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Sulfiredoxin-1 Enhances Cardiac Progenitor Cell Survival against Oxidative Stress via the Upregulation of the ERK/NRF2 Signal Pathway

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Abstract

Cardiac stem/progenitor cells (CPCs) have recently emerged as a potentially transformative regenerative medicine to repair the infarcted heart. However, the limited survival of donor cells is one of the major challenges for CPC therapy. Our recent research effort on preconditioning human CPCs (hCPCs) with cobalt protoporphyrin (CoPP) indicated that sulfiredoxin-1 (SRXN1) is upregulated upon preconditioning aldehyde dehydrogenase bright hCPCs (ALDH^{br}-hCPCs) with CoPP. Further studies demonstrated that overexpressing SRXN1 enhanced the survival capacity for ALDH^{br}-hCPCs. This was associated with the up-regulation of anti-apoptotic factors, including BCL2 and BCL-xL. Meanwhile, overexpressing SRXN1 decreased the ROS generation and mitochondrial membrane potential, concomitant with the up-regulated primary antioxidant systems, such as PRDX1, PRDX3, TXNRD1, Catalase and SOD2. It was also observed that overexpressing SRXN1 increased the migration, proliferation, and cardiac differentiation of ALDH^{br}-hCPCs. Interestingly, SRXN1 activated the ERK/NRF2 cell survival signaling pathway, which may be the underlying mechanism through which overexpressing SRXN1 lead to protection of hCPCs against oxidative stress-induced apoptosis. Taken together, these results provide a

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Disclosure

The authors indicate no potential conflicts of interest.

Author contributions

C.C., X.L.: conception and design, data analysis and interpretation, and manuscript writing and revision; X.L., P.H.: conduct most of experiments; X.W, S.Z.: part of data analysis and interpretation; N.D., E.B.: consenting, and collection of human cardiac tissues.

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rationale for the exploration of SRXN1 as a novel molecular target that can be used to enhance the effectiveness of cardiac stem/progenitor cell therapy for ischemic heart disease.

Graphical Abstract

Keywords

Sulfiredoxin-1; Human ALDH^{br} cardiac stem/progenitor cells; Cell survival; Oxidative stress; ERK/NRF2 signaling pathway

Introduction

Ischemic heart disease, particularly myocardial infarction (MI), is a worldwide health care problem and the leading cause of morbidity and mortality (1). MI results in loss of cardiomyocytes, scar formation, ventricular remodeling, and eventually heart failure (2). Cardiac stem/progenitor cells (CPCs), with the special ability of tissue regeneration, have been widely investigated in the past (3–5). Cell therapy is a very promising option for ischemic heart disease, but severely limited by many obstacles, including limited cell resource, poor donor cell survival, etc. It has been shown that more than 90% of transplanted cardiac stem cells died within a week (4,6–8). Thus, strategies to enhance the effectiveness of CPC therapy would lead to great therapeutic implications for patients with ischemic heart disease.

Recently, a new subpopulation of cardiac progenitor cells with the high enzyme activity of aldehyde dehydrogenase (ALDH^{br}-CPCs) have been well characterized for myocardial repair (9–11). Previous studies have shown that ALDHs play important roles in the maintenance and differentiation of stem cells as well as normal development. ALDHs also exhibit high activity and may serve as markers in cancer stem cells. (12–14). It has been revealed that cardiac-resident progenitor cells expressing high aldehyde dehydrogenase activity, named as ALDH^{br}-CPCs, have the capacity to differentiate into functional cardiomyocytes both *in vitro* and *in vivo* (10). It has also been shown that ALDH^{br}-CPCs preserve cardiac function by extensive engraftment and cardiomyogenic differentiation, indicating the enormous potential of ALDH^{br}-CPCs in myocardial repair in a mini-pig model (9). Recent clinical studies provided evidence that treatment with autologous

ALDH^{br}-CPCs is safe and may provide perfusion and functional benefits in patients with chronic myocardial ischemia (15).

Sulfiredoxin-1 (SRXN1), an endogenous antioxidant protein, belongs to the sulfiredoxin family and plays an important role in various physiological processes, including cell apoptosis, cell proliferation, invasion, and redox balance (16–20). SRXN1 is a part of the thiol-based antioxidant system; it can get preferentially oxidized under oxidative stress conditions and can decrease ROS levels (21,22). It has been suggested that the sulfiredoxin/ peroxiredoxins-IV axis is critical for lung cancer maintenance and metastasis (23). Recently, it was also reported that SRXN1 protected against simulated ischemia/reperfusion injury in H9C2 cells (24). However, it is unknown whether SRXN1 is cytoprotective for human ALDH^{br}-CPCs.

In the present study, we establish for the first time that the survival of human ALDH^{br}-CPCs upon increased oxidative stress is significantly affected by SRXN1 expression based on the gain- and loss-of-function studies. We also provide evidence that the upregulation of both anti-apoptotic and anti-oxidant molecules is associated with the cytoprotective effect of overexpressing SRXN1. In addition, we identified that the activated ERK/NRF2 survival pathway is the potential molecular mechanism associated with the cytoprotective effect of overexpressing SRXN1 in human ALDH^{br}-CPCs. These findings suggest that SRXN1 plays an important role in protecting ALDH^{br}-CPCs against cell death induced by oxidative stress and is a potential therapeutic target for enhancing the effectiveness of cardiac stem/progenitor cell therapy for ischemic heart disease.

Materials and methods

Reagents

Collagenase II was from Worthington Biochemical. Ham's F12 medium was from Invitrogen. Fetal bovine serum (FBS) was obtained from Hyclone. The primary antibodies were listed in the Supplemental Table S1. Quantitative PCR primers for target genes were obtained from Real Time Primers, LLC. Unless indicated otherwise, chemicals used in experiments were purchased from Sigma.

Sort and culture ALDH^{br}-hCPCs

The study was approved by the Institutional Committee on Research Involving Human Subjects in Albany Medical College (IRB#3728), and written informed consent was provided by the patients. All experiments were performed in accordance with relevant guidelines and regulations. ALDH^{br}-hCPCs were sorted from atrial appendages from patients during open-heart surgery at Albany Medical Center. The procedures for isolating ALDH^{br}-hCPCs were exactly followed as described previously (10,11). ALDH^{br}-hCPCs were sorted by LSRII flow cytometry (BD Biosciences) with ALDEFLUOR™ kits (Stem Cell Technologies), and characterized following live cell staining with antibodies against different surface markers (Supplemental Table S2). The experiments for preconditioning ALDH^{br}-hCPCs with CoPP were performed exactly as described previously (25).

Lentiviral product and infection of ALDH^{br}-hCPCs

ORF expression clone for SRXN1 (purified plasmid), empty control vector for pEZ-Lv105, shRNA scrambled control clone for psi-LVRH1GP, and shRNA clone set against human SRXN1 were purchased from GeneCopoeia. The second-generation lentiviral package system (psPAX2, pMD2.G, and target plasmid) was used to package the lentiviral particles at the ratio of 3:1:4 with the HEK293FT cells. Split ALDH^{br}-hCPCs were infected at 50% confluence in a 6-well tissue culture plate in 2ml of F-12 complete media at 12 hours. The proper controls and replicates were included. An infectious viral titer was determined prior to infection in order to calculate the amount of virus to be added. Polybrene was added to the 1mL of virus/media at a final concentration of 10 µg/mL. Finally, 1mL of media containing the virus particles was added to the plated cells. To avoid the viral toxicity, fresh Ham's F-12 completed media was added to replace the infection medium after 12–16 hours of adding viral particle. Cells were used for the following experiments after 48 hours of lentiviral infection.

Cell viability assay

LDH release assay, a simple approach to measure cellular membrane integrity, was applied to determine the oxidative stress-induced cell death in ALDH^{br}-hCPCs. The procedures were followed exactly according to the manufacturer's instructions from Cytotoxicity LDH Detection kit (Takara). The day before lentivirus infection, ALDH^{br}-hCPCs were seeded into 24 well plates with 50% confluence. After 48-hour viral infection, cells were exposed to 2 mM H₂O₂ for 3 hours in serum-free Ham's F-12 medium, for which conditions were optimized based on previous studies (26). The supernatant was collected and centrifuged at 250g for 5 min at 4 °C. 100 µl of supernatant was mixed with an equal volume of pre-made solution (catalyst/dye buffer 1:45) for 30 min protected from light at room temperature. The absorbance of samples at 490 nm was measured using a Bio-Rad iMarkTM microplate reader. The percentage of LDH release for each sample was exhibited by comparing to the absorbance value from cells pretreated with 0.5% Triton X-100.

FACS analysis for apoptotic assay

Cell apoptosis was investigated by dual staining of ALDH^{br}-hCPCs with Alexa Fluor 647-Annexin V and Propidium Iodide (PI), using Annexin V/Dead cell apoptosis kit (Invitrogen). Briefly, ALDH^{br}-hCPCs were plated at 50% confluence in 6-well plate prior to lentivirus infection. After 24 h of infection, ALDH^{br}-hCPCs were challenged with or without 1mM H₂O₂ in serum-free F12 medium for 90 min to induce early and late cell apoptosis; this is a relatively moderate condition compared to the harsh treatment with 2 mM H₂O₂ for 3 hours in the LDH release assay. Cells were then detached by TrypLE solution (Invitrogen), washed twice with PBS, and suspended in 100 µl of 1× annexin-binding buffer supplemented with 1 µg/ml PI and 5 µl of Annexin V, Alexa Fluor 647 conjugate solution (Invitrogen). After incubation at room temperature for 15 min, 400 µl of 1 annexin-binding buffer was added with gentle mixing, and samples were immediately analyzed by the Guava EasyCyteTM System (EMD Millipore Corporation, Inc). Data was analyzed by GuavaSoftTM Module software (EMD Millipore Corporation, Inc). The four quadrants (Q1, Q2, Q3, and Q4) are marked in all FACS analysis. Cells positive for Annexin V only (Q4), or double

positive for both Annexin V and PI (Q2) were defined as apoptotic cells (Q2+Q4); cells positive for PI only (Q1) were defined as necrotic cells; and cells negative for both Annexin V and PI (Q3) were defined as live cells.

Measurement of ROS and mitochondrial membrane potential (Ψ_m)

Intracellular ROS level was measured using a Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, ab113851) uses the cell permeant reagent 2',7'-dichlorofluorescein diacetate (DCFDA, also known as H2DCFDA), a fluorogenic dye that measures hydroxyl, peroxy and other reactive oxygen species (ROS) activity within the cell. Procedures were followed exactly according to the manufacturer's instructions. The intensity of DCF-DA fluorescence was detected by Guava EasyCyte™ System. Alternatively, the production of mitochondrial ROS was analyzed by labeling cells with dihydrorhodamine 123 (DHR123) (Sigma) described previously (27,28). Briefly, ALDH^{br}-hCPCs were seed in a 6-well plate with a confluence of 50%, and infected with lentivirus expressing SRXN1 or the control virus. 48 hours after lentivirus infection, cells were detached with 0.25% Trypsin-EDTA, and ROS were induced by 500μM TBHP for 1 hour at 37°C in the cell culture incubator. Cells were washed with PBS once, and suspended with 100μl assay buffer containing 100μM DHR123 Dye, and incubate at 37°C for 15 min. The intensity of green fluorescence for the DHR123 Dye was examined by Guava EasyCyte™ System. For the measurement of mitochondrial membrane potential, we followed the protocol described previously (29). The basal level of mitochondrial membrane potential was evaluated by incubating cells with potentiometric dye tetramethyl rhodamine methylester (TMRM; Sigma) at final concentration of 25 nM for 30min at 37 °C. The intensity of TMRM fluorescence was measured by Guava EasyCyte™ System.

RNA isolation and quantitative real time PCR

RNA isolation and quantitative real time PCR were performed to determine the change of oxidative stress associated genes, cardiac transcriptional genes and cardiac marker genes when overexpressing SRXN1 in ALDH^{br}-hCPCs. The total RNA of each sample was extracted and purified using Aurum™ Total RNA Mini Kit (Bio-Rad). The quality and quantity of RNA were detected by a NanoDrop 2000C spectrophotometer (Thermo Scientific). The reverse transcription of 0.5 μg of RNA to cDNA was established using Bio-Rad iScript™ cDNA synthesis Kit. Samples for Real-time PCR were prepared according to the manufacturer' instructions of the iQ SYBR Green Supermix kit (Bio-Rad), and the real-time PCR was run with a Bio-Rad CFX384 Touch™ Real-Time PCR detection system. For all qPCR data, GAPDH was used as internal control gene for quantitative analysis. Human ROS primer Library was purchased from Realtimeprimers.com (Supplemental Table S3). Primers for the other genes are listed in Supplemental Table S4.

Western Blotting

Western blotting analysis was performed according to a protocol as described previously (25). The primary antibodies were listed in the Supplemental Table S1. The chemiluminescent signals were detected using ECL-plus reagent (GE Healthcare) and imaged by the Image Quant LAS 4000 system. The α-tubulin detected in the same sample

was used as an equal loading control. Band density analysis was performed by Image J software.

Wound healing assay

Scratch wound healing assay is a typical method to examine the cellular ability of migration. Briefly, cells were seeded in a 12-well plate with 60% confluence and infected with lentivirus the next day. After 48 hours of viral infection, 4 straight lines were scratched with 1ml pipette tips to form a “#” like shape, and the medium was replaced with fresh growth medium. 10 images were taken at different sites of the wound area for each sample by Leica TCS SPE system immediately (0 hour) and 18 hours after wound injury. The number of migrated cells were calculated by Image J. The total number of cells in each dish were counted by a hemocytometer following cell collection. The number of migrated cells in 10 images were normalized to the total number of cells in the dish. This assay was repeated with three independent experiments.

BrdU staining for proliferation assay

To check the proliferation of ALDH^{br}-hCPCs with overexpression of SRXN1, we performed FACS analysis with BrdU staining. Briefly, ALDH^{br}-hCPCs were refreshed with growth medium supplemented with 10 μ M BrdU and cultured for another 12 hours after 48 hours of viral infection. Cells were collected and fixed with 4% PFA in PBS for 15 min at room temperature. Cells were then permeabilized with 0.5% Triton X-100 for 20 min, and treated with 2 M HCl for another 30 min. After sufficient 4 $\times$ PBS washes, cells were stained with BrdU antibody (Cell signal) for 2 hours at room temperature in 1% BSA/PBS, which was followed by the application of a secondary antibody (Alexa Fluo®-647, Life Technologies) for 1 h at room temperature. On final PBS wash, 1 μ g/ml PI was applied to 100 μ l of suspended cells for 5 min. Cells were subsequently diluted to an appropriate volume in PBS for the flow cytometry analysis.

Statistics

All the data are presented as mean $\pm$ SEM of results taken from at least three independent experiments. Statistical significance was calculated by Student's Test. A p-value less than 0.05 were considered as statistically significant.

Results

SRXN1 is associated with the survival of ALDH^{br}-hCPCs in response to oxidative stress

Our previous studies have shown that preconditioning hCPCs with CoPP promoted cell survival and enhanced the therapeutic efficacy of human cardiac progenitor cells in SCID mouse following MI model (6,25). Further studies with mRNA profiling identified a cluster of antioxidant genes, including SRXN1, HMOX1, SOD2, etc., that are associated with preconditioning hCPC with CoPP (unpublished data). Therefore, we hypothesized that SRXN1 is cytoprotective for hCPCs. Since there is debate regarding the capacity of c-Kit positive CPCs to regenerate new cardiomyocytes *in vivo* (30,31), we recently established a protocol to sort ALDH^{br}-hCPCs from human right atrial appendage derived cells (hRAACs) as described previously (10,11). As shown in the Supplemental Fig. S1, ~1.5% of hRAACs

are ALDH^{br}-hCPCs. The FACS analysis with live cell surface marker staining showed that these sorted ALDH^{br}-hCPCs are negative (<5%) for CD117(c-Kit), CD45, CD133, and CD146; but mainly positive (>80%) for stem/progenitor cell markers, including CD34, CD44, and CD90, which were consistent with previous report (11); these cells are partially positive (>30%) for CD172a, CD172b, CD309, CD31, CD184, CD140b, CD135, CD354, and CD71 (Supplemental Fig. S2). In order to test our hypothesis, we performed the gain- or loss-of-function studies for SRXN1 in ALDH^{br}-hCPCs, which were sorted from human right atrial appendage derived cells (Fig. 1A). Their cardiac regenerative potential, including their capacity for survival, proliferation, migration and differentiation, and the underlying mechanism were examined in this study (Fig. 1A).

First, results from Western blot analysis confirmed that preconditioning ALDH^{br}-hCPCs with CoPP led to a significant increase in expression of SRXN1, which is consistent with our unpublished microarray data (Fig. 1B). To examine whether SRXN1 plays a role in hCPC survival, we generated lentiviral particles to overexpress or knock down SRXN1 in ALDH^{br}-hCPCs. Western blot analysis showed that lentiviral-mediated overexpression of SRXN1 resulted in over 3-fold increase in SRXN1 protein levels (Fig. 1C). In contrast, over 80% knock-down was achieved in ALDH^{br}-hCPCs infected with a lentivirus expressing shRNA against SRXN1 (Fig. 1E, clone# 3). Next, ALDH^{br}-hCPCs infected with the SRXN1 overexpression or knockdown lentiviral systems were incubated with 2 mM H₂O₂ for 3 hours and cell viability was evaluated using an LDH release assay. We found ~13% increase in cell viability upon overexpression of SRXN1 (Fig. 1D), and a statistically significant decrease (~22%) in cell viability upon silencing of SRXN1 (Fig. 1F).

The impact of SRXN1 on hCPC apoptosis in response to oxidative stress

The effect of SRXN1 on hCPC apoptosis was evaluated next by Annexin V-APC and PI staining followed by flow cytometry. In these experiments, hCPCs were treated with 1 mM H₂O₂ for 90 min. Consistent with the results described for the LDH release assay, we found that overexpression of SRXN1 significantly enhanced the total number of live cells in the cells overexpressing SRXN1 ($77.2 \pm 1.42\%$) compared to the cells expressing the control empty vector ($69.8 \pm 0.93\%$) (Fig. 2A). Under these conditions, the number of apoptotic cells was decreased by 7% following overexpression of SRXN1. In addition, we found a significant decrease in the number of live cells and an increase in the number of apoptotic cells after SRXN1 was knocked-down (Fig. 2B). To evaluate the mechanism by which SRXN1 may be regulating cell death and apoptosis in hCPCs, we performed western blotting to check anti-apoptotic protein expression and found significant increased expression of BCL2 and BCL-xL at the protein level in SRXN1 overexpressed ALDH^{br}-hCPCs (Fig. 2C). Taken together, these results demonstrated that SRXN1 promotes the survival of ALDH^{br}-hCPCs in response to H₂O₂-induced oxidative stress, indicating it may play an important role as a cytoprotective molecule in ALDH^{br}-hCPCs.

Overexpressing SRXN1 results in increased proliferation and migration

The cell ability for proliferation and migration is essential for the regenerative potential of hCPCs in myocardial repair; therefore, we conducted the experiments to examine the cellular proliferation and migration for ALDH^{br}-hCPCs after overexpressing SRXN1. First,

the data from the FACS analysis with BrdU incorporation and staining showed that overexpressing SRXN1 enhanced the proliferation of ALDH^{br}-hCPCs by ~17%, compared with vector control cells (Fig. 3A–B). In addition, the wound healing assay suggested that overexpressing SRXN1 significantly promoted cell migration in ALDH^{br}-hCPCs in comparison to the vector control, as indicated by the increased number of migrated cells at the time point of 18 hours after scratching a wound gap (Fig. 3C–D). In this study, the migrated cells in 10 representative images are normalized to the total number of cells in each dish, which eliminates the effect from increased cell proliferation as shown in Fig. 3A–B.

Overexpressing SRXN1 enhances the cardiac differentiation

To understand whether overexpressing SRXN1 leads to any effects on the cellular ability for differentiation, we first examined the basal level of cardiac gene expression, including both cardiac transcriptional factors and mature cardiac marker genes. As shown in Fig. 4A, results from the quantitative PCR showed that overexpressing SRXN1 led to a significant increase in expression of NKX2.5, TBX5, and HAND2 in ALDH^{br}-hCPCs at the basal/undifferentiated stage, while the other cardiac transcriptional factors, including GATA4, GATA6, MEF2C, were not changed. Meanwhile, it was observed that overexpressing SRXN1 in ALDH^{br}-hCPCs did not alter their basal expression of cardiac marker genes, including MYH6, RYR2, cTNT and cTNI, indicating that SRXN1 did not promote the spontaneous cardiac differentiation (Fig. 4B). To examine whether SRXN1 overexpression promotes cellular capacity for differentiation *in vitro*, ALDH^{br}-hCPCs were infected with lentivirus expressing either SRXN1 or vector only for 24 hours, and then cells were refreshed in the differentiation medium for continuous culture for 7 days. In comparison to undifferentiated ALDH^{br}-hCPCs, there was a dramatic increase in gene expression of MYH6 and Troponin I after 7-day differentiation in both differentiated cell groups. Most importantly, it was observed that there was a 2~3-fold increase in the expression of MYH6, RYR2, and cTNI in SRXN1 overexpressed ALDH^{br}-hCPCs in comparison to the vector group (Fig. 4C). Taken together, the above results suggested that overexpressing SRXN1 has a positive effect on the cell regenerative abilities in terms of cell survival, proliferation, migration, and differentiation in ALDH^{br}-hCPCs.

Overexpressing SRXN1 lowers the ROS generation and mitochondrial membrane potential (Ψ_m)

In the next set of experiments, we examined whether altering SRXN1 expression levels could change the intracellular redox state of ALDH^{br}-hCPCs as previously shown for other systems. First, we evaluated the oxidation of the fluorescent indicator, carboxy-H2-DCF-DA (followed by analysis of flow cytometry). An increase in the fluorescence intensity for DCF-DA suggests an increase in oxidation. We found that overexpression of SRXN1 decreased the oxidation of DCF-DA (Fig. 5A); while silencing SRXN1 increased the oxidation of DCF-DA (Fig. 5B). Another ROS indicator, Dihydrorhodamine 123 (DHR123), has been widely used as an alternative probe for the assessment of ROS production (27,28). Interestingly, it was observed the mean fluorescence intensity is significantly decreased by overexpressing SRXN1 in hCPCs by measuring the DHR123 fluorescence (Supplemental Figure S3). Taken together, these results suggested that SRXN1 expression minimizes intracellular oxidation, possibly by reducing ROS bioavailability. It has been revealed that

progenitor cells with low mitochondrial membrane potential (Ψ_m) exhibit decreased ROS generation and maintain long-term survival of cells (29). Therefore, we subsequently measured the resting level of mitochondrial membrane potential by the analysis of flow cytometry after the live cell staining with the potentiometric dye tetramethyl rhodamine methyl ester (TMRM). The results showed that overexpressing SRXN1 in ALDH^{br}-hCPCs significantly decreased the mitochondrial membrane potential as indicated by the left-shift peak value and the reduced mean fluorescence intensity (Fig. 5C). Meanwhile, it was observed that knocking down SRXN1 in ALDH^{br}-hCPCs substantially increased their mitochondrial membrane potential as indicated by the right-shift peak value and the elevated mean fluorescence intensity (Fig. 5D). Taken together, these results suggested that SRXN1 expression minimizes intracellular oxidation, possibly by reducing ROS bioavailability.

To expand on these results, we screened for the expression of genes classically associated with anti- and pro-oxidant functions using an oxidative stress targeted PCR primer library (Table S3). We found that there was a global increase in antioxidant pathways upon overexpression of SRXN1 (Supplemental Fig. S3) and a 2~9-fold increase in expression of SOD2, CAT, TXNRD1, PRDX1, PRDX3, GPX4, GSTP1, and GSTT1 (Fig. 5E). We confirmed increased expression at the protein level for SOD2, CAT, TXNRD1, PRDX1, and PRDX3 (Fig. 5F). All together, these results support past studies in other cell systems and indicate that SRXN1 may control intracellular redox biochemistry, in part through regulation of primary intracellular antioxidant systems.

Up-regulation of ERK/NRF2 signal pathway following overexpressing SRXN1

Earlier studies have shown that the cellular antioxidant defense capacity is dependent on the activation of the ERK/NRF2 signal pathway (32,33). We have also previously shown that the activation of ERK/NRF2 signal pathway is associated with the cytoprotective effect of preconditioning hCPCs with CoPP (25). In the following experiments, we tested the specific hypothesis that the activation of ERK/NRF2 signal pathway is required for the cytoprotective effect associated with SRXN1 overexpression in hCPCs. Quantitative Western blot analysis confirmed a 3~4-fold increase in the protein levels of phosphorylated ERK1/2 and NRF2, while the total protein expression of ERK1/2 and NRF2 were not significantly altered upon overexpressing SRXN1 in hCPCs (Fig. 6A–B).

To further define the critical role of the ERK signaling pathway in the cytoprotection afforded by overexpressing SRXN1, we examined the effect of ERK inhibition on oxidative stress-induced cell apoptosis with FACS analysis following Annexin-V/PI staining. As shown in Fig. 6C–D, compared to the control cells (blue column), overexpressing SRXN1 in ALDH^{br}-hCPCs (green column) significantly increased the number of live cells and decreased the number of apoptotic cells in the vehicle-treated groups; on the other hand, the inhibition of ERK by U0126 significantly decreased the number of live cells and increased the number of apoptotic cells in comparison to the vehicle-treated groups ($^{\#}p<0.05$). However, this inhibition of ERK by U0126 significantly diminished the protective effect of overexpressing SRXN1 against total cell death, as indicated by the non-significant change of live cell number for ALDH^{br}-hCPCs expressing either vector and SRXN1 following the

treatment with U0126. Thus, these data suggested that the ERK signaling pathway plays an important role in SRXN1-mediated hCPC survival.

Down-regulation of NRF2 diminishes the cytoprotective effect of overexpressing SRXN1

As shown in Fig. 6A, the phosphorylation of both ERK and NRF2 were up-regulated following overexpressing SRXN1. To determine whether NRF2, which is downstream of ERK, played a role in the cytoprotective effect associated with SRXN1, we overexpressed SRXN1 in an ALDH^{br}-hCPC line with stable knockdown of NRF2 (NRF2-shRNA) as described previously (25). Decreased NRF2 protein expression in the NRF2 shRNA cell line was confirmed by WB blotting (Fig. 7A). In addition, we found that these cells expressed significant lower levels of SRXN1, indicating that SRXN1 is downstream of NRF2 signal pathway. As already shown, treatment of a control cell line (Scramble-shRNA; Fig. 7B–C) with H₂O₂ resulted in significant amounts of apoptotic and dead cells as assessed by Annexin-V/PI staining and flow cytometry, an effect partially inhibited upon overexpression of SRXN1. However, the cytoprotective effect of SRXN1 overexpression was lost in the NRF2-shRNA cells (Fig. 7B–C).

Based on the results collected in this study, we propose that overexpressing SRXN1 is capable of promoting hCPC survival against oxidative stress, and enhancing hCPC proliferation, migration, and cardiac differentiation. The involved mechanism seems to include initial phosphorylation of ERK, subsequent activation of NRF2, and eventual up-regulation of anti-oxidant proteins, including SOD2, CAT, TXNRD1, PRDX1, and PRDX3, as well as anti-apoptotic proteins BCL-2 and BCL-xL (Fig. 7D). Disruption of ERK and NRF2 impairs downstream anti-oxidant and anti-apoptotic gene expression and diminishes the cytoprotective effect of overexpressing SRXN1, demonstrating that ERK and NRF2 both possess essential roles in regulation of SRXN1-mediated cell survival.

Discussion

Various strategies to enhance the survival of donor cells have been reported, including hypoxia or anoxia conditioning (34), overexpression of growth factor (35), induction of anti-inflammation, and heat shock treatment (36), among others. In our previous study, we have demonstrated that hCPCs exposed to CoPP exhibited a better performance against apoptosis (25). The main and novel result presented in this study is that overexpressing SRXN1 in hCPCs leads to a significant increase in cell survival in response to H₂O₂ challenge. Accordingly, silencing of SRXN1 increases cell death upon treatment of hCPCs with H₂O₂. We have also established that overexpressing SRXN1 in hCPCs is capable of activating survival signaling molecules, including ERK and NRF2, as well as mediating the expression of anti-oxidant genes (SOD2, CAT, TXNRD1, PRDX1, and PRDX3) and anti-apoptotic genes (BCL-2 and BCL-xL), and eventually inhibiting apoptosis under oxidative stress. The present study is the first to undertake a comprehensive analysis of SRXN1 function in cardiac progenitor cells.

SRXN1 has been recognized as an endogenous antioxidant that protects against brain tissue damage in Parkinson's disease (37). Recent studies have confirmed the critical role of SRXN1 in regulating oxidative stress-triggered cell death in neuronal cells (20,38) and

cardiomyocytes (24). When SRXN1 expression was transiently knocked down in PC12 cells, cellular damage increased and cell viability decreased, indicating a potential target for neuroprotective intervention in response to oxidative stress (38). Furthermore, its silencing obviously decreases cell viability and its overexpression leads to anti-apoptotic and neuroprotective effects against oxidative stress-triggered cortical astrocyte injury, indicating a prominent role in cerebral ischemia (20). Upon ischemia-reperfusion (I/R) simulated injury, SRXN1 exhibited a cytoprotective effect in H9c2 cardiomyocytes by inhibiting PI3K/AKT-regulated mitochondrial apoptotic pathways (24). However, it remains unclear whether SRXN1 plays a cytoprotective role in hCPCs. In the present study, the gain- or loss-of-function studies for SRXN1 were conducted in order to explore the potential effect of SRXN1 in hCPCs against the oxidative stress-induced cell death. Interestingly, SRXN1 overexpression dramatically attenuated the oxidative stress-induced cell death, which was accompanied by a decrease in cell membrane damage as detected by LDH levels. Simultaneously, the number of apoptotic cells was remarkably decreased following SRXN1 upregulation. Additionally, SRXN1 down-regulation dramatically deteriorated cell death upon H₂O₂ treatment. Therefore, our results suggested a cytoprotective role of SRXN1 in hCPCs against oxidative stress, indicating a potential avenue in promoting the cardiac regenerative potential of hCPCs.

CPCs with c-kit positivity have been well-studied and exhibit the potential to improve heart function after myocardial infarction in various animal models (39–41) and clinical trials (42). However, there has been significant controversy in this field over whether the proportion of cardiomyocytes derived from c-kit⁺ cells is limited based on the genetic lineage-tracing studies (30,31). Aldehyde dehydrogenases (ALDH) are a group of enzymes catalyzing the conversion of aldehydes to the corresponding acids (43), which also play an important role in the maintenance and differentiation of stem cells as well as normal development (13,14,44). Recently, it has been reported that human ALDH^{br}-CPCs can differentiate into functional cardiomyocytes both *in vitro* and *in vivo* (10). And the transplantation of ALDH^{br}-CPCs preserved the cardiac function by extensive engraftment and cardiomyogenic differentiation in a pig model (9). Therefore, we sorted ALDH^{br}-hCPCs as a new source of cardiac stem/progenitor cells in the present study. Interestingly, these ALDH^{br}-hCPCs express stem/progenitor cell surface markers, including CD34, CD90, CD44, etc., but not express c-kit (CD117), CD45, and CD133, which is consistent with previous studies (11); More importantly, our results suggest that genetic modification of ALDH^{br}-hCPCs by overexpressing SRXN1 significantly enhances their cardiac regenerative potential, including proliferation, and migration.

Cellular redox state is critical for multiple functions of CPCs, including their survival ability, self-renewal/proliferation, and differentiation (45,46). Our results here show that overexpressing SRXN1 helps keep ROS levels low, and thus provides a novel molecular mechanism supporting the long-held view that SRXN1 protects against ROS-induced cell death. Although SRXN1 is known to inhibit ROS generation (21,47), the precise mechanisms by which this occurs remain unclear. NRF2 has been known as an inducible transcription factor that regulates ROS through activating a battery of genes encoding antioxidant proteins, resulting in a significant contribution to progenitor cell activity (48). While SRXN1 has been associated with the NRF2-mediated redox pathway (20,21), no

study to date has specifically addressed the role of SRXN1 in regulating the regenerative capacity of CPCs. In the present study, our data show that the activity of ERK is important for the cytoprotective role of SRXN1 because U0126, the specific ERK inhibitor, abrogated SRXN1-mediated protection from cell death after oxidative stress (H_2O_2). It has been shown that NRF2 is phosphorylated by ERK under conditions of oxidative stress, resulting in its nuclear translocation and the subsequent enhancement in the expression of antioxidant genes (49,50). We have previously showed that preconditioning of hCPCs with CoPP enhances their survival ability through activation of the ERK/NRF2 signaling pathway, where ERK is upstream of NRF2 (25). Results from this study showed that NRF2 transcriptional activity was elevated in SRXN1-overexpressed hCPCs, and partial reduction in NRF2 achieved by shRNA resulted in a corresponding partial reduction in SRXN1 expression. Thus, NRF2 is a candidate for an upstream factor in the pathway leading to induction of SRXN1, indicating a positive feedback upon the overexpressing SRXN1 toward its cytoprotective role in ALDH^{br}-hCPCs. Perhaps most importantly, the functional data showed that down-regulation of NRF2 with shRNA gene silencing abrogated the cytoprotective effect of overexpressing SRXN1 in hCPCs. Taken together, these results indicate that overexpressing SRXN1 activates the ERK/NRF2 signaling pathway, which then elicits a cellular anti-oxidant and anti-apoptotic response, and protects against oxidative stress-induced apoptosis in hCPCs. However, it is difficult to rule out the other signal pathways (e.g. PI3K/AKT, or NF κ B) that may be involved in SRXN1 mediated hCPCs regenerative potential. It would be interesting to investigate these alternative signal pathways in a separate study in the future.

Low mitochondrial membrane potential is normally associated with the poor quality of the mitochondria in somatic/adult cells, e.g., cardiomyocytes (51), or primary human fibroblasts (52). However, recent studies indicated that low mitochondrial membrane potential is related to the regenerative potential for resident stem/progenitor cells. Tissue-specific stem/progenitor cells, including CPCs, reside within *in vivo* niches with low oxygen tensions. This maintains stem cells in a glycolytic quiescent state and ensures tissue regenerative capacity by limiting oxidative metabolism and generation of reactive oxygen species (ROS) (53–55). Long-term hematopoietic stem cells (LT-HSCs) have been isolated from whole bone marrow by gating for low mitochondrial membrane potential. These LT-HSCs display greater colony formation capacity and bone marrow reconstitution capacity (56). In the present study, it was observed that the decreased mitochondrial membrane potential by overexpressing SRXN1 is beneficial for hCPCs, which is consistent with increased cell survival and proliferation, and better potential to differentiate into cardiomyocytes.

In summary, this is the first conclusive study to address the pivotal role of SRXN1 in suppressing oxidative stress-induced cell apoptosis and promoting the cardiac regenerative capacity, including proliferation, migration and cardiac differentiation, in hCPCs through genetically manipulating the expression of SRXN1. Our studies would also show for the first time that SRXN1 function may be involved in the activation of ERK/NRF2 signaling pathways that regulate downstream anti-oxidant and anti-apoptotic gene expression. We propose that this strategy can be used as a simple and effective approach to improve the efficacy of CPC-based therapies for heart disease. Future studies will be required to examine the *in vivo* survival capacity of hCPCs upon upregulation of SRXN1, and conduct a

thorough analysis of the molecular mechanisms underlying the potential cytoprotective function of SRXN1 following acute myocardial infarction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SRXN1	sulfiredoxin-1
ALDH	aldehyde dehydrogenase
CoPP	Cobalt protoporphyrin
Ψ_m	mitochondrial membrane potential
BCL2	B-cell CLL/lymphoma 2
BCL-XL	BCL2-like 1
FACS	fluorescence-activated cell sorter
ALDH^{br}-hCPCs	aldehyde dehydrogenase bright human cardiac stem/progenitor cells
HO-1	heme oxygenase 1
LDH	Lactate dehydrogenase
BrdU	5-Bromo-2'-deoxyuridine
MI	myocardial infarction
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PCR	polymerase chain reaction
PRDX1	peroxiredoxin 1
PRDX3	peroxiredoxin 3
ROS	Reactive oxygen species
shRNA	short hairpin RNA

SOD2

Superoxide Dismutase 2

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Highlights

- Sulfiredoxin-1 (SRXN1) promotes the survival of hCPCs in response to oxidative stress.
- Overexpressing SRXN1 increases cell proliferation, migration and cardiac differentiation.
- Overexpressing SRXN1 lowers the ROS generation and mitochondrial membrane potential.
- Up-regulation of ERK/NRF2 signal pathway is involved.

Figure 1. The expression of SRXN1 is associated with the cell survival ability in ALDH^{br}-hCPCs (A) Scheme of experiments using ALDH^{br}-hCPCs sorted from human atrial appendage derived cells. (B) Western blot and quantitative analysis showed the up-regulated expression of SRXN1 following preconditioning with 10 μ M CoPP for 12 hours. (C) Western blot and quantitative analysis confirmed that SRXN1 was overexpressed in ALDH^{br}-hCPCs after infected with SRXN1 lentiviral particles. (D) ALDH^{br}-hCPCs were infected with lentivirus expressing SRXN1 or vector for 36 h, challenged with 2 mM H₂O₂ for 3h, and then evaluated by LDH assay. (E) Western blot confirmed that the expression of SRXN1 was down-regulated in ALDH^{br}-hCPCs with shRNA for clone #3. (F) ALDH^{br}-hCPCs were infected with lentivirus expressing shRNA (clone #3) against SRXN1 or scramble shRNA for 36 h, challenged with 2 mM H₂O₂ for 3h, and then evaluated by LDH assay. Data are presented in this figure were mean values on a ratio of H₂O₂-induced LDH release to total LDH in cells with standard error of the mean (mean $\pm$ SEM). * indicates $p<0.05$ vs. control. Quantitative data analysis was performed with n=3 independent experiments.

Figure 2. SRXN1 promotes cell survival and exhibits anti-apoptotic effect in ALDH^{br}-hCPCs
 (A) ALDH^{br}-hCPCs were infected with lentivirus expressing SRXN1- or vector for 36 h, and treated with 1 mM H₂O₂ for 1.5 h, then evaluated by Annexin/PI FACS assay with quantitative analysis. (B) ALDH^{br}-hCPCs were infected with lentivirus expressing shRNA against SRXN1 or scramble shRNA for 36 h, and treated with 1 mM H₂O₂ for 1.5 h, then evaluated by Annexin/PI FACS assay with quantitative analysis. (C) Representative images and quantitative data of Western blot showing the expression level of anti-apoptotic proteins, including BCL2 and BCL-xL, after SRXN1 was overexpressed. Data were presented as mean ± SEM from 3 independent experiments (n=3); * indicates $p < 0.05$ vs. control.

Figure 3. Overexpressing SRXN1 enhances the capacity of cell proliferation and migration in ALDH^{br}-hCPCs

(A) Representative FACS analysis with BrdU incorporation and staining showed the increased proliferation ability of hCPCs after overexpressing SRXN1 in ALDH^{br}-hCPCs. (B) Quantitative data analysis for panel A. (C) Representative images for wound healing assay showing SRXN1-promoted cell migration in ALDH^{br}-hCPCs compared with vector control; (D) Quantitative data analysis for the ability of cell migration with counted cells that migrated to gap area by Image J software. Data were collected from 10 images per dish and normalized to the total number of cells in each dish, and presented as mean $\pm$ SEM from 3 independent experiments (*n*=3); * indicates *p* < 0.05 vs. vector control.

Figure 4. Overexpressing SRXN1 exhibits beneficial effect for promoting the cardiac differentiation of ALDH^{br}-hCPCs

(A) Real time qPCR was performed to examine the basal gene expression of cardiac transcriptional factors (*GATA4*, *GATA6*, *HAND2*, *MEF2C*, *NKX2.5*, and *TBX5*) at the mRNA level after SRXN1 was overexpressed in ALDH^{br}-hCPCs. (B) Real time qPCR was performed to examine the basal gene expression of mature cardiac markers (*MYH6*, *RYR2*, *cTNI*, and *cTNT*) at the mRNA level after SRXN1 was overexpressed in ALDH^{br}-hCPCs. (C) Real time qPCR was performed to examine the gene expression of mature cardiac markers (*MYH6*, *RYR2*, and *cTNI*) at the mRNA level in ALDH^{br}-hCPCs expressing SRXN1 or vector only following 7-day cardiac differentiation. Data were normalized to the same gene expression in undifferentiated ALDH^{br}-hCPCs (0-day), and presented as mean $\pm$ SEM from 3 independent experiments ($n=3$); * indicates $p < 0.05$ vs. vector control.

Figure 5. Overexpressing SRXN1 displays an anti-oxidant effect in ALDH^{br}-hCPCs
 Representative merged histogram and quantitative analysis of the mean fluorescence intensity (MFI) for ROS measurement by the FACS analysis with the DCF staining for ALDH^{br}-hCPCs under the condition of overexpressing SRXN1 (A) or knocking down the expression of SRXN1 with shRNA (B). Representative merged histogram and quantitative analysis of the mean fluorescence intensity (MFI) for assessing the basal level of mitochondrial membrane potential by the FACS analysis with the TMRM staining for ALDH^{br}-hCPCs under the condition of overexpressing SRXN1 (C) or knocking down the expression of SRXN1 with shRNA (D). (E) Real time qPCR was performed to examine the expression of anti-oxidant genes at the mRNA level after SRXN1 was overexpressed in ALDH^{br}-hCPCs. (F) Representative images and quantitative data of Western blot showing the expression level of anti-oxidant proteins, including SOD2, CAT, TXNRD1, PRDX1, and PRDX3 after SRXN1 was overexpressed in ALDH^{br}-hCPCs. Data were presented as mean $\pm$ SEM from 3 independent experiments ($n = 3$); * indicates $p < 0.05$ vs. control.

Figure 6. The cytoprotective effect of SRXN1 is associated with up-regulated ERK/NRF2 survival signaling pathway

(A) Representative images of Western blot showing the expression of total and phosphorylated ERK and NRF2 in SRXN1-overexpressed ALDH^{br}-hCPCs. (B) Quantitative data analysis for panel A. (C) Annexin/PI FACS assay for ALDH^{br}-hCPCs expressing SRXN1 or vector for 36 h, followed the treatment with U0126 or vehicle for 12 hours, and then challenged with oxidative stress induced by 1 mM H₂O₂ for 1.5 h. (D) Quantitative data analysis for panel C. Data were presented as mean $\pm$ SEM from 3 independent experiments ($n=3$); * indicates $p < 0.05$ vs. vector control.

Figure 7. Knocking down the expression of NRF2 diminishes the cytoprotective effect of overexpressing SRXN1 in ALDH^{br}-hCPCs

(A) Representative images and quantitative data of Western blot showing the expression level of NRF2 and SRXN1 in ALDH^{br}-hCPCs stably expressing the shRNA against NRF2. (B) Representative FACS analysis with annexin-V/PI staining showing H₂O₂-induced apoptosis in ALDH^{br}-hCPCs stably expressing scrambled or NRF2 shRNA following with or without SRXN1 overexpression. (C) Quantitative data analysis for panel B. (D) Descriptive diagram of proposed molecular mechanism for SRXN1 regulated ALDH^{br}-hCPCs survival. Data were presented as mean $\pm$ SEM from 3 independent experiments ($n=3$); * indicates $p < 0.05$ vs. control.