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Negative Regulation of miR-375 by Interleukin-10 Enhances Bone Marrow derived Progenitor Cell-mediated Myocardial Repair and Function after Myocardial Infarction

Venkata Naga Srikanth Garikipati[1], **Prasanna Krishnamurthy**[2], **Suresh Kumar Verma**[1], **Mohsin Khan**[1], **Tatiana Abramova**[3], **Alexander Mackie**[3], **Gangjian Qin**[3], **Cynthia Benedict**[1], **Emily Nickoloff**[1], **Jennifer Johnson**[1], **Ehre Gao**[1], **Douglas W Losordo**[3], **Steven R Houser**[5], **Walter Koch**[1,4], and **Raj Kishore**[1,3,4,*]

[1]Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA, 19140

[2]Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, TX, 77030

[3]Feinberg Cardiovascular Research Institute, Northwestern University, Chicago IL 60611

[4]Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA 19140

[5]Cardiovascular Research Center and Dept. of Physiology, Temple University School of Medicine, Philadelphia, PA 19140

Abstract

Poor survival and function of transplanted cells in ischemic and inflamed myocardium likely compromises the functional benefit of stem cell based therapies. We have earlier reported that co-administration of IL-10 and BMPAC enhances cell survival and improves LV (LV) functions after AMI in mice. We hypothesized that IL-10 regulates miR-375 signaling in BMPACs to enhance their survival and function in ischemic myocardium after MI and attenuates left ventricular dysfunction after MI. MicroRNA-375 expression is significantly up regulated in BMPACs upon exposure to inflammatory/hypoxic stimulus and also after MI. IL-10 KO mice display significantly elevated miR-375 levels. We report that ex vivo miR-375 knock down in BMPAC before transplantation in the ischemic myocardium after MI significantly improve the survival and retention of transplanted BMPACs and also BMPAC-mediated post-infarct repair, neovascularization and LV functions. Our in vitro studies revealed that knockdown of miR-375

[*]**Correspondence to:** Raj Kishore, PhD, FAHA, Center for Translational Medicine, Temple University School of Medicine, MERB-953, 3500 N Broad Street, Philadelphia, PA 19140, Phone: 215-707-2523, Fax: 215-707-9890, raj.kishore@temple.edu.

Author Contributions: Venkata Naga Srikanth Garikipati: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing.
Prasanna Krishnamurthy: Collection and analysis of data
Suresh K Verma, Mohsin Khan, Alexander Mackie: Conception and design
Gangjian Qin, Douglas Losordo, Erhe Gao, Walter Koch and Steven Houser: Conception and design
Cynthia Benedict: Manuscript writing
Tatiana Abramova, Emily Nickoloff and Jennifer Johnson: Collection and /or assembly of data
Raj Kishore: conception and design, final editing and approval of manuscript

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enhanced BMPAC proliferation and tube formation and inhibited apoptosis; over expression of miR-375 in BMPAC had opposite effects. Mechanistically, miR-375 negatively regulated 3-phosphoinositide-dependent protein kinase 1 (PDK-1) expression and PDK-1-mediated activation of PI3Kinase/AKT signaling. Interestingly, BMPAC isolated from IL-10-deficient mice showed elevated basal levels of miR-375 and exhibited functional deficiencies, which were partly rescued by miR-375 knockdown, enhancing BMPAC function in vitro and in vivo. Taken together, our studies suggest that miR-375 is negatively associated with BMPAC function and survival and IL-10-mediated repression of miR-375 enhances BMPAC survival and function.

Keywords

Bone marrow progenitor angiogenic cells; miRNA; inflammation; angiogenesis; left ventricular remodeling

INTRODUCTION

In the absence of effective endogenous repair mechanisms after cardiac injury, cell-based therapies have rapidly emerged as a potential novel therapeutic approach in ischemic heart disease. After initial characterization of putative bone marrow-derived angiogenic progenitor cells (BMPAC) or endothelial progenitor cells (EPC) and their potential to promote cardiac neovascularization and to attenuate ischemic injury, a decade of intense preclinical research led to BMPAC-based clinical trials which yielded promising yet modest results [1–5]. Preclinical studies suggest that microenvironment in the infarcted myocardium, including inflammation and oxidative damage, has adverse effects on transplanted stem cell survival and function [6, 7]. Thus enhancing survival and function of transplanted stem remains a recognized challenge.

We and others have established a cardio-protective role of anti-inflammatory cytokine interleukin-10 (IL-10) therapy in mouse models of AMI and pressure-overload, which in turn is mediated by signal transduction pathways including p38 MAP kinase, NF-kB and STAT-3[8–11]. Patients with high serum levels of pro-inflammatory cytokines have low circulating and functionally impaired BMPACs [6, 7]. We demonstrated that combined therapy with bone marrow progenitor cells and IL-10 is more effective in the improvement of post-AMI LV function than bone marrow progenitor cells alone[14]. Further role of the IL-10 on BMPAC biology, signaling and function has never been studied and warrants thorough investigation.

The discovery of microRNAs (miR) has opened a new approach regarding regulation of cellular processes such as proliferation, differentiation, cell metabolism, apoptosis and angiogenesis [12–14]. We reported that BMPACs from IL-10 knockout (IL-10 KO) mice are functionally impaired [15]. Schaefer et al reported that mononuclear cells from IL-10 KO mice express high levels of miR-375 [16]. The miR-375 gene has been shown to be found on chromosome 2 in humans and chromosome 1 in mice and located in an intergenic region between the cryba2 (b-A2 crystallin, an eye lens component) and Ccdc108 (coiled-coil domain-containing protein 108) genes and is highly conserved between humans and mice [17],[18, 19]. Emerging evidence suggests an association of decreased miR-375 expression

with tumourigenesis and progression in melanoma, carcinoma of the head and neck, esophageal, gastric or prostate cancer [17]. Furthermore, patients with heart failure and/or diabetes have high levels of miR-375[20, 21]. However, no prior study has established a role of miR-375 either in BMPAC/stem cell biology for ischemic tissue repair.

Here, we provide the first evidence of miR-375 to be an independent downstream target of IL-10 and miR-375 knockdown in BMPACs improve their retention and function in ischemic myocardium.

MATERIALS AND METHODS

All experiments conform to the protocols approved by the Institutional Animal Care and Use Committee. Eight-weeks-old Wild-type (WT) and IL-10 knockout (KO; IL10tm1Cgn) male mice of C57BL/6J background were procured from Jackson Research Laboratory (Bar Harbor, ME).

Bone marrow cell isolation and BMPAC culture

BMPAC isolation, *ex vivo* expansion and culture of BMPACs was performed as previously described (9). The BMPACs are phenotypically akin to mouse bone marrow derived endothelial progenitor cells and have widely published. Given the ambiguity over exact definition of mouse EPCs, we name these cells as BMPACs. In brief, bone marrow mononuclear cells were isolated from mice by density-gradient centrifugation with Histopaque-1083 (Sigma) and macrophage-depleted by allowing attachment to uncoated plate for 1 hour. The unattached cells were removed and plated on culture dishes coated with 5μg/ml human fibronectin (Sigma) and cultured in phenol red–free endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with 5% FBS, vascular endothelial growth factor (VEGF)-A, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. Cells were cultured at 37 °C with 5% CO_2 in a humidified atmosphere. After 4 days in culture, non-adherent cells were removed by washing with PBS, new medium was applied, and the culture was maintained through day 7. BMPACs, recognized as attaching spindle-shaped cells were used for further analysis and treatment. In all the experiments wherever mentioned, BMPAC were lipofectamine mediated transfected with miRNA inhibitor or mimic or respective controls (30nM) for 24 hrs. For lentiviral infection, BMPACs were transduced with lentivirus-GFP (3.2×10^5 PFU/ml) for 24 or 48 h.

LPS or hypoxia-induced miR-375 expression in BMPAC (*in vitro* studies)

BMPACs derived from bone marrow of WT (WT-BMPAC) and IL-10KO mice (IL-10-deficient; KO-BMPAC) were subjected to LPS (100ng/ml, sigma) insult or incubated in 1% o_2 for 24h and treated with or without IL-10 (10ng/ml, R&D systems). WT-BMPACs were treated with LPS or IL-10 or both before the addition of 5μg actinomycin D (Act-D). At increasing intervals (0, 30, 60 and 120 min) thereafter, cells were processed and changes in the amount of miR-375 were quantified by RT quantitative PCR.

Tube formation assay

WT-BMPAC and IL-10 KO- BMPAC were treated with scrambled ctrl or antimiR-375 for 24 h and media supernatant from the same was collected and added in equal quantities (1:1) of growth factor reduced EBM-2 media with 2% FBS (Lonza, Basel, Switzerland) to 1.5 × 10^4 human umbilical vein endothelial cells (HUVECs) plated on 40ul Matrigel (BD Falcon) in a 96 well plate. After incubation at 37°C in an atmosphere of 5% CO_2 gels were observed by using a phase contrast microscope (×4). The branch points for each tube structure were counted in each image. Results are represented as s.e.m for three independent experiments.

Apoptosis Assay—WT-BMPAC and IL-10 KO- BMPAC were treated with scrambled or Anti miR-375 for 24 h. Thereafter cells were subjected H_20_2 insult (100μm) for 2h and cells were evaluated for apoptosis by Tunel staining and activity of caspase-3/caspase-7. Apoptosis was measured with the cell death detection kit (Roche Diagnostics) following the manufacturer's instructions. The activity of caspase-3 and caspase-7 was detected in 96-well format by using the caspase-Glo 3/7assay kit (Promega) following the manufacturer's instructions. Results are presented as s.e.m for three independent experiments.

Proliferation assay

WT-BMPAC and IL-10 KO-BMPAC were treated with scrambled or Anti-miR-375 for 24 h. Thereafter, CyQuant and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay were performed in 96 well plates (Corning) with a cell seeding of $1X10^4$ cells/ 96 well, followed by incubation with CyQuant (Invitrogen) or MTT reagent (Sigma, St Louis, MO) following the manufacturer instructions. Results are presented as s.e.m for the three independent experiments.

Real time PCR

Expression levels of miR-375 were measured using quantitative miRNA stem loop RT-PCR technology (TaqMan miRNA assays; Applied Biosystems). This assay uses gene specific stem cell loop RT primers and TaqMan probes to detect mRNA or mature miRNA transcripts. Transcription was performed using 2ug or 10 ng total RNA and the TaqMan miRNA RT kit (Applied Biosystems). Real-Time PCR was performed on an applied biosystems 770 apparatus using the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). The amplification steps consisted of initial denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. The TaqMan specific primer 18S or U6 small nucleolar RNA was used for normalization with the threshold delta-delta cycle method [Gene Expression Macro; Bio-Rad, Hercules, CA, USA].

Luciferase assay

BMPACs were cultured (Standard BMPAC media). Further BMPACs were co-transfected with miRNA mimic mma-miR-375 or Anti miR-375 or corresponding controls (30nM) (Applied Biosystems) and a reporter plasmid containing the 3' UTR of PDK-1 inserted downstream of the luciferase reporter gene (pEZX-PDK-1-UTR; GeneCopoeia; Rockville, MD) using Lipofectamine 2000 (Invitrogen) in a 48-well plate. Twenty-four hours after

transfection, luciferase assay was performed on cell-culture supernatant using Secrete-Pair dual luminescence kit (GeneCopoeia; LabOmics).

SiRNA experiments

Small interfering RNA (siRNA) sequences targeting mouse PDK-1 were synthesized by Invitrogen (PDk-1 siRNA or a negative control (siRNA-NC) was used at a final concentration of 100n_M according to the manufacturer's instructions, and the cells were transfected for 24h. Subsequently, the knockdown efficiency in HUVECs was determined by Western blot assays. In addition, 30 n_M anti-miR-375 (Applied biosystems) was introduced alone or in combination with 100n_M PDK-1-1 SiRNA using Lipofectamine™ RNAiMAX (Invitrogen) in HUVECs. The tube formation assay and apoptosis assay was then performed as described above.

Myocardial infarction and study design

Mice were subjected to myocardial infarction (MI) by ligation of left anterior descending coronary artery (LAD) as described previously (8, 9). Immediately after LAD ligation, one set of mice received intramyocardial injection of $1x10^5$ GFP+ WT-BMPAC (n=22) or IL-10 KO-BMPAC (n=12) BMPAC with or without miR-375 knock down in a total volume of 15 μL at 5 different sites (basal anterior, mid anterior, mid lateral, apical anterior, and apical lateral) in the peri infarct area. All the mice were followed up for LV functional changes on 7, 14 and 28 days and structural remodeling at 28 days post-MI.

Echocardiography

Transthoracic two-dimensional M-mode echocardiogram was obtained using Vevo 770 (Visual Sonics, Toronto, Canada) equipped with 30 MHz transducer. Echocardiographic studies were performed before (baseline) and at 7, 14 and 28 day's post-MI on mice anesthetized with a mixture of 1.5% isoflurane and oxygen (1 L/min). M-mode tracings were used to measure LV wall thickness, end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD). Percent fractional shortening (%FS) was calculated as described (8, 9).

Morphometric studies

The hearts were perfusion fixed with 10% buffered formalin. Hearts cut into 3 slices (apex, mid-LV and base) and paraffin embedded. The morphometric analysis including infarct size and wall thickness and percent fibrosis was performed on Masson's trichrome stained tissue sections using Image-J software (NIH, version 1.30, http://rsb.info.nih.gov/ij/). Fibrosis area was measured to determine percent fibrosis (9).

Immunofluorescence for BMPAC retention and engraftment in myocardial tissue

Immunofluorescence staining for tissue sections was performed as described previously (9). Proliferation of BMPAC was assessed by GFP/ BrdU+ cells in the border zone of infarcted myocardium and expressed as number per HVF at 5 days. Whereas for the mice received anti miR-375 WT/IL-10 BMPAC therapy capillaries formation was (Lectin positive) assessed in 10 randomly selected low-power visual fields (LPF) 28 days post MI. Nuclei

were counter-stained with 4', 6-diamidino-2-phenylindole (DAPI, 1:10000, Sigma Aldrich, St Louis, MO), and sections were examined with a fluorescent microscope (Nikon, Japan).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labelling (TUNEL) staining for apoptosis in the myocardium

At 5 day's post-MI, myocardial apoptosis was determined by TUNEL staining on 4μm thick paraffin-embedded sections as per manufacturer's instructions (Cell death detection assay, Roche, Indianapolis, IN). Also BMPAC's were GFP^+. DAPI staining was used to count the total number of nuclei. Counting the number of GFP+/TUNEL+ cells per HVF assessed apoptosis of transplanted BMPACs at 5days post MI. Whereas mice received scrambled/ miR-375 knockdown BMPAC, Tunel assay was performed counting the number of a-sarcomeric actinin TUNEL+ cells per HVF assessed Apoptosis of transplanted BMPACs at 5 days post MI.

Isolation of neonatal rat cardiomyocytes and treatments

Isolation of neonatal rat ventricular myocytes and treatments NRCM were prepared by enzymatic digestion of hearts obtained from newborn (0–2 day old) Sprague–Dawley rat pups using percoll gradient centrifugation and plated on six-well cell culture grade plates (coated with collagen IV) at a density of 0.85 × 106 cells/well in DMEM/M199 medium and maintained at 37°C in humid air with 5% CO_2. Cells were treated with BMPAC control conditioned medium or anti-miR-375 conditioned medium and subjected to 100uM H202 stress and Tunel assay was performed as mentioned above.

Statistical analyses—Data are presented as mean±SE between two groups; unpaired Student's *t*-test determined significance. For >2 groups, ANOVA with Turkey post hoc was used. P values of <0.05, <0.01 and <0.001 were considered significant.

RESULTS

IL-10 regulates miR-375 expression in BMPAC

We assessed miR-375 levels in the LV tissue of the MI mice and found to be significantly higher compared to sham at 5 days post MI (***P*<0.01 vs sham**; Fig 1A). Exogenous recombinant IL-10 therapy substantially reduced miR-375 expression in the ischemic myocardium (Fig 1A; **##p<0.01 Vs MI**). As miR-375 has been identified to be robustly up-regulated in mononuclear cells from IL-10 KO mice [16] and the biological function of this miR has never been studied in cardiovascular physiology. We measured miR-375 levels after different stimulus in BMPAC. The LPS-dependent up-regulation of miR-375 observed in BMPAC was significantly higher in BMPACs obtained from IL-10 KO animals, which also showed higher basal levels of miR-375 (Fig 1B; ***p<0.001 Vs WT BMPAC ctrl**). Similarly compared to WT BMPAC, miR-375 was expressed at 15 fold higher level in IL-10 KO BMPACs cultured under hypoxic conditions (Fig 1C; ***p<0.001 Vs WT BMPAC Ctrl**) indicating that, miR-375 is a stress marker for BMPAC and induced by inflammatory (LPS) and hypoxia and is in turn subject to negative regulation by IL-10. In the attempt to investigate the mechanism whereby IL-10 decreases miR-375 levels, we examined the effect of IL-10 on miR-375 post-transcriptional stability. BMPACs were stimulated with LPS with

or without IL-10 and then were examined for the rate of miR-375 decay following blockade of further transcription using actinomycin D. At increasing intervals thereafter, cells were processed and changes in the amount of miR-375 were quantified by RT quantitative PCR (qRT-PCR). The LPS-dependent miR-375 stability was observed in BMPAC. Interestingly IL-10 markedly reduces miR-375 half-life compared with LPS (Fig 1D) these observations suggest that IL-10 controls miR-375 expression at post-transcriptional level and regulates inflammation and/or ischemia induced expression of miR 375.

Knockdown of miR-375 enhances BMPAC functions

To enable the detailed study of the role of miR-375 biological function in BMPACs, BMPACs were transfected with anti-miR-375 or scrambled non-specific anti miRs. Transfection significantly repressed miR-375 as compared to scrambled BMPAC. (Supplement Fig 1; **p<0.001 vs scrambled BMPAC**). We further assessed BMPACs functions: tube formation, cell viability and proliferation in both WT and IL-10 KO BMPACs. The exposure of BMPACs with anti-miR 375 significantly increased the tube formation ability compared to control cells. More interestingly, anti-miR-375 treatment to IL-10 KO BMPAC, which are functionally impaired and have poor tube formation ability, partially restored their tube formation ability suggesting IL-10 regulates miR-375 mediated BMPAC angiogenic activity (Fig 2A–B; **P<0.01 Vs Scrambled BMPAC**). Anti-miR-375 in WT-BMPAC significantly reduced apoptosis whereas miR-375 knockdown in IL-10 KO BMPAC partially reduced apoptosis exposed to H_20_2 evident by both decreased Tunel positive cells (Supplement Fig 2; **p<0.001 vs Scrambled BMPAC**) and quantification of tunel+ cells and caspase3/7 levels compared to their respective controls (Fig2C–D: **P<0.01 Vs Scrambled BMPAC**), suggesting IL-10 regulates miR-375 mediated survival activity. Treatment of BMPACs with anti-miR-375 enhanced proliferation of WT-BMPAC compared to scramble BMPACs revealed by Ciquant assay (Fig 2D) and MTT assay (Supplementary Figure-3). Whereas, anti-miR-375 treatment to IL-10 KO BMPAC partially restored proliferation compared to IL-10 KO scrambled BMPAC suggesting IL-10 regulates miR-375 mediated BMPAC proliferation. Interestingly miR- 375 mimic treatment to both wild type and IL-10 KO BMPAC rendered them more prone to apoptosis and impaired tube formation ability on matrigel (Supplement Fig-4 and Supplementary Figure-5). These observations suggest that IL-10 regulates miR-375 mediated BMPAC functions *in vitro*.

PDK-1 is a direct target of miR-375

To identify potential targets of miR-375, Target Scan program designed to predict mRNA targets of microRNAs was used. One of the predicted targets for miR-375 is PDK-1. To investigate whether miR-375 affects PDK-1, anti-miR-375 or scrambled oligo were transfected in BMPAC. Subsequent analysis by real-time PCR (Fig-3A; **P<0.01 Vs Scrambled BMPAC**) and western blot showed significant up-regulation of PDK-1 in cells transfected with anti-miR-375, suggesting PDK-1 as a potential target for miR-375 in BMPAC (Fig 3B–C; **P<0.01 Vs Scrambled BMPAC**). To further confirm if PDK-1 was a direct target of miR-375, luciferase assay was performed with the pEZX-PDK-1-UTR (vector with 3′-UTR of PDK-1) co-transfected into the BMPAC with miR-375 mimic or anti miR-375 or respective scrambled controls. We found significantly decreased luciferase activity with miR-375 mimic over expression as compared to scrambled negative control

(Fig 3D; **p<0.001 vs scrambled negative control**). Whereas anti miR-375 significantly increased luciferase activity (Fig 3D; **P<0.001 vs scrambled control**) suggesting that miR-375 directly targets *PDK-1*. Since miR-375 is predicted to target PDK-1/AKT cell survival signaling, we determined the effect of IL-10 on AKT -phosphorylation in BMPACs transfected with anti-miR-375 or pre-miR-375. In un-transfected control BMPACs, IL-10 stimulated significant AKT -phosphorylation within 15 min; overexpression of miR-375 inhibited IL-10 responsiveness to AKT -phosphorylation while down-regulation of miR-375 restored IL-10 sensitivity (Fig 3E).

Effects of miR-375 on angiogenesis and apoptosis are PDK-1 dependent

To investigate whether anti miR-375 exerts its angiogenic and anti-apoptotic effects via PDK-1, we knocked down PDK-1 in human umbilical vein endothelial cells (HUVECs) using siRNA (Fig. 4A–4B) and further assessed their functions in apoptosis and tube formation assay. PDK-1 silencing exaggerated H_20_2 induced apoptosis and inhibited tube formation compared to controls. Intriguingly in the co-transfection of PDK-1 siRNA and anti-miR-375 in HUVECs, the effect of anti-miR-375 on HUVECs apoptosis and tube formation ability was completely abolished by PDK-1 siRNA (Fig 4C–4D). These data confirm that miR-375 enhances angiogenesis and reduces susceptibility to apoptosis in a PDK-1 dependent manner.

Increased survival of miR-375 knockdown BMPACs in the heart following MI

Superiority of miR-375 knockdown BMPACs in the face of h202 challenge relative to scrambled ctrl BMPACs in terms of survival, we found *in vitro*, was further validated *in vivo*. BMPACs transduced with lentiviral GFP particles had >95% transduction efficiency (Supplement Fig-6). We examined the retention and survival of GFP+BMPACs after their transplantation in the ischemic myocardium (5 days after MI). As shown in (Fig 5A–C; **p<0.001 vs scrambled**), mice receiving miR-375 knockdown BMPACs had a higher number of GFP+BMPACs retained in the myocardium as compared with scrambled BMPAC ctrl ($P<0.001$). Interestingly, in scrambled BMPAC ctrl group, a large number of these GFP+ cells were undergoing apoptosis as compared with the mice that received miR-375 knockdown BMPACs. (Fig 5D; **p<0.01 vs scrambled BMPAC**). Further, BMPACs engineered with miR-375 knockdown showed typical characteristics of increased proliferation observed *in vitro* was further validated *in vivo*. $BrdU^+/GFP^+$ cells were also significantly increased in miR-375 knockdown BMPACs compared with scrambled ctrl BMPACs indicating increased proliferation of the transplanted BMPACs (Fig 5E–G;**P<0.001 Vs scrambled BMPAC**). We also examined the cardiomyocyte apoptosis after anti-miR-375 treated BMPACs transplantation in the border zone of the infarct (5 days after MI). Interestingly, in scrambled BMPAC group, a large number of these cells were undergoing apoptosis as compared with the mice that received anti-miR 375 BMPAC (Fig 5H–J; ***P*<0.01Vs scrambled BMPAC**). This data suggests that miR-375 knockdown BMPACs protects cardiomyocyte apoptosis in the ischemic myocardium. Collectively these data suggests that, miR-375 knockdown in BMPACs protects transplanted BMPAC in ischemic myocardium and thereby increases the numeric availability (retention) of live BMPACs leading to enhanced myocardial repair and LV function.

PDK-1 is up-regulated in the anti-miR-375 BMPACs transplanted hearts after MI

As PDK-1 is a potential target of miR-375 and also PDK-1 plays an important role in survival following MI [22, 23]. Therefore, we examined PDK-1 protein expression (Fig 6A–C) and its downstream target AKT in the border zone of infarct at 5 d post-MI. Cardiomyocyte survival was associated with increased PDK-1 levels and AKT phosphorylation after MI. These data suggest that the miR-375-knockdown-mediated increase in PDK-1 expression was directly associated with the suppression of post-MI apoptosis.

Anti-miR-375 BMPACs transplantation attenuates adverse LV remodeling and function after MI

To assess the influence of anti-miR-375 on BMPAC-mediated effects on LV remodeling; % fibrosis area was assessed at 28 days after MI. Scrambled BMPAC significantly attenuated % fibrosis (Fig 7A–D; **P<0.01vs MI**). Interestingly, anti-miRNA 375-BMPAC treated mice showed further reduction in fibrosis (Fig 7A–D; **p<0.001 vs scrambled BMPAC**). Whereas IL-10 KO BMPAC treated with anti-miR-375 partially restored reduction in % fibrosis compared to scramble IL-10 KO BMPAC suggesting the role of IL-10 in regulating miR-375 in BMPAC mediated improvement in cardiac functions. (Supplementary Fig. 7).

To determine the effect of miR-375 knockdown on BMPAC-mediated neovascularization, we assessed capillary density in the border zone of the infarct. As shown in (Fig 7E–H; **p<0.001 vs scrambled BMPAC**) the number of Lectin+ capillaries were significantly higher in mice receiving anti-miR-375 treated BMPACs compared to those receiving scrambled miR treated BMPACs. Interestingly IL-10 KO BMPAC treated with anti-miR-375 partially enhanced neovascularization compared to scramble IL-10 KO BMPAC suggesting the role of IL-10 in regulating miR-375 in BMPAC mediated improvement in cardiac functions. (Supplementary Fig.7).

Since our data in the preceding experiments established that IL-10 suppresses miR-375 expression, we determined if ex vivo knockdown of miR-375 in BMPACs before transplantation mimics IL-10 protective effects at 7, 14 and 28 days, post-MI. As expected and in support of number of published studies mice that received scrambled miR treated BMPAC (control) showed significantly increased %EF and %FS (**#P<0.001 vs MI**, Fig.7I and 7J) at 28d, post-MI as compared to placebo (saline). Interestingly, anti-miR-375 treated BMPACs robustly enhanced increase in %EF and %FS (P<0.001 vs control BMPAC; Fig.7I &7J). Interestingly IL-10 KO BMPAC treated with anti-miR-375 partially restored cardiac functions compared to control IL-10 KO BMPACs (supplementary Fig 7).

Interestingly anti miR-375 conditioned medium protected neonatal rat ventricular myocytes apoptosis (subjected to H_20_2 injury) compared to scrambled ctrl BMPAC (**p<0.01;** supplementary Fig. 8A–B). We further validated the paracrine activity of BMPAC in vivo and found VEGF, HGF, IGF-1, Ang-1 and SDF-1 were markedly increased in BMPAC treated with anti-miR-375 compared to scrambled ctrl and saline group in the myocardium at 28 days post-MI was assessed by quantitative RT-PCR (Supplementary Fig.9).

DISCUSSION

Cellular therapy has emerged as a potential regenerative strategy for patients with acute myocardial infarction (MI). In preclinical studies, BMPACs have been shown to enhance neovascularization and improve post-MI ventricular functions paving ways for BMPAC based clinical trials with modest success. However, the inflammatory and ischemic myocardial environment resulting in reduced survival and function of BMPAC/stem cells constitute important liabilities for autologous BMPAC/stem cell-based therapies, thereby compromising full benefits of post-infarct cardiovascular repair [7, 24]. We have shown that combined therapy with bone marrow progenitor cells and IL-10 attenuates inflammatory response in the myocardium and also enhanced neovascularization and further improvement in LV function [15]. We have previously shown that bone marrow progenitor cells from IL-10 KO mice display functional impairment and decreased survival when transplanted in ischemic myocardium [15].

In this study we observed that IL-10 KO BMPACs show high basal levels of miR-375 and that exposure of wild type BMPAC to stress leads to up-regulated miR-375 expression. Importantly we also observed miR-375 to be elevated in LV tissue after MI and exogenous IL-10 therapy has an inhibitory effect on the same. Since this has been an important miR related to cancers and since IL-10 deficient BMPAC show high levels of miR, we speculated this miR to play an important role in BMPAC-mediated angiogenesis and ischemic myocardium. Additionally, the biological function of this miR has never been studied in cardiovascular physiology although a single study reported elevated levels of miR-375 in HF patients [20]. Our results show that miR-375 indeed play a negative role both in the BMPAC survival and function as well in post-MI functional recovery by directly targeting PDK-1-Akt signaling pathways and IL-10 suppresses miR-375 expression. Several lines of evidence support our conclusions: (1) inflammation and hypoxia/ischemia up regulates miR-375 expression which is suppressed by IL-10; (2) Knockdown of miR-375 in BMPAC enhances their survival and functions functional rescue of IL-10 KO BMPAC, *in vitro*; (3) knockdown of miR-375 enhances the retention, survival and function of the intramyocardially transplanted BMPACs; (4) enhanced BMPAC survival in the ischemic myocardium is associated with augmentation of BMPAC-mediated neovascularization and further improvements in the LV function when compared with BMPAC transplantation alone (5) mechanistically, miR-375 effects on BMPACs appear to be mediated through PDK-1/AKT signaling mechanisms On a whole, the present study represents a logical extension of further mechanisms of IL-10 and is entirely novel since it is not focused upon IL-10 per se but on miR-375 as an independent downstream target of IL-10 activating PDK-1/AKT axis.

Prolonged inflammation has been implicated with reduced BMPAC mobilization, cell death, and functional impairment.[25, 26] Interestingly, Schaefer et al that reported that IL-10 KO mononuclear cells express high levels of miR-375[16], however whether miR-375 modulates BMPAC survival and function when subjected to inflammatory or hypoxia and the role of this miR in cardiovascular injury and repair has never been reported. Corroborating with Schaefer et al, we observed that, IL-10 KO BMPAC express significant up-regulation of miR-375 at basal level and the levels of miR-375 are significantly up-

regulated in WT-BMPACs both under inflammation, hypoxia or ischemia. Interestingly exogenous IL-10 therapy significantly reduced miR-375 expression; suggesting IL-10 regulates miR-375 levels in BMPAC. Most importantly, we identified miR-375 as an IL-10–regulated miRNA and IL-10 directly limits miR-375 at a post-transcriptional level and thereby reducing its expression. The present data suggests miR 375 as a downstream target of IL-10 and that IL-10 mediated inhibition of miR 375 may in part explain positive effects of IL-10 in BMPACs.

We confirmed that miR-375 directly interacts with PDK-1 by luciferase assay. This is consistent with a recent report showing that miR-375 directly targets PDK-1 at the protein level in gastric carcinoma cells [17]. Furthermore, we showed that knockdown of miR-375 increased the phosphorylation of Akt in BMPACs and post MI heart by targeting PDK-1. Therefore, we propose that repression of miR-375 may provide a survival advantage to BMPAC and cardiomyocytes via activation of the PDK-1/Akt survival pathway. Several reports suggest the importance of PDK-1 in cardiovascular biology. PDK-1-MCKCre mice showed impairment of LV contraction [22]. It was also reported that cardiomyocytes deficient for *PDK-1* were sensitive to hypoxia [23], and that ischemic preconditioning failed to protect *PDK-1*-hypomorphic mutant mice against myocardial infarction [27]. PDK-1 has been shown to be a pivotal effector to promote survival of cardiomyocytes in vivo [22]. Therefore it appears up-regulation of PDK-1, by targeting miR-375 inhibition, in the hearts may emerge as a potential therapeutic strategy for heart failure.

Further, we have shown that anti-miR-375 treatment enhances BMPAC functions such as tube formation ability, proliferation and reduction in apoptosis in vitro and transplantation of anti-miR-375 treated BMPAC enhanced neovascularization, reduced infarct size and attenuated LV dysfunction. Interestingly miR-375 knock down in IL-10 KO BMPAC partially restored their functions in *vitro* and *in vivo* as well, suggesting the observed functional benefits were IL-10 dependent. Our data demonstrating increased PDK-1 expression and AKT phosphorylation in miR-375 knockdown BMPACs and the ischemic myocardium suggests the role of PDK-1 in enhancing BMPAC functional benefits. PDK-1 plays an important role in promoting cell survival, as loss of PDK-1 has been implicated in endothelial cell apoptosis. Therefore, the poorer tube formation, cell proliferation and enhanced cell death of IL-10 KO BMPAC might be due to inactivation of AKT, which is well established to play an important role in endothelial cell biology and angiogenesis by activating anti-apoptotic, pro-survival signaling cascades [28, 29]. Our PDK-1 knock down experiments in this study further confirm its crucial role in modulating BMPAC functions.

Another explanation for this finding could be our observation of enhanced paracrine factors secretion by anti-miR-375 treated BMPAC compared to scrambled ctrl BMPACs contributing to not only vasculogenesis but also myocytes protection are in line with a recent report suggesting the role of miR-133a in enhancing CPCs paracrine effects [30].

CONCLUSION

In summary, our observations demonstrate that inhibition of IL-10 dependent miR-375 exhibits pleiotropic beneficial effects, which contribute to the enhanced BMPAC survival

and angiogenic potential. Thus, miR-375 knockdown BMPAC therapy appears to be feasible approach to limit ischemic injury and might prove to be an attractive therapeutic strategy for patients with MI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

We believe that our work identifies a novel signaling that determines the survival and tissue repair function of adoptively transferred stem cells in ischemic myocardium. Moreover, our findings have a clear translational value. Bone marrow derived progenitor cells have been used in clinical trials of heart failure with only modest success rate. One of the recognized limitations of stem cell based therapies is extremely low retention, survival and alteration in the function of transplanted stem cells in the ischemic and inflamed myocardium, thereby compromising the full functional benefits of cell based therapies. We provide evidence that ex vivo modulation of miR375 expression in EPCs before transplantation not only enhances survival but also repair function of these cells. Finally, ours is the first study to demonstrate a negative role of miR375 in cardiovascular injury context and potentially be applicable to other types of stem cell therapies for ischemic tissue repair.

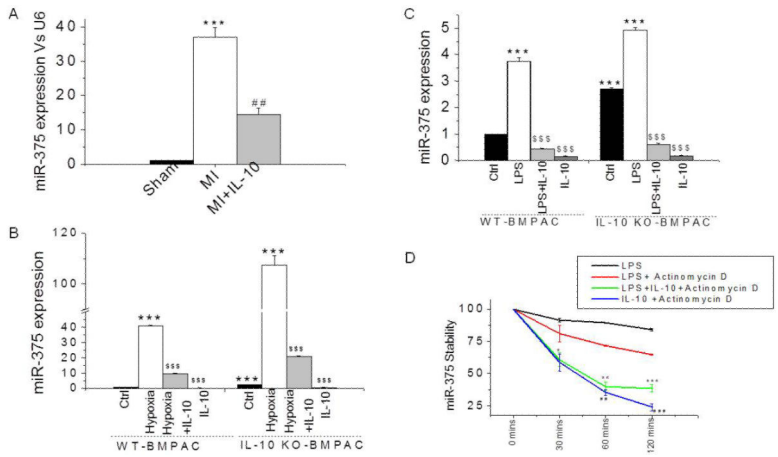

Fig. 1. IL-10 regulates miR-375 expression in BMPAC

(A) IL-10 inhibits MI-induced myocardial miR-375 expression. ***p<0.001Vs sham injured hearts; ##p<0.01Vs MI injured hearts (n=4) **(B)** WT-BMPAC/IL-10 KO BMPAC were stimulated with LPS, with LPS + IL-10 or IL-10 alone. Expression of miR-375 was measured by RT-PCR. **(C)** BMPAC were subjected to hypoxia, hypoxia +IL-10 or IL-10 alone and miR-375 expression was measured by RT-PCR normalized to U6 with or without IL-10. *n* = 3. ****P*< 0.001 Vs WT-Ctrl BMPAC; $$$.p<0.001 Vs WT/IL-10 KO BMPAC +LPS. **(D)** BMPACs were treated with LPS or IL-10 or both before the addition of 5μg actinomycin D (Act-D). BMPACs were harvested 30, 60, and 120 min after the addition of actinomycin D (time 0), RT-qPCR was performed for miR-375. Expression data are expressed as percent of miR-375 remaining at each time point vs. miR-375 levels at time 0. miR expression was normalized to U6 snRNA. ***P*< 0.01 ****P*< 0.001 Vs LPS alone; ##. P<0.01###.p<0.001 Vs LPS+ actinomycin-D.

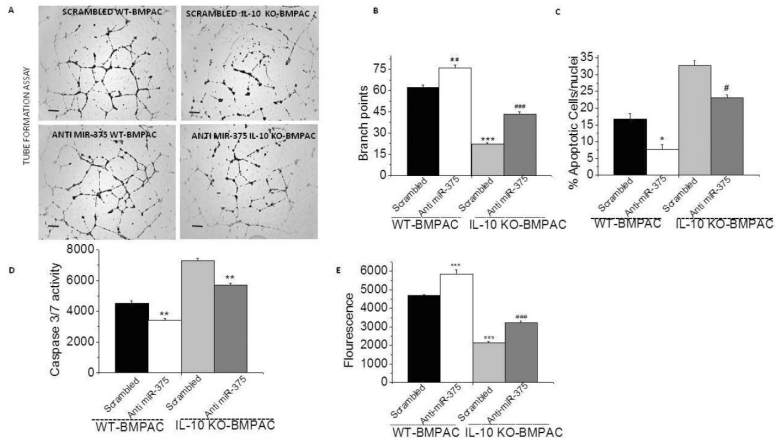

Fig.2. Knockdown of miR-375 enhances WT BMPAC functions and rescues IL-10 KO BMPAC phenotype

(A) The representative photomicrographs (10X, 20uM scale bar) of tube formation by matrigel angiogenesis assays in WT- BMPAC/IL-10 KO BMPAC transfected with scrambled or anti-miR-375. (B) Relative quantification of branch points, (C) Quantification of percentage of Tunel+ cells were measured by fluorescence microscopy after apoptotic stimuli of 100μmol/L H202 after WT- BMPAC/IL-10 KO BMPAC transfected with scrambled or anti-miR-375. (D) Quantification of apoptosis by caspase 3/7 assay. Quantification of BMPAC proliferation measured by (D) ciQuant assay after WT- BMPAC/ IL-10 KO BMPAC transfected with scrambled or anti-miR-375. Results are presented as s.e.m for three independent experiments. ***$P < 0.001$, **$P < 0.01$, *P<0.05 Vs WT Scrambled BMPAC; ###p<0.001, ##p<0.01, #p<0.05 Vs IL-KO scrambled BMPAC.

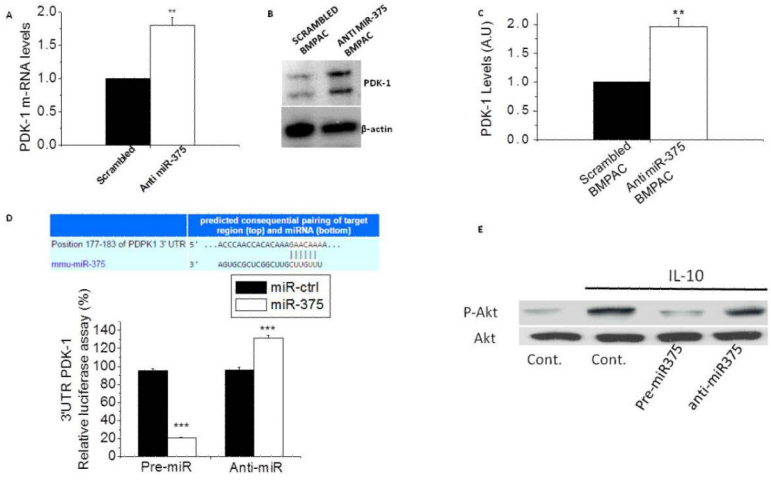

Fig.3. miR-375 directly targets PDK-1

(A) Relative mRNA expression of PDK-1 normalized to 18S (B) Representative immune-blot of PDK-1. *(C)* Relative quantification of PDK-1 protein. (D) Study of the interaction between miR-375 and 3′UTR of PDK-1 mRNA by luciferase assay (E) Overexpression of miR-375 attenuates AKT phosphorylation. Results are presented as s.e.m for three independent experiments. $n = 3$. ***$P < 0.00$, **$P < 0.01$, *$P < 0.05$ Vs WT Scrambled BMPACs.

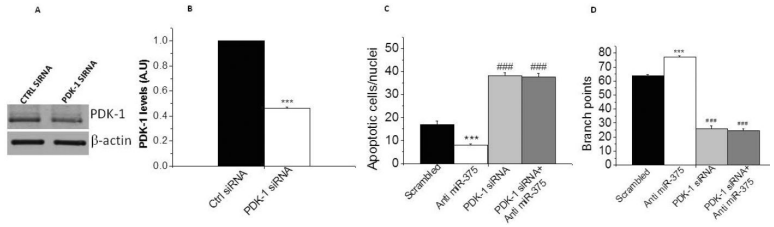

Fig.4. Down-regulation of PDK-1 inhibits the effects of anti-miR-375 on HUVECs apoptosis and tube formation

HUVECs were transfected with NC-siRNA or PDK-1 for 24h. (A) Representative PDK-1 protein levels (B) Quantification of PDK-1 levels normalized to β-actin. HUVECs transfected with NC-siRNA, anti-miR-375, PDK-1 siRNA, PDK-1 siRNA+ anti miR-375. (C) Quantification of apoptosis by tunel assay. (D) Relative quantification of branch points, Results are presented as s.e.m for three independent experiments. *n* = 3. ***p<0.001Vs Scrambled ctrl HUVECs. ### P<.001 Vs anti miR-375 treated HUVECs.

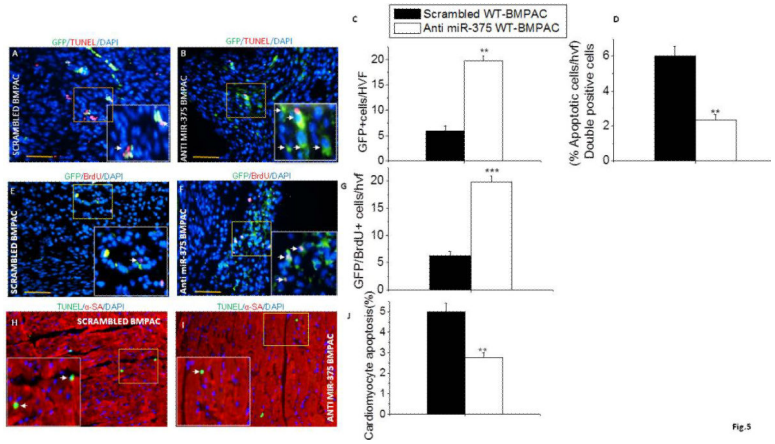

Fig.5. Increased survival of miR-375 Knockdown BMPACs in situ in the heart following myocardial infarction

BMPAC retention and survival in the myocardium at 5 days after MI in anti-miR-375 BMPAC or scrambled BMPAC treated mice. Tunel staining for detecting apoptosis (red) of BMPAC (GFP-positive, green florescence) and DAPI (blue) for nuclear staining. Inset is higher magnification of yellow-boxed area. Arrows indicate GFP+TUNEL+cells (40x, Scale bar 100μm). (A–C) Quantification of GFP+(BMPAC) at 5 days post MI. (D) Quantitative analysis of GFP/TUNEL double positive cells at 5 days after MI. (E–F) Increased GFP +BrdU+ cells (40x, Scale bar 100μm) within hearts treated with anti-miR-375 BMPAC compared scrambled BMPAC-treated hearts stained with green fluorescent protein (GFP; green), BrdU (red), DAPI (blue) for nuclei staining. Inset is higher magnification of yellow-boxed area. Arrows indicate GFP+BrdU+cells. (G). Quantification of BrdU+/ GFP+ cells in mice treated with anti-miR-375 BMPAC and Scrambled BMPAC (n=5). (H–I) Representative TUNEL staining image for cardiomyocyte apoptosis (green nuclei), alpha actinin (red), DAPI (blue) in border zone of LV infarct at 5 d post-MI. (*J*) Quantitative analysis of TUNEL^{+} cardiomyocytes at 5 d post-MI. *n* = 5/group. ***P<0.001, **P<0.05 vs scrambled WT-BMPAC treated groups.

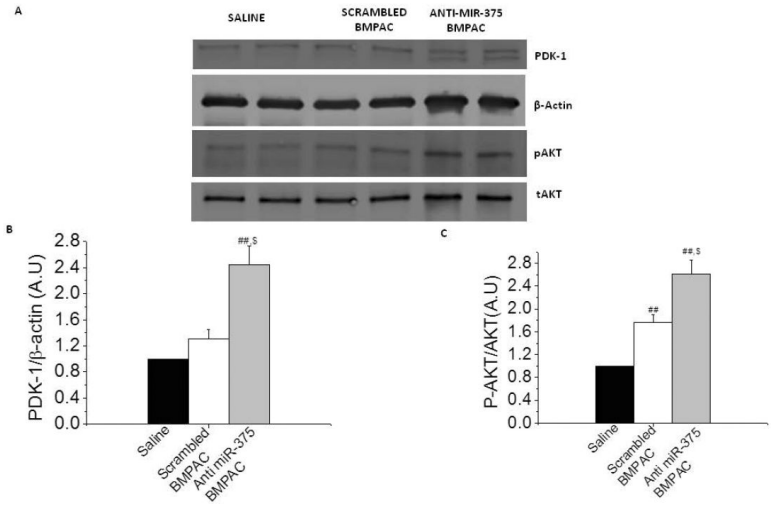

Fig.6. PDK-1 is up-regulated in the anti-miR-375 BMPACs transplanted hearts after MI

Representative western blot and its quantification for PDK-1, pAKT and total AKT protein expression in LV at 5 d post-MI normalized to β-actin. n = 5/group. ##P < 0.01 *vs* saline group and $ P<0.05 vs Scrambled BMPAC group.

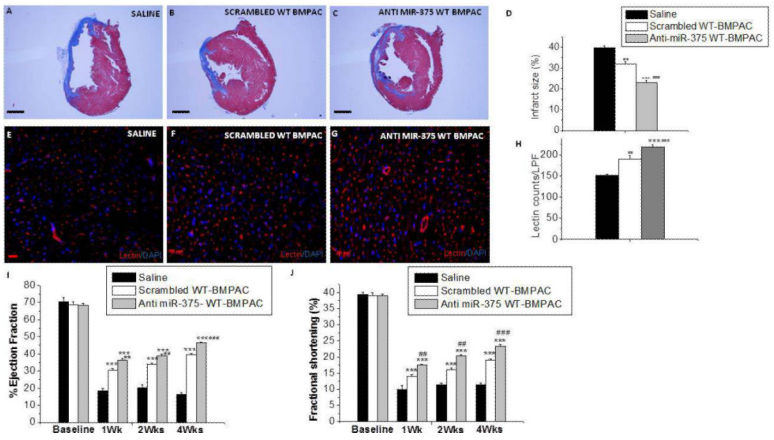

Fig.7. Transplantation of miR-375 Knockdown BMPACs reduces fibrosis and enhances neovascularization and LV functional recovery 4 weeks after MI

(A–C) Representative masons trichome stained heart (1X, 100μm scale bar) treated with saline or WT-BMPAC treated with scrambled or anti-miR-375. (*D*) Quantitative analysis of infarct size (%LV area). (E–G) Representative immunofluorescence (20x, Scale bar 100μm) capillaries images taken within the infarct border zone of mice treated with saline or WT-BMPAC treated with scrambled or anti-miR-375. Capillaries were stained with BS-lectin-Alexa-555 (red) and nuclei were counterstained with DAPI (blue). **(H)** Quantification of border zone capillary number across treatments presented as the number of isolectin B4–positive capillaries and DAPI-stained nuclei per high power field (LPF). Representative echocardiography analysis shown in bars, in the hearts treated with saline or WT-BMPAC treated with scrambled or anti-miR-375. Mice receiving miR-375 knockdown WT-BMPAC: (I) %EF, (J) FS %. n = 6/group*P< 0.05, **P< 0.01, ***P< 0.001 *vs* saline group; #P< 0.05, ##P< 0.01, ###P< 0.001 vs scrambled BMPAC group. (n=6).

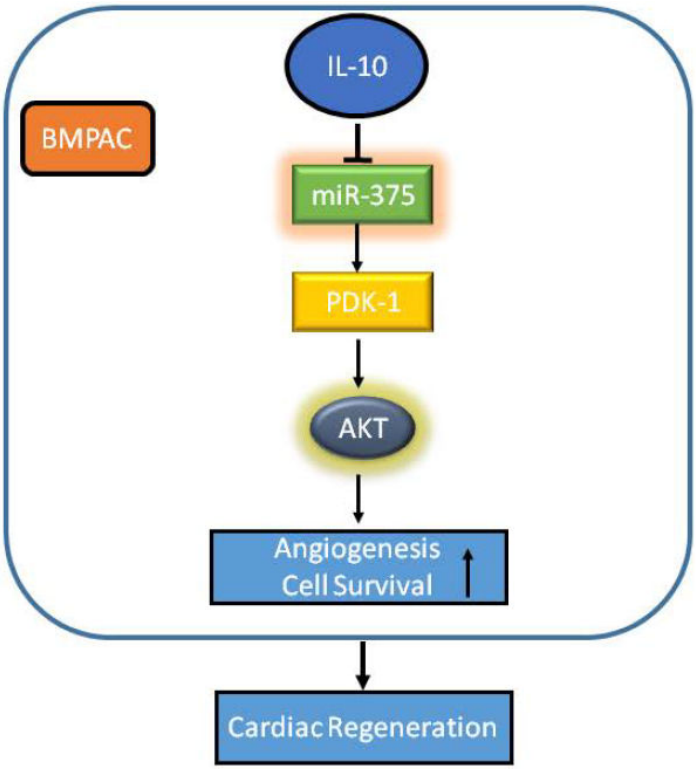

Fig.8. Proposed mechanisms demonstrating the role of miR-375 in BMPAC mediated cardiac regeneration

BMPACs inhibits IL-10 regulated miR-375 leading to activation of PDK-1/AKT signaling, PDK-1 (potential target of miR-375), thereby enhancing the neovascularization and also BMPAC survival post transplantation in MI mice. BMPAC indicates bone marrow progenitor angiogenic cell; IL-10, interleukin -10; PDK-1, 3-phosphoinositide-dependent protein kinase 1.