

# Histone Deacetylase-3/CAGE Axis Targets EGFR Signaling and Regulates the Response to Anti-Cancer Drugs

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We have previously reported the role of miR-326-HDAC3 loop in anti-cancer drug-resistance. CAGE, a cancer/testis antigen, regulates the response to anti-cancer drug-resistance by forming a negative feedback loop with miR-200b. Studies investigating the relationship between CAGE and HDAC3 revealed that HDAC3 negatively regulated the expression of CAGE. ChIP assays demonstrated the binding of HDAC3 to the promoter sequences of CAGE. However, CAGE did not affect the expression of HDAC3. We also found that EGFR signaling regulated the expressions of HDAC3 and CAGE. Anti-cancer drug-resistant cancer cell lines show an increased expression of pEGFR<sup>Y845</sup>. HDAC3 was found to negatively regulate the expression of pEGFR<sup>Y845</sup>. CAGE showed an interaction and colocalization with EGFR. It was seen that miR-326, a negative regulator of HDAC3, regulated the expression of CAGE, pEGFR<sup>Y845</sup>, and the interaction between CAGE and EGFR. miR-326 inhibitor induced the binding of HDAC3 to the promoter sequences in anti-cancer drug-resistant Malme3M<sup>R</sup> cells, decreasing the tumorigenic potential of Malme3M<sup>R</sup> cells in a manner associated with its effect on the expression of HDAC3, CAGE and pEGFR<sup>Y845</sup>. The down-regulation of HDAC3 enhanced the tumorigenic, angiogenic and invasion potential of the anti-cancer drug-sensitive Malme3M cells in CAGE-dependent manner. Studies revealed that PKC $\delta$  was responsible for the increased expression of pEGFR<sup>Y845</sup> and CAGE in Malme3M<sup>R</sup> cells. CAGE showed an interaction with PKC $\delta$  in Malme3M<sup>R</sup> cells. Our results show that HDAC3-CAGE axis can be employed as a target for overcoming resistance to EGFR inhibitors.

## INTRODUCTION

Multi drug-resistant phenotypes are under epigenetic regulation

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(Gao et al., 2009; To et al., 2008). It is known that HDAC3 negatively regulates the expression of MDR1 and confers sensitivity to anti-cancer drugs (Kim et al., 2015a; Park et al., 2014b). MicroRNAs (miRNAs) play essential roles in various cellular processes such as cellular proliferation and anti-cancer drug-resistance (Yoon et al., 2014). HDAC3, through a negative feedback loop with miR-326, regulates the response to the anti-cancer drugs (Kim et al., 2014). Through its interaction with tubulin  $\beta$ , HDAC3 regulates the response to anti-cancer drugs (Kim et al., 2015a). The expression level of HDAC3 has been shown to be down-regulated in various cancer cell lines that are resistant to anti-cancer drugs (Kim et al., 2014). The expression of HDAC3 is controlled via ubiquitination (Kim et al., 2015b). miR-335 increases the expression of HDAC3 by preventing SIAH2 from inducing ubiquitination of HDAC3 (Kim et al., 2015b). The selective inhibition of HDAC3 protects beta cells from cytokine-induced apoptosis (El-Khoury et al., 2007). Previous studies have shown that HDAC3 mediates beta cell apoptosis (Lundh et al., 2012). SAHA, an inhibitor of HDAC(s), induces MDR to confer anti-apoptotic effects (Xu et al., 2012).

The transcriptional repression of HDAC3 enhances tumorigenicity and invasiveness of lung cancer cells (Dhar et al., 2014). HDAC3 acts as a negative regulator of angiogenesis (Park et al., 2014a). The role of HDAC3 in cancer development is known to be tissue-specific. In gastric, prostatic, and colorectal cancer samples, HDAC3 overexpression was significantly associated with poor prognosis (Weichert et al., 2008a; 2008b). In agreement with these reports, it has been shown that HDAC3 appeared to be up-regulated and to repress the tumor suppressor gene *p21* in colorectal cancer cells (Wilson et al., 2006). Studies have shown that liver-specific HDAC3 knock-out mice develop hepatoma (Bhaskara et al., 2010). In gastric cancers, HDAC3 inhibits the expression of PUMA (p53-upregulated modulator of apoptosis) and the down-regulation of HDAC3 promotes interaction of p53 with the promoter sequences of PUMA (Feng et al., 2013).

HDAC3 inhibition by vorinostat reduces the EGFR expression level and attenuates cellular proliferation (Gilbert et al., 2011). HDAC inhibition decreases the expression of EGFR and causes dissociation of HDAC3 from the promoter sequences of EGFR in colorectal cancer cells (Chou et al., 2011). TSA, an inhibitor of HDAC activity, inhibits the activation of ERGFR by HIF1 $\beta$  (Robertsson et al., 2012). EGFR signaling down-regulates the expression of Runx2 by up-regulating the expression of HDAC4 and HDAC-6 (Zhu et al., 2011). EGFR signaling

regulates the multi drug-resistant phenotypes (Shi et al., 2009). The targeting EGFR in cancers are largely limited due to the status of KRAS mutation (Van Cutsem et al., 2009). The KRAS mutants bypass EGFR to activate the Ras/Raf/MEK/ERK signals, and significantly weaken the therapeutic effect of cetuximab (Walther et al., 2009). Targeting c-Met enhances the therapeutic effect in malignant colon cancer cells harboring KRAS mutation (Li et al., 2014). Thus, the inhibition of both c-Met and EGFR serves as an effective therapy for hepatocellular carcinoma (Steinway et al., 2015). However, the role of HDAC3 in imparting resistance to EGFR inhibitors has so far not been reported.

CAGE, cancer/testis antigen, was isolated by SEREX (serological analysis of recombinant expression library) from the sera of gastric cancer patients (Cho et al., 2002). CAGE, through interaction with HDAC2, decreases the expression of p53, which in turn regulates the response to anti-cancer drugs (Kim et al., 2010). The expression of CAGE is increased in anti-cancer drug-resistant cancer cell lines (Kim et al., 2013). CAGE, through a negative feedback loop with miR-200b, regulates the response to anti-cancer drugs (Kim et al., 2013). The expression of CAGE is under the epigenetic regulation (Cho et al., 2013). CAGE displays oncogenic potential and increases the expression of Cyclin D1 and -E in AP1 and E2F-dependent manner (Por et al., 2010). CAGE is present in the sera of various cancer patients (Iwata et al., 2005). The expression of CAGE is seen in most of the cancer tissues (Kim et al., 2010). The role of CAGE in conferring resistance to microtubule-targeting anti-cancer drugs, such as taxol and celastrol, has been reported (Kim et al., 2010; 2013). However, the effect of CAGE on the response to EGFR inhibitors has not been reported.

In this study, we show the direct regulation of CAGE expression by HDAC3. We show that HDAC3-CAGE axis regulates the activation of EGFR. HDAC3 targets CAGE to regulate the tumorigenic potential and angiogenic potential of cancer cells and the response to anti-cancer drugs. Our results show that the HDAC3-CAGE axis serves as a strategy for overcoming resistance to EGFR inhibitors.

## MATERIALS AND METHODS

### Materials

Anti-mouse and anti-rabbit IgG-horse radish peroxidase conjugate antibodies were purchased from Pierce Company. An ECL (enhanced chemiluminescence) kit was purchased from Amersham. Lipofectamin and Plus<sup>TM</sup> reagent were purchased from Invitrogen (USA).

### Cell lines and cell culture

Cancer cell lines made resistant to microtubule-targeting drugs were established by stepwise addition of the respective drug. Cells surviving drug treatment (attached fraction) were obtained and used throughout this study. SNU387/SNU387<sup>R</sup> or Malme3M/Malme3M<sup>R</sup> cells that stably express anti-sense HDAC3 cDNA or HDAC3-Flag were selected by G418 (400 µg/ml).

### Western blot analysis

Western blot analysis, immunoprecipitation and cellular fractionation were performed according to the standard procedures (Kim et al., 2013). For analysis of proteins from tumor tissues, frozen samples were grounded to a fine powder using a mortar and pestle over liquid nitrogen. Proteins were solubilized in RIPA buffer containing protease inhibitors, and insoluble material removed by centrifugation.

### Cell viability determination

The cells were assayed for their growth activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Viable cell number counting was carried out by trypan blue exclusion assays.

### Caspase-3 activity assays

Caspase-3 activity was measured according to the manufacturer's instructions (BioVision, USA). Cells were lysed in 0.1 M HEPES buffer, pH 7.4, containing 2 mM dithiothreitol, 0.1% CHAPS, and 1% sucrose. Cell lysates were incubated with a colorimetric substrate, 200 µM Ac-DEVD-p-nitroanilide, for 30 min at 30°C. The fluorescence was measured at 405 nm using a microtiter plate reader.

### Immunohistochemistry

Paraffin-embedded tissue sections were immunostained using the Vecta stain ABC Elite Kit (Vector Laboratories). Tissue sections were deparaffinized with xylene and washed in ethanol. Endogenous peroxidase activity is blocked with 3% hydrogen peroxide and H<sub>2</sub>O for 10min. Slides were then blocked with 5% normal goat serum in TBS containing 0.1% Tween-20 (TBS-T) for 1 h. For immunohistochemistry, a primary antibody to HDAC3 (1:100, Santa Cruz), CAGE (1:100, Santa Cruz), EGFR (1:100, Santa Cruz), pEGFR<sup>Y845</sup> (1:100, Santa Cruz) or IgG (1:100, Santa Cruz) was added and incubation continued at 4°C for 24 h. After washing with TBS-T, slides were treated with biotinylated secondary antibody for 30 min. After washing, slides were incubated in the ABC complex for 30 min, and then stained with diaminobenzidine (DAB, Sigma). Sections were counterstained with hematoxylin and finally mounted using Fixo gum rubber cement (Mercateo, Germany).

### Immunofluorescence staining

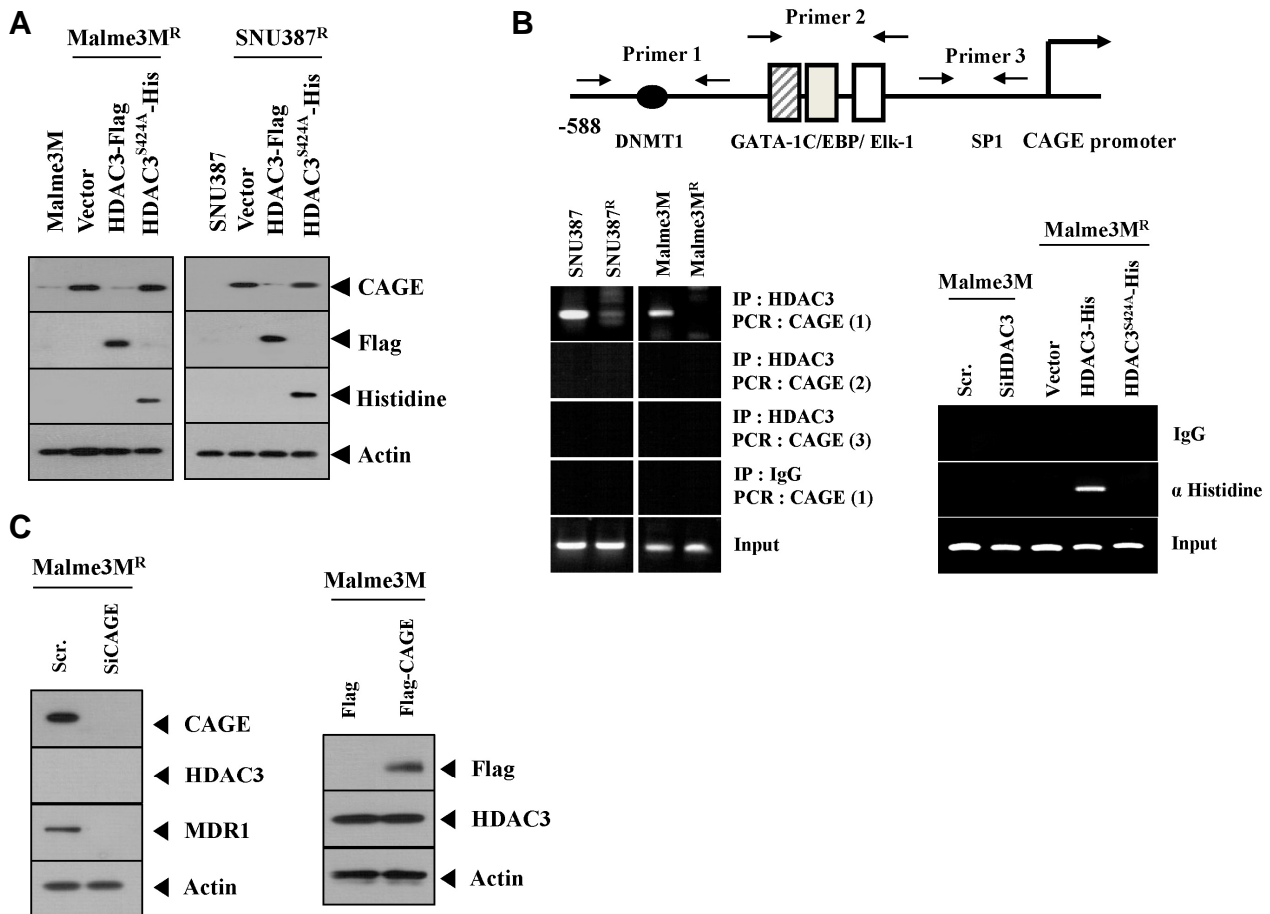
Cells were seeded onto glass coverslips in 24-well plates and fixed with 4% paraformaldehyde (v/v) for 10 min and then permeabilized with 0.4% Triton X-100 for 10 min. Nonspecific antibody binding sites were blocked by incubation with 1% BSA in TBST for 30 min. Cells were then incubated with primary antibody specific to CAGE (1:200; BD Biosciences) or pEGFR<sup>Y845</sup> (1:200; Santa Cruz) for 2 h, followed by washing with TBS-T three times. Anti-goat IgG-FITC (for detection of pEGFR<sup>Y845</sup>) or anti-rabbit Alexa Fluor 586 (for detection of CAGE) secondary antibody (Molecular Probes) was added to cells and incubated for 1 h. Cover slips were then washed and mounted by applying Mount solution (Biomedica, USA). Fluorescence images were acquired using a confocal laser scanning microscope and software (Fluoview version 2.0) with X 60 objective (Olympus FV300, Japan).

### Chromatin immunoprecipitation (ChIP) Assays

Assays were performed according to manufacturer's instruction (Upstate). For detection of binding of HDAC3 to CAGE promoter sequences, specific primers of CAGE promoter-1 sequences [5'-CCTGACAAAGTACTGTATTCACTCCA-3' (sense) and 5'-TGGCTCAGCTTGAGAGCAAC-3' (antisense)], CAGE promoter-2 sequences [5'-CGCAGAAGTTAAGGAGGCAG-3' (sense) and 5'-AAGTTGCCCCAGAAACAGT-3' (antisense)] and CAGE promoter-3 sequences [5'-ATGTGACTAGCACCGGAAA-3' (sense) and 5'-GGGATAGTGGGAGTATCGG-3' (antisense)] were used.

### Transfection

All transfections were performed according to the manufacturer's instructions. Lipofectamine and Plus reagents (Invitrogen)



**Fig. 1.** HDAC3 regulates the expression of CAGE. (A) The indicated cancer cells were transiently transfected with wild type HDAC3 or mutant HDAC3 (HDAC3<sup>S424A</sup>). At 48 h post transfection, cell lysates were subjected to Western blot analysis. Cell lysates from SNU387 or Malme3M cells were also subjected to Western blot analysis. (B) Cell lysates isolated from the indicated cancer cells were subjected to ChIP assays (left panel). The indicated cancer cells were transiently transfected with the indicated siRNA (10 nM) or construct (1  $\mu$ g). At 48 h post transfection, cell lysates were isolated and subjected to ChIP assays (right panel). (C) The indicated cancer cells were transiently transfected with the indicated siRNA (10 nM) or construct (1  $\mu$ g). At 48 h post transfection, cell lysates were subjected to Western blot analysis.

were used. The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, USA). For miR-326 knockdown, cells were transfected with 200 nM of oligonucleotide (inhibitor) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The sequences used were: 5'-CCUCUGGGCCCUUCUCG-3' (miR-326 inhibitor); and 5'-GCCUCCGGCUUCGCACCUCU-3' (control inhibitor).

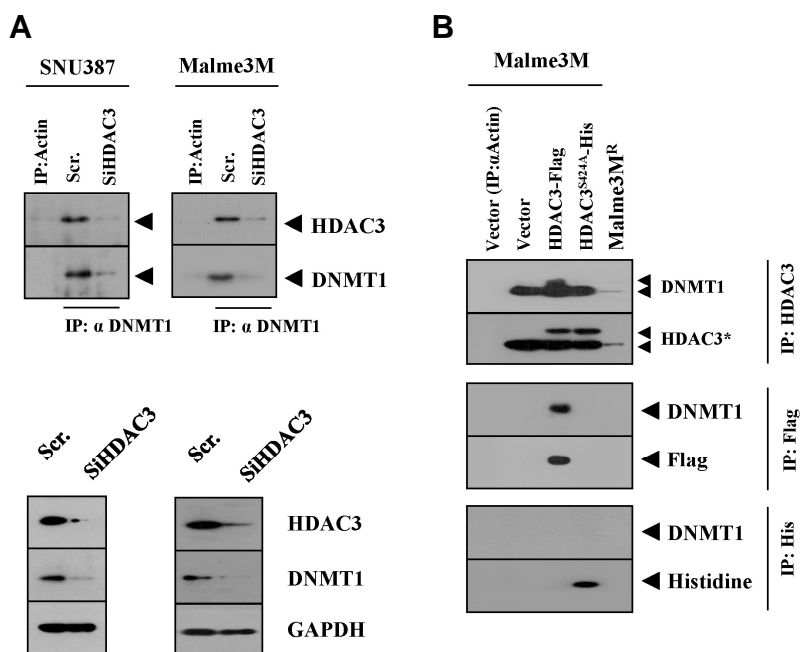
#### **In vivo tumorigenic potential**

Athymic nude mice (BALB/c nu/ nu, 5-6-week-old females) were obtained from Orient Bio Inc. (Korea) and were maintained in a laminar air-flow cabinet under aseptic conditions. Each cancer cells ( $1 \times 10^6$ ) were injected subcutaneously into the dorsal flank area of the mice. Tumor volume was determined by direct measurement with calipers and calculated by the following formula: length  $\times$  width  $\times$  height  $\times$  0.5. To determine the effect of HDAC3 on *in vivo* response to microtubule-targeting drugs, each cancer cell line expressing HDAC3-Flag or anti-sense HDAC3 cDNA were injected subcutaneously into

the dorsal flank area of the mice. Following the establishment of sizeable tumor, celestrol (1 mg/kg), taxol (1 mg/kg) or vinblastine (0.5 mg/kg) was administered via tail vein. Tumor volume was measured as describe above. To examine the effect of miR-326, control inhibitor (50  $\mu$ M/kg) or miR-326 inhibitor (50  $\mu$ M/kg) was administered via tail vein. To determine the effect of HDAC3 and CAGE on the *in vivo* tumorigenic potential, scrambled siRNA (100 nM), HDAC3 siRNA (100 nM) or CAGE siRNA (100 nM) was injected following the establishment of sizable tumor by Malme3M<sup>R</sup> cells, via tail vein 5 times in a total of 30 days.

#### **Chemo invasion assays**

The invasive potential was determined by using a transwell chamber system with 8- $\mu$ m pore polycarbonate filter inserts (CoSTAR, USA). The lower and upper sides of the filter were coated with gelatin and Matrigel, respectively. Trypsinized cells ( $5 \times 10^3$ ) in the serum-free RPMI 1640 medium containing 0.1% bovine serum albumin were added to each upper chamber of the transwell. RPMI 1640 medium supplemented with



**Fig. 2.** HDAC3 interacts with DNMT1. (A) SNU387 or Malme3M cells were transfected with scrambled siRNA (10 nM) or HDAC3 siRNA (10 nM). At 48 h after transfection, cell lysates were immunoprecipitated with the indicated antibody, followed by Western blot analysis (upper panel). Cell lysates were also subjected to Western blot analysis (lower panel). (B) Malme3M cells were transfected with the indicated construct (1  $\mu$ g each). At 48 h after transfection, cell lysates were immunoprecipitated with the indicated antibody, followed by Western blot analysis.

10% fetal bovine serum was placed in the lower chamber and cells were incubated at 37°C for 16 h. The cells were fixed with methanol and the invaded cells were stained and counted. Results were analyzed for statistical significance using the Student's *t* test. Differences were considered significant when *p* < 0.05.

### Intravital microscopy

Male BALB/c mice (6–8 week old) were obtained from Daehan Biolink (Korea). *In vivo* angiogenesis was assessed as follows. The mice were anesthetized with 2.5% avertin (v/v) via intraperitoneal injection (Surgivet, USA), and abdominal wall windows were implanted. Next, a titanium circular mount with eight holes on the edge was inserted between the skin and the abdominal wall. Growth factor-reduced matrigel containing the conditioned medium was applied to the space between the windows, and a circular glass cover slip was placed on top and fixed with a snap ring. After four days, the animals were anesthetized and injected intravenously with 50  $\mu$ l of 25 ng/ml fluorescein isothiocyanate-labeled dextran (molecular weight, Mr ~2,000,000) via the tail vein. The mice were then placed on a Zeiss Axiovert 200 M microscope. The epi-illumination microscopy setup included a 100-W mercury lamp and filter set for blue light. Fluorescence images were recorded at random locations of each window using an electron-multiplying charge coupled device camera (Photo Max 512, Princeton Instruments, USA) and digitalized for subsequent analysis using the Metamorph program (Universal Imaging, USA). The assay was scored from 0 (negative) to 5 (most positive) in a double-blinded manner.

### Statistical analysis

Statistical differences in this were determined by using the Student's *t* test.

## RESULTS

### HDAC3 directly regulates the expression of CAGE

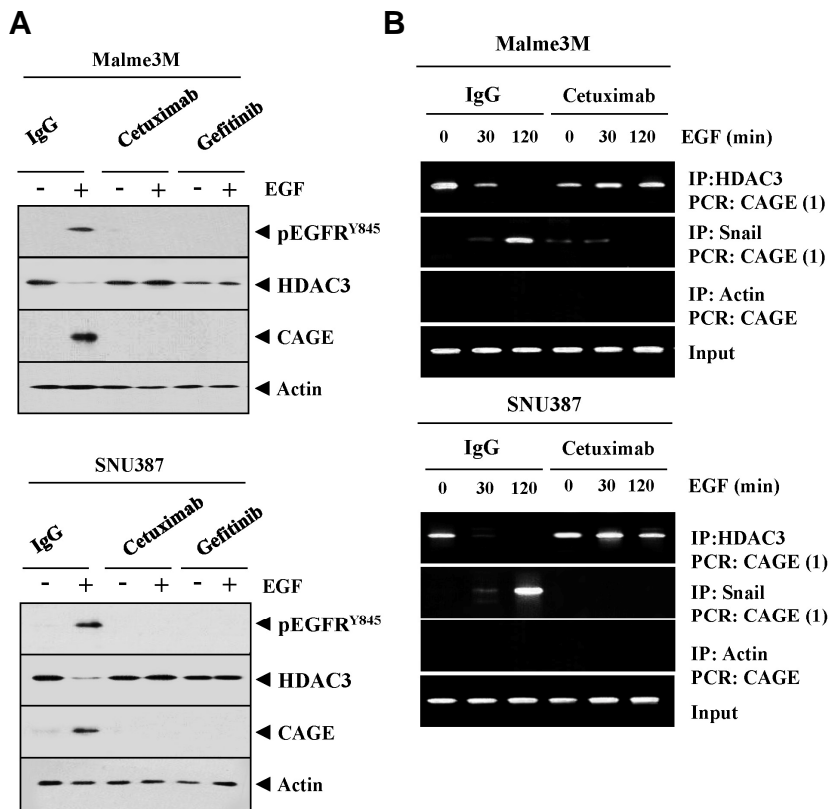
We have previously reported that miR-326-HDAC3 loop regu-

lates the response to anti-cancer drugs. We showed that the expression of HDAC3 is lower in cancer cells resistant to anti-cancer drugs than those that are sensitive to anti-cancer drugs (Kim et al., 2014). By forming a negative feedback loop with miR-200b, CAGE regulates the response to anti-cancer drugs (Kim et al., 2013). The expression of CAGE is higher in resistant cancer cells than those that are sensitive to anti-cancer drugs (Kim et al., 2010). Because HDAC3 and CAGE show an inverse relationship in resistant cancer cells (Kim et al., 2013; 2014), we were interested in examining the relationship between HDAC3 and CAGE. Wild type, but not the catalytically inactive mutant HDAC3 (HDAC3<sup>S424A</sup>), decreased the expression of CAGE in drug-resistant SNU387<sup>R</sup> and Malme3M<sup>R</sup> cells (Fig. 1A), suggesting that HDAC3 activity is necessary for regulating the expression of CAGE by HDAC3. Unlike wild type, the HDAC3<sup>S424A</sup> mutant does not show nuclear localization (Park et al., 2014b). CAGE promoter sequences contain putative binding sites for DNMT1, GATA-1 and SP1 (Fig. 1B). We know that the decreased expression of PSalpha gene involves the recruitment of HDAC3 by Sp1 in ER-positive HepG2 cells (Suzuki et al., 2010). This led us to hypothesize that HDAC3 may be exerting a direct regulation on the expression of CAGE. ChIP assays showed binding of HDAC3 to site 1, but not site 2 or site 3, of the CAGE promoter sequences in SNU387 and Malme3M cells (Fig. 1B). Wild type, but not the mutant HDAC3, showed binding to the site 1 of the promoter sequences of CAGE (Fig. 1B), suggesting that HDAC3 activity is necessary for direct regulation of CAGE by HDAC3. The down-regulation of CAGE or overexpression of CAGE did not affect the expression of HDAC3 in Malme3M<sup>R</sup> or Malme3M cells (Fig. 1C). Taken together, these results suggest the role of HDAC3 in regulating the expression of CAGE.

### HDAC3 interacts with DNMT1 to regulate the expression of CAGE

In our studies, we reported the decreased expression of DNMT1 in SNU387<sup>R</sup> and Malme3M<sup>R</sup> cells (Kim et al., 2010). DNMT1 regulates the sensitivity to cisplatin (Xiang et al., 2014).





**Fig. 3.** EGFR signaling regulates the expression of HDAC3 and CAGE. (A) Malme3M or SNU387 cells were pretreated with IgG (2 µg/ml), cetuximab (2 µg/ml) or gefitinib (10 µM) for 24 h. The next day, cells were then treated with EGF (50 ng/ml) for 1 h, followed by Western blot analysis. (B) Malme3M or SNU387 cells were pretreated with IgG (2 µg/ml), cetuximab (2 µg/ml) or gefitinib (10 µM) for 24 h. The next day, cells were then treated with EGF (50 ng/ml) for various time intervals, followed by ChIP assays.

The expression of CAGE is also known to be under epigenetic regulation (Cho et al., 2013). Site 1 of the CAGE promoter contains putative binding site for DNMT1 (Fig. 1B). It is thus reasonable that DNMT1 may regulate the expression of CAGE. We first examined the possibility of an interaction between HDAC3 and DNMT1. HDAC3 interacts with DNMT1 in SNU387 and Malme3M cells (Fig. 2A). Our studies revealed that the down-regulation of HDAC3 decreases the expression of DNMT1 in SNU387 and Malme3M cells (Fig. 2A). This implies that the down-regulation of HDAC3 may regulate the expression of the transcriptional factor that regulates the expression of DNMT1 either directly or indirectly. The inhibition of HDACs reduces the expression of DNMT1 (Brodie et al., 2014). HDAC3 activity is necessary for the interaction with DNMT1 (Fig. 2B). Taken together, these results suggest that HDAC3 regulates the expression of CAGE through interaction with DNMT1.

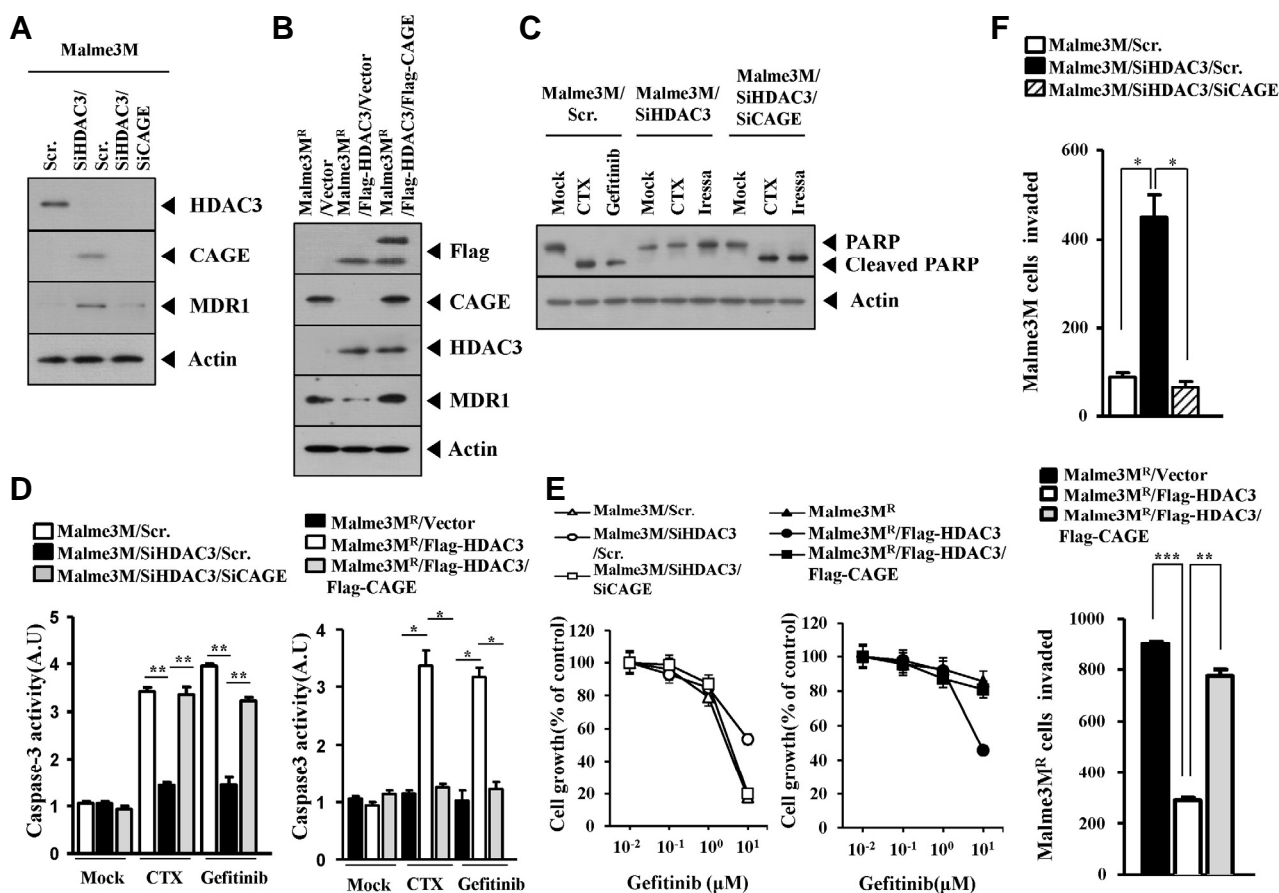
### EGFR signaling regulates the expression of HDAC3 and CAGE

CAGE increases the expression of Cyclin D1 (Por et al., 2010), and EGFR signaling regulates the expression of Cyclin D1 (Lee et al., 2010). Over-expression of EGFR is correlated with the resistance to taxol (Itamochi et al., 2012). EGFR tyrosine kinase inhibitors overcome this resistance to taxol (Kuang et al., 2010). Over-expression of ATP-binding cassette (ABC) transporters such as ABCB1, ABCC1 and ABCG2 is one of the main causes of multidrug resistance (MDR). AG1478, an inhibitor of EGFR tyrosine kinase, inhibits the function of ABCB1 and ABCG2 (Itamochi et al., 2009). These reports suggest that the MDR phenotype is closely related with EGFR signaling. P53 modulates resistance to EGFR inhibitors (Huang et al., 2011). CAGE, through interaction with HDAC2, regulates the expres-

sion of p53 to confer resistance to anti-cancer drugs (Kim et al., 2010). miR-126 regulates EGFR-Akt signaling through miR-126-DNMT1 circuit (Liu et al., 2015). These reports led us to hypothesize that the interaction between the HDAC3-CAGE axis and EGFR signaling regulates the response to anti-cancer drugs. We first examined the relationship between HDAC3-CAGE axis and EGFR signaling. We saw that the EGF treatment increased the expression of CAGE and pEGFR<sup>Y845</sup> while decreasing the expression of HDAC3 (Fig. 3A). EGFR inhibitors, such as cetuximab (CTX) and gefitinib, prevented EGF from regulating the expression of HDAC3 and CAGE in SNU387 and Malme3M cells (Fig. 3A). These results suggest that EGFR signaling may regulate the expression of HDAC3 and CAGE. ChIP assays also showed that EGF treatment prevented HDAC3 from binding to the promoter sequences of CAGE (Fig. 3B). Furthermore, we found that cetuximab prevented EGF from inhibiting the binding of HDAC3 to the promoter sequences of CAGE (Fig. 3B), and also EGF treatment prevented Snail from binding to the promoter sequences of CAGE (Fig. 3B). Taken together, these results suggest that EGFR signaling, in association with HDAC3-CAGE axis, regulates the response to anti-cancer drugs.

### HDAC3 targets CAGE to regulate the response to EGFR inhibitors and the invasion potential of cancer cells

The down-regulation of HDAC3 increased the expression of CAGE and MDR1 in CAGE-dependent manner (Fig. 4A). It is apparent that CAGE does not regulate the expression of HDAC3 (Fig. 4A). We studied the relationship between the expression of HDAC3, CAGE and MDR1 in Malme3M<sup>R</sup> cells. Over-expression of HDAC3 decreased the expression of CAGE and MDR1 in Malme3M<sup>R</sup> cells (Fig. 4B). HDAC3 decreased the expression of MDR1 while CAGE restored the



**Fig. 4.** HDAC3 targets CAGE to regulate the sensitivity to EGFR inhibitors and invasion potential of cancer cells. (A) Malme3M cells were transiently transfected with the indicated siRNAs (each at 10 nM). At 48 h after transfection, cell lysates were subjected to Western blot analysis. (B) Malme3M<sup>R</sup> cells were transiently transfected with the indicated constructs (each at 1 μg). At 48 h after transfection, cell lysates were subjected to Western blot analysis. (C) Malme3M cells were transiently transfected with the indicated siRNAs (each at 10 nM). Next day, cells were then treated with cetuximab (2 μg/ml) or gefitinib (10 μM) for 24 h, followed by Western blot analysis. (D) Malme3M cells were transiently transfected with the indicated siRNAs (each at 10 nM). The following day, cells were then treated with cetuximab (2 μg/ml) or gefitinib (10 μM) for 24 h, followed by caspase-3 activity assays (left panel). Malme3M<sup>R</sup> cells were transiently transfected with the indicated construct (each at 1 μg) and similarly treated (right panel). \*p < 0.05; \*\*p < 0.005. (E) Malme3M cells were transiently transfected with the indicated siRNAs (each at 10 nM). The next day, cells were then treated with various concentrations of gefitinib for 24h, followed by MTT assays (left panel). Malme3M<sup>R</sup> cells were transiently transfected with the indicated construct (each at 1 μg). The next day, cells were then treated with various concentrations of gefitinib for 24 h, followed by MTT assays (right panel). (F) The indicated cancer cells were transfected with the indicated siRNAs (each at 10 nM). At 48 h after transfection, cells were then subjected to chemoinvasion assays. \*p < 0.05; \*\* p < 0.005; \*\*\*p < 0.0005.

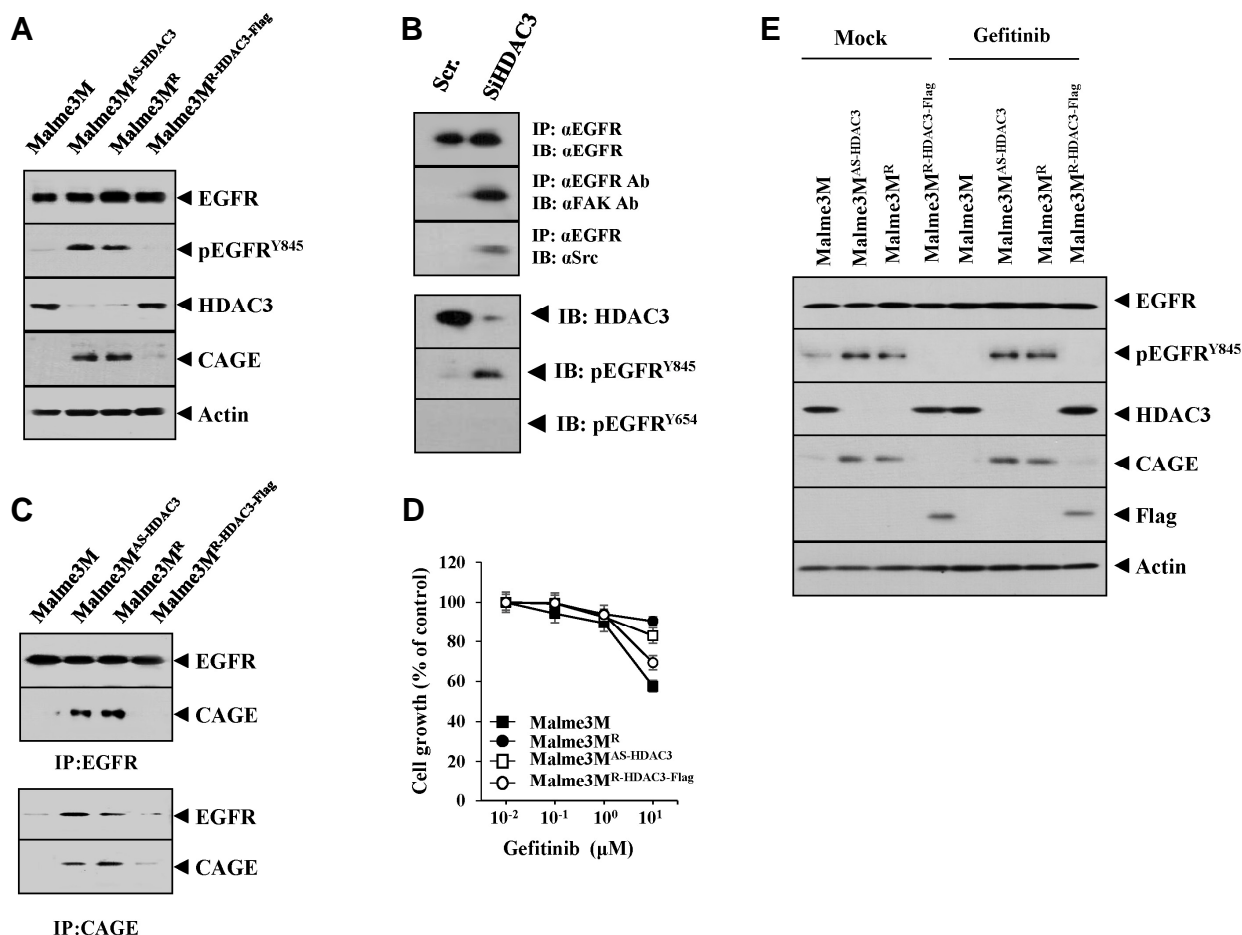
expression of MDR1 in Malme3M<sup>R</sup> cells transfected with HDAC3 (Fig. 4B). The down-regulation of HDAC3 prevented cleavage of PARP in response to EGFR inhibitors such as CTX and gefitinib in CAGE-dependent manner (Fig. 4C) and also prevented CTX and gefitinib from increasing caspase-3 activity in CAGE-dependent manner in Malme3M cells (Fig. 4D). We also observed that CAGE prevented HDAC3 from increasing caspase-3 activity in Malme3M<sup>R</sup> cells (Fig. 4D). The down-regulation of HDAC3 conferred resistance to gefitinib in CAGE-dependent manner in Malme3M cells (Fig. 4E). CAGE prevented HDAC3 from increasing sensitivity to gefitinib in Malme3M<sup>R</sup> cells (Fig. 4E). The down-regulation of HDAC3 enhanced the invasion potential of Malme3M cells in CAGE-dependent manner (Fig. 4F). The overexpression of CAGE restored the invasion potential of Malme3M<sup>R</sup> cells transfected with HDAC3 (Fig.

4F). Taken together, these results suggest that HDAC3 targets CAGE to regulate the response to EGFR inhibitor and the invasion potential of cancer cells.

#### HDAC3 regulates the expression of pEGFR<sup>Y845</sup> and an interaction between CAGE and EGFR

Given the fact that p53 negatively regulates the activation of EGFR and CAGE acts as a negative regulator of p53, we hypothesized that the HDAC3-CAGE axis regulates EGFR activation.

Using Malme3M cells as a control, the following observations were made: Malme3M<sup>R</sup> cells and Malme3M<sup>As-HDAC3</sup> that stably express anti-sense HDAC3 showed higher expression of CAGE and pEGFR<sup>Y845</sup> (Fig. 5A); Malme3M<sup>R-HDAC3-Flag</sup> cells that stably express HDAC3 showed a lower expression level of CAGE and pEGFR<sup>Y845</sup> than Malme3M<sup>R</sup> cells (Fig. 5A). The



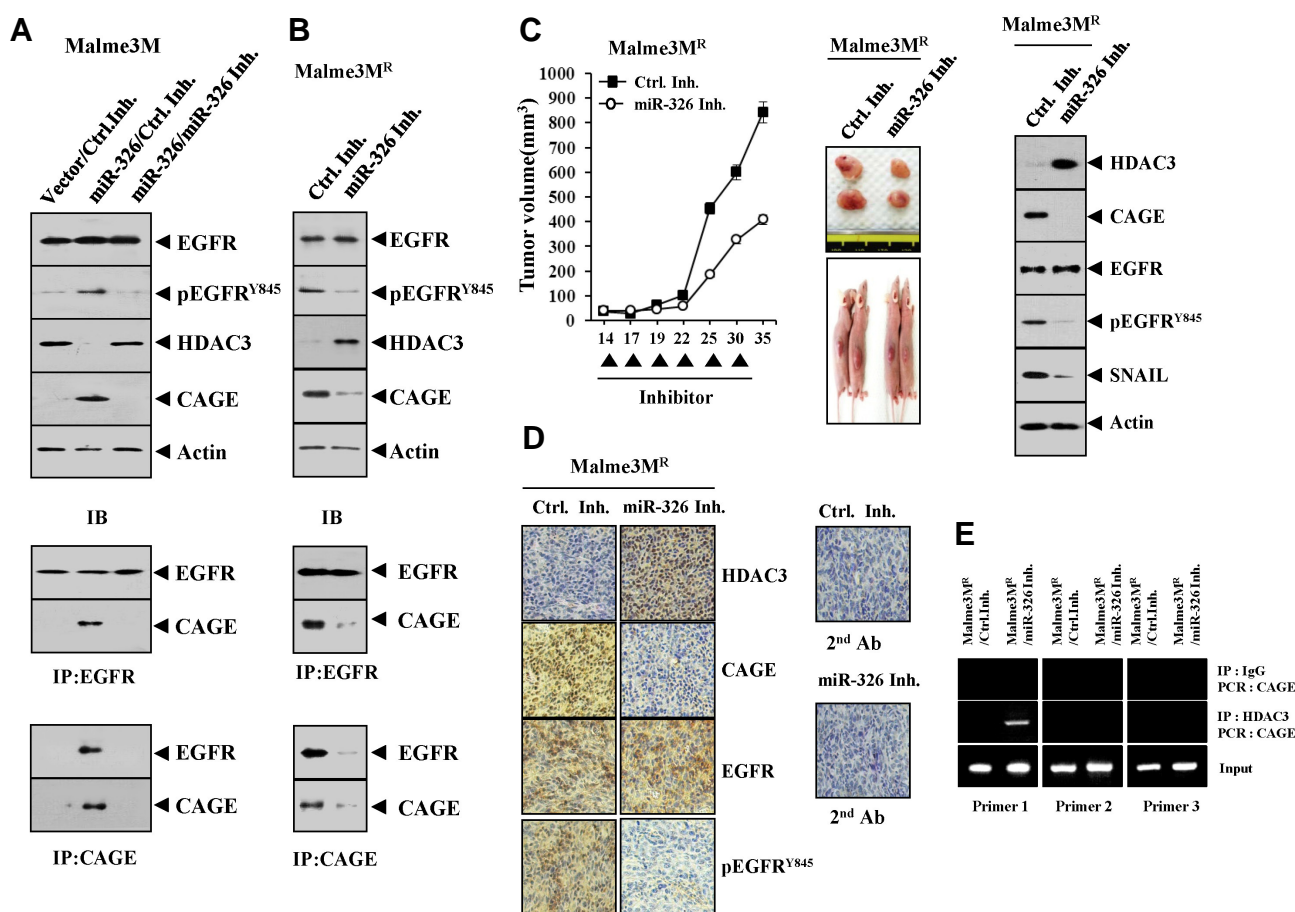
**Fig. 5.** HDAC3 regulates the activation of EGR and induces an interaction between CAGE and EGFR. (A) Cell lysates isolated from the indicated cancer cells were subjected to Western blot analysis. (B) Malme3M cells were transiently transfected with the indicated siRNA (each at 10 nM). At 48 h after transfection, cell lysates were immunoprecipitated with the indicated antibody (2  $\mu$ g/ml), followed by Western blot analysis. Cell lysates were also subjected to Western blot analysis. (C) Cell lysates isolated from the indicated cancer cells were immunoprecipitated with the indicated antibody (2  $\mu$ g/ml), followed by Western blot analysis. (D) The indicated cancer cells were treated with various concentrations of gefitinib for 48 h, followed by MTT assays. (E) The indicated cancer cells were treated with gefitinib (1  $\mu$ M) for 48 h, followed by Western blot analysis (lower panel).

down-regulation of HDAC3 increased the expression of pEGFR<sup>Y845</sup> and induced interaction of EGFR with FAK and Src (Fig. 5B). Some reports show the effect of HDAC inhibition on the expression of EGFR (Chou et al., 2011). However, in our studies, the down-regulation of HDAC3 did not affect the expression of EGFR (Fig. 5B). The down-regulation of EGFR did not affect the expression of HDAC3 (data not shown). Malme3M<sup>As-HDAC3</sup> and Malme3M<sup>R</sup> cells showed an interaction between CAGE and EGFR (Fig. 5C). Malme3M cells that stably express anti-sense HDAC3 (Malme3M<sup>As-HDAC3</sup>) showed resistance to gefitinib (Fig. 5D), whereas the Malme3M<sup>R</sup> cells that stably express HDAC3 (Malme3M<sup>R-HDAC3-Flag</sup>) showed sensitivity to gefitinib (Fig. 5D). Malme3M<sup>R</sup> and Malme3M<sup>As-HDAC3</sup> cells showed higher expression of CAGE and pEGFR<sup>Y845</sup> and lower expression of HDAC3, as compared to the Malme3M cells (Fig. 5E). However, Malme3M<sup>R-HDAC3-Flag</sup> cells showed lower expression of CAGE and pEGFR<sup>Y845</sup> than the Malme3M<sup>R</sup> and Malme3M<sup>As-HDAC3</sup> cells (Fig. 5E). Taken together, these results suggest that the HDAC3-CAGE axis

regulates EGFR signaling in relation to response to anti-cancer drugs.

**miR-326, a negative regulator of HDAC3, regulates the activation of EGFR and an interaction of EGFR with CAGE and the tumorigenic potential of cancer cells**

We reported that miR-326-HDAC3 negative feedback loop regulates the response to anti-cancer drugs (Kim et al., 2014). We therefore examined the effect of miR-326 on the activation of EGFR. We observed that miR-326 specifically decreased the expression of HDAC3 while increasing the expression of CAGE and pEGFR<sup>Y845</sup> in Malme3M cells (Fig. 6A). The down-regulation of miR-326 by miR-326 inhibitor increased the expression of HDAC3 while decreasing the expression of CAGE and pEGFR<sup>Y845</sup> in Malme3M<sup>R</sup> cells (Fig. 6B). miR-326 inhibitor inhibited an interaction between CAGE and EGFR (Fig. 6B), and also decreased the tumorigenic potential of Malme3M<sup>R</sup> cells (Fig. 6C). Western blot of tumor tissue lysates showed that miR-326 inhibitor increased the expression of HDAC3 while



**Fig. 6.** miR-326, a negative regulator of HDAC3, regulates the activation of EGFR and an interaction between CAGE and EGFR. (A) Malme3M cells were transiently transfected with control vector or miR-326 construct (each at 1  $\mu$ g) along with the indicated inhibitor (each at 10 nM). At 48 h after transfection, cell lysates were immunoprecipitated with the indicated antibody (2  $\mu$ g/ml), followed by Western blot analysis. Cell lysates were also subjected to Western blot analysis. (B) Malme3M<sup>R</sup> cells were transiently transfected with the indicated inhibitor (each at 10 nM). At 48 h after transfection, cell lysates were subjected to immunoprecipitation and Western blot analysis. (C) Malme3M<sup>R</sup> cells ( $1 \times 10^6$ ) were injected into the dorsal flank area of athymic nude mouse. Control inhibitor (40  $\mu$ g/kg or 50  $\mu$ M/kg) or miR-326 inhibitor (40  $\mu$ g/kg or 50  $\mu$ M/kg) was injected into each nude mouse after the tumor reached a certain size. Tumor volume was measured on the same day as injection of inhibitor. Each value represents an average obtained from five mice of each group. Tumor tissue lysates from each mouse of the experimental group were subjected to Western blot. (D) immunohistochemical staining of tumor tissue derived from Malme3M<sup>R</sup> cells treated with the indicated inhibitor was performed as described. Immunohistochemistry staining employing secondary antibody alone served as a negative control. Representative images from five animals from each experimental group are shown (magnification, 400X; Olympus). H&E staining was performed to check structural integrity. (E) Malme3M<sup>R</sup> cells were transiently transfected with the indicated inhibitor (each at 10 nM). At 48 h after transfection, cell lysates were subjected to ChIP assays.

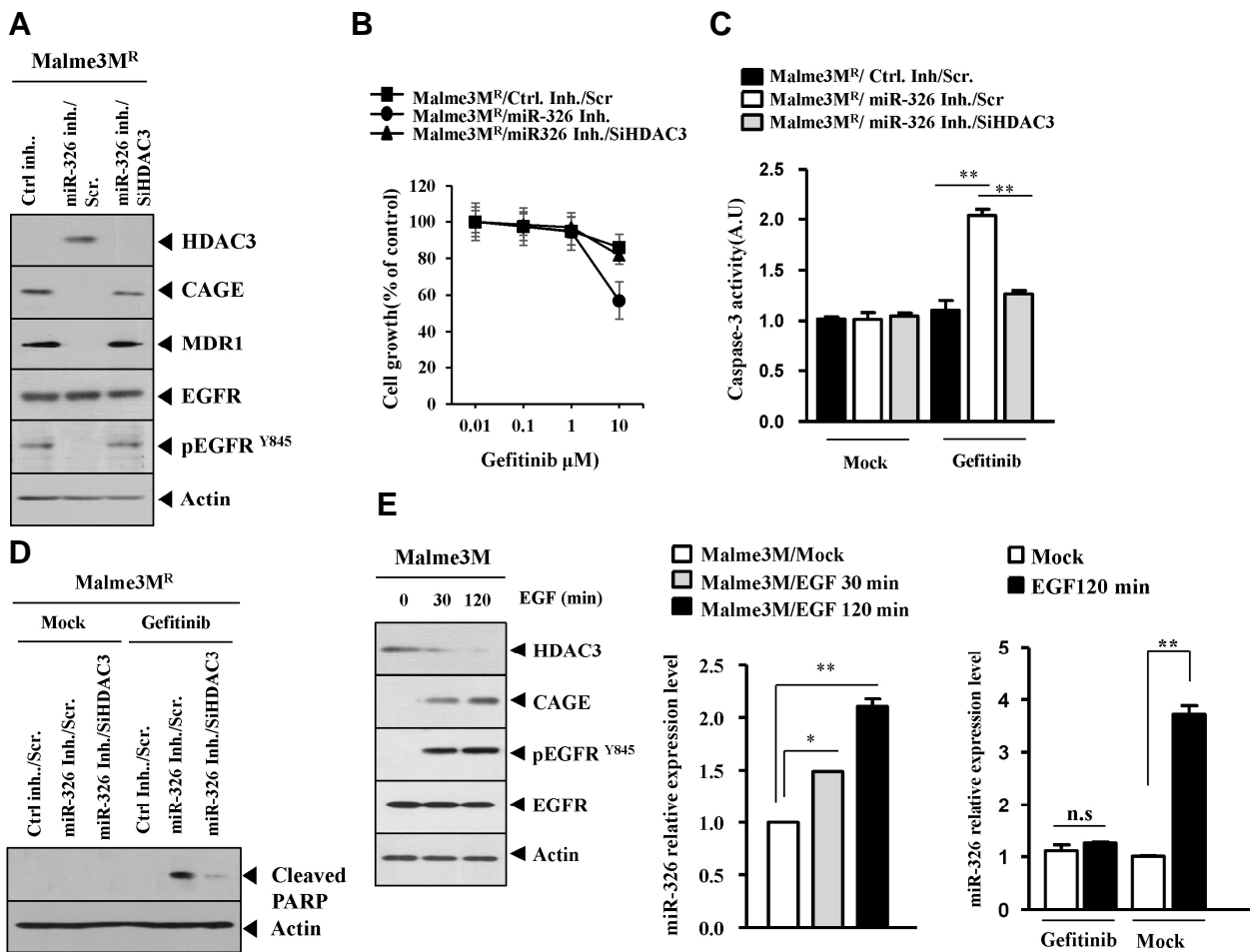
decreasing the expression of CAGE and pEGFR<sup>Y845</sup> (Fig. 6C). Immunohistochemical staining of tumor tissue showed that miR-326 inhibitor also showed similar results (Fig. 6D). ChIP assay showed that miR-326 inhibitor induced the binding of HDAC3 to the promoter sequences of CAGE (Fig. 6E). Taken together, these results suggest that miR-326 inhibitor decreases the tumorigenic potential of cancer cells by regulating the expression of HDAC3, CAGE and pEGFR<sup>Y845</sup>.

#### miR-326 inhibitor enhances sensitivity to EGFR inhibitor

miR-326 inhibitor increased the expression of HDAC3 while decreasing the expression of CAGE and MDR1 in Malme3M<sup>R</sup> cells (Fig. 7A). miR-326 inhibitor decreased the expression of pEGFR<sup>Y845</sup> (Fig. 7A). miR-326 inhibitor enhanced the sensitivity

to gefitinib by targeting HDAC3 in Malme3M<sup>R</sup> cells (Fig. 7B). miR-326 inhibitor increased caspase-3 activity (Fig. 7C) and PARP cleavage (Fig. 7D) in response to gefitinib in Malme3M<sup>R</sup> cells. Because miR-326 inhibitor regulated the expression of HDAC3, CAGE and pEGFR<sup>Y845</sup> (Fig. 6B), we examined whether EGFR signaling would affect the expression of miR-326. EGF treatment decreased the expression of HDAC3 while increasing the expression of CAGE and pEGFR<sup>Y845</sup> in Malme3M<sup>R</sup> cells (Fig. 7E). EGF treatment increased the expression of miR-326 in Malme3M cells (Fig. 7E). Gefitinib prevented EGF from increasing the expression of miR-326 in Malme3M cells (Fig. 7E). This suggests that EGFR signaling regulates the expression of miR-326. Taken together, these results suggest that miR-326 inhibitor enhances sensitivity to EGFR inhibitor by targeting HDAC3.





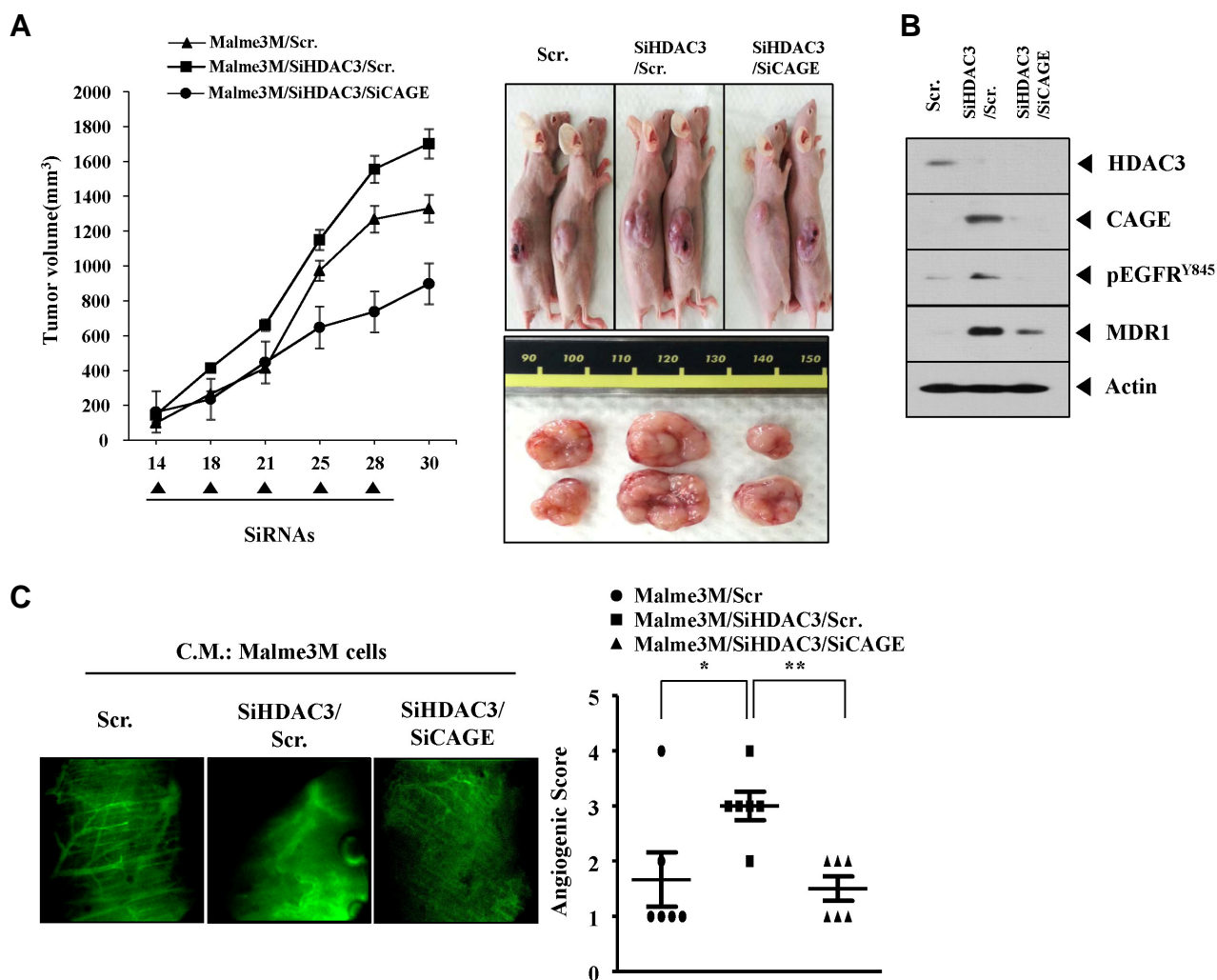
**Fig. 7.** miR-326 inhibitor enhances sensitivity to EGFR inhibitors. (A) Malme3M<sup>R</sup> cells were transfected with the indicated inhibitor (each at 10 nM) along with the indicated siRNA (each at 10 nM). At 48 h after transfection, cell lysates were subjected to Western blot analysis. (B) Malme3M<sup>R</sup> cells were transfected with the indicated inhibitor (each at 10 nM) along with the indicated siRNA (each at 10 nM). At 24 h after transfection, cells were treated with various concentrations of gefitinib for 24 h, followed by MTT assays. (C) Malme3M<sup>R</sup> cells were transfected with the indicated inhibitor (each at 10 nM) along with the indicated siRNA (each at 10 nM). At 24 h after transfection, cells were then treated with gefitinib (10 μM) for 24 h, followed by caspase-3 activity assays. \*\*p < 0.005. (D) Malme3M<sup>R</sup> cells were transfected with the indicated inhibitor (each at 10 nM) along with the indicated siRNA (each at 10 nM). At 24 h after transfection, cells were then treated with gefitinib (10 μM) for 24 h, followed by Western blot analysis. (E) Malme3M cells were treated with EGF (50 ng/ml) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis and qRT-PCR analysis. Malme3M cells were pretreated with gefitinib (10 μM). The next day, cell were then treated with EGF (50 ng/ml) for 2 h, followed by qRT-PCR analysis (right panel). \*p < 0.05; \*\*p < 0.005.

### The down-regulation of HDAC3 enhances the tumorigenic potential and angiogenic potential of cancer cells by targeting CAGE

The *in vivo* down-regulation of HDAC3 enhanced the tumorigenic potential of Malme3M cells in CAGE-dependent manner (Fig. 8A). The down-regulation of HDAC3 enhances the tumorigenic potential of lung cancer cells (Dhar et al., 2014). Western blot of tumor tissue lysates showed that the down-regulation of HDAC3 increased the expression of pEGFR<sup>Y845</sup> and MDR1 in CAGE-dependent manner (Fig. 8B). The conditioned medium of Malme3M cells transfected with HDAC3 siRNA showed enhanced angiogenic potential based on intravital microscopy (Fig. 8C). Taken together, these results suggest that HDAC3 regulates the tumorigenic and angiogenic potential of cancer cells in a manner associated with its effect on the expression of CAGE and pEGFR<sup>Y845</sup>.

### PKCδ interacts with CAGE and regulates the expression of CAGE and pEGFR<sup>Y845</sup>

Since the Malme3M<sup>R</sup> cells showed higher expression of pEGFR<sup>Y845</sup> than Malme3M cells (Fig. 5A), we investigated the mechanisms associated with the increased expression of pEGFR<sup>Y845</sup>. EGF treatment increased the expression of pPKCδ<sup>T505</sup> in SNU387 and Malme3M cells (Fig. 9A). The inactivation of PKCδ prevented EGF from regulating the expression of CAGE, HDAC3 and pEGFR<sup>Y845</sup> in SNU387 and Malme3M cells (Fig. 9B). The inactivation of PKCδ increased the expression of HDAC3 while decreasing the expression of CAGE and pEGFR<sup>Y845</sup> (Fig. 9C). CAGE also showed an interaction with PKCδ in Malme3M<sup>R</sup> cells (Fig. 9D). Taken together, these results suggest that the CAGE-PKCδ-EGFR axis regulates the response to anti-cancer drugs.



**Fig. 8.** The down-regulation of HDAC3 enhances the tumorigenic potential and angiogenic potential of cancer cells by targeting CAGE. (A) Malme3M cells ( $1 \times 10^6$ ) were injected into the dorsal flank area of athymic nude mouse. The indicated siRNA (each at 10 nM) was injected into each nude mouse after the tumor reached a certain size. Tumor volume was measured on the same day as injection of siRNA. Each value represents an average obtained from five mice of each group. (B) Tumor tissue lysates from each mouse of the experimental group were subjected to Western blot. (C) Malme3M cells were transiently transfected with the indicated siRNAs. Concentrated conditioned medium (10  $\mu$ l) obtained from the indicated cancer cell line was mixed with 100  $\mu$ l matrigel and intravital microscopy was performed as described.

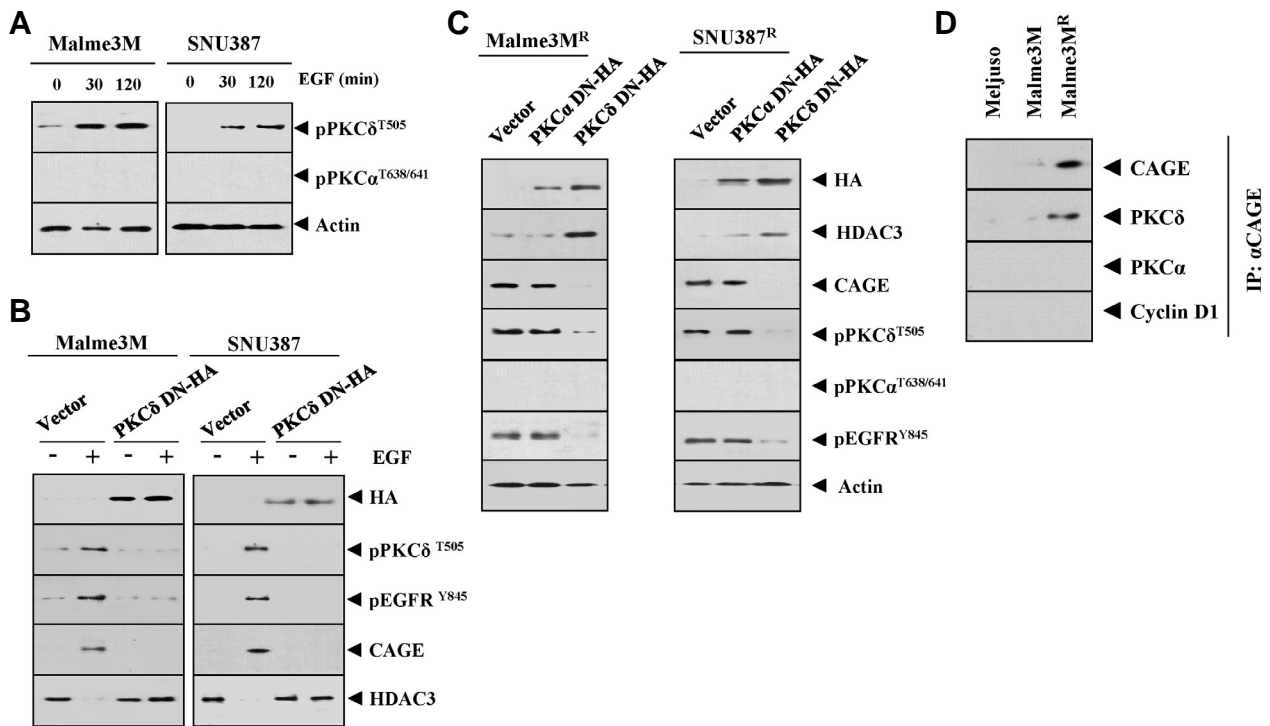
## DISCUSSION

Celastrol and taxol increase the expression of pEGFR<sup>Y845</sup> in SNU387 and Malme3M cells (personal observations). This suggests that resistance to these anti-cancer drugs is closely related to the resistance to EGFR inhibitors. Taxol, in combination with EGFR-targeted nanoparticle, regulates multi drug-resistant phenotype (Milane et al., 2011). Previously, we reported the identification of HDAC3 domain that regulates the expression of MDR1 (Park et al., 2014b). It will be interesting to further examine whether this domain regulates the expression of CAGE and pEGFR<sup>Y845</sup>.

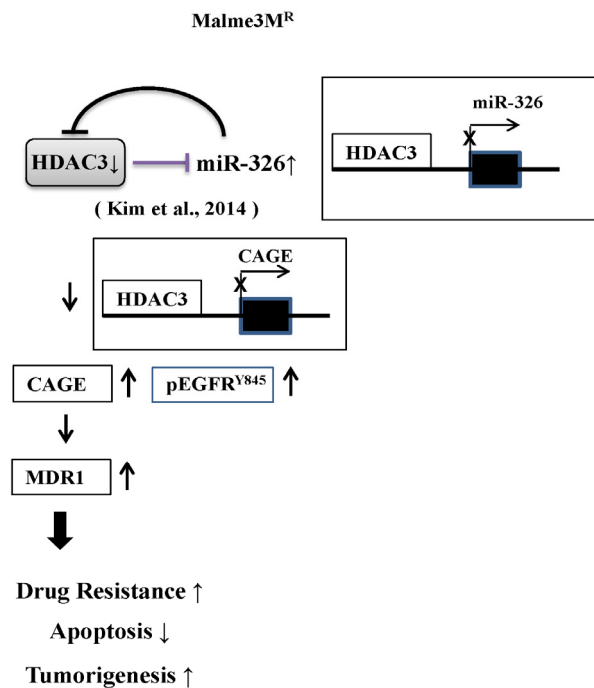
It is established that p53 regulates the activation of EGFR (Dong et al., 2009). In this study, we found that the down-regulation of p53 increases the expression of pEGFR<sup>Y845</sup> in Malme3M cells (personal observations). It would be interesting to examine whether HDAC3, through interaction with DNMT1 and p53, binds to the promoter sequences of CAGE.

Inhibition of class I HDACs suppresses EGFR phosphorylation as well as reduces its expression (Tang et al., 2014). In this study, we showed that the down-regulation of HDAC3 increases the expression of pEGFR<sup>Y845</sup> (Fig. 5A). Thus, further studies should be done to investigate whether HDAC2, increased in Malme3M<sup>R</sup> cells (Kim et al., 2010), regulates the expression of pEGFR<sup>Y845</sup>.

In this study, we found that CAGE confers resistance to trastuzumab, an inhibitor of HER2 (personal observations). It would be interesting to examine the effect of CAGE on the expression of HER2 and on the sensitivity to HER2 inhibitors. HDAC3 acts as a substrate of Src (Longworth and Laimins, 2006). C-Src-HDAC3 interaction decreases the expression of CXCR4 in highly invasive breast tumor cells (Matteucchi et al., 2007). Src kinase pathway is involved in trastuzumab-resistance in HER2-amplified breast cancers (Boyer et al., 2013). EGFR and HER2 are transactivated by c-Src and MMPs (Garcia-Recio et al., 2015). Thus, studying the role of



**Fig. 9.** PKC $\delta$  interacts with CAGE and regulates the expression of CAGE and pEGFR<sup>Y845</sup>. (A) The indicated cancer cells were treated with EGF (50 ng/ml) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis. (B) The indicated cancer cells were transfected with the indicated construct (each at 1  $\mu$ g). At 48 h after transfection, cells were treated with EGF (50 ng/ml) for 1h, followed by Western blot analysis. (C) The indicated cancer cells were transfected with the indicated construct (each at 1 $\mu$ g). At 48 h after transfection, cell lysates were subjected to Western blot analysis. (D) Cell lysates isolated from the indicated cancer cells were subjected to immunoprecipitation, followed by Western blot analysis.



**Fig. 10.** The proposed mechanism of anti-cancer drug-resistance regulated by HDAC-EGFR-CAGE axis.

Src in the increased phosphorylation of EGFR by HDAC3-CAGE axis, and the possible interaction between CAGE and Src, would reveal relevant information.

The down-regulation of HDAC3 enhances the angiogenic potential of Malme3M cells (Fig. 8C). We previously showed that recombinant CAGE protein enhances the angiogenic potential of cancer cells (Kim et al., 2013). HDAC3 negatively regulates the expression of PAI-1, a downstream target of TGF $\beta$  (Park et al., 2014a). We found that CAGE increases the expression of PAI-1 (personal observations). It would be interesting to examine whether PAI-1 affects the activation of EGFR. It is probable that PAI-1 confers resistance to EGFR inhibitors by activating EGFR.

TGF- $\beta$ 1/SMAD3 pathway activation in renal fibrosis (induced by ureteral ligation) is correlated with epidermal growth factor receptor (Y845) (EGFR (Y845)) and p53 (Ser15) phosphorylation and is responsible for the induction of PAI-1 (Samarakoon et al., 2013). HDACs, and in particular HDAC3, are required for activation of the ERK and PI3K signaling pathways by TGF- $\beta$  and for the subsequent gene induction dependent on these signaling pathways (Barter et al., 2010). It is probable that the resistance to EGFR inhibitors involves a cross-talk between EGFR and TGF $\beta$ R. In this study, we found an activation of TGF $\beta$ R in Malme3M<sup>R</sup> cells (personal observations). It would be necessary to examine the effect of HDAC3 on the activation of TGF $\beta$ R.

Given the fact that CAGE interacts with EGFR (Fig. 6A), it would be necessary to examine whether CAGE displays kinase

activity toward EGFR. PKC $\alpha$  mediates resistance to EGFR inhibitor (erlotinib) and enhances EMT in lung cancer cells (Abera and Kazanietz, 2015). Atypical protein kinase C (aPKC) confers resistance to EGFR inhibitors in gliomas (Kusne et al., 2014). Our studies revealed that Malme3M<sup>R</sup> cells show the activation of PKC $\delta$  (Fig. 9C), but not PKC $\alpha$ , and also an interaction between CAGE and PKC $\delta$  (Fig. 9D). It is probable that CAGE, through interaction with PKC $\delta$ , regulates the expression of EGFR.

In summary, the decreased expression of HDAC3 lead to the increased expression of miR-326 in Malme3M<sup>R</sup> cells (Fig. 10). HDAC3-miR326 negative feedback loop regulates the expression of CAGE and pEGFR<sup>Y845</sup> to regulate the response to anti-cancer drugs (Fig. 10). Therefore, we can conclude that the HDAC3-CAGE-EGFR axis serves as a target for overcoming resistance to EGFR inhibitors.

## ACKNOWLEDGMENTS

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