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Interaction of E2F3a and CASP8AP2 Regulates Histone Expression and Chemosensitivity of Leukemic Cells

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Summary: Low expression levels of E2F3a and caspase 8-associated protein 2 (CASP8AP2) are associated with poor outcomes in children with acute lymphoblastic leukemia. Our previous study showed that a combined assessment of E2F3a and CASP8AP2 expression was more accurate in predicting relapse in children with acute lymphoblastic leukemia. However, the underlying mechanism remains unclear. In this study, the interaction between E2F3a and CASP8AP2 and its role in the regulation of histone expression, cell proliferation, the cell cycle, and chemosensitivity were investigated. Exogenous E2F3a-GST was coprecipitated with CASP8AP2-FLAG in HEK-293T cells. E2F3a was colocalized with CASP8AP2-GFP in the nucleus. The replication-dependent histones H2A and H2B were significantly upregulated when E2F3a or CASP8AP2 was overexpressed in HEK-293T or 697 cells and downregulated by E2F3a or CASP8AP2 knockdown. E2F3a and CASP8AP2 could collaboratively enhance the transcriptional activity of *HIST1H2AG* and *HIST1H2BK*. Both CASP8AP2 and E2F3a are involved in S phase progression. E2F3a and CASP8AP2 also affected the sensitivity of leukemic cells to daunorubicin. Therefore, CASP8AP2 and E2F3a collaboratively regulated replication-dependent histone expression, cell cycle progression, and chemosensitivity of leukemic cells.

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Acute lymphoblastic leukemia (ALL) is the most common malignancy in children under 14 years old.[1] Over the past few decades, the overall survival of children with newly diagnosed ALL has dramatically improved.[2] However, 10% to 15% of patients still experience relapse, and an additional 1% of ALL patients are refractory to chemotherapy, which has been the main obstacle to further improvement in patient outcomes.[3–6] The mechanism of leukemic relapse and chemoresistance has not been fully clarified. Therefore, more molecular markers are needed to explore the role of recurrence and resistance in leukemia.

The transcription factor (TF) E2F family has been reported to play an essential role in the regulation of cell proliferation, differentiation, and apoptosis,[7,8] and E2F3a was found to be involved in the G1/S transition and cell proliferation.[9] We previously demonstrated that a low expression of *E2F3a* was associated with a poor prognosis in patients with childhood ALL.[10] Caspase 8-associated protein 2 (CASP8AP2), also known as FLICE-associated huge protein (FLASH), is considered a multifunctional protein involved in apoptosis mediated by Fas and tumor necrosis factor α, S phase progression, and 3' end processing of replication-dependent histone precursor mRNAs.[11–13] Similarly, a low expression of *CASP8AP2* was correlated with a high level of minimal residual disease and relapse in childhood ALL.[14] Furthermore, our previous study showed that a combined assessment of *E2F3a* and *CASP8AP2* expression was more accurate in predicting relapse in children with ALL.[15] In addition, E2F3a was proven to activate *CASP8AP2* gene transcription in leukemic cells.[16]

In this study, we investigated the interaction between CASP8AP2 and E2F3a and its role in regulating the expression of replication-dependent histones and the chemosensitivity of leukemic cells.

MATERIALS AND METHODS

Cell Culture

A human ALL cell line, that is, 697 cells (kindly provided by Professor Suning Chen, Jiangsu Institute of Hematology, the First Affiliated Hospital of Soochow University), was grown in RPMI 1640 medium (GIBCO, USA). The HEK-293T and COS-7 cell lines (both obtained from the National Infrastructure of Cell Line Resource, Beijing, China) were grown in DMEM (Corning, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air. All media were supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Viruses, Plasmids and siRNAs

Tet-on recombinant lentivirus for the overexpression of E2F3a, a negative control (lentivirus encoding red fluorescent protein), and recombinant plasmids for the overexpression of E2F3a and CASP8AP2 (E2F3a-GST, CASP8AP2-FLAG, and CASP8AP2-GFP) were products of Shanghai Genechem Co., Ltd.

HIST1H2AG (histone cluster 1 H2A family member g, -38 nt to -46 nt) and *HIST1H2BK* (histone cluster 1 H2B family member k, -198 nt to -206 nt and -304 nt to -312 nt) were chosen as representatives of histone genes. There are one and two E2F3a binding sites (TTTSSCGC) in the promotors of *HIST1H2AG* and *HIST1H2BK*, respectively. DNA fragments containing E2F3a binding sites were amplified using the following primers: 5'-CCCAAGCTT CGCAAATCCAGAAGACGCAC-3' as the forward primer, 5'-CCGCTCGAGGCAACCACAAAGTGAACG GG-3' as the reverse primer (*HIST1H2AG*); 5'-CCCAA GCTTACCCGCTCGGCATAATTACC-3' as the forward primer, 5'-CCGCTCGAGAGGGTGACGTCAGAGGTT AGT-3' as the reverse primer (*HIST1H2BK*). The underlined letters represent the introduced restriction digestion sites (HindIII in the forward primers and XhoI in the reverse primers). After digestion with HindIII and XhoI, the PCR products were subcloned into a pGL3-basic vector to generate the luciferase reporters *HIST1H2AG*-Luc and *HIST1H2BK*-Luc.

Small-interfering RNAs (siRNAs) for E2F3a and CASP8AP2 or control scrambled siRNA were combined with Lipofectamine RNAi/MAX reagent (Invitrogen), and the cells were transfected with siRNA (20 nM final concentration) according to the recommended protocol. All siRNA duplexes were purchased from Santa Cruz, USA.

Transiently Transfected or Stably Overexpressed Cells

Transient transfection was performed with X-treme HP Transfection Reagent (Roche, Germany) according to the manufacturers' instructions.

Then, 697 cells were infected with the Tet-on E2F3a recombinant lentivirus (multiplicity of infection = 100). After 48 hours of culture, puromycin (0.4 μg/ml) was added to the culture medium. The medium containing puromycin was changed every two days until the stably infected cells were screened. The overexpression of E2F3a could be induced by adding doxycycline (Dox, final concentration of 1 μg/ml) to the medium.

Western Blot Analysis and Immunoprecipitation

The total proteins were extracted from cells lysed with RIPA lysis buffer (Thermo Fisher Scientific, USA). The nuclear proteins were separated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The proteins were separated using a NuPAGE Bis-Tris Mini Gels System (Thermo Fisher Scientific). A Western blot was carried out with the following antibodies: histone H2A and H2B antibodies (CST, USA), GAPDH antibody (SAB, USA), anti-FLAG antibody (Sigma, USA), and anti-GST antibody (CST).

In HEK-293T cells, exogenous CASP8AP2-FLAG and E2F3a-GST were coprecipitated from nuclear lysate by anti-FLAG M2 agarose beads (Sigma) according to the manufacturer's instructions. The immune complexes were separated on a 4-12% SDS polyacrylamide gel electrophoresis gradient gel (Invitrogen, USA) and analyzed with anti-FLAG and anti-GST antibodies.

Immunofluorescence

Cells were grown on glass coverslips, washed twice with PBS, and fixed in 4% paraformaldehyde for 20 minutes. The fixed cells were permeabilized with 0.1% Triton X-100 for 10 minutes and then incubated with 2% BSA for 1 hour. After incubating overnight with antibodies and washing with PBS 3 times, the cells were incubated for 1 hour with goat antirabbit IgG-TRITC (KeyGEN BioTECH, China) and treated with mounting media DAPI (ZSGB-BIO, China). Laser confocal microscopy was used to show the localization.

Chromatin Immunoprecipitation (ChIP)

The ChIP assay was carried out with a ChIP assay kit (Beyotime Institute of Biotechnology, China), rabbit anti-E2F3 antibody (Santa Cruz), rabbit anti-CASP8AP2 antibody (Santa Cruz), or an unrelated rabbit IgG antibody (Santa Cruz). Then, the immunoprecipitated DNA was amplified with the *HIST1H2AG*- or *HIST1H2BK*-promoter specific primers mentioned above by 30 cycles of PCR.

Dual-luciferase Reporter Assay

HEK-293T cells were transfected with E2F3a-GST or a control vector along with *HIST1H2AG*-Luc or *HIST1H2BK*-Luc and a Renilla luciferase reporter. Twenty-four hours later, the luciferase activity was quantified using a Dual-Luciferase Reporter Assay (Promega, USA).

Determination of Cell Cycle Phases and Cell Survival Assay

HEK-293T cells with upregulated E2F3a and/or downregulated CASP8AP2 were fixed with 75% ethanol, digested with RNase A, and stained with propidium iodide. Then, the fraction of cells in the various phases of the cell cycle was determined by flow cytometry.

Cell proliferation was determined with the MTS method. Cells were plated into 96-well plates at 10^4 cells per well. After the treatment with Dox (1 μg/ml), daunorubicin (DNR) was added to the wells to final concentrations of 1×10^1, 1×10^2, 2×10^2, 4×10^2, 5×10^2, 8×10^2, 1×10^3, and 5×10^3 ng/ml. The cells were cultured for 48 hours at 37°C. CellTiter 96 AQueous One Solution Reagent (Promega) was used to determine the cell viability according to the manufacturer's instructions. A nontreatment control was regarded as 100% cell viability.

Statistics

A nonlinear regression curve fit was used to draw the curve of cell proliferation. A one-way analysis of variance and the Fisher least significant difference test were used to compare the regression coefficients of the curves, distribution of the cell cycle phases, and relative fluorescence intensities among groups of cells transfected with different plasmids. A P-value <0.05 was regarded as statistically significant. The statistical analyses were carried out using the SPSS 22.0 software package. GraphPad Prism 5 was used to plot the fitting curves of the inhibitory effects of DNR on cell proliferation and calculate the half maximal inhibitory concentration (IC50).

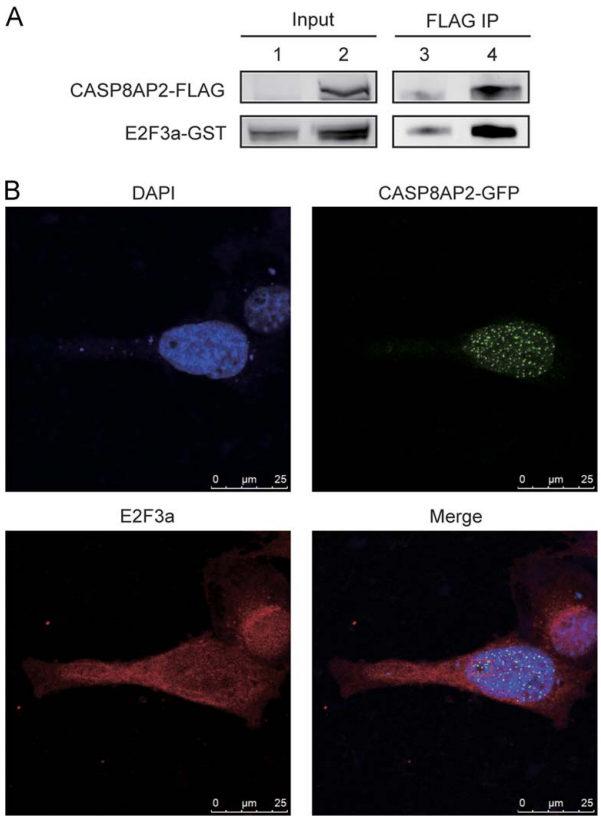

FIGURE 1. CASP8AP2 interacted with E2F3a and colocalized in the nucleus. A, E2F3a-GST was immunoprecipitated by CASP8AP2-FLAG in HEK-293T cells. Lane 1 and lane 3: transfected with the expression vector (upper) or the E2F3a-GST recombinant plasmid (lower), respectively. Lane 2 and lane 4: transfected with the CASP8AP2-FLAG (upper) or the E2F3a-GST recombinant plasmid (lower), respectively. B, CASP8AP2-GFP (green) and endogenous E2F3a (red) were colocalized in the nuclei of COS-7 cells.

RESULTS

CASP8AP2 Interacted With E2F3a in the Nucleus

We explored the interaction between CASP8AP2 and E2F3a using an immunoprecipitation assay and confirmed that exogenous E2F3a-Gst coprecipitated with CASP8AP2-flag overexpressed in HEK-293T cells (Fig. 1A). Then, we observed the subcellular distribution of CASP8AP2 and E2F3a through confocal microscopy. Since CASP8AP2 was difficult to observe using immunofluorescent staining because of its low expression, CASP8AP2-GFP was overexpressed in the COS-7 cells. Exogenous CASP8AP2-GFP was expressed in the nucleus and formed foci. The immunofluorescent staining of endogenous E2F3a showed that E2F3a was located in the cytoplasm and nucleus. Moreover, E2F3a was colocalized with CASP8AP2-GFP in the nucleus (Fig. 1B). These results indicate the interaction between E2F3a and CASP8AP2 in the nucleus.

E2F3a and CASP8AP2 Collaboratively Regulated the Expression of Replication-Dependent Histones

When E2F3a was overexpressed in HEK-293T cells, the expression of replication-dependent histone H2A or H2B was significantly increased (Figs. 2A–B). When CASP8AP2 was overexpressed, similar results were observed (Figs. 2C–D). Furthermore, the reduction in histones induced by the E2F3a knockdown could be reversed by the overexpression of CASP8AP2 (Fig. 2E). These results suggest that the interaction between E2F3a and CASP8AP2 was involved in regulating the expression of replication-dependent histones.

E2F3a and CASP8AP2 Activate the Transcription of Histone Genes

The results of the ChIP experiments showed that both E2F3a and CASP8AP2 could be coprecipitated with the promoter of the *HIST1H2AG* or *HIST1H2BK* gene in HEK-293T and 697 cells (Fig. 3A), indicating that E2F3a and CASP8AP2 could directly bind the

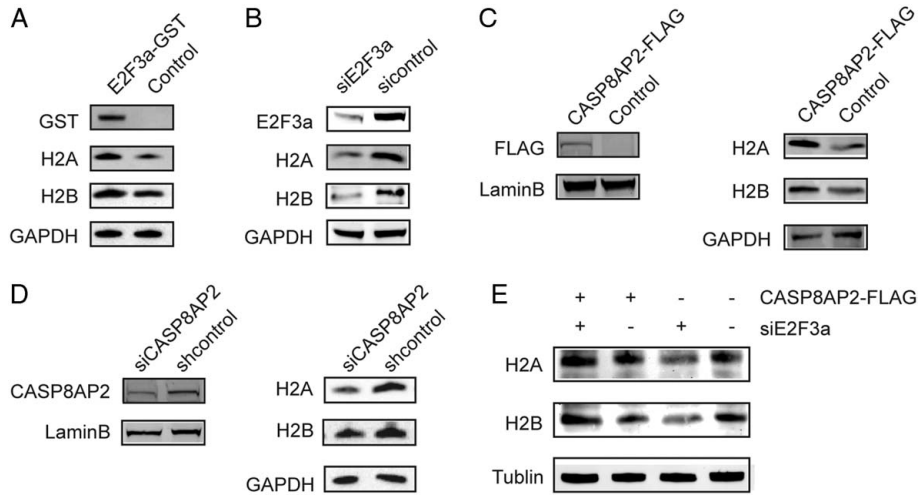

FIGURE 2. E2F3a and CASP8AP2 collaboratively affected H2A and H2B expression. A, Overexpression of E2F3a increased the expression of H2A and H2B in HEK-293T cells. B, E2F3a knockdown decreased the expression of H2A and H2B in HEK-293T cells. C, Overexpression of CASP8AP2 enhanced the expression of H2A and H2B. D, Downregulation of CASP8AP2 reduced the expression of histones in HEK-293T cells. E, The reduction in histones induced by the E2F3a knockdown could be reversed by the overexpression of CASP8AP2.

replication-dependent histone gene promoter. Furthermore, the overexpression of E2F3a increased the luciferase activity of the *HIST1H2AG* or *HIST1H2BK* promoter by the dual-luciferase reporter assay (both $P < 0.001$, Fig. 3B). Moreover, the CASP8AP2 knockdown attenuated the E2F3a-induced transcriptional activation of the replication-dependent histone gene promoter (*HIST1H2AG*, 9.1 ± 2.0 vs. 20.4 ± 0.8, $P < 0.001$; *HIST1H2BK*, 4.0 ± 0.3 vs. 7.9 ± 0.3, $P < 0.001$; Fig. 3C). Therefore, both E2F3a and CASP8AP2 could collaboratively enhance the transcriptional activity of the replication-dependent histone gene.

Interaction Between E2F3a and CASP8AP2 was Involved in S Phase Progression

CASP8AP2 was previously shown to play a crucial role in cell cycle progression at the S phase.[17] Here, the cell cycle analysis by flow cytometry showed that the proportion of cells in the S phase was significantly increased in the CASP8AP2 knockdown cells ($50.1\% \pm 1.8\%$ vs. $46.1\% \pm 1.3\%$, $P = 0.003$) and the E2F3a overexpressed cells compared with that in the control cells ($48.2\% \pm 1.0\%$ vs. $43.8\% \pm 1.2\%$, $P = 0.001$; Fig. 4A). In contrast, there was a significant reduction in the proportion of cells in the G0/G1 phases by the CASP8AP2 knockdown ($39.8\% \pm 1.6\%$ vs. $44.6\% \pm 0.9\%$, $P = 0.001$) or E2F3a overexpression ($37.8\% \pm 0.9\%$ vs. $43.8\% \pm 2.5\%$, $P < 0.001$; Fig. 4A). Furthermore, a cell cycle analysis was performed in simultaneous CASP8AP2 knockdown and E2F3a-overexpressing cells. When the cells were transfected with siCASP8AP2 and E2F3a plasmids simultaneously, there was a greater increase in the proportion of cells in the S phase and a greater reduction in the proportion of cells in the G0/G1 phases compared with the control cells (siCASP8AP2: $53.4\% \pm 1.5\%$ vs. $43.9\% \pm 0.8\%$, $P < 0.001$; E2F3a: $35.9\% \pm 1.9\%$ vs. $46.2\% \pm 0.9\%$, $P < 0.001$; Figs. 4A-B). These results demonstrate that both CASP8AP2 and E2F3a were involved in S phase progression.

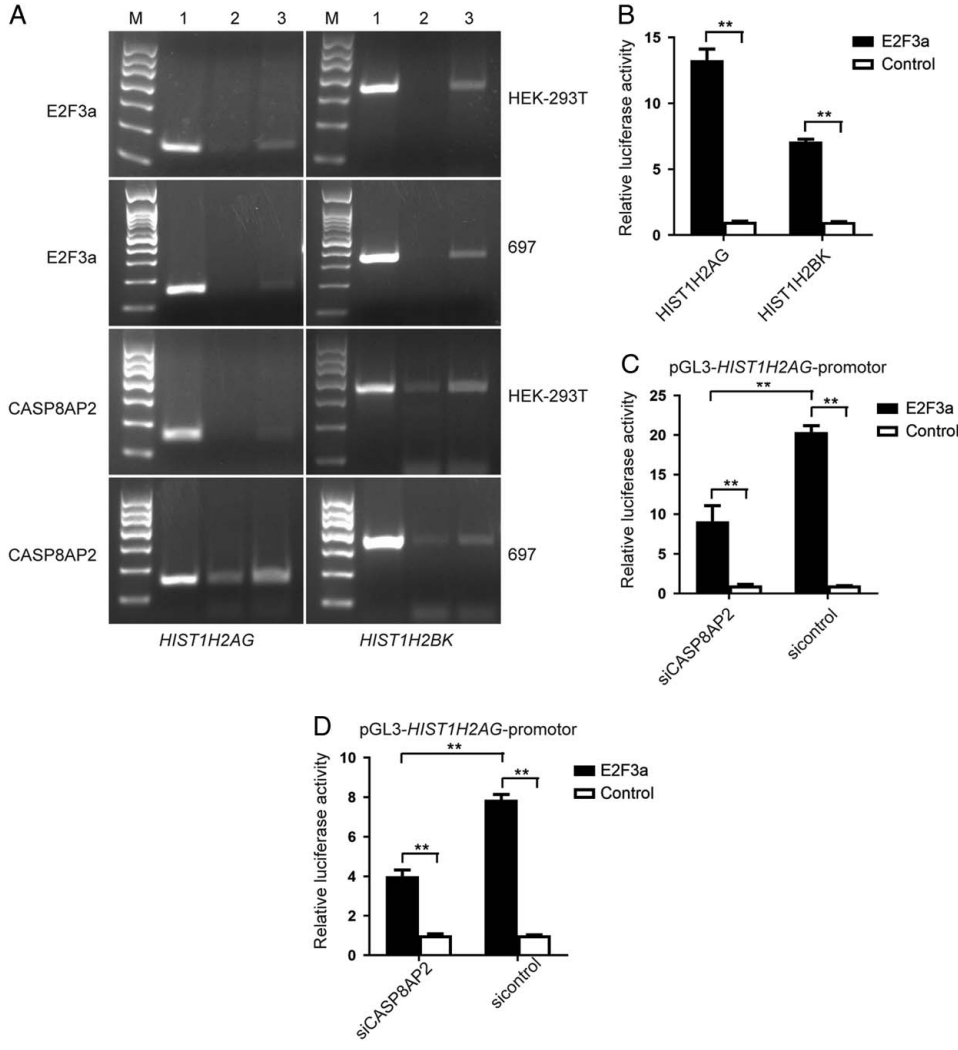

FIGURE 3. E2F3a and CASP8AP2 activate the transcription of histone genes. A, ChIP experiments showed that both E2F3a and CASP8AP2 could bind the promoter of *HIST1H2AG* or *HIST1H2BK*. Lane 1: positive control (DNA extracted from untreated cells); lane 2: IP with an unrelated normal rabbit immunoglobulin G; lane 3: IP with an anti-E2F3a antibody or anti-CASP8AP2 antibody. B, Overexpression of E2F3a significantly increased the relative luciferase activity of HIST1H2AG-Luc or HIST1H2BK-Luc. $^{**}P < 0.001$. C, CASP8AP2 knockdown attenuated the E2F3a-induced transcriptional activation of the replication-dependent histone gene promoter. $^{**}P < 0.001$.

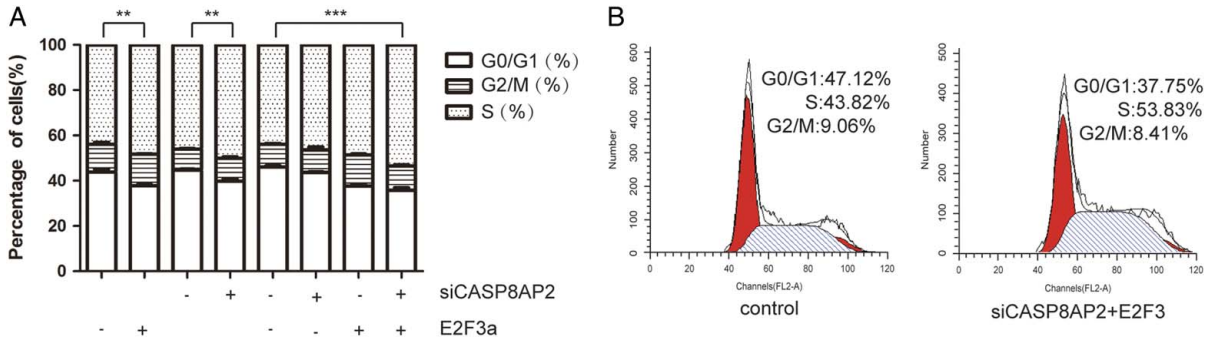

FIGURE 4. E2F3a and CASP8AP2 affected cell cycle progression. A, Overexpression of E2F3a or CASP8AP2 knockdown increased the proportion of HEK-293T cells in the S phase and decreased the proportion of cells in the G0/G1 phases. When cells were transfected with siCASP8AP2 and E2F3a plasmids simultaneously, there was a greater increase in the proportion of cells in the S phase and a greater reduction in the proportion of cells in the G0/G1 phases. B, The proportion of cells in the S phase was increased in the cells transfected with siCASP8AP2 and the E2F3a plasmid simultaneously.

E2F3a and CASP8AP2 Affected the Chemosensitivity of Leukemic Cells

DNR is an important chemotherapeutic drug used in induction therapy in childhood ALL. Our previous study demonstrated that a low expression of *E2F3a* and *CASP8AP2* was associated with relapse in children with ALL. Therefore, the proliferation ability was assessed by an MTS assay in cells treated with DNR. The upregulation of E2F3a resulted in a reduction in the IC50 in 697 cells (110.9 vs. 139.2 ng/ml, $P = 0.0053$, Fig. 5A). Similarly, the downregulation of CASP8AP2 led to a significant increase in the IC50 of DNR (250.3 vs. 141.4 ng/ml, $P < 0.0001$, Fig. 5B). We further found that the downregulation of CASP8AP2 reduced the increased sensitivity to DNR in the E2F3a-overexpressing cells (IC50 145.0 vs. 110.9 ng/ml, $P = 0.0046$, Fig. 5C). The upregulation of E2F3a increased the sensitivity to DNR in the CASP8AP2 knockdown cells (IC50 145.0 vs. 250.3 ng/ml, $P < 0.0001$; Fig. 5C). Thus, the interaction between E2F3a with CASP8AP2 probably affected the chemosensitivity of ALL cells.

DISCUSSION

E2F3a and CASP8AP2 are key regulators involved in the cell cycle.[18,19] Our previous study showed that E2F3a could directly bind the *CASP8AP2* promotor and activate its transcription.[16] This study further found that these two molecules were colocalized and interacted in the nucleus. CASP8AP2 interacts with several key TFs, including c-Myb,

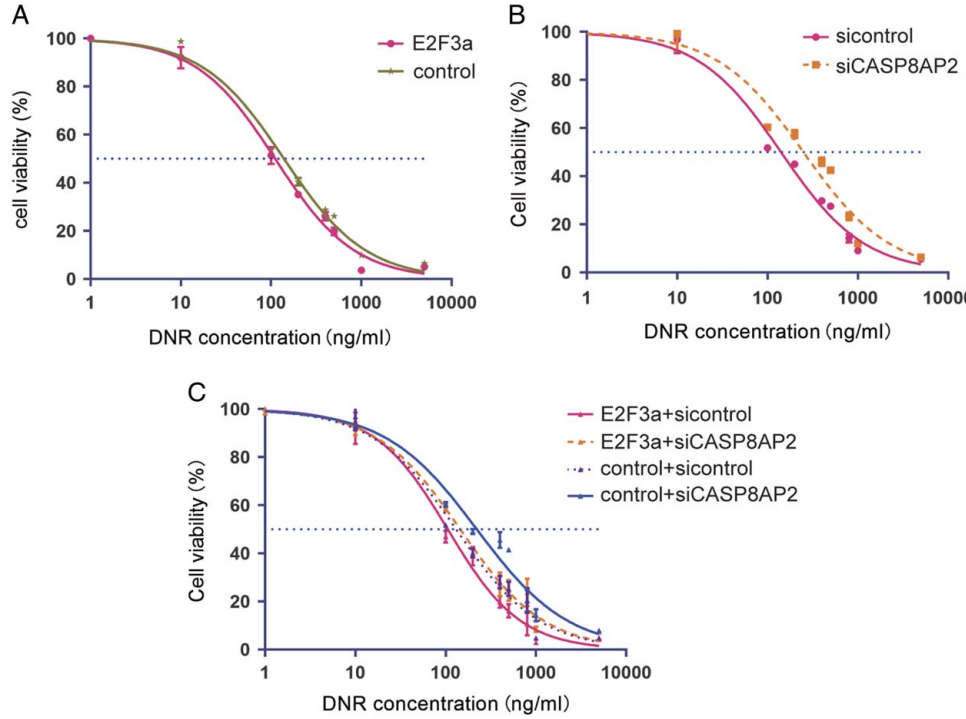

FIGURE 5. E2F3a and CASP8AP2 affected the chemosensitivity of leukemic cells. A, E2F3a overexpression enhanced the sensitivity of 697 cells to DNR. B, CASP8AP2 knockdown reduced the sensitivity of 697 cells to DNR. C, Downregulation of CASP8AP2 reduced the increased sensitivity to DNR in E2F3a-overexpressing cells. The SEM are shown (triplicate experiments per drug concentration).

p73, and E2A.[20–22] Thus, CASP8AP2 might play an essential role in histone gene transcription, cell proliferation, and senescence after transcriptional activation by E2F3a and other TFs, which probably depend on the interacting TFs.

The TF E2F family has been reported to be a central transcriptional regulator of replication-dependent histones.[23] The present study demonstrated that E2F3a could bind the promoters of histone genes, increase transcriptional activities and upregulate the expression of H2A and H2B. Meanwhile, CASP8AP2 is a key regulator of 3'-end processing of replication-dependent histone pre-mRNA.[13,24,25] Moreover, we found that CASP8AP2 could bind the promoters of *HIST1H2AG* and *HIST1H2BK*, activate transcription, and upregulate H2A and H2B expression collaboratively with E2F3a. Hence, CASP8AP2 was involved in histone biogenesis, including transcription regulation, indicating its influence on cell cycle progression. We speculate that the CASP8AP2 knockdown decreased the transcription of histone genes and pre-mRNA processing, which led to a reduction in histone biogenesis and S phase arrest. In contrast, the increased histone biogenesis by the E2F3a upregulation accelerated cell cycle progression.

We previously demonstrated the association between a low expression of E2F3a and CASP8AP2 and a poor prognosis in childhood ALL.[10,14,26] Consistent with these findings, the upregulation of E2F3a increased the sensitivity of leukemia cells to DNR, a noncell cycle-specific chemotherapeutic drug, while the downregulation of CASP8AP2 decreased chemosensitivity. Moreover, the CASP8AP2 knockdown reversed the increased chemosensitivity induced by the E2F3a upregulation, indicating the vital role of the interaction between the two molecules in chemotherapeutic resistance. Notably, our previous study reported that E2F3a and CASP8AP2 collaboratively affected the sensitivity of leukemic cells to vincristine,[16] a cell cycle-specific chemotherapeutic drug. Therefore, the effect of E2F3a and CASP8AP2 on chemosensitivity may rely on the regulation of cell cycle progression and other mechanisms that need further investigation.

In summary, CASP8AP2 interacted with E2F3a to regulate replication-dependent histone expression and cell cycle progression. They also affected the chemosensitivity of leukemic cells, and the underlying mechanisms were further studied.

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