IntroductIon

Nucleolin (NCL) accounts for 10% of all the nuclear proteins and is found in the nucleoplasm, cytoplasm, and cell membrane.\(^1\)\(^2\) As an abundant multifunctional shuttle protein, the primary function of NCL is to bind and transport ribosomal RNAs (rRNAs) that regulate ribosomal assembly and cell proliferation.\(^2\)\(^3\) Recent reports have shown that NCL is involved in the occurrence and development of several cancers, and it can be used as a promising biomarker during virus infection.\(^4\)\(^5\) NCL is also an important regulator of posttranscriptional RNA stability and translation efficiency, and NCL overexpression reduces apoptosis by regulating mRNA stability of apoptotic genes, such as \(Bcl-2\).\(^6\)-\(^8\) NCL also plays an essential role in maintaining the self-renewal capacity of embryonic stem cells by suppressing a p53-dependent pathway.\(^9\) Furthermore, our previous study demonstrated that NCL stabilizes and increases heat-shock protein (Hsp) 32 and Hsp70 expression during myocardial ischemic preconditioning.\(^10\)

MicroRNA Profiling of Transgenic Mice with Myocardial Overexpression of Nucleolin

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Abstract

Background: Nucleolin (NCL) is the most abundant RNA-binding protein in the cell nucleolus and plays an important role in chromatin stability, ribosome assembly, ribosomal RNA maturation, ribosomal DNA transcription, nucleocytoplasmic transport, and regulation of RNA stability and translation efficiency. In addition to its anti-apoptotic properties, the underlying mechanisms associated with NCL-related roles in different cellular processes remain unclear. In this study, the effect of NCL on microRNA (miRNA) expression was evaluated by generating transgenic mice with myocardial overexpression of NCL and by analyzing microarrays of mature and precursor miRNAs from mice.

Methods: Using microinjection of alpha-MyHC clone 26-NCL plasmids, we generated transgenic mice with myocardial overexpression of NCL firstly, and then mature and precursor miRNAs expression profiles were analyzed in NCL transgenic mice (\(n = 3\)) and wild-type (WT) mice (\(n = 3\)) by miRNA microarrays. Statistical Package for the Social Sciences version 16.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform Student’s t-test, and statistical significance was determined at \(P < 0.05\).

Results: Several miRNAs were found to be differentially expressed, of which 11 were upregulated and 4 were downregulated in transgenic mice with myocardial overexpression of NCL compared to those in WT mice. Several differentially expressed miRNAs were subsequently confirmed and quantified by real-time quantitative reverse transcription-polymerase chain reaction. Bioinformatics analysis was used for the prediction of miRNA targets. Furthermore, in vitro experiments showed that NCL regulated miR-21 expression following hydrogen peroxide preconditioning.

Conclusions: Myocardial-protection mechanisms exerted by NCL might be mediated by the miRNAs identified in this study.

Key words: MicroRNA microarray; Myocardium; Nucleolin; Transgenic mice

INTRODUCTION

Nucleolin (NCL) accounts for 10% of all the nuclear proteins and is found in the nucleoplasm, cytoplasm, and cell membrane.\(^1\)\(^2\) As an abundant multifunctional shuttle protein, the primary function of NCL is to bind and transport ribosomal RNAs (rRNAs) that regulate ribosomal assembly and cell proliferation.\(^2\)\(^3\) Recent reports have shown that NCL is involved in the occurrence and development of several cancers, and it can be used as a promising biomarker during virus infection.\(^4\)\(^5\) NCL is also an important regulator of posttranscriptional RNA stability and translation efficiency, and NCL overexpression reduces apoptosis by regulating mRNA stability of apoptotic genes, such as \(Bcl-2\).\(^6\)-\(^8\) NCL also plays an essential role in maintaining the self-renewal capacity of embryonic stem cells by suppressing a p53-dependent pathway.\(^9\) Furthermore, our previous study demonstrated that NCL stabilizes and increases heat-shock protein (Hsp) 32 and Hsp70 expression during myocardial ischemic preconditioning.\(^10\)
NCL is able to protect the heart from ischemia-reperfusion (IR) injury by upregulating Hsp32, and NCL expression confers pro-angiogenic effects during the recovery of heat-denatured human umbilical vein endothelial cells.[12] These results suggest that NCL may play an important role in regulating various biological processes, including apoptosis, oxidative stress, inflammation, angiogenesis, and other pathophysiological processes.

Several recent studies revealed that NCL was actively involved in mediating microRNA (miRNA)-directed mRNA deadenylation and subsequent translation of mRNAs encoding a number of important genes.[13-16] MiRNAs are small noncoding RNA molecules that regulate gene expression at the posttranscriptional level through base pairing at either the 3’-untranslated region (3’-UTR) or the 5’-UTR of target mRNAs.[8]

Given the importance of NCL in several physiological processes involving mediation of miRNA regulation of gene transcription and translation in other types of cells and tissues, it is likely that NCL is involved in mediating similar functions in cardiocytes. Our recent study demonstrated NCL involvement in regulating IR-induced cardiac injury and dysfunction by upregulating Hsp32 expression.[11] To expand on these findings and determine the specific functions of NCL in cardiocytes, we hypothesized that NCL regulated IR-induced cardiac injury and dysfunction by controlling the expression of miRNAs involved in posttranscriptional regulation of important genes in cardiocytes. To further examine NCL-related mechanisms involved in myocardial protection against IR injury in vivo, we investigated the roles of NCL in the regulation of miRNAs in the cardiocytes. We generated a cardiac-specific transgenic mouse model that overexpressed NCL and compared miRNA gene-expression profiles between transgenic mice with myocardial overexpression of NCL and wild-type (WT) mice.

**METHODS**

**Ethical approval**

The animal protocol was approved by the Animal Research Center of Nanjing University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Construction of the alpha-MyHc clone 26-nucleolin plasmids**

To amplify full-length NCL, cDNA from mouse somatic cells was used as a template, and the Pfu DNA polymerase was used with the following primers: forward, 5’-CCGCTCGAGATGGTGAAGCTCGCAAAG-3’; reverse, 5’-CGGAATTCTTCAAACTTCGTCTTTCC-3’ (underlined regions indicate XhoI and EcoRI restriction sites, respectively). Polymerase chain reaction (PCR) was conducted at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2.5 min. PCR products were double digested with XhoI and EcoRI, followed by insertion into the predigested alpha-MyHc clone 26 to produce alpha-MyHc clone 26-NCL. The recombinant plasmid was verified by DNA sequencing. To generate the transgenic mouse model, full-length NCL cDNA was inserted into the downstream region of the cardiac-specific promoter of alpha-MyHc to generate the α-myosin heavy chain-NCL recombinant vector. The cardiac-specific alpha-MyHc promoter was used to investigate the role of Yin Yang 1 in human heart failure.[21]
Generation of transgenic mice with myocardial overexpression of nucleolin

The transgenic mice were generated by the Animal Research Center of Nanjing University (Nanjing, China). Using microinjection, alpha-MyHc clone 26-NCL plasmids were injected into mouse eggs. Groups of founders from the F0 generation (line 6, 34, 37, 39, 46, 51, 52, 56, and 86) were genotyped and confirmed by PCR [Figure 1a]. Line 52 transgenic mice were backcrossed for more than 10 generations into the C57BL/6 background and used for analysis. Transgenic-negative (WT) siblings served as controls.

Cell culture and reagents

Rat myocardium H9C2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in the presence of 5% CO2 and 95% O2 for 12–20 h in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and 5 mmol/L glucose. Cellular oxidative stress was induced by exposing the cells to hydrogen peroxide (H2O2; 0.1 mmol/L) for different durations. H2O2 concentration was determined spectrophotometrically at 240 nm. A 10-mmol/L stock solution was prepared and diluted into the medium to a final concentration of 0.1 mmol/L.

Transient transfection of short interfering RNA against nucleolin

Short interfering RNA (siRNAs) specific for rat NCL and its control were purchased from GenePharm Suzhou (Jiangsu, China). Transfection of NCL siRNA and its control was performed using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Grand Island, NY, USA).

RNA isolation

Total RNA was extracted from transgenic mice with myocardial overexpression of NCL (n = 3) and WT mice (n = 3) using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. RNA concentration was determined by measuring absorbance at 260 nm (A260) using a spectrophotometer (Infinigen Biotech, City of Industry, CA, USA). The quality of the purified RNA was evaluated using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) for miRNA microarray and whole-genome expression analyses. Only RNA samples with a A260/A280 ratio between 1.8 and 2.0 were used for miRNA microarray analysis.

MicroRNA microarray analysis

Equal amounts of RNA samples from the transgenic and WT mice (n = 6) were subjected to miRNA profiling using a miRCURY LNA miRNA array (v11.0; Exiqon, Vedbaek, Denmark).

Quantitative reverse transcription-polymerase chain reaction

To validate the key microarray results, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City CA, USA) with miRNA-specific primers and primer kits (Applied Biosystems) on an Eppendorf Realplex thermal cycler (Mississauga, ON, Canada). The specific primers of miR-21, miR-208b, miR-218, and miR-153a and the miScript Universal Primer were synthesized by Qiagen (NASDAQ, New York, USA).

Western blot

Proteins were extracted using radioimmunoprecipitation buffer (10 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% NP-40, 1 mmol/L ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate, and 1 mmol/L dithiothreitol) after various treatments. Protein samples were mixed with sample buffer and boiled for 5 min. Equal amounts of clear lysates were resolved on 10% Tris-glycine polyacrylamide mini-gels and transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore, Billerica, MA, USA) using a tank-transfer system. Equal transfer was validated by staining with Ponceau red. The membranes were blocked overnight in phosphate-buffered saline containing 10% nonfat dry milk and 0.5% Tween-20 and were incubated with 1 μl rabbit anti-NCL polyclonal antibody (1:2000; Sigma-Aldrich, St. Louis, MO, USA) and 2 L rabbit anti-glyceraldehyde 3-phosphate dehydrogenase monoclonal antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. The membrane was then washed with Tris-buffered saline with Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The membrane was washed three times in TBST and developed using a DAB assay kit (Boster Biological Technology, Fremont, CA, USA) according to the manufacturer’s protocol.

Statistical analysis

Each experiment was performed at least three times; all data were presented as mean ± standard deviation (SD). Statistical Package for the Social Sciences version 16.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform a two-tailed Student’s t-test, and statistical significance was determined at P < 0.05.

Results

Generation of transgenic mice with myocardial overexpression of nucleolin

To investigate the protective role of NCL against myocardial IR injury, we generated transgenic mice with myocardial overexpression of NCL. The first group of mice (numbers 6, 34, 37, 39, 46, 51, 52, 56, and 86) were identified by PCR. NCL expression in transgenic mice was significantly (>2.5-fold, P < 0.05) higher than that in WT mice [Figure 1b and 1c]. No phenotypic changes in myocardial morphology, body weight, heart weight, or heart-weight index were observed in transgenic mice as compared with those in WT mice [Supplementary Figure 1]. These data indicated the successful generation of transgenic mice by exhibiting myocardial overexpression of NCL.
Table 1: MiRNAs of NCL exhibiting increased expression in transgenic mice with myocardial overexpression of NCL

| miRNA      | Fold change* | P     |
|------------|--------------|-------|
| miR-223    | 1.40         | <0.001|
| miR-214    | 1.67         | <0.001|
| miR-21     | 2.01         | 0.006 |
| miR-146b   | 3.85         | 0.007 |
| miR-199a-5p| 1.39         | 0.005 |
| miR-208b   | 3.14         | 0.006 |
| miR-29a    | 1.31         | 0.017 |
| miR-222    | 1.57         | 0.019 |
| miR-146a   | 1.52         | 0.023 |
| miR-34a    | 1.57         | 0.046 |
| miR-690    | 1.70         | 0.022 |

*NCL transgenic mice (n = 3) versus wild-type mice (n = 3). Statistical significance was determined at P<0.05. NCL: Nucleolin; miRNA: MicroRNA.

Table 2: MiRNAs exhibiting decreased expression in transgenic mice with myocardial overexpression of NCL

| miRNA      | Fold change* | P     |
|------------|--------------|-------|
| miR-582-5p | 21.33        | <0.001|
| miR-135a   | 2.10         | 0.006 |
| miR-145    | 1.32         | 0.004 |
| miR-218    | 2.01         | 0.006 |

*NCL transgenic mice (n = 3) versus wild-type mice (n = 3). Statistical significance was determined at P<0.05. NCL: Nucleolin; miRNA: MicroRNA.

Differentially expressed microRNA between transgenic and wild-type mice

We performed miRNA microarray analysis and compared expression profiles to investigate the differential expression of miRNAs between transgenic mice with myocardial overexpression of NCL and WT mice. The results revealed that 15 miRNAs were differentially expressed between transgenic and WT mice, and of these, 11 (miR-223, -214, -21, -146b, -199a-5p, -208b, -29a, -222, -146a, -34a, and -690) were upregulated by 1.3–3.8-fold (P < 0.05), whereas four (miR-582-5p, -135a, -145, and -218) were downregulated by 1.3–21.3-fold (P < 0.05) in the transgenic mice [Table 1 and Table 2]. A heat map was generated to visualize differentially expressed miRNAs [Figure 2].

Quantitative reverse transcription-polymerase chain reaction validation of microarray data

To validate miRNA microarray results, differentially expressed miRNAs were subsequently quantified by qRT-PCR. The expression levels of most miRNAs were consistent with those observed in the miRNA microarray between the transgenic and WT mice (data not shown). Specifically, qRT-PCR analysis of three upregulated miRNAs (miR-21, -208b, and -146b) and two downregulated miRNAs (miR-218 and miR-135a) indicated a high degree of variation between transgenic mice with myocardial overexpression of NCL and WT mice [Figure 3]. The expression of miR-21 and miR-208b was significantly higher (2–5-fold, P < 0.05), whereas expression of miR-218...
and miR-135a was significantly lower (4–5-fold, \( P < 0.05 \)) in transgenic mice compared to those in WT mice. Each miRNA potentially regulates hundreds of targets; therefore, to improve accuracy, the targets predicted here were considered as putative candidates. To determine the biological significance of these potential targets, we used the Kyoto Encyclopedia of Genes and Genomes pathway analysis. The results indicated that the miRNA-targeted genes were involved in several signaling pathways, such as those mediated by vascular endothelial growth factor (VEGF), mitogen-activated protein kinase (MAPK), or p53, and apoptosis, all of which play a role in regulating IR [Table 3].

**Effect of nucleolin-knockdown on the expression of microRNA-21 in H9C2 cells treated with \( \text{H}_2\text{O}_2 \)**

Recent studies have shown that miR-21 plays a vital role in cardiovascular diseases, such as myocardial infarction and cardiac fibrosis.\(^{[17,18]}\) In this study, we noted a significant increase in miR-21 expression in miRNA microarray analysis [Table 3]. To further investigate the role of NCL in the differential regulation of miRNA expression in cardiocytes under oxidative-stress conditions, we chose differentially expressed miRNAs for further functional experiments. The results of siRNA experiments showed that miR-21 expression increased significantly during \( \text{H}_2\text{O}_2 \) treatment. However, it decreased significantly (by 4.7-fold, \( P < 0.05 \)) in the \( \text{H}_2\text{O}_2 \)-preconditioned (\( \text{H}_2\text{O}_2 \)-PC) 6 h + NCL-siRNA group compared to that in the \( \text{H}_2\text{O}_2 \)-PC 6 h + ctrl-siRNA group [Figure 4].

**Discussion**

Our previous study indicated that NCL is an important anti-apoptotic protein in myocardial ischemia–reperfusion injury. However, the underlying molecular mechanisms are still unclear. In this study, we first generated transgenic mice with myocardial overexpression of NCL and then analyzed the miRNA-expression profiles of transgenic and WT mice using a microarray and found that 15 miRNAs were differentially expressed in transgenic mice, of which 11 were significantly upregulated and 4 were downregulated. The levels of differentially expressed miRNAs were further validated by qRT-PCR followed by functional prediction, indicating regulatory roles in a number of signaling pathways associated with myocardial ischemia or IR injury. These results suggest a potential protective mechanism associated with NCL during myocardial IR injury.

**Table 3: Predicted functions of genes targeted of differentially expressed miRNAs**

| KEGG pathway           | Hits | Total hits (%) | Enrichment | \( P \)  |
|------------------------|------|----------------|------------|---------|
| Apoptosis              | 11   | 95 (11.6)      | 0.0000     | <0.001  |
| Calcium signaling      | 21   | 204 (10.3)     | 0.0000     | <0.001  |
| Cell cycle             | 15   | 140 (10.7)     | 0.0001     | <0.001  |
| Chemokine signaling    | 24   | 203 (11.8)     | 0.0000     | <0.001  |
| ErbB signaling         | 16   | 93 (17.2)      | 0.0000     | <0.001  |
| Insulin signaling      | 21   | 146 (14.4)     | 0.0000     | <0.001  |
| Jak/STAT signaling     | 20   | 160 (12.5)     | 0.0000     | <0.001  |
| MAPK signaling         | 41   | 284 (14.4)     | 0.0057     | <0.001  |
| Notch signaling        | 6    | 58 (10.3)      | 0.0057     | 0.001   |
| \( P \) signaling      | 12   | 76 (15.8)      | 0.0000     | <0.001  |
| PPAR signaling         | 6    | 86 (7.0)       | 0.0300     | 0.003   |
| TGF-\( \beta \) signaling | 14  | 92 (15.2)     | 0.0000     | <0.001  |
| TLR signaling          | 10   | 104 (9.6)      | 0.0007     | <0.001  |
| Ubiquitin signaling    | 16   | 151 (10.6)     | 0.0000     | <0.001  |
| VEGF signaling         | 13   | 80 (16.3)      | 0.0000     | <0.001  |
| Wnt signaling          | 27   | 162 (16.7)     | 0.0000     | <0.001  |

KEGG: Kyoto encyclopedia of genes and genomes; ErbB: Epidermal growth factor receptor family; VEGF: Vascular endothelial growth factor; PPAR: Peroxisome proliferator-activated receptor; TGF-\( \beta \): Transforming growth factor-\( \beta \); TLR: Toll-like receptor; MAPK: Mitogen-activated protein kinase.
NCL is a nucleolar phosphoprotein that is also among the most abundant nonhistone proteins in the nucleus. Its biological functions include ribosome biosynthesis and maturation, cell proliferation, chromatin replication, and nucleolus formation, with its activity being dependent primarily on its RNA-binding domain. Recently, NCL was described as exhibiting anti-apoptotic properties, including regulation of expression of several key genes that play essential roles in apoptosis regulation. Sengupta et al. reported that NCL enhanced mRNA stability of Bcl-2, a key anti-apoptotic gene. NCL is an RNA-binding protein that contains an rRNA-specific binding sequence ([T/G] CCCC [A/G]), which enables it to bind to the 3′-UTR of Bcl-2 mRNA. Cluster of differentiation (CD) 154 is a member of the tumor necrosis factor superfamily and is primarily expressed on the surface of activated T-cells. Its mRNA turnover is partially regulated at the posttranscriptional level by a protein complex that binds to a highly CU-rich region of the 3′-UTR. Singh et al. demonstrated that NCL acts as a key component of the complex involved in maintaining CD154 mRNA stability and regulating mRNA turnover in activated T-cells. In addition, Zhang et al. reported that the mRNA stability of growth arrest and DNA-damage-inducible alpha (GADD45a) was also regulated by NCL through binding to the GADD45a mRNA-coding region. The tumor suppressor, p53, exhibits multiple functions in regulating apoptosis, genomic stability, and inhibition of angiogenesis. NCL overexpression suppresses p53 by binding to the 5′-UTR of p53 mRNA to decrease its stability, thereby controlling p53 translation and induction following DNA damage. Conversely, NCL downregulation promotes p53 expression and activity. Woo et al. found that NCL bind to both, the G quadruplex and AREs, thereby enabling NCL to either enhance CSF-1 mRNA deadenylation and promote CSF-1 mRNA decay or increase translation of CSF1 mRNA. Our previous studies also found that NCL could bind to the 3′-UTR of Hsp32 and Hsp70 to increase their mRNA stability and translation, thereby enhancing myocardial protection against oxidative stress and IR injury. These studies demonstrated that NCL might play a protective role in the myocardium by regulating the expression of targeted genes by binding to their mRNA stem-loop regions.

MicroRNAs are small regulatory molecules, approximately 21–24 nucleotides in length, that control the expression of >50% of human protein-coding genes at the posttranscriptional level, and play essential roles in cell signaling pathways. Additional miRNAs act as potential markers for early diagnosis, treatment, and prognosis of cancer, resulting in their application in cancer therapeutics. Recently, several miRNAs, including miR-21, -199a, -210, and -494, were reported to play roles in hypoxia or ischemia of myocardial cells. Zhu and Fan also reported that miR-1-133 and miR-208 regulated myocardial contractile function, whereas miR-21, -133, -195, and -214 regulated cardiomyocyte hypertrophy. Baek et al. also reported that miR-210, -373, and -21 were associated with hypoxia-regulated transcripts and could modulate the hypoxic gene-expression profile. As an RNA-binding protein, whether NCL can play a biological role by regulating miRNAs biosynthesis? Pickering et al. reported that NCL interacted with the microprocessor complex and was capable of regulating biogenesis of the pro-tumorigenic miRNAs, miR-15a and miR-16. Here, we found that the expression of 11 miRNAs, namely miR-223, -214, -21, -146b, -199a-5p, -208b, -29a, -222, -146a, -34a, and -690, was upregulated; whereas, miR-582-5p, -135a, -145, and -218 were downregulated in transgenic mice with myocardial overexpression of NCL compared to those in WT mice. These results suggest that NCL is involved in the regulation of some miRNAs, such as miR-21 and miR-214, involved in cardiomyocyte hypertrophy and IR. However, the extent of miRNA involvement in apoptosis-induced cardiomyocyte injury by IR remains unknown. Therefore, further studies are required to understand the role of NCL in controlling miRNA-mediated cardiovascular function and pathology. These may reveal novel targets for improving survival of myocardial cells under oxidative stress.

We recently investigated NCL expression in hearts subjected to IR and in neonatal rat cardiomyocytes subjected to hypoxia/reoxygenation. We found that NCL expression was significantly downregulated under these conditions. NCL overexpression exhibited cytoprotective effects, whereas NCL ablation enhanced both hypoxia- and H$_2$O$_2$-induced cardiomyocyte death. In this study, transgenic mice with cardiac-specific overexpression of NCL were resistant to IR injury as indicated by decreased cellular necrosis and infarct size. The cardioprotective roles of NCL in cardiomyocytes are attributable to the interaction between NCL and Hsp32 mRNA, resulting in increased Hsp32 mRNA stability and subsequent upregulation of Hsp32 expression. Given the essential roles of NCL in miRNA biogenesis, we hypothesize that NCL regulates the expression of both Hsp32 and Hsp70, presumably at the posttranscriptional level, via a number of miRNAs. In this study, we compared miRNA profiles between cardiac-specific NCL-overexpressing transgenic and WT mice to further investigate the protective role of NCL against myocardial IR injury at the mRNA level. Bioinformatics analysis and pathway enrichment analysis helped identify putative targets of differentially expressed miRNAs. Through this study, we identified 15 differentially expressed miRNAs in transgenic mice, of which 11 were upregulated and 4 were downregulated. Some miRNAs, such as miR-21, -208b, -146b, -135a, and -218, exhibited significant differential expression. In a more recent study, we showed that miR-21 expression in H9C2 cells was consistent with its expression in the myocardium in vivo. Further analysis of miR-21 revealed that NCL bound to the precursor of miR-21 and upregulated/increased its expression. These results indicated the involvement of NCL in myocardial protection. Moreover, we found that siRNA knockdown of NCL markedly decreased miR-21 expression in H$_2$O$_2$-PC H9C2 cells. These results suggest...
that NCL-related myocardial protection might occur by regulating the expression of miRNAs, including miR-21. miR-21 plays important roles in vascular smooth muscle cell proliferation and apoptosis, cardiac cell growth and death, and cardiac fibroblast function.\(^{[10]}\) Hsp70 also plays important protective roles against IR and chronic hypoxemia.\(^{[10]}\) Moreover, regulation of Hsp70 mRNA and subcellular distribution of inducible Hsp70 protein in chronically hypoxic rabbit hearts are influenced by protein kinase C and MAPK, specifically p38 MAPK and c-Jun N-terminal kinase (JNK).\(^{[32,33]}\) The predicted functions of the genes targeted by the differentially expressed miRNAs suggested the involvement of p38 MAPK and JNK [Table 3]. Furthermore, the targeted genes associated with NCL-mediated miRNAs are thought to participate in the Notch and VEGF signaling pathways, which play important roles in cardiac development,\(^{[32,33]}\) and in the Janus kinase/signal transducers and activators of transcription signaling pathway associated with cardiac dysfunction during IR.\(^{[34]}\) Therefore, the results of this study revealed that some differentially expressed miRNAs may contribute to the protection from myocardial IR by regulating several important signaling pathways previously implicated in the myocardium. However, the precise function of these differentially expressed miRNAs in protection against myocardial IR requires further investigation.

In summary, the results of this study demonstrated the functional significance of NCL in protecting cardiomyocytes through its ability to modulate the expression and activity of a number of miRNAs.

**Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.**

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**Conflicts of interest**

There are no conflicts of interest.

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Supplementary Figure 1: Transgenic mouse phenotype. (a) Representative myocardial morphology in NCL-overexpressing transgenic mice and WT mice (H and E; original magnification ×100). The effects of NCL-overexpression on mouse body weight (b), heart weight (c), and heart weight index (d) in NCL-overexpressing transgenic mice (n = 6 offspring from line 52). NCL: Nucleolin; TG: Transgenic mice; WT: Wild-type.