Int. J. Mol. Sci. **2014**, *15*, 18206-18220; doi:10.3390/ijms151018206

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

RETRACTED: Mipu1 Protects H9c2 Myogenic Cells from Hydrogen Peroxide-Induced Apoptosis through Inhibition of the Expression of the Death Receptor Fas

Guiliang Wang [1,2,†], Lei Jiang [1,*,†], Juan Song [1], Shu-Feng Zhou [3], Huali Zhang [1], Kangkai Wang [1] and Xianzhong Xiao [1,*]

[1] Laboratory of Shock, Department of Pathophysiology, Xiangya School of Medicine, Central South University, Changsha 410008, China;
E-Mails: guiliangwang@126.com (G.W.); sjblsl@hotmail.com (J.S.); csxyhualizhang@hotmail.com (H.Z.); wangkangkai@csu.edu.cn (K.W.)

[2] Department of Digestive Internal Medicine, Gannan Medical University Pingxiang Hospital, 128 Guangchang Road, Pingxiang 337055, China

[3] Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, FL 33612, USA; E-Mail: szhou@health.usf.edu

† These authors contributed equally to this work.

* Authors to whom correspondence should be addressed;
E-Mails: yjianglei@csu.edu.cn (L.J.); xiaoxianzhong@csu.edu.cn (X.X.);
Tel./Fax: +86-731-235-5019 (X.X.).

External Editor: William Chi-shing Cho

Received: 13 June 2014; in revised form: 4 September 2014 / Accepted: 11 September 2014 / Published: 10 October 2014; Retracted: 12 November 2025

Abstract: Mipu1 (myocardial ischemic preconditioning upregulated protein 1), a novel rat gene recently identified in our lab, was expressed abundantly and predominantly in the brain and heart and upregulated in myocardium during myocardial ischemia/reperfusion in rats. In our previous study we found that Mipu1 was an evolutionarily conserved zinc finger-containing transcription factor. However, whether Mipu1 confers myocardial protection remains unknown. In this study, H9c2 myogenic cells were treated with hydrogen peroxide (H_2O_2) to simulate oxidative stress during myocardial ischemia-reperfusion injury. The expression of Mipu1 at mRNA and protein levels was detected by RT-PCR and Western blotting analysis. To study the effect of Mipu1 on apoptosis and expression of *Fas* induced

RETRACTED

by H_2O_2, full-length Mipu1 cDNA and Mipu1-RNAi plasmids were transiently transfected into H9c2 myogenic cells, and flow cytometry was used to quantitate the percentage of apoptotic cells. The expression of *Fas* was analyzed by Western blotting assay. The DNA binding and transcription activities of Mipu1 to the *Fas* promoter were detected by chromatin immunoprecipitation and luciferase reporter assays. The results showed that exposure of H9c2 myogenic cells to H_2O_2 resulted in a dose- and time-dependent increase in Mipu1 mRNA and protein levels; Mipu1 over-expression inhibited H_2O_2-induced apoptosis and upregulation of Fas induced by H_2O_2 in H9c2 myogenic cells; and knockdown of Mipu1 by RNAi promoted apoptosis and upregulation of *Fas* induced by H_2O_2. The chromatin immunoprecipition and reporter assays showed the DNA binding and transcription suppressor activities of Mipu1 to *Fas* promoter region. These results indicate that Mipu1 protected H9c2 myogenic cells from H_2O_2-induced apoptosis through inhibiting the expression of *Fas*.

Keywords: apoptosis; *Fas*; gene expression regulation; myogenic cells

1. Introduction

Living myocardium possesses potent self-protective functions when subjected to various insults [1]. However, the mechanisms for this have not been completely clarified. During myocardial ischemia or reperfusion, the expression of many genes, such as c-fos, c-jun, junB, Egr-1 and HSP70, are upregulated [2], and some of them are considered to be involved in the endogenous cardioprotection against myocardial ischemia-reperfusion injury. Recently, Yuan and colleagues, at our lab, isolated and cloned a novel gene Mipu1 (Myocardial ischemic preconditioning upregulated protein 1, GenBank accession no. AY221750), which was presented as zinc finger protein 667 (ZFP667) in human containing a KRAB domain at the *N*-terminal and 14 successive C_2C_{12} type of zinc finger domains at the *C*-terminal and upregulated in rat heart after a transient ischemia-reperfusion procedure [3].
Jiang further confirmed that Mipu1 functioned as a transcription factor that bound to a specific DNA sequence TGTCTTATCGAA, with TCTTA as the core sequence and could suppress the expression of the reporter gene containing its binding sites on the promoter [4]. Further studies showed that over-expression of Mipu1 could reduce the activity of caspase-3 and caspase-8 and growth arrest induced by serum withdrawal in C2C12 myogenic cells [5]. However, the exact physiological and pathophysiological role of Mipu1 remains unclear.

Fas, a member of TNF receptor superfamily, also called "death receptor", is a 36 kDa surface protein containing a single transmembrane region that induces apoptosis by *Fas*-*Fas* ligand binding [6]. By using bioinformatics analysis, we found that there is a putative Mipu1 binding element (−346~−334 bp) in the promoter of *Fas*. Therefore, we hypothesized that Mipu1 might play an important anti-apoptotic role by inhibiting the expression of the *Fas* gene. In this report, we studied the expression of Mipu1 in response to H_2O_2 and the effects of Mipu1 over-expression and Mipu1 knockdown on H_2O_2-induced apoptosis in rat H9c2 myogenic cells. The effects of Mipu1 on the expression of *Fas* and its regulatory mechanism were also investigated.

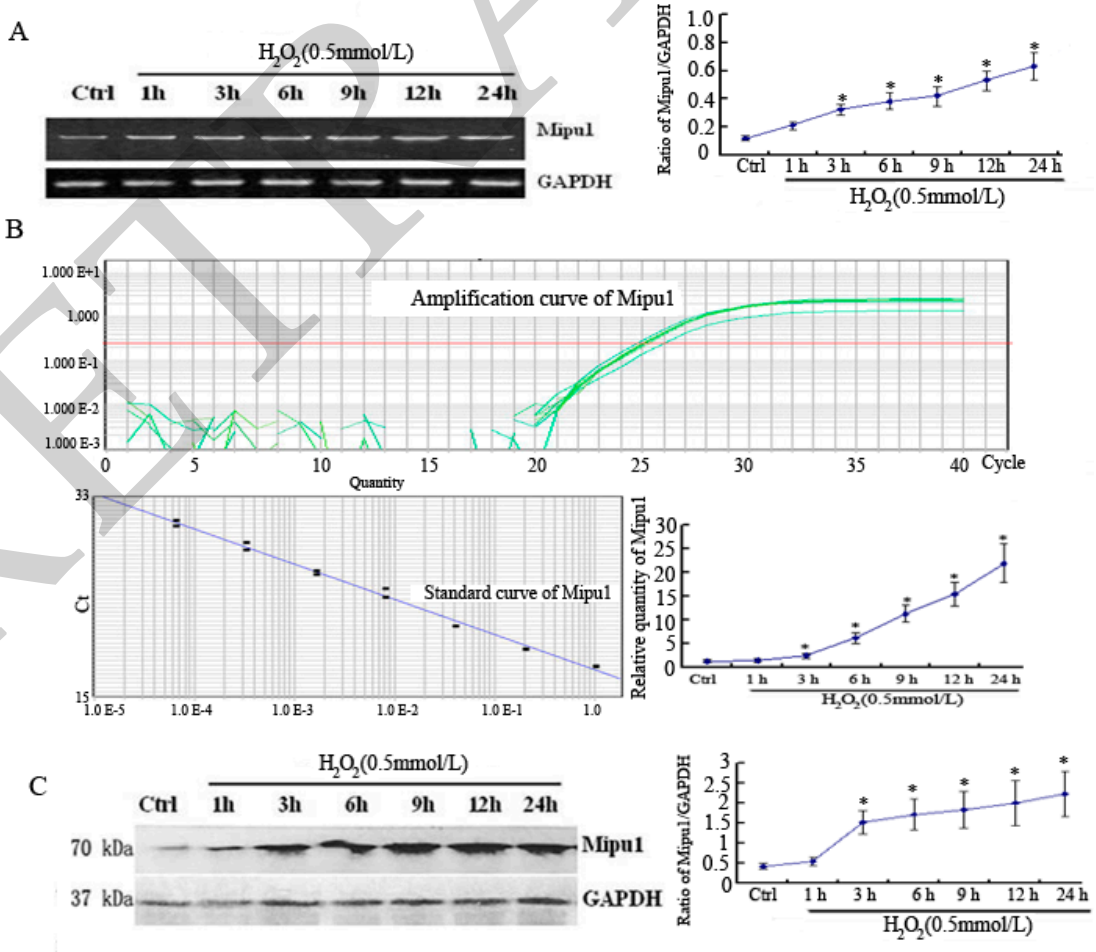
RETRACTED

2. Results

2.1. RT-PCR, Quantitative Real-Time RT-PCR and Western Blotting Showed Expression of Mipu1 in H9c2 Myogenic Cells after Exposure to H_2O_2

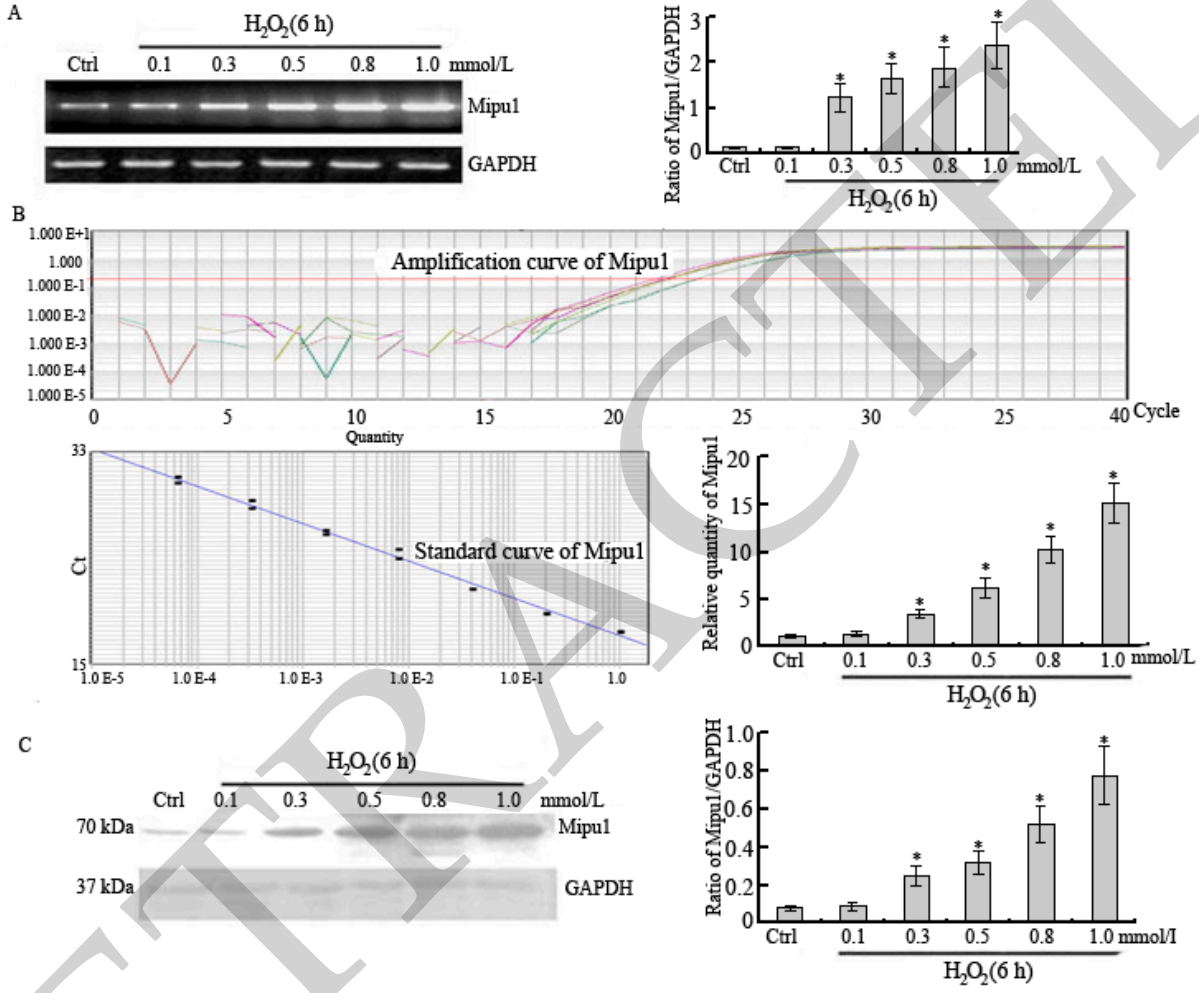
H_2O_2 treatment (0.5 mmol/L) led to a sustainable increase of Mipu1 RNA levels from 3 h (about 3.2-folds of basal level) to 24 h (about 6.3-folds of basal level) by RT-PCR (Figure 1A) and quantitative real-time RT-PCR (Figure 1B), a sustainable increase of Mipu1 protein levels from 3 h (about 3-folds of basal level) to 24 h (about 4.1-folds of basal level) by Western blotting (Figure 1C). H_2O_2 treatment for various doses led to a sustainable increase in the Mipu1 mRNA by RT-PCR (0.3 mmol/LH_2O_2 led to a five-fold increase compared to the basal level, and 1.0 mmol/L H_2O_2 led to an eight-fold increase) (Figure 2A) and quantitative real-time RT-PCR (Figure 2B), a sustainable increase of Mipu1 at the protein levels by Western blotting (0.3 mmol/L H_2O_2 led to a 4.5-fold increase compared to the basal level, and 1.0 mmol/L H_2O_2 led to a 7.8-fold increase (Figure 2C).

Figure 1. Expression of Mipu1 over H_2O_2-stimulated H9c2 myogenic cells over 24 h. (**A**) mRNA levels of Mipu1 over various periods of time were determined by RT-PCR; (**B**) mRNA levels of Mipu1 over various periods of time were determined by quantitative real-time RT-PCR; (**C**) Protein levels of Mipu1 in various periods of time were determined by Western blotting assay. The relative values of all results were determined and expressed as mean ± SEM of three experiments in duplicate. *, $p < 0.05$, *vs.* control group.



RETRACTED

Figure 2. Expression of Mipu1 in H_2O_2-stimulated H9c2 myogenic cells at indicated doses for 6 h. (**A**) mRNA levels of Mipu1 were determined by RT-PCR; (**B**) mRNA levels of Mipu1 were determined by quantitative real-time RT-PCR; (**C**) Protein levels of Mipu1 were determined by Western blotting assay. The relative values of all results were determined and expressed as mean ± SEM of three experiments induplicate. *, $p < 0.05$, *vs.* control group.

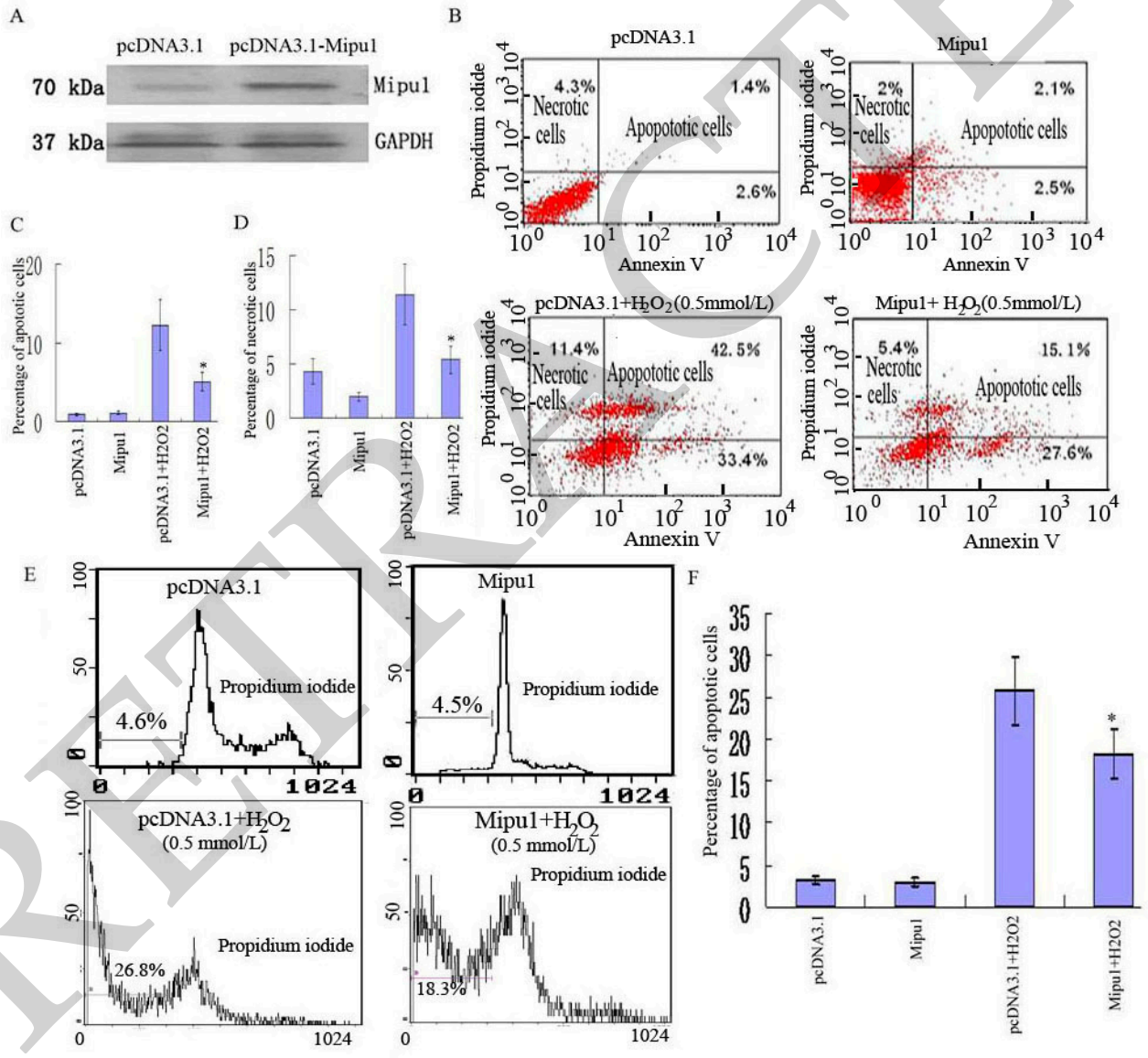


2.2. Effects of Mipu1 Over-Expression on Apoptosis Induced by H_2O_2

Using pcDNA3.1-Mipu1 construct, we over-expressed Mipu1 in H9c2 myogenic cells (Figure 3A), and the transfection did not reduce cell viability significantly (data not shown). As shown in Figure 3B,C, at 6 h after H_2O_2 exposure, the percentages of apoptotic cells detected using flow cytometry were also significantly lower in Mipu1 over-expressed cells than in the control cells transfected with pcDNA3.1 vector.

RETRACTED

Figure 3. Over-expression of Mipu1 inhibited apoptosis induced by H_2O_2 in H9c2 myogenic cells. (**A**) H9c2 myogenic cells were transfected with pcDNA3.1-Mipu1 and the expression level of Mipu1 was determined by Western blotting assay; (**B**) The percentage of apoptotic cells was determined by Annexin V and propidium iodide staining and flow cytometry; (**C**) Statistical analysis of the flow cytometry data of apoptotic cells; (**D**) Statistical analysis of the flow cytometry data of necrotic cells; (**E**) The percentage of apoptotic cells was determined by propidium iodide staining and flow cytometry; (**F**) Statistical analysis of the flow cytometry data of apoptotic cells. pcDNA3.1: the vector control group; Mipu1: Mipu1 over-expression group. Values represent the mean ± SEM. *, *vs.* pcDNA3.1 + H_2O_2 group, $p < 0.05$, $n = 5$.



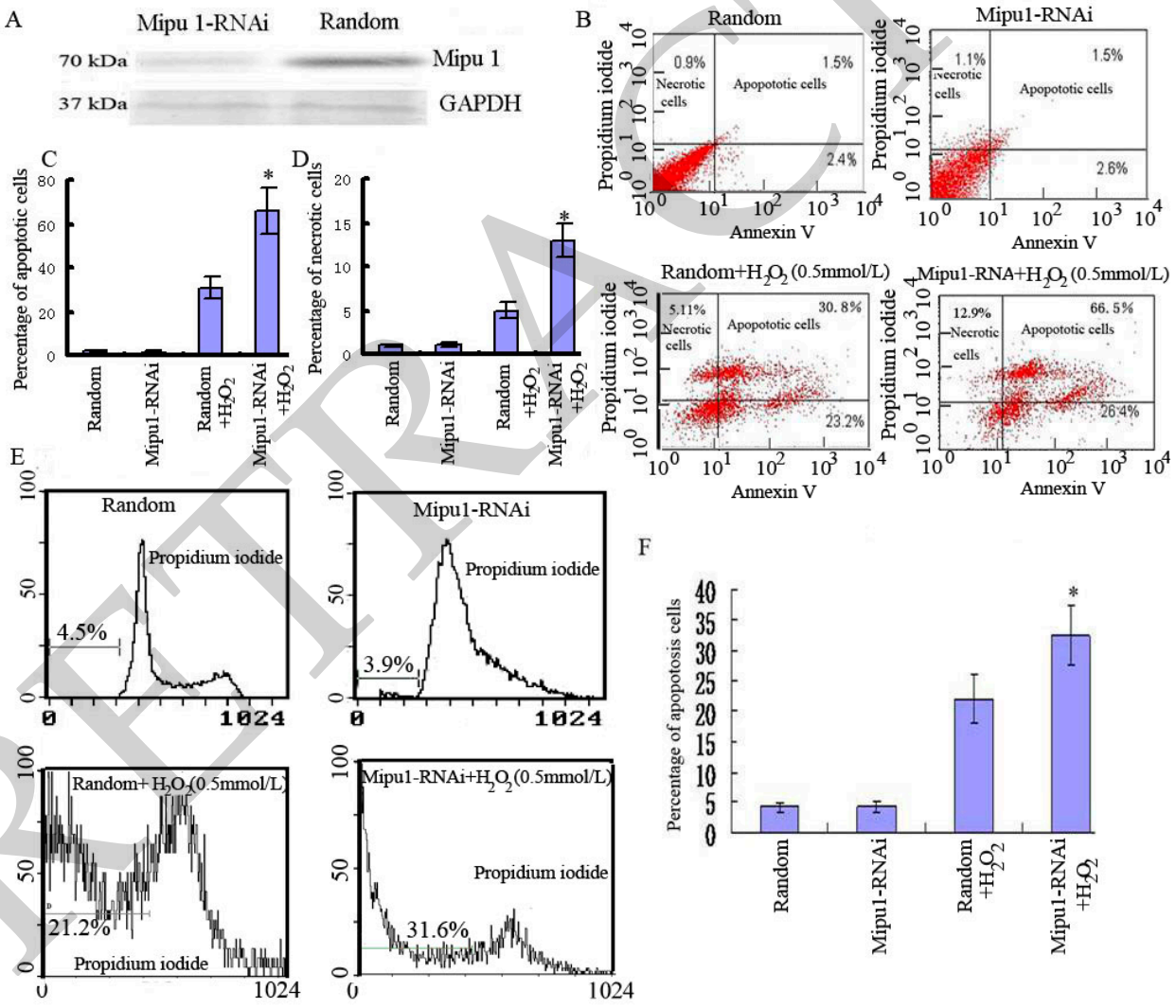
2.3. Effects of Mipu1 Inhibition by RNAi on Apoptosis Induced by H_2O_2

The percentage decrease of Mipu 1 protein by RNAi is about 85% compared to the basal expression (Figure 4A). At 6 h after H2O2 exposure, the percentages of apoptotic cells detected by flow cytometry

RETRACTED

were significantly higher in the cells transfected with Mipu1-RNAi than in the cells transfected with a random control plasmid (Figure 4B,C).

Figure 4. Mipu1-RNAi sensitized H9c2 myogenic cells to H_2O_2-induced apoptosis. (**A**) Effect of Mipu1-RNAi on the levels of *Fas* was measured by Western blotting assay; (**B**) The percentage of apoptotic cells were determined by Annexin V and propidium iodide staining and flow cytometry; (**C**) Statistical analysis of the flow cytometry data of apoptotic cells; (**D**) Statistical analysis of the flow cytometry data of necrotic cells. Random: H9c2 myogenic cells were transiently transfected with random oligonucleotide of Mipu1; (**E**) The percentage of apoptotic cells was determined by propidium iodide staining and flow cytometry; (**F**) Statistical analysis of the flow cytometry data of apoptotic cells. Mipu1-RNAi: H9c2 myogenic cells were transiently transfected with RNAi of Mipu1. Values represent the mean ± SEM. *, *vs.* Random + H_2O_2 group, $p < 0.05$, $n = 5$.

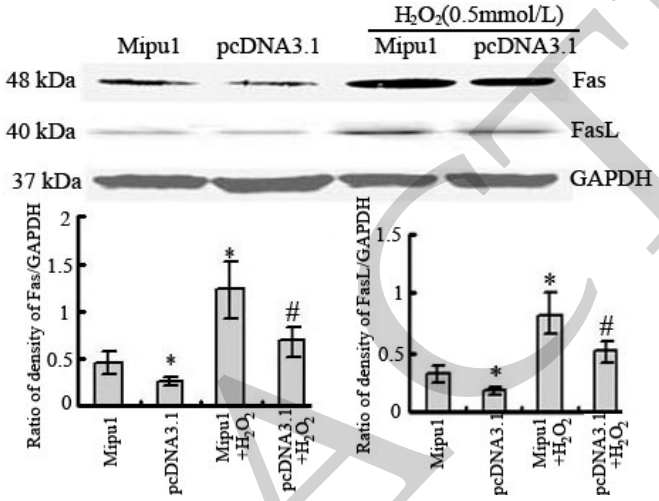


RETRACTED

2.4. Effects of Mipu1 Over-Expression on the Expression of Fas

By using bioinformatics analysis, we found that the promoter of *Fas* contains one putative Mipu1 binding site. Therefore, we deduced that Mipu1 might regulate the expression of *Fas* gene. As demonstrated in Figure 5, over-expression of Mipu1 led to a decreased basal expression of *Fas* and *FasL* protein in non-treated cells. After H_2O_2 treatment, the level of *Fas* and *FasL* protein was upregulated and Mipu1 over-expression significantly inhibited the upregulation of *Fas* and *FasL* induce by H_2O_2.

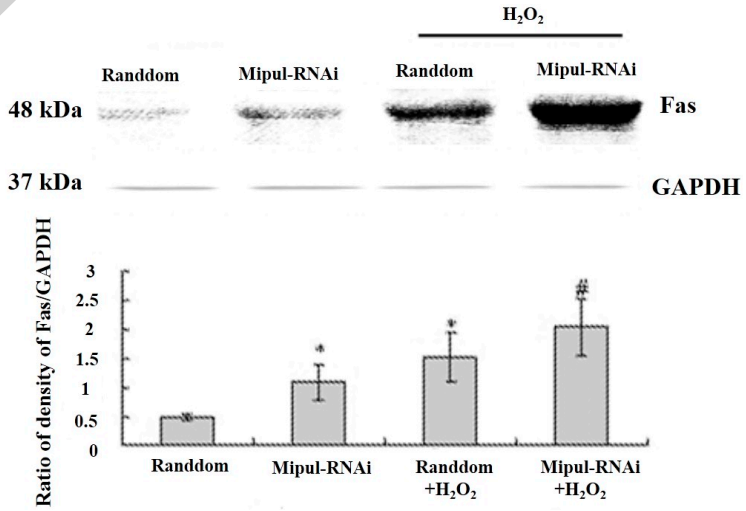
Figure 5. Effect of Mipu1 over-expression on the expression of *Fas* and *FasL* in H9c2 myogenic cells. *, *vs.* pcDNA3.1 group, $p < 0.05$; #, *vs.* pcDNA + H_2O_2 group, $p < 0.05$, $n = 5$.



2.5. Effects of Mipu1 Inhibition by RNAi on the Expression of Fas

In order to observe the effect of Mipu1 silencing on *Fas* expression, a plasmid designed for RNAi of Mipu1 was transfected into H9c2 myogenic cells. As shown by Figure 6, RNAi of Mipu1 led to an increased basal expression of *Fas* protein. After H_2O_2 treatment, the level of *Fas* protein was upregulated and the inhibition of Mipu1 by RNAi further promoter the upregulation of *Fas* gene.

Figure 6. Effect of RNAi of Mipu1 on expression of *Fas* in H9c2 myogenic cells. *, *vs.* Random group, $p < 0.05$; #, *vs.* Random + H_2O_2 group, $p < 0.05$, $n = 5$.

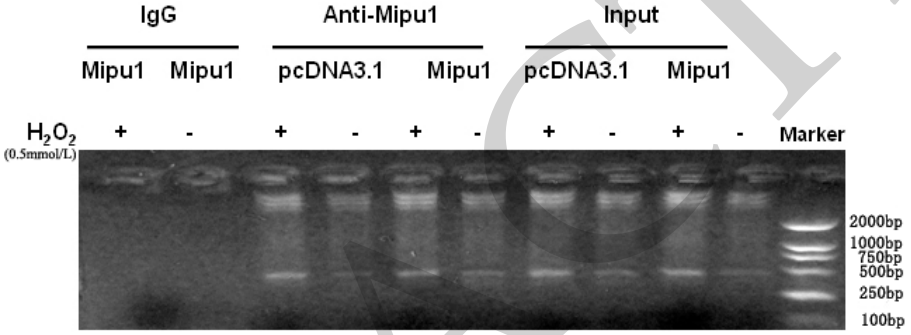


RETRACTED

2.6. Binding of Mipu1 to the Fas Promoter

To investigate whether Mipu1 directly binds to the *Fas* promoter, we performed chromatin immunoprecipitation assays. H9c2 myogenic cells were transfected with pcDNA3.1-Mipu1 or pcDNA3.1 as control, and the cell lysate prepared after fixation was immunoprecipitated with anti-Mipu1 antibody or anti-IgG isotype control antibody, followed by PCR with primers amplifying a fragment from −474 to −25 bp of the 5'-flanking sequence of the *Fas* gene. In cells transfected with pcDNA3.1-Mipu1, anti-Mipu1 antibody enriched the chromatin-containing DNA of the *Fas* promoter region as compared to incubation with a non-specific rabbit IgG (Figure 7). It was suggested that Mipu1 bound to the *Fas* promoter sequence between −474 to −25 bp upstream of the initiation site.

Figure 7. DNA binding activity of Mipu1 to *Fas* promoter shown by chromatin immunoprecipitation in H9c2 myogenic cells.



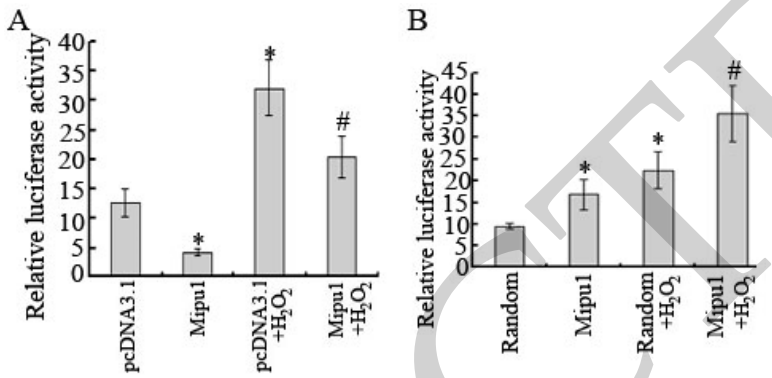
Mipu1 bound to its binding site in the region of −474 to −25 bp in *Fas* promoter. Mipu1 over-expression and H_2O_2 exposure promoted the binding of Mipu1 to *Fas* promoter containing Mipu1 binding site. pcDNA3.1: the vector control group; pcDNA3.1-Mipu1: Mipu1 over-expression group; H_2O_2: cells stimulated by H_2O_2 (0.5 mmol/L) for 6 hours; IgG: a non-specific rabbit IgG as control.

2.7. Inhibition of the Luciferase Reporter Activity of Fas Promoter by Mipu1

We further assessed the effect of Mipu1 on *Fas* promoter activity. Full length of Mipu1 showed a strong inhibitive effect on the *Fas* promoter, suggesting that the effects required intact Mipu1 (Figure 8A). The fact that RNAi of Mipu1 promoted the activity of *Fas* promoter provided further evidence for the above viewpoint (Figure 8B).

RETRACTED

Figure 8. Effect of Mipu1 on transcription activity of the *Fas* promoter in H9c2 myogenic cells. (**A**) H9c2 myogenic cells were co-transfected transiently with an expression plasmid of full length Mipu1 and the reporter driven by the *Fas* promoter; (**B**) H9c2 myogenic cells were co-transfected transiently with RNAi plasmid of Mipu1 and the reporter driven by the *Fas* promoter. pcDNA3.1: the vector control group; Mipu1: Mipu1 over-expression group. *, Statistically significant *vs.* the vector control group (pcDNA3.1 or Random), $p < 0.05$; #, Statistically significant *vs.* the vector control + H_2O_2 group (pcDNA3.1 + H_2O_2 or Random + H_2O_2), $n = 5$.



3. Discussion

Mipu1 is a novel gene that was found to be upregulated in rat heart after a transient myocardial ischemia-reperfusion procedure by Yuan *et al.*, at our laboratory. Jiang *et al.* further confirmed that Mipu1 functioned as a transcriptional repressor which could bind specifically to the consensus sequence 5'-TGTCTTATCGAA-3', with TCTTA as the core sequence. H_2O_2-induced upregulation of Mipu1 in H9c2 myogenic cells was mediated by cAMP/PKA-dependent CREB activation [7].

It was shown in our study that Mipu1 could be induced by H_2O_2 in H9c2 myogenic cells in a dose- and time-dependent manner, suggesting that oxidative stress is one of the important inducing factors in Mipu1 expression. We found that the percentage of apoptotic cells were decreased after the over-expression of Mipu1, but the percentage of apoptotic cells were significantly increased after the expression of Mipu1 was inhibited by RNAi, which suggested that Mipu1 can protect H9c2 myogenic cells from H_2O_2-induced apoptosis.

It is currently known that cardiomyocytes express all the apoptotic pathway proteins including death receptors (Fas, TNF-R), caspase family members, mitochondrial pathway family members (Apaf-1, AIF, Smac/Diablo) and Bcl-2 family members. Apoptosis is an important mechanism during occurrence and development of many cardiovascular diseases. As one of the most important pathways of apoptosis, *Fas*-*FasL* caspase signaling pathway could result in the recruitment and activation of several key proteins and caspases, chief of which are *Fas*-associated death domain-containing protein (FADD) and caspase-3. *Fas*/*FasL* system participates in the apoptosis of myocardial cells as signal transduction system. The combination of *FasL* to *Fas* switches *Fas*/*FasL* death signal transduction system and then activates sphingomyelin pathway, protease pathway and death domain associate protein (Daxx) pathway and leads to apoptosis of *Fas* positive target cell. *Fas* agonist causes trimerization of *Fas* and recruits a number of molecules to form a complex death-inducing signaling

RETRACTED

complex (DISC), starting with recruitment of the *Fas*-adapter protein FADD through *Fas* and FADD's mutual death domains. The other end of FADD contains two death-effector domains (DEDs) that recruit caspase-8 (FLICE) or its enzymatically inactive homologue, the *Fas* inhibitory FLICE inhibitor protein (FLIP). Procaspase-8 is cleaved and activated after binding to FADD, which starts the following cascade reactions toward apoptosis. Recent studies have shown that the *Fas* signal transduction pathway is involved in various types of stress-induced apoptosis in myocardium [8,9]. Furthermore, upregulation of *Fas* has been reported in a variety of cardiac pathologic processes, such as cardiomyopathy induced by rapid pacing in dogs, myocardial infarction in rats [10], or hypoxia in cultured neonatal rat cardiomyocytes [11]. Triggering of *Fas* by either agonistic antibodies or *FasL* results in receptor oligomerization and recruitment of the adaptor protein, *Fas*-associated death domain (FADD), which along with procaspase-8, forms a death-inducing signaling complex [12]. Then, the effectors caspases, including caspase-3, are activated [13,14].

In our previous study, we found that activities of caspase-3 and caspase-8 were both reduced by Mipu1, which showed that the major protective mechanisms against *Fas*-induced apoptosis in myogenic cells seem to be a caspase-dependent mechanism. To elucidate the role of Mipu1 in the *Fas* pathway, we over-expressed Mipu1 in H9c2 myogenic cells and detected that the basal and H_2O_2-induced *Fas* and *FasL* expression were both down-regulated, while if we suppressed Mipu1 expression in H9c2 myogenic cells the basal and H_2O_2-induced *Fas* and *FasL* expression were both upregulated. Chromatin immunoprecipitation and luciferase reporter gene assay showed that Mipu1 directly combined with the *Fas* promoter and negatively regulated its expression. The further mechanisms of this effect may involve the direct upregulation by Mipu1 of caspase-associated antiapoptotic proteins such as FLIP, Bcl-2, Bcl-XL, and also caspase-3 inactivation via upregulation of Redox factor-1, which are being under research in our laboratory.

4. Methods

4.1. Cell Culture and Reagent

H9c2 myogenic cells (ATCC, Manassas, VA, USA) were routinely grown in DMEM (Dulbecco's modified Eagle's medium, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum at 37 °C and 5% CO_2. Annexin V FITC Apoptosis Detection Kit I was purchased from BD Pharmingen (Franklin Lakes, NJ, USA).

4.2. Plasmids Construction

For the construction of pcDNA-Mipu1, PCR was used to amplify the coding sequence of rat *Mipu1* cDNA, yielding a 1.8 kb product with the following oligonucleotide primers: 5'-ATCTCTCGAGTAATGCCTGCAGCCCGAGGGA-3' (Forward) and 5'-ATCTGGTACCGCGGA CATTTCCTCCGAATGTATAC-3' (Reverse). The PCR product was electrophoresed onto 1.0% agarose and the 1.8 kb target fragment was purified with the DNA purification kit (Qiagen, Hilden, Germany). The fragment was then inserted into the pcDNA3.1 vector (Invitrogen). Plasmid containing Mipu1 gene deleted the DNA binding domain (pcDNA3.1-Mipu1KRAB) and plasmid merely

RETRACTED

containing the DNA binding domain of Mipu1 gene (pcDNA3.1-Mipu1ZnF) are also constructed. All the constructs were sequenced commercially (Invitrogen, Shanghai, China).

For construction of Mipu1 RNAi vector, ShRNA of Mipu1 was cloned into plasmid vector pRNA-U6.1/Neo (GenScript Corporation, Piscataway, NJ, USA) and specifically targeted to the Mipu1 mRNA; the target sequence was: 5'-AGTCACCGGATCTCTCTTA-3' (positions 1553–1572 of Mipu1). Sequence of random control: 5'-AGCCACCTGATCTCGCTTA-3', and mismatch nucleotides were underlined.

4.3. RNA Extraction and Reverse-Transcription PCR (RT-PCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. 1 μg of total RNA was reverse-transcribed by the reverse transcription kit (Promega, Madison, WI, USA) and PCR was performed using Cycler Apparatus (Bio-Rad, Hercules, CA, USA). For PCR amplification, the following primers were used: GAPDH (580 bp), 5'-AAGCCCATCACCATCTTCCA-3' (Forward) and 5'-CCTGCTTCACCACCTTCTTG-3' (Reverse); Mipu1 (1800 bp), 5'-ATGCCTGCAGCCCGAGGGAAATC-3' (Forward) and 5'-CTTAGGACAT TTCCTCCGAATG-3' (Reverse). The PCR reaction consisted of 26 cycles of denaturing at 95 °C for 30 s, annealing at 61 °C for 45 s, extension at 72 °C for 2 min, and a further 10 min at 72 °C. RT-PCR products were analyzed in a 1.0% agarose gel. For PCR amplification, the primers were shown in Table 1.

Table 1. The primers for the quantitative real-time RT-PCR.

Genes	Primers
Mipu1	Sence 5'-ATGCCTGCAGCCCGAGGGAAATC-3' Antisence 5'-CGATGATATTTGGCCTCCGGCAGGC-3'
GAPDH	Sence 5'-AACACAGTCCATGCCATCAC-3' Antisence 5'-TCCACCACCCTGTTGCTGTA-3'

4.4. Quantitative Real-Time RT-PCR

The mRNA expression of Mipu1 and GAPDH was analyzed in more detail by a quantitative real-time RT-PCR method using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd.), PCR reactions were performed in a 25 μL final volume containing 1× SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). The final primer concentrations for Mipu1 and GAPDH were 10 μmol/L. The amplification profiles for Mipu1 and GAPDH were 10 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. Each sample was analyzed in triplicate with each primer set. Data were analyzed using the absolute standard curve method. Standard curves were generated using a dilution series of corresponding purified PCR products. The intra- and interassay coefficients of variations were <2% and <3.3%, respectively (data not shown). GAPDH was used for normalizing the inefficiencies in cDNA synthesis and in the amount of RNA applied. Briefly, the copy numbers for GAPDH were divided by the highest GAPDH value obtained in the experiment, resulting in a correction factor for every sample. These correction factors were then used for normalizing the absolute copy numbers of Mipu1. The normalized copy numbers were obtained by dividing the copy numbers of Mipu1 by the corresponding correction factors. In addition, we analyzed the melting curve of each PCR product in each PCR session and

RETRACTED

confirmed that no non-specific products had been produced. There was rarely significant primer dimer formation during the numbers of cycles required for quantification of the PCR products from a range of experimental samples. Negative controls were checked with samples in which the RNA templates were replaced by nuclease-free water in the reactions. For quantitative real-time RT-PCR, the following primers were used: GAPDH (128 bp), 5'-AACACAGTCCATGCCATCAC-3' (Forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (Reverse); Mipu1 (190 bp), 5'-ATGCCTGCAGCCCGAGGG AAATC-3' (Forward) and 5'-CGATGATATTTGGCCTCCGGCAG-3' (Reverse). The scores of "relative Mipu1", which indicates the expression of Mipu1 relative to that GAPDH, were obtained by dividing the copy number of Mipu1 in a sample by the corresponding correction factor.

4.5. Western Blotting Analysis

After treatment, proteins in the whole-cell lysate were resolved on 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked overnight in phosphate-buffered saline containing 10% nonfat dry milk and 0.5% Tween-20, and incubated with primary antibodies for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. The immunoreactive bands were visualized using DAB (Boster Biological Technology, Beijing, China). Anti-GAPDH was used to normalize for equal amounts of proteins and calculate the relative induction ratio. The following antibodies were used: Rabbit anti-Mipu1 polyclonal antibody (Jingmei Biothech Co., Ltd., Beijing, China); Rabbit anti-*Fas* polyclonal antibody (StressGen, Victoria, BC, Canada); mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Sigma, San Francisco, CA, USA); and HRP-conjugated anti-mouse and anti-rabbit IgG (Boster Biological Technology, Wuhan, China).

4.6. Gene Transfection

Gene transfection of H9c2 myogenic cells was carried out using Lipofectamine 2000™ (Invitrogen) according the manufacturer's instructions. Briefly, about 5×10^5 cells per bottle containing 5 mL DMEM complete growth medium were seeded, and incubated at 37 °C with 5% CO_2 until the cells were 70% to 80% confluent (24 h). After the cells were rinsed with serum free and antibiotics-free medium, the cells were transfected separately with pcDNA3.1-Mipu1 10 μg/Lipofectamine 20 μL (experimental group), pcDNA3.1 10 μg/Lipofectamine 20 μL (vector control), followed by incubation at 37 °C in a CO_2 incubator for 6 h. The medium was then replaced with DMEM culture medium containing 10% FBS. After 48 h recovery, G418 (Gibco/BRL) was added at 1000 ng/mL for 20 days, when colonies were picked and expanded under G418 selection.

4.7. Quantification of Apoptotic Cells by Flow Cytometry

Early apoptosis and necrosis were identified by means of double fluorescence stained with Annexin V/propidium iodide. Apoptotic cells translocate phosphatidylserine from the inner site of the plasma membrane to the outer surface while the membrane remains physically intact. Apoptotic cells were therefore stained with Annexin V, which binds with high affinity to phosphatidylserine, resulting in a red fluorescence when excited at 450~480 nm, and exclude propidium iodide, a DNA dye unable to cross

RETRACTED

the plasma membrane. Necrotic cells lose the physical integrity of their plasma membrane and therefore stained with both Annexin V and propidium iodide. Cells that were neither apoptotic nor necrotic were not stained with either dye. The test described, discriminates intact cells ($FITC^-/PI^-$), apoptotic cells ($FITC^+/PI^-$), and necrotic cells ($FITC^+/PI^+$). H9c2 myogenic cells transfected with pcDNA3.1, pcDNA3.1-Mipu1, Random control, or RNAi of Mipu1 were, respectively, treated with 0.5 mmol/L H_2O_2 for 6 h and then were incubated with 10 μL PE-labeled Annexin V and 5 μL propidium iodide 2 h after H_2O_2 treatment for 10 min at 37 °C in the dark and then analyzed with a FACStar Plus flow cytometer (Becton–Dickinson, Mountain View, CA, USA) within 15 min and then the apoptotic cells were analyzed by flow cytometry.

4.8. Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed with the EZ-ChIP kit (Upstate Inc., Charlottesville, VA, USA) according to manufacturer's instructions. Whole cell extracts were prepared 2 days after transfection using RIPA buffer. Clear lysate (2 mg) was incubated with 10 μg of anti-Mipu1 polyclonal antibody for 1 h at 4 °C in a final volume of 1 mL PBS. Coimmunoprecipitated DNA was detected by PCR (449 bp) with the following primers: 5'-TAGAGTGTAGGCCTTACAAAG-3' (Forward), 5'-TGATTTCTGAACTAAGCACCG-3' (Reverse). The PCR reaction consisted of 26 cycles of denaturing at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s, and a further 10 min at 72 °C. The PCR products were analyzed in a 1.5% agarose gel. A parallel coimmunoprecipitation with a non-specific antiserum was used as control.

4.9. Luciferase Reporter Gene Assay

The assay was performed according to the instructions of Dual Luciferase Reporter System (Promega). Rat *Fas* promoter region (−1000~+1) was amplified by PCR using rat genomic DNA as template and cloned into plasmid pGL3-Basic, and the authenticity was verified by sequencing. For luciferase reporter assay, exponentially growing H9c2 myogenic cells were seeded in 24-well culture dishes. Transfections were done as described above. All transfections were performed in triplicate from at least three independent experiments. Each transfection experiment contained 500 ng of pGL3-*Fas* promoter reporter construct with 500 ng of pcDNA3.1-Mipu1 vector or RNAi of Mipu1 and with 20 ng of pRL-null vector (Promega) as an internal transfection control.

4.10. Statistical Analysis

Data are expressed as means ± SEM of the indicated number of separate experiments. Statistical comparison between experimental group and control was performed using unpaired two-tailed Student's T tests (for measurement data) or Chi-square (χ^2) test (for percentage). $p < 0.05$ was considered significant.

5. Conclusions

We found for the first time in this study that Mipu1, as a transcription suppressor, could partly inhibit apoptosis induced by H_2O_2 in H9c2 myogenic cells by inhibiting the expression of the pro-apopototic

RETRACTED

gene *Fas*, which may have important clinical implications for the prevention and treatment of ischemic and oxidative injury.

Acknowledgments

This work was supported by funding from the National Natural Science Foundation of China (81360080, 30971205, 30672149), the Major National Basic Research Program of China (2007CB512007).

Author Contributions

Guiliang Wang and Lei Jiang designed, performed the experiments and wrote the paper. Juan Song, Huali Zhang and Kangkai Wang reviewed the data. Shu-Feng Zhou and Xianzhong Xiao revised and finalized the paper.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Portt, L.; Norman, G.; Clapp, C.; Greenwood, M.; Greenwood, M.T. Anti-apoptosis and cell survival: A review. *Biochim. Biophys. Acta* **2011**, *1813*, 238–259.
2. Plumier, J.C.; Robertson, H.A.; Currie, R.W. Differential accumulation of mRNA for immediate early genes and heat shock genes in heart after ischaemic injury. *J. Mol. Cell Cardiol.* **1996**, *28*, 1251–1260.
3. Yuan, C.; Zhang, H.L.; Liu, Y.; Wang, Q.P.; Xiao, X.Z. Cloning and characterization of a new gene Mipu1 upregulated during myocardial ishemia-reperfusion. *Prog. Biochem. Biophys.* **2004**, *31*, 231–236.
4. Jiang, L.; Tang, D.L.; Wang, K.K.; Zhang, H.L.; Yuan, C.; Duan, D.Y.; Xiao, X.Z. Functional analysis of a novel KRAB/C2H2 zinc finger protein Mipu1. *Biochem. Biophys. Res. Commun.* **2007**, *356*, 829–835.
5. Yuan, C.; Liu, Y.; Wang, Q.P.; Xiao, X.Z. Effects of New Gene Mipu1 over-expression on the cell growth cycle of C_2C_{12} cells cultured under condition of serum withdrawl. *J. Clin. Res.* **2004**, *21*, 837–869.
6. Lavrik, IN.; Krammer, PH. Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ*. **2012**, *19*, 36–41.
7. Zhu, H.L.; Wei, X.; Qu, SL.; Zhang, C.; Zuo, X.X.; Feng, Y.S.; Luo, Q.; Chen, G.W.; Liu, M.D.; Jiang, L.; *et al.* Oxidative stress-mediated upregulation of myocardial ischemic preconditioning upregulated protein 1 gene expression in H9c2 myogenic cells is regulated by cyclic AMP-response element binding protein. *Free Radic. Biol. Med*. **2010**, *49*, 580–586.apoptosis in myocardium
8. Katsaros, K.M.; Wiesbauer, F.; Speidl, W.S.; Kastl, S.P.; Huber, K.; Zorn, G.; Niessner, A.; Glogar, D.; Maurer, G.; Wojta, J. High soluble Fas and soluble Fas Ligand serum levels before stent implantation are protective against restenosis. *Thromb. Haemost.* **2011**, *105*, 883–891.

RETRACTED

9. Fan, Q.; Huang, Z.M.; Boucher, M.; Shang, X.; Zuo, L.; Brinks, H.; Lau, W.B.; Zhang, J.; Chuprun, J.K.; Gao, E. Inhibition of Fas-associated death domain-containing protein (FADD) protects against myocardial ischemia/reperfusion injury in a heart failure mouse model. *PLoS One* **2013**, *13*, doi:10.1371/journal.pone.0073537.
10. Cardinal, H.; Brophy, J.M.; Bogaty, P.; Joseph, L.; Hébert, M.J.; Boyer, L.; Madore, F. Usefulness of soluble fas levels for improving diagnostic accuracy and prognosis for acute coronary syndromes. *Am. J. Cardiol.* **2010**, *105*, 797–803.
11. Niessner, A.; Hohensinner, P.J.; Rychli, K.; Neuhold, S.; Zorn, G.; Richter, B.; Hülsmann, M.; Berger, R.; Mörtl, D.; Huber, K.; *et al.* Prognostic value of apoptosis markers in advanced heart failure patients. *Eur. Heart J.* **2009**, *30*, 789–796.
12. Morgan, M.J.; Kim, Y.S.; Liu, Z.G. Membrane-bound Fas ligand requires RIP1 for efficient activation of caspase-8 within the death-inducing signaling complex. *J. Immunol.* **2009**, *183*, 3278–3284.
13. Liu, C.C.; Jung, S.M.; Orlandi, A.; Yeh T.S.; Lin Y.S.; Shiu T.F.; Wu H.H.; Chu J.J.; Lin P.J.; Chu P.H. The Fas-mediated apoptotic pathway in cardiac myxoma. *Int. J. Surg. Pathol.* **2010**, *18*, 493–498.
14. Li, C.P.; Li, J.H.; He, S.Y.; Li, P,; Zhong, X.L. Roles of Fas/Fasl, Bcl-2/Bax, and Caspase-8 in rat nonalcoholic fatty liver disease pathogenesis. *Genet Mol Res*. **2014**, *13*, 3991–3999.

© 2014 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/).

RETRACTED