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BTB and CNC homology 1 (Bach1) promotes human ovarian cancer cell metastasis by HMGA2-mediated epithelial-mesenchymal transition

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ABSTRACT

Transcriptional factor BTB and CNC homology 1 (Bach1) has been linked to tumor progression and metastasis, but the mechanisms underlying the effects of Bach1 on tumor growth and metastasis are largely uncharacterized. Here, we report that Bach1 expression was significantly higher in human epithelial ovarian cancer (EOC) tissues than in normal ovarian tissues and that higher levels of Bach1 were associated with tumor stage and poorer overall and progression-free survival. We found that Bach1 enhanced the expression of epithelial-mesenchymal transition (EMT) genes, including Slug and Snail, and promoted cell migration by recruiting HMGA2 in the human EOC cell line A2780. Bach1 overexpression enhanced and Bach1 knockout reduced the expression of Slug and the metastasis of EOC cells in a tumor metastasis mouse model. Bach1 expression was positively correlated with Slug and HMGA2 expression in human ovarian cancer tissues. In addition, Bach1 activated *p*-AKT and p-p70S6K, increased the expression of cyclin D1, and promoted the growth of ovarian cancer cells in vitro and tumor xenografts in vivo. Together, our findings reveal that Bach1 enhances tumor growth and recruits HMGA2 to promote EMT and ovarian cancer metastasis.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy. Due to the lack of effective screening methods for ovarian cancer, 75% of patients are diagnosed with advanced disease [1]. To date, the main treatment for ovarian cancer is surgery followed by combination chemotherapy. While the majority of patients respond to treatment, most patients will die from distant metastasis and relapse such that the survival rate for patients with advanced-stage disease remains low [2]. Therefore, a better understanding of the mechanisms of EOC metastasis may help provide a therapeutic strategy for metastatic ovarian cancer.

BTB and CNC homology 1 (Bach1) is a ubiquitously expressed transcriptional factor and a member of the Cap 'n' Collar and basic region leucine zipper factor (CNC-bZip) family. Bach1 acts as a transcriptional repressor by heterodimerizing with small Maf proteins and inhibiting the transcription of various genes dependent on Maf-recognition elements (MAREs), such as heme oxygenase-1 (HO-1). Bach1 plays a key role in the regulation of oxidative stress, heme oxidation, the cell cycle, hematopoiesis, and immunity [3–5]. We have shown that Bach1 impairs both developmental angiogenesis in zebrafish embryos and the angiogenic response to peripheral ischemic injury in adult mice [6,7]. In contrast with its role as a transcriptional repressor, Bach1 also acts as an activator of metastatic genes such as CXCR4 and MMP1, and it has been associated with bone metastasis in breast cancer [8,9]. The expression of Bach1 and its target genes has been linked to a higher risk of breast cancer recurrence in patients [9] as well as increases in cancer cell invasion and migration in human colorectal cancer and prostate cancer [10–12], whereas lower Bach1 levels have been associated with decreases in breast cancer metastasis [13]. However, the detailed role of Bach1 in the regulation of EOC metastasis and growth remains unclear.

Epithelial-mesenchymal transition (EMT) is an important process during embryonic cell layer movement and tissue repair. EMT contributes to cancer progression and metastasis and might also help chemoresistant cancer cells acquire enhanced motility [14]. During

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EMT, adherent epithelial cells are converted to motile mesenchymal cells characterized by increased expression of mesenchymal markers (vimentin, thrombospondin, N-cadherin, and vitronectin) and decreased expression of epithelial markers (E-cadherin, collagen IV, occludin, and desmoplakin) [15]. Several transcriptional repressors, such as the zinc finger factors Snail and Slug and the basic helix-loop-helix (bHLH) transcription factor Twist, have been implicated in the onset of EMT in mammary epithelial cells [16]. High-mobility group AT-hook protein 2 (HMGA2) is a critical factor for TGF-β-induced EMT in mouse mammary epithelial cells; it directly binds to the Snail promoter and acts as a transcriptional regulator of Snail expression [17]. HMGA2 has been shown to promote metastasis and drug resistance in cancers [18], and increased HMGA2 expression is associated with EMT in ovarian cancer [19].

Here, we explored the role of Bach1 in epithelial ovarian cancer (EOC). We found that Bach1 was highly expressed and positively correlated with Slug and HMGA2 expression in EOC and that higher levels of Bach1 were associated with invasive progression and poorer prognosis in human ovarian cancer. Bach1 promoted EMT and EOC metastasis in a mouse model. Mechanistically, Bach1 interacted with HMGA2 and promoted cell migration by recruiting HMGA2 in the human EOC cell line A2780. In addition, Bach1 increased the expression of *p*-AKT, p-p70S6K and cyclin D1 and promoted the growth of ovarian cancer cells and tumor xenografts. Thus, our findings identify Bach1 as a key transcriptional regulator of ovarian cancer growth and metastasis.

2. Materials and methods

2.1. Cell culture

The epithelial ovarian cancer cell line A2780 and human embryonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Life Technologies, Inc., Burlington, ON, Canada) and glutamine or in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics. Normoxic incubation (CO_2 water-jacketed incubator; Thermo Electron, Waltham, MA) was performed at 37 °C in 5% CO_2 with 95% humidity.

2.2. Transfection and establishment of a stable cell line

For the in vitro study, Bach1 expression in A2780 cells was transiently downregulated by small interfering RNA transfection, designated Bach1-siRNA (with Con-siRNA as the corresponding control), or upregulated by Bach1-adenovirus infection, designated Ad-Bach1 (with Ad-GFP as the corresponding control). A2780 cells were transfected with Bach1 siRNA or negative control siRNA (100 nmol/L) using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after transfection, the cells were used for different subsequent experiments. Recombinant adenoviruses encoding the human Bach1 gene (Ad-GFP-Bach1) or the GFP control (Ad-GFP) were purchased from GenePharma (Shanghai, China) and used to infect A2780 cells. For transduction, a multiplicity of infection (MOI) of 25 was used. To elucidate the molecular mechanisms underlying Bach1-mediated tumor cell migration and growth, a mixture of 3 pairs of HMGA2-siRNA or negative control siRNA was transfected into Ad-Bach1 A2780 cells. The siRNA sequences targeting Bach1 or HMGA2 in this study are listed in Supplementary Table 1.

For the in vivo study, Bach1 expression in A2780 cells was stably knocked out using the CRISPR/Cas9 genome editing technology, designated KO (with WT as the corresponding control), or overexpressed by Bach1-lentivirus, designated Lenti-Bach1 (with Lenti-Con as the corresponding control). The sgRNAs targeting Bach1 were designed using CRISPR DESIGN (http://crispr.mit.edu/). Then, sgRNA expression vectors (lenti crispr V2) were constructed and transfected into A2780 cells (5×10^5/well in 24-well plates) with Lipofectamine 2000. Twenty-four hours after transfection, puromycin (1 μg/ml) was added to select stably transfected cells. Positive clones were identified by sequencing the gene of interest and confirmed by analyzing protein levels. Similarly, Lenti-Bach1-transfected cells were isolated with puromycin selection, and clones were chosen for use in subsequent experiments.

2.3. Cell counting

Cell proliferation was evaluated by cell counting. A2780 cells were transfected with Bach1 siRNA (100 nmol/L) or control siRNA (100 nmol/L) using Lipofectamine 2000 transfection reagent and cultured for 48 h. Ad-GFP or Ad-Bach1 was used to infect the cells, and the cells were then transfected with HMGA2 siRNA (100 nmol/L) or control siRNA (100 nmol/L) for 24 h. Cells were seeded in 12-well plates (7×10^4 cells/well) and cultured for the indicated time periods; the cells were then counted with a cell counting chamber as described previously [20]. Each experiment was performed independently three times.

2.4. Soft agar colony formation

The anchorage-independent growth ability of cells was evaluated by a soft agar colony formation assay. In this assay, a bottom layer of 1.2% agar in complete media (1:1) was poured into 30-mm-diameter culture dishes and solidified, followed by an upper layer containing 1×10^3 cells suspended in a mixture of medium with 0.6% agar (1:1). After two weeks of incubation, the colonies were stained with a 0.5% crystal violet solution, photographed and counted using image analysis software [21].

2.5. Wound healing assay

In brief, 1.0×10^6 A2780 cells were seeded into each well of 6-well plates and incubated to form a confluent monolayer. Forty-eight hours after transient transfection with Con-siRNA, Bach1-siRNA, Ad-GFP or Ad-Bach1, the cell monolayer was scraped in a straight line with a p200 pipette tip to create a “scratch;” the debris was removed, and the edge of the scratch was smoothed by washing the cells twice with 1 ml of PBS. Then, the cells were cultured with serum-free medium and imaged at 0, 24, and 48 h after wounding. The wound area was calculated by manually tracing the cell-free area in the captured images using ImageJ public domain software (NIH, Bethesda, MD, USA) [22].

2.6. Transwell cell migration assay

Forty-eight hours after transient transfection with Con-siRNA, Bach1-siRNA, Ad-GFP or Ad-Bach1, A2780 cells were harvested using trypsin and counted. Forty thousand cells were added to the top chambers of the Transwell plates (8 μm pore size; Corning, NY, USA) in 200 μL of serum-free medium. In the lower compartments of the chambers, 500 μL of RPMI 1640 medium supplemented with 20% FBS was added as an inducer to promote cell migration. After 48 h of incubation at 37 °C in a 5% CO_2 incubator, the cells that migrated through the filters were fixed, stained with a 0.1% crystal violet solution, photographed, and counted. The experiment was repeated three times, and statistical significance was analyzed using Student's *t*-test [23].

2.7. Western blot analysis

Cell lysates (30 μg of protein) from each group were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a PVDF membrane (Invitrogen, Carlsbad, CA). Membranes were blocked

in 5% milk solution for 2 h at room temperature and were then incubated with primary antibody overnight at 4 °C with agitation. Detection was performed using an HRP-labeled secondary antibody and enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA) reagents according to the manufacturer's instructions. The antibodies used in this study are listed in Supplementary Table 2.

2.8. Xenograft experiments

All studies involving experiments with animals were approved by the Ethics Committee of Experimental Research at Fudan University Shanghai Medical College, adopting the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health.

Six-week-old nude mice (six mice per group, grouped as WT, KO, Lenti-Con and Lenti-Bach1) were injected subcutaneously in the bilateral flank area with 3×10^6 cells in 100 μL of phosphate-buffered saline (PBS) mixed with 100 μL of Matrigel. Tumor growth was monitored every 3 days, and the tumor volume was calculated according to formula $V(mm^3) = a \times b^2/2$, where a is the largest diameter and b is the perpendicular diameter. Three weeks after injection, the mice were euthanized with an intraperitoneal injection of 2% sodium pentobarbital (50 mg/kg), and the tumor weight was measured. A portion of the tumors was fixed in 10% buffered formalin solution for subsequent histological examination, and the remaining tissue was snap frozen in liquid nitrogen and stored at −70 °C for molecular studies.

For the in vivo tumor metastasis assay, eight nude mice per group were injected intraperitoneally (i.p.) with 5×10^6 cells in 200 μL of PBS [24,25]. Five weeks after i.p. injection, the visceral organs (liver, intestine, mesentery, kidney, ovary and diaphragm) were observed. The visceral organs were removed, and a portion of the liver tumors were fixed in 10% buffered formalin solution for subsequent histological examination. The remaining tissue was snap frozen in liquid nitrogen and stored at −70 °C for molecular studies.

2.9. Immunohistochemical (IHC) staining

Thirty paraffin-embedded specimens from tissues representing different stages of ovarian cancer and 5 specimens from adjacent noncancer tissues were obtained from the Obstetrics & Gynecology Hospital of Fudan University (Shanghai Red House Ob & Gyn Hospital). The study was approved by the Ethics Committee (permit no. 2017–25).

Slides of tissue from patients or xenografts were incubated with anti-Bach1 rabbit polyclonal (1:200 dilution; DF8317, Affinity), anti-Slug (1:100 dilution; DF6202, Affinity), anti-Ki67 (1:100 dilution; GB13030-2, Goodbiotechnology, Wuhan,China) or anti-HMGA2 (1:250 dilution; SAB2701959, Sigma-Aldrich, Buchs, Switzerland) antibodies at 4 °C overnight, with normal rabbit serum used as the negative control, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz, CA, USA) at 37 °C for 1 h. The signals were detected using a diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA). Counterstaining was performed with hematoxylin. An immunohistochemical score (IHS) was used to evaluate Bach1, Slug or HMGA2 expression in human specimens as previously described [26]. The expression of Bach1 and Slug in mouse xenografts was quantified using ImagePro Plus (IPP) according to three parameters: integrated optical density (IOD), area and mean optical density = IOD sum/area.

2.10. In vitro coimmunoprecipitation (co-IP) assay

The Bach1-FLAG and HMGA2-HA plasmids were cotransfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) and cultured in 10 cm dishes for 48 h to obtain 90% confluence. The cell lysates were transferred to microcentrifuge tubes and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatants were transferred to another tube and incubated with an anti-HA antibody in the presence of protein G beads using a Protein G Immunoprecipitation kit (Sigma-Aldrich, Buchs, Switzerland) overnight at 4 °C. The beads were then washed three times and boiled for use in the western blot analysis. For the IP assay using A2780 cells, the cells were lysed, and the supernatants were incubated with an anti-Bach1 or anti-HMGA2 antibody before the expression of Bach1 or HMGA2 was assessed by western blotting.

2.11. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as described previously [7]. In brief, cells were seeded into a 10 cm tissue culture dish, and one day later, they were cross-linked with 1% formaldehyde for 10 min at room temperature, followed by genomic DNA fragmentation using a sonication apparatus. The chromatin fragments derived from A2780 cells were immunoprecipitated with 5 μg of an antibody against Bach1 (AF5776, R&D Systems, Minneapolis, MN, USA) or an antibody against HMGA2 (SAB2701959, Sigma-Aldrich, Buchs, Switzerland). DNA extraction was performed using a Qiagen Purification kit. Real-time PCR analysis was performed with primers amplifying the promoters of Vimentin, Slug, Snail and Twist. The primers are listed in Supplementary Table 3.

2.12. Analysis of Slug promoter activity

A luciferase assay was performed as described previously [7]. Briefly, A2780 cells were transfected with the Bach1-FLAG or control plasmid. Twenty-four hours later, the cells were transfected with a β-galactosidase plasmid and the wild-type or mutated versions (gaaaggactaagcag→gaaaggactaGTAag) of the Slug promoter plasmid or the pGL3-basic luciferase reporter plasmid. Transfection was performed with Lipofectamine 2000, and the transfected cells were cultured for an additional 24 h; then, the cells were harvested, and luciferase activity was measured with a Luciferase Assay Kit (Promega, Madison, WI, USA). β-Galactosidase (β-gal) activity was measured. The relative Luc activity was calculated as the ratio of Luc/β-gal activity. Each experiment was performed independently three times.

2.13. Analysis of TCGA data for human ovarian cancer

Clinicopathological data were available from The Cancer Genome Atlas (TCGA) project. Bach1 expression in ovarian cancer was retrieved from the TCGA (594 patients, probe 204194_at). Then, the correlation between Bach1 expression and tumor stage was assessed. Survival curves were determined using the Kaplan-Meier method at the following website: http://www.kmplot.com. Progression-free survival (PFS, n = 1435) and overall survival (OS, n = 1656) were analyzed using TCGA data (GSE14764, GSE15622, GSE18520, GSE19829, GSE23544, GSE26193, GSE26712, GSE27651, GSE30161, GSE3149, GSE51373, GSE63885, GSE65986 and GSE9891).

2.14. Statistical analysis

The data are presented as the means ± SEMs. Differences among groups were determined with One-way analysis of variance (ANOVA) with post hoc test for multiple comparisons. Differences between two groups were assayed by two-tailed Student's t-test using the SPSS 19.0 software package. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Bach1 is highly expressed in ovarian cancers and predicts poor prognosis

By analyzing EOC and normal ovarian tissues, we found that Bach1 protein levels were low in normal ovarian tissues, relatively higher in

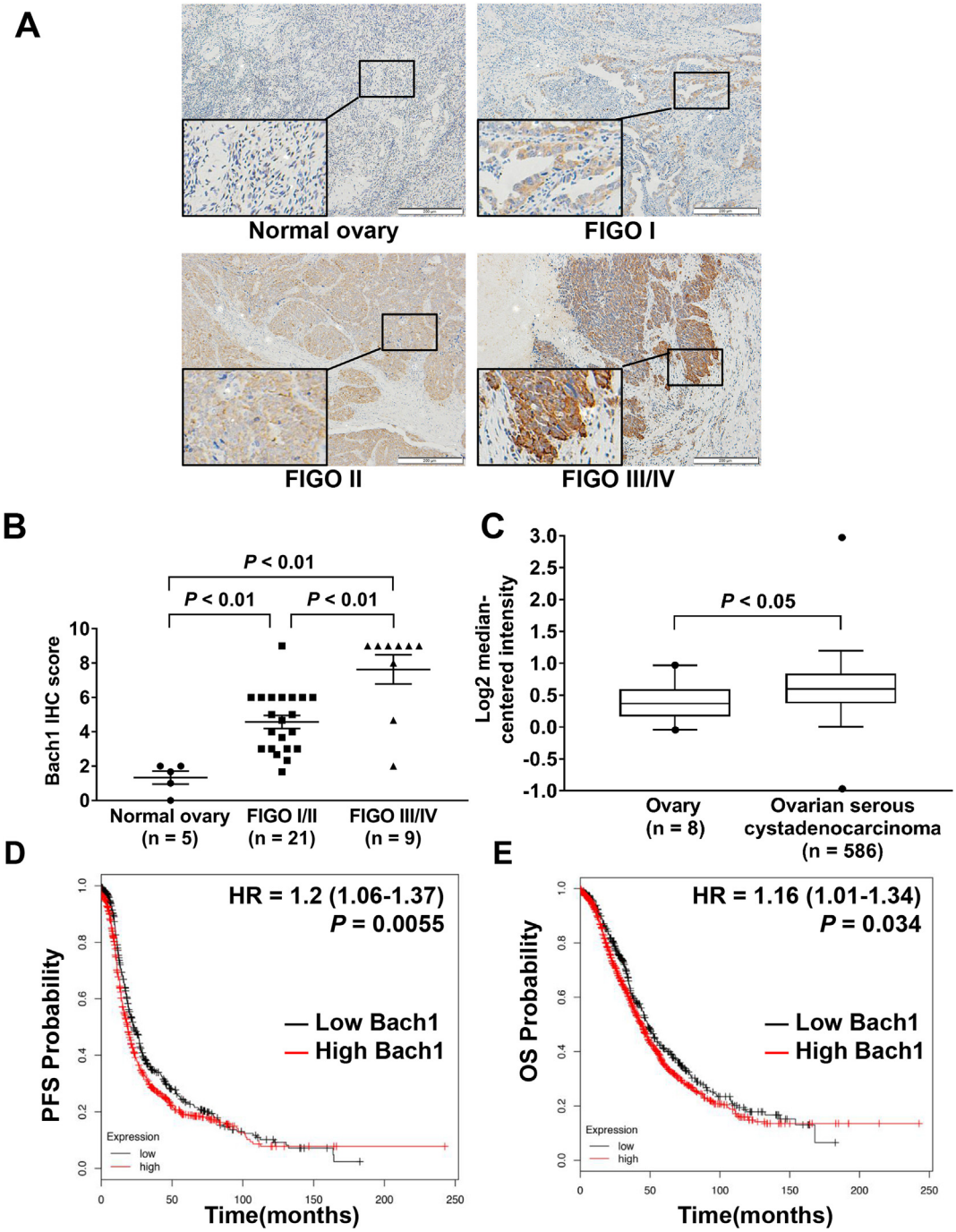

Fig. 1. Bach1 is highly expressed in ovarian cancers and predicts poor prognosis. A. Representative images of Bach1 immunostaining. Bach1 expression was determined by immunostaining in 5 normal ovary samples, 21 samples of early-stage EOC (stage I or II), and 9 samples of advanced-stage EOC (stage III or IV). The stage of EOC was defined using the International Federation of Gynecology and Obstetrics (FIGO) staging systems. Scale bar, 200 μm. **B.** The IHC scores for Bach1 in EOC and normal ovarian tissues. ANOVA with post hoc test. **C.** Bach1 mRNA expression in healthy ovaries (n = 8) and ovarian cancers (n = 586) of stage I-IV was retrieved from the TCGA dataset (probe 204194_at). *t*-test. **D-E.** The progression-free survival (PFS) rates of 1435 patients and overall survival (OS) rates of 1656 patients with ovarian cancer were compared between the low-Bach1 and high-Bach1 groups based on extracted clinical data from the TCGA (http://tcga-data.nci.nih.gov).

early-stage (stage I/II) patients, and further increased in advanced-stage (stage III/IV) patients, suggesting the potential role of Bach1 in the invasive progression of EOC (Fig. 1A and B). Consistent with this result, the average levels of Bach1 mRNA were significantly higher in ovarian serous cystadenocarcinoma tissues than in normal ovarian tissues (Fig. 1C) in TCGA data derived from a total of 594 ovarian cancer patient tumor samples. We next sought to determine whether Bach1 expression in human ovarian cancer is associated with poor survival. Patients in the high expression group had either shorter progression-free survival (PFS; log-rank test P = 0.0055, Fig. 1D) or poorer overall survival (OS; log-rank test P = 0.034, Fig. 1E) than patients in the low-expression group. Thus, these results suggest that higher levels of Bach1 are associated with invasive progression and poorer prognosis of human ovarian cancer.

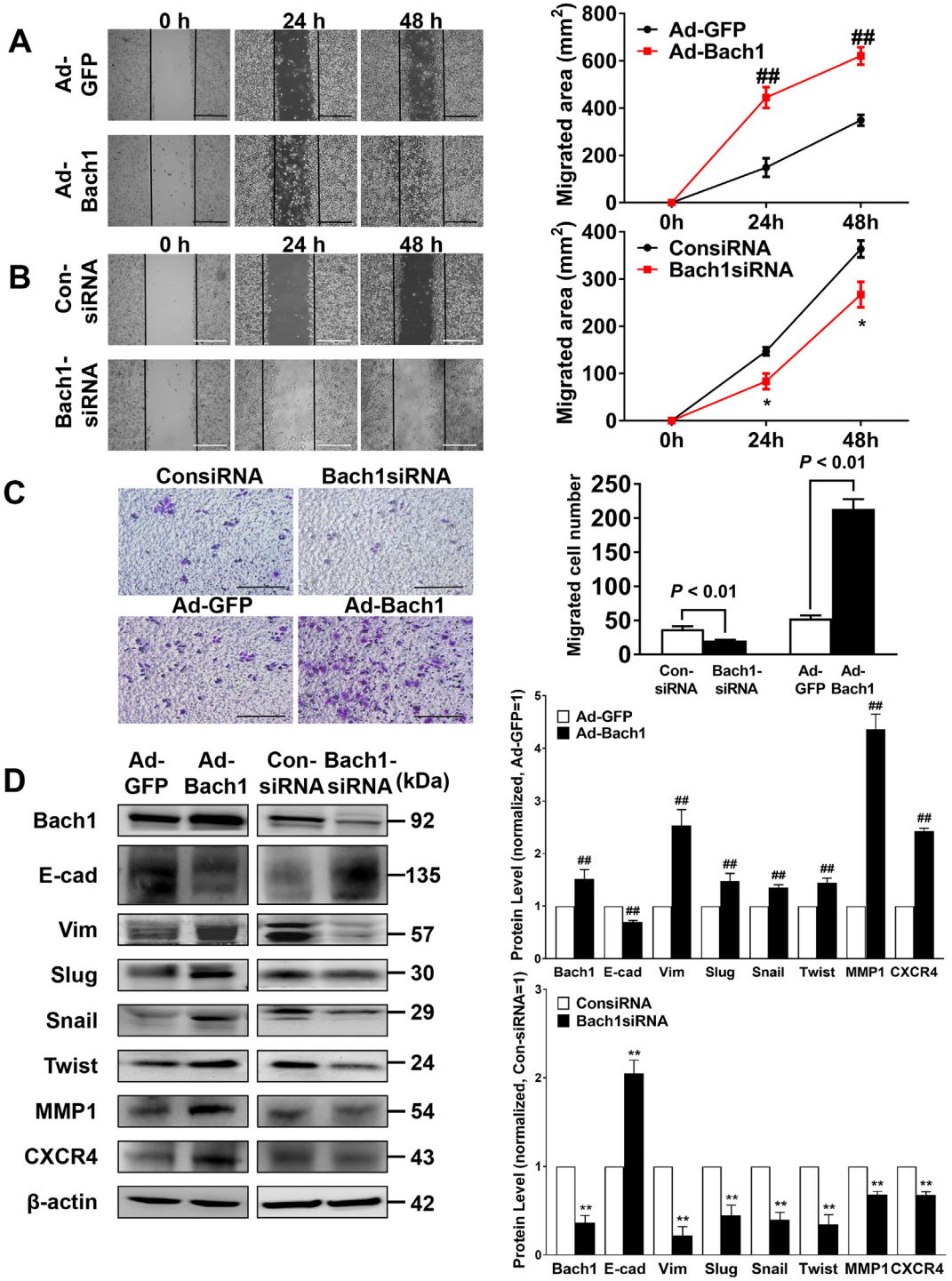

Fig. 2. Bach1 promotes cell migration and EMT in the epithelial ovarian cancer cell line A2780 in vitro. A-C. Assessments of wound healing **(A and B,** scale bar, 500 μm) and cell migration **(C,** scale bar, 200 μm). n = 3, $##P < 0.01$ vs Ad-GFP. $*P < 0.05$ vs Con-siRNA. *t*-test. **D.** Comparison by western blotting of the levels of the EMT-related proteins E-cadherin (E-cad), Vimentin (Vim), Slug, Snail, Twist, MMP1 and CXCR4 between AdBach1-cells and AdGFP-cells as well as between Bach1-siRNA cells and Con-siRNA-cells. The data are representative of three independent experiments. $##P < 0.01$ vs Ad-GFP. $**P < 0.01$ vs ConsiRNA. *t*-test.

3.2. Bach1 promotes cell migration and EMT in the epithelial ovarian cancer cell line A2780 in vitro

To determine the in vitro significance of the above clinical data, we generated adenoviruses expressing Bach1 and infected the epithelial ovarian cancer cell line A2780. The wound healing and cell migration abilities were significantly greater in AdBach1 cells than in AdGFP cells (Fig. 2A–C) as well as in Con-siRNA cells vs Bach1-siRNA cells (Fig. 2B–C). EMT is known to be involved in the progression and metastasis of various cancers. We then evaluated the expression of EMT-related proteins in A2780 cells. Higher levels of Bach1 expression were associated with significant increases in Vimentin, Slug, Snail, Twist, MMP1 and CXCR4 expression as well as with a significant reduction in E-cadherin expression. In contrast, silencing endogenous Bach1 in A2780 cells significantly reduced the expression of Vimentin, Slug, Snail, Twist, MMP1 and CXCR4 and increased E-cadherin expression

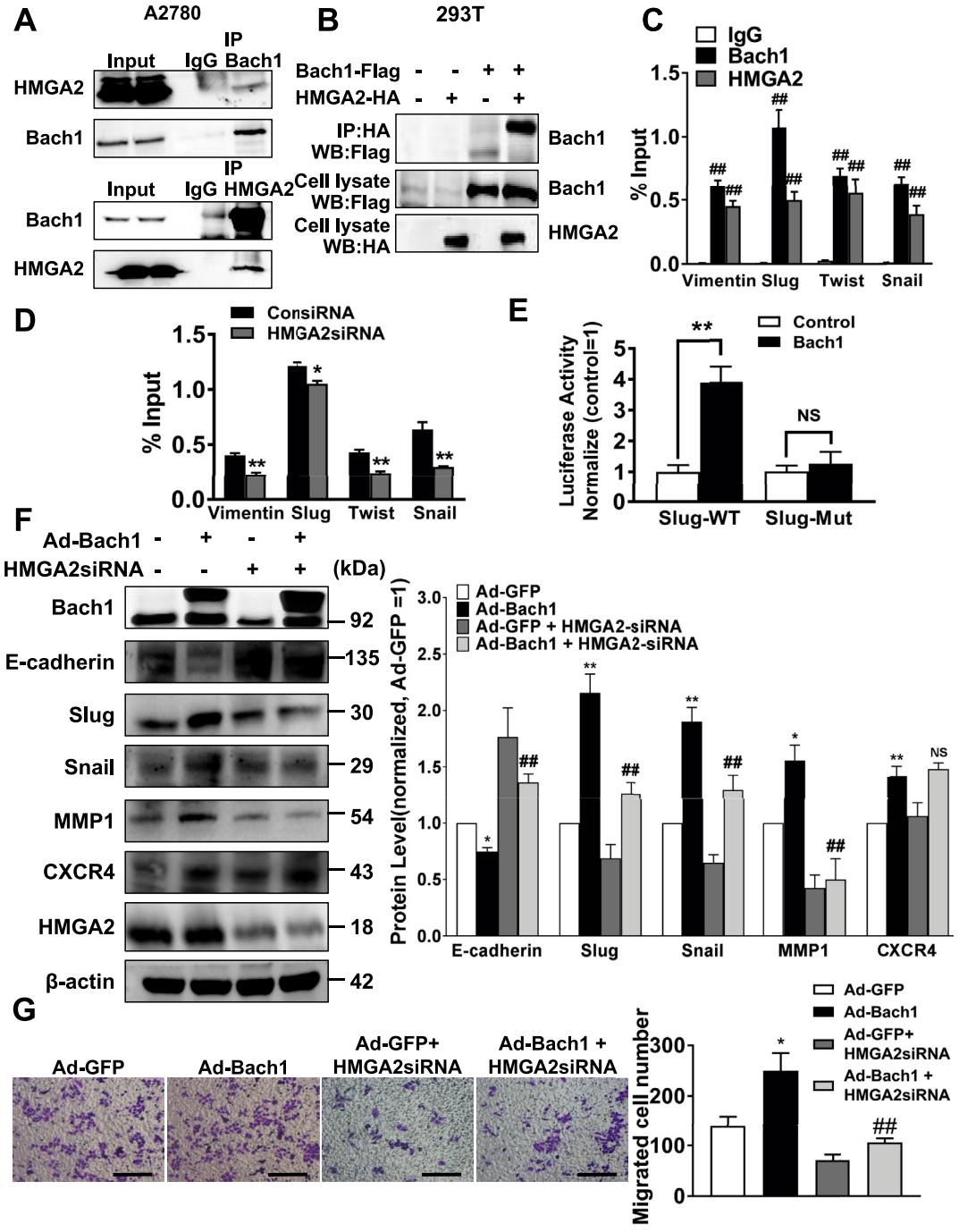

Fig. 3. Bach1 cooperates with HMGA2 in regulating EMT in human ovarian cancer cells. A-B. In vitro co-IP experiments. Endogenous Bach1 or HMGA2 was immunoprecipitated from lysates of A2780 cells **(A)** using a Bach1 or HMGA2-specific antibody, respectively, and immunoglobulin G (IgG) was used as the control. Co-IP proteins were analyzed by western blotting (WB) using the indicated antibodies. Input, 5% of material used for IP. Lysates from 293 cells **(B)** cotransfected with the Bach1-Flag and HMGA2-HA plasmids were immunoprecipitated with an anti-HA antibody. Total lysates and immunoprecipitates were then analyzed by WB. **C-D.** ChIP-qPCR was performed in A2780 cells with anti-Bach1 or anti-HMGA2 antibodies **(C)** and in HMGA2 siRNA-transfected A2780 cells with an anti-Bach1 antibody **(D)** to determine the enrichment of Vimentin, Twist, Snail and Slug promoter region sequences in the obtained ChIP DNA.$\#\#P < 0.01$ vs IgG, $^{**}P < 0.01$, $^{*}P < 0.05$ vs Con-siRNA. *t*-test. **E.** Analysis of Slug promoter activity. A2780 cells were transfected with the Bach1-FLAG or control plasmid for 24 h; the cells were then transfected with a Slug promoter plasmid or a plasmid containing a mutated Slug promoter sequence for an additional 24 h. Cells were lysed, and luciferase activity was measured. $^{**}P < 0.01$ vs control. *t*-test. NS, not significant. **F.** Assessments of EMT-associated protein expression by western blotting after HMGA2 knockdown in A2780 cells. The data are representative of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Ad-GFP; $\#\#P < 0.01$ vs Ad-Bach1. NS, not significant. ANOVA with post hoc test. **G.** Assessments of cell migration ability after HMGA2 knockdown in A2780 cells. HMGA2 downregulation reversed the effect of Bach1 overexpression, inhibiting EMT in A2780 cells. Scale bars, 200 μm n = 3. $^{*}P < 0.05$ vs Ad-GFP, $\#\#P < 0.01$ vs Ad-Bach1. ANOVA with post hoc test.

(Fig. 2D).

3.3. Bach1 promotes EMT and cell migration by recruiting HMGA2 in human ovarian cancer cells

HMGA2 was shown to directly bind to the Snail1 promoter, acting as a transcriptional regulator of Snail1 expression during the induction of EMT [16]. Therefore, we determined whether HMGA2 is involved in the regulation of Bach1-induced EMT in ovarian cancer. We found that Bach1 coimmunoprecipitated with HMGA2 in A2780 cells (Fig. 3A). Bach1 and HMGA2 also coprecipitated from the lysate of HEK293T cells engineered to express Flag-tagged Bach1 and HA-tagged HMGA2 (Fig. 3B). ChIP-qPCR assays revealed that Bach1 and HMGA2 co-occupy the promoters of EMT-related genes (Fig. 3C). Furthermore, downregulation of HMGA2 in A2780 cells impaired the binding of Bach1 to the promoter region of EMT-related genes (Fig. 3D). Bach1 overexpression significantly increased the luciferase activity of the Slug promoter but did not increase the luciferase activity of the Slug promoter when the Bach1 binding site was mutated (Fig. 3E). Importantly, the expression of these EMT-related genes and the cell migration induced by Bach1 overexpression were partially abolished by knockdown of HMGA2 (Fig. 3F–G), indicating that HMGA2 is required for the induction of EMT by Bach1. Interestingly, the enhancement of CXCR4 expression by Bach1 did not seem to be mediated by HMGA2 (Fig. 3F).

3.4. Bach1 promotes the metastasis of the epithelial ovarian cancer cell A2780 in vivo

We sought to determine whether Bach1 could promote the metastasis of EOC in an established mouse model of metastatic dissemination. We then generated Bach1 knockout A2780 cells (Bach1-KO A2780) using CRISPR–Cas9 genome editing (Fig. 4A–B) and generated a Bach1-overexpressing cell line by lentiviral infection (Fig. 4B). Accordingly, we injected A2780 cells into the peritoneal cavity of nude mice to mimic the route of dissemination of human ovarian cancer. We observed that the number and volume of metastatic liver tumors and the tumor volumes on the diaphragm were lower in the Bach1-KO group than in the WT group and higher in the LV-Bach1 group than in the LV-Con group (Fig. 4C–D); higher levels of Bach1 in metastatic tumors of the liver were associated with higher levels of Slug (Fig. 4E), indicating that Bach1 promotes the metastasis of EOC in vivo.

3.5. Correlation of Bach1 with Slug and HMGA2 in human ovarian cancers

To further confirm the relationship among Bach1, HMGA2 and Slug, we analyzed the expression of these proteins in normal ovarian tissues and EOC samples. The protein levels of Bach1 and the EMT markers Vimentin, Slug, Snail and Twist were significantly increased in human ovarian cancer samples compared with normal tissues (Fig. 5A–B); moreover, the expression of Slug and HMGA2 was significantly increased in the early stages (stage I and II) of EOC and further increased in the advanced stages (stage III and IV) of EOC (Fig. 5B–C). Combined with the expression of Bach1 in these samples (Fig. 1A), the results of the correlation analysis revealed that Bach1 expression was positively correlated with Slug and HMGA2 expression in human ovarian cancers (Fig. 5D). Furthermore, Fig. 5D shows a positive correlation between the expression of HMGA2 and Slug.

3.6. Bach1 enhances EOC cell growth in vitro and in vivo

We evaluated the role of Bach1 in ovarian cancer cell growth in vitro and in vivo. The numbers of Ad-Bach1 cells and colonies were significantly greater than those of Ad-GFP cells; those of Bach1-siRNA cells were significantly lower than those of Con-siRNA cells (Fig. 6A–C). HMGA2 downregulation did not impair the cell growth promoted by Bach1 (Fig. 6A), although HMGA2 knockdown alone slightly reduced cell growth, indicating that Bach1-mediated tumor cell growth was independent of HMGA2. The overexpression of Bach1 significantly upregulated the levels of *p*-AKT, p-p70S6K and Cyclin D1, and the opposite results were observed after silencing Bach1 expression by siRNA in A2780 cells (Fig. 6D). These results demonstrate that the AKT/p70S6K pathway is activated after Bach1 overexpression.

To further confirm the growth-promoting effects of Bach1, we performed an in vivo xenograft tumor experiment in nude mice. The tumor volumes, growth rates, and tumor weights were significantly increased in the tumors derived from Bach1-overexpressing cells and markedly decreased in the tumors derived from Bach1-KO cells (Fig. 7A–C). Tumors derived from Bach1-overexpressing cells exhibited an increase in Ki67, *p*-AKT and Cyclin D1 expression by immunohistochemistry or western blotting, whereas tumors derived from the Bach1-KO cells showed a reduction in the expression of these proteins (Fig. 7D–F).

4. Discussion

Ovarian cancer patients with metastasis have low survival rates [27]. The investigation of genes involved in ovarian cancer migration and metastasis is critical for understanding their therapeutic potential. However, the mechanisms of ovarian cancer metastasis have not been thoroughly elucidated. Although Bach1 functions as a regulator of metastasis, the role of Bach1 in ovarian cancer metastasis is unknown. Here, our data demonstrated that high levels of Bach1 were associated with metastasis, tumor growth, and poor prognosis in EOC. Bach1 promoted EMT and ovarian cancer metastasis by recruiting HMGA2. Moreover, Bach1 promoted ovarian cancer cell growth and tumorigenesis. Thus, our findings suggest that Bach1 is a key regulator of ovarian cancer metastasis and growth.

There is increasing evidence linking Bach1 to tumor metastasis. Bach1 has been shown to promote the metastasis of breast cancer [9], colon cancer [10], prostate cancer [11] and neuroendocrine tumors [28]. Bach1 forms a complex with MAFG and the DNA methyltransferase DNMT3B, leading to the hypermethylation and transcriptional silencing of tumor suppressor genes [29,30]. Furthermore, Bach1 both suppresses and is suppressed by the metastasis suppressor Raf kinase inhibitory protein (RKIP) and is a let-7-regulated transcription factor that promotes the metastasis of breast cancer by upregulating metastatic genes such as CXCR4 and MMP1 [13,31]. In the present study, we found that Bach1 was highly expressed in human ovarian cancers. The upregulation of Bach1 in ovarian cancer may be associated with decreases in the expression of metastasis-suppressors such as RKIP and let-7, but the specific mechanisms of the upregulation of Bach1 in ovarian cancers have yet to be determined.

Furthermore, Bach1 expression was positively correlated with EMT-related gene expression in human ovarian cancers, and the prometastatic activity of Bach1 was confirmed in the model of metastatic ovarian cancer in mice. Bach1 enhanced the expression of the EMT-related genes Vimentin, Slug, Snail, Twist, MMP1 and CXCR4, significantly reduced the expression of E-cadherin and promoted cell migration in the EOC cell line. EMT, a process by which epithelial cells lose their polarity and acquire a migratory mesenchymal phenotype, has been widely regarded as a process that initiates cancer metastasis [32]. Several EMT-associated genes have been identified, and their functions have been characterized in ovarian cancers [33]. Thus, the prometastatic properties of Bach1 in ovarian cancer may be mediated by its regulation of the genes involved in EMT.

HMGA2, a chromatin-binding protein, contains three AT-hook domains that enable its binding to the minor groove of DNA, which allows it to organize protein complexes on enhancers of various genes regulating EMT gene expression (e.g., Snail and Twist) [16,34] and cell differentiation [19,35]. HMGA2 overexpression is associated with aggressive tumor growth, early metastasis, and poor prognosis [36,37], whereas knockdown of HMGA2 suppresses tumor metastatic invasion, homing and osteolysis [38]; thus, the HMGA2 gene is a promising target

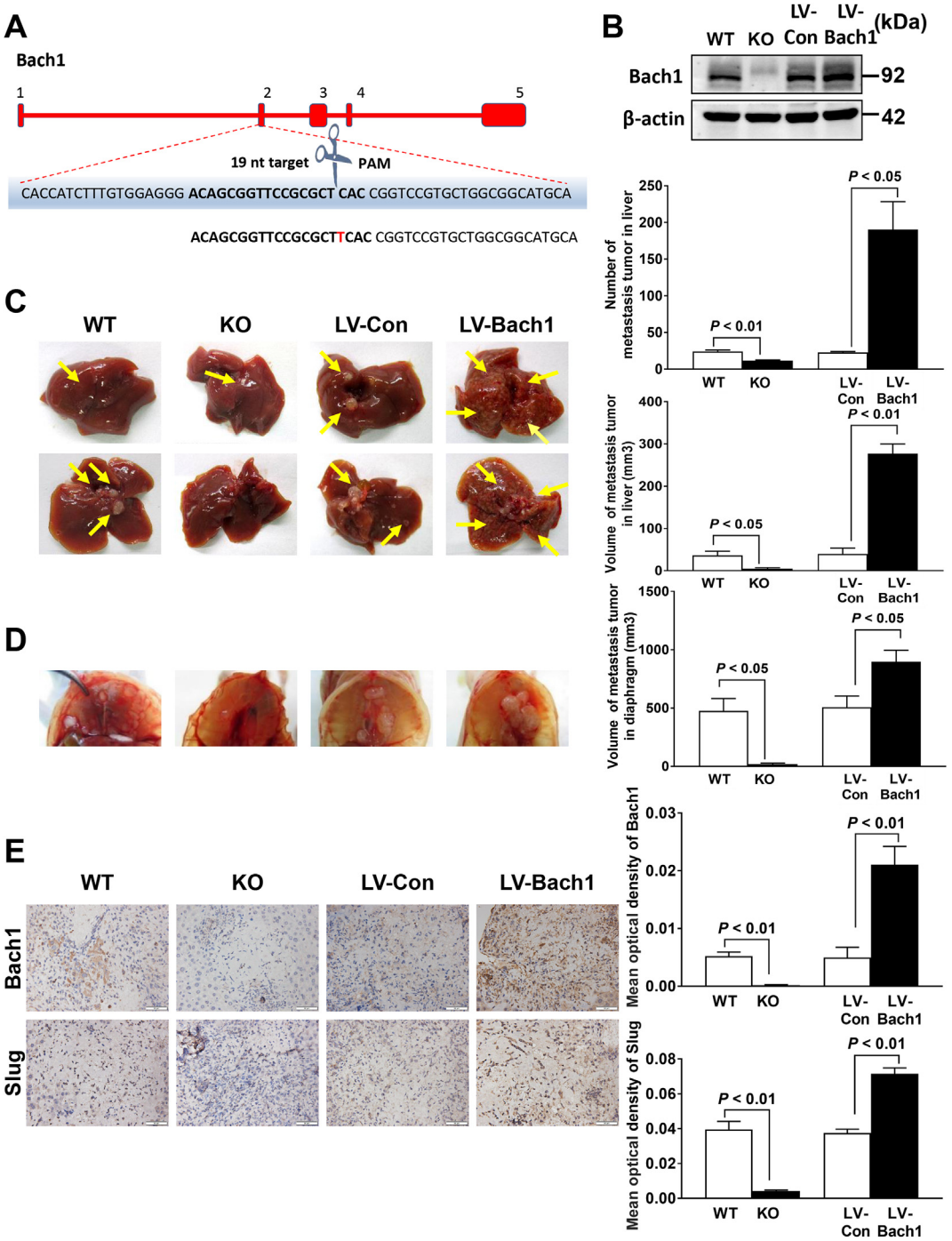

Fig. 4. Bach1 promotes the metastasis of the epithelial ovarian cancer cell A2780 in vivo. A. The exact loci of the CRISPR/Cas-9 genomic target within the Bach1 gene nucleotide sequences. **B.** Western blot validation of the construction of stable Bach1 knockout and Bach1 overexpression cell lines. **C-D.** Metastatic tumors in the liver **(C)** and diaphragm **(D)**. To establish the mouse model of dissemination, 5×10^6 A2780 cells were injected into nude mice by the i.p. route, resulting in tumor growth. Left: Representative images of metastatic tumors in the liver and diaphragm. Right: Number and volume of metastatic tumors in the liver and diaphragm. n = 8. *t*-test. **E.** IHC staining for Bach1 and Slug in mouse liver. Higher expression of Bach1 in metastatic tumors of the liver was associated with increased levels of Slug. Scale bar, 50 μm. *t*-test.

for ovarian cancer-silencing therapy [37]. Both HMGA2 and Bach1 are targets of let-7 [31], which is regulated by RKIP, but the relationship between HMGA2 and Bach1 is unknown. We found that HMGA2 is a binding partner of Bach1 in the EOC cell line and that Bach1 recruits HMGA2 to occupy the binding sites of the promoter regions of EMT-related genes. The effect of Bach1 overexpression on EMT-related genes and cell migration in the EOC cell line can be partially abolished by HMGA2 silencing, indicating that Bach1 promotes EMT and cell motility at least in part via HMGA2. Interestingly, the enhancement of CXCR4 expression by Bach1 does not seem to be mediated by HMGA2. Moreover, we observed that Bach1 expression was positively correlated with HMGA2 expression in human ovarian cancer tissues. Taken together, these results suggest that Bach1 plays an important role in ovarian cancer metastasis via HMGA2-mediated EMT. Both HMGA2 and Bach1 are targets of let-7 in breast cancer cells [39]. We found that HMGA2 knockdown repressed the protein expression of Bach1 in the

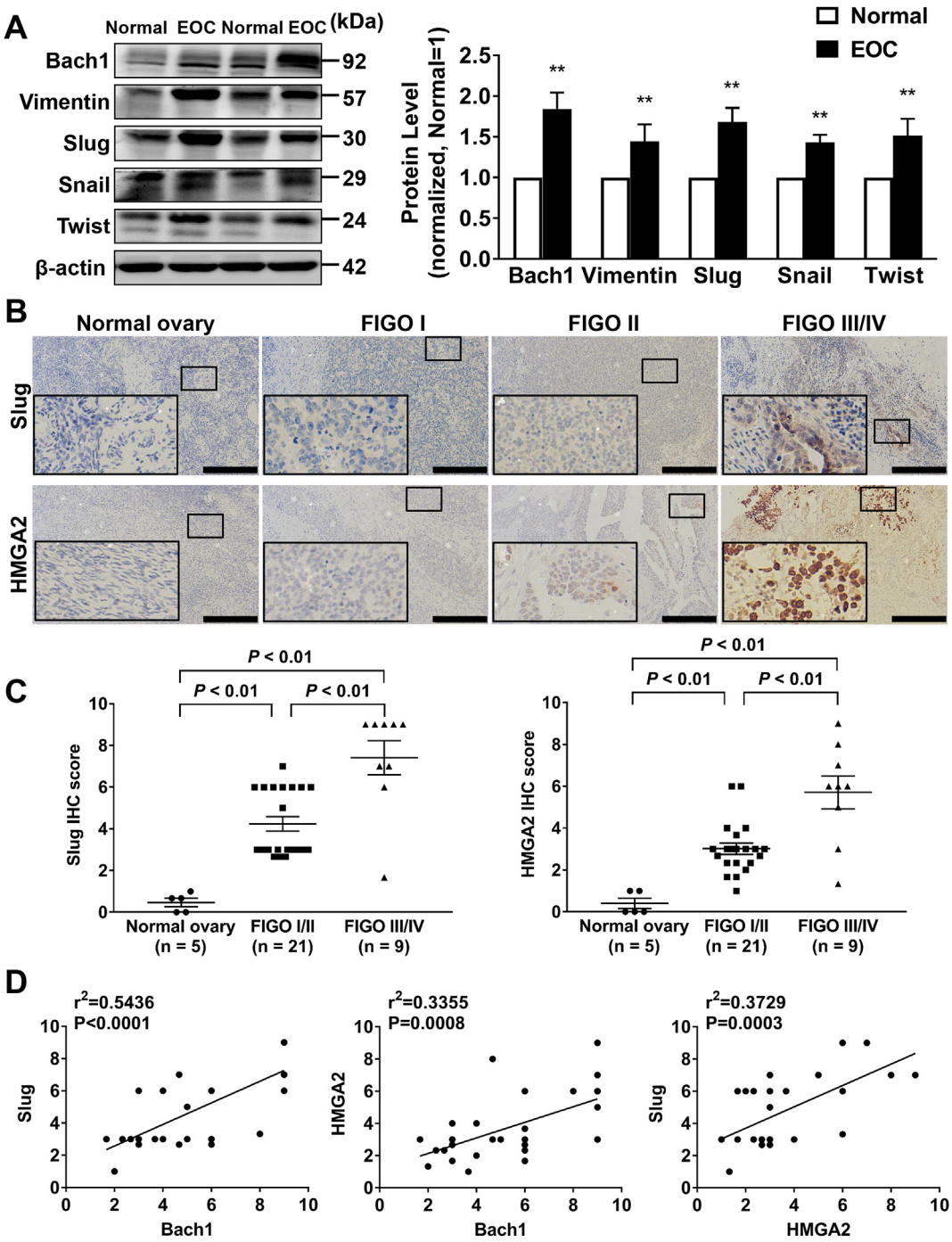

Fig. 5. Correlation of Bach1 with Slug and HMGA2 in human ovarian cancers. A. Comparison of the protein expression of Bach1 and EMT-related genes in normal ovarian tissues and EOC samples by western blotting. n = 5. $^{**}P < 0.01$ vs normal. *t*-test. **B–C.** The protein expression of Slug and HMGA2 was determined by immunostaining in 5 normal ovary samples, 21 samples of early-stage EOC (stage I or II), and 9 samples of advanced-stage EOC (stage III or IV). The stage of EOC was defined using the International Federation of Gynecology and Obstetrics (FIGO) staging systems. B: Representative images of immunostaining for Slug (upper) and HMGA2 (lower). Scale bar, 200 μm. C: The IHC scores for Slug and HMGA2 in EOC and normal ovarian tissues. ANOVA with post hoc test. **D.** Correlation analysis. Bach1 expression was positively correlated with Slug and HMGA2 expression in human ovarian cancers. Pearson's coefficient tests were performed to assess statistical significance.

EOC cell line (Fig. 3F). It is possible that HMGA2 positively regulates Bach1 transcription. Future studies are expected to elucidate the relationship between HMGA2 and Bach1.

Bach1 serves as an oncogene to promote tumor progression. Bach1 overexpression in human colorectal cancer accelerated the growth of tumor xenografts in vivo [10], and deficiencies in Bach1 expression have been associated with decreases in proliferation and migration of a human breast adenocarcinoma cell line [40]. Both of these observations are consistent with the results from our studies: higher levels of Bach1 expression were associated with increases in the proliferation of the EOC cell line and tumor xenograft growth in nude mice. Our results show that Bach1-mediated cell growth was not affected by HMGA2 silencing, indicating that HMGA2 is not required for the tumor cell growth induced by Bach1. We observed that Bach1 increased the expression of *p*-AKT, p-p70S6K and cyclin D1 in both the EOC cell line and xenograft tumors in mice; these increases may be responsible for the

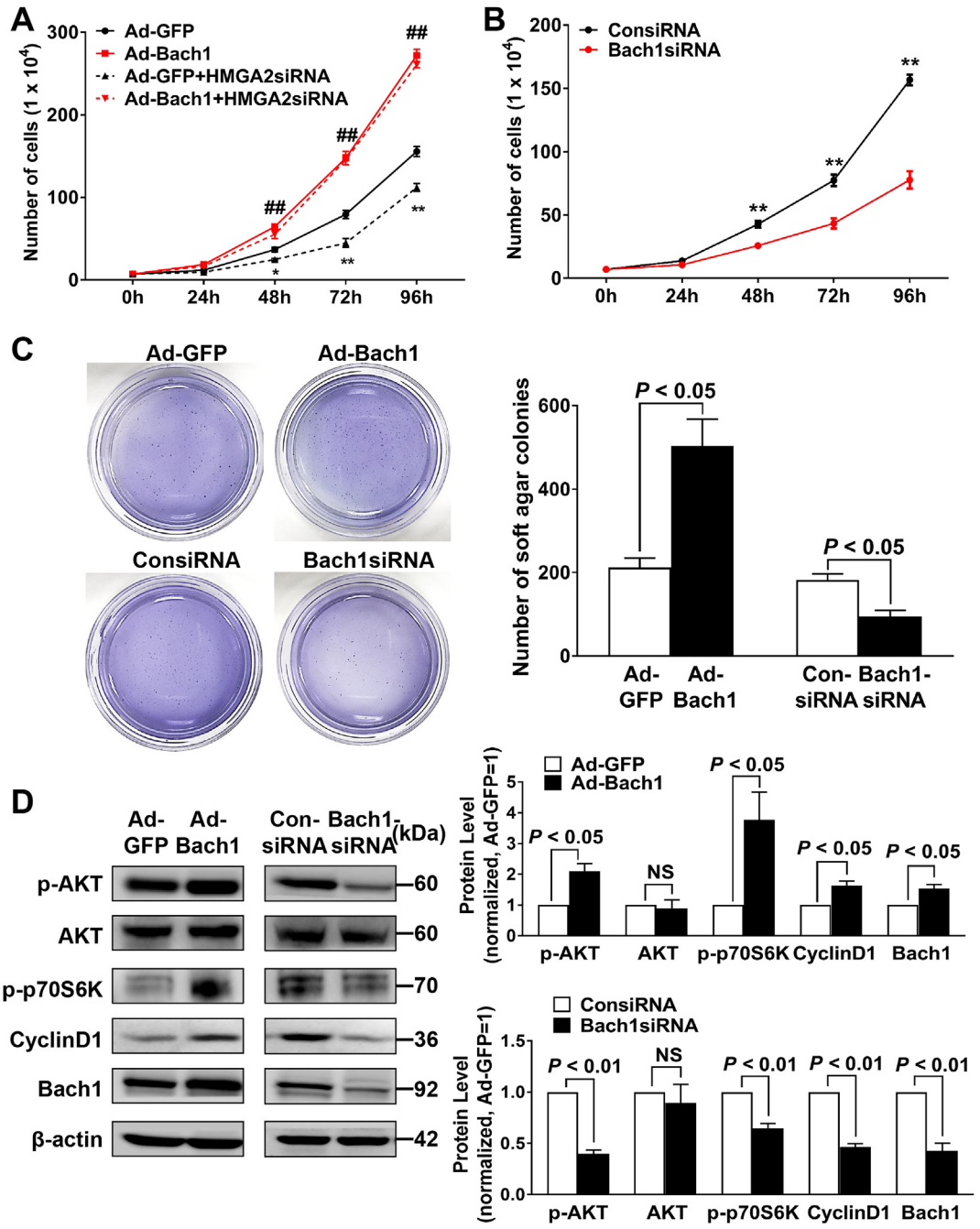

Fig. 6. Bach1 enhances EOC cell growth in vitro. A-C. Assessments of the numbers of cells **(A and B)** and colonies **(C)**. Colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and counted. n = 3, A: $^{\#\#}P < 0.01$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs Ad-GFP. ANOVA with post hoc test. B: $^{**}P < 0.01$ vs Con-siRNA. *t*-test. C: *t*-test. **D.** Comparison by western blotting of protein levels of AKT pathway proteins and Cyclin D1 between AdBach1-cells and AdGFP-cells as well as between Bach1-siRNA cells and Con-siRNA-cells. The data are representative of three independent experiments. NS, not significant. *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

accelerated tumor growth induced by Bach1. However, lower levels of Bach1 expression were associated with increases in HO-1 levels and cell viability after exposure to an anticancer drug in acute myeloid leukemia cells [41] and with increased cell proliferation in human umbilical vein endothelial cells [42], demonstrating that the effect of Bach1 on cell proliferation can differ profoundly among cell types. The contrasting roles of Bach1 in cell proliferation are most likely related to its role as a transcriptional repressor or as a transcriptional activator in different cells or under different experimental conditions.

In summary, we identified Bach1 as a key regulator that controls multiple factors essential for EOC metastasis and growth. Bach1 promotes EMT gene expression by recruiting HMGA2 in EOC cells, and HMGA2 plays a role in Bach1-induced EMT of epithelial ovarian tumor cells. On the other hand, it was demonstrated that the enhancement of ovarian tumor cell proliferation and CXCR4 expression by Bach1 does not seem to be mediated by HMGA2, suggesting different mechanisms by which Bach1 acts on ovarian tumor progression. Thus, Bach1 may be a novel candidate target for metastatic ovarian cancer therapy.

Author contributions

M.D. and L.Y. conceived and designed the project. W.H. performed

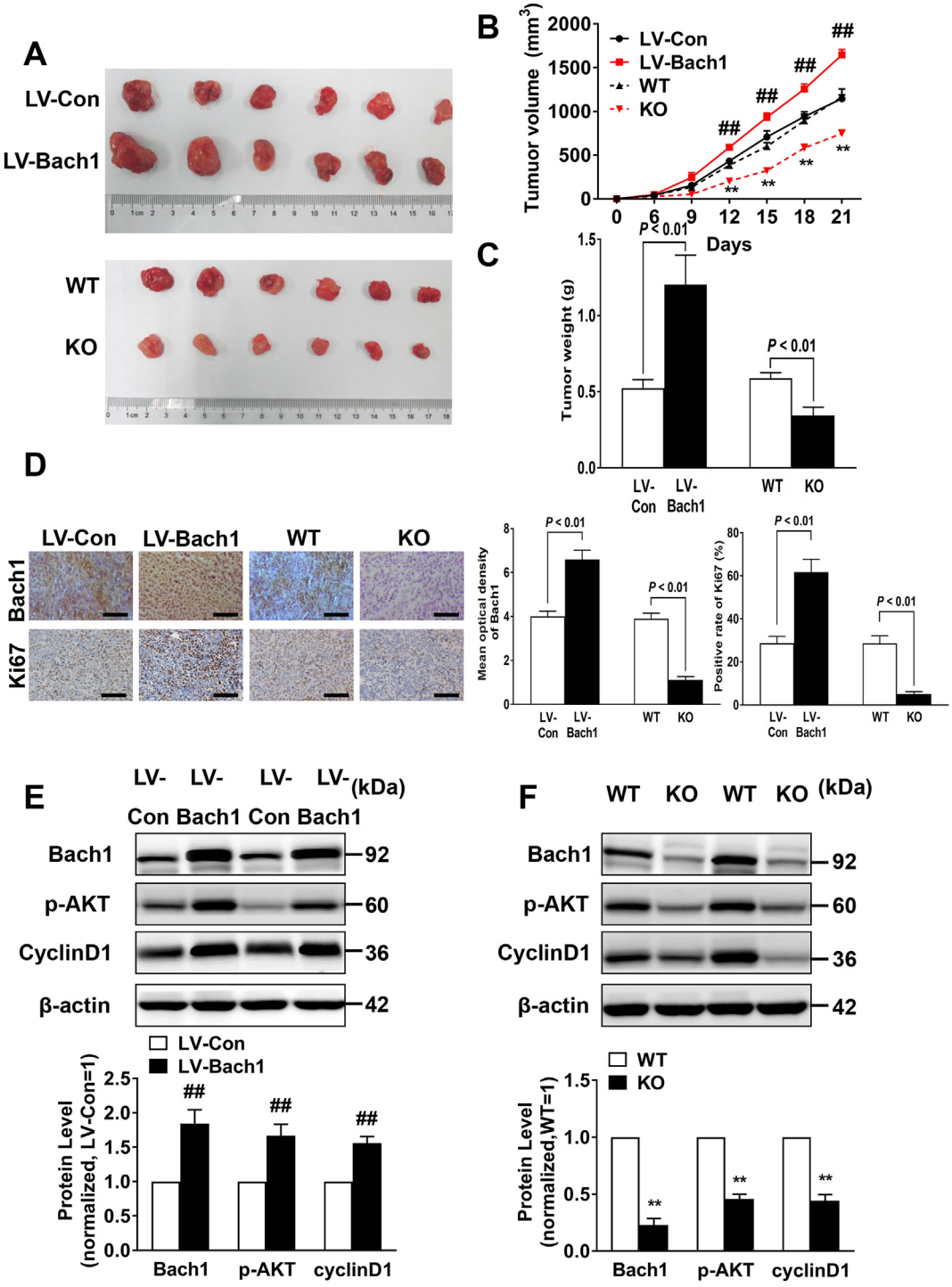

Fig. 7. Bach1 enhances EOC cell growth in vivo. A. Comparison of the gross morphology of the EOC tumors developed in nude mice injected subcutaneously with stably transfected A2780 cells (WT, KO, LV-Con and LV-Bach1). **B**–**C.** Comparison of tumor volumes by serial measurement **(B)** and tumor weights at the time of sacrifice **(C)** of the tumors derived from mice implanted with stably transfected A2780 cells. n = 6. $^{\#\#}P < 0.01$ vs LV-con, $^{**}P < 0.01$ vs WT. ANOVA with post hoc test. **D.** IHC staining for Bach1 and Ki67. Higher expression of Bach1 in xenograft tumors was associated with an increased number of Ki67-positive cells. Scale bar, 50 μm *t*-test. **E-F.** Comparison by western blotting of the levels of *p*-AKT and Cyclin D1 in xenograft tumors. Tumors from Bach1-overexpressing cells exhibited an increase in *p*-AKT and Cyclin D1 **(E)**, whereas tumors from Bach1-KO cells showed a reduction in the expression of these proteins **(F)**. $^{\#\#}P < 0.01$ vs LV-con, $^{**}P < 0.01$ vs WT. *t*-test.

most experiments and analyzed the results. Y.Z. performed the IHC experiments and data analysis. Y.Z. and C.N. assisted with and repeated some of the experiments. J.G., N.C., and Y.W. performed the western blot experiments. W.X., J.L. and M.J. performed the ChIP-qPCR experiments. M.D. and X.Z. supervised the whole study and wrote the manuscript.

Declarations of interest

None.

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Conflict of interest disclosure

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.003.

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