearts were harvested at day 28. Histology, TUNEL staining, and Caspase-3 activity were used to assess apoptosis and fibrosis. ERK1/2 phosphorylation was quantified using ELISAs. M-mode echocardiography fractional shortening was used to assess cardiac function. **Results**: Animals transplanted with ES cells, iPS cells, or both CMs showed a significant ($p<0.05$) reduction in interstitial fibrosis, and apoptosis compared with STZ

group.   ERK expression was not significantly different compared with STZ. Echocardiography showed a significant (p<0.05) improvement in fractional shortening in cell and media transplanted groups compared with STZ. **Conclusions**: Our data suggest that ES cells, iPS cells, and/or CMs inhibit apoptosis, reduce fibrosis, and improve cardiac function in STZ-treated diabetic rats.

Dedicated to John and Susan Neel

ACKNOWLEDGMENTS

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LIST OF ACRONYMS/ABBREVIATIONS

AGE	Advanced Glycation End Product
ANOVA	Analysis of Variance
bFGF	basic Fibroblast Growth Factor
CM	Conditioned Media
DCM	Diabetic Cardiomyopathy
DM	Differentiation Media
ES	Embryonic Stem
FS	Fractional Shortening
GM	Growth Media
H & E	Hematoxylin and Eosin
ip	Intraperitoneal
iv	Intravenous
iPS	Induced Pluripotent Stem
LVIDd	Left Ventricular Internal Diameter at Diastole
LVIDs	Left Ventricular Internal Diameter at Systole
PBS	Phosphate Buffered Saline
Pdx-1	Pancreatic and duodenal homeobox
PFA	Paraformaldehyde
Ptf1a	Pancreatic Transcription Factor 1 a
ROS	Reactive Oxygen Species

RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
STZ	Streptozotocin
VA	Vessel Area
VF	Vessel Fibrosis
WHO	World Health Organization

INTRODUCTION

Diabetes is a devastating disease affecting an estimated 170 million people worldwide (Wild et al 2004). The World Health Organization (WHO) predicts this number will more than double to an astounding 366 million people by the year 2030 (Wild et al 2004). Type I diabetes is an autoimmune disease characterized by destruction of insulin-producing beta cells of the islets of Langerhans in the pancreas. Normally, insulin functions to regulate blood glucose levels and to increase cellular absorption of glucose by enhancing membrane expression of glucose receptors. Loss of insulin production leads to high circulating glucose levels. High circulating blood glucose, or hyperglycemia, leads to many complications, including retinopathy, nephropathy, neuropathy, impaired wound healing and tissue damage, osteoporosis, and cardiomyopathy (Clark & Lee 1995). Previous estimations conclude that cardiovascular disease (CVD), including diabetic cardiomyopathy (DCM), account for more than 70% of all diabetic deaths (Geiss et al 1995) (Guddati et al 2009). Despite significant improvements in pharmalogical interventions, morbidity and mortality among diabetic patients remain significantly elevated (Standl & Schnell 2000). To that end, new therapeutic strategies for the treatment of DCM are imperative.

Diabetic Cardiomyopathy

DCM is defined as ventricular dysfunction without diagnosis of hypertension or atherosclerosis in persons affected by diabetes. Without appropriate interventions, DCM can lead to insufficient cardiac pumping capacity disallowing proper blood flow to tissues and organs, also known as heart failure (Boudina 2010). DCM results from several altered pathways, including lack of normal insulin signaling, oxidative stress, and hyperglycemia (Boudina 2007). Specifically, in DCM, high glucose conditions initiate cardiac myocyte internalization of glucose receptors. Following cellular uptake of glucose receptors, cardiac metabolism shifts from the normal state to free fatty acid oxidation.   This shift in energy production leads to increased generation of reactive oxygen species (ROS) and ceramide and cellular death via apoptosis (Tarquini et al 2010).

Apoptosis

Apoptosis is gene-regulated cell death that leads to cytoplasmic blebbing, cell shrinkage, protein cleavage by caspases and DNA fragmentation (Intengan et al 2001). Diabetes induces cardiac apoptosis through various mechanisms including oxidative stress, loss of normal insulin signaling, hyperglycemia and advanced glycation endproducts (AGEs). Following apoptotic cell death which is well documented in DCM, adverse ventricular remodeling occurs, including fibrosis (Cai et al 2001).

Fibrosis

Fibrosis results from an imbalance of excess extracellular matrix (ECM) protein production including collagen, elastin, laminin, and fibronectin and their degradation (Intengan et al 2001). Experimental evidence has shown fibrosis is due mainly to overproduction of type III collagen in DCM (Shimizu et al 1993). Both interstitial and vascular fibrosis occur as a maladaptive response in the DCM heart. Interstitial fibrosis occurs between cardiac myocytes whereas vascular fibrosis occurs in and around vessel walls (Intengan et al 2001). Fibrosis ultimately leads to stiffening of the heart and eventual poor cardiac function. Overall, in the pathogenesis of DCM involving apoptosis and fibrosis, we are still looking for new treatment options. In this regard, we would like to study the effects of transplanted stem cells and their conditioned media to treat and ameliorate DCM.

Embryonic Stem Cells

Stem cells are cells that replicate indefinitely and differentiate into most lineages from the three germ layers. Several types of stem cells have been considered for the repair and regeneration of damaged myocardium including mesenchymal stem cells, embryonic stem (ES) cells, hematopoietic stem cells, endogenous cardiac stem cells, and induced pluripotent stem (iPS) cells (Zhang et al 2008)(Kim et al 2009). Specifically, ES cells are derived from the inner cell mass of the pre-implantation blastocyst (Evans et al 1981). Previous studies indicate ES cells differentiate into all 3 cell types of the heart including smooth muscle cells, cardiac myocytes, and endothelial

cells (Singla et al 2006). Additionally, in vivo studies show transplanted ES cells integrate into the myocardium, facilitate cardiac repair and regeneration, and improve heart function (Singla et al 2007). Although bone marrow-derived mesenchymal stem cells have been attempted in the DCM animal model (Zhang et al 2008), there is no data which determines the potential of ES and iPS cells in cardiac repair and regeneration in STZ-induced diabetic heart.

Induced Pluripotent Stem Cells

Induced pluripotent stem (iPS) cells are somatic cells, dedifferentiated through forced gene expression of various human stemness factors including Oct3/4, Sox2, Klf4 and C-myc (Wernig et al 2007). Transduction of the various factors converts these differentiated cells into an ES cell-like state, capable of indefinite self-renewal and differentiation into most body cell types. However, the effects of iPS cells or released autocrine or paracrine factors have not yet been elucidated in the setting of DCM.

Conditioned Media

Conditioned media (CM) is the supernatant removed from growing particular cell populations after a period of time which contains various autocrine/paracrine/growth factors released from the cultured cells. Our laboratory used ES cells derived CM and demonstrated presence of released factors, including clusterin, TIMP-1, cystatin C, and osteopontin which have been identified as anti-apoptotic and anti-fibrotic (Singla et al 2008). In vitro and in vivo studies using CM have demonstrated cytoprotective effects in

isolated cardiac myocytes and the injured myocardium (Singla et al 2008) (Fatma et al 2010). Nevertheless, CM experiments have yet to be conducted in the DCM heart.

Use of Streptozotocin in the Induction of Diabetes

Streptozotocin (STZ) treatment induces destruction of the insulin-producing pancreatic beta cells leading to diabetes (Lenzen 2008). STZ or 2deoxy2 ([methylnitrosoamino) carbonyl] amino)-D-glucopyranose is taken up through the Glut-2 receptor, which is highly expressed in beta cells (Elsner et al 2007). Upon uptake into the cell, the methylnitrosourea moiety is separated from the glucose ring and methylates the beta cell DNA, which leads to cell death and induces diabetes (Lenzen 2008). STZ is a well documented and tested compound used by various investigators as an excellent method to simulate diabetes (Mihm et al 2001).   Moreover dose dependent diabetes can be given to stimulate the induction of either Type I or Type II (Chakrabarti et al 2003).

Hypothesis

Transplantation of iPS/Ptf1a ES cells and their CM reduce apoptosis and fibrosis and enhance cardiac function in the STZ-induced DCM rat heart.

Aims

1. Create a rat model of DCM that can be replicated and verified

2. Effectively inject various stem cell and CM into the tail vein of STZ-induced diabetic rats

3. Assess cardiac function through echocardiography

4. Evaluate apoptosis in the DCM heart using TUNEL staining and caspase-3 activity assay

5. Validate the role of the ERK pathway in DCM-induced apoptosis

6. Quantify interstitial and vascular fibrosis using Masson's trichrome staining and Image J software

MATERIALS AND METHODS

Ptf1a Cells

Ptf1a, or Pancreatic Transcription Factor 1, transfected ES cells were a kind gift from Mark Magnuson of Vanderbilt University. Ptf1a is a transcription factor for pancreatic and duodenal homeobox factor 1 (Pdx-1) (Wiebe). Increased expression of Pdx-1 in Ptf1a ES cells was verified by RT-PCR and gel electrophoresis.

Generation of Ips Cells

IPS cells were generated from transduced H9c2 cells (cardiomyoblasts). The pBluescript SK(-) vector from Stratagene was used to clone the 4 human stemness factors c-Myc, Klf4, Oct3/4 and Sox2. Primers for c-Myc: Forward 5' AGGCTGGATTTCCTTTGG 3' and Reverse 5' TTTATGCACCAGAGTTTCG 3', Klf4: Forward 5' CCTCTCTCTTCTTCGGACTC 3' and Reverse 5'AAAGTGCCTCTTCATGTGTAAG 3', Oct3/4: Forward 5' ATGGCTGGACACCTGGCTTC 3' and Reverse 5' CTCAGTTTGAATGCATGGGAG 3' and Sox2: Forward 5' ATGTATAACATGATGGAGACG 3' and Reverse 5' CATGTGCGACAGGGGCAGTGT 3'. To generate an expression vector allowing expression of all four genes, the cDNA sequences for Klf4, Sox2 and Oct3/4 were linked through the 2A sequence of foot-and mouth disease virus 5' AAAATTGTCGCTCCTGTCAAA CAAACTCTTAACTTTGATTTACTCAAACTGGCTGGGATGTAGAAAGCAATCCAGGT

CCA 3', which is a self-cleaving sequence. Because the pBluescript SK(-) vector lacked appropriate promoters, the Oct 3/4-2A-Klf4-2A-Sox2 was cleaved from pBluscript SK(-) vector and then cloned into the Eco-R1 sites of the pCX-EGFP vector (a kind gift from Masuru Okabe). In addition, c-Myc was also ligated into the EcoRI site of pCX-EGFP. Expression of the 4 human stemness factors was verified by RT-PCR and western blot analysis. Additionally, iPS cells were stained for Oct 3/4 and Alkaline Phosphatase to verify pluripotency. (iPS cell creation and verification has recently been submitted as a method for cardiac iPS cell generation) Transduced H9c2 cells, now referred to as iPS cells, were cultured in iPS growth medium (GM) containing 50% Mouse Embryonic Fibroblast-CM, 50% Embryonic Stem Cell-Growth Medium (Dulbecco's Modified Eagles Medium, supplemented with streptomycin, penicillin, non-essential amino acids, glutamine, ES qualified fetal bovine serum, leukemia inhibitor factor (LIF), and β-mercaptoethanol), 10 µg/ml bFGF and 10 µg/ml Activin A.

Generation of Conditioned Media

As described previously, CM was generated by plating 1.6×10^6 iPS cells or Ptf1a-ES cells in GM (see aforementioned description) (Singla et al 2008). The following day, GM was removed and replaced with differentiation media (DM) which lacks LIF and β−mercaptoethanol. Forty-eight hours later, media was removed, filtered to remove any cellular debris, and labeled as CM.

Stz-Induced Diabetes in the Rat

All procedures using animals were approved by the University of Central Florida Institutional Animal Care Use Committee. Male Sprague Dawley rats aged 8-10 weeks were purchased from Charles River. Animals were provided irradiated Rodent Diet from PicoLab and autoclaved water *ad libitum*, excepting short periods of fasting prior to blood glucose testing. Animals were divided into six study groups including; control, STZ, STZ + Ptf1a ES cells, STA + Ptf1a CM, STZ + iPS cells, and STZ + iPS CM. In all groups, prior to injections, blood sugar was measured using a One-touch Ultra glucose meter. Following glucose check, all groups except control, were injected with STZ intraperitoneally (ip) on day 1 (D1) and D2 at a dose of 65 mg/kg. In the control group, sodium citrate vehicle buffer only was injected ip. Twenty-four hours post the last STZ injection, blood sugar was measured again. All animals with circulating blood glucose exceeding 250 mg/dL were considered diabetic and continued in the study. On D3, D4, and D5, animals were injected with appropriate stem cells or conditioned media; 1 x 10^6/1 mL intravenously (iv) Ptf1a-ES cells or iPS cells and 2 ML iv Ptf1a-ES or iPS CM. Control animals on D3, D4, and D5 were given 1 mL saline iv. At D28, rats were sacrificed by isoflurane following by cervical dislocation. Hearts were removed, the top half placed in RNA later, and the bottom half in 4% paraformaldehyde (PFA) for further analysis.

Determination of Apoptosis

Heart preserved in 4% PFA were washed 3 times with PBS, and transferred to 70 percent ethanol. Hearts were dehydrated in a Leica tissue processor sequentially in 70, 80, 95, 100, and 100 percent ethanol, followed by Citrosolv, Citrosolv, paraffin and paraffin. Hearts were then made into paraffin blocks using a TissueTek embedding machine. Five micrometer sections were cut transversely from the paraffin blocks. Sections were deparaffinized and rehydrated by 3 minutes sequential incubation in xylene, fresh xylene, 100, 95, 70 percent ethanol at room temperature. Slides were then washed with distilled water, followed by PBS for 6 minutes. TUNEL staining for apoptotic nuclei was done with TMR Red In Situ Cell Death Detection kit from Roche. Sections were permeabilized with proteinase K (25µg/mL in 100 mM Tris-HCl). Vectashield mounting medium containing DAPI was used to stain total nuclei. Percent TUNEL positive nuclei = # of TUNEL positive nuclei/ total nuclei X 100. Apoptotic nuclei were quantified using sections from six different rat hearts per experimental group.

To confirm apoptosis using an additional method, a caspase-3 activity assay was performed. In brief, tissue from hearts preserved in RNAlater (Ambion) was homogenized in RIPA buffer with Protease Inhibitor Cocktail (Sigma), PMSF, Sodium Fluoride, and Sodium Orthovandate. Homogenates were centrifuged 10 minutes at 15871 rpm/ 13000 G. Supernatant was removed and stored at -20°C for later use. Tissue homogenates were assayed for protein concentration by Bradford assay using Bio-Rad Protein Assay Dye Reagent, and Bio-Rad Protein Assay Standard 11, Bovine Serum albumin. Biovision Caspase-3 Activity kit was used as per manufacturer's

directions, using 200 μg of each protein sample. Developed color was measured in a microtiter plate reader (Biorad) at 405 nm. Caspase-3 graph was plotted as arbitrary units.

ERK 1/2 Assay

Hearts were homogenized in RIPA buffer as previously mentioned. Phosphorylated ERK 1/2 level was assessed by ELISA using protocol provided by Exalpha Biologicals, Inc. Colorimetric changes were measured in a microtiter plate reader (Bio-Rad) at 450nm. ERK 1/2 graph was plotted as arbitrary units.

Fibrosis Quantification

Heart tissue were blocked and embedded as previously described. Heart sections were then stained with Masson's trichrome. To quantify interstitial fibrosis, sections were first qualitatively assessed, and then quantified directly using Image J software by measuring 10 microscopic fields from 6 different rats per experimental group, at 20 X magnification. Vascular fibrosis was also quantitated using the same Masson's trichrome stained sections. 10 vessels per section for 6 animals/experimental group were analyzed. Vascular fibrosis was plotted as a percent of vascular fibrosis/ total vessel area.

Assessment of Cardiac Function

At D28, fractional shortening (FS) (FS % = (Left Ventricular Internal Dimension at diastole (LVIDd) – Left Ventricular Internal Dimension at systole (LVIDs))/LVIDd x 100)

was measured transthoracically by 2D M-mode echocardiography. Rats were anesthetized with 2% isoflurane, place on a heating pad, and chest was shaved. Echocardiography was performed using a Philips Sonos 4500 or 5500 Ultrasound system and values for three consecutive heart beats were averaged.

Statistical Analysis

Statistical analysis was completed using t-test, Tukey, one-way analysis of variance (ANOVA) and the Bonferroni test. Statistical significance was assigned when $P < 0.05$.

RESULTS

Ptf1a/Ips Cells and CM Inhibit Apoptosis in Stz-Induced DCM Heart

To assess whether Ptf1a/iPS cells and CM inhibit apoptosis in the STZ-induced DCM heart, TUNEL staining was performed (Figure 1A-I) at D28. Our data suggests there was a significant increase in TUNEL-positive nuclei in the STZ alone treated group compared with control (STZ: 1.17 ± 0.21% vs. Control: 0.17 ± 0.03%, mean ± SEM, $p<0.001$) (Figure 2). However, rat hearts transplanted with Ptf1a/iPS cells demonstrated significantly reduced apoptosis compared to STZ rat hearts (Ptf1a cells: 0.35 ± 0.02%, and iPS cells: 0.39 ± 0.04%, vs. STZ, see aforementioned data, $p<0.05$) (Figure 2). Moreover, hearts transplanted with Ptf1a/iPS CM also had significantly reduced apoptosis compared to STZ (Ptf1a CM: 0.66 ± 0.07% and iPS CM: 0.65 ± 0.11% vs. STZ, see aforementioned data). Additionally, there was no significant apoptotic inhibition between groups treated with Ptf1a/iPS cells or CM.

To verify our TUNEL apoptotic data, a caspase-3 activity assay was performed. As with the TUNEL data, hearts from the STZ treated rats had a significant increase in caspase-3 activity compared to hearts from control rats (STZ: 0.22 ± 0.01 vs. Control: 0.12 ± 0.01, $p<0.001$) (Figure 3). Additionally, Ptf1a/iPS cell and CM transplanted hearts exhibited reduced capsase-3 activity when compared to STZ alone hearts (Ptf1a cells: 0.13 ± 0.02, Ptf1a CM: 0.14 ± 0.01, iPS cells: 0.13 ± 0.01, and iPS CM: 0.14 ± 0.01 vs. STZ, see aforementioned data, $p < 0.05$) (Figure 3). Moreover, there was no statistical

significance in caspase-3 activity between hearts transplanted with cells (Ptf1a and iPS) or their CM.

Ptf1a/Ips Cell And CM Cytoprotective Effects Are Not Mediated Through the Erk 1/2 Pathway

To determine whether the ERK 1/2 plays a role in the DCM apoptosis, an ERK1/2 ELISA was performed. Levels of pERK 1/2 were not significantly altered among any of the experimental groups (Control: 0.08 ± 0.01, STZ: 0.09 ± 0.01, Ptf1a cells: 0.08 ± 0.01, Ptf1a CM: 0.08 ± 0.01, iPS cells: 0.08 ± 0.01, and iPS CM: 0.09 ± 0.01, mean $\pm$ SEM) (Figure 4), thus indicating Ptf1a/iPS cell and CM cytoprotective effects are not mediated via the ERK 1/2 pathway.

Ptf1a/Ips Cells And CM Inhibit Fibrosis in the DCM Heart

To quantify cardiac fibrosis in the hearts transplanted with Ptf1a/iPS cells and CM, Masson's trichrome staining was performed (Figure 5). Interstitial fibrosis in the STZ-treated group was significantly increased compared to control (STZ: 0.38 ± 0.08 vs. control: 0.01 ± 0.00, mean $\pm$ SEM, $p<0.001$) (Figure 6). Following transplantation of Ptf1a/iPS cells and CM, interstitial fibrosis was significantly reduced compared to STZ (Ptf1a cells: 0.04 ± 0.01, Ptf1a CM: 0.11 ± 0.06, iPS cells: 0.01 ± 0.00, and iPS CM: 0.01 ± 0.00 vs. STZ, see aforementioned data, $p<0.05$) (Figure 6). However, there was no statistical significance in interstitial fibrosis between groups treated with cells (Ptf1a or iPS) or their CM.

Vascular fibrosis was also quantified and depicted in Figure 7 A-F. As observed with interstitial fibrosis, vascular fibrosis in the STZ treated groups was significantly increased compared to control (STZ: 44.61 ± 1.60% vs. control: 8.64 ± 1.24%, $p<0.001$) (Figure 8). Vascular fibrosis was significantly reduced in Ptf1a/iPS cell treated groups compared to STZ group: (Ptf1a cells: 22.85 ± 1.59% and iPS cells: 18.84 ± 1.94%, and vs. STZ, see aforementioned data, $p<0.05$) (Figure 8). As observed in hearts treated with cells, vascular fibrosis was also significantly reduced in hearts transplanted with Ptf1a and iPS CM compared to STZ treated hearts (Ptf1a CM: 22.47 ± 3.16% and iPS CM: 11.96 ± 1.60% vs. STZ, see aforementioned data, $p<0.05$). Nevertheless, hearts from rats transplanted with Ptf1a/iPS cells demonstrated no significant reduction in vascular fibrosis compared with hearts from rats transplanted with ptf1a/ips cm.

Echocardiography

Cardiac function was assessed by measuring fractional shortening transthoracically by M-mode echocardiography. Fractional shortening was significantly reduced in the STZ treated groups compared to control (STZ: 25.40 ± 1.28 % vs. control: 39.70 ± 0.80 %, mean ± SEM, $p<0.001$) (Figure 9). Fractional shortening was significantly increased in Ptf1a/iPS cell and CM treated groups compared to STZ group: (Ptf1a cells: 38.60 ± 1.52 %, Ptf1a CM: 38.90 ± 1.03%, iPS cells: 33.30 ± 2.14%, and iPS CM: 37.88 ± 1.51% vs. STZ, see aforementioned data, $p<0.001$). However, there was no statistical significance observed in fractional shortening between groups treated with Ptf1a/iPS cells or their CM.

DISCUSSION

Diabetes is a leading cause of death worldwide with the majority of these deaths attributable to CVD (Shimizu et al 1993). Although great progress has been made toward the development of therapeutic options for the treatment of DCM, novel, effective strategies remain vital. Stem cells and their CM have recently gained significant attention for their potential to repair and regenerate the injured myocardium. To that end, we have generated a STZ-induced diabetic rat in which we transplanted Ptf1a-ES cells and CM and iPS cells and CM to test their potential to inhibit apoptosis and fibrosis and enhance cardiac function in the DCM heart.

Our STZ-induced diabetic rat model possessed many characteristics previously published by other groups including hyperglycemia and cardiac fibrosis and apoptosis (Ares-Carrasco et al 2009). Following STZ injections, circulating blood glucose levels were measured and all mice continued in the study had a blood glucose level >250 mg/dL, a level well above the normal non-diabetic range. Additionally, 28 days following STZ injections, rats receiving only STZ had a significant amount of cardiac apoptosis and fibrosis compared with the control animals our data corroborates with previously published data showing STZ induces apoptosis in the heart. (Ares-Carrasco et al 2009). Moreover, our data confirmed that we generated a viable STZ-induced diabetic rat with characteristics of diabetic cardiomyopathy.

Cardiac apoptosis plays a critical role in the pathogenesis and progression of DCM. Apoptosis contributes to the loss of cardiac myocytes leading to adverse cardiac remodeling, including fibrosis and hypertrophy, poor function, and eventual heart failure. Previous studies have shown that transplanted ES and mesenchymal stem cells significantly inhibit apoptosis in the infarcted heart (Guo et al 2010). Additionally, ES and mesenchymal stem cell CM has been shown to inhibit apoptosis *in vitro* and in *in vivo* models of injured myocardium. However, Ptf1a ES cells and their CM and iPS cells and their CM have yet to be attempted to attenuate cardiac apoptosis in DCM. Upon transplantation of Ptf1a/iPS cells or their CM, the number of cardiac apoptotic nuclei and caspase-3 activity was significantly decreased. We have for the first time shown that Ptf1a/iPS cells and CM inhibit apoptosis in the DCM heart as mechanisms of apoptosis in DCM vary greatly from apoptosis induced by ischemic conditions. Although the exact mechanism of reduced diabetes-induced cardiac apoptosis has yet to be delineated, our data suggests that Ptf1a cells and CM and iPS cells and CM contain factors that protect host myocardium from apoptosis-causing insults. In comparison to a study which treated STZ-induced diabetic rats orally with garlic oil, stem cell and CM treated groups had percentages of TUNEL positive nuclei less than the highest dose of garlic oil (Ou et al).

One suggested mechanism, leading to cardiac apoptosis in DCM, involves mediation through the ERK 1/2 pathway, although this idea remains controversial. Previous

studies have shown that the ERK 1/2 pathway is involved in cell survival and proliferation (Keyes et al 2010). To validate the protective, anti-apoptotic effects of transplanted Ptf1a/iPS cell and CM, ERK 1/2 activity was quantified. However, we have determined that ERK 1/2 levels were not significantly altered in any of the experimental groups. In the current study, the cytoprotective mechanisms of Ptf1a/iPS cells and CM in the setting of DCM were not elucidated. However, additional anti-apoptotic/apoptotic pathways, such as Akt1, need to be investigated in future studies.

Although the exact mechanisms involved in the pathophysiology of DCM remain elusive, evidence of accumulated ECM proteins, including collagen type I, III, IV, VI, fibronectin, laminin, and elastin, is abundant (Asbun et al 2006). In addition to excess production of ECM proteins, nonenzymatic glycosylation and cross-linking of collagens, and degradation of collagen degrading matrix metalloproteinases, among others, are well documented contributors to fibrosis and are observed in DCM (Tschope et al 2004). Numerous studies have revealed that stem cells and their CM inhibit fibrosis and accumulated ECM proteins in pathological cardiac conditions (Boudoulas & Hatzopoulos 2009) (Singla 2010). However, no study defines the effects of Ptf1a/iPS cells and CM on fibrosis in DCM. Accordingly, in our study, interstitial and vascular fibrosis was quantified. As stated previously, rats receiving only STZ had significantly increased interstitial and vascular fibrosis compared to control animals. However, following transplantation of Ptf1a/iPS cells or CM, interstitial and vascular fibrosis were significantly inhibited. Endothelial to mesenchymal transition has been postulated to

contribute to larger numbers of fibroblasts and an increase in their secreted fibrotic matrix proteins (Widyantoro et al 2010). Stem cells or their conditioned media may act in reducing endocardial to mesenchymal transition, reducing numbers of fibroblasts and hence fibrosis (Zhao et al 2010). A few other possible mechanisms are cell fusion, mitochondrial transfer, and activation of endogenous cardiac progenitor cells (Passier et al 2008) (Spees et al 2009). Although our data suggests that transplanted Ptf1a ES and IPs cells and their CM inhibit adverse pathways leading to fibrosis in DCM, further studies will be required to elucidate mechanisms by which this occurs.

Poor cardiac function, attributable to apoptosis and fibrosis, has been well documented in the setting of DCM. Previous studies have reported improved cardiac function following ES, mesenchymal stem, and iPS cell transplantation in numerous diseased cardiac conditions (Passier et al 2008) Moreover, CM generated from mesenchymal stem and ES cells containing released paracrine factors were shown to increase fractional shortening in the infarcted heart (Timmers et al 2007). Compared with controls, rat hearts receiving only STZ demonstrated significantly reduced fractional shortening, indicative of poor cardiac function. However, following Ptf1a/iPS cell and CM transplantation, rats had a significant improvement in fractional shortening compared to rats only receiving STZ. Our data, for the first time, indicates that Ptf1a/iPS cells and their CM do enhance cardiac function in DCM.

Teratoma formation has long been a drawback and a source of controversy to stem cell therapy. However, in our studies, none of our animals receiving iPS cells or CM displayed teratoma formation. Numerous investigations have concluded several variables may affect teratoma formation in cell transplantation studies (Dengler et al 2010). These include cell type, dose, transplantation site, time from injury or disease progression, and culture conditions; such as culture dish material, three dimensional scaffolding or rotary culture, media composition, pretreatment with various factors and hypoxia (Passier et al 2008) (Befar et al 2007).

In this study, we have shown for the first time that Ptf1a cells and their CM inhibit apoptosis, fibrosis, and enhance cardiac repair in the STZ-induced diabetic rat model of DCM. Additionally, iPS cells and CM were equally effective in inhibiting adverse remodeling and improving fractional shortening in the DCM heart. Although cytoprotective mechanisms of Ptf1a/iPS cells and CM in the setting of DCM have not been delineated yet, we have provided the novel data demonstrating the potential of ES and iPS cells and CM for therapeutic use in treating DCM.

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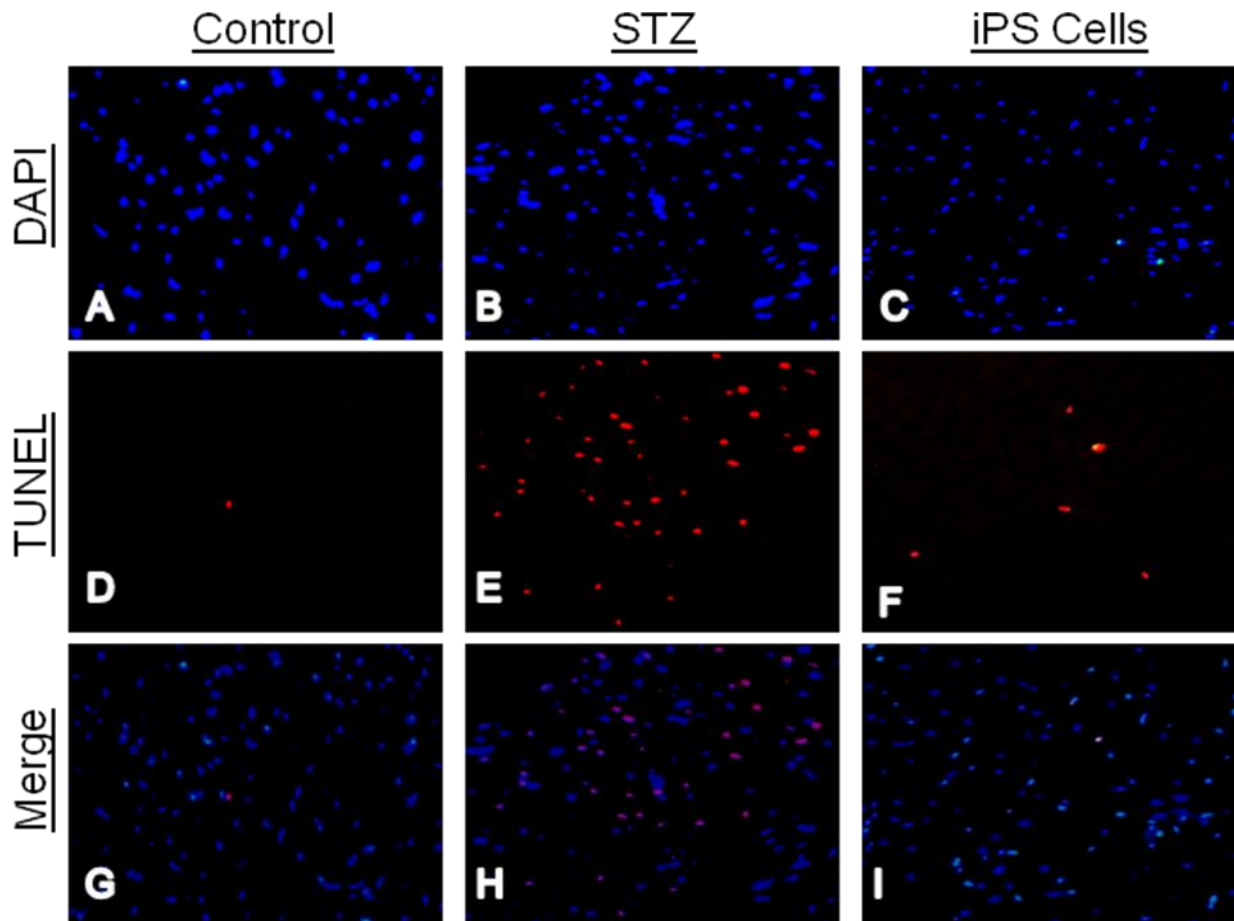# APPENDIX: FIGURES

APPENDIX: FIGURES

Figure 1: Transplanted Ptfl1a-ES cells, and their CM reduce apoptosis in the STZ-induced diabetic rat heart. Ptf1a/iPS cells and their CM were transplanted into STZ-induced diabetic rats and hearts were examined for apoptosis 28 days following cell transplantation. Representative photomicrographs of control, STZ, and iPS cell hearts demonstrating total nuclei stained with DAPI in blue (A-C), apoptotic nuclei stained with TUNEL in red (D-F), and merged nuclei in pink (G-I).
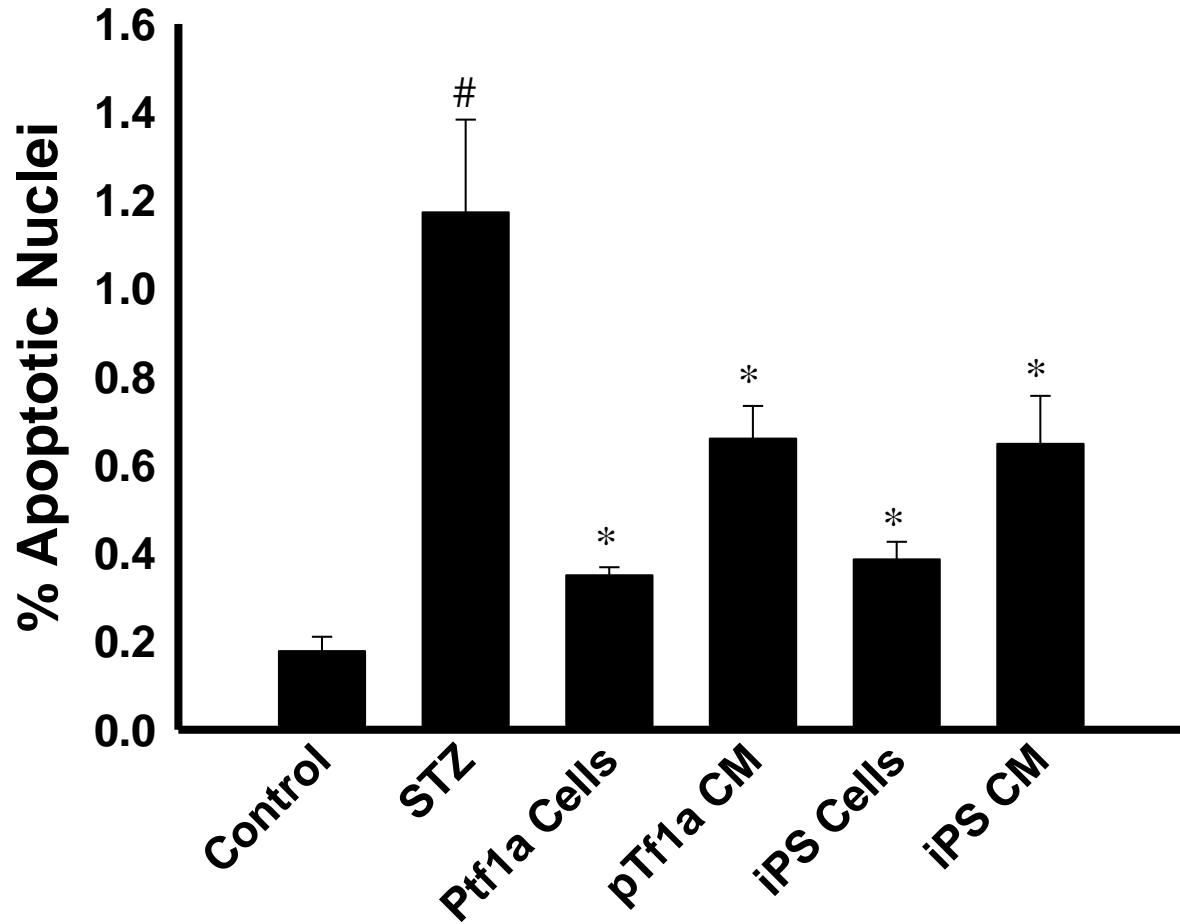
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Figure 2: Effects of transplanted Ptf1a/iPS cells and CM on apoptosis in the STZ-induced diabetic rat heart. Histogram data shows apoptosis was significantly inhibited in rat hearts transplanted with Ptf1a/iPS cells and CM compared with hearts treated only with STZ. #p<0.001 vs. control and *p<0.05 vs. STZ.
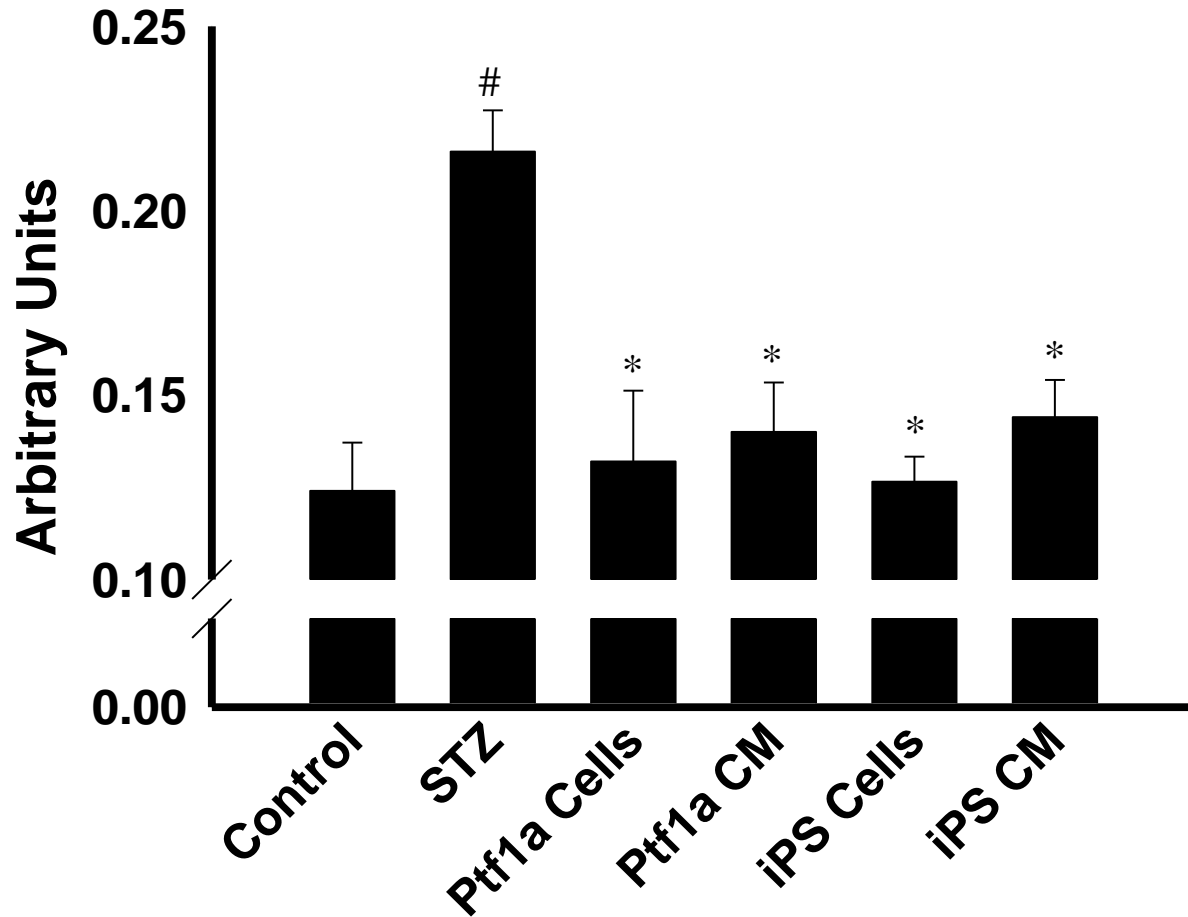
Figure 3: Caspase-3 activity is significantly enhanced in STZ treated diabetic rats. Histograms shows quantitative analysis of caspase-3 activity in hearts transplanted with and without Ptf1a/iPS cells and CM. #p<0.001 vs. control and *p<0.05 vs. STZ.
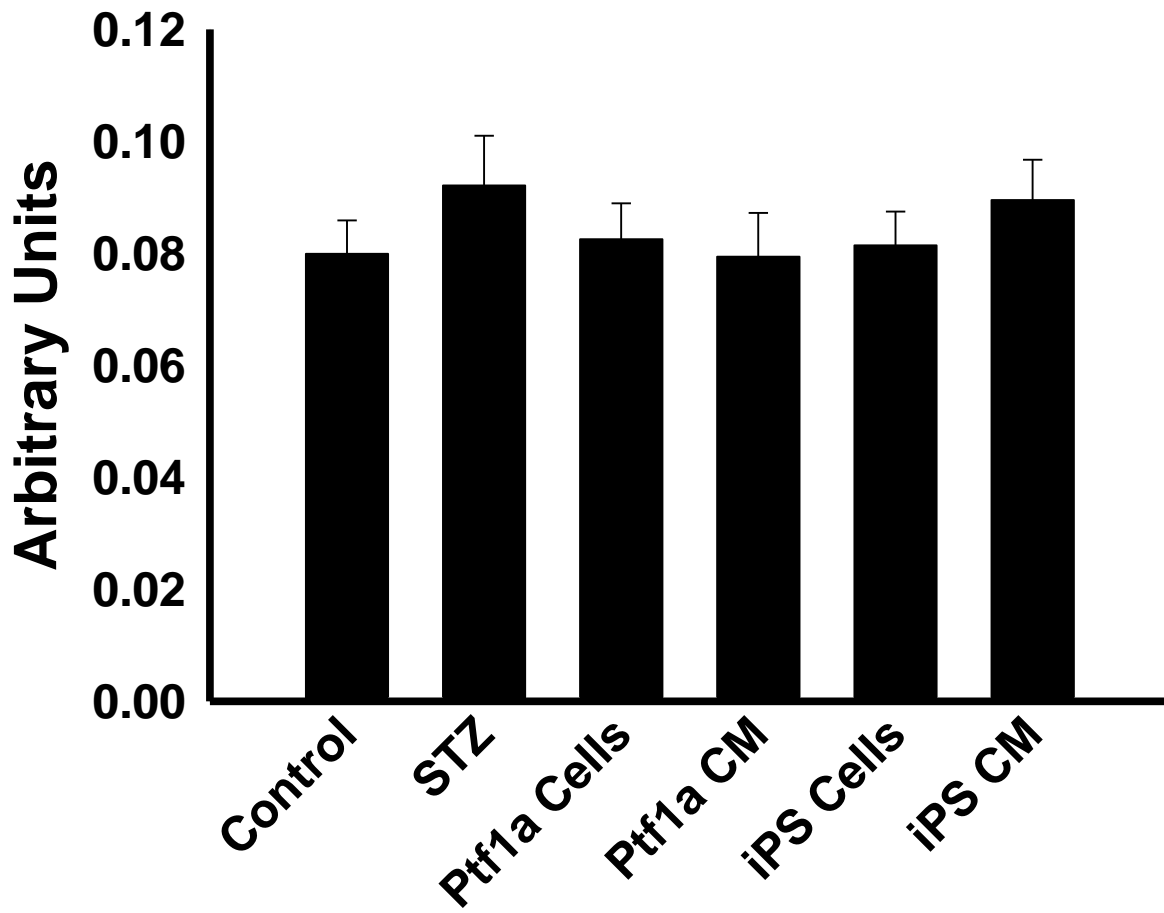
Figure 4: ERK 1/2 pathway is not involved in DCM apoptosis. Quantitative histogram reveals that there is no statistical significance in ERK 1/2 activity among any experimental groups, indicating Ptf1a/iPS cell and CM cytoprotective effects are not mediate
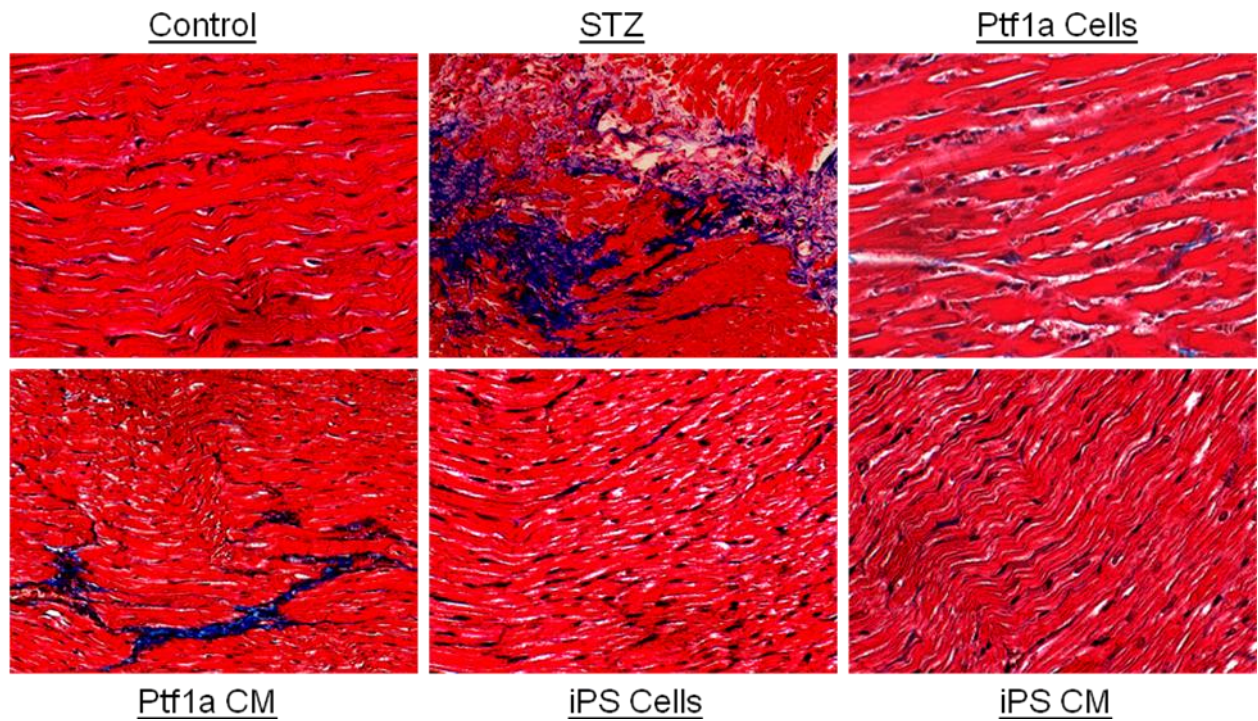
Figure 5: Effects of transplanted Ptf1a/iPS cells and CM on interstitial fibrosis.
Representative photomicrographs from sections stained with Masson's trichrome 28 days following cell and CM transplantation.   Magnification, 40X.
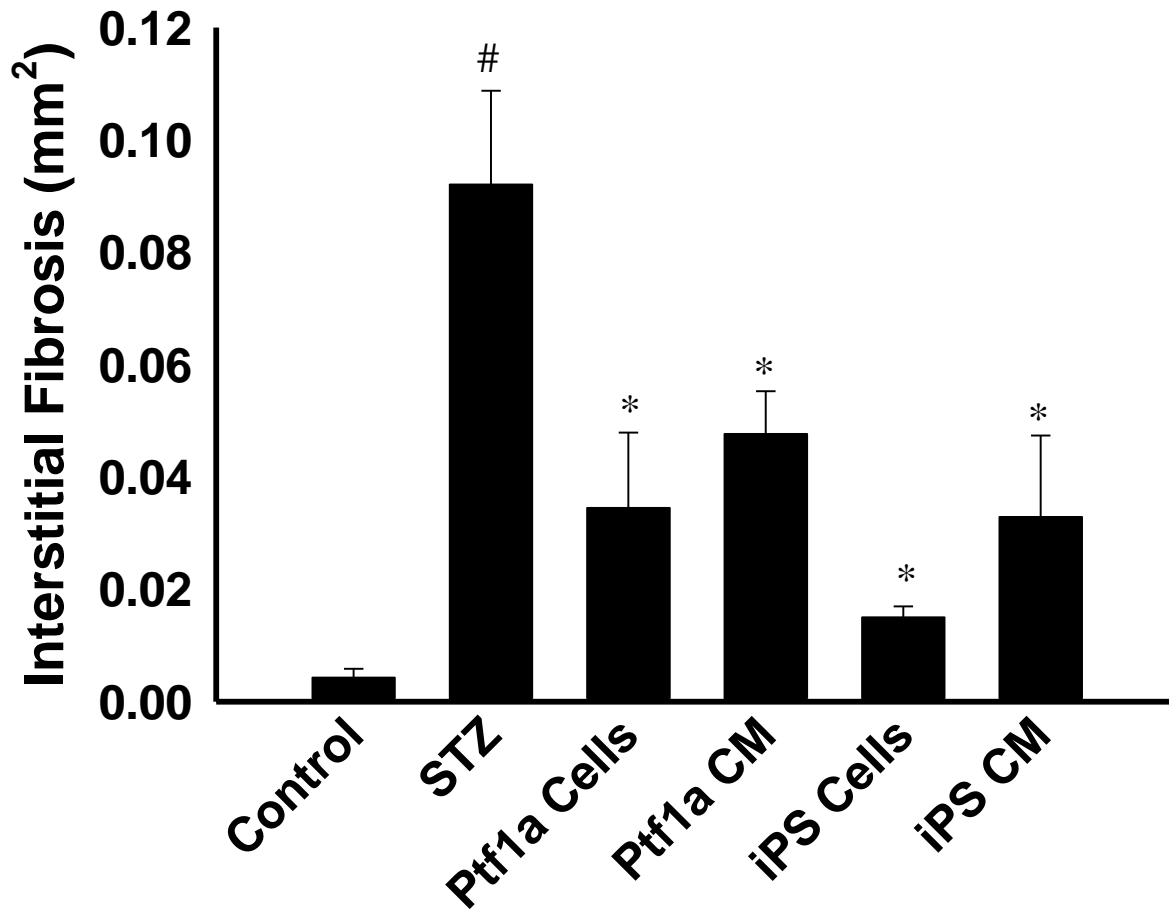
Figure 6: Cell and CM transplantation significantly inhibits interstitial fibrosis in the DCM rat heart. Histogram data demonstrates that STZ hearts have significantly enhanced interstitial fibrosis whereas; following Ptf1a/iPS cell and CM transplantation, this fibrosis is significantly inhibited. #p<0.001 vs. control and *p<0.05 vs. STZ.
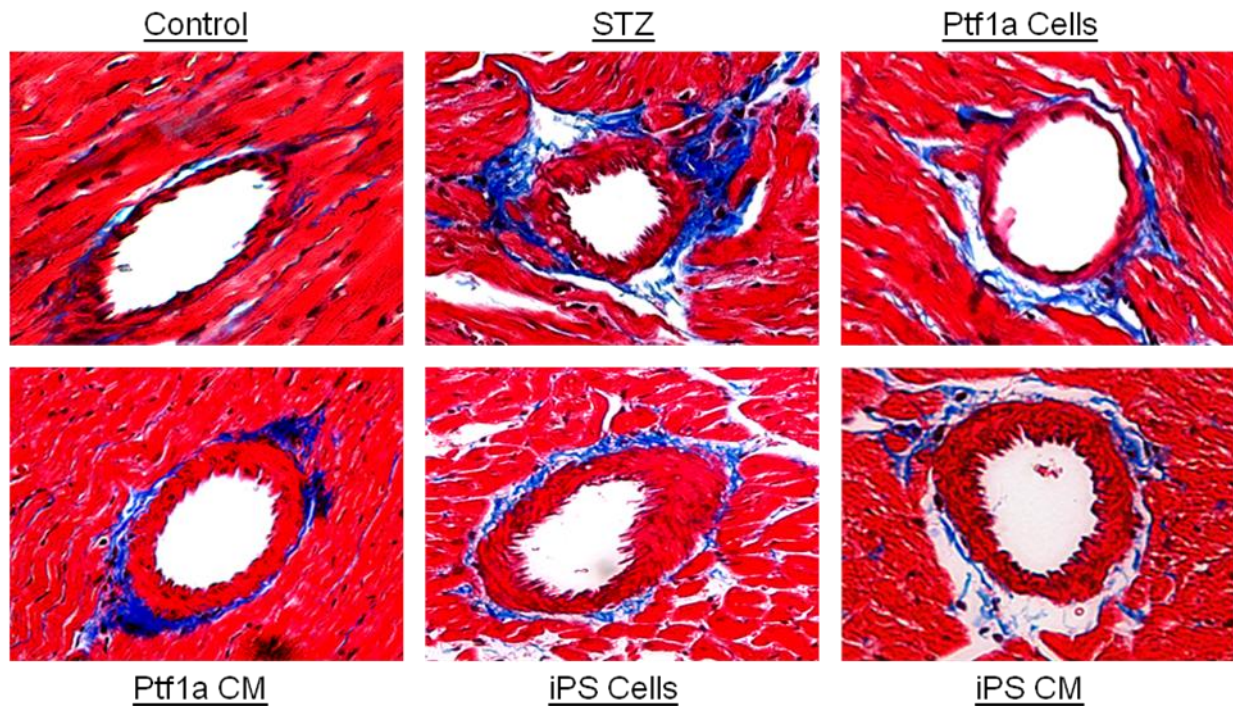
Figure 7: Effects of transplanted cells and CM on vascular fibrosis. Representative photomicrographs of sections containing large vessels stained with Masson's trichrome from the control, STZ, and Ptf1a cell experimental groups. Magnification, 20X.
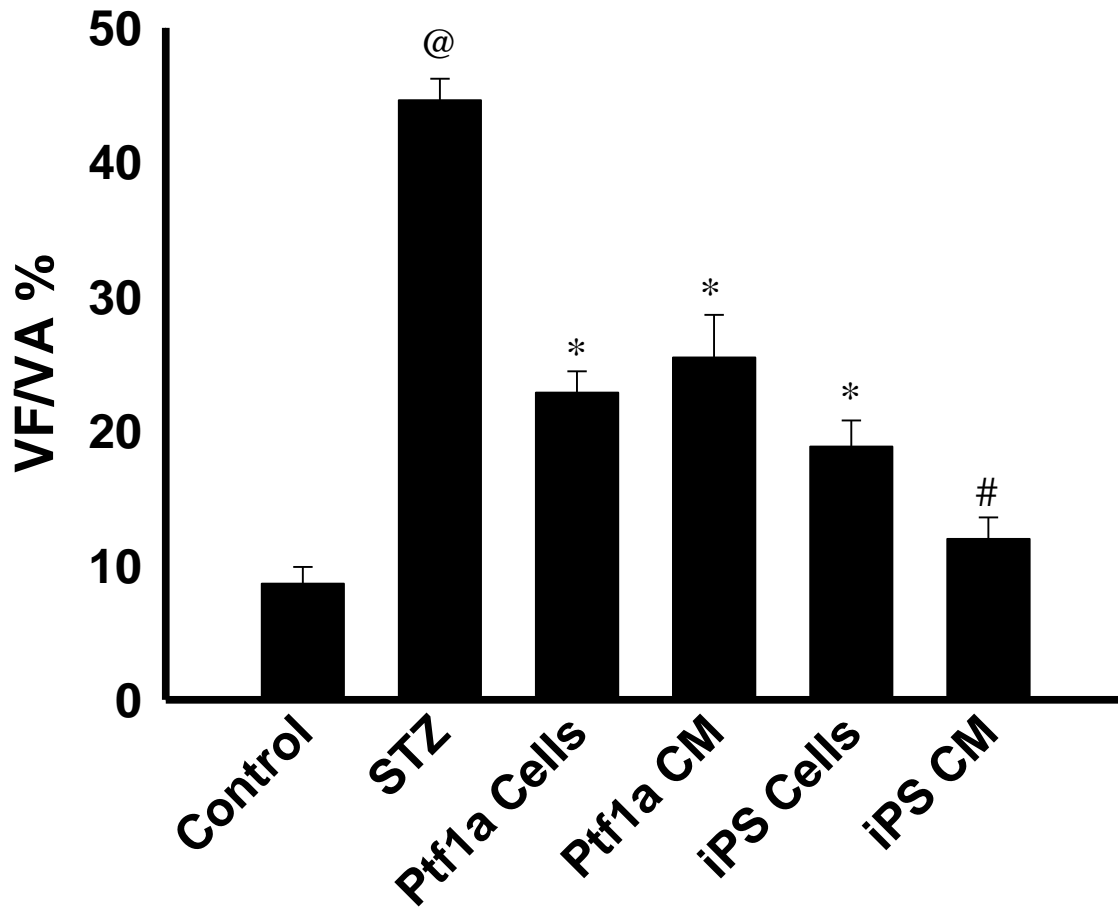
Figure 8: Transplanted Ptf1a/iPS cells and CM significantly reduce vascular fibrosis in the STZ-induced DCM rat heart. Histogram representing the quantitative analysis of vascular fibrosis in all experimental groups. @$p<0.001$ vs. control, #$p<0.001$ vs. STZ, and *$p<0.001$ vs. STZ, Ptf1a cells and Ptf1a CM. VF = vessel fibrosis, VA = vessel area
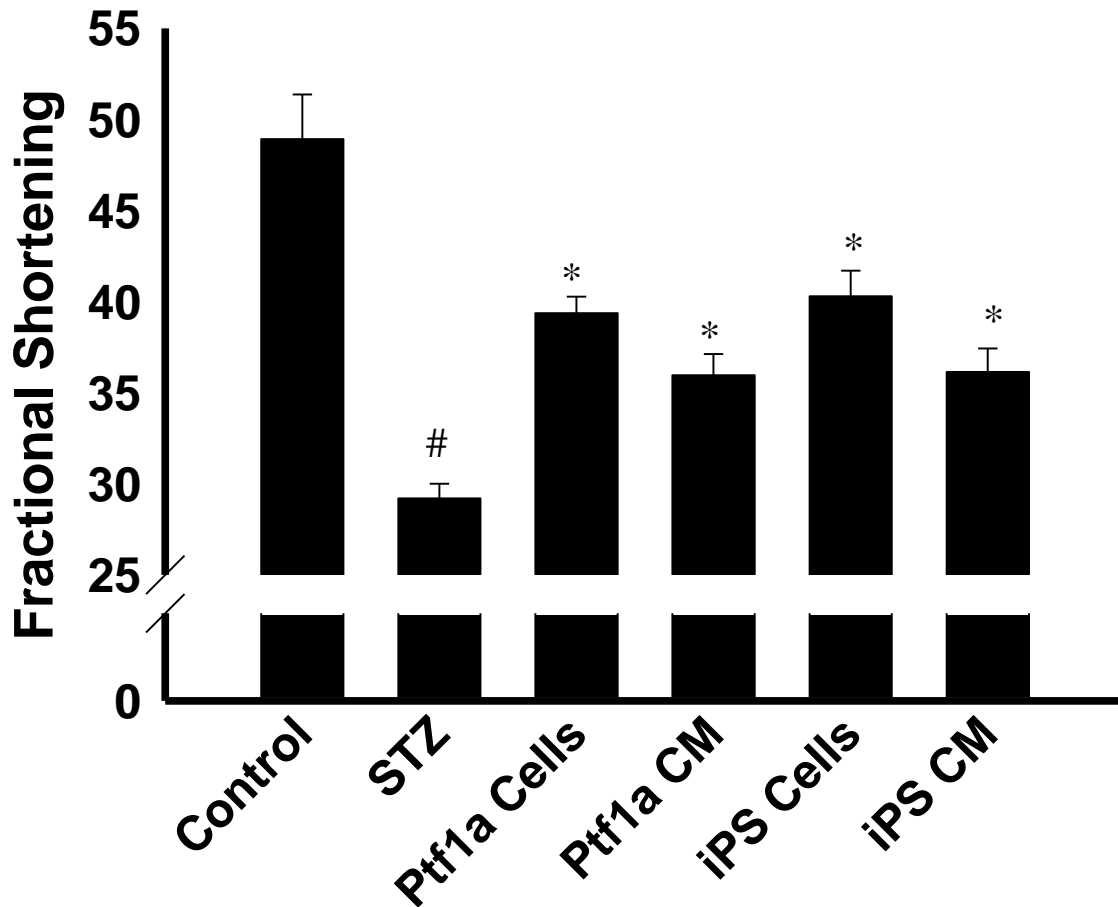
Figure 9: Transplanted Ptf1a/iPS cells and CM significantly improve heart function in the STZ-induced diabetic rat heart. Echocardiography was performed at D28 following cell transplantation and fractional shortening was determined. Histogram data shows average fractional shortening at D28 for all experimental groups. #p<0.001 vs. control and *p<0.001 vs. STZ.

Control **STZ** **Ptf1a Cells**

Ptf1a CM **iPS Cells** **iPS CM**
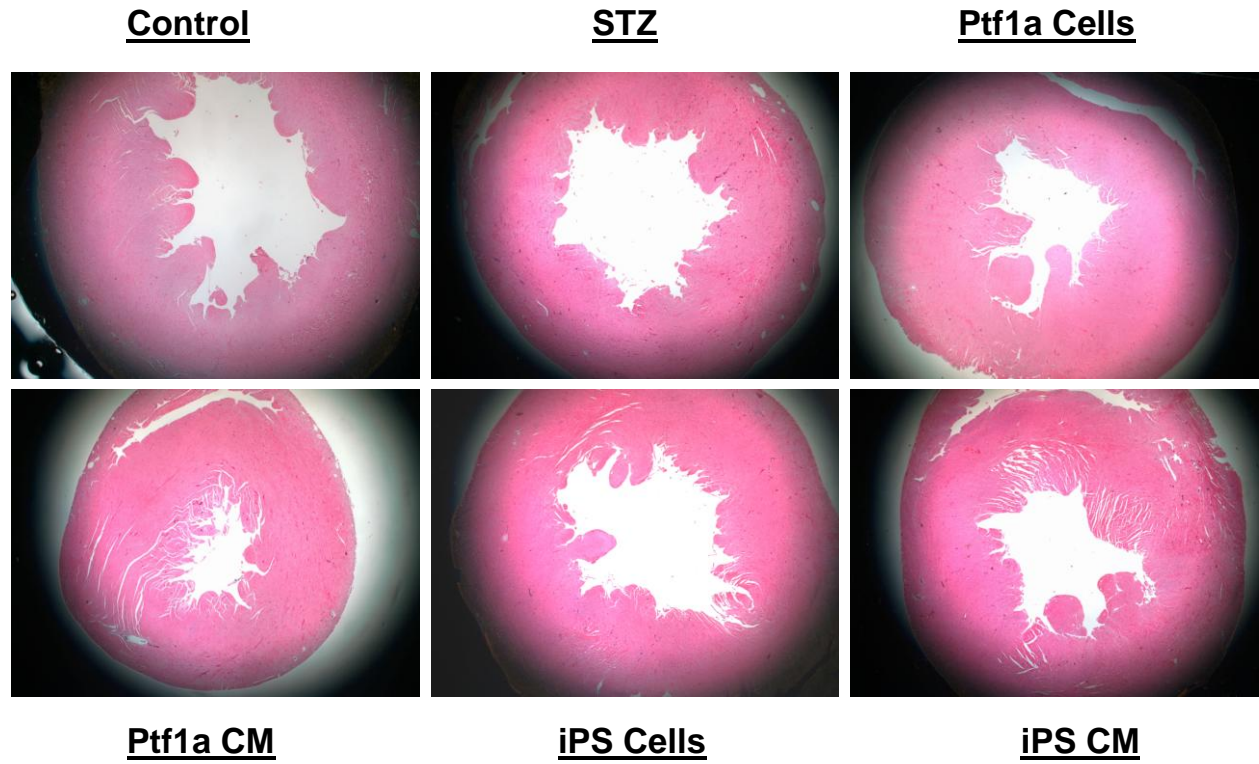
Figure 10: No teratoma formation in hearts transplanted with iPS cells or CM.
Representative photomicrographs of H&E stained whole heart sections demonstrating the absence of teratomas in all experimental. Magnification, 1.25X.

REFERENCES

1. Ares-Carrasco, S., Picatoste, B., Benito-Martin, A., Zubiri, I., Sanz, A. B., Sanchez-Nino, M... Lorenzo, O. (2009). Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes. *Am J Physiol Heart circl Physiol*, H2109-H2119.

2. Asbun, J., & Villarreal, F. (2006). The Pathogenesis of Myocardial Fibrosis in the Setting of Diabetic Cardiomyopathy. *Journal of the American College of Cardiology,* 47(4), 693-700.

3. Baraniak, P., McDevitt T., (2010). Stem cell paracrine actions and tissue regeneration, *Regenerative Medicine,* 5(1), 121-143.

4. Behfar A, Perez-Terzic C, Faustino RS, Arrell DK, Hodgson DM, Yamada S, Puceat M, Niederlander N, Alekseev AE, Zingman LV and others. 2007. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. *J Exp Med* 204(2), 405-20.

5. Brown, R., Ambler, S., Mitchell, M., & Long, C. (2005). The Cardiac Fibroblast:Therapeutic Target in Myocardial Remodeling and Failure. *Annual Review of Pharmacology and Toxicology,* 45, 657-687.

6. Boudoulas K & Hatzopoulos A. (2009). Cardiac repair and regeneration: the Rubik's cube of cell therapy for heart disease. *Dis Model Mech.*, 2(7-8):344-358.

7. Boudina, S., & Abel, E. (2007). Diabetic Cardiomyopathy Revisited. *Circulation,* 115, 3213-3223.

8. Boudina S, Abel E. (2010). Diabetic cardiomyopathy, causes and effects. (2010). Rev Endocr Metab Disord. 11(1):31-9.

9. Cai, L., & Kang, Y. (2001). Oxidative stress and diabetic cardiomyopathy. *Cardiovascular Toxicology,* 1(3), 181-193.

10. Chakrabarti, S., Biswas, T., Rokeya, B., Ali, L., Mosihuzzaman, M., Nahar, N., . . . Mukherjee, B. (2003). Advanced studies on the hypoglycemic effect of Caesalpinia bonducella F. in type 1 and 2 diabetes in Long Evans rats. *Journal of Ethnopharmacology, 84*(1), 41-46.

11. Clark, C., & Lee, A. (1995). Prevention and Treatment of the Complications of Diabetes Mellitus. *The New England Journal of Medicine, 332*, 1210-1217.

12. Dengler, J., Song H., Thavandiran, N., Massé, S., Wood, G., Nanthakumar, K., Zandstra, P., Radisic,. M. (2010) Engineered heart tissue enables study of residual undifferentiated embryonic stem cell activity in a cardiac environment. *Biotechnology and Bioengineering,* doi: 10.1002/bit.22987.

13. Elsner, M., Guldbakke, B., Tiedge, M., Munday, R., & Lenzen, S. (2000). Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. *Diabetologia, 43*, 1326–1333.

14. Evans, M., & Kaufman, M. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature, 292*, 154-156.

15. Fatma, S., Singla, R., Singla, D., (2009) Factors Released From Embryonic Stem Cells Stimulate c-kit-FLK-1+ ve Progenitor Cells and Enhance Neovascularization, *Antioxid. Redox Signal,* 13, 1857–1865.

16. Forcheron, F., Basset, A., Abdallah, P., Del Carmine, P., Gadot, N., & Beylot, M. (2009). Diabetic cardiomyopathy: effects of fenofibrate and metformin in an experimental model – the Zucker diabetic rat. *Cardiovascular Diabetology, 8*(16). doi:10.1186/1475-2840-8-16.

17. Fedak, P. (2008). Paracrine Effects of Cell Transplantation: Modifying Ventricular Remodeling in the Failing Heart. *Seminars in Thoracic and Cardiovascular Surgery, 20*(2), 87-3.

18. Geiss LS, Herman HW, Smith PJ, (1995). National Diabetes Data Group. *Diabetes in America*. Bethesda, MD: National Institute of Health, National Institute of Diabetes and Digestive and Kidney Diseases; 233-257.

19. Glass, C., Singal, P., & Singla, D. (2010). Stem cells in the diabetic infarcted heart. *Heart Failure Reviews, 15*(6), 581-588.

20. Guo HD, Wang HJ, Tan YZ, Wu JH. (2010). Transplantation of Marrow-Derived Cardiac Stem Cells Carried in Fibrin Improves Cardiac Function After Myocardial Infarction. *Tissue Eng Part A*. doi:10.1089/ten.tea.2010.0124.

21. Guddati AK, Otero JJ, Kessler E, Aistrup G, Wasserstrom JA, Han X, Webber MJ, Stupp SI, Lomasney JW, Kessler JA. (2009). Embryonic stem cells overexpressing Pitx2c engraft in infarcted myocardium and improve cardiac function. *Int Heart J. 50*(6), 783-99.

22. Hickson-Bick DL, Buja ML, McMillin JB (2000). Palmitatemediatedalterations in the fatty acid metabolism of rat neonatal cardiac myocytes. J Mol Cell Cardiol 32, 511–519.

23. Hofbauer, L., Brueck, C., Singh, S., & Dobnig, H. (2007). Osteoporosis in Patients With Diabetes Mellitus. *Jounal of Bone and Mineral Research, 22*, 1317–1328.

24. Intengan, H., & Schiffrin, E. (2001). Vascular Remodeling in Hypertension Roles of Apoptosis, Inflammation, and Fibrosis. *Hypertension, 38*, 581-587.

25. Keyes K., Ye Y., Lin Y., Zhang C., Perez-Polo J., Gjorstrup P., Birnbaum Y. (2010). Resolvin E1 protects the rat heart against reperfusion injury.*Am J Physiol Heart Circ Physiol. 299*(1), H153-H164.

26. Kim H, Kim S, Nam D, Kim S and Yoon Y. (2009). Cell therapy with bone marrow cells for myocardial regeneration. *Antioxid Redox Signal* 11: 1897-1911.

27. Kooreman, N., & Wu, J. (2010). Tumorigenicity of pluripotent stem cells: biological insights from molecular imaging. *Journal of the Royal Society Interface*. doi:10.1098/ rsif.2010.0353.focus.

28. Kuo W., Chung L., Liu C., Wu S., Kuo C., Tsai F., Tsai C., Lu M., Huang C., Lee, S. (2009). Effects of insulin replacement on cardiac apoptotic and survival pathways in streptozotocin-induced diabetic rats. *Cell Biochem Funct*. 27(7), 479-487.

29. Lenzen, S., (2008) The mechanisms of alloxan- and streptozotocin-induced diabetes *Diabetologia* 51, 216–226.

30. Lernmark, A. (1999). Type 1 Diabetes. *Clinical Chemistry, 45*, 1331-1338.

31. Leri, A., Kajstura, J., & Anversa, P. (2005). Cardiac Stem Cells and Mechanisms of Myocardial Regeneration. *Physiol Rev, 85*, 1373–1416.

32. Linthout, S., Seeland, U., Riad, A., Eckhardt, O., Hohl, M., Dhayat, N.,... Pauschinger, M. (2008). Reduced MMP-2 activity contributes to cardiac fibrosis in experimental diabetic cardiomyopathy. *Basic Research in Cardiology, 103*(4), 319-327.

33. Mihm, M., Seifert, J., Coyle, C., & Bauer, J. (2001). Diabetes related cardiomyopathy Time dependent echocardiographic evaluation in an experimental rat model. *Life Sciences, 69*, 527–542.

34. Ou, H., Tzang, B., Chang, M., Liu C., Liu, H., Lii, C., Bau, D., Chao P., Kuo, W., (2010). Cardiac Contractile Dysfunction and Apoptosis in Streptozotocin-Induced Diabetic Rats Are Ameliorated by Garlic Oil Supplementation. *J. Agric. Food Chem.* 58, 10347–10355 doi: 10.1021/jf101606s.

35. Passier, R., van Laake, L. W., & Mummery, C. L. (2008). Stem-cell-based therapy and lessons from the heart. *Nature, 453*, 322-329.

36. Shimizu, M., Umeda, K., Sugihara, N., Yoshio, H., Ino, H., Takeda, R., . . . Nakanishi, I. (1993). Collagen remodelling in myocardia of patients with diabetes, *Clin Pathol, 46*, 32-36.

37. Singla, D., Singla, R., & McDonald, D. (2008). Factors Released from Embryonic Stem Cells inhibit Apoptosis. *Am J Physiol Heart Circ Physiol, 295*, H907-H913.

38. Singla D. (2010) Stem cells in the infarcted heart. *J Cardiovasc Transl Res. 3*(1), 73-78.

39. Singla, D., Hacker, T., Ma, L., Douglas, P., Sullivan, R., Lyons, G., & Kamp, T. (2006). Transplantation of embryonic stem cells into the infarcted mouse heart: formation of multiple cell types. *Journal of Molecular and Cellular Cardiology, 40*(1), 195-200.

40. Singla, D., Lyons, G., & Kamp, T. (2007). Transplanted embryonic stem cells following mouse myocardial infarction inhibit apoptosis and cardiac remodeling. *Am J Physiol Heart Circ Physiol,* 293, H1308-H1314.

41. Standle, E., & Schnell, O. (2000). A New Look at the Heart in Diabetes Mellitus: From Ailing to Failing. *Diabetologia,* 43, 1455-1469.

42. Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM, Choo A, Lee CN, Pasterkamp G, de Kleijn DP.(2007). Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res.,* 1(2):129-37.

43. Tarquini, R., Lazzeri, C., Pala, L., Rotella, C., & Gensini, G. (2010). The diabetic cardiomyopathy. *Acta Diabetologica.* doi:10.1007/s00592-010-0180-x

44. Tschope C., Walther, T., Koniger, J., Spillman, F., Westermann, D., Escher, F., . ..Noutsias, M. (2004). Prevention of cardiac fibrosis and left ventricular

dysfunction in diabetic cardiomyopathy in rats by transgenic expression of the human tissue kallikrein gene. *The FASEB Journal, 18*(7), 828-836.

45. Volc H., Seidel C., Laohachewin D., Kaya Z., Müller O., Pleger S., Lasitschka F., Bianchi M., Remppis., …Andrassy M., (2010) HMGB1: the missing link between diabetes mellitus and heart failure. *Basic Res Cardiol.* doi 10.1007/s00395-010-0114-3.

46. Wernig M., Meissner A. Foreman R., Brambrink T., Ku M., Hochedlinger, K., Bradley E. Bernstein, B., Jaenisch, R., (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature, 448*, doi:10.1038/nature05944.

47. Westermann, D., Rutschow, S., Jager, S., Linderer, A., Anke, S., Riad, A.,... Tschope, C. (2007). Contributions of Inflammation and Cardiac Matrix Metalloproteinase Activity to Cardiac Failure in Diabetic Cardiomyopathy: The Role of Angiotensin Type 1 Receptor Antagonism. *Diabetes, 56*(3), 641-646.

48. Weibe, P., Kormish, J., Roper, V., Fujitani Alston, N., Zaret, K., Wright, C., Stein, R., Gannon M., (2007) Ptf1a Binds to and Activates Area III, a Highly Conserved Region of the *Pdx1* Promoter That Mediates Early Pancreas-Wide *Pdx1* Expression. *Molecular and Cellular Biology, 27*(11), 4093-4104.

49. Widyantoro B, Emoto N, Nakayama K, Anggrahini DW, Adiarto S, Iwasa N, Yagi K, Miyagawa K, Rikitake Y, Suzuki T, Kisanuki YY, Yanagisawa M, Hirata K (2010) Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic

hearts through stimulation of endothelial-to-mesenchymal transition.*Circulation*. *121*(22), 2407-18.

50. Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global Prevalence of Diabetes Estimates for the year 2000 and projections for 2030. *Diabetes Care, 27*(5), 1047-1053.

51. Zhao, M., Barron, M. R., Li, Z., Koprowski, S., Hall, C. L., & Lough, J. (2010). Making Stem Cells Infarct Avid. *Cell Transplantation,* 19, 245–250.

52. Zhang, R., Li, J., Luo, R., Jiang, J., & Wang, J. (2008). Bone Marrow Mesenchymal Stem Cells Induce Angiogenesis and Attenuate the Remodeling of Diabetic Cardiomyopathy. *Exp Clin Endocrinol Diabetes,* 116(2), 104-111.