Promoting differentiation and survival of human c-kit+ cardiac progenitor cells ex vivo.

Tareq Al-Maqtari

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PROMOTING DIFFERENTIATION AND SURVIVAL OF HUMAN C-KIT+ CARDIAC PROGENITOR CELLS EX VIVO

By

Tareq Al-Maqtari
B.S., Aleppo University, 2002
M.S., University of Louisville, 2012

A Dissertation Submitted to the Faculty of the
School of Medicine of the University of Louisville in
Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

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A Dissertation Approved on
June 8, 2015

By the following Dissertation Committee:

Dissertation Director: Dr. Kyung U. Hong

Dr. Steven P. Jones

Dr. Aruni Bhatnagar

Dr. Geoffrey Clark

Dr. Yongqing Liu
DEDICATION

This Dissertation is dedicated to my beloved kids

Bara’ah Al-Maqtari

Sundus Al-Maqtari

Mohammed Al-Maqtari

who filled my life with genuine joy and gave me unlimited love, trust, and support at all times.
ACKNOWLEDGEMENTS

It is my pleasure to express my gratitude to my mentor, Dr. Kyung U. Hong, for his continuous support and guidance. His motivation and insightful critiques were indispensable to help me finish this work. I would also like to thank my graduate committee members, Drs. Steven P. Jones, Aruni Bhatnagar, Geoffrey Clark and Yongqing Liu for their valuable comments and suggestions. I also appreciate the support and technical guidance of all my lab members.

Needless to say, without the funding and support of the Diabetes and Obesity Center, the Institute of Molecular Cardiology and the Department of Pharmacology and Toxicology (at University of Louisville), this work would not have been completed. Finally, I would like to express gratitude to my friends, colleagues, parents, and family whose continuous care, love, and encouragement throughout the years had a profound influence on my progress as a human being and as a knowledge seeker.
c-kit+ cardiac progenitor cells (CPCs) have recently gained much attention due to the therapeutic effects they exert on cardiac function following their administration into the infarcted heart as evidenced by animal studies and by a recent clinical trial (SCIPIO). However, injecting these cells in the heart is associated with poor differentiation into specialized cardiac cell types and with rapid death of the engrafted cells. With the ultimate goal of advancing cardiac stem cell therapy, we sought to facilitate the differentiation of human CPCs into cardiac cell types (e.g. cardiomyocytes, smooth muscle cells, endothelial cells and cardiac fibroblasts) by overexpressing selected cardiac transcription factors in vitro. To achieve that, Gata4, MEF2C, NKX2.5 and TBX5, were overexpressed in CPCs via lentivirus. When individually overexpressed, Gata4 upregulated some cardiomyocyte, smooth muscle cell, and fibroblast markers. TBX5, however, induced only few cardiomyocyte markers, indicating partial differentiation. In addition, these changes in CPC cardiac gene expression observed with Gata4 overexpression were accompanied by marked
morphological changes, manifested by the cells becoming wider and largely polygonal. However, introducing the aforementioned transcription factors in various combinations largely failed to further enhance the cardiac differentiation of CPCs induced by *Gata4* or *TBX5*, underscoring the complexity of the interaction between the cardiac transcription factors. Likewise, addition of the chromatin remodeling transcription factor *BAF60C* to *Gata4* and/or *TBX5* did not further potentiate their pro-differentiation effects in CPCs.

In addition to inducing differentiation, we also endeavored to promote CPC survival by overexpressing a pro-survival gene. To that end, a constitutively active mutant form of *Nrf2* (*caNrf2*) was overexpressed in CPCs. *caNrf2* overexpression protected CPCs against hydrogen peroxide- and 2, 3-dimethoxy-1, 4-naphthoquinone (DMNQ)-induced oxidative stress in vivo without altering the overall growth characteristics of the cells. Taken together, our results highlight the potential of *Gata4* in facilitating differentiation and the protective role of *caNrf2* in CPCs. These effects of *Gata4* and *caNrf2* may enhance the regenerative capabilities of CPCs and could thus be utilized to advance cell-based heart therapies.
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CHAPTER I
INTRODUCTION

Discovery of c-kit+ cardiac progenitor cells in the adult human heart

Myocardial infarction (MI) is a main cause of mortality and morbidity worldwide. In the USA, MI is responsible for approximately 50% of all cardiovascular deaths [1]. MI often occurs when an atherosclerotic plaque ruptures into a coronary artery, which may occlude the artery, and cause necrosis in the infarcted area [2]. Following infarction, both infarcted and non-infarcted regions of the heart undergo a remodeling process, causing the infarcted region to lose contractility. Eventually, the infarct region heals leaving a fibrotic non-contractile tissue which culminates in heart failure [3]. Although reperfusion and pharmacological interventions for acute MI patients have been successful in lowering mortality, they fall short in repairing the damaged cardiac tissue [4], underscoring the need for newer therapeutic approaches.

The long-standing notion of the heart being a terminally-differentiated organ has been challenged by cardiomyocyte-renewal findings in a number of studies [5-8]. The innate self-regenerative potential of the heart has been supported by the discovery of resident cardiac lin-/c-kit+ cardiac progenitor cells (CPCs) with stem cell characteristics (e.g. clonogenicity, self-renewal, and
multipotency) [9]. These CPCs have been isolated from multiple mammalian species including rats, pigs, dogs and humans [10-13]. The presence of CPCs that are positive for both the stem cell marker c-kit and one or more early cardiac cell markers (e.g. GATA4, MEF2C and ETS1) suggests that CPCs are capable of differentiating into committed cardiac cell types [13]. Indeed, the ability of c-kit+ CPCs present in embryonic hearts to generate specialized cardiac cell types including cardiomyocytes, smooth muscle cells and endothelial cells during cardiogenesis have been shown [14, 15]. Currently, however, a debate is rising in regards to whether these c-kit+ cells are bona fide cardiac stem cells or stromal cells that reside in the heart and can generate cardiac lineages under certain conditions [16]. Thus, different authors may have different names for these cells including: c-kit+ cardiac stem cells, CPCs and cardiac stromal cells. However, For the purpose of this work, these cells will be referred to as “cardiac progenitor cells or CPCs”.

**CPC therapy for ischemic cardiomyopathy following MI**

The intriguing properties of CPCs and the need for a treatment that can replace the infarcted portion of the myocardium with a functional viable tissue have led numerous groups to assess the therapeutic potential of these cells. Indeed, independent laboratories have shown promising findings in animal models. For instance, c-kit+ cells isolated from adult Fischer rats exhibited stem cell properties and appeared to give rise in vitro and in vivo to at least 3 cell types within the cardiac tissue, namely cardiomyocytes, smooth muscle cells and
endothelial cells [9]. When injected in two loci bordering the infarct region in a rat model of MI, CPCs appeared to generate small cardiomyocytes and new blood vessels, thereby promoting functional recovery [9]. In addition, Tang et al. have shown that intracoronary injection of CPCs one month after the induction of MI in rats, decreased fibrosis, increased the viable tissues in the risk region, and improved cardiac ejection fraction, a marker of cardiac function [17]. In a third study, MI was induced in a porcine model by a 90-minute coronary occlusion followed by reperfusion [18]. Three months later, autologous CPCs were injected into the coronary artery using a balloon catheter and the analysis at one month post-treatment showed that CPC-injected animals had improved cardiac function as shown by greater ejection fraction and lower left ventricular end-diastolic pressure, corroborating the therapeutic potential of CPCs.

These encouraging results in animal models led to the initiation of a phase I clinical trial [Cardiac stem cell infusion in patients with ischemic cardiomyopathy (SCIPIO)] in 2009. Albeit designed primarily to establish safety, SCIPIO has corroborated the positive therapeutic outcomes of the intracoronary infusion of CPCs as evidenced by improved left ventricular ejection fraction and smaller infarct size [19]. In this trial, $1.0 \times 10^6$ autologous CPCs were injected into 16 patients via the intracoronary route 4 months after coronary artery bypass grafting. When left ventricular ejection fraction was assessed 4 months after CPC infusion, there was a significant improvement (increasing from 30.3% before CPC infusion to 38.5%) while the control group exhibited no change. Also, twelve months post treatment, ejection fraction further improved by an additional 4%,
indicating a long-term beneficial effect. Moreover, infarct size, as assessed by cardiac magnetic resonance imaging (MRI), decreased by 24% at 4 months and by 30% at 12 months [20]. These positive effects on heart function were accompanied by an improvement in the overall quality of life as shown by the Minnesota Living with Heart Failure Questionnaire (MLHFQ).

Despite all the previous data that show the therapeutic potential of CPC for heart repair after MI, some studies have shown multiple shortcomings with the use of CPCs, including poor survival of the transplanted cells as well as lack of differentiation into mature cardiomyocytes [11, 17, 21, 22] (See below for more details). Addressing these issues may enhance the beneficial effects of CPCs for heart repair.

**CPCs do not differentiate upon injection in the infarcted heart**

The main underlying cause of heart failure following MI is the loss of viable contractile tissue [3] which is comprised of cardiomyocytes, smooth muscle cells and endothelial cells. Thus, it is conceivable that injecting cells that can generate functional cardiac cell types may be more conducive to heart recovery, conferring CPCs a critical advantage since they are considered, by many, as the progenitors of cardiomyocytes, endothelial cells and smooth muscle cells. However, only marginal differentiation of CPCs was noted upon intracoronary injection in a rat model of MI [17]. Indeed, although some injected cells coexpressed few cardiomyocyte-specific proteins, the cells were rather small and did not phenotypically resemble mature cardiomyocytes. In another study in
mice, GFP-labeled CPCs were traced following infusion via the intramyocardial and intracoronary routes [11] and their differentiation was assessed by expression of both GFP and the cardiomyocyte marker α-sarcomeric actin. Again, although few GFP-labeled cells co-expressed α-sarcomeric actin at 39 days post implantation, these cells did not exhibit identifiable sarcomeric structures and their sizes were much smaller than the endogenous cardiomyocytes. These studies constitute strong evidence that the injected CPCs do not spontaneously give rise to functional cardiac cell types upon introduction into the heart. That lack of differentiation into functional cardiac lineages is thought to limit the therapeutic potential of CPCs in repairing the injured myocardium.

Previous reports have demonstrated that multiple cell types can be directed to differentiate, at least partially, into cardiac cell lineages via introduction of cardiac transcription factors (TFs). For instance, cardiac TFs Gata4, Tbx5, and a subunit of the BAF chromatin-remodeling complex, Baf60c, directed ectopic differentiation of mouse mesoderm into beating cardiomyocytes [23]. Another combination of cardiac TFs (GATA4, TBX5, NKX2.5, and BAF60C) was sufficient to direct the differentiation of human embryonic stem cells into cardiomyocytes [24]. Also, both human embryonic stem cells and induced pluripotent stem cells generated cardiomyocyte-like cells following a plasmid-based transient overexpression of GATA4, BAF60C and the early cardiomyocyte marker MESP1 [25]. Even fibroblasts (cardiac and dermal) were reprogrammed into becoming functional cardiomyocyte-like cells in vitro and in vivo by
overexpressing Gata4, Mef2c, and Tbx5 [26]. However, these findings were challenged by a recent study [27], which could be ascribed to differences in reagents or methodology.

In addition, several reports have shown that cells which express higher levels of cardiac TFs (i.e. more cardiopoietic) exert superior therapeutic benefit for the infarcted heart. For instance, mesenchymal stem cells (MSCs) expressing Gata4 and Nkx2.5 (MSCs-GC) were superior to the naïve counterparts in promoting cardiac recovery after MI, in a mouse model [28]. Indeed, introducing MSCs-GC into the heart resulted in improved ejection fraction and fractional shortening, and led eventually to a thicker ventricular wall. In another study, a cocktail of TGF-β, activin, bone morphogenetic protein 4 (BMP4), retinoic acid, insulin-like growth factor-1 (IGF-1), alpha-thrombin, and interleukin-6 (IL-6) induced human MSCs to upregulate cardiac TFs [29]. These conditioned MSCs, which express higher levels of cardiac TFs, were superior to the naïve counterparts, leading to a pronounced improvement in cardiac structure and function upon injection into a mouse MI model. More recently, that cocktail of growth factors was used to create human cardiopoietic MSCs before injecting them into the endomyocardium of MI patients in a multi-center clinical trial [30]. Indeed, at 6 months post therapy, introduced MSCs improved multiple cardiac functional parameters such as ejection fraction, fractional shortening, and left-ventricular end-systolic volume. Taken together, these studies support two important notions: 1) Cells can be induced to differentiate into cardiac lineages and 2) Promoting the cardiopoietic capacity of cells enhances their therapeutic
potential. Thus, it is likely that overexpressing cardiac TFs in CPCs may prove advantageous in facilitating their differentiation and enhancing their therapeutic potential in the clinical setting.

**CPCs do not survive well in the host heart**

Another major challenge for cardiac cell-based therapy is that the majority of cells introduced into the heart die within a few days [31, 32]. For instance, more than 99.5% of MSCs were no longer detected in the left ventricle 4 days post-transplantation in the heart in a mouse model [33]. Consistent with that, the vast majority of the $5 \times 10^6$ neonatal rat cardiomyocytes injected in cryo-infarcted rats died within 7 days, as assessed by the TUNEL assay [34]. In order to assess whether CPCs undergo a similar fate, we previously monitored the retention and engraftment of CPCs following transplantation into the infarcted heart at serial time points. Of the $1.0 \times 10^5$ intramyocardially injected cells, less than 10% were still detected 24 hours post-injection [21]. Another study showed that injected CPCs were no longer detected at 35 days post injection in most of the investigated rats [17]. This profound loss of cells is thought to be caused by the poor viability of the transplanted cells in the harsh ischemic environment of the infarcted heart. Possible causes of premature cell death include: ischemia, host inflammatory response to infarction, and loss of matrix- and cell-cell interactions [34].

Thus, several attempts aimed at addressing the poor survivability of CPCs following transplantation have been carried out. For instance, Fischer et al.
implemented an *ex vivo* genetic engineering approach and injected CPCs overexpressing *Pim-1* (a prosurvival serine/threonine kinase) into the infarcted hearts of mice [35]. Indeed, *Pim-1* promoted proliferation of the implanted CPCs and enhanced their engraftment, leading eventually to a more pronounced improvement in cardiac function. Another approach utilized *in vitro* preconditioning of CPCs with cobalt protoporphyrin (a heme oxygenase 1 inducer). Indeed, the preconditioning promoted cell survival following exposure to H$_2$O$_2$-induced oxidative stress and resulted in the cells releasing higher levels of prosurvival cytokines, including epidermal growth factor (EGF), fibroblast growth factors 2 (FGF2), FGF3, BMP3 and erythropoietin [36].

With the ultimate goal of enhancing CPC therapeutic benefit in the treatment of MI, we sought in this work to address some of the limiting factors that seem to hinder the progress of CPC-based therapy, such as the poor survival and differentiation of CPCs. To facilitate CPC differentiation, cardiac TFs, including *Gata4, MEF2C, NKX2.5, TBX5* and *BAF60C* were overexpressed (will be discussed in Chapter II). Another genetic-engineering approach aimed at activating endogenous antioxidants and detoxifying enzymes within CPCs was utilized to promote cell survival (will be discussed in Chapter III).
Previous studies have shown that CPCs do not differentiate into functional cardiac cell types following their introduction to the infarcted heart [11, 17], which may limit their regenerative potential. On the other hand, cells which express higher levels of cardiogenic TFs (i.e. more cardiopoietic) have been shown to be therapeutically superior to less cardiopoietic cells [28, 29]. Thus, with the ultimate goal of promoting the regenerative potential of CPCs, we endeavored in this work to induce partial differentiation of CPCs into committed cardiac cell types in vitro. Once we achieve that goal and obtain more committed CPCs, it would be interesting down the road to test their therapeutic potential in vivo as compared to the naïve counterparts.

To promote differentiation of CPCs, the cardiopoietic effect of selected cardiac TFs was utilized. To that end, genes encoding cardiogenic TFs, namely GATA binding protein 4 (Gata4), myocyte enhancing factor 2C (MEF2C), a homeobox TF (NKH2.5), and T-box TF 5 (TBX5), were overexpressed in CPCs via a lentiviral system. First, we compared the ability of each TF individually to initiate the differentiation of CPCs into the four main cardiac cell types (i.e.
cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts). In the next set of experiments, all possible combinations of the aforementioned TFs were overexpressed in CPCs to determine whether the 4 aforementioned TFs synergize in promoting CPC differentiation. Also, the effect of differentiation media/dexamethasone on CPC differentiation was assessed. To assess the differentiation of CPCs caused by these methods, the changes in expression levels of markers associated with cardiomyocyte, smooth muscle cell, fibroblasts, and endothelial cells were analyzed. Quantitative real-time PCR (qRT-PCR) was used to monitor the changes in gene expression at the mRNA level, while immunocytochemistry and Western blot techniques were utilized to assess them at the protein level (mCherry-transduced CPCs served as a control). As an additional assessment of differentiation, microscopy was used to assess differentiation-associated morphological changes in size, shape, or formation of characteristic structural changes.

The results showed that Gata4 was most efficient in directing differentiation of CPCs into cardiomyocytes, smooth muscle cells and fibroblasts as evidenced by the upregulation of the correspondent cell-specific markers. However, when Gata4 was combined with differentiation medium and dexamethasone, CPCs seemed to initiate differentiation into cardiomyocytes only. These pro-differentiation effects of Gata4 may prove efficient in promoting complete differentiation of CPCs upon the in vivo transplantation and may thus have significant implications in maximizing the regenerative potential of CPCs.
MATERIALS AND METHODS

Isolation and culture of c-kit+/lin- CPCs

After obtaining a written consent, discarded right atrial appendage specimens from patients at Jewish Hospital in Louisville (KY) were collected according to a protocol approved by the Institutional Review Board (IRB) on human subject research at University of Louisville. Patients involved were undergoing open-heart, on pump, coronary artery bypass surgery and their ages ranged between 50 and 75 years. Briefly, right atrial appendages were transported under sterile conditions on wet ice before removal of gross blood and resection of adipose tissue [10]. The tissue was then washed with PBS, manually minced then enzymatically digested at 37°C using Worthington Collagenase type II/Hams F12 solution. The resulting cell suspension was centrifuged before discarding the supernatant. The cells were then transferred to T75 Flasks containing Ham’s F12 medium (Gibco), 10% FBS (Gibco), 10 ng/ml recombinant human basic FGF (PeproTech), 0.2 mM L-glutathione (Sigma), 0.005 U/ml human erythropoietin (Sigma) and 100 U/ml penicillin/streptomycin (Gibco). The adherent cells were continuously cultured with medium change every other day or subcultured every 4-5 days. Cells were passaged 1 time prior to c-kit magnetic activated cell sorting (MACS) kit using immunomagnetic beads (Miltenyi Biotec) to enrich for c-kit+/Lin- CPCs. The enriched CPCs were collected for expansion and/or analysis. Experiments shown in this study are performed on cells isolated from 1 patient but they have been repeated on isolates from at least one more patient to corroborate the findings. Figures depict the results from only 1 patient.
CPC immune magnetic-activated cell sorting (MACS)

70-75% confluent cells at passage 1 were sorted for c-kit using anti-c-kit (Miltenyi) microbeads and magnetic sorting apparatus. Cells were trypsinized and then washed twice in ice-cold MACS buffer made according to the manufacturer's specifications. All solutions were cooled on ice prior to the sorting protocol. Cells were immunomagnetically-sorted using MS columns (Miltenyi Biotec) and pre-separation filters with magnetic stands. To exclude the potential contamination with lin+ cells (such as T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, erythroid cells, and their committed precursors), a Lineage Cell Depletion Kit (Miltenyi Biotec) was used to purify c-kit+ cell populations as previously described [37]. The magnetically labeled Lin+ cells were depleted by retaining them on a MACS column in a magnetic field while unlabeled lin− cells passed through the column and were collected for further expansion and/or analysis. c-kit+ enriched cells were plated at subconfluence then cultured and expanded in vitro. Cells at passages 3-4 were fixed in 3.7% paraformaldehyde and assessed for c-kit positivity by flow cytometric analysis.

Flow cytometric analysis and immunocytochemistry

Enriched CPCs were trypsinized at passages 3-4, washed with 1x ice-cold buffer containing 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS) buffer followed by a second wash in cold PBS. Subsequently, cells were fixed for 15 minutes at room temperature (RT) using 3.7% paraformaldehyde. Following washing with PBS, fixed cells were stained for c-kit. Cells were then
blocked for 10 minutes at RT in 1% BSA buffer prior to staining for c-kit using c-terminal specific Santa Cruz C19 rabbit polyclonal IgG anti-human c-kit antibody for 1 hour in the dark at RT. Isotype rabbit polyclonal IgG was used in parallel as an isotype control. Cells were then washed with 1% BSA before adding the secondary antibody, FITC-conjugated donkey anti-rabbit IgG (Invitrogen) for 1 hour in the dark at RT. Flow cytometric analysis was performed using BD Accuri™ C6 flow cytometer. All analysis gates were set for false positivity of <1% in respective isotype controls. c-kit positivity was over 70%.

**Lentivirus expressing cardiac TFs**

Lentivirus expressing the cardiac TFs used in the current study was produced using ViraPower™ Lentiviral Expression System (Invitrogen) according to manufacturer’s instructions. The following MGC verified full-length cDNA clones for the TFs were purchased from OpenBiosystems: human *NKX2.5* cDNA (MHS1010-7430146; Clone ID: 5225103; NCBI Accession: BC025711), human *MEF2C* cDNA (MHS1010-7295133; Clone ID: 4815933; NCBI Accession: BC026341), and human *TBX5* cDNA (MHS1010-7430001; Clone ID: 5204163; NCBI Accession: BC027942). A retroviral expression construct for mouse *Gata4* (NCBI Accession: NM_008092.3) was a kind gift from Dr. Deepak Srivastava (UC San Francisco). The coding sequences for each TF or mCherry were PCR-amplified using Pfu high fidelity (HF) polymerase (Agilent) and subcloned into pLenti6/V5-D-TOPO vector (Invitrogen) according to the manufacturer’s instructions. Primer sequences used for the PCR were the following:
5' CACCATGTACAAAGCCTGGCCATG-3' and 5' - CGCGGTGATTATGTCCCCATGA-3' for Gata4; 5' - CACCATGTTCAGCCCT-3' and 5'-CCAGGCTCGGATACCATGC-3' for Nkx2.5; 5' - CACCATGGGGAGAAAAAAGATTCA-3' and 5' - TGTTGCCCATCATTCAGAAAGTC-3' for MEF2C; 5' - CACCATGGGACGACGAG-3' and 5' - GCTATTGTGCTCCACTCTGGC-3' for Tbx5; and 5' - CACCATGGTGAGCAAGGGC-3' and 5' - CTACTTTGACGCTCGTCCATGC-3' for mCherry. For generation of pLenti6-mCherry expression construct, pmCherry-C2 vector (K. U. Hong) was used as the PCR template. For generation of 3xFLAG constructs, the following oligos were synthesized, annealed and inserted into the BamHI site of pLenti6/V5-TOPO vector: 5' GATCCACCATGGATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATGAGGGG-3' and 5' GATCCCCTATCGTGCATCCTTTGTAATCCTATCGTGCATCCTTTGTAATACGACGATGAGGG-3'.

Each batch of virus was concentrated 10 times using Lenti-X Concentrator (Clontech) according to the manufacturer’s instructions and resuspended in complete CPC medium. Aliquots were made and stored at -80°C until use. Virus titers were determined by qRT-PCR-based measurement of integrated copies of viral genome following transduction of CPCs with varying dilutions of each virus. For calculation of the copy numbers of virus genome integrated into the host, serial dilutions of pLenti6 vector were used to generate
the standard curve. Briefly, CPCs transduced with varying dilutions of virus were harvested after 4 days. Genomic DNA was isolated, and 50 ng of DNA was analyzed by qRT-PCR as described below. The following primers were used for the qRT-PCR analysis: 5`GCTCAGTTCCAGTTGCTTG-3` and 5`GCAGTGAGCCAAGATTGCAC-3` for human HLA-A (for human/CPC genomic DNA) [21], and 5`-CATCTTGAGCCCCTGCAGACG-3` and 5`CCGTCGGCTGTCCATCACTGTC-3` for integrated lentiviral vector. For the assay, mCherry virus served as a reference. The efficiency of transduction with each dilution of mCherry virus was assessed by measuring the percentage of mCherry-positive cells, and it was plotted against the number of viral genomes integrated into CPCs to obtain a standard curve. Based on the curve, the volume of virus required to achieve 70-80% transduction efficiency was calculated for each virus batch.

**Lentivirus transduction of CPCs**

CPCs were plated on 12-well plates the day before transduction at a density of approximately 1.0 x 10^5 cells per well. Next day, the medium was replaced with 250 µl of complete medium containing appropriate dilution of virus and 6 µg/ml Polybrene (Sigma), and on the following day, the medium was replaced with fresh complete media. The cells remained on the same plate until harvest at specified time points (i.e., 7, 10, or 14 days post-transduction). The medium was refreshed every 3 days. Each treatment was done in quadruplicate.
Differentiation medium and dexamethasone treatment

On day 5 after Gata4 transduction of CPCs, regular culturing medium was replaced with differentiation medium (DMEM (Gibco), 5% FBS and Pen/strep) in the presence or absence of 10 nM dexamethasone (Sigma). Dexamethasone was added to the culture every other day for a total of 10 days. On day 14 post-transduction, cells were harvested for qRT-PCR, immunocytochemistry or Western blot analyses.

RNA isolation and qRT-PCR

Total RNA was isolated from CPCs using RNeasy Mini Kit (with DNase treatment) (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using 250 ng of total RNA using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent) according to the manufacturer's instructions. Samples were analyzed for mRNA levels of indicated markers using SYBR Green Master Mix (Applied Biosystems) and 7900HT Fast qRT-PCR System with SDS version 2.4.1 (Applied Biosystems). Each gene-specific primer set was initially validated based on the product size, and each PCR product was then sequence-verified (data not shown). Sequences of the primers used in the present study are listed in Table 1.
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**Immunofluorescence staining**

Cells grown on culture plates or glass coverslips were fixed in 3.7% formaldehyde in PBS for 15 minutes at RT and permeabilized using 0.25% Triton X-100 in PBS for 10 minutes. Following incubation in the blocking solution (5% BSA in PBS) for 30 minutes, the cells were incubated in primary antibody solution (diluted in the blocking solution) for 1 hour at RT and washed twice in PBS. They were then incubated in a solution of secondary antibodies conjugated to fluorochromes (diluted in the blocking solution) for 1 hour at RT and washed twice in PBS. They were finally counterstained with DAPI (4’, 6’-diamidino-2-phenylindole) and mounted on glass slides using Fluoromount (Sigma). Fluorescence images were viewed and acquired using EVOS® FL Cell Imaging System (Life Technologies).

**Western blot**

Total protein from transduced CPCs at serial time points was harvested using sample buffer and boiled for 15 min. Protein concentration was measured by BCA assay before loading onto a polyacrylamide gel. The proteins electrophoresed under reducing conditions were then transferred from the gel to a PVDF membrane. After blocking the membranes for 60 minutes with 5% dry milk powder and Tris-buffered saline, the membranes were incubated overnight at 4 °C with primary antibodies against the selected cardiac cell type markers. Following that, the membranes were washed and then incubated for an hour with horse radish peroxidase (HRP)-conjugated secondary antibody. Images were
developed using the ECL plus kit (Bio-rad) and an enhanced chemiluminescent
detection system (Pierce). Densitometry was executed using non-saturated
chemiluminescent membranes exposed and quantified using Fuji LAS-3000 bio-
imaging analyzer.

**Antibodies**

The antibodies used for the current study are listed below. KDR/VEFGR2
(mouse monoclonal; ab9530; Abcam); α-SMA (mouse monoclonal; A5228;
Sigma); troponin T (mouse monoclonal; clone 13-11; Thermo Fisher); α-
sarcomeric actin (mouse monoclonal; A7811; Sigma); FLAG tag (mouse
monoclonal; F-tag-01; Applied Biological Materials); Thy1/CD90 (mouse
monoclonal; clone 5E10; BD Pharmingen); smooth muscle myosin heavy chain
(rabbit polyclonal; Abcam); ANP (mouse monoclonal; clone 23/1; Santa Cruz);
BNP (mouse monoclonal; clone 50E1; Thermo Fisher); c-kit (rabbit monoclonal;
clone YR145; Epitomics); α-tubulin (mouse monoclonal; clone DM1A; Sigma); V5
tag (mouse monoclonal; clone E10; Applied Biological Materials).

**Phalloidin Staining**

To monitor potential changes in cell morphology induced by TF
overexpression, cultured cells were stained with Phalloidin CruzFluor™ 488
Conjugate (Santa Cruz) according to manufacturer’s instruction. Briefly, cultured
cells were washed twice with prewarmed PBS (pH 7.4) prior to fixation with 3.7%
formaldehyde in PBS for 10-15 minutes. Following 2 rounds of PBS wash, cells
were permeabilized using 0.125% Triton X-100 for 5-10 minutes. After another round of PBS washes, cells were incubated with the green fluorophore-conjugated phalloidin solution for 20 minutes at RT. Fluorescence images were viewed and acquired using EVOS® FL Cell Imaging System (Life Technologies).

**Statistical analyses**

All values are expressed as mean ± SE. Analysis of variance (ANOVA) was performed to compare data among the groups compared to control. Tukey test was used for other pairwise statistical comparisons. A *p* value of < 0.05 was considered statistically significant.
RESULTS

Introduction of cardiac TFs into CPCs

To promote differentiation of CPCs into functional cardiac cell types, selected TFs were overexpressed using a lentivirus delivery system. First, lentiviruses expressing one of the four selected cardiac TFs, $Gata4$, $MEF2C$, $NKX2.5$, and $TBX5$ were generated. These TFs either alone or in combination have been shown to direct differentiation of different cell populations into cardiomyocytes, including ES cells, extra cardiac mesoderm, and fibroblasts [23-26]. For ease of detection, The TFs used in this study were either FLAG-tagged at the N-terminus ($Gata4$, $MEF2C$, and $TBX5$) or V5-tagged at the C-terminus ($NKX2.5$). Following lentivirus production, CPCs were transduced with the viruses and the protein expression of the 4 TFs was verified by Western blot (Fig. 1). Also, immunocytochemical analysis showed a high efficiency of transduction ranging between 70 and 90% (Fig. 1). More importantly, the exogenous TFs were localized to the nucleus as expected (Fig. 1B).

Overexpression of individual TFs in CPCs

TFs were individually introduced into CPCs to assess whether they promote expression of markers associated with cardiac differentiation [note that endogenous transcripts of $GATA4$, $MEF2C$, and $TBX5$ but not $NKX2.5$ were detectable in naive CPCs (data not shown)]. Transduced cells were cultured for 1
Figure 1. **Lentivirus-mediated delivery of transcription factors to CPCs.** A, Images of untransduced and mCherry-transduced CPCs at 4 days post-transduction. Fluorescence of mCherry protein is shown in red. DAPI staining of nuclei was pseudo-colored in green. B, Immunostaining images for CPCs transduced with virus expressing 3xFLAG-tagged *Gata4*, *MEF2C*, *TBX5* or *BAF60C*, or V5-tagged *NKX2.5* and stained for the indicated epitope (i.e., FLAG or V5) which is shown in monochrome. DAPI images are shown in lower panels. C, Western blots confirming successful overexpression of the five TFs in CPCs.
or 2 weeks with medium change every 3-4 days before using qRT-PCR to detect
the changes in the transcript level of more than 30 different cardiac cell type-
specific markers (see Table 2 for the entire set of markers analyzed). In these
experiments, mCherry-transduced CPCs served as a control.

Among the 4 TFs investigated in the study, Gata4 overexpression was
associated with the most pronounced induction of cardiomyocyte, smooth muscle
cell and fibroblast (but not endothelial cell) markers both at 1 and 2 weeks.
Overexpression of Gata4 resulted in upregulating the mRNA transcripts of the
cardiomyocyte markers: brain natriuretic peptide (BNP) and troponin T (TNNT2),
within 1 week of expression (Fig. 2). However, not all cardiomyocyte markers
were upregulated by Gata4 overexpression. Examples include α- and β-MHC
and cardiac actin (ACTC) (data not shown). Interestingly, Gata4 induced the
expression of not only cardiomyocyte markers but also of other cardiac cell types’
markers. For instance, Gata4 overexpression upregulated the transcript levels of
smooth muscle cell markers, including calponin-1 and smooth muscle myosin
heavy chain (SM-MHC) within 2 weeks (Fig. 2). In addition, fibroblast markers
such as THY1/CD90 and fibroblast-specific protein 1 (FSP1; S100A4), were
significantly upregulated following Gata4 expression in CPCs (Fig. 2). However,
no significant induction of endothelial cell markers was detected in Gata4-
expressing cells (Fig. 2). In fact, Gata4 overexpression resulted in a marginal
decrease in the expression of the endothelial cell marker VE-Cadherin.
TABLE 2.
Markers of cardiac differentiation examined by qRT-PCR

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<tr>
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<td>THY1/CD90</td>
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Figure 2. **Effect of individual transcription factors on expression of cardiac differentiation markers in CPCs.** Transcription factors (*Gata4* [G], *MEF2C* [M], *NKX2.5* [N], and *TBX5* [T]) were overexpressed in CPCs via a lentivirus-based delivery system. Following transduction, cells were cultured for 1 or 2 weeks. At each time point, cells were harvested, and relative changes in mRNA levels of indicated genes were measured using qRT-PCR. The level of indicated transcript in each group was compared to that of mCherry-expressing control group (set at 1), and is expressed as a relative fold change. For each condition, n = 4. Bar graphs show mean ± SEM. *, p < 0.05 (vs mCherry-CPCs). †, not detected.
To assess any morphological changes in CPCs that may accompany Gata4-directed differentiation, phalloidin staining (which stains F-actin and shows the overall cytoskeleton), was utilized. Indeed, Gata4-overexpressing cells exhibited observable changes in cell morphology. The cells grew in size, resumed a polygonal morphology, and often exhibited prominent stress fibers, while mCherry-expressing cells retained their spindle-shaped morphology (Fig. 3A).

In addition to Gata4, TBX5 overexpression also exhibited a cardiogenic potential, albeit to a lesser extent. Genetic introduction of TBX5 induced few cardiomyocyte markers such as atrial natriuretic peptide (ANP) and TNNT2 (Fig. 2). However, TBX5 did not upregulate other cardiomyocyte markers such as α-, β-MHC and BNP by 2 weeks (data not shown and Fig. 2). Also, TBX5 transiently upregulated the endothelial cell marker KDR (vascular endothelial growth factor receptor 2; VEGFR2) but not VE-Cadherin, CD31 or vWF (Fig. 2 and data not shown). On the other hand, TBX5 expression resulted in downregulation of smooth muscle cell markers such as SM22α and αSMA (Fig. 2). Also similar but less pronounced than what was observed with Gata4 overexpression, TBX5 induced prominent morphological changes in some CPCs with the distinctive appearance of stress-fiber like structures and the enlarged cell phenotype (data not shown). Taken together, these findings show that Gata4 overexpression was associated with the most differentiation-promoting effect in CPCs, as reflected by induction of markers of at least three cardiac cell types (i.e. cardiomyocytes, smooth muscle...
Figure 3. **Gata4-induced changes in gene expression and morphology.** A, phase-contrast images of mCherry- and Gata4-overexpressing CPCs (upper panels). mCherry- and Gata4 virus-transduced CPCs were stained with Alexa Fluor 488-conjugated phalloidin to stain the cytoskeleton at 2 weeks post-transduction (lower panels). Gata4 overexpression changed the normal elongated shape of CPCs into a wide polygonal morphology. B, mCherry- and Gata4-transduced CPCs stained for the indicated markers at 2 weeks post transduction. Gata4-expressing cells exhibiting supranuclear staining of BNP are indicated by the arrows.
cells and fibroblasts). On the other hand, *TBX5* overexpression resulted in less pronounced induction of cardiomyocyte and endothelial cell markers.

With regard to *MEF2C* or *NKX2.5*, their overexpression unexpectedly did not significantly increase the expression of genes associated with cardiac differentiation. *NKX2.5* expression was associated with a trend of increasing the expression of the atrial cardiomyocyte marker connexin 40 and the endothelial cell marker *KDR* after 2 weeks (Figs. 2 and 4) but that induction did not reach statistical significance. *MEF2C* overexpression on the other hand was often associated with a decrease in the transcripts of smooth muscle cell markers (e.g., Calponin-1 and *SM22α*), compared to the control (Figs. 2 and 4). Also, *MEF2C* seemed to suppress some cardiomyocyte markers (e.g., *TNNT2* and connexins 40) and fibroblast markers (e.g., *Thy1/CD90*) although that did not reach statistical significance (Fig. 2 and 4).

To confirm the above observed changes in cardiac cell markers, protein expression of selected markers was assessed using immunofluorescence staining and Western blot analysis. For each assay, cells at 2 weeks post-transduction were analyzed. In line with the aforementioned qRT-PCR data, *Gata4*-overexpression in CPCs resulted in BNP upregulation and that was observed mainly in the supranuclear region (Fig. 3B), in consistence with a previous report (26). Also, *Gata4* overexpression was associated with a significant induction of the fibroblast marker Thy1/CD90 in CPCs, corroborating the qRT-PCR data (Fig. 3B). Interestingly, although αSMA mRNA level was not
Figure 4. Co-expression of transcription factors in combination in CPCs.

Cells were transduced with combinations of *Gata*4 [G], *MEF2C* [M], *NKX2.5* [N], and *TBX5* [T] and then cultured for 10 days prior to mRNA profiling using qRT-PCR. mCherry-transduced cells served as a negative control and was set at 1. The level of indicated transcript in each group was compared to that of mCherry control group, and is expressed as a relative fold change. For each condition, n = 4. Bar graphs show mean ± SEM. *, p < 0.05 (vs mCherry-CPCs).
upregulated by Gata4 (Fig. 2), its protein level was significantly upregulated as assessed by immunocytochemistry (Fig. 3B) and was confirmed by Western blot (data not shown), suggesting a Gata4-induced post-translational stabilization of αSMA. These observations are consistent with the previous qRT-PCR data mentioned earlier and further support the role of Gata4 in promoting differentiation of CPCs into cardiomyocytes, smooth muscle cells and fibroblasts. It should be noted that not all markers upregulated at the transcriptional level were associated by detectable protein expression (data not shown), suggesting that the level of induction of some markers by the TFs is minimal.

**Effects of overexpression of TFs in combination**

Based on the aforementioned data, we hypothesized that a combination of TFs may be needed for the TFs to achieve synergism and to further promote differentiation of CPCs. To test that, the four aforementioned TFs (i.e. Gata4 [G], MEF2C [M], NKX2.5 [N], TBX5 [T]) were introduced into CPCs in every possible combination via lentivirus. In total, 15 combinations of the aforementioned TFs were used (see Fig. 4). CPCs transduced with each combination of viruses were cultured for 10 days, and then analyzed for changes in mRNA expression of differentiation markers by qRT-PCR. The data show that overexpression of the correct set of TFs in each group was confirmed by quantitative as well as semi-quantitative RT-PCR (Fig. 5C and data not shown).
Figure 5. **Effect of transcription factor overexpression on the level of endogenous transcription factors.** A and B, Cells were transduced with combinations of *Gata4* [G], *MEF2C* [M], *NKX2.5* [N], and *TBX5* [T] and then cultured for 10 days prior to mRNA profiling using qRT-PCR. mCherry-transduced cells served as a negative control (not shown) and was set at 1. The level of indicated transcript in each group was compared to that of mCherry control group (set at 1), and is expressed as a relative fold change. For each condition, n = 4. Bar graphs show mean ± SEM. *, p < 0.05 (vs mCherry-CPCs).

C, Selected PCR products resulting from experiments described in Figures 4 and 5 were electrophoresed on agarose gel and stained by ethidium bromide.
Surprisingly, additive or synergistic effects of TFs were rarely observed. Only ANP, connexin 40 and α-actinin-2 were most upregulated in cells expressing all four TFs (Fig. 4). By and large, TFs overexpressed in combination were less effective in inducing cardiac differentiation markers when compared to single TFs. For example, induction of BNP, TNNT2, and THY1/CD90 mRNAs by Gata4 and induction of KDR by TBX5 were attenuated when other TFs were coexpressed (Fig. 4).

Next, the ability of overexpressed TFs to promote transcription of their endogenous counterparts was also assessed. Most of the TFs, whether alone or in combination, did not significantly induce their endogenous counterparts (Fig. 4). For instance, none of the 15 combinations tested was able to upregulate the transcript of endogenous Gata4 (Fig. 4). The only exception was the upregulation of the endogenous MEF2C transcript by MEF2C overexpression (Fig. 5A). Similarly, the combinations did not promote the transcription of other important cardiac TFs including serum response factor (SRF) and myocardin (MYOCD); a master regulator of smooth muscle gene expression (Fig. 5A). In fact, MYOCD mRNA level was suppressed by the overexpression of MEF2C and TBX5 (Fig. 5A). In fact, only Gata4 overexpression (individually) was able to reproducibly drive an upregulation in BAF60C, a chromatin remodeling TF, and in TEAD1 (a cardiac transcriptional enhancer) (Fig. 4 and data not shown). Taken together, these data show that only Gata4 overexpression (alone) activated some endogenous cardiac TFs.
**Overexpressing BAF60C in CPCs**

With the limited induction of the cardiac gene markers observed with overexpressing the selected 4 TFs and for the purpose of further attaining more pronounced differentiation of CPCs, BAF60C was added to the list of tested TFs. BAF60C possesses a helicase and an ATPase activity and is part of a chromatin remodeling SWI/SNF-like multi-subunit BAF chromatin remodeling complex. These activities enable Baf60c to play an important role in reorganizing the chromatin structure and facilitating the binding of TFs to their target genes [38]. Indeed, Baf60c has been shown to promote interactions between TFs (e.g., Tbx5, Nkx2.5, and Gata4) and the BAF complex, to enhance transactivation of cardiac genes during mammalian heart development [39]. In addition, Baf60c assisted Gata4 and Tbx5 to reprogram extra-cardiac mesoderm into heart tissue in mice [23].

The qRT-PCR analysis showed the presence of BAF60C transcript in undifferentiated CPCs (Fig. 6B). However, it was unknown if the protein was present at a sufficient level or if functional BAF complexes were present. Next, the role of BAF60C in promoting differentiation and its ability to potentiate the effects of Gata4 or TBX5 was investigated [The latter two TFs showed the most pronounced induction of differentiation markers and were thus included (Figs. 2 and 4)]. To that end, CPCs were forced to overexpress BAF60C [B], Gata4 [G], and TBX5 [T] individually and in every possible combination. Following two weeks of culture, cells were analyzed for changes in cardiac gene expression by qRT-PCR.
Figure 6. **Effect of overexpression of BAF60C, Gata4 and TBX5 on regulation of cardiac differentiation markers in CPCs.** A, CPCs transduced with different combinations of BAF60C [B], Gata4 [G], and TBX5 [T] via lentivirus and cultured for 2 weeks prior to mRNA profiling using qRT-PCR. mCherry-transduced cells served as negative control. The level of indicated transcript in each group was compared to that of mCherry-expressing control group (set at 1), and is expressed as a relative fold change. For each condition, n = 4. Bar graphs show mean ± SEM. *, p < 0.05 (vs mCherry-CPCs). B, Semi-quantitative qRT-PCR analysis showing increased expression of the indicated TFs in each treatment group. β-actin served as a loading control.
Unexpectedly, BAF60C expression did not upregulate any of the tested differentiation markers (Fig. 6). In fact, BAF60C had either no effect or downregulated the transcript levels of most investigated markers including the cardiomyocyte markers BNP and TNNT2, the smooth muscle marker α-SMA, the endothelial cell marker KDR, and the fibroblast markers Thy1/CD90 and FSP-1 (Fig. 6). In addition, BAF60C antagonized several Gata4 and/or TBX5 effects and in CPCs. For instance, BAF60C blunted Gata4-induced upregulation of BNP, TNNT2, THY1/CD90, and FSP-1 transcripts (Fig. 6). These observations suggest that BAF60C suppresses cardiac gene expression in CPCs and that downregulation of its expression may be needed for CPCs to differentiate into functional cardiac cell types.

Role of differentiation medium and/or dexamethasone

The use of a serum-reduced medium has been shown to drive the differentiation of multiple stem cells into cardiomyocytes [33, 40]. Similarly, adding dexamethasone to the culturing medium has been shown to direct cells into the cardiomyocyte lineage [41, 42]. With the limited CPC differentiation caused by TF-delivery in this study, we examined whether combining Gata4 overexpression and differentiation media/ dexamethasone have a synergistic effect in promoting differentiation of CPCs.

Interestingly, in comparison to the effect of differentiation media, Gata4 overexpression alone resulted in a more pronounced upregulation of cardiomyocyte markers. For instance, Gata4 overexpression caused a ~13 fold
induction in *BNP* whereas culturing in differentiation medium caused only a ~7 fold induction (Fig. 7). In line with that, *Gata4* overexpression had a consistent trend (yet not significant) of upregulating *TNNT2*, whereas the differentiation medium had no effect (Fig. 7). More importantly, a synergistic effect was observed when the differentiation medium was accompanied with *Gata4* overexpression. For instance, the ~7 fold induction in *BNP* caused by the differentiation medium tripled when *Gata4* was overexpressed (Fig. 7). Also, the combination of *Gata4/differentiation* was significantly synergistic in upregulating *TNNT2* mRNA level, increasing its level by 53 folds (Fig. 7). More interestingly, *Gata4* overexpression in CPCs that were cultured in differentiation medium containing dexamethasone had an even more pronounced synergistic effect in upregulating *TNNT2* expression, reaching 170 fold greater induction as compared to mCherry-expressing cells (Fig. 7). These findings suggest that *Gata4* combined with the dexamethasone/differentiation medium regimen has a strong synergistic effect in upregulating cardiomyocyte markers.
Figure 7. Effect of overexpression of Gata4, differentiation medium and/or 10 nM dexamethasone on regulation of cardiomyocyte markers in CPCs. CPCs transduced with Gata4 [G] via lentivirus and cultured for 2 weeks prior to mRNA profiling using qRT-PCR. Differentiation medium composed of DMEM medium containing 5% FBS was added on day 5 post Gata4 transduction and then changed freshly every other day. 10 nM dexamethasone was also added every other day starting on day 5 post-transduction. mCherry-transduced cells served as negative control and were set at 1. The level of indicated transcript in each group was compared to that of mCherry-expressing control group, and is expressed as a relative fold change. For each condition, n = 4. Bar graphs show mean ± SEM. *, p < 0.05 (vs mCherry-CPCs unless other way illustrated).
Discussion

Although CPCs have the potential to generate at least three of the cell types that reside in the heart muscle (cardiomyocytes, smooth muscle cells and endothelial cells) [9], they do not exhibit that ability upon reintroduction into the infarcted heart [11, 17]. Reasons for that could be related to the cell isolation process, culturing conditions or the introduction to harsh conditions within the infarcted myocardium. This limited differentiation of CPCs is thought to affect their regenerative potential in MI cell-based therapy, particularly because studies have shown therapeutic superiority of cells that are more committed to the cardiac cell lineages. For instance, MSCs expressing the cardiogenic TFs *Gata4* and *Csx/Nkx2.5* (*Csx* is the orthologue of *Nkx2.5* in mice) proved superior to their naïve counterparts in alleviating ischemic cardiomyopathy [28]. Indeed, the genetically-engineered MSCs improved ejection fraction and fractional shortening, and resulted in lower deposition of collagen within the myocardium. Upon analysis of the implanted cells, most MSCs overexpressing *Csx/Nkx2.5* and *GATA-4* also coexpressed the cardiomyocyte markers *TNNT2* and connexin 43, indicating partial differentiation into cardiomyocytes, and were associated with higher density of micro-vessels. In another study, bone marrow MSCs, isolated from 12 patients undergoing coronary artery bypass surgery, were injected into the infarcted hearts of 8- to 12-week-old immunocompromised mice [29]. Upon functional analysis, cells from only 2 patients led to functional cardiac benefit in the infarcted mice. These reparative MSCs were distinguished from the non-effective counterparts by the robust expression of the cardiac TFs *NKX-2.5,*
Interestingly, upon induction of the cardiac program in the non-reparative MSCs by a cocktail of growth factors (including TGFβ1, BMP-4, activin-A, retinoic acid, FGF-2, IGF-1, and IL-6), the cells upregulated *NKX2.5, TBX5 and MEF2C* expression and resulted in improved myocardial functional recovery when injected *in vivo*. These studies support that upregulating expression of cardiac TFs in CPCs (i.e. prompting them to acquire a more committed phenotype) is needed to promote their therapeutic benefit for heart cell-based therapy.

Protocols to facilitate differentiation of c-kit+ CPCs include the use of differentiation media, dexamethasone, 5-azacytidine followed by TGF-β1, or coculturing with neonatal rat cardiomyocytes [9, 10, 43-45]. However, these protocols have not been reproducibly effective in facilitating differentiation of CPCs. We, thus, sought to enhance the differentiation of CPCs into cardiac cell types via the introduction of selected cardiac TFs (*GATA4, MEF2C, NKX2.5, and TBX5*), which have shown to be able to drive cardiogenesis [23-26]. To that end, the 4 TFs were overexpressed individually and in various combinations in CPCs. Only Gata4 individually was able to reproducible induce significant effects. By mRNA analysis, Gata4 was shown to upregulate markers of cardiomyocytes including *BNP, connexin 40, BAF60C, and TNNT2*. Gata4 also upregulated the smooth muscle cell markers: *SM-MHC* and calponin 1, as well as the fibroblast markers: *THY1/CD90* and *FSP-1* (Fig. 2 and 4). However, there was no accompanying induction of endothelial cell markers, suggesting that Gata4 directs the differentiation of CPCs into cardiomyocytes, smooth muscle cells and
fibroblasts but not endothelial cells. It was also interesting to find that Gata4-induced upregulation of cardiomyocyte markers (such as BNP and TNNT2) were more pronounced than that of the differentiation medium (5% FBS-containing DMEM) (Fig. 7). That suggests that overexpressing Gata4 is more effective than the differentiation medium in directing differentiation of CPCs into the cardiomyocyte lineage. In line with these pro-differentiation roles of Gata4 in CPCs, isolated CPCs from adult rat hearts up-regulated GATA-4 expression over long-term culture and were then characterized by enhanced differentiation into cardiomyocytes [46].

Interestingly, a synergy between Gata4 and the differentiation medium was evident in facilitating differentiation of CPCs into cardiomyocytes, as evidenced by the induction of the cardiomyocyte markers BNP and TNNT2 (Fig. 7). There was an even more pronounced synergism in inducing cardiomyocyte markers (e.g. TNNT2), by adding dexamethasone to the differentiation medium and Gata4 overexpression, (Fig. 7). However, no induction of smooth muscle cell, endothelial cell, or fibroblast markers was noted with the aforementioned regimens (data not shown). These findings suggest that although Gata4 directs CPC differentiation into 3 cardiac cell lineages (cardiomyocytes, smooth muscle cells and fibroblasts), it directs the differentiation into only cardiomyocytes if combined with dexamethasone-containing differentiation media.

In addition to its role in driving differentiation, Gata4 has been reported to play other beneficial roles in the heart. For instance, Gata4 has been shown to possess a prosurvival effect, serving as an upstream activator of the
antiapoptotic gene $Bcl$-$X$ in differentiated postnatal cardiomyocytes [47]. Indeed, mouse heterozygotes for a null $Gata4$ allele had higher susceptibility to doxorubicin-induced cardiotoxicity which was rescued by genetic or pharmacological enhancement of $Gata4$ [47]. Also, $Gata4$ has also been shown to promote cardiac angiogenesis. For instance, conditional overexpression of $GATA4$ in adult cardiomyocytes increased myocardial capillary formation and increased coronary flow reserve and perfusion-dependent cardiac contractility [48]. These studies suggest that $Gata4$ activation in CPCs used for heart repair may have multi-faceted benefits, including promoting CPC differentiation and survival, as well as promoting cardiac angiogenesis.

Although $NKX2.5$ plays a pivotal role in early heart development and cardiac function [49-51], its overexpression in CPCs did not robustly upregulate differentiation markers. $NKX2.5$ led only to a slight increase in the mRNA level of connexin 40 and $KDR$ (Fig. 2 and 4). Also, $NKX2.5$ also did not synergize or potentiate the effects of the other TFs analyzed in the study. Although this was unexpected, a previous report has shown a similar antagonistic role of $NKX2.5$ in promoting differentiation into cardiomyocytes [26]. These data suggest specific requirements for the cardiogenic effect of $NKX2.5$ that were missing in the $in$ $vitro$ conditions of the current study or that $NKX2.5$ has a cell- or time- or developmental stage-specific effect.

Previous reports suggested that cardiac TFs interact with one another to co-regulate cardiogenesis. For instance, co-expression of $GATA4$ and $Nkx2.5$ resulted in a synergistic activation of the $ANP$ promoter in heterologous cells.
The synergy was associated with a physical interaction between the 2 TFs observed in vitro and in vivo [52]. Furthermore, Nkx2.5 has been shown to associate with Tbx5 to promote cardiomyocyte differentiation [53]. On the other hand, there are also indications in the literature suggesting antagonism between some cardiac TFs. For instance, the synergistic activation of the connexin 40 promoter induced by NKX2.5 and GATA4 was suppressed by co-expression of TBX5 [54]. In line with that, our results show that overexpressing TFs in combination did not synergize in directing CPC differentiation. Indeed, except for the markers ANP, connexin 40 and α-actinin2, expressing all the 4 TFs (Gata4, NKX2.5, MEF2C and TBX5) in CPCs led often to suppressing the transcription of differentiation markers compared to single TFs (Fig. 4). These mixed results observed when multiple TFs are expressed in CPCs, as shown in our study and in some previous reports, suggest a gene-specific synergism/antagonism of TFs, reflecting the complexity of the regulatory transcription network within CPCs.

BAF60C was added to the pool of investigated TFs in this study because it is a subunit of the BAF complexes that is involved in promoting interactions between cardiac TFs and the BAF complex, thereby promoting cardiogenesis [39]. To that end, BAF60C, Gata4 and/or TBX5 were overexpressed in every possible combination in CPCs. However, the qRT-PCR data (see Fig. 6) show that BAF60C suppressed the expression of most cell differentiation markers, suggesting that it inhibits CPC differentiation.

Although speculative, the inability of BAF60C, MEF2C, NKX2.5 and TBX5 (with the exception of Gata4) to promote robust CPC differentiation may be due
to one of the following: 1) it is possible that a precise temporal coordination is needed for these TFs to transactivate one another and drive cardiogenesis. In this study however, TFs were expressed at the same time and for the same period. 2) Duration of TF overexpression may need to be longer than the 2 weeks applied in this study 3) The chromatin epigenetic status (i.e. methylation and acetylation) of the promotor regions of the target genes of our investigated TFs may have not been optimal and may have inhibited differentiation [55]. 4) Protocols of isolation and culturing and other in vitro conditions of CPCs have affected their ability to differentiate. 5) CPCs used in the study are not clonally-expanded cells and thus may constitute a heterogeneous population of primitive and more committed progenitors [56, 57], with varying potentials of differentiation. 6) The cells used in this study are derived from relatively aged patients and thus may have lost their full differentiation potential. 7) Complete differentiation of CPCs may, in fact, never be achieved ex vivo due to a loss of one or more of cardiac tissue micro-environmental cues or because the surrounding milieu in vitro used in our experiments does not recapitulate the physiological cardiac tissue.

Nonetheless, despite the insufficiency of Gata4 to induce complete differentiation of CPCs, partial differentiation of CPCs may be all that is required to achieve a superior therapeutic effect for MI. It is possible that some Gata4-expressing CPCs may reach complete differentiation upon injection in the differentiation-conducive physiological environment of the mammalian heart. In support of that, Qian et al. have shown that TF-mediated reprogramming of
cardiac fibroblasts into cardiomyocytes is easier in the native cardiac tissue as compared to cells grown in culture [58].

In conclusion, overexpressing Gata4 alone or in the presence of differentiation medium and dexamethasone robustly upregulated markers of cardiomyocytes, smooth muscle cells, and fibroblasts, suggesting a pivotal role of Gata4 in facilitating CPC differentiation. Unexpectedly, however, BAF60C, MEF2C, NKX2.5, and TBX5 did not significantly synergize or facilitate Gata4 effects, underscoring the complexity of the interactions and effects of cardiac TFs.
CHAPTER III
PROMOTING SURVIVAL OF HUMAN C-KIT+ CPCS

Poor survival of cells engrafted in the heart is one of the major hurdles that slow the progress of heart cell-based therapies [21, 22]. That rapid cell loss does not allow implanted cells to remain long in the diseased cardiac tissue, and may thus limit their therapeutic benefit. Unfortunately, the exact cause of cell death is not well-defined although it is likely to be due to the ischemia within the infarcted heart, the host inflammatory response, and/or anoikis [34].

Ischemia in the infarcted heart has a deleterious effect not only on endogenous cardiac cells but also on cells introduced to the heart for therapeutic purposes. In addition to the ischemia-mediated oxygen and nutrient deprivation [34], ischemia generates detrimental high levels of reactive oxygen species (ROS) [59-61]. Indeed, Kolamunne et al. have shown that hypoxia produces high amounts of mitochondrial superoxide, which mediate the toxicity observed in cardiac progenitors [62]. Produced ROS could be damaging to stem cells in multiple ways. For instance, high levels of ROS injure cell membranes and increase the permeability of the mitochondrial membrane, leading to impairment of intracellular Ca\(^{2+}\) homeostasis [63]. ROS also transduce the deleterious effects caused by inflammatory cytokines [tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), IL-1\(\beta\) and IL-
through a TNF-α receptor/caspase pathway [63]. ROS can even impair other crucial stem cell properties such as migration and differentiation [64].

One interesting molecule that may be utilized to promote cell viability in the presence of high oxidative stress is Nrf2. Nrf2 is a TF that efficiently combats oxidative stress and may thus be utilized to enhance stem cell survival [65]. When activated, Nrf2 upregulates the transcription of various genes involved in cellular protection against oxidative stress, including heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (Nqo1), sulfiredoxin 1 (SRXN1), peroxiredoxin, epoxide hydrolase, peroxidases, and glutathione- (GSH)- synthesizing enzymes [66, 67]. Nrf2 also exerts additional beneficial effects by upregulating production of prosurvival cytokines and by moderating inflammation and immune response [68-70]. The significance of Nrf2 and its prosurvival role have been shown in multiple systems. For instance, preconditioning astrocytes by adding sulforaphane (a Nrf2 stabilizing isothiocyanate abundant in broccoli) upregulated the expression of the reactive quinone quencher Nqo1 and conferred protection against oxidative stress [71]. Another study showed that neurons that lack Nrf2 activity in knock-out mice were more susceptible to oxidative stress as compared to the wild type counterparts (Nrf2<sup>+/−</sup>) [72]. When Nrf2 activity was restored in the Nrf2<sup>−/−</sup> neurons by overexpressing the encoding gene, the neurons regained the ability to resist oxidative stress. Consistent with that, activating Nrf2 chemically or via adenovirus-mediated Nrf2 delivery protected neural stem cells from oxidative stress [64]. In the context of human hematopoietic stem progenitor cells, Nrf2 was shown to abrogate oxidative
stress-induced toxicity and to upregulate production of prosurvival cytokines such as BCL2A1 and IL-10 [68]. These studies prompted us to hypothesize that Nrf2 activation in CPCs is a good strategy to help promote survival of CPCs under high oxidative stress conditions \textit{in vitro}. The ultimate long-term goal is to obtain genetically-modified cells that can resist the oxidative stress within the infarcted heart \textit{in vivo}.

To enhance the activity of Nrf2 in CPCs, it is vital to understand how Nrf2 is regulated under both physiological and stressed conditions. Under normal conditions, Nrf2 is sequestered in the cytoplasm by binding to the actin-tethered Kelch like-ECH-associated protein 1 (Keap1) [73]. Keap1 recruits Cullin 3 (Cul3), a subunit of the E3 ligase complex, by interacting with its N-terminal region. Recruited Cul3, in turn, marks Nrf2 for proteosomal degradation and thus prevents Nrf2 translocation and subsequent activation into the nucleus [74]. In contrast, under stressful conditions, ROS and electrophiles disrupt critical cysteine residues (including Cys 151) within Keap1, which abolishes the interaction between Keap1 and Nrf2. This allows Nrf2 to freely translocate into the nucleus and bind to antioxidant response element (ARE) sites at the upstream promoters of Nrf2 target genes [75]. To bind to ARE, Nrf2 needs first to heterodimerize with its binding partner (small maf protein). DNA binding initiates transcription of several antioxidant genes [76]. In attempt to promote Nrf2 activity in CPCs, our lab generated a constitutively active form of \textit{Nrf2} (caNrf2) by deleting 88 amino acids at the N-terminus. The mutation stabilizes Nrf2 by abolishing the interaction of Nrf2 with Keap1, and thus activates Nrf2-mediated
antioxidant pathways [73]. Next, caNrf2 was expressed in CPCs and its ability to promote survival was assessed in vitro.
MATERIALS AND METHODS

Isolation and culture of c-kit+/lin- CPCs

See “methods and materials” section in Chapter II.

Immunofluorescence staining and Western blot analysis

See “methods and materials” section in Chapter II. Nrf2 antibody (rabbit polyclonal; GTX61763) was purchased from GeneTex.

Production of caNrf2 lentivirus and lentivirus-mediated transduction

Lentivirus expressing caNrf2 used in the current study was produced using ViraPowerTM Lentiviral Expression System (Invitrogen) according to manufacturer’s instructions. The pcDNA3-Myc3-Nrf2 (Addgene plasmid 21555) which contains the full-length human Nrf2 coding sequences was purchased from Addgene (www.addgene.org). caNrf2 was generated in our lab by deleting 88 amino acids at the N-terminus of Nrf2. That mutation is at the locus of Nrf2 responsible for Keap1 binding, leading to constitutive activation of caNrf2. The coding sequences for caNrf2 or mCherry were PCR-amplified using Pfu HF polymerase (Agilent) and subcloned into pLenti6/V5-D-TOPO vector (Invitrogen) according to the manufacturer’s instructions. Primers used for the PCR were the following: 5’-GTTTTTCTTAACATCTGGCTTCTTACTTTTG-3’ and 5’-CACCATGCAGCACATCCAGTCAGAAACCA-3’. For generation of pLenti6-mCherry expression construct, pmCherry-C2 vector (K. U. Hong) was used as the PCR template. For generation of 3xFLAG-caNrf2, the following oligos were
synthesized, annealed and inserted into the BamHI site of pLenti6/V5-TOPO vector:

\[
\text{5'}-\text{GATCGACCATTACAGGATGACGACGATAAGGATTACAGGATGACGACGATAAGGATTACAAGGATGACGACGATAAGG-3'} \quad \text{and} \quad \text{5'}-\text{GATCCCTTATCGTCGTCATCCTTGTAATCCTTATCGTCGTCATCCTTGTAATCCTTATCGTCGTCATCCTTGTAATCCATGGTC-3'}. 
\]

Each batch of virus was concentrated 10 times using Lenti-X Concentrator (Clontech) according to the manufacturer's instructions and resuspended in complete CPC media. Aliquots were made and stored at -80°C until use.

**Inducing oxidative stress in CPCs**

CPCs cultured in 6-well plates and supplemented with regular complete medium were transduced with mCherry- (control) or caNrf2-expressing lentiviruses. Transduced cells were cultured with medium change every 3 days for a total of 6 days to allow expression and activation of the inserted gene. The cells were then washed with PBS, trypsinized and counted using a hemocytometer. Subsequently, \(1.0 \times 10^4\) CPCs were plated per well of a 96 well plate. The cells were then exposed to oxidative stress-inducers such as 2, 3-dimethoxy-1, 4-naphthoquinone (DMNQ) or hydrogen peroxide (\(\text{H}_2\text{O}_2\)). DMNQ was used at either a concentration of 25 \(\mu\text{M}\) in regular complete medium or at a concentration of 12 \(\mu\text{M}\) in serum-free medium for a period of 4-5 days. \(\text{H}_2\text{O}_2\) was applied at a 1.5 mM concentration for a total of 6 hours. Both DMNQ and \(\text{H}_2\text{O}_2\) were freshly prepared from stock solutions prior to each experiment. Following
oxidative-stress exposure, viability assays were performed to determine the number of surviving cells at serial time points.

**PrestoBlue assay**

PrestoBlue (Invitrogen) cell assay was used to assess cell viability on $1.0 \times 10^4$ CPCs that were plated on a 96-well plate. PrestoBlue solution contains resazurin, which is a blue non-fluorescent substance that enters viable cells and then gets converted by the reducing intracellular environment into a red & fluorescent metabolite. Cell viability can thus be estimated by measuring absorbance or fluorescence. The PrestoBlue assay was performed according to manufacturer’s instructions. Briefly, the 10x reagent was diluted in the culturing medium to make a 1x reagent solution. At the time of analysis, culturing medium was replaced with the freshly prepared 1x PrestoBlue solution and incubated at 37°C for an hour. The viability was then assessed by reading the fluorescence at Ex/Em 560/590 nm. All experiments were done in quadruplicates.
RESULTS

**Overexpressing caNrf2 in CPCs**

Previous studies have shown that Nrf2 activation has conferred protection to multiple cell types exposed to high levels of oxidative stress [64, 68]. Thus, we hypothesized that Nrf2 activation by forced expression of the encoded gene will promote CPC survivability. To that end, FLAG-tagged caNrf2 was overexpressed in CPCs using a lentivirus delivery system. Western blot verified successful caNrf2 expression in CPCs and immunocytochemistry showed 70-90% transduction efficiency for both mCherry- and caNrf2-viruses (Fig. 8).

**caNrf2 induces multiple antioxidant genes in CPCs**

Following the successful expression of the caNrf2 construct, the effect of caNrf2 gene transfer on the expression of antioxidant genes was investigated. CPCs were transduced with mCherry- (control) or caNrf2-expressing lentiviruses. Six days post transduction, the gene expression of selected Nrf2 target genes were examined at the mRNA and protein levels using qRT-PCR and Western blot. Indeed, transduction of CPCs with caNrf2-lentivirus significantly increased the expression of Nrf2 target genes, most notably **HO-1** (Fig. 9). **HO-1** is a stress-inducible gene whose expression is upregulated in response not only to ROS but also to a large number of other internal and external factors that cause cellular stress [77]. In addition to **HO-1** induction, caNrf2 caused a significant upregulation of other Nrf2 target gene transcripts including NAD(P)H quinone
Figure 8. Lentivirus-mediated delivery of caNrf2 into CPCs. A, Western blot images confirming caNrf2 protein expression in CPCs compared to mCherry-transduced cells at day 5 post-transduction. GAPDH was used as a loading control. B, Immunostaining images showing level of forced expression in CPCs transduced with mCherry- (left panel) or 3xFLAG-tagged caNrf2-virus (right panel). DAPI images are shown in lower panels.
Figure 9. *caNrf2* overexpression in CPCs upregulates Nrf2 target genes. A, mCherry and *caNrf2* were overexpressed in CPCs via a lentivirus-based delivery system. At day 6 post-transduction, cells were harvested, and relative changes in mRNA transcripts of the indicated Nrf2 target genes were measured using qRT-PCR. The level of each transcript was compared to that of mCherry-expressing control group, and is expressed as a relative fold change. B, Western blot images confirming *caNrf2*-induced upregulation of HO-1 and SRXN1 at the protein level in CPCs compared to mCherry-transduced cells. For each group in A, n = 4. Bar graphs show mean ± SEM. *, p < 0.05.
oxidoreductase 1 (NQO1) [78], glutamate-cystein ligase catalytic (GCL-C), glutamate-cystein ligase modifier (GCL-M), GSH reductase (GSR), and SRXN1 (See Fig. 9A). Western blot analyses were also performed and corroborated the induction of HO-1 and SRXN1 at the protein level (Fig. 9B). These observations support that overexpressed caNrf2 bound to the promoter regions of target genes and upregulated the transcription of Nrf2 target genes.

**caNrf2 overexpression protects CPCs against oxidative stress**

After demonstrating the ability of caNrf2 to upregulate antioxidant genes, the ability of caNrf2 overexpression to protect CPCs from oxidative stress conditions was examined. To that end, 1.0 x 10^4 mCherry- and caNrf2-overexpressing CPCs were plated onto a 96-well plate and then cultured using regular 10% FBS-containing F12 medium. Next day, medium was replaced with regular F12 medium containing the redox cycling agent DMNQ at a concentration of 25 \( \mu \)M to induce oxidative stress before assessing cell viability. Indeed, significant protection was observed in caNrf2-overexpressing cells at 24 and 48 hours post-DMNQ treatment, as assessed by the Prestoblue cell viability assay (Fig. 10A). Next, caNrf2-expressing CPCs were subjected to a combination of DMNQ and serum-deprivation. To achieve that, regular CPC medium was replaced with serum-free F12 medium containing 12 \( \mu \)M DMNQ. caNrf2 overexpression, protected CPCs that are exposed to DMNQ and serum starvation at both 48 and 72 hours following DMNQ treatment (Fig. 10B), confirming the protective role of caNrf2 overexpression in CPCs.
Figure 10. *caNrf2* overexpression protects CPCs against oxidative stress.

A, CPCs transduced with mCherry- or *caNrf2*-encoding lentivirus were subjected to 25 μM DMNQ in regular CPC complete medium (A) or to 12 μM DMNQ in serum-free medium (B). PrestoBlue viability assay was performed at the indicated time points. For each group, n = 4. Bar graphs show mean ± SEM. *, p < 0.05.
To monitor viability of caNrf2-overexpressing CPCs at multiple time points, rather than taking a snap shot at 1 or 2 time points, a time-course analysis was performed by assessing viability at regular intervals. Twenty four hours after plating cells, 25 μM DMNQ was freshly added to the culturing medium and was renewed daily. Prestoblue assay was performed every 12 hours. Our results show that caNrf2 overexpression resulted in a significant protection in caNrf2-expressing CPCs starting at 24 hours and up to 60 hours post DMNQ treatment (Fig. 11A). Following that, the ability of caNrf2 to promote cell survival of CPCs was assessed upon exposure to a different source of oxidative stress. This time, caNrf2-expressing cells were incubated with 1.5 mM H2O2 prior to the cell viability assessment. As expected, caNrf2 gene delivery improved cell resistance to H2O2-induced toxicity at the time points analyzed (1, 2, 3, 4, 5 and 6 hours following H2O2 treatment) (Fig. 11B). Taken together, these data show a significant survival advantage of caNrf2-expressing CPCs under oxidative stress. Finally, to exclude any caNrf2-mediated impact on proliferation, mCherry- and caNrf2-overexpressing CPCs were seeded at a low density and proliferation was monitored at 12-hour intervals. Our data show that caNrf2-expressing CPCs proliferated in a manner comparable to mCherry-transduced cells (Fig. 12).
Figure 11. **Time-course cell viability analysis for caNrf2- and mCherry-overexpressing CPCs under oxidative stress.** CPCs transduced with mCherry- or caNrf2-encoding lentivirus, were subjected to 25 μM DMNQ (upper panel) or to 1.5 mM H2O2 (lower panel). PrestoBlue viability assay were performed at the indicated time points. The data indicate that caNrf2 overexpression protects CPCs from oxidative stress. For each time point, n = 4. Data are shown as mean ± SEM. *, p < 0.05.
Figure 12. **Effect of caNrf2 overexpression on CPC proliferation under normal conditions.** Five thousand CPCs transduced with mCherry- or caNrf2-encoding lentivirus were plated in a 96-well plate in regular complete medium. To assess proliferation, relative cell number at 12 hour intervals for 4 days was determined using the Prestoblu...
Discussion

It is accepted that the mammalian myocardium has very limited regenerative capacity after injury [79]. Thus, introducing exogenous cells that have the ability to regenerate or repair the heart constitutes a conceivable approach to treat MI-induced heart failure. To that end, several cell types have already been investigated, including cardiomyocytes [80], skeletal myoblasts [81], MSCs, [29] CPCs [9] or even smooth muscle cells [82] and fibroblasts [83]. Among these cell types, cardiomyocytes are theoretically the ideal candidate to replace lost cardiomyocytes. However, providing a reliable source of human cardiomyocytes remains unresolved. Thus, stem cells which have the ability to proliferate robustly and generate functional progeny provide an alternative to cardiomyocytes for heart cell replacement therapies.

Among stem cells that can be utilized for cardiac repair, CPCs offer a number of advantages. Unlike other types of stem cells, findings from independent laboratories have validated the ability of CPCs to generate not only cardiomyocytes, but also other cell types present in the cardiac tissue such as smooth muscle cells and endothelial cells [9, 35, 84]. Also, use of CPCs have not been associated with adverse side effects or oncogenic transformations, conferring them a significant safety advantage over the use of embryonic stem cells and induced pluripotent stem cells [85]. Indeed, these encouraging results observed with CPC grafting in animal models were conducive to the initiation of a phase I clinical trial, which corroborated both the safety and the therapeutic potential of CPCs [19].
Nevertheless, one of the remaining challenges for CPC-based therapy is that the majority of infused cells do not survive in the host tissue. For instance, our group has previously shown that ~99% of transplanted CPCs were undetected in the heart at 35 days post implantation [21, 22]. Thus, strategies that can enhance CPC survival after adoptive transfer are needed. To that end, a number of strategies have been utilized, including exposure to hypoxic preconditioning [86], chemical activation of HO-1 [36], delivery of growth factors genes [87], prosurvival genes and heat shock treatment [34]. In this study, we demonstrate for the first time in CPCs, that caNrf2 overexpression enhances the CPC resistance to oxidative stress. Our data show that caNrf2 protected CPCs against the redox cyler DMNQ with and without serum deprivation (Fig. 10 and 11). Furthermore, caNrf2 protected CPCs against another oxidative stress inducer, H$_2$O$_2$ (Fig. 11).

In line with our findings, previous studies have shown the beneficial effect of Nrf2 activation. For instance, sulforaphane (a Nrf2 activator) has been shown to upregulate Nqo1 and to protect astrocytes from oxidative stress [71]. Also, Nrf2 activation through selective deletion of Keap1 in Clara cells in the mouse lung upregulated the expression of Nqo1 and Gcl-m, and that was accompanied by a protection against oxidative stress ex vivo and boosted the resistance of lungs against cigarette smoke-induced inflammation [88]. In intact animals, boosting Nrf2 activity with pharmacological agents was also protective against oxidative damage [89]. More importantly, Nrf2 has also been shown to play a significant role in stem cell survival and function. For instance, hematopoietic
stem progenitor cells (HPSCs) from Nrf2-knockout mice had significantly higher rates of spontaneous apoptosis and had lower survival rates when exposed to oxidative stress [68]. The study also showed that Nrf2 is indispensable for myeloid development and stem cell function of HPSCs. Collectively, these lines of evidence, along with our findings, support a beneficial outcome of Nrf2 activation, which may be utilized to advance cell-based cardiac repair.

It is possible that caNrf2 overexpression in CPCs may affect their proliferation. For instance, Schafer et al. found that overexpressing caNrf2 in mice had a negative effect on keratinocyte proliferation [90]. In the present study, however, caNrf2 overexpression had no effect on the proliferation rate of CPCs under normal unstressed conditions (Fig. 12). These data suggest that caNrf2-induced effect on proliferation is cell-specific and that caNrf2-overexpressing CPCs retain their normal proliferative potential. This also indicates that the role caNrf2 plays in CPCs is protecting them against oxidative stress, rather than promoting their proliferation.

The cytoprotective effects of Nrf2 activation is thought to be mediated through a battery of antioxidant genes such as GCL, GSR, NQO1, HO-1, and SRXN1. These proteins play important roles in cellular responses to oxidative stress and thus help protect cells from oxidative stress [66]. Consistent with that, our findings show that caNrf2 overexpression in CPCs was associated with transcriptional induction of several Nrf2 target genes including GCL-C, GCL-M, GSR, NQO1, HO-1, and SRXN1 (Fig. 9). The qRT-PCR analysis revealed that the synthesizing components of GSH: GCL-C and GCL-M were significantly
upregulated (Fig. 9). GCL mediates the rate limiting step of GSH synthesis. This step involves the ATP-dependent condensation of cysteine and glutamate to form the dipeptide gamma-glutamylcysteine (γ-GC) [91]. Structurally, GCL is comprised of a 73 kDa heavy catalytic subunit (GCL-c) and a 31 kDa light modifier subunit (GCL-m) and genes encoding the two subunits have been shown to contain ARE sequences at their promoters [91]. Several studies have shown the transcriptional and functional association between Nrf2 and GSH, and the role of GSH in combating oxidative stress. For instance, Nrf2-knockout mice have been shown to express lower levels of GSH [92]. Also, N-acetyl L-cysteine (NAC), a precursor of GSH, was effective in protecting cells against Fas-mediated toxicity [93]. In addition to GCL, the GSH-replenishing enzyme GSR is also needed for GSH homeostasis and cellular activity. When GSH is oxidized, GSR is responsible for reducing the GSH oxidized form (GSSG) to the sulfhydryl form (GSH), to maintain a healthy reducing environment within the cell [94, 95]. Similar to what was observed with the GCL subunits, our data show a significant induction of GSR mRNA levels in caNrf2-overexpressing CPCs (Fig. 9).

In addition to the transcriptional elevation of enzymes that restore GSH levels, caNrf2 induced transcription of other Nrf2 target genes, most notably HO-1 and SRXN1 (Fig. 9). HO-1 is the inducible form of HO and is transcriptionally regulated by Nrf2 whereas HO-2 is the constitutive form. Both HO-1 and HO-2 mediate heme (pro-oxidant) breakdown, to produce biliverdin (an antioxidant that can be converted to bilirubin by biliverdin reductase) and carbon monoxide (an antioxidant exclusively synthesized by HO-1) [96, 97]. Previous studies have
shown the role of Nrf2 in upregulating *HO-1* transcripts and the importance of the latter in ameliorating ROS damage. For instance, conditional expression of a *Nrf2* dominant-negative mutant was accompanied by 85-95% reduction in the *HO-1* transcript in response to heme, cadmium, zinc, arsenite, and tert-butylhydroquinone [98], supporting that *HO-1* is one of the principal target genes of Nrf2. In addition, chemical activation of HO-1 through cobalt protoporphyrin was beneficial to CPCs by promoting ERK/Nrf2 signaling, and conferring protection against oxidative stress [36]. In accordance with that, our results show ~30 fold induction in the *HO-1* mRNA transcript in *caNrf2*-expressing CPCs (Fig. 9), corroborating the association between Nrf2 activation and *HO-1* expression.

Another target gene of Nrf2-activation is *SRXN1* which is believed to lower oxidative stress by re-activating peroxiredoxins (a family of peroxidases) that are inhibited by over-oxidation [99]. Our qRT-PCR revealed that *caNrf2*-expressing CPCs had a ~20 fold higher transcript level of *SRXN1* (Fig. 9). Although not tested, it is possible that that both HO-1 and SRXN1 play major roles in mediating caNrf2 protective effect in CPCs.

Like other Nrf2 target genes, mutational studies have identified the presence of ARE sequence in the promotor region of *NQO1* [100]. *NQO1* is a prototypical Nrf2 target gene that catalyzes the reduction and detoxification of highly reactive redox cycling quinones that can cause cellular damage [76]. Several studies have shown that Nrf2 activation promotes *Nqo1* expression, which in turn help promote cell survival. For instance, Nrf2 activation by *Keap1* selective deletion in the mouse lung was associated with increased levels of
Nqo1 [88]. When a pharmacological inhibitor of NQO1 was added, the cells were sensitized to Fas ligand-induced apoptosis [93], indicating a prosurvival role of NQO1. In line with these studies, there was a small but significant induction of NQO1 mRNA levels in caNrf2-overexpressing CPCs (Fig. 9).

Although this study did not look at the mechanisms by which caNrf2 promotes CPC viability under oxidative stress conditions, Nrf2 has been reported to possess multiple beneficial effects. 1) Nrf2 directly decreases oxidative stress by influencing homeostasis of ROS and reactive nitrogen species (RNS) [67]. For instance, Nrf2 promotes catabolism of superoxide and peroxides by increasing the cellular levels of superoxide dismutase (SOD), GSH peroxidase and peroxiredoxin. Nrf2 also promotes synthesis of reducing factors such as GSH and NADPH, and replenishes oxidized cofactors and proteins (including oxidized GSH) by upregulating the transcription of specific reductases [67, 101]. 2) Nrf2 activation has been shown to inhibit apoptosis [101, 102] although the precise mechanisms by which this occurs remain to be elucidated. Indeed, when Nrf2 was inhibited in HeLa cells by expressing an antisense Nrf2 cDNA or a membrane permeable dominant-negative polypeptide, the cells were sensitized to Fas-induced apoptosis and that was rescued by Nrf2 overexpression [93]. 3) Nrf2 induces the expression of a class of proteosomal proteins, and thus reduces protein aggregation that is detrimental to cells [103]. In support of that, sulforaphane protected murine neuroblastoma cells from amyloid beta (Aβ) aggregation-induced toxicity [104]. 4) Studies have revealed a beneficial anti-inflammatory effect for Nrf2. For instance, Nrf2 can attenuate NFkappaB-
inflammatory response *in vitro* and *in vivo* [105]. Also, inducing expression of *Nrf2* in microglia by using the phenolic antioxidant tBHQ, prevented LPS-induced microglial hyperactivation and attenuated overproduction of pro-inflammatory neurotoxic mediators like TNF-α, IL-1β, IL-6, prostaglandin E2 (PGE2), and nitric oxide (NO) [106]. Based on these reports, we speculate that in addition to caNrf2 ability to protect CPCs from oxidative stress, it may confer additional benefits to CPC survival by one or more of the following mechanisms: inhibiting apoptosis, reducing protein aggregation and/or reducing inflammatory mediators.

In summary, our data show that caNrf2 can protect CPCs against oxidative stress conditions *in vitro* with no apparent impact on cell proliferation. However, despite these promising results observed with *caNrf2*-expressing CPCs, it is important to be cautious when interpreting these data since all the experiments in this work were performed only under *in vitro* conditions. Therefore, whether *caNrf2*-overexpression in CPCs would be protective *in vivo* needs further investigation. It would be particularly interesting to test the prosurvival role of caNrf2 in cells upon implantation in an animal model of MI. If proved effective, *caNrf2* gene delivery may have significant implications for MI-induced heart failure.
CHAPTER IV
SUMMARY

MI-induced heart failure presents a major challenge because none of the existing therapies are capable of reversing myocardial death. Fortunately, newer approaches including CPC-based therapies are promising venues because they offer a potential to modify the underlying pathophysiology, either by replacing dead cells and/or by protecting the remaining ones. Indeed, this relatively new CPC field is advancing fast and is achieving encouraging results, albeit with a few unresolved concerns.

Among these concerns, poor survivability of transplants and their inability to generate functional cardiac cell types, hinder further advancement of the cell-based heart repair. It is thus imperative that more investigations be carried out to understand and resolve these issues. The present work focused on genetically engineering CPCs to facilitate their differentiation into committed cardiac lineages and to confer cells better protection against oxidative stress. The data indicate that Gata4 and caNrf2 gene delivery have the potential to address CPC poor differentiation and survival, respectively. Gata4 overexpression was able to direct the differentiation of CPCs into 3 out of the 4 main cardiac cell lineages (cardiomyocytes, smooth muscle cells and fibroblasts). Combining a differentiation medium (5% FBS-containing DMEM) and dexamethasone with
Gata4 overexpression shifted the differentiation into only the cardiomyocyte lineage. Determining the therapeutic advantage, if any, of CPCs committed to multiple cardiac cell lineages, versus those committed to cardiomyocytes only, need further investigation. With regard to promoting CPC survival, overexpressing caNrf2, which has the ability to activate several antioxidants and phase II detoxifying enzymes, protected CPCs against multiple oxidative stress conditions in vitro with no impact on cell proliferation.

Because the experiments performed in this work were performed under in vitro conditions, it would be interesting to confirm these findings by assessing whether Gata4-, caNrf2- or Gata4-caNrf2-overexpressing CPCs are therapeutically superior to naïve CPCs in an appropriate animal model of MI. Such studies need to analyze not only the differentiation and survivability of implanted cells in the infarcted heart, but also the ability of cells to result in improved cardiac function. Success of these genetically-engineered CPCs in vivo may have implications not only for ischemic cardiomyopathy but also in other diseases that may benefit from cell-based therapies.
REFERENCES


CURRICULUM VITAE

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EDUCATION AND TRAINING

2012-2015 Ph.D. in Pharmacology & Toxicology (GPA = 3.94/4)
University of Louisville, KY, USA
Dissertation “Genetic engineering of cardiac stem cells ex vivo to enhance their regenerative potential in vivo”

2010-2012 MSc. In Pharmacology & Toxicology (GPA = 3.93/4)
University of Louisville, KY, USA
Thesis “Therapeutic approaches in the treatment of Parkinson’s disease: A focus on stem cell-based therapies”

1997-2002 BSc. in Pharmacy (Rating = distinction)
Aleppo University, Aleppo, Syria
Graduation project “Components of the immune system”

TEACHING EXPERIENCE
Jan 2006-May 2010
Pharmacology lecturer
Several Medical Universities and community colleges:
- The National University (Al-Wataneya), Sana’a, Yemen
- Al-Rowad College, Sana’a, Yemen
- Higher Institute for Pharmacy, Sana’a, Yemen
- Al-Alamia Institute for Medical Training, Sana’a, Yemen
- International Medical Institute for Health Science, Sana’a, Yemen
- Al-Wihda Institute and College, Sana’a, Yemen
- Al-Jazeera Medical Institute, Sana’a, Yemen
- Ibn Seena Medical Institute, Sana’a, Yemen
- Al-Razi Medical Institute, Sana’a, Yemen
Taught junior and senior “pharmacy” students several pharmacology courses covering Principles of Pharmacology (e.g. mechanisms of drug action, drug pharmacokinetics & pharmacodynamics)

Taught “pharmacology” of agents involved in cardiovascular, nervous, endocrine, gastrointestinal systems

Taught “Pharmacology” of chemotherapeutics including antibiotics, antifungals, antivirals and anti-cancer agents.

Taught “phytochemistry” classes covering active ingredients of medicinal plants such as alkaloids, terpinoids, flavonoids, tannins and cardiac glycosides.

Taught “Medical Terminology” class for over 500 1st-year Pharmacy students.

Nov 2005 - May 2010
Teaching Assistant (Demonstrator)
Department of Pharmacognosy, School of Medicine
University of Sana’a, Yemen

Taught the curriculum of applied lab pharmacognosy for ~150 3rd-year Pharmacy students for 4 consecutive years.

Taught the curriculum of applied lab Phytochemistry for ~120 4th-year junior Pharmacy students for 4 consecutive years.

Supervised the Pharmacognosy class lab sessions that focused on microscopic analysis of miscellaneous medicinal plant parts for 4 years.

Conducted & assisted the Phytochemistry class applied lab sessions that focused on isolation, separation and detection of active ingredients of medicinal plants.

Assisted professors of the department in experimenting and characterizing medicinal plants.

RESEARCH EXPERIENCE

Aug 2012 - present
Graduate Research Assistant (PhD level)
Department of Pharmacology & Toxicology,
University of Louisville, USA
(Mentor: Dr. Kyung Hong)

Genetically-engineered human cardiac stem cells (hCSCs) to promote their regenerative potential in heart repair following myocardial infarction.
• Introduced cardiogenic transcription factors’ genes (Gata4, Nkx2.5, Mef2c, Tbx5, Baf60c) into hCSCs using lentivirus systems for the purpose of facilitating hCSCs differentiation in vitro
• Overexpressed prosurvival protein genes (telomerase & Nrf2) into hCSCs to enhance the survival of hCSCs under oxidative stress conditions.
• Investigated the role of epigenetic modulators such as the histone deacetylase inhibitor “trichostatin A” and the DNA methyltransferase inhibitor “5-azacytidine” in driving the differentiation of hCSCs.
• Designed protocols for cell culture, mRNA and protein profiling
• Troubleshooted and solved issues related to laboratory procedures & experiments
• Worked in a team and communicated effectively with lab members & collaborators
• Critically reviewed other lab members’ work

Aug 2010 – May 2012
Graduate Student (MSc level)
Department of Pharmacology & Toxicology, University of Louisville, USA
(Mentor: Dr. Uma Sankar)

• Investigated through a systematic library-based research the therapeutic options available for Parkinson’s disease patients. These treatments included the use of pharmacological agents, soluble growth factors, and surgical interventions. More focus was applied on the use of different types of stem cells (SCs) including embryonic SCs, induced pluripotent SCs, neural SCs and mesenchymal SCs.

AWARDS AND HONORS

Apr 2014 2nd Best Poster Presentation at University of Louisville 6th Annual Graduate Research Symposium, Louisville, KY, USA
Oct 2013 “BCVS Abstract Travel Grant” award to the American Heart Association (AHA) 2013 Scientific Sessions, Dallas, TX, USA
Aug 2013 International Academy of Cardiovascular Sciences Biomedical Award for cardiovascular students and fellows, Winnipeg, Canada
Aug 2013 Grant Pierce Biomedical Award recipient for Graduate Students and Postdoctoral Fellows, Univ. of Louisville, KY, USA
2012-2013 Integrated Programs in the Biomedical Sciences (IPIBS) Fellowship, Univ. of Louisville, KY, USA
June 2012 Graduate School Citation for Academic Excellence, Univ. of Louisville, KY, USA
May 2012 Top cumulative GPA in classes at masters graduation (GPA = 3.94/4), Univ. of Louisville, KY, USA
Aug 2011 Spotlight Student of the Month, Univ. of Louisville, KY, USA
2010-2012 Fulbright MSc Scholarship Grantee to Univ. of Louisville in the USA, Funded by the USA government (USA department of State)

Sep 2002 Second Best Cumulative GPA at College Graduation (rating: Distinction), College of Pharmacy, Aleppo Univ., Syria


TECHNICAL SKILLS

- **Cell and Molecular Biology**
  Protein and DNA electrophoresis, cell culture, cytotoxicity assays, ROS assay, immunostaining, cell transfection, gene cloning, real time PCR, western blotting, flow cytometry, confocal microscopy and lentivirus transduction.

- **In Vivo**
  Animal myocardial infarction models

- **Pharmacognosy**
  Harvesting and powdering medicinal parts of plants and analyzing the powders under the microscope.

- **Phytochemistry**
  Extraction techniques for active ingredients of medicinal plants, Chromatography-based methods in isolating active ingredients and chemical methods for identification.

- **Pharmaceutics**
  Designing and quality control tests for most pharmaceutical preparations such as syrups, elixirs, sugar- and film-coated tablets, capsules, suppositories, emulsions, suspensions, creams, ointments, effervescent tablets and granules.

- **Analytical chemistry**
  Acid-base titrations, Redox titration, PH indicators, Spectroscopy, HPLC, liquid and gas chromatography.

- **Biochemistry**
  Collecting blood samples from humans, determining glucose, cholesterol and lipids levels.

NON-ACADEMIC PROFESSIONAL POSITIONS

2009-2010 Facility and Curriculum Inspector
Department of Technical and Health Education, Sana’a, Yemen

- Responsible for Inspecting facilities’ infrastructure of medical, dental, medical assistant education centers, including classrooms, libraries and laboratories

- Reviewed the didactic curriculum and the staff qualifications of medical education centers throughout the country
• Wrote and submitted thorough analytic reports evaluating the inspected institutions to the Department of Technical and Health Education
• Co-approved licensing of newly-constructed medical education centers in Yemen

2002-2006  Medical Representative  
Headquarters in Lundbeck Pharma, Copenhagen, Denmark

• Raised product awareness through one-on-one and group meetings with physicians in hospitals, medical centers and private clinics in Yemen.
• Promoted the company’s sales in drug stores and hospitals by supervising purchases and distributing products brochures.
• Participated actively in organizing introductory seminars for the company’s newly-marketed drugs.
• Supervised company’s sales to big hospitals and drug stores throughout the country
• Managed financial issues regarding stipends, allowances, and other costs
• Aided efficiently in registering and introducing new products to the market
• Carried on business correspondence between the pharmaceutical company’s headquarters in Copenhagen (Denmark) and the importing agent in Sana’a (Yemen)

1996 & 2000-2001  Pharmacist  
Ibn-Hayyan Pharmacy, Sana’a, Yemen (2000 & 2001)  
Al-Sabeen Pharmacy, Sana’a, Yemen (1996)

• Prescribed over the counter (OTC) drugs to patients
• Read and dispensed prescribed medications.
• Explained the dosage and administration instructions to patients.
• Made sure drugs and doses in prescriptions are correct and optimal for patients.

MEMBERSHIP/AFFILIATIONS

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SELECTED WORKSHOPS

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<td>Mar 2012</td>
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<td>Jan 2012</td>
<td>Designing &amp; Presenting Research Posters, KY, USA</td>
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Nov 2011  Writing a Literature Review, KY, USA
Oct 2011  Grant application, KY, USA
Oct 2011  Effective Research Presentation Skills, KY, USA

PUBLICATIONS


ABSTRACTS & NATIONAL MEETING PRESENTATIONS


