LncRNA NORAD promotes the progression of myocardial infarction by targeting miR-22-3p/PTEN axis

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Abstract

Increasing evidences have demonstrated that lncRNAs are closely associated with the progression of acute myocardial infarction (AMI). Although lncRNA NORAD has been reported to be highly expressed in MI patients, its specific function remains unclear. Here, we found that downregulation of NORAD efficiently attenuated heart damage in an AMI rat model. Meanwhile, silencing of NORAD also inhibited hypoxia/re-oxygenation (H/R)-treated mouse cardiomyocyte cell line HL-1 cells. Further, bioinformatics analysis revealed that NORAD might act as a ceRNA of miR-22-3p, and PTEN was a potential target of miR-22-3p. Then luciferase reporter assay was performed to confirm that NORAD could upregulate PTEN by directly sponging miR-22-3p. Meanwhile, miR-22-3p inhibitor significantly reversed si-NORAD induced inhibitory effect on the apoptosis of H/R-treated HL-1 cells, and overexpression of PTEN could obviously reverse miR-22-3p mimics induced inhibitory effect on the apoptosis of H/R-treated HL-1 cells. Meanwhile, miR-22-3p mimics significantly reversed OE-NORAD (overexpression of NORAD) induced expression of p-AKT and p-mTOR. In a word, our results suggested lncRNA NORAD might promote the progression of myocardial infarction by promoting PTEN/AKT/mTOR signaling pathway through directly sponging miR-22-3p, suggesting that NORAD might be a potential target for AMI treatment.

Introduction

Acute myocardial infarction (AMI) has becoming a common and potential life-threatening disease with an increasing incidence and high mortality due to the acute interruption of myocardial blood flow which then lead to ischemic myocardial necrosis[1, 2]. Reperfusion therapy after AMI can effectively restore the blood supply and nutritional support in ischemic myocardium and even save the dying myocardium[3]. The pathogenesis of AMI was identified to be associated with multiple reasons and cardiomyocyte apoptosis was considered as the crucial components[4, 5]. Therefore, the well understanding of molecular mechanisms involved in cardiomyocyte apoptosis can contribute to identify potential and efficient molecular target for AMI.

Long chain non-coding RNAs (RNAs), a class of regulatory RNAs lacking protein coding function and approximately with 200 nucleotides in length, were previously considered as a type of transcriptional noise but have been identified as novel regulators in the tumor development[6–9]. Recently, a novel lncRNA, NORAD, has been reported to participate in the progression of a series of human cancers. For instance, the expression of NORAD was significantly upregulated in esophageal squamous cell carcinoma (ESCC) tissues compared to adjacent normal samples[10]. NORAD has been reported that can suppress the metastasis of lung and breast cancer through sequestering S100P, and the expression of NORAD was repressed by YAP pathway[11]. One previous study suggested that NORAD enhances the hypoxia-induced epithelial-mesenchymal transition (EMT) process to promote metastasis in pancreatic cancer[12]. NORAD has also been demonstrated to promote the progression of thyroid carcinoma by targeting miR-202-5p[13]. In addition, Yang et al. revealed that NORAD can enhance the TGF-β pathway to promote hepatocellular carcinoma development through directly sponging miR-202-5p[14]. Although NORAD has been found to be highly expressed in the myocardial infarction (MI) left ventricle tissues in mice[15], the specific mechanism has not been studied.

Increasing studies have demonstrated that the abnormal expression of microRNAs (miRNAs) are closely associated with biological processes such as neuroprotection, tumorigenesis and various other tumor progressions including miR-22-3p[16]. MiR-22-3p has been reported to control the amyloid β deposit in mice model of Alzheimer's Disease through targeting Mitogen-activated Protein Kinase 14 (MAPK14)[17]. MiR-22-3p can regulate the proliferation of retinoblastoma cells by targeting alpha-enolase 1[18]. In addition, Chen et al. revealed the mechanism of berberine treatment in hepatocellular carcinoma (HCC), specifically, berberine can upregulate the expression of miR-22-3p to inhibit the proliferation of HCC cells through targeting Sp1[19]. However, the function of miR-22-3p in AMI remains unclear.

Phosphatase and tensin homolog (PTEN), was firstly identified to act as a tumor suppressor gene by regulating the cycle of cell division and removing phosphate groups to modify proteins and fats[20, 21]. Recently, more and more studies have demonstrated that PTEN gene may trigger cardiomyocyte cells to undergo apoptosis and exacerbate myocardial

dysfunction, and the inactivation of cardiac-specific PTEN can protect the heart against functional failure, fibrosis and myocardial infarction in a mouse model[22, 23]. It has been reported that PTEN induces the apoptosis of cardiomyocyte cells might through modulating PI3K/AKT signaling pathways[24, 25], and the regulatory network of PTEN involved in cardiomyocyte apoptosis is lack.

In the present study, our results revealed that NORAD could promote the development of AMI by targeting miR-22-3p/PTEN axis to enhance the AKT/mTOR signaling pathway, which provided a novel therapeutic target for AMI.

Materials And Methods

Animal model

A total of 32 Sprague-Dawley (SD) rats (male, approximately 14 weeks) were obtained from Shanxi Bethune Hospital Shanxi Academy of Medical Sciences. Rats were kept on a 12-h light/dark cycle at room temperature (approximately 22-26˚C) with 50-70% humidity. A rat model of AMI was established through the ligation of the left anterior descending coronary artery as previously described[26]. After the ligation for 30 min, reperfusion was performed through cutting the knot in the ligature. For the sham group, the procedure was same with no transient artery ligation. All animal experiments were performed according to the institutional animal ethics guidelines for the Care and Use of Research Animals of Shanxi Bethune Hospital Shanxi Academy of Medical Sciences and this study was approved by Shanxi Bethune Hospital Shanxi Academy of Medical Sciences. To explore the specific function of NORAD *in vivo*, approximately 10mg/kg body lentivirus carrying si-NORAD (silencing of NORAD) or negative control was intravenously injected into rats through the tail vein for 24 h before surgery. Mice were randomly divided into four groups (n = 8): Sham si-NC group, Sham si-NORAD group, AMI si-NC group and AMI si-NORAD group.

The determination of myocardial infarction

At 24 h after AMI or Sham operation, mice were euthanized via the intraperitoneal injection of Avertin (2,2,2-Tribromoethanol) (Sigma-Aldrich) (20 mg/kg). The whole heart was cut into 2 mm thick slices and stained with 1% triphenyltetrazolium chloride (TTC) at 37°C for 20 minutes after washing out remaining blood. The sizes of the TTC-stained area (red staining, ischemic but viable tissue) and unstained area [infarct myocardium (INF)] were analyzed with the Image Pro Plus 6.0 software (Media Cybernetics Inc.).

The evaluation of cardiac function

The cardiac function of rats was evaluated by the motion-mode echocardiography under the VEVO 770 high-resolution *in vivo* imaging system (FUJIFILM VisualSonics Inc.). Of which, the mean value of left ventricular fractional shortening (LVFS) was analyzed by M mode, and left ventricular internal dimension (LVIDs) were evaluated at end-systole (s) through four-chamber view.

HE staining assay

After animal experiments were finished, the heart tissues of rats were rapidly removed, fixed with 4% paraformaldehyde, embedded in paraffin wax and sliced into 4–5 μm thicknesses following dehydration. Then the sections were stained with hematoxylin and eosin (H&E) staining as previously described[27] and observed under a light microscope (Olympus, Tokyo, Japan).

Cell culture

The mouse cardiomyocyte cell line HL-1 (SCC065) were obtained from Sigma-Aldrich (Merck KGaA). Cells were cultured with DMEM medium containing 10% FBS, and 1% penicillin-streptomycin (Nacalai Tesque Inc.) at 37˚C with 5% CO_2. To establish the cell model with hypoxia/re-oxygenation (H/R), HL-2 cells were firstly kept in a hypoxia incubator

(approximately 1% O_2, 5% CO_2, 94% N_2) at the density of 5 x 10^5 cells/ml for 20 h. Then cells were incubated under the normoxic conditions for 4 h, and collected for the subsequent experiments.

Cell transfection

Si-NORAD (silencing of NORAD), si-NC (control), miR-22-3p mimics (miR-22-3p overexpression) or miR-NC (control) was transfected into HL-1 cells by using Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA). Si-NORAD forward: 5'-GCUGUCGGAAGAGAGAAAUTT-3', reverse: 5'-AUUUCUCUCUUCCGACAGCTT-3'; si-NC forward: 5'-UUCUCCGAACGUGUCACGUTT-3', reverse: 5'- ACGUGACACGUUCGGAGAATT–3'; miR-22-3p mimics : 5'-GGCTGAGCCGCAGTAGTTCTTCAGTGGCAAGCTTTATGTCCTGACCCAGCTAAAGCTGCCAGTTGAAGAACTGTTGCCCTCTGCC-3'; miR-NC: 5'-UUC UCCGAACGUGUCACGUTT-3'. For NORAD overexpression, the cDNA fragments of NORAD was amplified and cloned into expression vector pcDNA3.1 (Richmond, BC, Canada). Of which, si-NORAD/si-NC was used at a concentration of 500 ng/well, and miR-22-3p mimic/miR-NC was approximately 100 nM (Shanghai GenePharma Co., Ltd.). After transfection for 48 h, cells were collected for the subsequent experiments.

Luciferase reporter assay

The cDNA fragments of NORAD/PTEN containing either the predicted potential miR-22-3p binding site (wild type, WT) or mutant type (MUT) sequences were amplified by PCR and cloned into Renilla luciferase reporter vector pmirGLO (Promega, Madison, WI, USA). Then HL-1 cells were transfected with luciferase reporter plasmids and miR-22-3p mimics or miR-NC. After transfection for 48 h, cells were lysed and the relative luciferase activity was evaluated by using dual luciferase reporter system.

qRT-PCR

Total RNA of heart tissues or cells was extracted by the TRIzol reagent. The quantitative polymerase chain reaction was performed by using 7500 Fast Real-time PCR system (Applied Biosystems) based on the SYBR Green PCR Kit (Toyobo). The relative fold changes were calculated by $2^{-\Delta\Delta Ct}$ method[28], with β-actin and U6 as the internal reference. The primers used in this study as follows: miR-22-3p forward: 5'-GCTGAGCCGCAGTAGTTCTT-3', reverse: 5'-GGCAGAGGGCAACAGTTCTT-3'; PTEN forward: 5'-TAGAGCGTGCGGATAATGAC-3', reverse: 5'-GATGGCTCCTCTACTGTTTT-3'; β-actin forward: 5'-CCTCTATGCCAACACAGTGC-3', reverse: 5'-CATCGTACTCCTGCTTGCTG-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGA ATTTGCG-3'.

Western blot

Total protein of cultured cells was isolated by using RIP lysis buffer according to the manufacturer's instructions. Approximately equal amounts of protein were separated by 10% SDS-PAGE and then transferred into PVDF membranes (Immobilon-P; EMD Millipore). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies including PTEN (1:1000, abcam), AKT (1:2000, CST), p-AKT (1:1000, CST), mTOR (1:2000, abcam), p-mTOR (1:1000, abcam), and internal reference β-actin (1:2000, abcam) overnight at 4˚C. After washing for three times, the membranes were incubated with HRP-conjugated secondary antibody for 2–3 h at room temperature. Finally, the images of bands were observed by a ImageJ2X software using the ECL reagent (Thermo).

RNA pull down assay

HL-1 cells were transfected with biotin-labeled negative control, biotin-labeled miR-22-3p WT or biotin-labeled miR-22-3p MUT. The probes used as follows, miR-22-3p WT: 5'-UGUCAAGAAGUUGACCGUCGAA-3'; miR-22-3p MUT: 5'-UGUCAAGGAGUUCAAACAGCGA-3'. After transfection for 48 h, the whole-cell extraction was incubated with M-280 streptavidin magnetic beads (Invitrogen, Carlsbad, CA, USA) at 4°C for 3-5 h. The co-precipitated RNA was purified by using TRIzol reagent supplementing with proteinase K, and analyzed by qRT-PCR.

The detection of L-lactate dehydrogenase (LDH) and malondialdehyde (MDA)

The LDH level and MDA level in the cell supernatant was detected by LDH assay kit (ab102526, Abcam) or Cellular Malondialdehyde Test kit (A003-2, Nanjing Jiancheng Bioengineering Institute) according to their corresponding manufacturer's instructions.

Flow cytometry

Cells were harvested after 48 h for transfection, and re-suspended in the precooled phosphate buffered saline. 5 μl Annexin V-FITC solution (Sangon Biotech) was added to each well and incubated for 15 min at room temperature in the dark. After centrifugation and washed with binding buffer, cells were re-suspended again in solution containing 190 μl binding buffer and 10 μl propidium iodide (PI, Sangon Biotech). Then apoptosis rate was analyzed by using flow cytometry (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

Data were presented as the mean ± SD which were derived from at least three independent experiments. Difference between two groups was determined by Student's t-test and that among multiple groups was analyzed by one-way ANOVA. $P < 0.05$ was considered as the significant threshold .

Results

Downregulation of NORAD inhibited AMI progression in mice model

To explore the function of NORAD in AMI, the mice model with AMI was firstly constructed and NORAD was silenced through si-NORAD transfection. QRT-PCR assay showed that si-NORAD could significantly decreased the expression of NORAD both in Sham group ($p < 0.001$) and AMI group ($p < 0.001$) (Fig. 1A). Further, the effect of NORAD in myocardial infarction of rat was evaluated, and the representative images was shown in Fig. 1B, suggesting that si-NORAD significantly decreased the infarction size compared with si-NC control in AMI group ($p < 0.001$), while exhibited no obvious change in Sham group (Fig. 1C). Meanwhile, downregulation of NORAD increased the LVFS ($p < 0.05$) (Fig. 1D) and decreased the LVIDs ($p < 0.05$) (Fig. 1E) compared with si-NC control in AMI group, while showed no obvious change in Sham group (Fig. 1D and E). In addition, histopathological examination by HE staining was carried out to confirm the protective effect of si-NORAD in AMI injury, and the representative images were shown in Fig. 1F. From the HE staining, we found that there was a normal histoarchitecture in Sham group, while the obvious necrosis of myofibers with cell infiltration was observed in ANI group, moreover, si-NORAD obviously ameliorated these damage induced by AMI compared with si-NC control. These results indicated that downregulation of NORAD could efficiently attenuate AMI-induced heart damage.

LncRNA NORAD acts as a ceRNA of miR-22-3p

To explore the specific mechanism of NORAD in AMI, the HL-1 cell model *in vitro* was established by using hypoxia/re-oxygenation (H/R) treatment. The results of qRT-PCR indicated that H/R stimulation significantly increased the expression of NORAD compared with negative control in HL-1 cells ($p < 0.001$) (Fig. 2A). Next, Starbase v2.0 was used to predict the potential targets of NORAD, and revealed that there was a putative binding site between NORAD and miR-22-3p (Fig. 2B), suggesting that miR-22-3p might a target of NORAD. Meanwhile, miR-22-3p mimics could significantly increase the expression of miR-22-3p compared with miR-NC control (Fig. 2C). Next, the luciferase reporter assay was performed to confirm the relationship between NORAD and miR-22-3p, and the results indicated that miR-22-2p mimics significantly decreased the relative luciferase activity of WT NORAD vector compared with miR-NC control ($p < 0.001$), while exhibited no obvious change in MUT NORAD vector (Fig. 2D). In addition, RNA pull down assay was also performed and the results showed that the expression of NORAD was significantly enriched in biotin-labeled WT miR-22-3p group compared with

biotin-labeled MUT miR-22-3p group ($p < 0.001$) (Fig. 2E). These results all confirmed that NORAD might act as a ceRNA of miR-22-3p.

MiR-22-3p inhibitor reversed si-NORAD induced inhibitory effect on the apoptosis of H/R-treated HL-1 cells

To further determine the negative correlation between NORAD and miR-22-3p, HL-1 cells were transfected with si-NORAD, OE-NORAD (overexpression of NORAD) or their corresponding negative controls, and qRT-PCR results showed that si-NORAD significantly increased the expression of miR-22-3p compared with si-NC control ($p < 0.001$), while OE-NORAD oppositely decreased the expression of miR-22-3p compared with negative control (empty vector pcDNA3.1) ($p < 0.001$) (Fig. 3A). Meanwhile, the levels of LDH and MDA in the cultured supernatants of H/R-treated HL-1 cells was evaluated. For LDH level, si-NORAD significantly decreased the LDH level compared with negative control ($p < 0.01$), while co-transfection with si-NORAD and miR-22-3p inhibitor obviously reversed si-NORAD induced inhibitory effect ($p < 0.01$) (Fig. 3B). For MDA level, si-NORAD significantly decreased the MDA level compared with negative control ($p < 0.01$), while additional miR-22-3p inhibitor obviously reversed si-NORAD induced inhibitory effect ($p < 0.01$) (Fig. 3C). In addition, the results of flow cytometry revealed that si-NORAD significantly inhibited the apoptotic rate of H/R-treated HL-1 cells ($p < 0.01$), while co-transfection with si-NORAD and miR-22-3p inhibitor could obviously reversed si-NORAD induced inhibitory effect on cell apoptosis ($p < 0.01$) (Fig. 3D). These data suggested that downregulation of NORAD significantly inhibited the apoptosis of H/R-treated HL-1 cells through sponging miR-22-3p.

PTEN was a target of miR-22-3p

To investigate the detailed molecular mechanisms of miR-22-3p in AMI, Targetscan was applied to predict the potential targets of miR-22-3p, and the results showed that miR-22-3p might potentially bind to the 3' untranslated region (3' UTR) of PTEN (Fig. 4A). Then PTEN was overexpressed through the transfection of OE-PTEN or empty vector pcDNA3.1, and qRT-PCR indicated that OE-PTEN significantly increased the expression of PTEN compared with empty vector control ($p < 0.001$) (Fig. 4B). To further confirm their interaction, the luciferase reporter assay was performed and showed that miR-22-3p mimics significantly decreased the WT 3' UTR of PTEN compared with miR-NC control ($p < 0.001$), while exhibited no obvious change in MUT 3' UTR of PTEN (Fig. 4C). Meanwhile, overexpression of NORAD increased the protein expression of PTEN ($p < 0.05$), and miR-22-3p mimics decreased PTEN expression ($p < 0.05$), while co-transfection with OE-NORAD and miR-22-3p mimics increased PTEN expression compared with miR-22-3p mimics group ($p < 0.001$) and decreased PTEN expression compared with OE-NORAD group ($p < 0.05$) (Fig. 4D). Meanwhile, miR-22-3p mimics significantly decreased LDH level compared with negative control ($p < 0.05$), while overexpression of miR-22-3p obviously reversed miR-22-3p mimics induced inhibitory effect on the LDH expression ($p < 0.05$) (Fig. 4E); Overexpression of miR-22-3p also decreased the MDA level ($p < 0.05$), while PTEN overexpression reversed miR-22-3p mimics induced inhibitory effect on MDA expression ($p < 0.05$) (Fig. 4F). In addition, miR-22-3p mimics significantly decreased the apoptosis of HL-1 cells ($p < 0.01$), while co-transfection with miR-22-3p mimics and OE-PTEN obviously reversed miR-22-3p mimics induced inhibitory effect on the apoptosis of H/R-treated HL-1 cells ($p < 0.05$) (Fig. 4G and H). These results suggested that overexpression of miR-22-3p could obviously inhibit the apoptosis of H/R-treated HL-1 cells by targeting PTEN.

Upregulation of NORAD promoted the mTOR/AKT signaling pathway through sponging miR-22-3p

Since PTEN induces apoptosis through mTOR/AKT signaling pathways, and we then explore the effect of NORAD/miR-22-3p axis in mTOR/AKT signaling in H/R-stimulated HL-1 cells by western blot (Fig. 5A). The results indicated that overexpression of NORAD significantly decreased the expression of p-AKT ($p < 0.05$) and p-mTOR ($p < 0.001$) compared with negative control, and miR-22-3p mimics increased the expression of p-AKT ($p < 0.05$) and p-mTOR ($p < 0.05$), while co-transfection with OE-NORAD and miR-22-3p mimics obviously reversed miR-22-3p induced increase on the expression of p-AKT ($p < 0.05$) and p-mTOR ($p < 0.05$) (Fig. 5B). These results suggested that upregulation of NORAD could significantly promote the activation of mTOR/AKT signaling pathway through sponging miR-22-3p.

Discussion

In the last decades, a large number of lncRNAs have been identified and demonstrated to be closely involved in the progression of AMI, and some of which may be considered as the potential diagnostic biomarkers or therapeutic targets for AMI. For example, Hu et al. found that knockdown of lncRNA MALAT1 attenuates AMI progression through targeting miR-320, and suggested that MALAT1 might be a potential therapeutic target[29]. Inhibition of the lncRNA Mirt1 can efficiently attenuate AMI via suppressing the activation NF-κB signaling[30]. One previous study revealed that downregulation of lncRNA ANRIL relieves the apoptosis of myocardial cell in AMI by regulating the expression of IL-33/ST2[31]. Zhuo et al. performed a RNA-seq analysis and constructed a lncRNA-miRNA-mRNA network potentially associated with AMI progression, and identified lncRNA SNHG8 might be a key regulator of AMI[32]. LncRNA ZFAS1 was found to act as a SERCA2a inhibitor to cause intracellular $Ca(2^+)$ overload and contractile dysfunction in a mice model of AMI[33]. Silencing of lncRNA XIST can significantly repress the apoptosis of myocardial cells in rats with AMI through targeting miR-449[34]. In addition, there were many other lncRNAs including Novlnc6, Mhrt and Tie-1-AS that play crucial regulatory functions in AMI development[35, 36]. Although NORAD have been observed to be highly expressed in AMI, its function and specific mechanism remains unclear. Here, we firstly found that downregulation of NORAD could efficiently attenuate AMI-induced heart damage in mice model, which is characterized by decreased LVIDs and necrosis of myofibers with cell infiltration as well as increased LVFS, suggesting that high level of NORAD was positively correlated to AMI progression.

As known, lncRNAs often act as competitive endogenous RNA (ceRNA) of miRNAs at for post-transcriptional control[37, 38]. To extend the regulatory mechanism of NORAD in AMI, Starbase v2.0 was used to predict the potential targets of NORAD, and revealed that there was a putative binding site between NORAD and miR-22-3p. Although the role of miR-22-3p in AMI has not been well studied, miR-22-3p has been identified to be direct target of many lncRNAs and participate the development of cardiovascular diseases. LncRNA MIAT functions as a ceRNA to upregulate DAPK2 through directly sponging miR-22-3p in diabetic cardiomyopathy[39]. LncRNA H19 has been demonstrated to ameliorate myocardial ischemia-reperfusion injury via targeting miR-22-3p[40]. Then luciferase reporter assay and RNA pull down assay were performed and confirmed that NORAD could sponging miR-22-3p and negatively regulate the expression of miR-22-3p. Moreover, co-transfection with si-NORAD and miR-22-3p inhibitor could obviously reversed si-NORAD induced inhibitory effect on cell apoptosis in H/R-stimulated HL-1 cells, as well as the production of LDH and MDA in the cultured supernatants. These data indicated that the effect of NORAD in AMI was mediated by miR-22-3p.

PTEN has been demonstrated to be closely related to the progression of AMI such as myocardial fibrosis and cardiomyocyte apoptosis, and always functions as a direct target of miRNAs. Li et al. found that miR-23a regulates AMI *in vivo* and *in vitro*, specifically, upregulation of miR-23a significantly increases the superoxide dismutase, glutathione and catalase activity levels, and decreases the malonaldehyde activity level through directly targeting PTEN[41]. MiR-26a has been found that can regulate myocardial fibrosis after myocardial infarction through targeting PTEN and then modulating PI3K/AKT signaling pathway[42]. In addition, MiR-214 suppresses left ventricular remodeling in an AMI mice model through inhibiting cellular apoptosis by targeting PTEN, suggesting a protective mechanism against AMI injury[43]. Here, PTEN was identified to be a target of miR-22-3p by using Targetscan software, and the luciferase reporter assay showed that miR-22-3p mimics significantly decreased the WT 3' UTR of PTEN, while exhibited no obvious change in MUT 3' UTR of PTEN. Meanwhile, overexpression of NORAD increased the protein expression of PTEN ($p < 0.05$), and miR-22-3p mimics decreased PTEN expression, while miR-22-3p mimics could significantly reversed the effect of NORAD overexpression on PTEN expression. Moreover, co-transfection with miR-22-3p mimics and OE-PTEN obviously reversed miR-22-3p mimics induced inhibitory effect on the apoptosis of H/R-treated HL-1 cells. All these data indicated that si-NORAD induced protective effect in AMI *in vitro* was mediated by miR-22-3p axis. In addition, based on the effect of PTEN in cardiomyocyte apoptosis though the modulation of mTOR/AKT signaling, we explore the effect of NORAD and miR-22-3p in the mTOR/AKT signaling pathway by western blot, and the results showed that NORAD overexpression markedly decreased the expression of p-AKT and p-mTOR, and miR-22-3p mimics increased p-AKT and p-mTOR expression, while co-transfection with OE-NORAD and miR-22-3p mimics obviously reversed miR-22-3p induced increase on the expression of p-AKT and p-

mTOR. These results confirmed that NORAD affect the cardiomyocyte apoptosis though regulating mTOR/AKT signaling by targeting miR-22-3p/PTEN axis.

However, whether the protective effect of si-NORAD *in vivo* could be reversed by miR-22-3p inhibitor or PTEN overexpression should be determined.

Conclusion

In summary, our study demonstrated that lncRNA NORAD could promote the progression of myocardial infarction by targeting miR-22-3p/PTEN axis, providing a novel therapeutic target for AMI.

Declarations

Acknowledgements

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Author contributions

CXL designed the experiment. CXL drafted the manuscript. CXL, LHZ, XPB, GFC, XFZ and YRL performed the experiment. All authors contributed towards the discussion and reviewed the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Ethics approval and consent to participate

All animal experiments were performed according to the institutional animal ethics guidelines for the Care and Use of Research Animals of Shanxi Bethune Hospital Shanxi Academy of Medical Sciences and this study was approved by Shanxi Bethune Hospital Shanxi Academy of Medical Sciences.

Consent for publication

All authors have consented the manuscript been published.

Competing interests

All authors declare no conficts of interest

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Figures

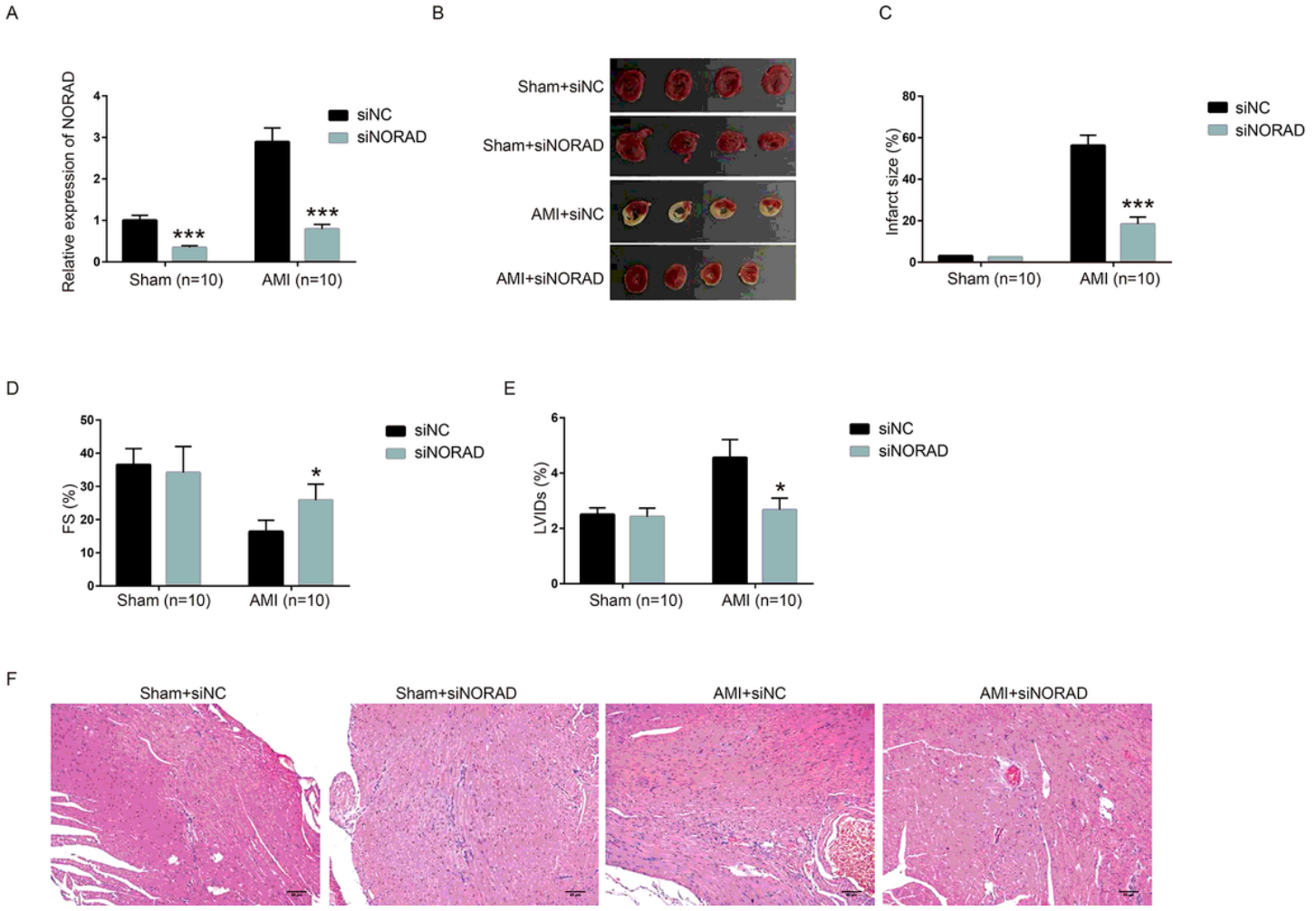

Figure 1

Silencing of NORAD attenuated AMI progression in vivo. Rats from Sham group and AMI group were all transfected with si-NORAD or si-NC control. (A) The mRNA level of NORAD in heart tissues was evaluated by qRT-PCR. (B) The infarcted size of heart was analyzed by using TTC staining. (C) Quantitative analysis of infarcted area. (D and E) The cardiac function of rats including FS (D) and LVIDs (E) was evaluated. (F) Histopathological examination of cardiac function was evaluated by HE staining assay. N = 8, * P < 0.05, *** P < 0.001. Scale bars, 5 mm. LVFS, left ventricular fractional shortening; LVID, left ventricular internal dimension.

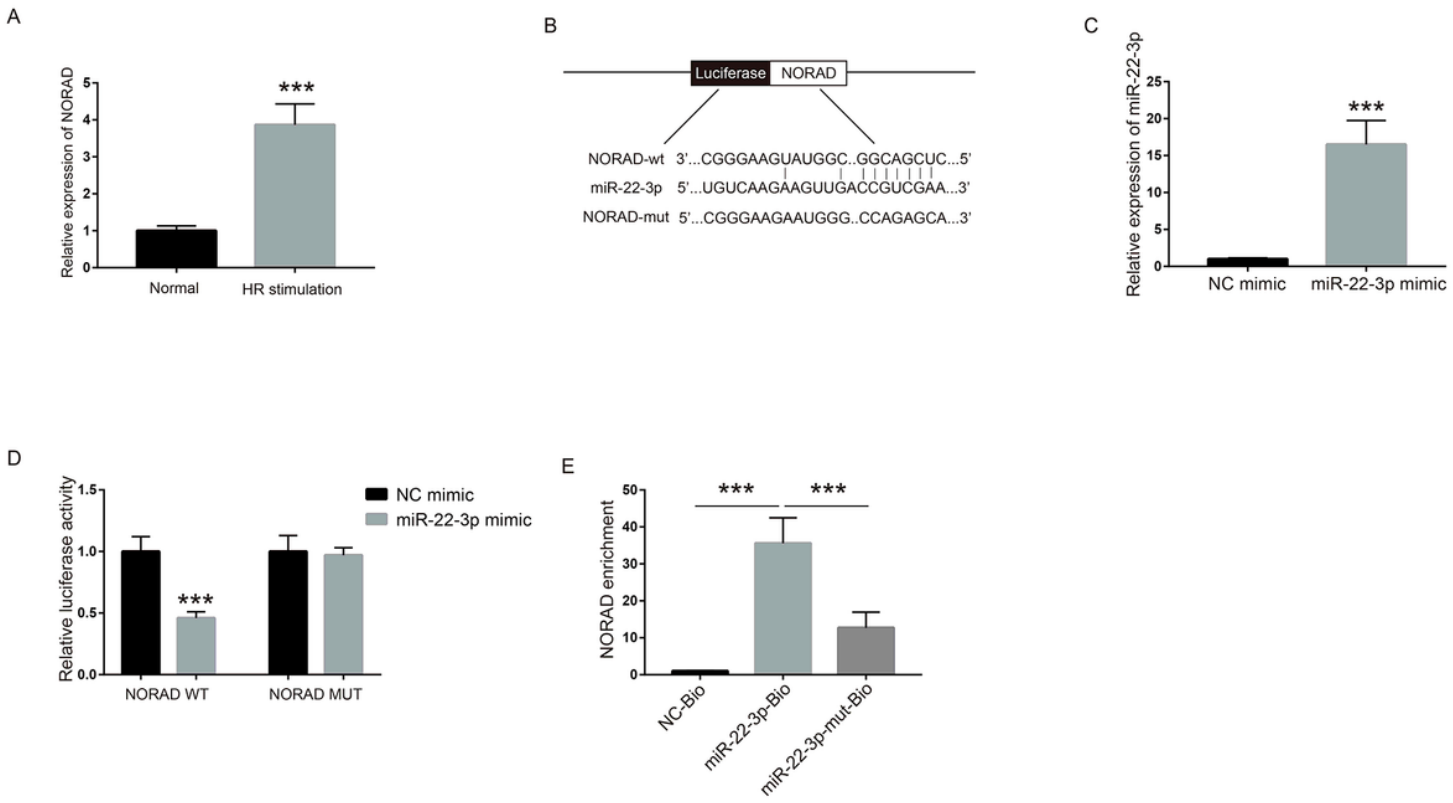

Figure 2

NORAD acts as a ceRNA of miR-22-3p. (A) HL-1 cells were stimulated with or without H/R stimulation, and the mRNA level of NORAD was evaluated by qRT-PCR. (B) The putative binding site between NORAD and miR-22-3p was predicted by Starbase v2.0. (C) HL-1 cells were transfected with miR-22-3p mimics or miR-NC control, and the mRNA level of miR-22-3p was evaluated by qRT-PCR. (D) HL-1 cells were co-transfected with luciferase reporter plasmids containing WT or MUT NORAD and miR-22-3p mimics or miR-NC control, and the relative luciferase activity was evaluated by dual luciferase reporter system. (E) RNA pull down assay was performed by using biotin-labeled WT miR-22-3p or biotin-labeled MUT miR-22-3p, and the enrichment fold of NORAD was analyzed by qRT-PCR. N = 3, * P < 0.05, ** P < 0.01, *** P < 0.001.

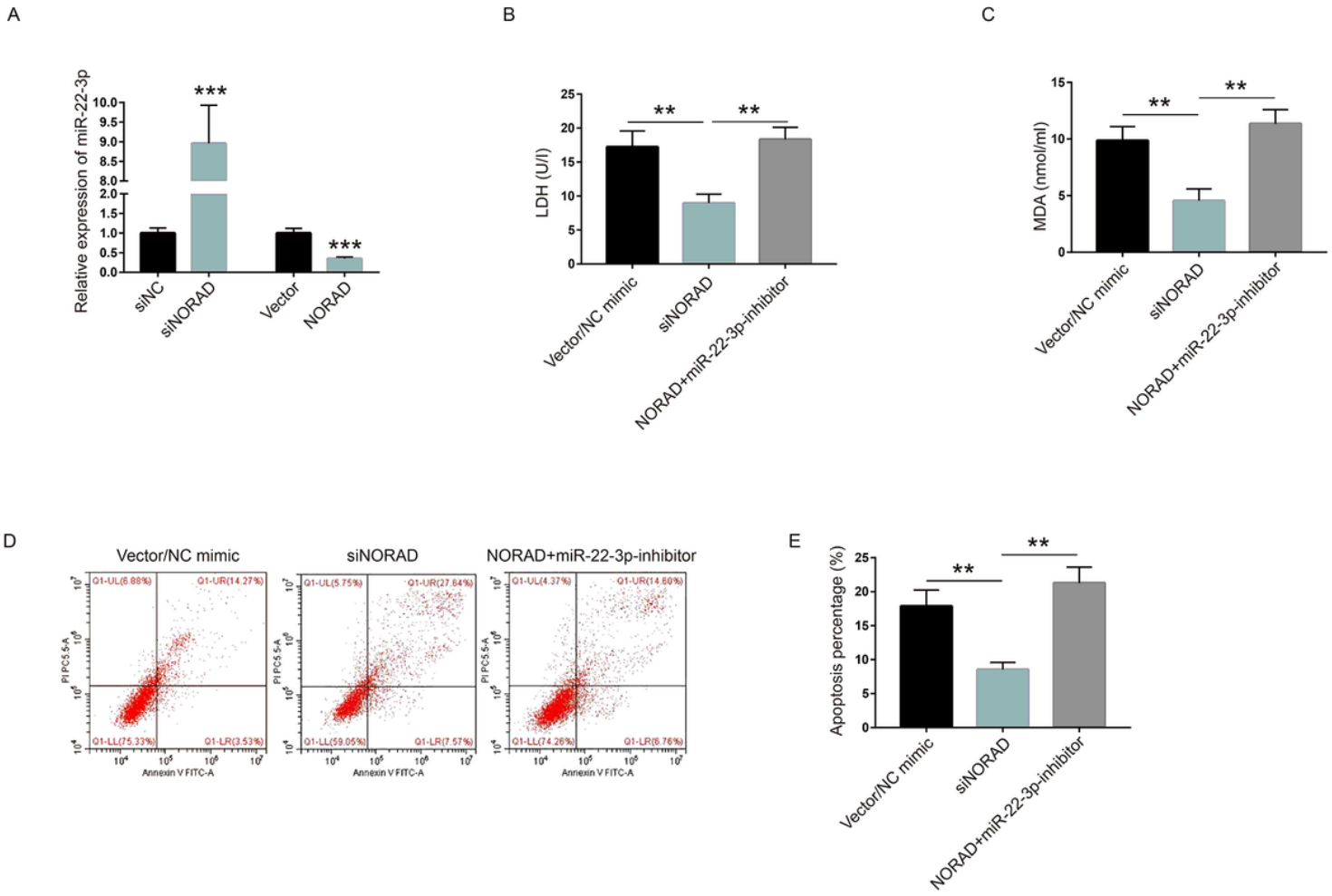

Figure 3

NORAD inhibited the apoptosis of H/R-treated HL-1 cells through sponging miR-22-3p. (A) HL-1 cells were transfected with si-NORAD, OE-NORAD, or corresponding negative controls, and then treated with H/R. The mRNA level of miR-22-3p was evaluated by qRT-PCR. (B-D) HL-1 cells were transfected with si-NORAD or co-transfected with si-NORAD and miR-22-3p inhibitor, and then treated with H/R. The LDH (B) and MDA (C) level in the cell supernatants was evaluated by corresponding detection kits. (D) The apoptotic rate was evaluated by flow cytometry. N = 3, ** P < 0.01, *** P < 0.001.

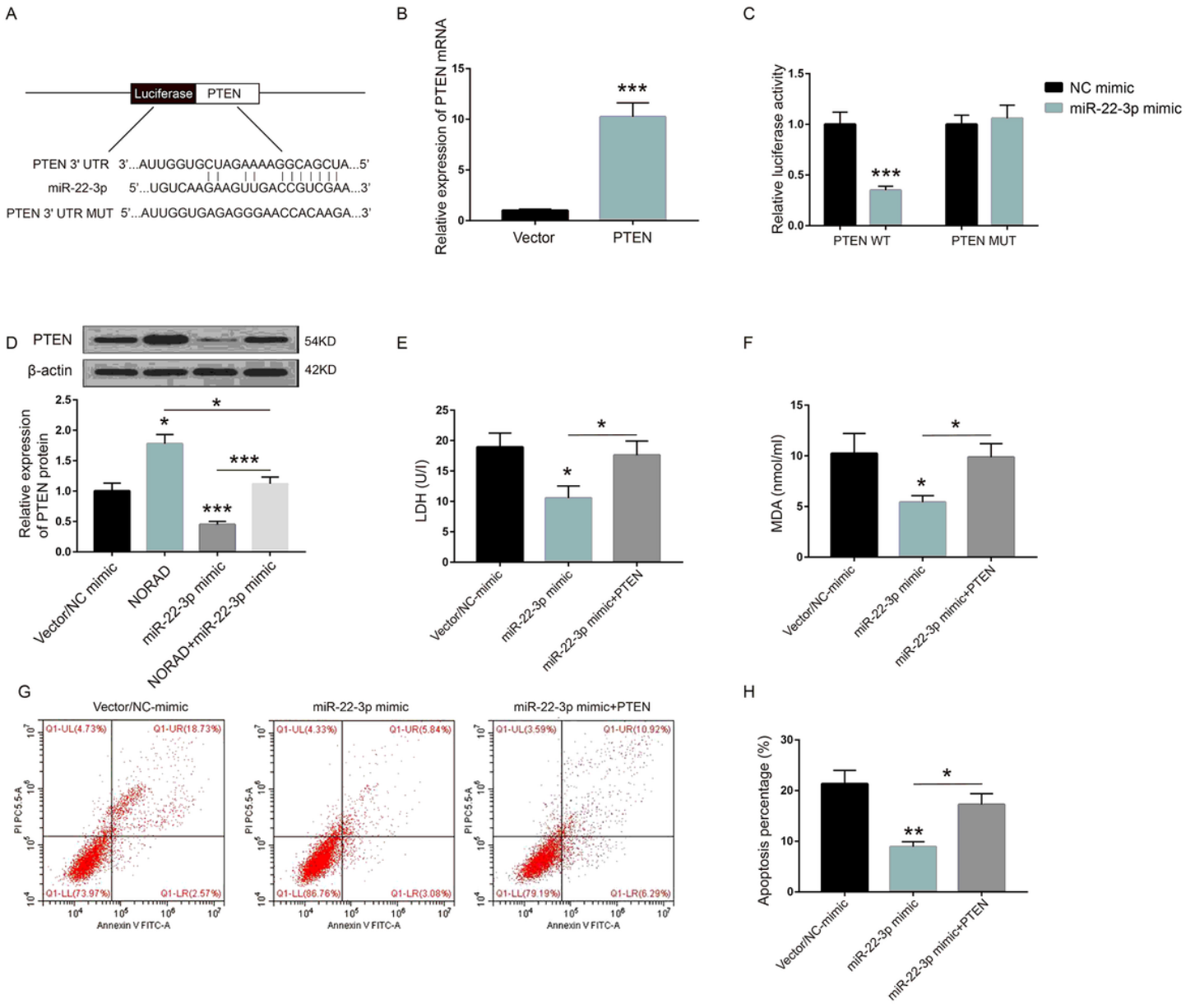

Figure 4

PTEN was a target of miR-22-3p. (A) The putative interaction between miR-22-3p and PTEN was predicted by Targetscan. (B) HL-1 cells were transfected with OE-PTEN or empty vector pcDNA3.1, and then treated with H/R. The mRNA level of PTEN was evaluated by qRT-PCR. (C) HL-1 cells were co-transfected with luciferase reporter plasmids containing WT or MUT PTEN against miR-22-3p and miR-22-3p mimics or miR-NC control, and the relative luciferase activity was evaluated by dual luciferase reporter system. (D) HL-1 cells were transfected with OE-NORAD, miR-22-3p, or co-transfected with OE-NORAD andmiR-22-3p, then treated with H/R. The protein level of PTEN was evaluated by western blot. (E-H) HL-1 cells were transfected with miR-22-3p mimics or co-transfected with miR-22-3p mimics and OE-PTEN, and then treated with H/R. The LDH (E) and MDA (F) level in the cell supernatants was evaluated by corresponding detection kits. (G and H) The apoptotic rate was evaluated by flow cytometry. N = 3, * P < 0.05, ** P < 0.01, *** P < 0.001.

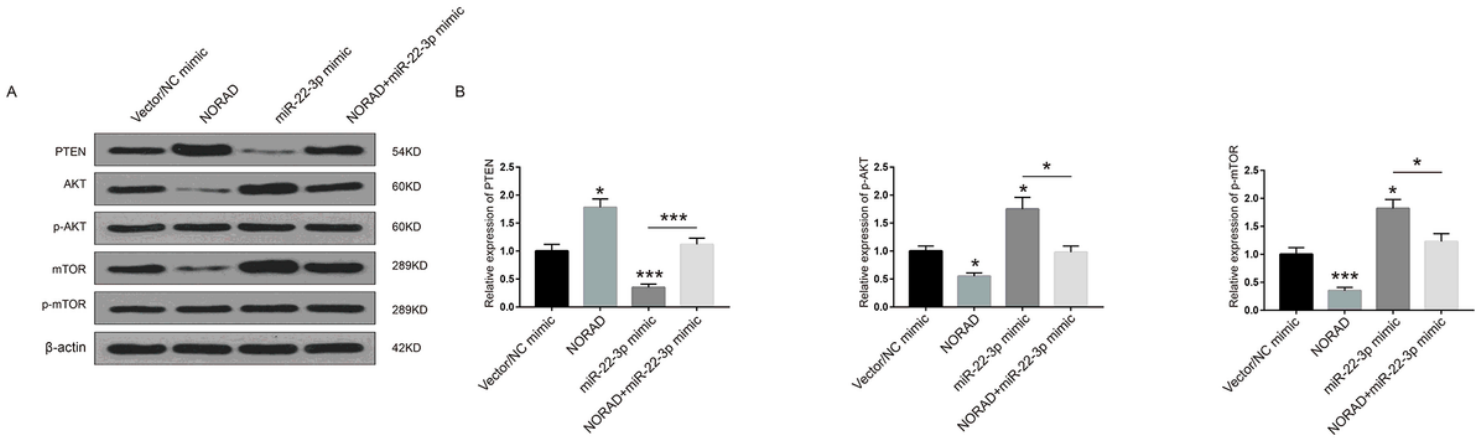

Figure 5

Upregulation of NORAD promoted the mTOR/AKT signaling pathway through sponging miR-22-3p. (A and B) HL-1 cells were transfected with OE-NORAD, miR-22-3p mimics, or co-transfected with OE-NORAD and miR-22-3p mimics, then treated with H/R. The protein expression of PTRN, AKT, p-AKT, mTOR, p-mTOR was evaluated by western blot with β-actin as the internal reference (A). (B) The data was analyzed by using the ImageJ2X software. N = 3, * P < 0.05, ** P < 0.01, *** P < 0.001.