

Acidic bile salt-induced DNA methylation promotes APE1 upregulation through miR-128-3p silencing in esophageal adenocarcinoma

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Received June 12, 2026; Accepted September 7, 2026

DOI: 10.3892/or.2026.9216

Abstract. Esophageal adenocarcinoma (EAC) is an aggressive malignancy with rapidly rising incidence in Western populations and a poor 5-year survival rate of <20%. It commonly develops from Barrett's esophagus, a metaplastic condition driven by chronic exposure to acidic bile salts (ABS). Apurinic/apyrimidinic endonuclease 1 (APE1) serves a central role in this process by coordinating DNA repair and redox signaling pathways that promote tumor cell survival, inflammation and progression. Despite its importance, the upstream regulatory mechanisms controlling APE1 in EAC remain incompletely understood. The present study aimed to identify a novel epigenetic mechanism linking reflux-induced stress to APE1 upregulation through microRNA (miR) regulation. Bioinformatics analysis of downregulated miRNAs in EAC and the 3'untranslated region of APE1 revealed that miR-128-3p has a predicted binding site within the APE1 3'untranslated region. Functional validation using luciferase reporter assays confirmed direct binding of miR-128-3p to APE1. Exposure to ABS resulted in marked suppression of miR-128-3p expression, concomitant with increased APE1 levels. Mechanistically, ABS induces DNA methyltransferase (DNMT1) expression, leading to hypermethylation of the

CpG-rich miR-128-3p promoter and transcriptional silencing. DNMT1 knockdown or inhibition reversed miR-128-3p suppression and decreased promoter methylation, restoring its regulatory effect on APE1. Collectively, these findings defined a DNMT1/miR-128-3p/APE1 regulatory pathway that links reflux-induced epigenetic modifications to oncogenic signaling in EAC, highlighting potential therapeutic targets for this malignancy.

Introduction

Esophageal adenocarcinoma (EAC) is an aggressive malignancy whose incidence has risen sharply over recent decades in Western countries, with a predicted increase from 2.12 in 2021 to 3.86 in 2040 (3.2%/year, 95% CI: -2.3% to 9.1%) (1). Although EAC accounts for a minority of esophageal cancers worldwide, it is the predominant histological subtype in many industrialized nations (1) and carries an overall 5-year survival rate <20% (2). EAC most commonly arises through the chronic acid and bile reflux which drives replacement of normal squamous epithelium by specialized intestinal metaplasia, known as Barrett's esophagus (BE), that can progress to low- and high-grade dysplasia and invasive cancer (3). Despite advances in endoscopic and multimodality therapy, most patients present with locally advanced or metastatic disease (4), underscoring the need to better understand the molecular determinants of EAC initiation and progression.

Apurinic/apyrimidinic endonuclease 1 (APE1) is increasingly recognized as a central mediator of BE-associated esophageal adenocarcinoma, where it couples base excision repair of reflux-induced oxidative DNA damage (5) with redox-dependent activation of oncogenic transcription factors such as NF- κ B, STAT3, hypoxia-inducible factor 1 α and yes-associated protein 1 (6-8). APE1 promotes survival of epithelial cells exposed to acidic bile salts (ABSs), enhances

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Key words: epigenetic regulation, microRNA, cancer, reflux conditions, methylation

proliferative and invasive programs and fosters a pro-inflammatory, pro-tumorigenic transcriptional landscape (5-7,9). Accordingly, dysregulated APE1 activity may represent a key molecular nexus linking chronic gastroesophageal reflux to EAC initiation, progression and therapeutic resistance.

APE1 expression and activity are tightly controlled at multiple levels, including stress-responsive transcriptional induction, post-translational modifications that tune its subcellular localization and redox/DNA repair functions and bidirectional crosstalk with microRNA (miRNA or miR) networks (10). APE1 directly regulates miRNA biogenesis and is post-transcriptionally controlled by tumor-associated miRNAs (10). This multilayered regulation positions APE1 as a nodal integrator of oxidative stress, DNA repair and non-coding RNA signaling, creating a permissive environment for esophageal adenocarcinoma initiation and progression.

To the best of our knowledge, the role of miRNAs in regulating APE1 in EAC is unknown. Analysis of our previously established databases of deregulated miRNAs in gastroesophageal and EAC (11) showed downregulation of miR-128-3p in EAC and a perfect match on APE1 3'untranslated region (UTR) supporting a link between miR-128-3p and APE1. The present study aimed to investigate the role of miR-128-3p in regulating APE1 in EAC.

Materials and methods

Reagents. The key resources for reagents, kits and primer sequences are provided in Tables SI-SIII.

Cell culture. Immortalized non-neoplastic esophageal epithelial cells (EPC2; provided by Dr. Anil Rustgi, Columbia University, NY, USA) and BE cells line BART (Dr. Rhonda Souza, Baylor University Medical Center, Dallas, TX, USA), CPA and CPB [both American Type Culture Collection (ATCC)] were cultured in DMEM/F12 supplemented with 5% FBS (both Gibco; Thermo Fisher Scientific, Inc.), 0.4 μ g/ml hydrocortisone (Sigma-Aldrich; Merck KGaA), 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 140 μ g/ml bovine pituitary extract (Thermo Fisher Scientific, Inc.), 20 mg/l adenine hydrochloride hydrate, insulin-transferrin-selenium supplement and 20 ng/ml epidermal growth factor (all Sigma-Aldrich; Merck KGaA). EAC cell lines FLO-1 (ATCC), OE33 (Dr David Beer, University of Michigan, Ann Arbor, MI, USA), SK-GT4 (Dr. Xiaochun Xu, MD Anderson Cancer Center, Houston, TX, USA) and OE19 (MilliporeSigma) were maintained in DMEM or RPMI-1640 with 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37°C in a humidified incubator with 5% carbon dioxide and tested for mycoplasma contamination every month using a mycoplasma detection kit (PCR; SouthernBiotech). Cell line authentication was conducted routinely every 6 months using Labcorp Cell Line Authentication Services, which utilizes the short tandem repeat DNA profiling technology. Cells were last authenticated in August 2025, prior to the experiments.

Acidic bile salts (ABS) treatment. To mimic gastroesophageal reflux episodes in patients, a bile salts cocktail was prepared by combining 40 μ M glycocholic acid, taurocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and deoxycholic

acid. Cells were treated with 200 μ M bile BS cocktail in acidic culture medium (DMEM or RPMI-1640; pH 4.0) for 20 min at 37°C based on our previous publications (12,13). The BS-containing medium was replaced with standard culture medium (DMEM or RPMI-1640) for 0-6 h.

Transfection. Scrambled small interfering (si)RNA (cat. no. sc-37007) was purchased from Santa Cruz Biotechnology, Inc. and DNA methyltransferase (DNMT)1 siRNA (ID: s4215; cat. no. #4390824) was obtained from Thermo Fisher Scientific, Inc. (sequences not available). The wild-type and mutated mammalian expression luciferase reporters containing the 3'UTR region of APE1 were generated by Vector Builder, Inc. The miR-128-3p mimic or miRNA mimic negative control was transfected into OE33 and FLO-1 cells and the miR-128-3p inhibitor or miRNA inhibitor negative control (all 50 nM) was transfected into CPA cells using PolyJet. For transient DNMT1 knockdown, siDNMT1 or scrambled siRNA (40 nM for OE33 and 80 nM for FLO-1) was transfected into OE33 and FLO-1 cells using LipoJet. Transfection was performed at 37°C. Cells were collected within 72 h post-transfection.

5-Aza-2'deoxyctidine (5'aza) treatment. FLO-1 and OE33 cells were treated with 2 μ M 5'aza (Sigma-Aldrich; Merck KGaA) for 72 h at 37°C in complete culture medium. 5'aza was replenished every 24 h.

DNA bisulfite treatment and pyrosequencing analysis. Genomic DNA was isolated from OE33 cells using a DNeasy Blood Tissue Kit (Qiagen GmbH) and bisulfite modified with an EZ DNA Methylation-Gold kit. The CpG island within the miR-128-3p promoter was identified using an online search tool (uscnorris.com). Pyrosequencing primers were designed with PSQ Assay Design Software version 3.0 (Biotage), including the forward primer 5' GAAAGGTTTAGGGAATGA 3' and the biotinylated reverse primer 5'-Biotin-TAAAAACATCCA AACTAACCCATCC-3'. Bisulfite-modified DNA (40 ng) was amplified by PCR (Bio-Rad Laboratories) using Platinum PCR SuperMix High Fidelity (Invitrogen; Thermo Fisher Scientific, Inc.). Thermocycling conditions:

Initial denaturation: 95°C for 5 min, followed by 50 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and final extension: 72°C for 5 min. PCR products were confirmed by 1.5% agarose gel electrophoresis and visualized using GelRed Nucleic Acid Gel Stain (MilliporeSigma). Quantitative methylation analysis was performed using the Biotage PyroMark MD System and the results were processed with Pyro Q-CpG 1.0.9 software (Biotage). A methylation threshold of 10% was used to define miR-128-3p promoter hypermethylation based on control normal samples and internal quality controls provided in the software analysis (14).

Sphere formation assay. Single-cell suspensions of OE33 cells transfected with miR-128-3p mimic or miRNA mimic negative control (both 50 nM) were resuspended in a 1:1 mixture of cold Growth Factor Reduced Matrigel™ (Corning, Inc.) and serum-free RPMI-1640 (Thermo Fisher Scientific, Inc.) to a final volume of 50 μ l/well. Each condition was performed in triplicate. The cell-Matrigel mixture was plated along the rim of

wells in a 24-well plate at 37°C for 45 min to allow solidification. Subsequently, RPMI-1640 supplemented with 5% FBS was added to the center of each well. Media was replenished every 2 days. Sphere formation was assessed by manual counting and imaging on day 10. The total number of spheres was counted manually under bright-field light microscopy (20X objective).

Esophageal tumoroid generation. Esophageal tumoroids were generated from patient-derived xenograft (PDX) sample PDX683-24-4. The PDX was developed at the University of Miami in collaboration with the Biospecimen shared resource of the Sylvester Comprehensive Cancer Center (Miami, FL, USA). Tissue samples were dissected into 2-3 mm fragments and incubated in 2 ml 5 mg/ml collagenase type II (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 17101-015) in Advanced DMEM-F12 (adDMEM/F12) for 45 min at 37°C. The pellet was digested with TrypLE followed by resuspension in adDMEM/F12. The resulting suspension was passed through 40 μ m cell strainers and centrifuged at 274 x g for 5 min at room temperature. The cell pellet was resuspended in 50 μ l Matrigel and plated into pre-warmed 24-well plates. Following Matrigel polymerization in a CO₂ incubator at 37°C for 20 min, 500 μ l IntestiCult Organoid Growth Medium was added. After 7-10 days of culture at 37°C, the total number of tumoroids was counted manually under bright-field light microscopy (20X magnification) and images were captured to calculate the mean diameter using the Perfect Screen Ruler program version 3.0 (perfect-screen-ruler.informer.com/download/).

For organoid transfection, organoids were dissociated into single cells using pre-warmed TrypLE Express (cat. no. 12604021, Thermo Fisher Scientific, Inc.) at 37°C for 10 min. The reaction was terminated by adding adDMEM/F12 and cells passed through a 30- μ m cell strainer. The miR-128-3p mimic or the miRNA mimic negative control (50 nM) was mixed with PolyJet. A total of 50 μ l reaction mixture was applied to 2x10⁵ organoids suspended with 450 μ l human organoid growth medium and incubated at 37°C overnight. Following overnight incubation, cells were pelleted by centrifugation at 274 x g for 5 min at room temperature and seeded in Matrigel in a 24-well plate as aforementioned. Organoid growth was monitored daily for 3-5 days following transfection.

Western blot analysis. FLO-1 and OE33 cells were lysed using RIPA buffer. Protein concentrations were determined with the DC Protein Assay (Bio-Rad Laboratories, Inc.) following the manufacturer's instructions. Samples were combined with 4X Laemmli buffer and heated at 85°C for 10 min prior to electrophoresis. Equal amounts of protein (30 μ g) were resolved on 10% SDS-PAGE at 100 V for 90 min and subsequently transferred onto nitrocellulose membranes. Membranes were blocked in 5% BSA (RPI Research Products; cat. no. A30075-250.0) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies. Following three washes with 1X TBST, membranes were incubated with secondary antibodies for 1 h at room temperature. Protein signals were detected using Immobilon Western Chemiluminescent HRP substrate (Thermo Fisher Scientific, Inc.). Images were captured and analyzed using a ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc.). Image Lab version 6.0.1 was used for analysis (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. The tissue samples utilized for RNA extraction were frozen de-identified human tissue samples that were collected from the archives of pathology at Vanderbilt University (Nashville, TN, USA) and the National Cancer Institute (Bethesda, Maryland, USA) Cooperative Human Tissue Network (11). The samples included 13 normal esophagus (NE), 7 isolated BE and 15 EAC tissue samples. The NE samples were matched whenever possible with EAC or isolated BE. Histopathological diagnosis was verified based on hematoxylin and eosin-stained sections. The tissue were obtained between January, 2010-June, 2017 from a total of 28 patients, aged 44-77 years. Tissue samples meeting the histopathological criteria of NE, BE, or EAC were randomly collected with no exclusion criteria

The total RNA was extracted using a miRNeasy kit according to the manufacturer's protocol. cDNA was synthesized from 1 μ g total RNA using miRNA All-In-One cDNA Synthesis kit, according to the manufacturer's instructions. RT-qPCR was performed using the GoTaq 2-Step RT-qPCR System (Promega Corporation; cat. no. A6020) on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). Thermocycling conditions were as follows:

Initial denaturation: 95°C for 3 min, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec 95°C for 10 sec. Normalization was performed using U6 small nuclear RNA (snRNA) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a reference gene. Relative gene expression levels were calculated using the 2^{- $\Delta\Delta C_q$} method (15).

Dual luciferase assay. Dual-luciferase reporter assays were performed following the manufacturer's protocol (Promega Corporation; cat. no. E397A). FLO-1 and OE33 cells were seeded in 6-well tissue culture plates overnight at 37°C; upon reaching 60-70% confluency, cells were transiently transfected with 1 μ g luciferase reporter plasmid/well using PolyJet, as aforementioned. After 48 h, luciferase activity was measured using a LUMIstar OPTIMA microplate luminometer (BMG LabTech) in 96-well plates (Dynex). Relative luciferase units were normalized to firefly luciferase, and results were expressed as relative luciferase activity. All transfections were performed in triplicate.

DNMT activity. OE33 cells were treated with ABS for 20 min at 37°C and allowed to recover in regular media for 3 h at 37°C. Cells were collected, and nuclear extracts were isolated using a nuclear extraction kit according to the manufacturer's instructions. DNMT activity was evaluated using a colorimetric DNMT activity assay, which measures methylation of CpG-rich DNA sequences immobilized on 96-well plates. DNMT1 catalyzes the transfer of a methyl group from S-adenosyl methionine to the substrate DNA and the resulting methylated cytosines are quantified via ELISA using a methyl-CpG-binding protein and HRP-conjugated antibody. DNMT activity was measured using a LUMIstar OPTIMA microplate luminometer.

Transwell invasion/migration assays. For the migration assay, 2x10⁵ transfected OE33 and FLO-1 cells were seeded in a serum-free medium (DMEM or RPMI-1640) in the top chamber of 24-well inserts (pore size, 8 μ m; Corning, Inc.) and

medium (DMEM or RPMI-1640) supplemented with 10% FBS was used in the lower chamber. Cells were allowed to migrate through the membrane at 37°C in a 5% CO₂ incubator for 48 h. Non-migratory cells in the upper chamber were scraped off with a cotton-tip applicator. Migrating cells on the lower surface of the membrane were fixed and stained with crystal violet for 20 min at room temperature. Images were captured using a bright-field light BZ-X710 KEYENCE All-in-One Microscope. For the invasion assay, the top chamber was freshly coated with 100 µl Matrigel at a dilution of 1:10 in cold PBS and was then air-dried overnight before the invasion assay.

Statistical analysis. All statistical analyses were conducted using GraphPad Prism (version 8.0; Dotmatics). Statistical significance was evaluated using unpaired student's t-test or one-way ANOVA followed by Bonferroni's post hoc test. Data are reported as the mean of three independent experiments ± SEM. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-128-3p has perfect match on APE1 3'UTR and is downregulated in EAC. To identify candidate miRNAs that target APE1, the present study analyzed all downregulated miRNAs in EAC and the 3'UTR of APE1 using our previously established database of deregulated miRNAs in EAC (11). miR-128-3p had a perfect match on APE1 3'UTR with 8 uninterrupted binding sites (Fig. 1A). miR-128-3p was significantly downregulated in EAC (Fig. 1B and C) with increased APE1 protein levels (Fig. 1D). The adenocarcinomas ranged from well to poorly differentiated with a mix of intestinal- and diffuse-type tumors.

miR-128-3p regulates APE1 in response to ABS. ABS induced an increase in APE1 protein (Fig. 1E). There were no significant changes in APE1 mRNA expression levels (Fig. 1F). There was complete silencing of miR-128-3p (Fig. 1G) that was concomitant with the observed increase in APE1 protein levels. These findings suggested a post-transcriptional regulation of APE1. To test this hypothesis, 3'UTR regions of APE1 containing the respective binding sites were cloned into a mammalian expression luciferase reporter (Fig. 2A). Treatment of OE33 and FLO-1 cells with ABS (pH 4.0, 20 min), followed by 3 h recovery, showed significant increase in the luciferase reporter activity, which was lost when combined with expression of miR-128-3p mimic (Fig. 2B). Successful transfection with miR-128-3p mimic was confirmed by RT-qPCR, which showed increased levels of miR-128-3p in the miR-128-3p mimic transfected group compared with the mimic negative control group in OE33 and FLO-1 cells (Fig. S1A). Inhibition of miR-128-3p in CPA cells, with constitutive high expression of miR-128-3p, enhanced the luciferase reporter activity (Fig. 2C). Unlike miR-128-3p mimic, successful transfection with miR-128-3p inhibitor cannot be confirmed by RT-qPCR because the inhibitor neutralizes its target miRNA by binding and does not degrade the mature miRNA so both free and inhibitor-bound miRNA will be equally detected by RT-qPCR, failing to reflect true functional neutralization (16). Therefore, in the present study, successful transfection of the miR-128-3p

inhibitor was confirmed via functional and reporter assays (Fig. 2C). To validate the functional binding of miR-128-3p on APE1 3'UTR, mutated binding sites on the APE1 3'UTR were cloned into the luciferase reporter (Fig. 2D). Unlike the wild-type APE1 3'UTR, no significant changes in luciferase activity were observed with the mutant APE1 3'UTR following ABS treatment in the presence or absence of miR-128-3p mimic expression (Fig. 2E). Notably, control untreated wild-type and mutant reporters showed no significant change in luciferase activity, confirming that the mutant construct did not affect the basal luciferase activity (Fig. S1B). Western blot analysis of APE1 in OE33 and FLO-1 cells following ABS treatment and miR-128-3p mimic expression showed a decrease in APE1 protein levels (Fig. 3A). Conversely, inhibition of miR-128-3p following ABS treatment in CPA cells induced APE1 protein levels (Fig. 3B). miR-128-3p mimic in OE33-derived spheroids (Fig. 3C) or patient-derived tumoroids (Fig. 3D) decreased their number and size and APE1 protein levels (Fig. S1C). Moreover, expressing miR-128-3p mimic in OE33 and FLO-1 cells reduced their migratory and invasive capacities (Fig. S2A and B). Taken together, these data suggested that ABS induced APE1 by silencing miR-128-3p.

miR-128-3p expression is regulated by methylation. Analysis of the miR-128-3p promoter demonstrated the presence of a CpG island (Fig. 4A), suggesting that miR-128-3p silencing may occur through an epigenetic mechanism. ABS decreased miR-128-3p expression (Fig. 1F) and induced DNMT1 protein levels and activity in EAC cells (Fig. 4B and C) with concomitant increases in APE1 protein. DNMT1 knockdown and ABS treatment restored the expression of miR-128-3p with downregulation of APE1 (Figs. 4D and E and S3). Similarly, inhibition of DNMT1 using 5'aza following ABS treatment rescued miR-128-3p expression and decreased APE1 protein levels (Fig. 5A and B). The 5'aza data were consistent with the DNMT1-specific siRNA results, although 5'aza inhibits all DNMT family members (17). To confirm the direct role of methylation in regulating miR-128-3p, quantitative methylation analysis of miR-128-3p was performed using pyrosequencing technology. ABS treatment was associated with increased methylation of miR-128-3p, whereas DNMT1 knockdown or inhibition in the presence or absence of ABS decreased miR-128-3p methylation (Fig. 5C). These findings suggest that ABS-induced DNMT1 could increase APE1 by downregulating miR-128-3p in a methylation-dependent manner (Fig. 5D).

Discussion

APE1 is a critical driver of EAC through its dual roles in DNA base excision repair and redox regulation of oncogenic transcription factors, enabling tumor cells to survive oxidative stress induced by chronic reflux (5,9). Its ability to promote inflammation, proliferation and therapeutic resistance positions APE1 as a key molecular link between BE and malignant progression (6,9,18,19,20). Despite this functional importance, the upstream mechanisms governing APE1 dysregulation in EAC remain poorly defined. Limited knowledge exists regarding how epigenetic and post-transcriptional pathways, including miRNA-mediated regulation, contribute to APE1 upregulation in response to reflux-associated stress (21).

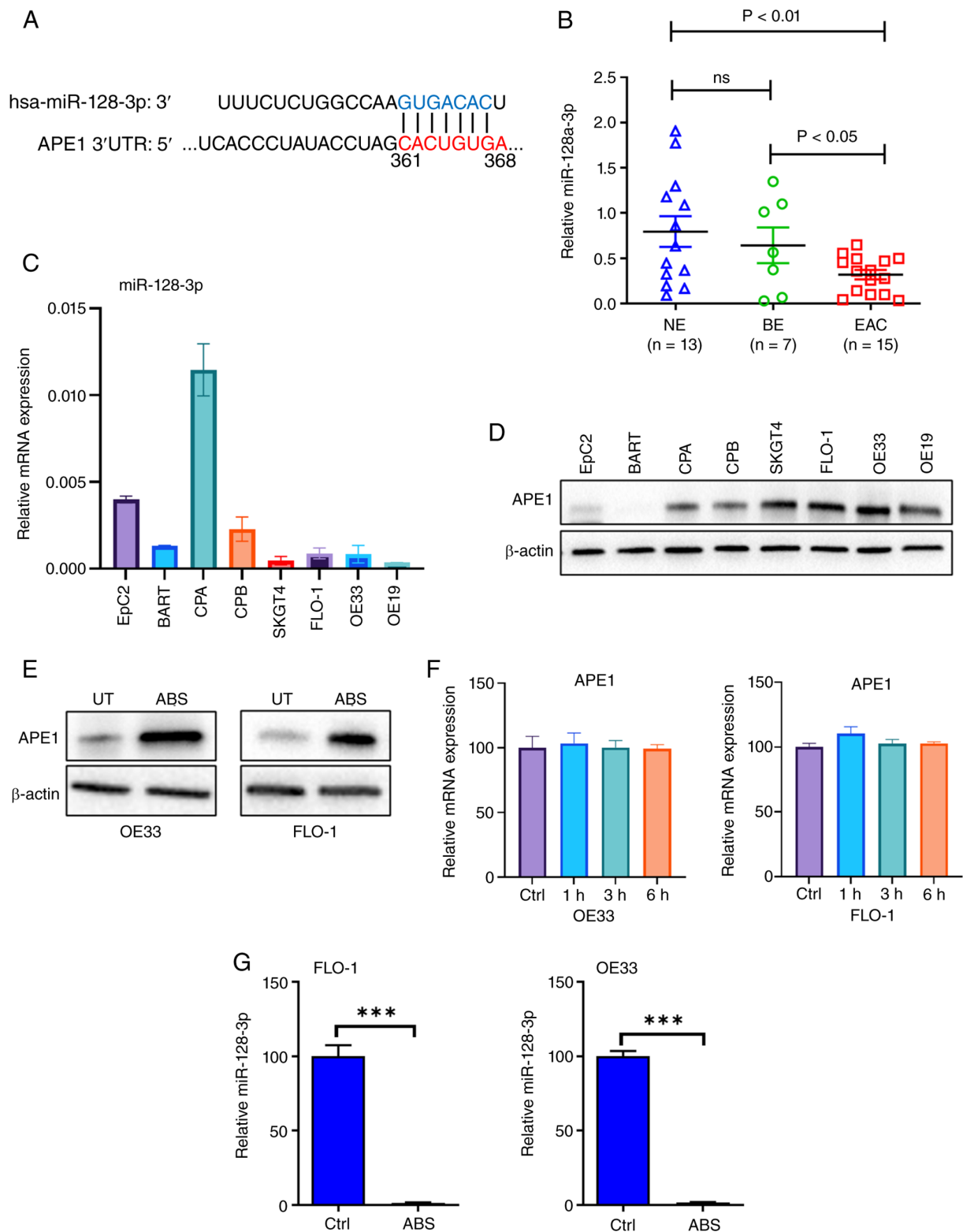


Figure 1. miR-128-3p binds perfectly to the APE1 3'UTR and is reduced in EAC in response to ABS. (A) Binding sites between miR-128-3p and APE1 3'UTR. RT-qPCR for miR-128-3p in NE, BE and EAC (B) tissue and (C) cell lines. (D) Western blot analysis of APE1 protein expression in NE, BE, and EAC cell lines. (E) Western blot analysis of APE1 protein expression following exposure to ABS in OE33 and FLO-1 cells. β -actin was used for normalization. RT-qPCR for (F) APE1 and (G) miR-128-3p in OE33 and FLO-1 cells following exposure to ABS. ***P<0.001. miR, microRNA; APE, apurinic/apyrimidinic endonuclease; UTR, untranslated region; EAC, esophageal adenocarcinoma; ABS, acidic bile salt; RT-q, reverse transcription-quantitative; NE, normal esophagus; BE, Barrett esophagus; UT, untreated; Ctrl, control; ns, not significant.

Addressing this gap is key for understanding EAC pathogenesis and for identifying new therapeutic strategies targeting APE1 regulatory networks.

In the present study, APE1 mRNA levels were unchanged, indicating that its upregulation is likely mediated through post-transcriptional mechanisms. Given the established role

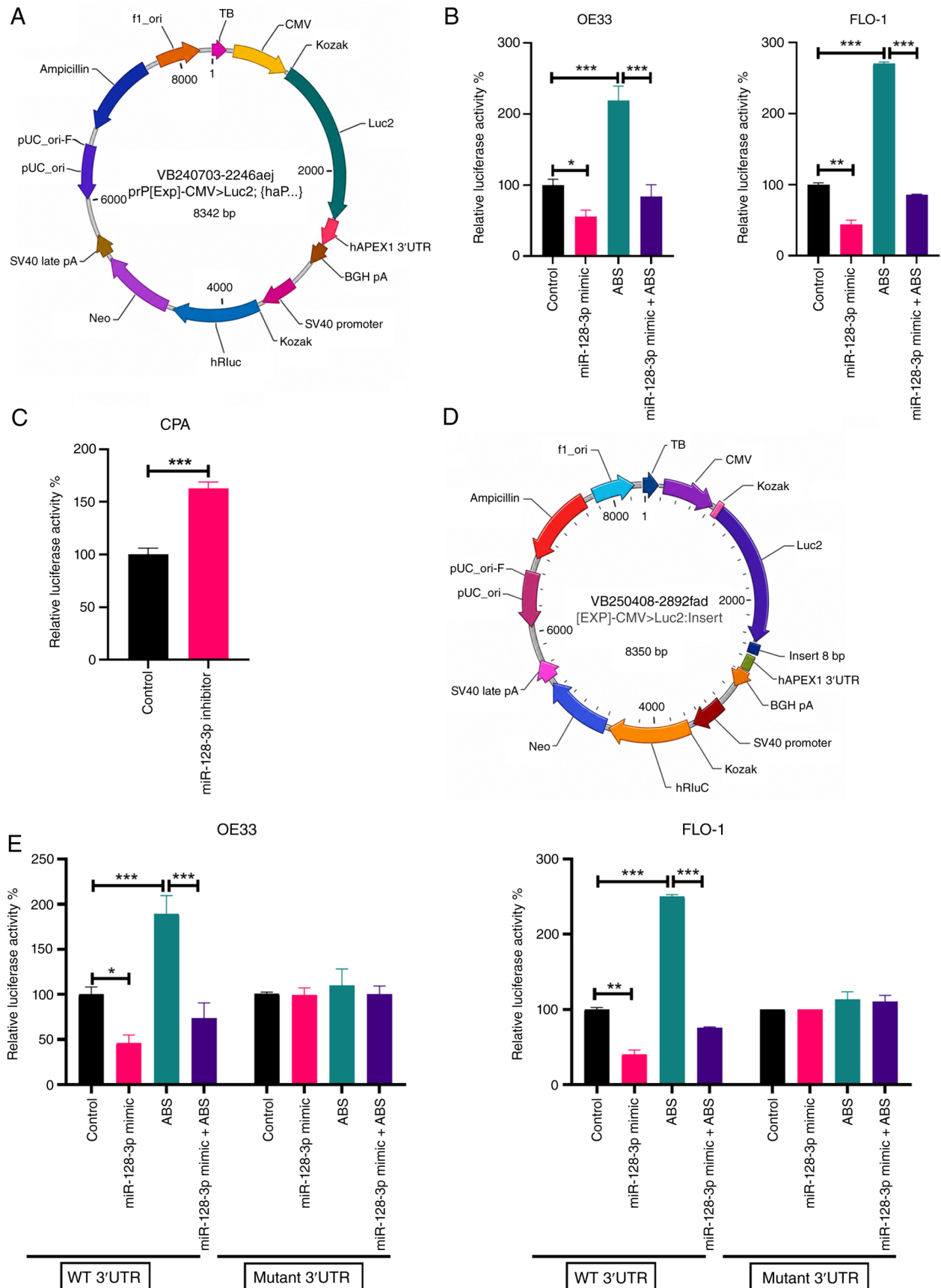


Figure 2. miR-128-3p regulates APE1 in response to ABS. (A) Map of luciferase reporter plasmid with the human APE1 3'UTR region corresponding to miR-128-3p binding sites. (B) Luciferase reporter assay with WT APE1 3'UTR in OE33 and FLO-1 cells in the presence or absence of miR-128-3p mimic and ABS exposure. (C) Luciferase reporter assay with WT APE1 3'UTR in CPA cells in the presence or absence of miR-128-3p inhibitor and ABS exposure. (D) Map of luciferase reporter plasmid with the mutant human APE1 3'UTR region corresponding to miR-128-3p binding sites. (E) Luciferase reporter assay with the mutant APE1 3'UTR in OE33 and FLO-1 cells following ABS treatment in the presence or absence of miR-128-3p mimic expression. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. miR, microRNA; APE, apurinic/apyrimidinic endonuclease; ABS, acidic bile salt; UTR, untranslated region; WT, wild-type.

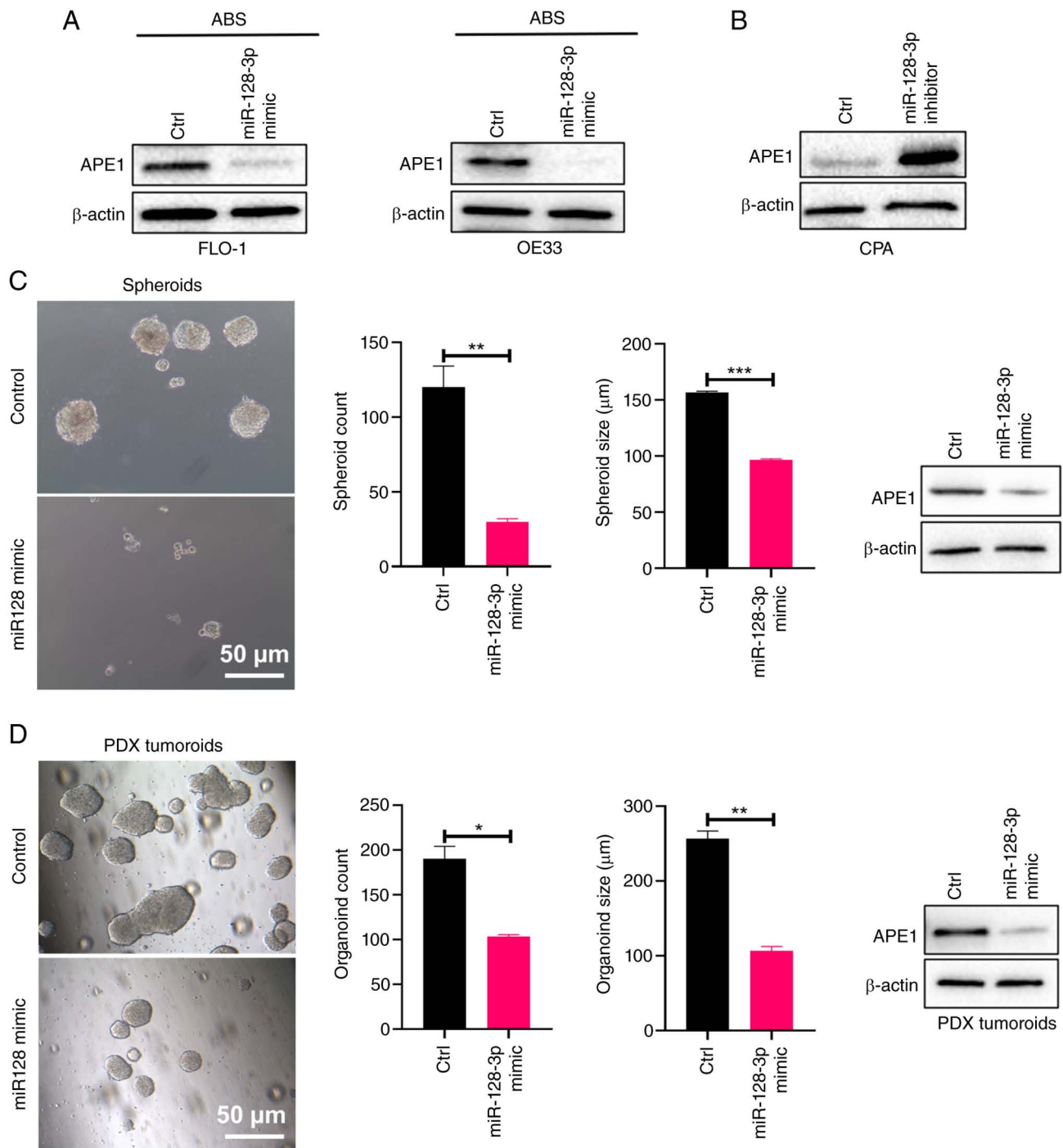


Figure 3. miR-128-3p modulates APE1 expression in response to ABS exposure. (A) Western blot analysis of APE1 protein following ABS treatment in the presence or absence of miR-128-3p mimic expression in OE33 and FLO-1 cells. (B) Western blot analysis of APE1 protein level following ABS treatment in the presence or absence of miR-128-3p inhibitor in CPA cells. Representative bright-field images of (C) OE33 spheroids and (D) PDX tumoroids and western blot analysis of APE1 protein in the presence or absence of miR-128-3p mimic. β-actin was used for normalization. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. miR, microRNA; APE, apurinic/apyrimidinic endonuclease; ABS, acidic bile salt; PDX, patient-derived xenograft; Ctrl, control.

of miRNAs in suppressing protein translation via binding to 3'UTRs, the present study investigated miRNA-mediated regulation of APE1. The best-established miRNAs that directly regulate APE1 in tumors are miR-27a-5p and miR-296-3p, both reported to target APE1 and decrease its expression in gastric (22) and non-small cell-lung cancer (23), respectively. Based on deregulated miRNAs in gastroesophageal cancer and EAC (11), the present bioinformatics analysis identified miR-128-3p as a strong candidate regulator, exhibiting

a perfect binding match to the APE1 3'UTR and significant downregulation in EAC. Expressing miR-128-3p mimic in established patient-derived tumoroids reduced both their number and size and was associated with lower APE1 protein levels. Notably, these tumoroids were derived from a single PDX line as a proof-of-concept model; therefore, further validation across multiple independent PDX lines is necessary to establish the generalizability of these findings to a broader patient population.

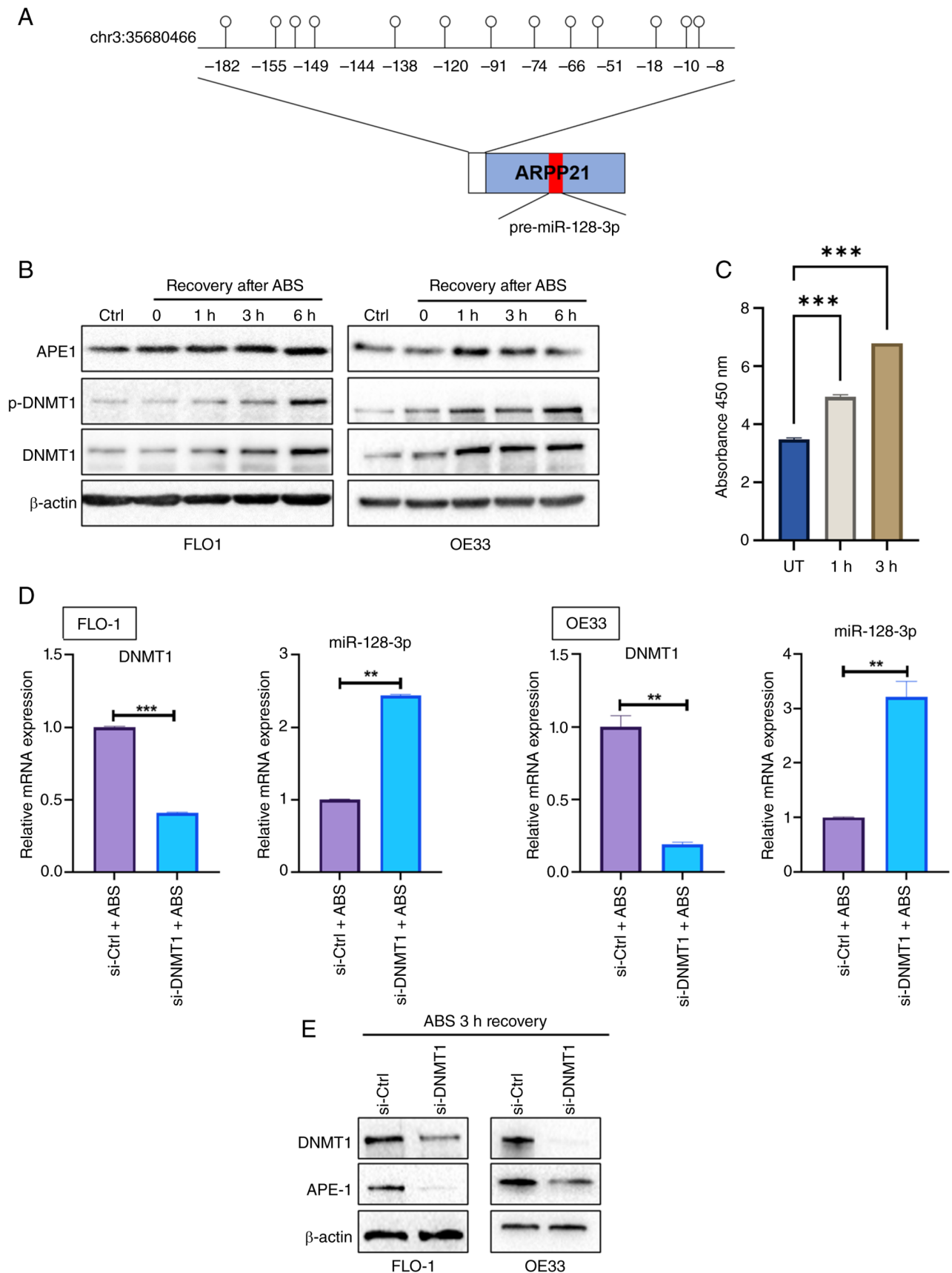


Figure 4. miR-128-3p expression is regulated by methylation. (A) Schematic diagram of the promoter of miR-128-3p showing CpG island with CpG nucleotides (open circles). (B) Western blot analysis of DNMT1 and APE1 protein levels following ABS exposure and recovery in FLO-1 and OE33 cells. (C) DNMT activity assay in OE33 cell nuclear extract in the presence or absence of ABS treatment. (D) Reverse transcription-quantitative PCR for DNMT1 and miR-128-3p in OE33 and FLO-1 cells following ABS treatment in the presence or absence of si-DNMT1. (E) Western blot analysis of DNMT1 and APE1 protein levels following ABS treatment in the presence or absence of si-DNMT1. β-actin was used for normalization. ** $P < 0.01$; *** $P < 0.001$. miR, microRNA; APE, Apurinic/Apyrimidinic Endonuclease; ABS, acidic bile salt; DNMT, DNA methyltransferase; si, small interfering; Ctrl, control; p-, phosphorylated; UT, untreated.

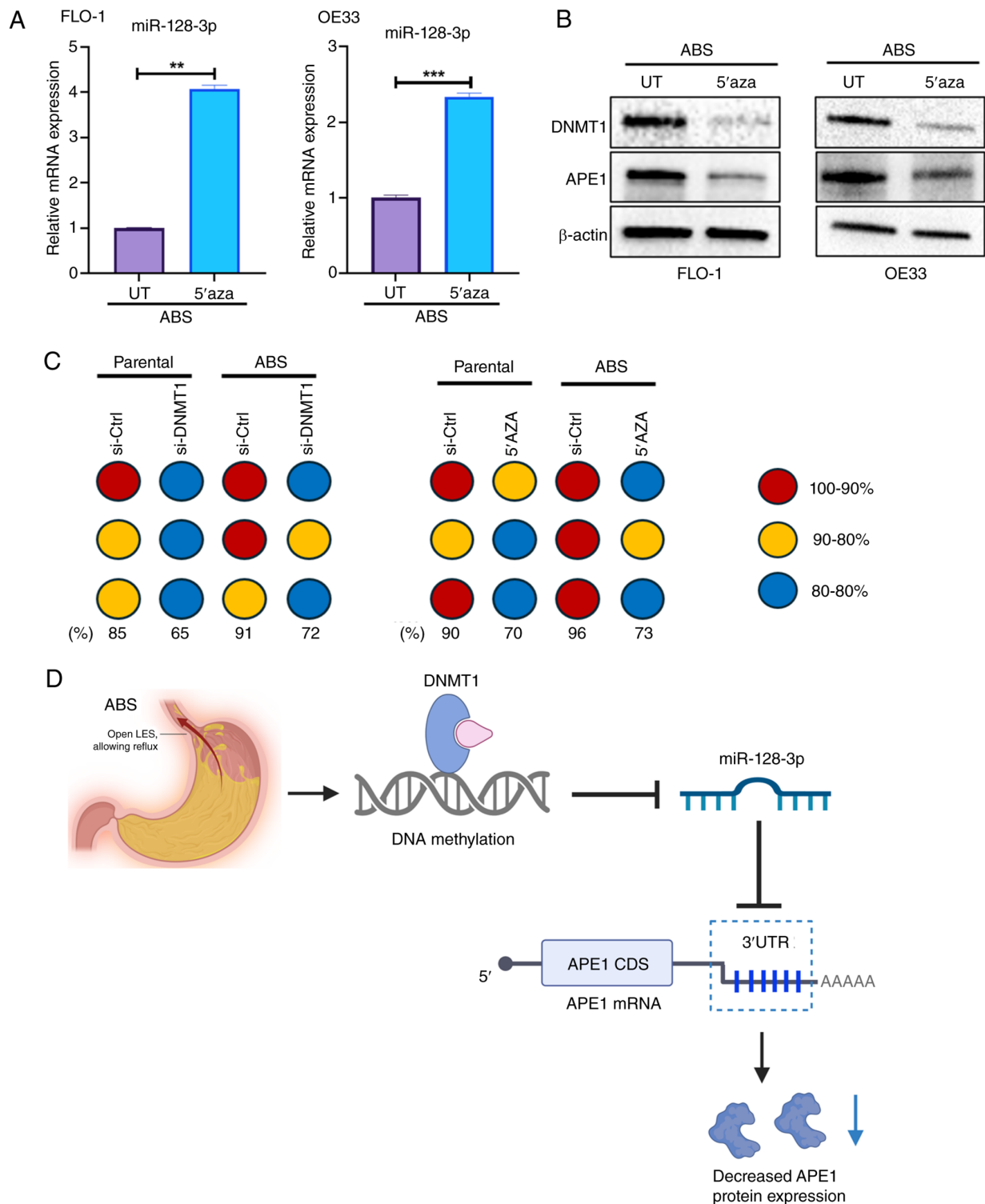


Figure 5. DNA methylation regulates miR-128-3p expression. (A) Reverse transcription-quantitative PCR for miR-128-3p and (B) western blotting of DNMT1 and APE1 in OE33 and FLO-1 cells following ABS treatment in the presence or absence of inhibition of DNMT1 using 5'aza. (C) Pyrosequencing analysis of miR-128-3p following ABS treatment in the presence or absence of knockdown or inhibition of DNMT1. Values indicate the average methylation percentage of CpG sites in control and 5'aza treated cells. (D) Schematic diagram showing reflux-induced DNMT1-mediated silencing of miR-128-3p promotes APE1 upregulation in esophageal adenocarcinoma. ** $P < 0.01$; *** $P < 0.001$. miR, microRNA; DNMT, DNA methyltransferase; APE, Apurinic/Apyrimidinic Endonuclease; aza, azacitidine; ABS, acidic bile salt; UT, untreated; si, small interfering; Ctrl, control; LES, lower esophageal sphincter; CDS, coding DNA sequence; ABS, acidic bile salt; UTR, untranslated region.

miR-128-3p is a context-dependent miRNA that is frequently downregulated in human cancers and typically functions as a tumor suppressor. In multiple tumor types, loss of miR-128-3p has been linked to increased cell proliferation, enhanced stem-like

properties, decreased apoptosis and greater resistance to therapy, primarily through upregulation of oncogenic targets involved in survival and cell cycle control (24-26). Hauser *et al* (24) showed that restoring miR-128-3p expression suppresses head

and neck squamous cell carcinoma growth and promotes apoptosis in cell culture and xenograft models. Mechanistically, miR-128-3p directly regulated several cancer-associated targets, including BCL2-associated athanogene 2 and BAX, supporting a tumor-suppressive role and suggesting potential value as a therapeutic or biomarker candidate in head and neck squamous cell carcinoma (24). Jin *et al* (26) showed that miR-128-3p functions as a tumor-suppressive microRNA in prostate cancer by restraining tumor-initiating, stem-like cell properties. miR-128-3p was reduced in prostate cancer, and its restoration inhibited proliferation, clonogenicity, sphere formation, and tumor regeneration in xenograft models by directly targeting the stemness regulator polycomb complex protein BMI-1 (26). miR-128-3p serves as an oncogenic miRNA in a context-dependent manner by suppressing tumor-suppressive targets and promoting cancer cell proliferation, migration, survival and therapy resistance. In osteosarcoma, for example, miR-128-3p downregulates zinc finger CCCH-Type Containing 12D (ZC3H12D), increases proliferation and migration and enhances cisplatin resistance, supporting its pro-tumor role (27). In glioblastoma, miR-128-3p is a tumor suppressor whose downregulation promotes malignancy and treatment resistance (28). In breast cancer, miR-128-3p is tumor-suppressive with inhibitory effects on proliferation, invasion, stemness and chemoresistance (25), while one study reported miR-128-3p as an oncomiR via hypermethylated in cancer 1 (HIC1) suppression (29). Although its expression pattern may vary across cancer, evidence (24-26,28) supports a tumor suppressor role for miR-128-3p in numerous malignancies, where restoration of its activity suppresses tumor progression and highlights its potential as both a biomarker and therapeutic target.

miR-128-3p is downregulated by DNA methylation in multiple cancers. For example, Yu *et al* (30) demonstrated that methylation silences miR-128-3p in gastric cancer, triggering epithelial-mesenchymal transition via the PI3K/AKT pathway. Similarly, Takahashi *et al* (31) reported that DNA methylation suppresses miR-128-3p in colorectal cancer, where it exerts effects through the NEK2 pathway. In line with these studies, the present analysis revealed the presence of a CpG island within the miR-128-3p promoter, suggesting epigenetic regulation. Under reflux-mimicking conditions, there was a marked increase in DNMT1 expression, accompanied by complete suppression of miR-128-3p and a corresponding increase in APE1 protein levels. Restoration of miR-128-3p expression following DNMT1 knockdown or inhibition using 5'aza resulted in decreased APE1 levels. Although 5'aza inhibits all DNMT family members, the present effects were consistent with those obtained using DNMT1-specific siRNA, supporting a DNMT1-dependent mechanism. These findings suggested that reflux-induced DNMT1 promoted APE1 upregulation through epigenetic silencing of miR-128-3p, highlighting a novel regulatory axis that may contribute to EAC pathogenesis.

Beyond DNA methylation, alternative epigenetic mechanisms may regulate miR-128-3p in a context-dependent and tissue-specific manner. Histone modifications are potential regulators, particularly histone 3-trimethylated lysine 27 residue (H3K27me3)-mediated suppression. Wang *et al* (32) showed that increased H3K27me3 at the miR-128-3p promoter is associated with reduced miR-128-3p expression, while lysine demethylase 6B-mediated removal of H3K27me3 promotes miR-128-3p

expression. This suggests that suppressive Polycomb-linked chromatin silences miR-128-3p, whereas histone demethylation could reactivate it (32). Chromatin remodeling is another potential mechanism of miR-128-3p regulation either by facilitating transcription factors binding at the miR-128-3p promoter or by enforcing suppression through maintaining a compact nucleosomal state. To the best of our knowledge, however, no direct evidence has been reported on this yet.

miR-128-3p has emerged as a key tumor-suppressive microRNA in cancer, where its reduced expression is typically associated with enhanced proliferation, survival and disease progression (24-26,28). In EAC, downregulation of miR-128-3p may relieve post-transcriptional suppression of APE1, thereby permitting APE1 upregulation and contributing to a pro-tumorigenic cellular state. Thus, loss of miR-128-3p may represent a key regulatory event linking epigenetic dysregulation to aberrant APE1 expression in reflux-associated EAC tumorigenesis. With several DNMT1 inhibitors such as azacitidine and decitabine approved by the US Food and Drug Administration (FDA) for treatment of certain hematological malignancies (17), DNMT1 inhibitors could be a plausible therapeutic strategy to target the oncogenic functions of APE1 in patients with EAC. Despite their therapeutic promise, systemic DNMT inhibitors are associated with potential toxicity and off-target effects, including global hypomethylation and unintended gene activation in normal tissue (33). These limitations underscore the need for evaluation of safety and specificity, particularly in the context of solid tumors such as EAC. Accordingly, rigorous preclinical validation in clinically relevant models, including PDXs, is essential to assess therapeutic efficacy, toxicity profiles and on-target epigenetic modulation prior to clinical translation.

Acknowledgements

Not applicable.

Funding

The present study was supported by the US National Institutes of Health (grant nos. P01CA268991 and R01CA224366).

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

FB, LF and WER conceived the study. FB, HL, LC and DP designed the experiments. FB, HL, ZC and WER analyzed and interpreted data. FB, LF, HL and WER wrote and revised the manuscript. AZ, JQ and OM analyzed and interpreted data. SC and XS performed bioinformatics analysis. All authors have read and approved the final manuscript. FB and WER confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All samples were de-identified and met the criteria for non-human subjects research in accordance with NIH

guidelines and institutional policy. The study used RNA isolated from frozen, de-identified human tissue specimens previously obtained from the pathology archives at Vanderbilt University (Nashville, TN, USA) and the National Cancer Institute (Bethesda, MD, USA) Cooperative Human Tissue Network. The original collection of coded specimens was approved by the Vanderbilt University (Nashville, TN, USA) Institutional Review Board (approval no. #VU111096). The use of de-identified specimens was approved by the University of Miami (Miami, FL, USA; approval no. #20240548), which determined that the work meets the criteria for exemption under 45 CFR 46.104. All patients provided written informed consent for use of tissue samples.

Patient consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

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