

## MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: protectomiRs

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**Varga ZV, Zvara Á, Faragó N, Kocsis GF, Pipicz M, Gáspár R, Bencsik P, Görbe A, Csonka C, Puskás LG, Thum T, Csont T, Ferdinandy P.** MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: protectomiRs. *Am J Physiol Heart Circ Physiol* 307: H216–H227, 2014. First published May 23, 2014; doi:10.1152/ajpheart.00812.2013.—We aimed to characterize early changes in microRNA expression in acute cardioprotection by ischemic pre- and postconditioning in rat hearts. Hearts isolated from male Wistar rats were subjected to 1) time-matched nonischemic perfusion, 2) ischemia-reperfusion (30 min of coronary occlusion and 120 min of reperfusion), 3) preconditioning (3 × 5 min of coronary occlusion) followed by ischemia-reperfusion, or 4) ischemia-reperfusion with postconditioning (6 × 10 s of global ischemia-reperfusion at the onset of reperfusion). Infarct size was significantly reduced by both interventions. Of 350 different microRNAs assessed by microarray analysis, 147–160 microRNAs showed detectable expression levels. Compared with microRNA alterations induced by ischemia-reperfusion versus time-matched nonischemic controls, five microRNAs were significantly affected by both pre- and postconditioning (microRNA-125b\*, microRNA-139-3p, microRNA-320, microRNA-532-3p, and microRNA-188), four microRNAs were significantly affected by preconditioning (microRNA-487b, microRNA-139-5p, microRNA-192, and microRNA-212), and nine microRNAs were significantly affected by postconditioning (microRNA-1, microRNA let-7i, microRNA let-7e, microRNA let-7b, microRNA-181a, microRNA-208, microRNA-328, microRNA-335, and microRNA-503). Expression of randomly selected microRNAs was validated by quantitative real-time PCR. By a systematic comparison of the direction of microRNA expression changes in all groups, we identified microRNAs, specific mimics, or antagomiRs that may have pre- and postconditioning-like cardioprotective effects (protectomiRs). Transfection of selected protectomiRs (mimics of microRNA-139-5p, microRNA-125b\*, microRNA let-7b, and inhibitor of microRNA-487b) into cardiac myocytes subjected to simulated ischemia-reperfusion showed a significant cytoprotective effect. This is the first demonstration that the ischemia-reperfusion-induced microRNA expression profile is significantly influenced by both pre- and postconditioning, which shows the involvement of microRNAs in cardioprotective signaling. Moreover, by analysis of microRNA expression patterns in cardioprotection by pre- and postconditioning, specific protectomiRs can be revealed as potential therapeutic tools for the treatment of ischemia-reperfusion injury.

microRNA; miRNA; miR; reperfusion injury; cardioprotection

MICRORNAS ARE SMALL (19–23 nucleotides) noncoding RNA molecules that are currently being recognized as endogenous physiological regulators of gene expression (1). It has been well established that microRNAs play an essential role in the posttranscriptional regulation of gene expression by repression or activation of translation/transcription (“RNA interference”) (5, 27). MicroRNAs are known to play important roles in many physiological and pathological processes in the heart, including cardiac fibrosis, the hypertrophic response, myocyte contractility, cardiac development, and arrhythmogenesis (36). Recently, several research groups have reported the involvement of some microRNAs in the pathophysiology of myocardial infarction (11, 31). The importance of microRNAs in cardiac pathologies suggests their promising therapeutic potential (15, 19). Modulation of microRNA expression in vivo with specific synthetic oligonucleotides is a feasible approach, as application of both microRNA inhibitors (antagomiRs) and microRNA mimics offer a potential therapeutic option for cardioprotection (35).

However, little is known on the role of microRNAs in the ischemic myocardium, particularly in the early phase of myocardial ischemia-reperfusion (I/R) injury and in endogenous cardioprotective maneuvers such as ischemic pre- and postconditioning. These cardioprotective interventions, especially ischemic postconditioning, has emerged as a clinically applicable approach for limiting myocardial injury in humans (30). However, despite of intensive research in the last decades, the exact biological mechanisms underlying the cardioprotective effect of pre- and postconditioning remain largely unclear due to the complexity of the cellular events and the interference of other comorbidities and risk factors of ischemic heart diseases (17, 30). Therefore, the use of systems biological tools seems necessary to reveal the complex molecular events. Accordingly, we have previously shown by cDNA microarrays that ischemic preconditioning (29) and ischemic postconditioning (10) markedly influenced the gene expression pattern of rat hearts.

In the present study, we aimed to identify microRNAs involved in pre- and postconditioning-induced cardioprotection by the application of a systematic comparison of the microRNA expression patterns of different myocardial phenotypes, including nonischemic, I/R, protected by preconditioning,

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and protected by postconditioning. In fact, preceding ischemic preconditioning and subsequent postconditioning stimuli can be considered as effective “inhibitors” of I/R injury. Therefore, by comparing microRNA expression changes and the direction of the changes (i.e., down- or upregulation) induced by I/R versus time-matched nonischemic controls and in I/R with pre- or postconditioning, respectively, we were able to reveal possible causative relationships between microRNA changes and endogenous cardioprotection. Using microRNA microarrays of 350 known rat microRNA genes, in the present study, we identified several microRNAs associated with cardioprotection by ischemic pre- and postconditioning. These microRNAs are potential therapeutic targets for cardioprotection, appropriate mimics, or antagomiRs, which were termed here as “protectomiRs.” Validation of four of the potential protectomiRs was also performed in cardiomyocytes subjected to simulated I/R *in vitro*.

## MATERIALS AND METHODS

This investigation conformed with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996) and was approved by local animal ethics committee of the University of Szeged.

### Experimental Protocol

Male Wistar rats (250–300 g) were anesthetized with diethyl ether and given 500 U/kg heparin intravenously. Hearts were then isolated and perfused according to Langendorff with oxygenated, normothermic Krebs-Henseleit buffer as previously described (9). Four different perfusion protocols were applied (Fig. 1). A time-matched nonischemic control group was aerobically perfused for 190 min. In the I/R group, regional ischemia was induced by 30 min of occlusion of the left anterior descending coronary artery (LAD) followed by 120 min of reperfusion. In the preconditioning group, hearts were subjected to a preconditioning protocol (induced by three intermittent cycles of 5 min of coronary occlusion/5 min of reperfusion) followed by I/R. In the postconditioning group, postconditioning was induced by six consecutive cycles of 10 s of global ischemia and 10 s of reperfusion applied immediately at the onset of the 120 min of reperfusion. Heart

rate and coronary flow were monitored throughout the perfusion protocol in all groups. To assess I/R injury and cardioprotection, infarct size was determined by planimetry (Infarctsize 2.4, Pharmahungary, Szeged, Hungary) after standard triphenyltetrazolium chloride staining at the end of reperfusion ( $n = 8–10/\text{group}$ ). Infarct size was expressed as a percentage of the area at risk (8). To further assess tissue injury, lactate dehydrogenase (LDH) release of hearts was measured using a LDH-P kit (Diagnosticum, Budapest, Hungary) from coronary effluents collected for 5 min upon reperfusion after test ischemia ( $n = 12/\text{group}$ ). LDH release was expressed as milliunits per minute per gram wet heart weight. In separate experiments, at the end of the 120 min of reperfusion, we sampled the LAD-supplied risk zone of the left ventricle for further analysis of microRNA expression. Sampling was performed by a single oblique cut with scissors from the origin of the LAD toward the right side of the apical area that involves the majority of the anterior wall of the left ventricle as well as the apex of the heart. Samples from all groups ( $n = 6/\text{group}$ ) were rapidly frozen in liquid nitrogen for microRNA isolation.

### MicroRNA Isolation

MicroRNA isolation was performed according to the protocol for isolation of microRNA from tissues as supplied in the manufacturer’s kit (Roche) with some modifications (14). RNA quality and quantity were assessed spectrophotometrically (Nanodrop) with a 2100 Bioanalyzer (Agilent).

### Microarray Analysis of MicroRNA Expression

MicroRNAs were labeled using the miRNA Complete Labeling and Hyb kit system (Agilent Technologies, Palo Alto, CA). Labeled samples were completely vacuum dried on the medium-high (45°C) heat setting and hybridized onto the surface of a  $8 \times 15\text{k}$  Rat miRNA Microarray (Agilent Technologies), which was done in microarray hybridization chambers (Agilent Technologies), as previously described (13). Each array was scanned as previously described (16). Statistical analysis was performed on fluorescent signal intensity data representing microRNA expression, as described below.

Changes in gene expression were determined as ratios of signal intensity values. For visual comparison and representation of both down- and upregulation, data were shown as  $\log_2$  changes. Averages of  $\log_2$  changes were plotted with SD values.

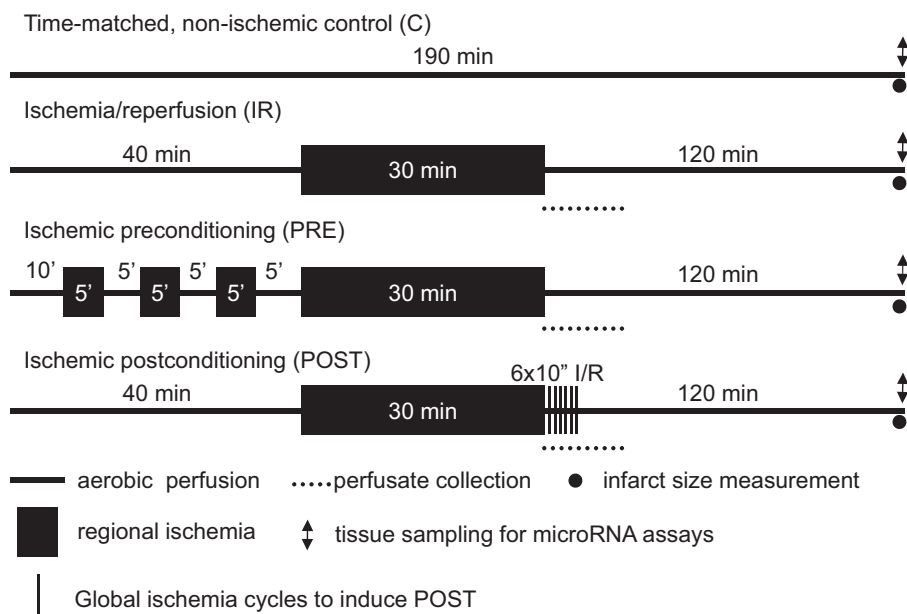


Fig. 1. Experimental protocol. Hearts isolated from male Wistar rats were subjected to either time-matched aerobic perfusion [i.e., time-matched nonischemic control (C)], 30 min of coronary occlusion and 120 min of reperfusion [ischemia/reperfusion (I/R; IR)],  $3 \times 5$  min of I/R followed by 30 min of coronary occlusion and 120 min of reperfusion [preconditioning (PRE)], or  $6 \times 10$  s of global I/R applied immediately after 30 min of coronary occlusion and followed by 120 min of reperfusion [postconditioning (POST)] protocols. Coronary effluent was collected upon the first 5 min of reperfusion after coronary occlusion for lactate dehydrogenase release detection. Infarct size was measured at the 120th min of reperfusion. For microRNA isolation, in separate experiments, left ventricles were frozen in liquid nitrogen at the end of the perfusion protocol.

### Validation of Microarray Data by Quantitative Real-Time PCR

To confirm microarray results, quantitative real-time PCR was used on randomly selected microRNAs, as previously described (12). Experiments were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and with TaqMan MicroRNA Assays (Applied Biosystems).

### Identification of ProtectomiRs

Ischemic preconditioning and postconditioning can be considered as effective “inhibitors” of I/R injury. Therefore, by comparing microRNA expression changes and the direction of the changes (i.e., down- or upregulation) induced by I/R versus time-matched nonischemic controls and in I/R with pre- or postconditioning, respectively, possible causative relationships between microRNA changes and endogenous cardioprotection can be revealed. Accordingly, we first selected microRNAs that were significantly affected by ischemic preconditioning and/or ischemic postconditioning compared with I/R versus nonischemic controls. These microRNAs were then divided into three groups according to the effect of I/R compared with time-matched nonischemic controls and further subdivided into two additional categories according to the direction of microRNA expression changes, thereby yielding the following six categories.

**Category 1.** MicroRNAs that were downregulated by I/R compared with time-matched nonischemic controls but their downregulation was inhibited by ischemic preconditioning and/or ischemic postconditioning were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or ischemic postconditioning. Therefore, these microRNAs or their mimics are considered as protectomiRs.

**Category 2.** MicroRNAs that were downregulated by I/R compared with time-matched nonischemic controls but their downregulation was further enhanced by ischemic preconditioning and/or ischemic postconditioning were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or ischemic postconditioning. Therefore, the antagonomiRs of these microRNAs can also be considered as protectomiRs. However, no example of this microRNA group was found.

**Category 3.** MicroRNAs that were upregulated by I/R compared with time-matched nonischemic controls but their upregulation was inhibited by ischemic preconditioning and/or ischemic postconditioning were considered to be associated with the development of I/R injury. Therefore, the antagonomiRs of these microRNAs can be also considered as protectomiRs.

**Category 4.** MicroRNAs that were upregulated by I/R compared with time-matched nonischemic controls but their upregulation was further enhanced by ischemic preconditioning and/or ischemic postconditioning were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or ischemic postconditioning. Therefore, these microRNAs or their mimics are considered as protectomiRs.

**Category 5.** MicroRNAs that were not affected by I/R alone compared with time-matched nonischemic controls but were significantly upregulated by ischemic preconditioning and/or ischemic postconditioning were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or ischemic postconditioning. Therefore, these microRNAs or their mimics are considered as protectomiRs.

**Category 6.** This group of microRNAs was not affected by I/R alone compared with time-matched nonischemic controls but was downregulated by ischemic preconditioning and/or ischemic postconditioning. Inhibition of these microRNAs was considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or ischemic postconditioning. Therefore, the antagonomiRs of these microRNAs can be also considered as protectomiRs.

### MicroRNA Transfection into Rat Cardiac Myocytes Subjected to Simulated I/R

To validate the cytoprotective effect of selected protectomiRs, neonatal cardiomyocyte cultures were prepared from newborn Wistar rats (40). Cells were kept in a standard CO<sub>2</sub> incubator (5% CO<sub>2</sub>) and supplied with proliferation medium for 1 day (DMEM + 10% FBS). Differentiation medium (DMEM + 1% FBS) was then administered for 2 days.

Synthesized microRNAs (Dharmacon) were transfected into cardiomyocytes using a previously described transfection protocol (40). Miridian microRNA mimics were diluted in antibiotic-free medium and were incubated with cells for 10 h with DharmaFect 1 transfection reagent, which was followed by a 10-h-long recovery period. For negative control transfection, cel-miR-67 (a nontargeting *Caenorhabditis elegans* microRNA) was used, which shows no sequence identity to known rat microRNAs. After completion of the transfection procedure, transfected cells were subjected to a simulated I/R protocol. To simulate ischemic conditions, the culture medium was changed to hypoxic solution, plates were placed into a hypoxic chamber, and cells were exposed to a constant flow of a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub> at 37°C for 4 h. Simulated ischemia was followed by 2 h of simulated reperfusion using differentiation medium and normoxia. To assess cell viability, cardiomyocytes were incubated with 1 μM calcein AM (Sigma, St. Louis, MO) at room temperature for 30 min. Fluorescence intensity was measured with a fluorescence plate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) with 490-nm excitation and 520-nm emission filters. The cytoprotective effect of different microRNA modulators was compared with the negative control, i.e., cardiomyocytes transfected with a nontargeting microRNA and subjected to simulated I/R. We expressed viability as a percentage of nontargeting microRNA-transfected normoxic control groups.

### Statistical Analysis

Microarray and quantitative real-time PCR data are presented as means ± SD; all other data are means ± SE. One-way ANOVA followed by Fisher's least-significant-difference post hoc test was used to evaluate differences in mean values of infarct size, LDH release, and hemodynamic parameters between groups.

Statistical analysis of microRNA microarrays were done as previously described using Feature Extraction software (Agilent Technologies) (14). All individual microRNAs were represented by 20 different probes on the array. A microRNA was considered as detected if at least 1 probe from all 20 probes was detected. The total gene signal is equal to the sum of all signals of the individual probes. Expressions of all 350 microRNAs found in the Sanger miRBase (version 10.1) were checked. Using a two-tailed two-sample unequal variance Student's *t*-test, the *P* value was determined to find significant gene expression changes. A corrected *P* value was determined for each gene to control the false discovery rate using the Benjamini and Hochberg multiple testing correction protocol. MicroRNA expression ratios with *P* values of <0.05 and log<sub>2</sub> changes of less than or equal to -0.586 or log<sub>2</sub> changes of ≥0.586 were considered as repression or overexpression, respectively.

## RESULTS

### Evaluation of I/R Injury and Cardioprotection

To confirm the cardioprotective effect of preconditioning and postconditioning in the present study, infarct size and LDH release were measured after I/R. Both ischemic preconditioning and ischemic postconditioning significantly decreased infarct size as measured at the 120th min of reperfusion compared with the I/R group (Table 1). LDH release measured in the first 5 min of reperfusion after 30 min of ischemia was

Table 1. *Markers of myocardial tissue injury*

	Time-Matched Nonischemic Control Group	Ischemia-Reperfusion Group	Ischemic Preconditioning Group	Ischemic Postconditioning Group
Animal weight, g	284 ± 10	282 ± 11	275 ± 9	276 ± 4
Area at risk, %	N/A	37.4 ± 1.7	40.1 ± 4.3	43.3 ± 3.5
Infarct size (%)	N/A	48.3 ± 3.5	15.3 ± 3.7*	21.7 ± 1.5*
Lactate dehydrogenase release, mU·min <sup>-1</sup> ·g <sup>-1</sup>	N/A	3.31 ± 0.45	0.82 ± 0.16*	1.51 ± 0.38*
Heart rate, beats/min	274 ± 8	283 ± 12	279 ± 8	295 ± 14
Coronary flow, ml/min	10.1 ± 1.1	9.9 ± 0.9	10.8 ± 0.7	12.2 ± 1.4

Values are means ± SE. The area at risk and infarct size were measured after 120 min of reperfusion. Lactate dehydrogenase release was measured from coronary effluent collected during the first 5 min of reperfusion. The heart rate and coronary flow data presented here were measured at the end of the perfusion protocols. N/A, not applicable. \* $P < 0.05$  vs. the ischemia-reperfusion group.

significantly reduced in both ischemic pre- and postconditioning groups (Table 1). Neither heart rate nor coronary flow was affected significantly by any of the interventions (Table 1).

#### *Global Expression Changes of MicroRNAs*

To determine microRNAs in 1) time-matched nonischemic control, 2) I/R, 3) preconditioning + I/R, and 4) I/R + postconditioning groups, microRNAs were isolated from the anterior wall of left ventricles after 2 h of reperfusion from all groups. Of the total 350 different rat microRNAs studied, 147 microRNAs in the time-matched nonischemic control group, 154 microRNAs in the I/R group, 160 microRNAs in the preconditioning group, and 156 microRNAs in the postconditioning group showed expression, respectively.

#### *Identification of MicroRNAs Associated With I/R Injury*

To determine microRNAs associated with I/R injury, microRNA expression induced in the I/R group was compared with the time-matched nonischemic control group as well as in ischemic preconditioning and postconditioning groups. Eight different microRNAs (microRNA-125a-5p, microRNA-322\*, microRNA-331, microRNA-378\*, microRNA-494, microRNA-652, microRNA-877, and microRNA-92a) were significantly altered by I/R compared with the time-matched nonischemic control group, and their alterations were not affected significantly either by pre- or postconditioning, which represented microRNAs associated with I/R injury alone without being associated with cardioprotective signaling (Fig. 2.).

#### *Identification of MicroRNAs Associated With Cardioprotection by Ischemic Pre- and/or Postconditioning*

To assess cardioprotective microRNAs associated with ischemic preconditioning, we analyzed expression alterations (including the direction of changes) induced by ischemic preconditioning followed by I/R compared with the I/R and time-matched nonischemic control groups, respectively. We found three different microRNAs that were significantly upregulated by preconditioning compared with both the time-matched nonischemic control group and the I/R group (microRNA-139-5p, microRNA-192, and microRNA-212; Fig. 3A). There was also one microRNA that was significantly upregulated by I/R compared with time-matched nonischemic controls; however, preconditioning inhibited this alteration, i.e., downregulated this alteration compared with I/R (microRNA-487b; Fig. 3B). We considered these microRNAs to be associated with the cardioprotective mechanisms of preconditioning.

To further assess cardioprotective microRNAs, the effect of ischemic postconditioning on myocardial microRNA expression was determined. We found three microRNAs that were not affected by I/R itself compared with nonischemic controls; however, their expression was significantly upregulated by the subsequent postconditioning stimulus compared with the I/R group (microRNA-1, microRNA let-7i, and microRNA let-7e; Fig. 4A). There were also six different microRNAs that were significantly altered (up- or downregulated) by I/R compared with time-matched nonischemic controls; however, postconditioning significantly inhibited these alterations (microRNA let-7b, microRNA-181a, microRNA-208, microRNA-328, microRNA-335, and microRNA-503; Fig. 4B). We considered these nine microRNAs to be associated with the cardioprotective mechanisms of postconditioning.

We found five different microRNAs (microRNA-188, microRNA-532-3p, microRNA-125b\*, microRNA139-3p, and microRNA-320) that showed similar expression patterns due to both ischemic preconditioning and postconditioning according to the following results. Both ischemic preconditioning and ischemic postconditioning significantly inhibited I/R-induced downregulation of microRNA-125b\*, microRNA-139-3p, and microRNA-320 (Fig. 5B). In the case of microRNA-188 and microRNA-532-3p, both pre- and postconditioning induced significant upregulation compared with I/R; however, I/R alone did not affect the expression of these microRNAs (Fig. 5A). We considered these five microRNAs to play a pivotal role in endogenous cardioprotective signaling in both pre- and postconditioning.

#### *Validation of Microarray Data by Quantitative Real-Time PCR*

To confirm the microarray data, we measured the expression of 12 randomly selected microRNAs using quantitative real-time PCR. The expression changes of 10 microRNAs were confirmed by quantitative real-time PCR and showed good reliability of the microarray data (Table 2). In addition, quantitative real-time PCR experiments were performed in the case of microRNA-125b\*, which showed the most pronounced alterations due to I/R and pre- and postconditioning as assessed by microarray. The quantitative real-time PCR results confirmed the microarray data (Table 2).

#### *Identification of ProtectomiRs*

To identify protectomiRs, we systematically compared microRNA changes and the direction of their changes in all

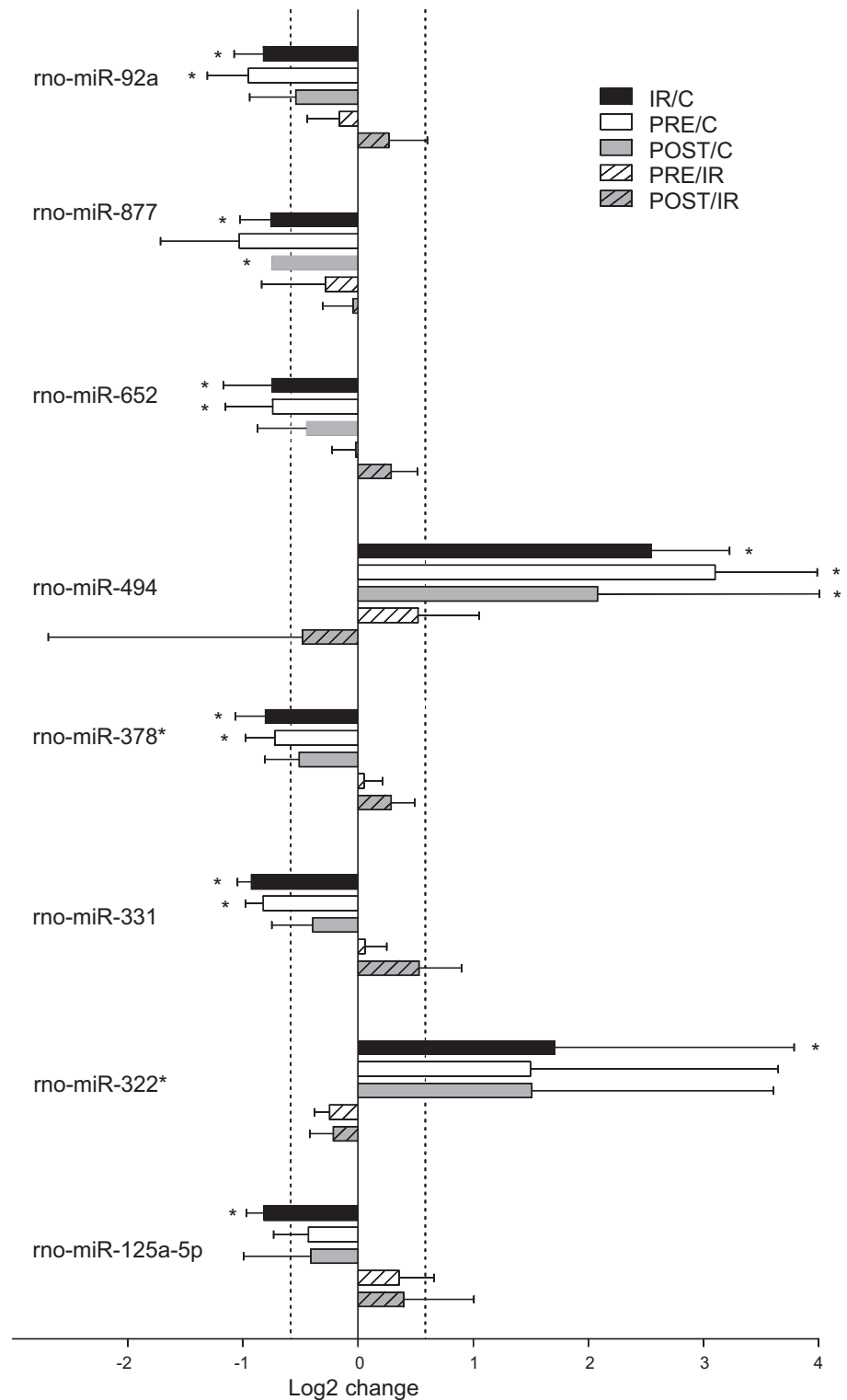


Fig. 2. Cardiac microRNAs (miR) associated with I/R injury. This graph shows microRNAs that were significantly changed by I/R compared with time-matched nonischemic controls but neither pre- nor postconditioning influenced their changes due to I/R. This shows that these microRNAs were associated with I/R and unaffected by endogenous cardioprotective mechanisms. Bars show alterations in microRNA expression in I/R versus time-matched nonischemic controls (IR/C), preconditioning versus time-matched nonischemic controls (PRE/C), postconditioning versus time-matched nonischemic controls (POST/C), preconditioning versus I/R (PRE/IR), and postconditioning versus I/R (POST/IR). Values are log<sub>2</sub> expression changes ± SD. \*P < 0.05 and a log<sub>2</sub> change of greater than or equal to ±0.585.

groups. Based on the comparative data, potential protectomiRs were classified into six theoretical categories, as described in METHODS (Fig. 6). The protectomiRs identified here belonged to three of the six theoretical categories, as follows. Mimics of microRNA-125b\*, microRNA-139-3p, microRNA-320, microRNA let-7b, microRNA-181a, microRNA-328, microRNA-335, and microRNA-503 were considered as protectomiRs as these

microRNAs were downregulated by I/R compared with time-matched nonischemic controls and their downregulation was inhibited by ischemic pre- and/or postconditioning. Further mimics of microRNA-188, microRNA-532-3p, microRNA-212, microRNA-192, microRNA-139-5p, microRNA let-7e, microRNA let-7i, and microRNA-1 were considered as protectomiRs as these microRNAs were not affected by I/R

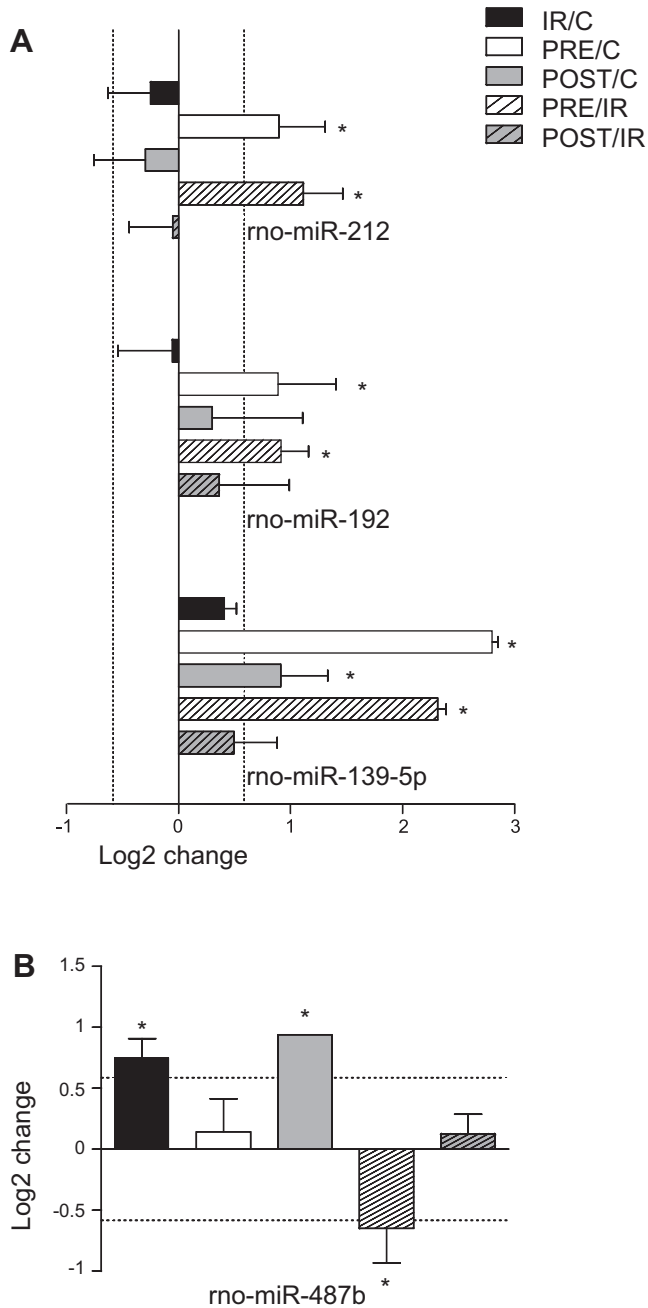


Fig. 3. MicroRNAs associated with cardioprotection by ischemic preconditioning. **A**: microRNAs that were significantly induced by preconditioning compared with both I/R and time-matched nonischemic controls, demonstrating that these microRNAs are specifically induced by preconditioning. **B**: microRNA-487b, which was significantly upregulated by I/R compared with time-matched nonischemic controls; however, preconditioning significantly inhibited its expression change due to I/R compared with nonischemic controls. Bars show alterations in microRNA expression in I/R versus time-matched nonischemic controls, postconditioning versus time-matched nonischemic controls, preconditioning versus I/R, and postconditioning versus I/R. Values are log<sub>2</sub> expression changes ± SD. \**P* < 0.05 and a log<sub>2</sub> change of greater than or equal to ±0.585.

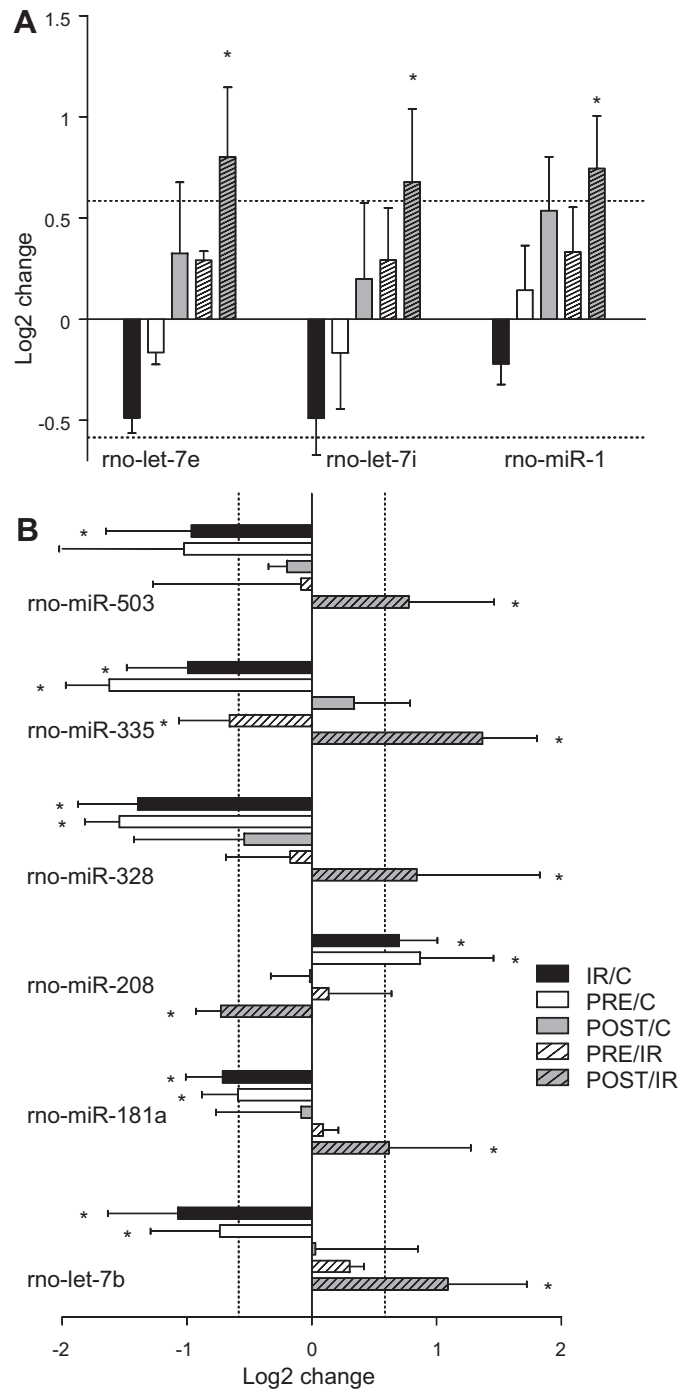


Fig. 4. MicroRNAs associated with cardioprotection by ischemic postconditioning. **A**: microRNAs that were significantly induced by postconditioning compared with I/R, demonstrating that these microRNAs are specifically induced by postconditioning. **B**: microRNAs that were significantly affected by I/R compared with time-matched nonischemic controls; however, their alterations were significantly inhibited postconditioning. Bars show alterations in microRNA expression in I/R versus time-matched nonischemic controls, postconditioning versus time-matched nonischemic controls, preconditioning versus I/R, and postconditioning versus I/R. Values are log<sub>2</sub> expression changes ± SD. \**P* < 0.05 and a log<sub>2</sub> change of greater than or equal to ±0.585.

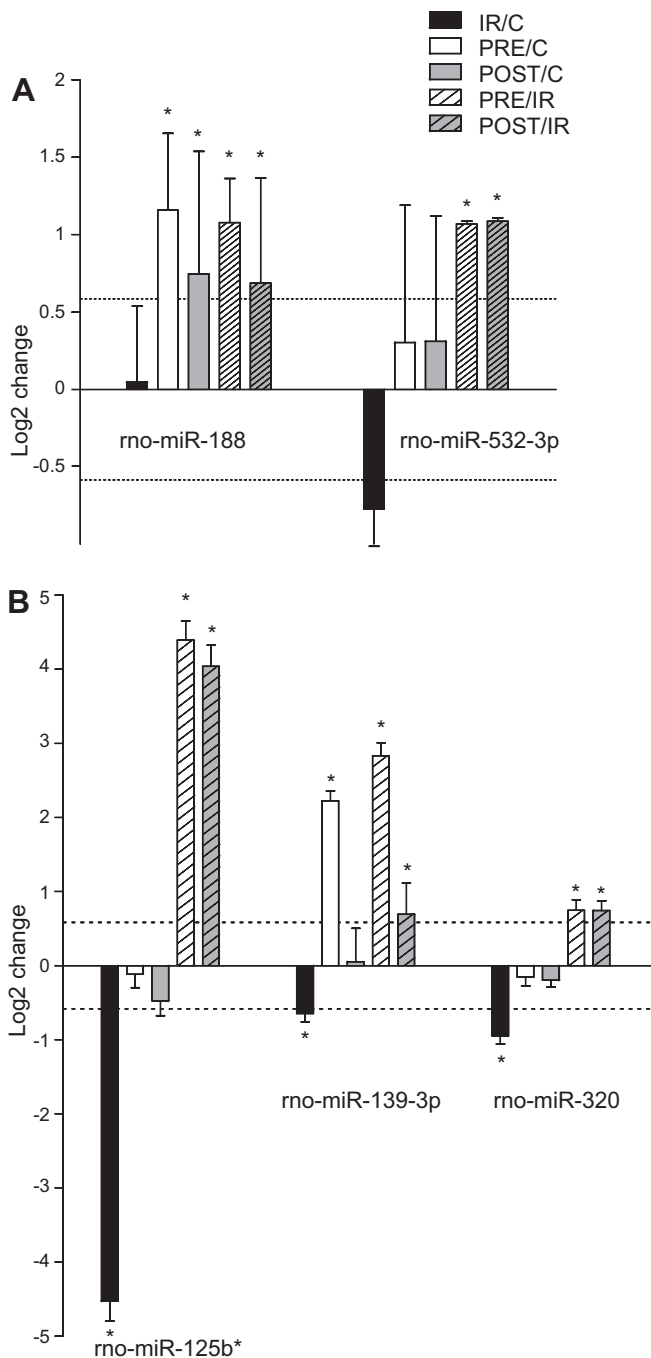


Fig. 5. MicroRNAs associated with cardioprotection by both preconditioning and postconditioning. *A*: microRNA changes induced by pre- and postconditioning stimuli per se. *B*: microRNAs that were significantly changed by I/R compared with time-matched nonischemic controls; however, both preconditioning and postconditioning significantly inhibited their expression changes due to I/R. This shows that these microRNAs are associated with cardioprotection by both pre- and postconditioning via a reversal of the deleterious mechanisms of I/R. Bars show alterations in microRNA expression in I/R versus time-matched nonischemic controls, preconditioning versus time-matched nonischemic controls, postconditioning versus time-matched nonischemic controls, preconditioning versus I/R, and postconditioning versus I/R. Values are log<sub>2</sub> expression changes  $\pm$  SD. \**P* < 0.05 and a log<sub>2</sub> change of greater than or equal to  $\pm 0.585$ .

compared with time-matched nonischemic controls; however, they were upregulated by ischemic pre- and/or postconditioning compared with I/R. AntagomiRs of microRNA-487b and microRNA-208 were considered as protectomiRs as these microRNAs were upregulated by I/R compared with time-matched nonischemic controls and their upregulation was inhibited by ischemic pre- and/or postconditioning.

#### Validation of Cardiocytoprotection by ProtectomiRs in Cardiomyocytes Subjected to Simulated I/R

Based on the microRNA microarray results, several microRNAs were identified to be associated with cardioprotection by pre- and/or postconditioning. To further validate the possible causative relationship between the affected microRNAs and cytoprotection, a postconditioning-associated mimic protectomiR (rno-let7b) and a preconditioning-associated mimic protectomiR (rno-miR-139-5p) as well as an inhibitor protectomiR (rno-miR-487b) and a mimic protectomiR associated with both pre- and postconditioning-induced cardioprotection (rno-miR-125b\*) were selected for further study in cardiac myocytes subjected to simulated I/R. Transfection of cardiomyocytes with any of the four selected protectomiRs significantly decreased cardiomyocyte cell death due to simulated I/R (Fig. 7).

#### DISCUSSION

In the present study, we have shown using a microarray containing all 350 rat microRNAs available in Sanger miRBase (version 10.1) that in the early phase of I/R injury (i.e., at 2 h of reperfusion) the microRNA expression pattern is altered in the rat heart, which is significantly influenced by preceding preconditioning and/or subsequent postconditioning stimuli. Moreover, by a systematic comparison of microRNA expression changes and the direction of the changes induced by I/R with or without pre- or postconditioning, we revealed possible causative relationships between microRNA changes and endogenous cardioprotection. This is the first demonstration that ischemic pre- and postconditioning significantly affect the I/R-induced global microRNA expression pattern. Furthermore, we validated the cardiocytoprotective effect of four of the potential cardioprotective microRNAs (i.e., the mimic of microRNA-125b\*, microRNA let-7b, microRNA-139-5p, and inhibitor of microRNA-487b) in cardiomyocytes subjected to simulated I/R in vitro. Therefore, we identified specific cardioprotective microRNAs as potential therapeutic targets, the modulation of which with specific microRNA mimics or antagomiRs may provide cardioprotection. These microRNA mimics or antagomiRs are commonly termed here as protectomiRs.

#### MicroRNAs Associated With I/R Injury

Some studies have previously reported alterations of global microRNA expression after myocardial infarction in different animal models; nevertheless, the majority of these studies focused on late stages of infarction (days after coronary artery ligation), thereby showing the possible involvement of microRNAs in cardiac remodeling and heart failure (32, 34, 39). However, there is limited information regarding microRNA alterations in the first few hours of I/R injury, when irreversible processes of cell injury are already triggered (11, 31, 41).

Table 2. Validation of the microarray data with quantitative real-time PCR

microRNA Name	Chip Result	Quantitative Real-Time PCR Result	Confirmed
Ischemia-reperfusion group versus time-matched nonischemic control group			
rno-let-7c	-0.92 ± 0.38	-1.25 ± 0.20	Yes
rno-let-7d	-0.66 ± 0.24	-1.65 ± 1.02	Yes
rno-miR-92a	-0.82 ± 0.25	0.69 ± 0.04	No
rno-miR-19a	0.60 ± 0.11	1.14 ± 0.72	Yes
rno-miR-19b	0.70 ± 0.25	1.33 ± 0.60	Yes
rno-miR-208	0.70 ± 0.30	1.30 ± 0.53	Yes
rno-miR-30c	0.32 ± 0.14	-0.07 ± 0.54	Yes
rno-miR-335	-0.98 ± 0.49	-1.91 ± 0.83	Yes
rno-miR-125b*	-4.53 ± 0.26	-5.02 ± 0.29	Yes
Ischemic preconditioning group versus ischemia-reperfusion group			
rno-miR-320	0.74 ± 0.13	1.75 ± 0.93	Yes
rno-miR-335	-0.65 ± 0.4	0.19 ± 0.32	No
rno-miR-125b*	4.39 ± 0.25	4.66 ± 0.36	Yes
Ischemic postconditioning group versus ischemia-reperfusion group			
rno-miR-1	0.74 ± 0.26	1.93 ± 0.47	Yes
rno-miR-335	1.36 ± 0.43	1.93 ± 0.75	Yes
rno-miR-125b*	4.03 ± 0.28	0.92 ± 0.29	Yes

Values are means ± SD of log<sub>2</sub> expression changes.

In the present study, we considered microRNAs to be associated with acute I/R injury (i.e., 2 h of reperfusion) when an alteration in the expression of a specific microRNA was significantly changed by I/R compared with time-matched nonischemic controls but when neither preceding preconditioning nor subsequent postconditioning influenced the I/R-induced change of the particular microRNA. This shows that these microRNAs were associated with I/R but unaffected by endogenous cardioprotective mechanisms. Such an expression pattern was characteristic in the case of eight different microRNAs (microRNA-125a-5p, microRNA-322\*, microRNA-331, microRNA-378\*, microRNA-494, microRNA-652, microRNA-877, and microRNA-92a). Accordingly, microRNA-92a, microRNA-494, and microRNA-378\* have been previously described to be affected by ischemia in different tissues of rodents (2, 26, 41). Nevertheless, to date, little is known on the role of these microRNAs in acute I/R injury.

#### MicroRNAs Associated With Ischemic Preconditioning

Our present study is the first in the literature focusing on the global microRNA expression pattern in the early phase of I/R injury preceded by ischemic preconditioning. Here, we found three different microRNAs (microRNA-139-5p, microRNA-192, and microRNA-212) that were significantly upregulated by preconditioning compared with both the I/R and time-matched nonischemic control groups, suggesting that these microRNAs are specifically induced by preconditioning stimuli. Furthermore, we found a single microRNA (i.e., microRNA-487b) that was significantly upregulated by I/R; however, preconditioning inhibited its upregulation, thereby demonstrating a specific counterregulation of this microRNA in I/R by preceding preconditioning. By this approach, we revealed a possible causative relationship between these microRNAs and the cardioprotective effect of preconditioning. To further validate the possible causative relationships, we selected the mimick of microRNA-139-5p and inhibitor of microRNA-487b as potential preconditioning-related protectomiRs for transfection into primary neonatal cardiomyocytes subjected to simulated I/R and found that both the mimick of microRNA-139-5p and inhibitor of microRNA-487b were cardiocytoprotective. These results con-

firmed a causative relationship between microRNAs identified by the above-mentioned approach and cardioprotection.

Among the microRNAs affected by ischemic preconditioning in the present study are several ones with unknown function. For instance, the function of microRNA-139 has been demonstrated only in mouse hepatocytes so far (21), i.e., microRNA-139 decreased the amount of FoxO1 protein, a key factor in the regulation of cardiomyocyte autophagy (33). Autophagy, on the other hand, has been shown to be involved in ischemic preconditioning-induced cardioprotection (25).

In a late preconditioning model, Yin et al. (44) investigated the involvement of microRNA-1, microRNA-21, and microRNA-24 in mice; however, we could not demonstrate a role of these microRNAs in our classic preconditioning model. Wang et al. (42) detected significant microRNA-144 and microRNA-451 upregulation immediately after preconditioning stimuli. Moreover, they showed that infarct size in microRNA-144/451 knockout mice is higher and cannot be preconditioned (42). In our present data set, microRNA-451 also showed some upregulation due to preconditioning compared with I/R; however, the level of upregulation did not reach statistical significance. These discrepancies could be due to differences in species and in the time of tissue sampling after ischemia.

#### MicroRNAs Associated With Ischemic Postconditioning

The present study is the first in the literature focusing on global microRNA expression changes in the early phase of ischemic postconditioning-induced protection. Here, we identified nine different microRNAs (microRNA-1, microRNA let-7i, microRNA let-7e, microRNA let-7b, microRNA-181a, microRNA-208, microRNA-328, microRNA-335, and microRNA-503) that were significantly affected by subsequent postconditioning compared with I/R, showing that these microRNAs are specifically regulated by the postconditioning stimuli. We found six different microRNAs (microRNA let-7b, microRNA-181a, microRNA-208, microRNA-328, microRNA-335, and microRNA-503) that were significantly altered (up- or downregulated) by I/R compared with time-matched nonischemic controls; however, postconditioning sig-



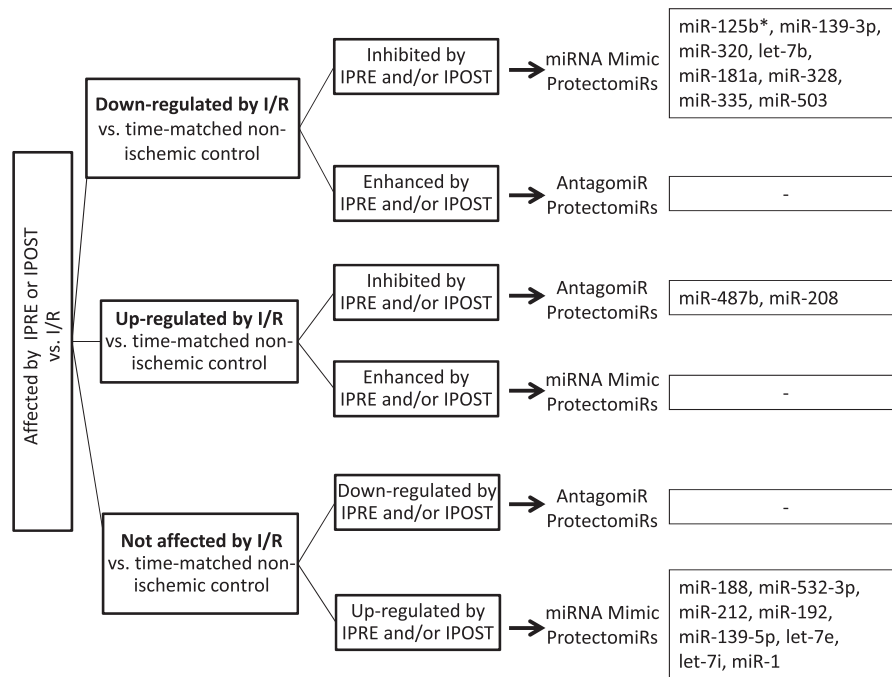


Fig. 6. Selection process of cardiac protectomiRs. MicroRNAs that were significantly affected by ischemic preconditioning (IPRE) and/or ischemic postconditioning (IPOST) compared with I/R alone were identified first. These microRNAs were then divided into three groups according to the effect of I/R compared with time-matched nonischemic controls and further subdivided into two additional categories according to the direction of microRNA expression changes, thereby yielding the following six categories. *Category 1* comprised microRNAs that were downregulated by I/R compared with time-matched nonischemic controls, but their down-regulation was inhibited by ischemic preconditioning and/or postconditioning. They were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or postconditioning; therefore, these microRNAs or their mimics are considered as protectomiRs. *Category 2* comprised microRNAs that were downregulated by I/R compared with time-matched nonischemic controls, but their down-regulation was further enhanced by ischemic preconditioning and/or ischemic postconditioning. They were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or postconditioning; therefore, the antagomiRs of these microRNAs can be also considered as protectomiRs. However, no example of this microRNA group was found. *Category 3* comprised microRNAs that were upregulated by I/R compared with time-matched nonischemic controls, but their upregulation was inhibited by ischemic preconditioning and/or postconditioning. They were considered to be involved in the development of I/R injury; therefore, the antagomiRs of these microRNAs can be also considered as protectomiRs. *Category 4* comprised microRNAs that were upregulated by I/R compared with time-matched nonischemic controls, but their upregulation was further enhanced by ischemic preconditioning and/or postconditioning. They were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or postconditioning; therefore, these microRNAs or their mimics are considered as protectomiRs. *Category 5* comprised microRNAs that were not affected by I/R alone compared with time-matched nonischemic controls but were significantly upregulated by ischemic preconditioning and/or postconditioning. They were considered to be associated with the cardioprotective mechanism of ischemic preconditioning and/or postconditioning; therefore, these microRNAs or their mimics are considered as protectomiRs. *Category 6* comprised a group of microRNAs not affected by I/R alone compared with time-matched nonischemic controls, but their expression was downregulated by ischemic preconditioning and/or postconditioning. Inhibition of these microRNAs was considered to be involved in the cardioprotective mechanism of ischemic preconditioning and/or postconditioning; therefore, the antagomiRs of these microRNAs can be also considered as protectomiRs.

nificantly inhibited these alterations. These findings demonstrate that I/R-induced expression changes of these six different microRNAs are specifically counterregulated by subsequent postconditioning stimuli. In addition, we found three different microRNAs (microRNA-1, microRNA let-7i, and microRNA let-7e) that were not affected by I/R compared with nonischemic controls; however, these microRNAs were up-regulated due to postconditioning compared with I/R. By this approach, we revealed a possible causative relationship between these microRNAs and the cardioprotective effect of postconditioning. To further validate this, we selected microRNA let-7b as a potential postconditioning-related protectomiR for transfection into primary neonatal cardiomyocytes subjected to simulated I/R and found that microRNA let-7b was cardiocytoprotective. This result confirmed a causative relationship between microRNAs identified by the above-mentioned approach and cardioprotection.

The exact role of most of the postconditioning-related protectomiRs in cardioprotection is unclear, but some of these

microRNAs have been previously studied in the heart. He et al. (24) showed that microRNA-1 and microRNA-133a were involved in the cardioprotective effect of ischemic postconditioning in the rat and found that these microRNAs were capable of decreasing cardiomyocyte apoptosis in vitro. Here, we found a similar upregulation of microRNA-1 after subsequent postconditioning stimuli. This microRNA has been shown to be involved in the regulation of apoptotic signaling by modulating the phosphatase and tensin homolog/Akt pathway (20), which is known to be a major participant of I/R injury and one of the intracellular mediator of cardioprotective signaling (18, 23). In the present study, microRNA-208 was significantly upregulated by I/R compared with nonischemic controls, which was inhibited by subsequent postconditioning stimuli. A similar upregulation of myocardial microRNA-208 has been previously described by Bostjancic et al. (3, 4) after myocardial infarction in humans. This microRNA is encoded in an intron of the  $\alpha$ -myosin heavy chain 6 gene, and it is believed to be cardiac specific (38). MicroRNA-208 mediates

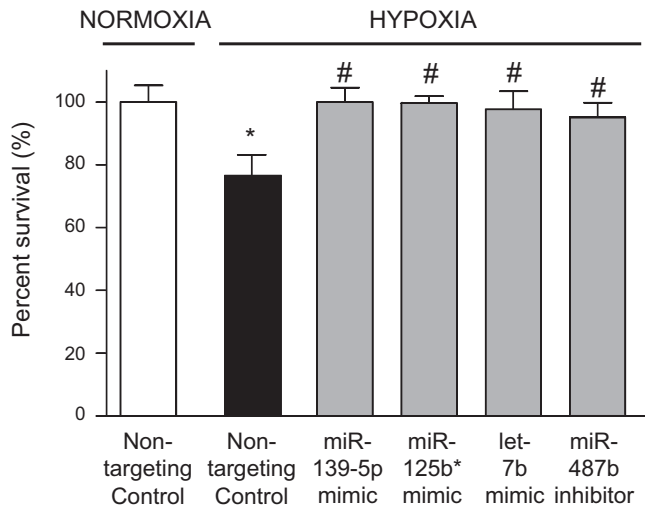


Fig. 7. Cardiocytoprotection by selected protectomiRs. Transfection of primary cardiomyocytes with the postconditioning-associated protectomiR mimic (rno-let7b), a preconditioning-associated protectomiR mimic (rno-miR-139-5p) and inhibitor (rno-miR-487b), and a protectomiR mimic associated with both pre- and postconditioning-induced cardioprotection (rno-miR-125b\*) showed that these protectomiRs were cardiocytoprotective. Values are means  $\pm$  SE. \* $P < 0.05$  vs. normoxic control; # $P < 0.05$  vs. hypoxic control.

downregulation of the  $\alpha$ -myosin heavy chain gene and upregulation of the  $\beta$ -myosin heavy chain (37), which are both involved in the development of hypertrophic response (43). In addition, some other targets of microRNA-208 have been determined by Callis et al. (7), including myostatin, a negative regulator of muscle growth and hypertrophy. These results, including those in the present study, suggest that an antagonomiR of microRNA-208 could be a promising protectomiR, having additional therapeutic value in cardiac hypertrophy. The 15 different protectomiRs identified in the present study to be involved in the cardioprotective effect of postconditioning represent a therapeutic target group against reperfusion injury.

#### MicroRNAs Commonly Associated With Cardioprotection by Both Pre- and Postconditioning

Our present study is the first to identify microRNAs commonly associated with cardioprotection induced by both pre- and postconditioning. These five different microRNAs (microRNA-188, microRNA-532-3p, microRNA-125b\*, microRNA-139-3p, and microRNA-320) represent especially important therapeutic targets for ischemia- as well as reperfusion-induced pathologies. In case of microRNA-188 and microRNA-532-3p, both pre- and postconditioning significantly induced upregulation compared with I/R; however, I/R alone did not affect the expression of these microRNAs. This is the first demonstration that microRNA-188 and microRNA-532-3p are specifically associated with both pre- and postconditioning stimuli. However, there are no data in the literature on the mRNA targets of microRNA-188 and microRNA-532-3p in the heart. In the case of microRNA-125b\*, microRNA-139-3p, and microRNA-320, this is the first demonstration that both preceding preconditioning and subsequent postconditioning stimuli resulted in a significant reversal of I/R-induced microRNA expression changes, suggesting the prominent role of these microRNAs in cardioprotective signaling. However, there are no data on the role of microRNA-139-3p, whereas

there are limited data on microRNA-320 and microRNA-125b\* in cardiac pathologies (6, 31). Ren et al. (31) showed that microRNA-320 was significantly downregulated in an ex vivo mouse model of 45 min of global ischemia and 2 h of reperfusion as well as in an in vivo mouse model of 30 min of coronary occlusion and 24 h of reperfusion. In the present study, we detected a similar downregulation of microRNA-320 after acute I/R. However, we have also shown here that both preconditioning and postconditioning inhibited the I/R-induced downregulation of microRNA-320, indicating that this microRNA plays an important role in cardioprotection. MicroRNA-125b\* showed the most significant changes in the present study, i.e., more than 20-fold down-regulation after I/R, while both preceding preconditioning and subsequent postconditioning stimuli markedly inhibited its downregulation. The exact role of microRNA-125b in cardioprotective cellular signaling remains to be investigated. Nevertheless, it should be mentioned that Busk et al. (6) reported a significant upregulation of microRNA-125b during the early hypertrophic growth in rat hearts after aortic banding.

According to the above-described approach, we revealed a causative relationship between microRNA-188, microRNA-532-3p, microRNA-125b\*, microRNA-139-3p, and microRNA-320 and the cardioprotective effect of both pre- and postconditioning. To further validate these causative relationships, we selected microRNA-125b\* as a potential pre- and postconditioning-related protectomiR for transfection into primary neonatal cardiomyocytes subjected to simulated I/R and found that microRNA-125b\* was cardiocytoprotective. This result further confirmed a causative relationship between microRNAs identified by the above-mentioned approach and cardioprotection.

#### ProtectomiRs

In the present study, we report a novel approach to identify therapeutically applicable microRNAs with cardioprotective features. The analysis of the direction of microRNA changes due to pre- and/or postconditioning allowed the identification of mimics or antagonomiRs of specific microRNAs, which were termed here as protectomiRs. Interestingly, all of the identified microRNA belonged to three major categories of the theoretically possible six categories, as described in METHODS. Moreover, the majority (18 of 20) of the identified protectomiRs were microRNA mimics, suggesting that the loss of specific microRNAs in the ischemic myocardium may play a role in I/R injury.

#### Limitations of the Study

In the present study, global microRNA expression was investigated in a single time point upon reperfusion; therefore, the time course of changes in microRNA expressions remained unknown. Thirty minutes of coronary occlusion followed by 120 min of reperfusion, when microRNA expression was measured, is considered an early phase of cell injury due to necrotic and/or apoptotic processes (28). However, it cannot be excluded that early cell death processes might affect microRNA alterations induced by I/R injury and cytoprotection by pre- and postconditioning.

Although we identified several protectomiRs in the present study, investigation of the exact role of each protectomiR in cardioprotective signaling was outside of the scope of this

study. Since the present project was performed in rats, determination of the translational value of the results needs further study. Moreover, it has to be noted that whereas the efficacy of preconditioning in humans is well established, the efficacy of postconditioning in humans is largely inconclusive as there are several negative data, probably due to different postconditioning protocols and the presence of multiple confounding factors (for extensive reviews, see Refs. 17, 22, and 30).

### Conclusions

This is the first comprehensive study on the global microRNA expression pattern of the heart in response to I/R with or without preceding preconditioning or subsequent postconditioning using a microarray of 350 different microRNAs. Here, we show that I/R induces acute alterations in the expression of several microRNAs of known and yet unknown function in the rat heart and that many of these alterations are significantly influenced by preceding preconditioning and/or subsequent postconditioning stimuli. Moreover, by a systematic comparative analysis of global microRNA expression patterns in time-matched nonischemic control, I/R, ischemic preconditioning, and ischemic postconditioning groups, microRNAs associated with endogenous cardioprotection can be identified.

These microRNAs can serve as potential targets for modulation by specific microRNA mimics or antagomiRs (termed protectomiRs) to achieve cardioprotection.

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### DISCLOSURES

Based partially on the present findings, Pharmahungary Group submitted a patent application in 2011.

### AUTHOR CONTRIBUTIONS

Author contributions: Z.V.V., A.G., C.C., T.C., and P.F. conception and design of research; Z.V.V., A.Z., N.F., G.F.K., M.P., R.G., and P.B. performed experiments; Z.V.V., A.Z., N.F., M.P., C.C., L.G.P., and T.C. analyzed data; Z.V.V., A.G., C.C., L.G.P., T.T., and T.C. interpreted results of experiments; Z.V.V. prepared figures; Z.V.V. drafted manuscript; Z.V.V., A.Z., N.F., G.F.K., M.P., A.G., C.C., L.G.P., T.T., T.C., and P.F. approved final version of manuscript; T.T., T.C., and P.F. edited and revised manuscript.

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