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ES cells overexpressing microRNA-1 attenuate apoptosis in the injured myocardium

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Abstract

MicroRNAs (miRs) are small, single-stranded, noncoding RNA's involved in post-transcriptional negative gene regulation. Recent investigations have underscored the integral role of miRs in various biological processes including innate immunity, cell-cycle regulation, metabolism, differentiation, and cell death. In the present study, we overexpressed miR-1, a muscle-specific miR, in embryonic stem cells (miR-1-ES cells), transplanted them into the infarcted myocardium, and evaluated their impact on cardiac apoptosis and function. We provide evidence demonstrating reduced apoptosis following transplantation of miR-1-ES cells 4 weeks post-myocardial infarction as compared to respective controls assessed by TUNEL staining and a caspase-3 activity assay. Moreover, we show significant elevation in p-Akt levels and diminished PTEN levels in hearts transplanted with miR-1-ES cells as determined by enzyme-linked immunoassays. Finally, using echocardiography, we reveal mice receiving miR-1-ES cell transplantation post-myocardial infarction had significantly improved fractional shortening and ejection fraction compared with respective controls. Our data suggest transplanted miR-1-ES cells inhibit apoptosis, mediated through the PTEN/Akt pathway, leading to improved cardiac function in the infarcted myocardium.

Keywords

Apoptosis; Heart; microRNA; Akt; PTEN

Introduction

Myocardial infarction (MI) induces cardiac myocyte cell death triggering left ventricular remodeling leading to hypertrophy, fibrosis, and dysfunction [3, 21]. Apoptosis, programmed cell death, is responsible for millions of cardiac myocytes lost following MI and subsequent ventricular remodeling [3, 7]. Taking into account cardiac myocytes are mostly terminally differentiated with minimal potential for cell division and the limited number of cardiac progenitor stem cells, intrinsic cardiac muscle regeneration is not sufficient to restore the heart to pre-MI homeostasis [15, 26]. Given this, the challenge to

develop effective therapies which protect the host myocardium from apoptosis following MI remains ever present.

Recent investigations have identified miRs as regulators of countless processes including cellular development, differentiation, metabolism, and death [4, 6, 9, 11]. miRs are small, single-stranded, noncoding RNAs which negatively influence gene expression through post-transcriptional modifications. miR manipulation to gain therapeutic effects for the treatment of various physiological and pathological conditions has gained notable attention within the research community.

Recently reported, miR-1 is a pro-cardiac miR which has been shown to enhance cardiac myocyte differentiation in the cell culture system [4, 27]. Whether miR-1-overexpressing embryonic stem (ES) cells following transplantation into the infarcted myocardium can inhibit apoptosis 4 weeks post-MI requires investigation. Therefore, we postulate that miR-1 will act as an anti-apoptotic miR when overexpressed in transplanted ES cells post-MI. Our data suggest that miR-1 over expressing ES cells following transplantation into the infarcted myocardium reduce total apoptotic nuclei. Furthermore, the observed decrease in apoptosis is mediated through up regulation of the Akt pathway and down regulation of PTEN in miR-1-ES cell-transplanted hearts. Finally, we illustrate that in vivo delivery of miR-1-ES cells 4 weeks post-MI improves cardiac function.

Materials and methods

ES cell culture

Mouse CGR8 ES cells were passaged and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 15% ES-qualified FBS, leukemia inhibitory factor (LIF), glutamine, nonessential amino acids, penicillin/streptomycin, β -mercaptoethanol, and sodium pyruvate as previously described [22].

miR-1-ES cell generation

Pre-miR-1 oligonucleotides (5'-tgc tgT GGA ATG TAA AGA AGT ATG Tag ttt tgg cca ctg act gac TAC ATA CTT TTA CAT TCC A-3' and 5'-cct gTG GAA TGT AAA AGT ATG Tag tca gtc agt ggc caa aac TAC ATA CTT CTT TAC ATT CCA c-3') were cloned into an expression vector (pcDNA 6.2-GW/EmGFP, Invitrogen). 48 h posttransfection into mouse CGR8 ES cells using Lipofectmaine 2000 (Invitrogen), media was changed and selection media containing 2 μ g/ml blasticidin (Invitrogen) was added and changed every 48 h thereafter for 2–3 weeks. Blasticidin resistant ES cell colonies (labeled miR-1-ES cells) were passaged and maintained in ES cell culture medium as described beforehand. The miR-1-ES cell cloning strategy is part of another submitted paper (Glass and Singla, unpublished data).

RNA extraction and real-time RT-PCR

Total RNA was extracted using RNA STAT-60 (Tel-Test). cDNA synthesis was performed with Taqman[®] MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Specific primers (Assay ID# 002222, Applied Biosystems) and Taqman[®] Universal PCR Master Mix (Applied Biosystems) were used for amplification and identification of miR-1. Real-time RT-PCR was performed using a CFX96[™] Real-Time System (Bio Rad). The relative amount of miR-1 was normalized against U6 snRNA (Assay ID# 001973, Applied Biosystems) and the fold change for miR-1 was calculated.

MI and ES cell transplantation

All experimental procedures were performed on 8–10 week old C57BL/6 mice (Jackson laboratories) and were approved by the University of Central Florida animal review board. Male and female mice were divided into four study groups ($n = 8$): Sham, MI + cell culture media (MI + CC), MI + ES cells, and MI + miR-1-ES cells. In brief, mice were intubated, ventilated using a rodent MiniVent (Harvard Apparatus), and anesthetized with 2.5% inhalant isoflurane throughout the procedure. Following a left thoracotomy, a permanent ligature was placed around the coronary artery. The peri-infarct region was identified and two separate intramyocardial injections totaling 20 μ l of media with or without 5.0×10^4 cells were delivered using a 29-gauge floating needle. After suturing the ribs, muscle, and skin, the lungs were expanded and mice were extubated. Sham animals received identical surgical procedures as detailed above while omitting the ligation. At D28 following surgery, mice were sacrificed using pentobarbital (80 mg/kg, ip) followed by cervical dislocation. Hearts were removed, transversely cut, and fixed in 4% paraformaldehyde for further analyses.

TUNEL staining

Heart tissue fixed in 4% buffered formalin was embedded in paraffin, cut into serial sections (5 μ m), and placed onto Colorfrost Plus slides (Fisher Scientific). TUNEL staining and analysis was performed as we previously reported [23, 24]. In brief, deparaffinized heart sections were permeabilized with proteinase K (25 μ g/ml in 100 mM Tris-HCl) and an in situ apoptotic cell death detection kit (TMR red; Roche Applied Biosystems) was used to identify apoptotic nuclei within the infarcted myocardium as per the manufacturer's instructions. Thereafter, heart sections were mounted with Anti-fade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Sections were observed under Olympus fluorescent and confocal microscopes. The percentage of apoptotic nuclei was determined in the infarct and periinfarct regions of heart tissue from $n = 5$ –8 animals/group. Percentage total apoptotic nuclei = (total number apoptotic nuclei)/(total number of DAPI) $\times$ 100%.

Caspase-3 activity assay

Caspase-3 activity was quantitated using a caspase-3 colorimetric activity assay kit from BioVision (K106-200). Heart tissue was homogenized in RIPA buffer containing phenylmethylsulfonyl fluoride, sodium orthovanadate, protease inhibitor cocktail, and sodium fluoride. Following centrifugation, supernatant was removed and protein concentration was estimated for each sample using the Bradford assay. Caspase-3 activity assay was performed following the manufacturer's instructions included with the kit. The resulting ODs at 405 nm were normalized to the protein concentration of each sample and plotted as arbitrary units (A.U.). Data were collected from heart homogenates of $n = 5$ –8 animals/group in duplicates.

p-Akt activity assay

p-Akt was quantified in heart homogenates using a phospho-Akt1 (PAN) ELISA kit (X1844k, Exalpha Biological) as detailed in the provided instructions. The developed color reaction was measured at 450 nm and the values obtained from the ELISA were normalized to the total protein concentration for each sample. Data were collected from heart homogenates of $n = 5$ –8 animals/group in duplicates.

PTEN ELISA

Phospho-PTEN (p-PTEN) was quantitated using a Phospho-PTEN Sandwich ELISA kit (#7285) from Cell Signaling Technology® as detailed in the provided manual. The

developed color reaction was measured at 450 nm and results were corrected for the protein concentration of each tissue sample. p-PTEN data were obtained from heart homogenates of $n = 5-8$ animals/group in duplicates and plotted as A.U.

Echocardiography

Cardiac function was assessed 4 weeks post-MI by noninvasive transthoracic echocardiography in short axis view at the mid-papillary muscle level using a Sonos 5500 Ultrasound system as we reported previously [24]. Obtained measurements include left ventricular internal dimension-diastole (LVIDd), left ventricular internal dimension-systole (LVIDs), fractional shortening (FS), and ejection fraction (EF).

Statistical analysis

Data are presented as a mean $\pm$ SEM. Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) and the Bonferroni test. $P < 0.05$ was considered statistically significant.

Results

Following transfection of ES cells with our generated miR-1 expression vector, blasticidin resistant cells were selected and maintained in cell culture for 2–3 weeks. To determine miR-1 expression in miR-1-ES cells and parental ES cells, RT-PCR was performed. Our data demonstrate more than a seven-fold increase in mature miR-1 expression in miR-1-ES cells compared to parental ES cells (Fig. 1).

Transplanted miR-1-ES cells inhibit apoptosis post-MI

To assess apoptosis within the infarcted myocardium, TUNEL staining was performed and representative photomicrographs are depicted in Fig. 2A–L. At day 28 (D28), there was a significant decrease in TUNEL-positive nuclei post-MI in miR-1 ES cell-transplanted hearts compared to ES cell and medium-transplanted hearts ($P < 0.05$, Fig. 2M). To support our TUNEL data, caspase-3 activity was quantified and a significant decrease in hearts transplanted with miR-1-ES cells post-MI compared to hearts transplanted with ES cells or medium alone resulted ($P < 0.05$, Fig. 3).

miR-1 enhances p-Akt activation and inhibits PTEN post-MI

Several mechanisms of apoptosis inhibition through various cell survival cascades have been reported including activation of the Akt pathway [25]. To quantitatively assess phospho-Akt (p-Akt), we performed an ELISA and were able to circumvent that hearts transplanted with miR-1-ES cells had significant p-Akt activation compared to hearts transplanted with ES cells and cell culture medium ($P < 0.05$, Fig. 4A). To evaluate whether enhanced p-Akt was consequent to inhibition of negative regulators of Akt activation, levels of PTEN, an antagonist of the Akt pathway, were quantitated. Our data demonstrate a significant reduction in PTEN in the miR-1-ES cell group versus the ES cell or cell culture medium group ($P < 0.01$, Fig. 4B).

Transplanted miR-1-ES cells improve cardiac function post-MI

Our echocardiography data at D28 illustrates mice receiving miR-1-ES cell transplantation following MI had significantly improved fractional shortening compared to ES cell and cell culture controls (mean $\pm$ SEM; MI $\pm$ miR-1-ES cells: 44.73 ± 0.72 , vs. MI $\pm$ ES cells: 39.51 ± 1.02 and MI $\pm$ CC 31.15 ± 1.69 , $P = 0.05$, Fig. 5A). Additionally, miR-1-ES cell-transplanted mice post-MI also had significantly improved ejection fraction when compared

with controls post-MI (mean $\pm$ SEM; MI $\pm$ miR-1-ES cells: 76.79 ± 0.86 , vs. MI $\pm$ ES cells: 70.46 ± 1.37 and MI $\pm$ CC 61.36 ± 2.39 , $P = 0.05$, Fig. 5B).

Discussion

Apoptosis, characterized by cell blebbing and shrinkage, chromatin condensation, and DNA fragmentation, plays an integral role in the pathogenesis of various cardiovascular diseases including MI [8]. Cardiac myocyte apoptosis following MI is a highly orchestrated programmed cell death initiated through various stressors including cytokines, oxidative stress, and DNA damage [1, 7, 8]. Although a multitude of attempts have been made to attenuate apoptosis in the infarcted myocardium through the use of various stem cell populations and genetic modifications, apoptosis remains a key factor in the adverse outcomes of MI [5, 10, 12, 14, 21].

In this study, we reveal a novel role of miR-1, when over expressed in ES cells, in the regulation of cardiac myocyte apoptosis in the infarcted myocardium. We illustrate miR-1-ES cells inhibit host myocardium apoptosis 4 weeks post-MI. Conversely, recent investigations have reported that in the infarcted myocardium, miR-1 promotes apoptosis in cardiac myocytes [20, 28]. However, the difference between the conflicting results lies within the cell type in which miR-1 is being expressed; that is, miR-1 has been shown to be apoptotic in endogenous cardiac cell types, whereas we are suggesting miR-1 is anti-apoptotic when miR-1 over expressing ES cells are transplanted into the infarcted heart [28]. Noticeably, these studies are unique from one another and demonstrate varying results. Nevertheless, this is not the first conflicting report in regards to the apoptotic nature of a particular miR. Consistent with our findings, it has been recently suggested that miRs can possess apoptotic, anti-apoptotic, or neither characteristic based solely on the type of cell in which they are being expressed [29]. These conclusions underscore the importance of defining the apoptotic function of a distinct miR to a specific cell type.

We next wanted to define mechanisms by which transplanted miR-1-ES cells obviate host myocardium apoptosis post-MI. Previous studies have identified the involvement of the Akt pathway in the regulation of cell growth, metabolism, proliferation, and survival [17, 25]. Moreover, the Akt pathway has been implicated in cardioprotection and apoptosis inhibition following MI through various secreted paracrine factors including secreted fibroblast growth factor 2 and follistatin-like 1 [2, 13, 16]. We evaluated the levels of activated Akt and noted a negative correlation between Akt activation and apoptosis inhibition. Specifically, we were able to illustrate a significant increase in p-Akt paralleled by a significant decrease in apoptosis in miR-1-ES cell-transplanted hearts compared to controls suggesting our data are in accordance with these previously published studies [2, 25]. We propose that increased Akt expression observed within the miR-1-ES cell-transplanted hearts leads to the observed cardioprotective effects through increased secreted paracrine factors. However, identification of exact paracrine mediators responsible for inhibited apoptosis is well beyond the scope of the current study and will require further investigation.

We next explored the potential effects of transplanted miR-1-ES cells on PTEN, an Akt signaling cascade inhibitor. We hypothesized that down regulation of PTEN might account for the up regulation of p-Akt observed in the miR-1-ES cell-transplanted hearts. We were able to elucidate that levels of PTEN were, in fact, significantly decreased in hearts transplanted with miR-1-ES cells compared to control hearts. Although PTEN is not a predicted mRNA target of miR-1, further investigation may determine whether miR-1 down regulates PTEN activators thus explaining the increased Akt activity and decreased PTEN observed within this study. In fact, a recent publication demonstrated a mechanistic

correlation between miR-101 and activation of Akt through down regulation of MAGI-2, a PTEN activator, as we suggest within the present study for miR-1 [19].

We also tested how inhibition of apoptosis by transplanted miR-1-ES cells would impact cardiac function 4 weeks following MI. In vivo delivery of miR-1-ES cells significantly increased fractional shortening and ejection fraction post-MI compared to respective controls. Conceivably, we suggest that inhibited apoptosis within the infarcted myocardium through miR-1 overexpression in transplanted ES cells obviated subsequent remodeling events post-MI leading to improved cardiac function. Our data are in agreement with a previously published study suggesting in vivo delivery of miR-24 into the mouse infarcted heart suppressed apoptosis thus attenuating contractility loss [18].

Next, our data suggest that the observed beneficial effects in the present study may not be the direct effects of released miR-1 from over expressing ES cells on reduced apoptosis. Rather, it may involve various factors released from transplanted miR-1 ES cells. Therefore, transplantation of LNA-miR-1 directly into the infarcted heart in comparison with the over expressing miR-1-ES cells may further shed light on the mechanisms of reduced apoptosis in this model.

In summary, we report that transplanted miR-1-ES cells inhibit apoptosis in the infarcted heart and these effects are mediated through the PTEN/Akt pathway. Furthermore, we illustrate the capability of transplanted miR-1-ES cells to improve cardiac function 4 weeks post-MI. Our data suggest the potential therapeutic applications using miR-1 modulation for the treatment of apoptosis-induced cardiac conditions. However, to understand how miR-1 exerts its anti-apoptotic effects in infarcted myocardium when overexpressed in ES cells, further studies are required to determine which mRNA targets are directly regulated by miR-1.

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Fig. 1. miR-1 overexpression confirmed by RT-PCR. Histogram shows quantitative RT-PCR data demonstrating overexpression of miR-1 in miR-1-ES cells. * $P < 0.001$ versus ES cells

Fig. 2. Transplanted miR-1-ES cells inhibit apoptosis. Representative photomicrographs of DAPI (A, D, G, J), apoptotic nuclei (B, E, H, K) and merged nuclei (C, F, I, L). Magnification, 40 $\times$. Right histogram (M) shows quantitative apoptotic nuclei. * $P < 0.05$ versus MI + CC, # $P < 0.05$ versus MI + ES cells and MI + CC

Fig. 3. miR-1-ES cells inhibit caspase-3 activity in the infarcted myocardium. Histogram shows significant decreases in caspase-3 activity in MI + miR-1-ES cells compared with MI + ES cells and MI + CC. * $P < 0.001$ versus MI + CC, # $P < 0.05$ versus MI + ES cells and MI + CC

Fig. 4. miR-1-ES cells exert apoptosis inhibition through PTEN/Akt pathway. Left histogram (A) reveals significant increase in p-Akt activity in hearts transplanted with miR-1-ES cells. * $P < 0.001$ versus MI + CC, # $P < 0.05$ versus MI + ES cells and MI + CC. Right histogram (B) shows quantitative PTEN expression. * $P < 0.05$ versus MI + CC, # $P < 0.01$ versus MI + ES cells and MI + CC

Fig. 5.

Transplanted miR-1-ES cells improve cardiac function 4 weeks following MI.

Echocardiography was performed at 4 weeks following MI. Left histogram (A) shows average fractional shortening for all treatment groups. * $P < 0.001$ versus MI + CC, # $P < 0.05$ versus MI + ES cells and MI + CC. Right histogram (B) shows transplanted miR-1-ES cells significantly improve fractional shortening. * $P < 0.01$ versus MI + CC, # $P < 0.05$ versus MI + ES cells and MI + CC