Knockdown of XIST up-regulates 263294miR-340-5p to relieve myocardial ischaemia–reperfusion injury via inhibiting cyclin D1

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Abstract

**Aim** Long non-coding RNAs (lncRNAs) are known to participate in various human diseases, while the role of X inactive-specific transcript (XIST) binding microRNA-340-5p (miR-340-5p) remains seldom studied. We aim to identify the role of the XIST/miR-340-5p/cyclin D1 (CCND1) axis in the myocardial ischaemia–reperfusion injury (MIRI).

**Methods and results** The mouse MIRI models were established. The expression of XIST, miR-340-5p, and CCND1 in mouse myocardial tissues in MIRI mice was assessed. The MIRI mice were respectively treated with altered XIST, miR-340-5p, or CCND1. The changes of myocardial enzyme activity were assessed, and the cardiac function was evaluated. Myocardial pathological changes, cardiomyocyte apoptosis and related apoptotic factors, oxidative stress and inflammatory factors were observed in myocardial tissues in mice with MIRI. The binding relationships between XIST and miR-340-5p, and between miR-340-5p and CCND1 were confirmed. XIST and CCND1 were up-regulated while miR-340-5p was down-regulated in MIRI mice. Silenced XIST could elevated miR-340-5p expression and reduced CCND1 expression, so as to promoted cardiac function and suppressed myocardial enzyme activity, ameliorated pathological changes, decelerated cardiomyocyte apoptosis by elevating Bcl-2 but reducing the levels of Bax and Caspase-3, attenuated inflammatory response by repressing IL-6 and TNF-α levels, and mitigated oxidative stress by reducing MDA contents and increasing CAT, GSH-Px, and SOD levels in MIRI mice. XIST sponged miR-340-5p and miR-340-5p targeted CCND1.

**Conclusions** Knockdown of XIST up-regulates miR-340-5p to relieve MIRI via inhibiting CCND1.

Keywords: Myocardial ischaemia–reperfusion injury; Long non-coding RNA X inactive-specific transcript; MicroRNA-340-5p; Cyclin D1; Cardiomyocyte

Introduction

Coronary artery disease (CAD), a main cause for morbidity and mortality throughout the world, is a common health problem.¹ The most common form of CAD is atherosclerosis. With the forming of lipid-rich plaques, atherosclerosis can gradually obstruct the vessel lumen, leading to coronary occlusion and cessation of blood flow.² Restoration of blood flow to ischaemic myocardium is usually used to treat ischaemic heart disease. Restoring blood flow minimizes infarct-induced damage, thereby decreasing the mortality. Nevertheless, this restoration may lead to additional cardiovascular trauma, namely, myocardial ischaemia–reperfusion (I/R) injury (MIRI).³ MIRI can result in cell death and cardiac dysfunction, and its potential mechanisms involve in oxidative stress, calcium overload, inflammation, neutrophil infiltration, and cytokine release.⁴ Although the treatments for this disease, such as percutaneous coronary intervention technology, antiplatelet agent, and antithrombotic agent, have been developed, there is still no effective therapeutic strategy to fight against MIRI.⁵ Therefore, it is necessary to explore novel therapeutic targets for MIRI.
Long non-coding RNA (IncRNA) is a group of functional RNA molecule with transcript length of >200 nt and is implicated in multiple physiological and pathological processes. Some particular IncRNAs were involved in MIRI. For instance, IncRNA RMRP up-regulation has been reported to aggravate MIRI, and it has been identified that knockdown of IncRNA AK139328 alleviated MIRI. LncRNA X inactive-specific transcript (XIST) is a major regulator of mammalian X chromosome inactivation, which has been demonstrated to regulate myocardial infarction (MI) and hypoxia-induced cardiomyocyte apoptosis. Interestingly, the reduction of XIST has been verified to improve MIRI. In recent decades, the competing endogenous RNA (ceRNA) hypothesis implies a regulatory circuitry that IncRNAs can serve as molecular sponges of specific microRNAs (miRNAs). MiRNAs are small non-protein-coding RNAs that regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of mRNAs and promoting degradation of transcripts or inhibiting translation. As one of the miRNAs, miR-340-5p has been clarified to inhibit hypoxia/reoxygenation (H/R)-induced apoptosis and oxidative stress in cardiomyocytes. However, the spongy relationship between XIST and miR-340-5p remains largely unknown. Cyclin D1 (CCND1) is a highly conserved cyclin family protein that has been identified as the indispensable for the transition of cell cycle from G1 phase into S phase. As reported, CCND1 was implicated in hypoxia-induced myocardial damage, and it has been elucidated that there exists a targeting relationship between miR-340-5p and CCND1 in multiple myeloma. However, this relationship in MIRI remains scarcely studied.

Thus, we aimed to explore the role of the XIST/miR-340-5p/CCND1 axis during the MIRI, and we inferred that XIST may act as a ceRNA of miR-340-5p to affect MIRI through targeting CCND1.

**Materials and methods**

**Ethics statement**

Animal experiments were strictly in accordance with the Guide to the Management and Use of Laboratory Animals issued by the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of Zhengzhou Central Hospital.

**Experimental animals**

Adult male-specific pathogen-free mice aged 10–12 weeks (the experimental animal centre of Zhengzhou University, Henan, China) were fed at 25°C and with 50% humidity.

**Animal grouping**

XIST shRNA, miR-340-5p antagonomir, CCND1 shRNA, or the negative control (NC) were added to lentiviral vectors PHLV-KDS.1 (Thermo Fisher Scientific Inc., MA, USA) and then were packaged into lentiviral particles. Mouse left ventricle was injected with 200 μL lentivirus (1 × 10⁹ TU/mL). The pulmonary artery was clamped for 20 s to maintain heartbeat in a closed system. Then, the air and blood were removed and the incision was sutured. Mice were classified into eight groups (n = 12): the sham, ischaemia–reperfusion (I/R), NC, sh-XIST, sh-CCND1, antagonomir NC, miR-340-5p antagonomir, and si-XIST + miR-340-5p antagonomir groups.

**Establishment of ischaemia–reperfusion mouse models**

Mice were modelled 5 days after lentivirus injection: mice were anaesthetized by 1% pentobarbital sodium (35 mg/kg, Qiaoxing Trading Co., Ltd., Shanghai, China). The left thoracic cavity was opened with a surgical scalpel, and the left anterior descending coronary artery (LAD) was ligated by 10-0 nylon suture to induce ischaemia for 30 min, then the suture was loosen for 60 min of reperfusion. After the I/R procedure, the chest was closed and the endotracheal intubation was removed. The sham group underwent the same surgery except the LAD ligation. After euthanasia, the blood and myocardial tissues were harvested.

**Color Doppler echocardiography**

Mice were intraperitoneally injected with 1% pentobarbital sodium (35 mg/kg) 48 h after reperfusion and then were fixed at a supine position to measure the left ventricular end-diastolic diameter (LVEDD), end-systolic dimension (LVESD), ejection fraction (LVEF), fractional shortening (LVFS), systolic pressure (LVSP), and end-diastolic pressure (LVEDP) by ultrasound Doppler examination.

**Detection of serum myocardial enzyme levels**

The determination of myocardial enzyme levels during MIRI has some intrinsic significance compared with the intuitive nature of microscopic analysis of myocardial sections. Myocardial enzyme is a general term for a variety of enzymes present in the myocardium, including aspartate aminotransferase, lactate dehydrogenase (LDH), creatine kinase (CK) and isoenzymes, and α-hydroxybutyrate dehydrogenase. The cardiac enzymes are released due to myocyte necrosis during MIRI, causing increased serum levels of cardiac en-
zymes. According to the instructions of relative cytotoxicity
detection commercial kits (Jining Industrial Co., Ltd., Shang-
hai, China), the serum levels of LDH and CK were determined
using spectrophotometry to measure the degree of myocar-
dial ischaemia or cardiomyocyte apoptosis.

Haematoxylin–eosin staining

Mouse hearts were harvested and fixed in 4% paraformalde-
hyde and then were dehydrated, paraffin-embedded, sec-
tioned, stained by haematoxylin and eosin (HE), and
observed under a microscope.

Masson staining

The staining was conducted as previously described, and
the sections were observed under a microscope.

Terminal deoxynucleotidyl transferase-mediated
deoxyuridine triphosphate nick end-labelling
staining

The prepared myocardial sections were used to assess cardio-
myocyte apoptosis using the terminal deoxynucleotidyl
transferase-mediated deoxyuridine triphosphate nick
end-labelling (TUNEL) kits. A light microscope was used for
observation, and TUNEL positive cells were counted.

Biochemical detection

Mouse myocardial tissues were made into homogenate,
which was centrifuged at 3773 g for 15 min to produce the
relative supernatant. The content of malondialdehyde
(MDA) and activities of catalase (CAT), glutathione peroxidase
(GSH-Px), and superoxide dismutase (SOD) were evaluated
using relative detection kits.

Reverse transcription quantitative polymerase
chain reaction

Total RNA in tissues was extracted by TRizol kits, purified
using RNeasy mini kits (Qiagen, MA, USA), and cDNA was syn-
thesized from total RNA by a PrimerScript RT Reagent kit
(TaKaRa, Shiga, Japan). miRNA from total RNA was reverse
transcribed using the Prime-Script miRNA cDNA Synthesis
Kit (TaKaRa). Real-time (RT)-PCR was performed with the
SYBR Green Premix Ex Taq II (TaKaRa), and data were
analysed by $2^{-\Delta\Delta Ct}$ method with glyceraldehyde phosphate
dehydrogenase (GAPDH) and U6 as the internal references.
The primers were shown in Supporting Information.

Western blot analysis

Total protein was extracted from tissues, which was lysed
by bicinchoninic acid kits and quantified. The proteins were
transferred onto membranes and conducted with gel elec-
trophoresis. The membranes were blocked for 1 h, incub-
bated with primary antibodies CCND1 and GAPDH (all
1:500 and from Abcam Inc., MA, USA) at 4°C overnight,
and incubated with relative secondary antibody for 2 h.
The enhanced chemiluminescent reagent (Pierce, IL, USA)
was used to analyse the membranes and the protein bands
were observed.

Dual luciferase reporter gene assay

XIST and CCND1 3’untranslated region (3’UTR) sequences
containing miR-340-5p binding site were synthesized and
subcloned to pGL4 luciferase reporter vector (Promega, WI,
USA), respectively, followed by construction of XIST and
CCND1 3’UTR wild-type (WT) and mutation (MUT) plasmids.
According to the manufacturer’s information, HEK239 cells
were co-transfected with constructed vectors together with
NC or miR-340-5p mimic using Lipofectamine 3000 (Thermo
Fisher) for 48 h. The luciferase activity was determined by
dual luciferase reporter gene detection kits (Promega) and
SpectraMax L (Molecular Devices, CA, USA).

RNA pull-down

HEK-293T cells were treated for 24 h with biotinylated miRNA
(200 nM). Next, the cells were gently rinsed two times with
PBS and then lysed for 10 min with ice-cold RNA pull-down ly-
sis buffer on ice. The obtained lysate was centrifuged, and the
supernatant that was aliquoted into 50 μL was used for subse-
quent input research. Afterward, streptavidin magnetic beads
(Thermo Scientific Fisher) were supplemented to the remain-
ing lysate, followed by incubation at room temperature and
addition of RNase-free bovine serum albumin (Sigma) and
yeast tRNA (Sigma). Subsequently, the mixture was incubated
for 3 h at 4°C. The beads were rinsed with ice-cold lysis buffer
and low-salt buffer for three times and lastly rinsed with high-
salt buffer to obtain a pure sample. At last, TRizol (Sigma) was
adopted for purifying the bound RNAs, and RT-qPCR was car-
ried out to analyze XIST expression.

Statistical analysis

All data analyses were conducted using SPSS 21.0 software
(IBM Corp., Armonk, NY, USA). The data were expressed as
mean ± standard deviation. The t-test was performed for
comparisons between two groups, analysis of variance
ANOVA) was used for comparisons among multiple groups, and Tukey’s post hoc test was used for pairwise comparisons after ANOVA. $P$ value < 0.05 was indicative of statistically significant difference.

**Results**

**Ischaemia–reperfusion induces mouse myocardial tissue injury**

Serum activities of LDH and CK were determined, and we found that I/R mice had higher LDH and CK activities than those in the sham group, indicating the formation of myocardial ischaemia (*Figure 1A*). Results of electrocardiogram suggested that LVEDD, LVESD, and LVEDP were increased while LVEF, LVFS, and LVSP were decreased in the I/R group versus the sham group (*Figure 1B–1D*). The HE staining showed that there were no characteristic pathological changes in mice of the sham group, while in the I/R group, there appeared congestion, haemorrhage, repairable inflammation, myocardial fibrosis, and even necrosis in mouse heart (*Figure 1E*). Moreover, in Masson staining, the normal cardiomyocytes were red while the fibrotic area was blue. We found that the blue area was nearly 0 in the sham group, while was increased in the I/R group (*Figure 1F*). TUNEL staining reflected that cardiomyocyte apoptosis was enhanced in I/R mice (*Figure 1G–1I*).

![Figure 1](image-url)

**Figure 1** I/R induces mouse myocardial tissue injury. (A) Detection of myocardial enzyme activity; (B–D) detection of haemodynamic indices; (E) representative images of HE staining; (F) representative images of Masson staining; (G) representative images of TUNEL staining; (H) mRNA expression of Bcl-2, Bax, and Caspase-3 assessed by RT-qPCR; (I–L) detection of oxidative stress-related factor levels; (M) levels of inflammatory factors; $n = 12$ in (A)–(D), $n = 6$ in (E)–(M); *$P < 0.05$ versus the sham group; the data were expressed as mean ± standard deviation, and the Student’s t-test was performed for comparisons between two groups.
Figure 2 XIST knockdown relieves MIRI and cardiomyocyte apoptosis. (A/B) XIST expression in mice detected using RT-qPCR; (C) detection of myocardial enzyme activity; (D–F) detection of cardiac blood flow parameters; (G) representative images of HE staining; (H) representative images of Masson staining; (I) representative images of TUNEL staining; (J) mRNA expression of Bcl-2, Bax, and Caspase-3 assessed by RT-qPCR; (K–N) detection of oxidative stress-related factor levels; (O) levels of inflammatory factors; n = 12 in (C)–(F), n = 6 in (A) and (B), (G)–(O); *P < 0.05 versus the sham group; #P < 0.05 versus the NC group; the data were expressed as mean ± standard deviation, and the t-test was performed for comparisons between two groups.
The apoptosis-related markers were detected as well and it was revealed that I/R mice had decreased B-cell lymphoma-2 (Bcl-2) level and increased Bcl-2-associated X (Bax) and Caspase-3 levels, indicating increased apoptosis (Figure 1H). The levels of oxidative stress-related factors and inflammatory factors were determined and it was discovered that the levels of MDA, interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) were increased whereas levels of SOD, CAT, and GSH-Px were decreased in I/R mice (Figure 1I–1M). The aforementioned findings implied that the I/R models were successfully established.

**XIST knockdown relieves myocardial ischaemia–reperfusion injury and cardiomyocyte apoptosis**

XIST expression in mouse myocardial tissues was detected and we found that it was overexpressed in I/R mice (Figure 2A). XIST was knocked down (Figure 2B) to observe its effect on MIRI, and results of our experiments indicated that XIST silencing suppressed myocardial enzyme activity, pathological changes, cardiomyocyte apoptosis, oxidative stress, and inflammatory response and promoted cardiac function in the I/R mice (Figure 2C–2O). These findings indicated that XIST knockdown relieved I/R-induced myocardial injury and cardiomyocyte apoptosis.

**XIST targets miR-340-5p**

It was predicated that there existed binding sites between XIST and miR-340-5p (Figure 3A), and it was further confirmed that the transfection of miR-340-5p mimic and XIST-WT suppressed the luciferase activity, which was not affected by transfection of miR-340-5p mimic and XIST-MUT (Figure 3B). Results of RNA pull-down assay indicated that XIST was enriched in the bio-miR-340-5p (Figure 3C), suggesting a direct binding relationship between XIST and miR-340-5p. Moreover, we found in RT-qPCR that miR-340-5p was down-regulated in I/R mice versus mice in the sham group, and miR-340-5p was up-regulated after XIST was knocked down (Figure 3D and 3E). These data showed that XIST negatively regulated miR-340-5p expression.

**MiR-340-5p reduction aggravates XIST-induced myocardial ischaemia–reperfusion injury**

MiR-340-5p was down-regulated to observe its role in XIST-induced MIRI, and we also found that the dual knockdown of miR-340-5p and XIST restored miR-340-5p expression (Figure 4A). It was discovered through a series of assays that miR-340-5p down-regulation aggravated myocardial enzyme activity, pathological changes, cardiomyocyte apoptosis, oxidative stress, and inflammatory response and...
promoted cardiac function in the I/R mice; the knockdown of XIST and miR-340-5p restored the levels of cardiac parameters to that in antagomir NC group in mice (Figure 4B–4N). The aforementioned data mirrored that miR-340-5p functioned in the downstream of XIST-induced MIRI regulatory axis.

**MiR-340-5p targets cyclin D1**

The binding sites between miR-340-5p and CCND1 were predicted (Figure 5A), and it was further confirmed that the co-transfection of miR-340-5p mimic and CCND1-WT inhibited the luciferase activity while that of miR-340-5p mimic and CCND1-MUT did not affect the luciferase activity (Figure 5B), indicating a direct interaction between miR-340-5p and CCND1. Furthermore, results of RT-qPCR and Western blot analysis reflected that CCND1 was up-regulated in I/R mice and its expression was negatively regulated by miR-340-5p (Figure 5C–5E). These findings suggested that CCND1 was a target gene of miR-340-5p.

**Cyclin D1 inhibition attenuates myocardial ischaemia–reperfusion injury**

MiR-340-5p was down-regulated to observe the impact of the functional axis of XIST, and we also found through
RT-qPCR and Western blot analysis that si-XIST down-regulated CCND1 (Figure 6A–6C). The outcomes of our experiments mirrored that the inhibition of CCND1 restrained myocardial enzyme activity, pathological changes, cardiomyocyte apoptosis, oxidative stress, and inflammatory response and improved cardiac function in the I/R mice (Figure 6D–6P). It can be concluded that XIST suppression ameliorated MIRI via targeting the miR-340-5p/CCND1 axis.

Discussion

While reperfusion is the main treatment of myocardial ischaemia in the setting of coronary occlusion, it can lead to serious myocardial damage through MIRI. We aimed to explore the effect of the XIST/miR-340-5p/CCND1 axis on the development of MIRI, and we found that XIST inhibition could up-regulate miR-340-5p to protect against the MIRI via reducing CCND1.

We assessed the expression of XIST in mouse myocardial tissues and found that the MIRI mice had higher XIST expression in comparison with the sham-operated ones. In line with this finding, a recent publication has indicated that the expression of XIST is increased in post-MI myocardial cells, and Zhou et al. have clarified that the expression of XIST is increased in post-MI myocardial cells. XIST was knocked down to figure out its role in the progression of MIRI, and the results of a series assays implied that the reduction of XIST promoted cardiac function and suppressed myocardial enzyme activity, ischaemia area, cardiomyocyte apoptosis, inflammatory response, and oxidative stress in MIRI mice. Consistent with our outcomes, it has been revealed that XIST silencing represses apoptosis of hypoxic cardiomyocytes in vitro and inhibits MI in a mouse model by regulating apoptosis-related proteins, Wang et al. have found that down-regulated XIST restrains inflammatory response in diabetic nephropathy, and decreased XIST expression has also been reported to attenuate oxidative stress and apoptosis in primary cultured rat hippocampal neurons. In addition, IncRNAs are known to serve as ceRNAs to sponge the miRNAs, and XIST has been identified to sponge miR-125b in hypoxia-induced cardiomyocyte injury and miR-133 in MIRI, while the binding between XIST and miR-340-5p still needs to be further explored.

MiR-340-5p expression was determined as well, and we found that it was down-regulated in myocardial tissues, and Zhou et al. have clarified that the expression of XIST is increased in post-MI myocardial cells. XIST was knocked down to figure out its role in the progression of MIRI, and the results of a series assays implied that the reduction of XIST promoted cardiac function and suppressed myocardial enzyme activity, ischaemia area, cardiomyocyte apoptosis, inflammatory response, and oxidative stress in MIRI mice. Consistent with our outcomes, it has been revealed that XIST silencing represses apoptosis of hypoxic cardiomyocytes in vitro and inhibits MI in a mouse model by regulating apoptosis-related proteins, Wang et al. have found that down-regulated XIST restrains inflammatory response in diabetic nephropathy, and decreased XIST expression has also been reported to attenuate oxidative stress and apoptosis in primary cultured rat hippocampal neurons. In addition, IncRNAs are known to serve as ceRNAs to sponge the miRNAs, and XIST has been identified to sponge miR-125b in hypoxia-induced cardiomyocyte injury and miR-133 in MIRI, while the binding between XIST and miR-340-5p still needs to be further explored.

MiR-340-5p expression was determined as well, and we found that it was down-regulated in myocardial tissues from MIRI mice. Consistently, Li et al. have unveiled that miR-340-5p was down-regulated after I/R in MI mice and H/R-induced cardiomyocytes, and it has been reported that miR-340-5p...
is poorly expressed in oxygen–glucose deprivation/reoxygenation (OGDR)-stimulated neurons. The expression of miR-340-5p was altered as well to investigate its impact on MIRI, and we discovered that miR-340-5p down-regulation impaired cardiac function and promoted myocardial enzyme activity, ischaemia area, cardiomyocyte apoptosis, inflammatory response, and oxidative stress in MIRI mice. Similarly, it has been clarified that miR-340-5p restricts H/R-induced apoptosis and oxidative stress in cardiomyocytes, and Qian et al. have elucidated that the up-regulation of miR-340-5p ameliorates spinal cord injury-induced neuroinflammation and apoptosis. In addition, Zheng et al. have illustrated that miR-340-5p alleviates OGDR-induced neuronal injury. Moreover, we confirmed using bioinformatic predic-
tion and dual luciferase reporter gene assay that miR-340-5p targeted CCND1 in MIRI. In accordance with our finding, it has been reported that there exists a targeting relationship between miR-340-5p and CCND1 in multiple myeloma.\(^3\) CCND1 expression was also assessed and it was revealed that in rats underwent I/R and partial hepatectomy injury, CCND1 showed an increased expression level in transplanted grafts with macrosteatosis.\(^3\) Additionally, we found through the gain-of-function and loss-of-function assays that CCND1 inhibition could relieve the MIRI. Similar to our result, Pei et al. have identified that the down-regulation of CCND1 protects against cerebral ischaemia in a middle cerebral artery occlusion model.\(^3\)

In conclusion, we discovered that the silencing of XIST up-regulates miR-340-5p to alleviate the MIRI in a mouse model through inhibiting CCND1. This study may provide novel biomarkers for MIRI treatment. However, the study sample can be further expanded in future studies to diminish the data errors in experimental results.

**Conflict of interest**

The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Primer sequence used for q-PCR.

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