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Long non-coding RNA FTX alleviates hypoxia/reoxygenation-induced cardiomyocyte injury *via* miR-410-3p/Fmr1 axis

L. LI[1], L. LI[1], Y.-Z. ZHANG[1], H.-Y. YANG[2], Y.-Y. WANG[1]

[1]Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[2]Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) are involved in the development of myocardial ischemia/reperfusion (I/R) injury. In this study, we aimed to investigate the roles and underlying mechanisms of five prime to Xist (FTX) in myocardial I/R injury using cardiomyocyte hypoxia/reoxygenation (H/R) model.

MATERIALS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to determine the expression of FTX, microRNA-410-3p (miR-410-3p) and fragile X mental retardation 1 (Fmr1) mRNA. Cell Counting Kit-8 (CCK-8) assay and flow cytometry analysis were employed to evaluate cell proliferation and apoptosis, respectively. Western blot assay was conducted to examine the protein levels of apoptosis-associated factors and Fmr1. Specific kits were used to detect the levels of oxidative stress-associated factors. Dual-luciferase reporter assay was performed to verify the association between miR-410-3p and FTX or Fmr1.

RESULTS: FTX was reduced in myocardial I/R injury patients' serum and H/R-stimulated H9c2 cells. FTX overexpression relieved cell damage caused by H/R treatment through inducing cell proliferation and repressing cell apoptosis and oxidative stress in H9c2 cells. FTX was a sponge for miR-410-3p and the impact of FTX overexpression on H/R-induced cell injury was abolished by miR-410-3p elevation in H9c2 cells. Fmr1 was identified as a target of miR-410-3p and Fmr1 knockdown reversed the effect on H/R-induced cell damage mediated by miR-410-3p inhibition in H9c2 cells. Moreover, FTX positively regulated Fmr1 expression through sponging miR-410-3p in H9c2 cells.

CONCLUSIONS: FTX regulated H/R-induced cardiomyocyte damage by upregulating Fmr1 *via* sponging miR-410-3p.

Key Words:
Myocardial ischemia/reperfusion (I/R) injury, Hypoxia/reoxygenation (H/R), FTX, MiR-410-3p, Fmr1.

Introduction

Acute myocardial infarction (AMI) is a local myocardial necrosis caused by acute and persistent ischemia and hypoxia, which can often endanger patients' life[1]. Reperfusion therapy is the main method for the treatment of myocardial infarction. However, cardiomyocytes may undergo drastic changes and aggravate the damage of myocardial during reperfusion, which is termed as myocardial ischemia/reperfusion (I/R) injury[2,3]. The main mechanisms of I/R injury are very complex and include increased oxygen free radical production, calcium overload, inflammatory response, mitochondrial damage, cell apoptosis and autophagy[4]. Although great efforts have been made by researchers, I/R injury remains a major obstacle for the treatment of myocardial infarction.

Long non-coding RNAs (lncRNAs), are a set of non-coding RNAs (ncRNAs) greater than 200 nucleotides (nts), without protein-coding potential[5]. Emerging studies have paid attention to the roles of lncRNAs in the pathogenesis of cardiovascular diseases[6,7]. More and more lncRNAs have been demonstrated to be involved in myocardial I/R injury. Ren et al[8] declared that NEAT1 was conspicuously elevated in myocardial I/R injury cells and knockdown of NEAT1 facilitated cell growth and hampered cell apoptosis in I/R injury H9c2 cells. Zhang et al[9] disclosed that ROR could aggravate I/R-induced myocardial damage *via* repressing cell growth, inducing cell apoptosis and elevating the release of oxidative stress-associated factors. Wang et al[10] referred that UCA1 was decreased in hypoxia/reoxygenation (H/R)-stimulated cardiomyocytes and UCA1 elevation repressed H/R-stimulated

Corresponding Author: Ling Li, MD; e-mail: zdliling@163.com

cardiomyocyte apoptosis. These data suggested that lncRNAs exert different roles in I/R-induced myocardial damage. Five prime to Xist (FTX) was demonstrated to be involved in the regulation of cardiomyocyte apoptosis induced by hydrogen peroxide[11]. However, the precise roles and underlying mechanisms of FTX in the development of myocardial I/R injury are still largely unknown.

MicroRNAs (miRNAs), a type of ncRNAs with 19-22 nts, exert as regulators of genes expression *via* recognizing the 3'-untranslated region (3'-UTR) of target genes[12]. Accumulating evidence demonstrated that diverse miRNAs take part in the regulation of myocardial I/R injury. For instance, Wu et al[13] uncovered that miR-335 was reduced in H/R-induced cardiomyocytes and I/R-exposed heart tissues, and its overexpression could relieve the injury mediated by H/R treatment. Sun et al[14] implicated that miR-148-3p was induced by H/R treatment and the elevation of miR-148-3p could repress the growth and facilitate the apoptosis of H/R-induced cardiomyocytes. Chen et al[15] demonstrated that H/R treatment increased miR-195 expression and enhanced cardiomyocyte apoptosis. MiR-410-3p was confirmed to be associated with the development of multiple human diseases[16,17]. Moreover, Teng et al[18] disclosed that miR-410-3p protected hypoxia-induced cardiomyocyte injury. However, it is still not very clear whether miR-410-3p is involved in the progression of myocardial I/R injury.

Fragile X mental retardation 1 (Fmr1) is located on chromosome Xq27.3 with 38 kb in length and it encodes a selective RNA-binding protein fragile X mental retardation protein (FMRP)[19]. A previous report claimed that Fmr1 could alleviate lipopolysaccharide (LPS)-induced myocardial injury[20]. Nevertheless, there has been no report on the roles of Fmr1 in the process of myocardial I/R injury.

Here, we explored the expression of FTX in myocardial I/R injury patients' serum and H/R-induced H9c2 cells. Additionally, the functional roles and underlying mechanisms of FTX in H/R-induced cardiomyocyte injury were determined.

Materials and Methods

Serum Samples Collection

After approval was obtained from the Ethics Committee of The First Affiliated Hospital of Zhengzhou University and written informed consents were signed by all participants, serum samples were collected from 20 patients with myocardial I/R injury and 20 healthy donors at The First Affiliated Hospital of Zhengzhou University. The collected serum samples were preserved at -80°C.

Cell Culture and H/R Treatment

Rat cardiomyocytes H9c2 cells were bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells grew in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South-Logan, UT, USA), including 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin (HyClone) in an incubator with 5% CO_2 at 37°C.

To establish H/R model, H9c2 cells were kept in an incubator with 95% N_2 and 5% CO_2 for 8 h to experience hypoxia. Then, cells were maintained at an atmosphere of 95% O_2 and 5% CO_2 for reoxygenation for 16 h. H9c2 cells in normoxia conditions were used as control.

Cell Transfection

The overexpression vector of FTX (FTX) and pcDNA3.1 empty vector (pcDNA); small interfering RNA (siRNA) against FTX (si-FTX), siRNA against Fmr1 (si-Fmr1) and their control (si-NC); mimics of miR-410-3p (miR-410-3p) and its control (miR-NC); inhibitors of miR-410-3p (anti-miR-410-3p) and its control (anti-miR-NC) were bought from GeneCopoeia (Guangzhou, China). The transfection of H9c2 cells was done with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of culture, cells were harvested.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The collected serum samples and H9c2 cells were lysed in TRIzol reagent (Beyotime, Shanghai, China) to extract total RNA. Then RNAs were transcribed into cDNAs with PrimeScript reverse transcriptase reagent Kit (TaKaRa, Dalian, China) or All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia). QRT-PCR was conducted using SYBR Premix EX Taq Kit (TaKaRa) on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression of FTX, Fmr1 and miR-410-3p was examined using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or small nuclear RNA U6 was regarded as the internal control. The prim-

ers were: FTX (Mouse): (F: 5'-ATCTTCTTG-CGCTCCTCCTT-3' and R: 5'-TGTGTC-CAGGGCTGTCTGT-3'); Fmr1 (Mouse): (F: 5'-GAGGGTGAGGATCGAAGCTG-3' and R: 5'-GTACCATCCCCCTCTGGACT-3'); miR-410-3p (Mouse): (F: 5'-CCGCCA ATATAACA-CAGATGGCC-3' and R: 5'-GGGTCCGAGG-TATTCGCACCAGAGC-3'); GAPDH (Mouse): (F: 5'-GGTCATGAGTCCTTCCACGATA-3' and R: 5'-ATGCTGGCGCTGAGTACGTC-3'); U6 (Mouse): (F: 5'-GCGCGTCGTGAAGCGTTC-3' and R: 5'-GTGCAGGGTCCGAGGT-3'); FTX (Human): (F: 5'-GAATGTCCTTGTGAGG-CAGTTG-3' and R: 5'-TGGTCACTCACATG-GATGATCTG-3'); Fmr1 (Human): (F: 5'-CAC-CATGGAAGATCTCCTCGTG-3' and R: 5'-GGACGTGCCATTGACCAG-3'); miR-410-3p (Human): (F: 5'-CGCGAATATAACACAGATG-GCCTGT-3' and R: 5'-CAGTGCGTGTCGTG-GAGT-3'); GAPDH (Human): (F: 5'-GGAGC-GAGATCCCTCCAAAAT-3' and R: 5'-GGCT-GTTGTCATACTTCTCATGG-3'); U6 (Human): (F: 5'-TGCGGGTGCTCGCTTCGGCAGC-3' and R: 5'-CCAGTGCAGGGTCCGAGGT-3').

Cell Counting Kit-8 (CCK-8) Assay

After relevant treatment, the proliferation ability of H9c2 cells was assessed through CCK-8 assay. In brief, H9c2 cells were plated into 96-well plates and then 10 μL CCK-8 (Solarbio, Beijing, China) was put into each well and kept for additional 2 h. The absorbance at 450 nm was determined with a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry Analysis

Following indicated treatment, H9c2 cells were harvested and cell apoptosis was evaluated *via* the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China). In brief, 5 μL AnnexinV-FITC and 5 μL PI were added to the cell suspension and incubated with cells for 15 min in the dark. Then the apoptosis of H9c2 cells was evaluated *via* a FACScan® flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h.

Western Blot Assay

Total protein was extracted from the harvested serum samples and H9c2 cells by using RIPA buffer (Beyotime) and quantified using a BCA Protein Quantification Kit (Vazyme). Then the same amount of proteins was subjected to sodium dodecyl sulfonate-polyacrylamide gel (SDS-PAGE; Solarbio) and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). Next, the membranes were blocked in non-fat milk for 2 h followed by incubation with primary antibodies against B-cell lymphoma protein 2 (Bcl-2; ab196495; Abcam, Cambridge, MA, USA), Bcl-2-associated X (Bax; ab32503; Abcam), cleaved-caspase-3 (cleaved casp-3; ab49822; Abcam), Fmr1 (ab10299; Abcam) or GAPDH (ab181602; Abcam) and corresponding secondary antibody (ab6728; Abcam). Finally, protein bands were visualized by an enhanced chemiluminescence reagent (Vazyme Biotech Co, Nanjing, China).

Detection of Oxidative Stress-Associated Factors

The levels of oxidative stress-associated factors were determined using specific kits based on the descriptions in the manual. The level of lactate dehydrogenase (LDH) was measured using Rat LDH enzyme linked immunoassay (ELISA) Kit (Sangon Biotech, Shanghai, China). The level of malondialdehyde (MDA) was determined using Rat MDA ELISA Kit (Sangon Biotech). The level of superoxide dismutase (SOD) was examined using Lipid Peroxidation MDA Assay kit (Beyotime). The level of glutathione peroxidase (GSH-PX) was detected with Rat GSH-Px ELISA Kit (Sangon Biotech).

Dual-Luciferase Reporter Assay

The fragments of FTX or Fmr1 3'-UTR including the wild-type or mutant binding sites of miR-410-3p were cloned into pmirGLO vector (Promega, Madison, WI, USA) to generate luciferase reporters FTX WT, FTX MUT, Fmr1 3'-UTR-WT and Fmr1 3'-UTR-MUT, respectively. Then the indicated vector was transfected into H9c2 cells together with miR-410-3p or miR-NC using Lipofectamine 2000 (Invitrogen). Dual-Luciferase Reporter Assay Kit (Promega) was utilized to analyze the luciferase reporter activity after cells were co-transfected for 48 h.

Statistical Analysis

The experiments in this research were repeated three times. The collected data were analyzed using GraphPad Prism 7 software (GraphPad Inc., La Jolla, CA, USA) and presented as mean ± standard deviation (SD). Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test was utilized for difference analysis. The linear relationships

among FTX, miR-410-3p and Fmr1 were analyzed through Spearman's correlation analysis. *p*-value less than 0.05 was considered as statistically significant.

Results

FTX was Downregulated in the Serum of Patients with Myocardial I/R Injury and H9c2 Cells Treated with H/R

To investigate the role of FTX in myocardial I/R injury, the expression level of FTX in the serum of myocardial I/R injury patients was determined *via* qRT-PCR. The data indicated that the level of FTX was conspicuously reduced in the serum of myocardial I/R injury patients compared to that in normal group (Figure 1A). Besides, the expression of FTX was drastically decreased in H9c2 cells treated with H/R when compared to cells under the condition of normoxia (Figure 1B). These findings suggested that FTX might be involved in the progression of I/R-induced myocardial injury.

Overexpression of FTX Relieved the Damage Caused by H/R Treatment in H9c2 Cells

To assess the functional role of FTX in H/R-induced injury in H9c2 cells, we treated H9c2 cells with normoxia, H/R, H/R+pcDNA or H/R+FTX. As presented in Figure 2A, the decreased expression of FTX mediated by H/R was partially reversed by FTX transfection in H9c2 cells. The results of CCK-8 assay implicated that H/R treatment led to a remarkable reduction in cell proliferation, while FTX overexpression distinctly reversed this reduction in H9c2 cells (Figure 2B). Flow cytometry analysis data showed that the apoptosis of H9c2 cells was evidently enhanced after H/R treatment, whereas this impact on cell apoptosis was partly restored by the transfection of FTX (Figure 2C). The protein levels of apoptosis-associated factors (Bcl-2, Bax and cleaved-casp-3) were examined through Western blot assay. The results implied that Bcl-2 was decreased, and Bax and cleaved-casp-3 were increased under the treatment of H/R in H9c2 cells, while the elevation of FTX abolished these influences (Figure 2D). Furthermore, the levels of oxidative stress-associated factors (including LDH, MDA, SOD and GSH-PX) were investigated *via* relevant kits. The data revealed that LDH, MDA, SOD and GSH-PX were all elevated in H9c2 cells treated with H/R compared to those in normal condition; however, the overexpression of FTX markedly overturned these effects (Figure 2E-H). All these results demonstrated that the injury caused by H/R treatment was markedly abolished *via* the elevation of FTX in H9c2 cells.

FTX Negatively Modulated miR-410-3p Expression via Directly Targeting miR-410-3p in H/R-Stimulated H9c2 Cells

In order to identify the underlying mechanism of FTX, online software LncBase Predicted v.2 was utilized to predict the target of FTX. As shown in Figure 3A, miR-410-3p was predicted to be a target of FTX and their

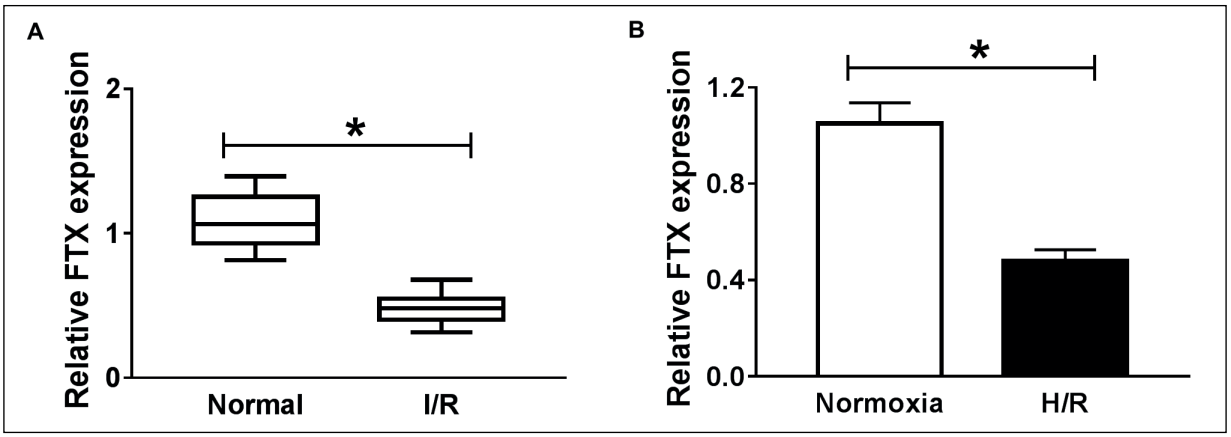

Figure 1. FTX was distinctly decreased in myocardial I/R injury patients' serum and H/R-treated H9c2 cells. **A,** The expression of FTX in the serum of I/R-induced myocardial injury patients and normal serum was determined through qRT-PCR. **B,** The expression of FTX in H9c2 cells after treatment of H/R and normoxia was measured via qRT-PCR. **p*<0.05.

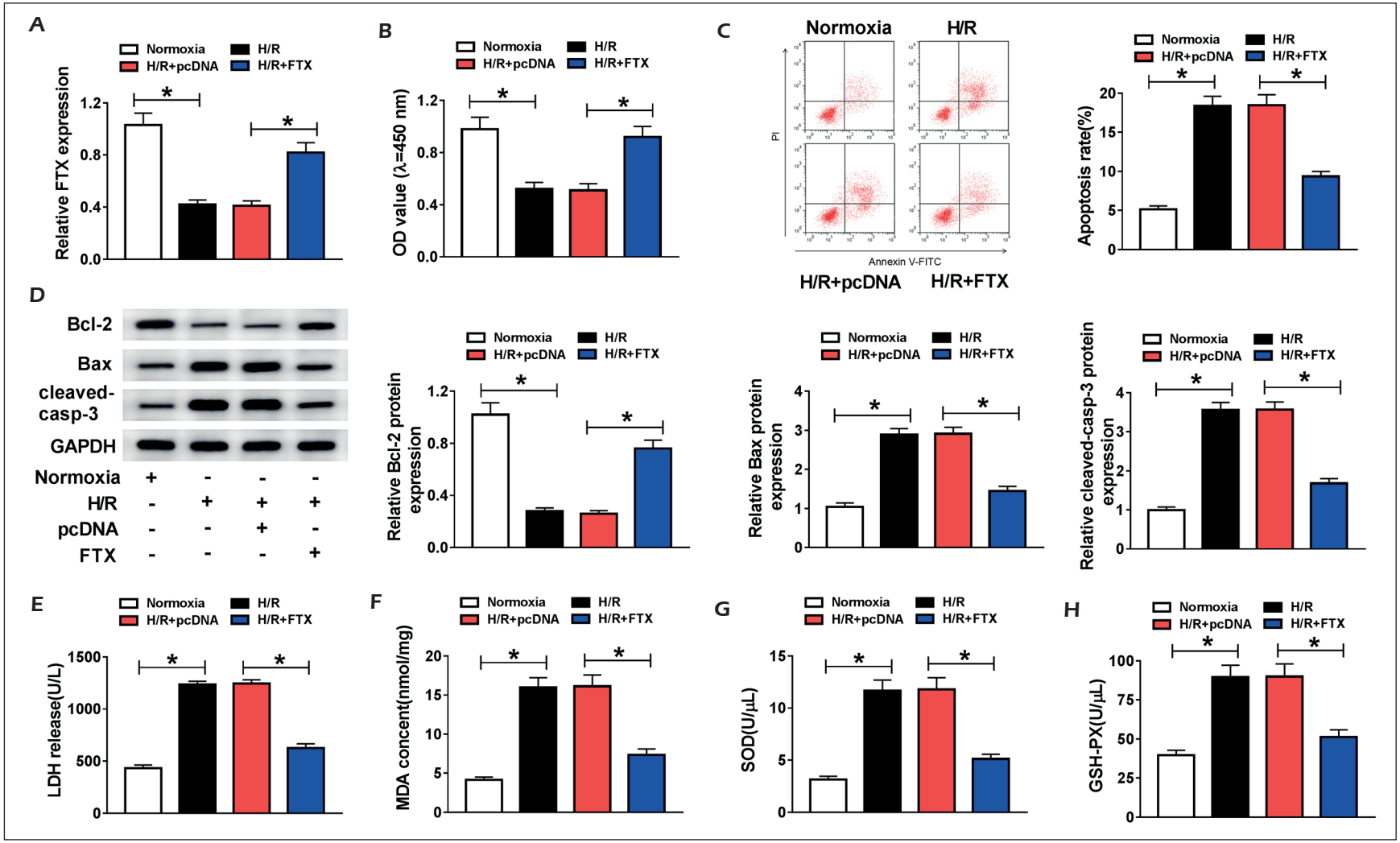

Figure 2. FTX overexpression weakened the damage caused by H/R treatment in H9c2 cells. H9c2 cells were assigned to normoxia, H/R, H/R+pcDNA and H/R+FTX groups. **A,** The expression of FTX in H9c2 cells was measured with qRT-PCR assay. **B,** The proliferation of H9c2 cells was evaluated via CCK-8 assay. **C,** The apoptosis of H9c2 cells was assessed through flow cytometry analysis. **D,** The protein levels of Bcl-2, Bax and cleaved-casp-3 in H9c2 cells were determined via Western blot assay. **E,-H,** The levels of oxidative stress-associated factors (including LDH, MDA, SOD and GSH-PX) in H9c2 cells were detected by specific kits. * $p<0.05$.

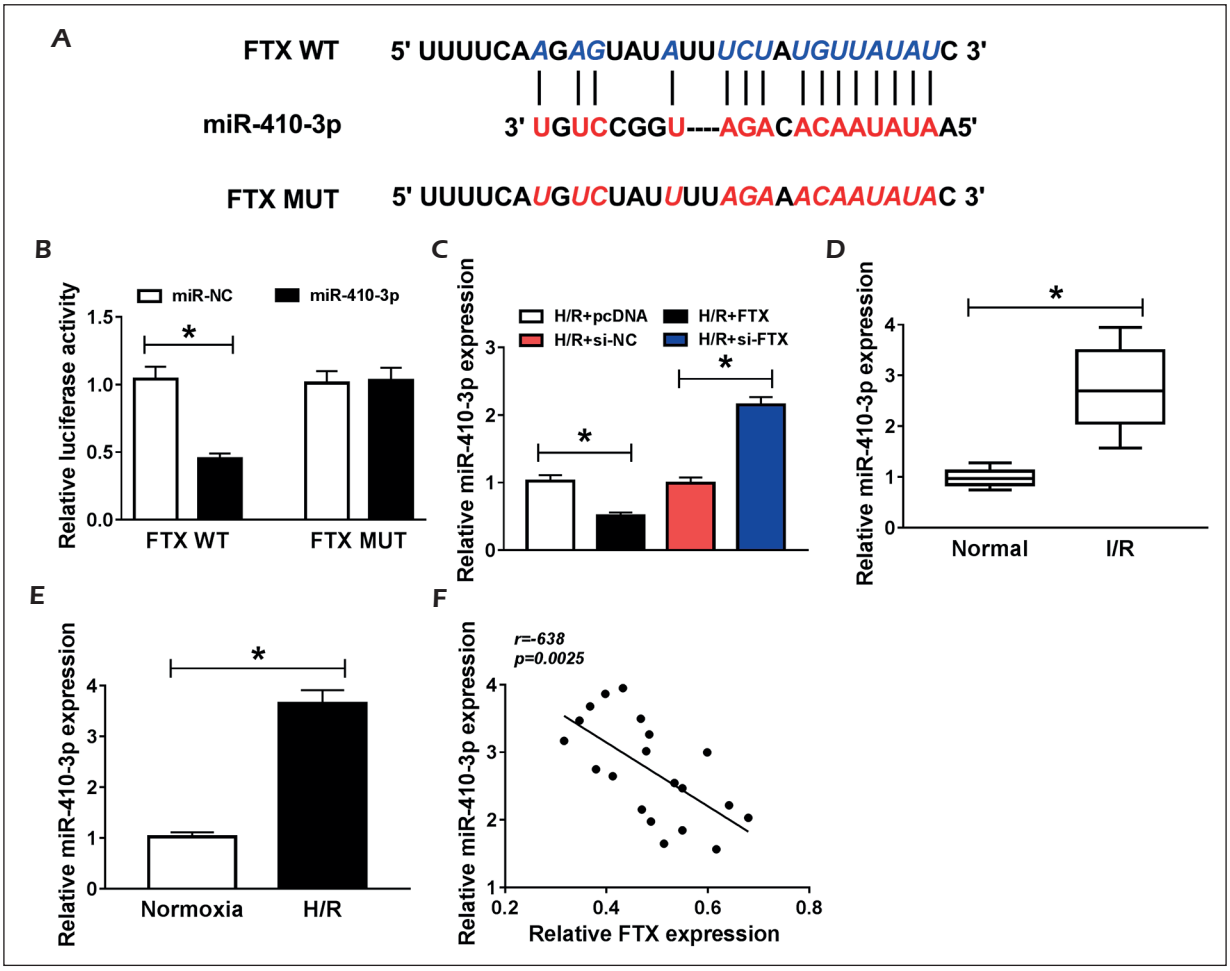

Figure 3. FTX sponged miR-410-3p to alter the expression of miR-410-3p in H/R-treated H9c2 cells. **A,** The potential binding sites between FTX and miR-410-3p were exhibited. **B,** The luciferase activity in H9c2 cells co-transfected with FTX WT or FTX MUT and miR-410-3p or miR-NC was examined by dual-luciferase reporter assay. **C,** The level of miR-410-3p in H9c2 cells treated with H/R+pcDNA, H/R+FTX, H/R+si-NC or H/R+si-FTX was detected via qRT-PCR. **D,-E,** The expression of miR-410-3p in myocardial I/R injury patients' serum and H/R-induced H9c2 cells was measured through qRT-PCR. **F,** The correlation between FTX and miR-410-3p was analyzed by Spearman's correlation analysis. *$p<0.05$.

potential binding sites were presented. Next, dual-luciferase reporter assay was employed to verify this prediction. The results implicated the luciferase activity was suppressed in H9c2 cells co-transfected with FTX WT and miR-410-3p in reference to that in H9c2 cells co-transfected with FTX WT and miR-NC, while the luciferase activity was not affected in FTX MUT group (Figure 3B). Subsequently, H9c2 cells were treated with H/R+pcDNA, H/R+FTX, H/R+si-NC or H/R+si-FTX and then the expression of miR-410-3p was determined by qRT-PCR. The results exhibited that FTX overexpression significantly inhibited the expression of miR-410-3p and FTX knockdown distinctly promoted the expression of miR-410-3p in H/R-induced H9c2 cells (Figure 3C). Moreover, we examined the expression of miR-410-3p in the serum of patients with myocardial I/R injury and H9c2 cells exposed to H/R. The data showed that miR-410-3p was notably elevated in myocardial I/R injury patients' serum and H/R-induced H9c2 cells (Figure 3D and E). As illustrated by Spearman's correlation analysis, miR-410-3p expression was negatively correlated with FTX expression in the serum of patients with myocardial I/R injury (Figure 3F). Taken together, FTX negatively regulated miR-410-3p expression by direct interaction in H/R-induced H9c2 cells.

MiR-410-3p Overexpression Ameliorated the Impact on the Injury Mediated by FTX in H/R-Induced H9c2 Cells

Based on the above results, we wondered whether FTX could alter the development of H/R-induced cell injury *via* targeting miR-410-3p. H9c2 cells were treated with normoxia, H/R, H/R+pcDNA, H/R+FTX, H/R+FTX+miR-NC or H/R+FTX+miR-410-3p. As we observed in Figure 4A, H/R treatment induced a remarkable increase in miR-410-3p expression but FTX transfection weakened this increase in H9c2 cells. Moreover, the effect of FTX transfection on miR-410-3p expression was partly abolished by miR-410-3p transfection. CCK-8 assay indicated that H/R treatment resulted in a reduction in the proliferation of H9c2 cells, while FTX overexpression restored this effect. Further, the elevated cell proliferation mediated by FTX overexpression was reversed by miR-410-3p (Figure 4B). As revealed by flow cytometry analysis, cell apoptosis was induced by H/R treatment in H9c2 cells, while FTX transfection partially restored this effect. In addition, miR-410-3p transfection rescued the impact of FTX transfection on cell apoptosis (Figure 4C). The data of Western blot assay displayed that the upregulation of Bcl-2 and the downregulation of Bax and cleaved-casp-3 mediated by FTX overexpression were partly relieved by the elevation of miR-410-3p in H/R-stimulated H9c2 cells (Figure 4D). Besides, the expression levels of LDH, MDA, SOD and GSH-PX were determined using specific kits. We found that LDH, MDA, SOD and GSH-PX were all increased by the treatment of H/R and FTX overexpression weakened these effects; moreover, miR-410-3p elevation further restored the impacts of FTX overexpression on LDH, MDA, SOD and GSH-PX levels in H9c2 cells (Figure 4E-H). Collectively, FTX could relieve H/R-induced cell injury by targeting miR-410-3p in H9c2 cells.

MiR-410-3p Negatively Regulated Fmr1 Expression by Direct Interaction in H/R-Induced H9c2 Cells

Through searching prediction software starBase v2.0, Fmr1 was predicted to be a target gene of miR-410-3p and the potential binding sites were shown in Figure 5A. To verify it, we performed dual-luciferase reporter assay. The data showed that compared to miR-NC and Fmr1 3'-UTR-WT co-transfected group, co-transfection of miR-410-3p and Fmr1 3'-UTR-WT led to an effective inhibition in the luciferase activity in H9c2 cells, while the luciferase activity was not affected in Fmr1 3'-UTR-MUT group (Figure 5B). Then we treated H9c2 cells with H/R+miR-NC, H/R+miR-410-3p, H/R+anti-miR-NC or H/R+anti-miR-410-3p to explore the association between miR-410-3p and Fmr1. As exhibited in Figure 5C and D, the mRNA and protein levels of Fmr1 were decreased in H/R-induced H9c2 cells following miR-410-3p transfection and were increased after anti-miR-410-3p transfection. As we expected, the mRNA and protein levels of Fmr1 in myocardial I/R injury patients' serum and H/R-stimulated H9c2 cells were dramatically reduced compared to control groups (Figure 5E-H). Furthermore, Spearman's correlation analysis showed that there was an inverse correlation between the expression of miR-410-3p and Fmr1 mRNA in the serum of myocardial I/R injury patients (Figure 5I). Taken together, miR-410-3p directly bound to Fmr1 and negatively modulated Fmr1 expression in H/R-treated H9c2 cells.

Silencing of Fmr1 Weakened the Influence of miR-410-3p Inhibition on the Progression of H/R-Induced Injury in H9c2 Cells

In order to reveal the association of miR-410-3p and Fmr1 in regulating H/R-induced injury in H9c2 cells, H9c2 cells were assigned to normoxia, H/R, H/R+anti-miR-NC, H/R+anti-miR-410-3p, H/R+anti-miR-410-3p+si-NC and H/R+anti-miR-410-3p+si-Fmr1 groups. As displayed in Figure 6A and B, the decreased expression of Fmr1 mRNA and Fmr1 protein induced by H/R was effectively increased following anti-miR-410-3p transfection in H9c2 cells, while si-Fmr1 transfection further abolished the effect of anti-miR-410-3p transfection on the expression of Fmr1 mRNA and Fmr1 protein. H/R treatment hampered the proliferation of H9c2 cells, whereas miR-410-3p knockdown significantly reversed the impact; moreover, silencing of Fmr1 further abolished the effect of miR-410-3p knockdown on H9c2 cell proliferation, as illustrated by CCK-8 assay (Figure 6C). Flow cytometry analysis showed that the increase of cell apoptosis mediated by H/R was weakened following miR-410-3p inhibition in H9c2 cells; however, the effect of miR-410-3p inhibition was overturned after the knockdown of Fmr1 (Figure 6D). Western blot assay displayed that Bcl-2 was reduced and Bax and cleaved-casp-3 were improved in H9c2 cells

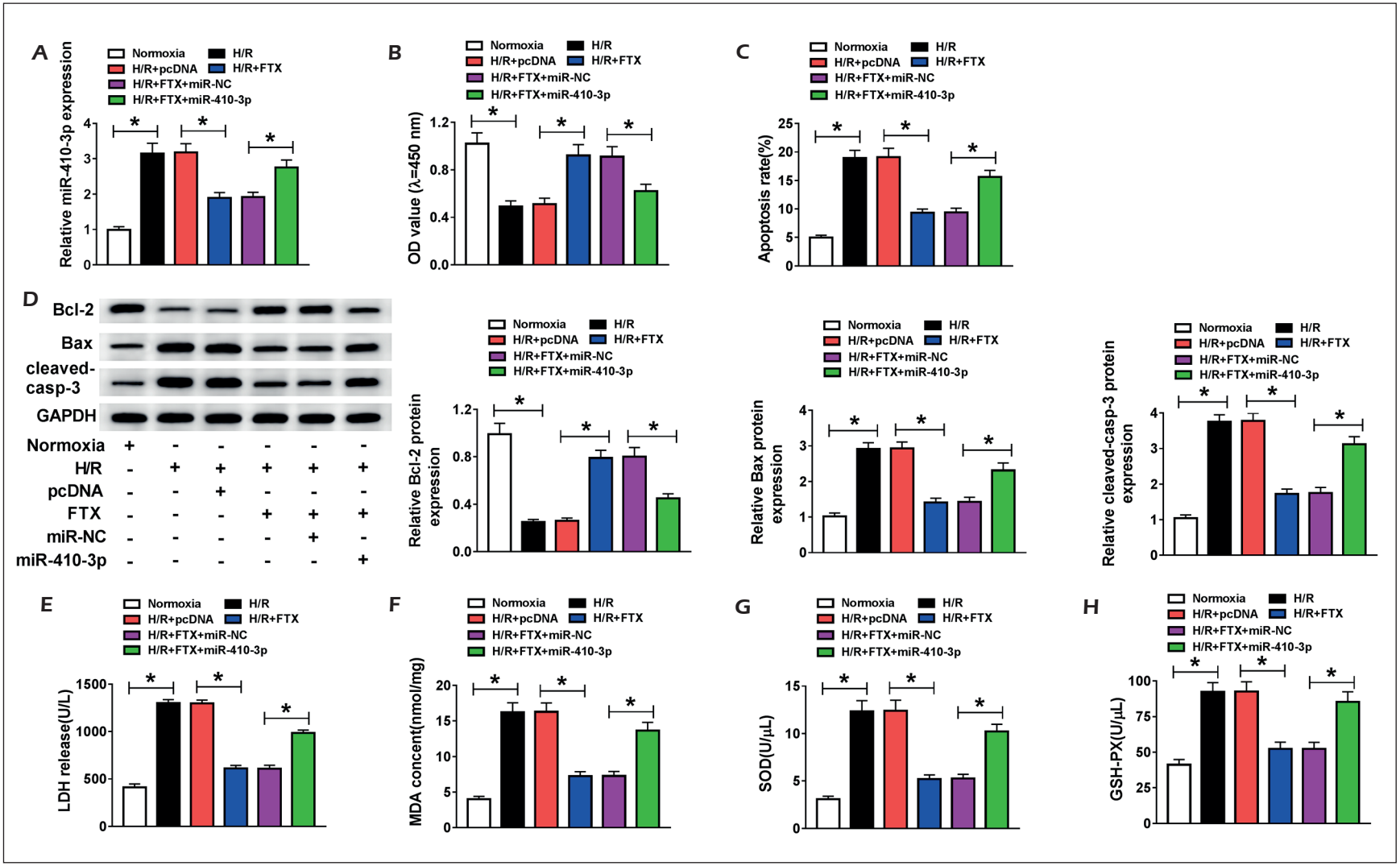

Figure 4. MiR-410-3p partially abrogated the injury mediated by FTX in H/R-induced H9c2 cells. H9c2 cells were divided into normoxia, H/R, H/R+pcDNA, H/R+FTX, H/R+FTX+miR-NC or H/R+FTX+miR-410-3p groups. **A,** The expression of miR-410-3p in H9c2 cells was examined through qRT-PCR. **B,** H9c2 cell proliferation was determined by CCK-8 assay. **C,** H9c2 cell apoptosis was analyzed via flow cytometry analysis. **D,** The protein levels of Bcl-2, Bax and cleaved-casp-3 were measured by Western blot assay. **E, H,** The levels of LDH, MDA, SOD and GSH-PX were detected using specific kits. *p<0.05.

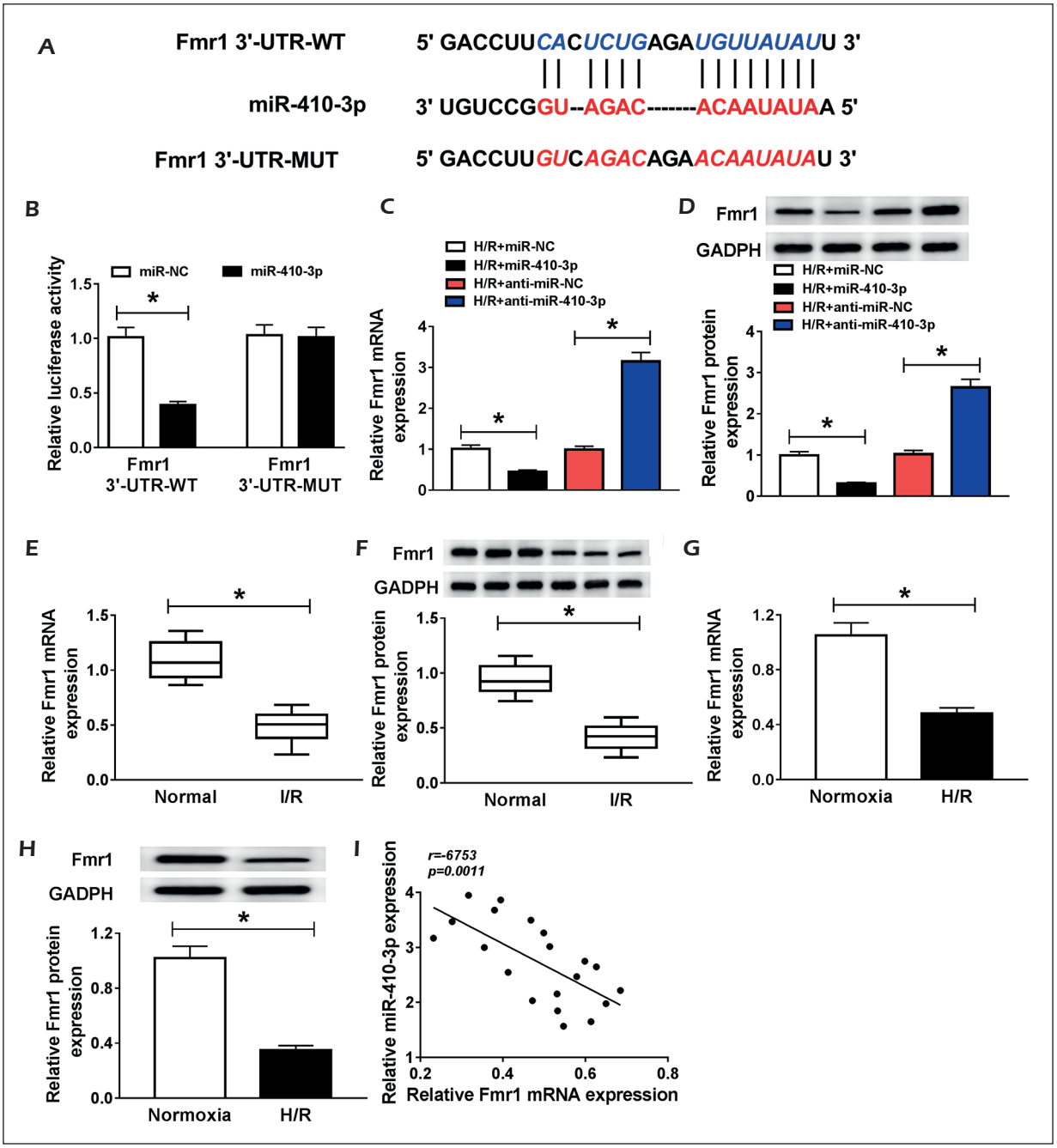

Figure 5. MiR-410-3p negatively modulated the expression of Fmr1 by directly targeting. **A,** The potential binding sites between miR-410-3p and Fmr1 were predicted by starBase v2.0. **B,** The targeting relationship was verified through dual-luciferase reporter assay. **C,-D,** The mRNA and protein levels of Fmr1 in H9c2 cells treated with H/R+miR-NC, H/R+miR-410-3p, H/R+anti-miR-NC or H/R+anti-miR-410-3p were determined via qRT-PCR assay and Western blot assay, respectively. **E,-H,** The mRNA and protein levels of Fmr1 in the serum of myocardial I/R injury patients, H/R-induced H9c2 cells and corresponding control groups were measured through qRT-PCR assay and Western blot assay, respectively. **I,** The correlation between miR-410-3p and Fmr1 was assessed by Spearman's correlation analysis. *p<0.05.

treated with H/R, while these effects were abolished by miR-410-3p inhibition. Also, the impacts of miR-410-3p inhibition on the levels of Bcl-2, Bax and cleaved-casp-3 were overturned following Fmr1 silencing (Figure 6E). MiR-410-3p inhibition restored the elevation in LDH, MDA, SOD and GSH-PX mediated by H/R treatment in H9c2 cells and silencing of Fmr1 further abrogat-

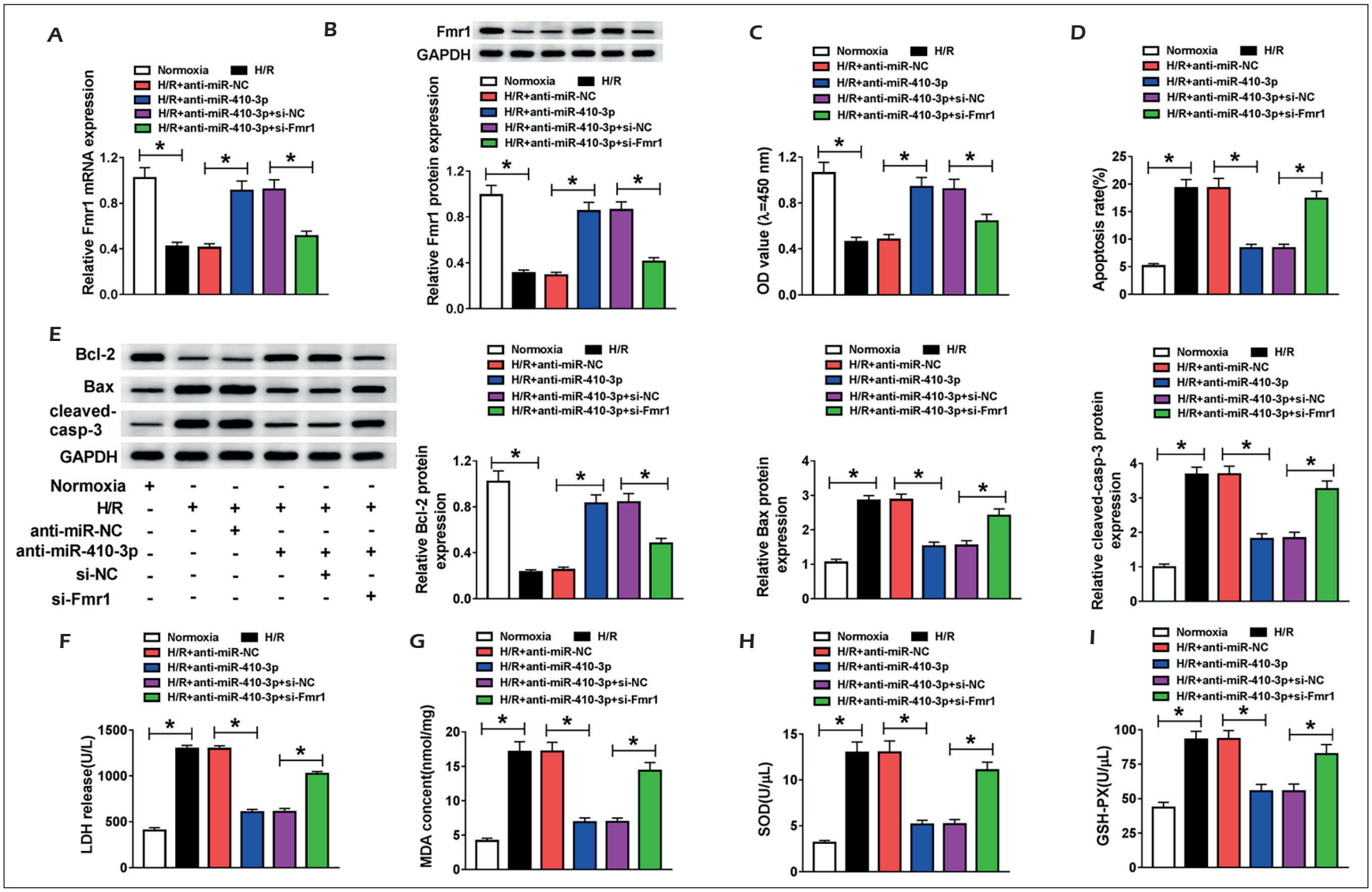

Figure 6. The damage mediated by miR-410-3p inhibition was rescued after Fmr1 knockdown in H/R-induced H9c2 cells. H9c2 cells were treated with normoxia, H/R, H/R+anti-miR-NC, H/R+anti-miR-410-3p, H/R+anti-miR-410-3p+si-NC or H/R+anti-miR-410-3p+si-Fmr1. **A,-B,** The mRNA and protein levels of Fmr1 in H9c2 cells were examined through qRT-PCR and Western blot assay, respectively. **C,** The ability of cell proliferation in H9c2 cells was detected by CCK-8 assay. **D,** The apoptosis of H9c2 cells was measured via flow cytometry analysis. **E,** The protein levels of Bcl-2, Bax and cleaved-casp-3 in H9c2 cells were determined through Western blot assay. **F,-I,** The levels of LDH, MDA, SOD and GSH-PX were measured using specific kits. *$p<0.05$.

ed the effects of miR-410-3p inhibition on LDH, MDA, SOD and GSH-PX levels (Figure 6F-I). The results revealed that miR-410-3p inhibition alleviated H/R-induced cell injury by targeting Fmr1 in H9c2 cells.

FTX Positively Regulated Fmr1 Expression via Sponging miR-410-3p in H/R-Induced H9c2 Cells

As shown in Figure 7A, Fmr1 mRNA expression was positively correlated with FTX expression in the serum of myocardial I/R injury patients. Next, to further confirm the association among FTX, miR-410-3p and Fmr1, H9c2 cells were treated with H/R+si-NC, H/R+si-FTX, H/R+si-FTX+anti-miR-NC or H/R+si-FTX+anti-miR-410-3p and then the mRNA and protein levels of Fmr1 were detected. The results exhibited that FTX depletion resulted in distinct reduction in the mRNA and protein levels of Fmr1 in H/R-induced H9c2 cell compared to control group, while the administration of anti-miR-410-3p markedly overturned the influences (Figure 7B and C). These findings demonstrated that FTX could modulate Fmr1 expression by sponging miR-410-3p in H/R-stimulated H9c2 cells.

Discussion

Numerous lncRNAs have been revealed to be aberrantly expressed in myocardial I/R injury patients. In this research, we investigated the functional roles and mechanisms of FTX in myocardial I/R injury by establishing a cardiomyocyte H/R model. We observed that FTX was elevated after I/R or H/R treatment. Mechanically, FTX alleviated H/R-induced injury *via* miR-410-3p/Fmr1 axis in H/R-stimulated cardiomyocytes.

Long et al[11] reported that FTX was weakly expressed subjected to I/R treatment and hydrogen peroxide treatment, and the apoptosis of cardiomyocytes induced by hydrogen peroxide treatment was repressed by FTX overexpression. Consistently, we found that FTX was reduced in the serum of patients with myocardial I/R injury and H/R-treated H9c2 cells. Furthermore, the decreased cardiomyocyte growth and increased cardiomyocyte apoptosis induced by H/R treatment were overturned by the overexpression of FTX. Besides, the levels of LDH, MDA, SOD and GSH-PX were increased after H/R treatment and further reduced in H/R-stimulated H9c2 cells treated with FTX. Zhang et al[9] disclosed that H/R treatment led to remarkable elevation in LDH, MDA, SOD and GSH-PX; and the enhanced expression of ROR further promoted their release. These different data indicated that different lncRNAs might play different roles in the process of myocardial I/R injury.

It is documented that FTX participates in the regulation of multiple human cancers *via* functioning as a miRNA sponge. For instance, FTX could accelerate the progression of glioma through binding to miR-342-3p[21]. FTX interacted with miR-215 to participate in the development of colorectal cancer[22]. Herein, FTX acted as a sponge for miR-410-3p in H/R-stimulated H9c2 cells. MiR-410-3p transfection restored the influences on cell growth, apoptosis and

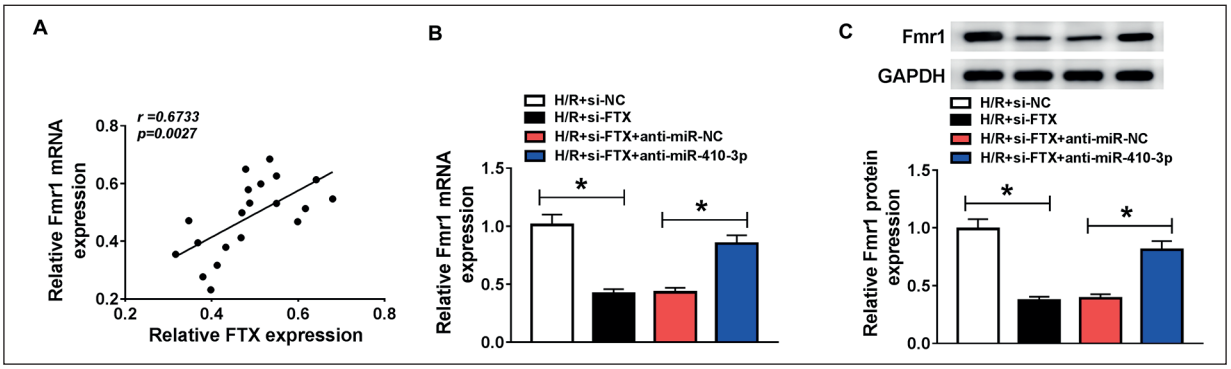

Figure 7. FTX acted as a sponge for miR-410-3p to regulate the expression of Fmr1 in H/R-induced H9c2 cells. (**A,** The correlation between Fmr1 mRNA and FTX in the serum of myocardial I/R injury patients was analyzed by Spearman's correlation analysis. **B,-C,** The mRNA and protein expression of Fmr1 in H9c2 cells treated with H/R+si-NC, H/R+si-FTX, H/R+si-FTX+anti-miR-NC or H/R+si-FTX+anti-miR-410-3p were determined through qRT-PCR and Western blot assay, respectively. *p<0.05.

oxidative stress-associated factors release mediated by FTX overexpression in H/R-stimulated H9c2 cells, suggesting that miR-410-3p could aggravate the development of H/R-induced cardiomyocyte injury. MiRNAs were revealed to aggravate or decelerate myocardial I/R injury *via* targeting genes. MiR-29 was improved in H/R-stimulated H9c2 cells and miR-29 could promote myocardial I/R injury by interacting with SIRT1[23]. MiR-374a was decreased in H/R-treated H9c2 cells and I/R-treated mouse, and miR-374a overexpression could relieve myocardial I/R injury through binding to MAPK6[24]. Yan et al[25] suggested that miR-410 was elevated and its elevation suppressed cell growth, ATP production and mitophagy level and induced cell apoptosis in H/R-induced HACMs. Our findings, in part, were consistent with the results in this research.

Here, we found that miR-410-3p could negatively modulate Fmr1 expression *via* interacting with Fmr1. It was well known that the aberrant expression of Fmr1 was associated with the function of cardiac[26]. Bao et al[20] disclosed that the overexpression of Fmr1 hampered LPS-induced oxidative stress and cell apoptosis, and enhanced cell growth in H9c2 cells. Herein, silencing of Fmr1 overturned the impact of miR-410-3p inhibition on H/R-induced damage in H9c2 cells through suppressing cell growth, promoting cell apoptosis and enhancing the release of oxidative stress-associated factors.

Conclusions

Taken together, FTX was decreased in patients with myocardial I/R injury and H/R-stimulated H9c2 cells. FTX overexpression could relieve H/R-induced cardiomyocyte damage by upregulating Fmr1 *via* sponging miR-410-3p. The study might be helpful for the treatment of myocardial I/R injury patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The present study was approved by the Ethical Review Committee of The First Affiliated Hospital of Zhengzhou University.

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