Identification of Therapeutic Covariant MicroRNA Clusters in Hypoxia-Treated Cardiac Progenitor Cell Exosomes Using Systems Biology


Rationale: Myocardial infarction is a leading cause of death in developed nations, and there remains a need for cardiac therapeutic systems that mitigate tissue damage. Cardiac progenitor cells (CPCs) and other stem cell types are attractive candidates for treatment of myocardial infarction; however, the benefit of these cells may be as a result of paracrine effects.

Objective: We tested the hypothesis that CPCs secrete proregenerative exosomes in response to hypoxic conditions.

Methods and Results: The angiogenic and antifibrotic potential of secreted exosomes on cardiac endothelial cells and cardiac fibroblasts were assessed. We found that CPC exosomes secreted in response to hypoxia enhanced tube formation of endothelial cells and decreased profibrotic gene expression in TGF-β-stimulated fibroblasts, indicating that these exosomes possess therapeutic potential. Microarray analysis of exosomes secreted by hypoxic CPCs identified 11 miRNAs that were upregulated compared with exosomes secreted by CPCs grown under normoxic conditions. Principle component analysis was performed to identify miRNAs that were coregulated in response to distinct exosome-generating conditions. To investigate the cue–signal–response relationships of these miRNA clusters with a physiological outcome of tube formation or fibrotic gene expression, partial least squares regression analysis was applied. The importance of each up- or downregulated miRNA on physiological outcomes was determined. Finally, to validate the model, we delivered exosomes after ischemia–reperfusion injury. Exosomes from hypoxic CPCs improved cardiac function and reduced fibrosis.

Conclusions: These data provide a foundation for subsequent research of the use of exosomal miRNA and systems biology as therapeutic strategies for the damaged heart. (Circ Res. 2015;116:255-263. DOI: 10.1161/CIRCRESAHA.116.304360.)

Key Words: exosomes ■ microRNA ■ systems biology

Cardiovascular disease is the leading cause of morbidity and mortality in developed nations, and acute myocardial infarction (MI) is a major contributor to poor outcomes, particularly in the United States and other developed nations. Beyond the acute treatment of restoring blood flow to hypoxic myocardium, subsequent measures focus on improving the contractility of noninfarcted tissue. Typically, the damaged, relatively nonregenerative myocardium undergoes a degenerative remodeling process that leads to heart failure. Furthermore, the economic burden of cardiovascular disease is profound. In 2010, costs for cardiovascular disease–related care totaled $315.4 billion, and by 2030, costs are expected to grow to $918 billion when 43.9% of the population is expected to have cardiovascular disease.1

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Editorial, see p 219
In This Issue, see p 215

Cell-based therapies to treat the damaged heart—including injection of stem cells from various sources—have yielded mixed results in several species.2–4 Cardiac progenitor cells (CPCs), a small population of stem-like cells residing in the heart, are of interest as they differentiate into cardiac lineages and can be isolated by tissue biopsy. Induced differentiation of stem cells into various cardiac cell types has shown changes in cell exocytosis in response to different growth conditions.1,5–7 However, whether these changes in stem cell exocytosis have a beneficial effect on the cardiac response through differentiation or paracrine signaling is unknown.
MicroRNAs (miRNAs) are recognized as important regulators of intracellular gene expression. Recently, they have been discovered extracellularly in the blood of mammals. Since then, stable extracellular miRNAs have also been identified in urine, saliva, semen, breast milk, and cerebrospinal fluid. Furthermore, distinct miRNA profiles in biofluids have been linked to disease pathologies, leading to interest in the use of extracellular miRNAs as disease biomarkers. Extracellular miRNA profiles have already been explored for a range of conditions, including cancer, diabetes mellitus, and cardiovascular diseases. Recent efforts have been made to explore the endogenous function of circulating miRNAs, especially in intercellular communication and gene regulation. Interestingly, the miRNA signatures are unique among different carriers and between carriers and parent cells, suggesting regulated export of miRNAs. Exosomes are secreted membrane-bound vesicles, with diameters ranging from 30 to 130 nm that carry a multitude of signals. Most cells secrete exosomes; those verified include platelets, lymphocytes, and adipocytes and muscle. Cardiac progenitor cells (CPCs) were isolated from neonatal adult Sprague–Dawley rats by removing the heart and homogenizing the tissue as described.

Exosome Generation
CPCs were grown to 90% confluence and quiesced for 12 hours. Plated cells were subjected to normoxic or hypoxic conditions for 3 or 12 hours. To generate hypoxic conditions, cells were transferred to an incubator chamber (Billups-Rothenberg MIC-101) and flushed with hypoxic gas mixture (95% N2 and 5% CO2). After conditioning, the media was subjected to sequential centrifugation (Optima XPN-100 ultracentrifuge; Beckman Coulter SW 41 Ti rotor) at 10,000 g for 35 minutes to remove cell debris and 100,000 g for 70 minutes followed by 2 washings in PBS (100,000 g for 70 minutes). The exosome pellet was isolated and the protein content of the exosome suspension was analyzed by Micro BCA Protein Assay kit (Thermo Scientific Pierce 23235) according to the manufacturer’s instructions.

Secreted miRNA Analysis
miRNA was isolated from conditioned media with the mirVANA PARIS kit (Invitrogen AM1556M) according to the manufacturer’s protocol. The miRNA solutions were analyzed (Agilent 2100 Bioanalyzer) for size, quality, and quantity of miRNA. After characterization, miRNA was subjected to analysis via Affymetrix MultiSpecies MicroRNA GeneChip array. Data were analyzed in Affymetrix Expression Console to determine levels of miRNA upregulation.

To evaluate levels of upregulated miRNA in exosomes, exosomal miRNA was isolated with the mirVANA PARIS kit and cDNA generated via NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen MIRC-50) according to the manufacturer’s protocol, cDNA samples were then subjected to qRT-PCR and relative miRNA levels ascertained by comparative CT method, with RNU6B as the housekeeping gene. The mean minimal cycle threshold values were calculated from triplicate reactions.

Principle Component and Partial Least Squares Regression Analysis
Principle component (PC) and partial least squares regression analysis were performed as described previously, using the SIMCA-P software (UMetrics) that solves the partial least squares regression (PLSR) problem with the nonlinear iterative partial least squares algorithm.

An expanded Methods section is available in the Online Data Supplement.

Results
Verification of Exosome Internalization
Exosomes were isolated and characterized as described in Online Figure I. To determine whether cardiac cell types of interest could internalize exosomes derived from CPCs subjected to 12 hours of hypoxia or normoxia, cardiac endothelial cells and fibroblasts were incubated for 12 hours with Acidine Orange or calcein-labeled exosomes and imaged by confocal microscopy (Figure 1A and 1E) or ImageStream flow cytometry (Figure 1B and 1F). Internalization of exosomes was confirmed visually by the presence of intracellular punctate fluorescence and also focally by localization of exosomes in the same plane as nuclei in z-stacks. Analysis of number of spots per cell revealed no difference in uptake between exosomes from hypoxic or normoxic CPCs in either cell type (Figure 1C and 1G). Furthermore, similar findings were observed for average fluorescence intensity per cell (Figure 1D and 1H). We also examined uptake of exosomes derived from CPCs subjected to 3 hours of hypoxia or normoxia and saw similar trends (Online Figure IIA–IIIF). Uptake by primary rat cardiomyocytes was minimal (Online Figure IIG).

Exosomes From Hypoxic CPCs Increase Tube Formation
To determine functional effects of exosomes on endothelial cells, we evaluated whether exosome internalization could induce endothelial tube formation. Cardiac endothelial cells were treated for 24 hours with exosomes from hypoxic or normoxic CPCs and then plated on GelTrex before imaging (Figure 2A). Treatment group tube lengths were normalized to tube formation measured in nontreated cells. Although exosomes from normoxic CPCs had no significant effect on tube formation, exosomes from hypoxic CPCs significantly enhanced formation of tube-like structures (Figure 2B). This response was dependent on exosome dose, and disruption of exosomes from hypoxic CPCs via sonication abrogated the effect. Furthermore, cotreatment with the RNA-induced silencing complex inhibitor
Gray et al: Cardiac Exosome Modeling and Therapy

aurintricarboxilic acid\(^29\) negated the effects of exosomes on tube formation. We also examined angiogenic gene expression in endothelial cells treated with exosomes and found modest changes in response to either exosome treatment (Online Figure III).

**Figure 1.** Cardiac cells internalize cardiac progenitor cells (CPC) exosomes. Confocal 2D-images (XY) from a central focal plane (with orthogonal XZ and YZ images on the bottom and right, respectively) from a single z-stack and differential interference contrast images of endothelial cells (A) or cardiac fibroblasts (E) with internalized Acridine Orange-stained CPC exosomes (blue, Hoechst; green, Acridine Orange). ImageStream flow cytometry images of cardiac endothelial cells (B) or cardiac fibroblasts (F) with internalized calcein-stained CPC exosomes (green=calcein). No difference was observed in the uptake of exosomes generated in normoxic or hypoxic conditions as evaluated by spots per cell (C and G) or total fluorescence per cell (D and H). More than 50,000 events were analyzed by IDEAS statistical software. n=6 for C and D, n=4 for G and H. Error bars represent SEM, unpaired t test comparison. All scale bars=10 \(\mu\)m.

**Figure 2.** Effects of cardiac progenitor cells (CPC) exosomes on tube formation. (A) Representative images of blood vessel-like structures stained with calcein on Geltrex. Scale bar=1 mm. (B) Tube formation relative to nontreated cells. Exosomes secreted by normoxic CPCs had no effect on tube formation, whereas exosomes from hypoxic CPCs increased tube formation in a positive dose-response manner. The effect plateaued after 0.1 \(\mu\)g/mL treatment, which elicited an increase in tube formation 2.1±0.12-fold over nontreated cells (mean±SEM). Negative controls included sonicated disrupted exosome, which elicited no change in tube formation. Co-treatment with RNA-induced silencing complex-inhibitor aurintricarboxilic acid neutralized the effect of exosomes from hypoxic CPCs. *\(P<0.05\), **\(P<0.001\). ***P<0.001 compared with nontreated cells (dotted line).

**Figure 3.** Exosomes generated from hypoxic cardiac progenitor cells (CPCs) in 12 h ameliorated cytokine stimulation of fibroblasts. Although tissue growth factor (TGF-\(\beta\)) increased fibrotic gene mRNA levels ≤3.6±0.28-fold (mean±SEM), only treatment with 0.1 \(\mu\)g/mL exosomes from 12 h hypoxic CPCs reduced stimulation: connective tissue growth factor (CTGF; A) to 1.7±0.27-fold (n=9–10); collagen I (COLI; B) to 1.3±0.3-fold (n=6); collagen III (COLIII; C) to 0.75±0.12-fold (n=5); vimentin (VIM; D) to 0.85±0.16-fold (n=7). *\(P<0.05\), **\(P<0.01\), ***P<0.001 compared with nontreatment mRNA levels. ANOVA followed by Tukey post test.
VIM (44%) mRNAs in cells treated with 12 hours hypoxic exosomes compared with TGF-β stimulation alone. COL1 was decreased 42% by 12 hours hypoxic exosomes, but this was not considered statistically significant.

CPC miRNA Secretome Is Altered in Response to Hypoxia

We isolated the small RNA fraction of CPC-conditioned media (3 and 12 hours normoxia and hypoxia) and performed Affymetrix GeneChip miRNA array. For the 12-hours time point, 11 miRNAs were upregulated ≥2-fold in response to hypoxic conditions. qRT-PCR analysis of small RNA isolated from pooled exosomes validated the upregulation of the 11 miRNAs by hypoxia (Figure 4A, light bars). Interestingly, 6 of the 7 miRNA upregulated in hypoxic exosomes have been previously shown to have a role in cardiac function (Online Figure IV). Only 2 of these miRNAs were upregulated in microvesicles (miR-17 and -210).

We also examined donor CPC miRNA levels after 12 hours of normoxia or hypoxia. We observed an inverse relationship between donor intracellular (Figure 4A, dark bars) and exosomal miRNA levels. To determine miRNA transfer, we measured the intracellular levels of 3 upregulated miRNAs in recipient endothelial cells and fibroblasts (Figure 4B and 4C) and we observed a 2.1-fold increase of miR-292 in fibroblasts from treatment with 12 hours hypoxic exosomes. Although not statistically significant, the trend suggests that 12 hours hypoxic exosomes increased miR-103 and -15b in endothelial cells.

Lack of Effect From 3 Hour Exosomes

We measured transfer of miRNA in recipient endothelial cells and fibroblasts treated with 3 hours exosomes (Figure 5A and 5B). In contrast to 12 hours exosomes, we found no significant increase of any of the examined miRNAs in recipient cells.

Based on earlier models, exosomes generated in 3 hours of hypoxia or normoxia were not projected to be a potent driver of fibrotic mRNA expression or tube formation. Out of the 4 fibrotic miRNAs examined (Figure 5C–5F), only COL1 decreased significantly (64%) when treated with 3 hours hypoxic exosomes (Figure 5D). No significant effect on tube formation was observed in cells treated with 3 hours exosomes (Figure 5G).

Principle Component Analysis Reveals Covarying miRNA Clusters

We used principle component analysis of the microarray data to investigate covarying relationships among the miRNAs that were up- or downregulated after pretreatment conditions. The...
first PC separated the fold change differences from the absolute values of the miRNA levels, as expected. PC 2 and 3 were far more interesting in that 4 distinct clusters of covarying miRNAs were identified as shown in Figure 6A.

To assign biological activity to each of these clusters, we used the miRNAs from the microarray that were validated by qRT-PCR (Figure 6A) as landmarks on the PC plot. miR-20a, miR-199a-5p, and miR-292-5p covary with the green cluster, miR-292-3p with the blue cluster, and miR-17 and miR-103 with the red cluster, and miR-17, miR-210, miR-15b, and miR-20a cluster with responses that are a combination of both the red and blue clusters.

Partial Least Square Regression Linked miRNA Clusters to Physiological Responses

PLSR was used to establish a relationship between the cue (pre-conditioning hypoxia/normoxia and time duration) and the signal (miRNA levels), which were then mapped onto a putative biological response in an unbiased approach (cue–signal–response paradigm). PLSR models were made using the entire 377 miRNAs from the microarray matched to the responses of tube formation and mRNA expression of CTGF, COLI, COLIII, and VIM as measured above, but then a reduced model was made with just the 11 miRNAs confirmed by miRNA array that were matched to physiologically relevant outcomes for angiogenesis and fibrosis. Scores plot shows that each treatment is separated across a PC with each time/treatment combination in a different quadrant (Figure 6B).

Separation across PC 1 (x-axis) indicates time difference as 3 and 12 hours timepoints are on opposite sides, and PC 2 (y-axis) separates the cues by oxygen treatment. In the loadings plot to the right in Figure 6B, CTGF and tube formation responses were segregated over PC 2 (oxygen treatment). Covarying miRNAs are shown as well on the loadings plot in PC space.

Refined PLSR Models Retained Integrity

Predictability of our model using a bootstrapping approach was determined for responses of tube formation and miRNA expression of CTGF, COLI, COLIII, and VIM. PLSR also determines the most important miRNA signals contributing to that response by calculating the variable importance for projection. The 211 miRNAs with variable importance for projection values ≥1 were chosen in an unbiased manner, and their model’s predictability was compared with the full model of all 377 miRNAs and against the 11 select miRNA model. The model trained only with significant variable importance for projections had the best predictability of 99%. However, the model with only the 11 miRNAs still maintained a 96.8% predictability (Figure 6C), slightly above the 96.5% of the full set of 377 miRNAs. This finding indicates that these 11 miRNAs may provide sufficient data to predict a biological response after exosome treatment or even that manipulating their relative levels may be sufficient to drive that particular biological response of CTGF expression or tube formation.

Figure 6. Computational modeling of covariant miRNAs using the cue–signal–response paradigm. A, Principle component (PC) modeling revealed 4 unique miRNA clusters that associate with either normoxia/hypoxia or other biological function. Labeled miRs are the key miRs verified by RT-PCR. B, Scores plot (left) and Loadings plot (right) from partial least squares regression (PLSR) analysis trained with 3 or 12 h normoxia or hypoxia treatment with only the 11 miRs of interest, matched to responses of tube formation and mRNA expression of connective tissue growth factor (CTGF), collagen I (COLI), collagen III (COLIII), and vimentin. CTGF and tube formation responses are clearly segregated over PC 2 (oxygen treatment), and covariance of hypoxia with tube formation and normoxia with CTGF were observed. Covarying miRs are plotted as well in PC space. C, Goodness of prediction for responses of tube formation and miRNA expression of CTGF, COLI, COLIII, and vimentin was tested on the models to compare reduced models of 11 select miR and 211 top variable importance for projection (VIP) miRs to the full model of all 377 miR. The refined models retained high $R^2$ values.
Unbiased Identification of Additional miRNAs That Significantly Contribute to Angiogenic and Antifibrotic Responses but not Identified by Microarray

To identify other miRNAs that may contribute to these responses, beyond the initial 11 chosen based on fold-change in the microarray, miRNA weighted coefficients of significant magnitude were investigated for being toward or against the physiological responses of CTGF and tube formation. Of these, 33 unique miRNAs were pro-tube formation (positive weighted coefficient) and 21 were anti-CTGF (negative-weighted coefficient) with 17 miRNAs fitting this category for both responses (Figure 7A). The list is shown in Figure 7B. Plotting only these miRNAs in PC space illustrates clustering with angiogenic response of tube formation and are antifibrotic, in that they are opposite CTGF mRNA expression (Figure 7C).

Hypoxic Exosomes Improve Function in the Infarcted Heart

We measured fractional shortening of the left ventricle 7 and 21 days after ischemia–reperfusion injury (Figure 8A and 8B). Ischemia–reperfusion significantly reduced function at both time points, but only treatment with 12 hour exosomes significantly improved function: in the acute phase (increased from 30.6% to 36.4%) and chronic phase (increased from 27.6% to 34.2%). We also measured fibrosis in reconstructed whole heart sections. Only treatment with 12 hours exosomes significantly reduced fibrosis (Figure 8C and 8D).

Discussion

Stem cell–based therapies to treat detrimental myocardial remodeling and cardiac dysfunction post-MI have shown promise, but significant obstacles to this approach remain. If possible, the amplification and delivery of beneficial paracrine signals generated by these cells could overcome obstacles associated with cell injection–based approaches to repair damaged myocardium.10,30 Because CPCs are specialized to function in the heart, CPC-generated signals may be particularly well suited to treat cardiac pathologies. Very few studies have investigated the therapeutic potential of CPC exosomes. In one, exosomes enhanced endothelial migration, indicating angiogenic effects.25 CPC exosomes were also shown to reduce myoblast apoptosis in vitro and decrease myocyte cell death in an animal MI model.23 However, in both of these studies, exosomes were generated under normoxic conditions, which likely did not reflect the state of postinfarct tissue. Importantly, hypoxic preconditioning enhanced the benefit of CPC therapy in an animal MI model.31 Here, exosomes generated by CPCs grown under normoxic conditions had a diminished reparative capacity compared with exosomes from hypoxic cells. This difference in physiological response was not caused by vesicle size, total RNA content, or protein levels because these values were similar between the different exosome groups. We found punctate (=1 μm) fluorescence in recipient cells treated by the different groups of exosomes, suggesting that exosomes deposit their cargo through endocytic pathways, which is then transported to the perinuclear region by the cytoskeleton.10,20

Figure 7. Partial least squares regression (PLSR) analysis identifies additional miRNAs from exosomes that covary with angiogenic (tube formation) and antifibrotic (anti-CTGF [connective tissue growth factor]) responses. Weighted coefficients of miRNAs determined to have significant variable importance for projection (VIP) values were investigated for their projections toward or against responses of CTGF and tube formation to identify other miRs beyond the initial 11 chosen based on fold change in the microarray. A, Venn diagram indicating that 33 unique miRs were pro-tube formation and 21 miRs were anti-CTGF, with 17 miRs projecting toward both responses. B, List of miRs associating with only protube formation, against CTGF, or both responses. C, Loadings plot of these 37 miRs with responses showing weighted coefficients and clustering of miRs identified through unbiased PLSR analysis.
We used microarray analysis to examine temporally dynamic extracellular miRNA release from CPCs after 3 and 12 hours of hypoxia. Of the 11 miRNAs upregulated by ≥2-fold by hypoxia at the 12-hour time point, qRT-PCR confirmed that 7 were encapsulated by exosomes. Interestingly, most of these have been shown to be involved in regulating cardiac functions of interest. One of the hypoxia-generated miRNAs, miR-15b, has been shown to be upregulated in the circulation of patients with critical limb ischemia. Here, we focused on miRNAs encapsulated within exosomes, although miRNAs may be transported extracellularly by other modalities, namely microparticles, proteins, and lipoproteins, which were not evaluated in this study. Indeed, we did detect 2 miRNAs upregulated in microparticles, and these also may have beneficial effects. Additionally, exosomes carry other molecules, including proteins, phospholipids, and carbohydrates. Although our data suggest exosomal miRNA played a critical role in the physiological response of recipient cells, we do not rule out the contribution of other exosomal cargo.

Computational modeling tools have been instrumental in evaluating large biological data sets, so they were well-suited to handle our wide array of dynamic exosomal content. We measured 377 mature miRNAs at both the 3- and 12-hour time points that may be difficult to attribute to specific functions. The cue–signal–response paradigm was able to extract important biological information by integrating known and unknown measurable variables. This powerful computational tool has been used to better understand data sets and develop predictive models in a variety of biological systems. The use of this paradigm was particularly important for miRNAs because 1 miRNA may affect hundreds of genes. miRNAs that changed covariantly will result in them clustering through principle component analysis. Indeed, principle component analysis on the normalized data from our microarray analysis identified miRNAs clusters that covary based on treatment condition. We were surprised to find that the majority of miRNAs clearly grouped into 4 major clusters. The black cluster is pro-hypoxic and blue is the opposite. Red and green clusters do not respond to hypoxia (zero projection onto PC2, the hypoxia axis), but are the effects caused by another currently unknown mechanism.

In the PLSR model trained with only the 11 miRNAs from the array, miR-292 had a high variable importance for projection scoring, indicating a potentially influential role in the response. To date, there are no reports of miR-292 as a regulator of cardiac function, and studies are underway to determine the role of this miRNA in the cardiovascular system. Since the functions of miR-20a and -17 have been described, other miRNAs closely clustering and covarying with these 2 may have similar roles in modulating cardiac function and repair. Overall, PLSR modeling is a helpful method to prioritize testing of new miRNAs that may be involved in certain biological outcomes. For example, if further analysis verifies an important role for miR-292 in improving cardiac function, then inclusion of other miRNAs that may be involved in certain biological outcomes. For example, if further analysis verifies an important role for miR-292 in improving cardiac function, then inclusion of other miRNAs from its cluster could amplify its protective effects. Indeed miR-292 was identified, unbiased, in the new group of miRNAs based on their weighted coefficients (Figure 7B), along with 36 others that might be beneficial to amplify the effect of tube formation although minimizing the profibrotic response. We initially examined only the miRNA that were upregulated >2-fold with RT-PCR, but the model was able to consider all
miRNA within the array. There were several pre-miRNA that were also increased, although none over 2-fold. Additionally, we did not examine the miRNA that were decreased in hypoxic exosomes. This is difficult to address because even if the miRNA are decreased in hypoxic-derived exosomes, they will still be transferred (albeit at a smaller level) to recipient cells. They could potentially explain why normoxic exosomes were less effective (a decrease in hypoxic-derived exosomes would mean they are enriched in normoxic-derived exosomes). We saw that exosomes from hypoxic CPCs had decreased levels of miR-320 (shown to be antiangiogenic),\textsuperscript{43,44} miR-222 (proapoptotic and antimigration),\textsuperscript{45} and miR-185 (profibrotic).\textsuperscript{46} The lack of these signals in hypoxic-derived exosomes and the enrichment of preregenerative miRNA could be an interesting area for future studies, and these types of modeling algorithms can provide information on potential biological effect.

Many of these 37 identified in Figure 7B were not identified by high-fold change in the microarray, providing a greater rationale for using multivariate analytical methods that consider the miRNA levels changing in response to a cue, but having an effect on several biological responses, instead of solely focusing on the miRNA signal changing as the final outcome. By mapping the signal changes to different biological responses (here CTGF, tube formation, COLI and COLIII, and VIM miRNA), the projection or contribution of that signal to several responses can be predicted, and their contributions to different aspects of a complex biological response may be parsed or integrated, providing insight into the same decisions cells must make.

Finally, delivery of exosomes derived from CPCs subjected to 12 hours of hypoxia improved both acute and chronic function, although inhibiting fibrosis. These data are in agreement with both our in vitro studies and our computational model. Recently, several studies have demonstrated that exosomes derived from stem cells cultured under normoxic conditions also have beneficial effects postinfarction. Although one study used cardio-sphere-derived cells,\textsuperscript{47} the other used CPCs similar to this study.\textsuperscript{48} One major difference is that the authors used total extracellular vesicles and not exosomes alone, thus it is unclear whether the other studies would see a benefit from subjecting those cells to hypoxia. What is interesting is that those studies, as well as the one presented here, demonstrate that vesicular transfer of miRNA is likely driving the protective/regenerative process.

In this report, we show that CPCs release a beneficial paracrine signal in response to hypoxia. We confirmed that exosomal miRNAs content is dynamically regulated based on the length of time CPCs are exposed to hypoxia. Based on our data, we developed a computational model to determine how exosome-generating conditions, miRNAs, and physiological responses covary. Together, our findings support the development of hypoxic CPC-derived exosomes as naturally derived therapeutics and lay the groundwork for statistical models that direct bio-inspired therapeutics of rational design.

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Disclosures

None.

References

Cardiac Exosome Modeling and Therapy

What Is Known?
- Stem cell therapy for myocardial infarction (MI) has provided mixed results, with beneficial paracrine signaling being a common explanation.
- A small population of cardiac progenitor cells (CPCs) resides in the heart, although the full understanding of these cells' function has yet to be fully elucidated.
- Ultimately, the body is unable to fully repair the heart post-MI, leading to significant long-term ailments.

What New Information Does This Article Contribute?
- CPCs secrete exosomes in response to hypoxic conditions that benefit cardiac cells and reduce deleterious effects in the post-MI heart.
- miR-15b, -17, -20a, -103, -19a, -210, and -292 are upregulated in exosomes generated in hypoxic conditions, which contribute to cardiac repair.
- Systems biology analysis correlates exosomal microRNA levels to CPC stimulation and physiological responses, which provides insight and guidance for biologically inspired therapies.

Novelty and Significance

CPCs are attractive candidates for treatment, and it is believed that a potential mechanism is paracrine signaling. The therapeutic potential and cargo of exosomes generated in conditions that mimic MI, such as hypoxia, are unknown. We found that CPC exosomes are internalized by cardiac fibroblasts and endothelial cells and subsequently regulate their function. These exosomes also decreased cardiac fibrosis in a rat MI model and improved cardiac function in the acute and chronic phases. We found 7 microRNAs to be upregulated in exosomes generated under hypoxia and identified clusters of co-varying microRNAs through systems biology analysis. This article demonstrates that CPCs tailor the contents of secreted exosomes in response to oxygen content toward a regenerative phenotype. This article is the first to show a beneficial effect of exosomes derived under hypoxic conditions, as well as model the correlation of time and oxygen levels with co-varying microRNAs and physiological responses. These findings suggest that the paracrine effect of CPCs may be enhanced by hypoxia and could be a potential mechanism of cell therapy in vivo. Furthermore, systems biology analysis can be used to determine potential microRNA mediators for future investigation.
Identification of Therapeutic Covariant MicroRNA Clusters in Hypoxia-Treated Cardiac Progenitor Cell Exosomes Using Systems Biology

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SUPPLEMENTAL MATERIAL

Methods

Cell isolation
The tissue homogenate was further digested with type-2 collagenase (1 mg/ml in Hank's balanced salt solution and passed through a 70 μm filter. Cells were incubated with Dynabeads (Dynal) conjugated to a c-kit antibody (Santa Cruz H-300) prior to magnetic sorting. Sorted cells were plated in a T-75 tissue culture flask and expanded to confluence. Following isolation, CPCs were characterized by flow cytometric analysis of c-kit (Santa Cruz H-300), multi-drug resistance protein (MDR) (Santa Cruz H-241), Gata-4 (Santa Cruz H-112) and Nkx2.5 (Santa Cruz H-114). Only clones with >90% c-kit expression were used for subsequent studies. The CPCs were used through passage 21.

Media components
The media that was used for the fibroblasts consisted for 10% FBS, 1% L-glutamine, 1% penecillin, and 1% streptavidin, and DMEM basal media. For DMEM quiescent media, FBS was reduced to 2%. The culture media that was used for the CECs consisted of animal cardiac endothelial cell basal media, 2% FBS, 1% L-glutamine and 1% antibiotic/amniotic and 0.1% mEGF. For CEC quiescent media, FBS was reduced to 0.04%. The CPC culture media, Ham’s F-12 basal media was used along with 10% FBS, 1% penecillin, 1% streptavidin, 1% L-glutamine, 0.1% Leukemia Inhibitory Factor, and 10% fetal bovine growth factor. For CPC quiescent media, no FBS was used and 1% ITS was added. For CPC treatment media, no FBS or LIF was used and 1% ITS was added.

Flow cytometry on exosomes
Pooled exosomes were incubated with 1 μL sulfate-aldehyde latex beads (Invitrogen A37304) for 2.5 hr at 37 C, after which 100 mmol/L glycine in 10% goat serum was added to quench the reactive groups and block. Exosome-bead complexes were centrifuged (4,000 x g, 5 min) and washed with 1% BSA in PBS. Complexes were resuspended and incubated with mouse anti-rat CD 1° antibody (BD Biosciences 551808) for 12 hr at 4 °C. Complexes were washed in 1% BSA/PBS and incubated in Alexa Fluor 488 goat anti-mouse IgG (H+L) antibody (Invitrogen A-11001). After incubation (2 hr at 37 °C), complexes were washed twice and subjected to flow cytometry (BD Biosciences BD LSR II), where at least 50,000 events were collected.

TEM
Exosome pellets were isolated. The samples were prepared by a negative staining method using 1% phosphotungstic acid. 5 μl of the sample was deposited onto carbon-coated 200 mesh cooper grids that have been treated by glow discharge. After 5 minutes, the grid was dragged on a peice of filter paper to remove excess liquid on grid. 5 μl of 1% aqueous phosphotungstic acid (PTS, pH6.5) was deposited onto the grid before sample on grid was dried. After 30 seconds, the grid wad dragged on a piece of filter paper to remove the PTA on the grid. In the end, the grid was left to air-dry. The samples were then imaged using the JEOL JEM-1400 transmission electron microscope.

Cellular uptake of exosomes
Exosomes were stained with calcein (2 uM final concentration) for 30 min at room temperature, followed by two washes in PBS (100,000 x g, 70 min). Pellets were then passed through 0.20 um filters. Rat cardiac fibroblasts and endothelial cells were treated with the stained exosomes (1 μg/mL) for 12 hour and washed before being trypsonized and subjected to flow cytometry
(Imagestream X Mark II), where 10,000 events were collected. Images were analyzed by Amnis IDEAS image analysis software using spot count and channel intensity wizards.

For confocal microscopy, exosomes were incubated with Acridine Orange for 1 hour prior to dialysis (3 changes over 12 hours in 500mL PBS) to remove free dye. Cells were incubated with exosomes for 12 hours at 37°C prior to staining with Hoechst and imaging. Free Acridine Orange was used as a control. Cells were imaged on an Olympus Fluoview FV1000 confocal microscope (Olympus, Melville, NY) with a 60x oil-immersion objective.

Tube Formation
Rat primary cardiac microvascular endothelial cells (CECs) (CellBiologics R2111) were plated on gelatin-coated 12 well plates. Following quiescence for 12 hour, cells were then treated with 0.01, 0.1 and 1 μg/mL hypoxic or normoxic exosomes for 24 hours. The cells were then lifted and counted so that 10,000 cells from each treatment group were plated onto 30 μl Geltrex (Invitrogen A1413202) thick gels in 96-well plates. The cells were then incubated for 6 hours and stained with 2 μmol/L calcein in PBS. Cell groups were imaged with a fluorescent microscope (Olympus IX71) and the tube length was quantified using ImageJ software analysis. Each experiment was normalized to an untreated control to account for variability in tube formation.

Rat cardiac fibroblast isolation
Excised hearts from adult male Sprague-Dawley rats were minced and subjected to trypsin digestion (1 mg/mL in HBSS-, 4 C, 6 hour), followed by collagenase digestion (0.8 mg/ml in HBSS-, 37 C, 15 min). Digestion solutions were quenched with culture media and cell suspension passed through a 100 um filter. Cells were pelleted and plated for 3 hours to allow adherence of fibroblasts before washing plates to remove non-fibroblasts.

Rat ventricular myocyte isolation
Adult rat ventricular myocytes were isolated from male rats using a modified enzymatic method. Rats were euthanized by intraplural injection of ketamine (100 mg/kg) and xylezene (10mg/kg). Hearts were excised, mounted on a Langendorff apparatus, and retrogradely perfused with nominally Ca-free Tyrode solution for 5 min followed by minimal essential medium Eagle (MEM) solution containing 20 μM Ca and 45 g/ml Liberase Blendzyme TH (Roche Applied Science, Indianapolis, IN) for 20 min at 37°C. Ventricular tissue was minced, filtered, and washed in MEM solution containing 50 μM Ca and 10 mg/ml BSA. Isolated cells were kept in MEM solution with 50 μM Ca at room temperature (20–24°C) until subsequent experimentation.

Sarcomere shortening and Ca transient measurements
To measure [Ca]i, cells were loaded for 20 min with 10 μM fluo-4/AM (Invitrogen) and washed for 20 min. Cells were plated on laminin-coated coverslips and imaged on an inverted microscope (IonOptix). Sarcomere shortening and Ca transients were simultaneously acquired using a video-based sarcomere length and Ca acquisition module system (IonOptix, Milton, MA) during 0.5 Hz electrical stimulation and perfusion with a 2 mM Ca tyrode solution. [Ca]i is expressed as F/F0 where F0 refers diastolic fluorescence levels measured under control steady-state conditions during electrical stimulation. Ca transient amplitudes were quantified as ΔF/F0.

TGF-β stimulation
Fibroblasts were quiesced for 12 hour and treated with 0.1 μg/mL exosome for 12 hour before the addition of TGF-β (10 ng/ml final concentration) for 12 hour. Cell mRNA was isolated via Trizol reagent (Invitrogen 15596-026) according to manufacturer’s instructions. cDNA was generated via M-MLV Reverse Transcriptase (Invitrogen 28025-013) and qRT-PCR (Power
SYBR Green, Invitrogen 43687-08) was performed to evaluate CT levels. Relative expression was calculated using the comparative CT method, with GAPDH as the housekeeping gene. The mean minimal cycle threshold values were calculated from triplicate reactions.

**Animal studies**
All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Emory University. Myocardial infarction was performed in male adult Sprague-Dawley rats (Charles River Laboratories) in a randomized double-blinded manner. Briefly, the rats were anaesthetized (1-3% isoflurane), intubated and heart exposed by separation of ribs. The left anterior descending (LAD) coronary artery was ligated for 30 minutes. During reperfusion, exosomes (5 μg/kg suspended in 100 μL of saline) were injected into the myocardium at 3 border zones. Cardiac function was evaluated at 7 and 21 days after treatment by echocardiography (Acuson Sequoia 512 with a 14 MHz transducer) to assess the functional effects of each treatment. All functional evaluations were conducted and analyzed by investigators blinded to the animal's treatment group. The rats were euthanized and the hearts were excised for histological analysis. The hearts were fixed in 4% paraformaldehyde, dehydrated in ethanol, embedded in paraffin, and sectioned at 7 μm thickness.

**Picosirius Red staining**
Tissue sections were dewaxed in Histoclear followed by a series of washes in ethanol and stained with pico-sirius red solution for 1 hour (Sigma). The sections were washed in acidified water and ethanol and mounted with resinous medium (Cytoseal). Images of the entire heart section were taken at 10x magnification on a bright field microscope (Olympus) and tiled together using Adobe Photoshop. The % fibrosis was quantified using Image J as the ratio of fibrotic tissue (stained red) to total tissue.

**Statistics**
Statistics were calculated with GraphPad Prism software. Unpaired student's T-test was used to compare two groups where appropriate. In cases of multiple groups, one-way ANOVA was employed with Tukey post-test.
Supplemental Figure I. Exosomes characterization. (A) Flow cytometric plot revealing the presence of CD9+ exosomes. (B) TEM images of exosomes generated from normoxic or hypoxic CPCs. Diameters were 102.0±3.1 nm and 96.1±6.1 nm, respectively (mean±SEM). Scale bar = 100 nm. No significant difference was found in small RNA (C) or protein content (D) between the exosome types. Unpaired t-test.
Supplemental Figure II. Uptake of exosomes by recipient cells. Confocal 2D-images (XY) from a central focal plane (with orthogonal XZ and YZ images on the bottom and left, respectively) and differential interference contrast images of endothelial cells (A-C), cardiac fibroblasts (D-F), or cardiac myocytes (G) with internalized Acridine Orange-stained CPC exosomes. Cardiac myocytes exhibited negligible uptake of exosomes. Blue=Hoechst; Green=Acridine Orange; scale bars=10μm.
Supplemental Figure III. Regulation of angiogenic genes by exosomes. Cardiac endothelial cells were incubated with exosomes derived CPCs under normoxic (N) or hypoxic (H) conditions for 3 or 12 hours. While changes were very moderate (<1.3-fold increase), there were significant differences as noted in the graphs for several genes. Mean±SEM, n=6; ANOVA followed by Tukey post-test, *p<0.05, **p<0.01, ***p<0.001.
Supplemental Figure IV. Literature review of upregulated exosomal miRNAs. Six of the upregulated exosomal miRNAs have been established in the literature to function in a manner of cardiac interest. miR-292 has not been explored, but predictive software indicates that it may regulate fibrosis. Thicker bars represent more literature to support relationship. Red bars indicate inhibition, green bars indicate promotion.
Supplemental Figure V. Sarcomere shortening and Ca transient amplitudes. Sarcomere shortening and Ca transient amplitudes were recorded from rat ventricular myocytes loaded with 10 μM fluo-4/AM after 12 hr treatment with exosomes. (A) Example traces of percentage of sarcomere length shortening (top) and Ca transient recordings (bottom) from control and the various treatment groups. Summary data for sarcomere shortening (B) and Ca transient amplitude (C) (Mean±SEM; n=4).