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Original Research

Cisplatin Disrupts Proteasome Bounce-Back Effect through Suppressing ZEB1/Nfe2l1 in Cholangiocarcinoma

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Abstract

Background: Bortezomib (BTZ) is a powerful proteasome inhibitor that has been approved for the treatment of haematologic malignancies. Its effectiveness has been assessed against different types of solid tumours. BTZ is ineffective in most solid tumours because of drug resistance, including cholangiocarcinoma, which is associated with a proteasome bounce-back effect. However, the mechanism through which proteasome inhibitors induce the proteasome bounce-back effect remains largely unknown. **Methods**: Cholangiocarcinoma cells were treated with BTZ, cisplatin, or a combination of both. The mRNA levels of Nfe2l1 and proteasome subunit genes (*PSMA1*, *PSMB7*, *PSMD1*, *PSMD11*, *PSMD14*, and *PSME4*) were determined using quantitative real time polymerase chain reaction (qPCR). The protein levels of nuclear factor-erythroid 2-related factor 1 (Nfe2l1) and proteasome enzyme activity were evaluated using western blotting and proteasome activity assays, respectively. Transcriptome sequencing was performed to screen for potential transcription factors that regulate Nfe2l1 expression. The effect of zinc finger E-box-binding homeobox 1 (ZEB1) on the expression of Nfe2l1 and proteasome subunit genes, as well as proteasome enzyme activity, was evaluated after the knockdown of ZEB1 expression with siRNA before treatment with BTZ. The transcriptional activity of ZEB1 on the Nfe2l1 promoter was detected using dual-luciferase reporter gene and chromatin immunoprecipitation assays. Cell viability was measured using the cell counting kit-8 (CCK-8) assay and cell apoptosis was assessed using western blotting and flow cytometry. **Results**: Cisplatin treatment of BTZ-treated human cholangiocarcinoma cell line (RBE) suppressed proteasome subunit gene expression (proteasome bounce-back) and proteasomal enzyme activity. This effect was achieved by reducing the levels of Nfe2l1 mRNA and protein. Our study utilised transcriptome sequencing to identify ZEB1 as an upstream transcription factor of Nfe2l1, which was confirmed using dual-luciferase reporter gene and chromatin immunoprecipitation assays. Notably, ZEB1 knockdown using siRNA (si-ZEB1) hindered the expression of proteasome subunit genes under both basal and BTZ-induced conditions, leading to the inhibition of proteasomal enzyme activity. Furthermore, the combination treatment with BTZ, cisplatin, and si-ZEB1 significantly reduced the viability of RBE cells. **Conclusions**: Our study uncovered a novel mechanism through which cisplatin disrupts the BTZ-induced proteasome bounce-back effect by suppressing the ZEB1/Nfe2l1 axis in cholangiocarcinoma. This finding provides a theoretical basis for developing proteasome inhibitor-based strategies for the clinical treatment of cholangiocarcinoma and other tumours.

Keywords: cisplatin; bortezomib; Nfe2l1; ZEB1; cholangiocarcinoma

1. Introduction

Cancer continues to be a leading cause of human mortality despite significant advancements in cancer prevention, early detection, and therapeutic strategies over the past decade. Various treatment approaches, including surgery, chemotherapy, radiation therapy, immunotherapy, and gene therapy, have been employed based on the patient's condition. Chemotherapy is the most commonly used treatment [1,2]. However, the effectiveness of chemotherapy is often hindered by inherent or acquired drug resistance, which greatly limits its clinical application [2]. Therefore, it is crucial to unveil the mechanisms underlying drug resistance and develop novel chemotherapeutic drugs to explore new cancer treatment strategies.

Bortezomib (BTZ), a chemotherapeutic drug targeting the active sites of the proteasome to disrupt intracellular protein degradation, is widely used to treat multiple myeloma and mantle cell lymphoma and different types of solid tumours [3,4]. Unfortunately, BTZ loses its inhibitory effect within a short time in most solid tumours because of strong drug resistance. This resistance is associated with the activation of antioxidative, anti-apoptotic, and mitogenic signalling pathways, involving nuclear factor-erythroid 2-related factor 2 (Nfe2l2)—haeme oxygenase 1, epidermal

growth factor receptor, extracellular signal-regulated kinase, and phosphatidylinositol 3 kinase/protein kinase B [3,4]. Recently, an *in vitro* experiment reconfirmed that the subunit genes of proteasome were upregulated when cells were subjected to proteasome inhibitors treatment, and this phenomenon is referred to as proteasome "bounce-back" effect [5]. Moreover, Nfe2l1 was identified as a direct upstream transcription factor regulating the expression of proteasome core subunit genes [6,7]. Of note, our previous work showed that Nfe2l1-mediated proteasome "bounce-back" effect was only activated in the presence of low-dose proteasome inhibitor [8]. However, the detailed mechanism through which proteasome inhibitors activate Nfe2l1 remains unknown.

Interestingly, a systematic review of clinical trials compared the effects of BTZ alone with that of other chemotherapy agents on patients with cholangiocarcinoma. The results showed that BTZ treatment alone resulted in a longer progression-free survival (PFS) of 5.8 months, compared with other treatments, such as gemcitabine (PFS, 5.0 months), which is commonly used in combination chemotherapy regimens [9]. This observation suggests that cholangiocarcinoma may be a vital tool for exploring the underlying mechanism of the BTZ-induced proteasome bounce-back effect. Importantly, our recent work showed that cisplatin decreased the abundance of Nfe2l1 by regulating both its transcription and post-translational modifications [10]. Therefore, it is necessary to determine the role of cisplatin in the BTZ-induced proteasome bounce-back effect and elucidate the underlying mechanism in cholangiocarcinoma.

2. Materials and Methods

2.1 Chemicals and Antibodies

BTZ (#HY-10227), cisplatin (#SC5170), dimethyl sulfoxide (DMSO, #ST038), and N,N-Dimethylformamide (68-12-2) were purchased from MedChemexpress (Shanghai, China), Solarbio (Beijing, China), Beyotime (Shanghai, China), and Merck KGaA (Darmstadt, Germany), respectively. The primary antibodies against zinc finger E-box-binding homeobox 1 (ZEB1; #21544-1-AP), poly (ADP-ribose) polymerase (PARP; #9542), and β-actin (#TA-09), were obtained from Proteintech (Wuhan, Hubei, China), Cell Signalling Technology (Shanghai, China), and ZSGB-BIO (Beijing, China), respectively. The antibody targeting Nfe2l1 was a kind gift from Professor Yiguo Zhang (Chongqing University, Chongqing, China). Secondary antibody against rabbit (#ZB-2301) and mouse (#ZB-2305) IgG was purchased from ZSGB-BIO (Beijing, China).

2.2 Cell Culture

Human cholangiocarcinoma cell line (RBE) and human embryonic kidney cell line (HEK293T) were purchased from NEWGAINBIO (Wuxi, Jiangsu, China), and ATCC (Zhong Yuan Ltd., Beijing, China), respectively. RBE cells were cultured with complete roswell park memorial institute (RPMI)-1640 medium (#8123203, Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (#2306018, VivaCell, Shanghai, China) and 1% Penicillin-Streptomycin Solution (#P1400, Solarbio, Beijing, China) at 37 °C in an environment of 5% CO_2 and 90% humidity. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and high-glucose medium (#C3113, VivaCell, Shanghai, China) under the same conditions. Additionally, all cell lines were validated by STR profiling and tested negative for mycoplasma.

2.3 Cell Viability Assay

The experimental cells were seeded in 96-well plates and subjected to the corresponding treatments upon reaching 80% confluence. After treatment, cell counting kit-8 (CCK-8) solution (#K009-500, Zetalife, Menlo Park, CA, USA) was added, and the cells were cultured for 1 h, followed by measurement of the optical density of each well at 450 nm using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA).

2.4 Transcriptome Sequencing

RBE cells were seeded in 6-well plates and treated with BTZ and/or cisplatin for 12 h once cell confluence reached 80%. Next, the cells were collected with 500 µL TRNzol Universal (#DP424, TIANGEN, Beijing, China) on the ice and immediately sent to the Novogene (Beijing, China) for transcriptome sequencing.

2.5 Bioinformatics Analysis

DESeq2 package in the R software (version 4.3.0; Lucent Technologies, Jasmine Hill, NJ, USA) was used to identify the differentially expressed genes (DEGs) from the sequencing data. "Adjusted p value ($p$adj) < 0.05 and $\log_2$ (Fold Change) > 1 or $\log_2$ (Fold Change) < -1" were defined as the threshold for DEGs. The expression heatmaps of the selected DEGs were drawn using the pheatmap package in R. Different colours represent the trend of gene expression in distinct samples, with red indicating higher expression levels and green indicating lower expression levels.

2.6 Small Interference RNA (siRNA) or Plasmid Transfection

Before transfection, the cells were seeded into 6-well plates at a density of 6×10^5/well and cultured until they reached 60%–70% confluence. Subsequently, the cells were transfected using a Lipofectamine 3000 Transfection Kit (#2533476, Invitrogen, Carlsbad, CA, USA). Briefly, siRNAs (20 nM/well) of negative control (5′-UUCUCCGAACGUGUCACG-3′), si-ZEB1 (5′-CCUCUCUGAAAGAACACAU-3′) [11], or si-

Nfe2l1 mixture (5′-GGGAUUCGGUGAAGAUUUGTT-3′, 5′-CUCCCAUCAAUCAGAAUGUTT-3′, 5′-GCAUAGACCUGGACAAUUATT-3′) were diluted with 100 µL transfection medium Opti-MEM (#31985070, Gibco, Grand Island, NY, USA) at room temperature for 5 min. The mixture was combined with 100 µL transfection medium containing 5 µL Lipofectamine 3000, followed by incubation for 10 min. Next, 800 µL transfection medium was added for an 8-h transfection period. Next, the transfection medium was replaced with normal culture medium, followed by incubation for 16 h to allow the cells to recover. For plasmid transfection, 5 µL P3000^{TM} reagent was added to plasmid diluted-transfection medium to enhance transfection efficiency. ZEB1 overexpression (pcDNA3.1-ZEB1-3×Flag) and vehicle (pcDNA3.1-3×Flag) plasmids were purchased from YouBio (Changsha, Hunan, China). Nfe2l1 overexpression (pcDNA3.1-Nfe2l1-V5-His B) and vehicle plasmids (pcDNA3.1-V5-His B) were kindly gifted form Prof. Yiguo Zhang (Chongqing University, Chongqing, China).

2.7 Quantitative Real-Time PCR (qPCR) Analysis

mRNAs of experimental cells were extracted with Eastep® Super Total RNA Extraction Kit (#LS1040, Promega, Beijing, China). Total RNA (1 µg) was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (#RR036, TaKaRa, Beijing, China). qPCR with a total capacity of 20 µL was carried out with CFX Connect™ Optics Module system (Bio-Rad, Hercules, CA, USA) using TB Green premix kit (#RR820a, TaKaRa, Beijing, China). Relative gene expression levels were calculated with $2^{-\Delta\Delta CT}$ and quantified by using GAPDH as a housekeeping gene. The primers used are listed in **Supplementary Table 1**.

2.8 Western Blotting

The experimental cells were lysed on ice with cell lysis/IP buffer (#P0013, Beyotime, Shanghai, China) for western blotting, and Inhibitor Cocktail (#HY-K0011, MedChemExpress, Shanghai, China) was added to the lysates on ice. The lysates were quantitatively detected using the Enhanced BCA Protein Assay Kit (#P0010, Beyotime, Shanghai, China). Equal amounts of total protein from each sample were separated by using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The protein samples were transferred onto polyvinylidene fluoride membranes before blocking with 5% non-fat milk. Next, the membranes were washed with 0.1% Tris Buffered Saline with Tween 20 (TBST) and incubated with primary antibody at 4 °C for 12 h and reacted with secondary antibody labelling horse radish peroxidase at room temperature for 2 h after being washed thrice in TBST. Signals were collected using a chemiluminescence instrument after the membranes were reacted with the enhanced chemiluminescence substrate for 30 s. ImageJ (version 1.54g) software (https://imagej.net/ij/) was used to quantify the protein band density.

2.9 Dual-Luciferase Reporter Gene Assay

The Dual-luciferase reporter gene plasmid, Renilla (pRL-TK), pGL3-Basic, and pGL3-Basic-Nfe2l1-Promoter containing the promoter region of *Nfe2l1* at –1– –2600 bp were obtained from Tsingke (Beijing, China). HEK293T cells were plated in 96-well plates and transfected with the indicated plasmids until the cells reached 70%–80% confluence. After treatment, the fluorescence intensity of each sample was determined using the Dual-Luciferase® Reporter Assay System (#E1910, Promega, Beijing, China). After removing the medium and being washed by phosphate buffer solution, 500 µL Passive Lysis Buffer was added to lyse the cells at room temperature for 15 min. The lysates were collected and centrifuged at 12,000 rpm for 1 min. Next, 20 µL of the supernatant was transferred into 96-well opaque white plate and mixed with 100 µL luciferase assay buffer before detecting the activity of firefly luciferase with Synergy H1 microplate reader (BioTek, Winooski, VT, USA). Subsequently, 100 µL stop & Glo reagent was added to detected the activity of renal luciferase.

2.10 Chromatin Immunoprecipitation (ChIP)

The RBE cells were cross-linked with 1% formaldehyde (final concentration) for 10 min at room temperature and quenched with 0.125 M glycine for 5 min. Next, the cells were collected with precooled TBS containing 0.5 mM PMSF and centrifuged at 2000 rpm for 5 min at 4 °C. The cell precipitates were washed with PBS and lysed with ChIP lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, complemented with a protease inhibitor cocktail) on ice for 10 min. Thereafter, the lysate was sonicated to remove the DNA. Some samples were retained as input controls and the rest were incubated overnight with IgG and ZEB1 bound-Protein A/G Agarose Beads (#78609, Thermo Fisher Scientific, Waltham, MA, USA). Next, the DNA fragment was eluted and subjected to reverse cross-linking (5 M NaCl) at 65 °C overnight before being purified for qPCR detection. The related primers are listed in **Supplementary Table 2**.

2.11 Proteasome Activity Assay

Proteasome enzymatic activity was detected according to the instructions of the Proteasome-Glo Cell-Based Assay Kit (#G8531, Promega, Beijing, China). Briefly, RBE cells (5×10^3/well) were seeded in 96-well plates with 100 µL medium and treated with siRNAs or chemicals (BTZ and cisplatin) for 12 h. Proteasome-Glo Cell-Based Reagents and sample plates were prepared and equilibrated at room temperature. Different aminoluciferin-linked enzyme substrates of the proteasome, namely Suc-LLVY, Z-LRR, and Z-nLPnLD, were added to 96-well plates and incubated at room temperature for 15 min. The reaction medium was then transferred into a 96-well opaque white plate, and luminescence intensity was measured using a Synergy H1 microplate reader (Biotek, Winooski, VT,

USA). The relative strength of luminescence was normalized to the cell number, which was confirmed using the CellTiter-Glo Luminescent Cell Viability Assay (#G7570, Promega, Beijing, China).

2.12 Flow Cytometry

RBE cells (5×10^5) were seeded in 6-well plates and cultured for 24 h in 2 mL/well of medium. Single-cell suspensions of treated and untreated cells were obtained and processed according to the instructions of the AnnexinV-FITC/PI Apoptosis Detection Kit (#556547, BD Biosciences, San Jose, CA, USA), followed by analysis using CytoFLEX (Beckman Coulter, Ltd., Miami, FL, USA) and CytExpert software (version 2.4.0.28, Beckman Coulter, ln; Brea, CA, USA).

2.13 Statistical Analysis

GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Significant differences between multiple groups were analyzed using one-way analysis of variance (ANOVA). All experiments were independently conducted at least thrice. Data are presented as mean $\pm$ standard deviation. For all data, $*p < 0.05$, $**p < 0.01$.

3. Results

3.1 Cisplatin Downregulates Nfe2l1 Expression in RBE Cells

To investigate the presence of a proteasome bounce-back effect in cholangiocarcinoma, we treated RBE cells with varying concentrations of the proteasome inhibitor BTZ for 12 hours. The results obtained from western blot analysis showed an increase in the four major isoforms of Nfe2l1 (isoform A, isoform B, isoform C, and isoform D) with increasing doses of BTZ (**Supplementary Fig. 1A**). Notably, the activated isoforms C and D were primarily accumulated at lower concentrations, while the non-activated isoform B accumulated at higher concentrations (**Supplementary Fig. 1A**). Additionally, when Nfe2l1 was silenced in RBE cells, there was a decrease in the expression of its downstream genes, both with and without BTZ treatment (**Supplementary Fig. 1B,C**). Furtherly, to confirm whether cisplatin could downregulate Nfe2l1 expression in cholangiocarcinoma, RBE cells were subjected to cisplatin treatment at different concentrations (0, 5, and 10 µg/mL) for 12 h in the presence and absence of BTZ. Western blotting results showed that cisplatin treatment reduced the protein level of Nfe2l1 in RBE cells (Fig. 1A), especially the isoform A and B at high cisplatin concentrations, compared with vehicle treatment (Fig. 1A, lane 5 *vs*. 1). Similar results were observed in the presence of BTZ (Fig. 1A, lane 6 *vs*. lanes 4 and 2). Next, to determine whether the mRNA level of *Nfe2l1* changed during low-dose BTZ-induced Nfe2l1 activation, RBE cells were treated with BTZ at low doses for different time points (0, 6, 12, and 18 h), followed by qPCR confirmation. The results showed that the mRNA level of *Nfe2l1* was significantly increased after treatment with BTZ for 12 h, compared with that in the vehicle control (Fig. 1B). Interestingly, the mRNA level of *Nfe2l1* gradually decreased with increasing duration of cisplatin treatment (Fig. 1C). Moreover, the transcriptional activation of *Nfe2l1* induced by BTZ was no longer observed with the combination of cisplatin and BTZ (Fig. 1D). These results imply that cisplatin reduces the abundance of Nfe2l1 by downregulating its transcripts.

3.2 Cisplatin Inhibits BTZ-Induced Proteasome Subunit Genes Upregulation in RBE Cells

It is well documented that Nfe2l1 is a key transcription factor responsible for the proteasome bounce-back effect through its ability to upregulate the expression of proteasome subunit genes during cellular insult by low doses of proteasome inhibitors [8]. Therefore, to understand the effect of cisplatin on BTZ-induced proteasome bounce-back effect, six typical proteasome subunit genes, proteasome 20S subunit alpha 1 (*PSMA1*), proteasome 20S subunit beta 7 (*PSMB7*), proteasome 26S subunit, non-ATPase 1 (*PSMD1*), proteasome 26S subunit, non-ATPase 11 (*PSMD11*), proteasome 26S subunit, non-ATPase 14 (*PSMD14*), and proteasome activator subunit 4 (*PSME4*), were detected with qPCR when RBE cells were treated with BTZ or cisplatin alone and in combination for 12 h. The results showed that all these proteasome subunit genes were significantly increased under BTZ treatment, except for *PSMA1*, whereas these genes were markedly decreased in the presence of cisplatin alone or in combination with BTZ (Fig. 2A). These data suggest that cisplatin inhibits the BTZ-induced proteasome subunit genes upregulation in cholangiocarcinoma. Next, to further confirm this finding, we assessed the enzyme activities of proteasome, including trypsin-like, caspase-like, and chymotrypsin-like enzymes, and the results showed that cisplatin markedly suppressed these three enzymes activities of proteasomes. However, the degree of inhibition did not reach the level achieved by BTZ (Fig. 2B). These results indicate that cisplatin downregulates Nfe2l1-mediated proteasome subunit gene expression, and it has the potential to offset the proteasome bounce-back effect when RBE cells are treated with a low dose of proteasome inhibitor.

3.3 Transcriptome Sequencing Reveals that ZEB1 is a Potential Transcription Factor Involved in Proteasome Bounce-Back Effect

Transcription factor is a crucial factor in the regulation of gene expression. Therefore, theoretically, there are transcription factors involved in the BTZ-induced transcriptional expression of *Nfe2l1,* which subsequently activate the proteasome bounce-back effect. To this end, RBE cells were treated with *Nfe2l1* expression inducer (BTZ) and *Nfe2l1* expression suppresser (cisplatin) alone or in

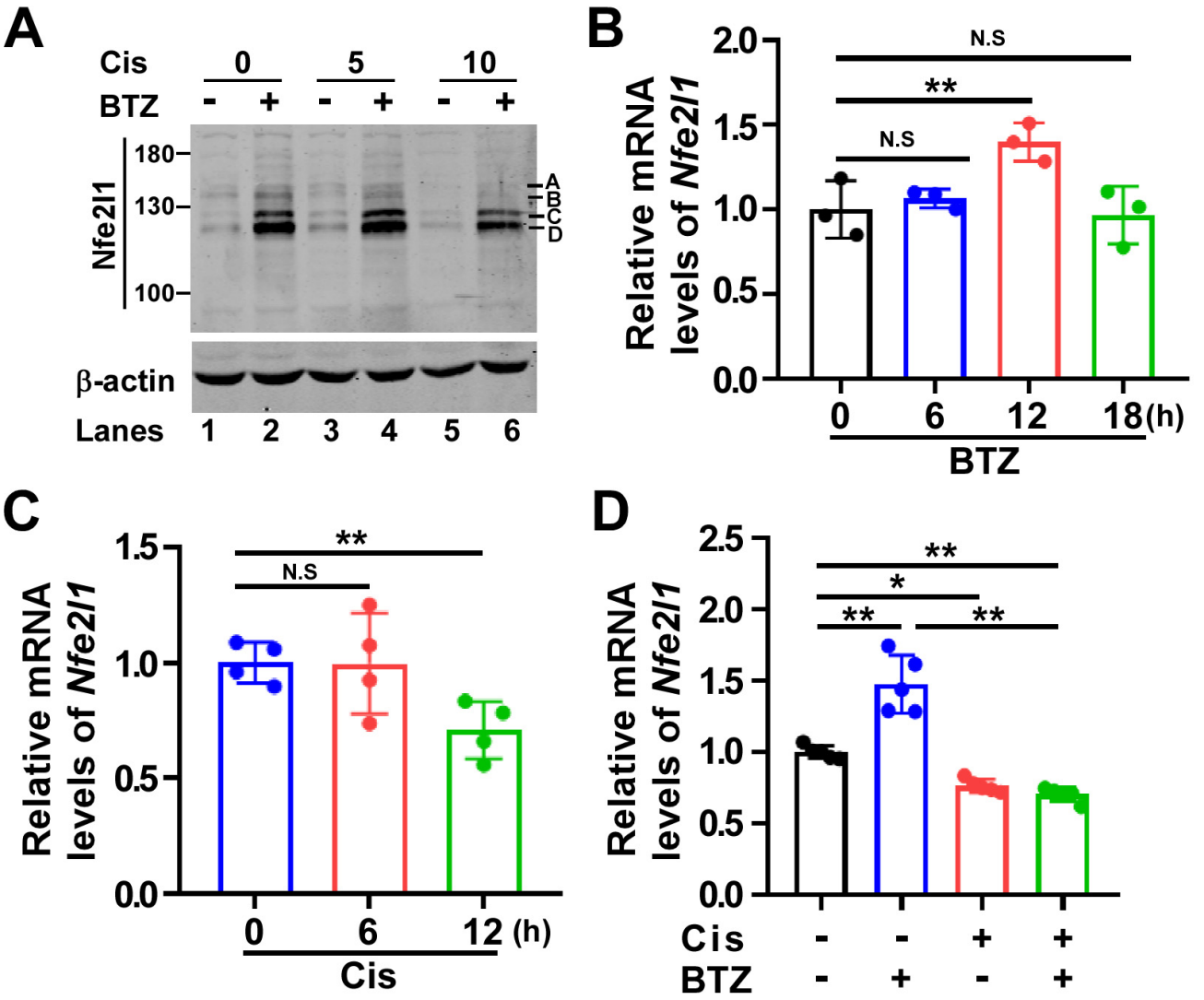

Fig. 1. The effect of cisplatin on the expression of *Nfe2l1* in human cholangiocarcinoma cells (RBE). (A) RBE cells were treated with bortezomib (BTZ, 0.1 μM) for the indicated time points, and the mRNA levels of *Nfe2l1* were detected by quantitative real time polymerase chain reaction (qPCR). (B) RBE cells were treated with BTZ (0.1 μM) and/or cisplatin (Cis) at different concentrations (0, 5, and 10 μg/mL) for 12 h, and the protein level of Nfe2l1 was evaluated by using western blotting. β-actin was used as a loading control. (C) RBE cells were treated with Cis (10 μg/mL) for the indicated time points, and the mRNA levels of *Nfe2l1* were detected by qPCR. (D) RBE cells were treated with BTZ (0.1 μM) and/or Cis (10 μg/mL) for 12 h, and the mRNA levels of *Nfe2l1* were detected by qPCR. *GAPDH* served as an internal reference gene. N.S, no significant difference; *$p < 0.05$, **$p < 0.01$. Bands (A–D) are the four major isoforms of Nfe2l1.

combination, after which the cells were subjected to transcriptome sequencing. In contrast to the control group, we screened the common differentially expressed genes (DEGs) that significantly changed after treatment with BTZ and cisplatin simultaneously using transcriptome sequencing. The results showed that 68 candidate genes were involved in the BTZ-induced proteasome bounce-back effect (Fig. 3A). Among these common DEGs, compared with the control group, 50 genes were upregulated by BTZ treatment and downregulated by cisplatin treatment alone and in the presence of BTZ, whereas the other 18 genes were downregulated by BTZ treatment and upregulated by cisplatin treatment alone and in the presence of BTZ (Fig. 3B). Subsequently, we screened the top four transcription factor-related genes with higher relative fold changes under BTZ treatment, cisplatin treatment, or a combination of the two treatments, namely *TFDP2* (transcription factor Dp-2), *ZEB1* (zinc finger E-box binding homeobox 1), *AFF1* (ALF transcription elongation factor 1), and *POU3F2* (POU class 3 homeobox 2) (Fig. 3C). Furthermore, we obtained the promoter region of *Nfe2l1* (from –1 bp to –2600 bp) and predicted the potential upstream transcription factors through JASPAR tool (https://jaspar.genereg.net/). There were 119 transcription factors with a predictive score of 1, as shown

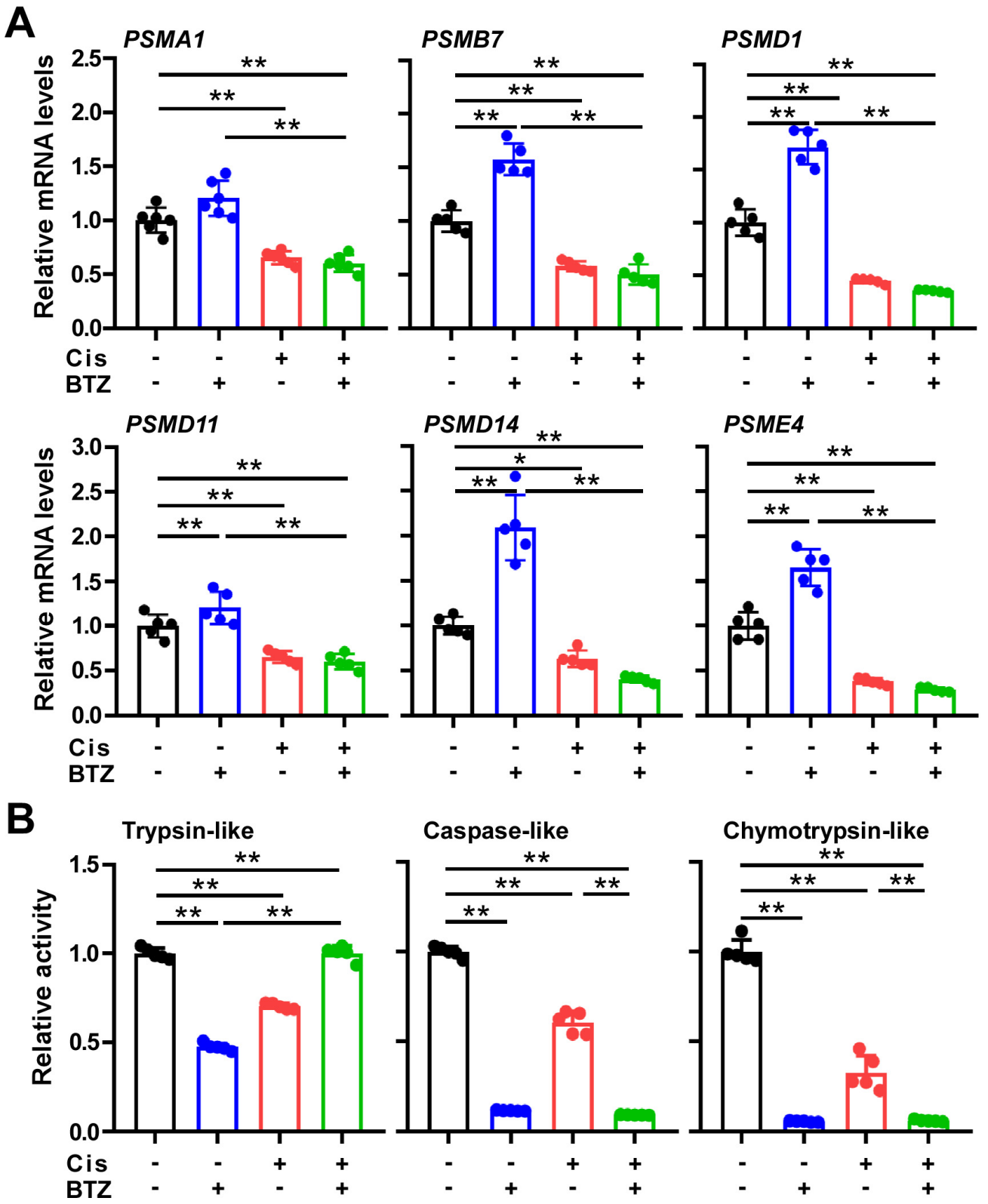

Fig. 2. Effect of cisplatin on the expression of proteasome subunit genes in RBE cells. (A) Six proteasome subunit genes (*PSMA1*, *PSMB7*, *PSMD1*, *PSMD11*, *PSMD14*, and *PSME4*) were detected with qPCR after RBE cells were treated with bortezomib (BTZ, 0.1 μM) and/or Cis (10 μg/mL) for 12 h. *GAPDH* was served as internal reference gene. (B) Meanwhile, the three enzyme activities of proteasomes, including trypsin-like, caspase-like, and cymotorypsin-like enzymes, were evaluated using specific fluorogenic substrates after RBE cells were treated with these compounds for 16 h. *$p < 0.05$, **$p < 0.01$.

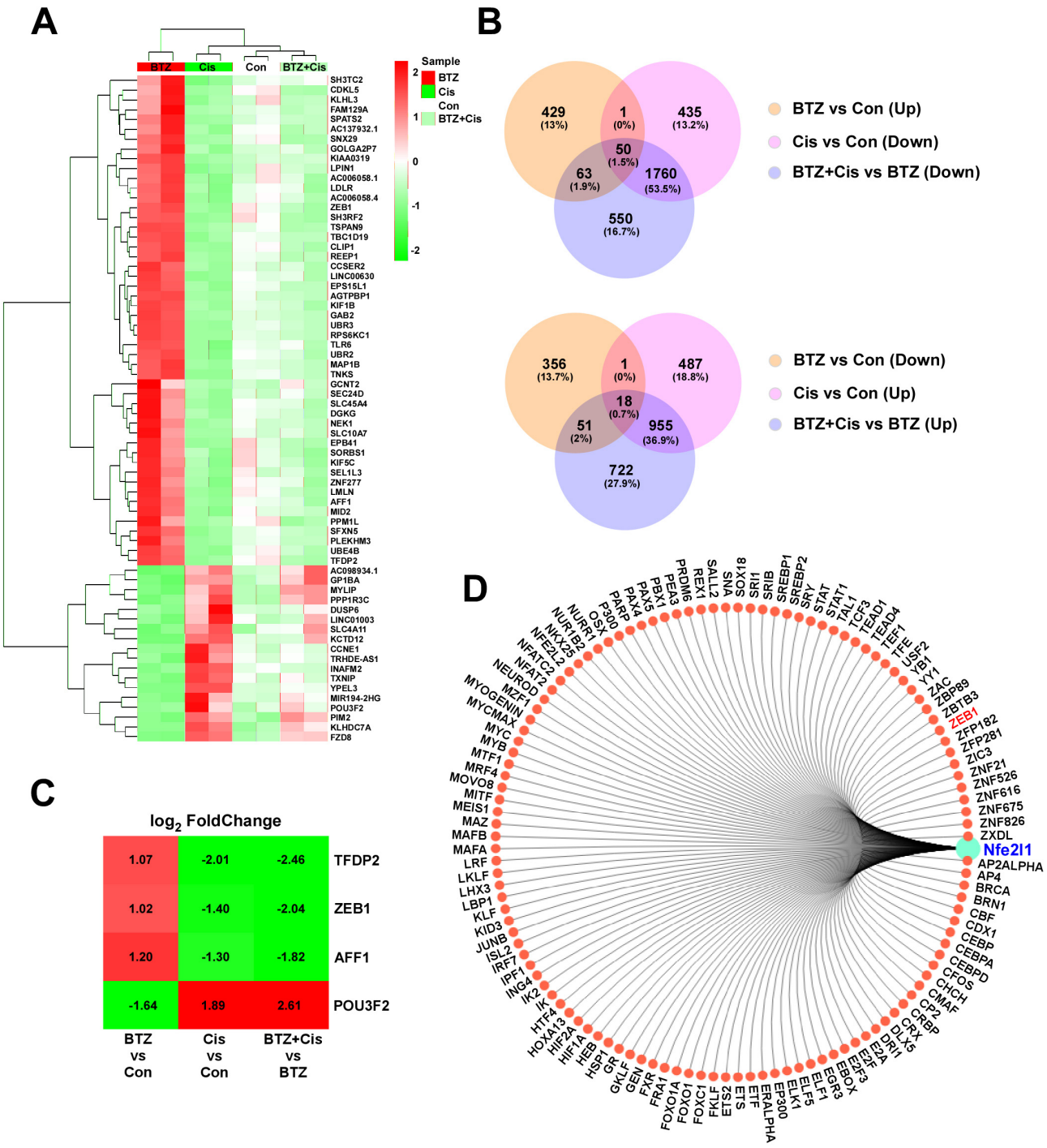

Fig. 3. Potential transcription factor mediating the regulation of proteasome bounce-back effect by Nfe2l1 in RBE cells. (A) The expression heatmap of screened differentially expressed gene candidates involved in bortezomib (BTZ)-induced proteasome bounce-back effect. These genes were selected because they exhibited contrasting expression changes in RBE cells when treated with the Nfe2l1 expression inducer (BTZ) and Nfe2l1 expression suppressor (cisplatin). A redder colour in the heat map indicates a higher expression level, whereas a greener colour indicates a lower expression level. (B) Venn diagram of differentially expressed genes with opposite trends in RBE cells treated with BTZ, cisplatin, or cisplatin combined with BTZ. (C) Expression heatmap of the top differentially expressed transcription factors with higher relative fold changes under BTZ, cisplatin, or a combination of these two treatments. (D) Potential upstream transcription factors targeting the promoter region of *Nfe2l1* (from –1 bp to –2600 bp) were predicted using the JASPAR tool (https://jaspar.genereg.net/). Transcription factors with a predictive score of 1 are shown in the chord diagram.

in the chord diagram (Fig. 3D). Next, by comparing them with the four screened genes from the sequencing data, we found that only *ZEB1* was present in both settings. Moreover, qPCR and western blotting results showed that the expression of ZEB1 demonstrated a trend similar to that of Nfe2l1 when the cells were treated with BTZ and cisplatin, that is, elevated by BTZ and decreased by cisplatin (**Supplementary Fig. 2**, Fig. 4A–C). These data imply that ZEB1 is a potential transcription factor involved in the Nfe2l1-mediated proteasome bounce-back effect.

3.4 ZEB1 Directly Regulates Nfe2l1 Expression

To confirm whether ZEB1 is an upstream regulatory factor that mediates *Nfe2l1* expression in BTZ-induced proteasome bounce-back effect in RBE cells, we silenced *ZEB1* expression with specific siRNA in RBE cells. Western blot results showed that the abundance of Nfe2l1 markedly declined in *ZEB1*-silenced cells treated with BTZ, compared to the group treated with BTZ alone (Fig. 4D, lane 4 *vs*. 3, Fig. 4E,F). Moreover, the overexpression of ZEB1 increased the abundance of Nfe2l1, especially in the presence of BTZ, compared with that in the control group (Fig. 4G,H, lane 2 *vs*. lane 1 and lane 4 *vs*. 3). These results imply that ZEB1 is an upstream transcription factor of Nfe2l1. However, the mRNA levels of *Nfe2l1* were not changed when *ZEB1* was silenced in RBE cells, even when the cells were treated with BTZ (Fig. 4I). To further determine the transcriptional regulatory relationship between ZEB1 and *Nfe2l1* expression, a dual-luciferase reporter gene assay was performed, which revealed that ZEB1 increased *Nfe2l1* promoter activity (Fig. 4J). Furthermore, the binding sites of ZEB1 in *Nfe2l1* promoter region were predicted using the JASPAR tool, and sites with a predicted score >6 are listed in **Supplementary Table 3**. Subsequently, we selected the site with the highest predicted score ($^{-2397}$TGCACCTGCTC^{-2387}, Score = 11.99) to perform the ChIP-qPCR assay. The results showed that, compared with the IgG group, all the three pairs of primers could obtain significantly augmented signals in ZEB1 pull-down samples (Fig. 4K). Taken together, these data reveal that ZEB1 can directly regulate the transcriptional expression of *Nfe2l1*.

3.5 Knockdown ZEB1 can Suppress BTZ-Induced Proteasome Subunit Genes Upregulation in RBE Cells

To identify the role of ZEB1 in BTZ-induced proteasome bounce-back effect, the mRNA levels of proteasome subunit genes induced by BTZ were tested by silencing *ZEB1* with specific siRNA (si-*ZEB1*) in RBE cells. As shown in Fig. 5A, after the cells were transfected with siRNA, the mRNA levels of *ZEB1* decreased to approximately 30% and did not show a marked increase in the presence of BTZ, compared with that in the negative control (si-*NC*) (Fig. 5A). When the siRNA-transfected cells were subjected to BTZ, all the typical proteasome core subunit genes, *PSMA1*, *PSMB7*, *PSMD1*, *PSMD11*, and *PSMD14*, were significantly increased, indicating that the BTZ-induced proteasome bounce-back effect was not affected by scramble siRNA. It is noteworthy that all these genes, except for *PSMD11* and *PSMD14*, demonstrated a salient decrease in the course of the BTZ treatment when *ZEB1* was silenced, compared with that observed in the scrambled siRNA group (Fig. 5A). This decrease was also observed in the si-*ZEB1* and si-*NC* groups, even without BTZ treatment. Moreover, proteasome analysis showed that proteasomal trypsin-like, caspase-like, and chymotrypsin-like activities were prominently blocked by silencing ZEB1 in RBE cells, particularly trypsin-like and caspase-like activities, which demonstrated greater and comparable inhibition to that induced by BTZ. Importantly, the inhibition of trypsin-like activity by BTZ was enhanced by the downregulation of *ZEB1* (Fig. 5B). These results indicate that *ZEB1* knockdown suppresses the proteasome function in RBE cells.

3.6 Cisplatin-Mediated ZEB1 Downregulation can Improve the Cytotoxicity of BTZ in RBE Cells

To evaluate whether targeting ZEB1 with cisplatin could improve the sensitivity of REB cells to BTZ, a CCK-8 assay was performed to evaluate cell viability upon treatment with BTZ and cisplatin. The results showed that combination treatment with BTZ and cisplatin could suppress the viability of RBE cells, compared with treatment with BTZ alone or cisplatin alone (Fig. 6A). Similar results were observed following the treatment with si-*ZEB1* (Fig. 6B). Further, the viability of RBE cells increased significantly after 36 h of BTZ treatment when *ZEB1* was overexpressed. Moreover, the average cell viability was higher in the *ZEB1* overexpression group compared to the vehicle group, although this difference was not statistically significant (**Supplementary Fig. 3A**). Additionally, a protective effect of *Nfe2l1* overexpression was observed in *ZEB1*-silenced RBE cells treated with BTZ, both in the si-*NC* and si-*ZEB1* groups (**Supplementary Fig. 3B**). In addition, western blotting results showed that cisplatin markedly increased the abundance of cleaved-PARP (c-PARP), an apoptosis marker, in RBE cells, compared with BTZ alone (Fig. 6C, lane 4 *vs*. 3, Fig. 6D). However, these results were not observed with the combination treatment of BTZ with *ZEB1* knockdown (data not shown). Further, flow cytometry also revealed that the BTZ-induced apoptotic effect was enhanced by cisplatin (Fig. 6E,F) but not by si-*ZEB1* (data not shown). Collectively, these results indicate that cisplatin-mediated downregulation of ZEB1 potentiates BTZ cytotoxicityin RBE cells.

4. Discussion

Cholangiocarcinoma is a highly lethal malignancy in the vast majority of patients with a poor prognosis [12,13]. For decades, the clinical treatment options for cholangio-

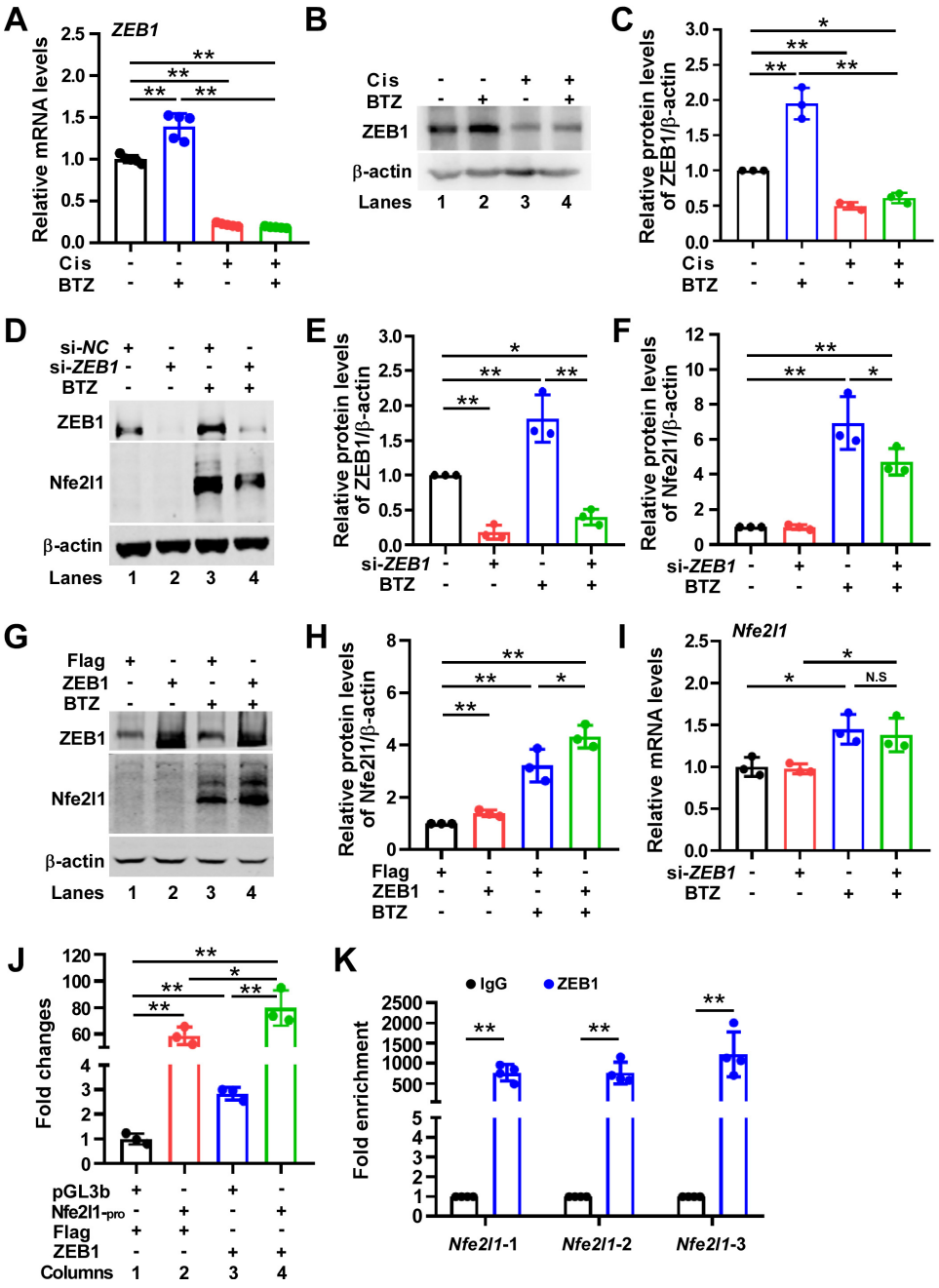

Fig. 4. The role of ZEB1 on the expression of Nfe2l1. (A–C) RBE cells were treated with bortezomib (BTZ, 0.1 μM) and/or Cis (10 μg/mL) for 12 h, and then the mRNA and protein levels of *ZEB1* were detected with qPCR (A) and western blot (B,C), respectively. *β*-actin was used as a loading control. (D–F) siRNAs specifically targeting *ZEB1* (si-*ZEB1*, 20 nM) and control scramble target (si-*NC*, 20 nM) were transfected into RBE cells for 8 h, and the cells were allowed to recover for 24 h before being treated with BTZ (0.1 μM) for 12 h, followed by western blot analysis to detect the abundances of ZEB1 and Nfe2l1. (G,H) *ZEB1* overexpression plasmid (pcDNA3.1-ZEB1-3×Flag, ZEB1, 2 μg) and vehicle plasmid (pcDNA3.1-3×Flag, Flag, 2 μg) were transfected into HEK293T cells for 8 h, followed by recovery culture for 24 h before the cells were subjected to western blot to detect the abundances of ZEB1 and Nfe2l1. (I) The mRNA levels of *Nfe2l1* were determined by qPCR under the same conditions as described in (D). (J) *ZEB1* overexpression plasmid (1.2 μg), pGL3-basic (pGL3b, 0.6 μg) or pGL3-basic-Nfe2l1-promoter (Nfe2l1-pro, 0.6 μg) and Renilla (pRL-TK, 60 ng) were transfected into HEK293T cells for 8 h. Next, the cells were allowed to recover from transfection for 24 h, followed by measurement of fluorescence intensity using the dual luciferase reporter assay. ZEB1-driven luciferase activity was normalized to the corresponding value of Renilla activity. (K) Chromatin Immunoprecipitation (ChIP)-qPCR results for the binding capacity of ZEB1 to the *Nfe2l1* promoter region. A 1% input sample was used as the control. Fold enrichment of ZEB1 on the *Nfe2l1* promoter was normalized to the corresponding value for IgG. N.S, no significant difference; *$p < 0.05$, **$p < 0.01$.

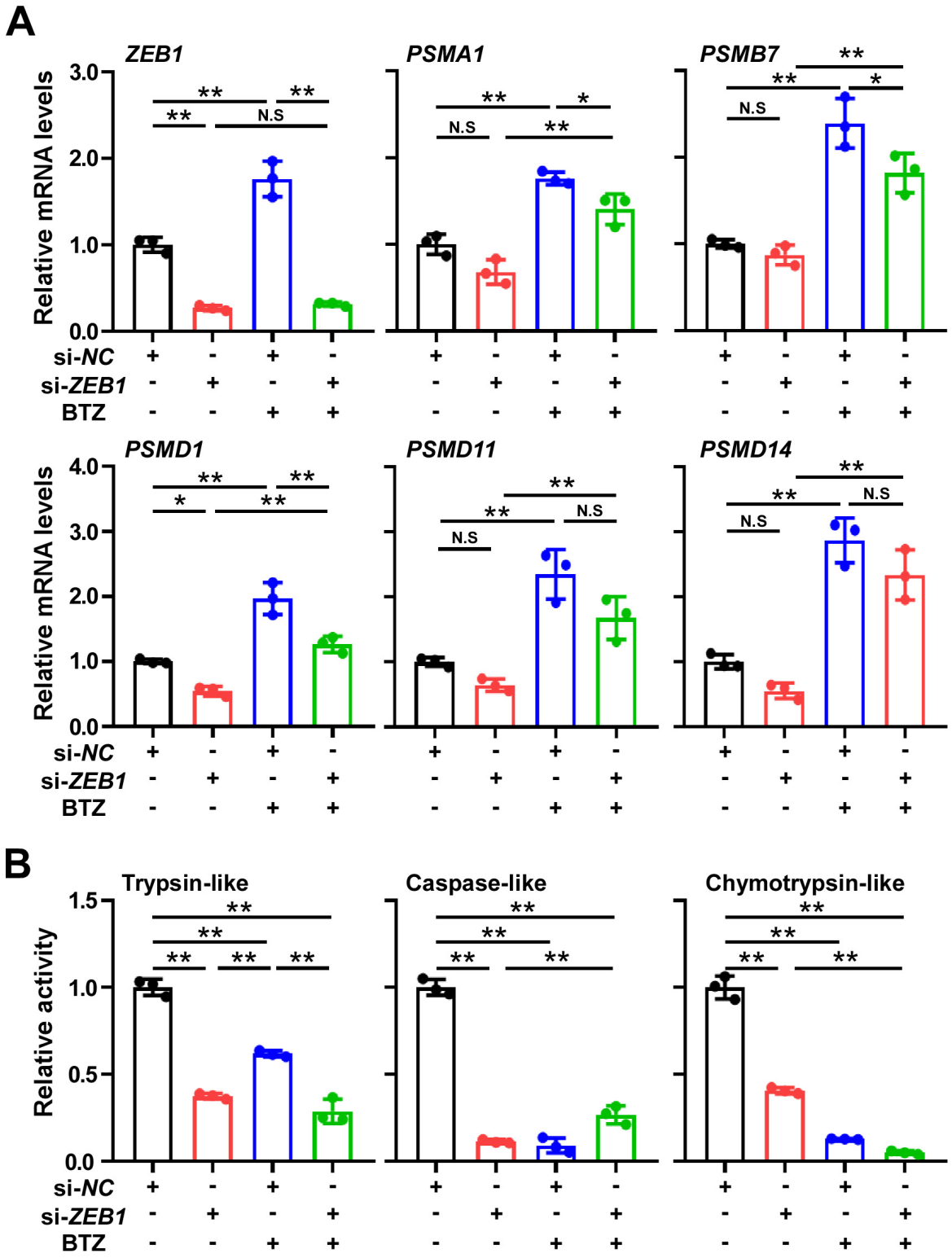

Fig. 5. The effect of ZEB1 on the expression of proteasome subunit genes in RBE cells. (A,B) siRNAs specifically targeting *ZEB1* (si-*ZEB1*, 20 nM) and control scramble target (si-*NC*, 20 nM) were transfected into RBE cells for 8 h, and the cells were allowed to recover for 24 h, before being treated with bortezomib (BTZ, 0.1 μM) for 12 h. Next, qPCR analysis was conducted to detect the mRNA levels of *ZEB1* and proteasome subunit genes (*PSMA1*, *PSMB7*, *PSMD1*, *PSMD11*, and *PSMD14*). *GAPDH* served as an internal reference gene. Meanwhile, the three enzyme activities of proteasomes, including the trypsin-like, caspase-like, and cymotorypsin-like enzymes, were evaluated using specific fluorogenic substrates after the cells were treated with si-*ZEB1* before treatment with BTZ for 16 h. N.S indicates no significant difference, *$p < 0.05$, **$p < 0.01$.

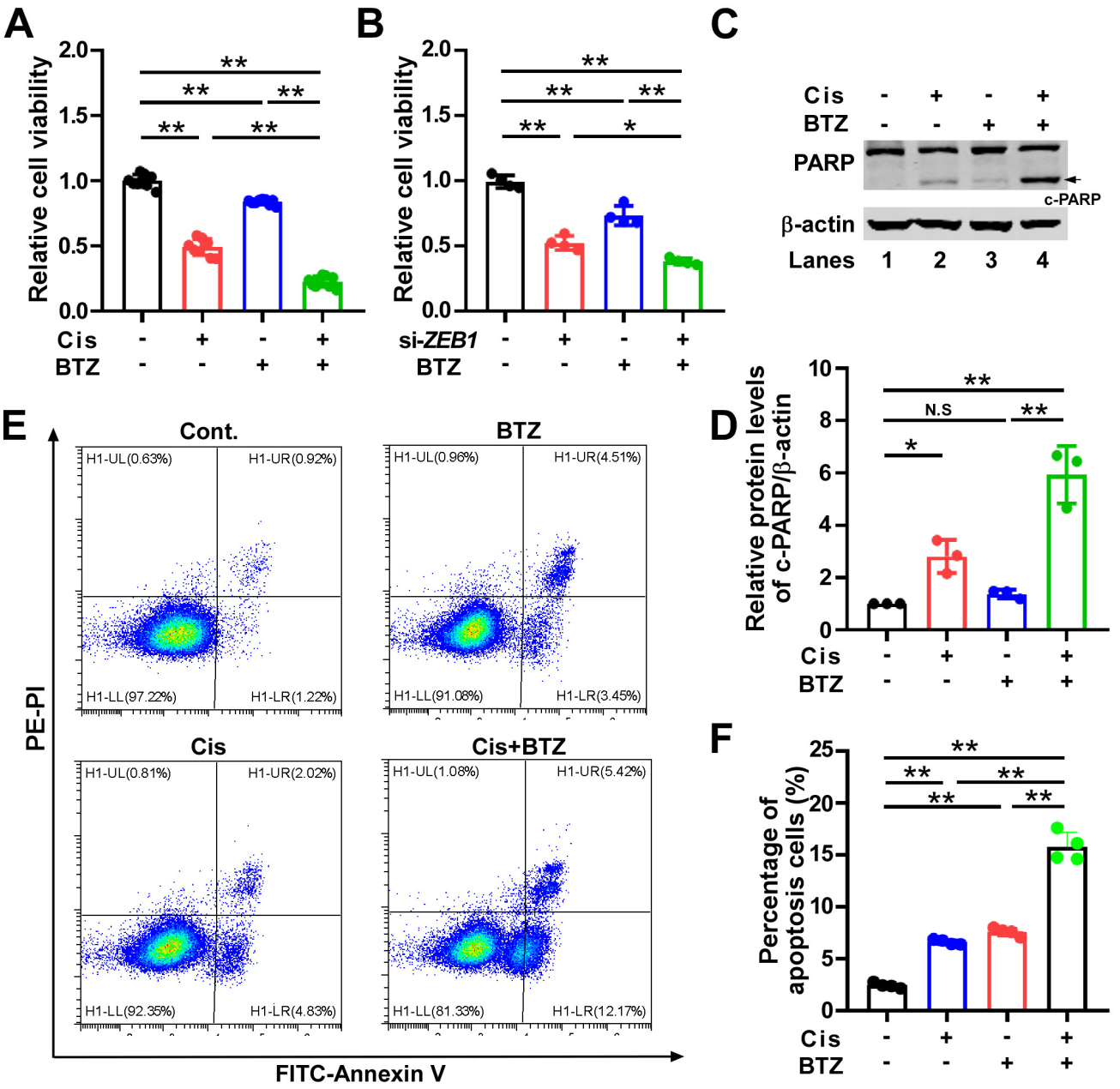

Fig. 6. The effect of cisplatin and BTZ combination treatment on the cytotoxicity of RBE cells. (A,B) RBE cells were treated with cisplatin (Cis, 5 μg/mL), bortezomib (BTZ, 0.1 μM) alone, and their combination for 36 h, and the cell viability were evaluated with CCK-8 assay. Relative cell activity was normalized to that of the control group (A). The cells were treated with si-*ZEB1* (20 nM) before being treated with BTZ (0.1 μM) for 36 h (B). (C) The abundance of the apoptosis marker, PARP, was determined by using western blotting after RBE cells were treated with BTZ for 36 h, and the cleaved-PARP (c-PARP) is indicated with an arrow. β-actin was used as a loading control. (D) Statistical bar chart of c-PARP levels normalized to those in the vehicle group. (E,F) RBE cells were treated with cisplatin (Cis, 5 μg/mL), bortezomib (BTZ, 0.1 μM) alone, and their combination for 36 h, and the apoptosis were evaluated using flow cytometry. N.S, no significant difference; *$p < 0.05$, **$p < 0.01$. FITC, Fluorescein Isothiocyanate.

carcinoma have been limited to surgery, chemotherapy and radiation therapy, lacking more effective treatment strategies [13,14]. As a chemotherapeutic drug that targets the active sites of the proteasome to disrupt intracellular protein degradation, BTZ has been used to treat multiple myeloma, mantle cell lymphoma, and various solid tumours [3,4]. Intriguingly, a systematic review summarising the current clinical trials of chemotherapeutics in cholangiocarcinoma treatment revealed that BTZ alone could extend the progression-free survival of patients with cholangiocarcinoma, compared with treatment with other chemotherapy agents alone, although no objective responses were ob-

served [9]. Unfortunately, the efficacy of BTZ in the treatment of most solid tumours is weakened by drug resistance, which is strongly associated with the Nfe2l1-mediated proteasome bounce-back effect. This indicated that proteasome subunit gene expression can be activated to alleviate the effect of proteasome inhibition when cells are treated with proteasome inhibitors [15,16]. However, strategies that can be employed to overcome this effect and improve the efficacy of BTZ in clinical practice have not been identified. Herein, we found, for the first time, that cisplatin suppresses the BTZ-induced proteasome bounce-back effect in human cholangiocarcinoma RBE cells by downregulating the ZEB1/Nfe2l1 signalling pathway, thereby eliminating the drug resistance of RBE cells to BTZ. This provides a new strategy for improving the efficiency of cholangiocarcinoma treatment.

Currently, our understanding of the regulatory mechanism of the proteasome bounce-back effect is mainly focused on changes in Nfe2l1-mediated proteasome subunit genes from the perspective of post-translational processing of Nfe2l1, including N/O-linked glycosylation, deglycosylation, ubiquitination, and phosphorylation [17,18]. Upon post-translational modification, the transmembrane-bound transcription factor Nfe2l1 can be released from the endoplasmic reticulum into the cytoplasm by proteasomes or proteases, such as DDI1/2 in a regulated juxtamembrane proteolysis way. Subsequently, it translocates to the nucleus to activate the expression of downstream genes, such as proteasome subunit genes [19]. Therefore, suppressing the abundance of Nfe2l1 by interfering with its post-translational processing is a promising method for the disruption of the proteasome bounce-back effect, which has been validated *in vitro* by several research groups [20]. Of note, in our previous study, we found that cisplatin could downregulate the mRNA levels of *Nfe2l1* [10], which prompted us to determine whether transcriptional regulation of *Nfe2l1* expression occurs during the BTZ-induced proteasome bounce-back effect. In the present study, we initially verified the presence of a proteasome bounce-back effect in RBE cells. Additionally, we found an obvious elevation in *Nfe2l1* mRNA levels in cholangiocarcinoma cells during treatment with BTZ, suggesting that the accumulation of Nfe2l1 protein contributed, at least partly, to its transcriptional regulation in the BTZ-induced proteasome bounce-back effect. Moreover, the inhibitory effect of cisplatin on the mRNA levels *Nfe2l1* and six selected typical proteasome subunit genes was observed after cisplatin treatment in qPCR and transcriptome sequencing data (**Supplementary Fig. 4**), implying that cisplatin has great potential to overcome the BTZ-induced proteasome-bounce-back effect. This conclusion is further substantiated by the discovery that cisplatin can significantly suppress the activities of three proteasomal enzymes (i.e., trypsin-like, caspase-like, and chymotrypsin-like enzymes), although the degree of inhibition was not comparable to that by BTZ.

The transcriptional regulation of genes is influenced by multiple factors, including methylated modification, histone modification, transcription factors, and RNA polymerase, during pre- and post-transcriptional regulation. In this study, we attempted to identify the transcription factors involved in BTZ-induced *Nfe2l1* transcriptional expression to activate the proteasome bounce-back effect. Therefore, we screened the potential genes using transcriptome sequencing and transcription factor prediction. Our results showed that ZEB1 may be the upstream transcription factor of *Nfe2l1*, which was validated by a dual luciferase reporter gene and ChIP assays. Moreover, the downregulation of ZEB1 decreased the expression of proteasome subunit genes. However, the mRNA levels of *Nfe2l1* did not markedly change after ZEB1 knockdown, even in the presence of BTZ, implying that ZEB1 is not a primary transcription factor that triggers *Nfe2l1* transcription. This notion is supported by the fact that the overexpression of ZEB1 increased the promoter activity of *Nfe2l1* by approximately 1.4-fold, compared with that observed in the control group overexpressing pcDNA3.1-3×Flag (Fig. 4J, column 4 *vs.* 2). Moreover, the basic activity of the *Nfe2l1* promoter was increased by approximately 58 folds, compared with the group transfected with an empty plasmid in the dual luciferase reporter gene assay (Fig. 4J, column 2 *vs.* 1). It is worth noting that the abundance of Nfe2l1 significantly decreased not only in RBE cells (Fig. 4D), but also in the other two CCA cell lines, QBC939 and HuccT1 (data not shown), when *ZEB1* was silenced. These results strongly suggest that ZEB1 may regulate the abundance of Nfe2l1 mainly in a post-transcriptional manner; however, a direct interaction between ZEB1 and Nfe2l1 was not found in co-immunoprecipitation assays (data not shown), indicating that ZEB1 regulates Nfe2l1 protein levels through other pathways during the course of the BTZ-induced proteasome bounce-back effect, such as NGLY1, p97, USP19, or DDI1/2-mediated post-translational modification [20], which warrants further research. Taken together, these data confirmed that ZEB1 is a vital factor in regulating the Nfe2l1-mediated proteasome bounce-back effect.

As an effector transcription factor regulating *Nfe2l1*, ZEB1 has also been reported to be a critical inducer of epithelial-to-mesenchymal transition (EMT) and orchestrates the transcription of genes involved in developmental processes and tumour metastasis via EMT process [21–23]. However, several studies have revealed that cisplatin decreases or increase the expression of ZEB1 in distinct tumour cells [24–27]. In this study, we found that cisplatin downregulates ZEB1 at both the mRNA and protein levels in RBE cells; however, the underlying mechanism remains unclear and requires further investigation. Based on the results of this study, we speculated that the regulatory mechanism of ZEB1 may be related to proteasome-mediated degradation because the protein levels of ZEB1 were significantly increased after BTZ treatment. Given the

role of ZEB1 in tumours, downregulation of ZEB1 is theoretically beneficial for killing cholangiocarcinoma cells. The results of this study confirmed that BTZ combined with cisplatin or si-*ZEB1* significantly augmented the cytotoxic effect of BTZ on cholangiocarcinoma cells, although these two treatments displayed different effects on cell apoptosis. These data suggest that the combination of cisplatin and BTZ is an effective strategy for the treatment of cholangiocarcinoma. However, this needs to be confirmed *in vivo*. A phase I/II trial showed that a proteasome inhibitor (ixazomib) combined with carboplatin was effective in patients with metastatic triple-negative breast cancer patients [28]. These data imply that a combination treatment with BTZ and cisplatin may be an effective therapeutic measure against cholangiocarcinoma. Additionally, it should be noted that the proteasome subunit genes remain unchanged when cisplatin-treated cells were subjected to BTZ treatment. This suggests the presence of alternative mechanisms through which cisplatin inhibits the proteasome bounce-back effect, necessitating further investigation and elucidation.

5. Conclusions

In this study, we found that cisplatin disrupts the proteasome bounce-back effect, at least partly, by suppressing the ZEB1/Nfe2l1 signalling axis, providing a theoretical basis for developing proteasome inhibitor-based strategies for the clinical treatment of cholangiocarcinoma and other tumours.

Availability of Data and Materials

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

Author Contributions

YX, MW and HM designed the study, interpreted data, and prepared manuscript. YX, MJ and YG performed the all the experiments on western blotting, qPCR, CCK-8 assay, and proteasome activity assay. MJ employed Dual-luciferase reporter gene assay and FCM assay, and YG repeated FCM assay. FY and TW carried out ChIP-pCR and repeated the qPCR results. YX, MJ, and YG analysed all the data. RD and MW analysed the sequencing data. RD, MW and HM discussed and revised the manuscript. All authors contributed to the editorial changes in the manuscript. All the authors have read and approved the final version of the manuscript. All authors have participated sufficiently in the study and agreed to be accountable for all aspects.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2903106.

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