C1q/TNF-related protein-2 improved angiogenesis to protect myocardial function during ischaemia–reperfusion

Mingfang Ye1,#, Qi-Hong Wu2,#, Ke Yang3 and Yukun Luo1

Abstract

Background: Collateral growth plays an important role in the recovery of acute myocardial infarction. C1q/TNF-related protein-2 (CTRP2), a CTRP family member, showed some protective effects on cell survival. In this study, the relationship between CTRP2 and collateral growth was examined.

Methods: C57BL/6 mice were subjected to myocardial ischaemia/reperfusion (I/R), and the expression of CTRP2 and the effect of CTRP2 on infarction size, cardiac function and angiogenesis were examined. The ischaemic hindlimb model was also used to examine the effect of CTRP2. In vitro, CTRP2-mediated regulation of angiogenesis, AKT activation and VEGFR2 expression in endothelial cells was examined. The CTRP2 level associated with good collateral growth was observed in a cohort.

Results: I/R reduced CTRP2 expression, and intraperitoneal injection of recombinant CTRP2 protein improved infarction size, cardiac function and angiogenesis. Overexpression of CTRP2 promoted blood refusion and collateral growth in ischaemic hindlimb mice. In vitro, CTRP2 enhanced tube formation and migration in a dose-dependent manner, while CTRP2 increased AKT phosphorylation and VEGFR2 expression. In an observational clinical cohort, CTRP2 levels were significantly increased in patients with good collateral growth, and CTRP2 was negatively associated with poor collateral growth in patients.

Conclusion: CTRP2 improved cardiac function by promoting collateral growth by promoting AKT-VEGFR2.

Keywords
CTRP2, collateral growth, angiogenesis, myocardial ischaemia/reperfusion

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Introduction

The main pathological change in coronary heart disease is coronary stenosis, which is an important cause of myocardial ischaemia, heart failure and even sudden death. Previous studies have shown that in acute coronary occlusion, good collateral circulation provides blood flow to the distal ischaemic myocardium, has significant cardioprotective effects, and improves clinical outcomes for patients. The formation of coronary collateral circulation is mainly related to angiogenesis. Many clinical factors affect the formation of coronary collateral circulation from various aspects. Thus, the present medicines and interventions only partially increase coronary collateral circulation, which is limited by multiple mechanisms of coronary collateral formation.

It is well known that inflammation and angiogenesis are involved in collateral circulation. Adiponectin, which is a protective factor, has been reported to promote the formation of collateral circulation. The adipokine complement C1q tumour necrosis factor-related protein (C1q and tumour necrosis factor-related protein, CTRP) family is a group of proteins that are structurally similar to adiponectin and closely associated with atherosclerosis. The CTRP family includes at least 15 members, and the expression level of CTRP1 is negatively correlated with the formation of coronary collateral circulation, while CTRP3 promotes microvascular endothelial function in diabetes. CTRP2, which is the most similar in structure to adiponectin, has been shown to be a significantly beneficial adipocytokine that regulates metabolism. A previous study showed that CTRP2 increased glucose uptake in cardiomyocytes via AMPK/Akt, the Akt signalling pathway is involved in angiogenesis by regulating VEGFR2. However, the relationship between CTRP2 and angiogenesis in ischaemia–reperfusion (I/R) during acute myocardial infarction (AMI) has not been examined.

In this study, the role of CTRP2 in collateral circulation after I/R was examined in I/R animals, and the effect of CTRP2 on angiogenesis in endothelial cells was measured. This study mainly explored the role of CTRP2 in the formation of collateral circulation in AMI.

Methods

Animals

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Fujian Medical University Union Hospital, China, which conforms to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Neonatal specific-pathogen-free (SPF) male C57BL/6 mice (background: C57BL/6N; 6–8 weeks of age) were purchased from Beijing Vital River Laboratory Animal Products Co., Ltd. The mice were housed in the laboratory animal facility and maintained at 25°C ± 1°C, with 65% ± 5% humidity, on a 12-hour light/dark cycle for one week for acclimatization prior to the experiments. The animal experiments followed the reduce, reuse, and recycle (3R) principle.

Myocardial ischaemia/reperfusion (I/R) animal model and treatment

The mice were anaesthetized by an intraperitoneal injection of sodium pentobarbital (20 mg/kg). Thoracotomy was performed through the 4th left intercostal space. Ligation of the left anterior descending (LAD) coronary artery was performed under a stereomicroscope. The I/R animal model was induced in C57BL/6 mice (n = 30) by ligating the LAD artery for 45 minutes followed by reperfusion. The mice underwent an identical procedure with the exception of I/R in the sham operation group (n = 10). I/R mice were randomized into 3 groups, and each group included 10 mice. Each group underwent different treatments: Group 1: only I/R (n = 10), Group 2: I/R with intraperitoneal injection of saline (n = 10), and Group 3: I/R with intraperitoneal injection of recombinant CTRP2 protein (250 pg/mL, n = 10). The recombinant CTRP2 protein was synthesized and purified by Yuanpeptide Company (China).

The cardiac function of I/R mice after 3 days of I/R was evaluated by two-dimensional echocardiography using a Vevo 2100 instrument (FUJIFILM Visual Sonics, ON, Canada) equipped with an MS-400 imaging transducer. To collect the whole heart from I/R mice, the mice were anaesthetized by isoflurane inhalation in combination with intraperitoneal injection of pentobarbital sodium (30 mg/kg weight) for euthanasia.

Model of ischaemic hindlimb and blood flow monitoring

The left femoral vessel (artery and vein) was removed, and all branches of the femoral bifurcation were resected to produce an ischaemic hindlimb animal model (n = 10). The ten ischaemic hindlimb mice were randomized into 2 groups, and each group contained 5 mice. Group 1 was intraperitoneally injected with saline (n = 5), and Group 2 was intraperitoneally injected with recombinant CTRP2 protein (250 pg/mL, n = 5) once every 2 days. The perfusion of the hindlimb was examined with laser Doppler perfusion imaging. The results are expressed as the ratio of perfusion in the ischaemic (left) versus nonischaemic (right) hindlimb.
Immunohistochemistry and immunofluorescence analysis

Myocardial and limb tissues were collected from each mouse and fixed in 4% paraformaldehyde overnight, and optimal cutting temperature compound (Sakura, MA, USA) was used to embed the tissues. All tissues were sliced into 6-μm-thick frozen sections that were then stained with haematoxylin and eosin (H&E) or for immunohistochemical analysis with the following antibodies: anti-CTRP (1:50, Santa Cruz, TX, USA) and anti-CD31 (1:100, Santa Cruz, TX, USA). The slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:100, Santa Cruz, TX, USA), exposed to diaminobenzidine and counterstained with haematoxylin. All sections were photographed with a microscope (Olympus Microsystems), and the levels of CTRP2 and CD31 were calculated in 5 different representative microscopic fields.

The myocardial tissues were sliced into 6-μm-thick frozen sections that were stained with anti-α-actinin (1:200, Santa Cruz, TX, USA) and anti-CD31 (1:200, Santa Cruz, TX, USA) and then incubated with the corresponding Alexa-555- or Alexa-488-conjugated secondary antibody (1:200) in the dark. DAPI (1:1000) was used to stain the nuclei, and the slides were mounted and photographed using a laser confocal microscope (Zeiss LSM 710).

Evans blue staining

As previously described, the area at risk (AAR) was determined by Evans Blue and TTC staining. The area at risk was measured using Image-Pro Plus software (v6.0; Media Cybernetics, Rockville, MD, USA).

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (MA, USA) and cultured in M200 medium with vascular growth supplement (VGS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, NY, USA). All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Tube formation assay

The angiogenic capacity of HUVECs was measured by using Matrigel (BD, CA, USA). HUVECs (1×10⁴ cells per well) were harvested and plated onto a 96-well glass slide that were precoated with Matrigel and incubated with different doses (50, 100 and 200 pg/mL) of CTRP2 for 8 hours. Experiments were performed, and three randomly selected fields were examined. All images were captured with Olympus Microsystems (Tokyo, Japan), and the length of tube formation was calculated.

Transwell assay

A 48-well transwell plate (Millipore, MA, USA) was used to examine cell migration according to the manufacturer's instructions.

Western blotting

The total proteins were collected with protein lysis buffer (CST, MA, USA), and 30 μg of total proteins was loaded on 10% SDS-PAGE for separation and subsequent electrophoretic transfer to polyvinylidene fluoride membranes. Then, the membranes were incubated with anti-phosphorylation-Akt (phosphorylation site was Thr308, 1:1000, CST, MA, USA), anti-total-Akt (1:1000, CST, MA, USA), anti-VEGFR2 (1:1000, CST, MA, USA) or β-actin (1:2000, CST, MA, USA) overnight. Then, the membranes were incubated with a secondary antibody (1:5000, CST, MA, USA) for 1 hour and exposed with an ECL kit (CST, MA, USA). The protein density was quantified with ImageJ software.

Human subjects

A cohort of 113 patients undergoing coronary angiography at Fujian Medical University Union Hospital with stable angina and CTO of at least 1 major epicardial coronary artery were enrolled in this study from July 2018 to July 2019. The study protocol was approved by the Ethics Committee of the Fujian Medical University Union Hospital, and written informed consent was obtained from all the patients before their participation in this study. Patients with a history of coronary artery bypass grafting surgery, acute coronary syndrome, chronic heart failure or pulmonary heart disease, and immune system disorder or tumour were excluded.

The assessment of collateral circulation was based on the Rentrop scoring system: 0 = none; 1 = filling of side branches of the artery; 2 = partial filling of the epicardial segment; and 3 = complete filling of the epicardial segment of the artery. A Rentrop score of 0–1 was regarded as poor, and a Rentrop score of 2–3 was identified as good.

Statistical analysis

The data are presented as the mean ± SD and were compared using Student’s t test (for two group comparisons) or one-way ANOVA (for >2 group comparisons). A p value <0.05 was considered statistically significant. Categorical variables were compared with the χ² test, and continuous variables were compared with the t test or the
Mann–Whitney U test as appropriate. Multivariable logistic regression and univariate analyses were performed to calculate the odds ratios (ORs) and 95% CIs for the association of specific CTRP2 levels with the risks of poor collateral growth. Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC).

Results

The level of CTRP2 was decreased in the infarction region of animals with myocardial ischaemia/reperfusion (I/R)

To investigate the effect of I/R on the level of CTRP2, mice were subjected to I/R, and CTRP2 was measured in the myocardial infarction region. Immunohistochemistry showed that I/R caused myocardial remodelling, and CTRP2 was significantly decreased (Sham = 0.180 ± 0.064 vs. I/R = 0.046 ± 0.021, p = .002, Figure 1. A and B). To further verify the immunohistochemistry results, western blotting was used to measure the protein expression of CTRP2, and the results showed that CTRP2 was significantly decreased in the I/R group, which was similar to the immunohistochemistry data (Sham = 1.132 ± 0.118 vs. I/R = 0.546 ± 0.152, p = .0001, Figure 1. C and D). Moreover, analysis of the noninfarction cardiac region showed a significant decrease in CTRP2 (Sham = 1.140 ± 0.095 vs. I/R = 0.636 ± 0.149, p = .0001, Figure 1. C and D). To measure the expression of CTRP2 at different times after I/R, the mRNA levels of CTRP2 were measured. The results showed that the greatest decrease in CTRP2 was on the 3rd day after I/R. Moreover, the expression of CTRP2 was recovered on the 7th day after I/R (Supplementary Figure 1).

CTRP2 reduced the I/R infarction size and promoted cardiac function and blood flow recovery

To verify whether CTRP2 affects I/R, recombinant CTRP2 protein or saline was injected into I/R mice, and infarction size, cardiac function and blood flow were examined. The infarct size/area at risk (IF/AAR) ratio after CTRP2 injection was significantly decreased following 45 minutes of ischaemia and 24 hours of reperfusion compared with that in response to saline injection (I/R+Saline group: IF/AAR = 41.60 ± 8.47% vs. I/R+CTRP2: IF/AAR = 23.00 ± 7.38%, p = .006, Figure 2. A-C). Echocardiography showed that at 3 days after I/R, the CTRP2 injection group exhibited significantly improved cardiac function.
function compared with the saline injection group, except for the heart rate (LVEF: I/R+Saline group = 43.00 ± 5.05% vs. I/R+CTRP2 = 57.00 ± 5.48%, p = 0.003; FS: I/R+Saline group = 20.48 ± 2.41% vs. I/R + CTRP2 = 28.50 ± 2.74%, p = 0.001; cardiac output: I/R + Saline group = 12.56 ± 2.29 mL/min vs. I/R + CTRP2 = 18.04 ± 2.12 mL/min, p = 0.004, Figure 2. D-H). The concentration of endothelial cells partially represents the degree of blood flow recovery; therefore, CD31, which is a marker of endothelial cells, was used to label endothelial cells in the myocardial infarction region.13 Compared with the I/R+Saline group, the I/R+CTRP2 group exhibited more CD31-positive cells (CD31: I/R+Saline=0.242 ± 0.069 vs. I/R+CTRP2 = 0.368 ± 0.089, p = .037, Figure 2. I and J).

**Figure 2.** The effect of CTRP2 on infarction size, cardiac function and collateral growth. C57BL/6 mice were subjected to 45 minutes of ischaemia and reperfusion, and saline or recombinant CTRP2 protein was injected into these mice. After three days, the mice were randomized into two groups to measure infarction size and cardiac function. (a) Cardiac tissues were stained with Evans Blue and TTC (n=5 for each group). (b) The ratio of the area at risk/left ventricle (AAR/LV) was calculated (n = 5 for each group). (c) The ratio of infarct size/area at risk (IF/AAR) was calculated (n = 5 for each group). (d) The left ventricular ejection fraction (LVEF), fractional shortening (FS), heart rate and cardiac output values of ischaemia/reperfusion mice were detected by echocardiography (n = 5 for each group). (i) The infarction areas were stained for immunofluorescence, and the fluorescence of CD31 was calculated. The nuclei were stained blue (DAPI), the endothelial cells were stained red (CD31), and the cardiomyocytes were stained green (α-actinin) (n = 5 for each group). (*p < .05, **p < .01, by Student’s t test, ns indicates not significant)

**CTRP2 promoted blood flow recovery in ischaemic hindlimb mice**

To further clarify the influence of CTRP2 on blood flow recovery in ischaemia, ischaemic hindlimb mice were treated with CTRP2 and saline, and the differences in blood flow were measured. The dose of CTRP2 that induced serum levels of CTRP2 that were associated with Good Collateral Growth in AMI was 218.87 ± 128.45 pg/mL. Compared with perfusion before surgery, the hindlimb ischemia severely blocked blood flow, and then perfusion was restored from 7 to 21 days. However, the I/R+CTRP2 group exhibited significantly higher recovery than the I/R+Saline group (p < 0.05, Figure 3. A and B). The levels of CD31-positive cells were also calculated in the ischaemic hindlimb, and I/R+CTRP2 significantly promoted CD31-positive cells (CD31: I/R+Saline = 0.176 ± 0.030 vs. I/R+CTRP2 = 0.270 ± 0.054, p = .009, Figure 3. C and D.)

**CTRP2 induced tube formation, migration, AKT activation and VEGFR2 expression in endothelial cells**

It is well known that angiogenesis in endothelial cells regulates blood flow after ischaemia14 and involves tube formation, migration, signalling pathway activation and...
VEGFR2 expression. Based on these points, HUVECs were incubated with different doses of CTRP2, and tube formation and migration were examined. The tube formation (Con = 5.52 ± 0.95 mm, 50 pg/mL CTRP2 = 7.95 ± 0.49 mm vs. Con \( p = .04 \), 100 pg/mL CTRP2 = 8.65 ± 0.66 mm vs. Con \( p = .01 \), 200 pg/mL CTRP2 = 11.59 ± 1.48 mm vs. Con \( p = .002 \), Figure 4. A and B) and Transwell (Con = 1.76 ± 0.08, 50 pg/mL CTRP2 = 2.48 ± 0.15 vs. Con \( p = .003 \), 100 pg/mL CTRP2 = 4.48 ± 0.15 vs. Con \( p < .0001 \), 200 pg/mL CTRP2 = 4.91 ± 0.27 vs. Con \( p < .0001 \), Figure 4. A and C) results showed a dose-dependent increase. Moreover, an AKT antagonist inhibited CTRP2-induced AKT phosphorylation, tube formation and migration in endothelial cells (Supplementary Figure 2).

**Increased CTRP2 was associated with good collateral growth**

To investigate the potential clinical association between CTRP2 and collateral development, an observational study was performed to measure serum CTRP2 levels and good collateral growth in patients with stable angina and CTO. The basic clinical characteristics of the patients are listed in Table 1. Patients with good collateral growth (\( n = 56 \)) had a lower incidences of diabetes mellitus (37.5% vs. 57.9%, \( p = .019 \)) and higher CTRP2 levels (218.87 ± 128.45 vs 149.31 ± 89.34 pg/mL, \( p = .001 \)) than those with poor collateral growth (\( n = 57 \)). Univariate analysis showed that CTRP2, which is a continuous variable, was associated with poor collateral growth (OR = 0.513; 95% CI 0.421–0.653, \( p = 0.001 \)). Multivariable regression analysis of candidate variables showed that diabetes mellitus and CTRP2 were independent risk factors for poor collateral growth in different models (Table 2).

**Discussion**

In this study, I/R reduced CTRP2 levels in the region of myocardial infarction, but overexpression of CTRP2 recovered the infarction size and improved cardiac function and blood flow after I/R. Further investigation found that CTRP2 promoted collateral growth in ischaemic hindlimb...
mice, and CTRP2 increased tube formation and migration in endothelial cells by activating AKT and upregulating VEGFR2 levels.

A previous study showed that PCI significantly improved myocardial perfusion and reduced the risk of coronary heart disease complicated with heart failure with coronary revascularization. However, good collateral growth was mediated by retrograde or forward perfusion to the distal end of the coronary occlusion, protecting the myocardium from ischaemia and necrosis and improving left ventricular function. Regieli found that although collateral growth did not completely restore myocardial perfusion to normal levels in the coronary occlusion area, collateral growth protected the ischaemic myocardium.
increased residual myocardial contractility, and reduced angina symptoms and cardiovascular events. Some meta-analyses also showed that patients with good collateral growth exhibited a 36% lower risk of death than those with poor collateral growth, possibly due to improved repolarization of the ischaemic myocardium during acute coronary occlusion and the avoidance of fatal arrhythmias. In these studies, good collateral growth was an important cardioprotective effect in AMI. In the present study, CTRP2 was decreased in the myocardial infarction area, which suggested that the decreased level of CTRP2 abrogated its cardioprotective effect and might be a protective factor. Moreover, the overexpression of CTRP2 precisely restored infarction size in the I/R mice and significantly promoted cardiac function, such as LVEF and FS. These results suggested that CTRP2 was a protective factor for AMI. Moreover, CTRP2 not only improved collateral growth in I/R and ischaemic hindlimb mice, such as increasing CD31-positive cells, but also promoted perfusion levels in the ischaemic area. In vitro, CTRP2 increased angiogenesis, including tube formation and migration, in endothelial cells. In an observation cohort study, CTRP2 levels were higher in good collateral growth patients than in poor collateral growth patients, and CTRP2 was negatively correlated with poor collateral growth. Thus, increased CTRP2 was related to good collateral growth after I/R and protected cardiomyocyte survival to improve cardiac function.

The CTRP family includes 15 members, and CTRPs exhibit different cellular functions. The cardioprotective effects of CTRP1, CTRP3, CTRP9 and CTRP15 have been reported. CTRP1 reduces apoptosis and the inflammatory response to protect against myocardial ischaemic injury. CTRP3 promotes survival and cardiac function in AMI and attenuates postischaemic pathological remodelling via Akt-HIF1-VEGF signalling. CTRP9 weakens myocardial infarction size and apoptosis in AMI, increases cardiac function in diabetic mice and inhibits adverse cardiac remodelling. CTRP15 improves acute myocardial ischaemic injury and overload-induced cardiac fibrotic remodelling. In our study, CTRP2 attenuated the infarction size in I/R mice, which suggests that CTRP2 was similar to other CTRPs and may be a novel cardioprotective factor. Furthermore, previous studies reported that CTRP9

### Table 1. Baseline characteristics.

|                          | Poor collateral growth (n = 57) | Good collateral growth (n = 56) | P     |
|--------------------------|---------------------------------|---------------------------------|-------|
| Age, years               | 66.1 ± 7.4                      | 65.2 ± 7.1                      | NS    |
| Male/female              | 37/20                           | 35/21                           | NS    |
| BMI                      | 25.0 ± 3.7                      | 25.4 ± 3.2                      | NS    |
| Hypertension, n (%)      | 35 (61.4)                       | 36 (64.3)                       | NS    |
| Diabetes mellitus, n (%) | 33 (57.9)                       | 21 (37.5)                       | 0.019 |
| Triglycerides, mmol/L    | 1.82 ± 0.84                     | 1.81 ± 0.83                     | NS    |
| Total cholesterol, mmol/L| 4.21 ± 1.13                     | 4.12 ± 0.92                     | NS    |
| HDL cholesterol, mmol/L  | 1.02 ± 0.21                     | 1.03 ± 0.20                     | NS    |
| LDL cholesterol, mmol/L  | 2.78 ± 1.13                     | 2.41 ± 1.12                     | NS    |
| Lipoprotein (a), g/L     | 0.26 ± 0.14                     | 0.23 ± 0.15                     | NS    |
| ACEI/ARB, n (%)          | 30 (52.6)                       | 28 (50.0)                       | NS    |
| β-bloker, n (%)          | 30 (52.6)                       | 31 (55.4)                       | NS    |
| Calcium channel bloker, n (%) | 14 (24.6) | 13 (23.2) | NS |
| Statin, n (%)            | 43 (75.4)                       | 48 (85.7)                       | NS    |
| Glucose lowering therapy, n (%) | 33 (57.9) | 21 (37.5) | 0.019 |
| CTRP2, pg/mL             | 149.31 ± 89.34                  | 218.87 ± 128.45                 | 0.001 |

Continuous variables were presented as Means±SD and categorical variables were expressed as n (%). For Continuous variables, Mann-Whitney U-tests were performed to assess differences. Differences in proportions were analyzed by 2×2 Chi-square tests. BMI = body mass index; HDL= high density lipoprotein; LDL= low density lipoprotein.

### Table 2. Multivariable regression analysis for the Risk of Poor Coronary Collateral Growth.

|                          | Crude model OR (95%CI) | P     | Model 1 OR (95% CI) | P     |
|--------------------------|------------------------|-------|---------------------|-------|
| Diabetes mellitus        | 2.426 (1.123–4.231)    | 0.034 | 1.876 (1.024–2.367) | 0.045 |
| CTRP2                    | 0.613 (0.232–0.826)    | 0.001 | 0.853 (0.453–0.975) | 0.021 |

Crude model: Unadjusted model. Model 1: adjusted for age and gender.
increased endothelial cell function and revascularization via the eNOS pathway in ischaemic vascular diseases. CTRP9 suppressed oxLDL-induced endothelial injury via PGC-1α/AMPK activation to induce antioxidant enzymes. CTRP3 enhanced the biogenesis of mitochondria to promote angiogenesis in AMI. Another study showed that CTRP3 increased the activation of AKT to enhance angiogenesis. Thus, CTRPs mediate different signalling pathways to restore angiogenesis after AMI. CTRP2 also promoted tube formation and migration of endothelial cells in vitro and increased the number of endothelial cells in the ischaemic area. Moreover, CTRP2 activated AKT phosphorylation and upregulated VEGFR2 expression. Collectively, CTRP2 exerted cardioprotective effects by improving angiogenesis-induced collateral growth via AKT-VEGFR2.

Conclusion

Ischaemia–reperfusion caused CTRP2 to decrease, but CTRP2 overexpression restored infarction size and promoted cardiac function in I/R mice. Furthermore, CTRP2 enhanced collateral growth to promote blood refusion in mice with I/R or ischaemic hindlimbs. In vivo, CTRP2 enhanced angiogenesis by increasing AKT activation and VEGFR2 expression. The clinical cohort showed that CTRP2 was associated with good collateral growth. This study sheds light on some novel characteristics of CTRP2 and the potential therapeutic value of CTRP2 in the treatment of AMI.

Declaration of conflicting interests

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Supplemental Material

Supplemental material for this article is available online.

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