MicroRNA-194-5p attenuates hypoxia/reoxygenation-induced apoptosis in H9C2 cardiomyocytes by inhibiting the over-activation of RAC1 protein

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Abstract. Ras-related C3 botulinum toxin substrate 1 (RAC1), a member of the Rac family of guanosine triphosphate phosphohydrolases, has been suggested to be a regulator of myocardial injury during ischemia and reperfusion (I/R). Whether microRNAs (miRs) are involved in the regulation of the aforementioned process remains to be elucidated. In the present study, an *in vitro* model of H9C2 cardiomyocytes was used to establish the overexpression of RAC1 following hypoxia and reoxygenation (H/R). Overexpression of RAC1 in H/R-cultured cardiomyocytes could lead to cellular accumulation of reactive oxygen species (ROS) and facilitate the induction of apoptosis of H9C2 cardiomyocytes during H/R. Subsequent bioinformatic analysis indicated that RAC1 was the target of miRNA-194-5p. Further experiments showed that miR-194-5p attenuated the accumulation of cellular ROS and alleviated the induction of apoptosis of H9C2 cardiomyocytes caused by H/R, which was accompanied by the reduction in the expression levels of the RAC1 protein. Taken together, these results indicated that upregulation of miR-194-5p may function as a self-regulated cardioprotective response against RAC1-mediated ROS accumulation and cardiomyocyte apoptosis. Exogenous administration of miR-194-5p may be a novel target to ameliorate I/R injury-induced myocardial apoptosis.

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Introduction

Currently, revascularization has become the most important therapy for patients with myocardial ischemia (1). Although the timely recovery of myocardial perfusion is related to long-term improved cardiac function (2), certain patients may suffer from continuous cardiac dysfunction despite of effective revascularization therapy, which is currently considered to be caused by the ischemia and reperfusion (I/R) injury (3). The pathogenesis of this disease is complicated and several mechanisms are involved, including conventional mechanisms of inflammatory response, oxidative stress, calcium overload, myocardial hibernation and stunning (4). Previous studies have shown that ras-related C3 botulinum toxin substrate (RAC) 1, a member of the RAC family of guanosine triphosphate phosphohydrolases, is a key regulator of myocardial I/R injury (5). Physiologically, RAC1 maintains the constitutive sarcomere reorganization (6) and the antioxidative efficacy of nicotinamide adenine dinucleotide phosphate oxidases (7) in cardiomyocytes. During I/R, RAC1 is overexpressed in cardiomyocytes and this induces I/R injury by stimulating reactive oxygen species (ROS)-mediated myocardial apoptosis via the inhibition of RAC1-driven processes (8,9). However, the potential regulatory mechanisms for the role of RAC1 during I/R remain to be elucidated.

MicroRNAs (miRs) are a cluster of small non-coding ribonucleotides (~22 nucleotides in length) which post-transcriptionally target mRNAs, suppress protein synthesis or increase mRNA degradation, thereby regulating important cellular processes, including myocardial I/R injury (10,11). It is notable that the post-translational modification of RAC1 has been highlighted to participate in several cellular processes (12), such as carcinogenesis and metastasis (13,14). Previous studies have also shown that the interactions of miRs with RAC1 may be important for the development of hypoxia (15) and hyperglycemia-induced injury of cardiomyocytes (16). Therefore, it was hypothesized that the interactions of miRs with RAC1 may also be involved in the pathogenesis of

myocardial I/R injury. Accordingly, the present study validated the changes in the expression levels of RAC1 during hypoxia and reoxygenation (H/R) in H9C2 cells by using isobaric tags used for relative and absolute quantification (iTRAQ)-based proteomic analysis (17). Moreover, bioinformatic analysis was used to identify the potential miRs targeting RAC1 in this process and to examine the associated molecular mechanisms.

Materials and methods

H9C2 cell culture and induction of H/R injury. The rat embryonic cardiac cell line H9C2 (Wuhan GeneCreate Biological Engineering Co. Ltd.), which was preserved in our institution, was used in the present study. The cells were routinely cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), penicillin/streptomycin and 4 mM L-glutamine and maintained in a CO_2 incubator (Shellab) at 37˚C. The medium was changed every 2-3 days and the cells were subcultured and passaged at 70-80% confluency. For subsequent experiments, the cells in the logarithmic phase of growth were selected. Following digestion with trypsin, the cells were harvested and seeded into 12-well plates for modeling of the H/R injury and subsequent molecular biological analyses. The processes of the induction of H/R injury in H9C2 cells were performed as previously reported (18). Briefly, prior to the incubation under hypoxic conditions, the regular medium was removed and replaced with DMEM under hypoxic conditions, which lacked glucose (pH 6.8). Subsequently, the cells were moved to a hypoxia chamber with 94% (v/v) N_2, 5% (v/v) CO_2 and 1% (v/v) O_2 for 6 h at 37˚C. Following incubation under hypoxic conditions, the medium was removed and changed to regular DMEM with 4.5 mM glucose (pH 7.4); the cells were maintained under normoxic conditions (5% CO_2 and 95% air) for 24 h.

iTRAQ-based proteomic analysis. Total protein was extracted by extraction buffer [7 M Urea/2 M Thiourea/4% SDS/40 mM Tris-HCl (pH 8.5)/1 mM PMSF/2 mM EDTA] from H9C2 cells of the control and the H/R injury groups for subsequent analyses. The concentrations of the proteins were determined with the Bradford assay according to the manufacturer's instructions (Abcam). For each sample, an aliquot of 100 μg protein was further digested with trypsin solution for 12 h at 37˚C to obtain the tryptic digested peptides, which were desalted, dried and reconstituted in a triethylammonium bicarbonate solution (0.5 M). Subsequently, an iTRAQ reagent kit (SCIEX) was used and the peptides were labeled according to the manufacturer's protocol. For nano-liquid chromatography (LC)-mass spectrometry (MS)/MS analysis, the iTRAQ-labeled plasma peptide mixtures were diluted in 20 mM ammonium formate (pH 10) and loaded onto a reverse phase column using an Eksigent ultra-performance liquid chromatography system. Subsequently, a 50 min linear gradient elution with 80% acetonitrile and 20% 20 mM ammonium formate (pH 10) was performed at a flow rate of 800 μl/min, leading to a collection of 50 separate fractions per min. These fractions were pooled, desalted, dried and stored at -80˚C for subsequent analyses as previously described (19). The Triple-time of flight (TOF) 5600 system (SCIEX) was used for MS and MS/MS analysis of the LC eluent. The key parameters and conditions for the working protocol of the Triple-TOF 5600 were set according to the manufacturer's instructions and previously published reports (20). Briefly, the peptides were labeled according to the ITRAQ-8 standard Kit instructions (SCIEX) and mixed. Next, these labeled peptides were segregated via the Ultimate 3000 HPLC system (Dionex; Thermo Fisher Scientific, Inc.). After labeling, the peptides were analyzed by 2D LC MS/MS analysis. The identification and quantification analysis of the significantly differentially expressed proteins detected in H9C2 cells was achieved between the H/R and the control groups following input of the iTRAQ-based proteomics data into the ProteinPilot software v4.5 (SCIEX) with the SwissProt Homo sapiens database (https://www.ebi.ac.uk/uniprot/) (release May 2022, containing 23,752 sequences) for bioinformatic analyses. Subsequently, the iTRAQ ratios and P values were estimated via the ProteinPilot software. The levels of significance were set at a ratio of >1.2 or <0.83 and $P<0.05$ was considered to indicate a statistically significant difference in the expression levels of the cellular proteins of the H/R and the control H9C2 cells. To further clarify the functional classifications of the differentially expressed proteins, the online DAVID tool (http://david.abcc.ncifcrf.gov) was searched for the Gene Ontology annotation. Moreover, the pathway-related information of the identified proteins with different expression levels was obtained with the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/). Subsequent analysis of the potential network of the protein-protein interactions was performed using the online tool of STRING 10.5 (http://string-db.org) as previously described (20).

Table I. Sequences of miR-194-5p mimics and controls.

Group	Sequence (5'-3')
miR-194-5p mimics	UGUAACAGCAACUCCAUGUGGA
Control mimics	UUGUACUACACAAAAGUACUG
miR-194-5p inhibitor	UCCACAUGGAGUUGCUGUUACA
Inhibitor control	CAGUACUUUUGUGUAGUACAA

miR, microRNA.

Identification of potential miRs which interact with RAC1 in rat and humans: TargetScan and miRDB. The miRs that potentially regulate RAC1 expression in rats and humans were identified by TargetScan 7.2 (www.targetscan.org). Subsequently, the putative binding sites were also predicted by miRDB (http://mirdb.org/miRDB/). The analysis was performed to identify common miRs between humans and rats, which may potentially interact with RAC1. It was considered that these candidate miRs are potential regulators of RAC1 protein expression by interacting with the 3'-untranslated region (UTR) of RAC1 mRNA.

Transfection of mimics and inhibitors of miR-194-5p and overexpression of RAC1 in H9C2 cardiomyocytes. Exogenous mimics, inhibitors of miR-194-5p and their controls were

Table II. Primer sequences.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
miR-194-5p	ACACTCCAGCTGGGTGTAACAGCAACTCCA	TGGTGTCGTGGAGTCG
RAC1	CTCTCCTACCCGCAAACAGA	TCAAGCTTCGTCCCCACTAG
GAPDH	GCAAGTTCAACGGCACAG	GCCAGTAGACTCCACGACAT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

miR, microRNA; RAC1, Ras-related C3 botulinum toxin substrate 1.

synthesized by the Wuhan GeneCreate Biological Engineering co. Ltd. The sequences are shown in Table I. Overexpression of the RAC1 proteins in H9C2 cells was achieved via the transfection of the plasmid vectors (6 μg) containing the cDNA of RAC1 (pcDNA3.1-GAPs), which was also constructed and obtained from the Wuhan GeneCreate Biological Engineering co. Ltd. Empty vector was used as the negative control. The transfection of 10 pmol mimics, inhibitors of miR-194-5p and their controls was performed with the Lipofectamine® RNAi MAX transfection medium (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following transfection at 37˚C for 20 min, the cells were cultured for 48 h prior to further experiments.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from $5x10^6$ H9C2c cells using total TRIzol® RNA extraction kit (AxyPrep; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, the removal of the genomic DNA and the reverse transcription reactions were performed using the gDNA Eraser and the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the instructions provided by the manufacturer. Following measurement of the concentrations of the obtained cDNA, the products were dispensed and stored at -20˚C for subsequent use. GAPDH was used as an internal control for the detection of the mRNA levels of miR-194-5p and RAC1. The process of the quantitative real-time PCR was performed with the PrimeScript RT reagent kit (Takara Bio, Inc.) with a total of 20 μl reaction mixture. The conditions for the quantitative real-time PCR were set as follows: 60 sec at 95˚C, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 45 sec. The $2^{-\Delta\Delta Cq}$ method was used to estimate the relative mRNA expression levels (21). The experiment was repeated three times. The sequences of the forward and reverse primers were shown in Table II.

Western blot analysis. To detect the protein expression levels of RAC1 compared with the internal control of GAPDH, western blot analysis was performed with an antibody against RAC1 (Abcam). Briefly, following the specific treatment in each group of H9C2 cells, the buffer of the radioimmunoprecipitation assay (Beyotime Institute of Biotechnology) was used to obtain the total protein of each group and the concentrations of the total proteins were determined by the bicinchoninic protein assay kit (Beyotime Institute of Biotechnology) as instructed by the manufacturer's protocol. The samples (15 μl/lane) were separated on 15% gels using SDS-PAGE, transferred to polyvinylidene fluoride membranes, incubated with 5% non-fat milk for 2 h at room temperature, washed with TBS plus 0.1% Tween 20 (TBST) and incubated with the primary rabbit antibodies against RAC1 (1:1,000; cat. no. ab155938; Abcam) and GAPDH (1,10,000; cat. no. ab37168; Abcam) overnight at 4˚C. The following day, the solution containing the primary antibody was removed and the membrane was washed with TBST six times (5 min each). Subsequently, the membranes were incubated with the HRP goat anti-rabbit secondary antibody (1:10,000; cat. no. AS1107; Wuhan Aspen Biotechnology Co., Ltd.) for 2 h at room temperature. Finally, the intensity levels of the bands corresponding to each protein were visualized using an ECL detection system (Amersham; Cytiva). The intensity levels of the bands corresponding to each protein of interest were analyzed using the Image Q analysis system (Storm Optical Scanner; Molecular Dynamics).

Immunofluorescence and cytochemistry. The cells in the exponential phase were cultured for 48 h, the culture medium removed, washed three times with PBS, then an appropriate amount of Fluo-4 AM (cat. no. S1060; Beyotime Institute of Biotechnology) original solution was diluted to 0.5-5 μM working solution with PBS. The Fluo-4 AM working solution to completely cover the cells and incubated at 20-37˚C for 10-60 min to load the fluorescent probe. After washing with PBS for three times, the fluorescence of Fluo-4 was observed in nine random fields using a fluorescence microscope (Olympus Corporation) to determine the change of intracellular calcium concentration.

Luciferase assay. The constructs of pGL3-RAC1 (RAC1-WT) or pGL3-RAC1-MUTANT (RAC1-MUT) (Wuhan GeneCreate Biological Engineering Co. Ltd.) were transfected into H9C2 cells with or without miR-194-5p mimics or control mimics by Lipofectamine® 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) prior to the H/R culture. Subsequently, the luciferase activity levels were measured by dividing the relative light unit (RLU) of firefly luciferase by the RLU of sea kidney luciferase (to represent the activation degree of the target report gene) at 6 h following transfection using the dual luciferase assay kit (cat. no. E1910; Promega Corporation) in accordance with the instructions of the manufacturer.

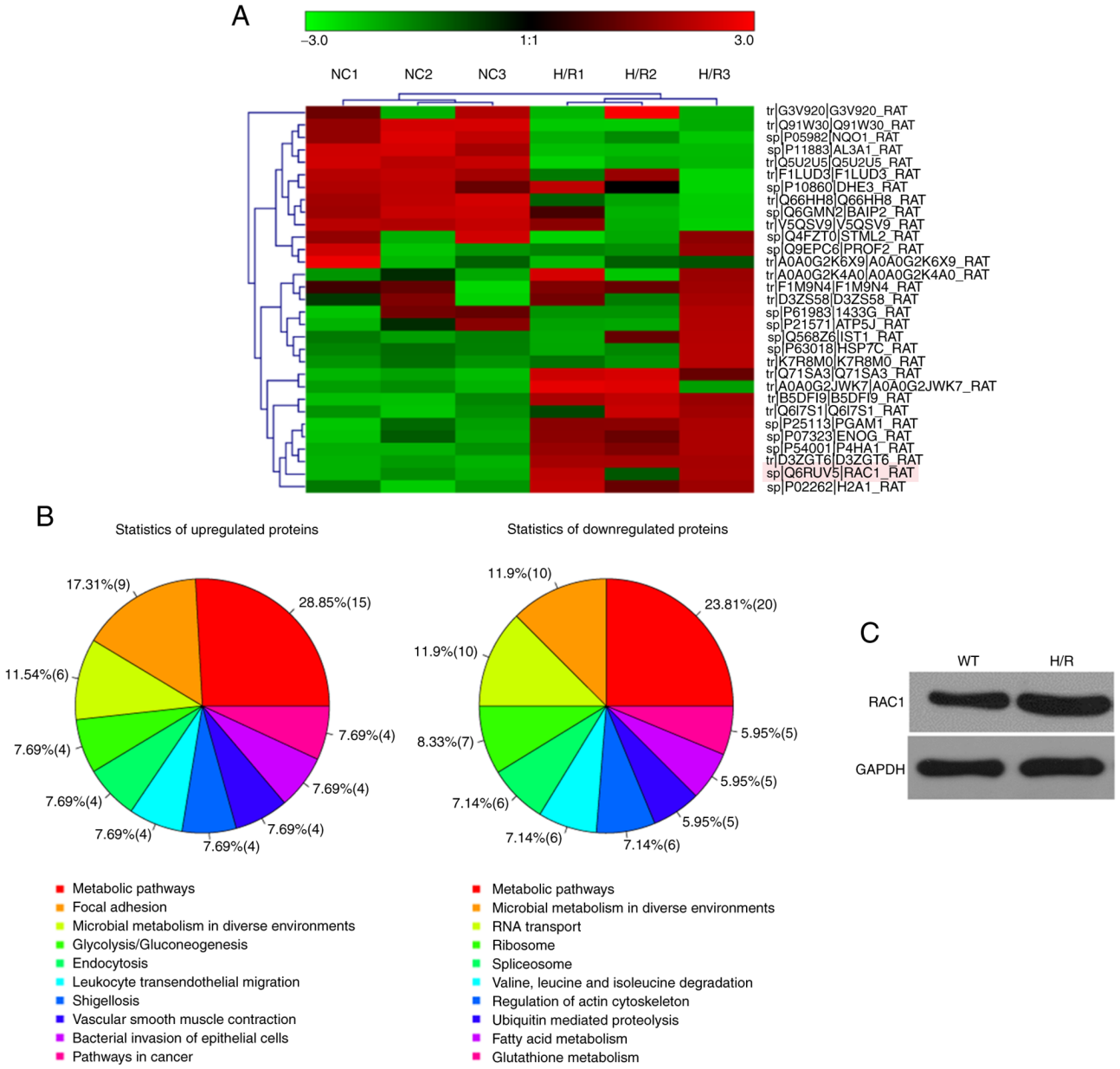

Figure 1. The results of the iTRAQ-based quantitative proteomic analysis for the differentially expressed proteins between H9C2 cardiomyocytes in the H/R and normal control groups. (A) The relative fold-changes in the protein expression levels for the top 30 proteins which were differentially expressed in H9C2 cardiomyocytes cultured under H/R conditions compared with the control samples, in which RAC1 is indicated with red background. (B) Pathway analyses for the 199 proteins that were differentially expressed in H9C2 cardiomyocytes cultured under H/R conditions compared with the control samples indicating that several proteins were involved in pathways regulating cellular metabolism and connection. (C) Western blot analysis was used to confirm that the protein levels of RAC1 were upregulated in H9C2 cardiomyocytes cultured under H/R conditions compared with those in the control samples of the cells grown under normoxic conditions. iTRAQ, isobaric tags used for relative and absolute quantification; H/R, hypoxia/reoxygenation; RAC1, Ras-related C3 botulinum toxin substrate 1; WT, wild-type.

Apoptosis detection. The induction of H9C2 cell apoptosis was evaluated in each group by an Annexin-fluorescein isothiocyanate (FITC) apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd.). The samples were analyzed with fluorescence-activated cell sorting (FACS) and flow cytometry. H9C2 cells were harvested from each group and washed with PBS twice. The cells were resuspended in 500 μl buffer and subsequently stained and incubated with Annexin V-FITC (5 μl) and propidium iodide (5 μl) at room temperature in the dark for 15 min. Subsequently, the cells were transferred into specific tubes for flow cytometry analysis using a BD Accuri C6 flow cytometer (BD Biosciences) and BD Accuri C6 Plus software (version C6; BD Biosciences). The apoptotic rate included the percentage of early and late apoptotic cells. A total of 10,000 cells were analyzed for each group.

ROS assay. To reflect the intracellular ROS levels in the H9C2 cells of each group, the cells were stained with 2,7-dichlorofluorescein diacetate (DCFH-DA; Nanjing Jiancheng Bioengineering Institute). In brief, the cells from each group were washed twice with PBS and incubated in DMEM, which contained 10 μM DCFH-DA, for 20 min at 37˚C in the dark. Subsequently, the cells were washed again,

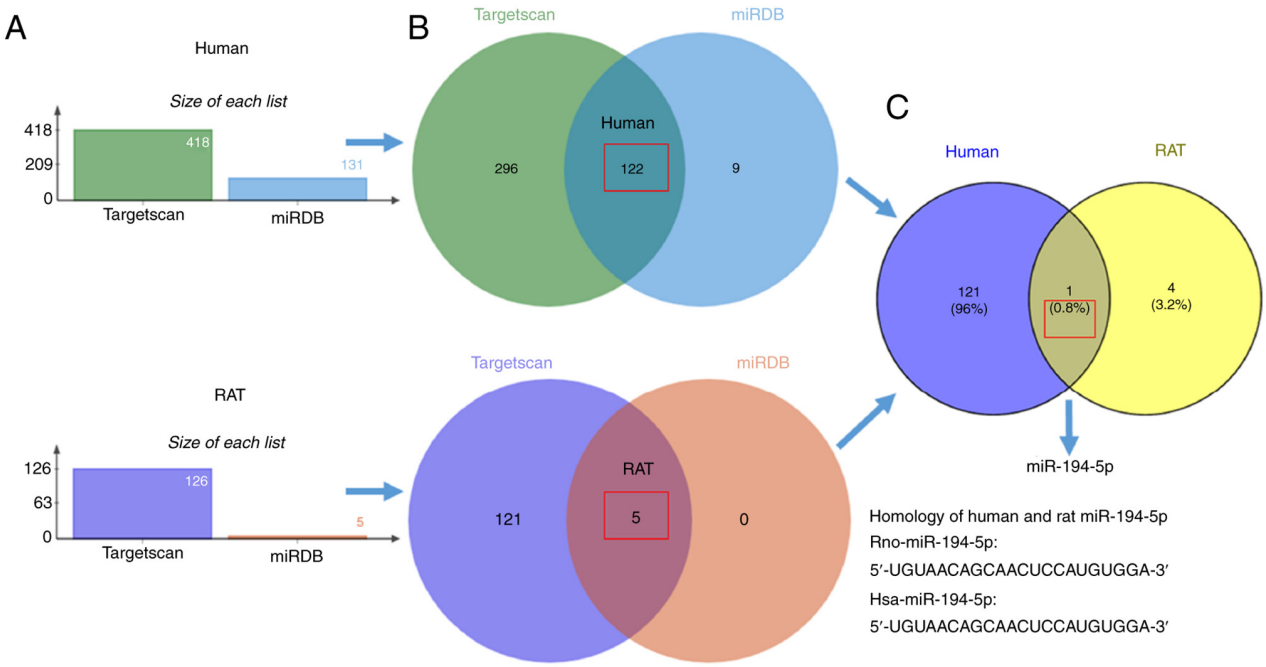

Figure 2. Bioinformatic analysis identifies miR-194-5p as the potential regulator for RAC1 in H9C2 cardiomyocytes cultured under H/R conditions. (A) TargetScan and miRDB database analyses predicted the cluster of miRs which could potentially interact with RAC1 in humans and rats. (B) The analysis further identified miRs, which were common between humans and rats and may potentially interact with RAC1. (C) Homogeneity analysis was performed for the sequences of miRs and RAC1 and the data indicated that miR-194-5p in humans and rats shared the same sequences which may target RAC1. miR, microRNA; RAC1, Ras-related C3 botulinum toxin substrate 1; H/R, hypoxia/reoxygenation.

collected following trypsin treatment (0.25% trypsin-EDTA), centrifuged at 225 x g for 3 min at 37˚C and resuspended in 500 μl PBS. FACS was used with an excitation wavelength of 488 nm and an emission wavelength of 521 nm.

Statistical analysis. The data are presented as mean ± SEM and compared with one-way analysis of variance among the groups. Post-hoc analysis with Fisher's least significant difference test, Dunnett's test or unpaired Student's t-test were used to compare the differences between the groups as appropriate with the SPSS software 20.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of RAC1 expression in H9C2 cardiomyocytes undergoing H/R. The relative fold-changes in the expression levels of the top 30 proteins, which were differentially expressed in H9C2 cardiomyocytes undergoing H/R compared with the control cells, were detected by the iTRAQ proteomic analysis (Fig. 1A). Subsequent pathway analyses for the 199 proteins that were differentially expressed between H9C2 cardiomyocytes undergoing H/R and those cultured under normoxic conditions indicated that several proteins were involved in pathways regulating cellular metabolism and communication (Fig. 1B). The protein levels of RAC1 were significantly higher in H9C2 cardiomyocytes undergoing H/R compared with those of the control cells cultured under normoxic conditions; these results were further validated by western blot analysis (Fig. 1C).

Identification of miR-194-5p as a regulator of RAC1 in H/R. Previous studies indicated that the activation of RAC1 is involved in H/R-induced cardiomyocyte injury by mediating sarcomere dysfunction and ROS accumulation (6,7). Since miRs have been involved in the pathogenesis of myocardial I/R injury at least partially via the regulation of the ROS pathway, the present study further explored using bioinformatic analysis the miRs, which may interact with RAC1 during the induction of H/R in cardiomyocytes. Firstly, TargetScan and miRDB database analyses were used to predict a cluster of miRs, which may potentially interact with RAC1 in humans and rats (Fig. 2A). Subsequent analysis identified the miRs, which were common between humans and rats and may potentially interact with RAC1 (Fig. 2B). Finally, sequence homogeneity analyses of miRs and RAC1 indicated that the miR-194-5p in humans and rats share similar sequences (Fig. 2C) and was likely to be the miR that regulated the expression of RAC1 in cardiomyocytes.

Effects of miR-194-5p on RAC1 expression in H9C2 cardiomyocytes. The changes in the expression levels of miR-194-5p in H9C2 cardiomyocytes during H/R were evaluated using qPCR analyses. The results indicated that the expression levels of miR-194-5p were significantly higher in H9C2 cardiomyocytes cultured under H/R conditions compared with those of the control cells cultured under normoxic conditions (P<0.001, Fig. 3A). Subsequently, exogenous miR-194-5p mimics and inhibitors were administered to evaluate the potential effects on the expression levels of RAC1. The results indicated that administration of 20 nM miR-194-5p mimics was associated with the most effective overexpression of cellular miR-194-5p (P<0.001, Fig. 3B), which caused inhibition of RAC1 expression. Accordingly, 20 nM miR-194-5p mimics was used as the optimal concentration for subsequent

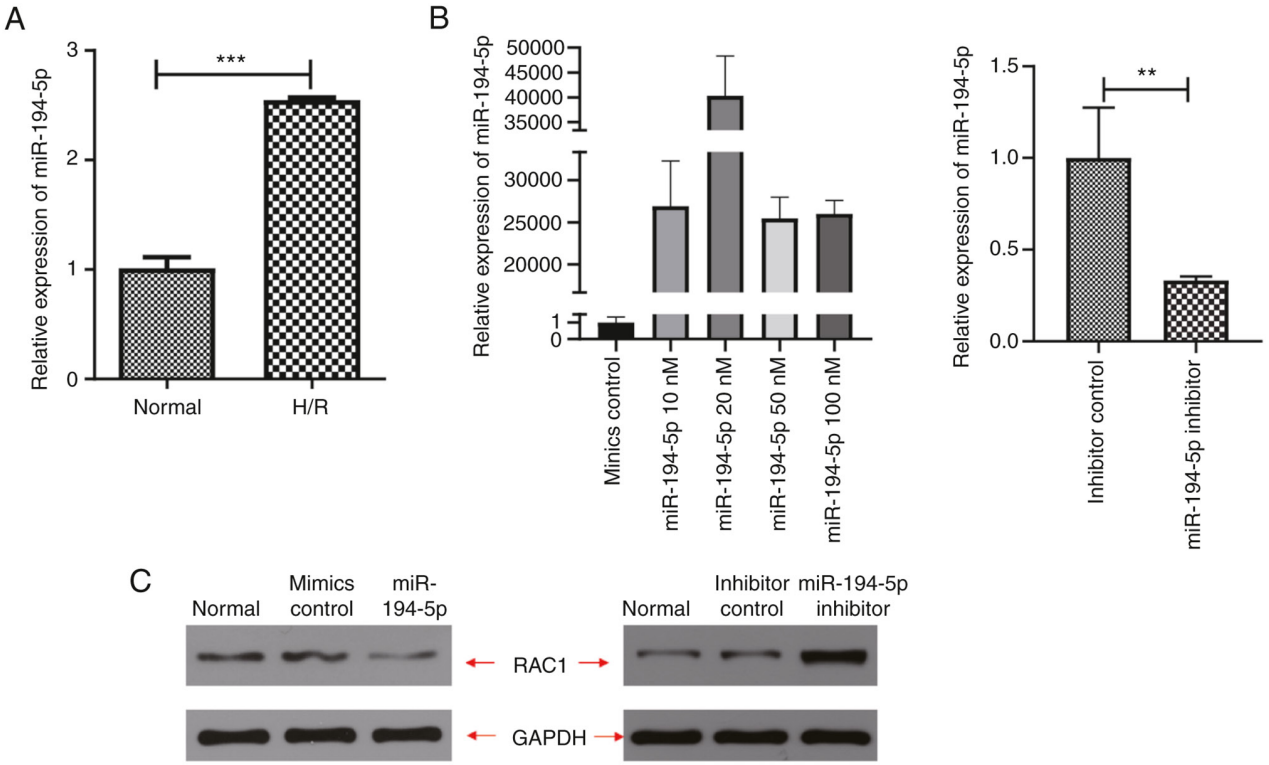

Figure 3. Effects of miR-194-5p on RAC1 expression in H9C2 cardiomyocytes. (A) qPCR confirmed that the levels of miR-194-5p were significantly upregulated in H9C2 cardiomyocytes (***P<0.001). (B) Effects of exogenous administration of miR-194-5p mimics and inhibitor in H9C2 cardiomyocytes. The results indicated that administration of 20 nM miR-194-5p mimics was associated with the most effective overexpression of cellular miR-194-5p; therefore, 20 nM miR-194-5p mimics and inhibitor was used for subsequent studies. Higher expression levels of miR-194-5p mimics (50 and 100 nM) may cause cellular injury, overactivation of oxidative stresses (**P<0.01). (C) Western blot analysis was used to assess the effects of miR-194-5p mimics and inhibitors on the protein expression of RAC1 in H9C2 cardiomyocytes. The results indicated that administration of 20 nM miR-194-5p mimics was significantly associated with inhibition of the protein levels of RAC1 compared with those noted following administration of mimic control, while administration of miR-194-5p inhibitors significantly increased the protein levels of RAC1 compared with those noted following administration of inhibitor controls. miR, microRNA; RAC1, Ras-related C3 botulinum toxin substrate 1; qPCR, quantitative PCR.

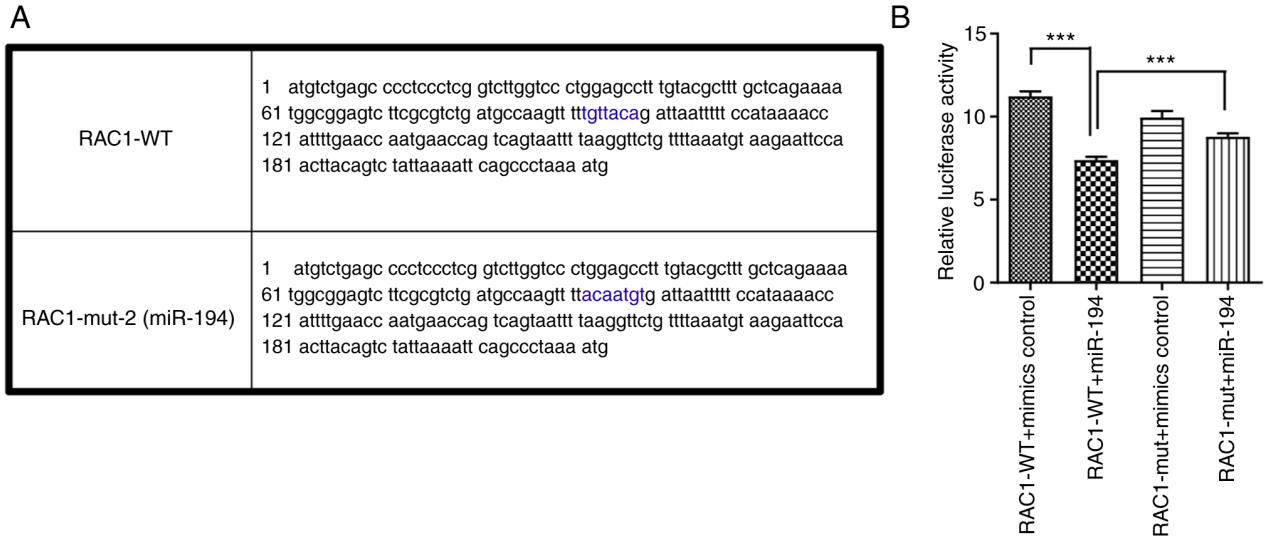

Figure 4. The luciferase activity assays indicates the potential targeting sites of miR-194-5p in the RAC1 miR sequences. (A) 3'-UTR sequences of WT and mut RAC1 constructs used in the luciferase activity assays. (B) Administration of miR-194 suppressed the relative luciferase activity levels of the constructs encompassing the binding sites in RAC1 3'-UTR (***P<0.0001), while administration of miR-194 did not significantly affect the relative luciferase activity levels of the constructs encompassing the mutant binding sites in RAC1 3'-UTR, suggesting that miR-194-5p may inhibit RAC1 expression by targeting the mutant sequences. miR, microRNA; RAC1, Ras-related C3 botulinum toxin substrate 1; UTR, untranslated region; WT, wild-type; mut, mutant.

studies. Higher concentrations of miR-194-5p mimics may cause cellular injury, overactivated oxidative stress and upregulation of RAC1 expression (Fig. 3C). These results were further validated by western blot analysis, which indicated

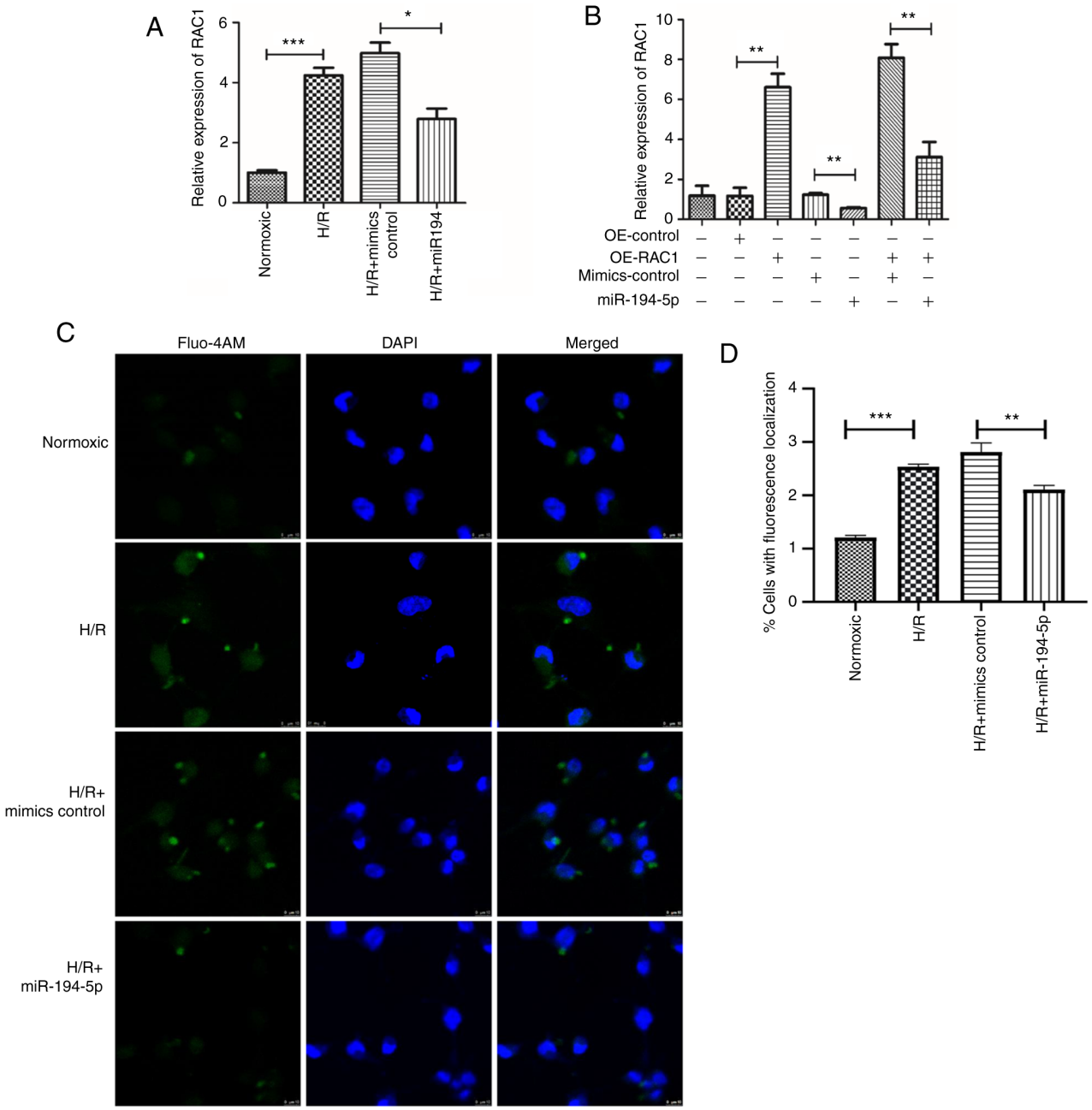

Figure 5. Effects of H/R and miR-194-5p on RAC1 expression in H9C2 cardiomyocytes. (A) qPCR indicated that RAC1 was significantly expressed in H9C2 cardiomyocytes undergoing H/R compared with those of the control cells cultured under normoxic conditions, while administration of exogenous miR-194-5p significantly attenuated the upregulation of RAC1 expression in H9C2 cardiomyocytes cultured under H/R conditions (***P<0.001, *P<0.05); (B) qPCR indicated that exogenous miR-194-5p significantly attenuated the upregulation of RAC1 expression in H9C2 cardiomyocytes cultured under H/R conditions and transfected with OE-RAC1 plasmids (**P<0.01). (C) and (D) The results of the immunofluorescence and cytochemistry analyses indicated that H/R significantly induced the cellular calcium concentration in H9C2 cardiomyocytes compared with control cells cultured under normoxic conditions (***P<0.001), while administration of exogenous miR-194-5p significantly attenuated the HR-induced upregulation of the cellular calcium concentration in H9C2 cardiomyocytes (**P<0.01). miR, microRNA; RAC1, Ras-related C3 botulinum toxin substrate 1; qPCR, quantitative PCR; H/R, hypoxia/reoxygenation; OE, overexpression.

that administration of miR-194-5p mimics (20 nM) was associated with significant inhibition of RAC protein expression levels compared with the administration of mimics control; however, administration of miR-194-5p inhibitors significantly increased the expression levels of the RAC1 protein compared with the expression levels of RAC1 noted following administration of inhibitor controls (Fig. 3C).

To further clarify the targeting sequence of RAC1 mRNA for miR-194-5p, the luciferase activity assay was performed. Administration of miR-194 suppressed the relative luciferase activity of the constructs encompassing the binding sites of RAC1 3'-UTR (P<0.001), while administration of miR-194-5p did not significantly affect the relative luciferase activity of the constructs encompassing the mutant binding sites of the RAC1 3'-UTR, suggesting that miR-194-5p may inhibit RAC1 expression targeting this sequence (Fig. 4).

To further validate the interactions between miR-194-5p and RAC1 in H9C2 cardiomyocytes undergoing H/R, specific plasmids were constructed to overexpress RAC1 (OE-RAC1) and exogenous miR-194-5p (miR-194 mimics) were transfected

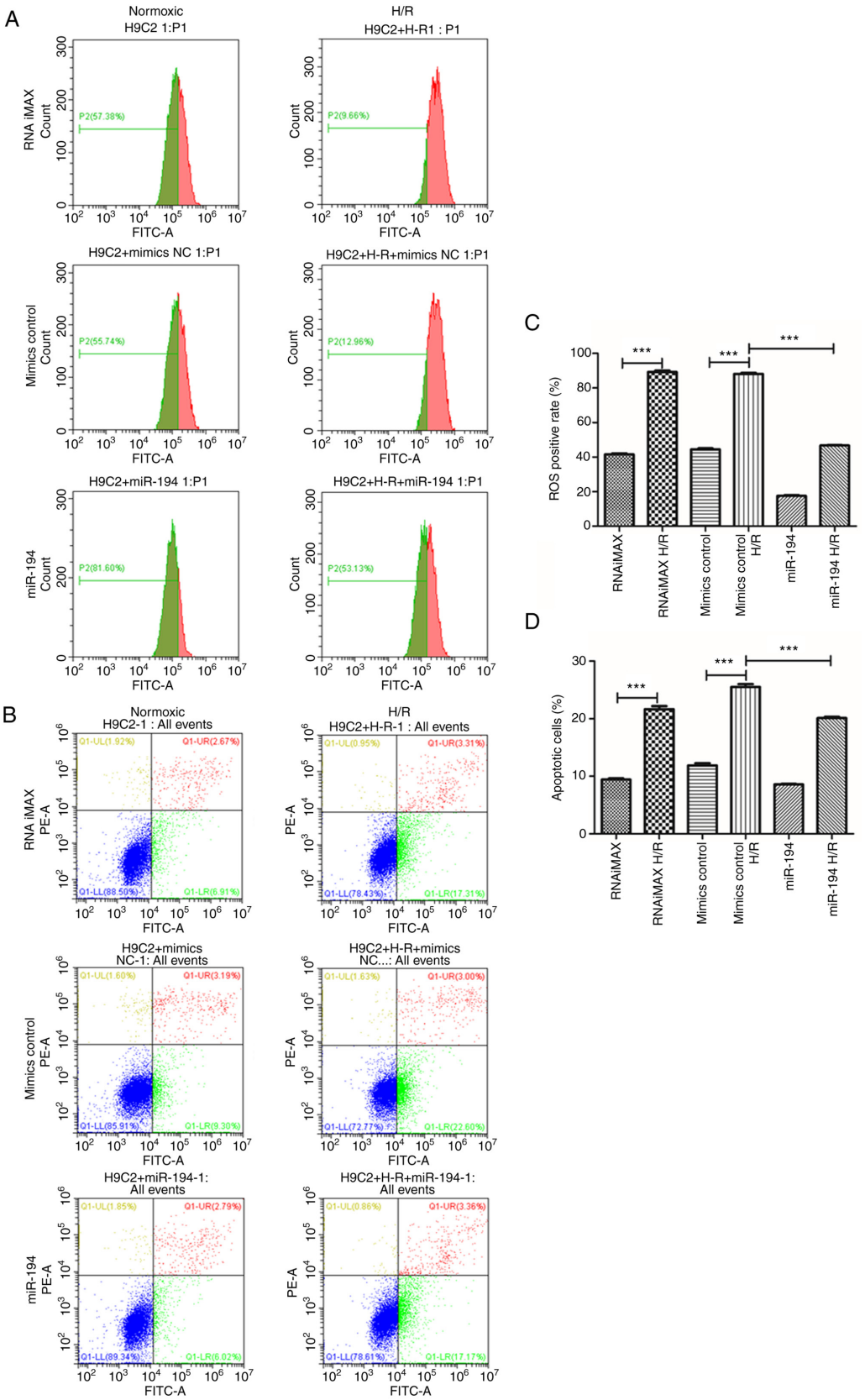

Figure 6. Effects of miR-194 on ROS production and induction of apoptosis in H9C2 cardiomyocytes undergoing H/R. (A) The results of the flow cytometry analysis indicated that H/R significantly increased the cellular ROS levels in H9C2 cardiomyocytes compared with those of the control cells grown under normoxic conditions ($P<0.05$), while exogenous administration of miR-194 significantly reduced the cellular levels compared with those of H9C2 cardiomyocytes undergoing H/R and treated with control mimics ($P<0.05$). (B) The results of the flow cytometry analysis indicated that H/R significantly induced apoptosis of H9C2 cardiomyocytes compared with those of the control cells cultured under normoxic conditions ($P<0.05$), while exogenous administration of miR-194 significantly reduced the apoptotic rate in H/R H9C2 cardiomyocytes treated with control mimics ($P<0.05$). (C) Quantitative assessment of the simultaneous effect of H/R and the exogenous administration of miR-194 on the ROS positive rates of H9C2 cardiomyocytes in each group. (D) Quantitative assessment of the effect of H/R and the exogenous administration of miR-194 on the apoptotic rates of H9C2 cardiomyocytes in each group. ***$P<0.001$. miR, microRNA; ROS, reactive oxygen species; H/R, hypoxia/reoxygenation.

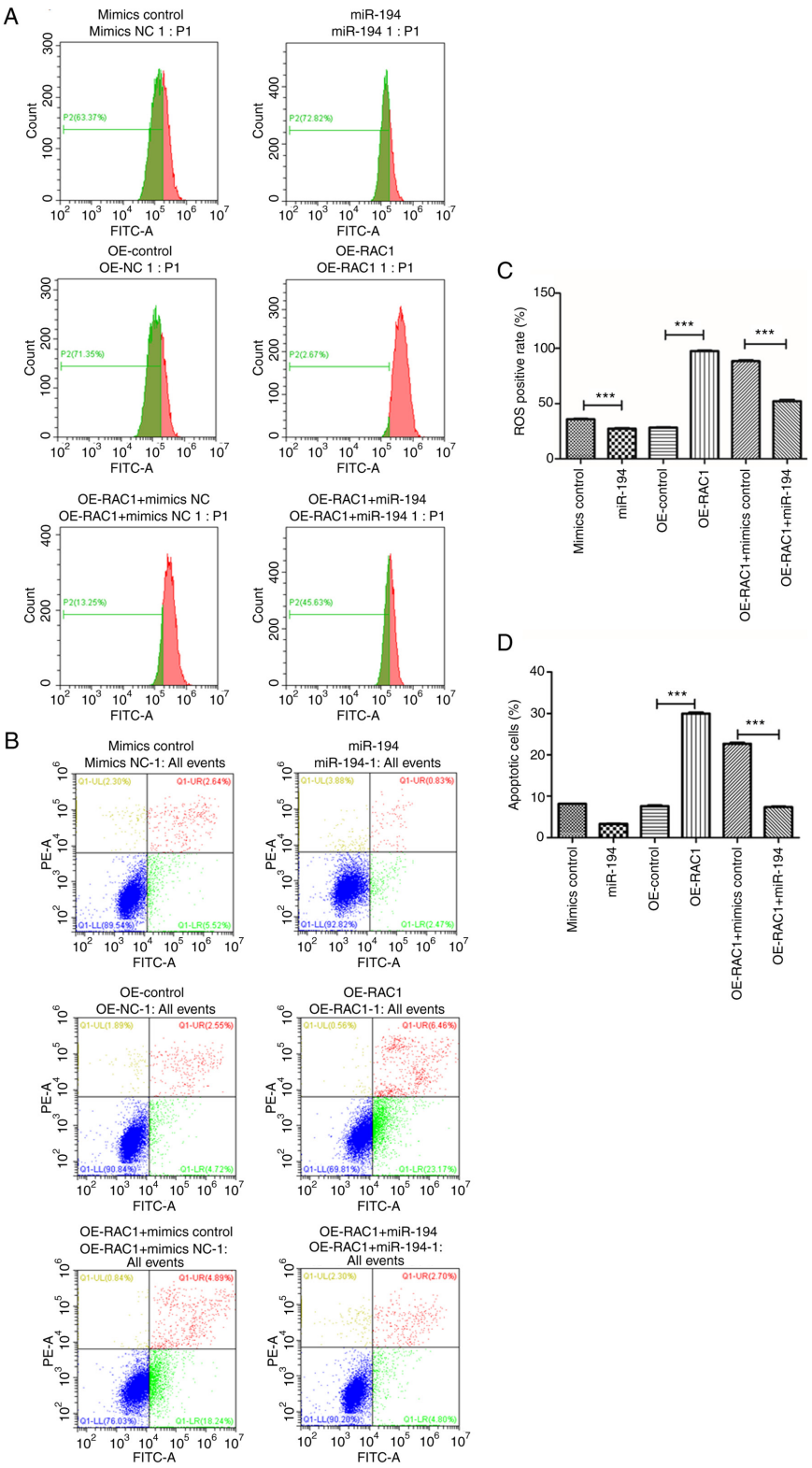

Figure 7. Effects of the overexpression of RAC1 and the exogenous addition of miR-194 on ROS production and the induction of H9C2 cardiomyocyte apoptosis undergoing H/R. (A) The results of the flow cytometry analysis indicated that exogenous administration of miR-194 significantly reduced the cellular ROS levels compared with those of the H9C2 cardiomyocytes undergoing H/R and treated with control mimics ($P<0.05$), while overexpression of RAC1 dramatically increased the cellular ROS levels compared with those noted in H9C2 cardiomyocytes undergoing H/R and treated with OE-control sequences. In addition, exogenous administration of miR-194 significantly reduced the cellular ROS levels induced by H/R and the overexpression of RAC1 in H9C2 cardiomyocytes. (B) The results of the flow cytometry analysis indicated that exogenous administration of miR-194 significantly reduced the rate of apoptotic cells compared with that of the H9C2 cardiomyocytes undergoing H/R and treated with control mimics ($P<0.05$), while overexpression of RAC1 dramatically increased the number of apoptotic cells compared with that of H9C2 cardiomyocytes undergoing H/R and treated with OE-control sequences. In addition, exogenous administration of miR-194 significantly reduced the rate of apoptotic cells induced by H/R and the overexpression of RAC1 in H9C2 cardiomyocytes. (C) Quantitative assessment of the simultaneous effects of H/R and the exogenous administration of miR-194 on the ROS positive rates of H9C2 cardiomyocytes in each group. (D) Quantitative assessment of the effects of H/R and the exogenous administration of miR-194 on the apoptotic rates of H9C2 cardiomyocytes in each group. ***$P<0.001$. RAC1, Ras-related C3 botulinum toxin substrate 1; miR, microRNA; ROS, reactive oxygen species; H/R, hypoxia/reoxygenation; OE, overexpression.

into H9C2 cardiomyocytes. The results of the qPCR analysis indicated a significant induction in RAC1 expression of H9C2 cardiomyocytes cultured under H/R conditions compared with the control cells cultured under normoxic conditions (P<0.001; Fig. 5A). However, administration of exogenous miR-194-5p significantly attenuated the upregulation of RAC1 expression in H9C2 cardiomyocytes cultured under H/R conditions (P<0.05; Fig. 5A). Further studies using qPCR indicated that exogenous expression of miR-194-5p significantly attenuated the upregulation of RAC1 expression in H9C2 cardiomyocytes cultured under H/R conditions and transfected with OE-RAC1 plasmids (P<0.01; Fig. 5B). In addition, the results of the immunofluorescence and immunocytochemistry analyses indicated that H/R significantly induced the cellular calcium concentration in H9C2 cardiomyocytes compared with the control cells cultured under normoxic conditions (P<0.01), while administration of exogenous miR-194-5p significantly attenuated the HR-induced upregulation in the concentration levels of cellular calcium in H9C2 cardiomyocytes (P<0.01; Fig. 5C).

Taken together, these results indicated that both miR-194-5p and RAC1 expressions were increased during H/R in cardiomyocytes and that miR-194-5p could attenuate the overexpression of RAC1 by targeting the 3'-UTR of RAC1 mRNA.

Effects of miR-194-5p on cardiomyocyte apoptosis and ROS accumulation in H9C2 cells during H/R. Compared with H9C2 cardiomyocytes cultured under normoxic conditions, H/R significantly increased the concentration levels of cellular ROS in these cells (P<0.001), while exogenous administration of miR-194 significantly reduced the cellular levels compared with those of the H9C2 cardiomyocytes cultured under H/R conditions and transfected with control mimics (P<0.001; Fig. 6A). Moreover, the results of the flow cytometry analyses indicated that H/R significantly induced apoptosis of H9C2 cardiomyocytes compared with the control cells cultured under normoxic conditions (P<0.001), while exogenous administration of miR-194 significantly reduced the apoptotic rate in H9C2 cardiomyocytes cultured under H/R conditions and transfected with control mimics (P<0.001; Fig. 6B). Subsequent quantitative analyses indicated similar results (Fig. 6C and D). These results indicated that miR-194-5p attenuated H/R-induced apoptosis in H9C2 cardiomyocytes by alleviating cellular ROS accumulation.

Role of RAC1 in the potential protective effect of miR-194-5p on H/R-induced apoptosis. The results of the flow cytometry analysis indicated that exogenous administration of miR-194 significantly reduced the cellular ROS levels compared with those of the H9C2 cardiomyocytes cultured under H/R conditions and transfected with control mimics (P<0.001), while overexpression of RAC1 dramatically increased the cellular levels compared with those of H/R-cultured H9C2 cardiomyocytes transfected with OE-control. Moreover, exogenous administration of miR-194 significantly reduced the cellular ROS levels induced by H/R and reduced the overexpression of RAC1 in H9C2 cardiomyocytes (Fig. 7A). Similarly, exogenous administration of miR-194 significantly reduced the apoptotic rate of the cells compared with that of the H/R-cultured H9C2 cardiomyocytes transfected with control mimics (P<0.001), while overexpression of RAC1 dramatically increased the apoptotic rate of the cells compared with that of the H/R-cultured H9C2 cardiomyocytes transfected with OE-control. Moreover, exogenous administration of miR-194-5p significantly reduced the apoptotic rate of H9C2 cells cultured under H/R conditions and transfected with RAC1 overexpressing plasmids (Fig. 7B). Subsequent quantitative analyses indicated similar results (Fig. 7C and D). Taken together, these results demonstrated that overexpression of RAC1 may mediate H/R-induced apoptosis in H9C2 cardiomyocytes by enhancing cellular ROS accumulation, whereas upregulation of miR-194-5p expression may function as a negative regulator of the aforementioned pathophysiological process.

Discussion

In the present study, an *in vitro* cultured H9C2 cardiomyocyte model was established under H/R conditions. The data indicated that RAC1 was significantly overexpressed. This effect facilitated the induction of apoptosis in cardiomyocytes by enhancing cellular accumulation of ROS. It is notable that bioinformatic and luciferase analyses confirmed that miR-194-5p could inhibit the translation of RAC1 in H9C2 cells. miR-194-5p expression was upregulated under H/R conditions in H9C2 cardiomyocytes, which subsequently ameliorated cellular ROS levels and the induction of apoptosis via inhibition of RAC1 overexpression. Taken together, these results indicated that the upregulated expression levels of miR-194-5p may function as a self-regulated cardioprotective response against RAC1-mediated ROS accumulation and cardiomyocyte apoptosis. Exogenous administration of miR-194-5p may be a novel target to ameliorate I/R-induced injury related to myocardial apoptosis.

Previous studies have indicated that constitutive expression of RAC1 in cardiomyocytes is essential for specific physiological functions including cytoskeletal formation, ROS clearance and energy biogenesis (8,22,23). However, overexpression of RAC1 in a mouse model of myocardial I/R injury has been attributed to causing exacerbation of myocardial injury and deteriorated cardiac function during follow-up (9). The results of the present study provided further information on the pathological role of RAC1 overexpression in the pathogenesis of myocardial I/R injury by indicating that H/R enhanced the protein levels of RAC1 in H9C2 cardiomyocytes and thereby led to cellular ROS accumulation and induction of cardiomyocyte apoptosis. Since myocardial apoptosis causes direct loss of functional cardiomyocytes, it has been shown to be a major factor of deteriorated cardiac function in an animal model and in patients with ischemic heart disease (24,25). These results further confirmed that overexpression of RAC1 in cardiomyocytes cultured under H/R conditions, is a key molecular pathway underlying the pathogenesis of I/R-mediated myocardial dysfunction.

Although previous studies have shown that miRs may interact with RAC1, the majority of these studies are focused on cancer (12-14). To the to the best of the authors' knowledge, a few studies have evaluated the potential interaction between miRs and RAC1 proteins in the pathogenesis of cardiovascular diseases. In an early study, which used streptozotocin-induced diabetic mice, the changes in the miR expression profiles and

their potentially targeted proteins were identified (26). The miR-mediated upregulation of RAC1 expression was proposed to be involved in the pathogenesis of diabetic cardiomyopathy (26). An additional study was performed in H9C2 cells cultured under persistent hypoxia and the results indicated that upregulation of miR-145 expression was associated with overexpressed RAC1; moreover, silencing of miR-145 expression protected H9C2 cells against hypoxia-induced injury by targeting RAC1 (15). Transfection of the cells with miR-182 mimics was shown to attenuate hyperglycemia-induced hypertrophy of cardiomyocytes via targeting the overactivated RAC1 protein (16). The present study, used a model with the mimics and the inhibitors of miR-194-5p and a luciferase reporter assay. The data indicated that the H/R-induced upregulation of miR-194-5p expression may be a self-protective mechanism which can target RAC1 overexpression and related ROS accumulation and apoptosis of H9C2 cardiomyocytes. Exogenous administration of miR-194-5p was shown to reduce the overactivated RAC1 protein and subsequently ameliorate the H/R-induced ROS accumulation and apoptosis of H9C2 cardiomyocytes. Future studies are required to determine the potential therapeutic efficacy of miR-194-5p upregulation on myocardial injury and cardiac function in animal model of I/R injury.

In conclusion, the present study indicated that upregulation of miR-194-5p may function as a self-regulated cardioprotective response against RAC1-mediated ROS accumulation and cardiomyocyte apoptosis, although animal and clinical experiments are needed to further confirm these results. Exogenous administration of miR-194-5p may be a novel target to ameliorate I/R injury-mediated myocardial apoptosis.

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Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD015991.

Authors' contributions

Material preparation, data collection and analysis were performed by CL, YaL, YiL, YW, YT and YH. All authors also contributed to the study conception and design. The manuscript was written by CL and YinL and all authors commented on the previous versions of the manuscript. All authors read and approved the final version of the manuscript. YT and YH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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