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MicroRNA-145 repairs infarcted myocardium by accelerating cardiomyocyte autophagy

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Highly conserved RNAs of around 22 nucleotides in length that negatively regulate messenger RNA (mRNA) expression by inhibiting translation or directly cleaving plural target mRNAs. Recent evidence has indicated that miRNAs play a fundamental role in a wide variety of biological processes, such as proliferation, cell death, and differentiation (1). Although more than 3,000 human miRNAs have been identified (miRBase, http://www.mirbase.org/), only a part of them have been characterized in detail (2). Recent knowledge regarding the roles of miRNAs in vascular remodeling is opening novel avenues for the treatment of cardiovascular diseases (21). Among most miRNAs, miRNA-145 (miR-145) has been found to be the most abundant miRNA in the vascular wall (11). It has also been reported that miR-145 inhibits the proliferation of vascular smooth muscle and progression of atherosclerosis (3). Furthermore, miR-145 has been reported to protect cardiomyocytes against hydrogen peroxide-induced apoptosis (13). However, it is still unclear whether miR-145 protects against ischemia-reperfusion injury. Therefore, the purpose of the present study was to investigate whether postinfarct treatment with miR-145 could reduce the size of a myocardial infarct and improve the left ventricle (LV) function and remodeling. We also investigated the mechanism by which miR-145 protects the heart by performing in vitro and in vivo experiments using a rabbit model of myocardial infarction (MI) and cultured rat cardiomyocytes.

METHODS

miR-145

The sequence of mature miR-145 used in this study was 5'–GUCAGUUUUCCAGAAUCCCU from Ambion (Life Technologies), which is conserved among human, rabbit, and rat miR-145s (miRBase, http://www.mirbase.org/). We used hsa-miR-145 from Ambion in all experiments in this study.

In Vivo Study

Animals. Male Japanese white rabbits weighing ~2.0–2.5 kg were used in this study. All rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication 85-23, revised 1996). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Surgical procedure. The surgical procedure was performed according to a method previously reported (16). Briefly, rabbits were anesthetized with an intravenous injection of pentobarbital sodium (30–40 mg/kg), and additional doses were given when required.
throughout the experiment. Once anesthetized, the rabbits were intubated and ventilated with room air supplemented with a low flow of oxygen using a mechanical ventilator (tidal volume of 25–35 ml, respiratory rate of 20–30 breaths/min; Shimano, model SN-480-5, Tokyo, Japan). Serial blood-gas analysis was performed, and ventilatory conditions were adjusted to maintain the arterial blood gas within the physiological range. Surgery was performed under sterile conditions. The carotid artery and jugular vein were cannulated to monitor the physiological range. Surgery was performed under sterile conditions.

Assessment of cardiac tissue levels of miR-145. To assess the cardiac tissue levels of miR-145, we subjected rabbits (n = 6) to 30 min of coronary occlusion, followed by 2 days of reperfusion with the intravenous administration of 2.5 nmol/kg of control miRNA or miR-145 encapsulated by liposomes immediately after the start of reperfusion. The rabbits were then euthanized under deep anesthesia at 2 days after reperfusion, and their hearts were removed. From the myocardial tissues, the level of miR-145 expression relative to that of RNU6B was obtained. Total RNA was isolated from tissues using a NucleoSpin miRNA isolation kit (TaKaRa, Otsu, Japan). RNA concentrations and purity were assessed by UV spectrophotometry. RNA integrity was checked by formaldehyde gel electrophoresis. To determine the expression levels of miR-145, we conducted quantitative RT-PCR using TaqMan MicroRNA Assays (Applied Biosystems) for hsa-miR-145 that can also detect mature rabbit miR-145 and THUNDER Probe qPCR Mix (TOYOBO, Osaka, Japan), according to the manufacturer’s protocol. The relative expression levels of miR-145 were calculated using the ∆∆Ct method. The plasma levels of encapsulated miR-145 were also examined using the same method as above after the collection of encapsulated miR-145 with an ExoMir kit (BioScientific, Austin, TX). The relative miR-145 levels were determined through normalization by miR-21, which is also conserved between humans and rabbits.

Protocol. As shown in Fig. 1, rabbits were subjected to 30 min of coronary occlusion, followed by 2 days or 2 wk of reperfusion. The control group (n = 10) was intravenously injected with 2.5 nmol/kg of control miRNA encapsulated within liposomes. In the miR-145 group, 2.5 nmol/kg of miR-145 encapsulated by liposomes (miR-145 group, n = 10) was intravenously injected immediately after the start of reperfusion. The rabbits were then killed at 2 days or 2 wk thereafter. Some of the rabbits treated with miR-145 were intramuscularly administered 10 mg/kg of chloroquine (Sigma; miR-145 + chloroquine group, n = 10) immediately after the start of reperfusion, beginning after 30 min of coronary occlusion. Chloroquine is a cell-permeable lysosomal inhibitor that acts by inhibiting vacuolar H⁺-ATPase and autophagosomal fusion to prevent the final digestion of apoptotic cells.

Table 1. Mortality rates of all rabbits in the control, miR-145, and miR-145 + chloroquine groups reperfused for 2 days or 2 wk after myocardial infarction

<table>
<thead>
<tr>
<th></th>
<th>No of Rabbits</th>
<th>No. of Successes</th>
<th>No of Dead Animals</th>
<th>Mortality</th>
</tr>
</thead>
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<tr>
<td>2 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
<td>17</td>
<td>6</td>
<td>26.0</td>
</tr>
<tr>
<td>miR-145</td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>23.0</td>
</tr>
<tr>
<td>miR-145 + chloroquine</td>
<td>16</td>
<td>12</td>
<td>4</td>
<td>25.0</td>
</tr>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>27</td>
<td>22</td>
<td>5</td>
<td>18.5</td>
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<tr>
<td>miR-145</td>
<td>14</td>
<td>11</td>
<td>3</td>
<td>21.4</td>
</tr>
<tr>
<td>miR-145 + chloroquine</td>
<td>19</td>
<td>15</td>
<td>4</td>
<td>21.0</td>
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</table>

miR-145, microRNA-145.
step during autophagy (10). In this study, we included a liposome-only group, although our laboratory recently reported that there is no difference in the effect on the myocardial infarct size, cardiac function or remodeling, or signal transduction between saline and liposome injection (23).

**Physiological analysis.** Echocardiography (SSD2000, Aloka) was performed before and 2 wk after MI. The ejection fraction, fractional shortening, and LV end-diastolic and end-systolic dimensions were obtained. The arterial blood pressure and heart rate were also measured via a catheter introduced into the carotid artery. After echocardiography, a micro-manometer-tipped catheter (SPR 407, Millar Instruments) was inserted into the LV to record positive and negative change in pressure over time (±dP/dt). All measurements were made by two persons blinded to the treatment.

**Determination of myocardial infarct size.** To measure the risk area, we mounted excised hearts on a Langendorff apparatus and then injected Evans blue dye (4°C) into the aorta for 1 min after reoclusion of the coronary branch. The LV was then weighed and sectioned in paraffin, and cut into 4-μm-thick sections with a microtome. These sections were stained with Masson’s trichrome. It is generally accepted that the Evans blue dye/TTC method is not reliable to evaluate the infarct size after 72 h of reperfusion because of remodeling due to scar shrinkage within the infarct. Therefore, we further assessed the infarct size histologically in samples obtained from rabbits with 2-wk reperfusion after MI. For transversely sliced preparations with infarction stained with Masson’s trichrome, the LV wall and infarcted and noninfarcted areas were calculated using an image analyzer connected to a light microscope (LUZEX-F, NIRECO, Tokyo, Japan), and the MI size was expressed as a percentage of the LV area at a papillary muscle level. The comparisons were made by two persons blinded to the treatment.

**In Vitro Study**

**Cell transfection with miR-145, anti-miR-145, or siR-FRS2.** H9c2 rat myoblasts for transfection with miR-145 were seeded in wells of six-well plates at a concentration of 5 × 10^4 cells/well on the day

### Table 2. Hemodynamic parameters before and during ischemia and after the start of reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Before Ischemia</th>
<th>10 min After Ischemia</th>
<th>20 min After Ischemia</th>
<th>30 min After Ischemia</th>
<th>10 min After Reperfusion</th>
<th>20 min After Reperfusion</th>
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<tr>
<td><strong>Systolic blood pressure, mmHg</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>103.9 ± 2.8</td>
<td>72.8 ± 4.4</td>
<td>75.5 ± 2.2</td>
<td>76.5 ± 2.1</td>
<td>73.7 ± 1.6</td>
<td>77.7 ± 2.7</td>
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<td>miR-145</td>
<td>98.7 ± 2.8</td>
<td>65.6 ± 1.2</td>
<td>79.0 ± 6.3</td>
<td>76.36 ± 6.4</td>
<td>73.8 ± 3.4</td>
<td>76.5 ± 4.0</td>
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<tr>
<td>miR-145 + chloroquine</td>
<td>92.3 ± 6.2</td>
<td>83.0 ± 7.0</td>
<td>90.0 ± 2.7</td>
<td>82.5 ± 4.0</td>
<td>73.8 ± 4.5</td>
<td>78.8 ± 4.3</td>
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<td><strong>Diastolic blood pressure, mmHg</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>84.1 ± 2.0</td>
<td>54.0 ± 4.7</td>
<td>56.8 ± 1.3</td>
<td>58.6 ± 1.3</td>
<td>54.5 ± 1.6</td>
<td>58.1 ± 2.6</td>
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<tr>
<td>miR-145</td>
<td>81.5 ± 2.4</td>
<td>46.7 ± 1.6</td>
<td>64.1 ± 6.3</td>
<td>60.0 ± 6.8</td>
<td>58.8 ± 4.0</td>
<td>60.4 ± 3.9</td>
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<tr>
<td>miR-145 + chloroquine</td>
<td>74.2 ± 6.1</td>
<td>61.6 ± 8.7</td>
<td>69.9 ± 3.5</td>
<td>63.4 ± 4.8</td>
<td>60.9 ± 5.2</td>
<td>59.4 ± 4.7</td>
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<tr>
<td><strong>Heart rate, beats/min</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>330 ± 7.5</td>
<td>264 ± 8.7</td>
<td>265 ± 9.9</td>
<td>264 ± 11.2</td>
<td>267 ± 13.5</td>
<td>253 ± 8.6</td>
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<tr>
<td>miR-145</td>
<td>346 ± 7.5</td>
<td>268 ± 6.5</td>
<td>260.6 ± 4.8</td>
<td>264.6 ± 9.6</td>
<td>256 ± 9.4</td>
<td>249 ± 7.4</td>
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<tr>
<td>miR-145 + chloroquine</td>
<td>362 ± 4.3</td>
<td>286 ± 12.3</td>
<td>284 ± 12.3</td>
<td>279 ± 2.9</td>
<td>264 ± 4.6</td>
<td>263 ± 4.0</td>
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Values are means ± SE.

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before transfection. The mature type of hsa-miR-145 (mirVana miRNA mimic; Ambion, Foster City, CA), anti-miR-145 (mirVana miRNA inhibitor; Ambion), or small interference RNA (siR) for rat fibroblast growth factor receptor substrate 2 (FRS2) (siR-FRS2; 5'-CAGCGCAGAGAGCUGACUAUU; NM_001108097; 1460–1484) was used for transfection of the cells, which was achieved using cationic liposomes, Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer’s lipofection protocol. The nonspecific control miRNA (HSS, Hokkaido, Japan) sequence was 5'-GUAGGUAGUGAAAGGCC-3', which was used as a control for nonspecific effects.

**Light microscopic observation.** H9c2 rat myoblasts were seeded at a concentration of 5 × 10⁴ cells/ml in wells of six-well (30-mm) dishes. The cells were examined morphologically by phase-contrast light microscopy (Olympus, Tokyo, Japan) at 2 days after the transfection with miR-145 or anti-miR-145.

**Cell viability assay.** The number of viable cells was determined by performing the trypan blue dye exclusion test. An autophagy inhibitor,
3-MA (Calbiochem, San Diego, CA) was used at the concentration of 100 μM to discriminate between autophagic cell survival and cell death. In the experiment, the cells were incubated with DMSO as a control.

**Assay for FRS2 overexpression.** The FRS2 expression vector was generated by inserting the open reading frame of rat FRS2 cDNA into the pIREShneo vector (Clontech). H9c2 cells were seeded in six-well plates at a concentration of 0.5 × 10^5/well and transfected at 0.4 μg/well with the control vector or pIREShneo-FRS2 expression vector using Lipofectamine 2000 (Invitrogen). Then, miR-145 transfection was performed at 23 h after the transfection. The effects manifested by FRS2 overexpression were assayed at 72 h after the transfection with plasmids.

**Western Blot Analysis**

*In vivo study.* Western blot analysis was carried out on lysates from heart tissues at 2 days, 2 wk, and 4 wk after MI. Proteins were separated and transferred to membranes using standard protocols. The phosphorylation of Akt and extracellular signal-regulated protein kinase (ERK) was assessed using antibodies against Akt, phosphorylated-(p)-Akt, ERK, and p-ERK (all from Cell Signaling). Expressions of LC3B I and II, and p62/SQSTM1 were assessed with antibodies against FRS2 (R&D Systems: AF4069) and Kruppel-like factor 4 (Klf4) (Cell Signaling: no. 4038) were used for the identification of target genes of miR-145 (miRBase, http://www.mirbase.org/), because both antibodies also react with rat and rabbit FRS2 and Klf4. The blots were visualized chemiluminescence (ECL, Amer- pharm). The signals were quantified by densitometry.

*In vitro study.* Whole cells were lysed with chilled lysis buffer comprising 10 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and total protein was extracted. Protein contents were measured with a DC protein assay kit (Bio-Rad, Hercules, CA). Lysates were then separated by SDS-PAGE using 7.5–15% polyacrylamide gels, elec-troblotted onto a polyvinylidene difluoride membrane (PerkinElmer Life Sciences, Boston, MA), and reacted with first antibodies specific for LC3B (Cell Signaling Technology, Danvers, MA), Akt (Cell Signaling Technology), phosphor-Akt (Ser473, p-Akt; Cell Signaling Technology), ERK-1/2 (Cell Signaling Technology), phosphor-ERK-1/2 (Cell Signaling Technology), and FRS2 (R&D Systems). The second antibodies used were horseradish peroxidase-conjugated horse anti-mouse or goat anti-rabbit IgG antibody (Cell Signaling Technology). The signals were visualized with the use of Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). The loading control was prepared by reincubating the same membrane with anti-β-actin antibody (Sigma-Aldrich). The signals were quantified by densitometry.

**Luciferase assay.** We constructed the sensor vector by joining the regions with a possible binding site from the 3′-UTR of rat FRS2 to a luciferase reporter pMIR-control vector (Applied Biosystems) to examine the target sequence recognized by miR-145. The seed sequence of the binding site in FRS2-3′-UTR (NM_001108097; 4216–4222) that recognizes miR-145 is highly conserved among species. The approximate 270-bp fragment, including a possible binding site for miR-145, was inserted into the 3′-UTR region of the sensor vector. To generate sensor vectors with mutations in the binding site for miR-145, we mutated seed regions from CUG to GCC (PrimeSTAR Mutagenesis Basal Kit; TaKaRa). The cells were seeded in 96-well plates at a concentration of 0.5 × 10^5/well the day before the transfection. The sensor vector (concentration: 0.1 μg/ml) and 20 nmol/l pre-miR-145 or nonspecific control miRNA (Dharmacon, Tokyo, Japan) were used for cotransfection of the cells using cationic liposomes of Lipofectamine RNAiMAX. Forty-eight hours after the cotransfection, luciferase activities were measured using a Dual-Glo

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**Fig. 4. MI size on day 2 post-MI.** A: the data for area at risk as a percentage of the LV were 27.2 ± 1.4% (control), 27.9 ± 1.4% (miR-145), and 30.0 ± 2.2% (miR-145 + chloroquine). On day 2 post-MI, there was no significant difference in the area at risk as a percentage of the LV among the three groups. B: the data for MI size as a percentage of the area at risk were 41.5 ± 1.8% (control), 29.0 ± 2.6% (miR-145), and 40.6 ± 3.4% (miR-145 + chloroquine). The MI size as a percentage of the area at risk was significantly smaller in the miR-145 group than in the control or miR-145 + chloroquine group. miR-145 significantly reduced the MI size compared with the size for the control group. The infarct size-reducing effect of miR-145 was reversed by chloroquine. Values are means ± SE. Values of P < 0.05 were considered significant. *P < 0.05 vs. the control and miR-145 + chloroquine groups. C: typical photomicrographs of triphenyl tetrazolium chloride (TTC)- and Evans blue dye-stained cross sections of the LV are shown. We determined the white region as the infarct area, red and white region as the area at risk, and blue region as the nonischemic area.
Luciferase Assay System (Promega, Madison, WI), according to the manufacturer’s protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

**Electron Microscopic Observation**

**In vivo study.** Rabbits underwent 30 min of coronary occlusion followed by 2 days or 2 wk of reperfusion. Control miRNA (2.5 nmol/kg; control group, \( n = 6 \)) or 2.5 nmol/kg of miR-145 encapsulated by liposomes (miR-145 group, \( n = 6 \)) was intravenously injected immediately after the start of reperfusion. The rabbits were euthanized under deep anesthesia, and their hearts were excised at 2 days (\( n = 3 \) in each group) or 2 wk (\( n = 3 \) in each group) after MI. The cardiac tissue was quickly cut into 1-mm cubes, immersion-fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (PB) (pH 7.4) overnight at 4°C, and postfixed in 1% buffered osmium tetroxide. The specimens were then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (90 nm), double-stained with uranyl acetate and lead citrate, were examined under an electron microscope (H-800, Hitachi, Tokyo, Japan). The EM analysis was directed at the border zone of the MI.

**In vitro study.** H9c2 cells that were or were not treated with miR-145 were harvested and rinsed with PBS. The cells were fixed for 2 h with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M PB (pH 7.4), rinsed in PB, and postfixed in 2% osmium tetroxide for 2 h. After having been washed with PB, the cells were progressively dehydrated in a 10% graded series of 30–100% ethanol and then cleared in QY-1 (Nissin EM, Tokyo, Japan). Thereafter, they were embedded in Epon 812 resin (TAAB Laboratories Equipment, Reading, UK), after which thin sections (70-nm thickness) were prepared, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy with a Hitachi-7650 (Hitachi, Tokyo, Japan), operating at 80 kV.

**Statistical Analysis**

All values are presented as means ± SE. Differences among the saline and miR-145 groups were assessed by ANOVA combined with
Fisher’s method (Stat View, J5.0 software). Values of $P < 0.05$ were considered significant.

RESULTS

In Vivo Study

Mortality rate after MI. The mortality rates of all rabbits in the control, miR-145, and miR-145 + chloroquine groups reperfused for 2 days or 2 wk after MI are shown in Table 1. There was no difference in mortality rates among these groups.

Profiles of plasma and cardiac tissue miR-145 levels and protein expression levels of FRS2 and Klf4 as predicted target genes of miR-145 after reperfusion. The half-life of miR-145 in the plasma was from 5 to 30 min after its intravenous injection (Fig. 2A). In the control group, the expression level of miR-145 was significantly decreased in the border and infarct areas of the myocardium on day 2 post-MI (Fig. 2B). However, on day 2 post-MI, this decrease was significantly attenuated by treatment with miR-145 (Fig. 2B). We examined the protein expression levels of FRS2 and Klf4 as predicted targets of miR-145. As a result, there was a decrease in the expression levels of both FRS2 and Klf4 in the miR-145 group at 2 days of reperfusion (Fig. 2C), whereas the control group showed an increased expression of FRS2. Thus these results reflected the expression profiles of miR-145 levels (Fig. 2B) in the samples from the border and infarcted areas (Fig. 2C).

Physiological findings. There was no significant difference in the heart rate or systolic and diastolic blood pressures among the three groups during the experiment in the 2-day reperfusion model (Table 2). At 2 wk after MI, there was no significant difference in the heart rate or systolic and diastolic blood pressures (Fig. 3A). The LV end-systolic and end-diastolic dimensions were significantly smaller (Fig. 3B), whereas the cardiac function, such as the LV ejection fraction, fractional shortening, and ±dP/dt, was significantly more favorable in the miR-145 group than in the control group (Fig. 3, C and D). However, treatment with chloroquine reversed these changes in the LV end-systolic and end-diastolic dimensions and LV ejection fraction, fractional shortening, and ±dP/dt caused by miR-145 (Fig. 3, B–D).

MI size and histological analysis. On day 2 post-MI, there was no significant difference in the area at risk as a percentage of the LV among the three groups (Fig. 4A). The MI size as a percentage of the area at risk was significantly smaller in the miR-145 group (29.0 ± 2.6%) than that in the control group (41.5 ± 1.8%; Fig. 4B). Treatment with chloroquine completely abolished the MI size-reducing effect of miR-145 (40.6 ± 3.4%; Fig. 4B). Typical results of the TTC staining and Evans blue dye staining of cross sections of the LV are shown in Fig. 4C.

At 2 wk post-MI, there was no significant difference in the area at risk as a percentage of the LV between the three groups (Fig. 5A). The MI size as a percentage of the area at risk was

Fig. 6. Ultrastructural findings of autophagy in cardiomyocytes at 2 days and 2 wk post-MI. Typical autophagosomes (arrows) were seen in the ischemic area of the myocardium after MI. Treatment with miR-145 accelerated the autophagy of cardiomyocytes, and increased numbers of autophagosomes were seen in the ischemic area of the myocardium after MI. Bar = 1 μm.
significantly smaller in the miR-145 group (20.4 ± 2.5%) than in the control group (31.2 ± 3.0%), and treatment with chloroquine completely abolished the MI size-reducing effect of miR-145 (31.9 ± 2.4%; Fig. 5B). At 2 wk post-MI, histological assessment of the MI size as a percentage of the LV assessed by Masson’s trichrome staining was also significantly smaller in the miR-145 group (12.7 ± 1.7%) than in the control group (20.8 ± 1.6%). Again, treatment with chloroquine completely abolished the infarct size-reducing effect of miR-145 (22.5 ± 3.6%; Fig. 5C). Typical photomicrographs of TTC-, Evans blue dye-, and Masson trichrome-stained cross sections of the LV are shown in Fig. 5, D and E.

Electron microscopic findings. Electron microscopy revealed the characteristic findings of autophagy, such as autophagosomes, including mitochondria, in the cardiomyocytes in both the control and miR-145 groups at 2 days and 2 wk post-MI (Fig. 6).

Western blot analysis. The transition of LC3 I to LC3 II (ratio of LC3II to LC3I) was significantly enhanced in samples from the miR-145 group compared with those from the control group, both at 2 days and 2 wk after MI (Fig. 7, A and B), but not at 4 wk after MI (Fig. 7C). The expression of p62/SQSTM1 was downregulated in the miR-145 group compared with that in the control group at 2 days or 2 wk after MI (Fig. 8, A and B), but not at 4 wk after MI (Fig. 8C). Treatment with chloroquine abolished the downregulation of expression of p62/SQSTM1 by miR-145 (Fig. 8A).

The level of p-Akt was significantly higher in hearts from the miR-145 group than in those from the control group at 2 days after MI (Fig. 9A), but not at 2 wk after MI (Fig. 9B). There was no significant difference in the expression of Akt, ERK, or p-ERK between the two groups at 2 days or 2 wk post-MI (Fig. 9, A and B).

**In Vitro Study**

**Effect of miR-145 on H9c2 rat cardiomyocytes.** The ectopic expression of miR-145 in H9c2 cells induced growth inhibition and loop formation (Fig. 10A). A part of the treated cells included vacuoles in their cytoplasm. Such morphological

![Fig. 7. Western blot analysis of LC3B I and LC3B II in the myocardium at 2 days, 2 wk, and 4 wk post-MI. A: the transition of LC3 I to LC3 II was significantly enhanced in samples from the miR-145 group compared with those from the control group at 2 days after MI. B: the transition of LC3 I to LC3 II was significantly enhanced in samples from the miR-145 group compared with those from the control group both at 2 wk after MI. C: There was no significant difference in the transition of LC3 I to LC3 II in samples between the control and miR-145 groups. Values are means ± SE. Values of *P* < 0.05 were considered significant. *P* < 0.05 vs. the control group.](http://ajpheart.physiology.org/)
changes partly disappeared on cotransfection with anti-miR-145. Electron microscopy indicated the characteristic findings of autophagy, which consisted of autophagosomes, including mitochondria, in the cells after transfection with miR-145 at 2 days after the transfection (Fig. 10B). To discriminate autophagic survival from cell death, we performed transfection with miR-145 in the presence of 3-MA. As a result, the viable cell number was slightly decreased in 3-MA-pretreated miR-145-transfectants compared with that in miR-145-transfectant alone (Fig. 10B). Biochemically, the LC3B I to II transition was clearly demonstrated by Western blot analysis (Fig. 11A).

In addition, the p-Akt but not p-ERK level was significantly enhanced (Fig. 11A), which indicated the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. On the other hand, the transition of LC3 I to II induced by miR-145 was reversed by cotransfection with anti-miR-145 (Fig. 11B). Thus the ectopic expression of miR-145 promoted autophagic cell survival in rat myoblasts.

FRS2 is a target mRNA of miR-145. Based on the results of the in vivo study and bioinformatic analysis (miRBase, http://www.mirbase.org/) of miR-145, we further examined the expression of a couple of candidate target proteins, such as Klf4 and FRS2, by Western blot analysis. As a result, the expression level of FRS2 was significantly decreased after the transfection of H9c2 cells with miR-145 (Fig. 11A). To confirm whether miR-145 directly targeted rat FRS2 (position 4201–4223 of...
FRS2 3’-UTR), we performed a luciferase activity assay for FRS2. Expectedly, compared with that of the control, the luciferase activity of the wild-type pMIR-FRS2 was significantly decreased after the introduction of miR-145 into H9c2 cells (Fig. 11C). Mutations of the FRS2 3’-UTR-binding site (seed sequence) significantly abolished the ability of miR-145 to regulate luciferase expression. These results demonstrated that miR-145 directly targeted FRS2. Next, we performed the silencing of FRS2 with the use of siR-FRS2, which resulted in the transition of LC3B I to LC3B II and dose-dependent growth suppression (Fig. 12 A and B). To further validate the target gene FRS2 of miR-145 in H9c2 cells, we conducted the cotransfection of the FRS2 expression vector with miR-145. Figure 12C clearly indicates that FRS2 overexpression significantly prevented the autophagy induced by miR-145. Thus these findings together indicate that one of the major target mRNAs of miR-145 in rat cardiomyoblasts is FRS2, promoting autophagic survival through the downregulation of FRS2.

**DISCUSSION**

The present study demonstrated that 1) in a rabbit model of MI, post-MI treatment with miR-145 significantly reduced the MI size, improved the LV function and remodeling, which were reversed by chloroquine, accelerated cardiomyocyte autophagy, accelerated the transition of LC3B I to LC3B II, and enhanced the phosphorylation of Akt in the myocardium; 2) and in the in vitro study using H9c2 rat cardiomyoblasts, miR-145 induced autophagy through targeting FRS2, accelerated the transition of LC3B I to LC3B II, and enhanced the phosphorylation of Akt.

**Behavior of Cardiac Tissue miR-145 Levels after MI**

In the present study, 30 min of coronary occlusion followed by 2 days of reperfusion caused a significant decrease in the cardiac tissue expression of miR-145 in the border and infarct areas of the myocardium compared with that in the remote noninfarct area of the myocardium (Fig. 2B). This result is consistent with a previous report demonstrating that the expression level of miR-145 in the myocardium is significantly decreased in mice with 50-min myocardial ischemia and 1- or 3-h reperfusion (13). However, the decrease in the miR-145 expression level in the border and infarct areas was significantly attenuated by treatment with
miR-145 at 2 days after MI (Fig. 2). This finding suggests that miR-145 in the border and infarct areas may have contributed to the pathogenesis of MI. In other words, cardiac tissue miR-145 in the border and infarct areas may have been lost during MI, probably for the repair of infarcted myocardium, and the replacement of miR-145 by the intravenous administration of liposomes encapsulating miR-145 after MI may have repaired the infarcted myocardium, reduced the MI size, and improved the LV function and remodeling (Figs. 3–5).

Fig. 10. A: morphological changes in H9c2 cells in response to miR-145 treatment. The ectopic expression of miR-145 in H9c2 cells inhibited the growth and induced loop formation. These morphological changes were abolished in part by anti-miR-145. B: ultrastructural findings of autophagy in H9c2 cells 2 days after miR-145 treatment. Electron microscopy indicated the characteristic findings of autophagy and autophagosomes. Arrow, autolysosome. C: the transfection of H9c2 rat cardiomyocytes with miR-145 in the presence of 3-MA (an autophagy inhibitor) was performed to discriminate autophagic survival from cell death. The viable cell number was slightly decreased in 3-MA-pretreated miR-145-transfectants compared with that in miR-145-transfectant alone. Values are means ± SE. Values of P < 0.05 were considered significant. *P < 0.05.

Fig. 11. A: Western blot analysis of LC3B I, LC3B II, FRS2, Akt, p-Akt, ERK, and p-ERK in H9c2 cells 2 days after miR-145 transfection. B: Western blot analysis of the transition of LC3B I to LC3B II by miR-145 in the presence of miR-145 inhibitor, anti-miR-145. C: luciferase assay. The activity was markedly decreased after the cotransfection with miR-145 and sensor vector with the wild-type region, including a possible binding site (rat FRS2: 4201–4223; top) in H9c2 cells, but the decrease in the activity was significantly inhibited by that with mutant vector. The effects were estimated at 48 h after the transfection. Values are means ± SE. Values of P < 0.05 were considered significant. *P < 0.05. Cont, control.
MI Size, LV Function, and Remodeling

In the present study, we found that the MI size at 2 days and 2 wk post-MI was significantly smaller in the miR-145 group than in the control group. This finding suggests that the infarct size-reducing effect of miR-145 after MI was elicited acutely within 2 days and lasted during the first 2 wk after MI. The window for myocardial salvage with reperfusion injury is usually within a few minutes, as was shown in the case of ischemic or pharmacological postconditioning (18). We believe that the effect of miR-145 on the myocardial infarct size was somewhat different from that of ischemic or pharmacological postconditioning. Since it has been reported that the myocardial infarct size becomes irreversible within 4 or 6 h after the onset of a MI (8, 19), the infarct size-reducing effect of miR-145 that targets FRS2 might have been due to the induction of autophagy during several hours after the start of reperfusion.

In a rabbit model of MI with 30-min coronary occlusion and 2 days of reperfusion, there was no significant difference in the heart rate, and systolic or diastolic blood pressure before or during ischemia or during reperfusion among the three groups (Table 2). The reduction in the MI size by miR-145 was completely abolished by treatment with chloroquine, an inhibitor of autophagy, suggesting that the MI size-reducing effect of miR-145 was caused by the acceleration of autophagy (Figs. 4 and 5). Concerning the LV remodeling and function, both LV systolic and diastolic dimensions were smaller in the miR-145 group than in the control group at 2 wk post-MI, suggesting that miR-145 improved LV remodeling. At 2 wk post-MI, +dP/dt, an indicator of the LV systolic function, and −dP/dt, an indicator of the LV diastolic function, were significantly greater in the miR-145 group than in the control group. The ejection fraction and fractional shortening were significantly greater in the miR-145 group than in the control group (Fig. 3). These data suggest that the treatment with miR-145 improved the LV function. The improvements in the ±dP/dt and LV ejection fraction and fractional shortening by miR-145 were abolished by treatment with chloroquine, suggesting that improvement of the LV function by miR-145 was caused by the acceleration of autophagy (Fig. 3). These effects may be at least in part associated with the reduction in the MI size caused by miR-145.

Cardiomyocyte Autophagy

In our in vivo study, MI by itself induced cardiomyocyte autophagic change, and treatment with miR-145 led to the accelerated induction of this cardiomyocyte autophagic change, as assessed by electron microscopy (Fig. 6). Western blot analysis demonstrated that the transition of LC3B I to LC3B II in the myocardium, a biochemical marker of autophagy, was significantly more enhanced in the miR-145 group than in the control group at both 2 days and 2 wk after MI (Fig. 7), suggesting that miR-145 enhanced cardiomyocyte autophagy. Furthermore, the expression of p62/SQSTM1, which binds directly to LC3, a marker of autophagic influx, was decreased at 2 days and 2 wk after MI but not at 4 wk after MI in the miR-145 group. The present results suggest that the miR-145-induced cardiomyocyte autophagy lasted for up 2 wk but not for 4 wk.

It has been reported that MI induces cardiomyocyte autophagy, and this autophagy, by itself, limits acute MI induced by permanent coronary occlusion (12). Autophagy is required for the maintenance of cardiomyocyte homeostasis (16a). However, abnormal autophagy could lead to the development of heart failure (17, 22). Our results are consistent with this report, although the MI model in the present study was not a permanent coronary occlusion but an ischemia-reperfusion model. Even though it has been established that one of the most powerful cardioprotective interventions is ischemic preconditioning, it has been reported that autophagy induced by ischemic preconditioning is essential for cardioprotection, and that autophagy is regarded as an end-effector in ischemic and pharmacological preconditioning (9). In the present study, we showed that miR-145 downregulated p62, and that chloroquine reversed its effect at 2 days of reperfusion. Therefore, autophagic flux may have been stimulated during a short period...
of time after the onset of infarction up to 2 days after reperfusion.

In our in vitro study, electron microscopy revealed that there were numerous cardiomyocytes containing autophagic vacuoles, autolysosomes, and autophagosomes, suggesting the autophagic morphology of cardiomyocytes in H9c2 cells 48 h after transfection with miR-145 (Fig. 10B). These autophagic changes were abolished by treatment with anti-miR-145, suggesting that miR-145 indeed induced cardiomyocyte autophagy. Western blot analysis demonstrated that miR-145 treatment induced the transition of LC3B I to LC3B II, suggesting that miR-145 induced an increase in the accumulation of autophagosomes (Fig. 10B). Furthermore, we determined FRS2 to be one of the major target genes associated with cardiomyoblast autophagy both in vivo (Fig. 2C) and in vitro (Fig. 12). FRS2 is an adaptor protein that plays a critical role in fibroblast growth factor receptor signaling (6, 28). FRS2 in fibroblast growth factor receptor signaling pathways induces downstream activation of the RAS-MAPK pathway. Therefore, FRS2 has been reported to be oncogenic and amplified in high-grade liposarcoma and serous ovarian cancers (14, 26).

The inhibition of FRS2-mediated signals increases autophagy, and the activation of autophagy promotes myocardial differentiation (24, 25). Furthermore, the cell growth-suppressing effect of miR-145, as shown in our in vitro study, may have led to improvements of the cardiac structure and function after MI, which may be explained by the induction of cardiomyocyte autophagy. As mentioned previously, it has been reported that autophagy of cardiomyocytes after MI leads to a reduction in the size of a myocardial infarct (12), and this reduction may be explained by the improvement of energy efficiency of the cardiac tissues afforded by autophagy of cardiomyocytes and the prevention of further cardiomyocyte death (12).

Generally, a reduction in MI size may be explained by a decrease in cardiomyocyte death, hypertrophy of cardiomyocytes, or the regeneration of cardiomyocytes after ischemia and reperfusion. There was no difference in the transverse myocyte size in the border zone area of infarction between the miR-145 and control groups (data not shown). We demonstrated that postinfarct treatment with miR-145 accelerated the autophagy of cardiomyocytes in the border zone areas as a cardioprotective adaptation, suggesting that surviving cardiomyocytes would have contributed to the increased area of viable tissue, to the reduction in MI size, and to the improvement of LV remodeling and function.

**Signal Transduction**

In our in vivo study, we observed significant phosphorylation of Akt but not of ERK-1/2 in the ischemic myocardium on day 2 post-MI in the miR-145 group. The activation of Akt has been reported to play a role in cardiac ischemic preconditioning, thereby reducing the MI size (7). The ischemic postconditioning effect is also a cardioprotective mechanism (27), which is mediated by the PI3K-Akt pathway (20), thereby protecting the heart against ischemia and reperfusion injury. In our in vitro study, the ectopic expression of miR-145 in H9c2 cells also activated Akt phosphorylation, but not that of ERK-1/2. Therefore, in the present study, the miR-145-PI3K/Akt signaling axis may also have contributed to the cardioprotective effects. Thus it is likely that miR-145 exerted its cardio-protective effects through an increase in autophagy and activation of the Akt signaling axis. On the other hand, some previous studies revealed that Akt inhibited autophagy (5, 15). However, this variation may have been due to differences in the cells, tissues, organs, and species studied.

**Study Limitation**

The present study demonstrated that miR-145 accelerated the autophagy of cardiomyocytes, enhanced the phosphorylation of Akt, reduced the MI size, and improved the cardiac function and remodeling. However, the mechanism by which miR-145 induced the autophagy of cardiomyocytes through targeting FRS2 and simultaneously activating PI3K/Akt survival signaling remains to be elucidated.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: K.H., Y.Y., Shingo Minatoguchi, S.B., M.I., H.K., G.T., M. Kumazaki, Y.A., and Shinya Minatoguchi performed experiments; K.H. and Shinya Minatoguchi prepared figures; K.H. and Shinya Minatoguchi drafted manuscript; K.H. and Shinya Minatoguchi edited and revised manuscript; K.H., Y.Y., Shingo Minatoguchi, S.B., M.I., H.K., M. Kawasaki, G.T., M. Kumazaki, Y.A., and Shinya Minatoguchi approved final version of manuscript; Shinya Minatoguchi conception and design of research.

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