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IRE1α-XBP1 signaling pathway regulates IL-6 expression and promotes progression of hepatocellular carcinoma

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Abstract. Of the three unfolded protein response pathways, which are activated by endoplasmic reticulum stress, inositol-requiring enzyme 1α (IRE1α)-X-box-binding protein 1 (XBP1) signaling is the most conserved. These pathways are implicated in a variety of types of cancer, including hepatocellular carcinoma (HCC). However, the role of IRE1α-XBP1 signaling in the development of HCC remains unclear. In the current study, reverse transcription-quantiative polymerase chain reaction was used to analyze the expression levels of *XBP1* and interleukin (*IL)-6* in human tissues and cells. ChIP and luciferase reporter assays were utilized to investigate the interaction between XBP1s and *IL-6* promoter DNA. It was revelaed that IRE1α-XBP1 signaling promotes the proliferation of HCC cells via regulating hepatic IL-6 expression. It was observed that the splicing levels of XBP1 and hepatic IL-6 content were increased and positively correlated with each other in human HCC tissues (r^2=0.5846, P=0.004). Ectopic expression of IRE1α or XBP1s increased IL-6 levels in LO2 and Hep3B cells. In addition, pharmacological inhibition of IRE1α reduced the levels of IL-6 expression and secretion through blocking the generation of XBP1s, which bound directly to the IL-6 promoter and activated its expression. Further investigation demonstrated that IL-6 driven by XBP1s was secreted outside of cells and activated signal transducer and activator of transcription 3 (STAT3) signaling in an autocrine/paracrine manner, to regulate the proliferation of Hep3B cells. Blockage of IL-6-STAT3 signaling with tocilizumab attenuated the effect of IRE1α-XBP1 signaling in promoting Hep3B cell proliferation. In conclusion, the present study revealed that IRE1α-XBP1 signaling promotes carcinogenesis of HCC by regulating the activation of the IL-6-STAT3 signaling pathway.

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Introduction

Liver cancer, one of the most malignant cancer types, is a leading cause of cancer-associated cases of mortality. It was responsible for 782,500 and 745,500 cases if mortality worldwide in 2012 (1). The majority (85-90%) of primary liver cancer cases are hepatocellular carcinoma (HCC) (2). Interleukin 6 (IL-6) is one of the best-characterized tumorigenic cytokines, particularly in promoting HCC progression (3-6). Expression levels of IL-6 were previously identified to be increased in liver cirrhosis and HCC (7,8). An increasing number of studies have demonstrated that following chronic liver damage or viral hepatitis, elevated IL-6 activates compensatory proliferation of quiescent hepatocytes, which eventually results in HCC (5,9,10).

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen causes ER stress and initiates the activation of the unfolded protein response (UPR). In mammals, UPR pathways are comprised of three branches, which are initiated by three ER-localized transmembrane signal transducers. These include activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and double-stranded RNA-activated protein kinase-like ER kinase (PERK). Among the UPR pathways, the IRE1α-XBP1 branch is the most conserved, indicating its essential role in cells (11-13). Upon activation, IRE1α catalyzes the non-conventional splicing of the mRNA encoding X-box-binding protein 1 (XBP1) by removing a 26-nucleotide intron, and thereby produces an active spliced form (XBP1s) to initiate a key UPR program (14).

Persistent activation of UPR is reported in various solid tumor types, including liver cancer tissue sections (15). An increasing number of reports have identified somatic IRE1α mutations in various types of cancer, including glioblastoma, adenocarcinoma in lung and stomach, renal clear cell carcinoma and serous ovarian cancer (16,17).

While signal transducer and activator of transcription 3 (STAT3) is transiently activated in normal cells, it is frequently reported to maintain a constitutively activated state and promote tumorigenesis by enhancing angiogenesis and cell proliferation and survival in different types of cancer, including colon cancer, melanoma and myeloma (18-20). Notably, IL-6 was revealed to act in an autocrine/paracrine manner to provide a pivotal survival signal via activation of STAT3 signaling in lymphoid malignancies (20) and melanoma (21). A previous study demonstrated that the spliced form of XBP1 may drive the transcription of IL-6 in macrophages upon lipopolysaccharide (LPS) stimulation (22). Notably, IL-6 was recently identified to induce the expression of XBP1 during liver regeneration (23). These results suggest a complex relationship between IL-6 and XBP1. However, the molecular mechanisms underlying the regulation of hepatic expression of IL-6 and XBP1 during the pathogenesis of HCC remain unclear.

The current study reports the critical role of the IRE1α-XBP1 branch of UPR in promoting the proliferation of HCC cells. Elevated expression of IL-6 driven by XBP1s led to HCC cell proliferation via activation of STAT3 signaling. This effect of IRE1α-XBP1 was abolished when IL-6-STAT3 signaling was blocked.

Patients and methods

Patient characteristics. Paired human non-cancerous liver tissues and HCC tissues were collected from 17 patients and analyzed in the current study. The patients were diagnosed with HCC from 2013 to 2016 in the Department of Pathology, The Second Affiliated Hospital and Yuying Children's Hospital, Wenzhou Medical University (Wenzhou, China). The information of each patient was recorded and could be accessed during and after the data collection in this study. The clinical characteristics of the patients are presented in Table I. The tissue sample collection was approved by the Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital, Wenzhou Medical University. Informed consent was obtained from all subjects.

Table I. Clinical characteristics of patients with hepatocellular carcinoma.

Characteristic	n (%)
Age, years	
≤45	6 (35.2)
45-65	8 (47.1)
≥65	3 (17.6)
Sex	
Male	11 (64.7)
Female	6 (35.3)
Risk factor	
HBV	8 (47.1)
HCV	4 (23.5)
Alcohol	3 (17.6)
Other	2 (11.8)

HBV, hepatitis B virus; HCV, hepatitis C virus.

Cell culture and ELISA. Normal hepatocyte cell lines (LO2 and THLE-2) and HCC cell lines (Hep3B, Huh7, SKHep-1, MHCC97L and MHCC97H) were obtained from Cell Bank of Shanghai, Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

To overexpress IRE1α or XBP1s, indicated plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For inhibition of IRE1α activity, 4μ8C (Selleck Chemicals, Shanghai, China) was dissolved in DMSO and added to the medium of indicated cells at a final concentration of 10 μM for 24 h. To block IL-6 receptors, cells were incubated with tocilizumab (Genentech; Roche Diagnostics, Basel, Switzerland) for 8 h prior to further assays and analysis.

To knockdown endogenous XBP1, 50 nM shXBP1 (Genepharma; Shanghai, China) were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. 48 h after transfection, cells were collected for further analysis. The sequences are as following:

shXBP1: Sense, 5'-CCAGUCAUGUUCUUCAAAUTT-3' and antisense, 5'-AUUUGAAGAACAUGACUGGTT-3'; Negative control shRNA for shXBP1: Sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC GUUCGGAGAATT-3'.

Cell culture medium of Hep3B cells was collected and used for the determination of IL-6 content using a human IL-6 ELISA kit (eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

CCK8 and BrdU assay. To determine the effects of IRE1α and XBP1s on cell proliferation, CCK8 and BrdU assays were performed as previously described. Briefly, $1x10^3$ cells were seeded onto 96-well culture plates at day 0. Then, cells were cultured for different time periods (1-5 days) and incubated with CCK8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 2 h at 37˚C on the indicated day. The staining intensity in the medium was measured by reading the absorbance at 450 nm. BrdU assays were performed using a BrdU Cell Proliferation assay kit (Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's protocol.

Luciferase reporter assay. The pGL3 basic plasmid was constructed with the insertion of the promoter of the human *IL-6* gene, corresponding to the region of -2000 to +100 bp with respect to the putative transcription start site (denoted nucleotide +1). The ACGT core from the IL-6 promoter was deleted under a PCR-based strategy. The designed plasmids were transfected into 293T cells and luciferase activities were measured using a Dual-Luciferase assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Renilla luciferase activity was used as an internal control for normalization.

Chromatin immunoprecipitation (ChIP). ChIP assays were conducted using an Agarose ChIP kit (Pierce; Thermo Fisher

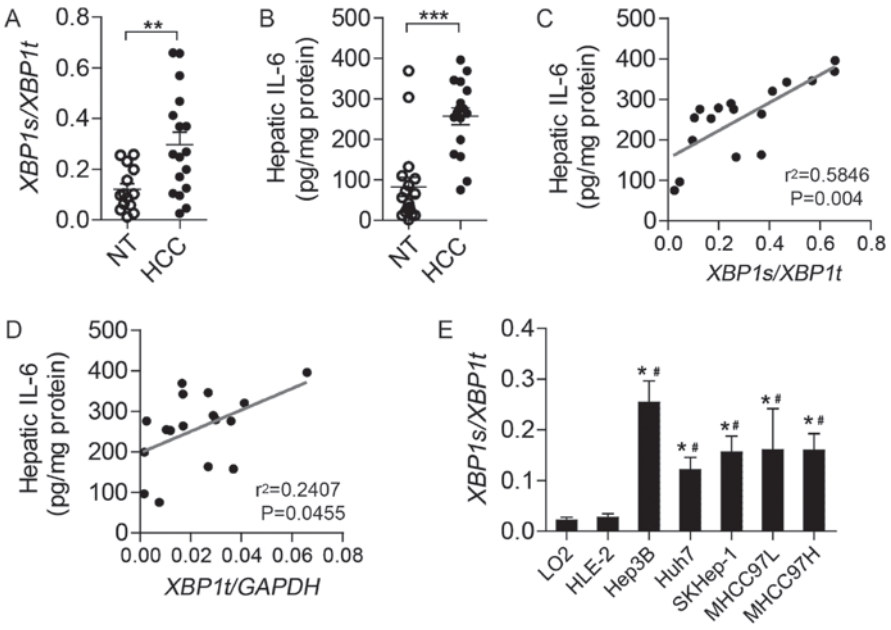

Figure 1. Positive correlation between *XBP1* splicing and IL-6 in HCC tissues. (A-C) Paired human normal liver and HCC tissues. (A) *XBP1* splicing levels were analyzed by quantitative PCR. (B) Hepatic IL-6 contents were tested by ELISA. (C) Pearson correlation analysis of hepatic IL-6 content and *XBP1* splicing levels. (D) Pearson correlation analysis of hepatic IL-6 content and *XBP1t*. (E) Quantitative PCR analysis of *XBP1* splicing levels in normal liver cell lines (LO2 and THLE-2) and HCC cell lines (Hep3B, Huh7, SKHep-1, MHCC97L and MHCC97H). Data are presented as the mean ± standard error of the mean. (A and B) **P<0.01, ***P<0.01 by two-tailed unpaired Student's t-test or (E) *P<0.05 vs. LO2 and $^{\#}$P<0.05 vs. THLE-2 by one-way analysis of variance. XBP1, X-box-binding protein 1; IL-6, interleukin-6; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction.

Scientific, Inc.) according to the manufacturer's protocol. Firstly, indicated cells were subjected to cross-linking with 1% formaldehyde. Glycine solution was added to stop the cross-linking process then the cells were lysed for the preparation of nuclear extracts. Subsequently, chromatin-XBP1s complexes were immunoprecipitated with anti-Flag (diluted 1:500; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or anti-XBP1s (diluted 1:100; BioLegend, Inc., San Diego, CA, USA) antibodies at 4˚C overnight, followed by incubation with beads from the kit at 4˚C for 1 h with gentle agitating. The complexes were eluted from the beads using several washes with the elution buffer, prior to being subjected to further PCR analysis.

Statistical analysis. All experiments in the current study were repeated more than three times. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using two-tailed unpaired Student's t-tests after a demonstration of homogeneity of variance with the F test, or one-way or two-way analysis of variance (ANOVA) for comparisons of more than two groups. Turkey post hoc test was used after one-way ANOVA and Bonferroni post hoc tests were used after two-way ANOVA. P<0.05 was considered to indicate a statistically significant difference. For correlation analysis, linear regression analysis is applied and the coefficient of determination (r^2) and P-value are indicated.

Results

Elevated XBP1 splicing in tumor tissues of patients with HCC and HCC cell lines. To investigate the expression of *XBP1*s and *IL-6* in human HCC tissues, splicing levels of XBP1 mRNA and IL-6 content were analyzed in normal liver tissues and tumor tissues of patients with HCC. Compared with normal liver tissues, HCC tumors exhibited markedly increased *XBP1* splicing (Fig. 1A) and IL-6 protein (Fig. 1B). Notably, further analysis revealed a positive correlation between hepatic IL-6 content and the level of *XBP1* splicing (Fig. 1C) as well as *XBP1t* mRNA levels (Fig. 1D), indicating a close association between IL-6 and XBP1 in HCC.

To explore the extent of *XBP1* splicing in HCC, *XBP1* splicing was evaluated in a series of cell lines, including human normal hepatocyte cell lines (LO2 and THLE-2) and HCC cell lines (Hep3B, Huh7, SKHep-1, MHCC97L and MHCC97H). Relative to LO2 and THLE-2 cells, almost all the HCC cell lines exhibited notably higher levels of *XBP1s* generated from the alternative splicing of *XBP1u* (unspliced *XBP1*) mRNA, indicating enhanced activation of the IRE1α-XBP1 branch of UPR in HCC cells (Fig. 1E).

IRE1α-XBP1 pathway regulates IL-6 expression in LO2 and Hep3B cells. To investigate the physiological functions of increased XBP1s in HCC cells, XBP1s was overexpressed in LO2 and Hep3B cells (Fig. 2A and B). Notably, mRNA levels of *Il-6* were markedly increased in XBP1s-overexpressing LO2 cells (Fig. 2A) and Hep3B cells (Fig. 2B). To explore the secretion of IL-6 by HCC cells, the cell culture medium of Hep3B cells was collected and subjected to ELISA in order to determine extracellular levels of IL-6. Extracellular IL-6 content also exhibited a marked increase following the overexpression of XBP1s in Hep3B cells (Fig. 2C). Consistent with these results, inhibition of XBP1 in Hep3B cells resulted in a significant reduction of both *Il-6* mRNA levels (Fig. 2D) and extracellular IL-6 content (Fig. 2E).

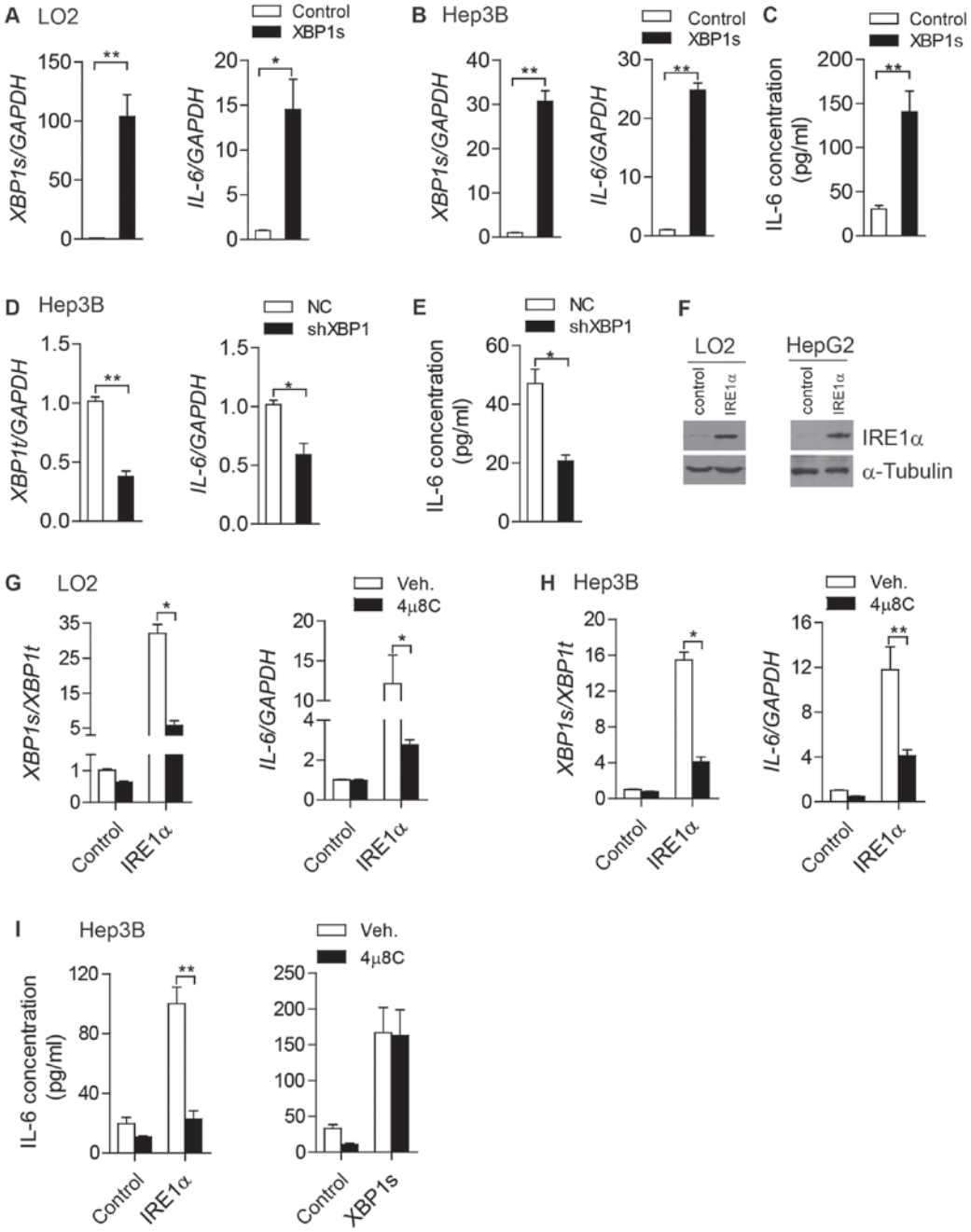

Figure 2. IL-6 expression is regulated by the IRE1α-XBP1 pathway in LO2 and Hep3B cells. (A and B) mRNA levels of *XBP1s* and *IL-6* in (A) LO2 and (B) Hep3B cells transfected with plasmids of pCMV-XBP1s or vector control. (C) IL-6 concentrations in cell-cultured mediums of XBP1s-overexpressed Hep3B cells. (D) Levels of *XBP1* splicing and *IL-6* mRNA in Hep3B cells transfected with shXBP1 or negative control shRNA. (E) IL-6 concentrations in cell-cultured mediums of shXBP1-transfected Hep3B cells. (F) Protein levels of IRE1α were analyzed by western blotting in LO2 and Hep3B cells transfected with plasmids of pCMV-IRE1α or vector control. α-tubulin was used as an internal control. (G and H) *XBP1* splicing and *IL-6* expression levels were determined by quantitative PCR in IRE1α-overexpressing (G) LO2 cells and (H) Hep3B cells with or without the presence of 4μ8C. (I) IL-6 concentrations in cell-cultured mediums of IRE1α-overexpressing or XBP1s-overexpressing Hep3B cells with or without the presence of 4μ8C. Results are from at least three independent experiments. Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01 by two-tailed unpaired Student's t-test or two-way analysis of variance. IRE1α, inositol-requiring enzyme 1α; XBP1, X-box-binding protein 1; IL-6, interleukin-6; PCR, polymerase chain reaction; ctrl, control; NC, negative control.

Next, IRE1α was overexpressed in LO2 and Hep3B cells to determine the role of IRE1α in regulating IL-6 expression (Fig. 2F). Trans-autophosphorylation and subsequent activation of RNase activity of IRE1α may occur following excess accumulation of the protein, which would catalyze the alternative splicing process of XBP1 mRNA (24). A significant increase in *XBP1s* mRNA was observed in IRE1α-overexpressing LO2 cells (Fig. 2G) and Hep3B cells (Fig. 2H). This effect was abolished when the RNase activity of IRE1α was inhibited by the addition of 4μ8C (Fig. 2G and H) (24). Consistent with this, ectopic expression of IRE1α increased the mRNA levels of *IL-6* in LO2 (Fig. 2G) and Hep3B cells (Fig. 2H). With decreased levels of XBP1 splicing, 4μ8C-treated LO2 cells and Hep3B cells exhibited attenuated *IL-6* expression even when

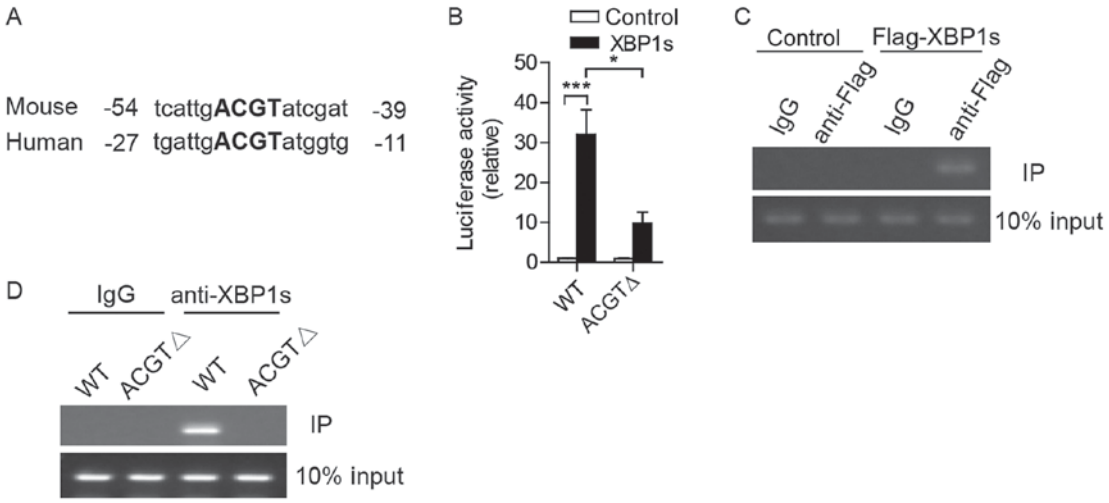

Figure 3. XBP1s binds to *IL-6* promoter to activate its expression in Hep3B cells. (A) Alignment of the sequences of *IL-6* promoters to find the putative UPR element from the mouse and human. The 'ACGT' core is indicated in bold. (B) Luciferase reporter assays were performed in 293T cells co-transfected with plasmids of pCMV-XBP1s together with luciferase reporter constructs controlled by the human *IL-6* promoter (WT) or *IL-6* promoter without ACGT core (ΔACGT). (C) ChIP assays were conducted using IgG as the control or anti-Flag antibody in extracts from Hep3B cells transfected with plasmids of Flag-tagged XBP1s or vector control. The figure indicates representative results of PCR, which was performed to amplify the indicated region of the *IL-6* promoter. (D) ChIP assays were performed in extracts from 293T cells co-transfected with plasmids of pCMV-XBP1s together with the plasmids of human *IL-6* promoter (WT) or *IL-6* promoter without ACGT core using IgG or anti-XBP1s antibodies. The figure indicates representative results of PCR, which was performed to amplify the indicated region of the *IL-6* promoter. Results are from more than three independent experiments. Data are presented as the mean ± standard error of the mean. *P<0.05, ***P<0.001 by two-way analysis of variance. UPR, unfolded protein response; XBP1, X-box-binding protein 1; IL-6, interleukin-6; PCR, polymerase chain reaction; WT, wild type; IgG, immunoglobulin G; ChIP, chromatin immunoprecipitation

IRE1α was overexpressed (Fig. 2G and H). Consistent with the intracellular changes of *IL-6* mRNA, extracellular secretion of IL-6 by Hep3B cells was markedly upregulated when IRE1α or XBP1s were overexpressed (Fig. 2I). Furthermore, 4μ8C treatment blocked the effects of IRE1α overexpression on IL-6 expression, but did not have an impact on the effects of XBP1s overexpression (Fig. 2I).

XBP1s binds to the IL-6 promoter and drives its expression in Hep3B cells. To explore the underlying mechanisms by which XBP1s promotes IL-6 expression, potential promoter sequences of mouse and human *IL-6* were analyzed. Notably, a putative UPR element for XBP1 binding was highly conserved in both mouse and human *IL-6*, and contained the 'ACGT' core sequence as reported previously (Fig. 3A) (25).

To investigate if this core sequence was important in XBP1s-activated *IL-6* expression, luciferase reporter plasmids were constructed containing human *IL-6* promoter of full length (WT) or with 'ACGT' deletion (ΔACGT). A reporter assay was performed in 293T cells. Transcriptional activity of the *IL-6* promoter was markedly enhanced in cells with ectopic expression of XBP1s, and this effect was diminished when the 'ACGT' core sequence was deleted (Fig. 3B).

To determine whether XBP1s directly binds to the IL-6 promoter, a ChIP assay was subsequently conducted. Notably, exogenous Flag-tagged XBP1s proteins were co-immunoprecipitated with chromatin, including a putative *IL-6* promoter, using anti-Flag antibodies in Hep3B cells (Fig. 3C). In 293T cells, exogenous XBP1s interacted with the DNA of the IL-6 promoter (Fig. 3C). Following the deletion of the 'ACGT' core sequence (ΔACGT) from the IL-6 promoter, XBP1s proteins lost the ability to bind to the *IL-6* promoter (Fig. 3D). In summary, these results demonstrate that XBP1s binds directly to the *IL-6* promoter and activates its transcription in human HCC cells.

Effect of the IRE1α-XBP1 branch of UPR on Hep3B cell proliferation is dependent on IL-6 signaling. To investigate the function of upregulated extracellular IL-6, CCK8 assays and BrdU assays were performed to assess the proliferation of Hep3B cells (Fig. 4). Notably, overexpression of XBP1s markedly elevated the proliferation of Hep3B cells, but this effect was diminished when tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, was added (Fig. 4A and B). Consistent with this, a similar phenomenon of cell proliferation was observed in IRE1α-overexpressing Hep3B cells (Fig. 4C and D). These results suggest a critical role of IL-6 signaling in HCC cell proliferation, which may be promoted by the IRE1α-XBP1 branch of UPR.

Tocilizumab attenuates the effect of the IRE1α-XBP1 branch on activating STAT3 signaling in Hep3B cells. To determine whether the IRE1α-XBP1 branch of UPR regulates the activation of IL-6 signaling, levels of STAT3 phosphorylation were evaluated in Hep3B cells that were overexpressing IRE1α or XBP1s. Notably, markedly increased STAT3 phosphorylation was detected following ectopic expression of IRE1α (Fig. 4F) or XBP1s (Fig. 4G). To determine if upregulated extracellular IL-6 activated STAT3 signaling, tocilizumab was added to block the interaction between IL-6 and its receptor. The addition of tocilizumab did not exhibit any effects on the intracellular levels of IRE1α protein (Fig. 4F), or *XBP1s* mRNA (Fig. 4E) or protein (Fig. 4G). As expected, increased STAT3 phosphorylation induced by overexpressed IRE1α or XBP1s was diminished following treatment of Hep3B cells with tocilizumab (Fig. 4F and G). In summary, these data demonstrate that the IRE1α-XBP1 pathway regulates

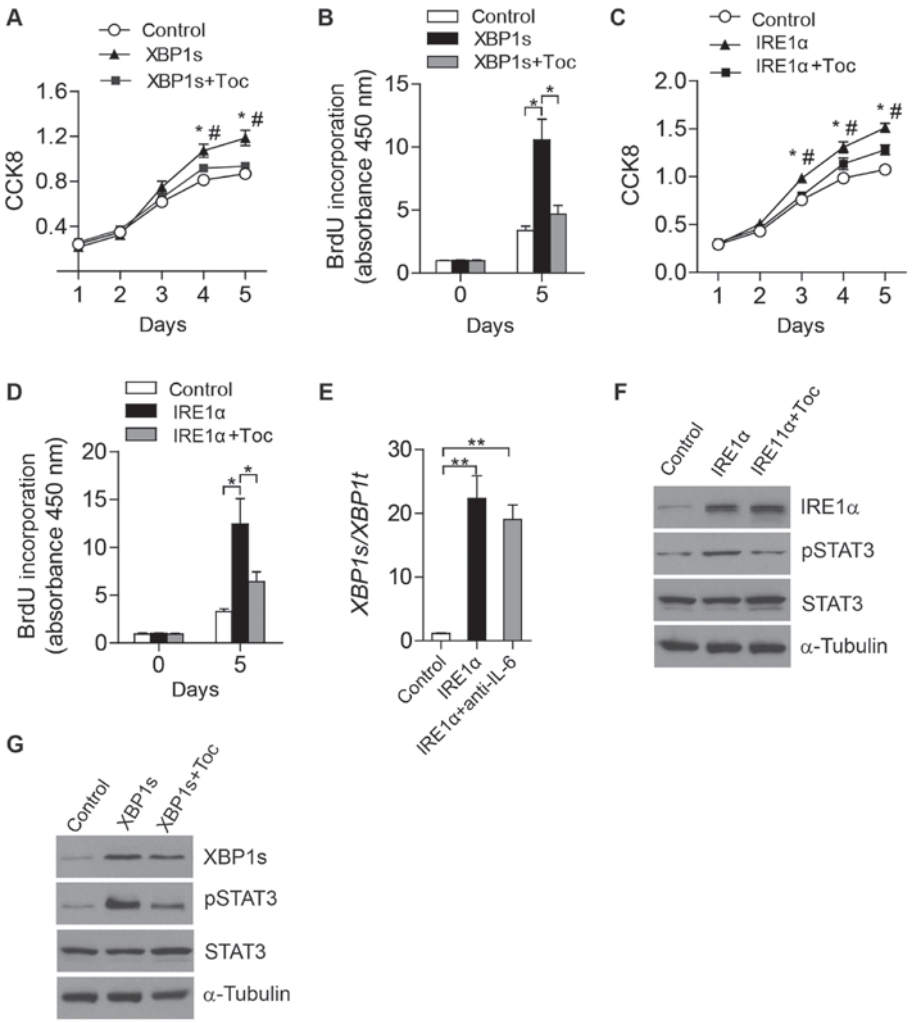

Figure 4. IRE1α-XBP1 pathway promotes Hep3B cell proliferation via regulating IL-6-STAT3 signaling. Cell proliferation was analyzed by (A) CCK8 assays and (B) BrdU assays in Hep3B cells that were transfected with plasmids of pCMV-XBP1s or vector control and then treated with Toc. Cell proliferation was analyzed by (C) CCK8 assays and (D) BrdU assays in Hep3B cells that were transfected with plasmids of pCMV-IRE1α or vector control and then treated with Toc. *XBP1* splicing levels were determined by (E) quantitative PCR and (F) levels of indicated proteins were analyzed by western blotting in Hep3B cells that were transfected with plasmids of pCMV-IRE1α or vector control and then treated with Toc. (G) Indicated protein levels were analyzed by western blotting in Hep3B cells transfected with pCMV-XBP1s or vector control and then treated with Toc. Results are from more than three independent experiments. Data are presented as the mean ± standard error of the mean. (A and C) *P<0.05, **P<0.01 vs. ctrl; $^{\#}$P<0.05 vs. XBP1s + Toc or IRE1α + Toc by one-way ANOVA with Tukey's post hoc tests. (B and D) *P<0.05, as indicated, by two-way ANOVA. (E) **P<0.01 vs. control, by one-way ANOVA relative to ctrl. IRE1α, inositol-requiring enzyme 1α XBP1, X-box-binding protein 1; PCR, polymerase chain reaction; Toc, tocilizumab; ANOVA, analysis of variance; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3.

the activation of STAT3 signaling by increasing IL-6 expression and secretion in HCC cells.

Discussion

ER stress and UPR pathways are implicated to be essential in the development of HCC (4), but the exact mechanisms of this have not yet been elucidated. The current study reveals a novel and critical function of IRE1α-XBP1 signaling in HCC progression. Liu *et al* (26) recently reported that IRE1α is essential in controlling hepatocyte proliferation and liver regeneration via regulation of the STAT3 pathway. Furthermore, IRE1α has also been reported to be implicated in promoting cell proliferation of obesityinduced pancreatic islet cells (27) and certain cancer cell lines (28). However, whether the IRE1α-XBP1 branch of UPR is linked to HCC cell proliferation remains unclear. To the best of our knowledge, the current study was the first to demonstrate a critical role of the IRE1α-XBP1 pathway in promoting the proliferation of HCC cells and the underlying molecular mechanism of this.

In the current study, increased splicing levels of XBP1 were detected in human HCC tissues and HCC cell lines compared with normal liver tissues or hepatocyte cell lines. Furthermore, hepatic IL-6 content exhibited a positive correlation with XBP1 splicing. Although IL-6 was mainly from resident immune cells, hepatocytes also contribute to the total IL-6 in the local microenvironment of the liver, which promotes the compensatory proliferation of hepatocytes, particularly during the progression of tumors (5).

In the current study, it was demonstrated that XBP1s could bind to the *IL-6* promoter and activate its transcription in human HCC cells, indicating a highly conserved role of XBP1 in controlling IL-6 expression. As a key component downstream of IRE1α signaling, XBP1 usually acts as a

potent transcription activator and mediates the transcription of numerous genes to relieve ER stress and restore ER homeostasis (11-13,29). An increasing number of studies has revealed that the IRE1α-XBP1 pathway is also involved in the regulation of various physiological processes, in addition to ER stress, via activation of gene expression, including fatty acid synthase (30), peroxisome proliferator-activated receptor α (31), protein disulphide isomerase (32) and UDP-galactose-4-epimerase (33), or via non-transcriptional activity, such as promoting degradation of the forkhead box O1 (34). Furthermore, in a study of murine macrophages in innate immunology, XBP1s was demonstrated to bind to the *Il-6* promoter and activate its transcription upon LPS stimulation (22). Consistent with these findings, it was also identified in the current study that XBP1s worked as a transcriptional activator in regulating IL-6 expression during the development of HCC. Additionally, 4μ8C blocked the generation of XBP1s and thus attenuated the IL-6 expression and secretion that was induced by IRE1α overexpression, indicating the importance of IRE1α RNase activity in controlling IL-6 expression. Argemí *et al* (23) demonstrated that IL-6 could induce the expression of XBP1 during liver regeneration. Combined with the current data, this indicates a positive feedback loop; XBP1s activates IL-6 expression and IL-6 induces more XBP1 to be spliced into XBP1s. Further studies are required to elucidate the complex interactions between IL-6 and XBP1 in the liver.

IL-6 mRNA transcription and secretion were increased in LO2 and Hep3B cells following ectopic expression of IRE1α and XBP1s. This induced the activation of intracellular STAT3 signaling in an autocrine/paracrine manner, which could be abolished by blocking the IL-6 receptor. The activation of IL-6-STAT3 signaling by the IRE1α-XBP1 pathway was also demonstrated to promote Hep3B cell proliferation. These results were consistent with the critical role of IL-6-STAT3 signaling in regulating cell proliferation and tissue regeneration (18-20), particularly in HCC (3,5,35). It is worth noting that XBP1s was recently identified to upregulate the expression of STAT3 during liver regeneration (23), suggesting that XBP1s could also amplify the activation of STAT3 signaling, as well as driving IL-6 expression in HCC. Furthermore, the addition of IL-6 receptor antibodies (tocilizumab) diminished the effect of IRE1α-XBP1 signaling in activating STAT3 phosphorylation and promoting the proliferation of Hep3B cells in the current *in vitro* results. An *in vivo* study is required to explore whether tocilizumab, an immunosuppressive drug for the treatment of rheumatoid arthritis, has a potential function in inhibiting the progression of liver cancer.

In summary, the present study reveals that the IRE1α-XBP1 branch of UPR promotes cell proliferation and progression of HCC via upregulation of IL-6 expression and activation of IL-6-STAT3 signaling. Although further research is required to verify the role of the IRE1α-XBP1 pathway in HCC development *in vivo*, the current study provides a novel promising therapeutic target for drug discovery and suggests that tocilizumab may have an application in the clinical treatment of patients with HCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

PF, LX, SH, CP and YZ conceived and designed the study. PF, LX, SH and CP conducted the majority of the experiments and analyzed the data. LJ, GZ and LZhu performed some of the cellular experiments. HF and LZho analyzed the data from the human tissues. PF, CP and YZ wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital, Wenzhou Medical University and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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