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Enhanced Cardiac Regenerative Ability of Stem Cells After Ischemia-Reperfusion Injury

Role of Human CD34$^+$ Cells Deficient in MicroRNA-377

Darukeshwara Joladarashi, PhD,* Venkata Naga Srikanth Garikipati, PhD,† Rajarajan A. Thandavarayan, PhD,* Suresh K. Verma, PhD,† Alexander R. Mackie, PhD,‡ Mohsin Khan, PhD,† Anna M. Gumpert, PhD,† Arvind Bhimaraj, MD,§ Keith A. Youker, PhD,§ Cesar Uribe, MD,§ Sahana Suresh Babu, PhD,* Prince Jeyabal, PhD,* Raj Kishore, PhD,† Prasanna Krishnamurthy, PhD*‖

ABSTRACT

BACKGROUND MicroRNA (miR) dysregulation in the myocardium has been implicated in cardiac remodeling after injury or stress.

OBJECTIVES The aim of this study was to explore the role of miR in human CD34$^+$ cell (hCD34$^+$) dysfunction in vivo after transplantation into the myocardium under ischemia-reperfusion (I-R) conditions.

METHODS In response to inflammatory stimuli, the miR array profile of endothelial progenitor cells was analyzed using a polymerase chain reaction–based miR microarray. miR-377 expression was assessed in myocardial tissue from human patients with heart failure (HF). We investigated the effect of miR-377 inhibition on an hCD34$^+$ cell angiogenic proteome profile in vitro and on cardiac repair and function after I-R injury in immunodeficient mice.

RESULTS The miR array data from endothelial progenitor cells in response to inflammatory stimuli indicated changes in numerous miR, with a robust decrease in the levels of miR-377. Human cardiac biopsies from patients with HF showed significant increases in miR-377 expression compared with nonfailing control hearts. The proteome profile of hCD34$^+$ cells transfected with miR-377 mimics showed significant decrease in the levels of proangiogenic proteins versus nonspecific control–transfected cells. We also validated that serine/threonine kinase 35 is a target of miR-377 using a dual luciferase reporter assay. In a mouse model of myocardial I-R, intramyocardial transplantation of miR-377 silenced hCD34$^+$ cells in immunodeficient mice, promoting neovascularization (at 28 days, post–I-R) and lower interstitial fibrosis, leading to improved left ventricular function.

CONCLUSIONS These findings indicate that HF increased miR-377 expression in the myocardium, which is detrimental to stem cell function, and transplantation of miR-377 knockdown hCD34$^+$ cells into ischemic myocardium promoted their angiogenic ability, attenuating left ventricular remodeling and cardiac fibrosis. (J Am Coll Cardiol 2015;66:2214–26)

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From the *Department of Cardiovascular Sciences, Center for Cardiovascular Regeneration, Houston Methodist Research Institute, Houston, Texas; †Center for Translational Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania; ‡Feinberg Cardiovascular Research Institute, Northwestern University, Chicago, Illinois; §Houston Methodist DeBakey Heart & Vascular Center, Houston Methodist Hospital, Houston, Texas; and the ‖Department of Cell and Developmental Biology, Department of Cardiothoracic Surgery, Weill Cornell Medical College, New York, New York. This work was supported, in part, by the National Institutes of Health grants 1R01HL116729 to Dr. Krishnamurthy; and HL091983, HL053354, HL108795, and HL108806 to Dr. Kishore; American Heart Association grant-in-aid GRNT 25860041 to Dr. Krishnamurthy; American Heart Association Postdoctoral Fellowship 15POST25710392 to Dr. Thandavarayan; and American Heart Association Post-doctoral Fellowship 15POST22720022 to Dr. Garikipati. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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Heart failure (HF) has been classified as an epidemic of the 21st century and is now the major cause of morbidity in the United States (1). A number of cardiac pathophysiological conditions, including myocardial infarction (MI) and ischemia reperfusion (I-R) injury that leads to HF, are associated with activation of inflammatory mediators in the heart (2).

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Despite our belief that the heart has limited regenerative capacity, murine endothelial progenitor cells (EPCs) and human CD34$^+$ (hCD34$^+$) cell-based therapy provide substantial clinical benefits for ischemic diseases, such as chronic angina, MI, and HF (3,4). These cells, through paracrine-mediated growth factor secretion, induce neoangiogenesis/vasculogenesis and therefore augment cardiac cell survival and function (5). The major tissue factors for poor clinical outcome include hostile ischemia and chronic inflammatory tissue microenvironments into which the cells are introduced.

Recently, microRNAs (miR; approximately 20 to 22 nucleotides long) have elicited substantial interest as regulators of and therapeutic targets for HF (6). miR dysregulation after cardiac injury has been implicated in several biological processes involved in cardiovascular diseases (7,8). However, studies to understand the deleterious effect of miR dysregulation on hCD34$^+$ cells or murine EPCs in response to an inflammatory stimulus and its implications on cardiac regeneration and repair after myocardial transplantation have been limited.

So far, the role of miR-377 in hCD34$^+$ cell or EPC biology and function has never been explored. Moreover, it is unknown whether altering miR-377 expression affects hCD34$^+$ cell-induced angiogenesis in ischemic myocardial tissue. Therefore, determining the role of miR-377 in hCD34$^+$ cell or EPC biology and function and its molecular mechanisms could be of major significance for stem cell-based therapy aimed at regeneration of heart tissue.

In this study, we first sought to determine the alterations of miR in mouse EPCs under inflammatory conditions and cardiac biopsies from patients with HF. We further tested whether knockdown of miR-377 in hCD34$^+$ cells could affect the angiogenic response in ischemic myocardium.

METHODS

EPCs isolated from the bone marrow of C57BL/6J male mice (Jackson Laboratories, Bar Harbor, Maine) were cultured as described previously (9). Detailed methodology is provided in the Online Appendix. EPCs were subjected to lipopolysaccharide (LPS) treatment (25 ng/ml) for 12 h (to stimulate inflammatory response). Untreated cells served as controls. miR expression was analyzed using a polymerase chain reaction (PCR)-based miR microarray platform covering a total of 352 mouse miR. Data analysis was performed using Internet-based software for miR PCR array systems.

Heart tissue samples were obtained from failing human hearts at the time of transplantation at the Houston Methodist DeBakey Heart & Vascular Center, Houston Methodist Hospital (Houston, Texas), immediately frozen in liquid nitrogen, and stored at −80°C until use. Normal tissue samples were obtained from donor hearts not used for transplantation and were collected and stored in the same manner. All tissues were collected under an approved protocol by the Houston Methodist Research Institutional Review Board.

ABBREVIATIONS AND ACRONYMS

EPC = endothelial progenitor cell

HF = heart failure

HUVEC = human umbilical vein endothelial cell

IHD = ischemic heart disease

I-R = ischemia-reperfusion

LPS = lipopolysaccharide

LV = left ventricular

MI = myocardial infarction

miR = microRNA

mRNA = messenger RNA

qRT-PCR = quantitative reverse transcriptase polymerase chain reaction

STK35 = serine/threonine kinase 35

UTR = untranslated region

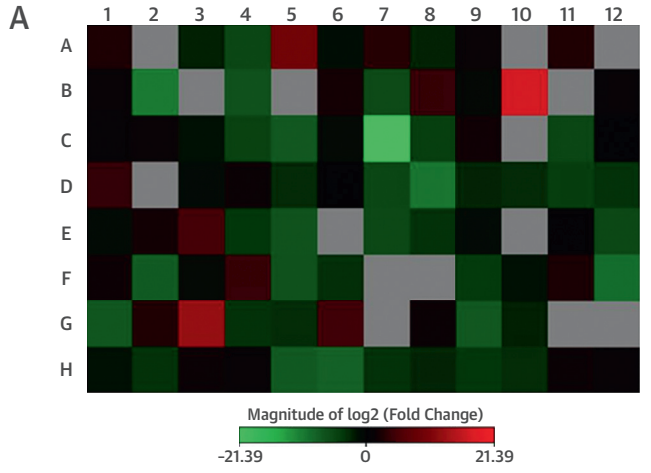
VEGF = vascular endothelial growth factor

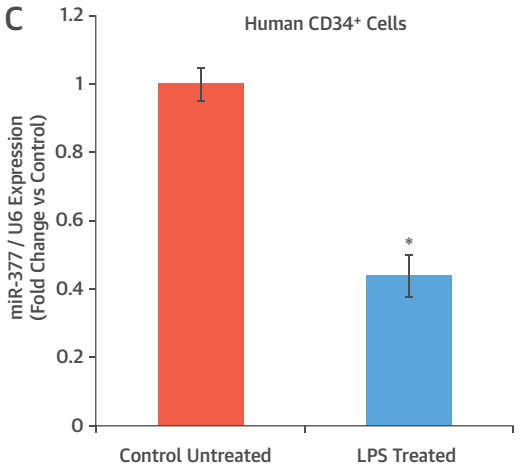
ISOLATION OF CELLS AND STUDY DESIGN. Cardiomyocytes were isolated from 12-week-old C57BL/6J male mice after 3 days of myocardial I-R by Langendorff perfusion and the Thompson procedure (10). For isolation of adult cardiac endothelial cells, CD31$^+$ cells were isolated via magnetic-activated cell sorting separation using anti-biotin CD31 microbeads (Miltenyi Biotec, San Diego, California), and CD31$^-$ cells were depleted. Purity of CD31$^+$ cells was confirmed by immunohistochemistry for CD31 on cytospins of freshly isolated CD31$^+$/CD31$^-$ cardiac cells.

To determine the role of miR-377 in hCD34$^+$ cells, the condition medium collected from culturing hCD34$^+$ cells transfected for 48 h with miR-377 mimic (60 nm/l), inhibitor (60 nm/l: anti-miR-377), miR mimic, or miR inhibitor negative control was analyzed using Proteome Profiler (human angiogenesis antibody array, R&D Systems, Minneapolis, Minnesota) per the manufacturer's instructions. Data were analyzed via ImageQuant LAS 4000 software (GE Healthcare, Waukesha, Wisconsin). The detailed transfection method is provided in the Online Appendix.

Human umbilical vein endothelial cells (HUVECs) were cotransfected with miR-377 mimic (60 nm/l), miR-377 inhibitor (60 nm/l), or miR mimic or inhibitor negative control (60 nm/l) and a reporter plasmid containing the 3′-untranslated region (3′-UTR) of serine/threonine kinase 35 (STK35; HmiT003085-MT06, 100 ng), mutated 3′-UTR of STK35 (CS-HmiT003085b-MT06-01, 100 ng), or corresponding control empty

FIGURE 1 miR Array Analysis of EPCs in Response to Inflammatory Stimuli





B

	1	2	3	4	5	6	7	8	9	10	11	12
A	miR-134 1.43	miR-137 1.06	miR-708 −1.42	miR-181b −2.19	miR-298 3.89	miR-101b −1.22	miR-195 1.55	miR-29c −1.45	miR-300 1.13	miR-471 1.06	miR-122 1.49	miR-302b 1.06
B	miR-30b 1.1	miR-33 −4.72	miR-547 1.06	miR-130b −2.75	miR-292-3p 1.06	miR-203 1.32	miR-301a −2.38	miR-431 1.75	miR-148a −1.08	miR-375 11.89	miR-135b 1.06	miR-449a 1.09
C	miR-20b 1.08	miR-151-5p 1.12	miR-152 −1.24	miR-103 −2.13	miR-34a −3.01	miR-7a −1.1	miR-377 −21.39	miR-652 −1.98	miR-9* 1.27	miR-381 1.06	miR-193 −2.2	miR-200a 1.03
D	miR-125a-3p 1.7	miR-376a 1.06	miR-378 −1.15	miR-467a* 1.22	miR-106a −1.57	miR-184 1.02	miR-291a-5p −2.23	miR-147 −4.46	miR-128 −1.51	miR-324-5p −1.59	miR-500 −1.94	miR-185 −1.68
E	miR-210 −1.14	miR-223 1.3	miR-21* 2	miR-301b −1.79	miR-202-3p −2.83	miR-208a 1.06	miR-324-3p −2.34	miR-335-3p −1.72	miR-98 −1.16	miR-153 1.06	miR-342-3p 1.06	miR-423-3p −2.26
F	miR-194 1.24	miR-222 −3.14	miR-370 −1.11	miR-465a-5p 1.72	miR-100 −2.73	miR-451 −1.67	miR-465a-3p 1.06	miR-878-3p 1.06	miR-320 −1.86	miR-188-5p −1.23	miR-192 1.39	miR-221 −4.02
G	miR-423-5p −2.93	miR-429 1.47	miR-672 6.24	miR-149 −1.7	miR-669a −1.63	miR-135a 1.92	miR-202-5p 1.06	miR-380-5p 1.2	miR-470 −2.99	miR-129-5p −1.44	miR-290-5p 1.06	miR-292-5p 1.06
H	miR-361 −1.25	miR-484 −1.72	miR-326 1.22	miR-350 1.1	snoRNA251 −3.11	snoRNA202 −3.43	snoRNA142 −1.69	Rnu6 −1.49	miRTC −1.79	miRTC −1.6	PPC 1.2	PPC 1.11

(A) Heat map shows microRNA (miR) expression in control and lipopolysaccharide (LPS)-treated endothelial progenitor cells. **(B)** Location of the miR in the heat map and their relative expression. **(C)** Validation of miR-377 expression in control and LPS-treated human CD34$^+$ cells by quantitative reverse transcriptase polymerase chain reaction (normalized to control U6; n = 3). *p < 0.05. EPC = endothelial progenitor cell.

luciferase reporter vector (CmiT000001-MT06, 100 ng, GeneCopoeia, Rockville, Maryland). The miR and reporter plasmid were mixed with Lipofectamine 2000 (Thermo Fisher Scientific, Tewksbury, Massachusetts) and added to a 48-well plate containing HUVECs (1.3×10^4). After a 24-h transfection, luciferase activity was assessed using a dual luciferase reporter assay kit (Promega, Madison, Wisconsin) per manufacturer's protocol.

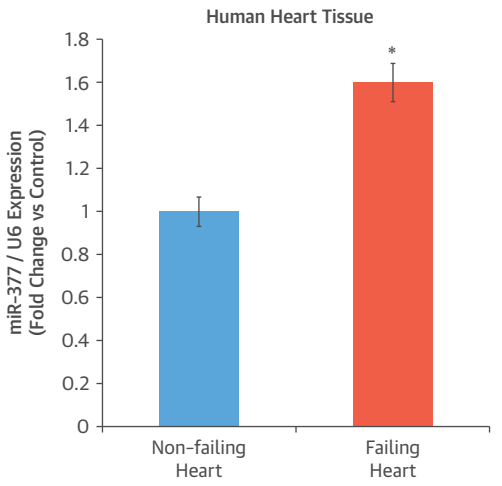
Twelve-week-old severe combined immunodeficiency (SCID) mice were subjected to myocardial ischemia for 30 min by temporary ligation of the left anterior descending coronary artery followed by reperfusion for 28 days as described previously (11,12). Immediately after ischemia was completed and reperfusion begun, mice received intramyocardial injection of hCD34$^+$ cells (5×10^4) transfected with either miR-377 inhibitor or miR inhibitor negative control at 3 different sites in the ischemia area. The hCD34$^+$ cells were labeled using a PKH26 red fluorescent cell linker kit (Sigma-Aldrich, St. Louis, Missouri) per manufacturer's protocol. Transplanted hCD34$^+$ cells were detected at 24 h after injection by immunofluorescence, and left ventricular (LV) functional changes and structural remodeling were evaluated 28 days after I-R.

STATISTICAL ANALYSIS. Data are presented as mean ± SE. An unpaired Student *t* test was performed between 2 groups of mice to determine statistical significance. When more than 2 groups were involved, analysis of variance with a Tukey post-hoc test was used to analyze the data. Probability (p) values of <0.05 were considered statistically significant.

RESULTS

Our previous study showed that prolonged inflammatory response in the myocardium is detrimental for EPC function (9). To determine the miR profile of EPCs under myocardial inflammatory conditions, we treated bone marrow–derived EPCs (mouse) with LPS (25 ng/ml) for 12 h and performed a quantitative

FIGURE 2 miR-377 Expression in Failing Human Hearts



As measured by quantitative reverse transcriptase polymerase chain reaction, microRNA 377 (miR-377) expression was significantly upregulated in failing versus nonfailing human heart samples (normalized to control group U6; n = 5). *p < 0.05.

reverse transcriptase PCR (qRT-PCR)-based miR array analysis. The miR array data analysis showed that several miR were differentially expressed with robust decreases in miR-377 expression in EPCs treated with LPS (**Figures 1A and 1B** [well 7C]). We further validated miR-377 expression in LPS-treated hCD34$^+$ cells (**Figure 1C**) using qRT-PCR. The results consistently showed significant decreases in miR-377 expression upon LPS treatment ($p < 0.05$ vs. control untreated cells). Further, we confirmed similar results in the mouse EPCs (Online Figure 1A) and HUVECs (Online Figure 1B) upon LPS treatment versus control ($p < 0.05$). The miR array data (heat map and expression) of all of the miR analyzed are depicted in Online Figure 2.

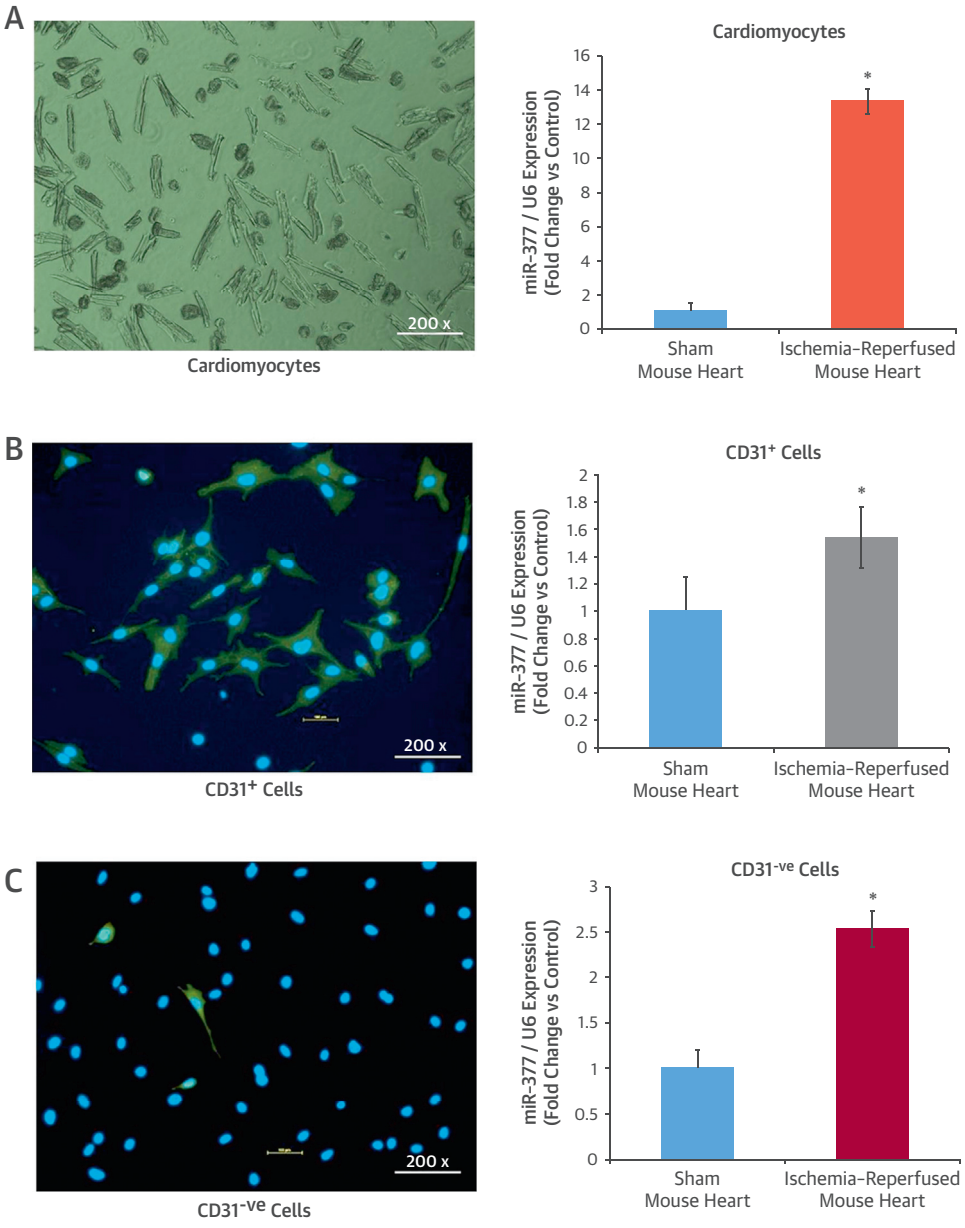
miR-377 EXPRESSION IN HUMAN FAILING HEARTS. To determine the effect of HF on miR-377 expression, cardiac biopsies were collected from the LV free wall of patients with ischemia at the Houston Methodist DeBakey Heart & Vascular Center. The qRT-PCR results showed that the miR-377 expression was significantly upregulated in human heart tissue samples from patients with HF compared with patients with non-cardiac-related ailments ($p < 0.05$) (**Figure 2**).

To determine ischemia-induced miR-377 expression in different cardiac cell types, we isolated cardiomyocytes, endothelial cells (CD31$^+$), and CD31$^-$ cells from mouse hearts with sham or I-R procedure, 3 days post-surgery, and assessed miR-377 expression by qRT-PCR analysis. I-R injury increased miR-377 expression in all of the cardiac cell types versus levels in sham-operated mice ($p < 0.05$) (**Figure 3**). These data, together with the miR-377 expression in the human failing hearts, prompted us to evaluate the effect of increased miR-377 expression in the myocardium on hCD34$^+$ cells that will be introduced into the myocardium for cell-based therapy.

Previous reports have suggested that the beneficial effect of hCD34$^+$ cell therapy is mediated through paracrine secretion of growth factors that aid in angiogenic response (13). To determine the effect of miR-377 on paracrine secretion of angiogenic factors in hCD34$^+$ cells, a proteome profile of condition medium of hCD34$^+$ cells transfected with miR-377 mimic, miR-377 inhibitor, or respective negative controls was performed using a human angiogenesis array. **Figure 4** shows that miR-377 mimic treatment significantly decreased secretion of various pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), matrix metalloproteinase 9, platelet-derived growth factor AA, hepatocyte growth factor, insulin-like growth factor-binding protein 1, endocrine gland-derived VEGF, endothelin 1, angiopoietin 1, and angiopoietin 2 compared with the conditioned media of the miR mimic negative control-transfected hCD34$^+$ cells ($p < 0.05$) (**Figure 4**). Some of the antiangiogenic proteins, like serpin E1, serpin F1, thrombospondin 1, urinary plasminogen activator, platelet factor 4, angiogenin, angiostatin, and artemin, were expressed at significantly higher levels in the conditioned media collected from miR-377 mimic-treated hCD34$^+$ cells compared with miR mimic negative control-transfected hCD34$^+$ cell-conditioned media. On the contrary, miR-377 inhibitor treatment showed higher proangiogenic factor and lower antiangiogenic factor secretion compared with miR-377 mimic-treated cell-conditioned media ($p < 0.05$) (**Figure 4**).

Vasculogenesis is a dynamic process involving migration, differentiation, endothelial cell engraftment, and vascular tube formation (14). To first determine the effect of miR-377 on migratory response of endothelial cells, we transfected HUVECs with miR-377 mimic, miR-377 inhibitor, or respective negative controls and assessed their migration toward a well-known stimulant VEGF using a modified Boyden chamber migration assay as described earlier (11). HUVECs transfected with miR-377 mimic demonstrated significantly lower levels of migration in response to VEGF stimulation compared with control nonspecific transfection ($p < 0.05$), whereas HUVECs transfected with miR-377 inhibitor showed significantly higher levels of migration in response to

FIGURE 3 Upregulated miR-377 Expression in Ischemic Mouse Hearts



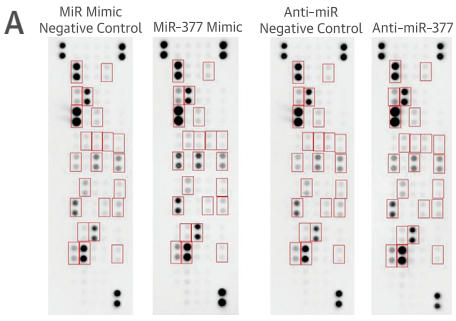
In **(A)** cardiomyocytes and **(B)** $CD31^+$ cells and **(C)** $CD31^-$ cells isolated from mouse hearts subjected to ischemia-reperfusion (I-R) or sham operation, I-R significantly upregulated microRNA 377 (miR-377) expression (normalized to control U6; $n = 6$). *$p < 0.05$.

VEGF stimulation compared with miR inhibitor negative control ($p < 0.05$) (Figure 5A).

Furthermore, we analyzed the effect of miR-377 on morphogenesis of HUVECs into vascular tubes. HUVECs transfected with miR-377 mimic showed significantly reduced ability and those transfected with miR-377 inhibitor displayed significantly increased ability to form vascular tubes compared with their respective negative controls (each $p < 0.05$) (Figure 5B).

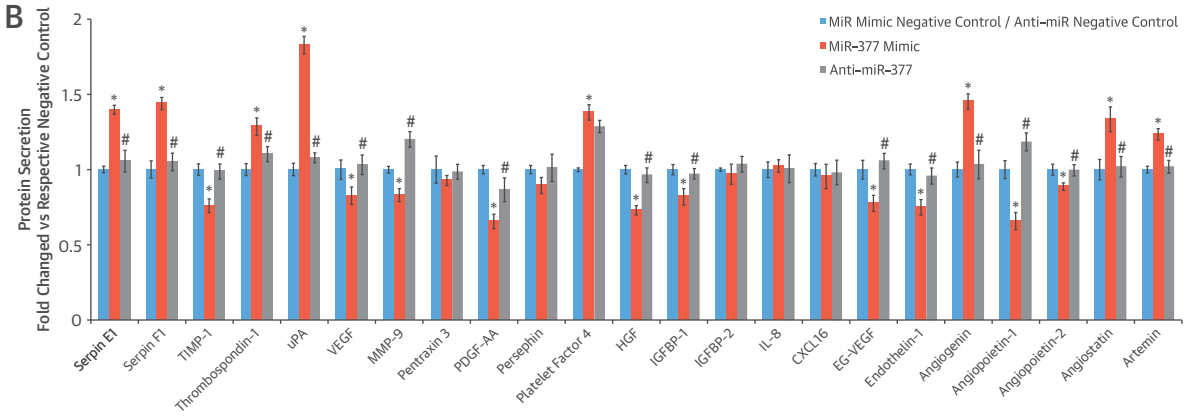
STK35 3′-UTR: DIRECT TARGET OF miR-377. miR bind to the 3′-UTR of target messenger RNA (mRNA) and regulate gene expression via post-transcriptional, translational repression or mRNA destabilization. To identify potential target genes involved in miR-377-mediated regulation, specifically

FIGURE 4 Proteome Profile of Conditioned Media From hCD34$^+$ Cells



C

+ control	Serpin B5	MIP-1a	GDNF	Coagulation Factor-III	+ control
	Serpin E1	MMP-8	GM-CSF	CXCL16	
	Serpin F1	MMP-9	HB-EGF	DPPIV	Activin
	TIMP-1	NRG1-β1	HGF	EGF	ADAMTS-1
	TIMP-4	Pentraxin 3	IGFBP-1	EG-VEGF	Angiogenin
	Thrombospondin-1	PD-ECGF	IGFBP-2	Endoglin	Angiopoietin-1
	Thrombospondin-2	PDGF-AA	IGFBP-3	Endostatin/CollagenXVIII	Angiopoietin-2
	uPA	PDGF-AB/PDGF-BB	IL-1β	Endothelin-1	Angiostatin/Plasminogen
	Vasohibin	Persephin	IL-8	FGF acidic	Amphiregulin
	VEGF	Platelet Factor 4	LAP (TGF-β1)	FGF basic	Artemin
	VEGF-C	PlGF	Leptin	FGF-4	
- control		Prolactin	MCP-1	FGF-7	+ control

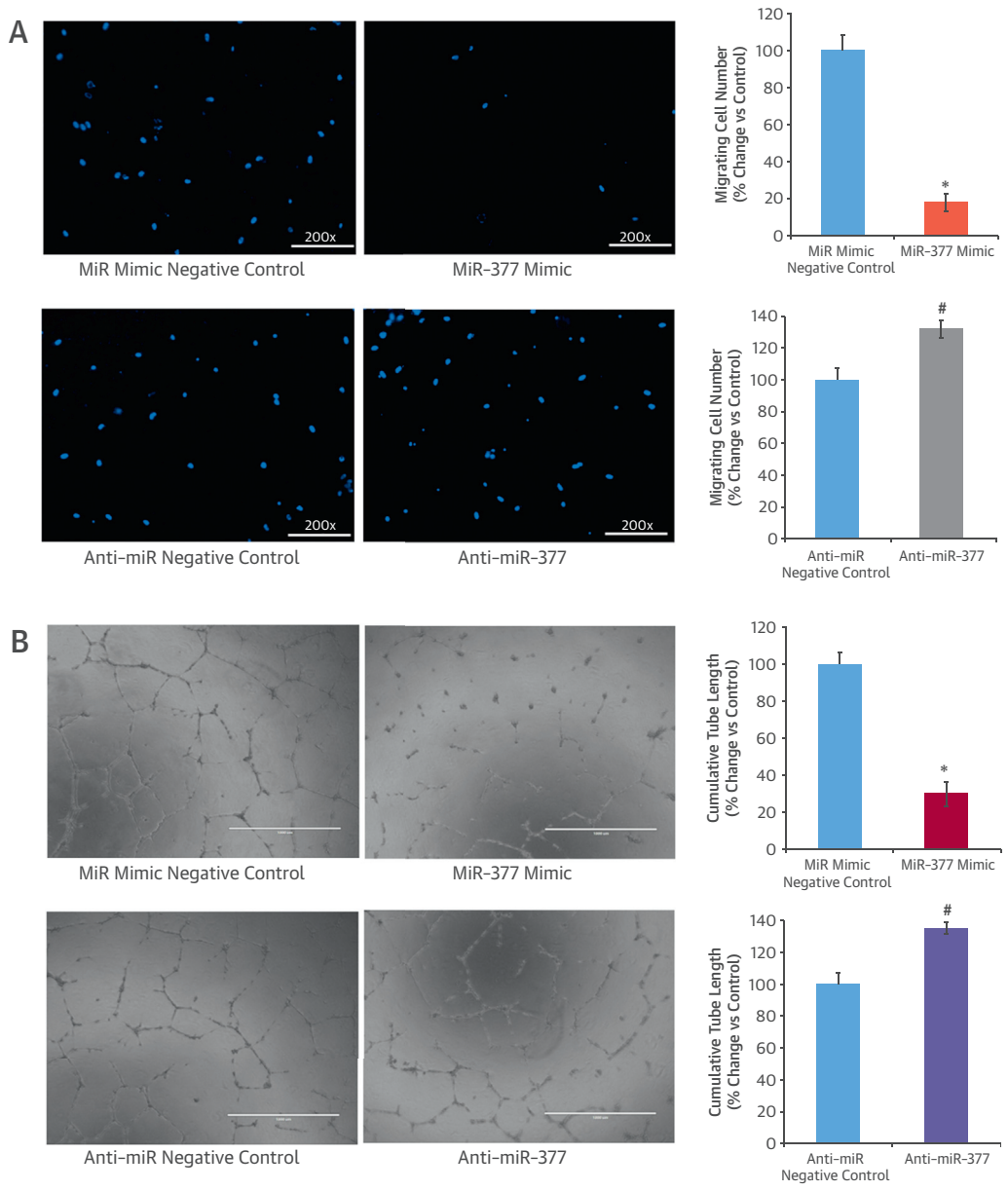


(A) Human angiogenesis antibody array analysis on condition media collected after 48 h from microRNA 377 (miR-377) mimic or inhibitor or respective negative control–transfected human CD34$^+$ (hCD34$^+$) cells. **(B)** Values were normalized to controls in the blot and expressed as fold change versus respective control (n = 3; miR-377 mimic vs. miR mimic negative control; miR-377 inhibitor vs. miR-377 mimic). **(C)** Layout of the antibodies on the human angiogenesis array blots. ADAMTS-1 = a disintegrin-like and metalloprotease with thrombospondin 1; CXCL16 = chemokine (C-X-C motif) ligand 16; DPPIV = dipeptidyl-peptidase 4; EGF = epidermal growth factor; EG-VEGF = endocrine gland–derived vascular endothelial growth factor; FGF = fibroblast growth factor; GDNF = glial cell–derived neurotrophic factor; GM-CSF = granulocyte macrophage colony-stimulating factor; HB-EGF = heparin-binding EGF-like growth factor; HGF = hepatocyte growth factor; IGFBP = insulin-like growth factor binding protein; IL = interleukin; LAP = latency-associated peptide; MCP = monocyte chemotactic protein; MIP-1A = macrophage inflammatory protein 1 alpha; MMP = matrix metalloproteinase; NRG-1-β1 = neuregulin 1 beta 1; PD-ECGF = platelet-derived endothelial cell growth factor; PDGF = platelet-derived growth factor; PlGF = placental growth factor; TGF-β1 = transforming growth factor beta 1; TIMP = tissue inhibitor of metalloproteinase; uPA = urinary plasminogen activator; VEGF = vascular endothelial growth factor. *p < 0.05. #p < 0.05.

those involved in angiogenic processes, we performed computational miR target prediction analysis using the miR databases and target prediction tools, TargetScan, miRBase, and PicTar. STK35, also known as CLP36-interacting kinase 1 or STK35 long form, is listed as one of the top potential targets for miR-377 and contains complementary seed sequence in its 3′-UTR that is highly conserved among human, chimpanzee, mouse, rat, guinea pig, rabbit, dog, cow, elephant, and horse RNA (**Figure 6A**). Unlike other cytoplasmic kinases, STK35 is a novel kinase, mainly localized to the nucleus and nucleolus, that binds to nuclear actin (15); thus, it might play a critical role in directly modulating gene transcription machinery (16). A previous study showed that VEGF stimulation in endothelial cells upregulated STK35 expression and that STK35 knockdown led to diminished angiogenic ability of endothelial cells (15).

We first examined the impact of miR-377 mimic and miR-377 inhibitor on STK35 mRNA and protein expression in HUVECs. The effect of miR-377 mimic or inhibitor transfection on miR-377 expression was confirmed by qRT-PCR in HUVECs and hCD34$^+$ cells (Online Figures 3A and 3B). The effect of miR-377 on STK35 expression was validated by both qRT-PCR and Western blot methods. Levels of STK35 mRNA and protein expression were decreased by miR-377 mimic and increased by miR-377 inhibitor compared with their respective negative controls ($p < 0.05$) (**Figures 6B and 6C**).

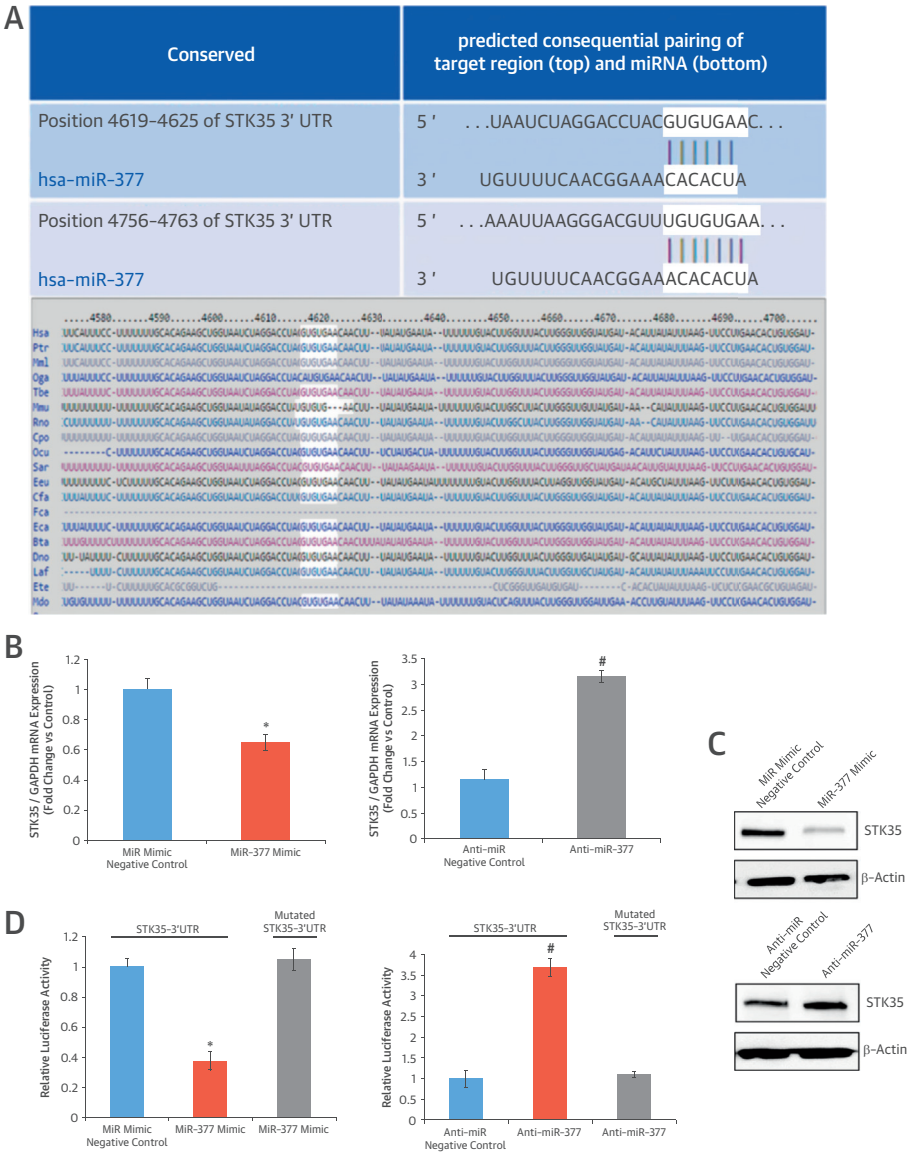
FIGURE 5 miR-377 Mimic Inhibits HUVEC Migration and Vascular Tube Formation



(A) Migratory response of HUVECs transfected with miR-377 mimic or inhibitor or respective negative control toward VEGF gradient was measured by modified Boyden chamber migration assay. **(B)** Matrigel angiogenesis assay, tube formation by HUVECs transfected with miR-377 mimic or inhibitor or respective negative control, and relative quantification of tube length. *$p < 0.05$ (miR-377 mimic vs. miR mimic negative control). #$p < 0.05$ (miR-377 inhibitor vs. miR inhibitor negative control). HUVEC = human umbilical vein endothelial cell; other abbreviations as in Figure 4.

Next, to validate whether STK35 3′-UTR is a direct target of miR-377, HUVECs were transfected with a dual luciferase reporter vector containing the 3′-UTR of STK35 or mutated 3′-UTR of STK35 along with miR-377 mimic, miR-377 inhibitor, or its respective negative controls. miR-377 inhibitor significantly increased luciferase activity versus miR inhibitor negative control-treated cells, whereas cells transfected with miR-377 mimic showed approximately 40% decrease in luciferase activity compared with miR mimic negative control-treated cells (both $p < 0.05$) (Figure 6D). No change was

FIGURE 6 Validation of STK35 as miR-377 Target

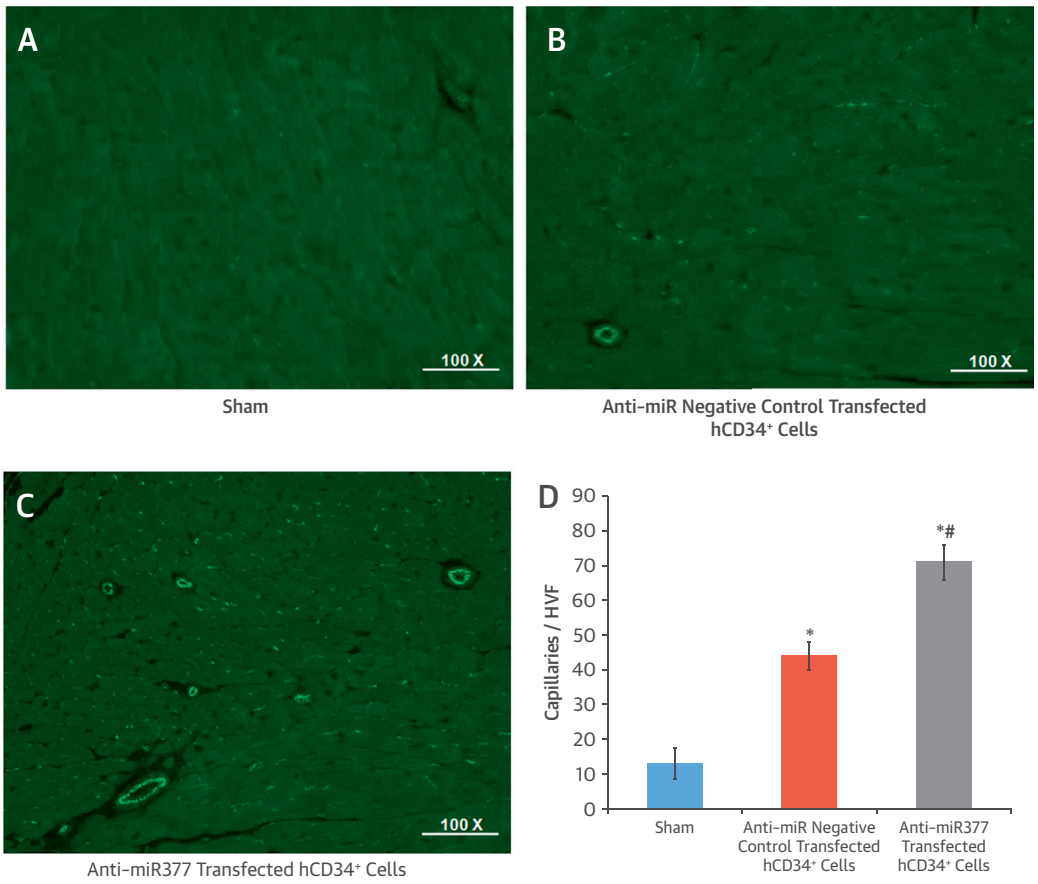


(A) Computational miR target prediction analysis shows that STK35 has complementary sequences for miR-377 that are highly conserved **(white fonts)** in most mammals. STK35 messenger RNA **(B)** and protein expression **(C)** were measured in the HUVECs transfected with miR-377 mimic or inhibitor or respective controls by quantitative reverse transcriptase polymerase chain reaction and Western blot. **(D)** Quantitative data for dual luciferase reporter assay (n = 3). *p < 0.05 (miR-377 mimic vs. miR mimic negative control). #p < 0.05 (miR-377 inhibitor vs. miR inhibitor negative control). GAPDH = glyceraldehyde-3-phosphate dehydrogenase; STK35 = serine/threonine kinase 35; 3′UTR = 3′-untranslated region; other abbreviations as in Figures 4 and 5.

observed in mutated STK35-treated cells with both miR-377 mimic/inhibitor compared with their respective negative control transfections. These data indicated that STK35 is a direct target of miR-377.

Our in vitro data revealed that miR-377 has a negative influence on hCD34$^+$ cell secretion of proangiogenic paracrine factors coupled with reduced endothelial migration and morphogenesis. To assess the effect of miR-377 inhibition on hCD34$^+$ cell-mediated cardiac repair, we evaluated capillary density and fibrosis in the myocardium and LV function on day 28 after myocardial I-R injury followed by PKH26-labeled hCD34$^+$ cell transplantation (Online Figure 4 showing transplanted cells in the

FIGURE 7 Myocardium Capillary Density



Representative immunofluorescence images taken within the infarct border zone of mice receiving **(A)** sham treatment or human $CD34^+$ ($hCD34^+$) cells treated with **(B)** anti-microRNA (miR) negative control or **(C)** anti-miR-377. **(D)** Graph depicting capillary density across treatments presented as the number of $CD31^+$ cells ($n = 4$). *$p < 0.05$ ($hCD34^+$ cells treated with anti-miR negative control or anti-miR-377 vs. sham-operated group). #$p < 0.05$ ($hCD34^+$ cells treated with anti-miR-377 vs. $hCD34^+$ cells treated with anti-miR negative control group).

myocardium). The mice receiving $hCD34^+$ cells treated with anti-miR-377 negative control showed a trend toward increased capillary density versus sham-operated mice ($p < 0.05$) (Figures 7A, 7B, and 7D); mice receiving anti-miR-377-treated $hCD34^+$ cells had a further increase in the number of capillaries compared with mice receiving $hCD34^+$ cells treated with anti-miR negative control ($p < 0.05$) (Figures 7B to 7D).

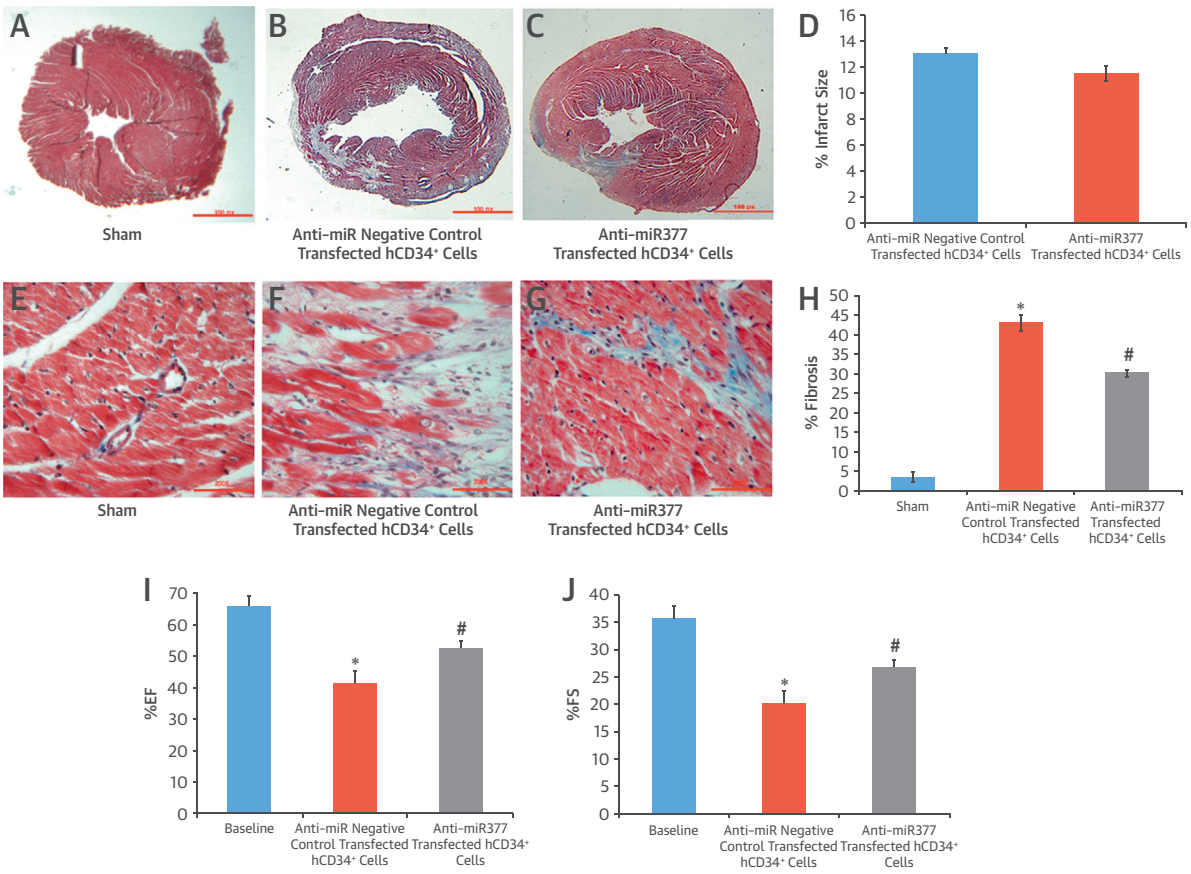
Furthermore, we assessed the relevance of anti-miR-377-transfected $hCD34^+$ cells on LV remodeling and functional recovery. Although not statistically significant, infarct size after 28 days post-I-R was reduced in mice receiving anti-miR-377 $hCD34^+$ cells (Figures 8A to 8D). Mice receiving anti-miR-377-treated $hCD34^+$ cells showed significantly lower interstitial fibrosis in the myocardium compared with controls ($p < 0.05$) (Figures 8E to 8H).

LV function analysis using echocardiography at 28 days post-I-R showed decreased LV function versus sham-operated mice ($p < 0.05$) (Figures 8I and 8J). Mice receiving anti-miR-377-treated $hCD34^+$ cells demonstrated significant improvement in LV function, with increased percent ejection fraction and fractional shortening compared with mice receiving negative control-treated cells ($p < 0.05$) (Figures 8I and 8J). These data suggest that miR-377 inhibition in $hCD34^+$ cells promotes their angiogenic ability in the myocardium and attenuates myocardial I-R-induced fibrosis and LV dysfunction.

DISCUSSION

Current medical therapies have improved the prognosis of patients with MI. Despite these advancements,

FIGURE 8 Cardiac Fibrosis and Left Ventricular Functional Recovery After I-R

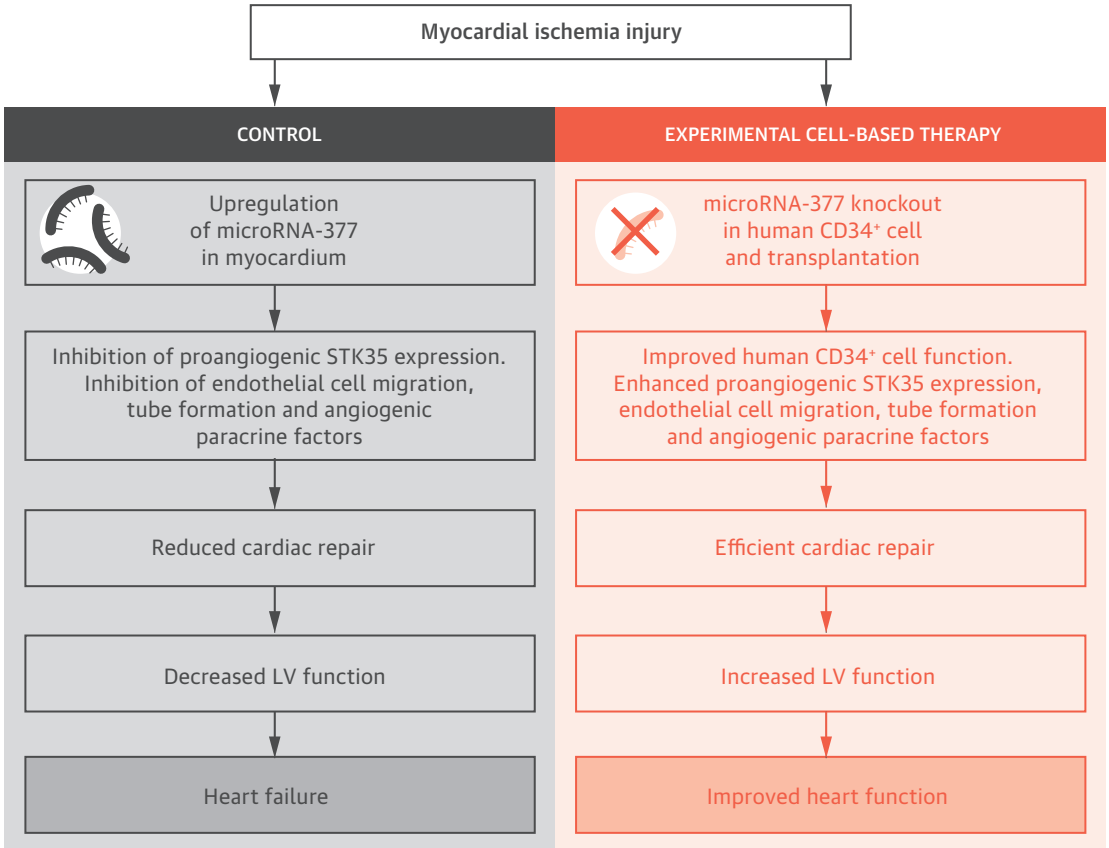


Trichrome-stained heart sections from mice with sham operations **(A and E)** or transplanted with hCD34$^+$ cells treated with anti-miR negative control **(B and F)** or hCD34$^+$ cells treated with anti-miR-377 **(C and G)** 28 days post-I-R. Quantitative analyses of infarct size **(D)**, fibrosis area **(H)**, percent ejection fraction (%EF) **(I)**, and percent fractional shortening (%FS) **(J)** were conducted 28 days post-I-R. n = 4. *p < 0.05 (hCD34$^+$ cells treated with anti-miR negative control vs. sham-operated group). #p < 0.05 (hCD34$^+$ cells treated with anti-miR-377 vs. hCD34$^+$ cells treated with anti-miR negative control). Abbreviations as in Figures 3 and 4.

significant mortality and increased risk for reoccurrence of HF remain in survivors (17). The underlying causes include microvasculature abnormalities due to endothelial dysfunction, loss of cardiomyocytes, and adverse remodeling due to fibrosis. Hence, developing therapeutics to preserve or regenerate damaged myocardial tissue might open novel avenues for treating patients with heart diseases. Accumulating evidence suggests that miR are involved in several pathophysiological processes, and their dysregulation has been implicated in cardiac diseases (18-20). Additionally, they are used as diagnostic or prognostic markers in various diseases (21). However, studies to determine the effect of cardiac miR (induced upon injury) on the stem/progenitor cells that are transplanted for tissue regeneration are limited. Therefore, therapeutic strategies to augment progenitor cell-based myocardial repair, especially in the context of tissue ischemia, might be clinically relevant and crucial to the success of cell-based therapy.

In the present study, we observed that patients with ischemic heart disease (IHD) have substantial upregulation of miR-377 in biopsy samples from myocardial tissue. In contrast, hCD34$^+$ cells exposed to inflammatory stimuli for 12 h show significant decreases in miR-377 expression. Interestingly, miR-377 mimic treatment diminished proangiogenic paracrine secretion in hCD34$^+$ cells and reduced endothelial cell tube formation ability. These data suggest that the decrease in miR-377 expression in response to inflammatory stimuli might be a

CENTRAL ILLUSTRATION **MicroRNA-377 Knockdown Human CD34+ Cells Enhance Cardiac Function**



Joladarashi, D. et al. J Am Coll Cardiol. 2015; 66(20):2214–26.

LV = left ventricular; STK35 = serine/threonine kinase 35.

compensatory mechanism to protect endothelial cell function.

Furthermore, reports have shown that miR-377 has been negatively implicated in various pathological conditions, such as diabetic nephropathy (22), oxidative stress (23), lung fibrosis, hydrogen peroxide-induced premature senescence (24), and cancer (25–28). Our findings, for the first time, demonstrated that miR-377 critically regulates endothelial cell function in an inflammatory setting. Therefore, we speculated that strategies to modulate miR-377 to enhance hCD34$^+$ cell-mediated myocardial repair might be suitable for therapeutic intervention in the setting of IHD. We demonstrated this by showing that transplantation of anti-miR-377-treated hCD34$^+$ cells enhanced neovascularization, reduced interstitial fibrosis, and attenuated LV dysfunction. These in vivo changes corroborated well with the in vitro observations that miR-377 inhibitor treatment enhanced hCD34$^+$ cell function. A recent study showed that miR-377 knockdown in mouse mesenchymal stem cells promoted the angiogenic ability in mouse MI models (29). Our data demonstrated that anti-miR-377 treatment enhanced the angiogenic potential of hCD34$^+$ cells, supporting Wen et al. (29), together demonstrating that miR-377 plays a negative role in IHD, in line with various other pathophysiological conditions.

We know that miR bind to target mRNA through imperfect base pairing and are involved in post-transcriptional regulation of gene expression by affecting mRNA stability and translation. On the basis of computational analysis, we identified STK35 as a potential target gene. We observed that miR-377

mimic treatment in hCD34$^+$ cells decreased the levels of proangiogenic paracrine factors in association with reduced STK35 expression. miR-377 inhibitor treatment had the opposite effect. More importantly, we validated that STK35 mRNA is a direct target of miR-377. These data, together with previous reports, suggest that the negative effects of miR-377 on hCD34$^+$ cells might be potentially mediated through STK35 signaling.

Enhanced levels of STK35 have been reported in tissues obtained from patients with colorectal cancer (30) and shown to be altered in a rodent model of Parkinson disease (31). Additionally, VEGF stimulates STK35 expression in endothelial cells (15). A well-known proangiogenic cytokine, VEGF promotes EPC migration, differentiation, and angiogenic function. Our data showed that VEGF treatment in HUVECs significantly decreased miR-377 expression; furthermore, *VEGF* mRNA expression decreased with miR-377 mimic treatment in HUVECs (Online Figure 5). Computational analysis revealed that VEGF-A is yet another potential target of miR-377, as validated in a previous study on mesenchymal stem cells (29). Taken together, these data suggest that a negative feedback loop might exist in regulating miR-377 and VEGF-A expression. Kinases are of great interest because they serve as targets in cancer therapeutics. Therefore, strategies involving pharmacological, genetic, or miR-based manipulation to modulate STK35-mediated angiogenesis in cardiomyopathies or cancer might be clinically relevant.

Beyond STK35, miR-377 has other experimentally verified targets relevant to cardiovascular diseases, such as VEGF-A (29) and hemeoxygenase 1 (23), all shown to be expressed in endothelial (progenitor) cells. Wang et al. (22) also demonstrated that miR-377 plays a role in the pathogenesis of diabetic nephropathy through translational repression of target genes superoxide dismutase 1 and 2 and p21/CDC42/Rac1-activated kinase 1 (22). Our study showed that miR-377 negatively regulated hCD34$^+$ cell function in association with reduced STK35 expression. Taken together, by regulating the expression of STK35, VEGF-A, hemeoxygenase 1, and superoxide dismutase 1/2, miR-377 has deleterious effects on endothelial cell biology and function, possibly through inflammation and oxidative stress.

Considering the potential role of miR in a multitude of human diseases (18–20,32–38), targeting miR-377 represents an exciting prospect for therapeutic applications to limit cardiac remodeling and enhance hCD34+ cell-based tissue repair post-MI, after stroke, or in other ischemic events or to inhibit aberrant angiogenesis in cancer.

STUDY LIMITATIONS. More studies are required to determine the role of miR-377 in inflammation/oxidative stress under myocardial ischemic conditions. We showed that miR-377 expression was increased in various cardiac cell types isolated from mouse hearts after I-R injury. However, we did not explore its role in different cell types. Previous studies have shown the possibility of a pleiotropic role of some miR in different cell types (39). Therefore, further work is warranted to clarify the role of miR-377 in progenitor cell mobilization, cardiomyocyte cell death, and function during HF, potentially uncovering therapeutic modalities to modulate the fibrogenesis process, LV remodeling, and repair of post-ischemic injury.

CONCLUSIONS

We demonstrated for the first time that: 1) miR-377, which has increased levels in the myocardium of patients with IHD, negatively influenced hCD34$^+$ angiogenic paracrine factors in vitro; and 2) anti-miR-377 treatment in hCD34$^+$ cells enhanced their function and attenuated LV dysfunction after intramyocardial transplantation in SCID mice with myocardial I-R injury (**Central Illustration**). Thus, knockdown of miR-377 appears to be a feasible approach to protect against LV remodeling and might prove to be an attractive therapeutic strategy for cell-based therapy in patients with HF.

REPRINT REQUESTS AND CORRESPONDENCE: Dr. Prasanna Krishnamurthy, Department of Cardiovascular Sciences, Center for Cardiovascular Regeneration, Houston Methodist Research Institute, 6670 Bertner Avenue, R10-214, Houston, Texas 77030. E-mail: pkrishnamurthy@houstonmethodist.org.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The microenvironment in ischemic tissue detrimentally affects survival of mobilized/transplanted hCD34$^+$ cells, potentially compromising the benefits of cell-based therapies for myocardial ischemia and infarction. Transplantation of miR-377 knockdown hCD34$^+$ cells into ischemic mouse myocardium promoted cardiac repair and attenuated ventricular dysfunction.

TRANSLATIONAL OUTLOOK: Further work is needed to develop clinically applicable strategies to preserve stem cell function and regenerate damaged myocardial tissue for evaluation in patients with ischemic heart disease.

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KEY WORDS endothelial progenitor cells, heart failure, neovascularization

APPENDIX For an expanded Methods section, and supplemental figures and references, please see the online version of this article.