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Epigenetic upregulation of Bak by ZBP-89 inhibits the growth of hepatocellular carcinoma

Cai Guo Ye[a,b], **George G Chen**[a], **Rocky LK Ho**[a], **Juanita L Merchant**[c,d], **Ming-Liang He**[e], and **Paul BS Lai**[a]

[a]Department of Surgery, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

[b]China-America Cancer Research Institute, Guangdong Medical College, Dongguan 523808, PR China

[c]Internal Medicine-Gastroenterology, University of Michigan, Ann Arbor, Michigan 48109, USA

[d]Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan 48109, USA

[e]Stanley Ho Centre for Emerging Infectious Diseases, School of Public Health and Primary Care, and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

Abstract

ZBP-89 regulates Bak to facilitate apoptosis in cancer cells. This study examined if ZBP-89 regulates Bak through an epigenetic mechanism in hepatocellular carcinoma (HCC). We first demonstrated that the expression of Bak was reduced but the levels of DNMT1 and HDAC3 were increased in HCC cancer tissues compared to the corresponding non-cancer tissues. Moreover, there was a negative correlation between Bak expression and DNMT1 levels in HCC. Administration of ZBP-89 downregulated HDAC3 expression and suppressed the activities of HDAC and DNMT, which led to maintenance of histone acetylation status, inhibited the binding of methyl-CpG-binding protein 2 to genomic DNA and demethylated CpG islands in the Bak promoter in HCC cells. Using the xenograft mouse tumor model, we demonstrated that ZBP-89 or inhibitors of either epigenetic enzymes could stimulate Bak expression, induce apoptosis, and arrest tumor growth and that the maximal effort was achieved when ZBP-89 and the enzyme inhibitors was used in combination. Conclusively, ZBP-89 upregulates the expression of Bak by targeting multiple components of the epigenetic pathway in HCC.

Keywords

Hepatocellular Carcinoma; ZBP-89; HDAC; DNMT; Bak

Corresponding author: George G Chen, Department of Surgery, Prince of Wales Hospital, Shatin, NT, Hong Kong. Tel: 852 26323934 Fax: 852 26450605, gchen@cuhk.edu.hk.

Conflict of Interest
All authors do not have the conflict of interest.

Appendix A. Supplementary data

1. Introduction

ZBP-89, a Kruppel-type zinc-finger transcription factor, binds to gene promoter GC-rich sequence to activate or suppress gene transcription which is closely related to cell growth and cell death [1,2]. Previously, we reported that ZBP-89 induced Bak gene expression, a pro-apoptotic protein known to stimulate apoptosis in hepatocellular carcinoma (HCC) cells [3]. In addition to its ability to bind to gene promoters, ZBP-89 also forms protein-protein interactions[2]. For example, ZBP-89 interacts with histone deacetylases (HDAC) to repress p16 and vimentin gene transcription [2,4], or interacts with p53, GATA-1 and/or FOG-1 to activate gene expression [5,6]. These studies propose a link between ZBP-89 and epigenetic regulation, though the detailed mechanism is not understood. Bak promoter methylation was reported previously [7,8]. Therefore we queried whether the regulation of Bak expression by ZBP-89 is subjected to epigenetic regulation.

HDAC and DNA methyltransferas (DNMT) inhibitors can synergically regulate the same genomic targets [9]. They may synergize when added together, although they activate different gene targets through different mechanisms [6]. An increase in the acetylated histone H3 and histone H4 is closely correlated with gene expression [10]. HDAC3 is regarded as a biomarker of HCC recurrence and therapy target [11,12]. The roles of HDAC and DNMT in the regulation of Bak are known.

CpG dinucleotide islands are usually located in the promoter region of given genes. At these locations, CG dinucleotides that become methylated suppress gene expression. DNA methylation may silence gene expression through either interfering with transcription factor binding or recruiting methylated DNA binding proteins [13,14]. Aberrant genome-wide DNA methylation mediated by DNMT has been thought to be related to tumorigenesis. Increased expression of DNMT in HCC is correlated with poor prognosis and survival [15,16].

Bak expression is suppressed in HCC. We previously reported that ZBP-89 induced Bak gene expression [3]. The aim of this study was to determine whether ZBP-89 regulated Bak gene expression through its ability to modulate HDAC and DNMT

2. Materials and methods

2.1. Chemicals and reagents

Antibodies against ZBP-89, PARP, methyl-CpG-binding protein 2 (MeCP2); protein A/G PLUS-agarose beads, ZBP-89 siRNA, HDAC3 siRNA and DNMT1 siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HDAC3, HDAC4, DNMT1 and DNMT3a, DNMT3b antibodies were provided by Cayman Chemicals (Ann Arbor, MI). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. HCC patient samples, cell lines, siRNA transfection, and adenovirus infection of cells

103 HCC tissue samples and the matched non-cancerous liver tissue samples were obtained from patients with HCC, who underwent surgical resection and the detailed information of

these samples was shown in Table S1. The study was approved by the local Clinical Research Ethics Committee. HCC cells, PLC/PRF/5 and HepG2 cells were purchased form ATCC. HepG2 cells are a low tumorigenic cells but they are positive for HBV antigens. PLC/PRF/5 cells positive for HBV show higher tumorigenic ability. MIHA cells are a SV40-immortalized normal liver cell line. 10 μM of each ZBP-89 siRNA, HDAC3 siRNA, or DNMT1 siRNA were transfected into the cells with Fugene reagent (Roche, Indianapolis, IN). The replication-deficient recombinant adenoviral expression vector Ad-ZBP-89 was used according to previous publications [3,5].

2.3. Activities of HDAC and DNMT

The effect of ZBP-89 on HDAC activity in cancer cells was assessed by the Fluor de Lys-Green HDAC Assay Kit (Enzo Life Science, Farmingdale, NY). The experiment was performed according to the manufacturer's protocol. DNMT was prepared according to a previous publication [17]. The S-adenosyl-L-methionine was used as the methyl group donor. The activity of DNMT was detected by the methyltransferase activity kit (Enzo Life Science, Farmingdale, NY).

2.4. Co-immunoprecipitation (Co-IP) and Western blotting

These experiments were performed according to the previous publications [3,5]. Briefly, cell lysates were incubated overnight with primary antibody after being precleared with protein A/G PLUS-agarose beads. Immunocomplexes were precipitated after incubating with Protein A/G PLUS-agarose beads. Immunocomplexes were released by boiling in 2×SDS sample buffer for Western blot analysis. The positive signals were visualized by ECL reagent kit (GE Healthcare).

2.5. Immunohistochemistry and Immunofluorescence

The immunohistochemical examination was carried out according to our previous publication [3,5]. Briefly, the tissue sections were incubated with a primary antibody followed by a HRP-labeled IgG secondary antibody. The antigen-antibody complex was visualized by the diaminobenzidine method. Phosphate-buffered saline was used as a negative control instead of the primary antibody. For immunofluorescent staining, after the cells were fixed with 4% paraformaldehyde, they were incubated with primary antibody, followed by a FITC- or Rhodamine-labeled secondary antibody. Fluorescent cells were visualized using the Zeiss Axioplan 2 microscope (Zeiss, Germany).

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed based on the protocol previously published with some modifications [18]. The following primers were used to detect precipitated sequence:

S1_F1: GGGAGGATGGGAGGTTGGGGG;

S1_R1: AGCCCCTGCCCTCAGGGTTC;

S2_F2:GAGGTGGGGGAGAAGATCAAAGACA,

S2_R2:CGGCACCCGGCTCCAATCAG.

2.7. Sodium bisulfite modification sequencing of Bak promoter

Sodium bisulfite modified sequencing was carried out using the 377DNA sequencer (ABI) and gene promoter primers were designed using MSPPrimer. Totally, 1 μg of genomic DNA was modified and cleared up by EZ DNA modification-Gold kit (Zymo Research, Irvine, CA). The following Bak gene promoter primers were used:

msp_F1: GTTTAGGTTGGAGTGTAGTGGGTTA,

msp_R1: CCAAAATTATACCACTACACTCCTACC;

msp_F2: GTGGTTATAGAGTAATTTTTTTTAGAGGGA,

msp_R2: ACCTACCCAAAAAAACCTTAAACTT;

2.8. Cell proliferation and Apoptosis detection

Cell proliferation was determined by MTT assay as described previously [3,5]. Apoptosis was determined by the Vybrant® Apoptosis Assay Kit (Invitrogen, Carlsbad, CA) and performed according to the introduction of the manufacture [3,5].

2.9. Xenograft animal model

The xenograft tumor model was generated by injecting 2×10^6 PLC/PRF/5 cells into the left axilla of nude mice and left to grow for 3 weeks. The control group received 0.9% saline by i.p. The Ad-ZBP-89 recombinant adenoviral vector (1×10^{10} pfu) was administrated by direct injection into tumor tissues. The first injection was done at the beginning of the implantation, and the second at 7 days later. Valproic acid (VPA) and/or zebularine (Zebu) was i.p. injected into mice at 50mg/kg/d and 100 mg/kg/d, respectively, for 7 days [19,20]. At the end of the experiment, the tumor was excised intact and weighted. The expression of Bak in tumor tissues was determined and apoptosis was analyzed by TUNEL [21]. The mouse experiment was approved by the local Animal Research Ethics Committee.

2.10. Statistical analysis

Analyses were performed using SPSS (SPSS Inc, Chicago, IL). Statistical analysis was performed by paired t-test, Mann-Whitney U test or one-way ANOVA. P values of less than 0.05 were considered statistically significant. Results were representative of triplicate experiments and expressed as mean ± SD.

3. Results

3.1. ZBP-89 formed a complex with DNMT1 and HDAC3

There are a number of epigenetic enzymes which are closely associated with hepato-carcinogenesis, including DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC3 and HDAC4. Therefore, we examined if ZBP-89 could interact with these epigenetic enzymes. Co-IP studies showed that ZBP-89 protein interacted with DNMT1 and HDAC3 but not the rest, ZBP-89 formed a complex with DNMT1 and HDAC3 that could be detected by ZBP-89 antibody as well as DNMT1/HDAC3 antibody (Fig. 1A, B and C). The confocal images showed that ZBP-89 and DNMT1 co-localized primarily in the nucleus (Fig. 1D and E). After treatment with the HDAC inhibitor TSA, ZBP-89 appeared to migrate from the

nucleus to the cytoplasm. Some DNMT1 proteins co-migrated with ZBP-89 to the cytoplasm, while some DNMT1 proteins remained in the nucleus.

3.2. Expression of Bak, DNMT1 and HDAC3 in HCC tissues

In order to elucidate the relationship between Bak, ZBP-89, and two chromatin-modifying enzymes, Western blot was performed on 103 liver cancer tissues and their non-caner liver tissues (Fig. 2A). We found that the levels of Bak, ZBP-89, DNMT1 and HDAC3 were significantly different between cancer tissues and paired non-cancer tissues ($P<0.05$, Fig. 2B, C, D and E). The levels of DNMT1 and HDAC3 proteins were much higher in the cancer tissues than the non-cancer tissues, whereas Bak expression was lower in the cancer tissues than in the non-cancer tissues. Furthermore, immunohistochemical staining confirmed that a lower level of ZBP-89, but a higher level of DNMT1 was found in poorly-differentiated HCC (Fig. S1).

3.3. Inhibition of HDAC and DNMT induced Bak expression and apoptosis

The results showed that VPA and Zebu inhibited the cell proliferation in a time- and dose-dependent manner (Fig. 3A, B and C). Similar results were also obtained when the cells were treated with TSA and 5-aza-dC (data not shown). VPA and Zebu in combination showed the greatest suppression on cell proliferation, compared with either agent alone ($P<0.05$). Furthermore, both VPA and Zebu induced Bak expression, and activated PARP cleavage-mediated apoptosis in PLC/PRF/5 and HepG2 cells (Fig. 3D, Fig. S2). Both agents also enhanced the acetylation of H4Lys12. These data suggest that Bak expression is regulated by HDAC3 and DNMT1, which is likely related to histone acetylation. In addition, ectopic expression of ZBP-89 clearly induced expression of the pro-apoptotic protein Bak (Fig. 3E).

3.4. ZBP-89 inhibited HDAC activity

Both TSA and VPA significantly suppressed the activity of HDAC in both nuclear and cytosol extracts *in vitro* ($P<0.001$, Fig. 4A and B). Surprisingly, ZBP-89 overexpression also inhibited HDAC activity ($P<0.05$). Furthermore, Western blot analysis showed that ZBP-89 could maintain acetylation at histones H3 lysine 9/14 and H4 lysine 12 (Fig. 4C, Lane 2 and 3, Fig. S3).

3.5. ZBP-89 suppressed DNMT activity and demethylated methyl-CpG islands

The overexpression of ZBP-89 significantly inhibited DNMT activity in the nuclear extract ($P<0.001$, Fig. 4D). DNMT1 has been reported to bind DNA at CpG islands [22]. Thus, the finding suggests that ZBP-89 might interfere with the promoter CpG methylation by decreasing DNMT1 activity. Two ZBP-89 DNA binding sites, GGGGGCAGGGG and GGGAGGTGGGG [23], were identified (Fig. 5A and B). Chromatin precipitation method was used to testify the ZBP-89 binding sites. Results showed that 204 bp S1 sequence which is 3 kbp away from exon1, and 228 bp S2 which is near exon1, were precipitated by ZBP-89 antibody and amplified by primers (Fig. 5C). Furthermore, using Co-IP, we measured the precipitated MeCP2, a protein that binds to promoter regions [24,25]. The precipitated products without proteinase K treatment were analyzed by SDS-PAGE and Western

blotting, and the result showed that ZBP-89 overexpression inhibited MeCP2 binding to genomic DNA (Fig. 5D). This finding indicated that ZBP-89 might modulate Bak expression by decreasing MeCP2 binding to DNA. Using two computer programs (http://www.mspprimer.org/cgi-mspprimer/design.cgi and http://www.urogene.org/methprimer/index1.html), we found two potential CpG islands in human Bak promoter (Fig. 5E). Bisulfite modification sequencing was performed on these 2 CpG islands and showed that ZBP-89 could demethylate them (Fig. 5F). In the first CpG island, ZBP-89 demethylated methyl-CpGs, but the second CpGs island was not affected.

3.6. Downregulation of HDAC3 and DNMT1 enhanced Bak expression

Although the activities of HDAC3 and DNMT1 are affected by ZBP-89, it is still unknown if they directly participate in Bak regulation. We therefore used HDAC3 siRNA and DNMT1 siRNA to knockdown HDAC3 and DNMT1 respectively. It showed that the downregulation of HDAC3 and DNMT1 enhanced Bak expression (Fig 4C and 4E).

3.7. ZBP-89 enhanced Bak expression, induced apoptosis and inhibited tumor growth

Thirty nude mice were randomly divided into 5 groups, saline, Ad-ZBP-89, VPA, Zebu, and VPA plus Zebu. The significant inhibition of tumor growth was observed in mice treated with Ad-ZBP-89, VPA and Zebu, compared with the saline control (Fig. 6A). The most obvious inhibition was observed in mice treated with VPA and Zebu in combination. The expression of Bak was enhanced by ZBP-89, VPA and Zebu and the TUNEL positive cells were much more in tumor tissues receiving ZBP-89, VPA and Zebu (Fig. 6B and C).

4. Discussion

The involvement of pro-apoptotic Bak in HCC development, progression and treatment has been documented. The level of Bak is reduced or even non-detectable in HCC cells [26,27]. The reduction of Bak contributes to the development and progression of HCC [28,29]. The significance of Bak in HCC cells is further supported by several experiments in which different agents induce apoptosis in HCC cells by stimulating Bak expression [30–33]. Although the significance of Bak in HCC has been reported, the mechanism responsible for its regulation in HCC is largely unknown. Our previous study has indicated that ZBP-89 may target a certain region of Bak promoter to induce its expression and subsequently apoptosis [3]. In this study we further demonstrated that ZBP-89 stimulated Bak expression through an epigenetic mechanism in HCC.

Sodium butyrate is known to promote ZBP-89-mediated p21waf1 activation in human colorectal cancer cells [34]. ZBP-89 may recruit HDAC1 to the vimentin promoter to repress the expression of vimentin in human cervical cancer HeLa cells [2]. Furthermore, ZBP-89 and HDAC3 may form a complex to help HDAC3 bind to the p16 promoter, resulting in p16 downregulation in human lung cancer cells [4]. To our best knowledge, there is no report on effect of ZBP-89 on the epigenetic regulation of Bak. In the present study, we have shown the involvement of ZBP-89 in the epigenetic regulation of Bak in HCC. First, our Co-IP experiments showed that ZBP-89 protein could form a complex with DNMT1 and HDAC3, and this finding was supported by the confocal examination showing

that ZBP-89 and DNMT1 were co-localized primarily in the nucleus. Interestingly, ZBP-89 along with a portion of DNMT1 migrated from the nucleus to the cytoplasm when HCC cells were treated with HDAC inhibitor TSA. Second, ZBP-89 could inhibit the activities of HDAC and DNMT. It was noted that such an inhibitory effect of ZBP-89 on DNMT was even much greater than the classic DNMT inhibitor, Zebu. However, the inhibition of HDAC by ZBP-89 was much weaker than the traditional HDAC inhibitors TSA and VPA. Third, ZBP-89 could maintain histone H3 lysine 9/14 residues and histone H4 lysine 12 residue acetylation status. Fourth, ZBP-89 could significantly inhibit the activity of DNMT that participate in DNA CpG islands *de novo* methylation. Finally, ZBP-89 could inhibit MeCP2 binding to genomic DNA and partially demethylate Bak promoter CpG islands. These findings evidently indicate that ZBP-89 is able to target multiple points in the epigenetic pathway to upregulate the expression of Bak in HCC.

Our study confirms that the level of Bak is reduced in HCC, especially in the poorly-differentiated HCC. However, a gradual decline in Bak with a corresponding increase in DNMT1 and HDAC3 was not found to be correlated with tumor progression from well/moderate differentiated to poorly differentiated. We do not have a good explanation for this. But it is possible that there are some unknown relevant co-factors in poorly differentiated HCC but not in well/moderately differentiated HCC. The size of the samples in each subgroup may not large enough to render a significant result. Nevertheless, ZBP-89 is able to promote the expression of Bak in HCC cells. Importantly, we have further demonstrated that the expression of Bak is controlled by epigenetic elements in HCC. First, the expression of Bak in liver tissues of HCC is negatively associated with two important epigenetic enzymes, DNMT1 and HDAC3, whose levels are increased in the HCC tumor tissues. Second, the inhibition of either DNMT1 or HDAC3 induces Bak expression and the maximal inhibition is achieved when both enzymes are simultaneously suppressed. These results clearly indicate that both DNMT1 and HDAC3 participate in the expression of Bak in HCC. Third, two CpG islands were located in the promoter of Bak gene as the result of our analysis of human Bak gene promoter. Further analysis showed that the Bak gene is methylated on the dinucleotide CpG islands found. It is well known that CpG island hypermethylation may inhibit transcription by interfering with the recruitment and function of basal transcription factors or transcriptional coactivators [24]. The hypermethylation of CpG islands near the transcriptional regulatory region can promote the recruitment of the methyl-CpG binding domain family proteins including MeCP2 to facilitate a repressive chromatin environment, resulting in silencing genes [24]. MeCP2 can also interact with HDAC-containing complexes to contribute to the histone deacetylation-mediated transcriptional repression [25]. These concepts are consistent with our finding that ZBP-89 inhibits MeCP2 binding to chromatin, and permits demethylation of the CpG islands within the Bak gene, and subsequently induces Bak expression. Though we do not have data to show how ZBP-89 inhibits MeCP2 binding to chromatin, it is possible that ZBP-89 may compete with MeCP2 to bind to genomic DNA, and dissociates MeCP2 from DNA (Fig. S5). Finally, our *in vivo* experiment demonstrated that the administration of ZBP-89 significantly inhibited the growth of xenograft HCC tumor and such a tumor-inhibitory effect was comparable to that of Zebu, a DNMT inhibitor, and VPA, a HDAC inhibitor.

Importantly, along with the reduction of the tumor size, there was an increase in Bak expression and occurrence of apoptosis.

In conclusion, we shows that the expression of Bak is reduced but the levels of DNMT1 and HDAC3 are increased in HCC. Bak expression is negatively associated with levels of these enzymes. ZBP-89 upregulates Bak expression via decreasing DNMT1 and HDAC3, inhibiting MeCP2 binding to genomic DNA and demethylating CpG islands in the Bak promoter. The application of ZBP-89 enhances Bak expression, induces apoptosis and inhibits the growth of HCC tumor *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations list

5-aza-dC	5-aza-2’-deoxycytidine
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
DNMT	DNA methyltransferase
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
MeCP2	methyl-CpG-binding protein 2
MIHA	immortalized non-tumorigenic hepatocyte cell line
VPA	sodium valproic acid
Zebu	zebularine

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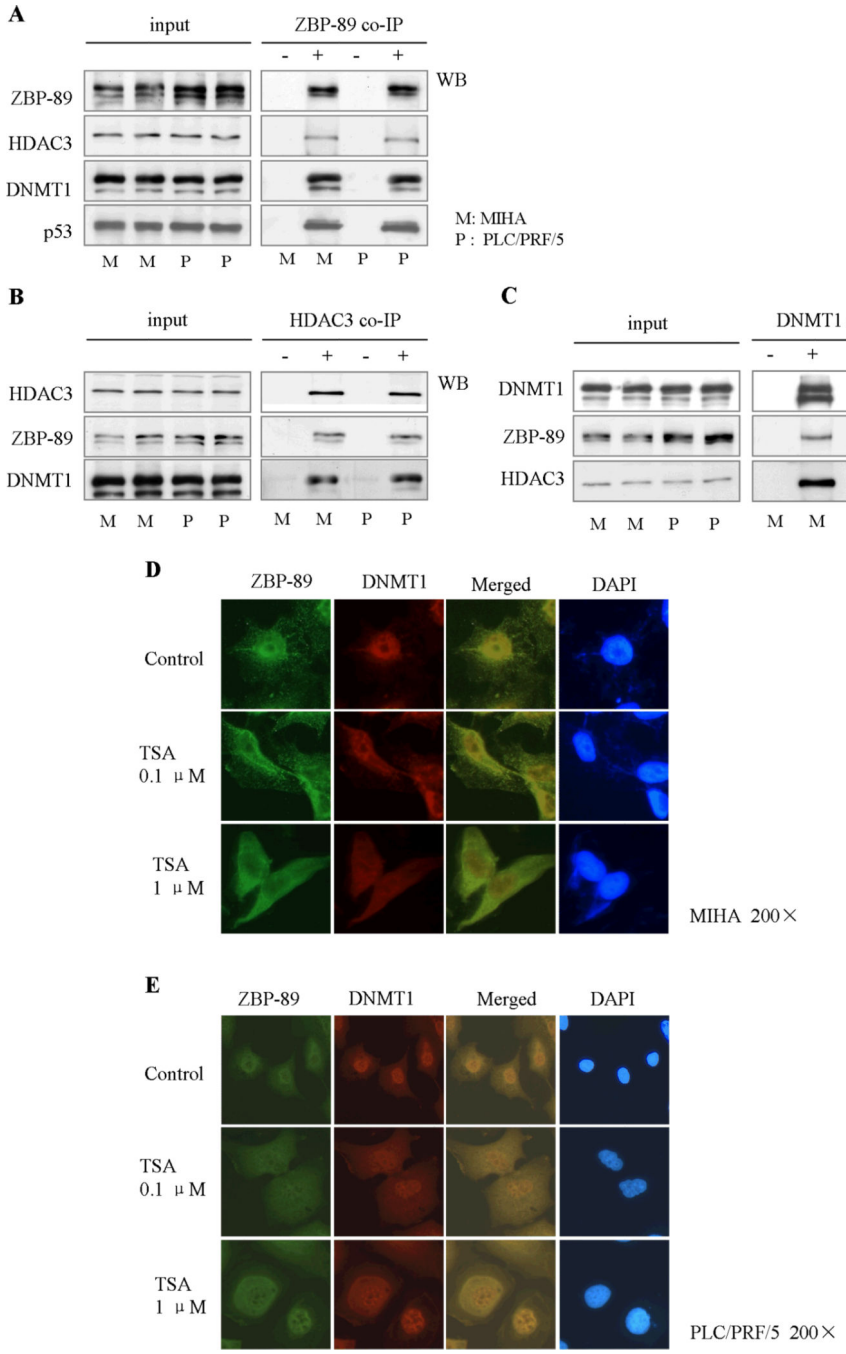

Fig. 1. ZBP-89 interacts with HDAC3 and DNMT1 to form a complex

ZBP-89 bound HDAC3 and DNMT1 to form a complex in MIHA and PLC/PRF/5 cells (A). The complex could be precipitated by either HDAC3 antibody or DNMT1 antibody (B, C). Original and merged confocal images showed that the complex formed by ZBP-89 and DNMT1 was primarily located in the nucleus (D, E). However, in the presence of TSA, a HDAC inhibitor, ZBP-89, was mainly found in the cytoplasm, and some DNMT1 was also present in the cytoplasm. “+” represents precipitation with labeled antibodies listed in the

left side; “−”represents precipitation with control isotype IgG rather than the main antibodies.

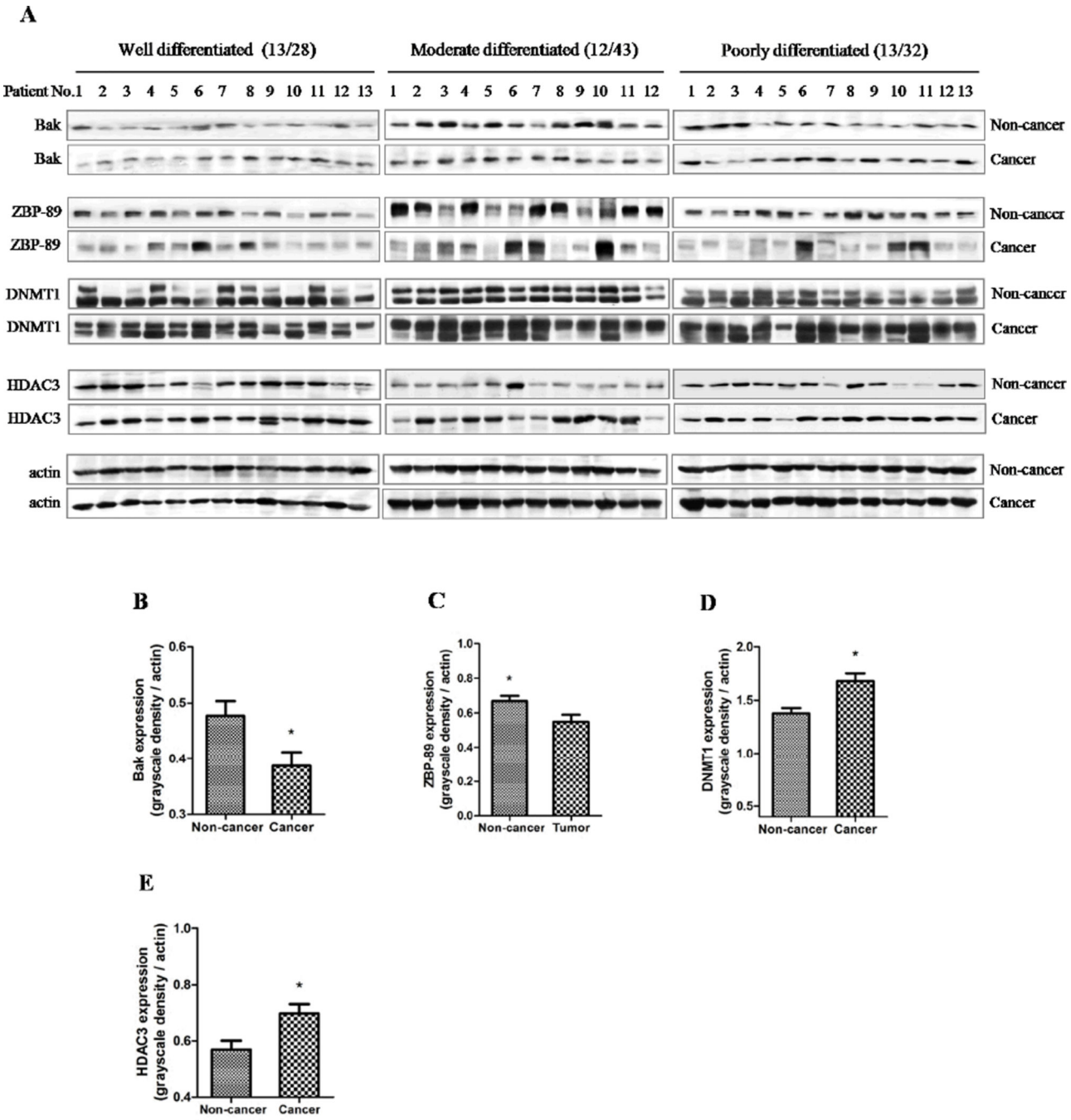

Fig. 2. Bak expression is strongly related to ZBP-89, DNMT1 and HDAC3 in HCC

The expression of Bak, ZBP-89, DNMT1 and HDAC3 in different differentiated HCC samples was examined. The grayscale density of proteins bands was shown in a bar chart, according to different HCC differentiation subtypes. Bak, ZBP-89, DNMT1 and HDAC3 levels were significantly different between cancer tissues and their paired non-cancer tissues (B, C, D and E).

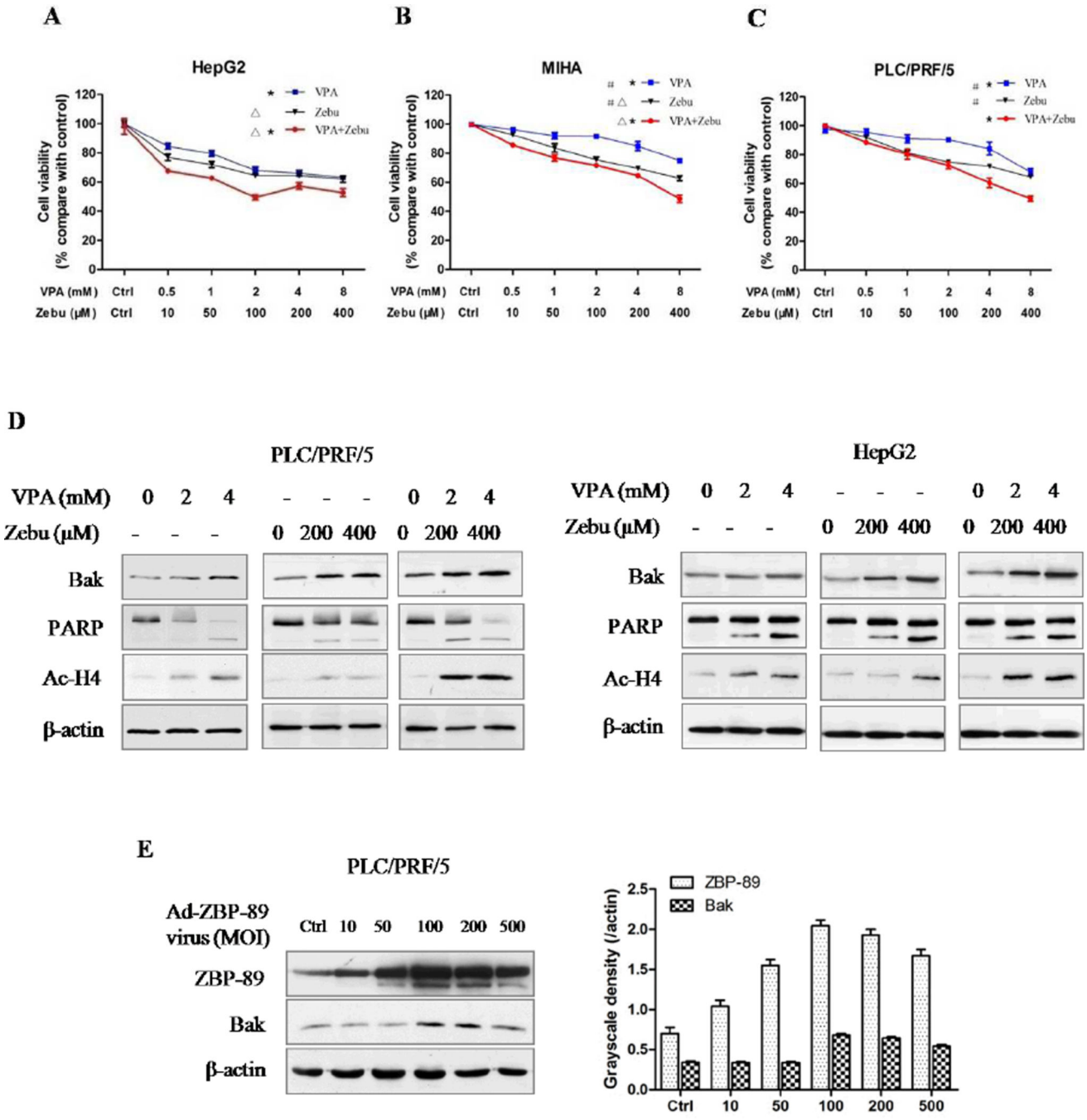

Fig. 3. HDAC and DNMT inhibitors, and ZBP-89 induce Bak expression

VPA and Zebu inhibited the proliferation of PLC/PRF/5, HepG2 and MIHA cells. Combination of VPA and Zebu significantly suppressed cells proliferation (A, B and C). Data were shown as Mean±SD. Both VPA acid and Zebu induced Bak expression and cleaved PARP. Treatment of VPA and Zebu increased histone H4 acetylation (D). Ad5-ZBP-89-mediated ZBP-89 overexpression up-regulated Bak expression in a dose-dependent manner after 24 h infection (E). *, Zebu+VPA vs VPA, $P<0.05$; △, Zebu+VPA vs Zebu, $P<0.05$; #, Zebu vs VPA, $P<0.05$, one-way ANOVA analysis.

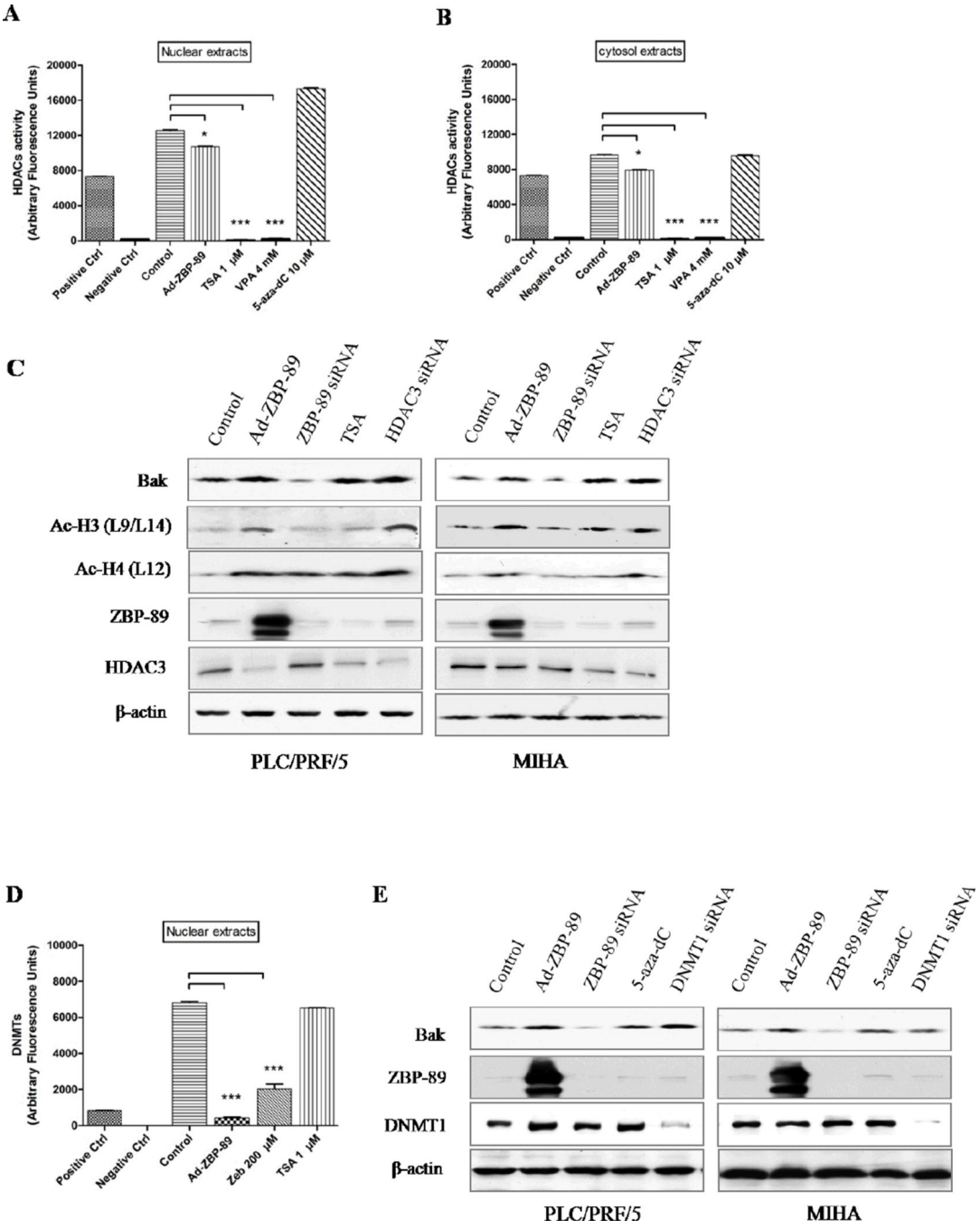

Fig. 4. ZBP-89 inhibits HDAC and DNMT activities, and increases histone acetylation level

Histone deacetylases activity analysis showed that adenovirus-mediated ZBP-89 overexpression inhibited HDAC activity in both nuclear and cytosol extracts (A and B). TSA (1 μM) significantly suppressed HDAC activity *in vitro*. Interestingly. Western blot analysis showed that ZBP-89 increased histone H3 acetylation (Lys9/Lys14) and histone H4 acetylation (Lys12), and decreased HDAC3 protein level (C, Lanes 2 and 3). HDAC3 siRNA upregulated the level of Bak (C, Row 1) and increased histone H3 and H4 acetylation in PLC/PRF/5 and MIHA cells (C, Rows 2 and 3). The overexpression of

ZBP-89 significantly inhibited DNMT1 activity in nuclear extracts (D). The activity was also suppressed by DNMT inhibitor Zebu. The downregulation of DNMT1 expression by siRNA increased Bak expression (E Lane 1). ZBP-89 siRNA decreased Bak expression in both cell lines. Data were shown as mean ± SD in triplicate experiments. *P<0.05, ***P<0.001.

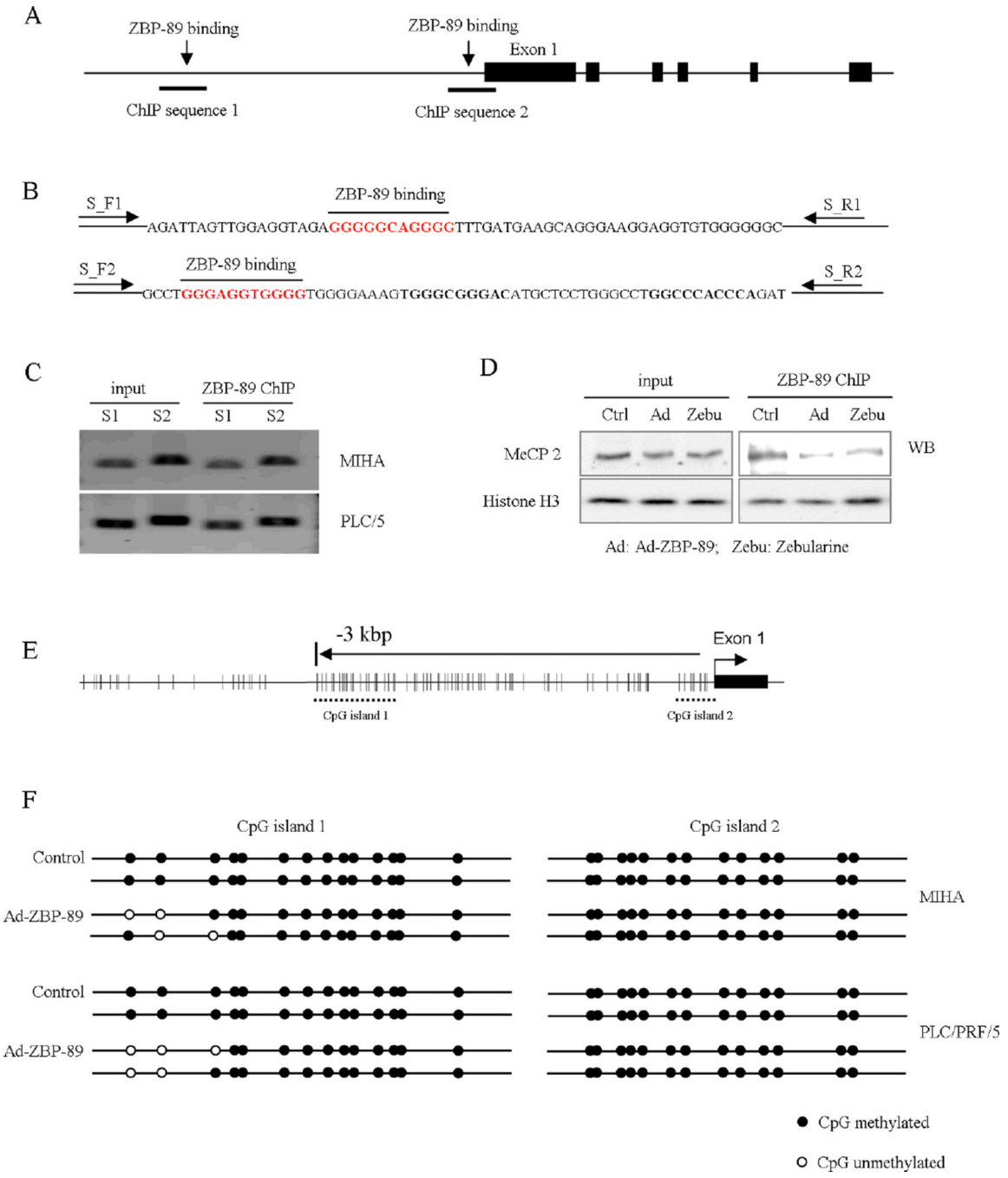

Fig. 5. ZBP-89 demethylates methyl-CpG dinucleotide

Schematic diagram of Bak promoter and ZBP-89 binding sites (A). ChIP experiment showed ZBP-89 binding to two regions. One is very close to exon 1 and the other is 3 kbp away form transcription start site (B). 204 bp S1 sequence which is 3 kbp away from exon1, and 228 bp S2 which is near exon1, were precipitated by ZBP-89 antibody and amplified by primers (C). MeCP2 binds to chromatin in a methylation-dependent manner and is associated with a core repressor complex to silence gene transcription. Results showed that adenovirus-mediated ZBP-89 overexpression decreased MeCP2 protein binding to

chromatin (D). CpG islands in Bak gene promoter were predicted by online software and 2 potential CpG islands in Bak promoter were found (E). When ZBP-89 was overexpressed, some methylated CpG regions became demethylation (F).

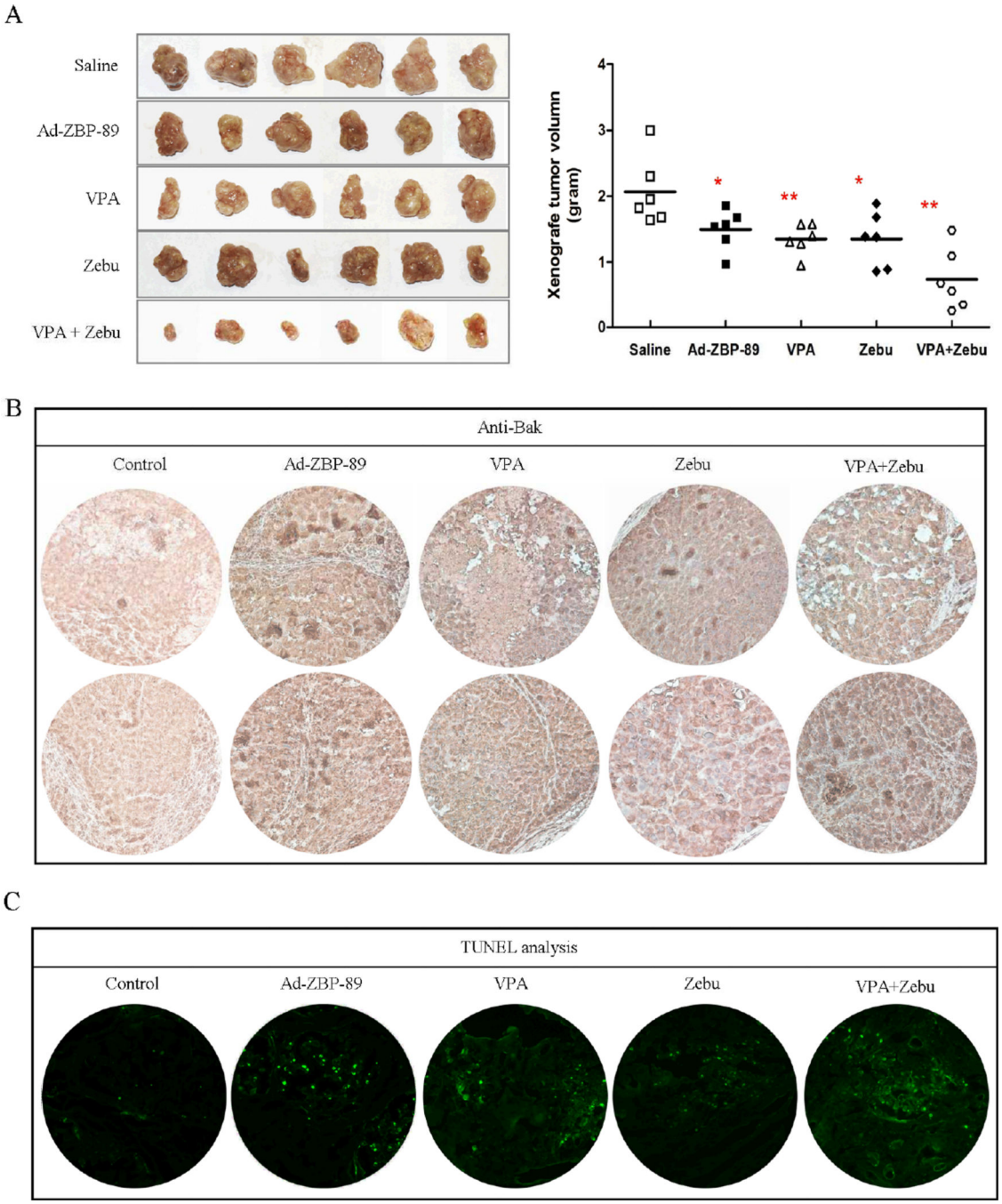

Fig. 6. ZBP-89, VPA and Zebu inhibit xenograft tumor growth

The tumor-bearing mice were given saline, Ad-ZBP-89, VPA, Zebu and VPA plus Zebu. Two weeks after the treatment, mice were sacrificed by cervical dislocation. Xenograft tumors were resected and weighed to determine the growth of tumor (A). The expression of Bak was examined by immunohistochemical staining (B). The apoptosis was analyzed by TUNEL assay (C). *P<0.05; **P<0.01. All tests were analyzed using Mann-Whitney U test and compared with saline control group.