LncRNA Hnf4aos exacerbates liver ischemia/reperfusion injury in mice via Hnf4aos/Hnf4α duplex-mediated PGC1α suppression

Chaoqun Wang a,b,1, Hongjun Yu a,b,1, Shounan Lu a,b,1, Shanjia Ke a,b,1, Yanan Xu b,c, Zhigang Feng b,d, Baolin Qian a,b, Miaoyu Bai a,b, Bing Yin a,b, Xinglong Li a,b, Yongliang Hua b,e, Liqian Dong a,b, Yao Li a, Bao Zhang b, Zhongyu Li a, Dong Chen a, Bangliang Chen a, Yongzhi Zhou a, Shangha Pan b, Yao Fu g, Hongchi Jiang b,c, Dawei Wang b,h,**, Yong Ma a,b,*

a Department of Minimal Invasive Hepatic Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China
b Key Laboratory of Hepatosplenic Surgery, Ministry of Education, Harbin, China
c The First Department of General Surgery, The Affiliated Hospital of Inner Mongolia Minzu University, Tongliao, China
d Department of Pediatric Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China
e Department of Ultrasound, The First Affiliated Hospital of Harbin Medical University, Harbin, China
f Department of Anorectal Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China

1. Introduction

Hepatic ischemia/reperfusion injury (HIRI) is a common pathological process that occurs in several clinical scenarios, such as complex liver resection, liver transplantation, and hemorrhagic shock. During this process, the initial ischemic injury causes direct hepatocyte damage, and subsequent blood flow reflux further aggravates liver dysfunction and injury due to the propagation of reactive oxygen species (ROS), macrophage activation and inflammatory cytokines, which trigger cell death [1,2]. However, the underlying molecular mechanisms of ischemia/reperfusion (I/R) injury remain largely unknown.

Long noncoding RNAs (lncRNAs) are defined as single-stranded RNA molecules spanning more than 200 nucleotides that are involved in multilevel gene expression regulation, including epigenetic modifications, transcriptional and posttranscriptional progression [3]. According to the proximity to protein coding genes in the genome, lncRNAs are generally placed into five categories: sense, antisense, bidirectional, divergent, and intergenic lncRNAs [4]. Currently, several studies have highlighted the significant roles of lncRNAs in the pathogenesis of liver injury/reperfusion models and patients who underwent liver resection surgery.

Chaoqun Wang a,b,1, Hongjun Yu a,b,1, Shounan Lu a,b,1, Shanjia Ke a,b,1, Yanan Xu b,c, Zhigang Feng b,d, Baolin Qian a,b, Miaoyu Bai a,b, Bing Yin a,b, Xinglong Li a,b, Yongliang Hua b,e, Liqian Dong a,b, Yao Li a, Bao Zhang b, Zhongyu Li a, Dong Chen a, Bangliang Chen a, Yongzhi Zhou a, Shangha Pan b, Yao Fu g, Hongchi Jiang b,c, Dawei Wang b,h,**, Yong Ma a,b,*

a Department of Minimal Invasive Hepatic Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China
b Key Laboratory of Hepatosplenic Surgery, Ministry of Education, Harbin, China
c The First Department of General Surgery, The Affiliated Hospital of Inner Mongolia Minzu University, Tongliao, China
d Department of Pediatric Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China
e Department of Ultrasound, The First Affiliated Hospital of Harbin Medical University, Harbin, China
f Department of Anorectal Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China

1. Introduction

LncRNAs are involved in the pathophysiologic processes of multiple diseases, but little is known about their functions in hepatic ischemia/reperfusion injury (HIRI). As a novel lncRNA, the pathogenetic significance of hepatic nuclear factor 4 alpha (Hnf4α) in hepatic I/R injury remains unclear. Here, differentially expressed Hnf4αos and Hnf4α antisense RNA 1 (Hnf4α-as1) were identified in liver tissues from mouse ischemia/reperfusion models and patients who underwent liver resection surgery. Hnf4αos deficiency in Hnf4αos-KO mice led to improved liver function, alleviated the inflammatory response and reduced cell death. Mechanistically, we found a regulatory role of Hnf4αos-KO in ROS metabolism through PGC1α upregulation. Hnf4αos also promoted the stability of Hnf4α mRNA through an RNA/RNA duplex, leading to the transcriptional activation of miR-23a and miR-23a depletion was required for PGC1α function in hepatoprotective effects on HIRI. Together, our findings reveal that Hnf4αos elevation in HIRI leads to severe liver damage via Hnf4αos/Hnf4α/miR-23a axis-mediated PGC1α inhibition.

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disease. For instance, IncRNA HULC is upregulated in hepatocellular carcinoma and enhances hepatocarcinogenesis by promoting the phosphorylation of YB-1 via the ERK pathway [5]; IncRNA ANRIL alleviates liver fibrosis and hepatic stellate cell (HSC) activation via the AMPK pathway [6]; and IncRNA CCAT1 promotes nonalcoholic fatty liver disease (NAFLD) by increasing LXR transcription [7]. Nevertheless, in the case of hepatic I/R injury, little is known about IncRNAs in hepatic I/R injury. Thus, a deeper understanding of the molecular mechanisms underlying the pathogenic process of hepatic I/R is required to uncover potential IncRNA-targets for developing promising therapeutic strategies.

Furthermore, we have identified a novel IncRNA hepatic nuclear factor 4 alpha, opposite strand (Hnf4αos), a natural antisense transcript (NAT) of hepatocyte nuclear factor 4 alpha (Hnf4α), which was aberrantly upregulated in mouse I/R models. Although Hnf4αos has been reported, little information is available for regarding its molecular function [8,9]. PPARγ coactivator 1 alpha (PGC1α) is well known as a metabolic regulator in the physiological process of oxidative phosphorylation (OXPHOS), the tricarboxylic acid (TCA) cycle and ROS metabolism [10–12]. Intriguingly, our previous studies have demonstrated that PGC1α is an important regulator of ROS metabolism that reduces cell death, ameliorates the sterile inflammatory response and alleviates oxidative stress-induced liver damage during hepatic I/R insult [13]. Moreover, several lines of evidence, including data from bioinformatic analysis and determination of oxidative stress levels, suggest a close link between the IncRNA Hnf4αos and PGC1α. Thus, we further investigated the effects of Hnf4αos on I/R progression and the underlying mechanisms between Hnf4αos and PGC1α.

2. Material and methods

2.1. Human liver samples

Human liver samples were obtained from subjects who underwent partial hepatectomy due to hepatic hemangioma. All procedures involving human samples were approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University and patient informed consent was obtained. We listed the detailed clinical information of the hemangioma patients in Supplementary Table S3.

2.2. Animals

Male C57BL/6 mice, hepatocyte-specific Hnf4α knockout (Hnf4αos-KO) mice and wild-type (WT) mice (8 weeks old) were housed in specific pathogen-free (SPF) conditions and raised following institutional guidelines for animal care. Hnf4αos-KO mice were obtained by CRISPR/Cas9 methods as described previously [14]. Hnf4αos-KO mice were generated by crossing Hnf4αos-floxed mice with Albumin-Cre mice (Jackson Laboratory. Bar Harbor, ME, USA) on the C57BL background. The donor vector containing the fourth exon of the Hnf4αos gene was floxed by two loxp sites. All animal experiments were performed in accordance with the standard protocols of the Committee on the Use of Live Animals in Teaching and Research of Harbin Medical University, Harbin, China.

2.3. Mouse hepatic I/R injury model

The procedures for partial hepatic ischemia have been described previously [15]. Mice were housed in a specific pathogen-free and temperature-controlled environment with a 12-h light/dark cycle. Briefly, the mice were anesthetized with pentobarbital sodium (50 mg/kg), and a midline laparotomy was performed. An atrumatic clip was placed across the left lateral and median lobes of the liver (~70%). After 75 min of partial hepatic ischemia, the clip was removed for initial reperfusion. Sham control mice underwent the same operation without vascular clamping.

2.4. Cell A/R treatment model

Cellular anoxic conditions were established and maintained in a modular incubator chamber (Biospherix, Lacona, NY, USA) by continuous gas flow with a 1% O2, 5% CO2 and 94% N2 gas mixture. After incubation under hypoxia for 6 h, the cells were incubated under normoxic conditions with 95% air and 5% CO2 for the indicated times (0, 3, 6, 12, 24 h). The medium and cells were collected for further analysis.

2.5. Cell culture and treatment

Mouse hepatocytes were isolated by a modified in situ collagenase perfusion technique as previously described [15]. Hepatocyte purity and viability typically exceeded 99 and 95%, respectively. Primary hepatocytes and L02 cell lines (Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO2/water-saturated incubator at 37 °C.

2.6. Immunofluorescence assay

Paraffin-embedded tissue sections were used for immunofluorescence as described previously [16]. The liver sections were incubated with primary antibody against Ly6G (Cell Signaling Technology) (1:500) (31469), and the slides were incubated with corresponding fluorescence-labeled secondary antibody (ThermoFisher) (1:1000) (A323744) for further staining.

2.7. ROS detection

Cellular reactive oxygen species (ROS) levels were estimated as previously described [17]. For intracellular ROS levels, cells were incubated in medium containing 10 μM dihydroethidium (DHE) (Invitrogen, USA) for 30 min at 37 °C in the dark. The medium was switched to fresh medium before fluorescence detection. The relative ROS levels, which are proportional to the fluorescence intensity, were quantified using Image-Pro Plus software.

2.8. Luciferase reporter assay

We predicted potential Hnf4α binding sites on the PGC1α and miR-23a promoters using the JASPAR database, and the PGC1α 3'-
untranslated region (UTR) contains conserved miR-23a binding sites as reported previously [18]. We then cloned the candidate binding sites in an SV40 driven luciferase reporter plasmid. Briefly, luciferase activity was assessed using a luciferase assay kit (Promega, Madison, WI, USA). HEK-293T cells containing specific plasmids and 1 ng pRL-TK Renilla luciferase plasmid were seeded into 24-well plates. After 48 h, we used the dual luciferase reporter assay system (Promega) to measure luciferase activity according to the manufacturer’s instructions.

2.9. Ribonuclease protection assay (RPA)

A ribonuclease protection assay (RPA) and quantitative RT-PCR were performed to detect the RNA-RNA duplex. Total RNA from primary hepatocytes was isolated as described previously [19]. The RNA samples were treated with DNase I (Sigma, 12.5 units/ml) and RNase A (QIAgen, 200 ng/ml) to remove residual DNA and single-stranded RNAs. Finally, the solutions were incubated for 40 min at 37 °C for further qRT-PCR.

2.10. Electrophoretic mobility shift assay (EMSA)

An electrophoretic mobility shift assay (EMSA) was performed as described previously [12]. The oligonucleotides used in EMSA were as follows: Hnf4α/miR-23a wt, 5’-GATCAGCTCCCCCTAAACCTTATTAAC-3’ and 3’-CTAGTGGGACCTTTTAAAAACATTG-5’. Hnf4α/miR-23a mut, 5’-GATCAGCTCCCCCTAAACCTTATTAAC-3’ and 3’-CTAGTGGGACCTTTTAAAAACATTG-5’.

2.11. Statistical analysis

All data are expressed as the mean ± SD. Significant differences between groups were determined by ANOVA, with Bonferroni correction for continuous variables and multiple groups. Two-tailed Student’s t-test was used for comparison of a normally distributed continuous variable between 2 groups. The level of significance was set at a p value less than 0.05 for all analyses.

Further details of the experimental materials and procedures are described in the Supplementary Files.

3. Results

3.1. LncRNA Hnf4αos is elevated during hepatic I/R injury

Several lncRNAs were differentially expressed in the GEO data-set (GSE15891) with exposure to chronic anoxia and our heatmap demonstrated the marked differentially expressed lncRNAs related to oxidative stress, inflammatory response and apoptosis pathways (Fig. 1A). For examining the relationships of lncRNAs and traget genes, the top-ranked lncRNAs and mRNAs correlated oxidative stress/inflammatory
Among the top-ranked differentially expressed lncRNAs, only Hnf4αos was enriched in adult mouse liver tissue (Supplementary Table S1). Thus, Hnf4αos was selected for further investigation during hepatic I/R injury.

To explore the role of lncRNA Hnf4αos in HIRI, we first detected the expression levels of Hnf4αos in murine hepatic I/R and hepatocyte A/R models, and Hnf4αos was found to be increased after reperfusion. The human-derived lncRNA, Hnf4α-as1, was also found to be differentially expressed in clinical liver samples from patients who underwent partial hepatectomy (Fig. 1C–D, Supplementary Fig. S1). Furthermore, cellular fractionation of hepatocytes followed by qRT-PCR implied that Hnf4αos was predominantly expressed in the nuclei of hepatocytes rather than other compartments, compared with U6 (localized in the nucleus) and 18S (localized in the cytoplasm) expression (Fig. 1E). Moreover, a fluorescence in situ hybridization (FISH) assay was performed to detect the locations of and changes in Hnf4αos in mouse hepatocytes after A/R treatment. The results showed that the fluorescence intensity of Hnf4αos was markedly enriched in hepatocyte nuclei and significantly elevated in the Hnf4αos-KO and WT mice subjected to I/R operation. n.s. P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 2. Hnf4αos deteriorates liver damage induced by hepatic I/R insult. (A) Images (100 × magnification) of H&E-stained liver sections and representative histopathological scores after the transfection of adenovirus vectors. The scale bar represents 200 μm. (B–C) Serum levels of aminotransferases (ALT and AST) were detected in the mice subjected to I/R after the transfection of adenovirus vectors. (D) Images of H&E-stained liver sections and representative histopathological scores in Hnf4αos-KO and WT mice. The scale bar represents 200 μm. (E) Serum levels of aminotransferases (ALT and AST) were detected in the Hnf4αos-KO and WT mice subjected to I/R operation. n.s. P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.
3.2. *Hnf4α*os exacerbates liver damage induced by hepatic I/R insult

To evaluate the potential effects of *Hnf4α*os on liver damage after hepatic I/R in mice, we altered the expression level of endogenous *Hnf4α*os by tail vein injection with *Hnf4α*os overexpression and down-regulation adenoviral vectors (Supplementary Fig. S2A). When we knocked down *Hnf4α*os expression in mice, no statistical significance in sham mice was found, and I/R induced tissue necrosis was markedly ameliorated in the liver by silencing *Hnf4α*os expression, whereas, *Hnf4α*os overexpression worsened pathological changes (hemorrhagic change, inflammatory cell infiltration and focal necrosis) in I/R liver tissue (Fig. 2A). Additionally, serum aminotransferase (ALT and AST) levels were also significantly decreased in *Hnf4α*os knockdown mice, and ectopic expression of *Hnf4α*os exhibited the opposite effect.
compared with control mice (Fig. 2B and C). Thus, we concluded that Hnf4αos exacerbated liver damage induced by HIRI insult.

To obtain more evidence supporting the role of Hnf4αos in I/R-induced liver injury, we generated Hnf4αos-knockout (Hnf4αos-KO) and Hnf4αos-wild-type (Hnf4αos-WT) mice (Supplementary Figs. S2B and C). Subsequently, Hnf4αos-KO mice were subjected to a 75-min I/R operation. As expected, histological H&E staining showed considerable amelioration of tissue necrosis levels by Hnf4αos knockout (Fig. 2D).

Moreover, Hnf4αos-KO mice exhibited reduced release of ALT and AST in serum compared with Hnf4αos-WT mice (Fig. 2E). Of note, serum
aminotransferases were significantly lower in the low Hnf4α-as1 group, suggesting less liver injury and better liver function after partial hepatectomy (Supplementary Fig. S2D). Overall, these observations suggest that Hnf4α inhibition plays a protective role in hepatic I/R injury.

3.3. Hnf4α-depletion inhibits the inflammatory response during hepatic I/R injury

The sterile inflammatory response plays a pivotal role in I/R injury, and the release of cytokines and chemokines is sustained throughout the entire pathophysiological processes of hepatic I/R. Therefore, we performed RNA-seq with I/R challenged liver samples of WT and Hnf4α-KO mice to detect whether Hnf4α can affect liver damage by modulating the inflammatory response. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated significantly enriched signaling pathways of inflammatory response, in particularly the NF-κB pathway (Fig. 3A). Moreover, heatmap of leading-edge enriched pathways showed that Hnf4α ablation mainly affected the expression of NF-κB signaling related molecules (Fig. 3B). The ELISA and qRT-PCR analysis suggested sham procedure did not induce basal inflammation changes in mice (Fig. 3C and D). Hnf4α-KO mice exhibited less inflammatory cytokine/chemokine (TNF-α, IL-1β, IL-6, and MIP-2) release than WT mice in the I/R model (Fig. 3C and D). In accordance with the data obtained in vivo, the medium collected from the primary Hnf4α-KO hepatocyte culture contained lower levels of cytokines/chemokines (Supplementary Fig. S3A). Tissue MPO activity, an indicator of neutrophil infiltration, was dramatically increased following I/R insult in WT mice. In contrast, Hnf4α-KO mice exhibited less neutrophil accumulation (Supplementary Fig. S3B). Moreover, tissue section immunofluorescence analysis demonstrated fewer Ly6G positive (a neutrophil biomarker) cells when comparing Hnf4α-KO versus WT-I/R mice (Fig. 3E). Gene set enrichment analysis (GSEA) also indicated that Hnf4α knockout reduced the apoptosis levels of hepatocytes subjected to I/R operation compared to Hnf4α-WT group (Fig. 4B). The results of the caspase-3 activity assay and DNA fragmentation ELISA also suggested dramatic decrease in apoptotic levels with Hnf4α depletion (Fig. 4C and D). As shown by qRT-PCR and Western blot, I/R-induced cell death was markedly blunted in the livers of Hnf4α-KO deficient mice, as evidenced by the expression of apoptotic markers (BCL-2, Bax and cleaved caspase-3) (Fig. 4E and F). Moreover, less LDH was released from Hnf4α-KO deficient hepatocyte cultures than from control hepatocytes (Fig. 4G). The CCK-8 assay results in Fig. 4H showed that Hnf4α-depletion enhanced cell viability and promoted cell proliferation in Hnf4α-KO mice, compared to control mice. In line with our observations in primary mouse hepatocytes, Hnf4α-as1-knockdown in human L02 hepatocytes also alleviated cell apoptosis and Hnf4α-as1-overexpression had the opposite effects (Supplementary Fig. S4A).

3.5. PGC1α mediates Hnf4α function in hepatic I/R injury

Based on the GEO data-set (GSE15891), we found the differentially expressed genes (DEGs) (Fig. 5A) are closely related to the regulation of cell death, oxidative and anti-inflammatory response according to the Gene Ontology (GO) analysis (Fig. 5B). Moreover, we established a module by bioinformatic methods to evaluate the potential correlation between the DEGs and differential expressed lncRNAs. The lncRNA-mRNA interaction network (Fig. 5C) surprisingly revealed a close correlation between Hnf4α and PGC1α. We previously reported that PGC1α protected the liver from I/R injury by attenuating hepatocyte death, reducing cytokine/chemokine release and alleviating oxidative stress [13]. GSEA also demonstrated that most genes affected by PGC1α overexpression were involved in the KEGG apoptosis pathway. More importantly, a dramatically negative correlation was found between Hnf4α and PGC1α pathway related molecules (Fig. 5D). Specifically, in Fig. 5E, the module enriched in multiple cell death, oxidative stress and inflammatory pathways also showed a high degree of correlation with Hnf4α and PGC1α expression. Thus, we confirmed an obviously negative association between Hnf4α and PGC1α by Western blot (Fig. 6A). Our previous study found that PGC1α can protect the liver against I/R insult by accelerating the clearance of ROS. Therefore, we hypothesized that Hnf4α-KO ameliorates liver damage in the I/R process by scavenging accumulated ROS. Subsequently, we detected a significant decrease in LDH activity in Hnf4α-KO mice compared to WT group (Fig. 6B). Flow cytometry analysis showed that Hnf4α depletion reduced the apoptotic levels of hepatocytes subjected to A/R operation compared to Hnf4α-WT group (Fig. 4B). The results of the caspase-3 activity assay and DNA fragmentation ELISA also suggested dramatic decrease in apoptotic levels with Hnf4α depletion (Fig. 4C and D). As shown by qRT-PCR and Western blot, I/R-induced cell death was markedly blunted in the livers of Hnf4α-KO deficient mice, as evidenced by the expression of apoptotic markers (BCL-2, Bax and cleaved caspase-3) (Fig. 4E and F). Moreover, less LDH was released from Hnf4α-KO deficient hepatocyte cultures than from control hepatocytes (Fig. 4G). The CCK-8 assay results in Fig. 4H showed that Hnf4α-depletion enhanced cell viability and promoted cell proliferation in Hnf4α-KO mice, compared to control mice. In line with our observations in primary mouse hepatocytes, Hnf4α-as1-knockdown in human L02 hepatocytes also alleviated cell apoptosis and Hnf4α-as1-overexpression had the opposite effects (Supplementary Fig. S4A).

3.4. Hnf4α depletion alleviates apoptosis in hepatic I/R injury

An excessive inflammatory response inevitably causes cell death, which is accompanied by varying degrees of liver damage [20]. Therefore, we further examined the effects of Hnf4α on cell apoptosis. As expected, the I/R model showed a significant elevation in apoptosis, and we found fewer TUNEL-positive cells in liver tissues from Hnf4α-KO mice than in liver tissues from Hnf4α-WT mice (Fig. 4A). Flow cytometry assay showed that Hnf4α depletion reduced the apoptotic levels of hepatocytes subjected to A/R operation compared to Hnf4α-WT group (Fig. 4B). The results of the caspase-3 activity assay and DNA fragmentation ELISA also suggested dramatic decrease in apoptotic levels with Hnf4α depletion (Fig. 4C and D). As shown by qRT-PCR and Western blot, I/R-induced cell death was markedly blunted in the livers of Hnf4α-KO deficient mice, as evidenced by the expression of apoptotic markers (BCL-2, Bax and cleaved caspase-3) (Fig. 4E and F). Moreover, less LDH was released from Hnf4α-KO deficient hepatocyte cultures than from control hepatocytes (Fig. 4G). The CCK-8 assay results in Fig. 4H showed that Hnf4α-deficiency enhanced cell viability and promoted cell proliferation in Hnf4α-KO mice, compared to control mice. In line with our observations in primary mouse hepatocytes, Hnf4α-as1-knockdown in human L02 hepatocytes also alleviated cell apoptosis and Hnf4α-as1-overexpression had the opposite effects (Supplementary Fig. S4A).
ROS levels by dihydroethidium staining (DHE) and DHE staining showed that in the livers of Hnf4αos-KO mice, intracellular concentrations of ROS were markedly decreased compared with those in control mice subjected to I/R operation (Fig. 6B). As indicators of oxidative stress damage, MDA and 4-HNE contents were tested in I/R-treated liver tissues. In line with the results of DHE staining, Hnf4αos knockout abrogated the I/R-induced increase in MDA/4-HNE contents and resulted in lower MDA/4-HNE contents (Fig. 6C; Supplementary Fig. S5). Next, we speculated whether the activities of ROS scavenging enzymes were increased, which were induced by Hnf4αos knockout-mediated PGC1α upregulation. The hepatic activities of ROS scavenging enzymes (SOD, CAT and GPX) were increased in the KO groups compared with the WT mice following the I/R operation (Fig. 6D). In line with the activities of antioxidative enzymes, the mRNA levels of Sod1, Sod2, Cat and Gpx1 were dramatically decreased after mice were subjected to the I/R procedure. However, Hnf4αos-KO enhanced the expression of those enzymes in the I/R model compared to that in WT mice (Fig. 6E). We then constructed an shPGC1α adenovirus and transferred PGC1α-deficient vectors into Hnf4αos-KO mice and primary hepatocytes (Supplementary Figs. S6A and B). Reversibility experiments ensured that PGC1α knockdown abrogated the reduced oxidative stress damage induced by Hnf4αos-KO and that Hnf4αos-KO-mediated protection against hepatic I/R injury was also reversed by PGC1α deficiency (Fig. 6F–L; Supplementary Figs. S6C–E).

3.6. Hnf4αos promotes the stability of Hnf4α mRNA

To determine how Hnf4αos manipulates hepatocyte viability by regulating PGC1α, we further conducted an in-depth study of the structural features of Hnf4αos. Hnf4αos is a natural antisense transcript (NAT) of Hnf4α known for its transcriptional regulation of several hepatic genes. As reported previously, antisense lncRNAs are used to bind
to the respective sense strand mRNA to form a duplex strand, which enhances the stability of the latter mRNA [21–23]. We further explored the mRNA and protein levels of Hnf4α accompanied by Hnf4α os alteration. As shown in Fig. 7A and B, downregulated Hnf4α os expression significantly decreased the mRNA and protein levels of Hnf4α. Conversely, Hnf4α os overexpression enhanced the expression levels of Hnf4α. Then, we constructed Hnf4α os overexpression and Hnf4α knockdown adenovirus vectors (Supplementary Figs. S7A–B). However, the variations in Hnf4α expression had no effects on the Hnf4α os transcript (Fig. 7C). To determine whether Hnf4α os regulated the stability of Hnf4α mRNA, we performed an RNA stability assay. Hnf4α os-KO and Hnf4α os-overexpressing hepatocytes were treated with actinomycin D (ActD) to inhibit mRNA transcription. qRT-PCR analysis showed that Hnf4α os downregulation markedly shortened the half-life of Hnf4α mRNA and that Hnf4α os overexpression elevated the level of Hnf4α mRNA (Fig. 7D). These findings indicate that Hnf4α os positively regulates Hnf4α mRNA expression.

In the case of the Hnf4α os/Hnf4α pair, complementarity was noted in both transcripts (Fig. 7E). To determine the existence of a sense-antisense RNA duplex, a ribonuclease protection assay (RPA) was performed and showed that the complementary region was protected from degradation by RNase, indicating an RNA duplex between lncRNA Hnf4α os and Hnf4α mRNA (Fig. 7F). Furthermore, the biotin-labeled RNA pulldown assay and tagged RNA affinity purification (TRAP)
assay revealed a strong interaction between Hnf4αos and endogenous Hnf4α mRNA (Fig. 7G and H). We noticed that enhanced expression of Hnf4α worsened liver injury (Fig. 7I–K, Supplementary Figs. S7C and E) and activated a sterile inflammatory response (Fig. 7K, Supplementary Fig. S7D), as evidenced by more severe tissue necrosis and cytokine/chemokine release, which could be ameliorated by Hnf4αos-KO. Collectively, these data support the conclusion that Hnf4αos increased the stability of Hnf4α mRNA, which was modulated by the duplex of Hnf4αos/Hnf4α.

3.7. Hnf4α mediates the suppressive effect of miR-23a on PGC1α

To further confirm the exact mechanism through which Hnf4αos regulated PGC1α expression, we speculated that Hnf4α exerted a directive transcriptional inhibitory effect on PGC1α by acting as a transcription factor (TF). In support of our hypothesis, we analyzed the PGC1α promoter sequences using the UCSC, JASPAR, SWISSREGULON and PROMO algorithms and surprisingly found that the promoter region of PGC1α has a candidate binding site for TF-Hnf4α (Fig. 8A). The luciferase reporter assay demonstrated no relationship between Hnf4α and the transcriptional activity of PGC1α (Fig. 8B). Numerous reports have shown that miR-23a is a key regulator of PGC1α expression [24–26], and we found a physical interaction between miR-23a and PGC1α through the miRDB, RNAinter, TargetScan and miRmap databases (Fig. 8C). The luciferase reporter assay confirmed that miR-23a was a negative regulator of PGC1α (Fig. 8D). Then, we performed qRT-PCR to detect the RNA level of miR-23a between Hnf4αos and Hnf4α (Supplementary Fig. S8). To confirm that miR-23a contributes to the function of PGC1α in hepatic I/R injury, we constructed miR-23a mimics and inhibitors. Western blot analysis showed that miR-23a and Hnf4α deficiency dramatically upregulated the protein levels of PGC1α, conversely, miR-23a/Hnf4α overexpression suppressed PGC1α protein expression (Fig. 8E). To confirm that miR-23a contributes to the function of PGC1α in hepatic I/R injury, we constructed miR-23a mimics and inhibitors. Western blot analysis showed that miR-23a and Hnf4α deficiency dramatically upregulated the protein levels of PGC1α, conversely, miR-23a/Hnf4α overexpression suppressed PGC1α protein expression (Fig. 8E). Given that the considerable lncRNA Hnf4αos enhances the stability of Hnf4α, we speculated whether TF-Hnf4α mediated the transcription of miR-23a and subsequently attenuated the expression of PGC1α. Intriguingly, based on the prediction by the database, we found that Hnf4α binding sites in the promoter of miR-23a and revealed that the transcription of miR-23a was dramatically
activated by TF-Hnf4α (Fig. 8F and G). Consistently, nuclear extracts were obtained and used for an electrophoretic mobility shift assay (EMSA), and the results identified marked DNA-protein binding activity in mouse primary hepatocytes (Fig. 8H). Furthermore, chromatin immunoprecipitation (ChIP) assays provided evidence for the direct interaction of Hnf4α with the miR-23a promoter (Fig. 8I). Together, the data above showed that a significant interaction between the promoter region of miR-23a and TF-Hnf4α.

As shown by confocal microscopy examination, both Hnf4αos and miR-23a levels were increased in A/R-treated cells compared with normoxic cells by dual-RNA FISH detection (Fig. 9A). Moreover, the primary hepatocytes subjected to Hnf4αos-KO exhibited almost no red/green fluorescence signals, while the fluorescence signals of WT-cells were much stronger (Fig. 9B). Further experiments validated that miR-23a overexpression attenuated the oxidative stress induced by Hnf4α overexpression (Fig. 9C). Importantly, both Hnf4α and miR-23a also abrogated the antioxidative effects induced by Hnf4αos-KO, and miR-23a knockdown suppressed the oxidative activation of Hnf4α overexpression as demonstrated by Fig. 9F–I. Finally, miR-23a deficiency ameliorated liver damage and the inflammatory response induced by Hnf4α overexpression (Supplementary Figs. S9D–G). These data suggest that Hnf4α mediates the suppressive effect of miR-23a on PGC1α.

4. Discussion

HIFI is the most important effector in liver surgery, particularly in liver transplantation. In the present study, we found a differentially expressed lncRNA – Hnf4αos during HIFI progression in both human and mouse models. Knocking out Hnf4αos in hepatocytes significantly suppressed the oxidative stress – induced hepatic injury and inhibited the inflammatory response during HIFI both in vitro and in vivo. Using the integrated approaches of bioinformatic analysis, we identify the potential interaction of Hnf4αos and PGC1α, and Hnf4αos facilitated the RNA decay of PGC1α by ceRNA function. Thus, Hnf4αos could be a promising therapeutic target of HIFI.

Oxidative stress – induced liver injury plays dominant roles during HIFI progression. The production of ROS caused by the oxidative stress response triggers peroxidation reactions, which activate the apoptotic pathway and decrease hepatocyte viability in hepatic I/R injury [27,28]. Therefore, regulation of ROS metabolism is expected to have the potential to effectively protect the liver against I/R injury. In the current study, we found that the reduced Hnf4αos level exhibited a significant antioxidative effects by regulating the balance of ROS scavenging and accumulation systems. Considering that PGC1α is key mediator of ROS metabolism [13,18], we speculated that Hnf4αos regulated the oxidative stress in HIFI by targeting PGC1α. To verify our hypothesis, Hnf4αos-KO mice were generated. Hnf4αos deficiency in vivo and in vitro reduces the
degree of hepatic I/R and improves hepatic function in mice by PGC1α elevation-mediated ROS scavenging compared to WT conditions. Functionally, lncRNAs can bind not only to proteins but also to DNA and RNA, rendering lncRNAs a crucial factor in protein-nucleic acid/nucleic acid-nucleic acid networks. Several studies, including ours, have provided strong evidence that NATs regulate the expression of their sense protein-coding mRNAs [21, 22]. Here, our findings demonstrated that Hnf4α-sos and Hnf4α formed an RNA – RNA duplex and further promoted Hnf4α mRNA stability, which consequently enhanced the protein level of Hnf4α as shown by Western blot.

Hnf4α generally functions as a transcription factor in the liver and has been reported to play prominent roles in cell proliferation, cell differentiation, lipid metabolism and gluconeogenesis [29–32]. Several studies have revealed that Hnf4α is a key regulator in inhibiting hepatoctye proliferation. Walesky et al. found that hepatocyte-specific depletion of Hnf4α induced increased levels of cell proliferation. Further microarray analysis demonstrated that a significant number of genes known to be promitogenic were upregulated by Hnf4α-deficiency [33]. It has been reported that Hnf4α promoted the transcriptional activity of ASK1, which is a typical proapoptotic mediator in MAPK pathway [34] and Mai et al. confirmed the antipotptic potential of Hnf4α-deficiency in endometriosis [35]. Furthermore, a study related to viral hepatitis suggested that knocking down Hnf4α markedly inhibited HBV RNA transcripts and respective DNA replication intermediates, which played a key role in delaying the progression of HBV-induced hepatitis [36]. Although Hnf4α exerts antineoplastic activity in HCC, Hnf4α was reported to act as an oncogene in gastrointestinal adenocarcinomas and pancreatic cancer [37,38], indicating multiple roles of Hnf4α. In our study, we found that Hnf4α served as a TF binding site in the promoter of miR-23a and subsequently further activated its transcription, showing antiproliferative and proapoptotic effects in HIRI accompanied by Hnf4α depletion.

Although our clinical data showed a downward trend of Hnf4α-as1 in patients who underwent partial liver resections, Hnf4α and miR-23a were also downregulated simultaneously during I/R insult, which may be due to the species differences between humans and mice [39] (Supplementary Fig. S10). More importantly, Hnf4α-as1 deficiency also exerted hepatoprotective effects on the process of HIRI, and Hnf4α-as1 overexpression had the opposite effects. Consequently, from this perspective, clinical therapeutic strategies targeting Hnf4α-as1 can be reasonably established.

5. Conclusions

In conclusion, our findings demonstrate a strategy to manipulate PGC1α activity by Hnf4α-sos. Specifically, Hnf4α-sos-mediated stabilization of Hnf4α mRNA reverses the protective effect of PGC1α by upregulating miR-23a expression, leading to a reduction in the scavenging levels of ROS and exacerbation of hepatic I/R injury. Thus, targeting Hnf4α-as1 may provide potential clinical benefits for liver I/R injury.

Declaration of competing interest

No potential conflicts of interest were disclosed.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102498.

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