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Cold-inducible RNA-binding protein, CIRP, inhibits DNA damage-induced apoptosis by regulating p53

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ABSTRACT

CIRP has been implicated in apoptosis, yet its mechanism of action remains unknown. To determine the role of CIRP in DNA damage-induced apoptosis, we performed CIRP overexpression and knockdown experiments to investigate the effects of CIRP on key molecules in apoptosis pathway. Etoposide treatment was used to induce DNA damage-induced apoptosis. We found that CIRP knockdown increased p53 level, which in turn up-regulated pro-apoptotic genes and down-regulated anti-apoptotic genes. In contrast, CIRP overexpression decreased p53 level, which in turn down-regulated pro-apoptotic genes and up-regulated anti-apoptotic genes. The change in the expression levels of pro-apoptotic and anti-apoptotic genes shifts the balance between life and death of cells. CIRP expression is upregulated by chronic inflammation, and this phenomenon provides an interesting interventional opportunity in cancers arising from chronic inflammation. Chronic inflammation up-regulates CIRP, which in turn inhibit apoptosis. Therefore, inhibiting the function of up-regulated CIRP may have a therapeutic value in cancer.

1. Introduction

Cold-inducible RNA-binding protein (CIRP) was initially screened as a gene transcript that is induced by DNA damage and plays a key role in controlling various cellular responses to stress induced by ultraviolet light, hypoxia, or hypothermia [1–4]. CIRP has an RNA-recognition motif (RRM) that binds to its target mRNAs [5]. Several studies have reported that CIRP migrates from nucleus to cytoplasm, regulating its target mRNAs at the post-transcriptional level. CIRP is constitutively expressed in various tissues and upregulated in human tumors [6,7].

CIRP has been implicated in various pathogenic conditions including apoptosis [8]. When CIRP is downregulated, pancreatic cancer cells significantly slowed down growth and colony formation, exhibiting enhanced chemo-sensitivity against Adriamycin and cisplatin [9]. In colitis-associated cancer mouse model, CIRP deficiency decreases the expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL23, attenuating the development of inflammation and tumors [8,10]. Moderate low temperature prevents epidermal growth factor (EGF) deprivation-induced apoptosis through upregulation of CIRP expression in neural stem cells [11]. In addition, overexpressing CIRP protects cells from TNF-α-induced apoptosis by activating extracellular signal-regulated kinase (ERK) pathways in CIRP-deficient mouse fibroblasts [8].

Chemotherapeutic drugs used in cancer treatment usually induce not only apoptosis but necrosis, autophagy, senescence and mitotic catastrophe [12]. Apoptosis is induced through two main signal pathways: the intrinsic and extrinsic pathways. The intrinsic pathway (mitochondrial mediated) is usually triggered by DNA damage, while the extrinsic pathway (receptor mediated) is initiated by the activation of death receptor [13]. Etoposide, anti-cancer drug that inhibits the topoisomerase II, leads to DNA strand breaks, inhibition of DNA replication, and apoptotic cell death [14]. Cellular stress causes an imbalance in cellular homeostasis and apoptosis if

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damage exceeds the capacity of repair systems [15–17]. Exposure to DNA damage, such as ionizing radiation or UV in cells, increases the level of p53, which regulates cellular apoptosis and cell cycle [17–19]. Puma and Noxa, p53-inducible genes, function as critical mediators of the apoptotic response induced by DNA damage [20]. Several studies have suggested that CIRP is involved in the DNA damage response process. Downregulating CIRP enhances DNA damage caused by drugs and inhibits the activation of p53 in prostate cancer cells [21]. Moreover, CIRP overexpression reduces testicular damage induced by cryptorchidism by downregulating the levels of p53 and Fas [21]. Despite experimental links between CIRP, DNA damage and apoptosis, how CIRP works in response to DNA damage remains unclear. In this study, we aimed to investigate the biological role of CIRP in apoptosis using an etoposide-induced apoptosis model.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from WelGENE (Daegu, Korea), and Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Anti-CIRP antibody was purchased from Proteintech (Chicago, IL, USA), and etoposide, β-actin, and Flag antibodies were purchased from Sigma (St. Louis, MO, USA). Anti-PARP-1 and anti-Caspase-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-Rabbit IgG-HRP and anti-Mouse-IgG-HRP were obtained from Jackson Immuno Research Laboratories, Inc. (West Grove, P, USA). An enhanced chemiluminescence system (ECL) was purchased from Intron Biotechnology. Control and CIRP small-interfering RNAs (siRNAs) were purchased from Genolution Pharmaceuticals (Seoul, Korea). A high-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Carlsbad, CA, USA), DNaseI and PCR Master Mix was purchased from Thermo Scientific, and an SYBR Green Quantitative PCR kit was purchased from Bio-Rad (Berkeley, CA, USA). The Ezway Annexin-V-FITC apoptosis detection kit was purchased from KOMA Biotech (Seoul, Korea).

2.2. Cloning, cell culture, and transfection

The human CIRP gene was amplified from a human placental cDNA library by polymerase chain reaction (PCR). The fragment was cloned into a pCS4-Flag vector and verified by sequencing. The human hepatoma HepG2 and SK-HEP-1 cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were cultured at 37 °C under 5% CO_2 conditions. pCS4-Flag vector or pCS4-Flag-CIRP plasmid was transfected with polyethylenimine (PEI) or 20 nM siRNA were transfected with Lipofectamine 2000, as suggested by the manufacturer. The sequences of the two CIRP siRNA (siCIRP) oligonucleotides were as follows: siCIRP#1, 5′-GACGCCAUGAUGGCUAUGAUU-3′; siCIRP#2, 5′-GGCGGCAGAUCAGAGUUGAUU-3’.

2.3. Western blotting

The cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM EDTA, 1% Triton X-100) at 4 °C for 10 min. The cell lysates were clarified by centrifugation at 13,000 rpm for 10 min, after which the supernatants were collected. Protein concentration in the supernatants was determined using the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of proteins were subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was then incubated with primary antibody in 5% milk followed by a horse radish peroxidase (HRP)-conjugated secondary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) system.

2.4. RNA expression analysis

HepG2 cells were incubated for the indicated concentrations and times with etoposide. Total RNA was extracted from cells using Trizol (Takara Bio Inc., Shiga, Japan), treated with DNaseI, and reverse-transcribed using High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. The pro-apoptotic genes (Puma, Noxa, and Bax) were amplified using PCR Master Mix. The expression of β-actin was monitored as an internal control. PCR products were separated by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. mRNA levels of anti-apoptotic genes and Bax were quantified by real-time PCR using an SYBR Green Quantitative PCR kit and C1000™ thermal cycler, following the manufacturer's protocol. Each sample was tested in triplicate, and human cyclophilin was used as a control for normalization of RNA expression.

2.5. Cell viability assay

Cell viability to etoposide treatment was measured using the Trypan blue exclusion assay. After 24 h of transfection, HepG2 cells were cultured on 24-well plates at a density of 5.0×10^4 cells/well and treated with etoposide in a dose- and time-dependent manner. Cells were then trypsinized and mixed with 0.2% M/W trypan blue reagent. The viability was then quantified by counting the viable and dead cells using a hemocytometer. The experiments were repeated three times independently.

2.6. Flow cytometry (FACS) analysis

FACS analysis was performed using the EzWay Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol. Briefly, at the indicated treatments, HepG2 cells were harvested through trypsinizaion and washed twice with cold PBS. The cells were centrifuged at 13,000 rpm for 5 min, after which the supernatant was discarded and the pellet was re-suspended in 1× Binding Buffer. Annexin V-FITC was added to each sample, and the cells were incubated for 15 min at RT in the dark. After centrifugation, the supernatant was removed and the pellet was re-suspended with 1× Binding Buffer. Then, propidium iodide (PI) was added to each cell suspension. The samples were analyzed immediately by FACS using Cell Quest Research Software. The apoptosis percentage was calculated as the number of PI positive and Annexin-V positive cells divided by the total number of cells. The experiments were repeated three times independently. The lower graph represents the average percentage of early-apoptotic cells for the three independent experiments.

2.7. Statistical analysis

Statistical significance was analyzed using Student's *t* test. Data are expressed as mean standard deviations (±SD) of three independent experiments.

3. Results

3.1. CIRP knockdown accelerates etoposide-induced apoptosis

To examine the apoptotic effect of etoposide on HepG2 cells, we

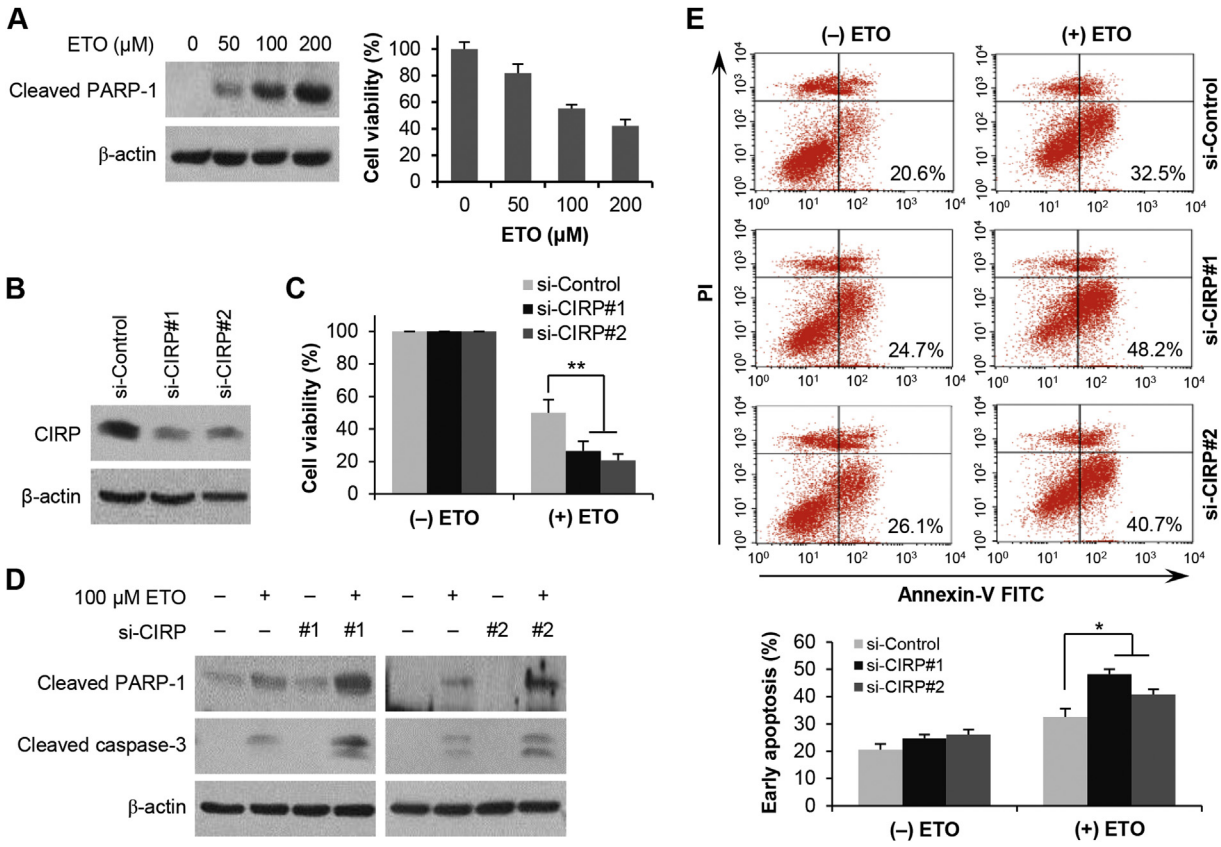

Fig. 1. CIRP knockdown enhances ETO-induced apoptosis. (A) HepG2 cells were exposed to increasing doses of ETO for 24 h. Western blotting was performed to detect cleaved PARP-1 as a marker of apoptosis (*left*). Cell viability was assessed by Trypan-blue exclusion and compared with untreated cells (*right*). (B) Efficient knockdown of CIRP in HepG2 cells is shown by Western blotting. (C) After transfection with si-Control or CIRP siRNAs, cells were treated with 100 μM of ETO for 24 h and viability was measured using Trypan-blue exclusion. (D) The levels of cleaved PARP-1 and caspase-3 were analyzed by Western blotting. (E) After 24 h of 100 μM ETO treatment in control siRNA or CIRP siRNA-transfected cells, the percentage of apoptotic cells was determined using the Annexin V/PI staining assay. Data are expressed as means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$).

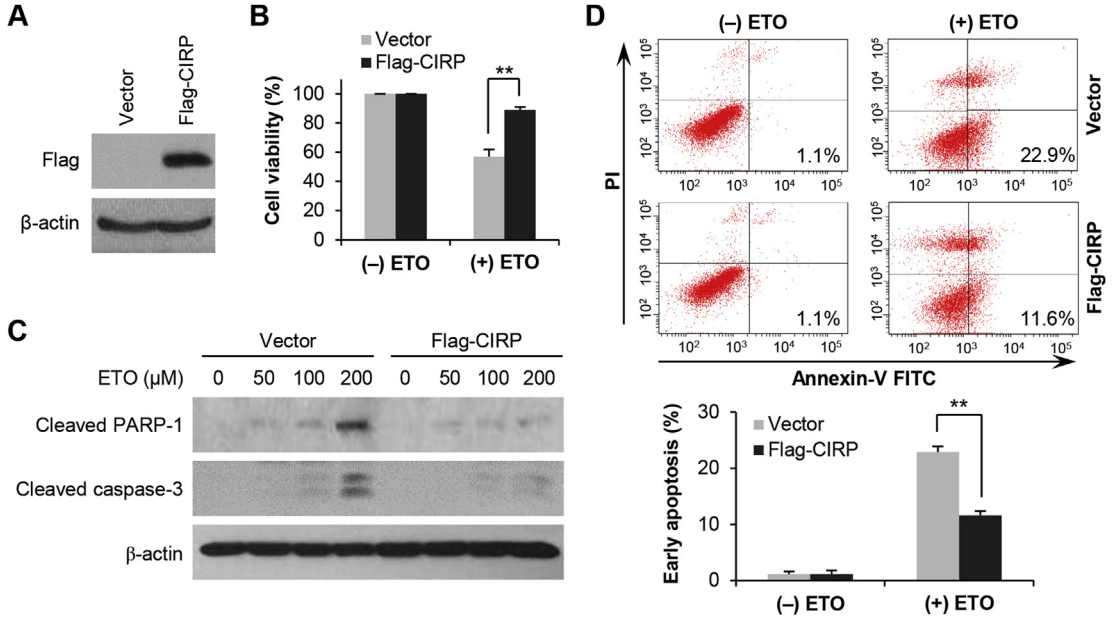

Fig. 2. CIRP overexpression suppresses ETO-induced apoptosis. (A) After transfection with pCS4-Flag vector (Vector) or pCS4-Flag-CIRP (Flag-CIRP), Flag-CIRP protein expression was examined by Western blotting. (B) After transfection with control vector or Flag-CIRP, cells were treated with 100 μM ETO for 24 h, and viability was measured by Trypan-blue exclusion. (C) After 24 h of ETO treatment at the indicated concentrations in control vector or Flag-CIRP-transfected cells, the levels of cleaved PARP-1 and caspase-3 were analyzed by Western blotting. (D) After 24 h of 100 μM ETO treatment in control vector or Flag-CIRP-transfected cells, the percentage of apoptotic cells was determined using the Annexin V/PI staining assay. Data are expressed as means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$).

first examined the cleavage of PARP-1 (an apoptotic marker) and cell viability upon etoposide treatment. When cells were treated with etoposide, PARP-1 cleavage increased and cell viability decreased in a dose-dependent manner (Fig. 1A). Based on this data, we chose 100 μM, LC_{50}, as an experimental concentration of etoposide to induce apoptosis in the following experiments.

To examine whether CIRP is involved in etoposide-induced apoptosis, we transiently silenced CIRP in HepG2 cells using two different siRNAs (Fig. 1B). After silencing CIRP, we measured the cytotoxic effect of etoposide on cells using Trypan-Blue exclusion assay. Cell viability was 25–30% lower in CIRP-silenced cells than in control cells (26.5 ± 4.6, 20.7 ± 3.2 vs. 50.0 ± 6.3, $P < 0.01$) (Fig. 1C). Upon apoptosis, caspase-3 is cleaved to be active, and the active caspase-3, in turn, cleaves PARP-1. Therefore, we measured the levels of PARP-1 and caspase-3 cleavage as an indicator of apoptosis, using Western blot analysis. CIRP knockdown increased the cleavage of both PARP-1 and caspase-3 upon etoposide treatment (Fig. 1D). We also counted apoptotic cells by flow cytometry. When HepG2 cells were treated with transfection reagents only, apoptotic cells accounted for 20.6 ± 3.2% of total. When HepG2 cells were treated with etoposide, the proportion of apoptotic cells increased to 32.5 ± 3.7% of total. When HepG2 cells were treated with etoposide, after silencing CIRP using either siCIRP#1 or siCRIP#2, the proportions of apoptotic cells increased to 48.2 ± 1.6% and 40.7 ± 2.1%, respectively ($P < 0.05$) (Fig. 1E). These findings indicate that CIRP knockdown synergizes etoposide-induced apoptosis in HepG2 cells.

3.2. CIRP overexpression inhibits etoposide-induced apoptosis

We examined the effect of exogenous CIRP overexpression on etoposide-induced apoptosis. After etoposide treatment, the proportion of viable cells was 33% higher in HepG2 cells transfected with Flag-CIRP than in HepG2 cells transfected with control vector (89.1 ± 2.1 vs. 57.2 ± 3.7%, $P < 0.01$) (Fig. 2A and B). Moreover, CIRP overexpression decreased the levels of cleaved PARP-1 and caspase-3 after etoposide treatment (Fig. 2C). We also performed flow cytometry to measure the effect of CIRP overexpression on apoptosis at the cellular level. At 24 h after etoposide treatment, the proportion of cells in the early stages of apoptosis was 11% lower in CIRP-overexpressing cells than in control cells (11.1 ± 1.7 vs. 22.9 ± 2.1%, $P < 0.01$) (Fig. 2D). These findings from CIRP overexpression experiments reconcile with our previous findings from CIRP knockdown experiments, solidating the link between CIRP and apoptosis.

3.3. CIRP suppresses p53 upregulation in apoptosis

DNA damage triggers apoptosis via signaling pathway that activate the p53 [22]. Thus, we hypothesized that p53 play a link between CIRP and etoposide-induced apoptosis (i.e., DNA damage-induced apoptosis). To address this hypothesis, we first measured the protein level of p53 and p21, a direct target of p53, in our *in vitro* models. The protein levels of p53 and p21 were higher in CIRP-knockdown HepG2 cells than in control HepG2 cells after etoposide treatment (Fig. 3A). In contrast, CIRP overexpression suppressed the elevation of p53 and p21 induced by etoposide treatment in HepG2 cells (Fig. 3B). This finding was further confirmed in SK-HEP-1 cells (Fig. 3B). These findings support our hypothesis that CIRP regulates p53.

3.4. CIRP regulates the expression of genes involved in apoptosis

In response to DNA damage, p53 activates the intrinsic mitochondrial apoptotic pathway by inducing the expression of pro-apoptotic family such as Puma, Noxa, and Bax [23]. In addition, p53 in apoptosis is directly and indirectly regulate the activity of anti-apoptotic gene such as Bcl-2, cIAP1, and Bcl-w [24]. Based on our finding that CIRP regulates p53 in apoptosis, we investigated whether the regulation has biological effects on downstream targets of p53. For this purpose, we measured the expression levels of pro-apoptotic genes (Puma, Noxa, and Bax) and anti-apoptotic genes (Bcl-2, cIAP1, and Bcl-w), all of which are downstream targets of p53. Etoposide treatment elevated the expression levels of Puma, Noxa, and Bax in control HepG2 cells. This elevation was further enhanced in CIRP-knockdown cells (Fig. 4A). In contrast, the expression levels of anti-apoptotic genes, represented as ratios to the level of Bax, decreased in CIRP-knockdown cells (Fig. 4B).

We then investigated the effect of CIRP overexpression on the expression of pro-apoptotic and anti-apoptotic genes. In HepG2 control cells, etoposide treatment elevated the expression levels of pro-apoptotic genes (i.e., Puma, Noxa, and Bax) in a dose-dependent manner. CIRP overexpression, however, suppressed the elevation of pro-apoptotic genes induced by etoposide treatment (Fig. 4C). In addition, CIRP overexpression increased the ratios

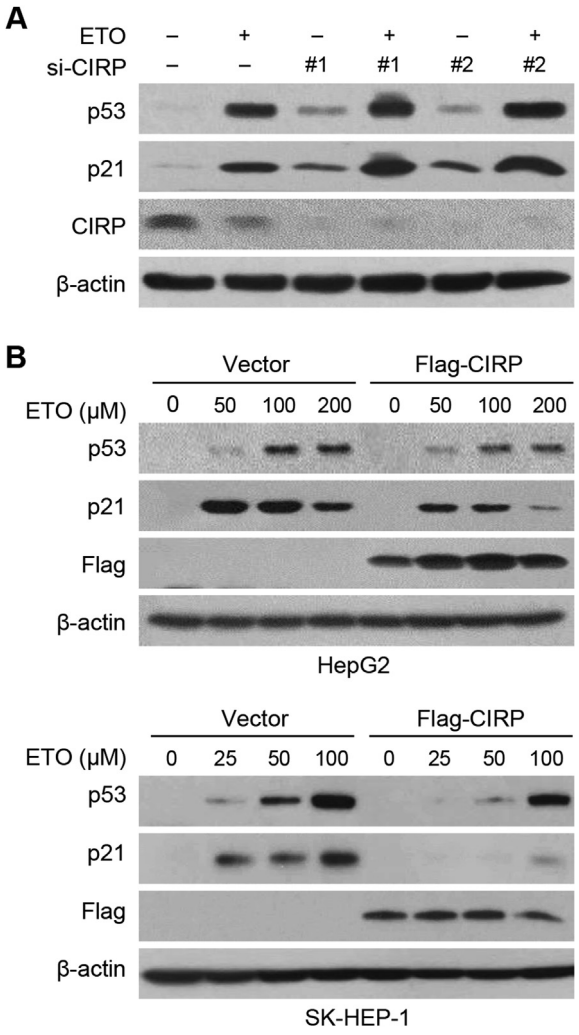

Fig. 3. CIRP regulates the level of p53 protein induced by etoposide. (A) HepG2 cells transfected with si-Control or CIRP siRNAs were treated with 100 μM of ETO for 24 h and the levels of p53 and p21 were analyzed by Western blotting. (B) After 24 h of ETO treatment at the indicated concentrations in control vector or Flag-CIRP-transfected HepG2 and SK-HEP-1 cells, the levels of p53 and p21 were analyzed by Western blotting.

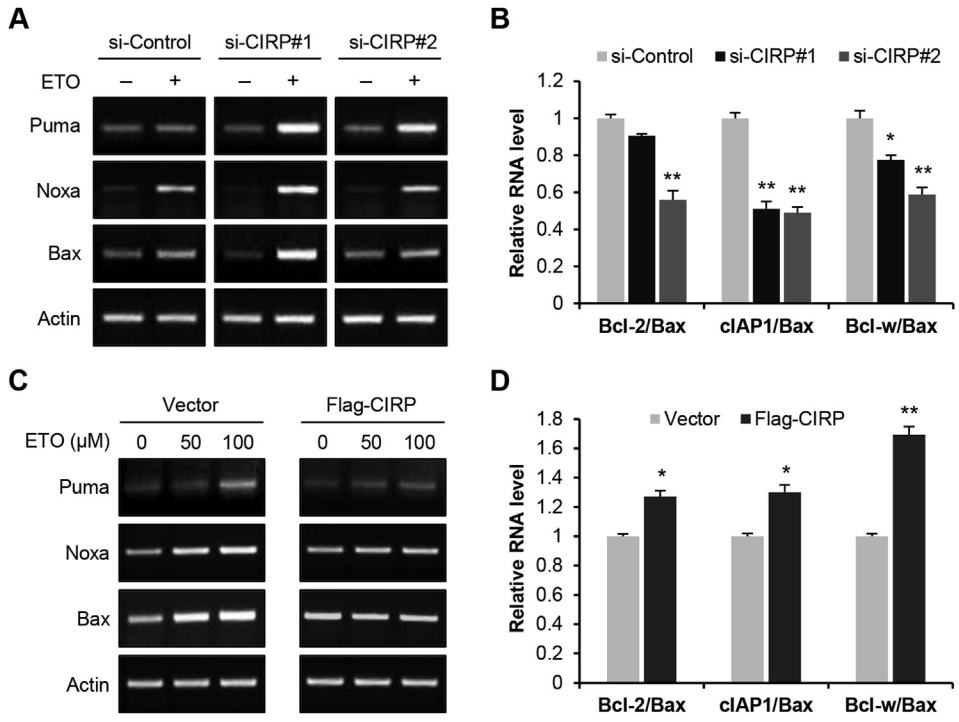

Fig. 4. CIRP regulates the expression of apoptosis-related genes. (A) Cells transfected with control siRNA or CIRP siRNAs were treated with or without 100 μM ETO for 24 h. mRNA levels of pro-apoptotic genes (Puma, Noxa, and Bax) were analyzed by RT-PCR. (B) Cells transfected with control siRNA or CIRP siRNAs were treated with 100 μM ETO for 24 h. Quantitative RT-PCR was performed to detect mRNA levels of anti-apoptotic genes (Bcl-2, cIAP1, and Bcl-w). These results are shown as Bcl-2/Bax, cIAP1/Bax, and Bcl-w/Bax mRNA ratios and fold changes compared with si-Control-transfected cells. The human cyclophilin gene was used for normalization. (C) Cells transfected with control vector or Flag-CIRP were treated with the indicated concentrations for 24 h. mRNA levels of Puma, Noxa, and Bax were analyzed by RT-PCR. (D) Cells transfected with control vector or Flag-CIRP were treated with 100 μM ETO for 24 h. The Bcl-2/Bax, cIAP1/Bax, and Bcl-w/Bax mRNA ratios were analyzed by qRT-PCR, and the results are shown as fold changes compared with control vector-transfected cells. The human cyclophilin gene was used for normalization. Data are expressed as means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$).

of Bcl-2/Bax, cIAP1/Bax, and Bcl-w/Bax (Fig. 4D). Altogether, these findings demonstrate that CIRP functions by regulating pro- and anti-apoptotic genes in apoptosis.

Altogether, these findings demonstrate a sequence of biological events. CIRP knockdown increases p53 level, which in turn up-regulates pro-apoptotic genes and down-regulates anti-apoptotic genes, shifting the balance between life and death towards death. In contrast, CIRP overexpression decreases p53 level, which in turn down-regulates pro-apoptotic genes and up-regulates anti-apoptotic genes, shifting the balance between life and death towards life.

4. Discussion

Our findings demonstrated that CIRP inhibits etoposide-induced apoptosis by regulating the levels of p53. In addition, we showed that this regulation has functional effects on the downstream targets of p53, shifting the balance between death and life of cells.

p53, the key molecule in apoptosis pathway, is regulated by a number of different genes. For example, RNF2, a component of the polycomb repression complex 1, decreases p53 protein level by binding directly with both p53 and MDM2 and promoting MDM2-mediated p53 ubiquitination [25]. DBC1 (deleted in breast cancer 1) deficiency decreases p53 protein level *in vitro* and *in vivo*. DBC1 binds to p53 and stabilizes it through competition with MDM2 [26]. In the present study, we demonstrated that CIRP is a novel regulator of p53-dependent pathway during apoptosis. Further studies are required to define the precise mechanism by which CIRP regulates p53 pathway.

CIRP expression is upregulated by chronic inflammation, enhancing the inflammatory response and tumorigenesis by increasing the levels of cytokines such as TNF-α and IL23 [10,27]. This phenomenon provides an interesting interventional opportunity in cancers arising from chronic inflammation such as HBV-associated hepatocellular carcinoma. As we demonstrated, CIRP overexpression inhibits apoptosis and reduces chemosensitivity. Chronic inflammation up-regulates CIRP, which may, in turn, inhibit apoptosis. Therefore, inhibiting the function of up-regulated CIRP may have a therapeutic value in cancer.

Conflict of interest

There are no conflicts of interest for all authors.

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Transparency document

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