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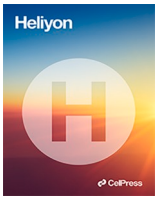

Research article

MAD1 deficiency accelerates hepatocellular proliferation via suppressing TGF-β signaling

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ABSTRACT

Numerous researches have reported on the regulatory network of liver regeneration induced by partial hepatectomy (PH). However, information on key molecules and/or signaling pathways regulating the termination stage of liver regeneration remains limited. In this study, we identify hepatic mitotic arrest deficient 1 (MAD1) as a crucial regulator of transforming growth factor β (TGF-β) in the hepatocyte to repress liver regeneration. MAD1 has a low expression level at the rapid proliferation phase but significantly increases at the termination phase of liver regeneration. We show that MAD1 deficiency accelerates hepatocyte proliferation and enhances mitochondrial biogenesis and respiratory. Mechanistically, MAD1 deficiency in hepatocytes enhances mitochondrial function and promotes hepatocyte proliferation by suppressing TGF-β signaling. Our study reveals MAD1 as a novel suppressor of hepatocyte proliferation, which may provide a new therapeutic target for the recovery of liver function after liver transplant and partial hepatectomy.

1. Introduction

The liver has an incredible ability to regenerate to restore its original quality and function following partial hepatectomy and damage caused by toxins and viral infections [1,2]. Liver regeneration (LR) is a precise and orderly regulatory process involving a variety of different cells, cytokines, and interactions between organ systems [3–5]. However, the mechanisms that regulate liver regeneration are not completely understood. Exploring the molecular regulatory mechanism of liver regeneration contributes to amplifying our understanding of the regulatory network of liver regeneration and further developing successful therapies to accelerate cell proliferation for liver cancer after hepatectomy or liver transplantation.

Many signaling pathways and key molecules contribute to liver regeneration, which involves three phases: initiation, proliferation, and termination [6,7]. Although the processes governing the termination of liver regeneration are not extensively studied or fully

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understood, the transforming growth factor β (TGF-β) signaling has been proven to suppress hepatocyte proliferation at the termination of liver regeneration [8,9]. TGF-β is purged from the liver at the initiation of regeneration after partial hepatectomy (PH) but begins to be activated in later stages [10]. However, it was reported that increased activation of TGF-β signaling is insufficient to block the proliferation of hepatocytes isolated between 24 and 72 h after PH [11]. Consequently, the pleiotropic roles of TGF-β signaling pathway in liver regeneration are not fully understood.

Mitotic arrest deficient 1 (MAD1) is reported to regulate cell proliferation, differentiation, and apoptosis [12,13]. Accumulated evidence reveals a non-negligible function of MAD1 in inhibiting tumor cell proliferation, such as repressing the proliferation of human melanoma cells, primary gastric cancer cells, and bladder cancer cells [14–16]. In addition, Mad1 overexpression in T cells leads to reduced thymic cellularity and impaired mitogen-induced proliferation [17]. Cao L. et al. found that the AR-PDEF pathway promotes tumor proliferation by promoting the degradation of MAD1 in estrogen receptor-negative breast cancer [18]. Notably, a recent high-throughput sequencing study showed that the expression of MAD1 is greatly changed in mice liver after PH [19]. However, whether and how MAD1 affects hepatocyte proliferation and liver regeneration after PH is unclear. Mitochondria provide energy in the process of liver regeneration, and a newly published study showed that increased mitochondrial activity promotes liver regeneration after partial hepatectomy [20–22]. MAD1 is reported to inhibit mitochondrial function, which may provide a novel link between MAD1-dependent mitochondrial function and liver regeneration [23]. We, therefore, sought to investigate whether MAD1 plays a role in regulating mitochondrial function and liver regeneration.

In this study, we identify MAD1 as a critical suppressor of hepatocyte proliferation. In addition, we demonstrate that MAD1 deficiency promotes hepatocyte proliferation by enhancing mitochondrial function and suppressing TGF-β signaling.

2. Results

2.1. MAD1 is down-regulated in the proliferating hepatocytes

Considering the inhibitory role of MAD1 in cellular proliferation, we sought to investigate whether MAD1 plays a role in liver regeneration and hepatocyte proliferation induced by PH in mice. To investigate the role of MAD1 in liver regeneration (LR), we first assessed the expression levels of MAD1 in mouse liver following 2/3 PH, which is the classic model for studying liver regeneration [1,7,24]. We observed a significant inhibition of MAD1 expression by 2/3 PH, with the lowest levels reached at approximately 24 h post 2/3 PH treatment (Fig. 1A). The period of bottom levels of MAD1 coincided with the peak period of liver regeneration (2–3 d) [25,26]. Subsequently, MAD1 exhibited a gradual increase and returned to basal levels by day 7 after 2/3 PH, and finally showed a two-fold increase on day 14 (Fig. 1A), suggesting a potential role of MAD1 in suppressing LR. To further test this hypothesis, we measured the levels of MAD1 as well as the hepatocyte proliferation marker gene Mki67 in mouse primary hepatocytes with EGF treatment. EGF treatment greatly inhibited MAD1 (Fig. 1B) but provoked Ki67 (Fig. 1C) expression in primary mouse hepatocytes, which further confirmed that MAD1 expression is suppressed in the proliferation phase of LR.

2.2. MAD1 deficiency accelerates hepatocyte proliferation

To further determine the role of MAD1 in liver regeneration, we first examined whether ablation of MAD1 affects the proliferation of primary hepatocytes isolated from the liver of C57BL/6 mice. We transfected primary hepatocytes with MAD1 siRNA or their control

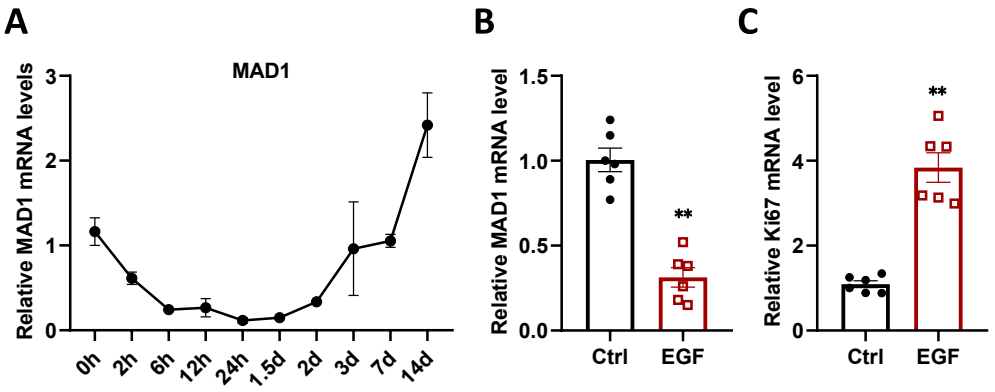


Fig. 1. MAD1 is down-regulated in the proliferating hepatocytes. (A) mRNA expression level of MAD1 at different time points (0 h, 2 h, 6 h, 12 h, 24 h, 1.5days, 2days, 3 days, 7days, 14days) after liver regeneration in 8-week old wild-type C57 BL/6J mice (n = 4–6/group). Primary hepatocytes were treated with EGF (final concentration: 30 ng/ml), and qRT-PCR analyses of MAD1 (B) and proliferation marker Ki67 (C) genes expression in primary hepatocytes (n = 6/group). Error bars in all experiments represent SEM; Significance was determined by unpaired 2-tailed Student's *t*-test. $^{**}P < 0.01$.

siRNA and confirmed that the mRNA level of Mad1 was suppressed by 70% with siRNA treatment (Fig. 2A). The successful knockdown of MAD1 has also been demonstrated by immunoblotting analysis (Fig. 2B). MAD1 deficiency promoted hepatocyte proliferation as demonstrated by increased Mki67 (Fig. 2C) and cyclins expression levels (Fig. 2D and E). In agreement with this result, suppressing MAD1 greatly increased the protein level of proliferating cell nuclear antigen (PCNA) (Fig. 2E), which is necessary for DNA synthesis during replication and hepatocyte proliferation [27]. In addition, the percentage of EdU-positive cells is 2-fold higher in MAD1-deficient hepatocytes compared to control hepatocytes (Fig. 2F). Taken together, these results suggest that MAD1 deficiency could accelerate hepatocyte proliferation.

2.3. *MAD1 deficiency enhances mitochondrial function*

Considering the crucial roles of mitochondrial biogenesis and activity in promoting hepatocyte proliferation during liver regeneration [21,28,29],we sought to investigate whether MAD1 deficiency facilitates hepatocyte proliferation through enhancing th mitochondrial function. As shown in Fig. 3A, MAD1 deficiency significantly increased oxygen consumption rates (OCR) in primary hepatocytes. By flow cytometry analysis, we found that MAD1 deficiency significantly increased mitochondrial mass (Fig. 3B) and decreased mitochondrial ROS (mtROS) production (Fig. 3C) in primary hepatocytes, suggesting that enhanced mitochondrial function in primary hepatocytes may play a critical role in MAD1 deficiency-induced hepatocyte proliferation.

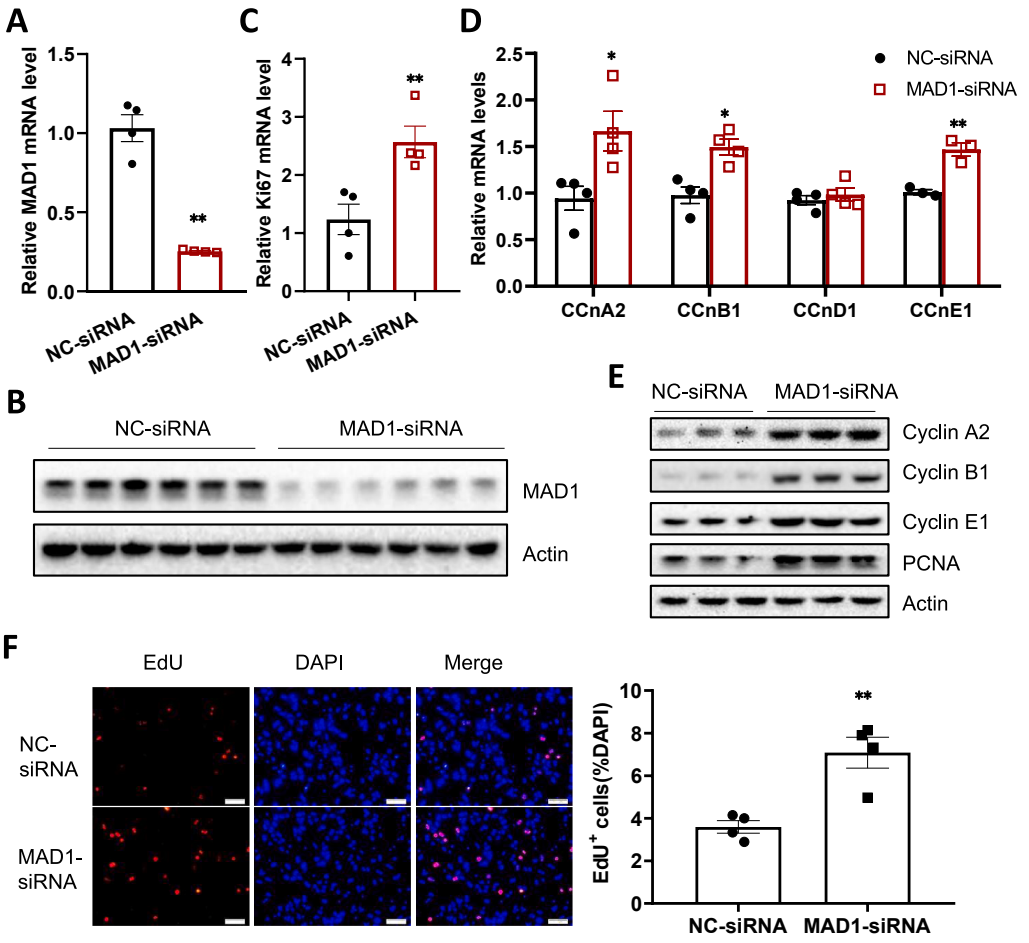
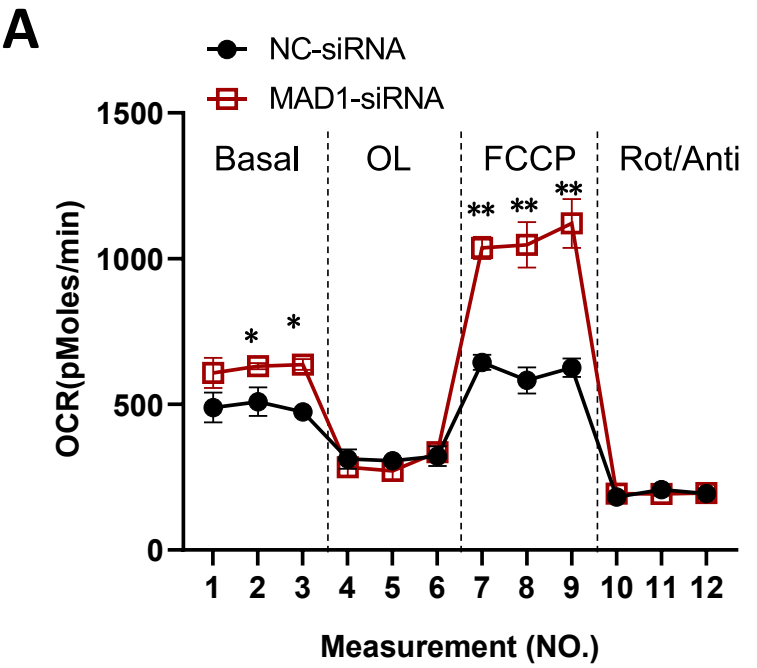
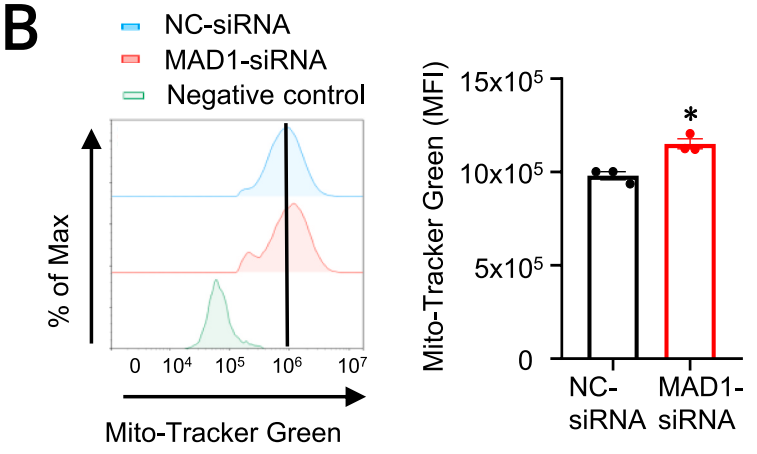


Fig. 2. MAD1 deficiency accelerates hepatocyte proliferation. MAD1 siRNA or control siRNA were treated in primary hepatocytes in the presence of EGF (final concentration: 30 ng/ml). (A and B) qRT-PCR and Western blot analyses of MAD1 in primary hepatocytes (n = 4 or 6/group). (C and D) qRT-PCR analyses of Ki67 and cyclin genes in primary hepatocytes (n = 4/group). (E) Western blot analyses of cyclin proteins and PCNA level (proliferating cell nuclear antigen) in primary hepatocytes (n = 3/group). (F) EdU immunostaining of primary hepatocytes (n = 4/group; scale bar: 100 μm). The uncropped images of (B) and (D) are provided in Supplementary Material. Error bars in all experiments represent SEM; Significance was determined by unpaired 2-tailed Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.





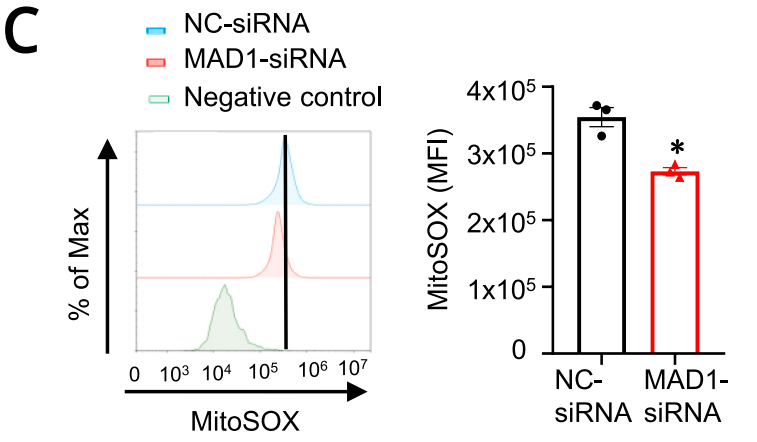


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Fig. 3. MAD1 deficiency enhances mitochondrial function. MAD1 siRNA or control siRNA were treated in primary hepatocytes in the presence of EGF (final concentration: 30 ng/ml). (A)OCR of NC-siRNA,MAD1-siRNA which plus and without EGF were measured under basal conditions and in response to indicated drugs (n = 4/group). OL, oligomycin; Rot, rotenone; Anti, antimycin. Mitochondrial mass (B) and mitochondrial ROS (C) of MAD-siRNA or NC-siRNA-treated primary hepatocytes were analyzed by Mito-Tracker Green and MitoSOX staining respectively (n = 4/group). Error bars in all experiments represent SEM; Significance was determined by unpaired 2-tailed Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

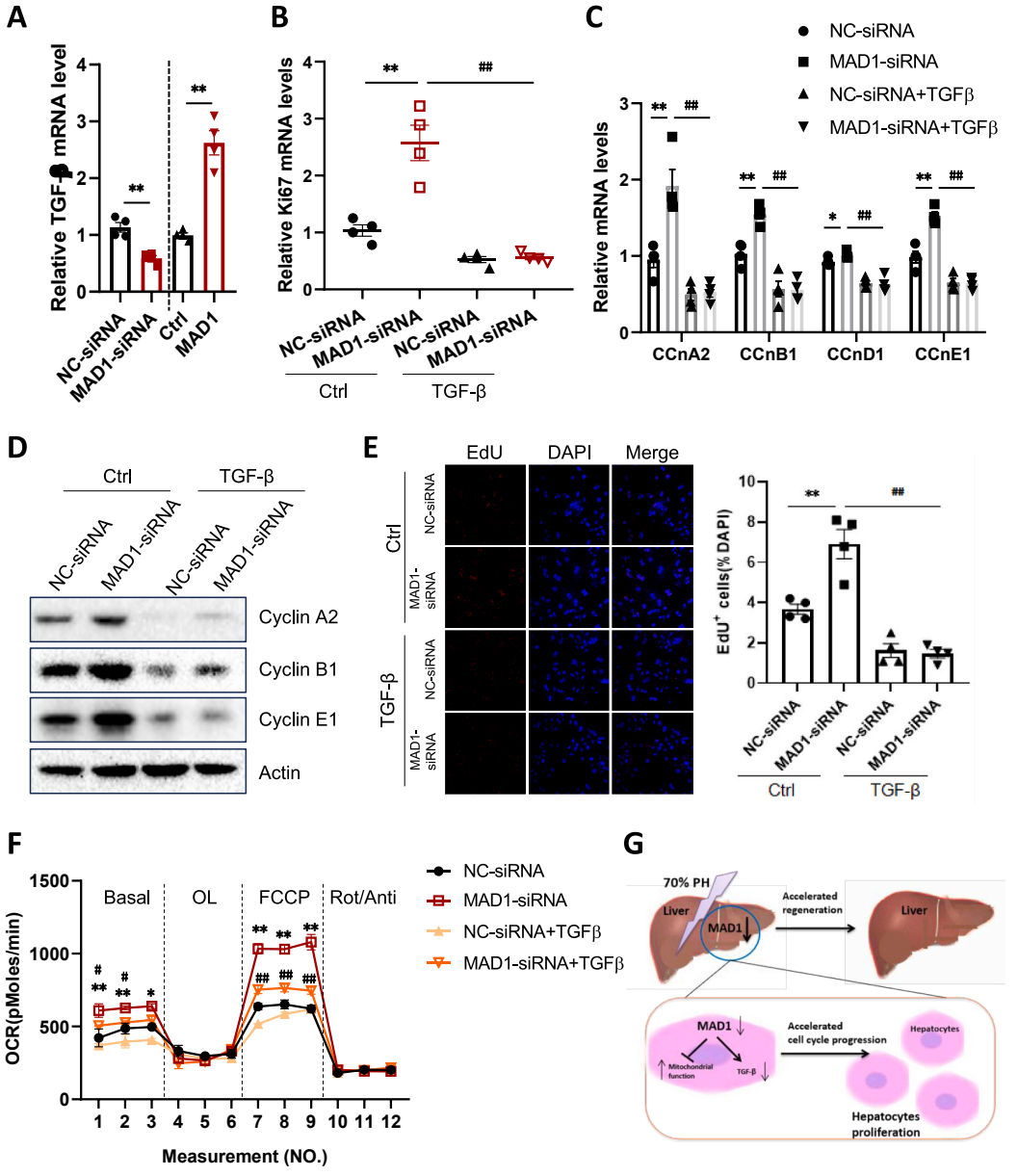

Fig. 4. MAD1 Suppresses hepatocytes proliferation via TGF-β signaling. Primary hepatocytes transfected with MAD1 siRNA and scramble control were treated with EGF (final concentration: 30 ng/ml). (A) qRT-PCR of TGF-β in primary hepatocytes. (B) qRT-PCR of Mki67 in NC-SiRNA, MAD1-SiRNA which plus and without TGF-β (30 ng/ml). (C and D) qRT-PCR and Western blot of cyclins (cyclinA2, cyclingB1, cyclinD1, cyclinE1) in NC-siRNA, MAD1-siRNA which plus and without TGF-β (30 ng/ml). (E) Edu staining and its quantification in NC-siRNA,MAD1-siRNA which plus and without TGF-β (30 ng/ml). (F) OCR of NC-siRNA, MAD1-siRNA which plus and without TGF-β (30 ng/ml) were measured under basal conditions and in response to indicated drugs. Oligo, oligomycin; Rot, rotenone; Ant, antimycin. (G) A proposed model for the mechanism by which hepatic MAD1 regulates hepatocytes proliferation. The uncropped images of (D) are provided in Supplementary Material. Error bars in all experiments represent SEM; Significance was determined by unpaired 2-tailed Student's *t*-test.$^{*}P < 0.05$, $^{**}P < 0.01$, MAD1-siRNA compared with NC-siRNA group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, MAD1-siRNA + TGF-β compared with MAD1-siRNA group.

2.4. MAD1 suppresses hepatocyte proliferation via TGF-β signaling

We next investigated the mechanism underlying MAD1 deficiency-mediated hepatocyte proliferation. Given the crucial role of the TGF-β signaling pathway in liver regeneration termination [25], we investigated the expression level of TGF-β in hepatocytes with siRNA-suppressed or plasmid-overexpressed MAD1. The results showed that ablation of MAD1 significantly inhibited the expression of TGF-β. However, MAD1 overexpression dramatically increased the TGF-β level in primary hepatocytes (Fig. 4A). To confirm the role of TGF-β signaling in MAD1 deficiency-induced hepatocyte proliferation, we treated MAD1 deficiency hepatocytes with or without TGF-β, and found that TGF-β effectively attenuated the stimulatory impact of MAD1 deficiency on hepatocyte proliferation (Fig. 4B–D). Furthermore, Edu staining also confirmed that supplementation of exogenous TGF-β could significantly inhibited the hepatocyte proliferation induced by MAD1 deficiency (Fig. 4E). Consistently, Seahorse experiments verified that TGF-β could effectively reverse the elevated OCR induced by MAD1 deficiency compared with the control group (Fig. 4F). Taken together, these results demonstrate that absence of MAD1 may promotes hepatocyte proliferation via inhibiting the TGF-β signaling.

3. Discussion

In the present study, we identified MAD1 as a critical suppressor of hepatocyte proliferation. In addition, we demonstrated that MAD1 deficiency promotes hepatocyte proliferation via the TGF-β signaling pathway. MAD1 expression is suppressed during the proliferation phase but markedly induced during the termination phase of LR, which regulates mitochondrial function and TGF-β signaling, orchestrating hepatocyte proliferation (Fig. 4F). Our study provides new evidence on a critical role of hepatocyte MAD1 in LR and uncovers a molecular mechanism by which PH-induced the proliferation and termination phase of LR.

Many studies highlight the critical role of MAD1 in cell proliferation [14–16,30], with the consistent finding that MAD1 deficiency promotes hepatocyte proliferation. Of note, the MAD1 level was low at the rapid proliferation phase (24–72 h after PH), but significantly increased at the termination phase (7–14 days after PH), which was consistent with the profile of liver regeneration suppressor. In addition, MAD1 is a core protein of the spindle assembly checkpoint which plays a vital role in error correct and faithful chromosome segregation to maintain chromosomal stability during mitosis [31,32], which affects cell proliferation and apoptosis by regulating mitotic processes [33,34]. Consistent with this, MAD1 deficiency exhibited increased hepatocyte proliferation, as evidenced by increased expression levels of Mki67 (Fig. 2B) and various cyclins such as cyclin E1, cyclin A2, and cyclin B1 (Fig. 2C).

Mitochondria provides energy for various cell physiological activities, including cell proliferation and mitosis, and provides normal mitochondrial complements for cells. On the one hand, proliferation produces high-energy demand, while mitochondrial function defects or lack of mitochondrial activity can cause impeded cell cycle progression, suggesting that mitochondria are important for cell cycle and proliferation during the proliferation process. Our research confirmed that the MAD1 deficiency not only promoted the proliferation of hepatocytes but also enhanced the mitochondrial function of hepatocytes. Firstly, we found MAD1 deficiency led to increased oxygen consumption rates (OCR) (Fig. 3A), indicating that inhibition of MAD1 enhanced mitochondrial respiration. Secondly, the deficiency of MAD1 resulted in the increase of mitochondrial mass (Fig. 3B) and the downregulated production of reactive oxygen species (ROS) (Fig. 3C), which improved mitochondrial oxidative metabolism. On the other hand, plenty of signaling pathways related to proliferation and cell cycle progress regulate mitochondrial function [35–38]. There is a positive feedback between the progression of the cell cycle and the mitochondrial function such that cell adaptation metabolism is caused when cell proliferation [39]. Thus, it is possible that enhanced mitochondrial function also promotes MAD1 deficiency-mediated hepatocyte proliferation. Further experiments will be needed to address these questions.

TGF-β is mainly produced by hepatic stellate cells (HSCs) and has been shown to play a role in the termination stage of regulating liver regeneration [7,40]. Meanwhile, previous researches reported that hepatocytes-derived TGF-β also plays vital role in hepatocytes proliferation [41]. Similar with these findings, we found that TGF-β expression level was decreased in MAD1-deficient hepatocytes and involved in MAD1 deficiency-mediated hepatocyte proliferation (Fig. 4B–D). These results suggested that the proliferation promoting effect of MAD1 deficiency was mainly attribute to the decreased TGF-β in the hepatic microenviroment. In addition, previous findings indicated that TGF-β can mediate the expression of MAD1, which means that MAD1 is a downstream target of TGF-β [42,43]. However, our data showed that TGF-β treatment could block MAD1 deficiency-enhanced mitochondrial respiration (Fig. 4E), which provides new evidence to support the negative regulatory mode of TGF-β in regulating mitochondrial function [44–46]. The subcellular localization of MAD1 protein was predominantly observed in the cytoplasm and nuclei, which could bind with Tpr (translocated promoter region) [47]. Our results showed that MAD1 regulates the mRNA level of *Tgfb* in hepatocytes, whether this modulatory function was mediated by directly DNA binding or indirect mechanisms is one of the major limitations of this study and remains to be further investigated.

In conclusion, we have identified hepatic MAD1 as a key suppressor of the hepatocyte proliferation termination phase that inhibits LR in mice after 2/3 PH. In addition, we uncover a new signaling mechanism underlying MAD1 deficiency that enhanced mitochondrial function and blocked TGF-β signaling pathway that is critical for PH-induced LR. Thus, MAD1 may provide a new therapeutic target for the recovery of liver function after liver transplant and partial hepatectomy.

4. Materials and methods

4.1. Partial hepatectomy mice model and tissue collection

Male C57BL/6J mice (Hunan SJA Laboratory Animal Co., Ltd, Changsha, China) were housed in a temperature-controlled

environment with a 12-h light/12-h dark cycle and had free access to water and food. Eight-week-old male mice were subjected to 2/3 partial hepatectomy as described by Higgins and Anderson (1931). After the surgery, mice were harvested at 0 h, 2 h, 6 h, 12 h, 24 h, 1.5 d, 2 d, 3 d, 7 d, 14 d to collect the liver tissues. Briefly, deep-anesthetized mice were subjected to cardiac puncture to collect blood samples. Mice were then sacrificed and liver tissue was collected and frozen in −80 °C for future study. All animal studies were performed under a protocol approved by the Central South University Animal Care and Use Committee (Permission number: CSU2020406).

4.2. Primary hepatocyte isolation and cell culture

The primary hepatocytes were isolated from male C57BL/6J mice. First, the liver was perfused with HBSS to washout blood and digested with IV collagenase to separate the cells. Then the cell suspension was filtrated and centrifuged to get the primary hepatocytes. The freshly isolated hepatocytes were seeded in 6-well plates pre-embedded with collagen type I, rat tail (Corning, Corning, NY) in William's E medium (Thermofisher Scientific, Waltham, MA) supplemented with 5% FBS (Thermofisher Scientific, Waltham, MA) and GlutaMax (Thermofisher Scientific, Waltham, MA). After culturing for 2–4 h, non-adherent cells and cell debris were washed off with PBS, and then replaced with William's E medium containing GlutaMax, 10 % FBS, and 30 ng/mL EGF from MedChemExpress (Monmouth Junction, NJ, USA) to establish a in vitro liver proliferation model.

4.3. siRNA transfection

The primary hepatocytes were transfected with MAD1-specific siRNAs (RiboBio Co., Ltd, Guangzhou, China) (Forward: 5′-GGAAAUGGCAAGAGGCAAATT-3'; Reverse: 5′- UUUGCCUCUUGCCAUUUCCTT-3′) or control NC-siRNA (Forward: 5′-UUCUCCGAACGUGUCACGUTT-3'; Reverse: 5′-ACGUGACACGUUCGGAGAATT-3′) using Lipofectamine 3000 (Thermofisher Scientific, Waltham, MA). Cells were harvested after 48 h transfection.

4.4. Quantitative RT-PCR

Total RNA was extracted from frozen liver tissues and cells by using Trizol reagent (Thermofisher Scientific, Waltham, MA) according to the manufacturer's instructions. RNA purity was analyzed using a spectrophotometer at with absorbance values of 260 nm and 280 nm (A260/280). Synthesized qualified RNA into cDNA following the reverse transcription kit (Accurate Biotechnology (Hunan) Co., Ltd, Changsha, China). Quantitatively amplified all cDNA by using SYBR Green (Accurate Biotechnology (Hunan) Co., Ltd, Changsha, China) and normalized by β-actin. The primers used were as follows: Actin (Forward: 5′- TTCTTTGCAGCTCCTTCG-3'; Reverse:5′-TTCTGACCCATTCCCACC-3′); CcnA2 (Forward: 5′-GTGGTGATTCAAAACTGCCA-3'; Reverse: 5′-AGAGTGTGAAGATGCCCTGG-3′); CcnB1 (Forward: 5′- AAATACCTACAGGGTCGTGAAG-3'; Reverse: 5′-CTCCTGAAGCAGCCTAAATTTC-3′); CcnD1 (Forward: 5′-AAGCATGCACAGACCTTTGTGG-3'; Reverse: 5′- TTCAGGCCTTGCATCGCAGC-3′); CcnE1 (Forward: 5′-TCCACGCATGCTGAATTATC-3'; Reverse:5′-TTGCAAGACCCAGATGAAGA-3′); Mki67 (Forward: 5′- CACAGAGAACAAAGGTGTGAAG-3'; Reverse: 5′- GGAGACTGCAGAGCTATTTTTG-3′); Tgfb1 (Forward: 5′-GCAGTGGCTGAACCAAGGA-3'; Reverse:5′- AGCAGTGAGCGCTGAATCG-3′); Mad1 (Forward: 5′-TCCGCTCAAAGTCCTACCTCATC-3'; Reverse: 5′-CGCTCGTAGTTCCTGGCATTG-3′).

4.5. Western blotting

Lysed cells and liver tissue with RIPA buffer (Beyotime, Shanghai, China) containing protease/phosphatase inhibitor (Merck Millipore, Billerica, MA). SDS buffer (Beyotime, Shanghai, China) was added to the lysate, followed by protein denaturation. Total protein was separated via SDS-PAGE and transferred to nitrocellulose membranes, the membrane was incubated with some primary antibodies against β-actin (Cat#A3854) from Sigma Aldrich (St. Louis, USA), MAD1 (Cat#4682S) from Cell Signaling Technology (MA, USA), PCNA (Cat#ab29) from Abcam (Cambridge, UK), Cyclin A2 (Cat#81754S), Cyclin B1 (Cat#4138S), Cyclin D1 (Cat#2978S), and Cyclin E1 (Cat#20808S) from Cell Signaling Technology (MA, USA), followed by anti-rabbit or anti-mouse secondary antibodies. Finally, the protein bands were developed with ECL chemiluminescence substrate (Thermofisher Scientific, Waltham, MA).

4.6. Edu staining

The number of proliferating hepatocytes were measured by Edu staining kit (RiboBio Co., Ltd, Guangzhou, China) after treated with EGF in vitro.

4.7. Flow cytometry

After primary hepatocytes were seeded into a six-well culture plate according toat the density of 1×10^6 cells per well, we set up the MAD1-siRNA group (hepatocyte transfected with MAD1-siRNA), NC-siRNA group (hepatocyte transfected with NC-siRNA), MAD1-siRNA + EGF group (hepatocyte transfected with MAD1-siRNA and treated with EGF (30 ng/ml)), NC-siRNA + EGF group (hepatocyte transfected with NC-siRNA and treated with EGF (30 ng/ml)). For mitochondrial mass and mitochondrial ROS measurements, primary hepatocytes were detached from the plates substrate by 0.25 % Trypsin-EDTA to get the single-cell suspension, and then incubated with 100 nM Mito-Tracker Green, 100 nM Mito-Tracker Red, and 5 μM mitoSOX, respectively (Thermofisher Scientific, Waltham, MA),

at 37 °C for 15–30 min and then analyzed by flow cytometry according to the manufacturer's instructions.

4.8. OCR analysis

Mitochondrial OCR in hepatocytes was measured using Seahorse Bioscience XF-24 analyzer (Agilent, Santa Clara, USA) according to the manufacturer's instructions. Seeding 4×10^4 primary hepatocytes/well into XF-24 microplates, basal mitochondrial respiration was measured in untreated cells, and the following reagents including 1 μM oligomycin (Sigma-Aldrich, St. Louis, USA), 1 μM FCCP (Sigma-Aldrich, St. Louis, USA) or 1 μM rotenone (Sigma-Aldrich, St. Louis, USA) were added to block state III respiration, induce uncoupling or shut down mitochondrial respiration, respectively. Data were normalized to protein content.

4.9. Statistical analysis

Statistical analysis was performed using SPSS statistics 19.0 (SPSS, Inc.) or GraphPad Prism 8.0 Software (GraphPad Software, Inc.). Data analysis involved unpaired 2-tailed Student's *t*-test for two groups and one-way ANOVA for more than two groups. All results were presented as the mean ± SEM and $P < 0.05$ was considered to be statistically significant.

Data availability statement

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

CRediT authorship contribution statement

Jiangming Deng: Investigation, Formal analysis, Data curation. **Jianhui Teng:** Software, Methodology. **Ting Xiao:** Visualization, Project administration. **Jie Wen:** Writing – original draft, Formal analysis. **Wen Meng:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31312.

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