Nucleolin protects the heart from ischaemia–reperfusion injury by up-regulating heat shock protein 32

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Received 28 January 2013; revised 26 March 2013; accepted 1 April 2013; online publish-ahead-of-print 16 April 2013

Time for primary review: 18 days

Aims Nucleolin plays important roles in a variety of cellular processes. In this study, we aimed to investigate the role of nucleolin in cardiac ischaemia–reperfusion (I–R) injury.

Methods and results We investigated the expression pattern of nucleolin in hearts subjected to I–R, or neonatal rat cardiomyocytes subjected to hypoxia–re-oxygenation. We found that nucleolin expression was significantly down-regulated and the cleaved protein was present, both in vivo and in vitro. Gene transfection and RNA interference approaches were employed in cardiomyocytes to investigate the function of nucleolin. Over-expression of nucleolin was cytoprotective, whereas nucleolin ablation enhanced both hypoxia- and H2O2-induced cardiomyocyte death. Furthermore, transgenic mice with cardi-specific over-expression of nucleolin were resistant to I–R injury as indicated by decreased cellular necrosis and decreased infarct size. The cardio-protective roles of nucleolin in cardiomyocytes, are attributable to the interaction of nucleolin with the mRNA of heat shock protein 32 (Hsp32), resulting in an increase of Hsp32 mRNA stability, and subsequent up-regulation of Hsp32 expression. The selective Hsp32 inhibitor, zinc protoporphyrin-IX, abrogated the cardiac protection mediated by nucleolin.

Conclusion This study has demonstrated that nucleolin is involved in the regulation of I–R-induced cardiac injury and dysfunction via the regulation of Hsp32, and may be a novel therapeutic target for ischaemic heart diseases.

Keywords Nucleolin • Cardioprotection • Ischaemia–reperfusion injury • mRNA stability • Hsp32

1. Introduction

Ischaemic heart disease is the leading cause of mortality, worldwide, and current projections indicate this trend to continue.1 Myocardial ischaemia, with subsequent progression to infarction is one of the most common causes of heart failure. While treatment of myocardial ischaemia aims to reduce morbidity and mortality by restoring tissue blood flow, it may, in fact, result in greater cellular damage. This phenomenon is known as ischaemia–reperfusion (I–R) injury. The fundamental mechanisms of myocardial I–R injury include calcium overload, increased generation of reactive oxygen species, apoptosis, and disregulated gene expression.2,3 Recently, some endogenous cardio-protective proteins, such as heat shock proteins, mitsugumin 53, hepatocyte growth factor, have been found.4,5

Nucleolin is the most abundant RNA-binding protein in the nucleolus. Its major functions are binding and transporting rRNAs, and regulating the assembly of ribosomes.6 Nucleolin is a multifunctional shuttle protein and additionally, plays varied roles in the replication and the infection of adeno-associated virus,7 angiogenesis,8 tumour growth,9 and apoptosis.10,11 Cell culture studies have shown that nucleolin is stable in proliferating cells, but undergoes self-cleavage in quiescent cells.12 Our previous studies have also shown that oxidative stress induces nucleolin cleavage and apoptosis in C2C12 myogenic cells13 and human umbilical vein endothelial cells.14 However, the potential role of nucleolin in the mechanism of myocardial injury is less well studied.

In this study, we investigated the expression pattern of nucleolin in hearts subjected to I–R in vivo. We observed that nucleolin expression was consistently decreased and protein cleavage was increased in rat...
hearts subjected to I–R. Over-expression of nucleolin was cytoprotective, whereas nucleolin ablation enhanced hypoxia- or H$_2$O$_2$-induced cardiomyocyte death. Using DNA arrays, bioinformatic analysis, and in vitro luciferase/green fluorescent protein reporter assays, we identified heat shock protein 32 (Hsp32) as an authentic target for nucleolin.

2. Methods

2.1 Animals

Male Sprague–Dawley rats and Neonatal Wistar rats (1–3 days), used as a source of primary cardiomyocytes, were purchased from the Animal Resource Center of Central South University. Transgenic (TG) mice with cardiac myocyte-specific over-expression of nucleolin were produced by microinjection of an expression vector fragment containing the full-length nucleolin cDNA (pcDNA3.1-Nuc; 2.2 kb) under the regulation of α-myosin heavy chain promoter (Model Animal Research Institute, Nanjing University). TG mice were backcrossed for >10 generations into the C57BL/6 background. Transgenic-negative (TGneg) siblings served as controls.

The investigation followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised in 1996) and the Animal Care and Use Committee of Central South University. The study protocol was approved by the Ethics Committee of Central South University, Medical Institution Animal Care and Research Advisory Committee (Changsha, China).

2.2 Cardiac myocyte culture and treatment

Primary neonatal rat cardiomyocytes were isolated from the hearts of 1–3-day-old Wistar rats by trypsin digestion as previously described.15 Hypoxia–reoxygenation (H–R) injury was achieved by placing the cells in a hypoxia chamber filled with 5% CO$_2$ and 95% N$_2$ at 37°C in a glucose-free Dulbecco’s modified Eagle medium (DMEM), and the cells were re-oxygenated with 5% CO$_2$ and 95% O$_2$ for 3–12 h in DMEM containing 10% serum and 5 mM glucose.15 Cellular oxidative stress was induced by exposure to hydrogen peroxide (H$_2$O$_2$; 0.5 mM) at different time points. The concentration of H$_2$O$_2$ was determined spectrophotometrically at 240 nm as described.15 A 10 mM stock solution was prepared and diluted into the medium.

2.3 Animal models of cardiac I–R injury

Cardiac I–R injury in animals was performed as described previously.18 In brief, the rats were anaesthetized with pentobarbitol (70–80 mg/kg ip) every 2 h. Under sterile conditions, the heart was exposed through a left thoracotomy in the fourth intercostal space. I–R was achieved by 30 min occlusion of the left anterior descending coronary artery (LAD), followed by 2–24 h reperfusion. The mice received a single ip injection of PBS (vehicle) or zinc protoporphyrin-IX [ZnPP, an haeme oxygenase 1 (HO-1) inhibitor; Sigma-Aldrich, St Louis, MO, USA] at a dose of 50 μmol/kg, and then underwent I–R 12 h later. The maximal slope of systolic pressure increment (+dP/dt) and diastolic pressure decrement (−dP/dt) was measured in mice anaesthetized with intraperitoneal injections of pentobarbitol (70 mg/kg) according to a previously described method.19 After the haemodynamic measurements were made, the mice were sacrificed, the blood was collected, and the hearts were excised. Creatine kinase (CK) release was measured using a commercially available kit (Sigma).

2.4 Measurement of myocardial infarct size

After reperfusion, the animals were anaesthetized, the LAD was re-occluded and 0.6 mL of 10% Evans blue dye (Sigma) was slowly injected into the aorta to define the ischaemic zone. The myocardial ischaemic area at risk (IAR) was identified as the region lacking blue staining. Serial, short-axis 1 mm thick sections were cut and incubated in 2% triphenyltetrazolium chloride (TTC, Sigma) at 37°C for 10 min for demarcation of viable and non-viable myocardium. Positive TTC staining was in red colour, and the infarcted area was pale. Images were analysed by Image-Pro Plus, and infarct size was expressed as a percentage of IAR (% IAR).

2.5 Cell viability assay and western blots

The caspase activity assay, cell viability assay, and western blots were performed as described previously.15,20

2.6 Measurement of Hsp32 mRNA stability

Cardiomyocytes were treated with either H$_2$O$_2$ or H–R injury, for the indicated time periods and the cells were then incubated with either 0.5% ethanol or 5 μg/mL actinomycin D in 0.5% ethanol. Aliquots were removed from the cultures at 30 min intervals over a 3 h time course. Actinomycin D at this concentration induced no DNA fragmentation during this period. At the indicated time points, 2 × 10$^5$ cells were harvested and total RNA was isolated using the QIAGEN RNeasy kit. Real-time PCR amplification of the cdNA pools was carried out with the following primer pairs: Hsp32 (5’-GAGCGAAAACAGCAGACAC-3’ and 5’-TGGCCAATCTTGTCAAGACC-3’) and GAPDH (5’-ACCCACGTCCATGCCATCAC-3’ and 5’-TCCACCAACCTTGTGCTGTA-3’).

2.7 Synthesis and purification of biotin-labelled RNA probe for Hsp32

The probe was synthesized and labelled in one step using the MAXiScript RNA Synthesis Kit according to the manufacturer’s instructions.

2.8 RNA-electrophoretic mobility shift assay

For in vitro binding of RNA to proteins, 10 μ binding buffer [500 mM KCl, 50 mM glycerol, 1% Non-ident P-40, 10 mM MgCl$_2$, 10 mM dithiothreitol, and 100 mM Tris—HCl (pH 8.0), 5 mg/mL heparin, and 5 mg/mL yeast tRNA] was used. The 20 μL binding reactions contained 1 × binding buffer, 35 μg S10 extracts, 2 μL RNase OUT (2 units/μL), and 50 pM biotin-labelled RNA probe. For the super-shift assay, anti-nucleolin or anti-GAPDH antibody (0.4 per 35 μg total protein) was added after the formation of RNA–protein complexes. All the binding reactions were incubated at room temperature for 30 min, and then 2 μL protein-loading buffer (50% glycerol, 0.02% bromophenol blue, and 0.02% dimethylaniline) was added. Samples were then separated by gel electrophoresis, transferred onto membrane, cross-linked, and biotin-labelled RNA was detected with the LightShift chemiluminescent EMSA kit.

2.9 Coimmunoprecipitation of nucleolin and Hsp32 mRNA

Heart tissues from TGneg and TG (Line #86) mice were homogenized in 1 mL lysis buffer containing protease inhibitors (10 μL/R0.1 g tissue weight, Sigma, USA). Soluble proteins in the lysate were collected after centrifugation at 12 000 g, 4°C for 15 min. An aliquot (500 μL) of tissue lysate was pre-cleared by incubation with 200 μL of washed protein A/G beads on ice for 60 min followed by centrifugation. Ten micrograms of anti-nucleolin antibody were added to the pre-cleared lysate and incubated at 4°C for 1 h, followed by the addition of 200 μL protein A/G beads, the lysate was incubated at 4°C, with shaking, and the immune complexes separated by centrifugation at 10 000 g for 30 s at 4°C. RNA was extracted from the immunoprecipitate and cdNA was prepared by reverse-transcription with random primers and subjected to PCR to amplify the target genes.

2.10 Hsp32 3′ UTR-dependent reporter gene assays

Cells cultured in 96-well plates were co-transfected with the Firefly-Luciferase pGL3-promoter vector (pGL3p; Promega) or pGL3p containing the 3′ UTR of Hsp32, as well as the Renilla-Luciferase pRL-TK vector (ratio
1:5) using the Lipofectamine LTX and Plus Reagent (Invitrogen Corporation) according to the manufacturer’s protocol. After 6 h, the transfection medium was removed and time for measurements started with the addition of fresh medium. The luciferase activity was detected using the Dual-Glo Luciferase Assay System (Promega) and a luminometer (Labsystems Luminoskan RS) programmed with individual software (Luminoscan RII, Ralf Mrowka). The co-transfection with the RenillaLuciferase expression vector served as a control.

2.11 Statistical analysis
All data are presented as mean ± SEM. The significance of differences in mean values for haemodynamics, infarct size, and nucleolin protein expression among groups was evaluated by one-way ANOVA, followed by post hoc testing (Newman–Keuls test). Differences between the two groups were evaluated by Student’s t-test, and P < 0.05 was considered as statistically significant.

3. Results

3.1 I–R-, H–R-, or H₂O₂-induced myocardial damage is accompanied with nucleolin cleavage and down-regulation
To investigate whether there is a correlation between nucleolin expression and cardiomyocyte integrity, we have used a rat in vivo myocardial I–R injury model (30 min ischaemia followed by reperfusion for the indicated times). I–R causes CK release and the subsequent development of a myocardial infarction in vivo (Supplementary material online, Figure S1A and B). Concurrently, I–R injury significantly reduces nucleolin expression at the mRNA level following 30 min of in vivo left coronary artery ischaemia and 30–6 h of reperfusion (Figure 1A). Importantly, I–R injury causes a significant decrease in the expression of the 110 kDa nucleolin protein in myocardial tissue and a subsequent increase in the expression of the 80 kDa fragment in a time-dependent manner (Figure 1B). These in vivo data suggest that I–R-induced cardiac injury is likely related to I–R-mediated down-regulation and cleavage of nucleolin.

Further, the cultured primary neonatal rat cardiomyocytes were treated with H–R or H₂O₂ injury. As shown in Figure 1C, untreated cardiomyocytes express only full-length nucleolin (apparent mass of 110 kDa). In cardiomyocytes treated with H–R, the amount of full-length nucleolin progressively decreases, while the 80 kDa proteolytic fragment increases in a time-dependent manner. The change of nucleolin expression correlates with the decrease in myocyte viability over the same time course (Figure 1E and Supplementary material online, Figure S2A). Similarly, oxidative stress with H₂O₂ causes a significant decrease in the expression of the 110 kDa nucleolin protein and a subsequent increase in the expression of the 80 kDa fragment in a concentration-dependent manner (Figure 1B). Concurrently, H₂O₂ treatment triggers cell death in a concentration-dependent manner (Figure 1F and Supplementary material online, Figure S2B). These data indicate that H–R- and H₂O₂-induced cardiomyocyte deaths are closely accompanied by the down-regulation and cleavage of nucleolin.

3.2 Cardiac myocyte-specific nucleolin TG mice are protected from I–R injury
In the above experiment, we found that treatment with H–R, H₂O₂, or I–R insults could result in the down-regulation and cleavage of nucleolin; it is, therefore, rational to propose that nucleolin may have a protective effect on cardiomyocyte integrity. To test this hypothesis, we generated TG mice with cardiac myocyte-specific over-expression of nucleolin under the control of the α-myosin heavy chain promoter. Forty-eight mouse lines were generated, 5 of which were positive for the transgene. Two of these five lines (Line #52 and Line #86) show 2.3- and 5.5-fold elevations in nucleolin protein expression, respectively, compared with control animals (Figure 2A).

Under normal physiological conditions, there were no morphological or functional differences between TG neg and TG mice (data not shown). However, I–R-induced myocardial damage is markedly alleviated in the heart of TG mice. The appearance of CK in the serum after I–R injury provides a direct index of damage to the cardiomyocytes in the injured heart (Figure 2B). Furthermore, TG mice exposed to 30 min of cardiac ischaemia and 24 h of reperfusion show a substantial reduction in infarct size compared with control mice (P < 0.01; Figure 2C and Supplementary material online, Figure S3).

Haemodynamic measurements were recorded to determine the protective effect of nucleolin on LV function in mice. There was no significant difference in the baseline haemodynamic indices between the TG neg and TG groups, but TG neg mice treated with I–R had a significant decline in haemodynamic indices compared with the sham group in +dp/dt (3321 vs. 4996 mmHg/s, P < 0.05; Figure 2D) and −dp/dt (3012 vs. 4638 mmHg/s, P < 0.05; Figure 2E). However, the I–R-induced degeneration of +dp/dt and −dp/dt was significantly alleviated in TG mice compared with the TG neg mice (P < 0.05). Together, these results indicate that over-expression of nucleolin provides protection for I–R-induced myocardial injury.

3.3 The effect of nucleolin on cell death induced by H–R or H₂O₂
To further establish the causative relationship between nucleolin expression and cardiomyocyte viability, we have acutely up-regulated or down-regulated nucleolin expression in cardiomyocytes. Transient transfection (30–50% efficiency) of pcDNA3.1-Nuc in isolated rat cardiomyocytes was used to overexpress nucleolin. Western blot of cell lysates (Figure 3A) shows that nucleolin is overexpressed in pcDNA3.1-Nuc-transfected cells, compared with vector control cells. Nucleolin over-expression profoundly reduces H–R- or H₂O₂-induced cell death as demonstrated by two independent readouts: lactate dehydrogenase (LDH) release and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Figure 3B and C and Supplementary material online, Figure S4A and B). In contrast, siRNA-mediated knockdown of nucleolin (psiRNA-Nuc) effectively reduced nucleolin expression (Figure 3D) and exacerbated H–R- or H₂O₂-induced cell death, as indicated by LDH release and MTT assays (Figure 3E and F and Supplementary material online, Figure S4C and D).

3.4 Over-expression of nucleolin up-regulates the expression of Hsp32 in vivo and in vitro
To elucidate the potential cellular mechanism underlying nucleolin-mediated cardioprotection, we screened nucleolin target genes by microarray analysis after co-immunoprecipitation of protein and RNA with anti-nucleolin antibody in nucleolin overexpressed cardiomyocytes (Supplementary material online, Table S1). Among these genes, Hsp32 (also known as HO-1) mRNA is identified as a putative nucleolin target as it contains the putative nucleolin-binding sequence.

We next determined whether nucleolin affects Hsp32 expression in nucleolin TG hearts. As shown in Figure 4A, Hsp32 levels show a 4.35 ±
0.22-fold elevation in Line #52 and 6.61 ± 0.51-fold elevation in Line #86 relative to TGneg hearts, respectively. Similarly, in cardiomyocytes, over-expression of nucleolin (pcDNA3.1-Nuc) significantly elevates the levels of Hsp32 protein expression 5.21 ± 0.56-fold over the vector control (n = 8; P, 0.05). In contrast, nucleolin ablation markedly down-regulates the protein levels of Hsp32 in the psiRNA-Nuc-transfected cardiomyocytes (Figure 4B). These results indicate that nucleolin regulates Hsp32 expression, both in vitro and in vivo.

### 3.5 Binding of nucleolin to Hsp32 mRNA in vivo and in vitro

To detect the potential binding of nucleolin to Hsp32 mRNA, we performed RNA–protein coimmunoprecipitation (co-IP). S100 fractions were extracted from the hearts of TGneg and TG mice (Line #86), and the nucleolin–Hsp32 mRNA complexes were isolated by co-IP with the anti-nucleolin monoclonal antibody. The immunoprecipitates were analysed by RT–PCR. As shown in Figure 5A, a small amount of Hsp32 mRNA coimmunoprecipitated with nucleolin from TGneg hearts, but significantly more is obtained from TG hearts (Line #86). This result suggests that binding of nucleolin to Hsp32 mRNA is increased in TG (Line #86) over-expressing nucleolin. The binding between Hsp32 mRNA and nucleolin is specific, since the anti-nucleolin antibody fails to co-IP GAPDH mRNA fragment in the control. Western blot analysis reveals the amount of nucleolin in the immunoprecipitates, further confirming the specificity of the antibody (data not shown).

The RNA-electrophoretic mobility shift assay (RNA-EMSA) is used to detect binding of a biotin-labelled Hsp32 mRNA 3′ UTR fragment (the
Figure 2  Nucleolin over-expression in heart attenuates I–R-induced injury in vivo. (A) Western blots showed that nucleolin was overexpressed in the TG hearts. * \( P < 0.01 \) vs. TGneg group (n = 4). (B) Change of CK concentration in the serum from TGneg and TG (Line #52 and Line #86) mice subjected to 30-min ischaemia and followed by 4 h reperfusion (\( P < 0.05 \) vs. TGneg + I–R group; \( P < 0.01 \) vs. TGneg + I–R group, n = 8). (C) The infarct size in the hearts of TGneg and TG (Line #86) mice subjected to I–R (\( P < 0.01 \) vs. TGneg + I–R group, n = 8). (D and E) The maximal slope of systolic (D) and diastolic (E) pressure decrement were observed (\( P < 0.05 \) vs. TGneg + sham group; \( P < 0.01 \) vs. TGneg + I–R group, n = 8). TGneg, TG-positive animals; TG, TG-positive animals.
probe) to nucleolin. S100 fractions were extracted from the hearts of TGneg and TG mice (Line #52 and Line #86). As shown in Figure 5B, a shifted probe band is detected in protein extract from TGneg hearts, indicating the basal level of nucleolin binding to Hsp32 mRNA 3′ UTR. Nucleolin over-expression (Line #52 and Line #86) results in a significantly increased band intensity. Adding the anti-nucleolin antibody to the binding reaction results in a new super-shifted band of higher molecular weight. A control antibody against GAPDH fails to react with the shift complex. These results confirm that nucleolin specifically binds the 3′ UTR of Hsp32 mRNA in vitro.

3.6 Nucleolin up-regulates the stability of Hsp32 mRNA through interacting with 3′ UTR of Hsp32

The present study has further investigated whether nucleolin regulates Hsp32 expression through regulation of its mRNA stability. After transfection with pcDNA3.1-Nuc for 48 h, cells are treated with actinomycin D (5 μg/mL), and the decay of Hsp32 mRNA is assayed. Compared with the control group, over-expression of nucleolin slows down the degradation of Hsp32 mRNA, i.e. increases Hsp32 mRNA stability (Figure 5C).
To further investigate whether nucleolin regulates Hsp32 mRNA expression through interacting with 3′ UTR of Hsp32 mRNA, we use pGL3p and the same vector containing Hsp32 mRNA 3′ UTR (pGL3p–Hsp32), in which the 3′ UTR of luciferase gene has been replaced with the specific 3′ UTR of Hsp32 mRNA. The transcription rate is controlled by the constitutive SV40 promoter. In this experiment, differences in luciferase activity are dependent upon a post-transcriptional control mechanism, mediated by the 3′ UTR, which involve mRNA stability and translational efficiency. Luciferase activity from the control vector (pGL3p) shows no change upon over-expression of nucleolin, whereas the luciferase activity in pGL3p–Hsp32 transfected cells is significantly enhanced. As shown in Figure 5D, such post-transcriptional control depends on the 3′ UTR of Hsp32 mRNA.

### 3.7 The Hsp32 selective inhibitor ZnPP negates the protective effect of nucleolin

To determine whether the Hsp32 expression mediates the protective effect of nucleolin, mice were treated with the ZnPP (an Hsp32 selective inhibitor; 50 μmol/kg) for 12 h prior to in vivo myocardial I–R injury. As shown as in Figure 6A, in response to myocardial I–R injury, nucleolin over-expressing mice (Line #86) demonstrated a significant decrease in infarct size per area-at-risk and CK release; however, pre-treatment of nucleolin over-expressing mice with ZnPP negated the myocardial protection conveyed by nucleolin over-expression. Similarly, +dP/dt\textsubscript{max} was elevated in the nucleolin over-expressing mice (TG) compared with controls (TG\textsubscript{neg}) after myocardial I–R injury. However, ZnPP pre-treatment alleviated the magnitude of +dP/dt and −dP/dt in the TG group (Figure 6B and C).

### 4. Discussion

The present study provides multiple lines of evidence to define nucleolin as an important myocardial protection protein by up-regulating Hsp32. First, over-expression of nucleolin protects cardiomyocytes against H–R- and oxidative stress-induced cell death. Secondly, nucleolin-ablated cardiomyocytes become susceptible to the injury induced by H–R and H\textsubscript{2}O\textsubscript{2}. Thirdly, the hearts from mice expressing cardiomyocyte-specific nucleolin transgene are more resistant to I–R injury. Fourthly, nucleolin stabilizes Hsp32 mRNAs by binding to its 3′ UTRs and up-regulates Hsp32 expression in vivo and in vitro. Finally, ZnPP, a selective inhibitor of Hsp32, negates the protective effect of nucleolin.

The precise molecular mechanism by which nucleolin in myocytes protects the heart from I–R injury remains to be determined. Nucleolin is an RNA-binding protein containing four, centrally located, consensus RNA-binding domains (CS-RBDs). These CS-RBDs specifically bind to pre-rRNA-splicing elements ‘(T/G)CCCG(A/G)’ in pre-rRNA and promote splicing to generate mature rRNA. Nucleolin is involved in several aspects of DNA metabolism, and participates extensively in RNA regulatory mechanisms, including transcription, ribosome assembly, mRNA stability and translation, and microRNA processing.21 Recent studies have shown that nucleolin binds various mRNA molecules to regulate mRNA stability, and subsequent protein expression of many genes, such as IL-2, bcl-2, GADD45\textsubscript{a}, and P53.22–26 We first screened nucleolin target genes using microarray analysis after co-IP of protein and RNA with anti-nucleolin antibody to identify the genes that contain the nucleolin-binding sequence in their mRNAs. We found that the 3′ UTR of Hsp32 mRNA contains a consensus nucleolin-binding sequence and is likely a nucleolin target gene.

Hsp32, also known as HO-1, is the rate-limiting enzyme catalysing haeme degradation to carbon monoxide, free iron, and biliverdin. These end-products are responsible for much of the biological activity of HO-1, including anti-inflammatory, anti-apoptotic, antioxidant effects, and the regulation of complement activation.27,28 Hsp32 is normally expressed at low levels in most tissues/organs except for spleen; however, it is highly inducible in response to a variety of stimuli, such as ischaemia, I–R, high-oxygen, or hypoxia.29 Hsp32 has been suggested to be a critical component of the stress response in the cell.
Hsp32 is documented to have cytoprotective effects against oxidative injury in mice. It has been shown that I–R substantially enhances Hsp32 expression in the porcine heart, suggesting a potential role of Hsp32 in the defence against pathophysiological stress. Jancsó G et al. observed the up-regulation and an important role of Hsp32 in the delayed phase myocardial preconditioning. The absence of Hsp32 exacerbates I–R-induced myocardial damage. In the present report, we have shown that nucleolin induces Hsp32 gene expression in hearts and in cardiomyocytes. However, the mechanisms of nucleolin-dependent Hsp32 up-regulation in cardiomyocytes are still unknown.

Hsp32 gene expression is regulated mainly at the transcriptional level. Transcription factors, such as NF-κB, AP-1, NF-E2-related factor 2, and hypoxia-inducible factor-1, bind to the promoter region of the gene, activate Hsp32 transcription, and increase the protein expression. In addition, Hsp32 can be regulated at the post-transcriptional level through mRNA stability. Nitric oxide (NO) has been shown to stabilize Hsp32 mRNA in human fibroblast cells IMR-90. Simvastatin induced the up-regulation of haeme oxygenase-1 via mRNA stabilization in human endothelial cells. Then, Kneten et al. observed that activation of PPARγ in response to oxidative stress stabilized Hsp32 mRNA.

Figure 5 Nucleolin increases the Hsp32 mRNA stability by binding to Hsp32 mRNA in vivo and in vitro. (A) IP-RT–PCR analysis demonstrated the binding of nucleolin with Hsp32 mRNA in TGneg or TG (Line #86) hearts. Representative of three-independent experiments. Input: positive control; IgG: negative control; (B) RNA-EMSA demonstrated the binding of nucleolin to Hsp32 3′ UTR. (C) Effect of nucleolin on Hsp32 mRNA stability. Cardiomyocytes were transfected with pcDNA3.1 vector or pcDNA3.1-Nuc and then incubated with actinomycin D (5 μg/mL) for various time (0, 0.5, 1, 2, and 3 h). The mRNA levels of Hsp32 were determined by real-time quantitative RT–PCR. Vect: transfected with pcDNA3.1 plasmid; Nuc: transfected with pcDNA3.1-Nuc plasmid. *P < 0.05 vs. Vect group; **P < 0.01 vs. Vect group (n = 6). (D) The effect of nucleolin on Hsp32 mRNA stability depended on the 3′ UTR of Hsp32 as demonstrated by the luciferase reporter assay.
prevention and treatment of myocardial ischaemia and perhaps provide ideas for clinical applications of nucleolin.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

**Acknowledgements**

We thank Daniel R. McMillan (UT Southwestern Medical Center) for his critical reading and revision.

**Conflict of interest:** none declared.

**Funding**

This work was supported by the grants from National Basic Research Program of China (2007CB512007) and The National Natural Science Foundation of China (81170113; 81071556).

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