Cross Talk of Combined Gene and Cell Therapy in Ischemic Heart Disease

Role of Exosomal MicroRNA Transfer

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Background—Despite the promise shown by stem cells for restoration of cardiac function after myocardial infarction, the poor survival of transplanted cells has been a major issue. Hypoxia-inducible factor-1 (HIF1) is a transcription factor that mediates adaptive responses to ischemia. Here, we hypothesize that codelivery of cardiac progenitor cells (CPCs) with a nonviral minicircle plasmid carrying HIF1 (MC-HIF1) into the ischemic myocardium can improve the survival of transplanted CPCs.

Methods and Results—After myocardial infarction, CPCs were codelivered intramyocardially into adult NOD/SCID mice with saline, MC-green fluorescent protein, or MC-HIF1 versus MC-HIF1 alone (n=10 per group). Bioluminescence imaging demonstrated better survival when CPCs were codelivered with MC-HIF1. Importantly, echocardiography showed mice injected with CPCs+MC-HIF1 had the highest ejection fraction 6 weeks after myocardial infarction (57.1±2.6%; *P*=0.002) followed by MC-HIF1 alone (48.5±2.6%; *P*=0.04), with no significant protection for CPCs+MC-green fluorescent protein (44.8±3.3%; *P*=NS) when compared with saline control (38.7±3.2%). In vitro mechanistic studies confirmed that cardiac endothelial cells produced exosomes that were actively internalized by recipient CPCs. Exosomes purified from endothelial cells overexpressing HIF1 had higher contents of miR-126 and miR-210. These microRNAs activated prosurvival kinases and induced a glycolytic switch in recipient CPCs, giving them increased tolerance when subjected to in vitro hypoxic stress. Inhibiting both of these miRs blocked the protective effects of the exosomes.

Conclusions—In summary, HIF1 can be used to modulate the host microenvironment for improving survival of transplanted cells. The exosomal transfer of miRs from host cells to transplanted cells represents a unique mechanism that can be potentially targeted for improving survival of transplanted cells. **(*Circulation*. 2014;130[suppl 1]:S60-S69.)**

Key Words: exosomes ■ genetic therapy ■ hypoxia-inducible factor-1 ■ microRNAs ■ stem cells

Despite significant advances in treatment, coronary heart disease is the leading cause of morbidity and mortality worldwide, accounting for the deaths of 3.8 million men and 3.4 million women annually according to the World Health Organization.[1] Although current therapeutic interventions for coronary heart disease improve clinical outcomes and prolong life, they are palliative in nature because they fail to address the fundamental issue of the loss of myocardium. In light of this, stem cell–based therapies have gained increasing interest as a potential therapy for not only attenuating cardiac dysfunction but also affording myocardial regeneration.

Unfortunately, progress in stem cell–based therapies has been hindered by the low percentages of transplanted cells that engraft and survive long term (<1%). This is of particular concern because early stem cell engraftment has been shown to have a direct correlation to late cardiac functional recovery.[2] One possible reason for the poor survival of transplanted cells is the hostile ischemic and inflammatory environment into which the cells are introduced. Hypoxia-inducible factor-1 (HIF1) is an oxygen-sensitive transcription factor, decreasing via proteolysis in response to normoxia and increasing because of stabilization under hypoxic conditions. Stabilization of HIF1 has been shown to mediate activation of various adaptive responses under low levels of oxygen, including glucose metabolism, cell proliferation, neovascularization, inflammation, and cellular differentiation.[3] Previously, we have demonstrated the use of a novel vector expression system termed minicircles, which provided long-term transgene expression

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Presented at the 2013 American Heart Association meeting in Dallas, TX, November 16–20, 2013.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.113.007917/-/DC1.

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of HIF1 (MC-HIF1) in vivo in the murine heart.[4] We have also shown that prosurvival microRNA (miR) cocktail involving miR-21, miR-24, and miR-221 can be used to improve the engraftment of transplanted cells and therapeutic efficiency for ischemic heart diseases.[5]

This follow-up study investigates our hypothesis that codelivery of cardiac progenitor cells (CPCs) together with MC-HIF1 into the ischemic heart can improve the potency of CPCs for cardiac repair. We tested our hypothesis by determining the survival of CPCs after transplantation with or without MC-HIF1 and by monitoring cardiac function, infarct size, and vascularity. The effects of MC-HIF1 on the host microenvironment were investigated to identify molecules that could potentially mediate cross talk between local transfected cells and transplanted CPCs. Finally, in vitro assays were performed to determine the molecular mechanisms that could give cultured CPCs increased resistance against ischemic stress.

Methods

An extended Methods is available in the online-only Data Supplement.

Isolation and Maintenance of Sca1$^+$ CPCs

Heart tissue explants were isolated from transgenic L2G mice with a ubiquitin promoter constitutively driving firefly luciferase (Fluc) and green fluorescent protein (GFP). The minced heart pieces were enzymatically dissociated into a single-cell suspension. Enrichment of Sca1$^+$ cells was achieved by sorting using the Magnetic Cell Sorting system (Miltenyi Biotec, Sunnyvale, CA). Whole primary cell suspension was incubated with phycoerythrin-conjugated anti-Sca1 Miltenyi beads in PBS+0.5% BSA, and then washed and isolated on a magnetic column to extract Sca1$^+$ CPCs according to manufacturer's instructions. To increase the purity of the Sca1$^+$ cells, magnetic sorting was performed one more time. The Sca1$^+$ cells were cultured on 1% gelatin-coated dishes in CPC media (DMEM/F12, 10% Embryonic Stem Cell-Grade FBS, Penicillin-Streptomycin-Glutamine, Insulin-Transferrin-Selenium, 1000 U/mL leukemia inhibitory factor, 40 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor) and passaged no more than 4 times.

Murine Myocardial Infarction and Cell Delivery

All animal research protocols were approved by the Stanford Animal Research Committee. Ligation of the mid-left anterior descending artery was performed in 8 to 10 weeks-old female NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME) under anesthesia (2% inhaled isoflurane) by a single experienced microsurgeon. Mice were randomized into 4 groups: (1) saline, (2) 1×10^6 CPCs with 20 μg MC-GFP; (3) 25 μg MC-HIF1 alone, and (4) 1×10^6 CPCs with 25 μg MC-HIF1 (n=10 per group). The animals were injected in the peri-infarct zone with a total volume of 25 μL using a 31-gauge Hamilton syringe.

Preparation of Conditioned Medium and Exosomes

Conditioned medium (CM) collected from endothelial cells (ECs) transfected with MC-GFP or MC-HIF1 were named EC^{GFP}-CM or EC^{HIF}-CM, respectively. Cells and debris were removed by differential centrifugation at 300*g* for 10 minutes, 2000*g* for 10 minutes, and at 13 000*g* for 15 minutes, followed by filtration (0.2 μm). The filtrated CM was then concentrated using an Ultracel-10K (Millipore, Billerica, MA) centrifugal device, to a protein concentration of ≈0.1 mg/mL before being resuspended in a 1:9 ratio with CPC medium. Protein concentration was determined using a Micro BCA Assay Kit (Thermo Scientific, San Jose, CA). For isolation of exosomes, EC^{GFP}-CM or EC^{HIF}-CM were filtered (0.2 μm) and concentrated using Ultracel-100K (Millipore). Exosomes in CM were then precipitated using Invitrogen's Total Exosome Isolation system according to manufacturer's protocol overnight at 4°C, followed by centrifugation at 12 000*g* for 1 hour and resuspension in PBS.

Exosomal MicroRNA Array Profiling

Exosomal RNA from EC^{GFP}-Exo versus EC^{HIF}-Exo was quantitated using an UV–Vis spectrophotometer, and quality was assessed using the Agilent 2000 Bioanalyzer. On the basis of obtained concentration, we used 10 ng of input RNA from each group (n=4 per group) as starting material for a quantitative polymerase chain reaction–based microRNAs (miRs) array from System Biosciences performed according to manufacturer's instructions.

Statistical Analysis

Data are expressed as mean±SEM, and statistical analyses were performed using SigmaStat 3.5 (SPSS Inc, Chicago, IL). For a 2-group comparison, a Student *t* test was applied if the pretest for normality (Shapiro–Wilk normality test) was not rejected at 0.05 significance level; otherwise a Mann–Whitney test for nonparametric data were used. Multiple group comparisons were performed using ANOVA followed by Tukey post-test. Kruskal and Wallis test followed by Dunn test comparison of pairs was used to analyze data that did not show normal distribution. *P* values of <0.05 indicate statistical significance.

Results

Codelivery of MC-HIF1 Improved Survival of Sca1$^+$ CPCs

HIF1 is known to mediate protective paracrine signaling in the ischemic heart.[6] Hence, we evaluated whether codelivery of stem cells together with MC-HIF1 could improve the survival of transplanted cells in the ischemic myocardium. Sca1$^+$ CPCs isolated from transgenic L2G mice expressed both Fluc and GFP and were amenable to lineage commitment, including differentiation into cardiac-like lineage (Figure 1A–1F). In vitro characterization demonstrated a robust linear correlation between bioluminescence signal intensity and cell numbers (R^2=0.99), indicating the validity of this imaging method for assessing cell survival in vivo (Figure 1G). Next, we assessed the survival of these CPCs noninvasively by bioluminescence imaging when acutely codelivered with MC-HIF1 or MC-GFP into mice subjected to myocardial infarction (MI). Bioluminescence imaging showed CPCs codelivered with MC-HIF1 was readily detectable as late as 42 days after injection ($3.3\times10^4\pm2.7\times10^3$ photons per second per square centimeter per steradian), in contrast to a barely detectable signal from CPCs codelivered with MC-GFP ($2.7\times10^4\pm1.7\times10^3$ p/s/cm^2/sr; P<0.05) at day 21 and undetectable by day 42 (Figure 1H). These results were also confirmed by quantitative analysis of Fluc activity (Figure 1I) and immunofluorescence staining (Figure IA in the online-only Data Supplement).

Combined Cell and Gene Therapy Improves Cardiac Outcomes

To determine whether enhanced survival of CPCs by codelivery with MC-HIF1 could improve cardiac function, 10-week-old female NOD/SCID mice subjected to MI were divided into 4 groups and received (1) saline control, (2) CPCs+MC-GFP, (3) MC-HIF1 alone, or (4) CPCs+MC-HIF1 by intramyocardial injection into the peri-infarct area. Ejection fraction and fractional shortening before MI were comparable across all groups when measured by echocardiography (Figure 2A and 2B). In the saline control group, a significant decrease

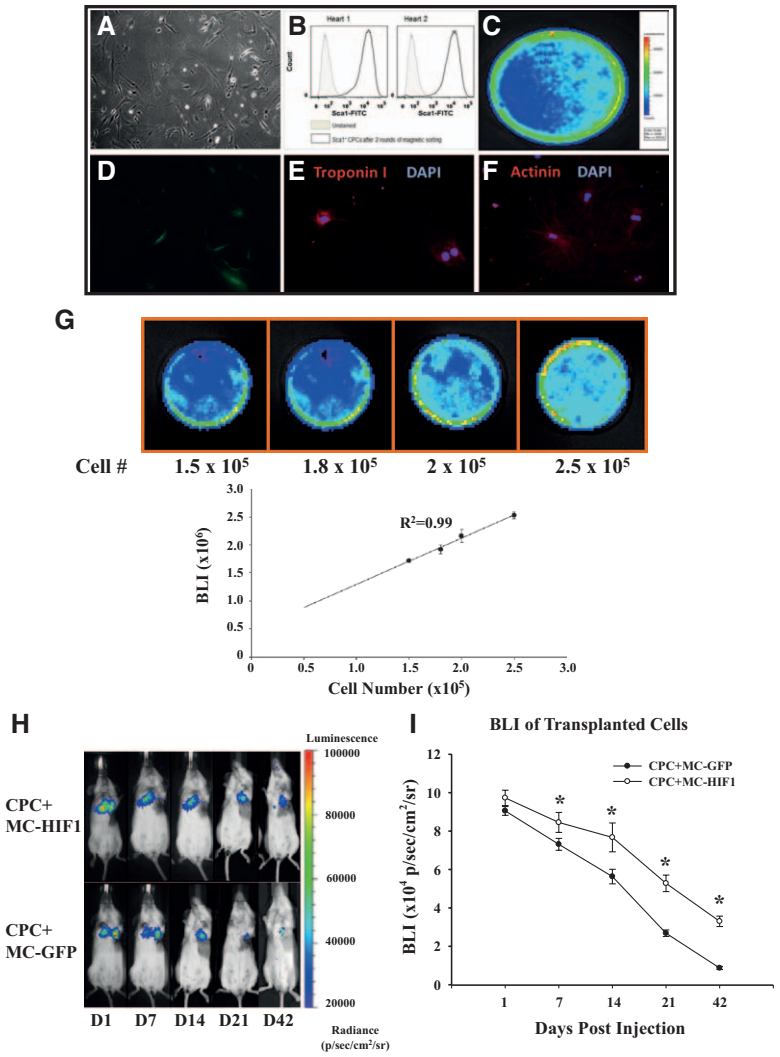

Figure 1. Codelivery of hypoxia-inducible factor-1 (HIF1) driven by minicircle (MC) plasmid promotes survival of transplanted Sca1⁺ cardiac progenitor cells (CPCs) in the ischemic heart. **A**, The morphology of isolated CPCs growing on gelatin-coated dish. **B**, Flow cytometric analysis of purified Sca1⁺ CPC population from 2 preparations. Typical purity of isolation is >95%. CPCs isolated from transgenic mice express robust (**C**) firefly luciferase (Fluc) and (**D**) green fluorescent protein (GFP) expression. After culturing in cardiac differentiation induction medium, some of the differentiated CPCs stained positively with (**E**) cardiac Troponin I and (**F**) α-actinin demonstrating its amenability to lineage commitment. **G**, CPCs were seeded into dishes with increasing cell numbers. Assessment of bioluminescence imaging (BLI) signals showed a robust linear correlation (R^2=0.99) between the cell number and Fluc expression, which is crucial for accurate tracking of cell survival by in vivo imaging. **H**, Representative BLI of animals injected with CPCs intramyocardially together with either MC-GFP or MC-HIF1 after myocardial infarction at indicated time points. **I**, Quantitative analysis of longitudinal BLI signal demonstrates that CPCs codelivered with MC-HIF1 had better survival when compared with CPCs codelivered with MC-GFP during a period of 42 days. *P<0.05 (n=10 per group). DAPI indicates 4',6-diamidino-2-phenylindole.

in ejection fraction and fractional shortening were observed, suggesting compromised cardiac function. At all measured time points, the CPCs+MC-GFP group showed no significant improvement in ejection fraction and fractional shortening when compared with saline control group. By contrast, mice receiving MC-HIF1 alone or CPCs+MC-HIF1 had significantly improved ejection fraction and fractional shortening as early as 2 days after MI, and these changes were sustained through 6 weeks after MI. Importantly, these changes were greater in CPCs+MC-HIF1 group when compared with that in MC-HIF1 alone. These results suggest that codelivery of CPCs together with MC-HIF1 provided superior therapeutic effects among all groups after MI.

Because both CPCs+MC-HIF1 and MC-HIF1–alone groups demonstrated preserved cardiac functions as early as day 2, we were interested to evaluate the infarct size. Six mice were randomly selected from each of the 4 groups to undergo infarct size analysis by tetrazolium chloride staining at 3 days after MI. Although there was no discernible difference in the infarct size between saline-injected or CPCs+MC-GFP–injected mice, infarct size was significantly reduced in both CPCs+MC-HIF1–injected and MC-HIF1–injected MI mice, albeit to a lesser extent in the latter group (Figure 2C; Figure IB in the online-only Data Supplement). Immunofluorescence staining with an endothelial marker (CD31) in the peri-infarct areas of hearts collected 7 days after MI also revealed increased capillary density for CPCs+MC-HIF1 and MC-HIF1–alone groups when compared with saline group. CPCs+MC-GFP group failed to show similar improvement when compared with saline group (Figure 2D; Figure IC in the online-only Data Supplement).

HIF1 Modulates the Ischemic Milieu Into a More Hospitable Environment

HIF1 is a transcription factor that activates various adaptive responses against ischemic stress.[3] Therefore, we reasoned that sustained expression of HIF1 would modulate the ischemic milieu by rendering it less hostile for transplanted CPCs. To test this hypothesis, myocardial tissue surrounding the transplanted Fluc⁺/GFP⁺ CPCs codelivered with either MC-GFP or MC-HIF1 was laser microdissected 5 days after MI, and quantitative polymerase chain reaction for angiogenic-related genes (*Hif1α, Vegfa, Fgf2, Angpt2*, and *Tgf*) was performed (Figure 2E). An additional group of MC-HIF1 delivery alone without cells was also included, and the remote nonischemic tissue of hearts from MC-HIF1–alone group was used as controls. A significant upregulation of several

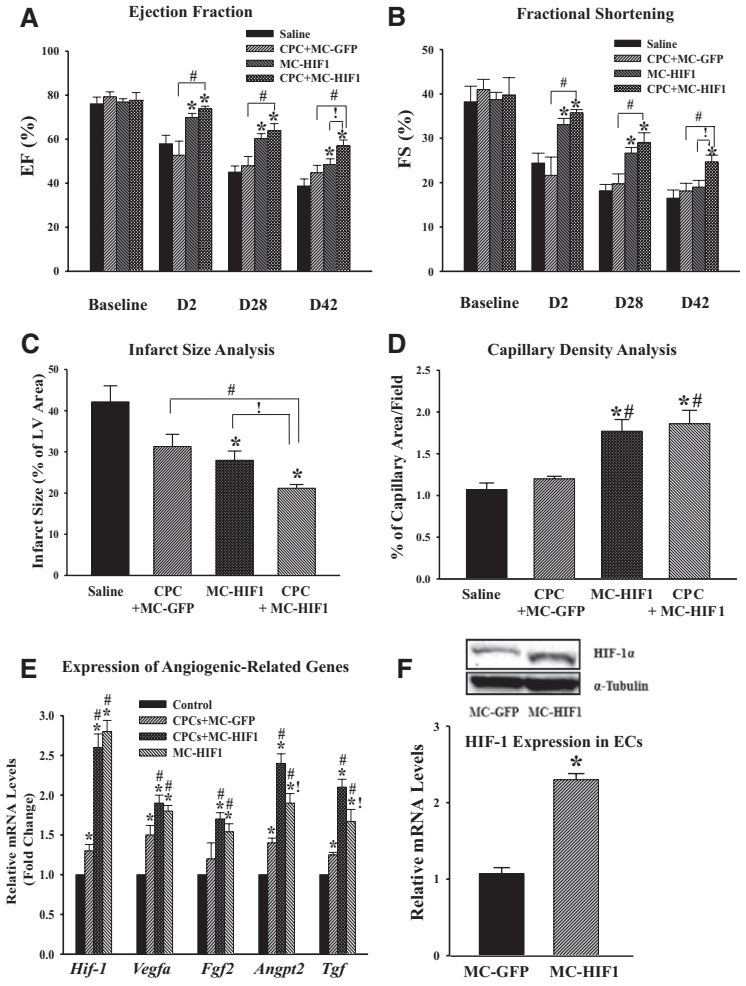

Figure 2. Combination of cell and gene therapy provides synergistic therapeutic effects after myocardial infarction (MI). Comparison of (**A**) ejection fraction (EF) and (**B**) fractional shortening (FS) among all 4 groups at indicated time points revealed that cardiac progenitor cells (CPCs) codelivered with minicircle plasmid carrying hypoxia-inducible factor-1 (MC-HIF1) had superior therapeutic effects among all groups. **P*<0.05 vs saline group; #*P*<0.05 vs CPCs+MC-GFP; !*P*<0.05 vs MC-HIF1 (n=10 per group). **C**, Three days after MI, mice from each group were euthanized and hearts were collected for determination of infarct size by tetrazolium chloride staining. **P*<0.05 vs saline group; #*P*<0.05 vs CPCs+MC-GFP; !*P*<0.05 vs MC-HIF1 (n=6 per group). **D**, Vascular density in each group was determined by CD31 staining at 7 days after MI. **P*<0.05 vs saline group; #*P*<0.05 vs CPCs+MC-GFP (n=6 per group). **E**, Areas close to engrafted GFP+ CPCs were laser microdissected to assess levels of angiogenic gene activation. Samples were collected 5 days after MI. Quantitative polymerase chain reaction showed that MC-HIF1 upregulates the expression of angiogenic genes, some of which were further augmented with the presence of CPCs. **P*<0.05 vs control (nonischemic remote zone of MC-HIF1); #*P*<0.05 vs CPCs+MC-GFP; !*P*<0.05 vs CPCs+MC-HIF1 (n=5 per group). **F**, In a separate set of experiments, mice subjected to MI received either MC-GFP or MC-HIF1 only. Three days later, cardiac ECs were isolated from the peri-infarct region, ECs from MC-HIF1 group were found to have higher expression of HIF1 at both protein (**top**) and gene levels (**bottom**) when compared with ECs from MC-GFP group, indicating that cardiac ECs are receptive to MC-HIF1 transfection. **P*<0.05 vs MC-GFP (n=6 per group). LV indicates left ventricular.

angiogenic genes was observed in the peritransplant tissues of CPCs+MC-GFP group when compared with controls, and this upregulation was significantly augmented in the CPCs+MC-HIF1 group (Figure 2E). Interestingly, although all these angiogenic genes were significantly upregulated in the MC-HIF1–alone group when compared with control or CPCs+MC-GFP, expression of *Angpt2* and *Tgf* were significantly lower when compared with CPCs+MC-HIF1 group (Figure 2E). These results suggest that the presence of HIF1 modifies the local microenvironment of transplanted CPCs at least through upregulation of angiogenic-related genes and the presence of CPCs helps to augment the upregulation of selected genes further, such as *Angpt2* and *Tgf*, hence conferring additional protection. To investigate whether host ECs could have taken up MC-HIF1, leading to a modified microenvironment, we next injected MC-GFP or MC-HIF into the peri-infarct zone of murine hearts (n=6 per group). Three days later, hearts were explanted, digested into single-cell suspensions, and ECs were isolated using CD31 magnetic beads. ECs from MC-HIF1–injected hearts were found to have significantly elevated expression of HIF1 both at gene and at protein levels when compared with MC-GFP (Figure 2F), indicating that ECs from host myocardium are receptive to injected MC-HIF1.

Molecular Cross Talk Between ECs and CPCs Involves Transfer of Exosomes

Because we had demonstrated that MC-HIF1 modifies the local ischemic milieu, we investigated the possibility that molecular cross talk between host ECs and transplanted CPCs may lead to improved survival. Recently, it has been shown that cell–cell communication can be mediated by a class of extracellular vesicles termed exosomes.[7,8] To study the potential involvement of exosomes in molecular cross talk, we first determined whether cardiac ECs produce them. We isolated exosomes from the supernatants of cultured ECs through a series of microfiltration and centrifugation steps (Figure 3A). Exosome identity was assessed by electron microscopy, which demonstrated a cup-shaped morphology, ≈110 nm in size, as usually observed in exosomes (Figure 3B). Dynamic light scattering analysis further confirmed a size distribution consistent with exosomes vesicles in the range of 30 to 110 nm (Figure 3C). Comparison of cell lysate with exosomal preparations by immunoblotting revealed the enrichment of exosomal markers CD63 and CD9 confirming their purity (Figure 3D).[9] We next determined the capacity of ECs transferring exosomes to recipient CPCs by prelabeling isolated exosomes with the fluorescent dye PKH26 before adding them to the medium of CPCs. Typical labeling efficiency of exosomes is ≈85% as determined by fluorescence-activated cell

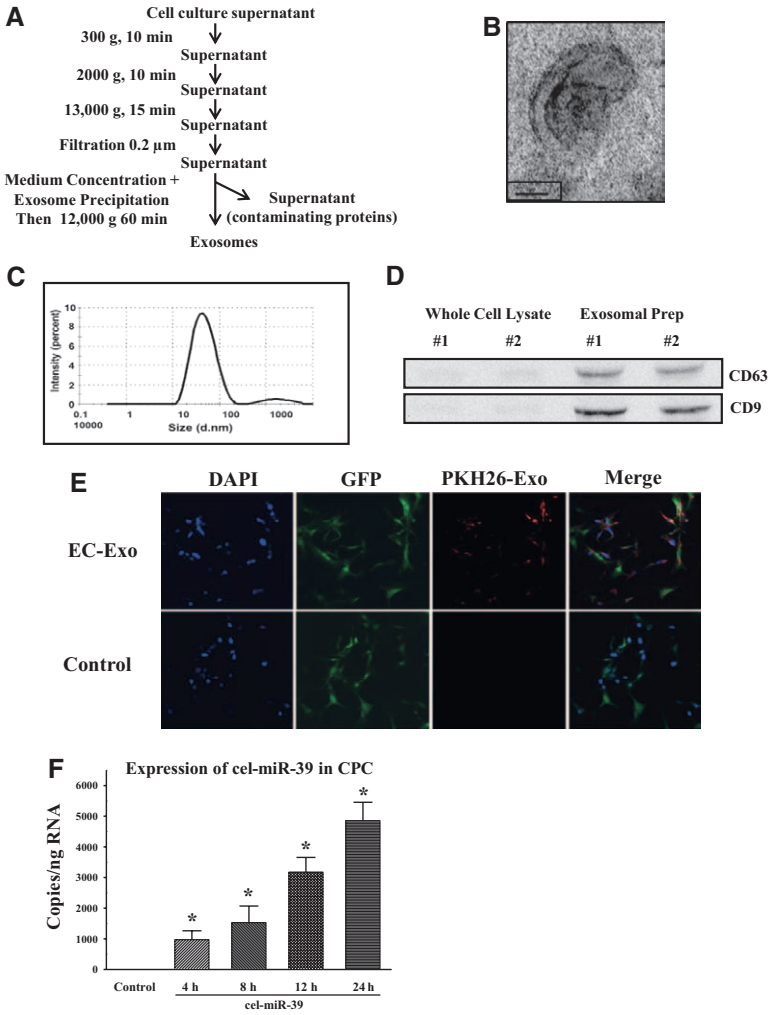

Figure 3. Purified exosomes produced by endothelial cells (ECs) are actively internalized by cardiac progenitor cells (CPCs) in vitro. **A**, Purification scheme of exosomes from EC culture supernatant. **B**, Cup-shaped morphology of purified ECs' exosomes assessed by transmission electron microscopy. **C**, Dynamic light scattering analysis of purified ECs' exosomes demonstrating a physical size distribution of 10 to 110 nm. **D**, Immunoblotting revealed an enrichment of exosomal markers CD63 and CD9 in purified exosomal fraction compared to whole EC lysates. Two separate preparations were shown. **E**, CPCs were cultured in the presence of EC-derived PKH26-labeled exosomes or absence (control; same volume of PBS labeled similarly) for 6 hours. Exosomes were taken up by CPCs as shown by confocal microscopy. **F**, ECs were transfected with cel-miR-39 or left untransfected. CPCs were then treated with exosomes isolated from untransfected (control) or cel-miR-39 transfected ECs for the indicated times. **P*<0.05 vs control (n=4 per group). DAPI indicates 4',6-diamidino-2-phenylindole.

sorter (Figure IIA in the online-only Data Supplement). After 6 hours of incubation, confocal imaging revealed the ability of CPCs to uptake the fluorescently labeled exosomes in a time-dependent manner (Figure 3E; Figure IIB in the online-only Data Supplement). To confirm these findings further, we transfected ECs with a miRNA that is naturally present only in *Caenorhabditis elegans* (cel-miR-39). Forty-eight hours later, exosomes from these cel-miR-39–transfected ECs were isolated and added to CPCs. Analysis of cel-miR-39 expression levels in CPCs demonstrated the presence of cel-miR-39, and that cel-miR-39 is transferred from ECs to CPCs in a temporal manner (Figure 3F).

Overexpression of HIF1 Alters the MicroRNA Contents of EC-Derived Exosomes

We next investigated the effects of MC-HIF1 transfection on the EC-derived exosome composition. HIF1 overexpression did not lead to a change in the amount of exosomes released from ECs (Figure IIC in the online-only Data Supplement). We next studied the repertoires of microRNAs (miRs) contained in exosomes secreted by both groups of cells (EC^{GFP}-Exo versus EC^{HIF}-Exo) to determine potential miRs that might be regulated by overexpression of HIF1. Using a quantitative polymerase chain reaction array of 380 mouse miRs, profiling of RNA isolated from exosomes showed that overexpression of HIF1 led to the upregulation of several exosomal miRs (Figure 4A). We chose to focus on 2 specific miRs, namely the endothelial-specific miR-126 and also miR-210, which has been previously reported to be regulated by HIF1[10] for subsequent experiments. We confirmed the involvement of HIF1 in upregulating these miRs by obtaining similar results using a pharmacological activator of HIF1, dimethyloxalylglycine (Figure IID in the online-only Data Supplement). In addition, the requirement for exosome biogenesis in the increased expression of secreted miRs was demonstrated. Knockdown of sphingomyelinase (nSMase2), which has been shown to inhibit exosome generation,[11] reduced the presence of both exosomal miR-126 and miR-210 from EC^{GFP} cells or EC^{HIF} cells (Figure 4B).

After incubation with control EC^{GFP}-CM, CPCs expressed significantly higher levels of miR-126 but levels of miR-210 remain unchanged, consistent with the fact that miR-126 is predominantly expressed in ECs (Figure 4C) rather than CPCs. In keeping with the profiling results, CPCs incubated with EC^{HIF}-CM had a significantly higher expression of both miR-126 and miR-210 when compared with CPCs supplemented with EC^{GFP}-CM or untreated cells, indicating that HIF1 leads to enriched expression of these miRs in

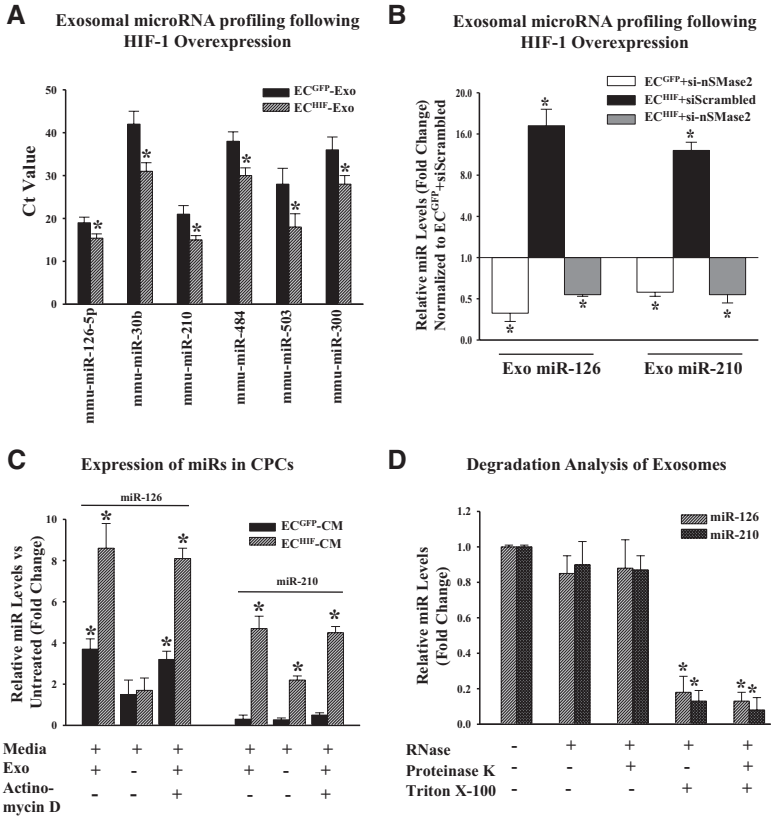

Figure 4. Hypoxia-inducible factor-1 (HIF1) modulates the microRNA (miRs) repertoire in endothelial cell (EC)–derived exosomes (Exo), which are transferable to cardiac progenitor cells (CPCs). **A**, ECs were transfected with either minicircle-green fluorescent protein (MC-GFP; control) or MC-HIF1. After transfection, Exo were purified from both groups and subjected to miRs profiling. Expression of selected exosomal miRs was increased after MC-HIF1 transfection. **P*<0.05 vs EC^{GFP}-Exo (n=4). **B**, On the basis of profiling results, we chose miR-126 and miR-210 for detailed analysis. EC^{GFP} and EC^{HIF} were transfected with either scrambled siRNA or siRNA targeting nSMase2. Exo were then purified from each group, and the expression of miR-126 and miR-210 was determined by quantitative polymerase chain reaction (qPCR) using Taqman probes and expressed as relative fold-change normalized to EC^{GFP} transfected with scrambled siRNA. **P*<0.05 vs EC^{GFP}+siScrambled (n=6 per group). **C**, Recipient CPCs were treated with vehicle or actinomycin D (5 μg/mL). CPCs were then grown in normal growth medium or supplemented with either EC^{GFP}-CM or EC^{HIF}-CM in the absence or presence of Exo as indicated. After 24 hours, the expression of miR-126 and miR-210 in CPCs was determined by qPCR and expressed as fold-change normalized against CPCs grown in normal growth medium. **P*<0.05 (n=6). **D**, Exo were isolated from EC^{HIF} and incubated with the indicated reagents for 45 minutes at 37°C before isolation of RNA and measurement of expression levels of miR-126 and miR-210 by qPCR. **P*<0.05 vs untreated (n=4).

target cells (Figure 4C). To investigate the requirement for exosomes in shuttling miRs directly, we depleted EC^{GFP}-CM and EC^{HIF}-CM of exosomes through ultracentrifugation. The depletion of exosomes considerably abrogated the expression of miR-126 and miR-210 in target CPCs (Figure 4C). The presence of a transcription inhibitor, actinomycin D, also did not affect levels of miR-126 and miR-210 in the treated CPCs, confirming the aforementioned effects as direct consequences of exosomal-mediated transfer (Figure 4C). Finally, degradation analysis of exosomes using a combination of ribonuclease (RNase), proteinase K (for degrading proteins), and Triton X-100 (disrupts phospholipid membranes) further demonstrated that the majority of endothelial-derived miR-126 and miR-210 are transmitted into CPCs through vesicle-protected RNA (Figure 4D), instead of forming soluble ribonucleoprotein complexes, as recently shown.[12–14]

Transferred MiR-126 Modulates Biological Properties of CPCs

Next, to test the biological functionality of the transferred miR-126 and miR-210 in CPCs, cells were transfected with a 3′-untranslated region luciferase reporter vector harboring a binding site for either miR-126 or miR-210, respectively. We also included a second group with a control reporter vector without a miR recognition site. As shown in Figure 5A, when CPCs were cultured with EC^{HIF}-CM, luciferase activity was decreased significantly in cells transfected with specific miR recognition sequence vector, but not the control vector or when exosomes were depleted from the CM through ultracentrifugation. Transfected CPCs in media alone displayed robust luciferase production (data not shown). These results show the transferred miRs were functional in the recipient CPCs. We then examined the biological effect of these transferred miRs that could potentially confer the demonstrated in vivo tolerance against ischemic stress in the recipient cells. We found that CPCs given EC^{GFP}-Exo had increased phosphorylated levels of prosurvival kinases phospho-ERK and phospho-AKT and that these effects were further augmented when CPCs were given EC^{HIF}-Exo, consistent with the established role of miR-126 in regulating these kinases (Figure 5B).[15] Silencing of miR-126 blunted the phosphorylation of phospho-ERK and phospho-AKT, lending support to the importance of miR-126 in EC^{HIF}-exosomes (Figure 5B). Furthermore, CPCs given EC^{HIF}-Exo had elevated expression of several angiogenic-related genes, such as *Vegfa* and *Fgf2*, when compared with untreated or EC^{GFP}-Exo cells (Figure 5C). These angiogenic factors have been previously shown to regulate phospho-ERK and phospho-AKT.[16] However, the upregulation of *Vegfa* is in contrary to the previously described role of miR-126 as a direct repressor of this gene. Hence, we subjected CPCs to culture conditions in which they were supplemented with exosomes derived from EC^{HIF} cotransfected with antagomirs targeting miR-126, and we found that these cells had significantly higher expression of both *Vegfa* and *Fgf2* but not *Il-8* and *Kdr* when compared with EC^{HIF} transfected with scrambled antagomirs (we did not observe a difference between EC^{HIF} versus EC^{HIF}-scrambled). These data suggest that although there were still partial repressive effects of miR-126, it was insufficient to lead to downregulation of *Vegfa* presumably because of the presence of other factors contained within

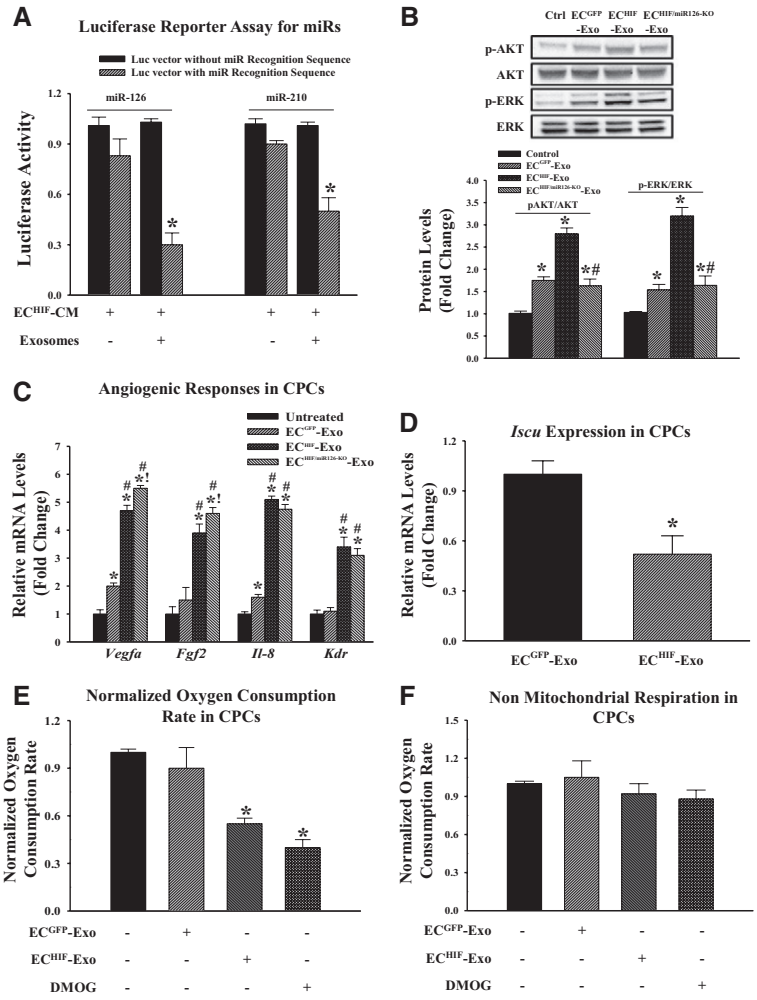

Figure 5. Transferred exosomal miRs regulate biological properties of recipient cardiac progenitor cells (CPCs). **A**, CPCs were transfected with a luciferase reporter vector containing either miR-126 or miR-210 recognition sequence or a control luciferase vector lacking the sequence. CPCs were then given endothelial cell (EC)^{HIF}-conditioned medium (CM) in the presence or absence of exosomes (Exo) as indicated. CPCs were then cultured for another 24 hours, and luciferase activity was determined and expressed as fold-decrease of cells transfected with the luciferase reporter containing specific miR recognition sequence over the vector lacking the recognition sequence for each culture condition. *P<0.05 (n=4) vs cells transfected with vector lacking miR recognition sequence. **B**, Representative immunoblots and densitometry quantification of indicated proteins in CPCs grown in either normal conditions, or supplemented with EC^{GFP}-Exo, EC^{HIF}-Exo, or EC$^{HIF/miR126\text{-}KO}$-Exo. *P<0.05 vs untreated CPCs; #P<0.05 vs EC^{HIF}-Exo (n=4). **C**, Expression of angiogenic genes in CPCs grown in each culture condition as indicated was determined by quantitative polymerase chain reaction. *P<0.05 vs untreated; #P<0.05 vs EC^{GFP}-Exo; !P<0.05 vs EC^{HIF}-Exo (n=8). **D**, Expression of *Iscu*, a validated miR-210 target gene, was determined in CPCs supplemented with either EC^{GFP}-Exo or EC^{HIF}-Exo. *P<0.05 (n=4) vs CPCs supplemented with EC^{GFP}-Exo. CPCs were cultured in conditions as specified for 24 hours. **E**, Oxygen consumption and (**F**) nonmitochondrial respiration were then determined and expressed as fold-change when compared with untreated control cells. Dimethyloxalylglycine (DMOG), a known hypoxia-inducible factor-1 (HIF1) activator was used as positive control for reduced oxygen consumption. *P<0.05 vs untreated controls (n=6). KO indicates knockout.

EC^{HIF}-Exo. We further confirmed the functionality of miR-126 in EC^{HIF}-Exo through additional experiments by measuring the expression of *Spred1*, a validated miR-126 target gene in untreated CPCs, or CPCs given EC^{GFP}-Exo, EC^{HIF}-Exo, or EC$^{HIF\text{-}miR126\text{-}KO}$-Exo. We found that exosomes derived from EC^{GFP} led to partial suppression of *Spred1* when compared with untreated CPCs; these effects were further augmented when EC^{HIF}-Exo were added and reversed when CPCs were treated with antagomirs targeting miR-126 (Figure IIE in the online-only Data Supplement).

Transferred MiR-210 Modulates Biological Properties of CPCs

Having shown that miR-126 affects CPCs, we postulated that transferred miR-210 would also have a biological relevance in CPCs. Iron-sulfur cluster scaffold homolog (*Iscu*) is a validated target of miR-210, which on repression, reduces mitochondrial metabolism.[17] A significant reduction of *Iscu* was observed in CPCs treated with EC^{HIF}-Exo indicative of repression through miR-210 (Figure 5D). To assess the metabolic profile of CPCs as a consequence of increased miR-210/repressed *Iscu*, we measured oxygen consumption rate in these cells by SeaHorse XF bioenergetic system. CPCs treated with EC^{HIF}-Exo displayed a significant reduction in basal oxygen consumption (normalized against cell numbers) when compared with untreated CPCs or CPCs treated with EC^{GFP}-Exo, showing that increased miR-210 leads to reduced mitochondrial metabolism in cells (Figure 5E). In contrast, nonmitochondrial respiration was similar among all groups, suggesting that EC^{HIF}-Exo act primarily in modulating mitochondrial respiration (Figure 5F). In addition, we noted an increase in intracellular lactate with CPCs treated with EC^{HIF}-Exo, indicating increased glycolysis as usually seen along reduced mitochondrial metabolism (Figure IIF in the online-only Data Supplement).

Exosomes Reduce Cellular Damage of CPCs Under Ischemic Conditions

Given the known evidence for increased levels of prosurvival kinases, enhanced angiogenic response and reduced metabolic demand as adaptive beneficial responses,[18,19] we hypothesized that these effects could collectively provide CPCs with increased tolerance to ischemic stress. Indeed, CPCs grown in EC^{HIF}-CM had significantly reduced cellular damage as assessed by lactate dehydrogenase release when subjected to in vitro hypoxia when compared with untreated or cells grown in EC^{GFP}-CM (Figure 6A). The protective effects of EC^{HIF}-CM were dependent on the presence of exosomes as ultracentrifugation abrogated the aforementioned effects. Importantly, CM derived from ECs overexpressing HIF1 that

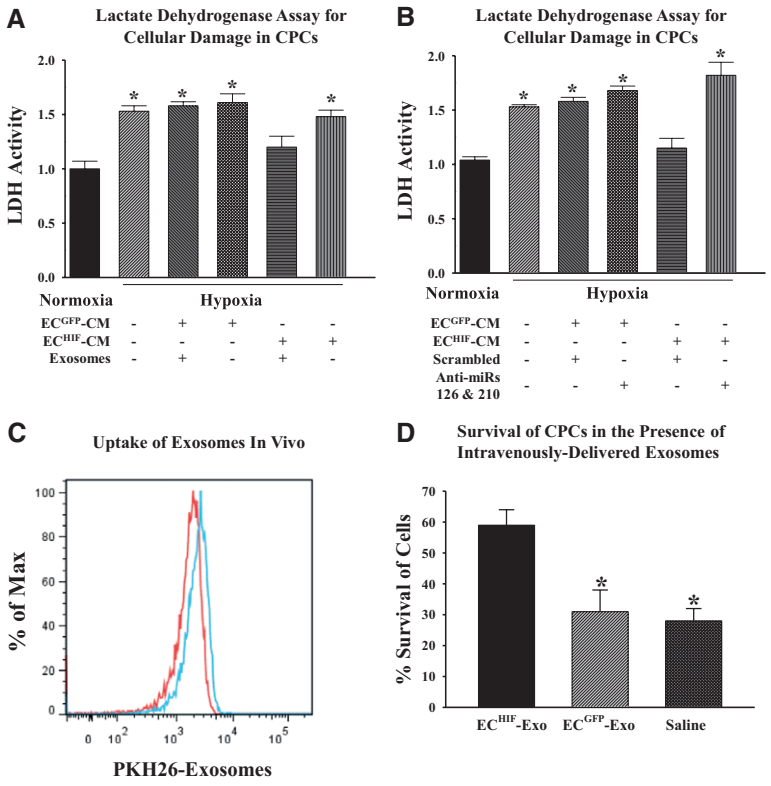

Figure 6. Exosomes (Exo) directly provide cardiac progenitor cells (CPCs) with increased tolerance against ischemic stress both in vitro and in vivo. **A**, CPCs were grown in either normal conditions or supplemented with endothelial cell (EC) ^{GFP}-conditioned medium (CM) or EC^{HIF}-CM with or without the presence of Exo as indicated, before being subjected to in vitro hypoxic stress. Lactate dehydrogenase (LDH) was then measured as an indicator of cellular damage and expressed as fold-change when compared with untreated cells kept in normoxic conditions. *P<0.05 (n=6) vs normoxic cells. **B**, EC^{HIF} were cotransfected with either scrambled antagomirs or antagomirs targeting miR-126 and miR-210. CPCs were then grown in conditioned medium from each group as indicated before being subjected to LDH assay to assess the importance of miR-126 and miR-210 in EC^{HIF}-CM–mediated protection seen in **A**. *P<0.05 vs normoxic control (n=6). **C**, After MI, CPCs were intramyocardially injected into mice hearts, and a bolus of PKH26-labeled ECs-Exo was delivered intravenously. After 12 hours, the animals were euthanized and CPCs were reisolated from the hearts. Samples were measured by flow cytometry for green fluorescent protein (GFP)$^+$ and PKH26$^+$ events. The histogram shows that PKH26-Exo (blue lines) were detectable in ≈10% of GFP$^+$ CPCs (red lines) demonstrating that CPCs are capable of uptaking Exo in vivo. **D**, To determine whether EC^{HIF}-Exo could confer increased tolerance to CPCs in vivo, cells were delivered intramyocardially after MI into mice concomitantly with intravenous injection of saline, EC^{GFP}-Exo, or EC^{HIF}-Exo. BLI was then performed at day 1 and day 7 after MI, and survival of CPCs was expressed as a percentage of signals intensity at day 7 when compared with initial signals intensity of day 1. *P<0.05 vs EC^{HIF}-Exo (n=6 per group).

were additionally transfected with antagomirs against miR-126 and miR-210 failed to provide similar therapeutic effects when given to CPCs, indicating that these miRs are crucial for EC^{HIF}-CM to afford CPCs with resistance against ischemic stress (Figure 6B). Finally, to determine whether EC^{HIF}-Exo can directly protect CPCs after transplantation into the ischemic heart, we delivered CPCs into mice after MI concomitant with intravenous delivery of saline, PKH26-labeled EC^{GFP}-Exo, or PKH26-labeled EC^{HIF}-Exo, respectively. Flow cytometry analysis of isolated CPCs from explanted hearts demonstrated the presence of PKH26 exosomes, confirming the uptake of these exosomes by CPCs in vivo (Figure 6C). Longitudinal bioluminescence imaging of mice showed that CPCs that received EC^{HIF}-Exo had better survival in the group when compared with saline or EC^{GFP}-Exo groups at 1 week (Figure 6D).

Discussion

This study revealed a multifaceted mechanism by which delivery of HIF1 via a MC plasmid platform enhances survival of cotransplanted Sca1$^+$ CPCs. First, using a murine model of MI followed by codelivery of CPCs with MC-GFP or MC-HIF, we observed that a combinatorial delivery with MC-HIF resulted in prolonged survival of transplanted CPCs, prevented cardiac remodeling, reduced infarct size, and enhanced vascularity. Second, HIF1 modulates the local milieu of the ischemic heart at least partly by effects on host cardiac ECs. Third, we found that these cardiac ECs generate and release exosomes in vitro, which are actively internalized by CPCs when present in the growth medium. Fourth, HIF1 modifies the biological contents of these exosomes, resulting in a phenotypic change in recipient cells. Fifth, in vitro hypoxia assays revealed that exosomes derived from ECs overexpressing HIF1 contributed to CPCs' increased tolerance under hypoxic stress. These findings were validated in vivo because intravenous delivery of EC^{HIF}-Exo significantly increased the survival of transplanted CPCs in the ischemic heart as determined by bioluminescence imaging.

HIF1 is a master transcriptional activator that mediates various physiological responses to hypoxia.[3] Here, we demonstrated that codelivery of CPCs together with MC-HIF1 led to better survival of transplanted cells and was associated with preserved cardiac function. The synergistic effects of this combinatorial approach were evident early on, as documented by a smaller infarct size and increased vascularity when compared with other treatment groups. It is well known that stem/progenitor cells in adult organs reside in specialized niches that provide an ideal microenvironment for their maintenance.[20,21] In this context, these cells are often found in close proximity to blood vessels, suggesting a role for these vessels in the regulation of stem cell self-renewal and differentiation. After an ischemic event, ECs are highly susceptible to undergoing necrosis, potentially compromising the maintenance and expansion of resident stem cells. Using laser microdissection analysis, we observed that MC-HIF1 changes the gene

expression landscape of the local milieu proximal to transplanted cells, including resident cardiac ECs.

Given that HIF1 affects local cardiac ECs and improves survival of transplanted CPCs, it is highly plausible that potential beneficial cross talk could occur between these 2 cell types. In recent years, exosomes have emerged as potential candidates for mediating cell–cell communication in various physiological or pathophysiological conditions through transfer of proteins, mRNAs, and miRs.[7,8] We confirmed that cardiac ECs promoted release and transfer of exosomes to CPCs in vitro and demonstrated differences in miR profiling of EC^{GFP}-Exo (control) versus EC^{HIF}-Exo, most prominently miR-126 and miR-210. Our degradation analysis data also revealed that a significant amount of miR-126 and miR-210 is exosome-enclosed (unaffected by RNase treatment but susceptible to blockade of nSMase2), distinct from some previous studies indicating that miR-126 is transferred in a vesicle-free form or through apoptotic bodies.[13,14] These data might reflect that additional mechanisms regulating the packaging of biological contents into exosomes are cell-type dependent. Recipient cell types might also differ in their ability to respond to exosomes because previous studies have reported receptor specificity as a crucial factor for internalization of exosomes.[22]

Endothelial miR-126 has been shown to regulate multiple pathways, including the regulation of prosurvival kinases phospho-ERK and phospho-AKT. Our findings demonstrated that CPCs internalizing EC^{GFP}-Exo had a transient activation of these kinases that were further increased by EC^{HIF}-Exo. The transient phosphorylation of these kinases is known to be protective in the settings of ischemia by limiting both the apoptotic and the necrotic components of cell death. Likewise, miR-210, a known HIF1–regulated miR, was also shown to be upregulated preferentially in exosomes by HIF1, switching recipient CPCs to a preferentially glycolytic state (reduced oxygen usage). This is consistent with previous studies documenting that one of the many targets of miR-210 is *Iscu*,[17] and repression of this gene leads to lower mitochondrial metabolism; both phenomena were observed in our study. This metabolic adaptation to oxygen conservation reflects a lower use of the mitochondrial electron transport chain, which reduced the generation of reactive oxygen species. Indirectly, this phenomenon could potentially function as a preemptive measure against ischemic stress because the production of reactive oxygen species is known to be greatly increased during ischemia and is highly detrimental to cellular health. In keeping with our hypothesis, we observed that CPCs in EC^{HIF}-CM had less cellular damage when exposed to in vitro hypoxic stress. This increased tolerance is attributable to exosomes because we found that depletion of exosomes from the CM abrogated the protective effects. In addition, the importance of both miR-126 and miR-210 mediating the protective effects of the EC^{HIF}-CM was corroborated because such effects were abolished when the donor cardiac EC cells were transfected with antagomirs targeting both miRs.

In the present study, a full comparison of the abundance of exosomes in vitro and in vivo was not feasible because the concentrations and transfer efficiencies are probably different. Nevertheless, we have generated evidence to support a physiological role for exosomes in mediating increased tolerance against ischemic stress, which helps to improve survival of transfected cells. Although we focused on the transfer of genetic material after HIF1 overexpression from cardiac ECs to CPCs, we think that reciprocal cross talk could also occur as well with other resident cell types, including fibroblasts and host cardiomyocytes, and even the possibility of CPCs themselves taking up MC-HIF1 that warrants deeper investigation. Likewise, although our data indicated a physiological role for exosomal miR-126 and miR-210 in recipient cells that was reversed on inhibition of both, this does not preclude the involvement of other miRs and proteins; further investigation is needed to show whether these proteins can improve survival of transplanted cells because various studies have demonstrated the complexity of biological molecules enclosed within exosomes.[23] Finally, our results demonstrating the upregulation of *Vegfa* despite being a direct target of miR-126 reveals the intricate mechanisms mediated by exosomes.

Collectively, we show for the first time an intricate exosome-mediated cross talk interface via HIF1 between the vascular endothelium and the transplanted stem cells. The improved tolerance against ischemic stress is afforded through activation of prosurvival kinases, increased angiogenic responses, and reduced metabolic demand, and involves, at least partially, miR-126 and miR-210 (Figure III in the online-only Data Supplement). Importantly, these results suggest that the combination of gene- and cell-based therapies should be explored in future clinical trials.

Sources of Funding

We are grateful for the funding support by National Institutes of Health U01 HL099776, R01 EB009689, R01 HL093172, R01 HL095571, American Heart Association Established Investigator Award, and Fondation Leducq (Dr Wu).

Disclosures

None.

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DATA SUPPLEMENT

Sang-Ging Ong, PhD[1,2]*; Won Hee Lee, PhD[1,2]*; Mei Huang, PhD[1,3]*; Devaveena Dey, PhD[1,3]; Kazuki Kodo, MD, PhD[1,2]; Veronica Sanchez-Freire, PhD[1,2]; Joseph D. Gold, PhD[1,4]; Joseph C. Wu, MD, PhD[1,2,3]

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SUPPLEMENTAL METHODS

Preparation of Minicircles. Minicircles are the product of site-specific intramolecular recombination between the attB and attP sites driven by bacteriophage ΦC31 integrase. The DNA fragment containing enhanced green fluorescent protein (MC-GFP) or HIF1α (MC-HIF1α) were bluntly ligated between the attB and attP sites of the minicircle plasmid. Minicircle DNA plasmids were prepared as described previously[1]. Briefly, *Escherichia coli* Top10 were transformed by parental plasmids. Cells from one transformed colony were inoculated into 5 ml of LB with Kanamycin (50 μg/ml) and incubated at 37°C with shaking at 250 rpm. Eight hours later, the bacteria was amplified by combining 100 μL of culture to every 400 mL LB containing Kanamycin (50 μg/ml) and continued incubation for 16 hours. The next day, a minicircle induction mix comprising of 400 mL fresh LB, 16 ml 1N sodium hydroxide, and 0.4 % L-arabinose were added to the overnight culture and allowed to grow at 32°C for another 8 hours. Minicircles were then isolated using Qiagen's Plasmid Plus Maxi Kit according to manufacturer's protocol.

***In Vitro* Cardiac Differentiation of Sca1$^+$ CPCs.** To induce cardiac differentiation, cultured Sca1$^+$ CPCs were differentiated into cardiomyocytes by culture in Cardiomyocyte Differentiation Medium (Millipore, Temecula, CA) for 12-15 days as previously described[2]. Characterization of the differentiated cardiomyocytes was done by immunostaining of Troponin I (Millipore, MAB1691-50UG) and Actinin (Millipore, MAB1682-25UL) according to manufacturer's instructions.

***In Vitro* and *In Vivo* Optical Bioluminescence Imaging (BLI).** CPCs were plated in increasing numbers in 10 cm dishes and cultured overnight. BLI was then performed the following day using the Xenogen IVIS 200 System (Xenogen, CA) and signals quantification was performed to determine the correlation between signals intensity and cell numbers. For *in vivo* BLI, recipient mice were anesthetized with isoflurane, and were intraperitoneally injected with D-Luciferin (200 mg/kg body weight). Peak signals from a fixed region of interest (ROI) were obtained and signals quantified in photons/s/cm^2/sr as previously described[3].

Echocardiographic Analysis of Left Ventricular Function. Echocardiography was performed before (day -2) and after (days 2, 28 and 42) the LAD ligation using a Vevo 2100 device equipped with 18-38 MHz linear-array transducer with a digital ultrasound system (Visualsonics) (N=10/group). A left ventricular M-mode tracing was obtained using the 2D parasternal short axis imaging as a guide. From these images, fractional shortening (FS) and ejection fraction (EF) were calculated. Left ventricular end-diastolic diameter (EDD) and end-systolic diameter (ESD) were measured and used to calculate fractional shortening (FS) by the following formula: FS (%) = [(EDD - ESD)/EDD] x 100%.

Triphenyltetrazolium Chloride (TTC) Staining for Determination of Infarct Size. Mice were anesthetized and hearts were rapidly removed and perfused with saline to rinse out residual blood (N=6/group). Hearts were then frozen and sectioned into 2 mm transverse sections from apex to occlusion site (4 slices/heart). Heart slices were stained in 1% TTC for 15 min at 37°C and then fixed in 10% formalin overnight. The area of infarction was demarcated as a white area whereas viable myocardium was stained red. Photographs were taken for all sections. Infarct size

was determined by computerized planimetry using the NIH Image J 1.63 software and expressed as a percentage of the left ventricle as previously described[4].

Determination of Capillary Density. The capillary density was determined as described previously[5]. Tissue sections were stained using CD31 antibody (BD Biosciences) (N=6/group). Capillary density in the peri-infarct zone was expressed as capillaries area per field. To obtain the average vessel area per cross-sectional area, a minimum of five individual fields per slide were sampled, and Image J was used to measure the counted field area in each field.

Analysis of Angiogenesis Genes using Laser-Capture Microdissection (LCM). Mice hearts were excised, rinsed, embedded in optimal cutting temperature, and immediately frozen in liquid nitrogen (N=5/group). Ten micron thick tissue sections of left ventricle were prepared on polyethylene napthalate membrane-coated slides (MicroDissect GmbH). For LCM, slides were thawed briefly and air dried 5 minutes before dissection. Green fluorescence observed under laser microscopy was used as a landmark for microdissection. Tissues near to the fluorescence area (engrafted cells) were dissected out by applying Leica LCM Systems (MicroDissect GmbH) into the caps of microcentrifuge tubes as previously described[6].

Quantitative Real-Time PCR. RNA was isolated from laser microdissected tissue using miRNeasy Mini Kit (Qiagen), from whole cells using RNeasy Mini Kit (Qiagen) and from exosomes using miRCURY™ RNA Isolation Kit (Exiqon), respectively, according to manufacturer's instructions. Total RNA was reverse transcribed with using the SuperScript first-strand synthesis system for RT-PCR (Applied Biosystems) or using Taqman microRNA reverse

transcription kit (Applied Biosystems). All primers and probes were obtained from Applied Biosystems. Reactions were analyzed using the ABI Prism 7300 sequence detection system and data were normalized to GAPDH or miR-16 levels and quantified using the comparative threshold cycle (Ct) method.

Isolation of Cardiac Endothelial Cells (ECs). Cardiac ECs were isolated from heart explants as described in the main text with slight modifications. Enrichment of ECs was performed using magnetic beads against CD31. Characterization of ECs was performed using an Endothelial Cell Characterization Kit (Millipore) according to manufacturer's instructions. To exclude contamination from serum-derived exosomes, exosome-depleted serum (System Biosciences) was used for propagation of ECs and the collection of conditioned medium (CM) and exosomes. ECs were transfected using Lipofectamine LTX with Plus reagent (Applied Biosystems) with MCs, and using Lipofectamine RNAiMAX reagent with cel-miR-39 (100 nM) and with siRNA targeting nSMase2 (50 nM), miR-126 (50 nM), miR-210 (50 nM), or scrambled (50 nM).

Uptake of Exosomal miRs by CPCs. Following transfection of ECs with cel-miR-39, MC-GFP, MC-HIF or siRNAs, conditioned media or purified exosomes (as indicated in the text) was added to CPCs. Expression of target miRs was then determined by qPCR.

Exosomal Degradation Analysis. Exosomes purified from ECs media were treated with either RNase, Proteinase K or Triton X-100 for 45 minutes at 37ºC. Expression levels of selected miRs were then determined by qPCR.

Electron Microscopy. For transmission electron microscopy, exosomes were fixed in 2% formaldehyde, loaded on 300-mesh formvar/carbon-coated electron microscopy grids (Electron Microscopy Sciences, PA), post-fixed in 1% glutaraldehyde, and then contrasted and embedded as described previously[7]. Transmission electron microscopy images were then obtained using a Jeol 1230 transmission electron microscope operating at 120 kV.

Immunoblotting. A total of 10-50 μg of protein of each sample was loaded onto a 10% Bis-Tris gel (Applied Biosystems). Primary antibodies used are as follows: HIF-1α (Novus Biologicals), CD63 and CD9 (System Biosciences), p-ERK, pan-ERK, p-AKT and pan-AKT (all from Cell Signaling). Equal protein loading was confirmed by α-Tubulin (Cell Signaling).

Dynamic Light Scattering. Dynamic light scattering analysis of exosomes was performed with a Zetasizer Nano ZS (Malvern Instruments, U.K.). Measurements were collected on a continuous basis for 3 minutes in sets of two using two different sets of samples from each exosome preparation.

Exosome Labeling and Uptake Assay. Exosomes were labeled with PKH26 red fluorescent membrane linker-dye (Sigma) according to manufacturer's protocol. Briefly, exosomes were labeled with PKH26 for 5 mins, before stopping the reaction with addition of exosomes-free FBS. Labeled-exosomes were then concentrated through an Ultracel-100K device (Millipore) and added to $Sca1^+$ CPCs in a 24-well plates. Six hours after addition, $Sca1^+$ CPCs were observed by confocal microscopy to visualize for uptake of PKH26-labeled exosomes. Labeling efficiency of

exosomes was also determined by flow cytometry using Exo-FLOW Exosome Purification Kit with slight modifications to manufacturer's instructions (System Biosciences, CA).

Luciferase Reporter Assay. CPCs were transfected with 100 ng of 3'-UTR luciferase reporter vector containing either a miR-126 or miR-210 target seed sequence compared to an empty vector (SwitchGear Genomics). Sixteen hours later, cells were washed and supplemented with conditioned medium as indicated for 24 hours before measuring luciferase activity with LightSwitch Assay Reagent (SwitchGear Genomics).

Oxygen Consumption Measurement. Oxygen consumption was measured using a Seahorse Bioscience XF96 Analyzer. Briefly, cells were seeded 24 hours before the day of experiment with treatments as indicated. Assays were initiated by replacing the growth medium from each well with unbuffered assay medium pre-warmed at 37°C. The cells were incubated at 37°C for 60 min to allow media temperature and pH to reach equilibrium before the first rate measurement. Basal respiration rates is defined as the initial oxygen consumption rate (OCR) taken after equilibration. All data were adjusted for the non-mitochondrial respiration rate (lowest rate after rotenone/antimycin injection). Total cellular protein was measured following each experiment by the Bradford method. All OCRs were normalized to total cellular protein and expressed as fold-change relative to control.

Intracellular Lactate Measurement. CPCs with or without CM treatment were cultured for 16 hours. Upon completion of treatment, cells were lysed, centrifuged, and supernatant was collected for intracellular lactate measurements using Lactate Colorimetric Assay Kit II

(Biovision Inc) according to manufacturer's instructions. Results were normalized against cell number as determined by trypan blue assay using the Countess Automated Cell Counter (Applied Biosystems).

***In Vitro* Hypoxia Assay.** CPCs were exposed to hypoxia (BD GasPak EZ system) for 8 hours in the presence or absence of CM, or the exosome-depleted fraction of EC^{HIF}-CM, or CM derived from ECs transfected with MC-HIF1 and also antagomirs against miR-126 and miR-210. The lactate dehydrogenase (LDH) release, as a marker for cell injury was quantified using CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) according to manufacturer's protocol.

Uptake of Exosomes by CPCs *In Vivo.* To analyze exosomes uptake by CPCs, cells were injected intramyocardially into NOD/SCID mice following MI (N=3/group). Exosomes pre-labeled with PKH26 were then injected intravenously through the tail vein (10 µg in 50 µl PBS). After 24 hours, hearts were explanted, dissociated into single cells, and analyzed by flow cytometry. Digested cells were first sorted for GFP^+ signals representing transplanted CPCs, followed by $PKH26^+$ signals representing uptake of labeled exosomes *in vivo*. A total of 10, 000 events were recorded in each analysis. Data analysis was performed using FlowJo software (Tree Star, OR).

Direct Effects of Exosomes on CPCs *In Vivo*. To determine whether exosomes can directly confer protection to CPCs *in vivo*, intravenous delivery of saline, EC^{GFP}-derived exosomes (EC^{GFP}-Exo), or EC^{HIF}-derived exosomes (EC^{HIF}-Exo) (20 µg protein in 50 µl PBS) was

delivered through the tail vein concomitantly with transplantation of CPCs following LAD ligation (N=6/group). BLI was then performed to determine cell survival 1 week post-injection.

SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure I. MC-HIF1 enhances cell survival and promotes cardioprotection. **(A)** Immunostaining of GFP^+ $Sca1^+$ cardiac progenitor cells (CPCs) at 7 days post-injection. **(B)** Representative images of infarct size analysis by tetrazolium chloride staining. **(C)** Representative images of vascularity analysis using an endothelial marker CD31.

Supplemental Figure II. Uptake of endothelial cell-derived exosomes by CPCs *in vitro*. **(A)** Labeling efficiency of exosomes was determined by flow cytometry. Typical efficiency was around 85%. **(B)** Flow cytometry analysis of exosomal uptake by CPCs. **(C)** Effects of HIF-1 overexpression on the production of exosomes from endothelial cells (ECs). **(D)** Pharmacological activation of HIF-1 increases exosomal miRs expression. $^*P<0.05$ vs. untreated (N=4). **(E)** Expression levels of *Spred1* in CPCs grown in indicated conditions were determined by qPCR. $^*P<0.05$ vs. untreated (N=5); $^\#P<0.05$ vs. EC^{HIF}-Exo (N=5). **(F)** Measurement of intracellular lactate in CPCs grown in conditioned medium from ECs. $^*P<0.05$ vs. EC^{GFP}-Exo (N=4).

Supplemental Figure III. Schematic diagram describing the modulation of ischemic microenvironment by HIF-1 leads to transfer of exosomal miRs from host endothelial cells to transplanted CPCs and promoting their survival.

SUPPLEMENTAL REFERENCE

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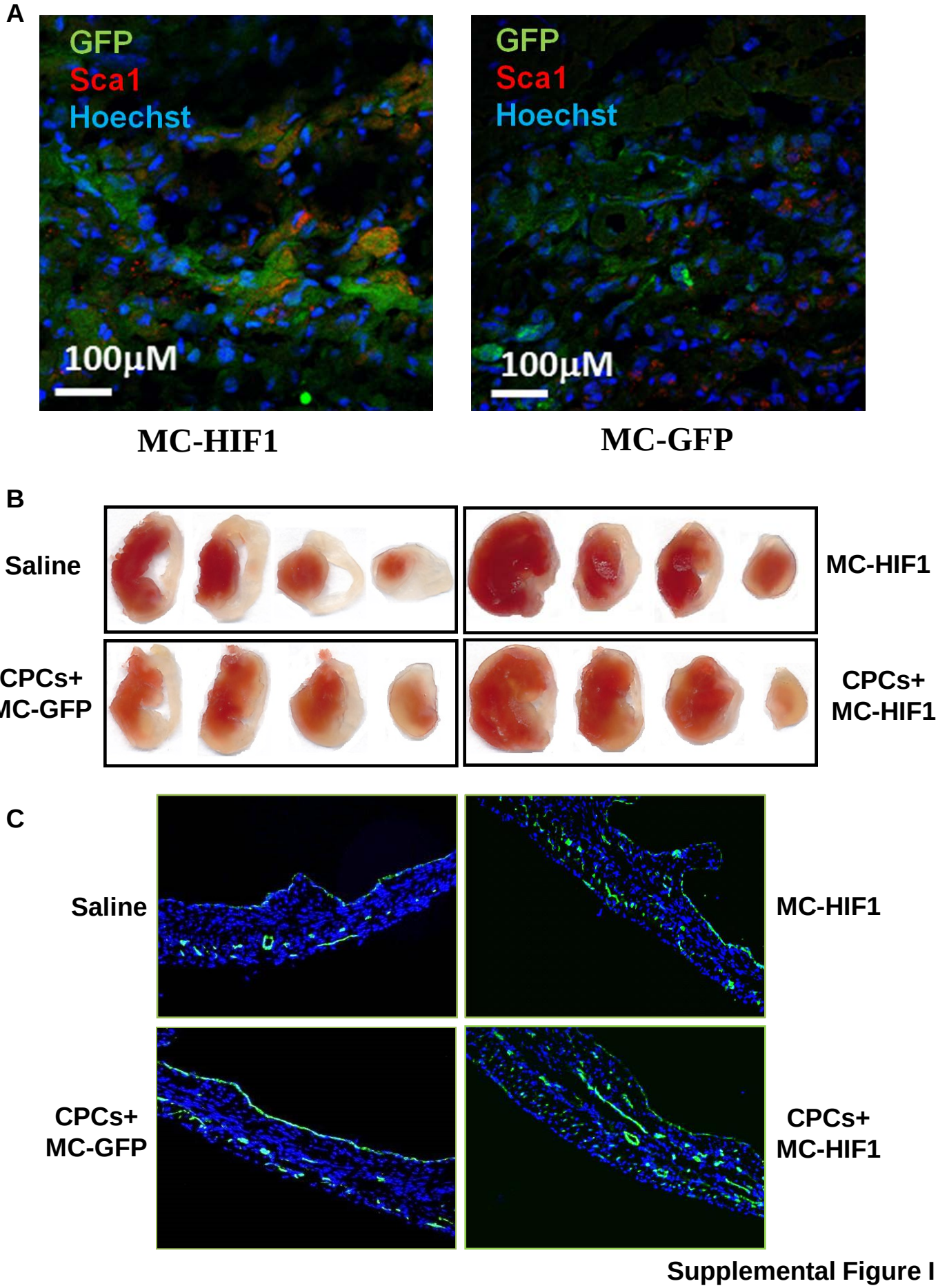
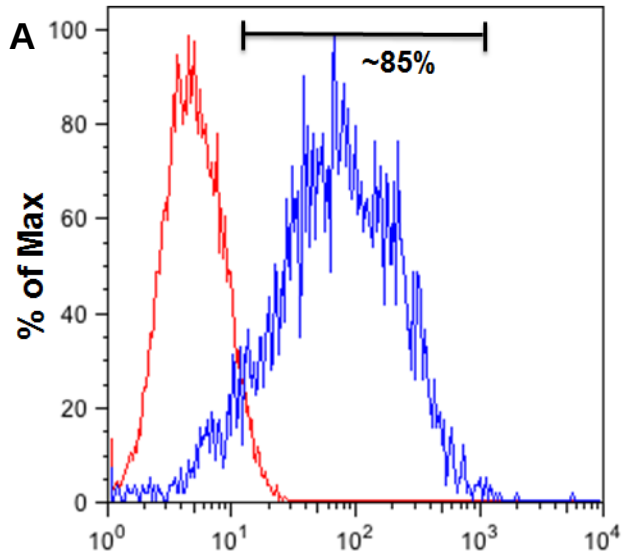
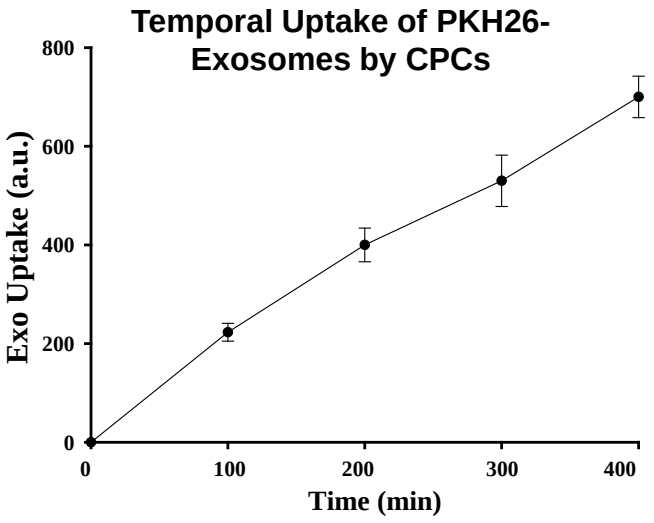
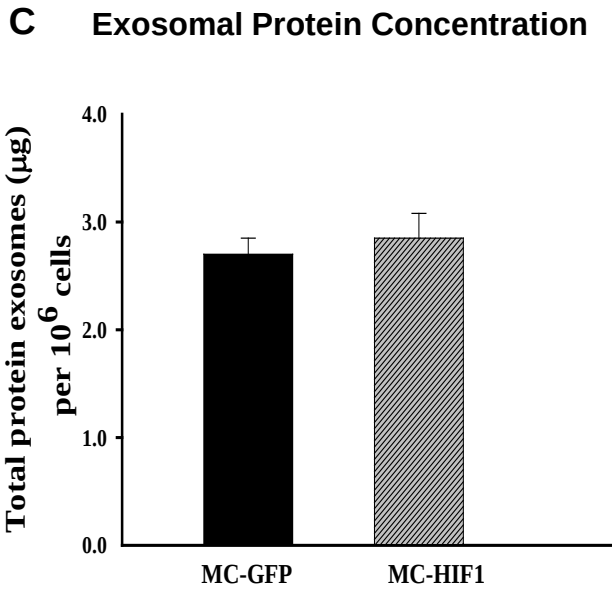
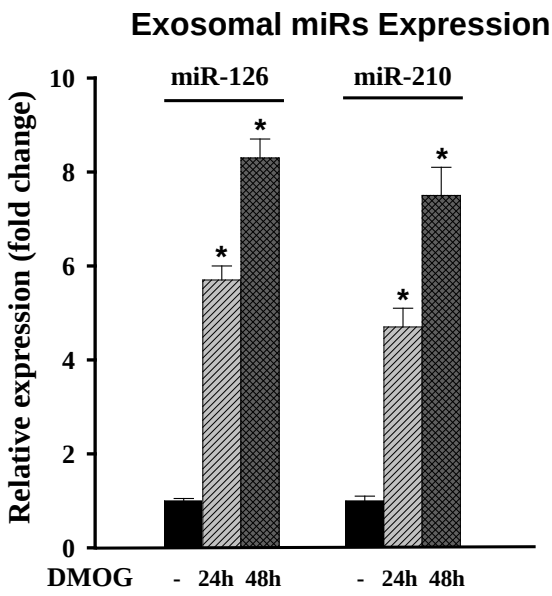


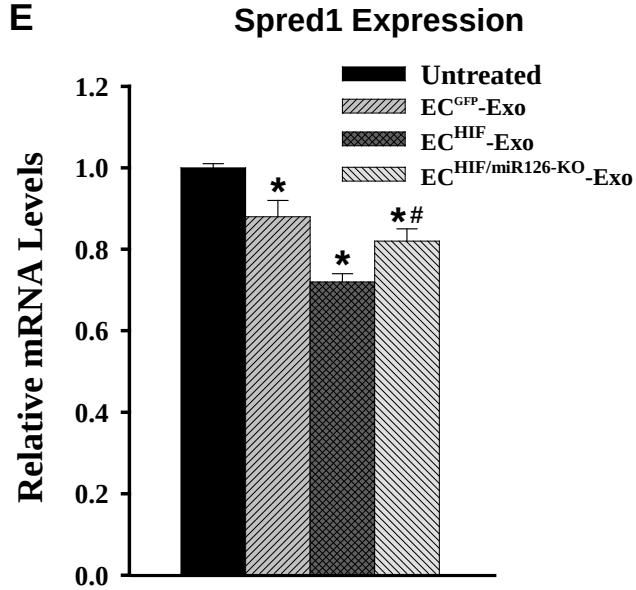
Supplemental Figure I

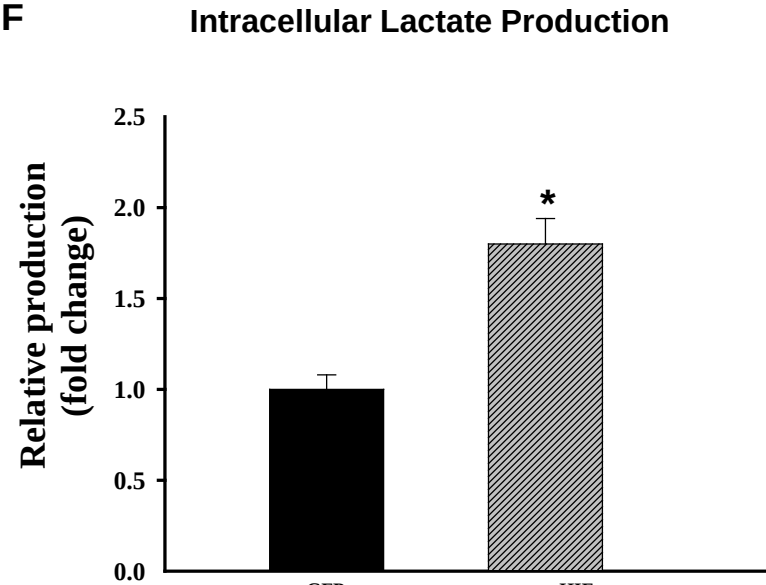




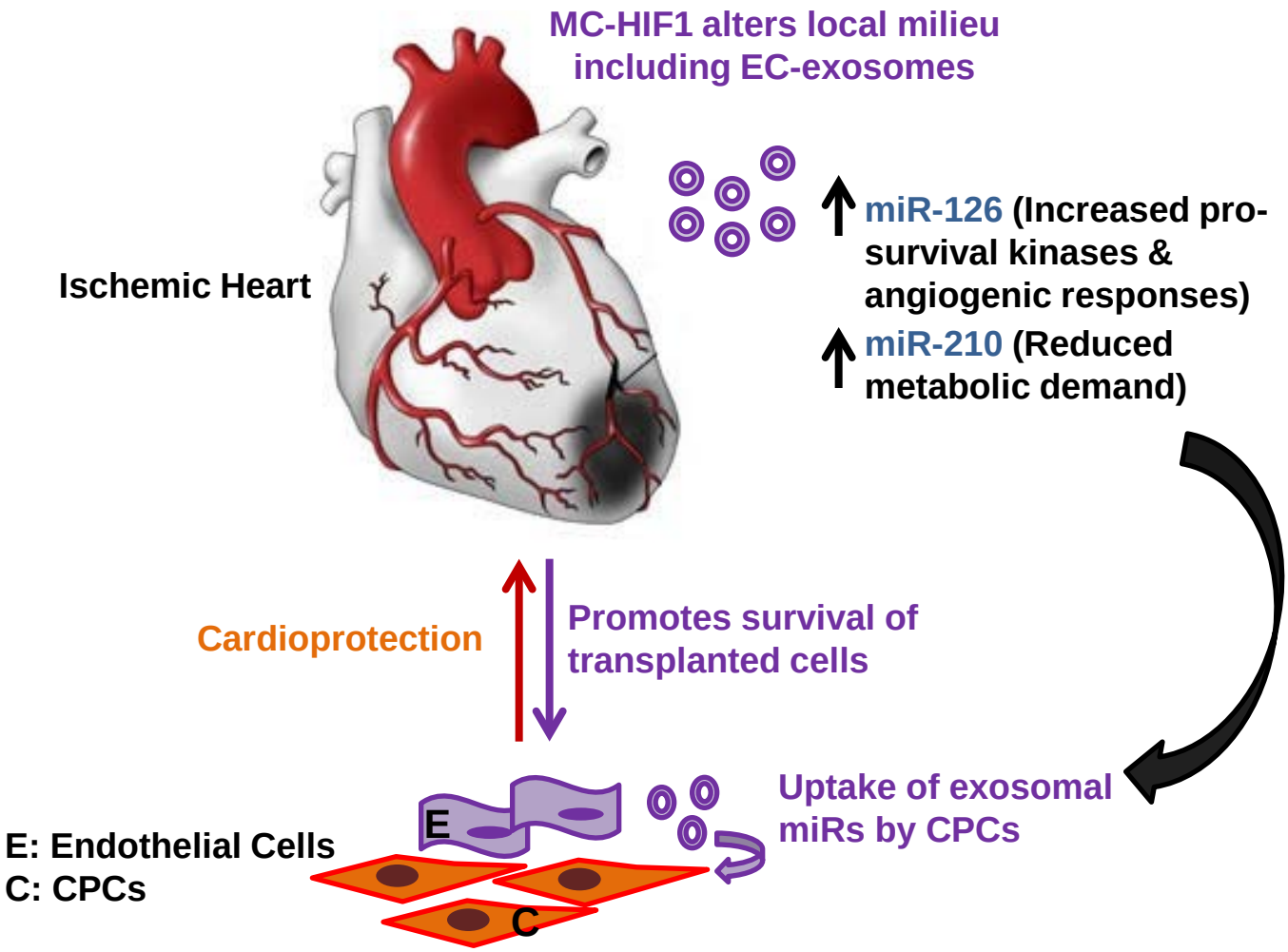








Supplemental Figure II



Supplemental Figure III

Cross Talk of Combined Gene and Cell Therapy in Ischemic Heart Disease: Role of Exosomal MicroRNA Transfer

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Circulation. 2014;130:S60-S69
doi: 10.1161/CIRCULATIONAHA.113.007917
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539