

LncRNA PVT1 Mediated by ZFP36L2 Regulates Myocardial Ischemia/Reperfusion Injury and Attenuates Mitochondrial Fusion and Fission via Activating miR-21-5p/MARCH5 Axis

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Research

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Abstract

Background

Among several leading cardiovascular disorders, ischemia-reperfusion (I/R) injury causes severe manifestations including acute heart failure, inflammation, and systemic dysfunction. Recently, there has been increasing evidence suggesting that alterations in mitochondrial morphology play a role in the prognoses of cardiac disorders. Long non-coding RNAs (lncRNAs) form major regulatory networks to modify gene transcription and translation. While several roles of lncRNAs have been explored in cancer and tumor biology, their implications on mitochondrial morphology and functions remain to be elucidated.

Methods

The functional roles of ZFP36L2 and lncRNA PVT1 were determined by a series of cardiomyocyte hypoxia/ reoxygenation (H/R) *in vitro* and myocardial I/R injury *in vivo* experiments. Quantitative Reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis were used to detect the mRNA levels of ZFP36L2 and mitochondrial fission and fusion markers in the myocardial tissues and cardiomyocyte. Cardiac function was determined by immunohistochemistry, H&E, Masson's staining and echocardiogram. Ultrastructural analysis of mitochondrial fission was performed using transmission electron microscopy (TEM). The mechanistic model of PVT1 with ZFP36L2 and miR-21-5p with MARCH5 was detected by subcellular fraction, RNA pull down, FISH, and luciferase reporter assays.

Results

In this study, we report a novel regulatory axis involving lncRNA PVT1, microRNA miR-21-5p, and E3 ubiquitin ligase MARCH5, which alters mitochondrial morphology during myocardial I/R injury. Using an *in vivo* I/R injury mouse model and *in vitro* cardiomyocyte H/R model, we observed that zinc finger protein ZFP36L2 directly associated with PVT1 and altered mitochondrial fission and fusion. PVT1 also interacted with miR-21-5p and suppressed its expression and activity. Furthermore, we identified MARCH5 as a modifier of miR-21-5p, and expression of MARCH5 and its effect on mitochondrial fission and fusion were directly proportional to PVT1 expression during H/R injury.

Conclusions

Our findings demonstrated that manipulation of PVT1-miR-21-5p-MARCH5-mediated mitochondrial fission and fusion via ZFP36L2 may be a novel therapeutic approach to regulate myocardial I/R injury.

Background

Hypoperfusion of the heart for short periods of time in response to sepsis, transplantation, or other syndromes is known as ischemia. Subsequent reperfusion or restoration of blood flow in some conditions causes injuries to ischemic tissues, known as ischemia-reperfusion (I/R) injury. Prolonged I/R

injury can lead to myocardial infarction and acute coronary syndrome [1]. Prolonged hypoxic conditions lead to anaerobic respiration and dysregulation of the electron transport chain in mitochondria. These results in lower levels of ATP, release of stress factors, and generation of antioxidative reactive oxygen species [2], could trigger cellular dysfunction, DNA damage, and apoptosis [3]. In the process of apoptosis, mitochondria are fragmented (mitoptosis), which is regulated by several proteins including dynamin related protein 1 (Drp1) and fission 1 (Fis1) [4].

Plasmacytoma variant translocation 1 (Pvt1) is a form of long non-coding RNA (lncRNA) that are classically differentiated from other non-coding microRNAs (miRNAs) based on its size, where lncRNAs are typically greater than 200 bp in length whereas miRNAs are approximately 22 nucleotides in length. Pvt1 has been identified in Burkett's lymphoma and has been implicated for decades in its oncogenic functions [4-7]. Aberrant expression of Pvt1 and its ability to regulate several miRNAs are hallmarks of cancer invasion and progression [8, 9]. In the context of cellular degradation, lncRNAs have been extensively characterized for its role in autophagy through interaction with with miRNAs. For example, miR-30a inhibition promoted autophagy protecting neuronal fibers from I/R injury [10]. However, the role of lncRNAs in specifically regulating mitochondrial fission/ fusion process has not been explored so far. In a study by Cho *et al*, microarray analysis in single cell muscle fibers identified Pvt1 as a regulator of mitochondrial respiration, fission/fusion, and mito/autophagy [11].

Zinc finger RNA binding protein ZFP36L2 is part of a family of proteins that contain tandem zinc finger domains that can associate with adenine-uridine rich elements present most often in the 3'- untranslated region (UTR) of mRNAs thereby interfering with posttranscriptional modifications and as a result affecting protein translation [12]. It causes cell cycle arrest, especially during embryonic development and has recently been shown to alter immune functions in T cells [13-15]. ZFP36L2 can also positively and negatively regulate adipogenesis and development of B cells. So far, regulatory roles of ZFP36L2 in cardiomyocytes and in the context of I/R injury have not been described.

On the other hand, miRNAs and their role in altering mitochondrial morphology or cardiomyocyte functions have been broadly studied. Mitochondrial miRNAs are more specifically targeted towards regulating pathways related to cell apoptosis, proliferation and differentiation [16]. More specifically to processes related to mitochondrial morphogenesis, miR-200a-3p has been shown to promote mitochondrial elongation by targeting mitochondrial fission factor (Mff) [17]. Using an anti-tumor drug, doxorubicin-induced cardiomyopathy can be counteracted using miR-532-3p, which targets mitochondrial fission and fusion processes in cardiomyocytes [18]. In the context of I/R injury, several miRNAs including miR-140 and miR-15 have been shown to alter mitochondrial fission/fusion and apoptosis [19-23]. MARCH5 (or MITOL) is a mitochondrial RING-finger E3 ubiquitin-protein ligase that regulates mitochondrial morphogenesis by direct ubiquitination and degradation of proteins Fis1, Mfn1 and Mfn2 [24, 25]. Under hypoxia, MARCH5 ubiquitinates mitochondrial receptor FUNDC to prevent hypoxia-induced mitophagy [26, 27].

In this study, we investigated the novel role of miR-21-5p in regulating PVT1 expression, and identified an E3 ubiquitin ligase MARCH5 that played a role in PVT1-mediated alterations of mitochondrial fission and fusion processes during I/R injury.

Materials And Methods

Cardiomyocyte culture and treatment

Murine cardiomyocytes were harvested from hearts of 2-day-old mice. Suspension cultures were treated with HEPES solution containing 12 mg/mL pancreatin and 0.14 mg/mL collagenase (Worthington, Freehold, NJ, USA). Cells were cultured *in vitro* in Dulbecco's Modified Eagle's Medium supplemented with 5% heat-inactivated horse serum and antimycotic cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and plated on laminin-coated (10 µg/mL) culture dishes. Cells were transduced with non-targeting shRNA (NC), or shRNA against ZFP36L2, PVT1, or MARCH5 for 48 h, or transfected with a PVT1 overexpression construct using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the hypoxia/reoxygenation (H/R) based injury model, myocytes were stimulated with palmitate (100 µM) for 2 h followed by induction of hypoxia (1% O₂) for 4 h and reoxygenation for 1 h. Cells were then stained for further mitochondrial analyses or harvested to evaluate mRNA and protein expressions.

Myocardial IR injury model

All *in vivo* animal experiments were conducted in accordance with the requirements and principles of the Animal Care and Use Committee of Shanghai Jiaotong University Affiliated Sixth People's Hospital and performed according to established guidelines. Eight-week-old C57BL/6 wild-type mice were anesthetized using isoflurane to perform thoracotomy. To assess myocardial I/R injury, mice were subjected to 45 min myocardial ischemia followed by 4 h reperfusion. Sham-operated group underwent the same procedure except that the snare was left untied. After reperfusion, Evans blue dye (1 ml of a 2% solution; Sigma-Aldrich) was injected through jugular vein to delineate the ischemic area at risk. The mice were euthanized by cervical dislocation. Then the heart was rapidly excised and sectioned. The heart slices were incubated in 1.0% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) for 15 min at 37°C to differentiate live (red) from dead or infarcted myocardium (white). After washing in ice-cold sterile saline, the slices were fixed in 10% formaldehyde, weighed and photographed from both sides. The infarct area (INF) and the risk zone were assessed using computer-assisted planimetry by a histologist blinded to treatment conditions. The INF/LV ratio (%), LVIDd (mm) and the infarct size (defined as % of risk zone) were calculated.

For lncRNA delivery, sh-PVT1 or sh-NC was administered by intravenous injection at a dose of 30 mg/kg per day for three consecutive days. The mice were then subjected to I/R treatment. For intracoronary delivery of adenoviruses, adenoviruses carrying ZFP36L2 shRNA (200:1 m.o.i.), shRNA-NC (200:1 m.o.i.),

sh-MARCH5 (250:1 m.o.i.), or sh-NC (250:1 m.o.i.) were injected with a catheter from the LV apex into the aortic root. The mice were subjected to I/R treatment five days after injection of adenoviruses.

Quantitative reverse transcription-PCR

Total RNA was extracted from heart samples or cells using a RNEase kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A total of 500 ng of RNA was used for subsequent cDNA preparation and quantitative PCR using SYBR-Green (Thermo Fisher Scientific), and the results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used for amplification of DRP1, Fis1, Mff, Mfn1, Mfn2, ZFP36L2, PVT1, miR-21-5p, MARCH5, and GAPDH are listed in Table S1.

Western blot analysis

Protein lysates from cells or tissues were quantified using a BioDrop μ LITE analyzer (BioDrop, Cambridge, UK). A total of 50 μ g total protein was loaded onto 10% SDS gels and blotted onto nitrocellulose membranes. The membranes were incubated in primary antibodies against Drp1 (1:1000, Abcam, UK), Fis1(1:500, Abcam, UK), Mff(1:1000, Cell signaling Technology, USA), Mfn1(1:2000, Abcam, UK), Mfn2(1:1000, Abcam, UK), ZFP36L2(1:500, Proteintech, China), MARCH5(1:1000, Cell signaling Technology, USA), and GAPDH(1:500, Abcam, UK) overnight at 4°C. After washing in phosphate-buffered saline – Tris (PBS-T), the membranes were incubated in fluorescent secondary antibodies for 1 h and visualized using an Odyssey Imager (LiCor, Lincoln, NE, USA). Image analysis and quantification were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Histology

Heart sections were analyzed after hematoxylin and eosin (H&E) staining and by immunohistochemistry (IHC). Samples were prepared for histology by infusing 4% paraformaldehyde into the heart. 5- μ m microsections were then subsequently stained with H&E or Masson's trichome to assess infiltration and fibrosis. For IHC, tissue sections were stained using primary antibodies against ZFP36L2 or MARCH5 overnight at 4°C, then imaged using a light microscope.

Subcellular fractionation

Cells were washed with fresh PBS and resuspended in fractionation buffer [20 mmol HEPES (pH 7.5), 10 mmol KCl, 1.5 mmol MgCl₂, 1 mmol EGTA, 1 mmol EDTA, 1 mmol DTT, and 0.1 mmol phenylmethanesulfonyl fluoride], 250 mmol sucrose, and 20mmol protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The suspension was homogenized using a Dounce homogenizer and centrifuged at 750 $\times g$ for 5 min. The nuclei were pelleted, leaving the cytosolic and ER fraction in the supernatant. The supernatant was subsequently centrifuged at 10,000 $\times g$ for 15 min to pellet the mitochondrial fraction. A third centrifugation step of the supernatant yielded the cytosolic fraction. The fractions were analyzed for proteins using western blotting.

Biotinylated miRNA pull-down assay

A RNA pull-down assay was performed as described previously [28]. Briefly, cells were transfected with biotinylated miR-21-5p, miR-21-5p-mut, or ZFP36L2, and harvested 48 h later for lysis. Cell lysates were incubated with streptavidin magnetic beads for 3 h at 4°C. The beads were thoroughly washed with a low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl) and high salt buffer ((0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0 and 500 mM NaCl). All bound RNA molecules were analyzed by northern blotting.

DNA pull-down assay

A complementary DNA probe (biotinylated) to CARL was synthesized (Sigma-Aldrich) and incubated with streptavidin-coated magnetic beads in binding buffer. DNA bound beads were then used to pull-down RNA from lysates prepared from cardiomyocytes. RNA bound to the probe was eluted and analyzed using northern blotting.

Luciferase assay

PVT1 and MARCH5 wild-type and mutant sequences were expressed using a pGL3 vector (Promega, Madison, WI, USA) encoding the firefly luciferase gene. Cardiomyocytes were co-transfected with the luciferase constructs using Lipofectamine 2000 (Invitrogen), and cells were harvested 48 h post-transfection for analysis of the Dual luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. 30 µL protein samples were analyzed in a luminometer. Firefly luciferase activities were normalized to Renilla luciferase activity.

Northern blot analysis

Samples were subjected to polyacrylamide-urea gel electrophoresis, blotted onto positively charged nylon membranes, and cross-linked using UV irradiation. Membranes were hybridized using 100 pmol 30-digoxigenin (DIG)-labeled probes against ZFP36L2, PVT1 or miR-21-5p overnight at 4°C, and detected using a DIG luminescent detection kit (MyLab) according to the manufacturer's instructions.

Immunofluorescence assay

Cells were seeded onto poly-L-lysine coated coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked using standard 5% bovine serum albumin in PBS blocking solution and incubated with primary antibody against Drp1 (1:1000, Abcam, UK) for 1 h at room temperature. After washing three times using PBS, the coverslips were incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:1,000, abcam, UK) for 1 h at room temperature. The antibody was removed by washing three times with PBS and the samples were mounted for visualization and imaged using confocal microscopy.

Fluorescence in situ hybridization (FISH)

End-labeled 6-carboxyfluorescein (FAM) probes were synthesized for PVT1 and ZFP36L2 (Invitrogen). Cells on coverslips were fixed using 10% neutral-buffered formalin and resuspended in hybridization buffer [0.7 M NaCl, 0.1 M Tris (pH 8.0), 0.1% SDS, and 10 mM EDTA] containing the probes. Samples were heated at 55°C for 30 min and unbound probes were washed using probe-free hybridization buffer. Cells were counterstained and mounted using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). Coverslips were analyzed by fluorescence microscopy (Carl Zeiss, Jena, Germany).

Mitochondrial staining

Cells were seeded onto poly-L-lysine coated coverslips, stained with MitoTracker Red CMXRos (0.02 µM; Molecular Probes, Eugene, OR, USA), and analyzed using a confocal microscope (LSM 510 META; Zeiss, Oberkochen, Germany). Total cells with fragmented mitochondria were represented as a percentage by counting at least 300 cells per treatment group, from six different fields of view.

Electron microscopy

Ultrastructural analysis of mitochondrial fission was performed using transmission electron microscopy (TEM) as described previously [29]. Tomograms from sections were obtained using a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) and analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). More than 1200 mitochondrion were analyzed to determine the mitochondria sizes and structures < 0.6 µm², which were classified as fission mitochondria.

Statistical analysis

All data are expressed as the mean (±SD) of at least three independent experiments, and statistical significance was calculated using Student's *t*-test to compare two groups, and one-way analysis of variance for comparing multiple groups. A value of *P* < 0.05 was considered statistically significant.

Results

ZFP36L2 knockdown inhibited myocardial ischemia/reperfusion (I/R) injury and attenuated mitochondrial fission *in vivo*.

Regulatory functions are imposed by RNA binding proteins in response to cellular stress, which alters transcription and translation [30]. In an *in vivo* mouse model, upon induction of ischemia followed by reperfusion, a 3-fold increase in ZFP36L2 protein level was observed. This increase was attenuated upon treatment with a shRNA targeted against ZFP36L2 (Fig. 1A). Reducing the levels of ZFP36L2 significantly lowered infarction size, infarct area/left ventricle (INF/LV), and improved the functioning of the left ventricle as measured by the left ventricular internal diastolic diameter (LVIDd) (Fig. 1B-C). An echocardiogram showed reduced capacity of ejection and fractional shortening upon I/R induction, which was significantly improved upon knockdown of ZFP36L2 (Fig. 1D). Analyses of cardiomyocytes observed by H&E and Masson's staining revealed that IR injury caused discontinuous tissue architecture due to

extensive apoptosis, but knockdown of ZFP36L2 alleviated this effect (Fig. 1E). Ultrastructural analyses using TEM showed that mitochondrial fission was significantly increased upon I/R injury, as indicated by increased expression of Drp1, Fis1, and Mff, and a decrease in Mfn1 and Mfn2 fusion proteins. This effect was dependent on ZFP36L2 (Fig. 1F-G). Together, these results identified ZFP36L2 as a key regulator of cardiomyocyte injury during I/R.

ZFP36L2 knockdown attenuated cardiomyocytes hypoxia/reoxygenation (H/R) injury and attenuated mitochondrial fission *in vitro*.

To further extend our findings *in vitro*, we used a H/R model with cardiomyocytes treated with 1% O₂ (hypoxia) for 4 h followed by reoxygenation. As previously observed, ZFP36L2 levels were increased upon H/R injury (Fig. 2A). Mitochondrial fission, as observed by staining of mitochondria with MitoTracker Red, revealed a 50% increase in fission upon H/R induction. This effect was significantly reduced in the ZFP36L2 knockdown group when compared to the non-targeting control-treated group (Fig. 2B). Similarly, western blotting and immunofluorescence assays showed that proteins mediating fission or fusion were significantly decreased or increased, respectively, upon knockdown of ZFP36L2 (Fig. 2C-E). Together, these results showed that *in vitro* regulation of ZFP36L2 levels altered mitochondrial fission and fusion processes in response to H/R injury.

ZFP36L2 bound to lncRNA PVT1 and regulated its expression.

To identify the mechanism responsible for ZFP36L2-mediated regulation of I/R injury, we performed RNA pull-down and immunoprecipitation experiments. The lncRNA PVT1 was bound to ZFP36L2 and was significantly enriched (Fig. 3A-C). DNA FISH and subcellular fractionation assays showed that a fraction of PVT1 was localized in the cytoplasm, while ZFP36L2 was localized in the nucleus (Fig. 3D-E). We also observed that knockdown of ZFP36L2 reduced the expression of PVT1 and overexpression increased PVT1 levels (Fig. 3F). However, knockdown and overexpression of PVT1 did not affect ZFP36L2 levels (Fig. 3G). These results indicated that ZFP36L2 bound to PVT1 and directly regulated its expression.

PVT1 knockdown suppressed I/R injury and attenuated mitochondrial fission.

To verify the functional implication of the association between PVT1 and ZFP36L2, we used an *in vivo* I/R injury model to determine the effects of PVT1. Upon intraperitoneal injection of shRNA against PVT1 at 24 h post-I/R injury, we observed a reduction in endogenous levels of PVT1 in cardiomyocytes when compared to increased expression upon I/R induction or treatment with control shRNA (NC) (Fig. 4A). This also significantly reduced infarction size, INF/LV ratios, and diastolic diameter of the left ventricle (LVIDd) when compared to the NC-treated group (Fig. 4B-D). Tissue analyses showed improved cell architecture and reduced apoptosis with low levels of PVT1 (Fig. 4E). In addition, TEM and qPCR analyses showed decreased levels of mitochondrial fission and improved fusion upon knockdown of PVT1 in cardiomyocytes, in both the *in vivo* and in *in vitro* H/R injury models (Fig. 4G and Supplementary Fig. S1).

PVT1 directly bound to miR-21-5p and suppressed its activity.

PVT1 is known to regulate functions of microRNAs (miRNA) during tumorigenesis [31, 32]. We identified a region in miR-21-5p having a complementary binding sequence to PVT1, using Starbase 3.0 [33], and also generated a binding-deficient mutant of PVT1 (PVT1-mut) (Fig. 5A). Luciferase assay results showed that treatment with a miR-21-5p mimic significantly reduced luciferase activity, indicating an inhibition in wild-type PVT1 (wt-PVT1) expression. This effect was lost in mut-PVT1, and when an inhibitor against miR-21-5p was used (Fig. 5B). A biotin-based pull-down assay with miR-21-5p and a mutant version that did not associate with PVT1 showed an enrichment of PVT1 specifically in the miR-21-5p wild-type fraction (Fig. 5C). Additionally, we performed DNA pull-down experiments and observed that miR-21-5p was specifically expressed in the pull-down fraction, using PVT1 (Fig. 5D). Fractionation experiments revealed that miR-21-5p specifically associated with PVT1 in the cytoplasm and not the nucleus (Fig. 5E-F). To test for the effect of miR-21-5p-mediated regulation of PVT1 expression in mitochondrial fission upon I/R injury, we treated I/R induced mice with a miR-21-5p mimic alone or in combination with PVT1 overexpression and/or the respective controls. TEM and tissue analyses revealed that treatment with a miR-21-5p mimic significantly reduced I/R induced effects on mitochondrial fission and cell apoptosis, and that this effect was lost when PVT1 was co-expressed (Fig. 5G-H and supplementary Fig. S2A). *In vitro* analyses also showed the total number of cells with fragmented mitochondria was below 20% in the control and miR-21-5p mimic-treated cells, and over 40% when H/R was induced and PVT1 was overexpressed (Fig. 5I and Supplementary Fig. S2B-C). Upon knockdown of PVT1, expression of miR-21-5p was significantly higher and overexpression reduced these levels, using both *in vivo* and *in vitro* analyses (Fig. 5J-K). Taken together, these results showed that miR-21-5p and PVT1 were negatively regulated by direct association and altered mitochondrial morphology during I/R injury.

The miR-21-5p associated with and regulated MARCH5 expression.

To further understand how miR-21-5p regulated PVT1 mediated mitochondrial fission, we looked for known components binding to miR-21-5p using Starbase 3.0, an online omics database for miRNA networks [33], and identified MARCH5, an E3 ubiquitin ligase that promotes mitochondrial fission by Drp1 [34, 35]. MARCH5 has a complementary sequence to miR-21-5p in its 3'-UTR region (Fig. 6A). We verified binding by treating cells encoding luciferase under the promoter of wild-type MARCH5 (MARCH5-wt) or a mutant version that could not associate with miR-21-5p (MARCH5-mut). Luciferase assays in cardiomyocytes showed reduced expression of MARCH5-wt upon treatment with a miR-21-5p mimic, and this effect was lost in the MARCH5-mut (Fig. 6B). Luciferase expression was increased when an inhibitor against miR-21-5p was used (Fig. 6C). Both *in vitro* and *in vivo*, cardiomyocytes responded to treatment with the miR-21-5p mimic by showing increased mRNA levels of miR-21-5p when compared to treatment with the NC mimic, which significantly reduced the infarction size 3-fold (Fig. 6D-E). Treatment with the miR-21-5p mimic also reduced MARCH levels in cardiomyocytes, and treatment with an inhibitor or overexpression of MARCH5 increased protein levels (Fig. 6F-G). Together, these results identified MARCH5 to be negatively regulated by miR-21-5p in cardiomyocytes.

MARCH5 promoted mitochondrial fission in cardiomyocytes.

We next tested the *in vivo* and *in vitro* importances of MARCH5 expressions in I/R or H/R injury. Intraperitoneal treatment of shRNA MARCH-treated cardiomyocytes 24 h post-I/R injury significantly reduced MARCH5 expression compared to shNC-treated mice (Fig. 7A). Infarction size decreased more than 50% in the shMARCH5 treated group compared to the controls (Fig. 7B). A significant decrease in LVIDd and infarction areas was also observed upon shMARCH5 treatment (Fig. 7C). Ultrastructural analyses of TEM images revealed reduced mitochondrial fission, and histology also showed improved tissue architecture and reduced apoptosis in the shMARCH5-treated group (Fig 7D-E). Knockdown of MARCH5 significantly reduced Drp1 mRNA expression along with Fis1 and Mff. However, Mfn1 and Mfn2 levels were increased (Fig. 7F). *In vitro* analyses also showed decreased fragmentation in mitochondria upon treatment with shMARCH5, and reduced Drp1, Fis1, and Mff mRNA and protein expressions along with increased Mfn1, Mfn2 mRNA, and protein levels (Fig. 7G and Supplementary Fig S3). These results showed that MARCH5 was functionally implicated in mitochondrial dysfunction during I/R injury.

PVT1 regulated mitochondrial fusion and fission through the miR-21-5p/MARCH5 axis.

To assess the roles of lncRNA PVT1, miR-21-5p, and MARCH5 in I/R injury, we tested different conditions *in vitro* using miR-21-5p mimics, PVT1 overexpression, and shRNA-mediated MARCH5 knockdown. Luciferase expression in response to MARCH5 3'-UTR in 293T cells was increased upon PVT1 overexpression, and this effect was reduced when treated with a miR-21-5p mimic (Fig. 8A). *In vitro*, cardiomyocytes showed decreased MARCH5 expression when PVT1 was targeted by shRNA (sh-PVT1), and these levels increased when treated with a miR-21 inhibitor, whereas overexpression of PVT1 and treatment with miR-21-5p showed an opposite effect (Fig. 8B-C). Overexpression of PVT1 also increased mitochondrial fragmentation, altered associated genes, mRNA levels, and protein expression levels (Fig. 8D-G). However, this effect was lost upon co-expression of shMARCH5 because mitochondrial fission was reduced and Drp1, Fis1, Mff mRNA, and protein levels decreased along with an increase in Mfn1 and Mfn2 (Fig. 8D-G). Taken together, these results showed that MARCH5 played a key role in the interplay between miR-21-5p-mediated regulation of PVT1 expression and its effects on mitochondrial morphology.

Discussion

Mitochondria are enriched in cardiomyocytes and undergo constant fission and fusion in response to physiological conditions. I/R causes changes in structure and function that determine various cell functions [36, 37]. In this study, we showed that lncRNA PVT1 played a critical role in altering mitochondrial fission and fusion processes. The levels of PVT1 were regulated by the presence of the zinc finger protein, ZFP36L2, which directly associated with PVT1. Furthermore, we also showed that miR-21-5p negatively regulated PVT1 and MARCH5 levels, thereby reducing mitochondrial fission during I/R injury.

Mitochondrial division during mitoptosis involves pro-apoptotic BAX and BAK proteins along with Drp1, in contrast to the roles of Fis1 and Mdv1 (mitochondrial division 1) proteins during the fission processes [38]. High levels of Drp1 result in morphologically altered mitochondria and decreased total numbers per cell, along with increased cytochrome c levels and cell death [39]. We observed that Drp1 levels along with Mff and Fis1 were increased upon induction of I/R or H/R. These levels were decreased upon knockdown of ZFP36L2 or PVT1. LncRNAs are classical regulators of gene expression. ZFP36L2 is a nuclear-DNA encoded lncRNA that acts in the mitochondria. Transport of these lncRNAs by RNA binding proteins during I/R injury remains to be elucidated. LncRNA can also form complex three dimensional RNA-RNA hybrid structures to which zinc finger proteins such as PVT1 can bind and regulate their trans-modulatory effects [40]. Future studies directed at identifying the binding motif of ZFP36L2, which associates with lncRNA PVT1, may provide the possibility to regulate its effects on mitochondrial fission/fusion processes.

In other cell types such as skeletal muscles, PVT1 is localized to both nuclear and cytoplasmic fractions [11], whereas in cardiomyocytes it was largely expressed in the cytoplasm. We found that miR-21-5p preferentially associated with PVT1 in the cytoplasm and reduced its expression. This effect was rescued by treatment with a miR-21-5p inhibitor. Although it is known that PVT1 regulates functions of miRNA to promote cell proliferation and invasion in cancer [41, 42], it is still not clear how I/R injury, and interference with mitochondrial fission/fusion processes are directed by PVT1 and miR-21-5p. The miR-21-5p could bind to fission proteins such as Fis1 or Mff to inhibit its translation as shown previously for miR-484 and miR-761 in cardiomyocytes and specific to I/R injury respectively [43, 44]. Indirectly, it could inhibit Drp1-mediated pathway thereby suppressing p53 mediated triggering of apoptosis.

MiRNAs are capable of interacting with the 3'-UTR regions of mRNAs to alter posttranscriptional modifications or translation [45], and several miRNAs, such miR-27 and miR-30 have been shown to alter the mitochondrial fission/fusion process [46, 47]. We identified MARCH5 as possibly binding to miR-21-5p, and previous studies have shown that it alters Drp1-mediated mitochondrial fission by redirecting Drp1 from fission sites [35]. We showed that miR-21-5p bound to the 3'-UTR region of MARCH5, and its knockdown reduced mitochondrial fission in I/R injury. Knockdown of MARCH5 may prevent ubiquitination and subsequent proteasomal degradation of miR-21-5p, thereby retaining fusion properties of mitochondria [48]. We observed that in the presence of MARCH5 and overexpression of PVT1, mitochondrial fission was significantly induced, and these effects were lost upon MARCH5 knockdown. While our data suggests that regulation of mitophagy by MARCH5 is via targeting miR-21-5p, MARCH5 is also capable of directly ubiquitinating fission/ fusion proteins [24, 25]. Therefore there is a possibility of additional mechanisms and direct targets for MARCH5 to coordinate mitochondrial morphogenesis during I/R injury in cardiomyocytes.

Hence, we have described a novel mechanism of miR-21-5p/ MARCH5-mediated regulation of mitochondrial morphology by PVT1 during I/R injury. Among increasing data describing a role for miRNAs as a link between I/R injury and mitochondrial dysfunction, our study contributes to a further

understanding of these mechanisms, which may eventually translate into improved therapeutic approaches.

Abbreviations

I/R: ischemia-reperfusion; H/R: hypoxia/ reoxygenation; lncRNAs: Long non-coding RNAs; qRT-PCR: Quantitative Reverse transcription-polymerase chain reaction; TEM: transmission electron microscopy; Drp1: dynamin related protein 1; Fis1: fission 1; PVT1: Plasmacytoma variant translocation 1; Mff: mitochondrial fission factor; TTC: 2, 3, 5-triphenyltetrazolium chloride; FISH: Fluorescence in situ hybridization; INF: infarct area; INF/LV: infarct area/left ventricle; LVIDd: left ventricular internal diastolic diameters.

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Experimental Animal Care Commission of Shanghai Jiaotong University Affiliated Sixth People's Hospital.

Consent for publication

Not applicable. All data presented in this article is non-identifiable.

Availability of data and materials

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

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' Contributions

WW and YL participate in the processes of the conception and design of the study. WH, QT, YG, YC and JS participate in the processes of experiment execution, data collection. WH, QT, FW and FP participate in the processes of data analysis and interpretation of data. WH, QT, WW and YL participate in the

processes of manuscript preparation and the final manuscript improvement. All authors read and approved the final manuscript.

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Not applicable.

Authors' information

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Figures

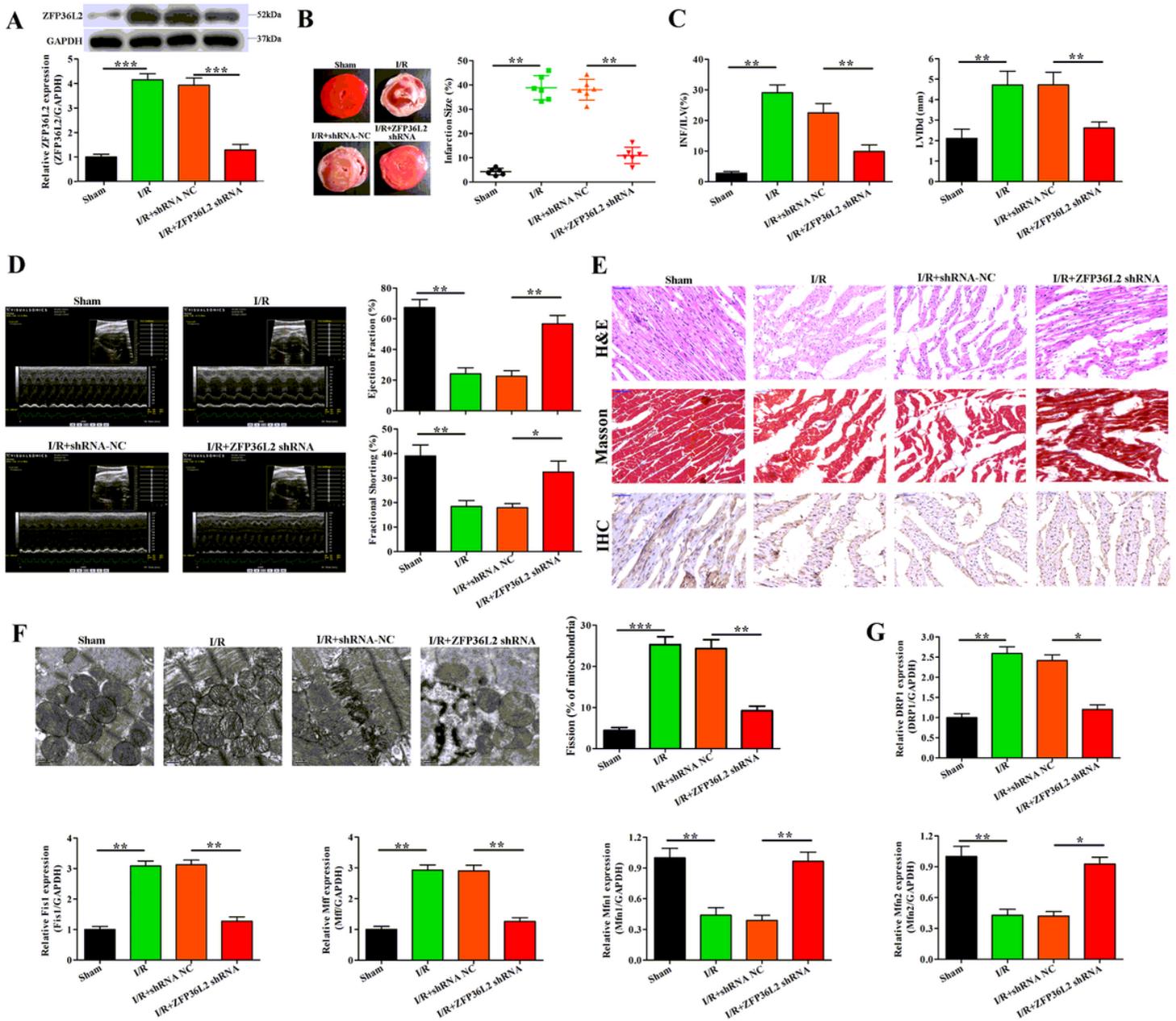


Figure 1

ZFP36L2 knockdown inhibited myocardial I/R injury and attenuated mitochondrial fusion and fission in vivo. (A) A myocardial I/R injury model was established by inducing ischemia for 45 min following 4 h of reperfusion (n = 6 per group). ZFP36L2 shRNA and shRNA-NC were intraperitoneally administered 24 h before induction of I/R injury. RNA was isolated from cardiomyocytes and mRNA levels of ZFP36L2 were determined by qRT-PCR. (B) The left panel shows representative images of tissue samples. The right panel indicates infarction size (%). (C) Quantification of the extent of injury measured by infarction area/left ventricle (INF/LV) and diastolic diameter of the left ventricle (LVIDd). (D) Representative echocardiograms at 4 h after post-I/R injury. The percentage of ejection fraction (EF) and fractional shorting [48] were shown. (E) Representative images of hematoxylin and eosin and Masson's trichrome staining and immunohistochemistry staining for ZFP36L2 in LV sections (Scale bar=50µm). (F)

Representative transmission electron microscopy images of heart tissues shown in the left panel and the corresponding quantification of fragmented mitochondria shown in the right panel (Scale bar=0.5 μ m). (G) Expression of mitochondrial fusion-related genes Drp1, Fis1, Mff, Mfn1, and Mfn2 after myocardial I/R injury as evaluated by qRT-PCR. Data are represented as the mean \pm SD (n = 3, technical replicates). Statistical significance was calculated by one-way analysis of variance with multiple comparisons. *P < 0.05; **P < 0.01 and ***P < 0.001.

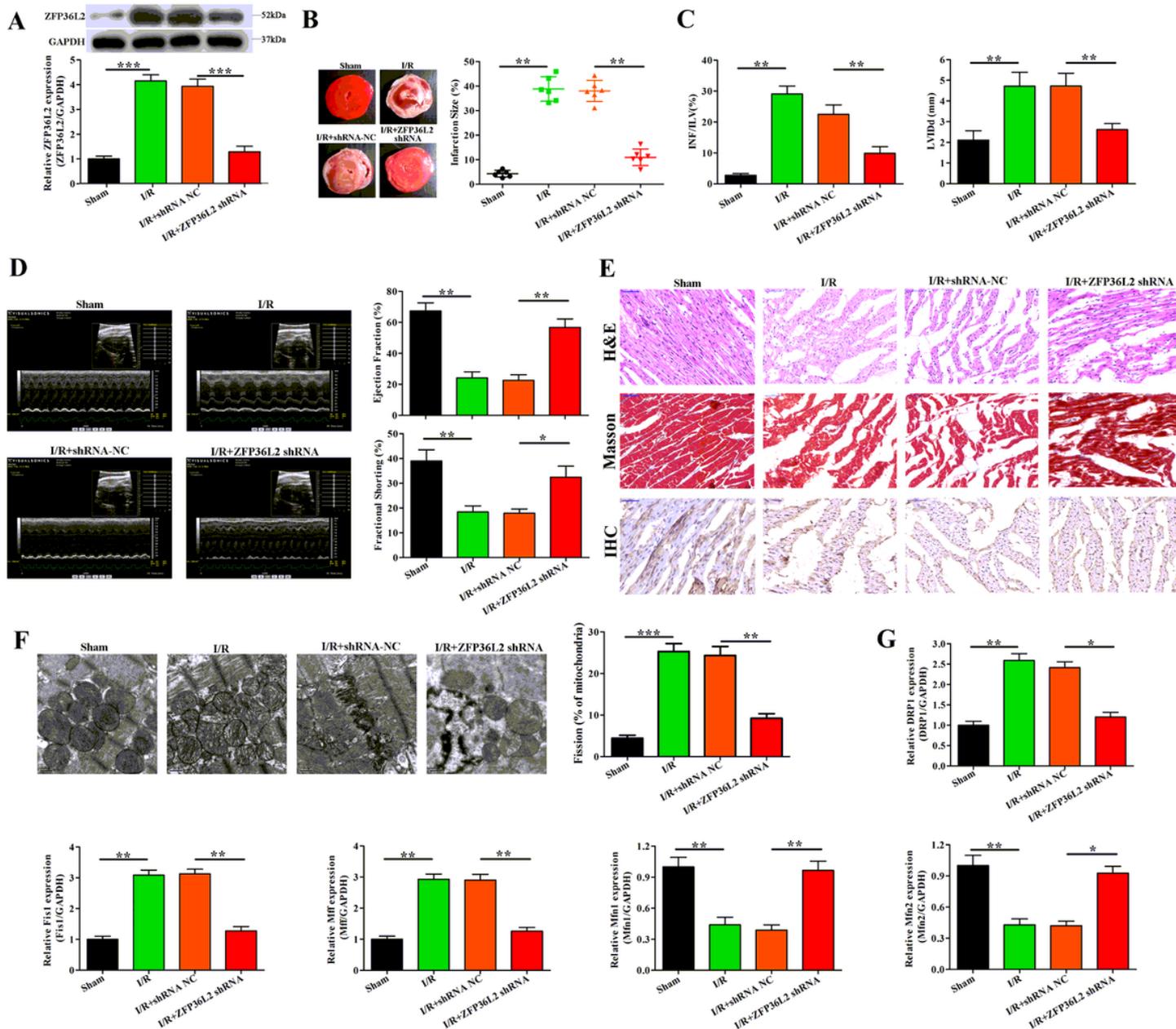


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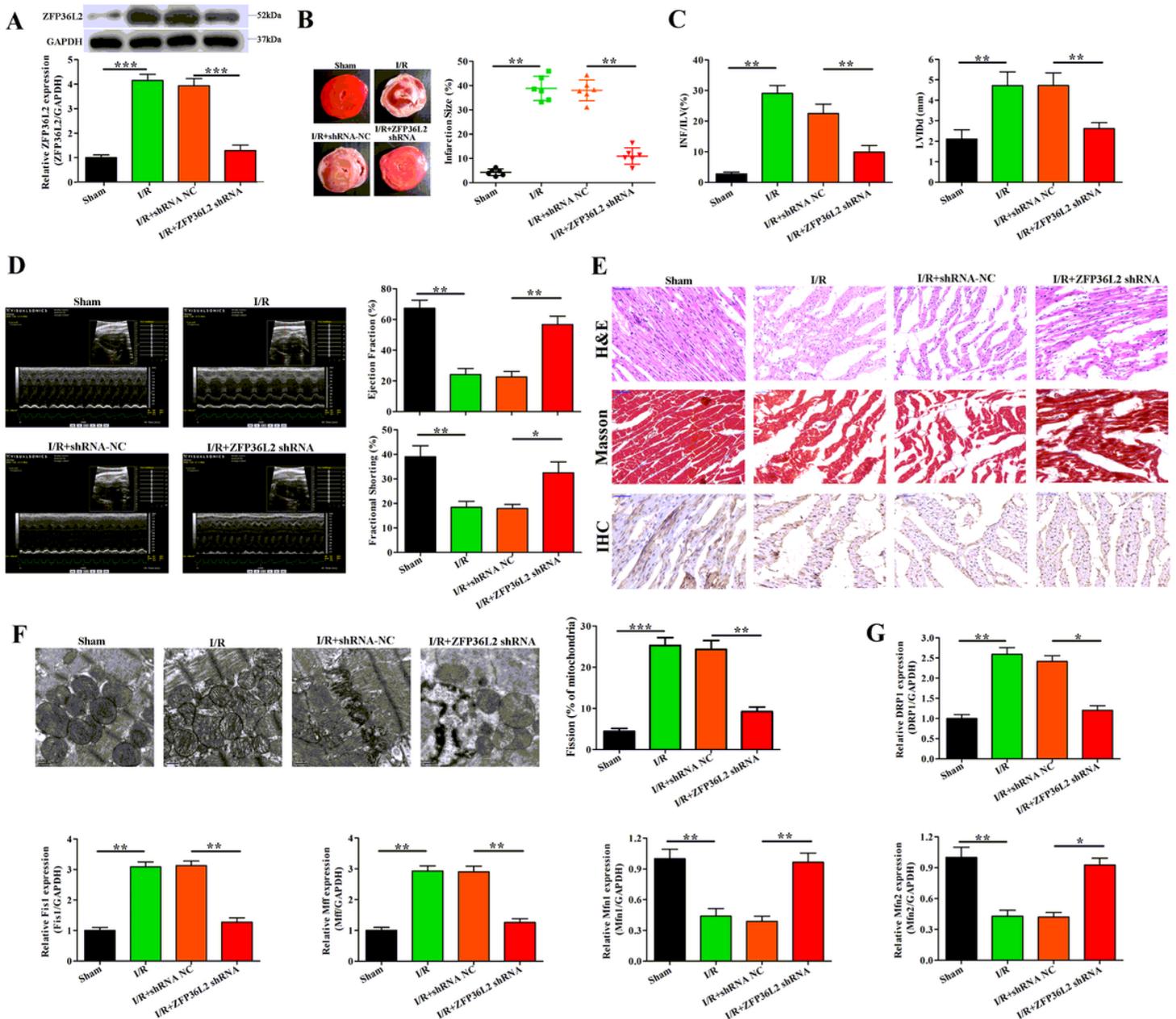


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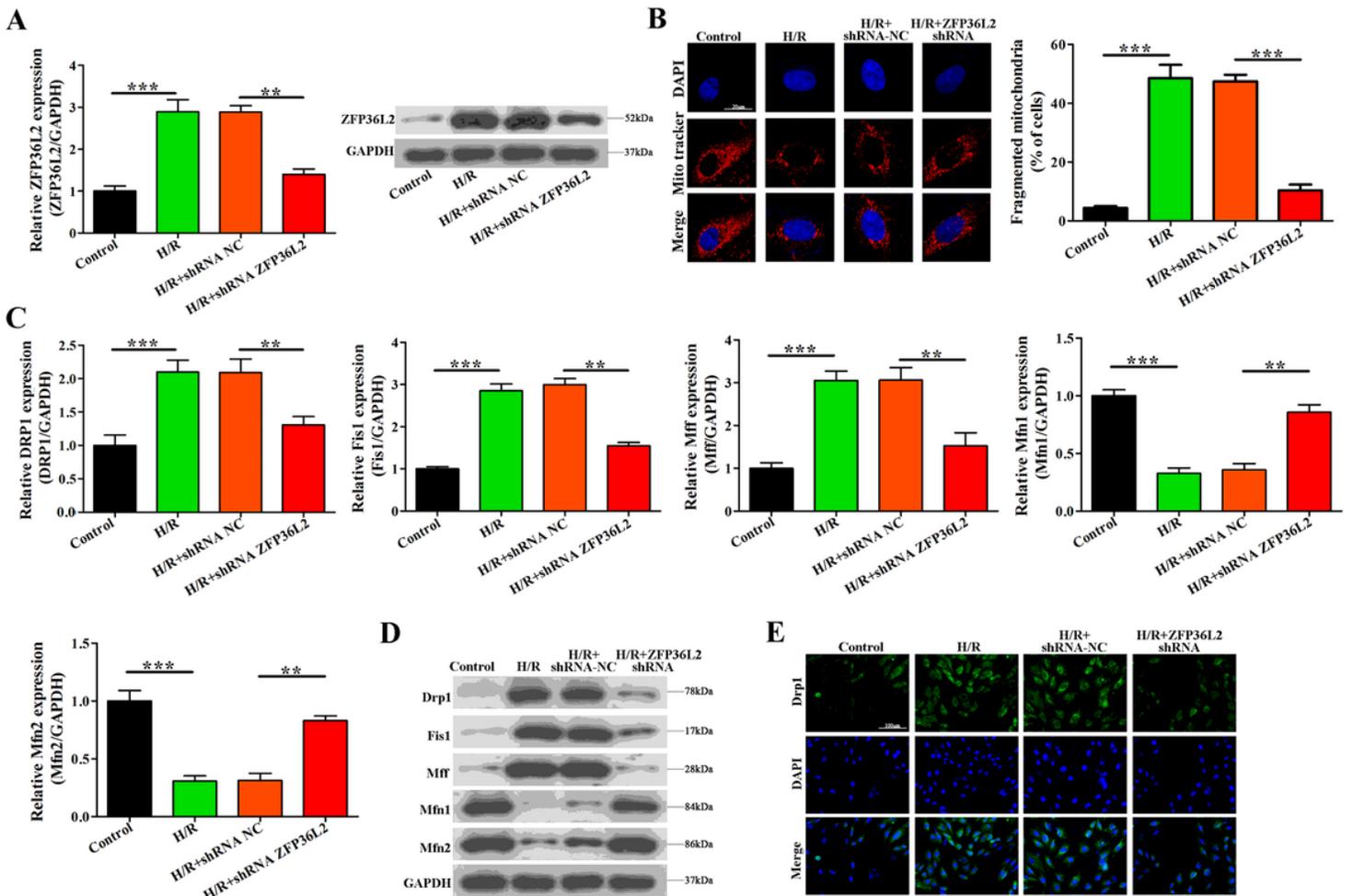


Figure 2

ZFP36L2 knockdown suppressed hypoxia/reoxygenation (H/R) injury and alleviated mitochondrial fusion and fission in vitro. (A) An H/R injury model was established by inducing hypoxia for 45 min followed by reoxygenation for 4 h. The mRNA and protein expressions of ZFP36L2 in cardiomyocytes were determined by qRT-PCR and western blotting, respectively. (B) Representative micrographs of cardiomyocytes after H/R injury expressing normal control shRNA or shRNA against ZFP36L2 stained with MitoTracker Red (mitochondria) and 4', 6-diamidino-2-phenylindole (DAPI) (nucleus). Quantification of cells with fragmented mitochondria was detected under different conditions. At least 100 cells were counted per condition (Scale bar=20 μ m). (C-D) Expression of mitochondrial fusion-related genes Drp1, Fis1, Mff, Mfn1, and Mfn2 after myocardial I/R injury as evaluated by qRT-PCR and western blotting (for respective proteins). (E) Representative micrographs from immunofluorescence assays of cardiomyocytes stained for Drp1 (green) and the nucleus (DAPI) (Scale bar=100 μ m). Data are represented as the mean \pm SD (n = 3, technical replicates). Statistical significance was calculated by one-way analysis of variance with multiple comparisons. **P < 0.01 and ***P < 0.001.

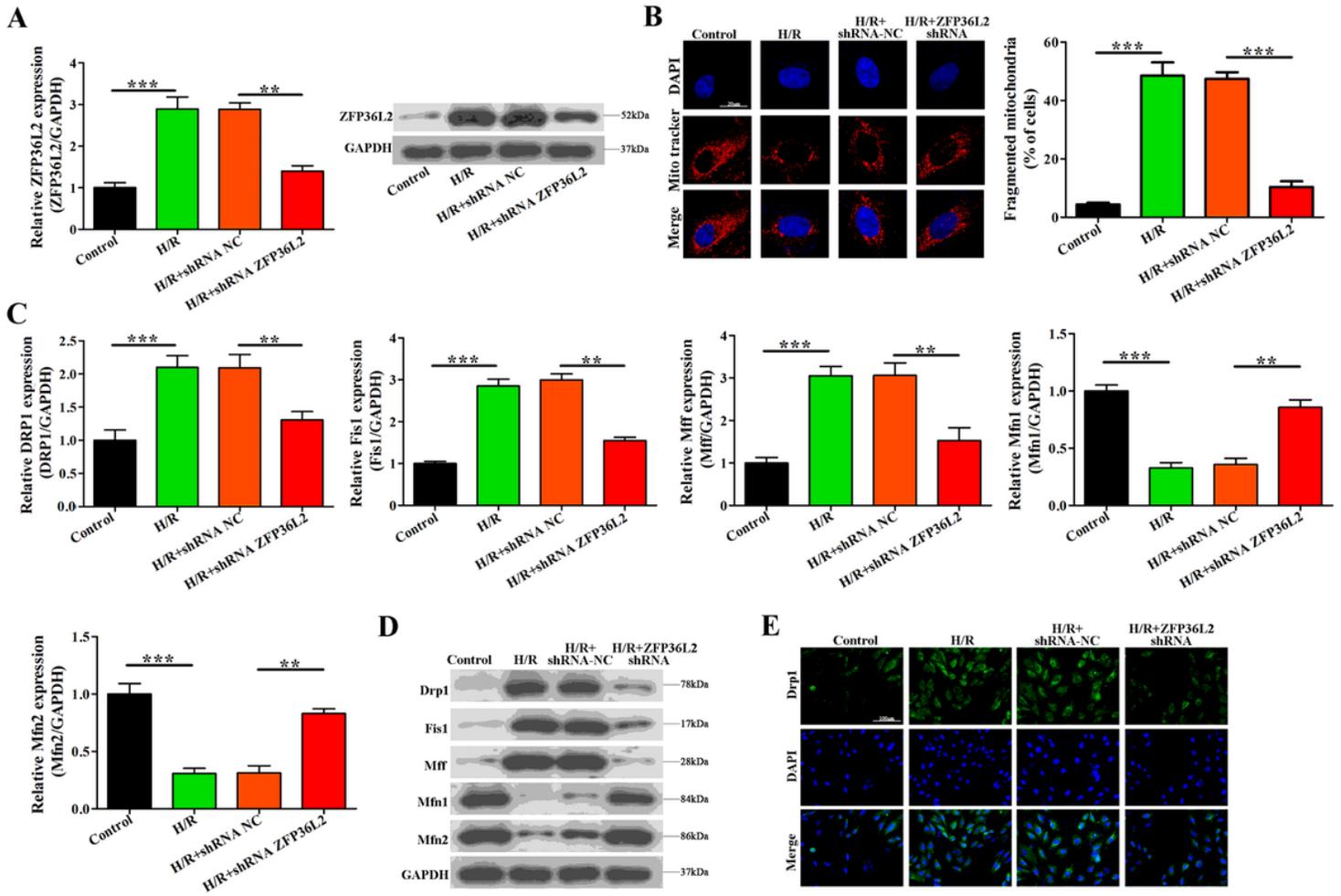


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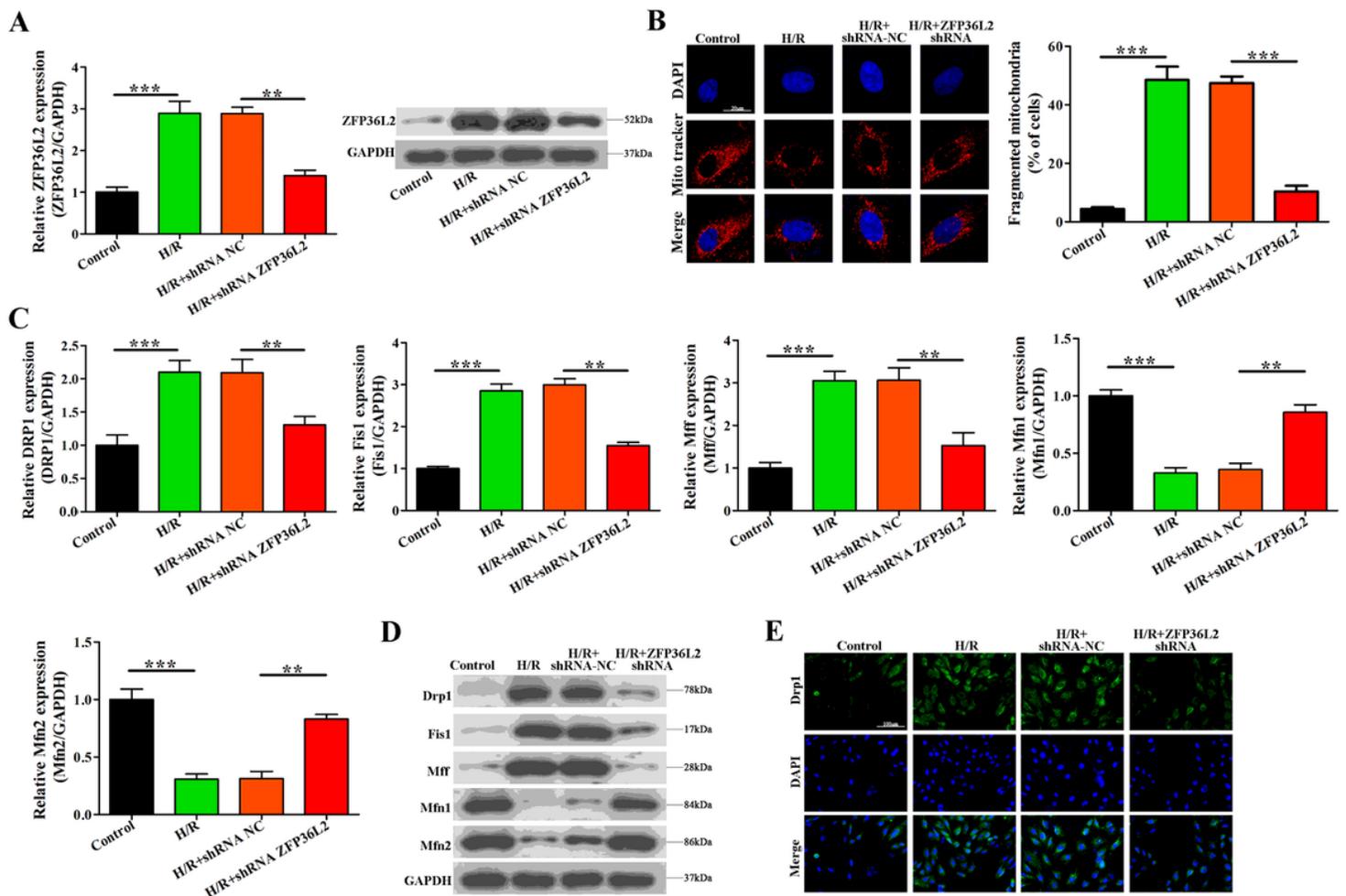


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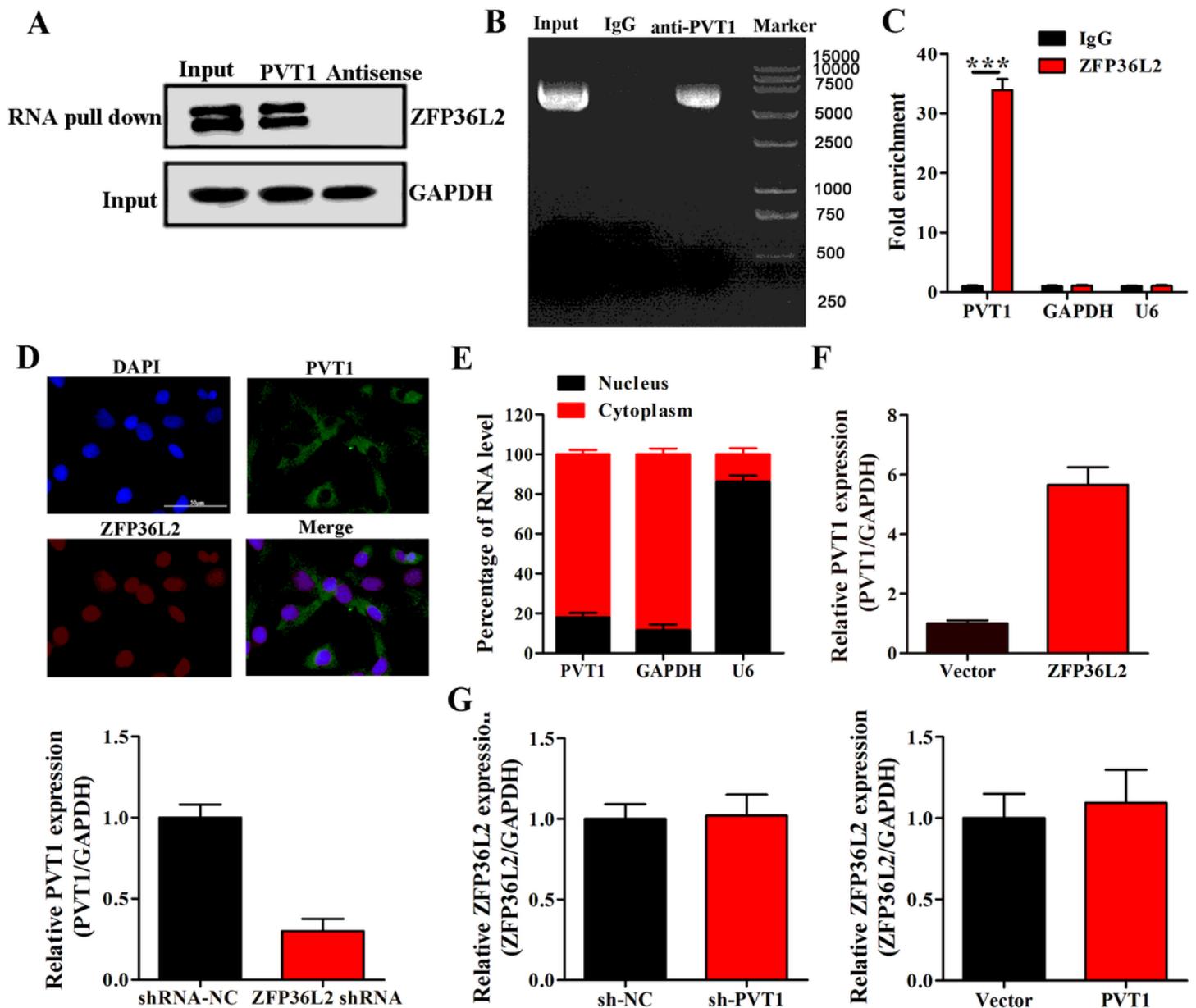


Figure 3

ZFP36L2 bound to lncRNA PVT1 and regulated PVT1 expression. (A) Western blot analyses of association between ZFP36L2 and lncRNA PVT1 using a RNA pull-down assay using PVT1 or antisense RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. (B-C) Representative northern blots from RIP experiments performed using ZFP36L2 or IgG, indicating the expression of PVT1. Quantification of fold enrichment of PVT1, GAPDH, and U6 in ZFP36L2 RIP fractions relative to input RNA was shown. (D) Representative confocal micrographs from fluorescence in situ hybridization with probes against PVT1 (green) and ZFP36L2 (red). The nucleus was stained with DAPI (Scale bar=50 μ m). (E) Total percentage of PVT1 RNA in nuclear and cytoplasmic fractions in cardiomyocytes determined by subcellular fractionation. (F) PVT1 mRNA levels were detected by qRT-PCR in cardiomyocytes expressing shRNA-NC/ZFP36L2 shRNA and Vector/ZFP36L2. (G) The mRNA levels of ZFP36L2 in cardiomyocytes were detected by qRT-PCR. Data are represented as the mean \pm SD (n = 3, technical replicates). Statistical significance was calculated using Students t-test. ***P < 0.001.

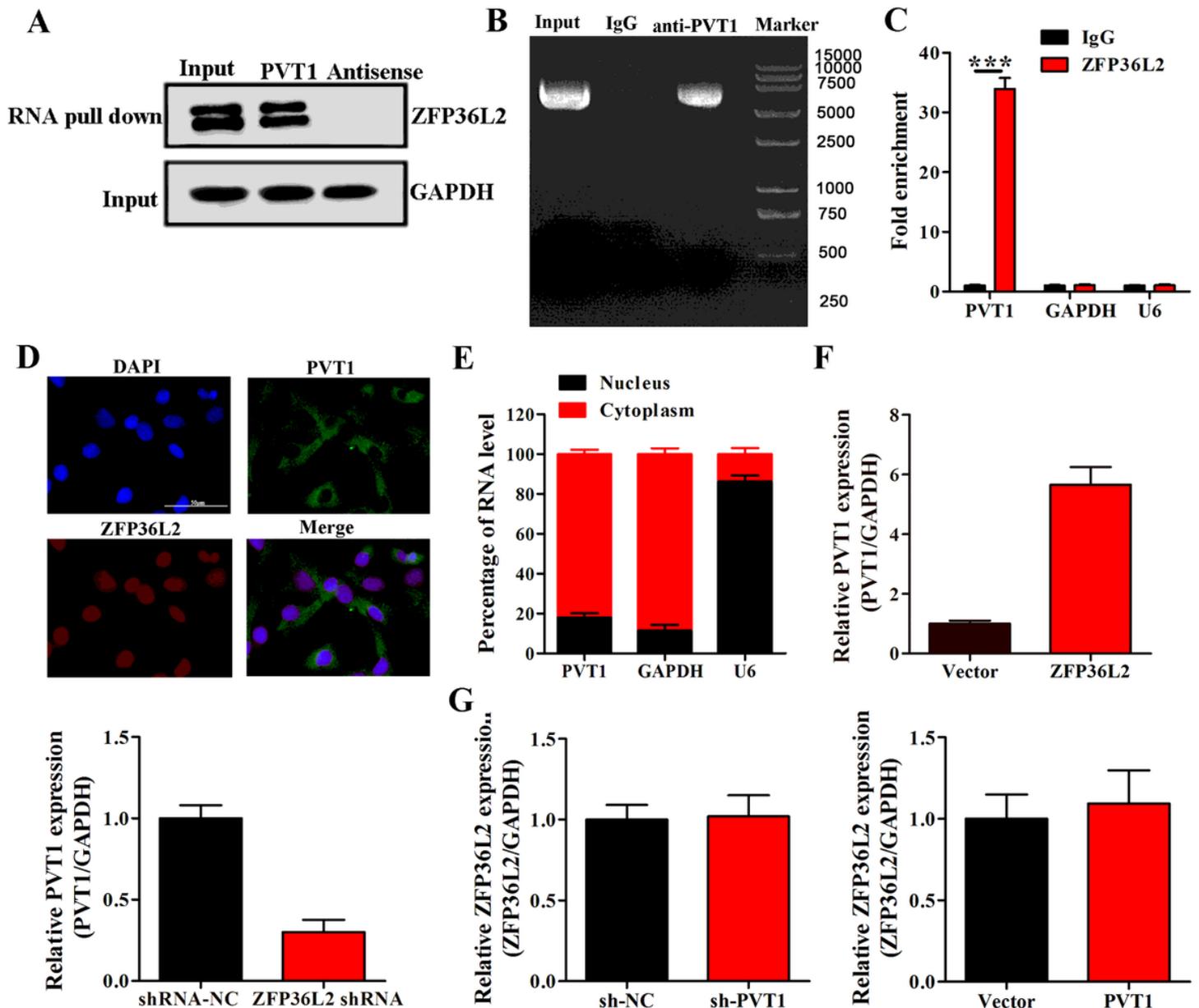


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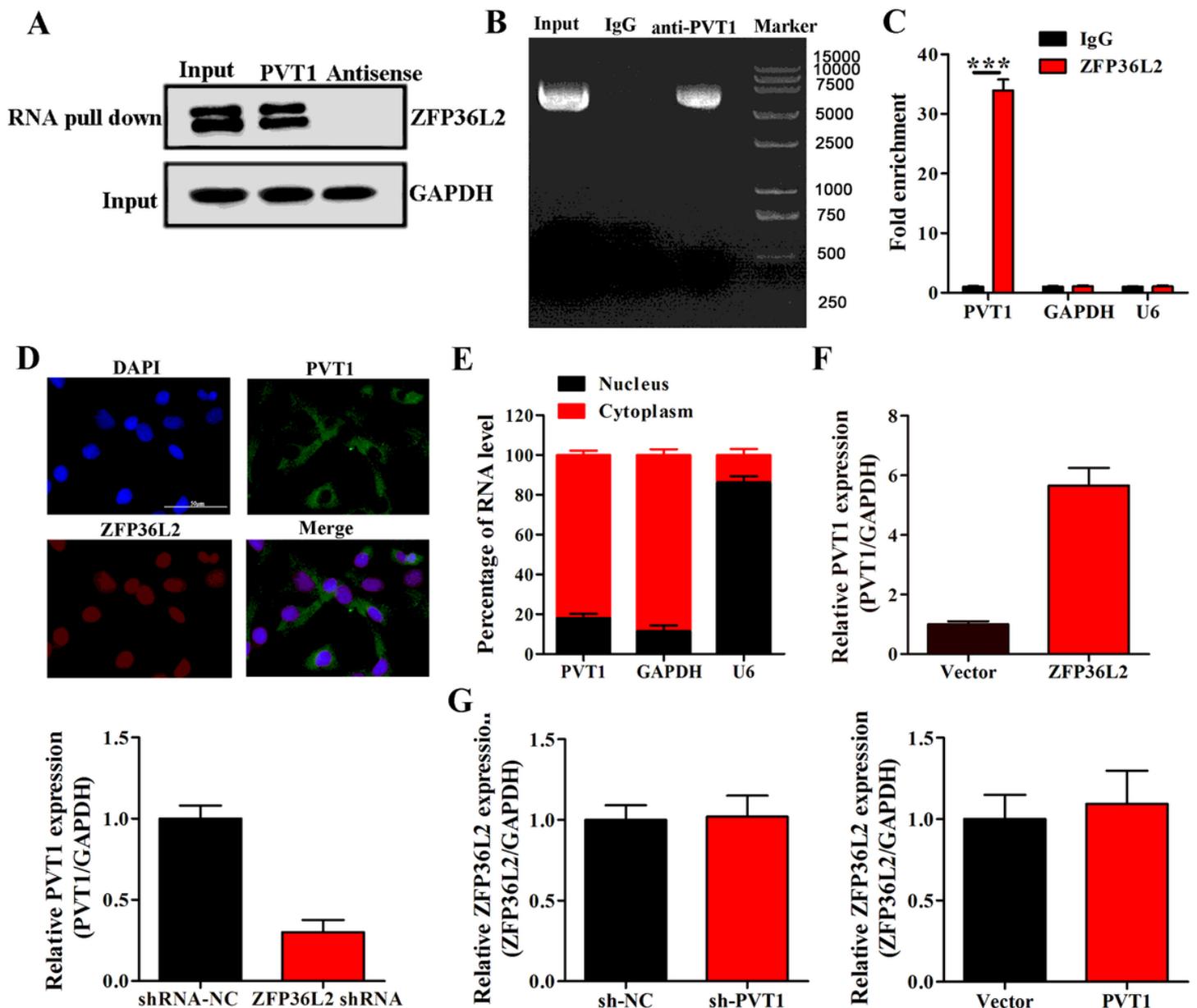


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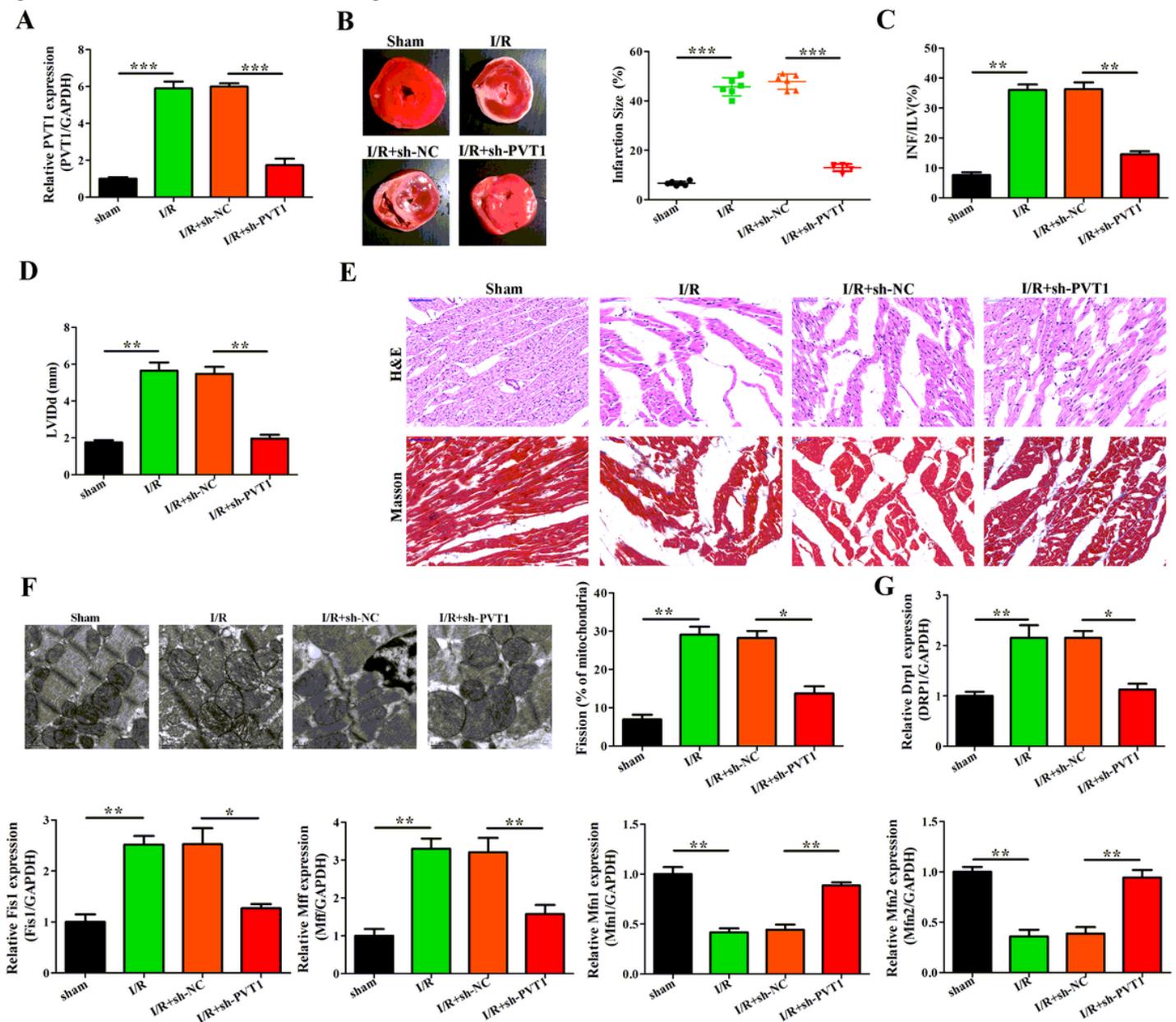


Figure 4

LncRNA PVT1 promoted myocardial ischemia/reperfusion (I/R) injury and promoted mitochondrial fusion and fission in vivo. (A) The sh-PVT1 and sh-NC were intraperitoneally administered 24 h before myocardial ischemia/reperfusion (I/R) injury. Post-I/R injury, RNA was isolated from heart tissues and expression of PVT1 was determined by qRT-PCR. Relative fold change of PVT1 expression with respect to glyceraldehyde 3-phosphate dehydrogenase was shown. (B-D) Quantification of I/R injury. Representative images of heart tissues in the left panel for different groups were shown. Other panels indicate infarction size (%), INF/LV (%), and LVIDd (mm). (E) Representative images of hematoxylin and eosin and Masson's trichrome staining of left ventricle [41] sections (Scale bar=50 μ m). (F) Representative transmission electron microscopy images of heart tissues are shown in the left panel. Quantification of fragmented mitochondria is shown in the right panel (Scale bar=0.5 μ m). (G) Expression of mitochondrial fusion-related genes Drp1, Fis1, Mff, Mfn1, and Mfn2 after myocardial I/R injury as evaluated by qRT-PCR. Data are represented as the mean \pm SD (n = 3, technical replicates). Statistical significance was calculated by one-way analysis of variance with multiple comparisons. *P < 0.05; **P < 0.01 and ***P < 0.001.

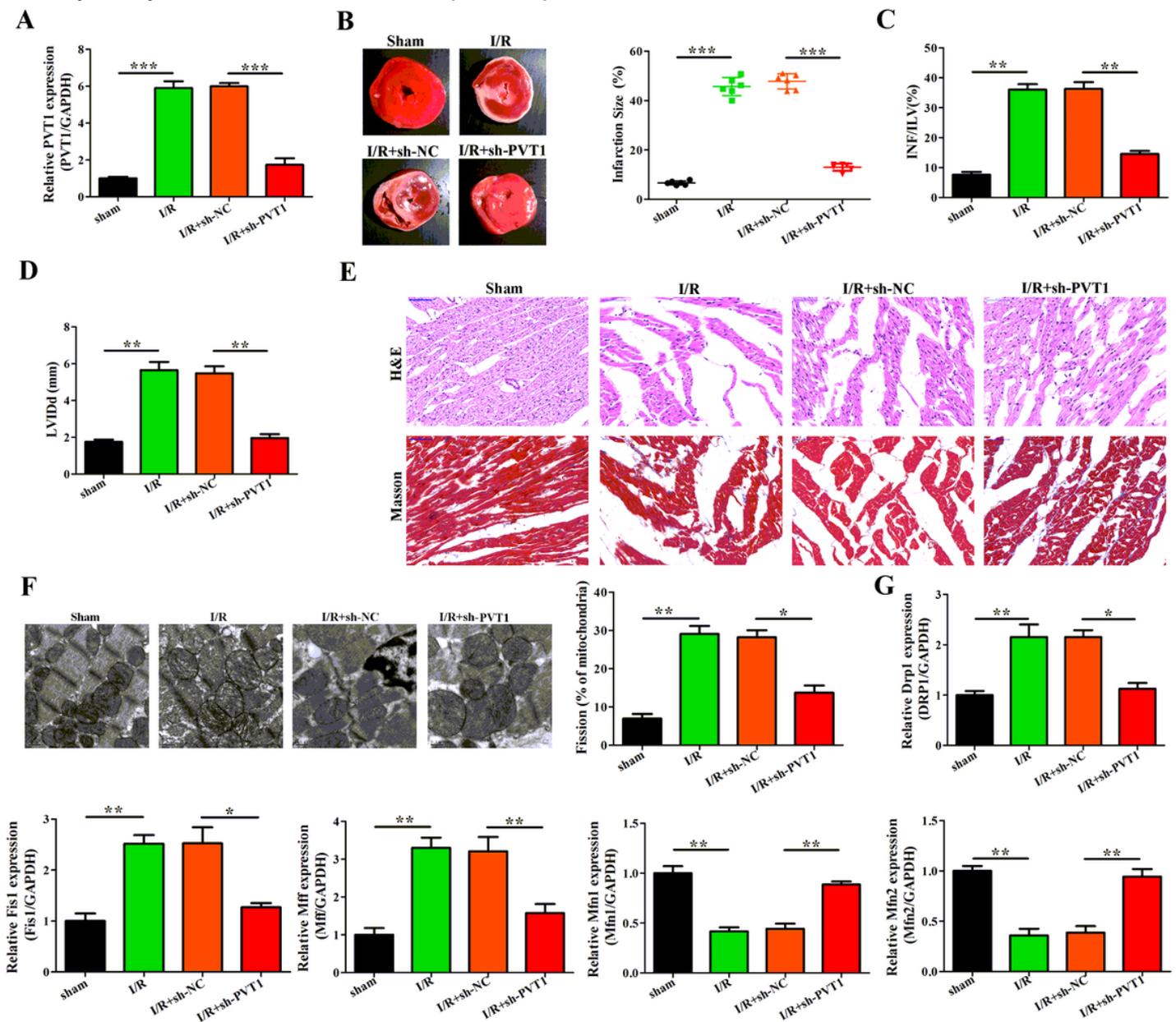


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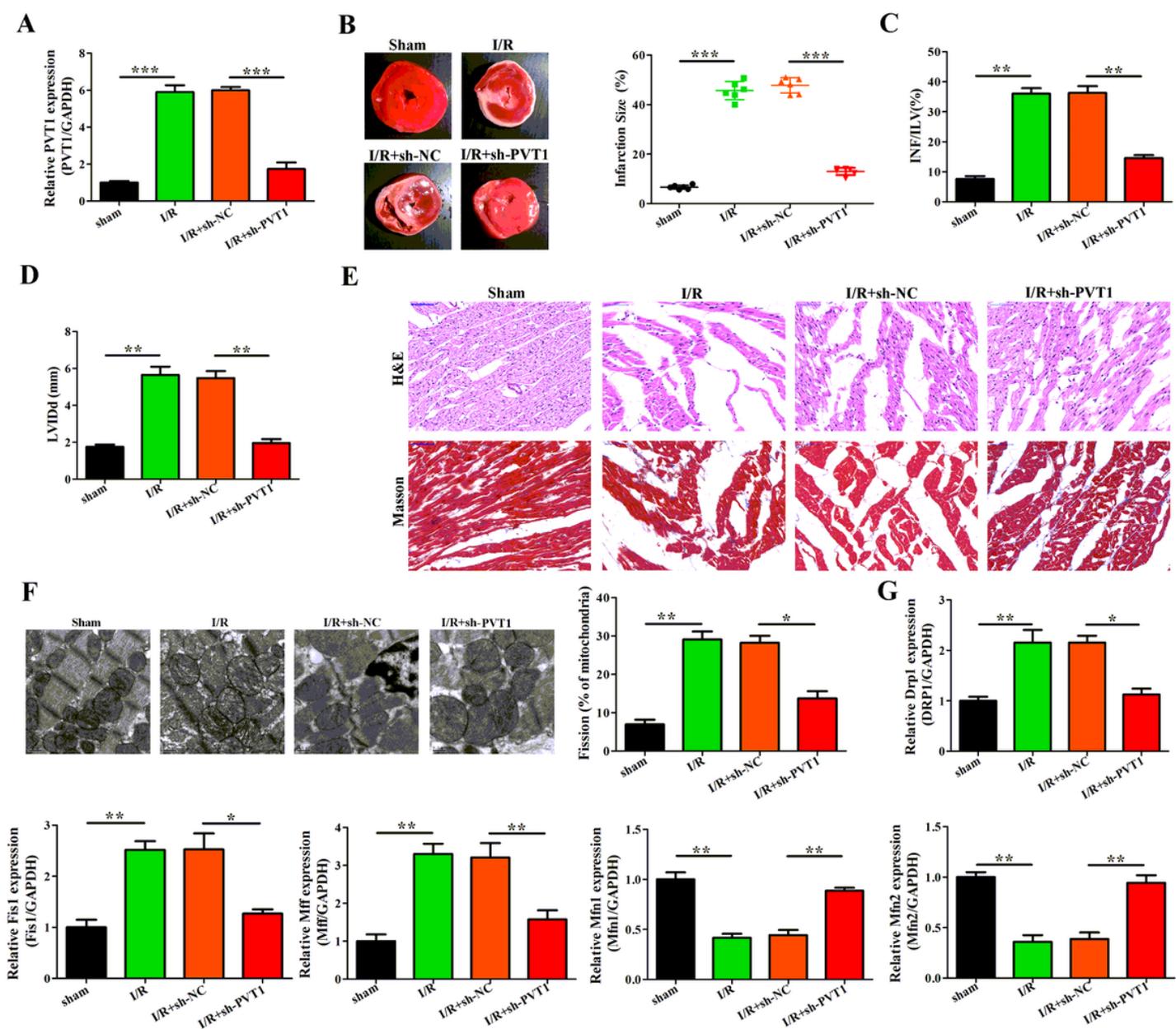


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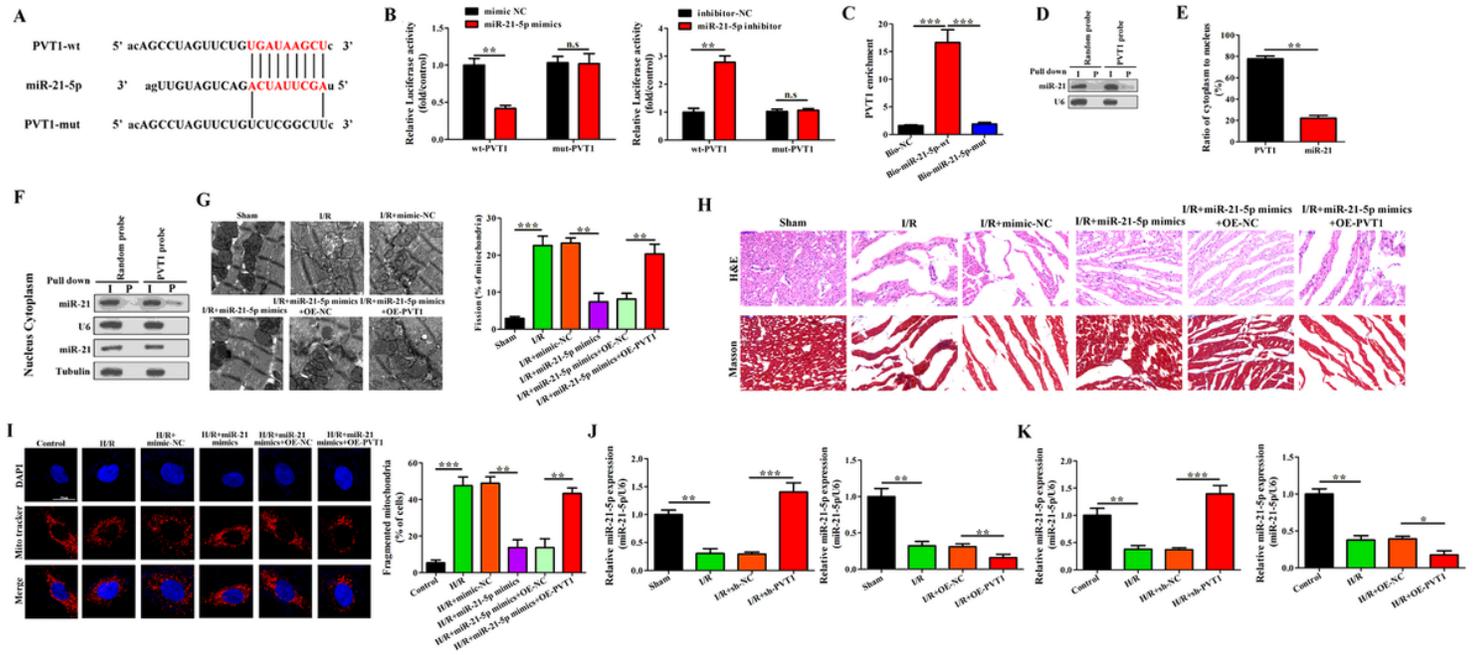


Figure 5

PVT1 directly binds to miR-21-5p and suppresses its activity. (A) Predicted binding sites of PVT1 and miR-21-5p using Starbase 3.0. (B) The luciferase reporter assay was performed in cardiomyocytes co-transfected with mimic-NC, miR-21-5p mimics, or inhibitor-NC, miR-21-5p inhibitors, and PVT1-wt or PVT1-mut. (C) Cardiomyocytes were transfected with biotinylated WT miR-21-5p (Bio-miR-21-5p-wt) or biotinylated mutant miR-21-5p (Bio-miR-21-5p-mut). A biotinylated miRNA that was not complementary to PVT1 was used as the normal control (Bio-NC). At 48 h after transfection, the cells were harvested for a biotin-based pull-down and PVT1 expression levels were analyzed by qRT-PCR. (D) Representative northern blot evaluating expressions of miR-21-5p in random or in CARL DNA probe pull-down fractions. (E, F) Detection of nuclear or cytoplasmic fractions of PVT1 and miR-21-5p in cardiomyocytes was determined by northern blotting. Tubulin and proliferating cell nuclear antigen (PCNA) were used as controls for cytoplasmic and nuclear fractions, respectively. (G) Representative transmission electron microscopy images of heart tissues under different conditions of miR-21-5p mimic treatment and PVT1 overexpression (OE-PVT1). Quantification of fragmented mitochondria is shown in the right panel (Scale bar=0.5 μ m). (H) Representative images of hematoxylin and eosin and Masson's trichrome staining of LV sections (Scale bar=50 μ m). (I) Representative micrograph of cardiomyocytes transfected with mimic-NC/miR-21-5p mimics, followed by PVT1 overexpression and induction of H/R stained with MitoTracker Red and 4', 6-diamidino-2-phenylindole. Quantification of cells with fragmented mitochondria was shown. At least 100 cells were counted per condition (Scale bar=20 μ m). (J) Relative miR-21-5p expression evaluated by qRT-PCR in cardiomyocytes harvested from mice treated with sh-PVT1/sh-NC or OE-PVT1/OE-NC. (K) Relative miR-21-5p expression evaluated by qRT-PCR in cardiomyocytes co-transfected with sh-PVT1/sh-NC and OE-PVT1/OE-NC followed by induction of hypoxia/reoxygenation. Data are

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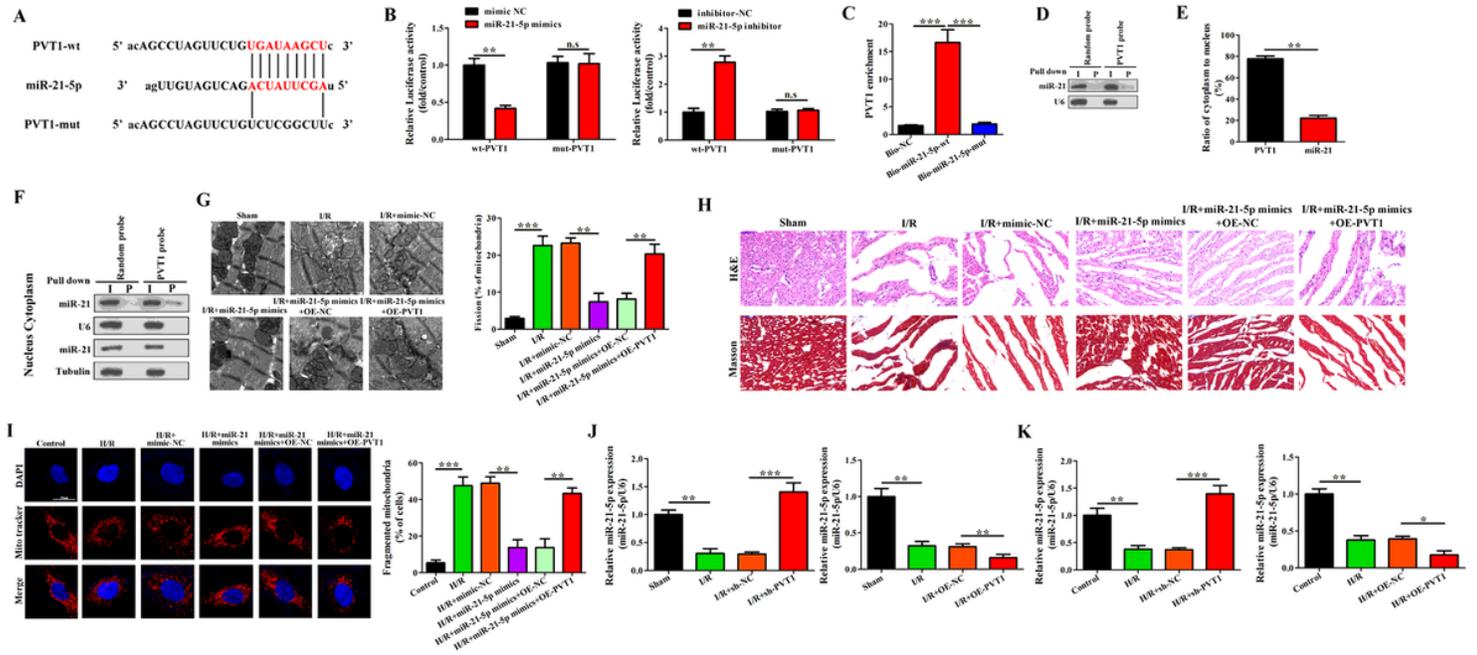


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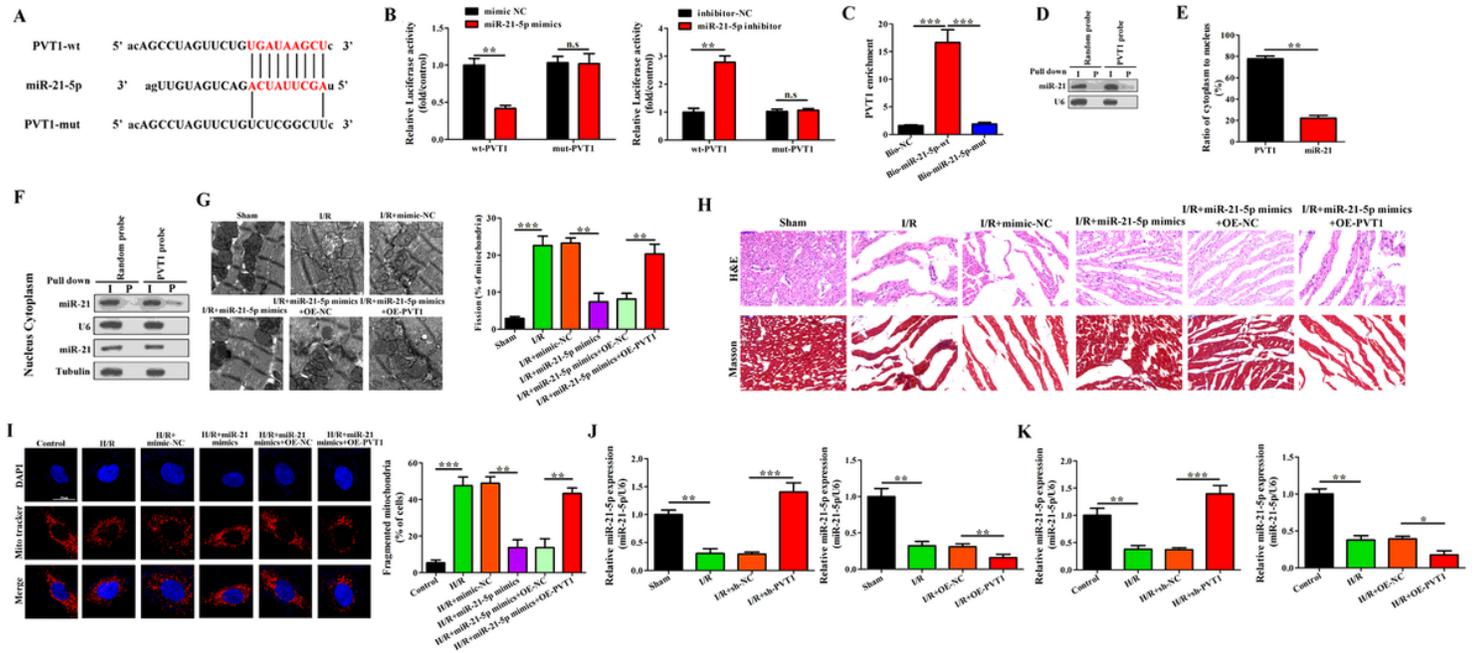


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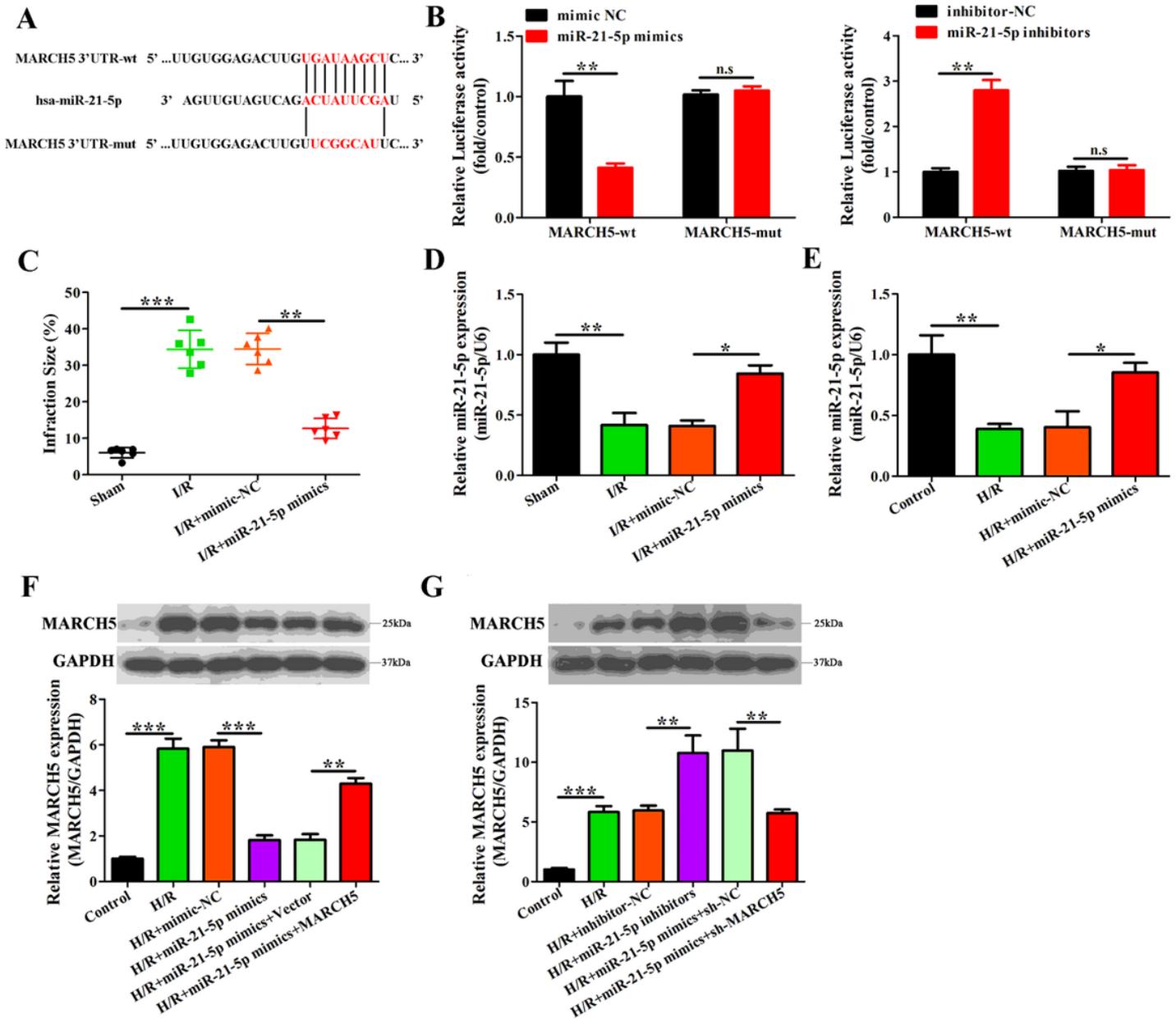


Figure 6

The miR-21-5p interacted with MARCH5 and regulated its expression. (A) Binding sites of miR-21-5p and MARCH5 using Starbase 3.0. (B) Relative luciferase activity from reporter assays in cardiomyocytes co-transfected with mimic-NC/miR-21-5p mimics or inhibitor-NC/miR-21-5p inhibitors along with MARCH5-wt or MARCH5-mut. (C,D) The mimic-NC and miR-21-5p mimics were intraperitoneally administered 24 h before myocardial hypoxia/reoxygenation (H/R) injury and infarction size (%) and levels of miR-21-5p expression were evaluated. (E) Cardiomyocytes were transfected with mimic-NC and miR-21-5p mimics, followed by induction of H/R. Expression of miR-21-5p was evaluated by qRT-PCR. (F) Cardiomyocytes were co-transfected with mimic-NC/miR-21-5p mimics and Vector/MARCH5 or inhibitor-NC/miR-21-5p

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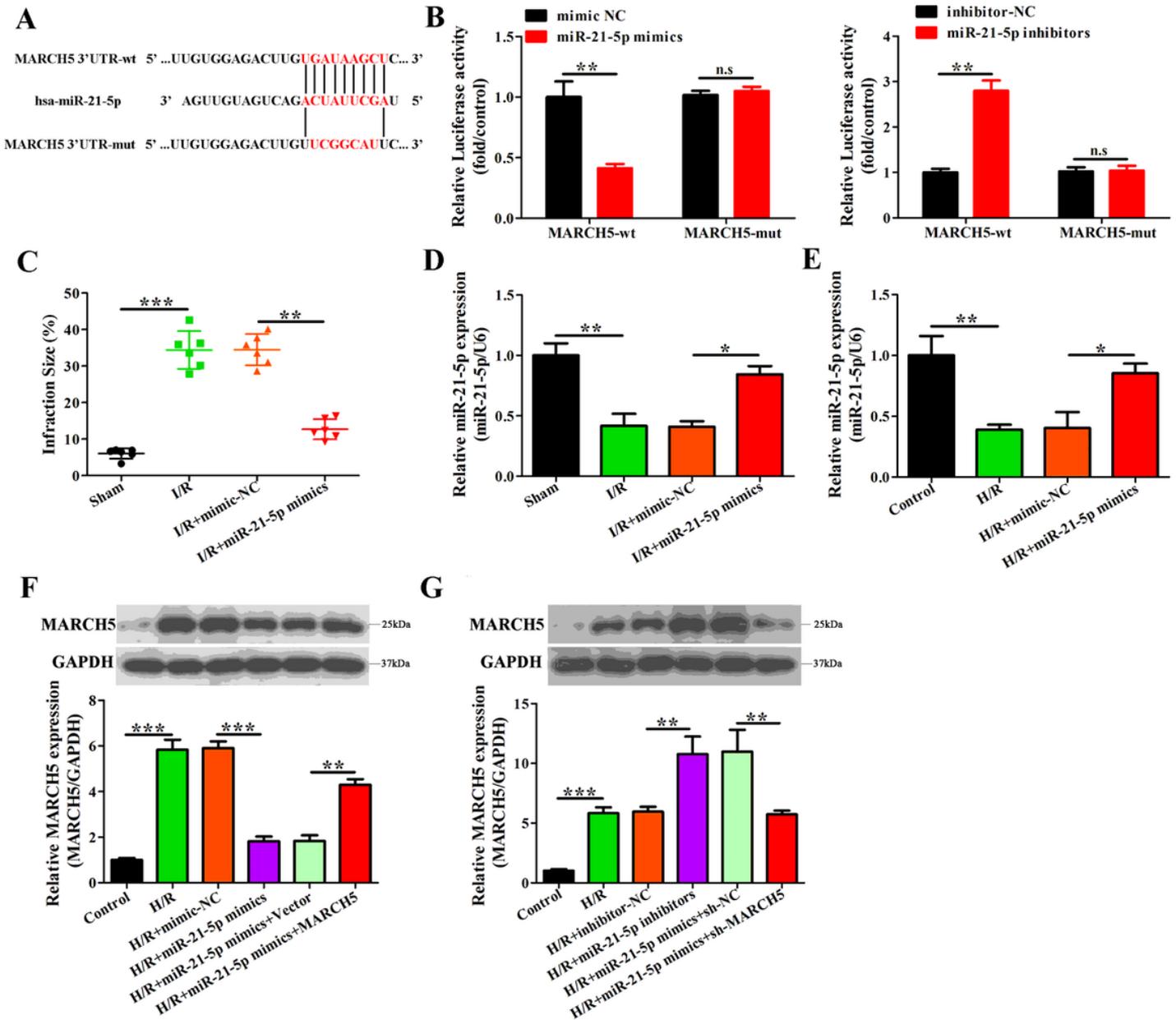


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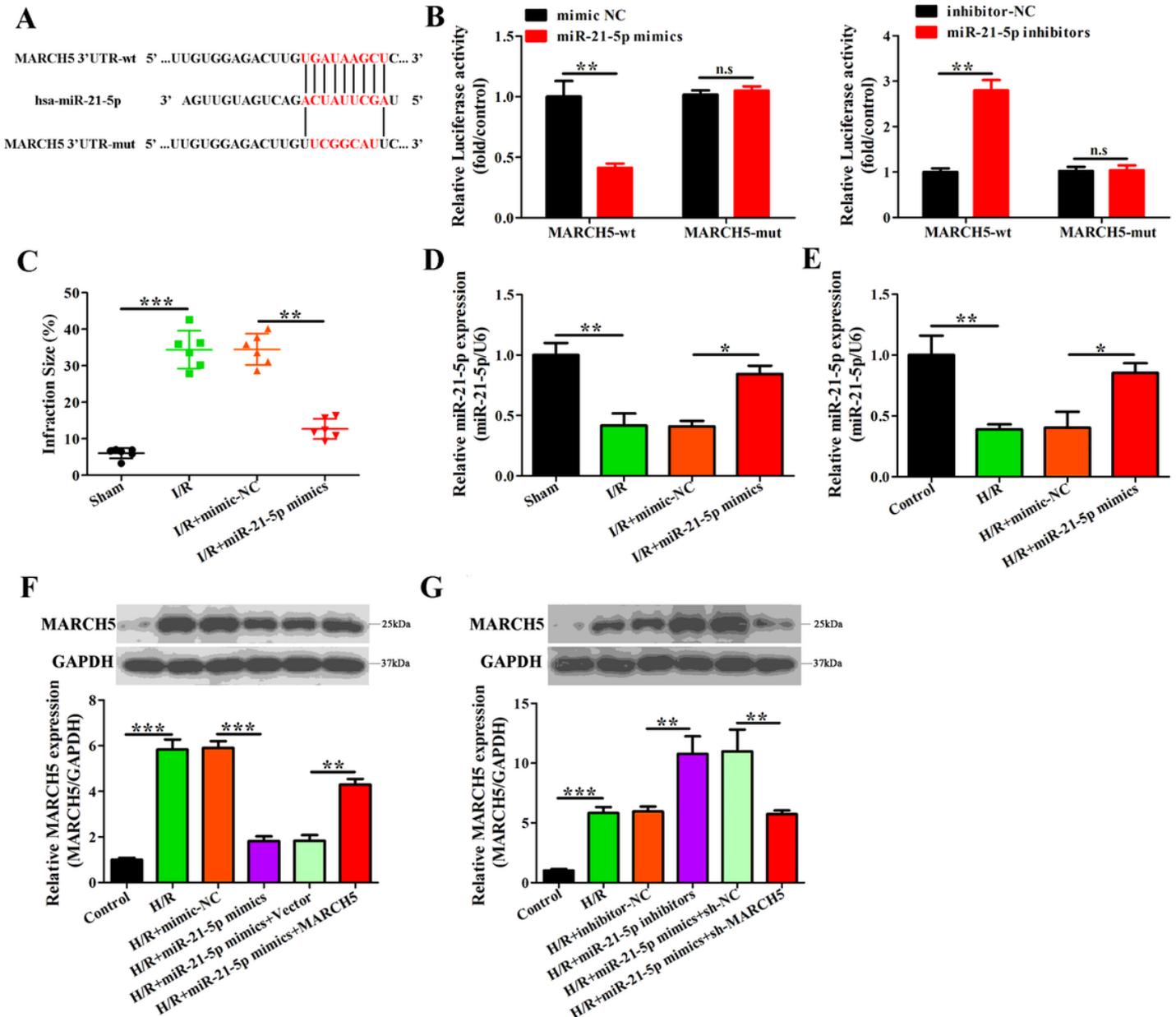


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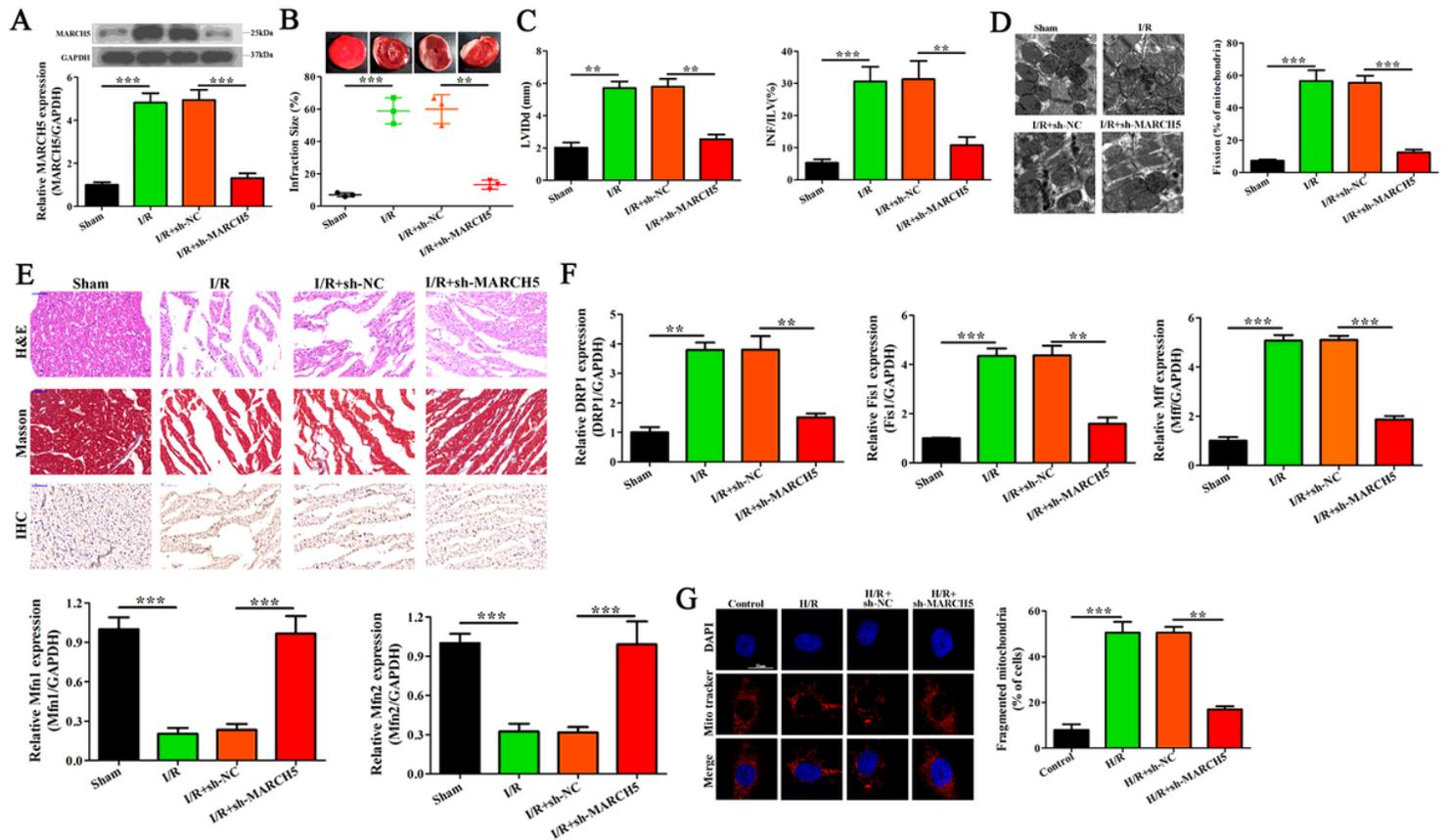


Figure 7

MARCH5 promoted myocardial ischemia/reperfusion (I/R) injury and regulated mitochondrial fusion and fission in vivo. (A) The sh-MARCH5 or sh-NC was intraperitoneally administered 24 h before myocardial I/R injury. Post I/R injury, RNA was isolated and the expression of MARCH5 was determined by qRT-PCR and western blotting. (B-C) The left panel shows representative images of heart tissues. The right panels show the infarction size (%), infarct area/left ventricle (INF/LV), and LVIDd. (D) Representative transmission electron microscopy images of heart tissues and quantification of fragmented mitochondria (%) (Scale bar=0.5 μ m). (E) Representative images of hematoxylin and eosin and Masson's trichrome staining or immunodetection of MARCH5 expression in LV sections (Scale bar=50 μ m). (F) Expression of mitochondrial fusion-related genes Drp1, Fis1, Mff, Mfn1, and Mfn2 after myocardial I/R injury as evaluated by qRT-PCR. (G) Representative micrographs of cardiomyocytes transfected with sh-MARCH5 or sh-NC followed by induction of H/R and stained with MitoTracker Red and DAPI (Scale

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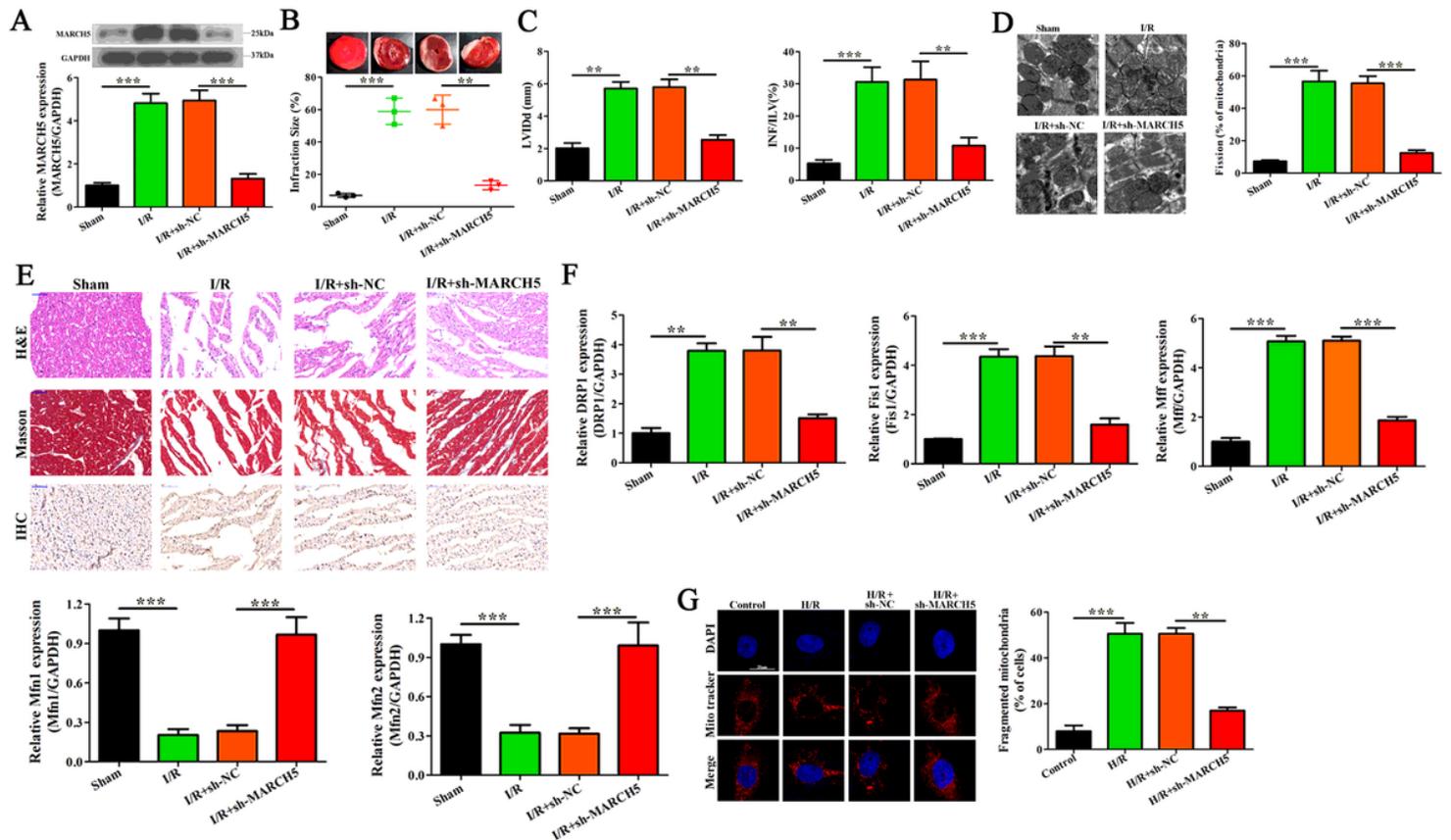


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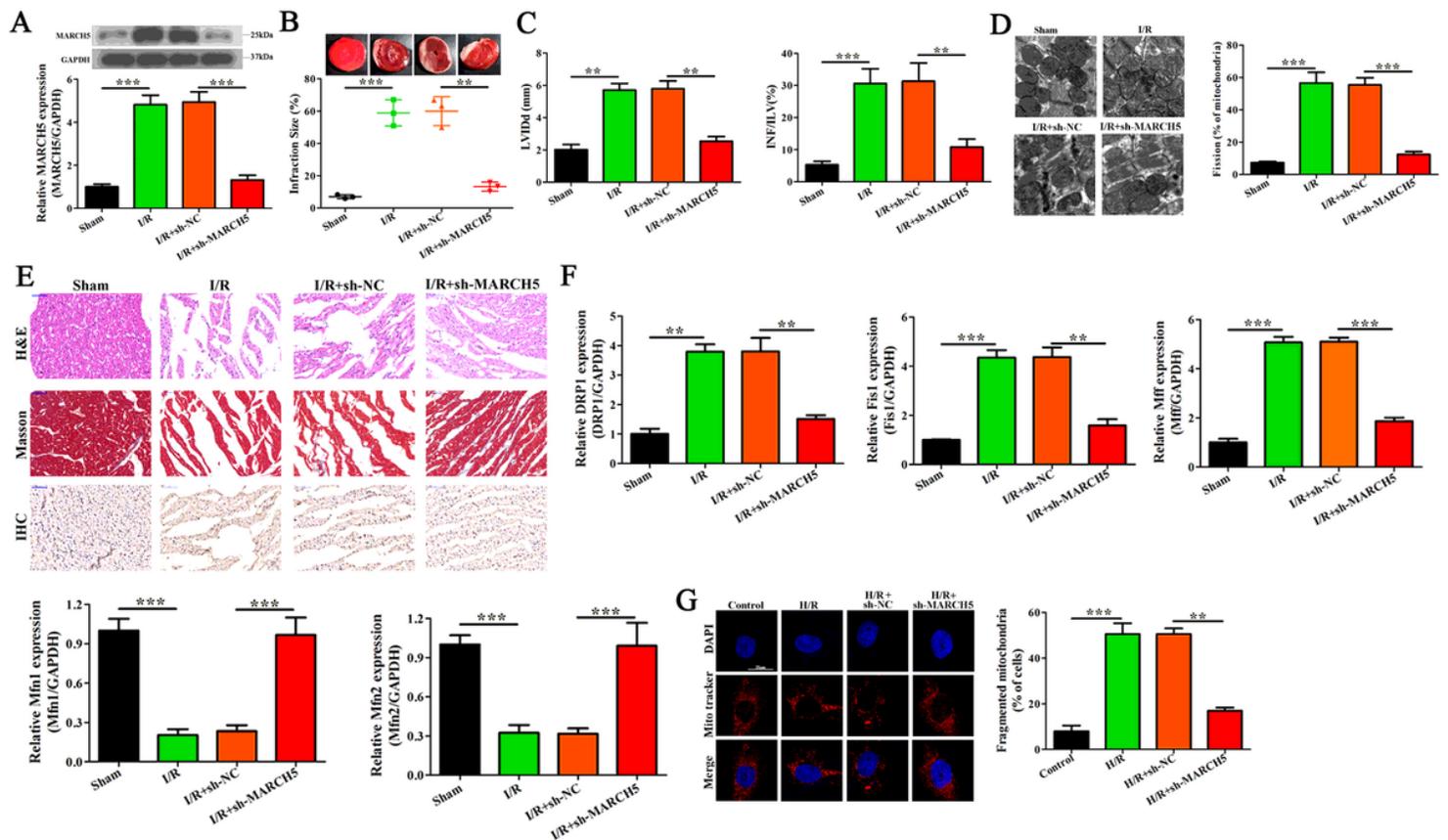


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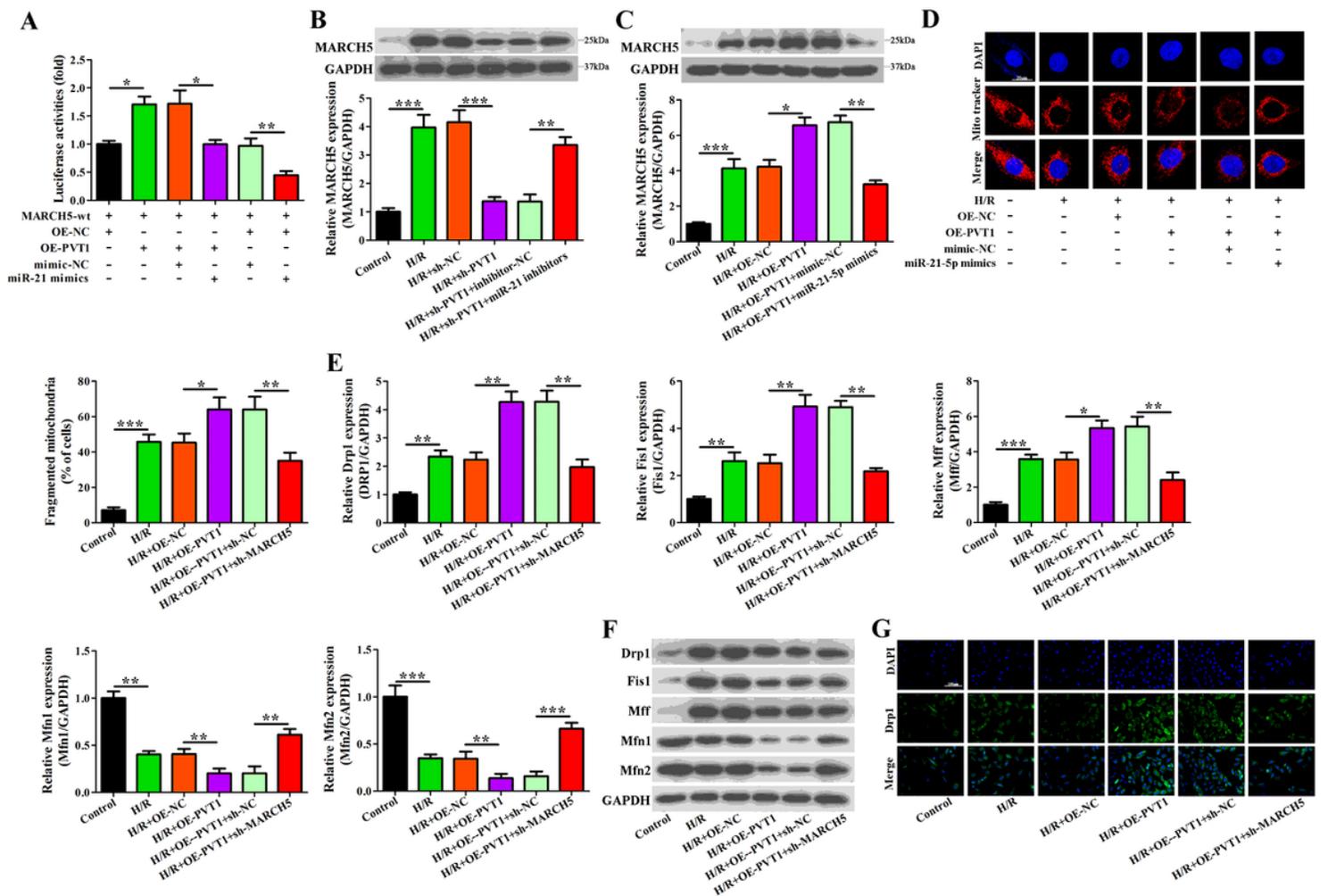


Figure 8

PVT1 regulated mitochondrial fusion and fission through the miR-21-5p/MARCH5 axis. (A) Relative luciferase activities analyzed in 293T cells co-transfected with OE-PVT1 or OE-NC, miR-21-5p mimics, or mimic-NC along with luciferase reporter vector MARCH5 3'-UTR-wt. (B, C) Cardiomyocytes were co-transfected with inhibitor-NC/miR-21-5p inhibitors and sh-NC/sh-MARCH5 or OE-NC/OE-PVT1 and mimic-NC/miR-21-5p mimics followed by exposure to hypoxia/reoxygenation (H/R). MARCH5 expression was evaluated by qRT-PCR and western blotting. (D) Mitochondrial fission induced by hypoxia/reoxygenation was determined in cardiomyocytes co-transfected with OE-NC/OE-PVT1 and mimic-NC/miR-21-5p mimics. The % cells with fragmented mitochondria were counted (Scale bar=20 μ m). At least 100 cells were counted per condition (E-F) Expression of mitochondrial fusion-related genes Drp1, Fis1, Mff, Mfn1, and Mfn2 after hypoxia/ reoxygenation (H/R) injury as evaluated by qRT-PCR and western blotting. (G) Representative confocal micrographs of immunofluorescence staining for Drp1 in cardiomyocytes co-transfected with OE-NC/OE-PVT1 and sh-NC/sh-MARCH5 during H/R injury. The nucleus was stained with DAPI (Scale bar=100 μ m). Data are represented as the mean \pm SD (n = 3, technical replicates). Statistical significance was calculated by one-way analysis of variance with multiple comparisons. **P < 0.01 and ***P < 0.001.

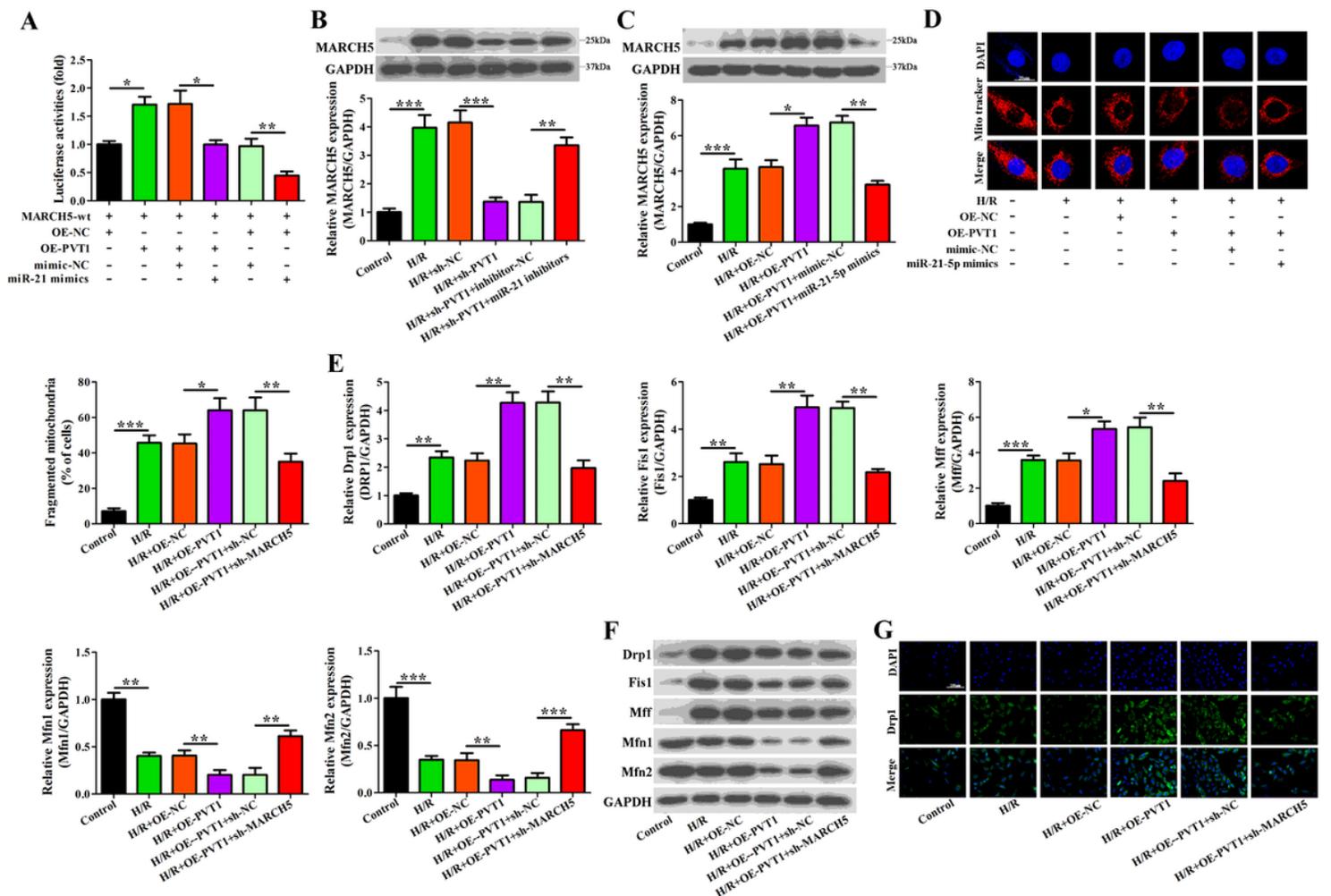


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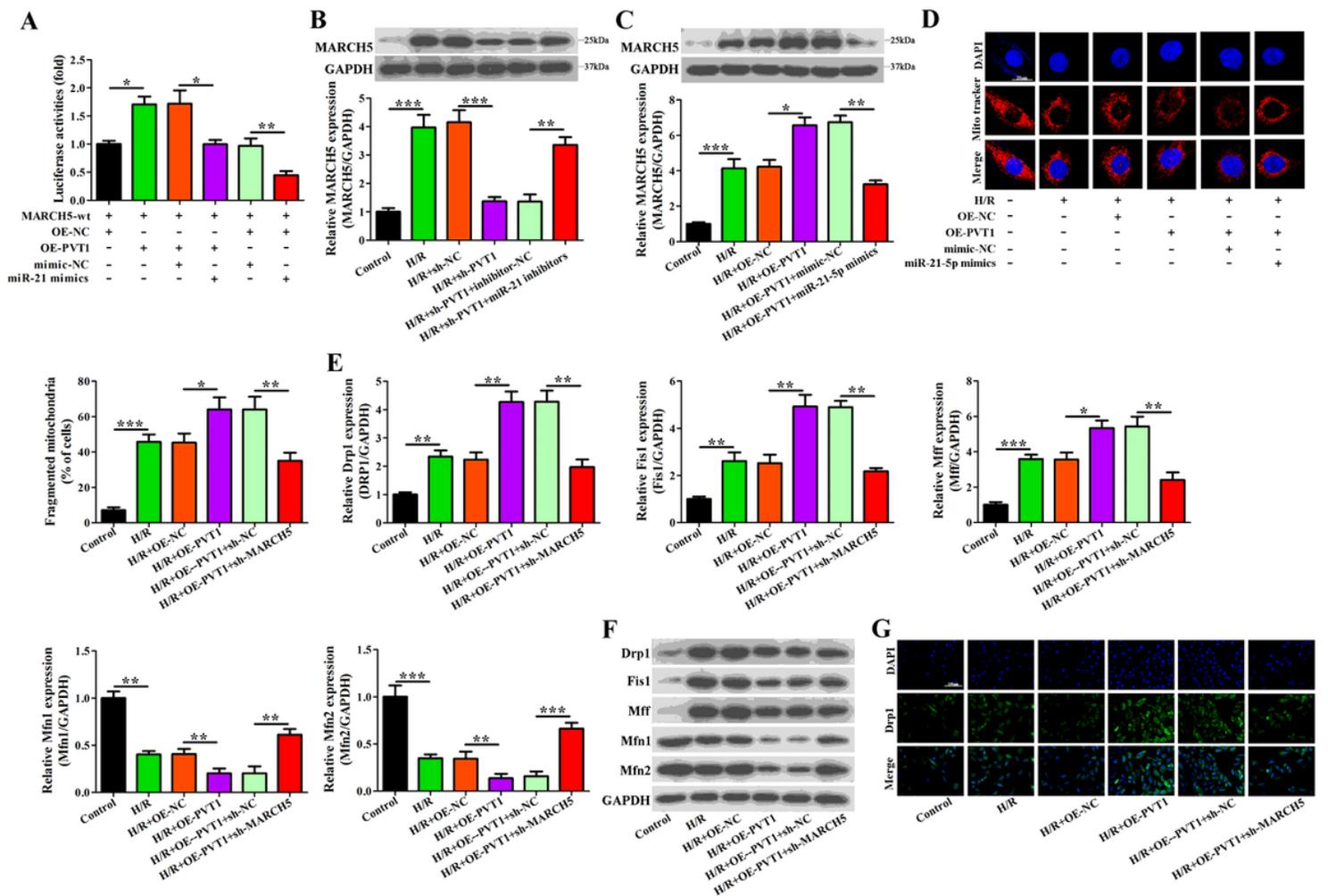


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