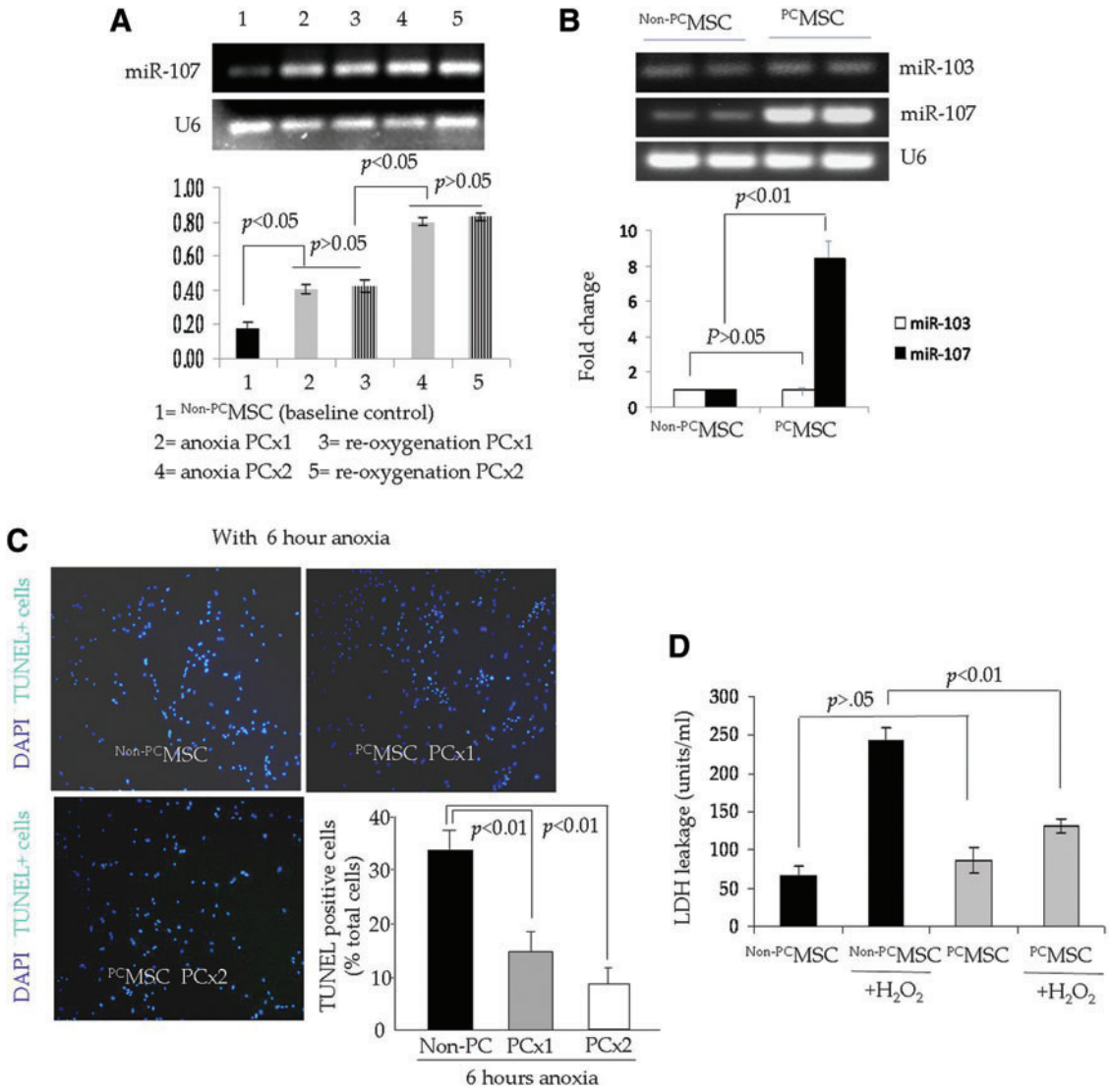
Supplementary Data

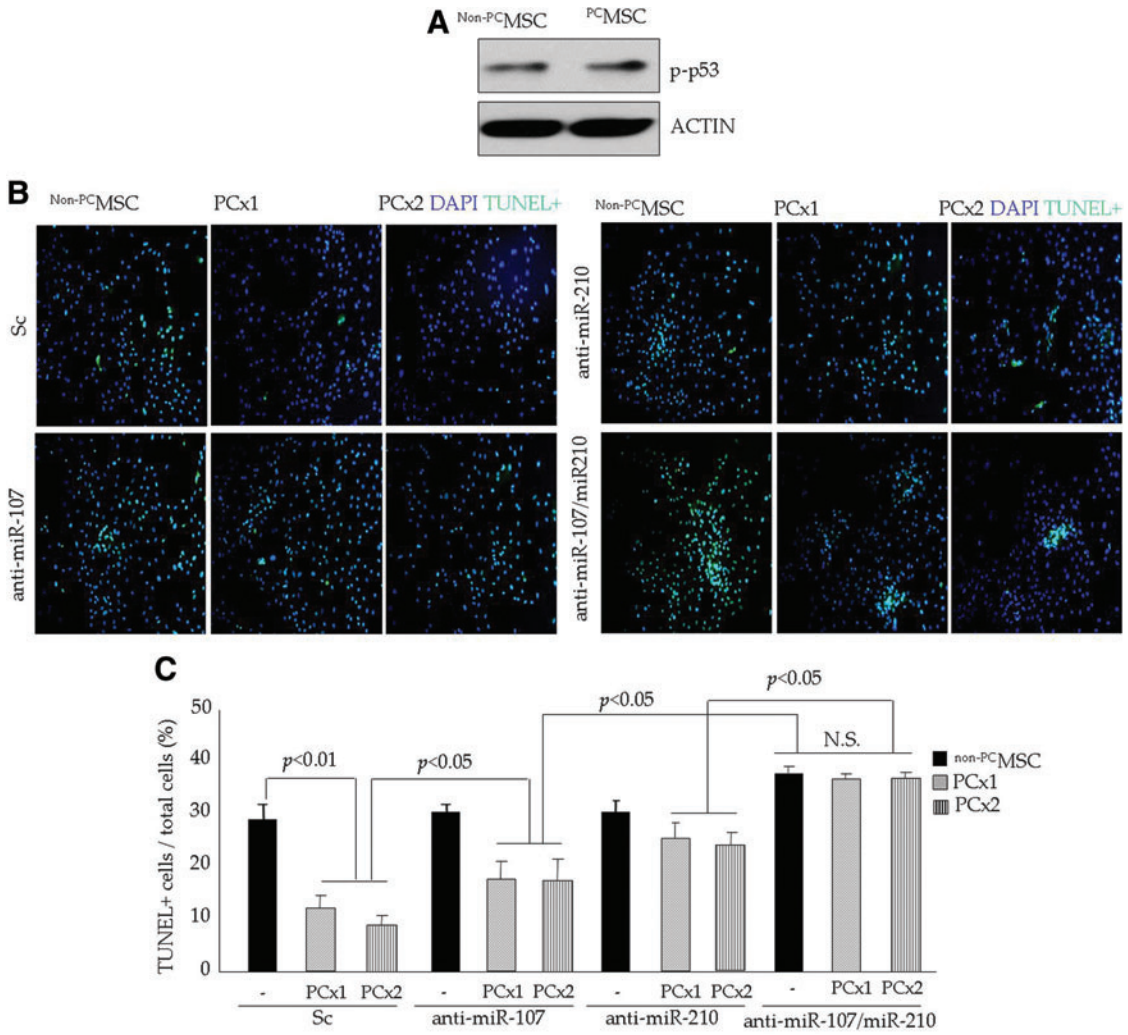
Supplementary Methods

Isolation and culture of mesenchymal stem cells

Mesenchymal stem cells (MSC) were purified from young male Fischer-344 rat bone marrow by flushing the cavity of femurs and tibias with basal Dulbecco's Modified Eagle Medium (DMEM) (2). The cells were seeded into 100 mm^3 dishes and cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37°C and 5% CO_2. Non-adherent cells were removed during routine fresh medium



SUPPLEMENTARY FIG. S1. Time course of miR-107 induction during preconditioning and its protective effects on MSC. **(A)** A study of the time course of miR-107 induction during anoxia/re-oxygenation phases of one cycle and two cycles of preconditioning. The cells were subjected to one cycle and two cycles of anoxia/re-oxygenation, and the cells were later harvested at the completion of anoxia and the re-oxygenation phases of each cycle and used to determine the expression of miR-107. Reverse transcription polymerase chain reaction showed that both PCx1 and PCx2 had a significantly higher level of miR-107 as compared with the $^{non\text{-}PC}$MSC baseline control. Although miR-107 expression was slightly increased in the samples collected at the completion of re-oxygenation phase of both PCx1 and PCx2 as compared with the samples collected at the completion of the anoxia phase of the respective cycle, the difference was negligible and statistically insignificant. **(B)** The preconditioning of MSC (PCx2) did not alter miR-103 expression in ^{PC}MSC as compared with $^{non\text{-}PC}$MSC ($p > 0.05$). **(C)** Representative fluorescence images and quantification of TUNEL positivity showing a significantly decreased number of TUNEL$^+$ cells in ^{PC}MSC preconditioned by PCx1 as well as PCx2 as compared with the $^{non\text{-}PC}$MSC after 6 h of lethal anoxia treatment. (magnification = ×200, green = TUNEL$^+$ nuclei; blue = DAPI). **(D)** LDH release assay using conditioned medium samples was obtained from various treatment groups of cells subsequent to the treatment with 100 μM H_2O_2. LDH leakage under oxidant stress was significantly reduced in ^{PC}MSC as compared with $^{Non\text{-}PC}$MSC. LDH, lactate dehydrogenase; miR, microRNA; MSC, mesenchymal stem cells; ^{PC}MSC, preconditioned MSC; $^{Non\text{-}PC}$MSC, non-preconditioned MSC; PCx1, preconditioning with A/R 30 min 1 cycle; PCx2, preconditioning with A/R 30 min 2 cycles; TUNEL, transferase-mediated dUTP nick-end labeling.



SUPPLEMENTARY FIG. S2. Role of p53 during IPC and the effect of miR-107/miR-210 inhibition on cell survival under lethal anoxia. (A) Western blot studies showed that the activation of p53 did not change in response to the preconditioning of MSC in ^{PC}MSC as compared with $^{non\text{-}PC}$MSC. **(B, C)** Representative images and quantification of TUNEL$^+$ cells after their respective treatment. The number of TUNEL$^+$ ^{PC}MSC pretreated with anti-miR-107 and anti-miR-210 was significantly increased under 6 h of lethal anoxia as compared with Sc siRNA-transfected cells. TUNEL positivity increased further when ^{PC}MSC were pretreated with both anti-miR-107 and anti-miR-210 ($p < 0.05$). $^{Non\text{-}PC}$MSC with and without anti-miR-107, anti-miR-210, and simultaneous anti-miR-107/miR-210 subjected to 6 h lethal anoxia were used as baseline control. Sc, scramble.

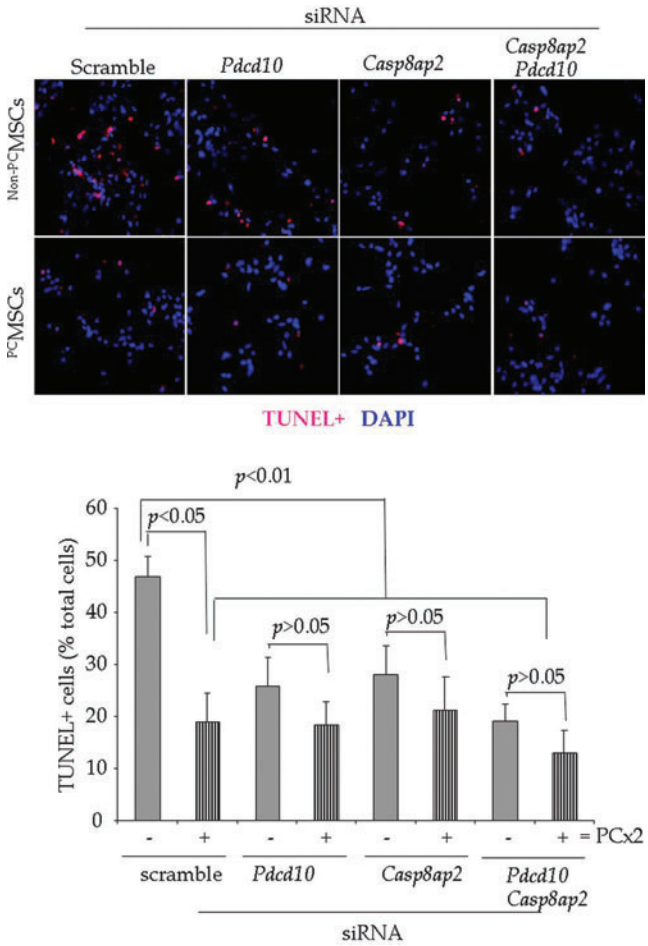
replacement every 3 days in culture. The adherent, spindle-shaped MSC were expanded and cultured for no more than two to three passages before use in experiments.

Preconditioning of MSC

The cells were preconditioned by exposure to intermittent cycles of anoxia/re-oxygenation (3). Briefly, native MSC were seeded at 5×10^5 cells/60 mm cell culture dish. The cells were starved overnight for serum and glucose before preconditioning. For ischemic preconditioning, the cells were subjected to repeated cycles of anoxia (30 min) with intermittent re-oxygenation (10 min) for specific cycles (one or two cycles) in an anoxia chamber (Forma-1025 Anaerobic System). At the end of the required number of cycles, the cells were either harvested for molecular studies or subjected to lethal anoxia for 6 h for survival studies *in vitro*. Cell survival was assessed by lactate dehydrogenase (LDH) release assay with a conditioned medium and transferase-mediated dUTP nick-end labeling (TUNEL) of cells from each treatment group, as described in Supplementary Data.

Western blotting

Western blotting was performed as described earlier (4). The cells with their respective treatment were lysed in lysis buffer, pH 7.4 (in m*M*) 50 HEPES, 5 EDTA, and 50 NaCl), 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 m*M* phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin), and phosphatase inhibitors ([in m*M*] 50 sodium fluoride, 1 sodium orthovanadate, and 10 sodium pyrophosphate) (3). Cell lysates (40 μg) were electrophoresed on 4%–12% precast SDS-polyacrylamide gel (Invitrogen) and transferred on to nitrocellulose membranes. The membrane was blocked for 1 h with 5% nonfat dry milk in PBS and 0.1% Tween-20. For detection of the antigen of interest, the membrane was incubated



SUPPLEMENTARY FIG. S3. Representative images of TUNEL$^+$ cells after their respective treatment. The number of TUNEL$^+$ ^{PC}MSC pretreated with *Casp8ap2, Pdcd10,* and *Casp8ap2/Pdcd10* siRNA was significantly reduced as compared with the Sc siRNA-transfected ^{PC}MSC on subsequent exposure to 6 h of lethal anoxia. TUNEL positivity was significantly reduced when ^{PC}MSC were pretreated with both *Casp8ap2/PDCD10* siRNA as compared with either *Casp8ap2* or *Pdcd10* alone. The Sc-transfected $^{non\text{-}PC}$MSC subjected to 6 h of lethal anoxia were used as controls and showed highest TUNEL positivity.

overnight with specific primary antibodies. The primary antigen-antibody reaction was detected by incubation of the membrane with specific secondary antibody for 1 h and detected by the Chemiluminescence ECL system (Amersham Corp.).

Transfection with microRNA inhibitors

To knock down microRNA (miR)-107 and miR-210, the siPORT™ NeoFx™ transfection agent was used for the transfection of respective anti-miR (Ambion, Inc.), as described earlier (3). Transfection was performed with miR inhibitors and NeoFx. Briefly, 5 μl NeoFx was diluted with 100 μl Opti-MEM for each sample and incubated for 10 min at room temperature. The specific inhibitor (5 pmol) was diluted with 100 μl Opti-MEM and added to the diluted NeoFx mixture. After incubation for 10 min at room temperature, the transfection mixture was added to 2.3 ml of the cell suspension (3×10^5 cells). The cell suspension containing the transfection mixture was then plated into 35 mm cell culture plates and used for further experimentation after 48–72 h of incubation.

LDH leakage and TUNEL assays

LDH leakage was measured using an LDH Assay Kit (Diagnostic Chemicals Ltd.) as per the manufacturer's protocol (1). Apoptotic cells were detected with the *In Situ* Cell Death Detection Kit (Roche Diagnostics) as per the manufacturer's protocol (1). The total number of cells and TUNEL$^+$ cells were counted using a fluorescence microscope (Olympus BX-41) after staining with DAPI.

Luciferase reporter assays

Precursor miR-107 and miR-210 expression clones were constructed in a feline immunodeficiency virus-based lentiviral vector system (pEZX-miR-107) and (pEZX-miR-210), respectively. Luciferase reporter constructs containing 3′-UTR of *Pdcd10* and *Casp8ap2* were designed to encompass the miR-107 and miR-210 binding sites (GeneCopoeia). For transfection, the cells were plated into 24-well plates in triplicate and co-transfected with 0.8 μg of pEZX-miR-107 or pEZX-miR-210 (or pEZX-miR-scramble) and the reporter construct with Lipofectamine 2000™ (Invitrogen). Transfection efficiency was normalized on the basis of *Renilla* luciferase activity. Firefly and *Renilla* luciferase activities were measured with the Dual Luciferase Reporter Assay System kit (Promega) as per the manufacturer's instructions.

Supplementary References

1. Afzal MR, Haider HKh, Idris NM, Jiang S, Ahmed RP, and Ashraf M. Preconditioning promotes survival and angiomyogenic potential of mesenchymal stem cells in the infarcted heart via NF-kappaB signaling. *Antioxid Redox Signal* 12: 693–702, 2010.
2. Jiang S, Haider H, Idris NM, Salim A, and Ashraf M. Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. *Circ Res* 99: 776–784, 2006.
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4. Lu G, Ashraf M, and Haider KH. Insulin-like growth factor-1 preconditioning accentuates intrinsic survival mechanism in stem cells to resist ischemic injury by orchestrating protein kinase cα-erk1/2 activation. *Antioxid Redox Signal* 16: 217–227, 2012.