Augmenter of Liver Regeneration Alleviates Renal Hypoxia–Reoxygenation Injury by Regulating Mitochondrial Dynamics in Renal Tubular Epithelial Cells

Rui-ting Long[1], Jun-bo Peng[1], Li-li Huang[1], Gui-ping Jiang[1], Yue-juan Liao[1], Hang Sun[2], Yu-dong Hu[1], and Xiao-hui Liao[1],*

[1]Department of Nephrology, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China, [2]Key Laboratory of Molecular Biology for Infectious Diseases (Ministry of Education), Institute for Viral Hepatitis, Department of Infectious Diseases, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China
*Correspondence: lxh@hospital.cqmu.edu.cn
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Mitochondria are highly dynamic organelles that constantly undergo fission and fusion processes that closely related to their function. Disruption of mitochondrial dynamics has been demonstrated in acute kidney injury (AKI), which could eventually result in cell injury and death. Previously, we reported that augmenter of liver regeneration (ALR) alleviates renal tubular epithelial cell injury. Here, we gained further insights into whether the renoprotective roles of ALR are associated with mitochondrial dynamics. Changes in mitochondrial dynamics were examined in experimental models of renal ischemia-reperfusion (IR). In a model of hypoxia-reoxygenation (HR) injury *in vitro*, dynamin-related protein 1 (Drp1) and mitochondrial fission process protein 1 (MTFP1), two key proteins of mitochondrial fission, were downregulated in the Lv-ALR + HR group. ALR overexpression additionally had an impact on phosphorylation of Drp1 Ser637 during AKI. The inner membrane fusion protein, Optic Atrophy 1 (OPA1), was significantly increased whereas levels of outer membrane fusion proteins Mitofusin-1 and -2 (Mfn1, Mfn2) were not affected in the Lv-ALR + HR group, compared with the control group. Furthermore, the mTOR/4E-BP1 signaling pathway was highly activated in the Lv-ALR + HR group. ALR overexpression led to suppression of HR-induced apoptosis. Our collective findings indicate that ALR gene transfection alleviates mitochondrial injury, possibly through inhibiting fission and promoting fusion of the mitochondrial inner membrane, both of which contribute to reduction of HK-2 cell apoptosis. Additionally, fission processes are potentially mediated by promoting tubular cell survival through activating the mTOR/4E-BP1 signaling pathway.

Keywords: acute kidney injury, augmenter of liver regeneration, hypoxia-reoxygenation, ischemia-reperfusion, mitochondrial dynamics

INTRODUCTION

Acute kidney injury (AKI), a common clinical critical illness with high morbidity and mortality rates, imposes a heavy health burden to society (Kaddourah et al., 2017; Mehta et al., 2015). Severe AKI can lead to end-stage renal disease, considered one of the primary risk factors for chronic kidney

disease (Leung et al., 2013). Ischemia-reperfusion (IR) injury is one of the most common causes of AKI. The kidney requires an abundance of mitochondria to provide sufficient energy for multiple physiological functions, especially in the proximal tubule. Compelling evidence has established mitochondrial damage as the core pathogenic mechanism of renal tubular cell injury and death in AKI (Szeto, 2017). Mitochondria are a class of highly dynamic organelles that undergo constant fission and fusion, and these complementary processes are fine-tuned and balanced by a series of key proteins of mitochondrial dynamics to regulate morphology (Dietz et al., 2019). Accumulating studies in recent years have shown that mitochondrial dynamics is closely related to mitochondrial function and homeostasis (Bhargava and Schnellmann, 2017). Fusion contributes to the maintenance of oxidative phosphorylation and is associated with redistribution of metabolites, proteins and mtDNA among mitochondria (Ishihara et al., 2013). Fission eliminates damaged mitochondria that are subsequently cleared by mitophagy to preserve the health of the mitochondrial network (Ishimoto and Inagi, 2016). However, under cellular stress or injury conditions, this dynamic fission-fusion balance is disrupted. Alterations in mitochondrial dynamics have been demonstrated in the experimental models of AKI, which are characterized by excessive division and reduced fusion, resulting in mitochondrial fragmentation (Bhargava and Schnellmann, 2017; Zhan et al., 2013). Eventually, fragmentation of mitochondria triggers increased mitochondrial membrane permeability, release of proapoptotic factors and consequent cell injury and death (Youle and Karbowski, 2005; Zhan et al., 2013). In view of these findings, it is proposed that maintenance of mitochondrial dynamics may have protective effects against AKI. Elucidation of the signaling pathways related to mitochondrial dynamics should aid in clarifying the pathogenesis of AKI and developing effective prevention and therapeutic strategies.

Augmenter of liver regeneration (ALR) is a widely distributed growth factor frequently expressed in human liver and kidney. ALR exists as two splice isoforms 15 and 23 kDa in size. The 23 kDa species is predominantly localized in the intermembrane space of mitochondria where it mediates import of mitochondrial proteins (Mordas and Tokatlidis, 2015). ALR has additionally been identified in different subcellular structures, including cytosol, endoplasmic reticulum, and nucleus (Gandhi, 2012). Recently, we showed that knockdown of ALR induces apoptosis and mitochondrial damage in HK-2 cells in a model of HR injury by interfering with mitochondrial biogenesis (Huang et al., 2018). In AKI, mitochondrial dynamics is disrupted and mitochondrial fragmentation occurs, leading to mitochondrial damage and cell death (Ishimoto and Inagi, 2016). In the current study, alterations in mitochondrial dynamics in a renal IR injury mouse model were evaluated along with HK-2 cells were transfected with ALR-containing lentiviral particles for overexpression of ALR and experiments conducted to examine the effects and underlying mechanisms of ALR on mitochondrial dynamics in HK-2 cells *in vitro* for the first time. Our collective data suggest that ALR exerts a renoprotective effect and promotes renal tubular epithelial cell survival by regulating mitochondrial dynamics.

MATERIALS AND METHODS

Mice and renal IR model
All experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and all procedures and protocols were approved by Chongqing Medical University Animal Care and Use Committee (approval No. 2018,146). Male 8- to 10-week-old C57BL/6 mice (Experimental Animal Center of Chongqing Medical University, China) were used in the present study. Mice were raised in standard vivarium with a 12-h light/dark cycle, and a standard diet and water were freely available.

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and placed on a thermoregulated heating pad to maintain body temperature at 34°C to 36°C. Following bilateral flank incisions, right nephrectomy was performed. Then left renal pedicle was exposed and cross-clamped for 26 min using a nontraumatic microvessel clamp. Clamp was then removed, and restoration of blood flow was visually confirmed. Sham-operated mice underwent the same surgical procedures without renal pedicle clamping. Twenty-four hours after reperfusion, mice were euthanized, and blood samples and kidneys were harvested for further analyses.

Assessment of kidney function and histology
The blood samples were centrifuged (3,000 rpm for 15 min) to separate serum. The serum samples were analyzed for blood urea nitrogen (BUN) and serum creatinine (Scr) levels using a Hitachi 747 Automatic Analyser (Hitachi, Japan). For histology, kidneys were fixed overnight in 4% paraformaldehyde and embedded in paraffin, subsequently sectioned at 4-μm thickness. Sections were stained with periodic acid-Schiff (PAS).

Electron microscopy
For each kidney, a tissue block of approximately 1 mm^3 was collected and rapidly fixed in fixative (Wuhan Servicebio Technology, China) at 4°C for 2-4 h, and then rinsed three times using 0.1 M phosphate buffer (pH 7.4), followed by incubating in 1% osmium (in 0.1 M phosphate buffer [pH 7.4]) for 2 h, washing and dehydrating. Then the samples were embedded in EmBed812 embedding resin and ultrathin sections (60-80 nm) were obtained and stained with uranyl acetate and Reynolds lead citrate. The tissue block was examined at magnification (×8,000). The lengths of individual mitochondria in a cell were measured by tracing using NIH ImageJ software (http://rsbweb.nih.gov/ij/), and length > 2 μm as elongated mitochondria.

Immunohistochemical analyses
Paraffin-embedded kidney sections (4 μm) were deparaffinized in xylenes, rehydrated in a graded series of ethanol (100%-75%), and washed in distilled water. Then, antigen retrieval was performed and sections were incubated in 3% H$_2$O$_2$ for 25 min. After blocking with 3% bovine serum albumin for 30 min at room temperature, sections were incubated in specific primary antibodies overnight at 4°C. Sections

were washed with phosphate buffer (pH 7.4) three times and incubated with anti-goat secondary antibody for 50 min. After another three washes with phosphate buffer, the sections were visualized by incubating with diaminobenzidine, counterstained by hematoxylin, and dehydrated.

Immunohistochemistry was performed with antibodies for DRP1 (OTI4F6; Abcam, UK), OPA1 (EPR11057; Abcam), Mitofusin1 (3C9; Abcam), and Mitofusin2 (D2D10; Cell Signaling Technology, USA). And Image-Pro Plus 6.0 (Media Cybernetics, USA) was used to analyze image of Immunohistochemistry.

Cell culture

Human kidney proximal tubular (HK-2) cells (ATCC, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen, USA) at a general chamber under an atmosphere of 95% air and 5% CO_2 at 37°C.

Cell transfection

ALR-containing lentiviral particles (Lv-ALR GV287; GFER(4589-41); Shanghai GeneChem, China) and vector-only lentiviral particles (Lv-vector; CON145 [Ubi-MCS-3FLAG-SV40-EGFP]) were transfected into HK-2 cells for 8-16 h following the manufacturer's instructions. In order to obtain the target gene fragment, the following primer sequences were used: GFER(4589-41)-P1: 5′-ATCGGGATCCCGCCACCAT-GGCGGCGCCCGGCGA-3′; GFER(4589-41)-P2: 5′-CGGG-TACCGGTGTCACAGGAGCCATCCTTC-3′. At 72 h after transduction, the successfully transduced cells were selected under a fluorescence microscope to observe GFP fluorescence, and cells with an infection efficiency of about 80% were included in the subsequent experiments.

HR treatment *in vitro*

For *in vitro* HR injury, confluent cells were serum-deprived overnight to synchronize cell growth under 95% air and 5% CO_2 at 37°C. On the next day, HK-2 cells were washed with sterile phosphate-buffered saline (PBS) twice and then maintained in Hank's balanced salt solution (Gibco) in a hypoxia chamber (Thermo Fisher Scientific, USA) with 94% N_2, 5% CO_2 and 1% O_2 at 37°C for 6 h. Six hours later, HK-2 cells were removed to a general chamber and cultured in RPMI 1640 complete medium under an atmosphere of 95% air and 5% CO_2 at 37°C for indicated time point. Finally, cells were collected at the indicated time point for the subsequent testing purpose.

Western blot analysis

Proteins were separated by 6% to 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, USA). After blocking with 5% bovine serum albumin for 1 h at room temperature, the membranes were incubated with specific primary antibodies overnight at 4°C. After washing with Tris-buffered saline containing Tween 20 three times, the membranes incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactions were visualized by using ECL

chemiluminescence substrate kit (Shanghai Genechem). The resulting bands were quantified using the ChemiDoc Imaging System (Bio-Rad Laboratories, USA).

The following antibodies were used: DYKDDDDK Tag (D6W5B) Rabbit mAb (1:1,000; Cell Signaling Technology), mTOR (7C10) Rabbit mAb (1:1,000; Cell Signaling Technology), Phospho-mTOR (Ser2448) (D9C2) XP® Rabbit mAb (1:1,000; Cell Signaling Technology), 4E-BP1 (53H11) Rabbit mAb (1:1,000; Cell Signaling Technology), Phospho-4E-BP1 (Ser65) (174A9) Rabbit mAb (1:1,000; Cell Signaling Technology), Anti-MTP18 antibody (ab198217) (1:500; Abcam), Anti-DRP1 antibody (ab56788) (1:500; Abcam), Anti-DRP1 (phospho S637) antibody (ab193216) (1:500; Abcam), OPA1 (D6U6N) Rabbit mAb (1:1,000; Cell Signaling Technology), Mitofusin-1 (D6E2S) Rabbit mAb (1:1,000; Cell Signaling Technology), Mitofusin-2 (D2D10) Rabbit mAb (1:1,000; Cell Signaling Technology), β-Actin (13E5) Rabbit mAb (1:1,000; Cell Signaling Technology). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse were used as secondary antibodies (1:10,000; Cell Signaling Technology).

STRING v10

The potential target protein was predicted using STRING v10 (http://string-db.org/cgi/input.pl), a database dedicated to protein-protein interactions. It integrates information and gives the interaction score to judge the available evidence (von Mering et al., 2003). In the present study, the search term was set as 'Mtfp1' and organism as 'Homo sapiens'.

Apoptosis assay

Apoptotic cells were determined using Annexin V-APC detection Kit (BD Bioscience, USA). Briefly, cells were harvested using 0.2% trypsin free EDTA, then washed twice with cold PBS and resuspended cells in 1× binding buffer at a concentration of 1×10^6 cells/ml. Transfer 100 μl of the solution (1 × 10⁵ cells) to a 5 ml culture tube. Annexin V-APC (5 μl) and DAPI (5 μl) were added and incubated for 15 min at 25°C in the dark and suspended in 300 μl 1× binding buffer. Then all the samples were analyzed by flow cytometry within 1 h.

Statistical analysis

Data analysis was performed using Prism software (GraphPad Software, USA). Parametric data in multiple groups were performed with one-way ANOVA followed by Tukey's post hoc test to evaluate the significance of the differences among the multiple groups, or statistical analyses were performed using the Student's *t*-test for individual comparisons. All data presented as mean ± SD. It was considered statistically significant when $P < 0.05$.

RESULTS

Renal dysfunction, histologic alterations and morphological changes in mitochondria *in vivo* following renal IR injury

C57BL/6 mice exhibited elevated Scr and BUN levels after right nephrectomy and 26 min of contralateral renal pedicle clamping to induce renal ischemia followed by 24 h reperfusion, compared with the sham group ($P < 0.05$; Figs. 1A and

A

B

C

Sham | IR

D

Sham | IR
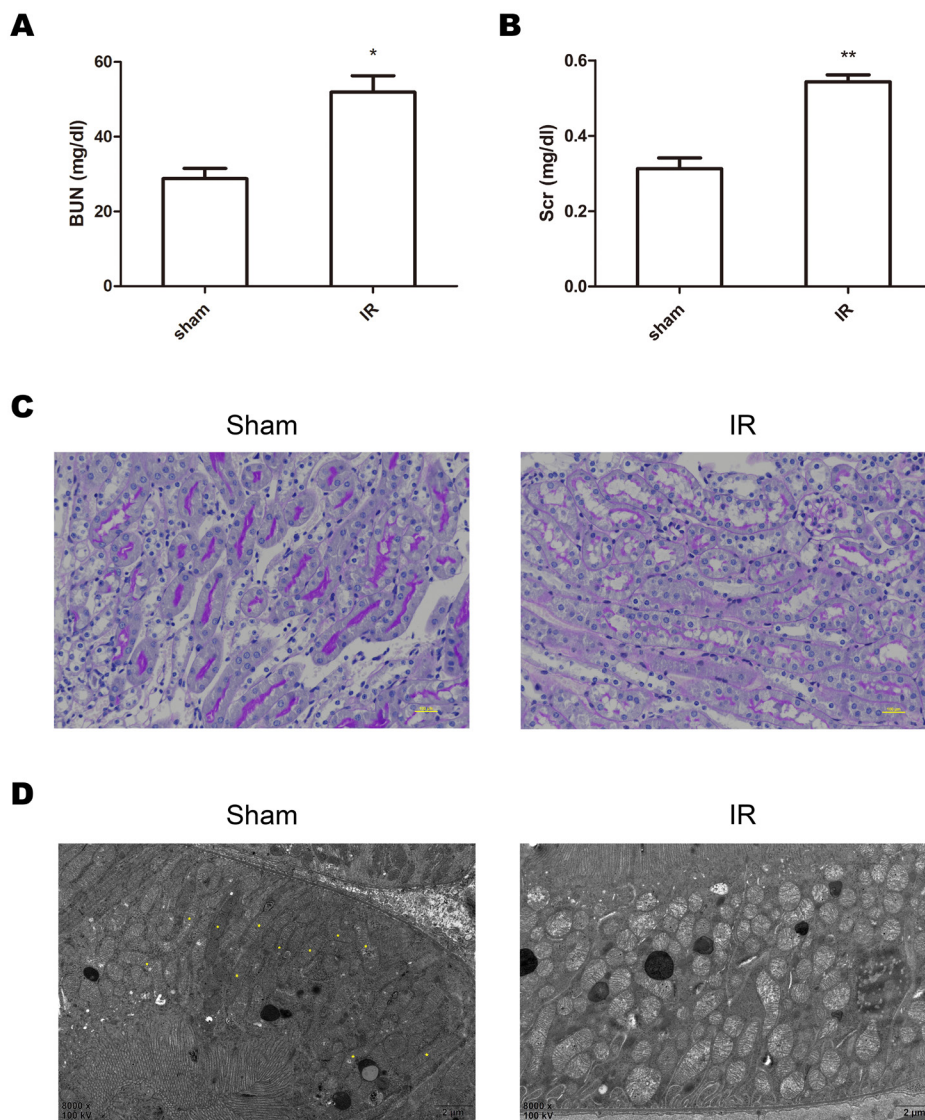
Fig. 1. Renal dysfunction, histologic alterations and morphological changes in mitochondria *in vivo* following renal IR injury. (A and B) Mice were subjected to IR or sham operation, and BUN and Scr levels measured 24 h after reperfusion (n = 3-4 per group). Parametric data were analyzed with the Student's *t*-test and presented as the mean ± SD (*$P < 0.05$, **$P < 0.01$, compared to the sham group). (C) Representative PAS-stained renal sections from sham and IR groups (×400 magnification, scale bars = 100 μm). (D) Electron microscopic analysis of mitochondrial morphology in sham and IR groups (scale bars = 2 μm). Asterisks indicate elongated (> 2 μm) mitochondria.

1B). Histological analysis of PAS-stained renal sections at 24 h was conducted (Fig. 1C). Renal IR treatment caused disordering of renal tubular epithelial cells, necrosis and shedding into the renal tubule lumen and luminal congestion with loss of brush borders, indicating successful establishment of a model of renal IR injury. Electron microscopy was performed to examine potential alterations in mitochondrial morphology *in vivo* after renal IR injury. Micrographs of proximal tubular cells from sham-operated mice displayed elongated (> 2 μm) mitochondria whereas higher numbers of small and short rod-shaped mitochondria appeared after IR treatment (Fig. 1D) characteristic of increased mitochondrial fission in renal tubular cells during IR injury *in vivo*.
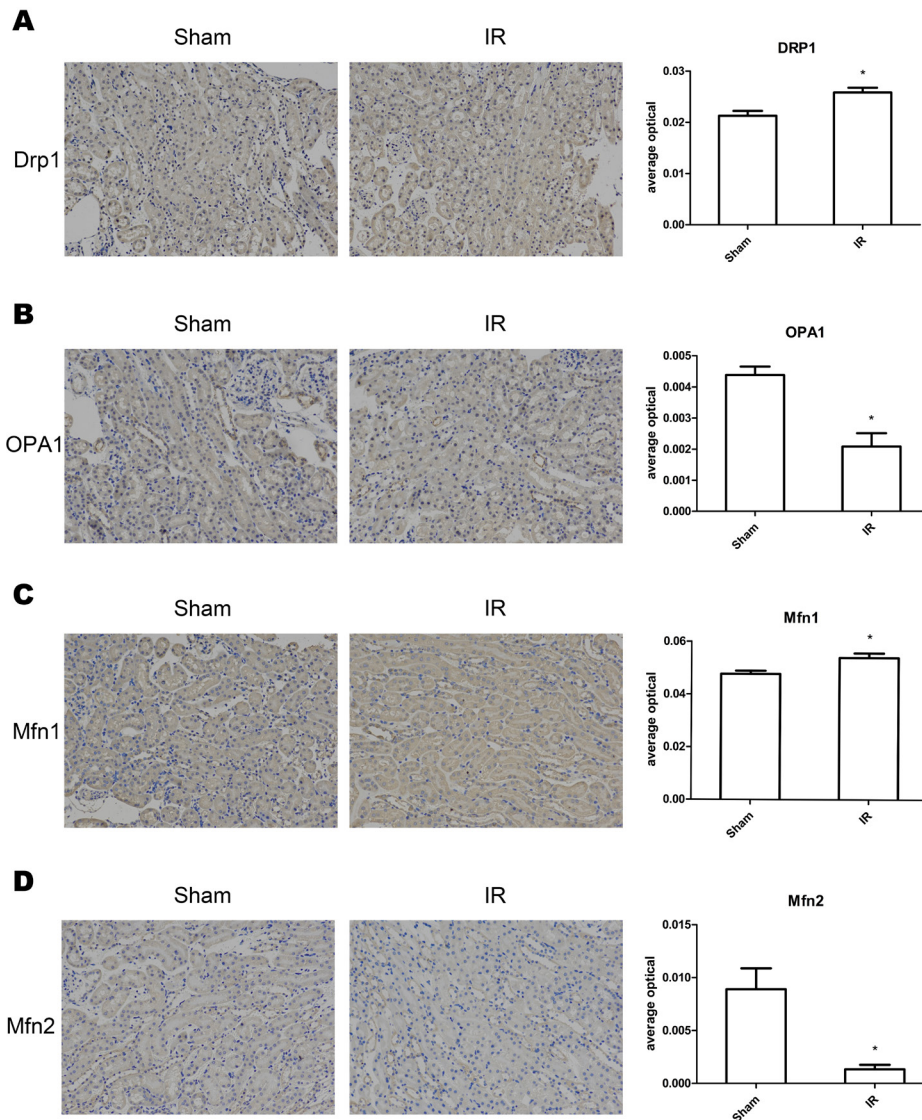
Expression of mitochondrial dynamic proteins in kidney
Dynamin related protein 1 (Drp1: Dynamin 1-like [Dnm1l] in yeast), a key protein of mitochondrial fission, is activated and mediates fragmentation in response to mitochondrial dysfunction during tubular cell injury (Brooks et al., 2009).

Immunohistochemical staining revealed higher Drp1 expression after 24 h of IR injury, compared with the sham group ($P < 0.05$; Fig. 2A), indicative of increased mitochondrial fission in the renal IR injury model. We further investigated the levels of the key mediators of mitochondrial fusion, including the outer membrane fusion proteins Mitofusin-1 and -2 (Mfn1, Mfn2) and the inner membrane fusion protein Optic Atrophy 1 (OPA1). OPA1 expression was significantly decreased in the IR relative to the sham group ($P < 0.05$; Fig. 2B). Interestingly, the IR group exhibited an increase in Mfn1 expression along with a significant decrease in Mfn2 expression, compared with the sham group ($P < 0.05$; Figs. 2C and 2D). Our results indicate that mitochondrial fusion is partly suppressed after IR.

HR treatment induces mitochondrial fission *in vitro*
To observe mitochondrial fission in HK-2 cells at different time-points after HR injury, Drp1 levels were assessed via western blot (Fig. 3). Notably, Drp1 expression in HK-2 cells

Fig. 2. Expression of mitochondrial dynamic proteins in kidney. (A) Drp1 immunohistochemistry in sham-operated mice and 24 h after reperfusion in IR mice. (B-D) Representative images of immunohistochemical staining of OPA1, Mfn1, and Mfn2 in sections of kidney tissues from sham-operated and IR mice (× 200 magnification). Average optical quantitative values for immunohistochemical images. Parametric data were analyzed with the Student's t-test. Data are presented as the mean ± SD (*P < 0.05, compared to the sham group).

subjected to HR injury was gradually upregulated, peaking at 12 h, and maintained for 24 h. No significant differences in Drp1 expression were observed between the H6R12 and H6R24 groups (P > 0.05). Our results indicated a mitochondrial fission peak during hypoxia (6 h) and reoxygenation (12 h) (H6R12) in HK-2 cells. Accordingly, H6R12 was selected as an observation point.

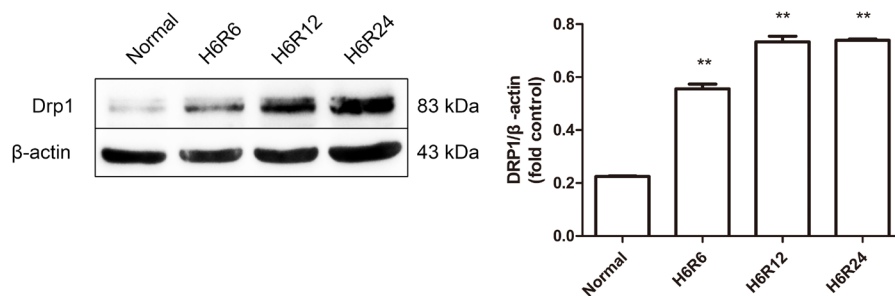### Effects of ALR gene transfection on ALR expression
After transfection of ALR in HK-2 cells, the ALR level was examined via western blot using an anti-Flag antibody. Additionally, expression of exogenously delivered ALR after *in vitro* H6R12 injury was detected. Our findings demonstrated that exogenously delivered Lv-ALR is efficiently expressed in HK-2 cells (Fig. 4).

Overexpression of ALR inhibits mitochondrial fission in renal HR injury *in vitro*
In addition to Drp1, mitochondrial fission process protein 1

(MTFP1) is a nuclear-encoded mitochondrial membrane protein implicated in control of mitochondrial fission in mammalian cells (Tondera et al., 2004; 2005). To clarify the molecular mechanisms underlying mitochondrial fission and potential functional correlations between these proteins in the experimental model of AKI, we performed protein interaction analyses using STRING v10. As illustrated in Figure 5A, Drp1 displayed the highest confidence interaction score (0.701) and was thus predicted as a MTFP1 target protein with a strong correlation. Accordingly, we selected Drp1 and MTFP1 that appeared to have strong correlation with mitochondrial fission for further investigation.

To elucidate the role of ALR in mitochondrial fission, immunoblot analysis of Drp1 and MTFP1 was conducted in each group exposed to H6R12. The results showed an increase in Drp1 expression after HR treatment, compared with the normal group. Notably, Drp1 expression was lower in the Lv-ALR + HR than Lv-vector + HR group (P < 0.01; Figs. 5B and 5C). Regulation of Drp1 is dependent on post-translational mod-

Fig. 3. HR treatment induces mitochondrial fission *in vitro*. Western blot analysis of Drp1 expression in HK-2 cells. Parametric data were analyzed with ANOVA followed by Tukey's post hoc test. Bar graphs represent the mean ± SD (**$P < 0.01$ vs the normal group).
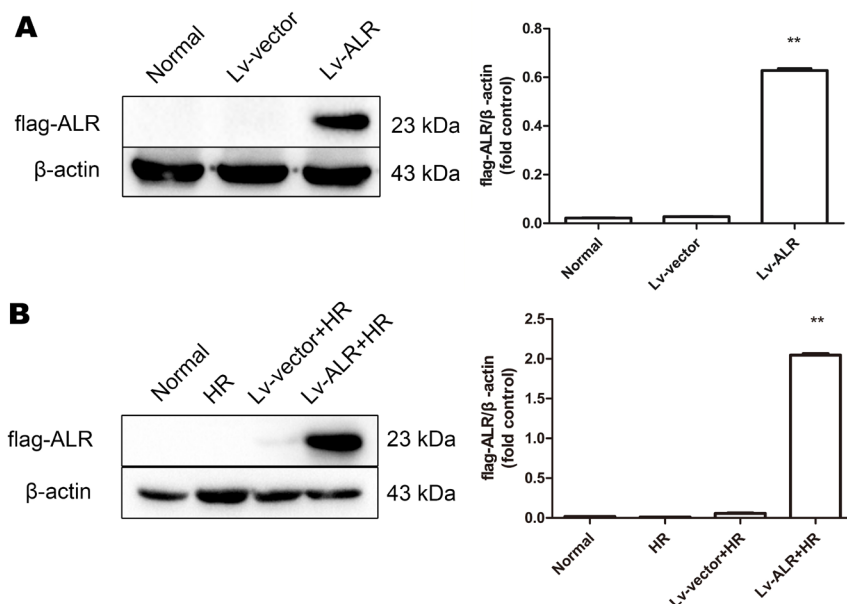

Fig. 4. Effects of ALR gene transfection on ALR expression. (A and B) Western blot analysis of expression of exogenously delivered ALR in HK-2 cells using an anti-Flag antibody. Parametric data were analyzed with ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SD (** $P < 0.01$, vs Lv-vector or Lv-vector + HR group).

ifications including phosphorylation, SUMOylation and ubiquitylation. Since mitochondrial fission is reported to be partly regulated by Drp1 phosphorylation, we performed western blot analysis to establish whether ALR affects phosphorylation at Ser637. As shown in Figures 5B and 5D, in response to HR injury, the p-Drp1/Drp1 ratio was decreased in the HR and Lv-vector + HR groups, compared with the normal group. Overexpression of ALR promoted rephosphorylation of Drp1. Meanwhile, expression of MTFP1 was increased in the HR and Lv-vector + HR groups. Relative to the Lv-vector + HR group, the MTFP1 level was significantly lower in the Lv-ALR + HR group ($P < 0.01$; Figs. 5E and 5F). These results suggested that mitochondrial dynamics was shifted to fission following renal HR treatment *in vitro*. Furthermore, overexpression of ALR played an important role in phosphorylation/dephosphorylation of Drp1 and inhibited mitochondrial fission in HK-2 cells.

Potential mechanisms underlying ALR-mediated control of mitochondrial dynamics

Recent studies have provided evidence that mitochondrial dynamics is associated with the mTOR/4E-BP signaling pathway via regulation of Drp1 and MTFP1 (Morita et al., 2017). To ascertain whether overexpression of ALR has a role in the mTOR/4E-BPs signaling pathway in renal HR injury *in vitro*, immunoblot analysis of mTOR and 4E-BP1 was conducted (Fig. 6). HR conditions led to near-complete blockage of expression of phosphorylated mTORC1 (p-mTORC1) at Ser2448 and phosphorylated 4E-BP1 (p-4EBP1) at Ser65. Conversely, the Lv-ALR + HR group displayed an increase in active p-mTORC1 (Ser2448), p-mTOR/mTOR ratio (Figs. 6A and 6B), active p-4E-BP1 (Ser65) and p-4E-BP1/4E-BP1 ratio (Figs. 6A and 6C), compared with the Lv-vector + HR group. Our results suggested that ALR overexpression activated mTOR/4E-BP1 signaling in HR injury *in vitro* and we hypoth-
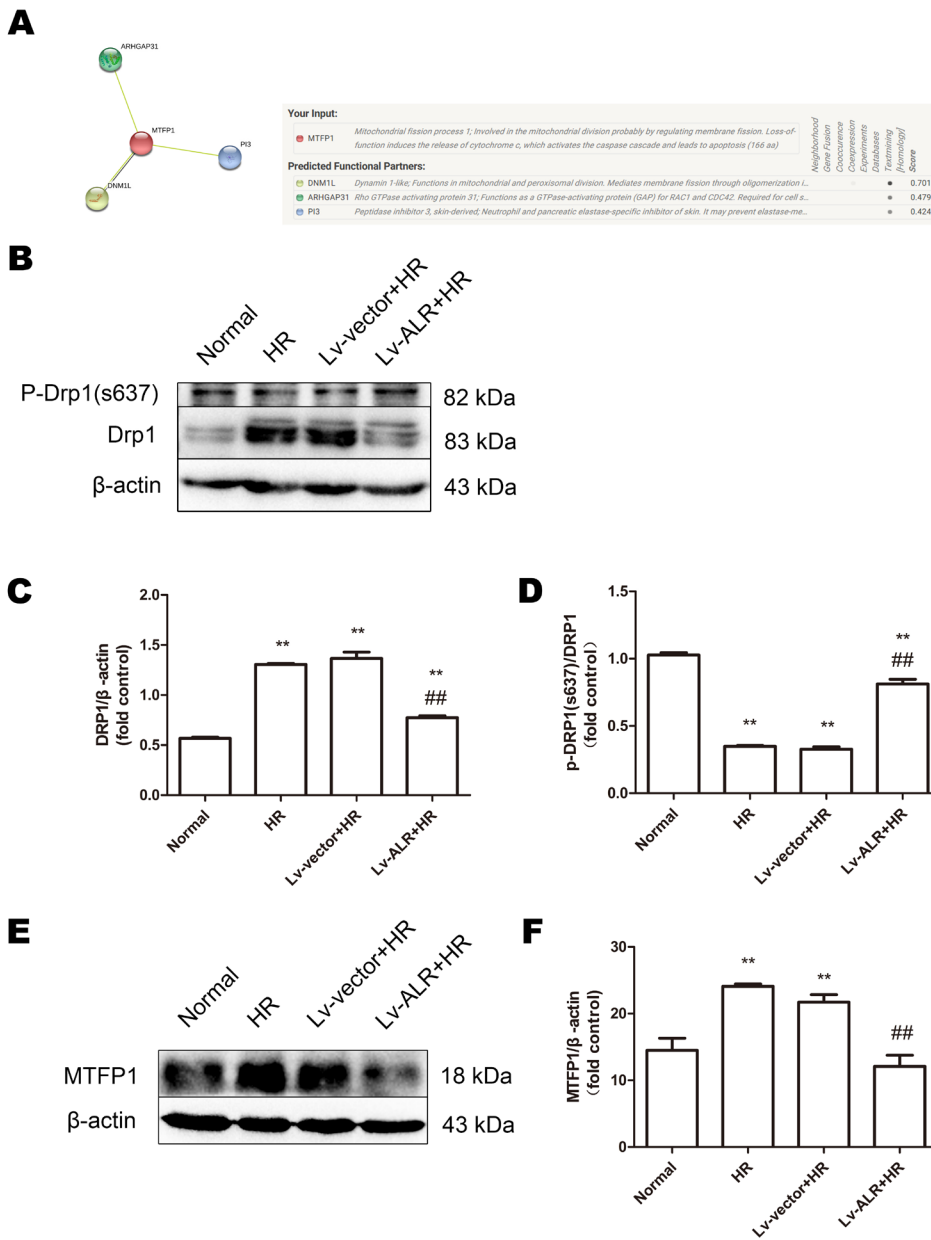
Fig. 5. Overexpression of ALR inhibits mitochondrial fission in renal HR injury *in vitro*. (A) Schematic representation of the MTFP1 target protein interaction network and confidence interaction scores of potential functionally associated proteins. The protein interaction network was constructed using STRING v10. (B) Western blot analysis of Drp1 and p-Drp1 (Ser637) levels in HK-2 cells. (C and D) Drp1 expression was normalized to that of β-actin and the p-Drp1: total Drp1 ratio quantified based on immunoblot images. (E and F) MTFP1 expression was verified via western blots and normalized to that of β-actin. Parametric data were analyzed with ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SD (*$P < 0.05$, **$P < 0.01$, compared to the normal group; #$P < 0.05$, ##$P < 0.01$, compared to the Lv-vector + HR group).

esized that ALR may regulate key mediates of mitochondrial fission via this signaling pathway.

Effect of ALR overexpression on mitochondrial fusion

To further investigate whether overexpression of ALR affected mitochondrial fusion in renal HR injury *in vitro*, we examined the key mediators of mitochondrial fusion via western blots. Both long and short isoforms of OPA1 were significantly decreased in the HR and Lv-vector + HR groups, compared with the normal group. Overexpression of ALR induced an increase in OPA1 under HR injury conditions (Fig. 7A). The results indicated that mitochondrial inner membrane (MIM) fusion was severely impaired in AKI and restored by ALR. Interestingly, Mfn1 expression was significantly enhanced after HR treatment, compared with that in the normal group ($P <$

0.01). However, no marked differences in Mfn1 expression were evident between the Lv-vector + HR and Lv-ALR + HR groups ($P > 0.05$; Figs. 7B and 7C). In addition, no significant differences in Mfn2 expression were evident among the four groups ($P > 0.05$; Figs. 7B and 7D). Based on these findings, we propose that ALR may specifically promote fusion of the MIM at this time-point.

Overexpression of ALR suppresses HR-induced apoptosis of HK-2 cells

Since mitochondrial fragmentation was closely related to apoptosis, the apoptotic rate of HK-2 cells was measured in response to HR treatment using flow cytometry (Fig. 8). The allophycocyanin (APC) label and proliferation dye, eFluor 450 (PB450-A), were used to identify different cell populations as
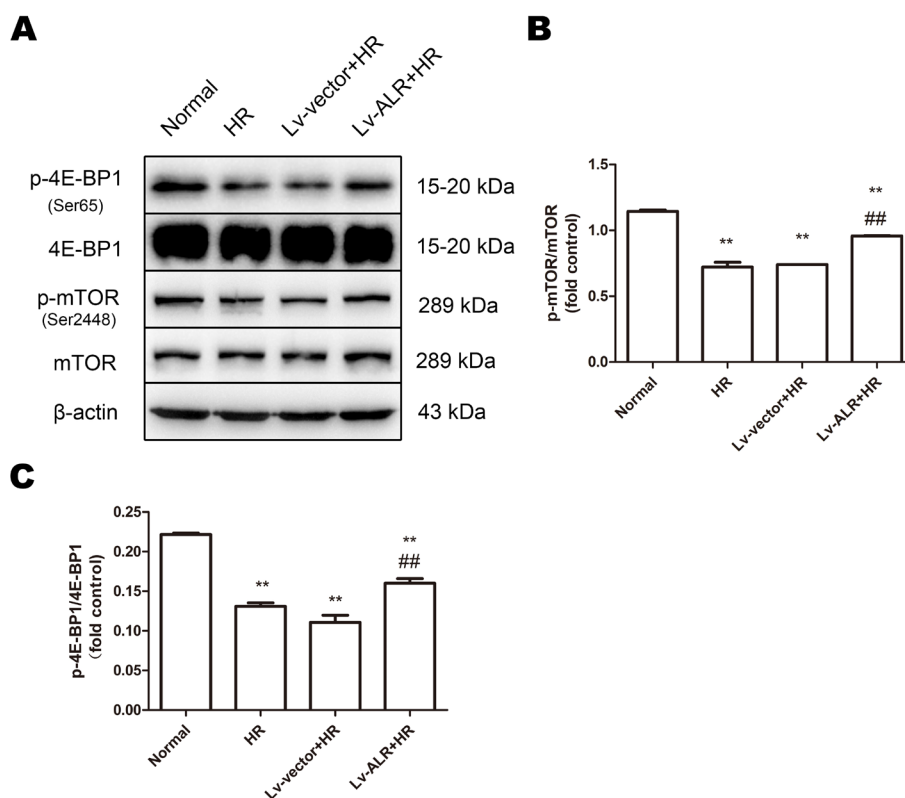
Fig. 6. Potential mechanisms underlying ALR-mediated control of mitochondrial dynamics. (A) Representative images from immunoblot assays against phospho-mTOR (Ser2448), mTOR, phospho-4E-BP1 (Ser65), 4E-BP1, and β-actin. (B and C) p-mTOR: total mTOR and p-4E-BP1: total 4E-BP1 ratios were quantified based on immunoblot images. Parametric data were analyzed with ANOVA followed by Tukey's post hoc test. Bar graphs represent the mean ± SD (*$P < 0.05$, **$P < 0.01$ vs the normal group; #$P < 0.05$, ##$P < 0.01$, compared to the Lv-vector + HR group).

follows: viable cells (low APC/low PB450-A), early apoptotic cells (low APC/high PB450-A), late apoptotic cells (high APC/high PB450-A), and necrotic cells (high APC/low PB450-A). Early and late apoptotic cells were combined as the total apoptotic cell population. The rate of apoptosis was markedly lower in the Lv-ALR + HR than Lv-vector + HR group (9.74 ± 1.15% vs 26.88 ± 2.66%, respectively; $P < 0.01$).

DISCUSSION

Mitochondrial dysfunction plays a crucial role in the pathogenesis of AKI, especially in the proximal tubule (Emma et al., 2016). Maintenance of mitochondrial homeostasis is therefore considered essential for repair of AKI. A balance between mitochondrial dynamics, mitophagy and mitochondrial biogenesis is required for a healthy mitochondrial network. These processes act in concert to maintain mitochondrial homeostasis (Bhargava and Schnellmann, 2017). Potential therapeutic strategies targeting the above pathways may effectively prevent IR-induced mitochondrial and cellular injury of renal tubular epithelial cells. Previous experiments by our group demonstrated renoprotective and prosurvival effects of ALR in AKI, with inhibition of apoptosis (Liao et al., 2012), prevention of inflammation (Yan et al., 2015), attenuation

of fibrosis (Liao et al., 2014) and regulation of mitochondrial biogenesis (Huang et al., 2018). In the present study, we assessed alterations in mitochondrial dynamics in a renal IR injury mice model and revealed a role of ALR in regulating mitochondrial dynamics during renal HR injury for the first time.

In AKI, the dynamic fission-fusion balance is disrupted, resulting in mitochondrial fragmentation (Ishimoto and Inagi, 2016; Zhan et al., 2013). We observed increased mitochondrial fission in the kidney following ischemia-reperfusion injury via electron microscopy. Importantly, Drp1 expression was increased and expression levels of proteins involved in mitochondrial fusion were partly suppressed after IR treatment *in vivo*. Our results provide compelling evidence of renal IR injury-induced changes in mitochondrial dynamics *in vivo*.

Mitochondrial fragmentation is strongly associated with apoptosis (Suen et al., 2008; Youle and Karbowski, 2005). Drp1, a large GTPase of the dynamin superfamily, is responsible for outer mitochondrial membrane scission in mammalian cells, similar to yeast and *Caenorhabditis elegans* (Smirnova et al., 2001). During mitochondrial fission, cytoplasm-localized Drp1 is recruited to fission sites of the mitochondrial outer membrane (MOM) where oligomerization occurs, leading to assembly into a helical structure to mediate membrane scis-
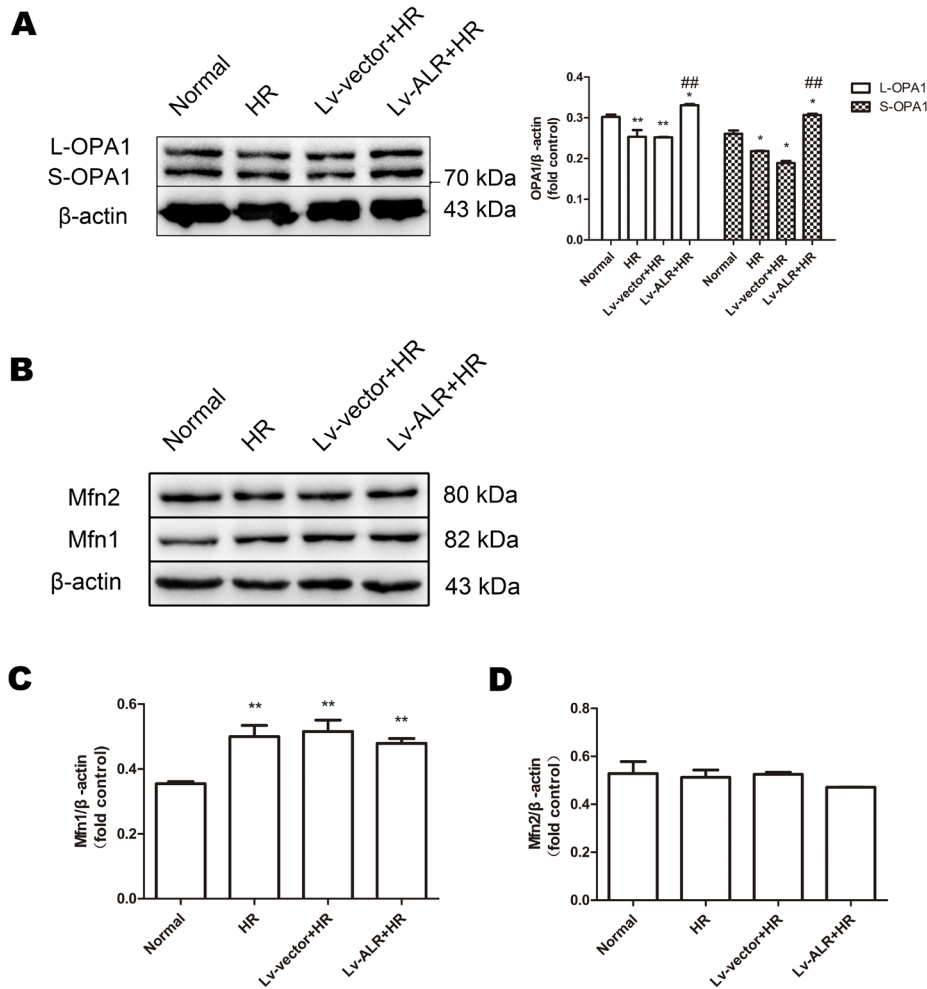
Fig. 7. Effect of ALR overexpression on mitochondrial fusion. (A) OPA1 expression was determined using western blots and normalized to that of β-actin. Parametric data were obtained with ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SD (*P < 0.05, **P < 0.01, vs the normal group; #P < 0.05, ##P < 0.01, compared to the Lv-vector + HR group). (B) Representative images from immunoblot assays against Mfn1, Mfn2, and β-actin. (C and D) Mfn1 and Mfn2 levels were normalized to that of β-actin. Parametric data were analyzed with ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SD (**P < 0.01, vs the normal group).

sion (Mears et al., 2011). Previously, Brooks et al. (2009) reported rapid mitochondrial fragmentation upon ischemic and cisplatin nephrotoxic AKI at an earlier time-point than cellular apoptosis. Inhibition of Drp1 blocked mitochondrial fragmentation and protected the kidney against AKI. Consistent with these results, Funk and Schnellmann (2012) subsequently reported elevation of Drp1 after injury in ischemic AKI, indicating that Drp1 is correlated with apoptosis. Perry et al. (2018) demonstrated that proximal tubule–specific genetic deletion of Drp1 was associated with maintenance of mitochondrial morphology and prevention of IR-induced fragmentation. Moreover, loss of Drp1 prevented renal IR injury, inflammation and apoptosis. These results indicated a requisite and specific role of Drp1 in mitochondrial fission, supporting its utility as an attractive therapeutic target for AKI. In our experiments, Drp1 expression levels peaked at hypoxia (6 h) and reoxygenation (12 h; H6R12) in HK-2 cells. This time-point was parallel with the previous report by our group that

mitochondrial injury is more pronounced at H6R12 (Huang et al., 2018). In view of the finding that Drp1-induced mitochondrial fission occurs during HR injury *in vitro* and peaks at H6R12, potentially resulting in mitochondrial dysfunction and cell death, H6R12 was selected for subsequent experiments.

Numerous studies have confirmed the importance of Drp1 in regulating MOM fission (Galvan et al., 2019; Kamerkar et al., 2018; Perry et al., 2018; Sumida et al., 2015). However, the mechanisms by which the MIM divides are yet to be established and it is unclear whether the molecular events at MOM are coordinated with those at MIM. MTFP1, a nuclear-encoded protein of the MIM, has been shown to contribute to mitochondrial fission in mammalian cells (Tondera et al., 2004; 2005). Recently, Aung et al. (2017) reported that MTFP1 induces pro-mitochondrial fission and plays a pro-apoptotic role in doxorubicin (DOX)-induced cardiotoxicity that may be associated with regulation of Drp1 in the mitochondrial membrane. The study showed that knockdown of
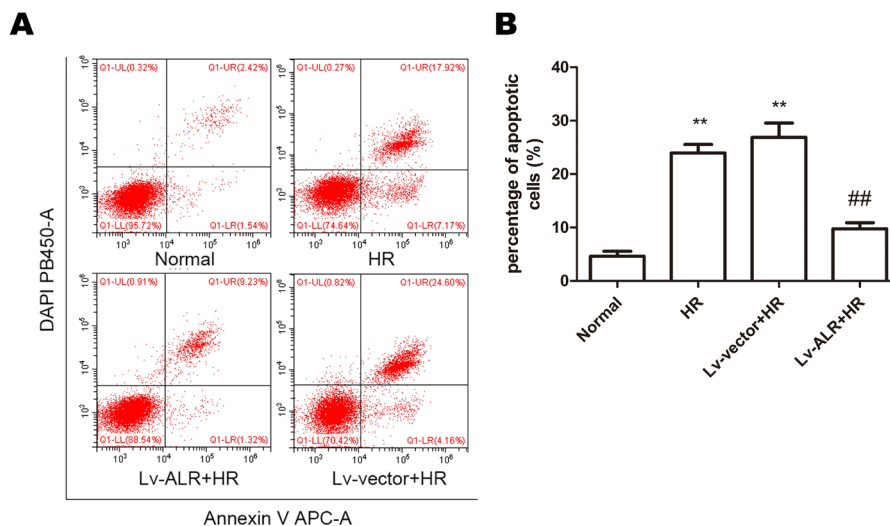
Fig. 8. Overexpression of ALR suppresses HR-induced apoptosis of HK-2 cells. (A) Annexin V-APC/DAPI staining for detection of apoptotic HK-2 cells. (B) Mean percentage of positively stained cells. Parametric data were analyzed with ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SD (∗∗$P < 0.01$, ##$P < 0.01$).

MTFP1 led to decreased cardiomyocyte loss and suppressed mitochondrial fission. In the present study, we constructed a protein interaction network using STRING v10, with a view to predicting the potential functional correlations between Drp1 and MTFP1 in our model. Owing to the strong correlation, Drp1 was predicted as a MTFP1 target and both proteins selected for further investigation. Our results showed upregulation of both Drp1 and MTFP1 following H6R12 treatment *in vitro*, suggesting that HR-induced mitochondrial fission was associated with increased levels of Drp1 and MTFP1. Compared to the Lv-vector + HR group, expression of total Drp1 and MTFP1 was significantly decreased in the Lv-ALR + HR group ($P < 0.01$), indicating that overexpression of ALR prevented HR-induced mitochondrial damage in tubular cells, probably though inhibiting mitochondrial fission. This finding was consistent with earlier studies on the heart.

Mitochondrial fission is partly regulated by Drp1 phosphorylation. Two main phosphorylation sites (Ser637 and Ser616) are critical in modulating human Drp1. Phosphorylation at Ser616 of Drp1 by Cdk1/Cyclin B promotes mitochondrial fission during mitosis (Taguchi et al., 2007). Consequently, mitochondria are divisive and transmit to daughter cells. In contrast, Drp1 phosphorylation at Ser637 appears more pathological and is associated with apoptosis induction. Phosphorylation of Drp1 Ser637 by protein kinase A leads to elongation of mitochondria and resistance to apoptosis. Conversely, dephosphorylation of Drp1 Ser637 by calcineurin promotes mitochondrial fragmentation and increases apoptotic sensitivity (Cribbs and Strack, 2007). In the current study, we detected a significant decrease in phosphorylation of Drp1 Ser637 during renal HR injury, which was effectively reversed in the Lv-ALR + HR group, suggesting that ALR overexpression restores the normal phosphorylation level of Drp1. Our findings were consistent with data from a similar study by Cho et al. (2010) showing that Drp1 dephosphorylation

at Ser637 during ATP depletion in renal tubular cells and activation contribute to mitochondrial fragmentation, outer membrane permeabilization and consequent apoptosis. Moreover, calcineurin inhibitors prevented Drp1 dephosphorylation and suppressed mitochondrial fragmentation. The collective data support the theory that ALR overexpression inhibits HR-induced mitochondrial fission potentially though downregulation of total Drp1 and MTFP1 levels. Additionally, ALR appears to regulate phosphorylation/dephosphorylation of Drp1 at Ser637 for mitochondrial fission during AKI in a MTFP1-dependent manner. However, the precise pathways by which MTFP1 controls activation of Drp1 Ser637 and whether its involvement is direct or indirect remain to be established.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that exists as two functionally and structurally distinct complexes, mTOR complex 1 and 2 (mTORC1 and 2) (Zoncu et al., 2011). mTORC1 controls mRNA translation through phosphorylation of downstream targets, such as eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BP) and ribosomal protein S6 kinases (S6K) (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017; Zoncu et al., 2011). The 4E-BPs act as translation initiation repressors. Hypophosphorylated 4E-BPs bind with high affinity to eIF4E whereas phosphorylation of 4E-BPs by mTORC1 leads to their dissociation from eIF4E, thereby allowing assembly of the eIF4F complex at the mRNA 5' end and promotion of translational initiation. Translational control has important implications for cell growth, homeostasis, survival and apoptosis. Additionally, hyperactivation of mTOR signaling frequently occurs in cancer (Laplante and Sabatini, 2012). Unsurprisingly, cancer cells can successfully evade apoptosis. Activation of mTOR signaling or eIF4E (either via direct overexpression or release from 4E-BPs) has been associated with resistance to apoptosis and tumor formation (Robert and

Pelletier, 2009). Accordingly, we further focused on whether the mTOR/4E-BP1 signaling pathway was involved in the renoprotective role of ALR in AKI. A recent study by Morita et al. (2017) linked the mTOR/4E-BP signaling pathway with mitochondrial dynamics and cell survival. The results have supported the conclusion that the mTOR/4E-BP axis controls mitochondrial fission via MTFP1 through Drp1 phosphorylation and localization. Maintenance of the pro-fission state in active-site mTOR inhibitor (asTORi)-based therapy could present a novel strategy for promoting cancer cell death. In the present study, HR injury led to significant suppression of p-mTOR and p-4E-BP1 protein expression. Conversely, a distinct increase in phosphorylation of mTOR and 4EBP1 was observed following ALR overexpression. Confocal microscopy data showed colocalization of ALR with mitochondria in HK-2 cells and expression in the cytoplasm, mitochondria and endoplasmic reticulum fraction using western blots (Jiang et al., 2019). We further transfected HK-2 cells with ALR-containing lentiviral particles for overexpression of ALR in the cytoplasm. The mTOR signaling pathway is a complex process. mTOR is expressed in the cytoplasm and limited endogenous mTORC1 is detectable in the nucleus (Laplante and Sabatini, 2012). Thus, overexpression of ALR may affect the mTOR signaling pathway and delivery of the *ALR* gene into HK-2 cells presents a potential therapeutic strategy. Our data indicate that ALR overexpression activates the mTOR/4E-BP1 signaling pathway during renal HR injury, leading to the speculation that ALR-mediated modulation of the translation of key proteins of mitochondrial fission involves this signaling pathway, resulting in resistance to AKI. Further studies are required to ascertain whether a direct molecular link exists between ALR and this machinery or simply a bystander effect.

Mitochondrial fission and fusion are two complementary processes essential for fine-tuning mitochondrial morphology. Fragmentation of mitochondria is a result of increased fission, suppressed fusion or a combination of both. Our experiments revealed an impact of ALR overexpression on mitochondrial fusion. OPA1 is a known key inner membrane fusion protein in mammalian cells. Due to proteolytic processing, OPA1 is expressed as multiple isoforms, whereby the long (L-OPA1) isoform mediates MIM fusion and preserves cristae structure, whereas the short (S-OPA1) isoform generated by proteolysis of L-OPA1 facilitates mitochondrial fission (MacVicar and Langer, 2016). Levels of both isoforms of OPA1 were decreased in the HR and Lv-vector + HR groups, in our study. Surprisingly, however, total OPA1 expression was significantly increased after overexpression of ALR in HR injury conditions. These results suggested that MIM fusion was severely impaired in our experimental model and was restored upon ALR overexpression in HR injury conditions *in vitro*. In addition to its pro-fusion activity, OPA1 contributes to maintenance of cristae integrity, keeping the junctions tight during apoptosis (Frezza et al., 2006). Loss of OPA1 increases susceptibility to cytochrome c release from the cristae folds and cell death in the setting of ischemia-reperfusion (Calo et al., 2013). Therefore, it is reasonable to speculate that ALR plays an essential protective role in renal HR injury partly by enhancing OPA1-mediated mitochondrial fusion and preservation of cristae structure. Furthermore, Mfn1

and Mfn2 localized on MOM function as key determinants of MOM fusion. Interestingly, ALR overexpression did not induce alterations in either Mfn1 or Mfn2 levels in HK-2 cells subjected to HR. One possibility to explain this finding is that ALR itself has no effect on Mfn proteins in the kidney. Another likely reason is the limited observation time-points in our study. Additionally, Mfn1 was upregulated whereas Mfn2 displayed no significant alterations in expression in whole cell lysates after HR treatment, compared with the normal group. A previous study by Brooks et al. (2007) demonstrated that Bak (a proapoptotic multidomain Bcl-2 protein) regulates mitochondrial morphology and pathology during apoptosis through interactions with Mfns. Upon apoptotic induction, Bak dissociates from Mfn2 and interacts with Mfn1, leading to decreased mitochondrial fusion activity of Mfn2, cessation of fusion, and consequently, mitochondrial fragmentation. Moreover, Mfn1 exhibits significantly higher GTPase activity than Mfn2 during membrane tethering and OPA1 functionally requires Mfn1, but not Mfn2, to regulate mitochondrial fusion (Zhan et al., 2013). In the current study, we speculated that enhanced levels of Mfn1 interacted with Bak to trigger mitochondrial damage and renal tubular epithelial cell apoptosis during HR injury *in vitro*. Further investigation is required to examine this possibility. Under these conditions, it is plausible that ALR overexpression may promote tethering of the MIM.

At H6R12, the level of apoptosis in the Lv-ALR + HR group was decreased, concurrent with suppressed mitochondrial fission and increased mitochondrial fusion. Based on these results, we speculate that the renoprotective effects of ALR overexpression are associated with regulation of mitochondrial dynamics. In addition, we observed no significant differences in apoptotic rates between the HR and Lv-vector + HR groups ($P > 0.05$), indicating that lentiviral transfection does not affect cell survival.

In summary, we conclude that ALR overexpression preserves mitochondrial functionality, possibly by inhibiting mitochondrial fission and promoting fusion of MIM, both of which aid in resistance to apoptosis in HK-2 cells. Moreover, fission processes are potentially mediated by promoting cell survival through the mTOR/4E-BP1 signaling pathway. Further studies are required to elucidate the mechanisms by which this pathway is integrated into mitochondrial dynamics during renal HR injury. Our collective results provide a basis for the development of novel therapeutic strategies to ameliorate IR-triggered renal injury.

Disclosure
The authors have no potential conflicts of interest to disclose.

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ORCID

Rui-ting Long	https://orcid.org/0000-0003-4468-4734
Jun-bo Peng	https://orcid.org/0000-0003-4943-6203
Li-li Huang	https://orcid.org/0000-0003-3752-2585
Gui-ping Jiang	https://orcid.org/0000-0002-5661-3864
Yue-juan Liao	https://orcid.org/0000-0002-3928-5483
Hang Sun	https://orcid.org/0000-0003-3342-6545
Yu-dong Hu	https://orcid.org/0000-0003-3972-2257
Xiao-hui Liao	https://orcid.org/0000-0002-2329-0456

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