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BATF is involved in the malignant phenotype and epithelial-mesenchymal transition of colon cancer cells via ERK/PD-L1 signaling

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Summary. Objective. Transcription factors have emerged as primary regulators in colon cancer. Basic Leucine Zipper Transcription Factor (BATF) was found to be differentially expressed in colon cancer. This study aimed to explore the impact of BATF on the malignant phenotype and epithelial-mesenchymal transition (EMT) process.

Methods. Based on The Cancer Genome Atlas (TCGA) data, the correlation between BATF and patients' overall prognosis was analyzed. BATF expression in epithelial and colon cancer cells was evaluated. By knocking down its levels in colon cancer cells, its effects on the malignant phenotype, apoptosis, EMT progression, and ERK/PD-L1 were evaluated. Cells were treated with ERK/PD-L1 agonists, and the BATF cell regulation was re-examined.

Results. BATF levels were negatively correlated with patients' overall survival. BATF is upregulated in colon cancer cell lines, and BATF knockdown in HCT116 cells suppressed the malignant cellular phenotypes (proliferation, migration, and invasion) and increased apoptosis. BATF knockdown inhibited EMT and ERK/PD-L1 signaling activation, whereas upon agonist treatment, BATF potency was disrupted.

Conclusion. This study revealed that BATF is involved in the malignant phenotype and EMT of colon cancer cells, and this process may be mediated by ERK/PD-L1 signaling.

Key words: Colon cancer, EMT, ERK, PD-L1, Transcription factor

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Introduction

According to Cancer Statistics 2023, colorectal cancer ranks third among new cancers and third among cancer-related deaths globally in both men and women (Siegel et al., 2023). The causes of colon cancer are complex, and there is no clear conclusion regarding its etiology (Shang et al., 2023). Various factors such as environment, genes, disease, inflammation, and lifestyle habits have been confirmed as high-risk factors for colon cancer (Esmeeta et al., 2022). In addition to traditional drug treatment, surgery, radiotherapy, chemotherapy, molecular targeted gene therapy, and immunotherapy, such as signal transduction and cell receptors, can significantly improve the quality of life and prolong the survival time of patients with colon cancer (Lichtenstern et al., 2020; Ghonim et al., 2021). Therefore, finding more efficient and accurate diagnostic methods and treatments has become the focus of research. Notably, transcription factors have emerged as key regulators of colon cancer, with numerous studies demonstrating their role in cancer progression and drug resistance. For example, C-X-C chemokine receptor type 4 regulates the transcription factors Snail, Twist, and zinc finger E-box-binding homeobox protein 1/2, which contribute to epithelial-mesenchymal transition (EMT) in colorectal cancer (Wynendaele et al., 2022). Activating the transcription factor CP2 can upregulate pro-adipogenic factors and stimulate tumor angiogenesis, and reducing its levels can improve the survival of colorectal cancer mouse models (Hsu et al., 2023). Regulation of FOXO3 sensitizes 5-Fluorouracil (5-FU)-resistant cancer cells to 5FU treatment (Ghosh et al., 2022). These studies highlight the critical role of transcription factors in colon cancer and their potential as therapeutic targets.

Basic Leucine Zipper Transcription Factor (BATF) belongs to the AP-1/AFT transcription factor superfamily, it can serve as an inhibitor of AP-1 activity and plays a key role in various cancers. Its expression level can predict cancer immunotherapy and

chemotherapy response (Jia et al., 2022). In models of chronic infection and CAR-T cell treatment of solid tumors, BATF was revealed to block the path of T-cell exhaustion and enhance the anti-viral and anti-tumor functions of T cells (Chen et al., 2021; Seo et al., 2021). This provides new ideas for curing chronic infections and solid tumors. The Gene Expression Profiling Interactive Analysis (GEPIA) database shows that BATF is differentially expressed in colon cancer and is negatively correlated with prognosis, that is, the higher the expression, the worse the prognosis. Existing research shows that BATF can regulate TGF-β to promote EMT in breast cancer cells (Zhang et al., 2021). ERK signaling can affect the EMT effect of colorectal cancer cells (Xie et al., 2022), and ERK activation can promote the expression of the PD-L1 signal (Lee et al., 2020). BATF can affect the ERK signaling pathway (Xu et al., 2010) and was also found to be enriched in the PD-L1 signaling pathway according to KEGG analysis. Meanwhile, previous studies have indicated that PD-L1 signaling correlates with survival in patients with colon cancer (Jung et al., 2020). Therefore, this study aimed to explore whether BATF could regulate the biological characteristics of colon cancer and modulate ERK/PD-L1 signaling.

This study explored the impact of BATF on the malignant phenotype and EMT process by knocking down BATF in colon cancer cells. Applying signaling agonists, the mediation of ERK/PD-L1 signaling in BATF regulation was initially revealed. The findings provide a theoretical basis for subsequent research on BATF in colon cancer.

Materials and methods

Cell culture and treatment

Human intestine epithelial cell HIEC-6 (ATCC), colon cancer cells HCT116, LS174T, HCT8, SW620 (all from the ECACC) were maintained in a humidified atmosphere with 5% CO_2 at 37°C, with complete medium comprising DMEM (#11965126; Gibco), 10% fetal bovine serum (FBS, Gibco) and 1% penicillin /streptomycin (Meiji Seika Pharma Co., Ltd.). To determine ERK/PD-L1 signaling, HCT116 cells were treated with ERK agonist t-butylhydroquinone (tBHQ, 50 μM, Selleck) or PD-L1 agonist IFN-γ (10 ng/ml, PeproTech) for 24h.

Cell transfection

HCT116 cells at ~70% confluence were transfected with short hairpin RNAs (shRNAs) for 48h using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). shRNAs targeting BATF (shRNA-BATF-1/2) and non-target shRNAs for negative controls (shRNA-NC), were procured from GenePharma (Shanghai, China). At 48h post-transfection, cells were harvested for the following assays.

Western blot analysis

Cell lysates were centrifuged at 12,000 g for 20 min, and the supernatant was subjected to protein concentration quantification. A mixture of equal parts of an aliquot of protein and sample buffer was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated protein was then transferred onto a PVDF membrane (Millipore Corporation). After the membrane was blocked in skim milk, it was incubated with primary antibodies overnight at 4°C. Primary antibodies were procured from Proteintech (Wuhan, China), including BATF (13507-1-AP), MMP2 (10373-2-AP), MMP12 (22989-1-AP), Bcl2 (12789-1-AP), Bax (50599-2-Ig), cleaved caspase-3 (25128-1-AP), E-cadherin (20874-1-AP), N-cadherin (22018-1-AP), vimentin (10366-1-AP), p-ERK (80031-1-RR), ERK (11257-1-AP), PD-L1 (17952-1-AP), GAPDH (10494-1-AP). Thereafter, the membrane was incubated with HRP-conjugated secondary antibody (RGAR001, Proteintech) for 2h at room temperature. Signals were visualized by enhanced chemiluminescence reagents (Proteintech) and grayscale values were analyzed by ImageJ software.

Cell Counting Kit-8 (CCK8)

Cell viability was assessed through the CCK-8 assay. After 24h of incubation, HCT116 cells in 96-well plates were supplemented with the CCK-8 reagent (Selleck). After 2h of incubation, absorbance at 450nm was measured using a microplate reader (DeTie, Nanjing, China).

EdU staining

Cell proliferation was assessed through the BeyoClick™ EdU assay kit (Beyotime, Shanghai, China). EdU working solution was added to HCT116 cells at a final concentration of 10 μM. After 2h of incubation, the working solution was removed and cells were fixed with 4% paraformaldehyde for 15 min. Cells were treated with 0.3% Triton X-100 for 12 min and then incubated in 3% H_2O_2 for 20 min at room temperature. According to the kit's instructions, Click reaction solution, Streptavidin-HRP working solution, and DAB chromogenic solution were prepared and incubated with cells for 30 min in the dark. DAPI solution was used for counterstaining and images were captured under a fluorescence microscope (Olympus, Tokyo, Japan).

Wound healing

The culture medium was replaced by a medium with 1% FBS. The cell monolayer at the center of the well

was scraped and the floating cells were removed. Before and after 24h incubation, photographs were captured under a microscope (Olympus, Tokyo, Japan). Cell migration was determined by the number of cells that migrated into the scraped area.

Transwell assay

The invasion of HCT116 cells was assessed using Corning Transwell multiwell plates (Fisher Scientific). HCT116 cells were added to the top chamber, which was pre-plated with Matrigel. The bottom chamber was filled with culture medium containing 20% FBS. After 24h, the invaded cells were fixed and stained with crystal violet (Sigma-Aldrich), and photographs were captured under a microscope (Olympus, Tokyo, Japan)

TUNEL assay

Cell apoptosis was assessed using the TUNEL assay kit (Beyotime). HCT116 cells were fixed with 4% paraformaldehyde for 20 min and permeated with 0.3% Triton X-100 for 10 min. Cells were incubated with TUNEL working solution for 1h away from light. After washing with PBS thrice, the cell nuclei were counterstained with DAPI solution, and images were captured under a fluorescence microscope (Olympus, Tokyo, Japan).

Bioinformatics and statistical analysis

The differential expression and prognostic relationship of BATF in colon cancer were obtained from the GEPIA tool, and the database was derived from TCGA. SPSS software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. All quantitative data were given as the mean ± standard deviation (SD) from independent bio-triplications. Statistical comparisons applied one-way ANOVA followed by Tukey's test. $P<0.05$ was considered statistically significant.

Results

BATF levels in colon cancer

Data based on TCGA showed that patients with low BATF expression had better overall survival than those with high expression (Fig. 1A). Compared with the control group, BATF was upregulated in patients with colon cancer (Fig. 1B). Western blotting examined BATF levels in several cell lines; it was increased in colon cancer cell lines compared with the epithelial cell

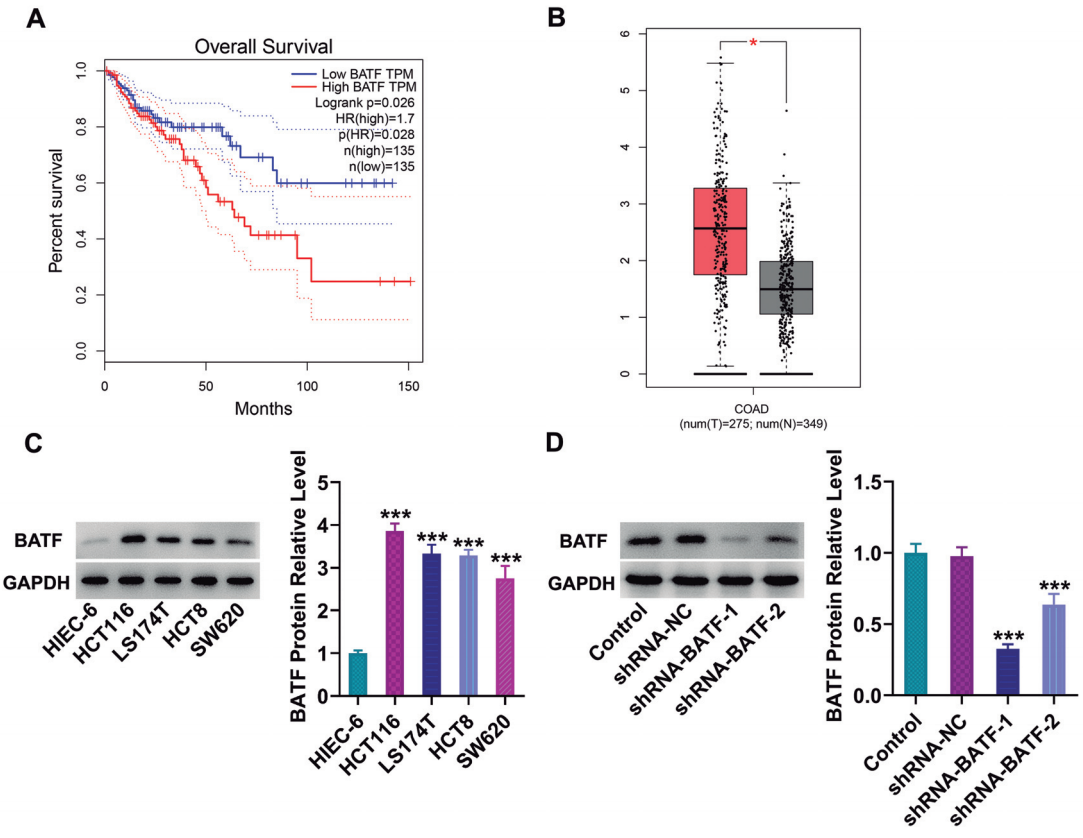

Fig. 1. BATF levels in colon cancer. **A.** Correlation between BATF levels and overall survival. **B.** BATF levels in patients with colon cancer and controls; red represents tumors and gray represents the control group. **C.** Western blotting examined BATF levels in several cell lines. **D.** HCT116 was transfected with shRNAs to knock down BATF and the transfection efficacy was identified using western blotting. ***$P<0.001$ vs. HIEC-6 or shRNA-NC.

line HIEC-6 (Fig. 1C). Given that BATF was highest in HCT116 cells, these cells were selected for subsequent experiments to highlight the role of the gene. HCT116 was transfected with shRNAs to knock down BATF. Western blotting demonstrated that BATF levels in the shRNA-BATF-1/2 group were successfully reduced (Fig. 1D). shRNA-BATF-1 was used in subsequent experiments because of better transfection efficiency.

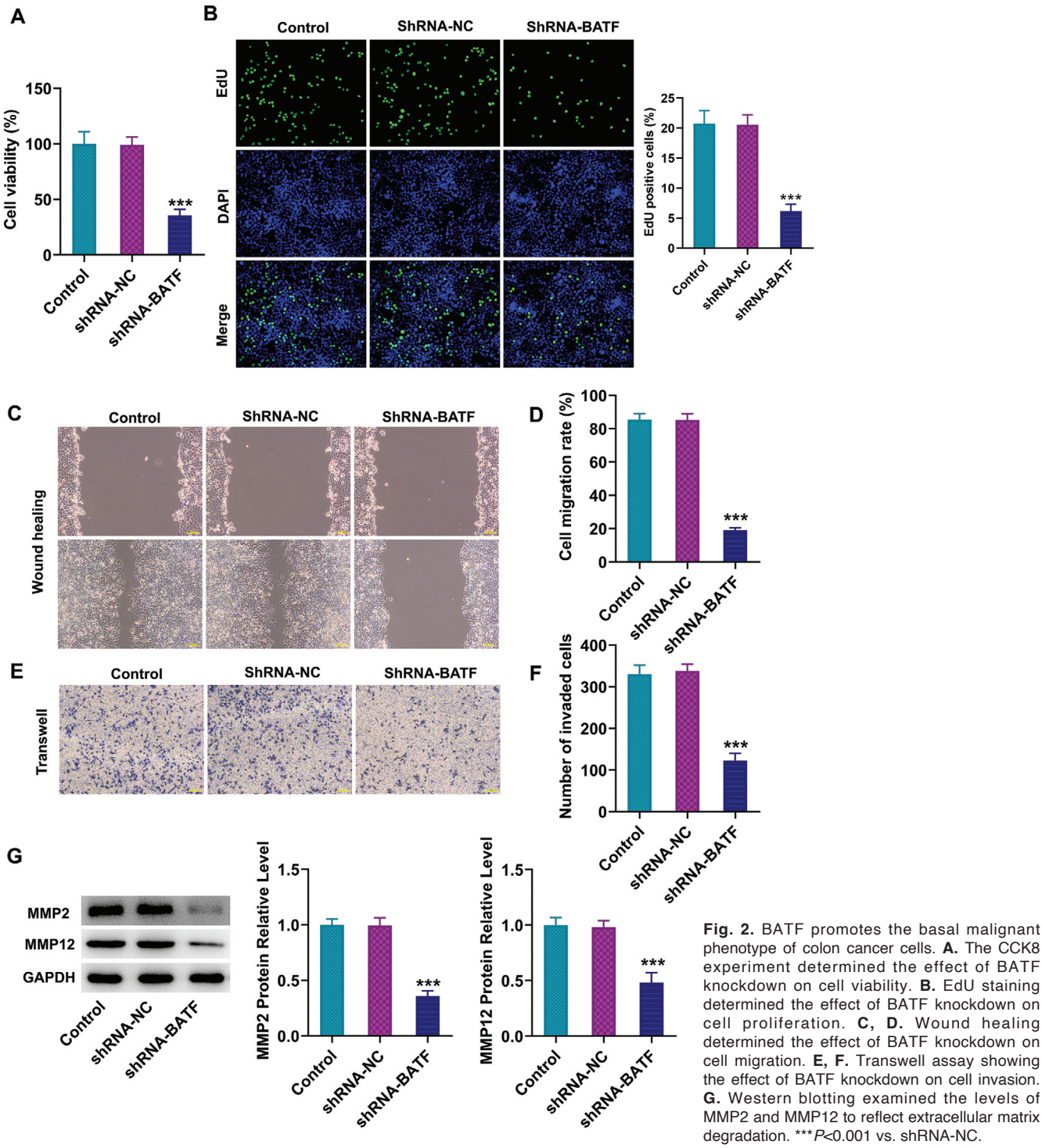

Fig. 2. BATF promotes the basal malignant phenotype of colon cancer cells. **A.** The CCK8 experiment determined the effect of BATF knockdown on cell viability. **B.** EdU staining determined the effect of BATF knockdown on cell proliferation. **C, D.** Wound healing determined the effect of BATF knockdown on cell migration. **E, F.** Transwell assay showing the effect of BATF knockdown on cell invasion. **G.** Western blotting examined the levels of MMP2 and MMP12 to reflect extracellular matrix degradation. ***P<0.001 vs. shRNA-NC.

BATF promotes the basal malignant phenotype of colon cancer cells

The CCK8 experiment determined cell viability, revealing that cell viability in the shRNA-BATF group decreased (Fig. 2A). EdU staining showed that the fluorescence of the shRNA-BATF group decreased compared with shRNA-NC, indicating that BATF knockdown reduced cell proliferation (Fig. 2B). Wound healing showed that BATF knockdown induced a decrease in the cell migration rate (Fig. 2C,D), and the Transwell assay reflected a decrease in cell invasion (Fig. 2E,F). In addition, compared with the shRNA-NC group, the levels of MMP2 and MMP12 in the shRNA-BATF group decreased, indicating that extracellular matrix degradation was weakened (Fig. 2G).

BATF inhibits colon cancer cell apoptosis and promotes EMT

TUNEL staining exhibited an increase in apoptotic cells in the shRNA-BATF group compared with the shRNA-NC group (Fig. 3A,B). Western blotting revealed that BATF knockdown reduced Bcl-2 protein levels and increased Bax and cleaved caspase-3 protein

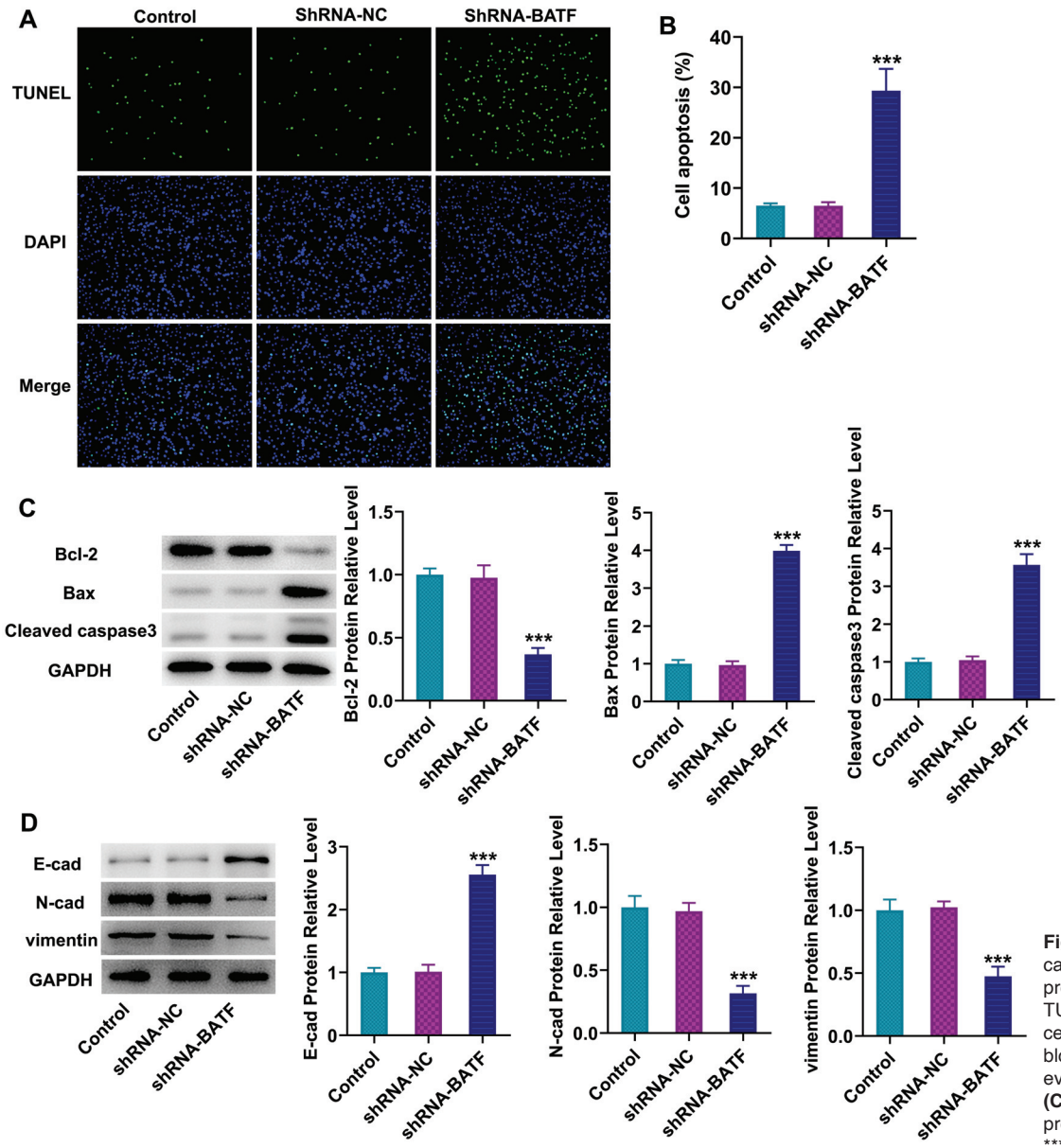

Fig. 3. BATF inhibits colon cancer cell apoptosis and promotes EMT. **A, B.** TUNEL staining reflected cell apoptosis. Western blotting was used to evaluate apoptosis-related **(C)** and EMT-related protein **(D)** levels. ***P<0.001 vs. shRNA-NC.

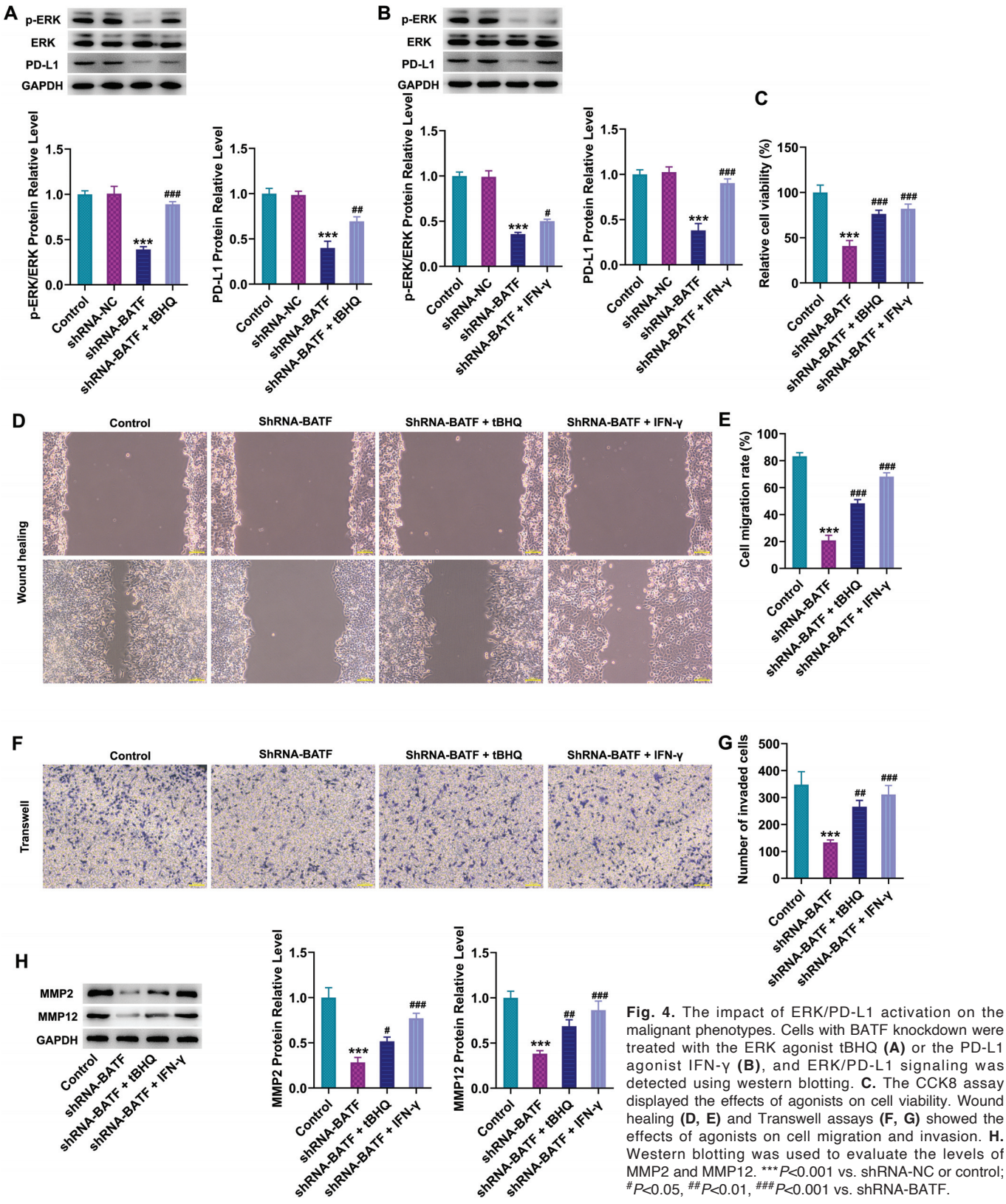

Fig. 4. The impact of ERK/PD-L1 activation on the malignant phenotypes. Cells with BATF knockdown were treated with the ERK agonist tBHQ **(A)** or the PD-L1 agonist IFN-γ **(B)**, and ERK/PD-L1 signaling was detected using western blotting. **C.** The CCK8 assay displayed the effects of agonists on cell viability. Wound healing **(D, E)** and Transwell assays **(F, G)** showed the effects of agonists on cell migration and invasion. **H.** Western blotting was used to evaluate the levels of MMP2 and MMP12. $^{***}P<0.001$ vs. shRNA-NC or control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. shRNA-BATF.

levels (Fig. 3C). Additionally, western blotting was used to evaluate EMT-related protein levels. Compared with the shRNA-NC group, the E-cadherin protein increased in the shRNA-BATF group, while N-cadherin and vimentin protein levels decreased (Fig. 3D).

The regulatory impact of ERK/PD-L1 signaling activation on BATF knockdown

To evaluate the mediation of BATF regulation by ERK or PD-L1 signaling, cells with BATF knockdown

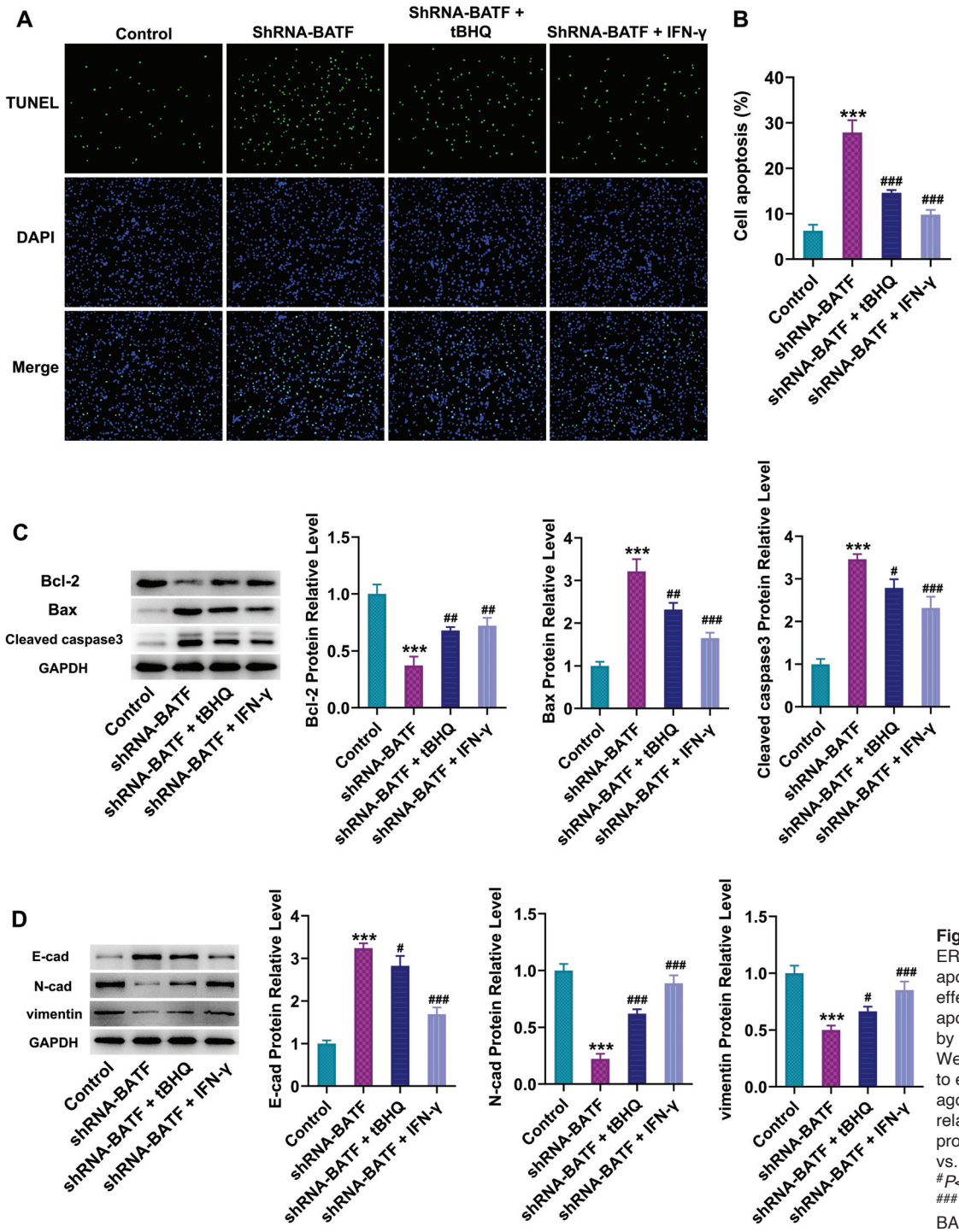

Fig. 5. The impact of ERK/PD-L1 activation on apoptosis and EMT. **A.** The effects of agonists on apoptosis were analyzed by the TUNEL assay. **C.** Western blotting was used to evaluate the effects of agonists on apoptosis-related **D.** and EMT-related protein levels. ***P<0.001 vs. shRNA-NC or control; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. shRNA-BATF.

were treated with the ERK agonist tBHQ or the PD-L1 agonist IFN-γ, and ERK/PD-L1 signaling was detected. Western blot analysis showed that p-ERK/ERK and PD-L1 were decreased in the shRNA-BATF group, indicating that ERK/PD-L1 was inhibited. Upon agonist treatment, compared with the shRNA-BATF group, the levels of p-ERK/ERK and PD-L1 showed a responsive increase (Fig. 4A,B). Afterward, the effects of both agonists on the malignant phenotype of HCT116 cells were evaluated. CCK8 assays displayed that both tBHQ and IFN-γ could increase cell viability, compared with the shRNA-BATF group (Fig. 4C). Wound healing and Transwell assays showed that agonists promoted cell migration and invasion (Fig. 4D-G), along with the elevation of MMP2 and MMP12 levels (Fig. 4H). The effects of agonists on apoptosis were analyzed by TUNEL and western blotting (Fig. 5A-C). The decrease in TUNEL fluorescence, increase in Bcl2, and decrease in Bax and cleaved caspase-3 indicated that agonists reduced cell apoptosis. Compared with the shRNA-BATF group, E-cadherin protein levels decreased, and N-cadherin and vimentin levels increased, indicating that agonists promoted the cellular EMT process (Fig. 5D).

Discussion

Colon cancer initiates with the formation of polyps, which then transform into cancerous tumors that can metastasize to other parts of the body (Dornblaser et al., 2024). Postoperative adjuvant chemotherapy is commonly used for patients with stage 3 cancer, while it is still controversial for those with stage 2 (Taieb and Gallois, 2020). Even with these treatments, the number of deaths and new cases is rising. The cost of treatment may be an important factor, however, the major reason is that treatment is ineffective in treating all cases (Ros et al., 2022). It is a heterogeneous disease attributed to the accumulation of intracellular molecular changes (Zygulska and Pierzchalski, 2022). These genetic and epigenetic mutations vary from patient to patient, which could explain differences in the effectiveness of the same treatment in different individuals. Therefore, personalized treatment is highly recommended at this stage, and targeted therapy is the basis of personalized medicine (Dey et al., 2023). Novel targets and clear regulatory mechanisms will facilitate the development of new therapies. This study focuses on BATF and analyzes its role in colon cancer. BATF knockdown in HCT116 cells inhibited cell proliferation, migration, and invasion, indicating that BATF has the potential to promote tumor growth and metastasis.

Additionally, BATF knockdown also reduced N-cadherin and vimentin proteins in cells. Tumor invasion and metastasis are complex processes involving multiple factors, nonetheless, EMT is considered to be its core initial step (Pastushenko and Blanpain, 2019). The occurrence of EMT is also accompanied by changes in cell morphology, and is closely related to the molecular mechanism regulating multiple signaling pathway proteins, transcription factors, and growth factors (Ramesh et al., 2020; Huang et al., 2022). EMT in tumor cells is accompanied by the loss of the epithelial marker E-cadherin, and the expression of the mesenchymal marker proteins N-cadherin, Vimentin, and MMPs, is increased. The results of this study reveal the role of BATF in promoting EMT. Furthermore, BATF knockdown inhibited ERK phosphorylation and PD-L1 expression. Treating cells with ERK and PD-L1 signaling agonists influenced the effects of BATF knockdown, which suggests that ERK/PD-L1 signaling mediates the regulatory mechanism of BATF. In fact, PD-L1 has received widespread attention in colon cancer, and clinical trials have shown that patients with metastatic colorectal cancer can benefit from PD-L1 monoclonal antibody treatment (Manca et al., 2023). Whether targeting BATF could enhance the therapeutic effect of monoclonal antibodies is not yet confirmed, and translational research is still required to highlight the value of biomarkers.

In summary, this study discovered that BATF is involved in the malignant phenotype and EMT of colon cancer cells. Furthermore, this process may be mediated by ERK/PD-L1 signaling. Transcription factors have been found to play a role in influencing tumor growth and guiding prognosis in colon cancer (Liu et al., 2020). This study initially revealed the role of BATF in colon cancer and provides a new marker or target for reference.

Data availability. The datasets utilized and/or analyzed in this study are available from the corresponding author upon reasonable request.

Ethics approval. Not applicable.

Authors' contributions. XC contributed to conceptualization, investigation, visualization, and drafting. HD contributed to conceptualization, investigation, visualization, and analysis. LJ contributed to investigation and analysis. All authors approved the final version.

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Conflicts of Interest. All authors have no conflicts of interest to declare.

Informed consent. Not applicable.

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