

## Basic Research

# MicroRNA-208a Increases Myocardial Endoglin Expression and Myocardial Fibrosis in Acute Myocardial Infarction

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*See editorial by Nattel, pages 591-592 of this issue.*

### ABSTRACT

**Background:** MicroRNAs (miRs) play a role in cardiac remodelling, and acute myocardial infarction (AMI) can regulate miR expression. MiR-208a is essential for the expression of the genes involved in cardiac hypertrophy and fibrosis. MiR-208a activates endoglin expression and may result in cardiac fibrosis. The role of miR-208a and endoglin in AMI is not known. We sought to investigate the regulation of miR-208a and endoglin in AMI.

**Methods:** Ligation of the proximal left anterior descending artery was performed in adult Sprague-Dawley rats to induce AMI. Echocardiography was used to measure heart size and left ventricular function. The TaqMan miR real-time quantitative assay was used to quantitate miR-208a. Myocardial fibrosis was detected by Masson trichrome staining.

**Results:** AMI and overexpression of miR-208a in the sham group without infarction significantly increased myocardial miR-208a, endoglin, and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) expression. Overexpression of

### RÉSUMÉ

**Introduction :** Les microARN (miARN) jouent un rôle dans le remodelage cardiaque, puis l'infarctus du myocarde aigu (IMA) peut réguler l'expression des miARN. Les miARN-208a (miR-208a) sont essentiels à l'expression des gènes impliqués dans l'hypertrophie et la fibrose du cœur. Les miR-208a activent l'expression de l'endogline et peuvent entraîner la fibrose cardiaque. On ne connaît pas le rôle des miR-208a et de l'endogline dans l'IMA. Nous avons pour objet d'examiner la régulation des miR-208a et de l'endogline dans l'IMA.

**Méthodes :** La ligature de la partie proximale de l'artère interventriculaire antérieure était réalisée chez des rats adultes Sprague-Dawley pour provoquer l'IMA. L'échocardiographie était utilisée pour mesurer la taille du cœur et la fonction du ventricule gauche. Le dosage quantitatif en temps réel des miARN en sonde TaqMan était utilisé pour quantifier les miR-208a. La fibrose myocardique était détectée par coloration au trichrome de Masson.

Acute myocardial infarction (AMI) caused by coronary artery occlusion is a major cause of morbidity and mortality in humans. The loss of blood flow to the myocardium results in cardiomyocyte death and myocardial remodelling. This remodelling after AMI can induce myocardial fibrosis and reduce left ventricular contractility and finally cause heart failure.<sup>1</sup> Reduction or prevention of myocardial fibrosis after AMI may improve cardiac function and reduce heart failure events and represents an important therapeutic strategy in management after MI.

A microRNA (miR) is a small 22-nucleotide non-protein-coding RNA that usually inhibits transcription or translation by interacting with the 3'-untranslated regions (UTRs) of target

mRNA and promoting target mRNA degradation (gene silencing).<sup>2</sup> However, Place et al.<sup>3</sup> have demonstrated that miR functioning induces gene expression, not gene silencing. Because of their capability to monitor the expression levels of the genes that control both adaptive and maladaptive cardiac remodelling processes, MiRs may be significantly involved in the pathogenesis of heart failure.<sup>4,5</sup> MiRs might be used as novel therapeutic agents or as novel biomarkers of disease, or both.

MiRs are significantly dysregulated in AMI.<sup>6</sup> Some MiRs—such as miR-21, miR-1, miR-216, and miR-29—have been reported to be dysregulated in AMI.<sup>7,8</sup> Recently, miRs have been used as a new biomarker in the early diagnosis of AMI.<sup>6,9-11</sup> However, some miRs have not been consistently found in AMI, especially miR-208a.<sup>6</sup> This miR seems to be fundamental for the expression of genes involved in cardiac fibrosis and hypertrophic growth.<sup>12,13</sup> MiR-208a is upregulated in pressure overloading with thoracic aortic banding and volume overloading with aortic-caval shunting and is activated by mechanical stress.<sup>13-16</sup>

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See page 689 for disclosure information.

antagomir-208a significantly inhibited the increase of myocardial endoglin and  $\beta$ -MHC protein expression induced by infarction. Overexpression of mutant miR-208a in the sham group did not induce myocardial endoglin and  $\beta$ -MHC expression. Pretreatment with atorvastatin and the angiotensin-receptor antagonist valsartan significantly attenuated the increase of endoglin and  $\beta$ -MHC induced by infarction. AMI and overexpression of miR-208a in the sham group significantly increased the area of myocardial fibrosis compared with the sham group. Overexpression of antagomir-208a and pretreatment with atorvastatin and valsartan in the AMI group significantly decreased the area of myocardial fibrosis induced by infarction.

**Conclusions:** MiR-208a increases endoglin expression to induce myocardial fibrosis in rats with AMI. Treatment with atorvastatin and valsartan can decrease myocardial fibrosis induced by AMI through attenuating miR-208a and endoglin expression.

Endoglin is a homeodimeric membrane glycoprotein that is a coreceptor of transforming growth factor (TGF)- $\beta$ 1 and TGF- $\beta$ 3.<sup>17</sup> Endoglin is a potent mediator of profibrotic effects of angiotensin II on cardiac fibroblasts and can modulate the effect of TGF- $\beta$ 1 on extracellular matrix synthesis.<sup>18,19</sup> These data indicate that endoglin may play an important role in fibrogenesis in cardiac remodelling. We have previously demonstrated that miR-208a can increase endoglin expression in cardiac myoblasts and in volume-overloading heart failure to modulate myocardial fibrosis.<sup>14,15</sup> The role of miR-208a and endoglin in AMI is not known. Because AMI can lead to heart failure and myocardial fibrosis, we sought to investigate the regulation of miR-208a and endoglin in AMI.

## Materials and Methods

### Rat model of acute myocardial infarction

A rat model of left anterior descending (LAD) coronary artery occlusion was used as previously described.<sup>20</sup> Sprague-Dawley rats weighing 250-300 g were used. After induction of anaesthesia with 2% isoflurane and after confirming a fully anaesthetized state (no response to toe pinching), a tracheotomy was performed, and the animal was ventilated on a Harvard rodent respirator (Harvard Apparatus, Holliston, MA). The heart was then rapidly exteriorized, and a 6-0 silk suture was tightened around the proximal LAD artery (before the first branch of the diagonal artery). Sham-operated control animals were prepared in a similar manner, except that the LAD artery was not occluded. After the ischemic insult, the wound and tracheotomy were closed so that spontaneous respiration occurred. For the AMI study, rats were randomly divided into 5 groups: (1) sham-operated, (2) sham-operated and treatment with miR-208a dominant vector, (3) sham-operated and transfection with control vector, (4) AMI and treatment with antagomir-208a dominant vector, and (5) AMI and treatment

**Résultats :** L'IMA et la surexpression des miR-208a dans le groupe recevant le traitement fictif sans infarctus a montré une augmentation significative de l'expression myocardique des miR-208a, de l'endogline et de la chaîne lourde de la  $\beta$ -myosine ( $\beta$ -MHC :  *$\beta$ -myosin heavy chain*). La surexpression de l'antagomir-208a inhibait significativement l'augmentation de l'expression myocardique de l'endogline et de la protéine  $\beta$ -MHC induite par l'infarctus. La surexpression des miR-208a mutants dans le groupe recevant le traitement fictif n'induisait pas l'expression myocardique de l'endogline et de la  $\beta$ -MHC. Le prétraitement par atorvastatine et par valsartan, un antagoniste des récepteurs de l'angiotensine, atténuait significativement l'augmentation de l'endogline et de la  $\beta$ -MHC induite par l'infarctus. L'IMA et la surexpression des miR-208a dans le groupe recevant le traitement fictif augmentaient significativement l'étendue de la fibrose myocardique comparativement au groupe recevant le traitement fictif. La surexpression de l'antagomir-208a et le prétraitement par atorvastatine et valsartan dans le groupe d'IMA diminuaient significativement l'étendue de la fibrose myocardique induite par l'infarctus.

**Conclusions :** Les miR-208a augmentent l'expression de l'endogline pour induire la fibrose myocardique chez les rats souffrant d'IMA. Le traitement par atorvastatine et valsartan peut diminuer la fibrose myocardique induite par l'IMA en atténuant l'expression des miR-208a et de l'endogline.

with control vector. Atorvastatin at 30 mg/kg, valsartan at 30 mg/kg, or *N*-acetylcysteine (NAC) at 250 mg/kg was given by oral gavage for 7 days after induction of infarction. At the end of the experiment, rats were killed by decapitation under anaesthesia with an overdose of isoflurane, and the heart was quickly removed and stored in liquid nitrogen. Left ventricular tissue was obtained for Western blot analysis and immunohistochemical staining. Serial sections (5- $\mu$ m thickness) of heart were performed with Masson's trichrome stain to delineate fibrotic tissue from viable myocardium in the peri-infarct zone. Infarct size was measured by the triphenyltetrazolium chloride method as described previously.<sup>20</sup> Computerized morphometry was used to calculate the fibrotic area and infarct size as the ratio of fibrotic area, infarct size, and total left ventricular area. All animal procedures were performed in accordance with institutional guidelines and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### Construction and delivery of miR-208a expression vector

A 71-bp rat-miR-208a precursor construct was generated as follows. Genomic DNA was amplified with forward primer CAACAGAAGTGCTTGGAAG and reverse primer GGCTGATCGACGGTAGCT. The 165-bp amplified product was digested with EcoRI and BamHI restriction enzymes and ligated into pmR-ZsGreen1 plasmid vector (coexpression miR-208a and green fluorescent protein; Clontech Laboratories, Mountain View, CA) digested with the same enzymes. The constructed plasmid (coexpression miR-208a and green fluorescent protein) was transfected into left ventricular myocardium using a low-pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan) essentially following the protocol from the manufacturer. In brief, 2 mg of plasmid DNA was suspended in 5 mL of phosphate-buffered saline (PBS) and then 100  $\mu$ L was added to the

loading hole near the nozzle. Pushing the trigger of the low-pressure gene gun released the DNA-containing solution, which was directly propelled by helium at a pressure of 15 psi into the left ventricular myocardium of the rat. The distribution of the fluorescent image in the treated rat was visualized by a dissecting fluorescence microscope with high-resolution CCD (Hamamatsu Photonics, Hamamatsu, Japan). After 3 days, the rat chest was reopened, and the fluorescent image on the left ventricular myocardium was detected. If the fluorescent image was able to be visualized, it was regarded as a successful transfection. The efficiency of using this method is about 30%. The miR-208a antagomir precursor construct was generated in pmR-ZsGreen 1 plasmid vector as described previously.<sup>16</sup> The sequence of miR-208a antagomir is ACAAGCTTTTTGCTCGTCTTAT. The sequence of mutant miR-208a (mut-208a) is ATAACAGCACGAAAAAGCTTGT.

### Hemodynamic monitor

Hemodynamic monitoring of rats was performed with polyethylene catheters to measure through a Grass model tachograph preamplifier as previously described.<sup>21</sup>

### Assessment of cardiac function

The cardiac function of rats was evaluated noninvasively by echocardiography performed with an Acuson Sequoia 512 ultrasound system (Siemens Medical Solutions, Malvern, PA) using a 15-MHz probe on the day the rats were killed, 7 and 14 days after the surgery as previously described.<sup>21</sup> The sonographer was blinded to the randomization of the rats.

### Western blot analysis

Western blot was performed as previously described.<sup>14</sup> Monoclonal rat anti-mouse endoglin antibody and polyclonal myosin heavy chain (MHC) and collagen type I antibodies (Santa Cruz Biotechnology, Paso Robles, CA) were used. Equal protein loading of the samples was verified by staining monoclonal antibody glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma, St Louis, MO). Signals were visualized by chemiluminescent detection. All Western blots were quantified using densitometry.

### Quantitative analysis of miRs

TaqMan miR real-time quantitative assays were used to quantitate miR as previously described.<sup>11</sup> All fold changes between samples were determined using the  $\Delta\Delta CT$  method.<sup>15</sup> In brief, each 15- $\mu$ L reverse transcription (RT) reaction contained 10 ng of purified total RNA, 3  $\mu$ L miR-208 RT primer (Applied Biosystems, Life Technologies, Grand Island, NY), 1  $\times$  RT buffer (Applied Biosystems), 0.25 mM each of deoxynucleotide triphosphates, 3.33 U/ $\mu$ L MultiScribe reverse transcriptase (Applied Biosystems), and 0.25 U/ $\mu$ L RNase inhibitor (Applied Biosystems). The reactions were incubated in an Applied Biosystems 9700 Thermocycler in a 96-well plate for 30 minutes at 16°C and 30 minutes at 42°C, followed by 5 minutes at 85°C, and were then held at 4°C. Each real-time polymerase chain reaction (PCR) for each miR assay (20- $\mu$ L volume) was carried out in triplicate, and each 20- $\mu$ L reaction mixture

included 1.33  $\mu$ L of RT product, 10  $\mu$ L of 2  $\times$  TaqMan Universal PCR Master Mix, 1  $\mu$ M 20  $\times$  TaqMan miR assay. The reaction was incubated in an Applied Biosystems 7300 Real-Time PCR system in a 96-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The expression levels of target MiRs were normalized to U6.

### Immunohistochemical analysis

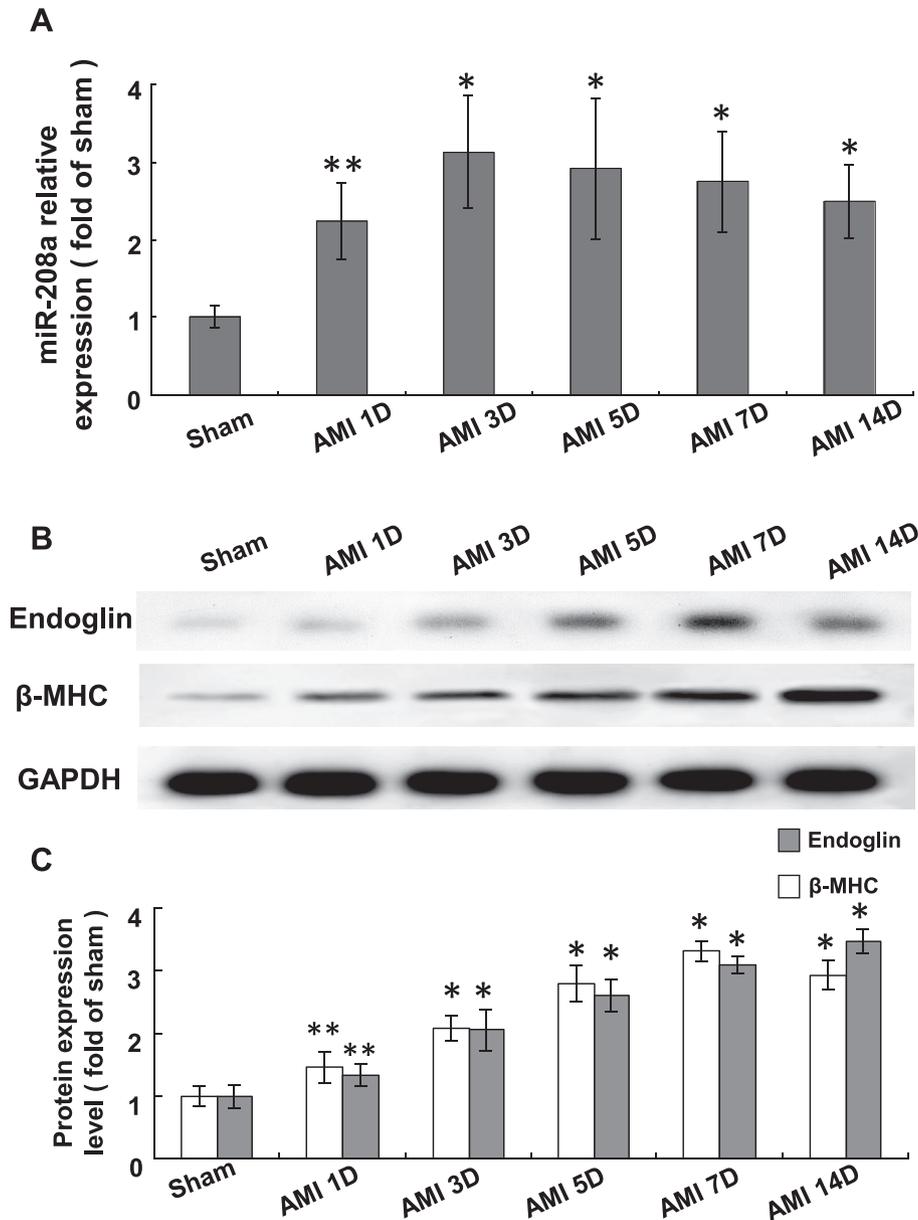
The left ventricle was harvested and fixed in 10% formaldehyde and sliced into 5- $\mu$ m paraffin sections. For immunohistochemical staining, the slides were postfixed in 4% paraformaldehyde for 20 minutes, treated in 3% hydrogen peroxide/PBS for 25 minutes, blocked in 5% normal rabbit serum for 20 minutes, blocked with biotin/avidin for 15 minutes each, and incubated with fluorescent isothiocyanate-conjugated rat monoclonal anti-endoglin antibody and polyclonal MHC antibody (Santa Cruz Biotechnology) for 2 hours at room temperature, biotinylated rabbit-anti mouse IgG at 1:400 for 30 minutes, and Vector Elite ABC biotin-avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 minutes. Sections were then developed with diaminobenzidine and diaminobenzidine enhancer (Vector Laboratories) and counterstained with hematoxylin. Images were examined with a fluorescent microscope.

### Promoter activity assay

A -700 to -1 bp rat endoglin promoter construct was generated as follows. Rat genomic DNA was amplified with forward primer 5'-CTTCAGAAAGGCCATGAAGGT-3' and 5'-AGAATGAAGGCAGGGGTCTG-3'. The amplified product was digested with MluI and BglII restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Mission Biotech, Taipei, Taiwan) digested with the same enzymes. The endoglin promoter contains miR-208a conserved sites (CCC) at -636 to -634 bp and -626 to -624 bp. For the mutant, the miR-208a binding sites were mutated using the mutagenesis kit (Stratagene/Agilent Technologies, La Jolla, CA). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into H9c2 cells (cardiac myoblast, ATCC CRL1446, passage numbers 16-21) using a low-pressure-accelerated gene gun (Bioware Technologies) essentially following the protocol from the manufacturer. In brief, 2  $\mu$ g of plasmid DNA was suspended in 5  $\mu$ L of PBS and was delivered to the cultured H9c2 cells at a helium pressure of 15 psi. The transfection efficiency using this method is 30%. After 2 hours of hypoxic (2.5% oxygen) stimulation, cell extracts were prepared using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and measured for dual luciferase activity by a luminometer (Turner Designs, Sunnyvale, CA).

### Statistical analysis

The data were expressed as mean  $\pm$  standard deviation. Statistical significance was performed with analysis of variance (GraphPad Software, San Diego, CA). The Tukey-Kramer comparison test was used for pairwise comparisons between multiple groups after analysis of variance. A value of  $P < 0.05$  was considered to denote statistical significance.



**Figure 1.** Effect of acute myocardial infarction (AMI) on miR-208a, endoglin, and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) expression in rat myocardium. **(A)** Quantitative analysis of miR-208a levels. \* $P < 0.01$  vs sham group; \*\* $P < 0.05$  vs sham group ( $n = 6$  per group). **(B)** Representative Western blot for endoglin and  $\beta$ -MHC protein expression in rat myocardium after different days of AMI. **(C)** Quantitative analysis of endoglin and  $\beta$ -MHC protein levels. The values from myocardium after AMI have been normalized to matched glyceraldehyde-3-phosphate dehydrogenase (GAPDH) measurement and then expressed as a ratio of normalized values to protein in sham group. \* $P < 0.01$  vs sham group; \*\* $P < 0.05$  vs sham group ( $n = 6$  per group). 1D, 1 day; 3D, 3 days; 5D, 5 days; 7D, 7 days; 14D, 14 days.

## Results

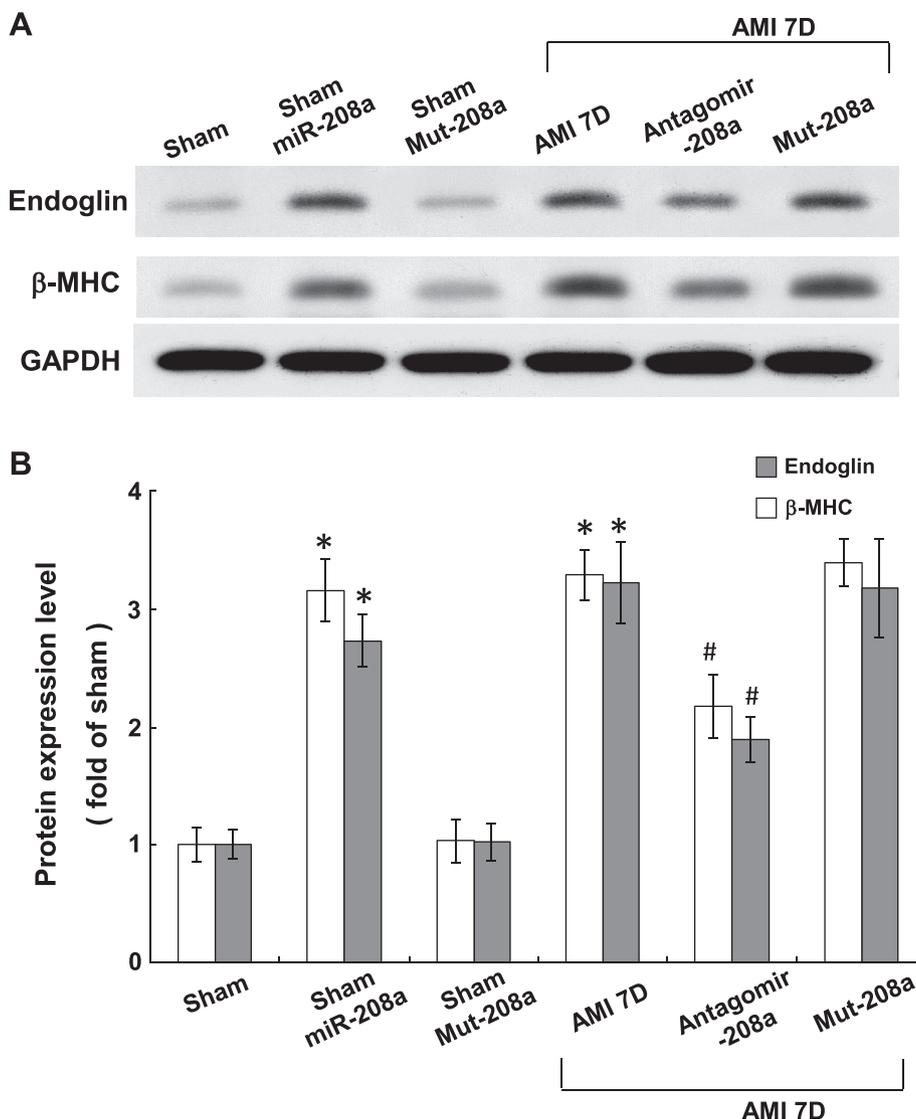
### AMI increases myocardial miR-208a and endoglin expression

As shown in Figure 1A, AMI significantly increased myocardial miR-208a expression at 1 day after infarction, reached a maximum of  $3.1 \pm 0.7$ -fold at 3 days, and remained elevated for up to 14 days after infarction. As shown in Figure 1, B and C, AMI significantly increased myocardial endoglin protein expression from 1 day up to 14 days. The cardiac hypertrophic marker  $\beta$ -MHC was also

significantly induced by AMI from 1 to 14 days as was endoglin protein.

### MiR-208a mediates myocardial endoglin expression

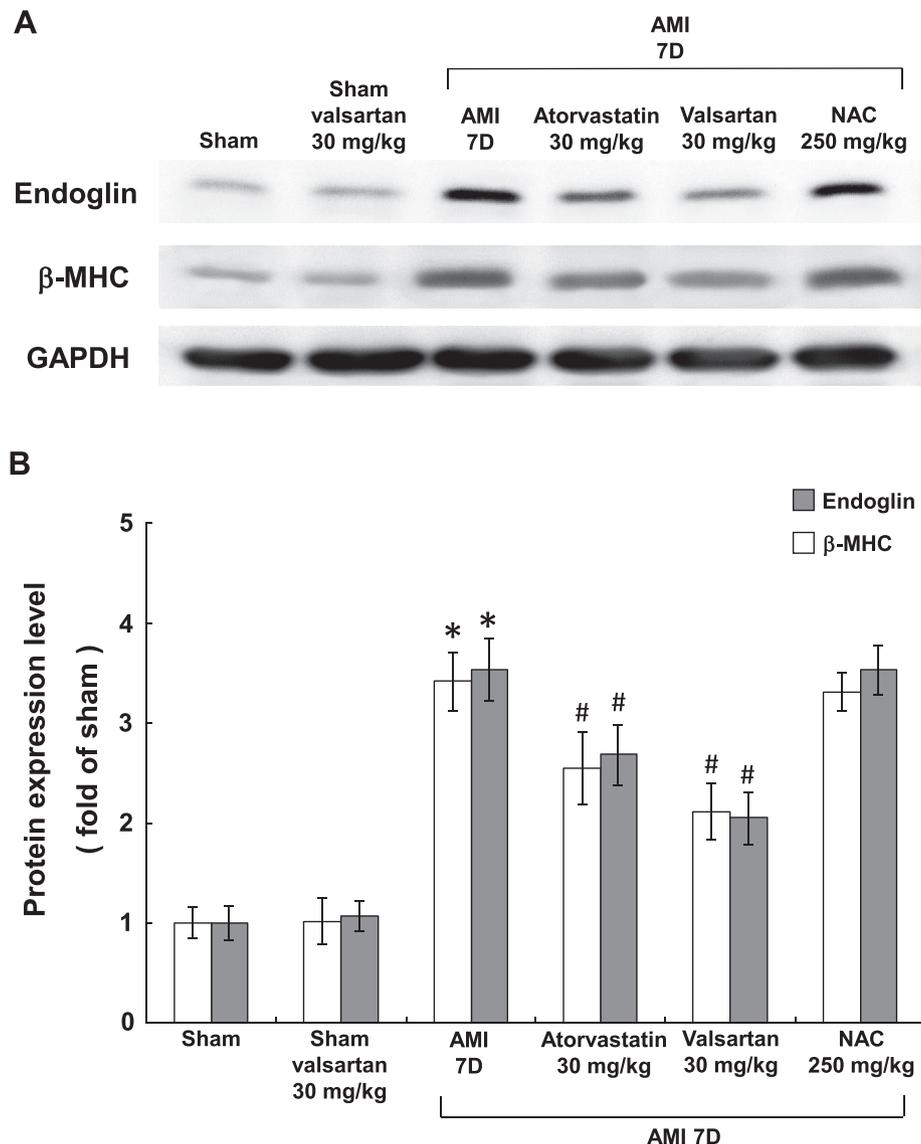
To investigate the effect of miR-208a on myocardial endoglin expression, overexpression of antagomir-208a and mut-208a in the left ventricle was induced. AMI at 7 days significantly increased myocardial endoglin and  $\beta$ -MHC protein expression, and overexpression of antagomir-208a significantly inhibited the increase of myocardial endoglin



**Figure 2.** MiR-208a mediates myocardial endoglin expression in rats with acute myocardial infarction (AMI). **(A)** Representative Western blot for endoglin and β-myosin heavy chain (β-MHC) protein expression in the rat myocardium after 7 days of myocardial infarction. MiR-208a expression vector was transfected into left ventricular myocardium by low-pressure–accelerated gene gun. **(B)** Quantitative analysis of endoglin and β-MHC protein levels. The values from myocardium after AMI have been normalized to matched glyceraldehyde-3-phosphate dehydrogenase (GAPDH) measurement and then expressed as a ratio of normalized values to protein in sham group (n = 6 per group). Overexpression of miR-208a in the sham group significantly increased endoglin and β-MHC protein expression. Overexpression of antagomir-208a significantly reduced endoglin and β-MHC protein expression induced by AMI. \*P < 0.001 vs sham group; #P < 0.001 vs AMI at 7 days (7D) (n = 6 per group).

and β-MHC protein expression induced by MI (Fig. 2). Overexpression of mut-208a did not have the effect on myocardial endoglin and β-MHC expression that was induced by MI. Overexpression of miR-208a in the sham group without MI significantly increased myocardial endoglin and β-MHC protein expression, whereas overexpression of mut-208a in the sham group did not induce myocardial endoglin and β-MHC protein expression (Fig. 2). Pretreatment with valsartan, an angiotensin-receptor antagonist, significantly attenuated the increase of myocardial endoglin and β-MHC protein expression induced by MI at days 3 (Supplemental Fig. S1) and 7 (Fig. 3). Pretreatment with atorvastatin did not significantly change the myocardial endoglin and β-MHC protein expression induced by MI at

day 3 but significantly attenuated the increase of myocardial endoglin and β-MHC protein expression induced by MI at day 7. Pretreatment with NAC, an oxidative scavenger, did not change myocardial endoglin and β-MHC protein expression induced by MI. The transfection of miR-208a into myocardium was monitored by a dissecting fluorescence microscope as shown in Supplemental Figure S2. The presence of miR-208a in the cytoplasm of cardiac myocytes was confirmed by an in situ hybridization assay (Fig. 4). Immunohistochemical staining showed increased myocardial endoglin and β-MHC expression after MI and overexpression of miR-208a in the sham group (Fig. 5). Mutant miR-208a did not change myocardial endoglin and β-MHC expression after MI. Overexpression of mut-208a decreased the signals of



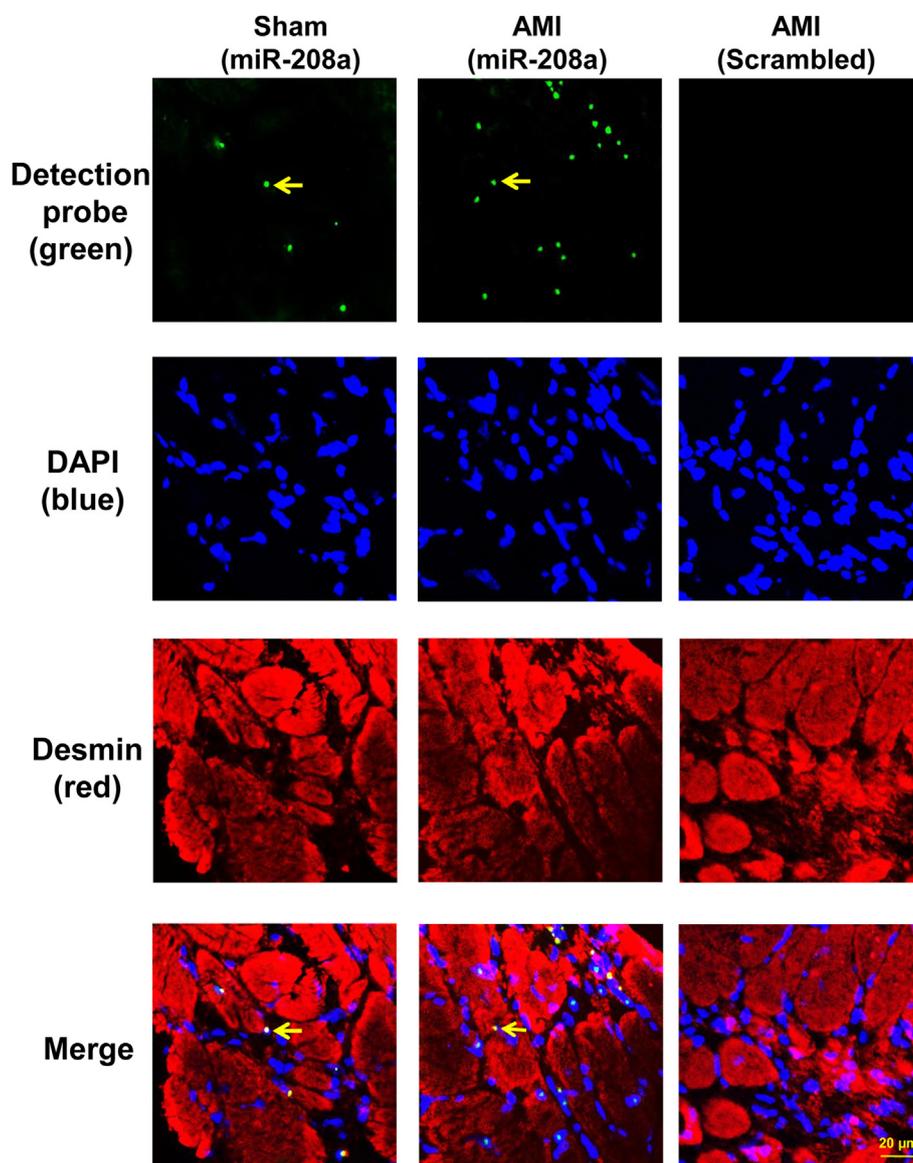
**Figure 3.** Effect of atorvastatin and valsartan on myocardial endoglin and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) expression after acute myocardial infarction (AMI). **(A)** Representative Western blot for endoglin and  $\beta$ -MHC protein expression in the rat myocardium after 7 days of myocardial infarction. **(B)** Quantitative analysis of endoglin and  $\beta$ -MHC protein levels. The values from myocardium after AMI have been normalized to matched glyceraldehyde-3-phosphate dehydrogenase (GAPDH) measurement and then expressed as a ratio of normalized values to protein in sham group ( $n = 6$  per group). Pretreatment with atorvastatin and valsartan but not *N*-acetylcysteine (NAC) significantly attenuated the increase of endoglin and  $\beta$ -MHC protein expression induced by myocardial infarction. \* $P < 0.001$  vs sham group; # $P < 0.001$  vs AMI at 7 days (7D) ( $n = 6$  per group).

myocardial endoglin and  $\beta$ -MHC induced by MI. Myocardial endoglin and  $\beta$ -MHC were not stained in the control sham group. The expression efficiency of miR-208a and antagomir-208a in myocardium is shown in [Supplemental Figure S3](#).

### MiR-208a increases myocardial fibrosis

AMI and overexpression of miR-208a in the sham group significantly increased the myocardial fibrotic area compared with the sham group ([Fig. 6](#)). Overexpression of mut-208a in the sham group did not change the fibrotic area compared with the sham group. Overexpression of antagomir-208a and pretreatment with atorvastatin and valsartan in the AMI group significantly decreased the myocardial fibrotic area induced by MI. AMI significantly increased infarct size and collagen type I protein expression

compared with the sham group, and overexpression of antagomir-208a in the AMI group significantly decreased infarct size and collagen type I protein expression induced by MI ([Supplemental Figs. S4 and S5](#)). Overexpression of mut-208a in the AMI group did not decrease the fibrotic area and infarct size induced by MI. Pretreatment with NAC did not change the fibrotic area induced by MI. These findings indicate that miR-208a plays a crucial role in myocardial fibrosis after MI, and pretreatment with atorvastatin and valsartan can decrease the myocardial fibrotic area induced by MI. AMI increased left ventricular end-systolic dimension and decreased fractional shortening, whereas treatment with atorvastatin or valsartan decreased left ventricular end-systolic dimension and increased fractional shortening ([Table 1](#)).



**Figure 4.** In situ hybridization assay detects the presence of miR-208a in the cardiac myocytes. Representative microscopic images showing the presence of miR-208a (**green, arrows**) in the cytoplasm of cardiac myocytes from left ventricular myocardium in acute myocardial infarction (AMI) shunted rats. DAPI stain was used to image nuclei (**blue**) and desmin fluorescence stain was used to image cardiac myocytes (**red**). Merge means image combined of detection probe, DAPI and desmin stain. The sham group or scrambled probe did not detect the presence of miR-208a. DAPI, 4',6-diamidino-2-phenylindole.

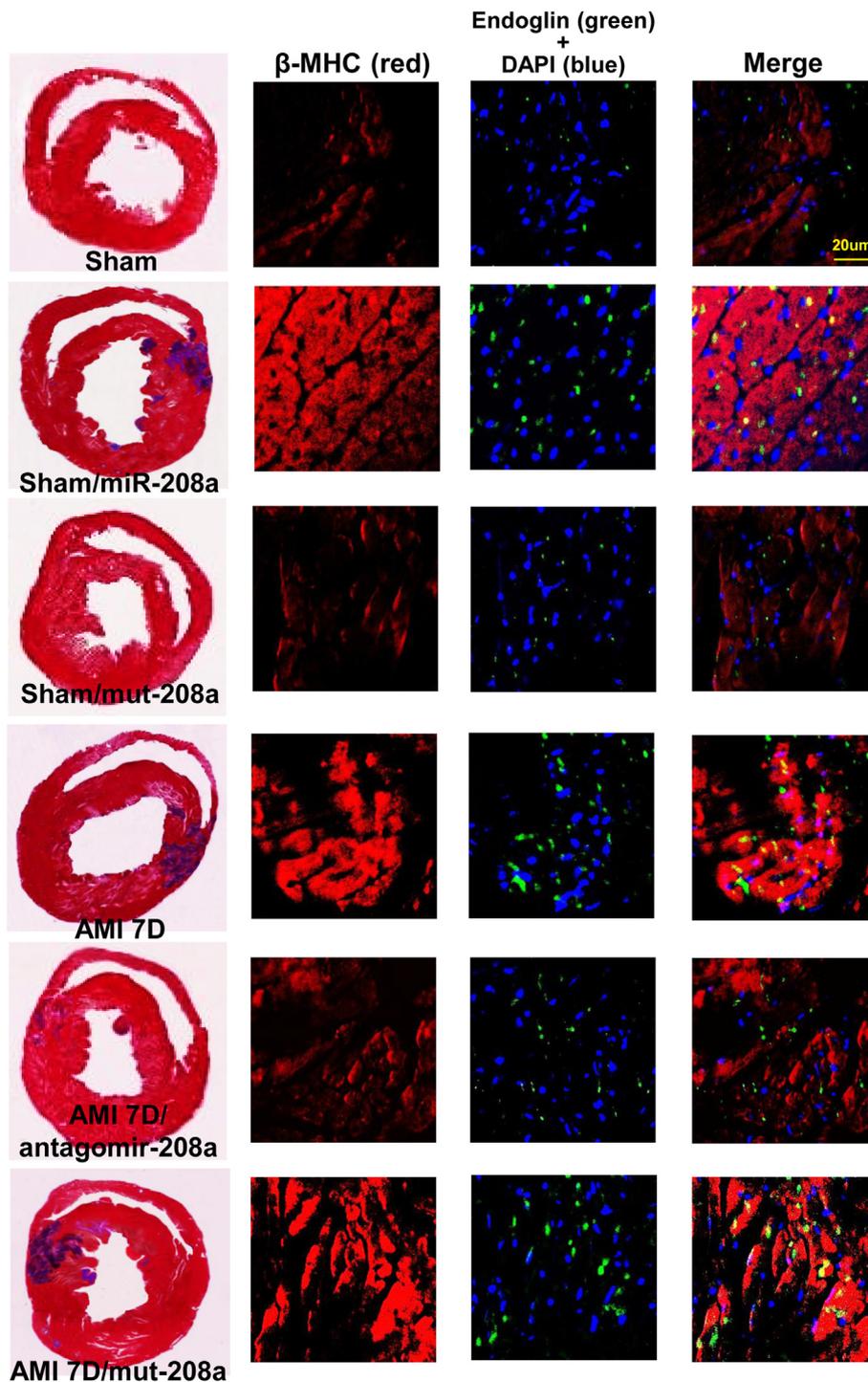
### MiR208a increases endoglin promoter activity

To study whether the endoglin expression induced by miR-208a is regulated at the transcriptional level, we cloned the promoter region of rat endoglin (−700 to −1) and constructed a luciferase reporter plasmid (pGL3-Luc). The endoglin promoter construct contains miR-208a, AP-1, Stat3, and Smad3/4 binding sites (Fig. 7A). As shown in Figure 7B, a transient transfection experiment in H9c2 cells using this reporter gene revealed that hypoxia at 2.5% oxygen or overexpression of miR-208a alone without hypoxia for 2 hours significantly increased endoglin promoter activity. This result indicates that endoglin expression is induced at the transcriptional level by miR-208a. When the miR-208a binding sites were mutated, the increased promoter

activity induced by endoglin was abolished. Moreover, addition of valsartan and antagomir-208a caused an inhibition of transcription. Mut-208a did not have an effect on endoglin promoter activity compared with the control group. Addition of antagomir-208a plus mut-208a in the hypoxic state for 2 hours also caused an inhibition of transcription.

### Effect of atorvastatin or valsartan on miR-208a and endoglin expression in cultured cardiac myoblasts and cardiomyocytes

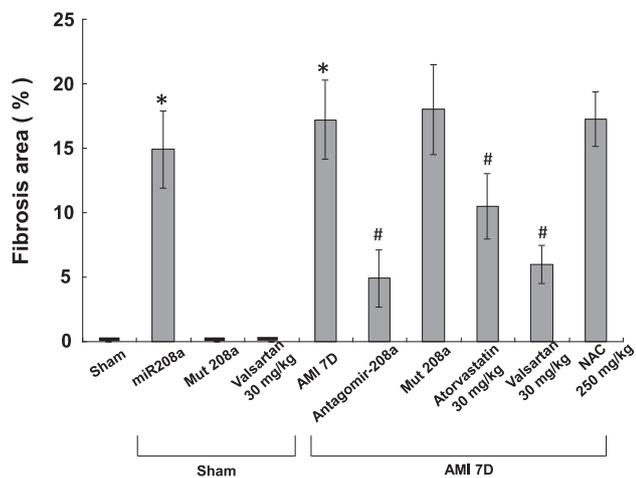
As shown in Supplemental Figure S6A, hypoxia at 2.5% oxygen for 2 hours significantly increased miR-208a



**Figure 5.** Immunohistochemical staining of left ventricular myocardium after induction of acute myocardial infarction (AMI) with or without antagomir-208a treatment. There are significantly increased immunoreactive signals for endoglin (**green**) and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) (**red**) after overexpression of miR-208a in AMI for 7 days (7D). Antagomir-208a significantly decreased the immunoreactive signal induced by MI. Rare endoglin signals were seen in the sham group.

expression in both cardiac myoblasts (H9c2 cells) and neonatal cardiomyocytes compared with the control group. The addition of antagomir-208a and valsartan significantly attenuated the increase of miR-208a expression induced by hypoxia. The exogenous addition of angiotensin II at 10 mM also significantly increased miR-208a expression. Endoglin

expression was not detected by real-time PCR in neonatal cardiomyocytes (data not shown). Hypoxia and overexpression of miR-208a significantly increased endoglin mRNA in cardiac myoblasts, and antagomir-208a and valsartan significantly attenuated the increase of endoglin mRNA induced by hypoxia ([Supplemental Fig. S6B](#)).



**Figure 6.** Antagomir-208a, atorvastatin, and valsartan decreased myocardial fibrotic area induced by acute myocardial infarction (AMI). Masson's trichrome staining was performed to delineate fibrotic tissue from viable myocardium in the peri-infarct zone (n = 6 per group). \*P < 0.01 vs sham group; #P < 0.01 vs AMI at 7 days (7D). Treatment with N-acetylcysteine (NAC) did not change the myocardial fibrotic area induced by myocardial infarction.

### Discussion

Endoglin expression is increased in human hearts with severe left ventricular systolic dysfunction and in heart failure induced by transaortic constriction, a pressure overload in mice. Kapur et al.<sup>22</sup> have demonstrated that reduced endoglin activity by soluble endoglin could decrease cardiac fibrosis and improve survival in heart failure. Endoglin is also increased in volume-overload heart failure induced by aorta-caval shunting.<sup>14</sup> Endoglin was demonstrated to regulate angiotensin-mediated fibrosis through the angiotensin receptor.<sup>15</sup> Endoglin has been used as a noninvasive measure of left ventricular filling pressure in heart failure and has been regarded as a novel biomarker for acute heart failure.<sup>17,23</sup> Therefore, targeting endoglin to prevent fibrosis and heart failure may improve clinical outcomes in patients with heart failure caused by AMI, in addition to the current clinical benefits of  $\beta$ -adrenergic receptor antagonists, angiotensin-converting enzyme or angiotensin-receptor blockers, and aldosterone antagonists.<sup>24</sup> The AMI model in our study increased left ventricular size and decreased fractional shortening, indicating a myocardial remodeling process. Endoglin and miR-208a were activated after AMI, and overexpression of miR-208a increased endoglin expression, whereas antagomir-208a attenuated the increase of endoglin induced by MI. Overexpression of miR-208a in the sham group without MI significantly increased myocardial endoglin expression. Overexpression of antagomir-208a in the AMI group significantly decreased the myocardial fibrotic area induced by MI, indicating that miR-208a plays a crucial role in myocardial fibrosis after MI. The increased endoglin to induce myocardial fibrosis induced by MI was mediated by miR-208a, indicating that endoglin is a downstream target of miR-208a in AMI. In addition to endoglin,  $\beta$ -MHC is also a target of miR-208a. Overexpression of miR-208a in cardiac myocytes increases  $\beta$ -MHC protein expression, and the addition of antagomir-208a

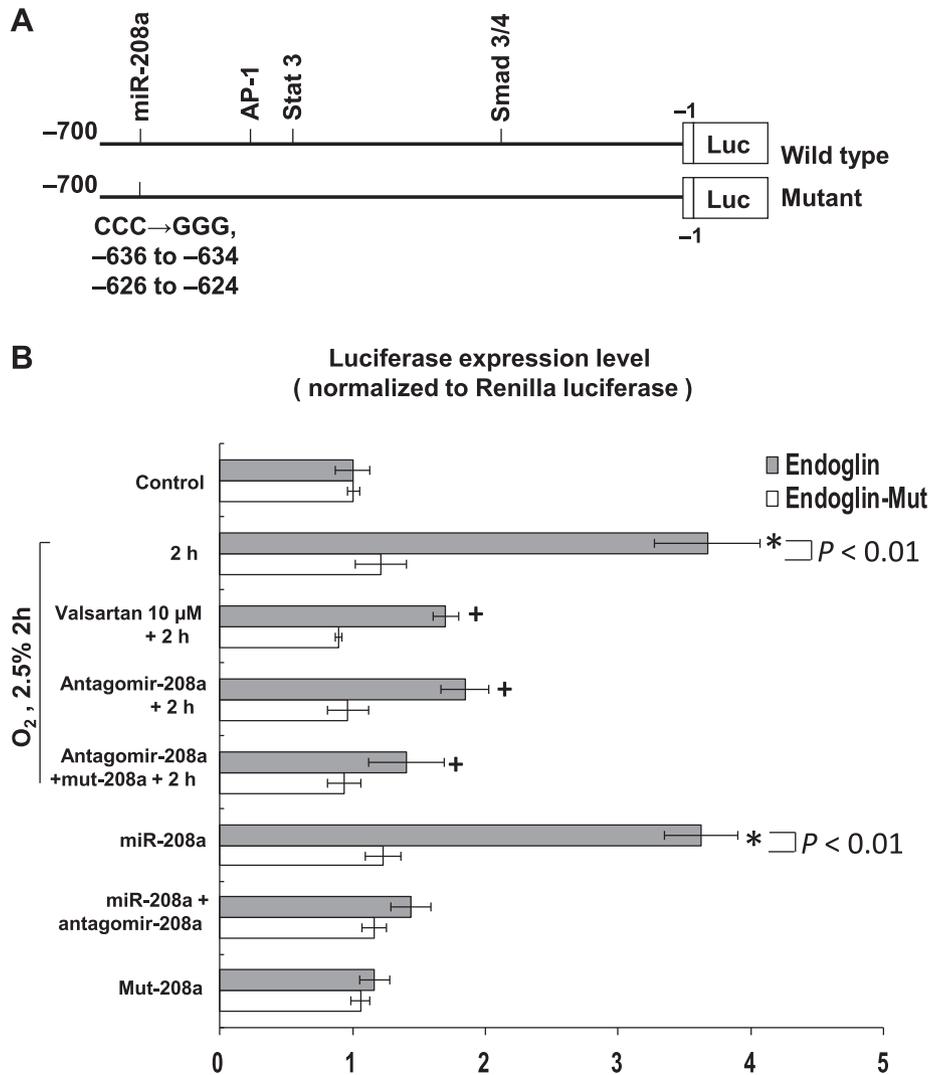
**Table 1.** Hemodynamic and echocardiographic parameters

Variable	Sham	AMI at 7 d	AMI at 14 d	Sham/miR-208a	Sham/mut-208a	Sham/valsartan 30 mg/kg	AMI at 7 d/ antagomir-208a	AMI at 7 d/ atorvastatin 30 mg/kg	AMI at 7 d/ valsartan 30 mg/kg	AMI at 7 d/NAC 250 mg/kg
No.	6	6	6	6	6	6	6	6	6	6
Body weight, g	314 ± 21	302 ± 16	311 ± 22	299 ± 18	297 ± 20	284 ± 29	309 ± 14	274 ± 9	277 ± 10	279 ± 22
Heart weight, mg	809 ± 37	875 ± 42	914 ± 51*	845 ± 44	802 ± 34	781 ± 30	854 ± 49	742 ± 34*	757 ± 47*	790 ± 34
Heart weight/body weight, mg/g	2.5 ± 0.6	2.9 ± 0.5	3.0 ± 0.5	2.8 ± 0.6	2.7 ± 0.3	2.6 ± 0.3	2.7 ± 0.5	2.8 ± 0.4	2.7 ± 0.3	2.9 ± 0.7
body weight, mg/g	307 ± 23	352 ± 29	310 ± 21	336 ± 20	312 ± 22	294 ± 31	328 ± 34	326 ± 35	322 ± 33	338 ± 20
Heart rate, min	90 ± 9	77 ± 6†	72 ± 5†	84 ± 8	89 ± 6	82 ± 7	79 ± 5	79 ± 5	82 ± 7	78 ± 6
MAP, mm Hg	6.4 ± 0.5	7.2 ± 0.5	7.4 ± 0.6	6.9 ± 0.6	6.5 ± 0.6	6.3 ± 0.5	6.3 ± 0.5	6.5 ± 0.6	6.6 ± 0.7	6.7 ± 0.5
LVEDD, mm	3.0 ± 0.3	4.2 ± 0.4†	4.5 ± 0.3†	3.8 ± 0.2	3.1 ± 0.4	3.0 ± 0.3	3.1 ± 0.3*	3.5 ± 0.2	3.1 ± 0.5*	3.7 ± 0.4
LVESD, mm	53 ± 5	42 ± 5†	39 ± 6†	43 ± 5†	52 ± 3	53 ± 4	51 ± 4†	44 ± 6	53 ± 4†	45 ± 7
FS, %										

AMI, acute myocardial infarction; FS, fractional shortening; LVESD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; MAP, mean arterial pressure; NAC, N-acetylcysteine.

\* P < 0.05 for AMI at 7 days.

† P < 0.05 for sham group.



**Figure 7.** Hypoxia and miR208a increase endoglin promoter activity in H9c2 cells. **(A)** Constructs of wild-type and mutant endoglin promoters. Mutant endoglin promoter indicates mutation of miR-208a binding sites in the endoglin promoter as indicated. **(B)** Quantitative analysis of endoglin promoter activity. Cultured H9c2 cells were transiently transfected with Endoglin-Luc by gene gun. The luciferase activity in cell lysates was measured and was normalized with Renilla activity by dual-luciferase reporter assay system (n = 3 per group). \*P < 0.01 vs control group; +P < 0.01 vs hypoxia at 2 hours or miR-208a.

significantly attenuates the increase of  $\beta$ -MHC induced by overexpression of miR-208a.<sup>16</sup> The expression of miR-208a peaks at days 3 and 5 after AMI, whereas the expression of endoglin and  $\beta$ -MHC increases over the entire time. Delayed degradation of endoglin and  $\beta$ -MHC after AMI may possibly explain our finding. MiR induces gene expression by targeting specific sites in gene promoters.<sup>3</sup> The endoglin promoter sequences have specific sites that are complementary to miR-208a.<sup>15</sup> We did not find the seed region of miR-208a in any region of the 3'-UTR of endoglin. The elevated levels of miR-208a induced by AMI increase the expression of endoglin. We also performed a TargetScan search and found that no other proteins that regulate endoglin are potential targets of miR-208a. Our promoter assay result indicates that miR-208a-dependent positive regulation of endoglin is indeed mediated by the direct targeting of miR-208a to its binding site within the

endoglin promoter. Our in vitro hypoxic model demonstrated that hypoxia increased miR-208a expression in neonatal cardiomyocytes and cardiac myoblasts and increased endoglin expression in cardiac myoblasts to confirm the results of the AMI model in the present study. MiRs have emerged as powerful regulators of a wide range of cellular processes by modulating gene expression.<sup>2,4</sup> MiRs can mostly downregulate target sequences by inhibiting their translation and in some cases destabilizing their mRNA,<sup>2</sup> but some miRs can induce gene expression if the miR binds to the specific promoter sites of target genes.<sup>3</sup> There is increasing evidence that miRs can also serve as activators of gene expression by targeting gene regulatory sequences.<sup>25-28</sup>

Several miRs are reported to be involved in cardiac remodelling after MI.<sup>29</sup> The cellular origins of the related miRs after MI are different, ranging from cardiomyocytes to

fibroblasts to endothelial cells.<sup>29</sup> As a new biomarker for the diagnosis of AMI, miR-208a was found to be elevated in plasma or serum in 4 clinical studies<sup>10,30-32</sup> but was found to be normal in 1 clinical study.<sup>11</sup> In animal studies, miR-208a was reported to be elevated in a rat model of MI<sup>32,33</sup> but was found to be decreased in a mouse model of AMI.<sup>9</sup> In our study, miR-208a was found to be elevated after AMI, and we used an in situ hybridization assay to confirm the presence of miR-208a in the cytoplasm of cardiac myocytes. The reason for the discrepancy between different studies is not known. Different animal models may partially explain the discrepancy.

Statin, a 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor, has been demonstrated to improve survival in patients with ischemic and nonischemic heart failure.<sup>34</sup> Recently, high-dose atorvastatin was found to significantly reduce hospitalization for heart failure in patients with stable coronary heart disease.<sup>35</sup> Atorvastatin is able to reduce endoglin expression in the endothelium of apo-E-deficient mice and C57BL/6J mice.<sup>36,37</sup> The antifibrotic effect of statins was found to occur through blocking of angiotensin II-mediated oxidative stress and procollagen type 1 expression in cardiac fibroblasts.<sup>38</sup> Recently, we have demonstrated that atorvastatin can inhibit endoglin expression induced by TGF- $\beta$ 1 in cultured cardiac fibroblasts.<sup>15</sup> The antifibrotic effect of statins has also been demonstrated in cardiac myocytes through the RhoA-extracellular signal kinase-serum response factor signaling pathway.<sup>39</sup> In this study, we have proved that atorvastatin can reduce myocardial fibrosis through reducing endoglin expression in AMI. We have previously demonstrated that TGF- $\beta$ 1 can activate miR-208a expression in cardiac myocytes and atorvastatin can inhibit TGF- $\beta$ 1 expression.<sup>16</sup> Therefore, the reason that pretreatment with atorvastatin in an AMI model can reduce miR-208a expression is possibly through the anti-inflammatory or pleiotropic effect of atorvastatin and partially through the anti-TGF- $\beta$ 1 effect. Endoglin is a potent mediator of profibrotic effects of angiotensin II on cardiac fibroblasts and can modulate the effect of TGF- $\beta$ 1 on extracellular matrix synthesis.<sup>18,19</sup> In this study, we have demonstrated that pretreatment with valsartan, an angiotensin-receptor antagonist, can reduce myocardial fibrosis through reducing endoglin expression after MI. The effect of inhibition of miR-208a induced by MI occurred earlier with valsartan than with atorvastatin because valsartan, but not atorvastatin, had an inhibitory effect on miR-208a expression after 3 days of AMI. Both valsartan and atorvastatin had an inhibitory effect on miR-208a expression after 7 days of AMI. Statin and angiotensin-receptor antagonist therapy may become another therapeutic strategy for controlling endoglin-associated pathologic cardiovascular disease in humans.

## Conclusions

We have demonstrated for the first time, to our knowledge, that miR-208a increases endoglin expression to induce myocardial fibrosis after acute MI. Treatment with atorvastatin or valsartan can attenuate myocardial fibrosis induced by MI through inhibition of endoglin expression.

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## Disclosures

The authors have no conflicts of interest to disclose.

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### Supplementary Material

To access the supplementary material accompanying this article, visit the online version of the *Canadian Journal of Cardiology* at [www.onlinecjc.ca](http://www.onlinecjc.ca) and at <http://dx.doi.org/10.1016/j.cjca.2014.12.026>.

## Erratum



In the December 2014 issue, the authors of the article by Liu et al., “Micro-RNA 21 Targets Dual Specific Phosphatase 8 to Promote Collagen Synthesis in High Glucose-Treated Primary Cardiac Fibroblasts,” omitted the name of their funding source. The funding source should have been listed as follows:

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