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β2-adrenergic receptor promotes liver regeneration partially through crosstalk with c-met

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The β2-adrenergic receptor (β2AR) is a G protein-coupled receptor (GPCR) that mediates the majority of cellular responses to external stimuli. Aberrant expression of β2AR results in various pathophysiological disorders, including tumorigenesis, but little is known about its role in liver regeneration. This study aims to investigate the impact and the underlying mechanism of β2AR in liver regeneration. Here, we found that β2AR was upregulated during liver regeneration induced by 70% PH. Deletion of β2AR in mice resulted in 62% mortality 2 days post-PH, decreased proliferative marker expression and impaired liver function throughout regeneration. Moreover, AAV8-mediated overexpression of β2AR in hepatocytes accelerated the regeneration process and increased target gene expression. Mechanistically, β2AR recruited G-protein-coupled receptor kinase 2 (GRK2) to the membrane and then formed a complex with c-met to transactivate c-met signaling, which triggered downstream extracellular regulated protein kinase (ERK) signaling activation and nuclear translocation. Inhibition of c-met with SU11274 or ERK with U0126 decreased β2AR overexpression-induced hepatocyte proliferation. Our findings revealed that β2AR might act as a critical mediator regulating liver regeneration by crosstalk with c-met and activation of ERK signaling.

INTRODUCTION

The liver has considerable regenerative capacity [1]. In response to partial hepatectomy (PH) or acute injury, quiescent hepatocytes can be triggered to re-enter the cell cycle and divide to restore tissue homeostasis [2–4]. Understanding the mechanisms during liver regeneration is crucial to promote regeneration and prevent clinical complications during liver transplantation. Previous studies have shown that a large number of genes are involved in liver regeneration, but the essential circuitry required for the process may involve the participation of cytokines, growth factors and metabolic networks [1]. However, the molecular regulatory mechanisms of liver regeneration are still elusive.

Three types of transmembrane receptors transmit extracellular mitotic signals, including ion channel-linked receptors, enzyme-linked receptors, and G protein-coupled receptors (GPCRs). The roles of enzyme-linked receptors (including growth factor receptors and cytokine receptors) and ion channel-linked receptor cascades involved in liver regeneration have been studied extensively [5–8]. However, little is known about the role of GPCRs in the regenerating liver.

β2-Adrenergic receptors (β2ARs) are prototypical GPCRs mediating diverse cellular signals, often in a ligand-specific manner [9]. Several studies in mice and humans reinforce the growing recognition of the role of β2AR in cancer progression, metastasis, and drug resistance in various tissues [10–12]. Other evidence has demonstrated that β2AR activation can promote cell proliferation to improve functional recovery from injuries in skeletal muscle, vasculature and heart [13–15]. Moreover, β2AR is closely involved in the regulation of cytokines, growth factors and metabolic networks that are essential for liver regeneration. Although the levels of β2AR are elevated in hepatocytes from regenerating livers [16], the detailed mechanisms remain unclear. In the present study, we used a β2AR knockout mouse model and adeno-associated virus 8 (AAV8)-mediated overexpression of β2AR in mouse liver to define the biological function of β2AR in liver regeneration post-PH.

RESULTS

β2AR was upregulated during liver regeneration induced by 70% PH

Partial hepatectomy is regarded as a classic model that has been widely used in the experimental analysis of the molecular mechanisms underlying liver regeneration [3]. To investigate the role of β2AR in liver regeneration, we determined changes in the expression of β2AR in C57BL/6J mice over a time course post-PH

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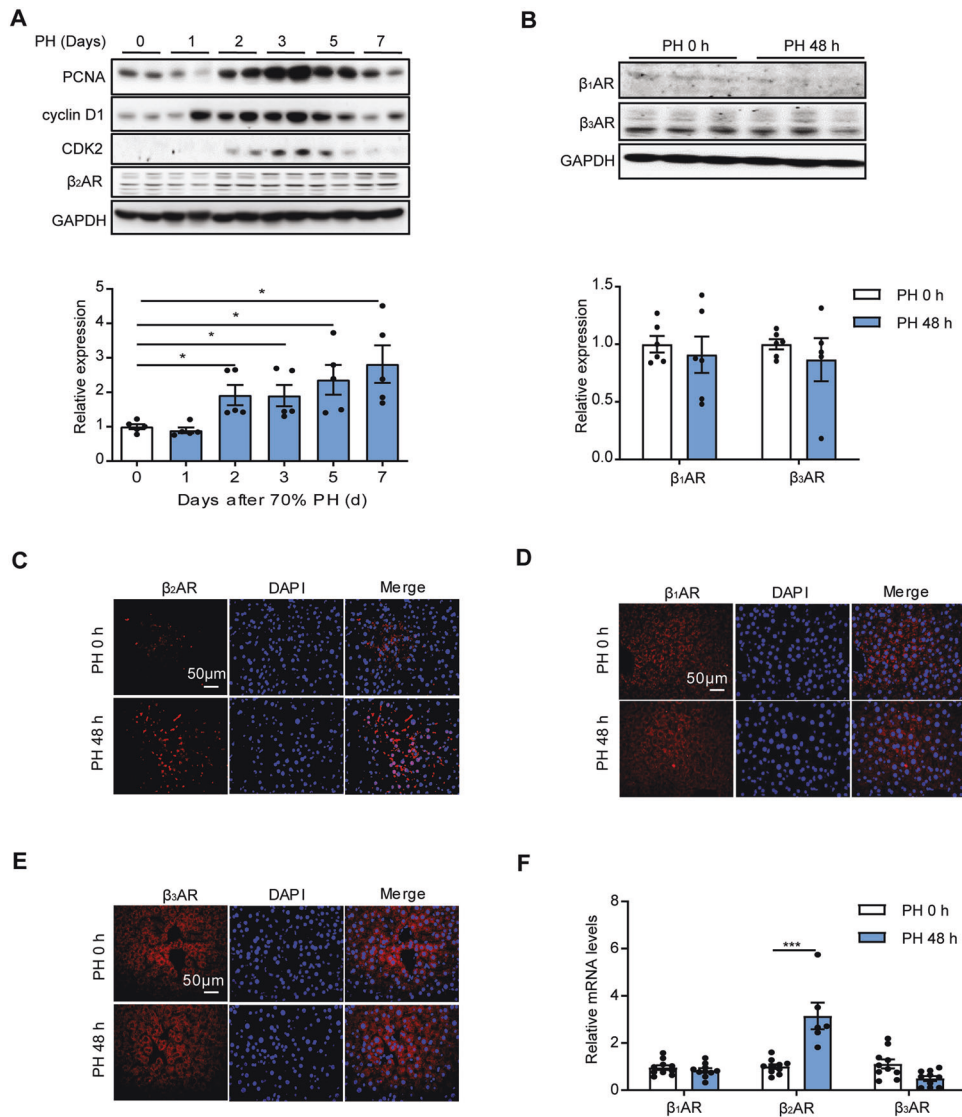Edited by Dr Francesca Bernassola

Fig. 1 **β₂AR was upregulated during liver regeneration induced by 70% PH. A** Protein expression levels of β_2AR, PCNA, cyclin D1 and CDK2 in the liver at the indicated times post PH. **B** Protein expression levels of β_1AR and β_3AR 48 h post PH ($n = 5–6$). **C–E** Immunofluorescence staining of PH mouse livers for the indicated proteins. **F** The mRNA levels of βARs 48 h post-PH. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-tailed Student's t test.

using Western blotting. Concomitant with increased expression of proliferation markers such as proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin-dependent kinase 2 (CDK2), β_2AR protein levels started increasing at 48 h post-PH (Fig. 1A). Nevertheless, the protein levels of β_1AR and β_3AR were not changed by PH (Fig. 1B). Additionally, β_2AR was upregulated post-PH by immunofluorescence staining, while β_1AR and β_3AR levels were not changed (Fig. 1C, E). Consistently, the mRNA expression of β_2AR but not β_1AR and β_3AR was increased at 48 h post PH (Fig. 1F). Thus, these findings indicated that β_2AR was upregulated during liver regeneration after 70% PH in mice.

β₂AR translocated to the nucleus during liver regeneration induced by 70% PH

β_2AR is a member of the 7-transmembrane family of receptors. Intracellular signaling after β_2AR activation is largely affected through cyclic adenosine monophosphate and protein kinase A [17]. To clarify the precise distribution of β_2AR in liver tissues, we analyzed the subcellular localization of β_2AR in normal human tissues by fluorescence immunohistochemistry. Notably, β_2AR

displayed a membrane location in mature hepatocytes marked by hepatocyte nuclear factor 4α (HNF4α) (Fig. 2A). In addition, β_2AR showed little colocalization with stellate cells, liver sinusoidal endothelial cells and Kupffer cells, which were marked by desmin, CD31 and CD68, respectively (Fig. 2B–D). Most remarkably, 48 h after PH, β_2AR colocalized with Ki67 in the nucleus, indicating that it was translocated to the nucleus in proliferating mouse liver cells (Fig. 2E). To further validate the migration of β_2AR, nuclear and non-nuclear components of liver tissues from Sham and PH groups were isolated and the expression of β_2AR was detected by western blot (Fig. 2F). Thus, these findings indicated that β_2AR was translocated to the nucleus during liver regeneration induced by 70% PH.

β₂AR deficiency impaired liver regeneration following 70% PH challenge

To investigate the role of β_2AR in liver regeneration, we performed 70% PH on wild-type (WT) and β_2AR knockout (β₂ARKO) mice. To our surprise, approximately 62% of the β₂ARKO mice died within 48 h after surgery, and the surviving mice exhibited much slower
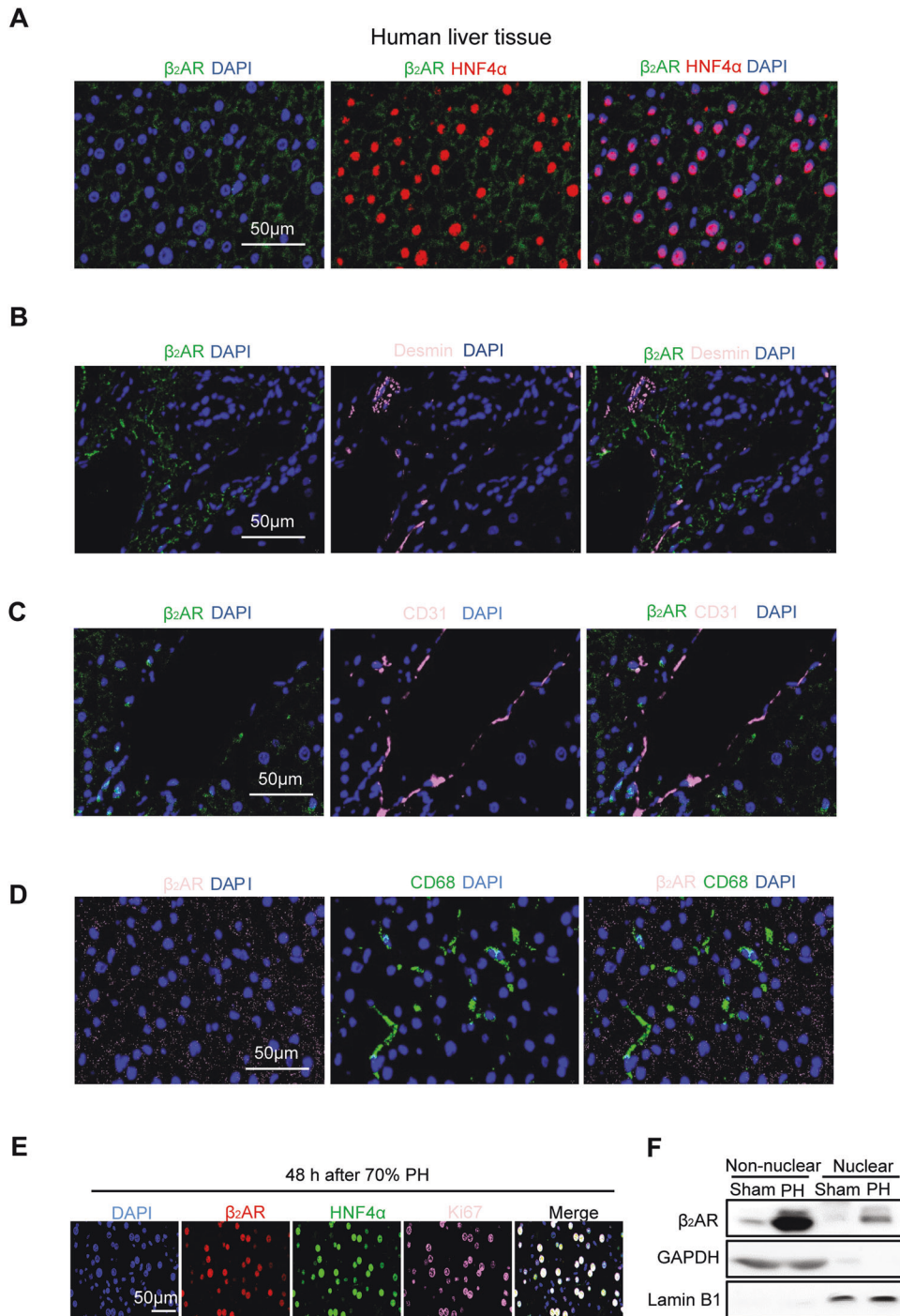
Fig. 2 β_2AR translocated to the nucleus during liver regeneration induced by 70% PH. A–D Immunofluorescence staining of β_2AR and DAPI in human liver with the indicated cell markers HNF4α (hepatocytes), desmin (hepatic stellate cells), CD31 (sinusoidal endothelial cells) and CD68 (Kupffer cells). **E** Immunofluorescence staining of β_2AR, Ki67, HNF4α and DAPI in mouse livers 48 h post PH. **F** The nuclear and non-nuclear components of liver tissues from Sham and PH groups were isolated and the expression of β_2AR were detected by western blot.

liver recovery rates. The hepatic index (ratio between liver and body weight) was found to be slightly decreased in β_2ARKO mice (Fig. 3A, B). As indicated by hepatic tissue section staining, local necrosis was observed in β_2ARKO mouse livers 48 h postoperation (Fig. 3C). Since transient steatosis is a metabolic hallmark of the early phase of liver regeneration [18], we performed oil red O staining and found that hepatic triglyceride levels were significantly decreased in β_2ARKO mouse livers compared with WT mice (Fig. S1A). Moreover, β_2AR deficiency decreased hepatocyte

proliferation as evidenced by decreased Ki67 staining (Fig. 3C). Compared with WT mice, β_2ARKO mice showed more serious liver injury in response to 70% PH, as evidenced by a slight rise in plasma levels of alanine transaminase (ALT) and aspartate transaminase (AST) (Fig. 3D).

Next, we investigated the underlying mechanisms of the decreased liver regeneration phenotypes in β_2ARKO mice. Given that activation of the cell cycle plays an important role in the stimulation of robust hepatocyte proliferation during liver
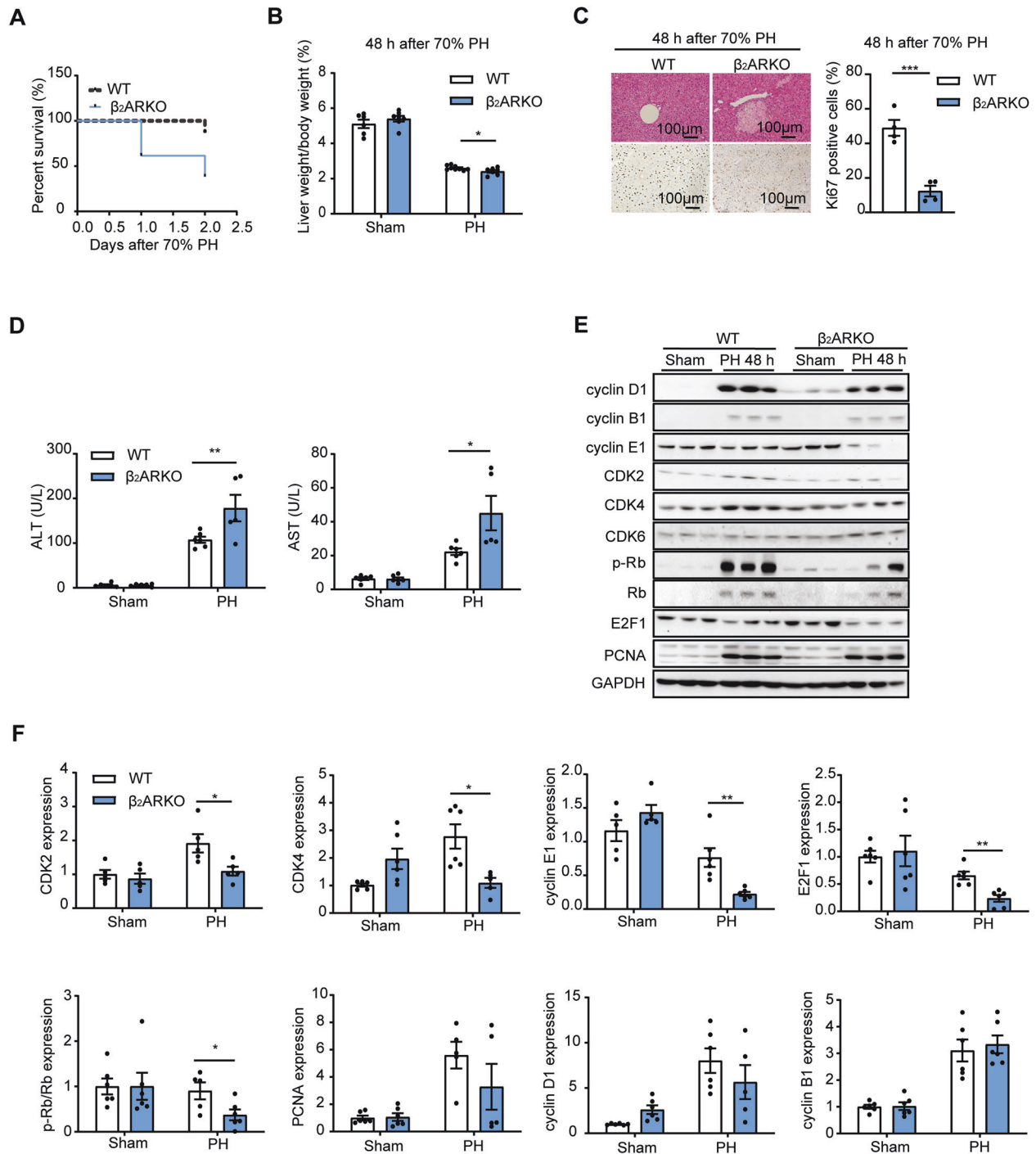
Fig. 3 β₂AR deficiency impaired liver regeneration following 70% PH challenge. β₂ARKO and WT mice were challenged with 70% PH, and the remaining livers were collected at the indicated times. The survival curve after PH of β₂ARKO and WT mice (**A**) and liver weight to body weight ratio ($n = 6$–9, **B**). **C** HE staining (upper panel) and Ki67 (lower panel) staining of β₂ARKO and WT mouse livers 48 h post PH. The percentage of Ki67-positive cells was measured ($n = 4$). **D** The levels of ALT and AST in the serum of β₂ARKO and WT mice 48 h post PH. **E** The protein levels of cell cycle markers in β₂ARKO and WT mouse livers 48 h post PH ($n = 5$–6). **F** Quantitative analysis of immunoblotting. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-tailed Student's t test.

regeneration [4], the expression levels of checkpoint components of the cell cycle machinery were measured. As shown in Fig. 3E, F, the expression levels of CDK4, which appeared in the early G1 phase, were low in the livers of β₂ARKO mice. Furthermore, cyclin E1, which interacts with CDK2 to form a complex that promotes the G1/S transition [19–21], was dramatically lower in β₂AR-

deficient livers. Consistently, impaired Rb phosphorylation and subsequent E2F1 translocation to the nucleus were found in β₂ARKO mouse livers (Figs. 3E, F and S1B). These results suggested that β₂AR deficiency resulted in a decreased G1 to S transition. Thus, these findings indicated that β₂AR deficiency impaired liver regeneration following 70% PH challenge.

Adeno-associated virus-mediated overexpression of β₂AR promoted liver regeneration post PH

To further investigate the gain-of-function effects of β₂AR on liver regeneration, adeno-associated virus 8 (AAV8)-mediated gene delivery in the liver was performed in mice. Four weeks later, we performed surgery on AAV8-GFP- and AAV8-β₂AR-treated mice. As shown in Fig. 4A, the mRNA levels of β₂AR in AAV8-β₂AR-treated mouse livers were nearly 50 times higher than those in control GFP mice. Moreover, the hepatic index was slightly higher 48 and 72 h post PH after AAV8-β₂AR treatment (Fig. 4B). Consistently, overexpression of β₂AR resulted in increased hepatocyte proliferation at 24, 48, and 72 h post-PH (Fig. 4C, D). As shown in Fig. 4E, F, immunoblots showed that AAV8-β₂AR treatment caused a profound increase in cell cycle genes in the liver of mice 48 h post PH, such as CDK2, CDK4, p-Rb/Rb and cyclin E1, while other genes were not significantly changed. Together, these results indicated that adeno-associated virus-mediated overexpression of β₂AR promoted liver regeneration post PH.

β₂AR played an important role in primary hepatocyte proliferation

To further explore the role of β₂AR in cell proliferation, primary hepatocytes from WT and β₂ARKO mouse livers were isolated and cultured. In β₂ARKO hepatocytes, HGF- and βAR agonist clenbuterol-induced cell proliferation was blocked compared to WT hepatocytes according to the CCK-8 assay (Fig. 5A, B). Moreover, as shown in Fig. 5C, adenovirus-mediated β₂AR overexpression was found to promote hepatocyte proliferation in both WT and β₂ARKO hepatocytes. In line with these findings, the number of EdU-positive nuclei was increased by virus infection in the presence or absence of HGF (Fig. 5D).

Next, we investigated the underlying mechanisms of the decreased cell proliferation phenotypes in β₂ARKO hepatocytes. Expression levels of checkpoint components of the cell cycle machinery were measured at different time points in response to HGF treatment. Interestingly, we found that in WT hepatocytes, the level of cyclin E1 was increased in a time-dependent manner induced by HGF, while it was unchanged in β₂ARKO hepatocytes. Furthermore, loss of β₂AR led to a delay in CDK2 and PCNA induction in response to HGF (Fig. 5E). We also examined MAPK/ERK and AKT signaling in WT and β₂ARKO hepatocytes. As shown in Fig. 5F, the lack of β₂AR caused a decrease in the phosphorylation of ERK and AKT at several early time points (Fig. 5F). Together, these findings indicated that β₂AR played a crucial role in hepatocyte proliferation.

β₂AR signaling and c-met signaling crosstalk during liver regeneration

Our previous studies suggested the existence of a novel insulin receptor-induced and G-protein-coupled receptor kinase 2 (GRK2)-mediated transactivation of a β₂AR-β-arrestin2-ERK signaling cascade that was the primary mediator of insulin-induced ERK activation in cardiomyocytes [22]. Inspired by these findings, we determined the GRK2-β₂AR-β-arrestin2 signaling cascade 48 h post PH. Notably, GRK2-mediated activation of a β₂AR-β-arrestin2 signaling cascade was observed in livers post PH (Fig. 6A). We further examined the signaling cascade in primary hepatocytes after HGF treatment. Indeed, the levels of GRK2-mediated phosphorylation of β₂AR and β-arrestin2 expression were significantly increased by HGF (Fig. 6B). Moreover, in primary hepatocytes, introduction of β₂AR but not mutant β₂AR lacking GRK2 phosphorylation sites promoted hepatocyte proliferation in the presence or absence of HGF (Fig. 6C). Since HGF/c-met signaling is an essential growth factor signaling pathway during liver regeneration [6], these data supported the existence of a link between β₂AR signaling and HGF/c-met signaling. Thus, we determined the HGF levels in the serum and liver between WT and β₂ARKO mice post PH. Interestingly, no difference was found

between these two groups (Fig. S1D–F), which prompted us to consider whether β₂AR could form a complex with the HGF receptor (c-met) to regulate downstream signaling pathways. As shown in Fig. 6D, β₂AR could bind to c-met in primary hepatocytes, and the binding of the two receptors was significantly increased after HGF treatment. Consistently, in regenerating livers, a large number of β₂AR and c-met complexes were noticed 2 days post-PH, while during the termination of liver regeneration, they disassociated from each other (Fig. 6E). Furthermore, immunofluorescence staining showed colocalization of β₂AR and c-met in the nucleus in mouse livers after surgery (Fig. 6F).

To further investigate the relationship of β₂AR and the HGF receptor c-met, we determined the phosphorylation of c-met in β₂AR-overexpressed hepatocytes. Interestingly, the protein levels of p-c-met and c-met were both upregulated after β₂AR introduction. Meanwhile, the expression levels of CDK2, CDK4, p-Rb/Rb and cyclin E1 were significantly increased upon β₂AR virus treatment (Fig. 6G). Furthermore, the upregulation of these cell cycle markers and cell proliferation was abolished when c-met was inhibited with SU11274 (Fig. 6G–H). These data suggested a crosstalk between β₂AR and c-met signaling during liver regeneration.

β₂AR-mediated hepatocyte proliferation was ERK-dependent

Cell cycle arrest in the proliferating hepatocytes of β₂ARKO mice prompted us to consider whether a growth factor signaling pathway was disrupted, since β₂ARKO mice showed a similar phenotype to that of mice lacking c-met [6]. Thus, we analyzed the most important downstream pathways of growth factor receptors, including the MAPK/ERK cascade, SAPK/JNK cascade, P38-MAPK cascade and Akt signaling. Notably, the levels of p-ERK/ERK were reduced in β₂ARKO mouse livers postsurgery, while the levels of p-P38/P38, p-JNK/JNK and p-AKT/AKT were not significantly changed (Figs. 7A, B and S1C). In addition, Western blotting and immunofluorescence showed that the levels of p-ERK in the nucleus were decreased in β₂ARKO mouse livers 48 h post PH (Fig. 7C, D). Together, these results indicated that loss of β₂AR impaired the MAPK/ERK pathway during liver regeneration.

To further explore the role of ERK signaling in β₂AR-induced cell proliferation, we next disrupted this signaling with the specific inhibitor U0126 in β₂AR-overexpressing hepatocytes. A Cell Counting Kit-8 (CCK-8) assay indicated that β₂AR-mediated cell proliferation was prevented by U0126 treatment (Fig. 7E). Similar to the results obtained in vivo, the expression levels of CDK2, CDK4, p-Rb/Rb and cyclin E1 were significantly increased upon β₂AR virus treatment, while only CDK2 and CDK4 were reduced by ERK inhibition (Fig. 7F). Moreover, HGF-induced CDK2 and CDK4 expression was reduced by ERK inhibition (Fig. 7G). These data suggested that β₂AR-induced cell proliferation was partly dependent on ERK activation.

DISCUSSION

Previous findings have shown the great significance of α1-adrenergic receptors in the regulation of liver regeneration [23]. The effect of nonselective β-blocker propranolol and highly selective β₁-receptor antagonist nebivolol on liver regeneration has been studied but is controversial [24–26], which may be related to the dosage form, time of administration, dosage, method of administration and drug selectivity and so on. However, these studies did not really clarify the role of β₂AR in regenerating livers and proliferating hepatocytes. In our study, β₂AR gene knockout mice and AAV8-β₂AR were used to further investigate the loss- and gain-of function effects of β₂AR on liver regeneration. In future studies, liver-specific gene knockout mice will be used to reinforce our conclusions. In this study, we demonstrate that β₂AR is a critical factor contributing to liver
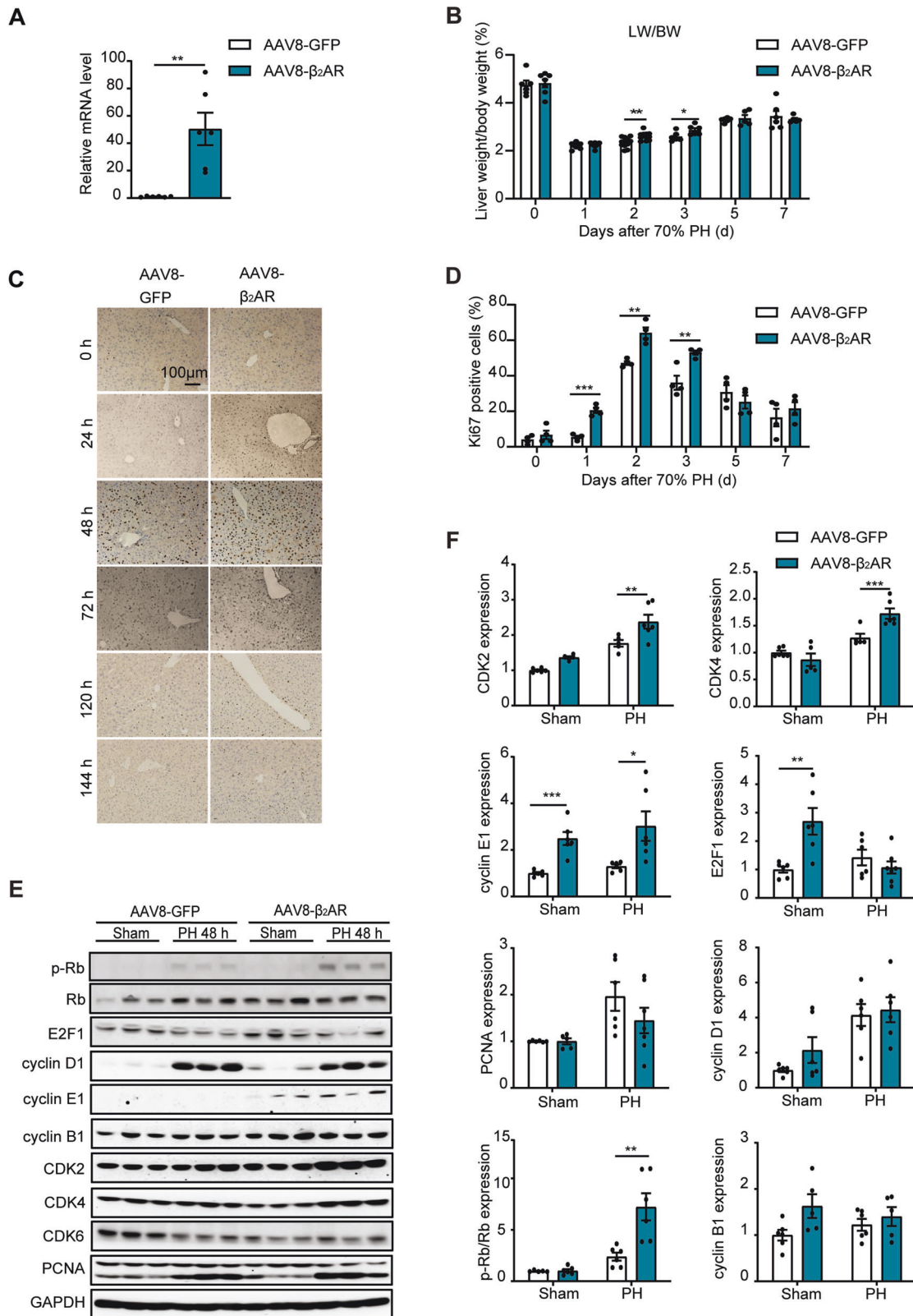
Fig. 4 Adeno-associated virus-mediated overexpression of β₂AR promoted liver regeneration post PH. A RT–qPCR analysis of the mRNA expression of β_2AR ($n = 6$). **B** Liver weight to body weight ratio ($n = 5$–11). **C** Ki67 staining of mouse livers at the indicated times post PH ($n = 4$). **D** The percentage of Ki67-positive cells was measured ($n = 4$). **E** The protein levels of cell cycle markers in AAV8-GFP and AAV8-β_2AR mouse livers 48 h post PH ($n = 5$–7). **F** Quantitative analysis of immunoblotting ($n = 5$–7). Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with a two-tailed Student's t test.

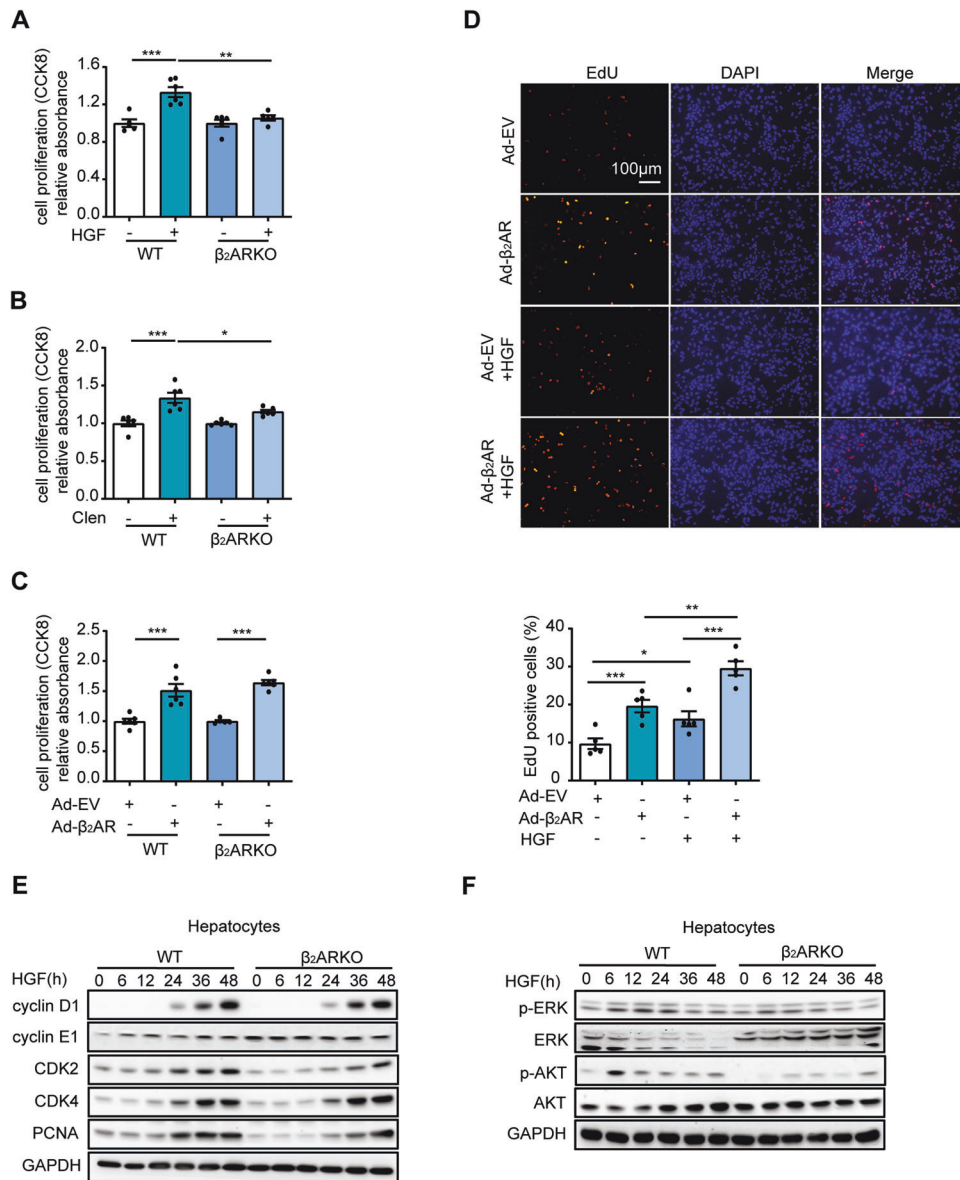**Fig. 5 β₂AR was essential for the regulation of hepatocyte proliferation. A** Primary hepatocytes from WT and β₂ARKO mice were treated with HGF (40 ng/ml) for 24 h, and then a CCK-8 assay was performed. **B** Primary hepatocytes from WT and β₂ARKO mice were treated with Clen (clenbuterol, 10^{-6} mol/L) for 24 h, and then a CCK-8 assay was performed. **C** Primary hepatocytes from WT and β₂ARKO mice were treated with Ad-EV or Ad-β₂AR for 24 h, and then a CCK-8 assay was performed. **D** EdU staining was performed in Ad-β₂AR- and Ad-EV-treated hepatocytes in the presence or absence of HGF. The percentage of EdU-positive cells was analyzed. **E** Primary hepatocytes from WT and β₂ARKO mice were treated with HGF for the indicated times. Western blot analysis of cell cycle markers was performed. **F** Western blot analysis of p-ERK/ERK and p-AKT/AKT. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way ANOVA and post hoc Tukey's test.

regeneration. β₂AR deficiency leads to a low survival rate after PH. Moreover, both loss- and gain-of-function studies revealed that β₂AR is necessary for regular cell cycle progression and proliferation of hepatocytes. Mechanistically, GRK2-mediated activation of β₂AR recruits the HGF receptor (c-met) complex to initiate the phosphorylation of ERK and subsequent cell proliferation. Finally, mutation of the GRK2 phosphorylation site on β₂AR or ERK inhibition robustly disrupted hepatocyte proliferation mediated by Ad-β₂AR or HGF treatment. Therefore, β₂AR promotes liver regeneration partially through crosstalk with c-met and subsequent activation of ERK signaling. Besides, nuclear transfer of β₂AR during liver regeneration was found in this study, and β₂AR can bind and phosphorylate hepatocyte growth factor receptor c-met, which has not been reported. Furthermore, how β₂AR transposes and regulates downstream signals needs further study. The

research results obtained in this project will expand the understanding of the regulation mechanism of liver regeneration and provide new targets and new ideas for the diagnosis and treatment of related liver diseases.

Early lipid accumulation is a hallmark of liver regeneration, and fatty acids serve as an energy source during the initial steps of regeneration [27]. Moreover, another study showed that transient attenuation of hepatic lipid accumulation in response to propranolol treatment impaired liver regeneration after PH [25]. Consistently, in our present study, β₂ARKO mice displayed a significant reduction in lipid formation post-PH compared to WT mice. In addition, glucose availability is another key factor during liver regeneration [28] and it has been suggested that inactivation of β₂AR disrupted glucose homeostasis in mice [29]. Together, these findings suggest that aberrant hepatic energy status may be
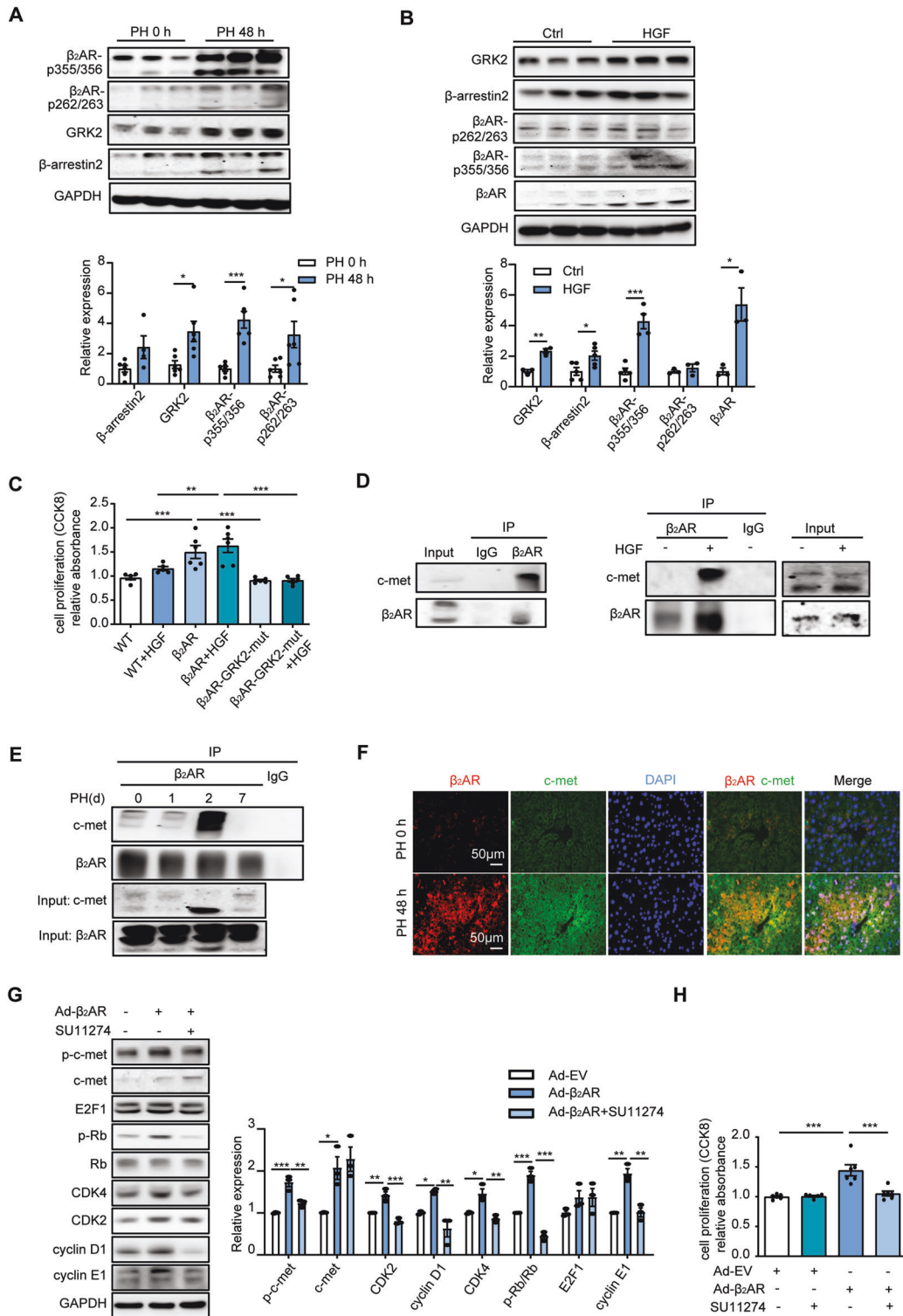
Fig. 6 β₂AR signaling was associated with c-met signaling during liver regeneration. A Western blot analysis of the protein levels of the β₂AR signaling cascade in mouse livers 48 h post PH ($n = 4–6$). **B** Western blot analysis of the protein levels of the β₂AR signaling cascade in hepatocytes treated with HGF for 24 h. **C** Primary hepatocytes were treated with β₂AR or β₂AR-GRK2-mut virus in the presence or absence of HGF, and then a CCK-8 assay was performed. **D** Coimmunoprecipitation assays demonstrated that β₂AR could bind to c-met in primary hepatocytes, and the binding of the two receptors was significantly increased in response to HGF treatment. **E** Coimmunoprecipitation assay showed the combination of β₂AR with c-met at the indicated time post PH. **F** Immunofluorescence of β₂AR, c-met and DAPI in mouse livers post PH. **G** Western blot analysis of the protein levels of cell cycle markers in hepatocytes treated with β₂AR virus in the presence or absence of the c-met inhibitor SU11274. **H** Hepatocytes treated with β₂AR virus in the presence or absence of the c-met inhibitor SU11274, and then a CCK-8 assay was performed. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with a two-tailed Student's t test in (**A**, **B**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way ANOVA and post hoc Tukey's test in (**C**, **G**, **H**).
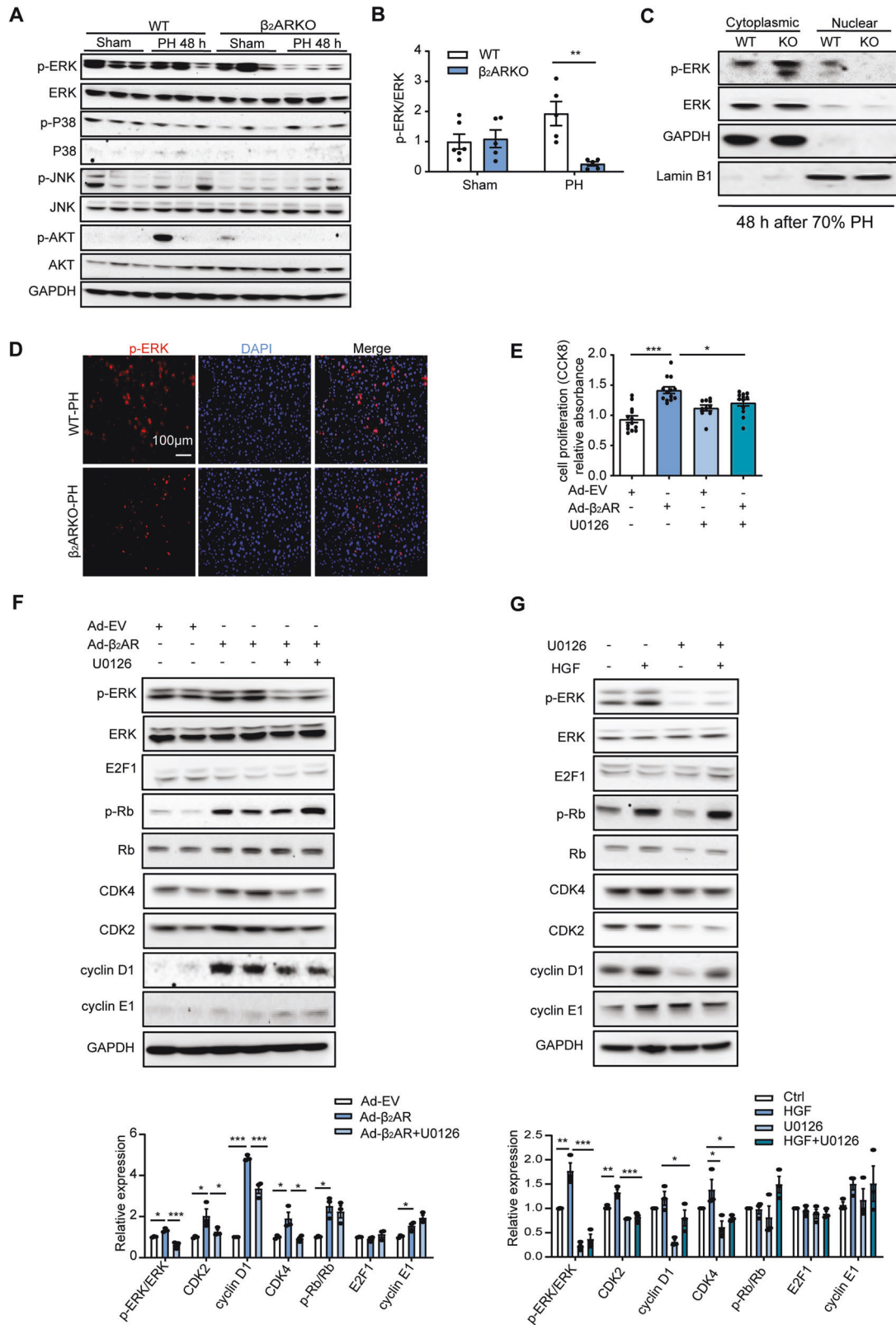
associated with high mortality and disrupted liver regeneration in β2ARKO mice.

Liver regeneration is known to be a process involving highly organized and ordered tissue growth upon surgical resection and following viral or drug-induced liver injury. In contrast to all other organs, liver-to-body-weight ratio needs to be maintained always at 100% of what is required for body homeostasis [30]. A large number of genes are involved in liver regeneration, and the overall process includes three phases. The initial step is characterized by priming of quiescent hepatocytes by factors

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Fig. 7 β₂AR-mediated cell proliferation was partially dependent on ERK signaling. A The protein levels of p-ERK/ERK, p-P38/P38, p-JNK/JNK and p-AKT/AKT in β₂ARKO and WT mouse livers 48 h post PH. **B** Quantitative analysis of immunoblotting ($n = 5–6$). **C** Immunoblotting for cytoplasmic and nuclear p-ERK and ERK in β₂ARKO and WT mouse livers 48 h post PH. **D** Immunofluorescence of p-ERK demonstrated that p-ERK failed to enter the nucleus in β₂ARKO mouse livers post PH. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with a two-tailed Student's t test in (**B**). **E** Primary hepatocytes were treated with β₂AR virus in the presence or absence of the ERK inhibitor U0126, and then a CCK-8 assay was performed. **F** Western blot analysis of the protein levels of cell cycle markers in hepatocytes treated with β₂AR virus in the presence or absence of the ERK inhibitor U0126. **G** Western blot analysis of the protein levels of cell cycle markers in hepatocytes pretreated with U0126 in the presence or absence of HGF. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way ANOVA and post hoc Tukey's test in (**E–G**).

such as TNF-α and IL-6. The proliferative stage is the step during which hepatocytes enter into the cell cycle's G1 phase with the stimulation of complete mitogens including HGF, TGF-α and EGF. Non-mitogenic cytokines, insulin, TNF-α, bile acids and IL-6 have also been implicated in regulating the regeneration process. To avoid excessive liver regeneration, there are many signals associated with regeneration termination, such as C/EBP transcription factors and Glypican-3 [4]. Therefore, in our study, we suggested that β₂AR accelerated liver regeneration during the proliferation step, but it did not increase the final level of liver regeneration.

In proliferating cells, E2F1 dissociates from Rb before translocating to the nucleus when Rb is phosphorylated and then initiates the transcription of cyclin E1 and other cell cycle markers [31]. Cyclin E1 interacts with CDK2 to form a complex that promotes the G1/S transition [21]. Our data, both in vivo and in vitro, demonstrated that β₂AR signaling might regulate hepatocyte proliferation by modulating the G1/S transition. Furthermore, some of the protein changes that were not as expected could be attributed to the choice of processing timing.

Movement of plasma membrane receptors to the nucleus has also been described for some other GPCRs, for receptor tyrosine kinases (RTKs), and in various cell types, which have been reported to play essential roles in cell proliferation [6, 32–35]. A similar phenomenon of β₂AR has been observed in liver regeneration, suggesting that there is a link between β₂AR translocation and hepatocyte proliferation. Our previous study showed that the insulin receptor induces arrestin-biased transactivation of β₂AR in a GRK2 phosphorylation-dependent manner [22]. Previous research has also uncovered a novel function for c-met in regulating hepatic glucose metabolism by directly interacting with and regulating the insulin receptor [36]. Inspired by these findings, our data demonstrate crosstalk between β₂AR signaling and c-met signaling. However, the possibility of forming a complex of these three receptors in the regulation of liver regeneration could not be ruled out. Since β₂AR-mediated epidermal growth factor receptor (EGFR) transactivation in DNA synthesis has been well documented in recent studies [37–39], the association of β₂AR with other RTKs, including EGFR, post PH should be further explored.

The MAPK/ERK pathway, which regulates cell proliferation, displayed significant disruption in β₂AR-deficient livers after PH and hepatocytes in response to HGF treatment. β₂AR-mediated cell proliferation is prevented by U0126 treatment. Furthermore, β₂AR-mediated upregulation of most of the cell cycle markers can be reduced by ERK inhibition. Nevertheless, the expression manner of p-Rb/Rb, E2F1 and cyclin E1 in response to β₂AR and U0126 treatment is complicated, which demonstrates that β₂AR-mediated cell proliferation is partially dependent on ERK signaling, and the manner by which these signaling pathways synergistically promote subsequent cell cycle progression is much more complicated than we have so far understood.

In conclusion, our study provides new insights into the dynamic nature of β₂AR in the regulation of liver regeneration, as presented in Fig. 8. Our findings highlight the importance of β₂AR in the promotion of cell cycle progression in proliferating cells and

support the β₂AR-c-met complex as a novel therapeutic target for abnormal proliferative diseases, such as benign and malignant tumors.

MATERIALS AND METHODS
Animals and treatments
Animal experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Animal Experimentation Ethics Committee of Huazhong University of Science and Technology. Eight-week-old male C57BL/6J mice (HFK Bioscience, Beijing, China) and β₂ adrenergic receptor (β₂AR) global knockout mice (a gift from Dr. Yang K. Xiang, UC Davis, USA) were challenged by 70% partial hepatectomy (PH). All mice were housed under a 12:12 h light/dark cycle at controlled temperature.

For PH, mice ($n = 21$ per group) received food and water *ad libitum*. PH was performed under 2,2,2-tribromoethanol anesthesia. Three liver lobes were removed [40]. As a control, sham operations were performed, but the livers were not removed ($n = 6$ per group). Animals were randomly assigned to sham or PH group. Mice were killed, and liver samples were harvested at different time points specified in each experiment. The hepatic index (liver weight/body weight) was monitored for each mouse over 7 days.

Human tissues
All human tissue samples were obtained by surgery from Tongji Hospital of Tongji medical college and informed consents were obtained from the patients. All study methodologies were strictly in accordance with Helsinki declaration for the Use of Human Subjects and were approved by the Ethics Committee at Tongji Medical College, Huazhong University of Science and Technology.

Cell culture and treatments
Primary hepatocytes were isolated by a collagenase IV (0.5 mg/ml, Worthington, NJ) perfusion method as previously described [41]. Hepatocytes were treated with hepatocyte growth factor (HGF, 40 ng/mL) for the indicated hours to mimic an in vivo model of 70% PH. The c-met inhibitor SU11274 (Selleck, Shanghai, China), ERK inhibitor U0126 (Selleck, Shanghai, China) and βAR agonist clenbuterol (Selleck, Shanghai, China) were used at 1, 10, and 1 µM, respectively. The adenovirus encoding β₂AR (Ad-β₂AR) (a gift from Dr. Yang K. Xiang, UC Davis, USA) was used to overexpress β₂AR in vitro.

Cell proliferation assay
Cell growth was determined using the CCK-8 assay (Cell Counting Kit-8, Dojindo, Japan) following the manufacturer's instructions. Briefly, primary hepatocytes seeded in 96-well plates were treated with HGF for 24 h. CCK-8 (10%) was added to the culture medium for 2 h, and then the absorbance value was measured at 450 nm.

Histological analysis
Liver tissues were fixed in formalin. Sections were stained with H&E and Ki67. Hepatic lipid content was analyzed on frozen sections of livers by Oil Red O staining (O0625, Sigma, St. Louis, MO) and counterstained with Mayer's hematoxylin.

AAV8-mediated overexpression of β₂AR in mouse liver
To overexpress β₂AR in mouse livers, recombinant β₂AR plasmids were driven by a liver-specific promoter (thyroxine-binding globulin, TBG) and
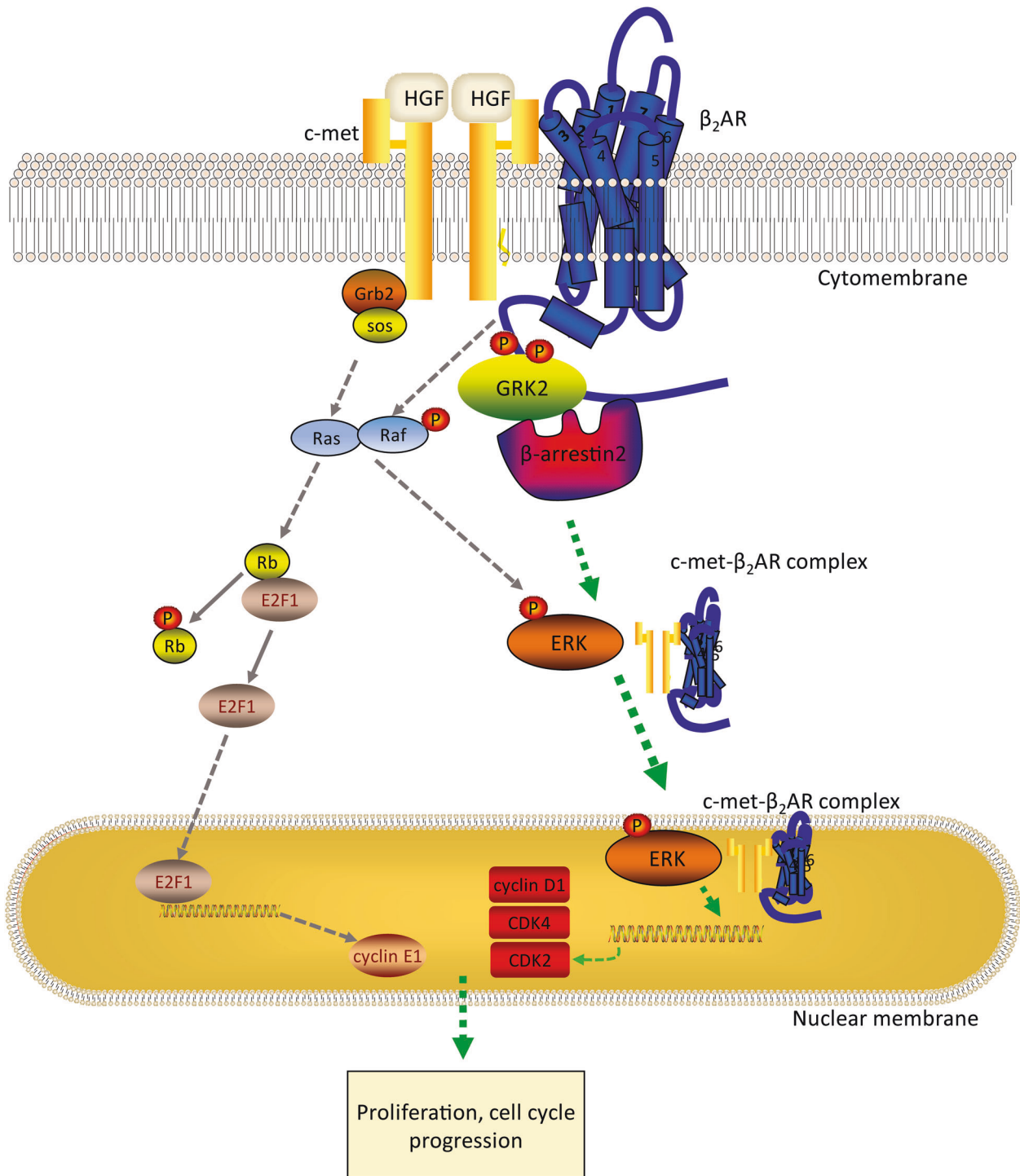
Fig. 8 β2-adrenergic receptor promotes liver regeneration partially through crosstalk with c-met. Model of β_2AR as a critical factor that regulates hepatocyte proliferation during liver regeneration.

packaged into adeno-associated virus 8 (AAV8) particles (Genechem, Shanghai, China). A total of 1×10^{11} plaque-forming units (pfu) of AAV8-β_2AR or AAV8-GFP in 200 µl saline were injected into mice via the tail vein, and 4 weeks later, 70% PH was performed.

Western blot analysis
Tissue extracts or mouse primary hepatocytes were lysed using RIPA buffer, and total protein was extracted and separated by SDS–PAGE and then transferred onto a PVDF membrane. Membranes were probed with antibodies. Antibodies used in this study are listed in Table S1.

Chemiluminescent detection was performed with horseradish peroxidase-coupled secondary antibody (Cell Signaling, Danvers, MA) and Super Signal West Femto reagent (Servicebio, Wuhan, China). Band densities were quantified using ImageJ software.

RNA extraction and quantitative real-time PCR analysis
Total RNA was isolated with TRI Reagent (D9108A, Takara Bio). cDNA was synthesized using an RNA PCR Kit (RR036A, Takara Bio) and analyzed by real-time PCR using SYBR Green Premix Ex Taq (Takara, Japan). The primers for the PCR and siRNA targeting sequences used in this study are listed in Table S2.

Statistical analysis

Data were tested for normality using Shapiro–Wilk and Kolmogorov–Smirnov normality tests. All normally distributed data were analyzed using Student's t test, one-way ANOVA, followed by Tukey's post hoc analysis. For data not normally distributed, statistical analyses were performed using the non-parametric Kruskal–Wallis test. All experiments were performed at least in triplicate, and representative data are shown. All analysis was performed using Prism (GraphPad Software, San Diego, CA). $P < 0.05$ was defined as statistically significant.

DATA AVAILABILITY

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

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AUTHOR CONTRIBUTIONS

XT., KH, and QF performed study concept and design. XT, CC, and YC performed the experiments and collected the data. XT, KH, HY, LZ, and JH analyzed the data. XT, KH, ML, and QF provided technical and material support and revised the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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