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Alpha-fetoprotein/endoplasmic reticulum stress signaling mitigates injury in hepatoma cells

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Alpha-fetoprotein (AFP) and endoplasmic reticulum (ER) stress play multiple roles in hepatocellular carcinoma. Here, we analyzed the crosstalk between AFP and ER stress in human hepatoma cells. We induced ER stress in human hepatoma cell lines (HepG2 and SK-Hep1 cells) with thapsigargin (TG, an ER stress inducer), and mitigated ER stress with 4-phenylbutyrate acid (4-PBA, an ER stress inhibitor). AFP expression was knocked down by AFP short hairpin RNA and rescued by the pCI-AFP vector. AFP expression and ER stress were examined, and their roles in apoptosis, necroptosis, and proliferation were analyzed. TG significantly induced ER stress, apoptosis, necroptosis, and intracellular AFP protein levels, and reduced proliferation and AFP mRNA expression as well as supernatant AFP protein levels in HepG2 and SK-Hep1 cells. 4-PBA pretreatment partially reversed those changes in HepG2 cells. By contrast to AFP overexpression, knockdown of AFP significantly exacerbated TG-induced ER stress, apoptosis, and necroptosis, and decreased proliferation and the expression of activating transcription factor 6 alpha. In conclusion, ER stress causes the accumulation of AFP protein, which may be related to the reduction of AFP secretion. Accumulated AFP mitigates apoptosis and necroptosis and restores the proliferation of hepatoma cells by reducing ER stress.

Key words: alpha-fetoprotein, endoplasmic reticulum stress, apoptosis, necroptosis, hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is among the leading causes of cancer-related mortality worldwide [1]. Alpha-fetoprotein (AFP) has long been regarded as a classical tumor marker for the clinical diagnosis of HCC. In particular, AFP levels can reflect the development of liver cancer [2]. AFP has multiple biological functions in hepatocellular carcinoma including promoting cell regeneration, inhibiting apoptosis, regulating the immune response, carrier, and hasten functions [3, 4]. Moreover, AFP promotes malignant behavior in hepatoma cells by regulating autophagy, apoptosis, and cell migration [5].

Endoplasmic reticulum (ER) stress plays a crucial role in the pathogenesis of several types of liver diseases [6]. During tumor development, tumor cells lack oxygen and nutrients and accumulate nitrogen [7, 8]. In response to those microenvironment changes, tumor cells often trigger ER stress, which is an ER defense mechanism that occurs secondary to physiological and pathological insults [9]. In tumors, the tumor microenvironment can promote ER stress, an event that can promote cancer progression; however, the mechanisms underlying the adaptation of tumor cells to ER stress are still unclear [10]. During ER stress, accumulated unfolded proteins bind to glucose-regulated protein 78 (GRP78), leading to phosphorylation of protein kinase R-like ER kinase (PERK). Consequently, PERK phosphorylates serine 51 of the eukaryotic initiation factor 2 alpha subunit (eIF2α) [11]. Alternatively, ER stress activates activating transcription factor 6 alpha (ATF6α) and inositol-requiring enzyme 1 (IRE1). Interestingly, eIF2α phosphorylation can repress protein synthesis and mitigate ER stress by decreasing the protein-folding load [12]. Following the attenuation of ER stress, phosphorylated eIF2α (p-eIF2α) can upregulate the expression of ATF4, which in turn induces the expression of growth arrest and DNA damage 34 (GADD34), GRP78, and C/EBP homologous protein (CHOP) [13]. Thus, PERK, ATF6α, and IRE1 are key molecules in ER stress as they can inhibit protein synthesis, upregulate the expression of ER response proteins, activate ER-associated degradation (ERAD), and promote cell survival [14]. Furthermore, ER stress can activate pro-apoptotic and pro-necroptotic signaling [15].

Apoptosis and necroptosis are two important modes of liver cancer cell death. CHOP, a growth arrest and DNA damage-inducible gene, is involved in ER stress-related apoptosis [16]. Caspase-3 is a key executor of apoptosis and its activation requires a proteolytic process that transforms its inactivated zymogen form into an activated p17 fragment [17]. Necroptosis is a regulated pathway for necrotic cell death. This process is negatively regulated by caspase and is initiated by a complex containing receptor-interacting protein 1 (RIP1) and receptor-interacting protein 3 (RIP3) kinases. Mixed lineage kinase domain-like protein (MLKL), a pseudokinase, is a downstream target of RIP3 in the necroptosis pathway. In necroptosis, RIP3 is phosphorylated, which recruits MLKL and causes its phosphorylation [18].

To the best of our knowledge, the crosstalk between AFP and ER stress and its impact on liver cancer have not yet been established. Here, we investigated the effects of crosstalk between ER stress and AFP on liver cancer apoptosis, necroptosis, and proliferation using thapsigargin (TG)-induced ER stress model. By inhibiting the calcium pump in the ER membrane, TG disrupts the calcium balance and induces ER stress [19]. The chemical chaperone 4-phenylbutyric acid (4-PBA) promotes protein folding and reduces ER stress and apoptosis [20].

Materials and methods

Cell culture and induction of ER stress. The HepG2 and SK-Hep1 human hepatoma cell lines were obtained from the Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HepG2 and SK-Hep1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. To induce ER stress, 2×10^5 HepG2 or SK-Hep1 cells/well were treated with TG (HepG2 cells: 0.5 μmol/l and SK-Hep1 cells: 0.25 μmol/l; Sigma, St. Louis, MO, USA; SK-Hep1 cells are more sensitive to TG-induced ER stress) for 12, 24, and 48 h in a 6-well plate. Alternatively, those cell lines were treated with dimethyl sulfoxide (DMSO) as an experimental control group. To understand the effects of ER stress on the expression of AFP, 4-PBA (10 mmol/l; Sigma) or phosphate-buffered saline (PBS) was added to HepG2 cells 2 h before treatment with TG (0.5 μmol/l; Sigma), or DMSO (control) was added for 24 and 48 h. 4-PBA reportedly decreases ER stress in various cell lines [21].

Table 1. Primers for qPCR.

Gene name	Sequence (5'-3')	
AFP	Forward	CTTTGGGCTCGCTATGA
	Reverse	GCATGTTGATTTAACAAGCTGCT
ATF6α	Forward	AGGCTGCCCTCTCAGAAAAC
	Reverse	TTTGCAGGGCTCACACTAGG
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

AFP short hairpin RNA transfection and AFP overexpression. A total of 1.2×10^6 HepG2 cells/well were plated in a 6-well plate and cultured in RPMI at 37 °C, 5% CO_2. The plated cells were transfected at 80% confluence. Short hairpin RNA (shRNA) targeting the human AFP gene (AFP shRNA target sequence: 5'-GCTTCCATATTGGATTCTTAC-3') and pCI-AFP vector (plasmid containing full-length human AFP open reading frame) were purchased from Syngentech Co., Ltd. (Beijing, China). For the knockdown or overexpression of AFP, HepG2 cells (1×10^6 cells/well) were cultured in a 6-well plate overnight at 37 °C and were subsequently transfected with AFP shRNA (2.5 μg/ml) or pCI-AFP vector (2.5 μg/ml), using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells transfected with an empty pCI vector (2 μg/ml) or control shRNA (2.5 μg/ml; sequence, 5'-TTCTCCGAACGTGTCACGTTT-3') served as the control groups. Transfection was performed according to the Lipofectamine® 3000 protocol. At 36 h post-transfection, ER stress was induced by the addition of 0.5 μmol/l TG for another 36 h. Finally, cells were harvested for the subsequent experiments.

Western blot analysis. HepG2 and SK-Hep1 cells were lysed in the immunoprecipitation assay lysis buffer (R0010; Solarbio, Beijing, China) for 1 h, on ice. Lysates were clarified by centrifugation for 5 min at 10,000×g and the protein contents were determined in the supernatants using the BCA method (GENEray, Shanghai, China). Then, equal amounts of total proteins in the supernatant were boiled under non-reducing conditions. Cell lysates (40 μg/lane) were separated by 10–12% SDS-PAGE followed by western blotting. Next, membrane blots were incubated with 5% fat-free dry milk in Tris-buffered saline with Tween 20 to prevent nonspecific binding and probed with mouse monoclonal antibodies against AFP (sc-130302; Santa Cruz, Dallas, TX, USA), ATF6α (MA1-25358l; Thermo Fisher Scientific), β-actin (sc-376421; Santa Cruz), CHOP (ab11419; Abcam, Cambridge, MA, USA), eIF2α (sc-133132; Santa Cruz), cyclin D1 (sc-8396; Santa Cruz), or MLKL (sc-293201; Santa Cruz); and rabbit monoclonal antibodies against ATF4 (11815, Cell Signaling Technology [CST], Danvers, MA, USA); cleaved caspase-3 (9664; CST), p-eIF2α (3398; CST), or XBP1s (83418, CST). The relative level of each target protein was compared to the control band using Quantity One software (Bio-Rad, Hercules, CA, USA).

Quantitative PCR. Quantitative polymerase chain reaction (qPCR) assays were used to evaluate AFP, ATF6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in HepG2 or SK-Hep1 cells (Table 1). Total RNA was extracted using TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Tokyo, Japan) following the manufacturer's instructions. The collected RNA was quantitated using Ultra micro spectrophotometer N60 series (IMPLEN Germany). Subsequently, the isolated RNA (500 ng) underwent reverse transcription with PrimeScript™ RT reagent Kit (TaKaRa), and qPCR was completed on the

CFX96 PCR system (Bio-Rad). Relative AFP expression was estimated with the delta-delta-comparative threshold cycle (ΔΔCt) method with GAPDH as the reference control [22].

Cell viability assay. The MTS assay was used to detect cell viability colorimetrically with the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Fitchburg, WI, USA) following the manufacturer's protocols. Briefly, 20 µl HepG2 or SK-Hep 1 culture medium was replaced with an MTS reagent and incubated at 37 °C for 3 h. Next, cell viability was detected colorimetrically on a microplate reader (Bio-Rad) at 490 nm. The percentage of cell viability was calculated as previously described [23].

Cell apoptosis analysis. The number of apoptotic cells was detected using the TransDetect Annexin V-FITC/PI Cell Apoptosis Detection Kit (TransGen Biotech, Beijing, China). Next, the apoptotic index was calculated relative to the total number of cells as previously described [24].

Analysis of supernatant AFP levels. AFP levels in the supernatant were measured by the chemiluminescence immunoassay on the Beckman Coulter Auto Analyzer (Model DX1800) as previously described [25]. Samples were centrifuged to remove the remaining cells and possible cell debris before testing the AFP concentration.

Statistical analyses. Differences among the experimental groups were statistically analyzed using a one-way analysis of variance with Tukey's post hoc analysis. The one-sample Kolmogorov-Smirnow test was used to analyze the normal distribution for continuous variables. The normally distributed data are expressed as the mean ± standard deviation (SD). A p-value <0.05 was considered statistically significant.

Results

TG induces ER stress, apoptosis, and necroptosis, and inhibits proliferation in hepatoma cell lines. TG significantly reduced cell viability ($p<0.05$; Figures 1A, 1B), upregulated the protein levels of ATF6α, CHOP, and cleaved caspase-3, and the phosphorylation levels of eIF2α and MLKL; and reduced the protein level of cyclin D1 in HepG2 cells ($p<0.05$; Figure 1C) and SK-Hep1 cells ($p<0.05$; Figure 1D).

TG increases intracellular AFP accumulation in hepatoma cells. TG treatment significantly increased intracellular AFP protein levels in HepG2 cells ($p<0.05$; Figure 2A) and SK-Hep1 cells ($p<0.05$; Figure 2B). In contrast, TG significantly reduced intracellular AFP mRNA levels ($p<0.05$; Figures 2C, 2D) as well as supernatant AFP protein levels ($p<0.05$; Figures 2E, 2F) in HepG2 and SK-Hep1 cells.

4-PBA pretreatment reduces TG-induced ER stress, apoptosis, necroptosis, and intracellular AFP protein levels, and restores proliferation in HepG2 cells. Compared to TG-treated HepG2 cells, pretreatment with ER stress inhibitor 4-PBA significantly improved HepG2 cell viability ($p<0.05$; Figure 3A). Compared to the control, PBA alone had no significant effect on intracellular AFP protein level ($p>0.05$; Figure 3B). On the other hand, it reduced the AFP, CHOP, cleaved caspase-3 p-eIF2α, and p-MLKL protein levels, and increased cyclin D1 protein levels at 24 and 48 h in the PBA+TG-treated HepG2 cells ($p<0.01$; Figure 3C). Furthermore, 4-PBA pretreatment partially restored intracellular mRNA levels ($p<0.01$; Figure 3D) as well as supernatant AFP protein levels at 24 and 48 h ($p<0.01$; Figure 3E). Interestingly, compared with the control, PBA alone had no significant effects on HepG2 cell viability, AFP mRNA level, and supernatant AFP protein levels ($p>0.05$).

AFP downregulation exacerbates TG-induced apoptosis and necroptosis, and reduces proliferation in HepG2 cells. Compared to control cells, AFP shRNA pretreatment alone significantly reduced the cell viability ($p<0.05$; Figure 4A) and downregulated the expression of AFP ($p<0.01$; Figure 4B) in HepG2 cells without TG treatment. The downregulation of AFP significantly increased CHOP, cleaved caspase-3, and p-MLKL protein levels, and reduced cyclin D1 protein levels, with or without TG treatment.

The impact of AFP knockdown suggested that the exacerbation of ER stress may be related to the increase of apoptosis, necroptosis, and inhibition of cell proliferation. Compared to TG-treated cells, knockdown of AFP with shRNA increased the expression of p-eIF2α, ATF4, and XBP1s, and reduced ATF6α (90 kDa and 50 kDa) protein expression ($p<0.05$; Figure 4C).

Furthermore, AFP overexpression significantly promoted cell proliferation ($p<0.05$), reduced CHOP, cleaved caspase-3, and p-MLKL protein levels, and increased cyclin D1 protein levels ($p<0.05$; Figure 4D). Finally, compared to the TG group, the apoptotic index was significantly increased in the AFP shRNA+TG group, and reduced in the pCI-AFP+TG group ($p<0.05$; Figure 4E). Similarly, the relative ATF6α mRNA expression was reduced in the AFP shRNA+TG group and increased in the pCI-AFP+TG group ($p<0.01$; Figure 4F).

Discussion

In this study, we investigated the possible crosstalk between AFP expression and ER stress, as well as its impact on apoptosis, necroptosis, and proliferation of hepatoma cells. Our results showed that the ER stress inducer TG, significantly increased apoptosis, necroptosis, as well as intracellular AFP protein levels, decreased cell proliferation, and AFP mRNA as well as supernatant AFP protein levels in HepG2 and SK-Hep1 cells. Further reducing the TG-induced ER stress of HepG2 cells by 4-PBA can partially reverse these changes. These results suggest that ER stress induces apoptosis, necroptosis, and inhibits proliferation as well as the accumulation of AFP protein in hepatoma cells. The accumulation of AFP in hepatoma cells could possibly lead to the decreased AFP secretion. Furthermore, pre-transfection of AFP shRNA significantly decreased AFP accumulation,

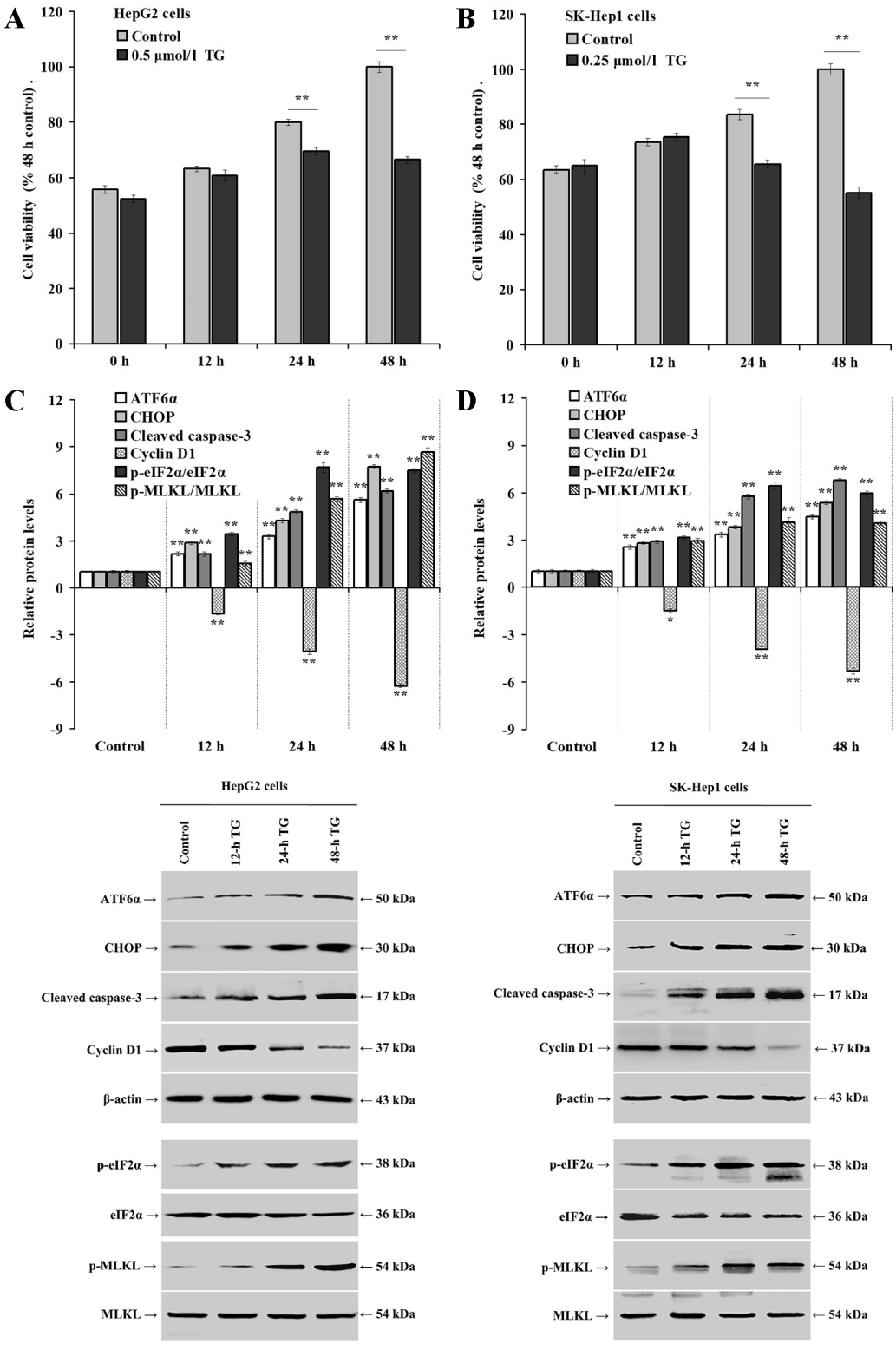

Figure 1. TG induces ER stress, apoptosis, necroptosis, and inhibits proliferation of HepG2 and SK-Hep1 cells. HepG2 and SK-Hep1 cells were incubated with TG or DMSO (control) for 12, 24, and 48 h. A) MTS cell viability assay post-TG treatment of HepG2 and B) SK-Hep1 cells. C) Western blot demonstrating the upregulated expression of ATF6α, CHOP, cleaved caspase-3, p-eIF2α, and p-MLKL, and the downregulated expression of cyclin D1 post-TG incubation in HepG2 and D) SK-Hep1 cells. Histograms representing the mean ± SD of five independent experiments. *p<0.05, **p<0.01 vs. the control group.

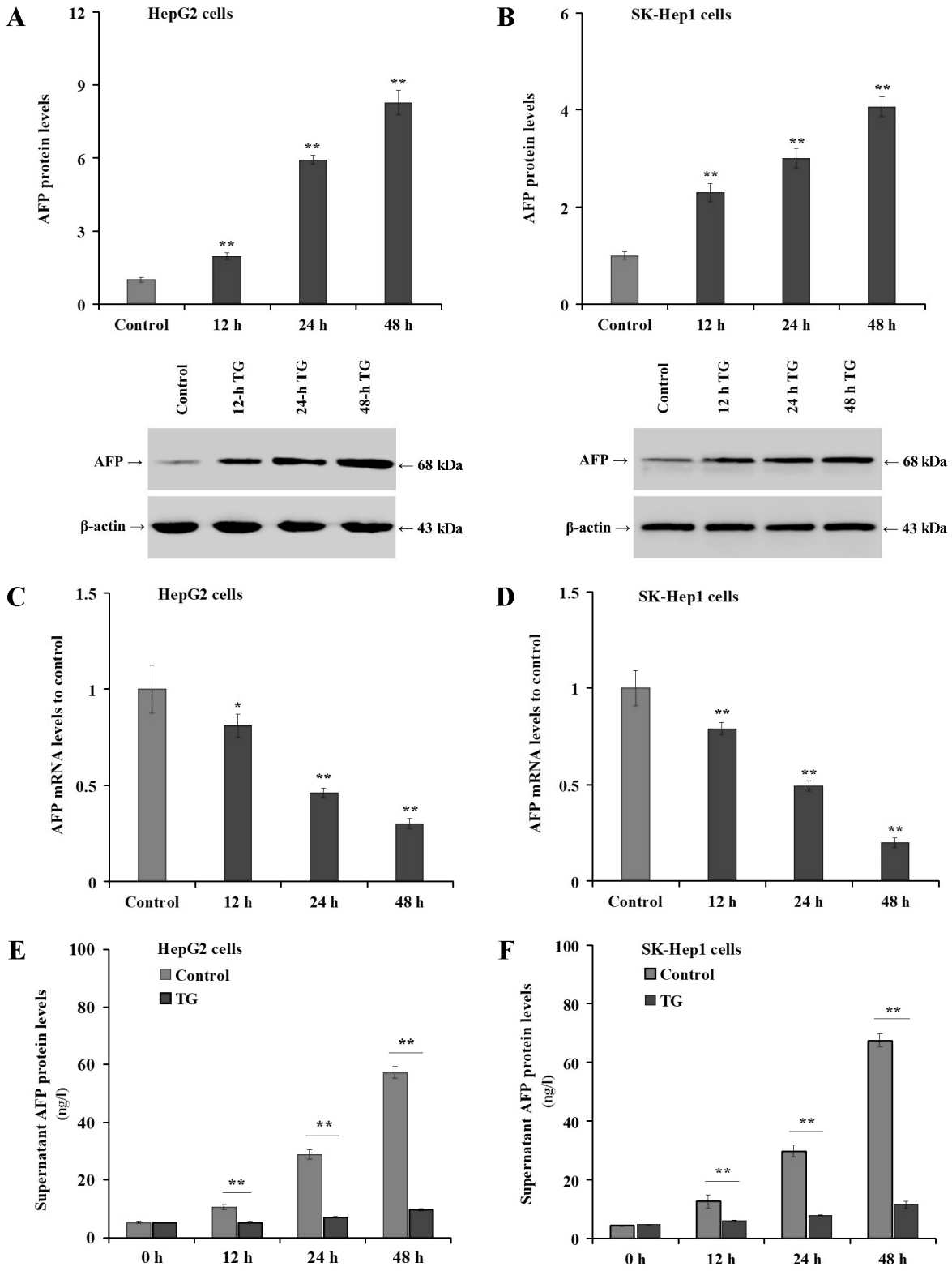

Figure 2. TG increases intracellular AFP protein levels in hepatoma cells. HepG2 and SK-Hep1 cells were incubated with TG or DMSO (control) for 12, 24, and 48 h. A) The protein levels of intracellular AFP post-TG incubation in HepG2 and B) SK-Hep1 cells. C) Bar chart demonstrating the relative mRNA levels of AFP in HepG2 and D) SK-Hep1 cells post-TG incubation. E) The supernatant protein levels of AFP in HepG2 and F) SK-Hep1 cells post-TG incubation. Histograms representing the mean ± SD of five independent experiments. *p<0.05, **p<0.01 vs. the control group.

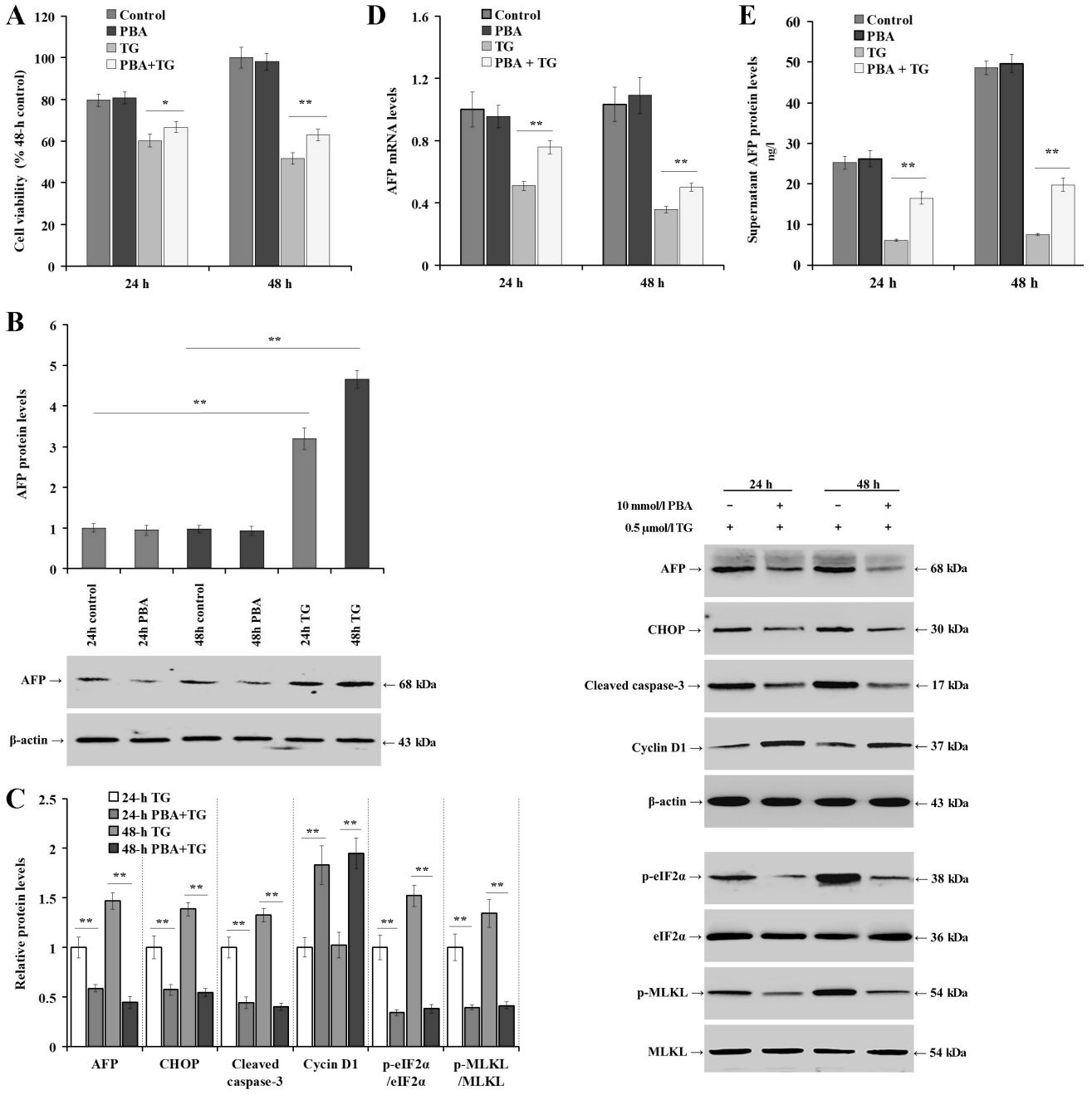

Figure 3. 4-PBA pretreatment reduces TG-induced ER stress, apoptosis, necroptosis, and intracellular AFP protein levels, partially restores proliferation in HepG2 cells. HepG2 cells were pretreated with 4-PBA or PBS for 2 h and were then incubated with or without TG for 24 and 48 h. A) Estimation of HepG2 cell viability among the different experimental groups by the MTS assay. B) Western blot demonstrating alterations in protein levels of AFP in PBA-treated HepG2 cells. C) Western blot demonstrating alterations in protein levels of AFP, CHOP, cleaved caspase-3, cyclin D1, p-eIF2α, eIF2α, p-MLKL, and MLKL in HepG2 cells. D) Bar chart representing relative AFP mRNA levels and E) bar chart representing supernatant AFP protein levels in control, PBA, TG, and PBA+TG HepG2 cells at 24 and 48 h. Histograms representing the mean ± SD of five independent experiments. *p<0.05, **p<0.001.

increased apoptosis and necroptosis, and decreased cell proliferation in HepG2 cells induced by TG. Simultaneously, lower AFP expression reduced the expression of ATF6α and aggravated ER stress, suggesting that AFP accumulation reduced apoptosis, necroptosis, and partially restored cell proliferation, which may be related to the feedback mechanism to reduce ER stress. Furthermore, the overexpression of AFP rescued the phenotype of AFP knockdown in the context of ER stress. In summary, the crosstalk between AFP and ER stress promotes the survival of hepatoma cells. Blocking the

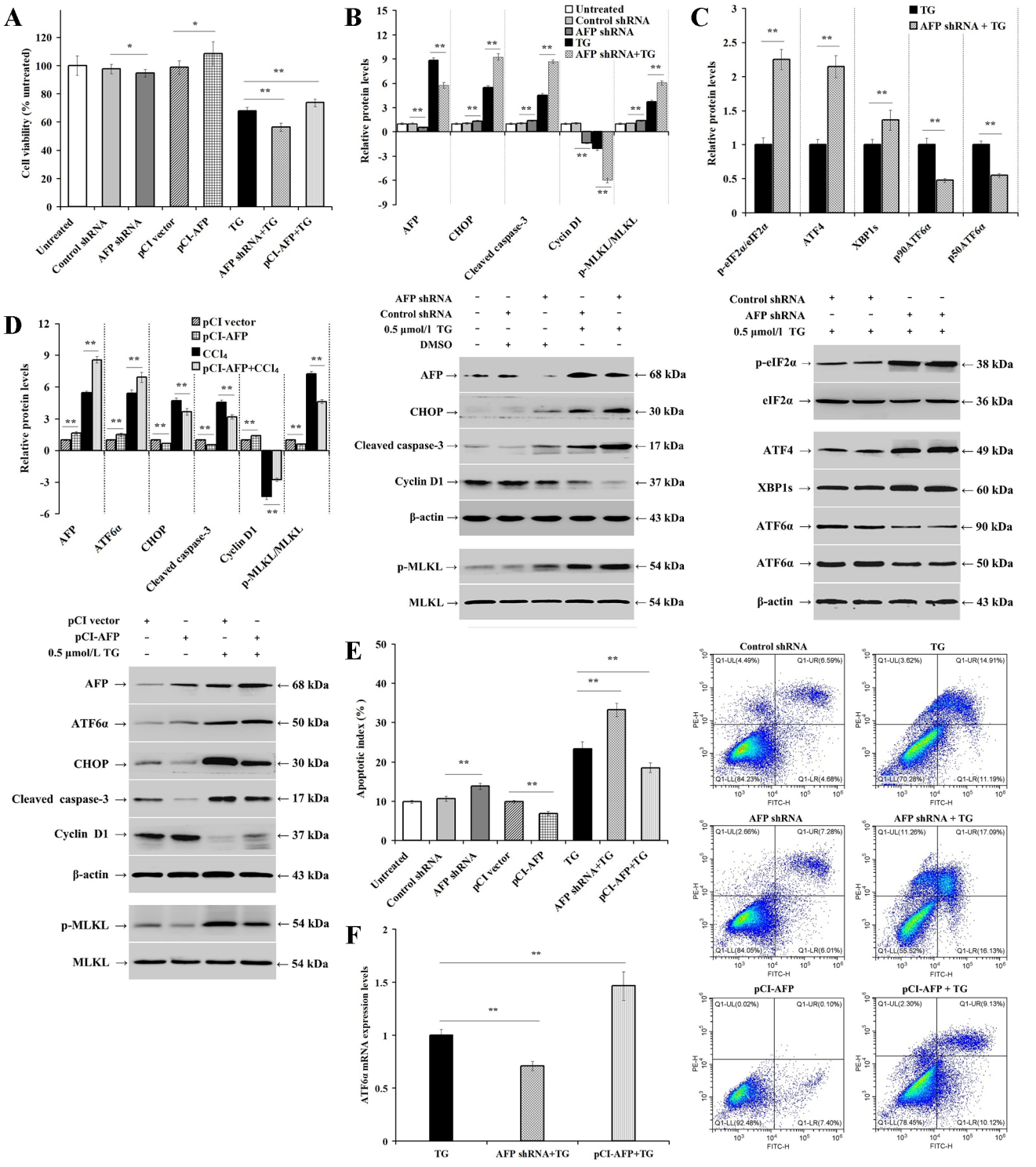

Figure 4. AFP downregulation aggravates TG-induced apoptosis and necroptosis, and reduces proliferation in HepG2 cells. HepG2 cells were pretreated with control shRNA, AFP shRNA, pCI vector, or pCI-AFP for 48 h, and then incubated with or without TG for another 36 h. A) Bar chart representing the viability of HepG2 cells in the different experimental groups as determined by the MTS assay. B) Western blot demonstrating alterations in protein levels of AFP, CHOP, cleaved caspase-3, cyclin D1, p-MLKL, and MLKL after AFP knockdown in HepG2 cells. C) Western blotting demonstrating alterations in the protein levels of p-eIF2α, eIF2α, ATF4, XBP1s, p90ATF6α, and p50ATF6α in HepG2 cells. D) Western blot demonstrating alterations in protein levels of AFP, ATF6α, CHOP, cleaved caspase-3, cyclin D1, p-MLKL, and MLKL in pCI-AFP-treated HepG2 cells. E) Bar chart representing the apoptotic index of HepG2 apoptosis as determined by flow cytometry. F) Bar chart demonstrating the relative mRNA levels of ATF6α. Histograms represent the mean ± SD of five independent experiments. *p<0.05, **p<0.01.

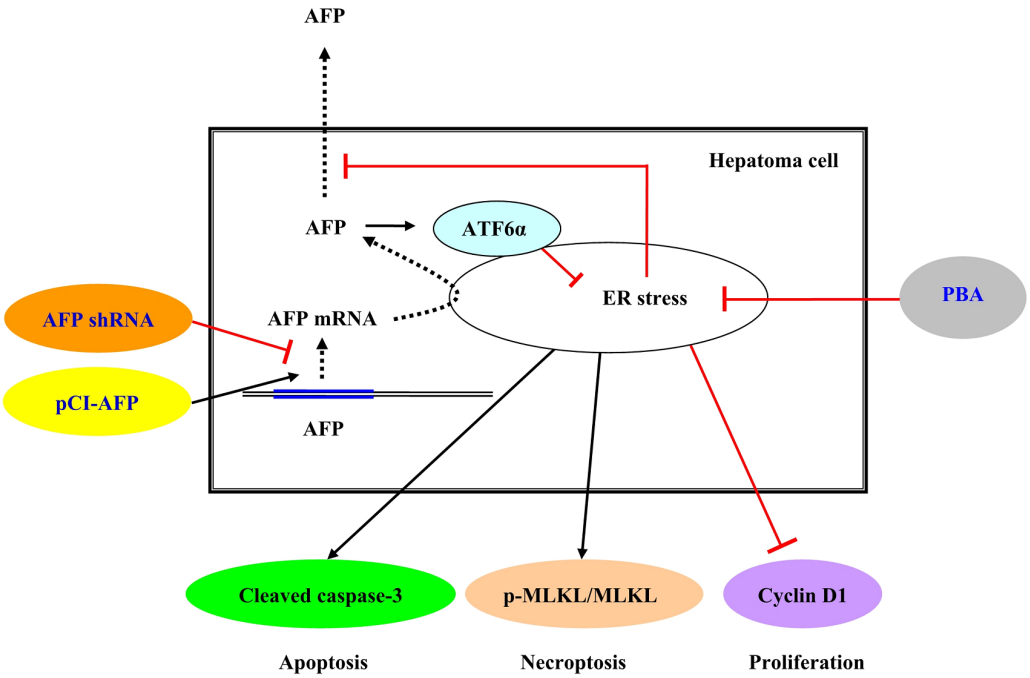

Figure 5. Schematic diagram representing the AFP-ER stress-apoptosis, necroptosis, proliferation signaling in hepatoma cells. ER stress induces apoptosis and necroptosis, inhibits cell proliferation in hepatoma cells, as well as the accumulation of AFP protein. The accumulation of AFP is beneficial to reduce ER stress-mediated apoptosis and necroptosis, and partially restores cell proliferation. Blocking the expression of AFP may be a potential treatment strategy for hepatocellular carcinoma.

expression of AFP may be a new and promising therapeutic target for the treatment of hepatocellular carcinoma.

ER stress is closely related to cell apoptosis, necroptosis, and cell proliferation [26–28]. Earlier, we reported that ER stress affects hepatocyte apoptosis [24], necroptosis [22], and cell proliferation [23, 29]. In this study, TG induced ER stress, triggered apoptosis and necroptosis, and inhibited cell proliferation in hepatocellular carcinoma cells. These effects were partially reversed by the ER stress protectors, PBA. Taken together, these results support that ER stress mediates liver cancer cell apoptosis and necroptosis, and inhibits cell proliferation.

ER stress can suppress protein synthesis by phosphorylated eIF2α, thus reduce the protein-folding load in the ER lumen [30]. Simultaneously, ER stress can also selectively upregulate the expression of specific proteins to better cope with cellular stress [31]. In addition, ER stress reportedly inhibits the secretion of certain proteins such as apolipoprotein A-I in HepG2 cells [32] and adiponectin in subcutaneous adipose tissue [33]. Our recent study also found that ER stress causes intracellular accumulation of hepatitis B surface antigen [34]. However, it is currently unclear if ER stress can regulate AFP expression. In this study, our results indicated that TG induced ER stress and in HepG2 and SK-Hep1 cells. Interestingly, the intracellular AFP protein levels were significantly elevated, while the mRNA expression and protein secretion of AFP were decreased during ER stress. These results suggest that ER stress causes the accumulation of AFP protein in hepatoma cells, which may be related to the reduction of AFP secretion. The exact mechanism should be clarified in future studies. The decrease of AFP mRNA expression may be related to the activation of IRE1 endonuclease by ER stress.

AFP is instrumental in diagnosing cancer, monitoring cancer progression, and predicting tumor recurrence [35, 36]. Further, AFP expression regulates multiple signaling pathways at the gene and protein levels. AFP was suggested to serve as a critical signaling molecule in the development of HCC that can regulate various biological processes in hepatoma cells [37]. The upregulation of AFP can help HCC cells resist apoptosis [38]. Moreover, it was also shown to stimulate HCC growth, thereby acting as a growth regulator in tumor progression [39]. The imbalance of cell proliferation, apoptosis, and necroptosis are among the most crucial events in HCC. ER stress can activate apoptotic pathways and block the cell cycle [40]. Therefore, it is plausible to speculate that the accumulation of AFP is beneficial for hepatoma cells to adapt to changes in the tumor microenvironment; AFP changes the balance of apoptosis, necroptosis, and cell proliferation by reducing the damage response of ER stress. On the other hand, AFP protein accumulation may also increase the ER protein-folding load, aggravate ER stress, and promote apoptosis. In this study, ER stress induced apoptosis and necroptosis of liver cancer cell lines and decreased cell proliferation. AFP shRNA increased

apoptosis, necroptosis, and decreased HepG2 cell viability. More importantly, the decrease of AFP accumulation caused the subsequent promotion of TG-induced apoptosis, necroptosis, and decreased HepG2 cell proliferation. In addition, the overexpression of AFP increased apoptosis and necroptosis, and promoted proliferation in both non-ER stressed and ER stressed HepG2 cells. Our results demonstrated that the accumulation of AFP reduced apoptosis, necroptosis, and promoted liver cancer survival through negatively regulating ER stress.

ATF4, ATF6α, and XBP1 are the main regulators of the transcriptome activation by UPR [41, 42]. ATF6α is a member of the basic leucine zipper family of transcription factors, with two main subtypes. ER stress causes cleavage of ATF6α and the translocation of the activated ATF6α to the nucleus [43]. Activated ATF6α promotes the ER stress response elements (ERSE-I, ERSE-II), UPR elements (UPRE), and cAMP response elements (CRE) gene transcription, upregulates the expression of ER-related protein degradation, and enhances the cell's ability to fold protein [44–46]. ATF6 signaling can promote the activity of malignant glioblastoma through ER stress [47]. In this study, AFP knockdown aggravated ER stress-related apoptosis, and selectively reduced ATF6α expression in HepG2 cells. These results show that the accumulation of AFP reduces ER stress through a negative feedback mechanism, reduces apoptosis, necroptosis, and inhibits proliferation. Promoting the expression of ATF6α may be one of its mechanisms and AFP overexpression verified this hypothesis. Therefore, the downregulation of AFP may be a potential treatment strategy for liver cancer in the future.

In conclusion, ER stress induces apoptosis and necroptosis, inhibits cell proliferation in hepatoma cells, as well as the accumulation of AFP protein. The accumulation of AFP may be related to the reduction of AFP secretion, and its effect is beneficial to reduce ER stress-mediated apoptosis, necroptosis, and partially restore cell proliferation. Therefore, blocking the expression of AFP may be a potential treatment strategy for hepatocellular carcinoma (Figure 5).

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