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Stem cell based delivery of Hypoxamir-210 to the infarcted heart: implications on stem cell survival and preservation of infarcted heart function

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Abstract

Aims—To test our hypothesis that transgenic induction of miR-210 in mesenchymal stem cells (MSC) simulate the pro-survival effects of ischemic preconditioning (IPC) and that engraftment of ^{PC}MSC help in functional recovery of ischemic heart by miR-210 transfer to host cardiomyocytes through gap junctions.

Methods and Results—miR-210 expression in MSC was achieved by IPC or nano-particle based transfection of miR-210 plasmid (^{miR}MSC), and functional recovery of the infarcted heart of rat transplanted with ^{PC}MSC or ^{miR}MSC were evaluated. Both ^{PC}MSC and ^{miR}MSC showed higher survival under lethal anoxia as compared to ^{non-PC}MSC and scramble-transfected MSC (^{Sc}MSC) controls with concomitantly lower CASP8AP2 expression. Similarly, both ^{PC}MSC and ^{miR}MSC survived better and accelerated functional recovery of ischemic heart post-transplantation. To validate our hypothesis that MSC deliver miR-210 to host cardiomyocytes, *in vitro* co-culture between cardiomyocytes and ^{PC}MSC or ^{miR}MSC (using ^{non-PC}MSC or ^{Sc}MSC as controls) showed co-localization of miR-210 with gap-junctional connexin-43. MiR-210 transfer to cardiomyocytes was blocked by heptanol pre-treatment. Moreover, higher survival of cardiomyocytes co-cultured with ^{PC}MSC was observed with concomitant expression of CASP8AP2 as compared to cardiomyocytes co-cultured with ^{non-PC}MSC thus suggesting that miR-210 was translocated from MSC to protect host cardiomyocytes.

Conclusions—Induction of miR-210 in MSC promoted their survival post-engraftment in the infarcted heart. Moreover, direct transfer of pro-survival miR-210 from ^{miR}MSC to host cardiomyocytes led to functional recovery of the ischemic heart.

Keywords

Connexin-43; miR-210; myocardium; preconditioning; stem cells

Introduction

MicroRNAs (miRs) are critical regulators of diverse physiological and pathological processes, including proliferation, apoptosis, differentiation, metabolism and development [1,2]. These regulatory effects of miRs are affected *via* repression or inhibition of translation of specific mRNAs involved in a specific cell function [3]. In this regard, a functional link

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Disclosure Statement

Nothing to disclose.

has been established between a discrete group of hypoxia inducible factor-1 α (HIF-1 α) dependent hypoxia-regulated miRs (HRM) and cell survival signaling [4,5]. More specifically, miR-210 from amongst the HRM has been identified as a universal responder to hypoxia in different cell types including endothelial cells and cancer cells [6–9]. We have reported that ischemic preconditioning (IPC) of mesenchymal stem cells (MSC) with intermittent short cycles of ischemia/re-oxygenation, like any other cellular or sub-cellular preconditioning strategy [10–13], promoted their survival upon subsequent exposure to lethal anoxia as well as post-engraftment in the infarcted heart [14]. While elucidating the mechanism, we demonstrated that stem cell survival was mediated through HIF-1 α dependent miR-210 which targeted pro-apoptotic gene, *Caspase8-associated protein-2* (*Casp8ap2*) [14]. In this study, we hypothesized that transfer of miRs from MSC to neighboring cardiomyocytes was the novel mechanism by which miRs affect survival under ischemic condition. We showed overexpression of miR-210 in MSC by IPC (^{PC}MSC) or by transfection with nanoparticle-based miR-210 plasmid (^{miR}MSC) contributed to functional recovery of the ischemic heart by transferring miR-210 to the cardiomyocytes through gap-junctions.

Methods

In vitro data represent average of at least three independent experiments.

Isolation and Culture of MSC

Bone marrow derived MSC were purified from young male Fischer-344 rats by flushing the cavity of femurs and evaluated as previously described [15].

Preconditioning of MSC

MSC were preconditioned using our optimized protocol [14]. Briefly, native MSC were seeded at 5×10^5 cells/60mm cell culture dish. After overnight serum and glucose starvation, cells were subjected to repeated 2 cycles of 30 minutes anoxia with intermittent 10 minutes re-oxygenation in an anoxic chamber (InVivo 500, Ruskin Life Science). Subsequently, the cells were either harvested for *in vitro* experiments or transplantation into the infarcted heart.

Nanoparticle-mediated delivery of miR-210 plasmid into MSC

Plasmids containing miR-210 (pEZX-miR-210) and scrambled miRNA (pEZX-Sc) were obtained from Genecopoeia (Rockville, MD) and delivery of these plasmids into MSC was performed using polyethyleneimine (PEI) nanoparticles (Polyscience, IL) [16]. Briefly, plasmid was mixed with PEI in 1:3 ratio and incubated in Opti-MEM medium for 10 minutes at room temperature. The PEI:pEZX-miR complex was directly added to cells and incubated for 4 hours followed by washing with PBS to remove free PEI nanoparticles. The cells were incubated for 48–72 hours and used for further experimentation.

For determination of polyplex resistance to nuclease digestion, 50 μ l DNase buffer (10X; Promega, WI) was mixed with 450 μ l corresponding solution containing 10 μ g naked plasmid DNA or PEI-DNA. After withdrawing 100 μ l for the time 0, 2U DNase-I (Promega, Madison, WI) was added into the mixture and incubated at 37°C. Samples (100 μ l) were collected at 30 minutes time intervals until 2 hours after incubation. To inactivate DNase-I, all samples were treated with 10 μ l of EDTA (20mmol/l) for 10 minutes at 65°C. Finally, 10 μ l of heparin (4 i.u./ μ l) was added to each sample and incubated at 65°C for 24 hour to facilitate dissociation of DNA from nanoparticles. The samples were later analyzed by electrophoresis system using 1% agarose gel.

Isolation and detection of miR

Extraction of miRs was performed using *mirVana*TM miR Isolation and qRT-PCR miR Detection Kits (Ambion Diagnostics, TX)[14]. Specific miR primers provided by Ambion were used during qRT-PCR per manufacturer's instructions.

FISH for miR-210

In situ detection of miR-210 was performed on MSC plated on chamber slides or cryosections. Samples were fixed in 4% paraformaldehyde for cell culture or acetone for cryosections at room temperature for 20 minutes followed by two washes in PBS. Fixed cells or 6µm of cryosections were incubated in hybridization solution (BioChain Inc., CA) for 3 hours at room temperature. Probe (3 pmol; LNA-modified and FITC-labeled oligonucleotide; Exiqon) complementary to miR-210 was hybridized to cells or tissue sections for 13–16 hours at 25°C lower than predicted T_m of the probe. *In situ* hybridization signals were detected with fluorescent microscope (BX41, Olympus, Japan).

Luciferase reporter assay

Luciferase assay was performed using the Dual-Luciferase Reporter Assay System kit (Promega), according to the manufacturer's instructions[14].

Lactate dehydrogenase (LDH) release and TUNEL assays

Intracellular LDH leakage and TUNEL assays were performed using LDH Assay Kit (Diagnostic Chemicals, Charlottetown, Canada) and TMR Red (Roche Applied Science, Germany) per manufacturer's protocol [14].

Experimental model of acute myocardial infarction and cell transplantation

Our study conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH-Publication#85-23, revised-1985) and protocol approved by the Institutional Animal Care and Use Committee, University of Cincinnati.

In vivo experiments were performed in a rat model of acute myocardial infarction using young female Fisher-344 rats [14]. Briefly, rats were anesthetized by intraperitoneal injection of ketamine/xylazine (24mg/kg and 4mg/kg body weight respectively). After endotracheal intubation and mechanical ventilation using Rodent Ventilator (Harvard Apparatus Model-683), the heart was exposed by left-sided minimal thoracotomy. Left anterior descending coronary artery was permanently ligated with prolene#6.0 suture. Immediately after ligation, 60µl basal DMEM without cells (group-1) or containing 1×10^6 cells (^{non-PC}MSC group-2; ^{PC}MSC group-3; ^{Sc}MSC group-4, ^{miR}MSC group-5 and heptanol pretreated animals treated with ^{PC}MSC group-6) were injected intramyocardially at multiple sites in and around the infarct zone. The chest was closed and animals were allowed to recover. During post-operation care, Buprenex (0.1mg/kg b.i.d) was administered for 24 hours to alleviate pain. For post-mortem studies, animals were sacrificed with an overdose of sodium pentobarbital. The heart tissue was collected and washed with PBS before use for molecular studies.

Histological and immunocytochemical analyses

For measurement of infarction size and area of fibrosis, the hearts were arrested in diastole by intravenous injection of cadmium chloride and fixed in 10% buffered formalin. The heart was excised, cut transversely, and embedded in paraffin for Masson's trichrome staining. Immunostaining was performed as described previously[14]. The primary antibodies used were specific for α -sarcomeric actin, α -smooth muscle actin (SMA, Sigma-Aldrich, MO), vonWillebrand Factor-VIII (vWF, Dako, CA), GFP (Abcam, MA) and Ki67 (BD

Pharmingen, CA). The samples were examined using fluorescence microscope (BX41, Olympus, Japan). For blood vessel density, blood vessels positive for vWF were counted in both infarct and peri-infarct areas. At least 32 high power microscopic fields ($\times 400$) each in the infarct and peri-infarct areas were randomly selected and counted in each treatment group of animals ($n=8$ animals/group). Blood vessel density was expressed as number of vessels/surface area (0.155mm^2).

Physiological assessment of the heart function

Echocardiography was performed using Compact Linear Array probe CL10-5 on HDI/5000 SONOS CT (HP) to study change in the heart function[10].

Polymerase chain reaction (PCR)

The cell and myocardial specimens from various treatment groups of animals on day 4 after cell transplantation were used for molecular studies by PCR[10]. The primer sequences were: *sry* (forward;5'-gaggcacaagttggctcaaca-3', reverse;5'-ctcctgcaaaaaggccttt-3') and *actin* (forward;5'-agccatgtacgtacccatcc-3', reverse;5'-ctctcagctgtggtggtgaa-3').

Western blot analysis

MSC were lysed in lysis buffer, pH7.4 [(in mM) 50 HEPES, 5 EDTA, 50 NaCl], 1% Triton X-100, protease inhibitors [(10 $\mu\text{g}/\text{mL}$ aprotinin, 1mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ leupeptin) and phosphatase inhibitors [(in mM) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate]. The protein samples (40 μg) were electrophoresed using SDS-polyacrylamide gel and electro-immunoblotted[14]. The specific antibody used for the detection of CASP8AP2/FLASH was purchased from Santa Cruz Biotechnology, CA.

In vitro co-culture studies

MSC were preconditioned as previously described [14]. Cardiomyocytes were isolated from the ventricles of 1 day-old Fisher-344 rat by using Neonatal Cardiomyocyte Isolation System (Worthington Biochemical, NJ) according to manufacturer's protocol. PC^{MSC} or miR^{MSC} (using non-PC MSC or Sc^{MSC} as controls) (2×10^5 , GFP-/DAPI $^{-}$) co-cultured with cardiomyocytes (4×10^5 , labeled with DAPI prior to co-culture) were seeded on 60mm culture dishes and incubated for 24 hours at 37°C in humidified atmosphere of 5% CO_2 . After cells were fixed with 4% paraformaldehyde, TUNEL and LDH assay were performed for cell survival studies. Protocols for FISH and immunostaining were combined to detect *in situ* miR-210 and Cx-43 or CASP8AP2 by using miR-210 hybridization probe together with specific antibodies for CX-43 (Abcam, Cambridge, MA) and CASP8AP2 (Santa Cruz Biotechnology, CA). miR-210 was first detected by FISH as described above. Expression of proteins was detected by immunohistochemistry[10].

Statistical analysis

All values were expressed as mean $\pm$ SE. Comparison between 2 mean values was evaluated by an unpaired Student 2-tailed *t*-test, and between 3 or more groups was evaluated by 1-way ANOVA followed by Bonferroni post-hoc analysis. Statistical significance was accepted at $p<0.05$.

Results

miR-210 transgene overexpression in MSC

The plasmid pEZXX-miR-210 encoding for miR-210 (Online Figure-I) was complexed with polyethyleneimine (PEI) nanoparticles at different PEI:plasmid ratios and highest

transfection efficiency (34.2%) was achieved at 3:1 PEI:plasmid ratio which was used during subsequent experiments (Figure-1A). *In vitro* characterization showed that PEI protected the encapsulated plasmid from degradation for over 120 minutes as compared to naked plasmid which fully degraded after 30 minutes by DNase-I (Figure-1B). The expression efficiency of miR-210 in MSC transfected with PEI:pEZX-miR-210 (^{miR}MSC) increased in time-dependent manner for up to 9 days of observation (Figure-1C). We observed 8.3±1.9 fold and 8.9±1.7 fold higher expression of miR-210 in ^{PC}MSC and ^{miR}MSC as compared to ^{non-PC}MSC and scramble (Sc) transfected MSC (^{Sc}MSC) respectively (Figure-1D). Successful transfection of MSC for miR-210 overexpression was substantiated by fluorescent *in situ* hybridization (FISH) using FITC-labeled miR-210 specific probe which showed that 38.7±2.6% ^{miR}MSC were positive for miR-210 expression (*vs* 36.5±6.5% ^{PC}MSC, *p*>0.05) (Figure-1E). These were significantly higher as compared to 3.5±0.9% ^{Sc}MSC and 3.2±1.2% ^{Nat}MSC staining positive for miR-210 expression respectively.

To test our hypothesis that overexpression of miR-210 in MSC simulated the cytoprotective effects of IPC, cells after their respective treatment were subjected to 12 hours lethal anoxia. The number of TUNEL⁺ cells was lower in ^{miR}MSC (*p*<0.01 *vs* ^{Sc}MSC; Figure-2A). Nevertheless, pro-survival effects of miR-210 overexpression were comparable to IPC as indicated by insignificant difference in the number of TUNEL⁺ cells between ^{miR}MSC and ^{PC}MSC (Figure-2A). LDH leakage, an indicator of cellular injury, confirmed pro-survival effects of miR-210 and showed that LDH release increased significantly under 12 hours anoxia in ^{Sc}MSC and ^{non-PC}MSC in comparison to ^{miR}MSC and ^{PC}MSC respectively (Figure-2B). However, no significant difference in LDH leakage was observed between ^{miR}MSC and ^{PC}MSC which indicated that cytoprotective effects of IPC were simulated by transgenic overexpression of miR-210. Moreover, both strategies to enhance miR-210 expression in MSC were consistent and equally potent in protecting MSC against lethal anoxia.

In terms of molecular signaling, miR-210 overexpression led to inactivation of CASP8AP2 similar to observed in ^{PC}MSC (Figure-2C)[14]. Western blotting confirmed CASP8AP2 abrogation in both ^{PC}MSC and ^{miR}MSC as compared to their respective controls under lethal anoxia (Figure-2D). We also observed higher caspase-8 (CASP8) in ^{non-PC}MSC and ^{Sc}MSC under lethal anoxia as compared to ^{non-PC}MSC cultured under normoxia whereas CASP8 was significantly reduced in ^{PC}MSC as well as ^{miR}MSC (Online Figure-IIA). Pre-treatment with 2.5 mM caspase-8 inhibitor (Granzyme-B II, EMD Millipore) simulated the cytoprotective effects of preconditioning and significantly increased the survival of MSC under lethal anoxia (Online Figure-IIB). On the contrary, pre-treatment of ^{PC}MSC with anti-miR-210 abrogated CASP8AP2 (Figure-2E) which was associated with significant increase in TUNEL⁺ cells (Figure-2F). These data clearly showed that induction of miR-210 *via* IPC or by transgenic overexpression blocked CASP8AP2 under ischemic stress.

miR-210 and stem cell survival in the infarcted heart

Concomitant with *in vitro* pro-survival effects of miR-210 in ^{PC}MSC and ^{miR}MSC, we investigated their post-transplantation survival in sex-mismatched female rat model of acute coronary artery ligation on day 4 after transplantation. *Sry*-gene analysis (n=4/group) by PCR showed that male ^{miR}MSC and ^{PC}MSC had multifold higher survival in female recipient rat heart as compared to ^{Sc}MSC and ^{non-PC}MSC (Figure-3A). DMEM injected female rat hearts did not show *sry*-gene and served as negative control. Fluorescence immunostaining of histological sections for Ki67 (red), a marker for proliferating nucleus, showed significantly higher proliferating GFP⁺ ^{miR}MSC at the site of cell graft as compared to ^{Sc}MSC (Online Figure-IIC). The higher *sry*-gene expression observed in Figure-3A in

fact reflected the combined effect of improved survival and enhanced proliferation of the transplanted cells. qRT-PCR also showed higher miR-210 expression in ^{miR}MSC transplanted group-5 hearts in comparison to ^{Sc}MSC treated group-4 hearts (Figure-3B). We did not observe miR-210 expression in DMEM treated group-1 hearts. On the contrary, the LV samples from ^{miR}MSC transplanted hearts exhibited abrogation of CASP8AP2 (Figure-3C), thus implying a major contribution of miR-210 signaling for improved survival of ^{miR}MSC. TUNEL positivity in the peri-infarct region was markedly reduced in ^{miR}MSC group-5 hearts as compared to ^{Sc}MSC group-4 thus suggesting a critical role of miR-210 in cell survival (Figure-3D).

Functional recovery of the infarcted heart

To investigate functional consequence of miR-210 induction and activation of its downstream signaling, animals were maintained for 4 weeks after their respective treatment. Transplantation of both ^{miR}MSC and ^{PC}MSC in and around the infarcted myocardium attenuated infarct size expansion (n=4/group; Figure-4A, 18.3±7.8% and 19.6±8.3% in ^{miR}MSC and ^{PC}MSC groups respectively) as compared to 32.6±6.5% and 31.4±9.6% in ^{non-PC}MSC and ^{Sc}MSC treated groups. The largest infarction size was observed in DMEM treated hearts (38.2±17.2%; $p<0.05$ vs all other groups).

The indices of LV contractile function were well-preserved in ^{PC}MSC group-3 and ^{miR}MSC group-5 (Figure 4B–C, Table-1). The LV fractional shortening (LVFS) and LV ejection fraction (LVEF) in ^{miR}MSC group-5, determined by transthoracic echocardiography, were 25.3±7.2% and 52.1±8.5% which were moderately significant as compared to ^{Sc}MSC group-4 21.1±3.4% and 43.4±3.2% respectively. The DMEM group-1 and ^{PC}MSC transplanted animals pretreated with heptanol group-6 had lowest preserved cardiac function (LVFS: 19.5±2.9% and 16.8±1.2%; LVEF: 38.2±4.2% and 35.1±3.2% respectively). These data showed that pretreatment of animals with heptanol significantly abrogated the beneficial effects of ^{PC}MSC treatment. Furthermore, LVFS (29.1±7.8%) and LVEF (55.6±7.4%) in ^{PC}MSC group-3 were significantly higher than those of ^{non-PC}MSC group-2 (22.4±3.1%, and 41.3±4.5% respectively).

Double fluorescence immunostaining of histological tissue sections from cell transplanted animal hearts showed myogenic differentiation of the transplanted GFP expressing MSC (Figure-5A). Immunostaining for vWF-VIII and α -SMA antigens revealed significantly higher number of capillaries and arterioles in ^{PC}MSC group-3 and ^{miR}MSC group-5 when compared to ^{non-PC}MSC group-2 and ^{Sc}MSC group-4 respectively (Figure-5B). Capillary density in ^{miR}MSC group-5 was 25.5±4.8 (infarct area) and 45.2±6.7 (peri-infarct area) which was similar to ^{PC}MSC treated group-3 in the infarct (25.2±5.1) and peri-infarct (49.3±6.6) areas and markedly higher than ^{non-PC}MSC group-2 in the infarct (18.3±4.1) and peri-infarct (37.6±4.3) areas respectively (Figure-5C). A similar trend was also observed in maturation of the newly formed blood vessels. Arteriole density in ^{miR}MSC group-5 was 6.9±2.4 and 9.2±2.4 in the infarct and peri-infarct areas as compared to 6.8±2.2 and 7.3±1.9 in ^{PC}MSC group-3 respectively. However, arteriolar density in these groups was higher as compared to 5.3±1.7 and 4.7±1.8 in ^{Sc}MSC group-4 and 3.7±1.5 and 4.2±1.8 in ^{non-PC}MSC group-2 in the infarct and peri-infarct areas respectively (Figure-5D).

Transfer of miR-210 from ^{PC}MSC and ^{miR}MSC and cardiomyocyte survival

FISH was performed on cell transplanted heart sections to examine miR-210 localization using miR-210 specific FITC-labeled probe (green). The results showed extensive localization of miR-210 (green) in the cardiomyocytes (visualized by actinin specific immunostaining; red) in ^{PC}MSC group-3 and ^{miR}MSC group-5 in animal hearts on day 4 after cell transplantation (Figure-6A). On the contrary, no miR-210 expression was observed

in the cardiomyocytes from ^{non-PC}MSC group-2 and ^{Sc}MSC group-4 (Figure-6A). A significantly higher number of miR-210 positive myocytes per 25mm² of the left ventricle in ^{PC}MSC (24.2±6.8) and ^{miR}MSC (23.8±4.3) transplanted groups as compared to their respective controls (Figure-6B). The expression of miR-210 in animal hearts from various treatment groups was confirmed by qRT-PCR which showed multiple-fold increase in miR-210 as compared to respective controls (Figure-3B). Therefore, we hypothesized that therapeutic benefits of transplanted stem cells with manipulated miR-210 expression was attributed to miR-210 transfer to the cardiomyocytes.

This hypothesis was validated in an *in vitro* co-culture model between different treatment groups of MSC and neonatal cardiomyocytes. FISH specific for miR-210 revealed that DAPI stained cardiomyocytes (blue nucleus, indicated by blue arrows) and ^{non-PC}MSC (without DAPI staining, indicated by red arrows) in their respective mono-cultures exhibited negligible level of miR-210 expression in comparison with ^{PC}MSC which was confirmed by RT-PCR (Figure-6C). However, using a co-culture system between DAPI labeled cardiomyocytes (blue nuclei, indicated by blue arrows) and ^{non-PC}MSC and ^{Sc}MSC (with unstained nuclei, indicated by white arrows) as controls wherein cardiomyocytes did not show miR-210 expression (Figure-6D and Online Figure-III), the cardiomyocytes in co-culture system with ^{PC}MSC (green; Figure-6E) and ²¹⁰MSC (Online Figure-III) showed marked expression of miR-210. Counter-immunostaining for connexin-43 (Cx-43; red), a gap-junction protein, revealed that miR-210 was co-localized with Cx-43 (Yellow; Figure-6E and Online Figure-IVA–B). However, pre-treatment with heptanol, a gap-junction uncoupler, blocked miR-210 transfer from ^{PC}MSC into the juxtaposed cardiomyocytes (Figure-6F) thus suggesting that miR-210 transfer from ^{PC}MSC to the cardiomyocytes was mainly *via* Cx-43.

To determine cytoprotective effects of the translocated miR-210 on the cardiomyocytes in co-culture system, we exposed the co-cultured cells to lethal anoxia for 12 hours. We observed that TUNEL⁺ cardiomyocytes co-cultured with ^{PC}MSC were significantly lower than the cardiomyocytes co-cultured with ^{non-PC}MSC (Figure-7A). These data were substantiated by lower LDH release from cardiomyocytes co-cultured with ^{PC}MSC as compared to the one co-cultured with ^{non-PC}MSC (Figure-7B). On the same note, transfection with Casp8ap2 specific siRNA recapitulated the preconditioning effects and improved cardiomyocyte survival under lethal anoxia ($p < 0.05$ vs scramble transfected control cells; Figure-7C). Luciferase assay confirmed that Casp8ap2 was a putative target of miR-210 in cardiomyocytes (Figure-7D). Immunostaining showed that CASP8AP2 was markedly inhibited in the cardiomyocytes co-cultured with ^{PC}MSC and ^{miR}MSC under lethal anoxia for 12 hours (Figure-7E). These results suggested that miR-210 delivered from ^{PC}MSC critically participated in cardiomyocyte protection in co-culture as well as in the infarcted heart.

Discussion

The significant findings of our study were; 1) Nanoparticle based transgenic overexpression of miR-210 in MSC simulated the cytoprotective effects of IPC to support their survival under lethal anoxia and post-transplantation in the infarcted heart. 2) Stem cells with miR-210 overexpression (either by IPC or genetic manipulation) rescued cardiomyocyte survival in the co-culture system under lethal anoxia and in the ischemic heart by miR-210 transfer. 3) Transfer of miR-210 from MSC overexpressing miR-210 to the cardiomyocytes. 4) *Ex vivo* stem cell-based delivery of miR-210 prevented infarct size expansion and successfully preserved the LV function.

Hypoxamir miR-210 is a universal responder to hypoxia in different cell types [6,7,17]. Consistent with its role in adaptive response to hypoxia, miR-210 was up-regulated in placenta from patients with pre-eclampsia which was characterized by insufficient angiogenesis and consequent hypoxia [18]. Similarly, miR-210 level has been correlated with adverse clinical outcome in both breast and pancreatic cancers and with their metastatic potential [19]. Giannakakis *et al.* reported that miR-210 was located at the telomeric portion of the chromosome segment 11p15.5 within a minimal region of loss of heterozygosity and was confined between polymorphic marker *Hras* and dinucleotide repeat marker D11S1363 (~250Kb) [20].

Several target genes of miR-210 have been identified so far including *EfnA3*, *E2f3*, *Neuronal pentraxin*, *Rad52*, and *Casp8ap* [7,14,20–22], indicating a diverse role of miR-210 in cell cycle, DNA repair, angiogenesis and apoptosis. Given the functional diversity of miR-210, data from tumor cells is extrapolated for manipulation of stem/progenitor cells to support their survival and functionality [6,7,23]. Minicircle system based transgenic delivery of miR-210 curtailed cardiomyocyte apoptosis and supported angiogenesis *via* involvement of Ephrin A3 and Protein tyrosine phosphatase-1B as the target genes [23]. As our study focused on pro-survival effects of miR-210, we did not study the mechanism of improved angiogenic response observed in the animal heart after treatment with ^{PC}MSC and ^{miR}MSC. However, there is mounting evidence that native MSC secrete plethora of angiogenic growth factors as part of their paracrine activity which stimulates angiogenic as well as cytoprotective responses in the heart after MSC transplantation. Besides, these bioactive molecules secreted as part of the paracrine activity of the ^{PC}MSC or ^{miR}MSC may also alter the miR expression profile in the cardiomyocytes (i.e., miR-210) to regulate their function including survival. However, future in-depth studies would be required to dissect the mechanism by which MSC modified for miR-210 expression (either by IPC or overexpression of miR-210) enhance angiogenesis in the infarcted heart as well as to identify the factors, if any, responsible for changing miR-profile of cardiomyocytes in general and that of miR-210 in particular.

Our strategy of stem cell based delivery of exogenous miR-210 to the heart has multiple advantages over direct injection of vector encoding for miR-210. Firstly, myogenesis combined with angiogenesis is required to regenerate the infarcted heart. Angiogenesis alone would be insufficient to improve contractile function of the infarcted heart as it would not compensate for the cardiomyocyte loss subsequent to infarction. Secondly, MSC release copious amounts of pleiotropic factors as part of their paracrine activity. Thirdly, MSC with miR-210 upregulation/ overexpression show better survival post-transplantation. Moreover, stem cells with miR-210 overexpression serve as reservoir for miR-210 for delivery to host cardiomyocytes in the harsh microenvironment of the infarcted heart.

There are several studies that show transfer of small molecules such as siRNA and miRs between adjacent cells *via* microvesicles [24] as well as through gap-junctions [25]. In the same context, cardiomyocytes also exchange small RNAs through intercellular gap-junctions [25]. While our manuscript was in preparation, two publications reported the role of gap-junctional translocation of miRs between adjacent cells [26,27]. Using a co-culture system between breast cancer cells and bone marrow stromal cells, miRs including miR-127, -197, -222, and -223 have been implicated for role in cell proliferation by targeting *Cxcl12* and these miRs were transported from bone marrow stroma to breast cancer cells *via* gap-junctions [28]. Similarly, in both *in vitro* and *in vivo* assay systems, miR-499 was actually translocated from cardiomyocytes into cardiac stem cells to repress Rod1 and Sox6 and promoted their cardiogenic differentiation [27]. These findings substantiate our hypothesis that such mechanism also exists between cardiomyocytes and stem cells with miR-210 overexpression to regulate *Casp8ap2* target gene expression in the

cardiomyocytes. CASP8AP2 is a multifunctional protein which is not only involved in apoptosis but also influences S-phase progression and transcriptional regulation [29,30]. We have identified *Casp8ap2* as a putative target of miR-210, abrogation of which significantly improved stem cell survival under lethal anoxia [14]. While elucidating the effect of concomitant activation of HIF-1 α dependent miRs, i.e., miR-017 and miR-210, we have recently shown that *Casp8ap2* specific siRNA interference, either alone or together with *PDCD10* (a putative target of miR-107) replicated the cytoprotective effects of IPC and led to improved survival of the cells lethal anoxia [31].

An interesting finding of our present study was that pre-treatment of stem cells with heptanol, an inhibitor of Cx-43 pores, blocked miR-210 transfer between cardiomyocytes co-cultured with ^{miR}MSC/^{PC}MSC which indicated that miR-210 transfer occurred through gap-junctions. However, this did not exclude the possibility that miR-210 transfer or induction also occurred by other mechanisms and therefore warranted in-depth studies for systematic analysis of the contribution of each of these different mechanisms of miR transfer. In the same context, future studies would be required to exclude the possible exchange of some as yet undefined factor/s between co-cultured cardiomyocytes and ^{miR}MSC/^{PC}MSC that accentuated miR-210 induction or transfer in the cardiomyocytes. More importantly, induction of miR-210 in stem cells improved their survival under ischemic stress and translocation of miR-210 reduced TUNEL positivity in recipient cardiomyocytes. Transplantation of ^{miR}MSC in the infarcted heart increased their survival as demonstrated by higher *sry*-gene expression, increased number of Ki67⁺ proliferating cells and lower TUNEL positivity as compared to ^{Sc}MSC. Higher *sry*-gene expression in ^{PC}MSC group-3 and ^{miR}MSC group-5 observed may be attributed to pro-survival effects of miR-210 combined with proliferative activity of the surviving cells in the heart. Taken together, direct transfer of miRs from MSC to the cardiomyocytes is a possible new strategy in cell-based therapy for the ischemic myocardium. Further in-depth mechanistic studies in this regard are therefore warranted to explore this intriguing possibility. Although, our data provide evidence regarding myogenic differentiation of the transplanted stem cells with higher miR-210 expression, future in-depth studies would be required to exclusively study the role of miR-210 in cardiac differentiation of stem cells.

In conclusion, stem cell-based miR-210 delivery to the infarcted heart is novel and effective strategy to preserve the LV function through multiple mechanisms. The induction of pro-survival miRs not only addresses the issue of donor cell survival, the direct cell-to-cell transfer of the “repair” miRs from donor cells to host cardiomyocytes also lead to repair of the damaged heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure-1. Transfection of MSC for miR-210 overexpression

(A) Determination of optimal PEI:pEZX-miR-210 ratio for transfection of MSC. The optimal PEI: pEZX-miR-210 ratio was 3:1 with highest transfection efficiency of >34% without apparent cellular injury. (B) Complexation of PEI with pEZX-miR-210 imparted stability to plasmid DNA and protected its degradation against DNase-I for 120 minutes as compared to the un-complexed plasmid DNA which fully degraded within 30 minutes. (C) qRT-PCR for time-dependent expression of miR-210 in MSC. Significantly higher expression of miR-210 continued for 9 days of observation *in vitro* as compared to *Sc*MSC ($p < 0.05$ on all time-points of observation). (D–E) qRT-PCR and *in miR*MSC and *PC*MSC showed higher miR-210 expression as compared to their respective controls *Sc*MSC

and non-PCMSC. (E) *In situ* detection of miR-210 using FITC-labeled miR-210 specific probes showing expression of miR-210 in miR^RMSC and PCMSC. Whereas the percentage of miR^RMSC and PCMSC positive for miR-210 was comparable, these were significantly higher as compared to ScMSC and non-PCMSC.

Figure-2. MiR-210 overexpression simulated the cytoprotective effects of IPC

(A) Transgenic overexpression of miR-210 significantly reduced TUNEL⁺ miRMSC upon subsequent exposure to lethal anoxia which was comparable to PCMSC ($p > 0.05$ miRMSC vs PCMSC). ScMSC were used as control which showed higher apoptosis under lethal anoxia. (B) LDH release was lower in miRMSC which was similar to PCMSC ($p > 0.05$). ScMSC were used as controls and showed extensive LDH release under lethal anoxia. (C) *In situ* detection of miR-210 (green; using miR-210 specific FITC-labeled probes) and immunostaining for CASP8AP2 (red) in miRMSC and ScMSC. CASP8AP2 was abrogated by miR-210 overexpression in miRMSC. (D–E) Western blots showing (D) abrogation of CASP8AP2 in both PCMSC and miRMSC as compared to their respective

control ($p < 0.01$ vs $^{non-PC}MSC$ and ^{Sc}MSC respectively). (E) Pretreatment of ^{PC}MSC with anti-miR-210 abolished IPC effects and increased CASP8AP2 ($p < 0.01$ vs ^{Sc}MSC). The cells in D&E were subjected to 6 hour anoxia after their respective treatment. (F) TUNEL showing significant increase in TUNEL⁺ ^{PC}MSC pretreated with anti-miR-210.

Figure-3. MSC manipulated for miR-210 expression showed improved survival post-transplantation in the infarcted heart

(A) PCR for *sry*-gene in the female recipient rat heart at 4 days after cell transplantation. Both miRMSC and PCMSC showed more than 8-fold and 10-fold higher survival respectively post-transplantation in the infarcted female rat heart which was significantly higher as compared to ScMSC and non-PCMSC controls. DMEM treated rat hearts did not show *sry*-gene expression and served as negative control. (B–C) qRT-PCR showed higher miR-210 expression in the LV of miRMSC group-5 hearts on day 4 post-transplantation with concomitant abrogation of CASP8AP2 as compared to ScMSC group-4 controls ($p < 0.05$). (D) TUNEL staining of the infarcted rat heart histological sections on day 4 after ScMSC

and ^{miR}MSC transplantation. TUNEL positivity was significantly reduced in ^{miR}MSC group-5 hearts.

Figure-4. MSC manipulated for miR-210 expression attenuated infarct size and preserved infarcted heart function

(A) Infarct size was significantly reduced in miRMSC group-5 and PCMSC group-3 hearts as compared to ScMSC group-4 and non-PCMSC group-2 hearts 4 weeks after their respective treatment. Largest infarct size was observed in DMEM group-1 hearts. (B–C) Transthoracic echocardiography showed well-preserved LVFS and LVEF at 4 weeks after PCMSC and miRMSC transplantation. Table-1 shows improved indices of wall thickness and chamber dimensions in PCMSC group-3 and miRMSC group-5 animal hearts.

Figure-5. Angiomyogenesis in the infarcted heart with MSC manipulated for miR-210 expression
 (A) Representative images of double fluorescence immunostaining of histological sections for desmin expression (red fluorescence) at 4 weeks after treatment with GFP⁺ miR²¹⁰MSC (green fluorescence) in group-5, using ScMSC as a control. Co-localization of red (desmin) and green (GFP) fluorescence showed myogenic differentiation of the transplanted cells.
 (B–D) Blood vessel density analysis after vWF-VIII and smooth muscle specific immunostaining of the heart tissue sections at 4 weeks in different treatment groups of animals using DMEM group-1 as control. PCMSC group-3 and miR²¹⁰MSC group-5 transplantation led to significantly higher capillary density as compared to ScMSC group-4 and non-PCMSC group-2 transplantation.

Figure-6. miR-210 transfer from PCMSC and miRMSC to cardiomyocytes

(A) Fluorescence *in situ* hybridization (FISH) on cell transplanted rat heart tissue sections to examine miR-210 localization using FITC-labeled probe. Transplantation of PCMSC and miRMSC increased miR-210 expression in cardiomyocytes of the infarcted heart whereas non-PCMSC and ScMSC transplantation showed no miR-210 expression in the host cardiomyocytes. Left panel of images were taken at low magnification (4×) to show pan view of the left ventricle probed positively for miR-210 (green) whereas right panel shows magnified images (40×) of the same heart tissue sections for clarity. Arrows indicate co-localization of cardiomyocytes and miR-210. (B) Graph showing the number of miR-210

positive cardiomyocytes in different treatment groups of animals on day 4 after their respective treatment. (C) FISH and RT-PCR for miR-210 showed higher miR-210 expression in ^{PC}MSC as compared to ^{non-PC}MSC and cardiomyocytes in their mono-cultures. (D–G) ^{non-PC}MSC (D) or ^{PC}MSC (without DAPI label) (E) were co-cultured with cardiomyocytes (DAPI labeled; blue) for 12 hours. miR-210 was un-detected in cardiomyocytes co-cultured with ^{non-PC}MSC (D). However, cardiomyocytes co-cultured with ^{PC}MSC stained positively for miR-210 (green; E). Counter-immunostaining for Cx-43 expression (red) revealed that miR-210 was co-localized with Cx-43 (E). Pretreatment with a gap-junction uncoupler, heptanol, blocked miR-210 expression in cardiomyocytes co-cultured with ^{PC}MSC thus indicating that ^{PC}MSC transferred miR-210 into neighboring cardiomyocytes through gap junctions (F). White arrows indicate MSC and blue arrows show cardiomyocytes.

Figure-7. miR-210 transfer from MSC enhances cardiomyocyte survival

(A–B) Cardiomyocytes co-cultured with ^{PC}MSC showed decreased TUNEL positivity (A) and significantly reduced LDH release (B) as compared to ^{non-PC}MSC. (C) Transfection of *Casp8ap2* specific siRNA abrogated *Casp8ap2* in cardiomyocytes and improved their survival under lethal anoxia as compared to scramble transfected cardiomyocytes. (D) Luciferase assay confirmed *Casp8ap2* as a putative target of miR-210 in cardiomyocytes (1=3'UTR only; 2=3'UTR+ Sc; 3=3'UTR+ miR-210) (E) CASP8AP2 induction after 12 hours lethal anoxia was inhibited in cardiomyocytes co-cultured with ^{PC}MSC or ^{miR}MSC as compared to their respective controls ^{non-PC}MSC or ^{Sc}MSC. Red arrows indicate MSC

(without DAPI labeling), and blue arrows show DAPI stained cardiomyocytes (blue nuclei) in the co-culture system.

Table-1

Echo function studies in various treatment groups of animals.

	LVFS	LVEF	LVDD	LVDs	IVSTd	PWTd
DMEM	19.5±2.9	38.2±4.2	8.5±0.05	6.71±0.26	0.61±0.08	1.56±0.07
non-PC ₁ MSC	22.4±3.1	41.3±4.5	8.02±0.45	6.54±0.58	0.56±0.12	1.56±0.05
PC ₁ MSC	29.1±7.8	55.6±7.4	7.32±0.68	5.33±0.69	0.75±0.45	1.48±0.06
S ₀ MSC	21.1±3.4	43.4±3.2	7.95±0.59	6.46±0.18	0.53±0.06	1.51±0.04
miR ₁ MSC	25.3±7.2	52.1±8.5	7.64±1.02	5.79±0.54	0.77±0.08	1.46±0.08
Hep ₁ MSC	16.8±1.2	35.1±3.2	8.48±0.44	7.05±0.44	0.49±0.11	1.52±0.04