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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Apolipoprotein A-1 Accelerated Liver Regeneration Through Regulating Autophagy Via AMPK-ULK1 Pathway

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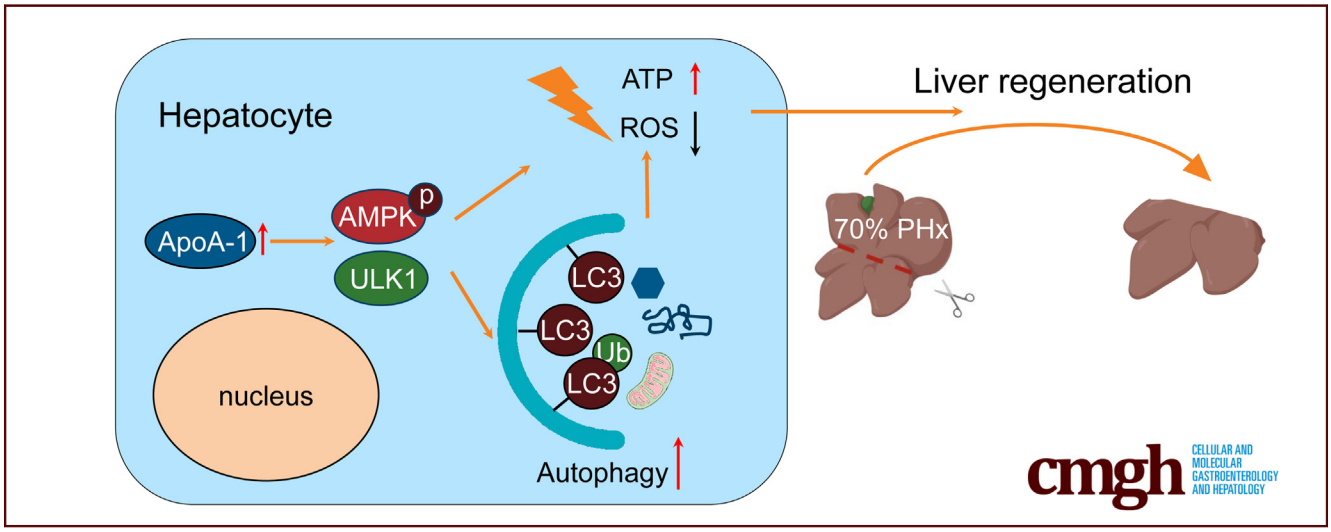

SUMMARY

Apolipoprotein A-1 accelerated liver regeneration through promoting autophagy in hepatocytes via adenosine 5'-monophosphate-activated protein kinase–unc-51-like autophagy activating kinase 1 pathway, providing a potential therapeutic target for liver injury.

BACKGROUND & AIMS: Apolipoprotein A-1 (ApoA-1), the main apolipoprotein of high-density lipoprotein, has been well studied in the area of lipid metabolism and cardiovascular diseases. In this project, we clarify the function and mechanism of ApoA-1 in liver regeneration.

METHODS: Seventy percent of partial hepatectomy was applied in male ApoA-1 knockout mice and wild-type mice to investigate the effects of ApoA-1 on liver regeneration. D-4F (ApoA-1 mimetic peptide), autophagy activator, and AMPK activator were used to explore the mechanism of ApoA-1 on liver regeneration.

RESULTS: We demonstrated that ApoA-1 levels were highly expressed during the early stage of liver regeneration. ApoA-1 deficiency greatly impaired liver regeneration after hepatectomy. Meanwhile, we found that ApoA-1 deficiency inhibited autophagy during liver regeneration. The activation of autophagy protected against ApoA-1 deficiency in inhibiting liver regeneration. Furthermore, ApoA-1 deficiency impaired autophagy through AMPK-ULK1 pathway, and AMPK activation significantly improved liver regeneration. The administration of D-4F could accelerated liver regeneration after hepatectomy.

CONCLUSIONS: These findings suggested that ApoA-1 played an essential role in liver regeneration through promoting autophagy in hepatocytes via AMPK-ULK1 pathway. Our findings enrich the understanding of the underlying mechanism of liver regeneration and provide a potential therapeutic strategy for liver injury. *(Cell Mol Gastroenterol Hepatol 2024;17:539–551; https://doi.org/10.1016/j.jcmgh.2023.12.004)*

Keywords: Liver Regeneration; ApoA-1; Autophagy; AMPK Pathway.

The liver is a unique organ with remarkable regenerative ability to restore liver mass and function after surgical resection, viral infection, or toxic exposure.[1,2] Based on this remarkable feature, partial hepatectomy (PH) and liver transplantation have become the effective strategy to treat end-stage liver diseases. Therefore, elucidating the molecular basis and signal transduction mechanisms of liver regeneration will provide a new intervention strategy for liver functional recovery after hepatectomy of malignant tumors and cirrhotic liver tissues.[3]

Apolipoprotein A-1 (ApoA-1), the main apolipoprotein in high-density lipoprotein, has been well studied in the area of

lipid metabolism and cardiovascular diseases. Previous studies have demonstrated that ApoA-1 could mediate reverse cholesterol transport through interactions with ATP-binding cassette transporter A1 and ATP-binding cassette transporter G1.[4,5] Moreover, ApoA-1 also played an essential role in energy metabolism and homeostasis. Recent studies suggested that ApoA-1 could regulate mitochondrial function to protect against myocardial infarction.[6] ApoA-1 also promoted mitochondrial biogenesis in skeletal muscle via AMPK pathway.[7] Our previous study demonstrated that ApoA-1 protected against hepatic ischemia-reperfusion injury by inhibiting macrophages pyroptosis through TLR4- nuclear factor-κB pathway.[8] In addition, ApoA-1 enhanced cell proliferation through the cells surface ATP synthase.[9,10] Our previous study also demonstrated that ApoA-1 could promote small-for-size fatty liver graft regeneration after liver transplantation.[11] However, the role and mechanism of ApoA-1 in liver regeneration after PH were still unclear.

Autophagy is a kind of catabolic process that degrades longevous proteins and cellular organelles, such as endoplasmic reticulum and mitochondria, for an alternative energy source during nutrient deficiency.[12] Recent studies reported that autophagy also played a crucial role in liver regeneration. Regeneration of the liver demands massive energy and cellular materials for DNA replication and cell division.[1] Autophagy efficiently provides substances required for the regeneration process and removes dysfunctional organelles and proteins, which contributed to the coordinated hepatocytes proliferation during regeneration.[13-15]

In this research, we intended to clarify the function and mechanism of ApoA-1 in liver regeneration after PH. We found that ApoA-1 was obviously upregulated during the early stage of liver regeneration, and ApoA-1 deficiency greatly impaired liver regeneration. Moreover, ApoA-1 deficiency diminished autophagy level during liver regeneration, and the activation of autophagy protected against ApoA-1 deficiency in inhibiting liver regeneration. Furthermore, the upregulated autophagy process during liver regeneration was demonstrated to be regulated by ApoA-1 through AMPK-ULK1 pathway, and the activation of AMPK significantly promoted liver regeneration. The administration of ApoA-1 mimic peptide D-4F could accelerate liver regeneration after PH. Taken together, this study revealed that ApoA-1 accelerated liver regeneration through regulating autophagy via AMPK-ULK1 pathway.

Results

ApoA-1 Was Upregulated During Liver Regeneration

To clarify the expression level of ApoA-1 in liver regeneration, 70% PH was performed on wild-type (WT) mice, and the levels of ApoA-1 were measured at indicated time points. Remarkably, the mRNA level of ApoA-1 reached its peak at Day 1 after PH, and descended to its baseline at Day 7 (Figure 1*A*). Meanwhile, the ApoA-1 protein level gradually increased, and reached its maximum at Day 2 after PH (Figure 1*B* and *C*). The expression of ApoA-1 in liver sections after PH was confirmed by immunohistochemistry staining (Figure 1*D*). It could be observed that the time when ApoA-1 expression reached its maximum was at the same time when the peak of DNA synthesis in hepatocytes took place, which was about 40 hours following PH.[16] It was also showed that ApoA-1 was mainly induced in hepatic parenchymal cells according to the immunohistochemistry and immunofluorescence staining results (Figure 1*D* and *E*). Briefly, ApoA-1 was upregulated in hepatocytes at the early stage of liver regeneration.

ApoA-1 Deficiency Impaired Liver Regeneration After Partial Hepatectomy

To fully elucidate the pathophysiological role of ApoA-1 in liver regeneration, ApoA-1 whole-body knockout (KO) mice were generated via targeted mutation and PH was performed on ApoA-1 KO and WT mice. It was obvious that the survival probability after PH was lower in KO group than WT group (Figure 2*A*). Meanwhile, compared with WT group, the liver weight/body weight ratio (LW/BW) of KO group significantly decreased within 14 days after PH (Figure 2*B*). Notably, the relatively higher alanine transaminase (ALT) and aspartate transaminase (AST) levels were detected in KO group at the early stage after PH (Figure 2*C*), indicating the delayed liver function recovery. Immunohistochemistry staining showed Ki-67 positive rate was higher in WT group than KO group, especially at 2 days after PH (Figure 2*D*). Other cell proliferation and cell cycle markers were also detected via real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting. The mRNA and protein levels of PCNA and Cyclin D1 were significant lower in KO group at early stage after PH (Figure 2*E* and *F*). Taken together, these results demonstrated that ApoA-1 KO impaired liver regeneration after PH.

ApoA-1 Deficiency Impaired Proliferation of Hepatocytes In Vitro

To further investigate the effect of ApoA-1 on hepatocyte proliferation, AML-12 cells were transfected with siRNA targeting ApoA-1 to reduce its level. The transfection efficiency was verified via RT-qPCR and Western blotting (Figure 3*A*). Cell counting kit (CCK)-8 and colony formation assays demonstrated that ApoA-1 deficiency significantly

***Authors share co-first authorship.**

Abbreviations used in this paper: **ALT, alanine transaminase; ApoA-1, apolipoprotein A-1; AST, aspartate transaminase; CCK-8, cell counting kit-8; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EdU, 5-Ethynyl-20-deoxyuridine; KO, knockout; LW/BW, liver weight/body weight ratio; mTOR, mammalian target of rapamycin; PH, partial hepatectomy; RT-qPCR, real-time quantitative polymerase chain reaction; WT, wild-type.**

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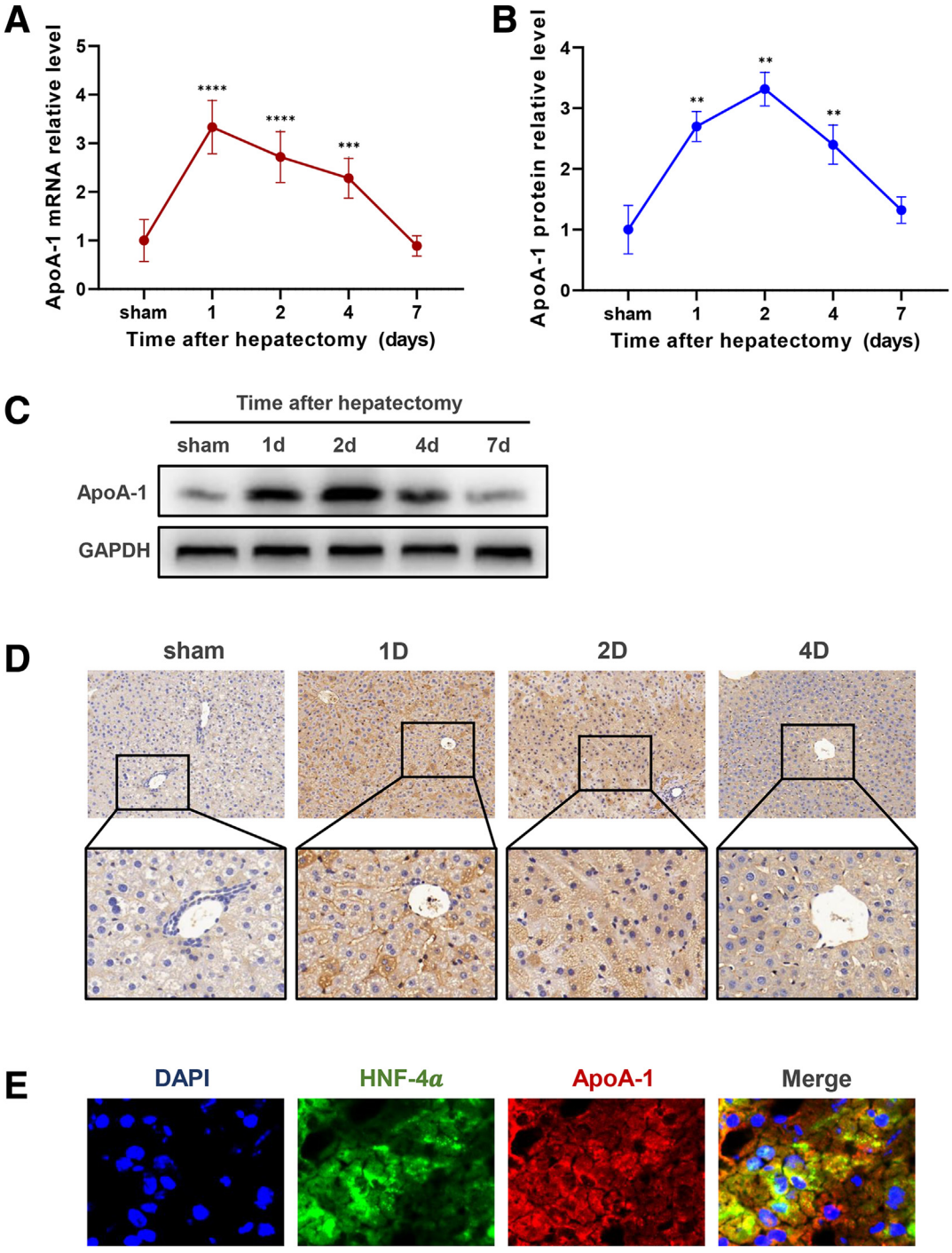

Figure 1. ApoA-1 was upregulated in hepatocytes after PH. (*A*) The RT-qPCR analysis of the ApoA-1 expression in livers from WT mice at the indicated time points after PH (n = 3–7). (*B*, *C*) Western blot analysis of the ApoA-1 expression in livers from WT mice at the indicated time points after PH (n = 3). (*D*) Immunohistochemical staining of ApoA-1 (*brown*) in liver sections from WT mice at the indicated time points after PH. (*E*) Immunofluorescence staining of ApoA-1 (*red*) in liver sections from WT mice 2 days after PH. All nuclei were stained with DAPI (*blue*). Cytoplasm of hepatocytes were stained with HNF-4α (*green*). $^{**}P < .01$. $^{***}P < .001$. $^{****}P < .0001$.

reduced hepatocytes proliferation in vitro (Figure 3*B* and *C*). The protein levels of PCNA and Cyclin D1 were down-regulated in ApoA-1 deficiency group (Figure 3*D*). Similarly, 5-ethynyl-20-deoxyuridine (EdU) incorporation assay revealed that the number of EdU-positive cells was decreased in ApoA-1 deficiency AML-12 cells (Figure 3*E*). Altogether, these observations suggested that ApoA-1 deficiency impaired hepatocytes proliferation in vitro.

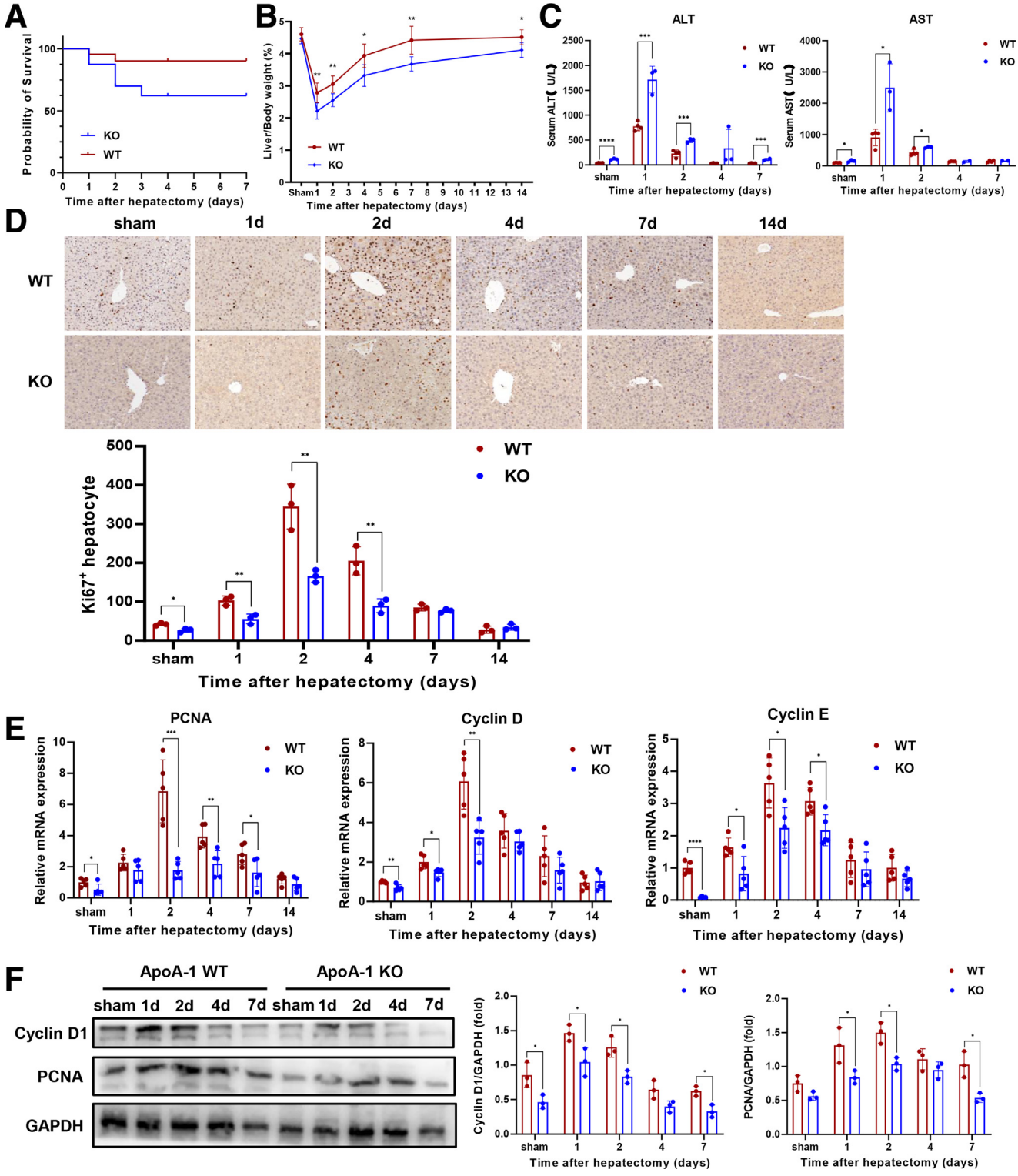

Figure 2. ApoA-1 deficiency impaired liver regeneration after PH. WT and KO mice were subjected to PH. (*A*) The probability of survival of WT and KO mice at the indicated time points after PH. (*B*) The liver weight/body weight ratio of WT and KO mice at the indicated time points after PH (n = 5–6). (*C*) Serum levels of ALT and AST of WT and KO mice at the indicated time points after PH (n = 3–4). (*D*) Representative images of immunohistochemical staining of Ki-67 (*brown*) in liver sections from WT and KO mice at the indicated time points after PH (n = 3). (*E*) The mRNA levels of PCNA, Cyclin D1, Cyclin E in livers of WT and KO mice at the indicated time points after PH (n = 5). (*F*) The protein levels of PCNA, Cyclin D1 in livers of WT and KO mice at the indicated time points after PH (n = 3). $^{*}P < .05$. $^{**}P < .01$. $^{***}P < .001$.

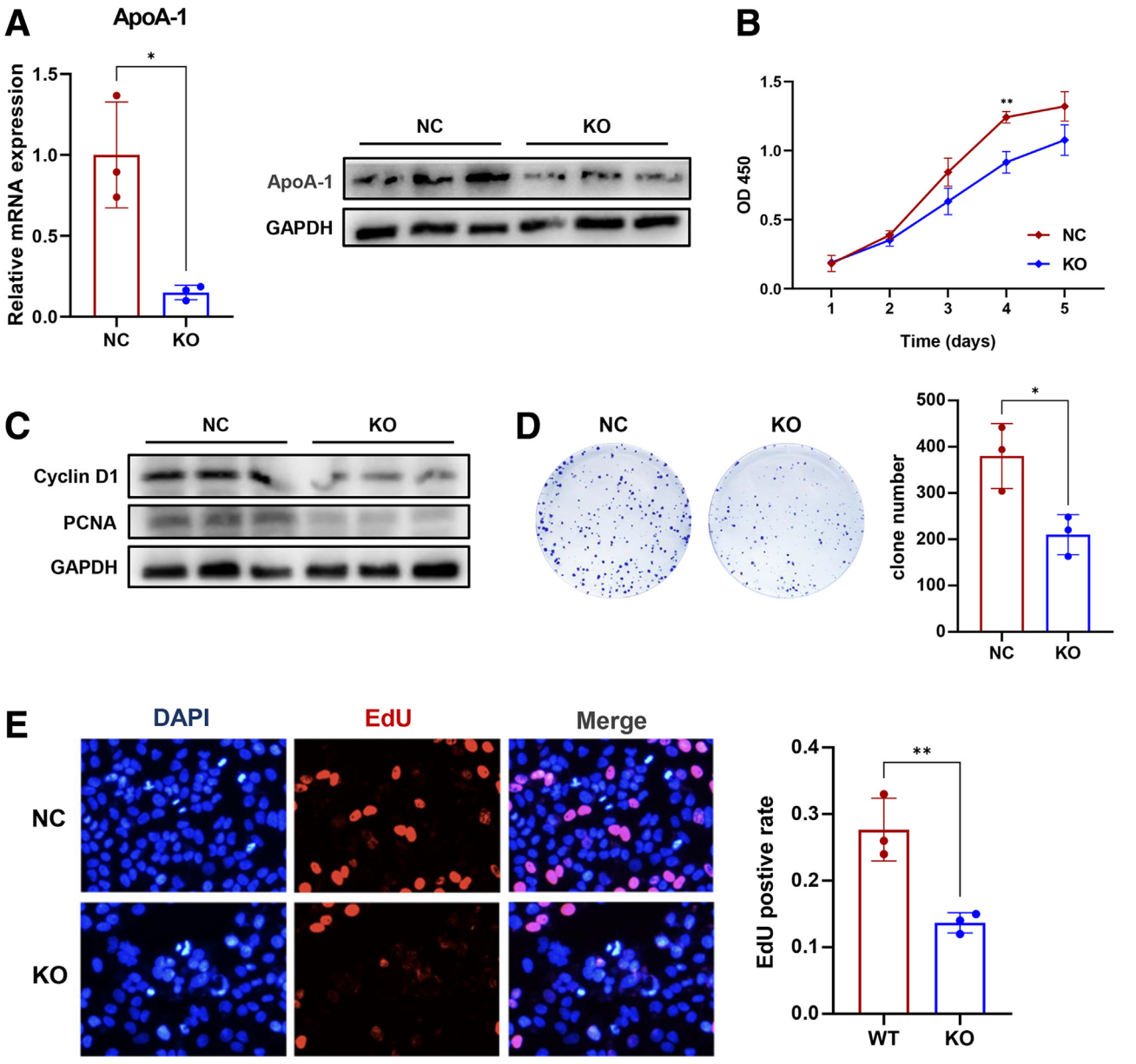

Figure 3. ApoA-1 deficiency impaired proliferation of hepatocytes in vitro. AML12 cells were transfected with ApoA-1 siRNA (KO) or control siRNA (NC). (*A*) mRNA and protein expression of ApoA-1 in KO and NC group of AML12 cells (n = 3). (*B*) The proliferation of KO and NC cells was determined by CCK8 assay at 1, 2, 3, 4, and 5 days (n = 3). (*C*) The colony-formation assay determines the proliferation of KO and NC cells. (*D*) The protein levels of PCNA, Cyclin D1 in KO and NC group of AML12 cells (n = 3). (*E*) EdU incorporation assay determines the proliferation of KO and NC cells (n = 3). $^{*}P < .05$. $^{**}P < .01$.

ApoA-1 Deficiency Inhibited Autophagy During the Early Stage of Liver Regeneration

To elucidate the mechanisms of the impaired liver regeneration caused by the deficiency of ApoA-1 in hepatocytes, we observed liver samples 2 days after PH via transmission electron microscopy. The pictures of transmission electron microscopy revealed that the number of autophagosome and autolysosome were less in KO group than WT group. Meanwhile, the structure of the mitochondria crest was indistinct in KO group, suggesting that mitochondria underwent more severe damage in KO group (Figure 4*A*). Afterward, we analyzed the expression levels of several major proteins involved in autophagy. As shown in Figure 4*B*, ATG5, ATG7, and LC3II/LC3I ratio were lower in KO group, whereas p62 were highly expressed in KO group. Furthermore, we used chloroquine to inhibit lysosomal degradation and confirmed that the lower LC3II/LC3I ratio in KO group was caused by decreased autophagy flux (Figure 4*C*). Because the process of liver regeneration requires energy, we measured ATP levels and found that the ATP levels were both downregulated in ApoA-1 deficiency mice (Figure 4*D*) and AML12 cells (Figure 4*E*). Moreover,

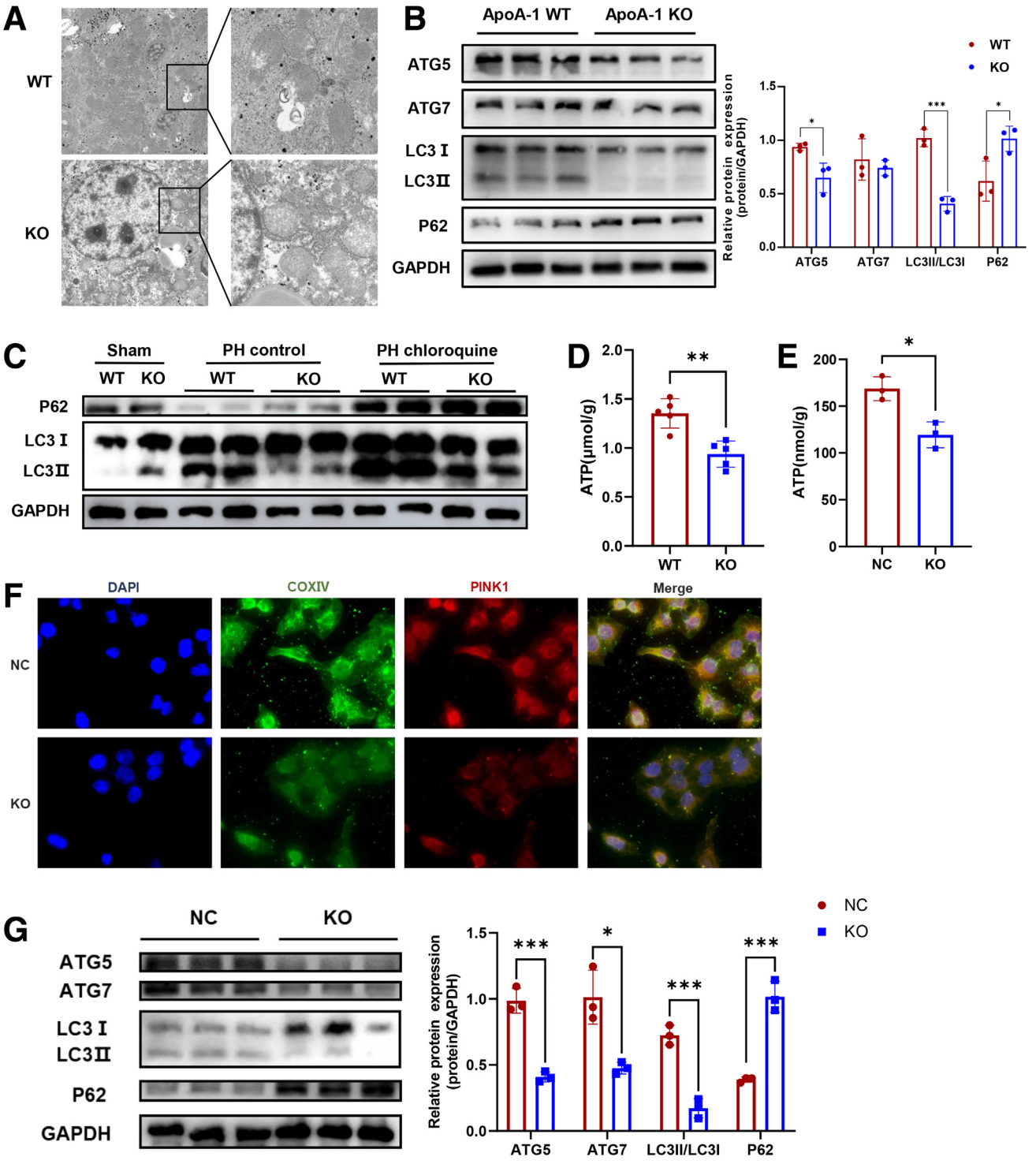

Figure 4. ApoA-1 deficiency inhibited autophagy during the early stage of liver regeneration. (*A*) Transmission electron microscopy images of liver sections from WT and KO mice 2 days after PH. (*B*) The protein levels of autophagy related proteins in livers of WT and KO mice at 2 days after PH (n = 3). (*C*) The protein levels of P62 and LC3B in livers of sham group and PH group injected with chloroquine or control. (*D*) ATP levels in WT and KO mice at 2 days after PH (n = 5). (*E*) ATP levels in KO and NC group of AML12 cells (n = 3). (*F*) Representative images of immunofluorescence staining of COXIV (*green*) and PINK1 (*red*) in KO and NC group of AML12 cells. The nuclei were stained with DAPI (*blue*). (*G*) The protein levels of autophagy-related proteins in KO and NC group of AML12 cells (n = 3). $^{*}P < .05$. $^{**}P < .01$. $^{***}P < .001$.

the immunofluorescence staining of the AML12 cells showed that the mitochondrial marker COXIV and mitophagy marker PINK1 expression were lower in ApoA-1 deficiency group than that in NC group (Figure 4*F*). The expression of proteins involved in autophagy also showed that ApoA-1 deficiency reduced autophagy process in the AML12 cells (Figure 4*G*). These results demonstrated that ApoA-1 KO inhibited autophagy during the early stage of liver regeneration.

Activation of Autophagy Protected Against ApoA-1 Deficiency in Inhibiting Liver Regeneration

To further verify whether ApoA-1 deficiency inhibited liver regeneration through regulating autophagy, we used autophagy activator MG-132 to eliminate the inhibitory effect of ApoA-1 deficiency on autophagy (Figure 5*A*). As shown in Figure 5*B*, pretreatment with MG-132 increased the ATG5, ATG7, and LC3II/LC3I ratio, whereas it decreased the expression of p62. The results showed that the LW/BW ratio were higher, whereas ALT and AST levels were lower in KO mice treated with MG-132 than that treated with dimethyl sulfoxide (DMSO) at 2 days after PH (Figure 5*C* and *D*). Meanwhile, MG-132 treatment also eliminated the inhibitory impact of ApoA-1 KO on liver regeneration, because there was no significant difference between WT and KO mice in MG-132 treated group (Figure 5*C* and *D*). Similarly, the same effects of MG-132 were also observed by Ki-67-positive cell rate and protein levels of PCNA and Cyclin D1 in mice treated with MG-132 or DMSO (Figure 5*B* and *E*). Altogether, these results suggested that ApoA-1 KO inhibited liver regeneration via impairing autophagy.

ApoA-1 Deficiency Impaired Autophagy Through AMPK-ULK1 Pathway During Liver Regeneration

Previous studies have shown that autophagy was regulated via mammalian target of rapamycin (mTOR)-dependent and mTOR-independent pathways.[15] To explore the signal transduction pathways underlying ApoA-1-mediated autophagy, Western blotting analysis on liver samples from WT and KO mice 2 days after PH was performed. As shown in Figure 6*A*, there was no significant difference in the protein levels of p-mTOR/mTOR, whereas there was a significant decrease in p-AMPK and ULK1 levels between WT and KO group. These results indicated that ApoA-1 KO impaired autophagy through mTOR-independent AMPK-ULK1 pathway during liver regeneration.

To further verify whether ApoA-1 deficiency inhibited autophagy via AMPK-ULK1 pathway, we used AMPK activator COH-SR4 (Figure 6*B*). As shown in Figure 6*C*, COH-SR4 administration significantly increased the p-AMPK/AMPK and p-ULK1/ULK1 ratio. Furthermore, COH-SR4 treatment significantly upregulated the expressions of autophagy-associated proteins ATG5, ATG7, and LC3II/LC3I ratio in KO group (Figure 6*C*). The activation of AMPK-ULK1 pathway significantly promoted the liver regeneration after PH, as shown by the increased expression of PCNA and Cyclin D1, LW/BW ratio, and Ki-67-positive cells rate, and decreased ALT and AST levels in KO mice compared with corresponding control groups (Figure 6*C-F*). Meanwhile, COH-SR4 treatment can also protect against ApoA-1 deficiency in inhibiting liver regeneration, because there was no significant difference between WT and KO mice in COH-SR4 administrated group (Figure 6*C-F*). Altogether, these results suggested that ApoA-1 KO impaired liver regeneration and autophagy through inhibiting AMPK-ULK1 pathway.

ApoA-1 Mimetic Peptide D-4F Administration Accelerated Liver Regeneration After PH

D-4F is an 18-amino acid peptide that mimics the tertiary structure of ApoA-1, and is easily absorbed without liver toxicity.[17] Previous studies have showed that administration of D-4F can enhance the physiological effects of ApoA-1 in different models.[18,19] Thus, we used D-4F to activate the physiological effects of ApoA-1 during liver regeneration. D-4F or DMSO control was injected introperitoneally into KO mice 1 and 24 hours after PH (Figure 7*A*). An increase in the LW/BW ratio was observed in D-4F treated mice (Figure 7*B*). Meanwhile, D-4F treatment promoted the recovery of liver function, as shown by the decreased levels of ALT and AST in D-4F treated group 2 days after PH (Figure 7*C*). Notably, D-4F treatment accelerated liver regeneration, as shown by the increased Ki-67-positive cells rate, hepatic expressions of proliferation-related genes, and proteins (Figure 7*D-F*). Furthermore, D-4F treatment significantly upregulate the expressions of autophagy-associated proteins ATG5, ATG7, and LC3II/LC3I ratio in liver at 2 days after PH (Figure 7*F*). These results indicated that the administration of D-4F had the potential to accelerate liver regeneration after PH.

Discussion

Liver regeneration is a complex physiological process regulated by various cytokines and growth factors. Elucidating the mechanisms of liver regeneration may provide potential therapeutic targets for the treatment of liver diseases. In this study, we first investigated whether ApoA-1 was involved in the regulation of liver regeneration after hepatectomy. The results showed that ApoA-1 level was upregulated during the early stage of liver regeneration, and was mainly expressed in hepatocytes. In vivo and in vitro studies demonstrated that ApoA-1 deficiency greatly impaired liver regeneration after hepatectomy and hepatocytes proliferation. Furthermore, D-4F administration accelerated liver regeneration after hepatectomy. These results suggested that ApoA-1 may play an important role in liver regeneration after hepatectomy.

Autophagy is an important physiological process in which eukaryotic cells digest and metabolize their own aged organelles and proteins. Recently studies have showed that autophagy played an important role in various physiological processes of liver regeneration.[15] Toshima et al[14] found that hepatic autophagy levels increased with the initiation of liver regeneration and decreased with the cessation of regeneration. PH can lead to substantial mitochondrial damage and a significant decrease in hepatic ATP reserves,

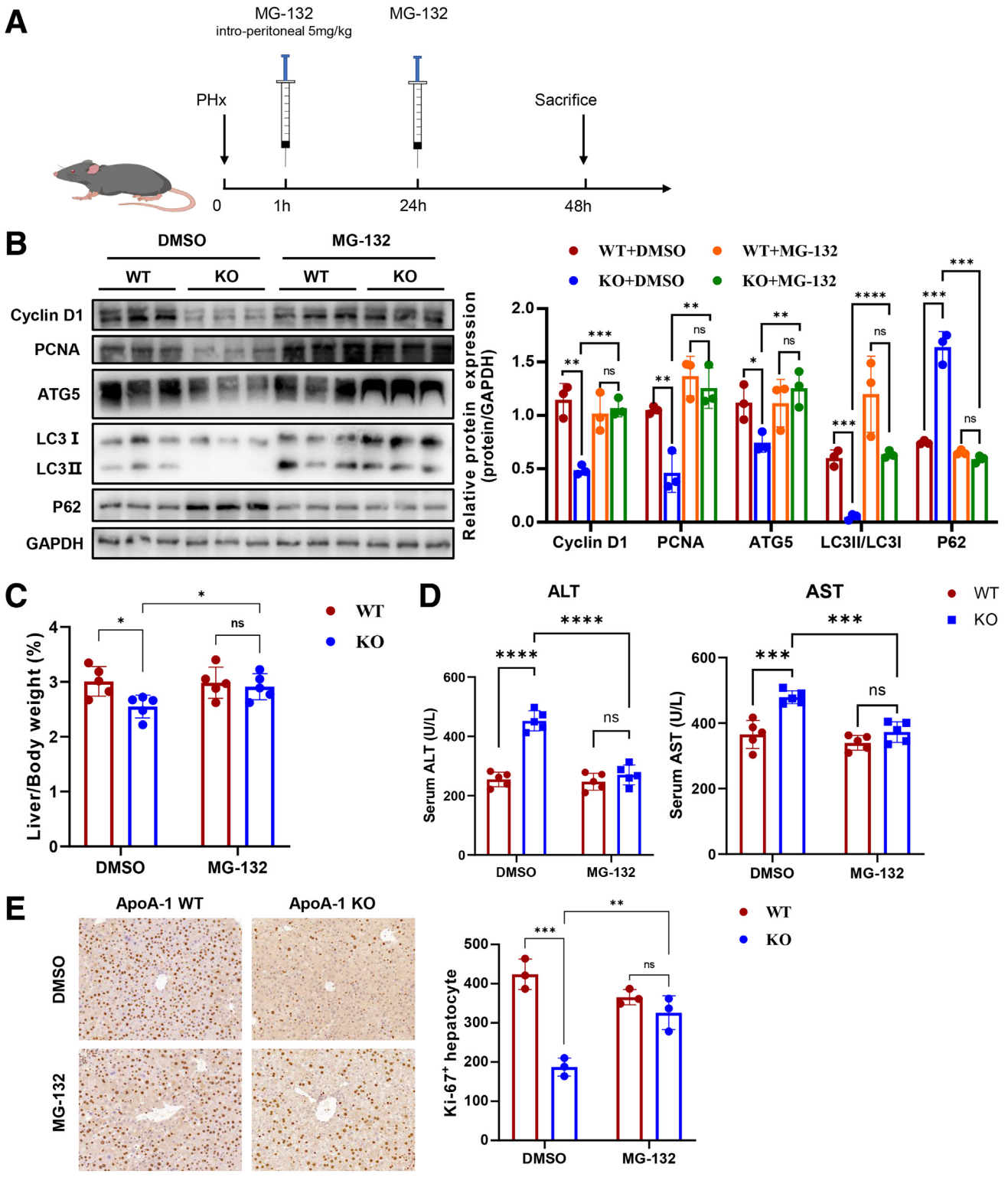

Figure 5. Activation of autophagy protected against ApoA-1 deficiency in inhibiting liver regeneration. Autophagy activator MG-132 or DMSO (control group) was administrated intraperitoneally in WT and KO mice 1 and 24 hours after PH. (*A*) Schematic flowchart of the injection of autophagy activator MG-132. (*B*) The protein levels of Cyclin D1, PCNA, ATG5, LC3B, and P62 in livers from mice of different groups at 2 days after PH (n = 5). (*C*) The liver weight/body weight ratio of mice of different groups at 2 days after PH (n = 5). (*D*) Serum levels of ALT and AST of different groups at 2 days after PH (n = 5). (*E*) Immunohistochemical staining of Ki-67 (*brown*) in liver sections from mice of different groups at 2 days after PH (n = 3). *$P < .05$. **$P < .01$. ***$P < .001$. ****$P < .0001$.

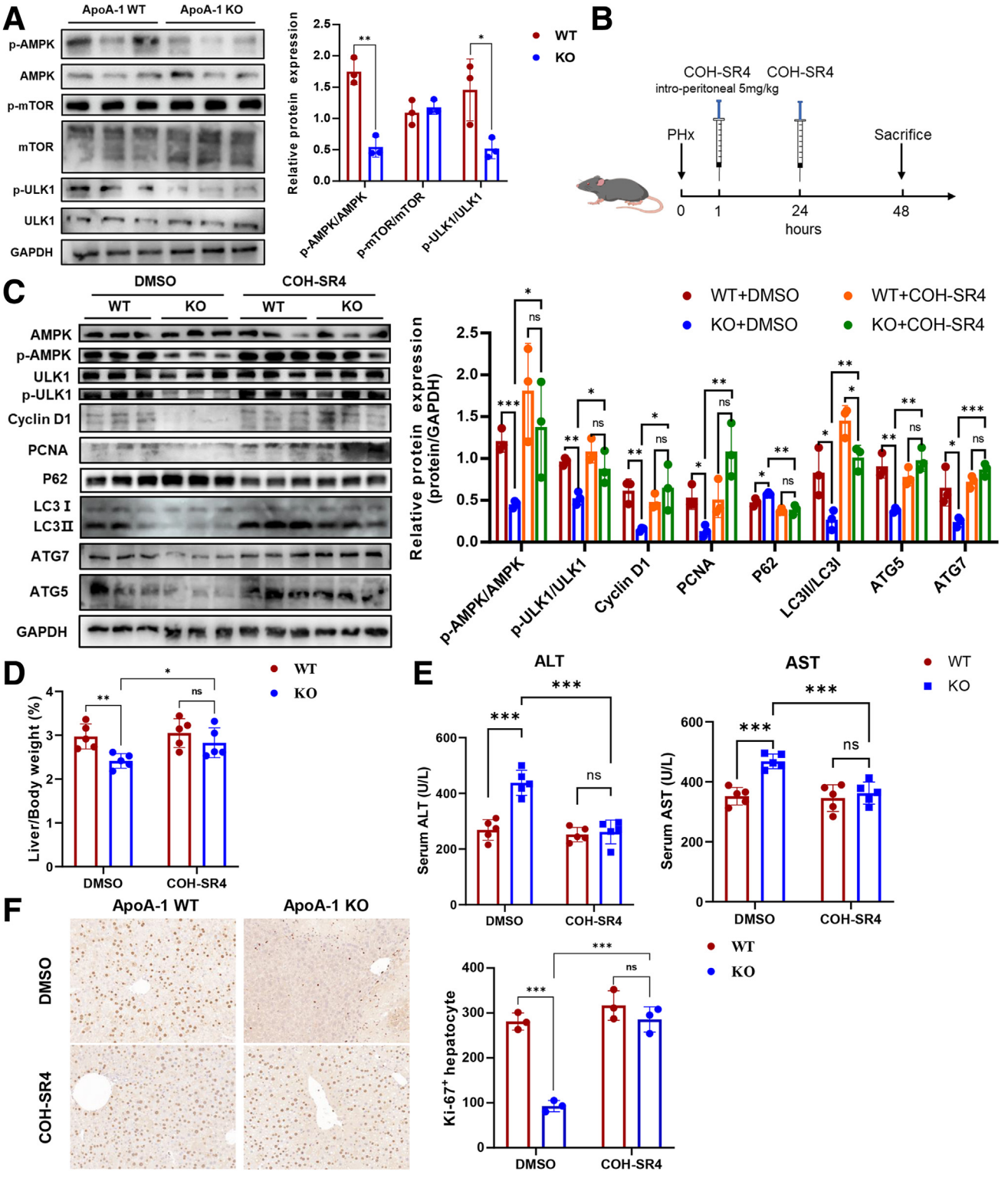

Figure 6. ApoA-1 increased the activation of AMPK-ULK1 pathway during liver regeneration. AMPK activator COH-SR4 or DMSO (control group) was administrated intraperitoneally in WT and KO mice 1 and 24 hours after PH. (*A*) Protein levels of AMPK pathway in livers of WT and KO mice 2 days after PH (n = 3). (*B*) Schematic flowchart of the injection of AMPK activator COH-SR4. (*C*) The protein levels of AMPK pathway, proliferation, and autophagy related proteins in livers of WT and KO mice 2 days after PH (n = 3). (*D*) The liver weight/body weight ratio of mice of different groups at 2 days after PH (n = 5). (*E*) Serum levels of ALT and AST of different groups at 2 days after PH (n = 5). (*F*) Immunohistochemical staining of Ki-67 (*brown*) in liver sections from mice of different groups at 2 days after PH (n = 3). *$P < .05$. **$P < .01$. ***$P < .001$.

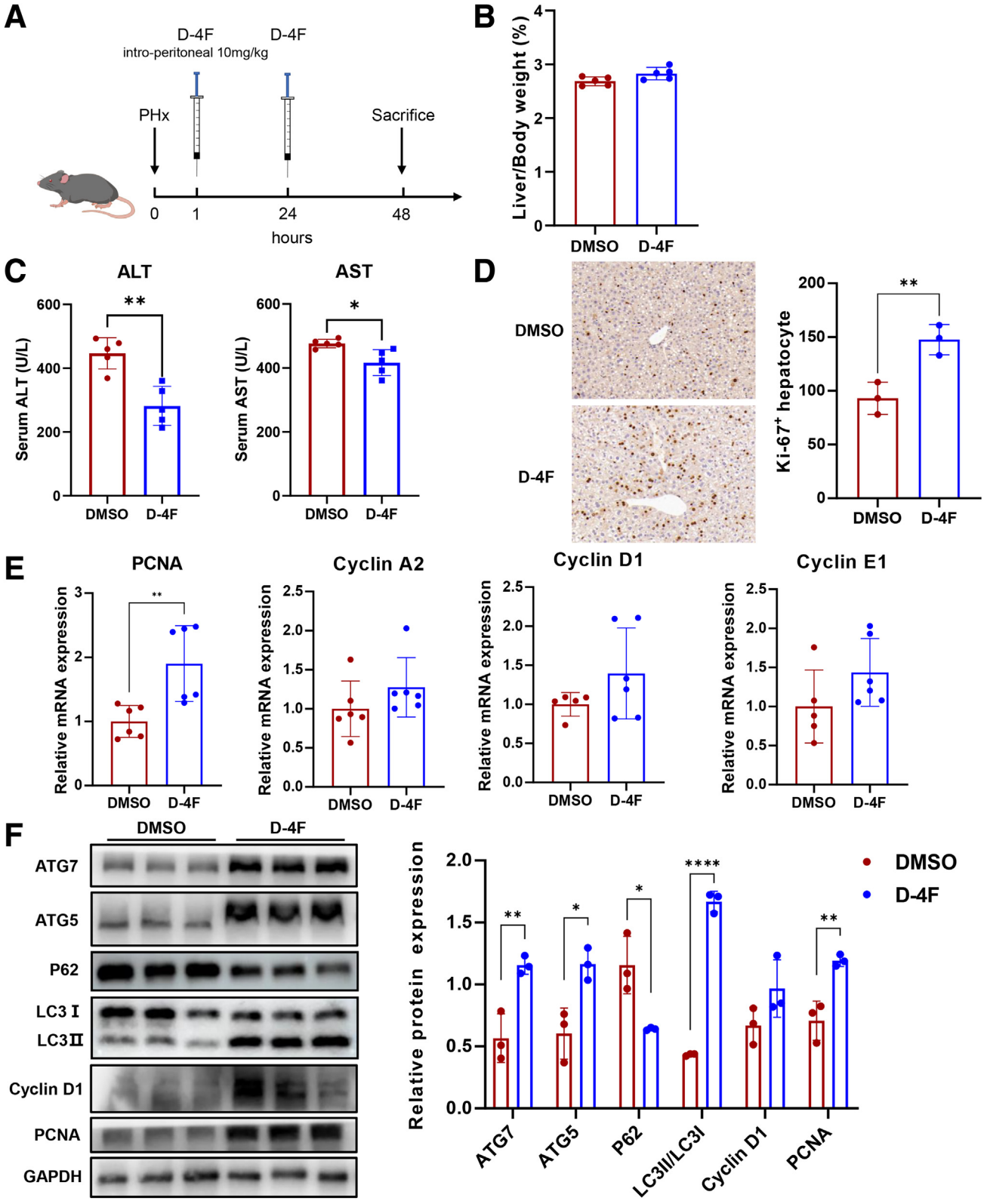

Figure 7. ApoA-1 mimic peptide D-4F accelerated liver regeneration after PH. ApoA-1 mimic peptide D-4F or DMSO (control group) was administrated intraperitoneally in KO mice 1 and 24 hours after PH. (*A*) Schematic flowchart of the injection of D-4F. (*B*) The liver weight/body weight ratio of different groups at 2 days after PH (n = 5). (*C*) Serum levels of ALT and AST of different groups (n = 5). (*D*) Immunohistochemical staining of Ki-67 (*brown*) in liver sections from mice of different groups at 2 days after PH (n = 3). (*E*) The mRNA levels of PCNA, Cyclin A2, Cyclin D1, and Cyclin E in livers from mice of different groups at 2 days after PH (n = 5–6). (*F*) The protein levels of proliferation and autophagy related proteins in livers from mice of different groups at 2 days after PH (n = 3). $^{*}P < .05$. $^{**}P < .01$. $^{****}P < .0001$.

whereas autophagy, especially mitochondrial selective autophagy, is vital to maintain the health of ATP-producing mitochondria during the initial stages of liver regeneration.[20] In this process, mitochondrial selective autophagy degrades dysfunctional mitochondria to facilitate mitochondrial regeneration and maintain the synthesis of ATP, which provides the energy required for liver regeneration.[21,22] Previous studies have shown that ApoA-1 has the potential to regulate autophagy in liver. Rao and Wang[23] found that ApoA-1 relieved nonalcoholic steatohepatitis by promoting hepatocyte autophagy. In the present study, we detected a significant decrease in the ATP levels and amount of autophagosomes and autophagic lysosomes and a severe mitochondrial damage in the livers of ApoA-1 KO mice 2 days after PH. The protein levels of autophagy-related markers also suggested a reduced level of autophagy in the KO group. Furthermore, the activation of autophagy protected against ApoA-1 deficiency in inhibiting liver regeneration. These results demonstrated that the KO of ApoA-1 inhibited liver regeneration after hepatectomy through impairing hepatic autophagy.

Autophagy can be regulated through mTOR-dependent and mTOR-independent signal transduction pathways. Previous studies have reported that the mTOR-dependent signaling pathway has antiproliferative effect, whereas the mTOR-independent signaling pathway has the potential to promote liver regeneration.[15] AMPK played important roles in regulating mTOR-independent signaling pathway.[15] In the mTOR-independent AMPK-ULK1 pathway, AMPK phosphorylates ULK1 directly, which in turn activated autophagosomes formation.[24] In this study, our results revealed that KO of ApoA-1 significantly reduced the phosphorylation and expression of AMPK-ULK1 pathway, while having no effect on p-mTOR/mTOR level, suggesting that the mTOR-independent AMPK-ULK1 pathway may be the key target of ApoA-1 in regulating hepatocyte autophagy and downstream liver regeneration. Further experiments indicated that treatment of mice with the AMPK activator COH-SR4 significantly increased the level of hepatic autophagy after PH, while almost eliminating the inhibitory effect of knocking out ApoA-1 on liver regeneration. These results suggested that ApoA-1 promoted hepatocyte autophagy through upregulating AMPK-ULK1 pathway levels, thereby promoting liver regeneration after hepatectomy.

In addition, we noticed that ApoA-1 KO mice had some ongoing liver injury and impaired hepatocyte proliferation even at basal level in Figure 2*C-E*. These could be explained by the function of ApoA-1 as an important factor in the process of reverse cholesterol transport. As a result of the long-term dysfunction of lipid metabolism in ApoA-1 KO mice, the liver phenotype showed ongoing liver injury and impaired hepatocyte proliferation at basal level. In fact, these factors could be associated with the impaired liver regeneration in ApoA-1 KO mice after PH. Our study concluded that ApoA-1 accelerated liver regeneration through promoting autophagy in hepatocytes via AMPK-ULK1 pathway, which was the focus on its role in autophagy after PH. Both of these factors contribute to the result of impaired liver regeneration.

In conclusion, we found that ApoA-1 played an essential role in liver regeneration through promoting autophagy in hepatocytes via AMPK-ULK1 pathway. The present study revealed the novel biologic role and molecular mechanism of ApoA-1 in regulating liver regeneration, which enriched the understanding of the underlying molecular mechanism of liver regeneration and provided a potential therapeutic target for liver injury.

Materials and Methods

Animal Models

Male, 6- to 8-week-old, WT (Charles River, China) and ApoA-1 whole-body KO mice (The Jackson Laboratory) on C57BL/6J background were used in the animal model. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. All mice were housed and maintained under standard animal care conditions and had free access to water and food with a 12-hour light/12-hour dark schedule.

Seventy percent of PH was conducted as the in vivo model of liver regeneration.[25] Mice were deeply anesthetized and exposed by midventral laparotomy. After ligation and resection of the left and median hepatic lobes, the peritoneal and skin were then closed in 2 layers. Mice were sacrificed at specific time points after PH. The blood and liver tissues were collected immediately for further research. Autophagy activator MG-132 (5 mg/kg; HY-13259, MCE) or AMPK activator COH-SR4 (10 mg/kg; HY-124822, MCE) was dissolved in 1% DMSO and injected intraperitoneally 1 hour and 24 hours after PH. The same volume of 1% DMSO was administered as control. ApoA-1 mimetic peptide D-4F (Ac-DWFKAFYDKVAEKFKEAF-NH2, 10 mg/kg; Scilight Biotechnology, China) dissolved in phosphate-buffered saline was injected via the tail vein at 1 hour and 24 hours after PH. The same volume of phosphate-buffered saline was administered as control.

Liver Function Examination

Serum samples were obtained from mice blood centrifuged for 8 minutes at 8000 *g*. The levels of serum ALT and AST were measured by AU5800 instrument (Beckman Coulter) to assess liver injury.

Cell Culture

The mouse hepatocyte cell line AML12 cells was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The cell line was cultured in Dulbecco's modified eagle medium with 10% fetal bovine serum and maintained at 37°C in an incubator supplemented with 5% CO_2.

EdU Incorporation Assay

EdU incorporation assay was used to detect the exact proliferation rates of AML12 cells with the Cell-Light EdU DNA Cell Proliferation Kit (C0075S, Beyotime) following the instructions. The number of proliferating cells was counted using a fluorescence microscope in 3 random fields.

CCK-8 Assay

One thousand cells were seeded per well of the 96-well plates. After incubation for required time, CCK-8 solution (Japan) was added after 24, 48, 72, or 96 hours. Cells were incubated in the dark for 2 hours at 37°C. After shaking the plates for 5 seconds, each well was subjected to absorbance measurement at 450 nm in a microplate reader (Bio-Tek Elx 800).

Western Blotting

Western blotting was performed as usual and representative immunoblot images were presented. The primary antibodies included anti-ApoA-1 (14427-1-AP), anti-COXIV (66110-1-Ig), anti-PINK1 (23274-1-AP; ProteinTech Group), anti-GAPDH (ab9485), anti-PCNA (ab152112), anti-HNF-4α (ab201460), anti-Ki-67 (ab16667), anti-ATG5 (ab228668), anti-ATG7 (ab133528), anti-AMPK-α1 (ab3759), anti-p(phosphorylated)-AMPK-α1 (ab194920), anti-ULK1 (ab167139), anti-p-ULK1 (ab203207; Abcam), anti-Cyclin D1 (#55506), anti-LC3B (#43566), and anti-p62 (#39749; Cell Signaling). The secondary antibody was horseradish peroxidase–conjugated goat antirabbit IgG (#7074; Cell Signaling).

Quantitative RT-PCR

Total mRNA was extracted from the tissues or cells by TRIzol reagent (Invitrogen) and was reverse-transcribed into cDNA using HiScript II Q RT SuperMix for qPCR kit (R223-01, Vazyme, China) according to the manufacturer's instructions. The levels of relative mRNA were quantified by qRT-PCR using SYBR Green (Q141-02, Vazyme, China) and normalized against GAPDH levels. The primers used were as follows:

GAPDH forward: 5'- AGGTCGGTGTGAACGGATTTG-3'
GAPDH reverse: 5'- GGGGTCGTTGATGGCAACA-3'
ApoA-1 forward: 5'- GGCACGTATGGCAGCAAGAT-3'
ApoA-1 reverse: 5'- CCAAGGAGGAGGATTCAAACTG-3'
Cyclin D1 forward: 5'- GCGTACCCTGACACCAATCTC-3'
Cyclin D1 reverse: 5'- CTCCTCTTCGCACTTCTGCTC-3'
Cyclin E forward: 5'- GAAAAGCGAGGATAGCAGTCAG-3'
Cyclin E reverse: 5'- CCCAATTCAAGACGGGAAGTG-3'
PCNA forward: 5'- TTGCACGTATATGCCGAGACC-3'
PCNA reverse: 5'- GGTGAACAGGCTCATTCATCTCT-3'

Immunohistochemistry and Immunofluorescence Staining

The levels of ApoA-1 and Ki-67 in mouse liver sections were detected by immunohistochemical staining or immunofluorescence staining. The levels of COXIV and PINK1 in AML12 cell line were detected by immunofluorescence staining, and the nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI).

Determination of ATP Content

ATP content was determined by an ATP Assay Kit (S0026, Beyotime, China) based on the need of ATP when luciferase catalyzes luciferin to produce fluorescence. Samples were lysed according to the manufacturer's instructions and subjected to a microplate reader (Bio-Tek Synergy H1) to measure luciferase activity. The ATP levels were calculated according to a generated standard curve.

Statistical Analysis

Statistical analyses were performed using GraphPad prism 8.0 (GraphPad Software, CA) and the results were expressed as the mean ± standard deviation. The results were tested for normal distribution and followed with unpaired Student t test. A difference was considered statistically significant if $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, or $^{****}P < .0001$.

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