APE1 Upregulates MMP-14 via Redox-Sensitive ARF6-Mediated Recycling to Promote Cell Invasion of Esophageal Adenocarcinoma

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Abstract

Esophageal adenocarcinoma (EAC) is an aggressive malignancy with poor clinical outcome. The incidence of EAC has been rising rapidly in the past three decades. Here, we showed that apurinic/apyrimidinic endonuclease (APE1) is overexpressed in EAC cell lines, and patients' samples of dysplasia and EAC. Downregulation of APE1 or inhibition of its redox function significantly repressed invasion. Overexpression of a redox-defective mutant, C65A, abrogated the proinvasive phenotype of APE1. APE1 regulated invasion via upregulation of matrix metalloproteinase 14 (MMP-14), which subsequently activated MMP-2, leading to degradation of the extracellular matrix in a redox-dependent manner.

Introduction

The incidence of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) has increased at an alarming rate over the past few decades in the United States and the Western world (1, 2). Chronic gastroesophageal reflux disease (GERD) is the main risk factor for the development of BE and its progression to EAC with invasive and metastatic features (3–6). Previous studies have shown that acidic bile salts (ABS), due to GERD, are implicated in the development of BE and EAC by inducing sustained high levels of oxidative stress, DNA damage, and mitochondrial damage (7–12).

APE1 (apurinic/apyrimidinic endonuclease), also known as redox factor 1 (REF-1), is a dual functional protein. On one hand, APE1 is a key enzyme in DNA base excision repair (BER) pathway, acting as a major apurinic/apyrimidinic endonuclease in the repair of damaged or mismatched nucleotides; on the other hand, APE1 regulates the activity of various redox-dependent transcription factors, such as p53, NF-κB, and STAT3 (13–15) by main-23). MMP-14 expression and protein levels are regulated by multiple molecular mechanisms (24).

In this study, we report a novel function of APE1 involved in regulating cellular invasion. We demonstrate that APE1 upregulates MMP-14 protein levels via ARF6-mediated endocytosis/recycling. We demonstrate that APE1 interacts with ARF6 and induces ARF6 activity in a redox-dependent manner.
Collectively, our findings uncover a novel redox-dependent signaling axis of APE1/ARF6/MMP-14 in promoting invasion of EAC cells.

Materials and Methods

Cell lines and reagents

Human CP-B cells were obtained from ATCC. OE19 and ESO26 cell lines were purchased from Sigma-Aldrich. FLO-1 and OE33 cell lines were a kind gift from Dr. David Beer (University of Michigan, Ann Arbor, MI). SK-GT-4 cell line was kindly provided from Dr. Xiaochun Xu (MD Anderson Cancer Center, Houston, TX). OE19, OE33, and ESO26 cells were maintained in RPMI medium (GIBCO), supplemented with 10% FBS (Invitrogen Life Technologies), 1% penicillin/streptomycin (GIBCO), and 2 mmol/L L-glutamine (GIBCO). FLO-1 and OE-GT-4 cells were maintained in DMEM (GIBCO) containing 10% FBS and 1% penicillin/streptomycin. CP-B cells were maintained in Keratinocyte-SFM (GIBCO) containing 5% FBS and 1% penicillin/streptomycin. The cells were periodically tested for Mycoplasma contamination, using mycoplasma detection Kit (PCR) purchased from SouthernBiotech, last checked in December 2018. All cell lines were ascertained to conform to the original in vitro morphologic characteristics and were authenticated by using short tandem repeat profiling (Genetica DNA Laboratories). All cell lines were used between passages 4 and 15 from the time of their arrivals.

Antibodies and reagents

Anti-MMP-14 antibody for Western blot analysis was purchased from Abcam. Anti-APE1 antibody (MA1-440) and Alexa Fluor 488 Phalloidin (A12379) were obtained from Thermo Fisher Scientific. Anti-actin antibody was purchased from Sigma-Aldrich. E3330 (APE1 redox-specific inhibitor) was purchased from Novus Biologicals, and APE1-i3 (APE1 DNA repair-specific inhibitor) was purchased from MilliporeSigma. The usage of inhibitors were following pharmacologic studies with recommended doses for the E3330 (25 μmol/L), E3464 (Abcam) and IHC Select Immunoperoxidase Secondary Detection System (DAB500, MilliporeSigma) were utilized for staining, and specimens were counterstained with hematoxylin, following manufacturer’s instructions. Specificity of immunostaining was checked by replacing the primary antibody with nonimmune serum. IHC results were evaluated for intensity and frequency of the staining and an index score was applied as described previously (29).

3D organotypic culture

3D organotypic cultures of shAPE1 knockdown cells and control cells (shCtrl) in CPB or FLO-1 cells were performed, as described previously (30). Briefly, human esophageal fibroblasts (ScienCell) were seeded into a 3D matrix (75,000 cells/well) containing collagen 1 (high concentration rat-tail collagen; Corning) and Matrigel (BD Biosciences) and incubated for 7 days at 37°C. Following incubation, the cells were seeded (500,000 cells/well) on top of the fibroblast matrix. After culturing for an additional 7 days, the cells were harvested, fixed in 70% ethanol, and processed for hematoxylin and eosin staining and immunocytochemistry.

Immunocytochemistry of 3D organotypic cell cultures

Paraffin-embedded organotypic culture slides were deparaffinized and rehydrated following standard protocols. Antigen retrieval was performed by boiling the slides in 1 mmol/L Tris EDTA, pH 8.0 for 10 minutes. Slides were allowed to cool down to room temperature before incubation in 10% normal goat serum blocking solution (Thermo Fisher Scientific) for 30 minutes. Primary antibodies of anti-APE1 (diluted at 1:500) and anti-MMP-14 (diluted at 1:200) were added to the slides and incubated overnight at 4°C in a humidified chamber. The next day following incubation, the slides were washed with PBS and incubated with Alexa Fluor conjugated anti-mouse or anti-rabbit secondary antibody (Alexa Fluor-488 or Alexa Fluor-568) for 1 hour at room temperature, protected from light. The slides were washed again with PBS and mounted with a Vectashield mounting medium with DAPI (Vector Laboratories). Images were obtained with an Olympus microscope with ImageJ software (National Institutes of Health, Bethesda, MD). Images were
In-gel zymography
MMP activities were assayed following the manufacturer’s instructions (Invitrogen Novex Zymogram Gels; Thermo Fisher Scientific). Briefly, 5 × 10⁵ cells in a 6-well plate were cultured in serum-free medium for 16 to 24 hours, and the conditioned medium was separated on a SDS-PAGE gel containing 1 mg/mL gelatin. The gel was washed with buffer I [Tris-HCl (pH 7.5) and 2.5% Triton X-100] incubated overnight in buffer II [150 mmol/L NaCl, 5 mmol/L CaCl₂, 50 mmol/L Tris-HCl (pH 7.6)] at 37°C and stained with Coomassie blue. The clear bands indicate where MMPs degraded gelatin.

In situ zymography
Total 10⁶ cells were washed with DMEM and seeded on DQ gelatin fluorescein conjugate (Thermo Fisher Scientific) coated cover glass and cultured with serum-free media for 16 hours. The fluorescence release resulting from MMP cleavage of the matrix was visualized by fluorescence microscopy.

Endocytosis and recycling assays
Cell surface protein biotinylation was performed as described previously (31). The cells were transfected with HA-tagged MMP-14 for overexpression. Subconfluent cells grown on 60-mm dishes were washed twice with ice-cold PBS and incubated for 15 minutes. Biotinylation was performed by incubating cells in PBS containing 0.5 mg/mL of the EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) for 30 minutes at 4°C, followed by washing and quenching of free biotin. Cells were then incubated at 37°C for different time lapses to allow endocytosis, before 2 successive reduction reagent (glutathione) treatments, which can remove the biotin from the cell surface. The cells treated with glutathione without 37°C incubation were used as a negative control, whereas the cells without glutathione treatment were used as a positive control. To determine endocytic levels of HA-MMP-14 protein, the cells were lysed and the biotinylated surface proteins were precipitated with high-capacity streptavidin agarose (Thermo Fisher Scientific) and eluted with 0.5% Triton X-100 incubated overnight in buffer I [150 mmol/L NaCl, 5 mmol/L CaCl₂, 50 mmol/L Tris-HCl (pH 7.6)] at 37°C and stained with Coomassie blue. The clear bands indicate where MMPs degraded gelatin.

Cycloheximide chase assay
CPB-scramble and CPB-shAPE1 knockdown cells were treated with 100 μg/mL cycloheximide (CHX) before lysates were collected at different time points and analyzed for MMP-14 protein levels by Western blot analysis. FLO-1 cells were pretreated with 40 μmol/L E3330 for 48 hours followed by treatment with CHX. The half-life of MMP-14 protein was determined by relative MMP-14 protein band intensity (MMP-14/actin) at different time points.

ARF6 activity assay
ARF6 activation assay was performed following the manufacturer’s instructions (Cell Biolabs, Inc.). Briefly, whole cell lysates from APE1 knockdown cells and scramble shRNA control cells or E3330-treated cells were prepared in RIPA buffer and the active form of ARF6 (GTP-ARF6) was specifically pulled down by GGA3 protein-binding domain (PBD) agarose beads. The effect of APE1 on ARF6 activity was further validated by Western blot analysis. Quantification was based on relative ARF6 protein band intensity (GTP-ARF6/total ARF6).

Proximity ligation assay
In situ protein–protein interactions were detected using the Duolink In Situ Proximity Ligation Assay (PLA) Detection Kit (Sigma-Aldrich) following the manufacturer’s instructions. Cells were cultured in 8-well chamber slides for 24 hours and then washed in PBS and fixed with 4% paraformaldehyde buffer for 45 minutes at room temperature. Cells were then permeabilized in 0.5% Triton X-100 in PBS for 5 minutes, blocked for 45 minutes at room temperature with gentle shaking, and incubated overnight at 4°C with the 2 primary antibodies raised against the 2 proteins of interest, each from a different host species. The primary antibodies (APE1, Mouse monoclonal; Thermo Fisher Scientific; ARF6, Rabbit Monoclonal; Abcam) were used. Hybridizations, ligations, washings, and detection steps were performed following the supplier’s protocol. After final washes in buffer B, cells were mounted using the Duolink in situ mounting medium with DAPI, sealed with nail polish, and allowed to dry for 15 minutes at room temperature before imaging using the All-in-One Fluorescence Microscope (BX-700; Keyence Corp.).

Statistical analysis
All the results were expressed as mean ± SEM. Differences were analyzed by Student t test or 1-way ANOVA followed by the Bonferroni post hoc test. All statistical analyses were performed using the GraphPad Prism, version 5.0 (GraphPad Software). P < 0.05 was considered statistically significant.

Results
APE1 silencing decreases cell invasion capabilities
Earlier studies have shown that APE1 is highly expressed in tissue samples and representative cell lines of dysplastic BE and EAC (13, 19). To study the functional role of APE1 in EAC, we generated stable shAPE1 knockdown cell lines and relevant control knockdown cells (shCtrl) in CPB and FLO-1 cells (Fig. 1A). Interestingly, we observed that shAPE1 knockdown cells changed the cellular shape from spindle-like to a cobblestone-like shape (Fig. 1B). This remarkable morphology change is usually associated with enhanced invasion capacities. Therefore, we performed in vitro Matrigel invasion assay to determine the impact of APE1 knockdown on cell invasion. APE1 silencing significantly diminished relative invasion ratio (P < 0.005) in CPB and FLO-1 cells, as compared with control cells (Fig. 1C), consistent with cell morphology changes. Additionally, APE1 knockdown by siRNA confirmed that APE1 is required for FLO-1 cell invasion (Supplementary Fig. S1A and S1B).

APE1 redox function is required for cell invasion
APE1 is a multifunction protein with DNA base excision repair and redox functions. To determine which function of APE1 is involved in APE1-dependent cell invasion capabilities, we treated CPB, FLO-1, OE19, and ESO26 cells with specific APE1 inhibitors,
E3330 or APE1-i3, respectively. E3330, known as a redox-specific inhibitor, whereas APE1-i3 is mainly an inhibitor of DNA damage repair function of APE1 (28). By using in vitro Matrigel invasion assays, we found that E3330 treatment significantly diminished cell invasion ($P < 0.01$), whereas APE1-i3 treatment had no significant effect on invasion, as compared with control cells (Fig. 2A; Supplementary Fig. S2A). Interestingly, we also observed that E3330 treatment changed FLO-1 cell morphology (spindle-like) to a less invasive shape (cobblestone-like), whereas APE1-i3 did not induce these changes (Supplementary Fig. S2B). To avoid their cytotoxic effects on cell viability, we treated cells with relatively low doses of these inhibitors on our invasion experiments (Supplementary Fig. S2C). To verify the inhibitors’ functions, we reconstituted APE1 expression in shAPE1 FLO-1 cells by overexpression of control vector (Ctrl), FLAG-tagged wild-type APE1, APE1-C65A (redox-defective mutant), or APE1-H309N (DNA-repair-defective mutant; ref. 32). APE1 silencing decreased FLO-1 cell invasion, comparing shAPE1 to shCtrl. More importantly, we detected a significant rescue of cell invasion by reconstitution of wild-type APE1 or H309N mutant, as compared with the control vector (Ctrl; $P < 0.05$). However, unlike wild-type APE1 or H309N mutant, redox-defective C65A mutant failed to rescue cell invasion ($P < 0.01$). The ectopic expression of flag-tagged wild-type APE1 or relevant mutants was confirmed by Western blot analysis (Fig. 2B). Additionally, we found that the C65A mutant did not promote cell invasion as wild-type APE1 or H309N mutant in OE33 and SK-GT-4 cells (Supplementary Fig. S2D). Taken together, these results indicate that the redox function of APE1, not DNA repair function, is required for cellular invasion.

APE1 upregulates MMP-14 protein levels in dysplastic BE and EAC

Overexpression of MMP-14 is known to mediate invasion in several cancer types. Western blot analysis suggested a correlation between APE1 and MMP-14 in several esophageal cell lines; CPB, FLO-1, and ESO26 cells showed higher protein levels of APE1 and MMP-14, whereas OE19, OE33, and SK-GT-4 cells exhibited lower levels of both proteins (Fig. 3A). Stable APE1-silencing induced a consistent decrease in MMP-14 protein levels in several cell models, including CPB, FLO-1, OE33, OE19, and ESO26 cell lines (Fig. 3B). We also observed similar results in transient APE1 knockdown by siRNA in FLO-1 cells (Supplementary Fig. S1B). Conversely, overexpression of APE1, by an adenoviral expression system, in cell models with low endogenous levels consistently increased MMP-14 protein levels in OE33 and SK-GT-4 cells (Fig. 3C). Furthermore, we investigated the protein levels of APE1 and MMP-14 in normal esophagus, non-dysplastic BE, dysplastic BE, and EAC tissues by IHC in TMAs (Fig. 3D). The results demonstrated similar protein expression profiles of MMP-14 and APE1, as indicated by the lack of expression in normal esophagus tissues, weak expression in non-dysplastic BE, high expression in dysplastic BE and EAC. A summary of IHC scores is given in Supplementary Table S1. Of note, dysplastic BE and EAC tissue samples demonstrated significantly higher protein levels of MMP-14 ($P < 0.01$) and APE1 ($P < 0.01$) than normal and non-dysplastic BE tissues. In dysplastic BE and EAC tissues, MMP-14 IHC staining was on the cell surface and cytosol, whereas APE1 staining was diffuse nuclear and cytosolic. Pearson correlation analysis indicated a trend of association between MMP-14 and APE1 in EAC tissues, although not statistically significant ($P = 0.149$), possibly due to small sample size.
APE1 upregulates MMP-14 protein level on cell surface via its redox function

The cell surface expression of MMP-14 is critical for ECM degradation and cancer cell invasion. We, therefore, performed immunofluorescence staining to examine the role of APE1 on MMP-14 cell surface localization and expression. APE1 silencing significantly decreased MMP-14 protein levels on the cell surface in CPB and FLO-1 cells, as compared with control cells (P < 0.05, Fig. 4A). Next, we examined the effects of APE1 redox function on MMP-14 cell surface expression. Immunofluorescence data indicated that inhibition of APE1 redox activity by E3330 had no effect on APE1 protein levels or cellular localization, as expected, but significantly diminished MMP-14 on cell surface in CPB and FLO-1 cells, relative to control cells.
Moreover, Western blot analysis (Supplementary Fig. S3A) and immunofluorescence staining (Supplementary Fig. S3B) showed C65A mutant could not rescue MMP-14 expression as wild-type APE1 or H309N in shAPE1 FLO-1 cells. By using organotypic 3D culture system, we confirmed that APE1-silencing decreased MMP-14 protein levels, as compared with control cells (Fig. 4C). Additionally, hematoxylin and eosin staining showed APE1 silencing (shAPE1) greatly reduced invasive cells (indicated with red arrows) that breached into the basement membrane and infiltrated into the Matrigel containing fibroblasts layer in the organotypic 3D culture (Supplementary Fig. S3C). Collectively, our results suggest that APE1 upregulates MMP-14 protein on cell surface through APE1-dependent redox function.

APE1 redox function is required for MMP-14 activity

MMP-14 is known to cleave MMP-2 pro-form (72 kDa) to generate MMP-2 active-form (62 kDa; ref. 33). To investigate whether APE1 and its redox function are required for MMP-14 activity, we examined APE1 effects on MMP-14 activation of MMP-2 proteinase. We concentrated conditioned media (C.M.) to detect total MMP-2, and collected whole cell lysates (WCL) for APE1 and MMP-14 protein analysis. Western blot analysis results indicated that APE1 knockdown decreased protein levels of MMP-2 (pro-form, 72 kDa), as well as MMP-14 in CPB and FLO-1 cells, as compared with the relevant control cells (Fig. 5A). Additionally, in-gel zymography assay data revealed that APE1 silencing significantly decreased active-MMP-2 levels in C.M. in CPB and FLO-1 cells relative to control cells, respectively \( (P < 0.01, \text{Fig. 5B}) \). At the same time, we found that APE1 knockdown had no effect on MMP-9 activation, suggesting that MMP-2, not MMP-9, is the downstream effector of APE1-MMP-14-dependent invasion. Furthermore, in situ zymography assay data indicated that APE-1 knockdown or inhibition of APE1-specific redox function by E3330 significantly reduced ECM degradation activities, as demonstrated by levels of quenched fluorescein-labeled gelatin (DQ-gelatin), in CPB and FLO-1 cells, as compared with control cells, respectively \( (P < 0.01, \text{Fig. 5C and D}) \).

APE1 regulates MMP-14 protein stability

To determine how APE1 regulates MMP-14, we first investigated if APE1 affects MMP-14 mRNA levels. qPCR data indicated that APE1 silencing had no significant effect on MMP-14 mRNA levels in CPB, FLO-1, or OE33 cells, as compared with control cells,
respectively (Supplementary Fig. S4A). These results suggested that APE1 regulates MMP-14 through a post-transcriptional mechanism at the protein level. By using CHX chase assay to determine possible changes in MMP-14 half-life, we found that APE1 silencing decreased MMP-14 protein half-life from ~4 to ~1 hour in CPB cells (Fig. 5E) and from ~11 to ~4 hours in FLO-1 cells (Fig. 5F). Additionally, inhibition of APE1 redox activity by E3330 significantly decreased MMP-14 protein half-life from ~10 to ~4 hours in FLO-1 cells as compared with control cells (Supplementary Fig. S4B). Taken together, these data indicated

![Image](https://cancerres.aacrjournals.org/content/79/17/4432/F4.large.jpg)
Figure 5. Knockdown of APE1 reduces active MMP-2 form and ECM degradation by downregulating MMP-14 protein level. A, Conditioned medium (CM) from CPB, FLO-1, or relevant APE1 knockdown cells was collected, concentrated, and examined by Western blot analysis for MMP-2. The whole cell lysates from the relevant dishes were collected for Western blots to detect MMP-14, APE1, and β-actin. B, In-gel zymography assay. The concentrated conditioned medium was loaded in gelatin gel to test MMP-2 activities. Arrows, pro- or active-forms of MMPs. Active MMP-2 levels were measured by the intensity ratio of active MMP-2/actin. C, In situ zymography assay was performed by using APE1 knockdown cells or control cells on DQ-gelatin precoated slides at 48 hours after cell seeding. D, In situ zymography assay by the cells treated with 40 μmol/L E3330 or vehicle control (Ctrl). ECM degradation activities were measured by immunofluorescent intensities. E and F, CHX chase assays were performed by using 100 μg/mL CHX in CPB (E) and FLO-1 (F) cells. The cell lysates were collected at the indicated time points for Western blot analysis of APE1 knockdown cells (shAPE1) or control cells (shCtrl). Measurement of MMP-14 protein stability was based on the intensity ratio of MMP-14/actin. All quantification analyses were based on independent triplicate experiments. *, P < 0.05; **, P < 0.01.
that APE1 mediated MMP-14 protein stability, not mRNA expression in a redox-dependent manner.

APE1 redox function is required for MMP-14 endocytic trafficking

Endocytosis and recycling are the most critical regulatory processes of cellular MMP-14 protein in cancer invasion. To investigate the role of APE1 in the endocytic trafficking of MMP-14, we monitored endocytosis and recycling of exogenously expressed HA-MMP-14 protein following APE1 knockdown in FLO-1 cells. The internalized HA-MMP-14 protein was detected by biotin-streptavidin pull-down at different time points. APE1 knockdown completely abolished MMP-14 endocytosis at 5 minutes ($P < 0.01$) and dramatically decreased it at 7.5 minutes ($P < 0.05$), relative to control cells (Fig. 6A). At 10 minutes, we observed a decrease in endocytic MMP-14 in control cells (shCtrl, as compared with 7.5 minutes time point, suggesting that endocytic MMP-14 has been partially recycled back to the cell surface at 10 minutes. The results strongly suggested that APE1 silencing significantly slowed down the rate of MMP-14 endocytosis in EAC cells. Moreover, consistent with APE1 knockdown data, inhibition of APE1 redox function by E3330 significantly enhanced endocytosis of MMP-14 at 5 minutes ($P < 0.01$) and at 7.5 minutes ($P < 0.05$) in FLO-1 cells, relative to control cells (Fig. 6B). These results suggested that APE1 regulation of the rate of MMP-14 endocytosis involved its redox function. Notably, recycling assay data indicated that APE1 knockdown, as well as APE1 redox function inhibition (E3330), significantly diminished MMP-14 recycling at 10 minutes ($P < 0.05$) and 15 minutes ($P < 0.01$) in FLO-1 cells, as compared with control cells, respectively (Fig. 6C and D). The results indicated that APE1 silencing or inhibition of APE1-redox function significantly delayed MMP-14 recycling. Taken together, our data demonstrated that APE1 and its redox function are required to maintain a high rate of MMP-14 endocytic trafficking (endocytosis and recycling), a critical factor for cancer invasion.

APE1 promotes ARF6 activity via interaction and redox function

To further examine the role APE1 has in MMP-14 endocytic trafficking, we screened known major regulators of endocytosis and recycling, such as EHD1, ARF6, Rab5, and Rab4 for protein interaction with APE1 by immunoprecipitation (IP). The results demonstrated a novel protein interaction of ARF6 and APE1, which was strong in FLO-1 cells and consistent in CPB cells (Fig. 7A; Supplementary Fig. S5A). In line with these results, PLA data confirmed the close proximity of ARF6 and APE1 proteins in the cytoplasm in both FLO-1 and CPB cells, as compared with negative control by a single antibody (Fig. 7B). As APE1 silencing had no significant effect on ARF6 protein levels in our in vitro models (Supplementary Fig. S5B), we examined ARF6 activity by GGA3 PBD agarose beads that specifically pull-down GTP-binding ARF6, which is the active form of ARF6. Both stable shRNA knockdown (Fig. 7C) and transient shRNA knockdown (Supplementary Fig. S5C) of APE1 significantly attenuated ARF6 activities ($P < 0.05$) in CPB and FLO-1 cells relative to control cells, respectively. In addition, APE1 redox inhibition (E3330) significantly decreased active-ARF6 levels ($P < 0.05$) in CPB and FLO-1 cells as compared with control cells, respectively (Fig. 7C). However, E3330 treatment or FLAG-tagged APE1-C65A (redox-defective) mutant had no significant effect on APE1-ARF6 interaction (Supplementary Fig. SSD and SSE). Interestingly, ARF6-specific activity inhibitor, NAV-2729 (34), inhibited active ARF6 (GTP-ARF6) and abolished MMP-14 protein elevation induced by APE1-overexpression (Ad-APE1) in OE33 and SK-GT-4 cells (Fig. 7D), suggesting that APE1 upregulates MMP-14 in an ARF6-dependent manner. Further, we found NAV-2729 or MMPs inhibitor, GM6001, abolished APE1-elevated invasion capability ($P < 0.05$) in SK-GT-4 cells (Fig. 7E). Collectively, our data revealed a novel redox-sensitive signaling axis of APE1/ARF6/MMP-14 in cancer cell invasion. A cartoon summarizing our data is shown in Fig. 7F.

Discussion

Chronic gastro-esophageal reflux disease is the main risk factor for the development of BE and its progression to EAC. Exposure of esophageal cells to ABs, the mimicry of GERD episodes, can induce overexpression of APE1 in BE and EAC cells (13, 19). In this study, we uncovered a novel function of APE1 in promoting cancer cell invasion and report a molecular signaling axis that includes APE1, ARF6, and MMP-14 in cancer cell invasion.

Our findings suggest that overexpression of APE1 is required to promote invasion abilities in EAC cells. We consistently found that knockdown of APE1 or inhibition of its redox function remarkably diminished invasion. In fact, APE1 is known as a reducing donor and its cysteine 65 (cys65)-redox activity is essential to maintain a reduced status on specific cysteine residues of APE1-associated transcriptional factors and other signaling proteins (13, 35). Unlike wild-type APE1 or H309N (DNA-repair-deficient mutant), overexpression of a redox-deficient APE1 mutant (C65A) failed to promote invasion in ARF6 levels of EAC, confirming that APE1 redox activity is required for APE1 pro-invasion function. The role of APE1 in promoting cell migration or invasion has been described in other cancer types such as breast cancer cells (36), pancreatic cancer cells (37), and hepatocellular carcinoma cell lines (38). However, the mechanism by which APE1 mediates invasion, remains unclear and largely unknown.

To our knowledge, our study is the first to describe the role of APE1 in promoting cell invasion in EAC or Barrett’s tumorigenesis. Therefore, we initiated investigations of the underlying mechanism by which APE1 mediates cell invasion. It is widely known that the persistent high levels of MMP-14 on cell surface enhances MMP-2 activation (39, 40) and ECM degradation (21–23), facilitating cancer cell migration and invasion (24, 41–43). Here we present several lines of evidence indicating that APE1 is required for increasing MMP-14 and active MMP-2, and ECM degradation. IHC staining of tissue array indicated a possible positive correlation between MMP-14 and APE1 protein levels in dysplastic BE and EAC. Indeed, we demonstrated that APE1 silencing or blockade of APE1 redox function decreased MMP-14 protein levels, MMP-2 activity and ECM degradation. The results confirm that APE1 regulates MMP-14/MMP-2/ECM degradation signaling cascade. Among ECM components, multiple targets of MMP-14 and MMP-2 cleavage activities include CD44 (44), Notch1 (45), VEGF (46) and latent TGF-β (47), suggesting that APE1-redox/MMP-14/MMP-2 axis could activate multiple signaling pathways associated with cancer cell invasion.

Although APE1 is recognized as a transcriptional cofactor for its redox regulation on important transcriptional factors, such as p53, NF-κB and STAT3 (13–15), our study clearly indicated that APE1 elevated MMP-14 protein levels on cell surface by
modulating endocytosis and recycling, not through mRNA regulation. Our results demonstrate a different signaling axis for APE1, not reported in earlier invasion studies (38). Furthermore, proximity ligation and IP assays revealed a close proximity and an interaction between APE1 and ARF6 proteins. Notably, the main cellular functions of ARF6 include regulation of endocytosis and recycling of plasma membrane proteins (48, 49). ARF6 plays a critical role in regulating MMP-14 endocytic trafficking, required for maintaining high protein levels of MMP-14 on cancer cell surface that mediates ECM degradation/remodeling and invasion (50–52). We demonstrated that APE1 redox function is critical for ARF6 activity and ARF6-mediated MMP-14 endocytic trafficking. Genetic knockdown or redox-specific inhibition (E3330) of APE1 repressed ARF6 activity and consequently

Figure 6.
APE1 is required for the rapid MMP-14 endocytic recycling in EAC cells. A and B, Endocytosis assays of HA-tagged MMP-14 in FLO-1 cells. Cells were transfected with HA-tagged MMP-14. The cell lysates were collected for biotin pulldown at the indicated time points of endocytosis (37°C incubation). C and D, Recycling assays of HA-tag-labeled MMP-14 in FLO-1 cells. Recycling assays were performed after 10 minutes endocytosis (37°C incubation). The cell lysates were collected for biotin pulldown at indicated time courses of recycling (second 37°C incubation). APE1 knockdown cells (shAPE1) and control cells (shCtrl) of FLO-1 were used (A and C). FLO-1 cells were pretreated with 40 μmol/L E3330 for 24 hours before experiments (B and D). Relative endocytic MMP-14 levels were measured by the ratio of endocytic MMP-14/total MMP-14. All quantification analyses were based on independent triplicate experiments. *, P < 0.05; **, P < 0.01.
decelerated MMP-14 endocytosis/recycling, suggesting that over-expression of APE1 activates ARF6 through its redox-function in cancer cells. We also observed clear effects of APE1 and redox on MMP-14 protein stability, which may be due to repressed recycling, increasing the chance of uptake of MMP-14 by lysosome for degradation. In addition, ARF6 activity inhibitor, NAV-2729, abrogated APE1-induced MMP-14 protein accumulation, further confirming the APE1–ARF6 axis in upregulating MMP-14.

Figure 7.
APE1 promotes ARF6 activity through interaction and redox function. A, IP of APE1 in CPB and FLO-1 cells. IgG IP works as a negative control. Western blots of APE1 and ARF6 were performed. B, PLA in FLO-1 and CPB cells were performed by using anti-ARF6 and anti-APE1 antibodies. Single antibody (anti-ARF6) was used as negative control. C, ARF6 activity assays in CPB and FLO-1 cells. The active ARF6 form, GTP-ARF6, was specifically pulled down from whole cell lysates by GGA3 PBD agarose beads. APE1, total ARF6, and β-actin in whole cell lysates, and GTP-ARF6 in pull down products were examined by Western blots. Stable APE1 knockdown cells (shAPE1), control cells (Ctrl), or the cells with 24 hours pretreatment of 40 μmol/L E3330 were harvested for cell lysates. Relative ARF6 activation was measured by the ratio of (GTP-ARF6)/total ARF6. D, APE1 was overexpressed in OE33 and SK-GT-4 cells by adenoviral infection (Ad-APE1). Cells were treated with 1 μmol/L specific ARF6 inhibitor, NAV-2729, for 48 hours. Whole cell lysates were collected for GTP-ARF6 pulldown and Western blot analysis. E, Representative images (magnification, ×200) of the invasion assays in SK-GT-4 cells. Control vector (Ctrl) or wild-type APE1 was overexpressed in the cells. APE1-overexpressed cells were pretreated and maintained with 10 μmol/L MMPs inhibitor, GM6001, or 1 μmol/L NAV-2729 through invasion assay. UT, untreated. All quantification analyses were based on independent triplicate experiments. F, Schematic summary of APE1-mediated MMP-14 endocytosis and recycling. MMP-14 is efficiently internalized from plasma membrane by clathrin-dependent or caveolar endocytosis. Active endocytosis and recycling of MMP-14 is critical for ECM degradation and cell invasion. APE1 promotes ARF6 activation through interaction and APE1-redox function. Active ARF6 (GTP-ARF6) in recycling endosome drives quick recycling of MMP-14. APE1 redox inhibitor, E3330, or ARF6-specific inhibitor, NAV-2729, can significantly decrease MMP-14 recycling to cell surface and subsequent cell invasion. Moreover, MMPs inhibitor, GM6001 can inhibit MMP-14 and MMP-2 activities and repress cell invasion. *, P < 0.05.
APE1 Promotes Cell Invasion via Activation of ARF6-MMP14

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Declarations
Ethics approval and consent to participate
All de-identified tissue samples were obtained from the archives of pathology at Vanderbilt University (Nashville, TN). The use of specimens from the tissue repository was approved by the Vanderbilt Institutional Review Board.

Authors’ Contributions
Conception and design: H. Lu, J. Hong, A. Belkhiri, W. El-Rifai
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In summary, our study identified a novel signaling axis for APE1 in promoting cancer cell invasion by activating ARF6-mediated MMP-14 recycling via APE1-redox function. This signaling axis may be critical in promoting invasion and progression not only in Barrett's tumorigenesis and EAC, but possibly in other cancer types.

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APE1 Upregulates MMP-14 via Redox-Sensitive ARF6-Mediated Recycling to Promote Cell Invasion of Esophageal Adenocarcinoma

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