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Downregulation of CFTR promotes epithelial-to-mesenchymal transition and is associated with poor prognosis of breast cancer

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ABSTRACT

The epithelial-to-mesenchymal transition (EMT), a process involving the breakdown of cell–cell junctions and loss of epithelial polarity, is closely related to cancer development and metastatic progression. While the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- and HCO_3^- conducting anion channel expressed in a wide variety of epithelial cells, has been implicated in the regulation of epithelial polarity, the exact role of CFTR in the pathogenesis of cancer and its possible involvement in EMT process have not been elucidated. Here we report that interfering with CFTR function either by its specific inhibitor or lentiviral miRNA-mediated knockdown mimics TGF-β_1-induced EMT and enhances cell migration and invasion in MCF-7. Ectopic overexpression of CFTR in a highly metastatic MDA-231 breast cancer cell line downregulates EMT markers and suppresses cell invasion and migration *in vitro*, as well as metastasis *in vivo*. The EMT-suppressing effect of CFTR is found to be associated with its ability to inhibit NFκB targeting urokinase-type plasminogen activator (uPA), known to be involved in the regulation of EMT. More importantly, CFTR expression is found significantly downregulated in primary human breast cancer samples, and is closely associated with poor prognosis in different cohorts of breast cancer patients. Taken together, the present study has demonstrated a previously undefined role of CFTR as an EMT suppressor and its potential as a prognostic indicator in breast cancer.

1. Introduction

The epithelial-to-mesenchymal transition (EMT) is one of the most critical processes that occur during the progression of tumor metastasis [1,2]. In breast carcinomas, acquisition of a mesenchymal-like phenotype that is reminiscent of an EMT, designated as oncogenic EMT, is associated with pro-metastatic properties, including increased motility, invasion, and cancer stem cell characteristics [3,4]. EMT has been characterized as a complicated multistep process rather than simply a morphological transition. Besides the cytoskeleton rearrangement and the subsequent morphological changes, EMT is often originated from the disruption of epithelial adhesion and other polarized structures, such as tight junctions and adherens junctions, followed by the acquisition of migratory and invasive properties of the cells [5]. While the full spectrum of signaling agents that contribute to the EMT of carcinoma cells remains unclear, numerous lines of evidence have revealed an essential role of NFκB during distinct steps of EMT and cancer progression [6]. One of the major transcriptional targets of NFκB activation is uPA. It has been well established that the uPA/uPAR axis plays a central role in the EMT process during cancer development [7]. Moreover, overexpression of uPA and uPAR has been shown to be associated with adverse prognosis in various malignancies, including breast cancer [8–10]. Interestingly, apart from NFκB, it has been shown recently that uPA can be directly targeted by a tumor suppressive miRNA, miR-193b [11,12].

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated anion channel expressed in the epithelial cells of a wide variety of tissues, mutations of which cause cystic fibrosis (CF), a common life-threatening autosomal recessive disease in Caucasian populations [13]. Of note, there is an increasing interest in the association of cancer incidence with the genetic variations in the *CFTR* gene. Large cohort studies in North American and European patients with CF found that there was a marked increase in the risk of malignancies affecting the gastrointestinal tract [14–16]. Particularly, in a recent study involving 41,188 cases of CF patients followed from 1990 to 2009 in the US,

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Maisonneuve P et al. reported a 17 times higher risk of digestive cancer with most cases arising in the bowel [16]. However, *CFTR* gene mutations have also been reported to be associated with a lower risk of several cancers, such as melanoma [17], prostate cancer [18] and lung cancer [19]. On the other hand, CFTR has been suggested to interact with various cancer-related kinases [20,21]. Despite all these observations, the biological role of CFTR in cancer development is still unclear.

Interestingly, various studies have reported that defect/suppression of CFTR in the epithelial cells results in aberrant activation of NFκB [22–24]. Given that the uPA/uPAR axis plays a central role in EMT, and uPA is known to be activated by NFκB during cancer development [7], we reasoned that CFTR might also be involved in the regulation of EMT and cancer metastasis through the NFκB/uPA pathway. Thus, we undertook the present study to investigate the role and the underlying mechanisms of CFTR in breast cancer EMT and metastasis. Our results show that CFTR suppresses breast cancer metastasis both *in vitro* and *in vivo* through modulation of EMT. Moreover, we demonstrate that low expression of CFTR is associated with cancer progression and poor survival of breast cancer patients.

2. Materials and methods

2.1. Cell lines and functional studies

We obtained human breast adenocarcinoma cell lines MCF-7 and MDA-231 from the American Type Culture Collection (Manassas, VA, USA) and cultivated them in DMEM/F12 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 unit/ml penicillin. EMT was induced using TGFβ$_1$ (2 ng/ml). CFTR channel function was blocked by CFTRinh-172 (10 μM) or GlyH101 (5–10 μM). For wound healing assay, cells were seeded at 1×10^6 cells/well in 6-well plates and then pre-incubated for 24 h before creating a wound across the cell monolayer with a plastic tip. Cells were then grown in culture medium with 1% FBS in the presence or absence of 10 μM inh172 or 10 μM GlyH101. The closure of the wound *via* the migration of cells into the wound was tracked and recorded using a Time Lapse Imaging System (CarlZwiss) at 1 hour intervals for 48 h. Cell migration was determined by measuring distances between parallel lines from the initial sites to the migrated sites. The experimental procedure was repeated three independent times. We tested cell invasion in modified Boyden chambers containing porous (8 μm), polycarbonate membranes (Corning Incorporated) coated with 500 μg/ml matrigel (BD biosciences, San Jose, USA). A total of 20,000 cells were added to the transwell inserts over the top of the artificial basement membrane. The plate was then incubated for 72 h for MCF-7 or 24 h for MDA-231 at 37 °C, 5% CO_2 and 95% humidity. After 72 h or 24 h, the inserts were removed from the plate and the inside of the insert was cleaned thoroughly with tissue paper. Any cells which had invaded through the membrane and passed to the underside of the insert were fixed in 4% paraformaldehyde (v/v) in PBS for 5 min before being stained in 0.5% crystal violet solution (w/v) in distilled water. At least five random fields per insert were counted and triplicate inserts were set up for each test sample. The experimental procedure was repeated for three times. Cell growth rate was measured with an MTS proliferation assay. The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) was conducted according to the manufacturer's instructions.

2.2. CFTR overexpression and knockdown

The pEGFPC3 plasmid expressing wild-type CFTR was kindly provided by Professor Tzyh-Chang Hwang (University of Missouri-Columbia). For overexpression experiments, the MDA-231 cells were transfected with 3 μg DNA and 6 μl Lipofectamine 2000 (Invitrogen, Camarillo CA), the transfected MDA-231 cells were selected in full medium containing G418 (Calbiochem, Schwalbach, Germany) at 1200 μg/ml. To knock down CFTR expression in MCF-7 cells, miRNA duplex specific to human CFTR was synthesized by Lift Technologies. The miRNA sequence is as follows: 5′-TTG GAA AGG AGA CTA ACA AGT-3′. MiR expression vector, named as pcDNA™6.2-GW/EmGFP, containing a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA sequence was established using BLOCK-iT™ Pol II miR RNAi Expression Vector kits (Life Technologies, Rockville, MD) following the standard protocol. Lentiviral particles were produced by transient transfection of 293FT (Invitrogen) cells using Lipofectemin 2000 (Invitrogen) reagent. Viral supernatants were collected 24 h post-transfection. One day before transduction, MCF-7 cells were seeded into 35 mm culture dishes at 60% to 80% confluence. Viral supernatants were mixed with fresh medium at the ratio of 1:1, and incubated with cells for 24 h. Blasticidin at the final concentration of 5 μg/ml was used to select the stable clones.

2.3. Primary breast cancer samples

To determine the clinical relevance of CFTR in breast cancer, tissue samples were collected from patients ($n = 17$) with primary breast cancer from the Department of Pathology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China. All samples were collected with the informed consent of the patients and the study was approved by the Ethics Committee. The clinical and histopathologic characteristics of patients with breast cancer are presented in Appendix A: Supplementary Table 2.

2.4. Immunohistochemistry and immunofluorescent staining

Primary breast cancer sections (5 μM) were deparaffinized, rehydrated and rinsed with PBS. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide and rinsed with PBS. Antigen retrieval was carried out in a retrieval citrate buffer (pH 6.0) at 95 °C for 15 min. The sections were rinsed with PBS and then incubated in 10% normal goat serum blocking solution in a humidified box at room temperature for 1 h. After blocking, anti-CFTR (Santa Cruz, 1:200) in PBS-T solution was added and the sections were incubated in a humidified box at 4 °C overnight. As negative staining controls, separate slides of tissue sections were incubated in PBS containing 1% goat serum in the absence of primary antibodies. The staining was visualized using DAB Plus Chromogen, followed by counterstaining with hematoxylin. Slides were evaluated for the staining intensity (0, none; 1, weak; 2, moderate; and 3, strong) of positively stained cells. Two investigators independently evaluated the tissue sections. For immunofluorescent staining, cells were fixed in 4% paraformaldehyde and blocked with 1% bovine serum albumin, and incubated overnight with primary antibodies at 4 °C. Then secondary Alexa fluor 568 goat anti-mouse IgG, fluor 488 goat anti-rabbit IgG or 568 donkey anti-rabbit IgG at a dilution of 1:500 was loaded on cells for 1 h. Samples were all co-stained with Hoechst 33342. Following three washings with PBS, the slides were mounted and visualized with fluorescence microscopy.

2.5. Animal studies

Nude mice were provided by the Laboratory Animal Service Center of the Chinese University of Hong Kong. They were maintained in an air-conditioned room with controlled temperature of 24 ± 2 °C and humidity of $55 \pm 15\%$, in a 12 h light/darkness cycle regulation and were fed laboratory chow and water *ad libitum*. All animal experiments were conducted in accordance with the University Laboratory Animals Service Center's guidelines on animal experimentation with approval from the Animal Ethnics Committee of the University. Tumorigenicity was investigated by tumor xenograft experiments. The athymic female nude mice of 6–8 weeks old were injected with 100 μl suspension of CFTR overexpressing cells or vector control MDA-231 cells (about 5×10^6) subcutaneously. Mice injected with saline were used as sham control. Tumor formation in nude mice was monitored about a 6 week

period and ratios of tumor weight to body weight were measured. Mice with tumor size larger than 1 cm in any dimension were terminated. The tumor size was calculated according to the following formula: $0.5234 \times [\text{long diameter} (\text{short diameter})^2]$. For metastatic model, six- to eight week old female nude mice were transplanted with 1×10^6 MDA-231 cells through the lateral tail vein under sterile conditions. All mice were sacrificed 35 days after injection by perfusion. Lungs were collected, fixed and sectioned (5 μm) for HE (hematoxylin–eosin) staining. Number and size of tumor foci were countered and calculated.

2.6. Correlation with breast cancer prognosis

Breast cancer tissues and normal background tissues were collected in the Department of Surgery, Cardiff University School of Medicine, Cardiff, UK. These tissues were collected immediately after mastectomy and snap frozen in liquid nitrogen, with approval of the local ethics committee. Background normal mammary tissues were removed from the same patients. The pathologist verified the normal background and cancer specimens, and it was confirmed that the background samples were free from tumor deposits. The median follow-up period was 120 months (June 2004). We subgroup the patients to Surv 1, 2, 3, and 4. Surv 1 represents those patients alive and well 10 years after surgery, Surv 2 and 3 represent patients with metastasis and local recurrence, respectively within the follow-up period. Surv 4 represents patients who died from breast cancer. Thus, Surv 2, 3, and 4 indicate patients in disease, including those with metastasis, local recurrence, and death compared to Surv 1, which is considered as disease free. RNA extraction, reverse transcription–PCR and quantitative PCR were performed as described before [25]. Statistical analysis was performed using the Minitab (Minitab Ltd., Coventry, UK) statistical software package (version 14). The two sample *t* test was used for normally distributed data. Kaplan–Meier survival analysis was performed using SPSS statistical software (version 11; SPSS. Chicago, IL, USA). Differences were considered to be statistically significant at $P < 0.05$.

2.7. Statistical analyses

Data are presented as the mean ± SEM and Student's unpaired *t* test was used for 2 groups of statistical analysis. Differences between groups were analyzed using Prism 4 (GraphPad, Inc., San Diego, CA). A p value <0.05 was considered statistically significant.

3. Results

3.1. TGF-β_1-induced EMT in breast cancer cells involves downregulation of CFTR

TGF-β is considered as a key mediator of EMT during physiological processes which is frequently and abundantly expressed in various tumors and induces EMT in cancer cells during cancer progression [26]. In addition, TGFβ signaling has been linked to CF pathogenesis [27,28]. As a first step to investigate the role of CFTR in the EMT process, we treated non-invasive breast cancer cell line MCF-7 with TGF-β_1, which has been shown to be a potent EMT inducer in MCF-7 cells, for 48 h. As shown in Fig. 1a a′ & b′, 2 μg/ml TGF-β_1 dramatically downregulated epithelial marker, E-cadherin, in MCF-7 cells. Interestingly, along with the loss of E-cadherin, the expression of CFTR was significantly suppressed by TGF-β_1 suggesting that downregulation of CFTR is associated with EMT (Fig. 1a & b). In order to investigate whether suppression of CFTR function *per se* could induce an EMT phenotype in MCF-7, the cells were treated with specific CFTR inhibitors, inh172 or GlyH101, in the absence of TGF-β_1. Our immunofluorescent staining result showed that treatment with 10 μM inh172 mimicked the effect of TGF-β_1, downregulating the expression of E-cadherin (Fig. 1c). The downregulation of epithelial marker and upregulation of mesenchymal marker by CFTR inhibitors (inh172 and GlyH101) were further verified by our western blot analysis (Fig. 1d & Appendix A: Supplementary Fig. 1). In addition, we demonstrated that suppression of CFTR function by its inhibitors promoted both cell invasion and migration of MCF-7 cells (Fig. 1e & f). Taken together, these results indicate that suppression of CFTR function elicits an EMT phenotype with enhanced metastatic capability in MCF-7 cells.

3.2. Knockdown/overexpression of CFTR induces/inhibits EMT and malignant phenotypes of breast cancer cells

The observed involvement of CFTR in mediating the effect of TGF-β_1 on EMT prompted us to ask whether CFTR gene manipulation could alter EMT and malignant phenotypes in breast cancer cells. First, we established MCF-7 cells with CFTR knockdown using a lentiviral expression system. As shown in Fig. 2a, CFTR knockdown significantly inhibited the expression of E-cadherin and Occludin, whereas it upregulated the expression of Laminin γ2 at the protein levels in MCF-7 (Fig. 2a). In addition, whereas CFTR knockdown did not have any effects on cell proliferation (Appendix A: Supplementary Fig. 2), suppression of CFTR dramatically increased the invasive and migratory capacity of MCF-7 cells (Fig. 2b & c). Taken together, these data suggest that either the inhibition of CFTR channel function or the knockdown is sufficient to trigger EMT.

As EMT has been considered as a reversible processing, we overexpressed wild-type CFTR in MDA-231, which is known as a highly invasive breast cancer cell line with typical mesenchymal features, to explore the possible effect of CFTR on its reversion to epithelium phenotype. As shown in Fig. 2d, CFTR expression was significantly increased in CFTR overexpressing stable cells compared to control vector transduced cells. Consequently, we observed that the overexpression of CFTR caused an increased level of epithelial marker E-cadherin and a decreased level of mesenchymal marker vimentin in the MDA-231 cells (Fig. 2d). In addition, functional assay demonstrated that the overexpression of CFTR repressed invasive and migratory ability in MDA-231 cells (Fig. 2e & f). Taken together, these data strongly suggest an important role of CFTR in regulating the EMT process and malignancy in breast cancer.

3.3. CFTR inhibits lung metastasis in vivo

We further tested whether we could suppress metastatic potential of breast cancer *in vivo* by overexpressing CFTR in nude mice. First, to test the tumorigenic capability, we established xenograft models by subcutaneous injection of MDA-231 cells transduced with CFTR or the corresponding empty vector into the right flanks of mice, and the primary tumor mass of tumor-bearing mice were monitored until sacrifice. Our results exhibited no differences in primary tumor growth between control vector transfected and CFTR overexpressing MDA-231 injected mice (Fig. 3a–c). We then tested the possible effects of CFTR overexpression on the metastatic potential of MDA-231 cancer cells. We injected MDA-231 cells expressing either control vector or CFTR into the tail vein of nude mice and examined signs of metastasis in the lungs 35 days after injection. Detailed quantification was conducted with H&E staining on the lung sections. We found that while 100% (6/6) of the mice injected with MDA-231-empty vector-transfected cells developed metastases at the end of the experiments, only 50% (3/6) of the mice injected with CFTR overexpressing MDA-231 cells presented lung metastases (Fig. 3e). Moreover, the tumor burden in the control vector group and the CFTR overexpression group had a significant difference after 35 days (Fig. 3f). These data, along with the results of *in vitro* analysis, indicate the role of CFTR as a metastasis suppressing gene.

3.4. Anti-metastatic effect of CFTR is mediated by NFκB targeting uPA

The urokinase-type plasminogen activator (uPA) is a secreted serine proteinase that converts plasminogen to plasmin. The binding of uPA to its specific membrane receptor, uPAR, initiates a proteolytic cascade for degrading ECM which in turn regulates cell migration and invasiveness.

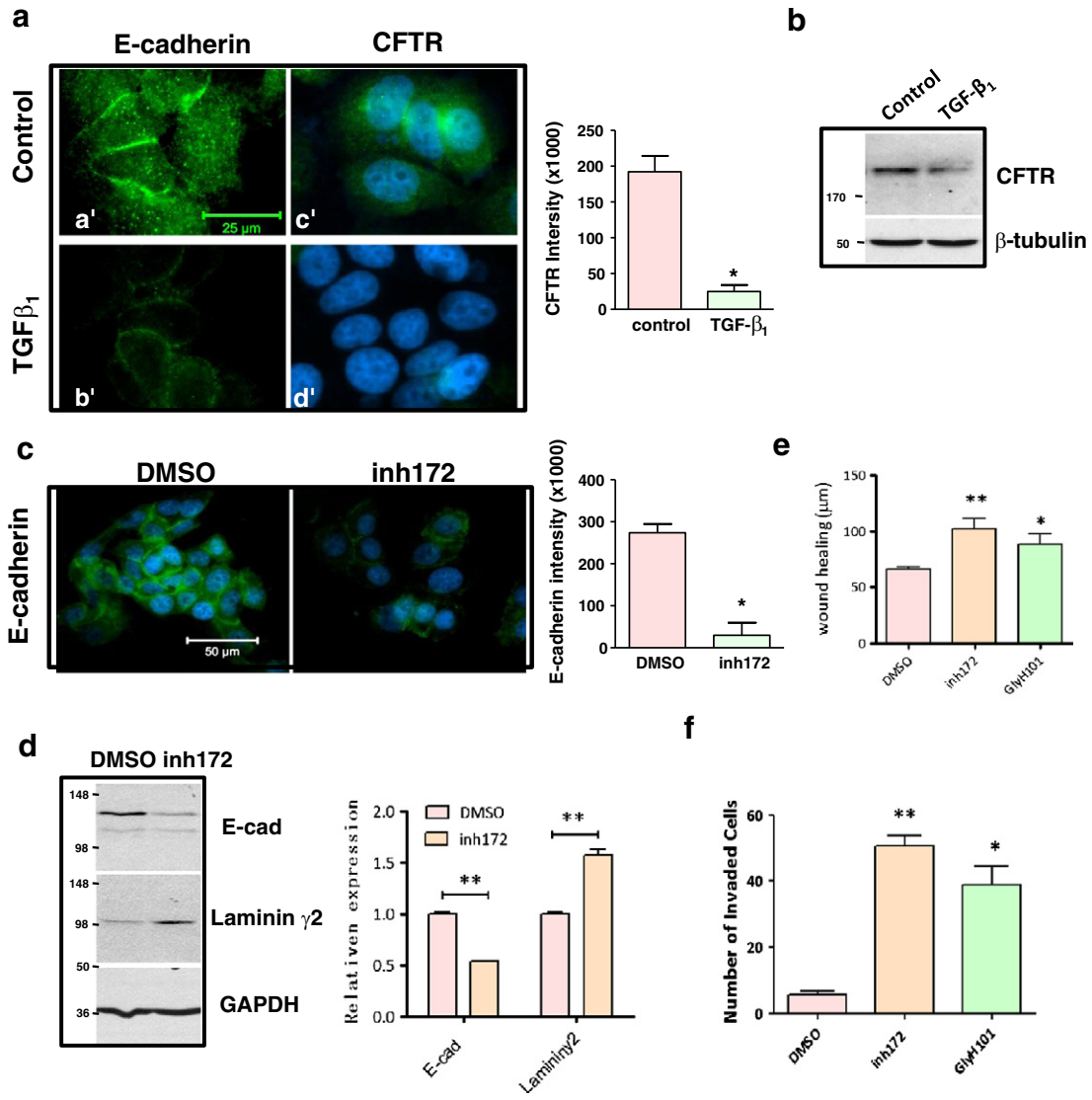

Fig. 1. CFTR suppression induces EMT in MCF-7 cells. (a–d) MCF-7 cells were treated with 2 ng/ml TGF-β_1 for 2 days. (a) Cells were stained with CFTR (ACL-006) (scale bar = 25 μm), the quantified intensity change is expressed as Mean ± SEM from three independent experiments,*$p < 0.05$. (b) Representative western blot showing TGFβ_1 downregulates CFTR expression from two independent experiments (c) Immunofluoresence staining shows that 24 h treatment with inh172 (10 μM) suppresses the expression of E-cadherin in MCF-7, green, E-cadherin; blue: DAPI (scale bar = 50 μm). The quantified intensity change is expressed as Mean ± SEM from three independent experiments,*$p < 0.05$. (d) MCF-7 cells were treated with 10 μM inh172 for 48 h. Western blot analysis shows inhibition of CFTR upregulates mesenchymal marker laminin γ2, whereas decreases epithelial marker E-cadherin and Occludin. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, ** $p < 0.01$. (e) Inhibition of CFTR with two inhibitors significantly enhances cell migration in MCF-7 cells. MCF-7 cells were incubated in 1% FBS with or without 10 μM inh172 or GlyH101 for 24 h. Wound healing assay was used to determine cell migration. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments,*$p < 0.05$, **$p < 0.01$. (f) Inhibition of CFTR enhances cell invasion. MCF-7 cells were incubated with DMSO or 10 μM inh172 or GlyH101, and modified Boyden chamber invasion assay was used to examine the effect of CFTR inhibition on cell invasion for 72 h as described in Materials and Methods. Note that while there was barely detectable cells migrated in control MCF-7 cells, treatment with inh172 or GlyH101 significantly promoted cell invasion. At least 4 random fields per insert were counted and triplicate inserts were set up for each test sample. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, *$p < 0.05$, **$p < 0.01$.

UPA and uPAR have been implicated as critical components in the regulation of metastatic phenotype of cancer including EMT [29,30]. In an attempt to explore by which mechanism(s) CFTR suppresses EMT and metastatic phenotype of breast cancer, we found that CFTR knockdown significantly increased uPA expression and secreted uPA activity in MCF-7 cells (Fig. 4a & b), whereas overexpression of CFTR was sufficient to decrease uPA activity in MDA-231 cells (Fig. 4c), indicating that uPA activity is CFTR dependent in these cancer cells. Since uPA is known to be activated by NFκB and defective CFTR has been shown to result in endogenous activation of NFκB [24,31], we further tested whether the effect of CFTR on uPA was mediated by NFκB. Indeed, in this study, we found that CFTR knockdown leads to enhanced expression of NFκB p65 and p50 in MCF-7 (Fig. 4d). In addition, we showed that the transcriptional activity of both p65 and p50 was significantly increased in CFTR knockdown MCF-7 cells compared to control cells (Fig. 4e). More importantly, we demonstrated that NFκB inhibitor curcumin reversed the upregulation of uPA in CFTR knockdown MCF-7 cells (Fig. 4f). If the tumor-suppressing effects of CFTR are largely due to alteration in NFκB activities, the effects observed with either CFTR knockdown or overexpression should be reversed by altering the NFκB activity. To test this, we used CFTR knockdown MCF-7 cells as our model system to test whether CFTR repression-induced EMT may be reversed by inhibiting NFκB activity. We treated both control and CFTR knockdown MCF-7 cells with different concentrations of curcumin and evaluated their changes in EMT markers and metastatic phenotypes. As shown in Appendix A: Supplementary Fig. 3, curcumin significantly reversed the molecular changes of EMT in the CFTR knockdown cell. More importantly, the inhibition of NFκB activity completely abrogated the CFTR

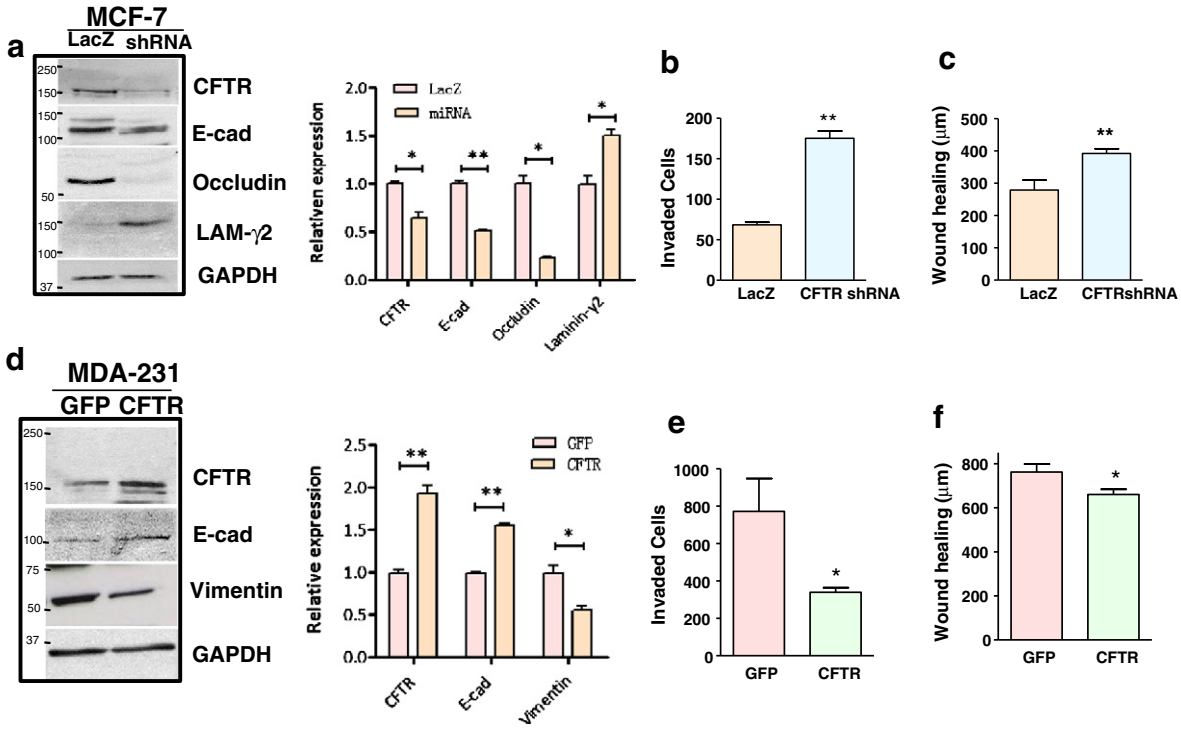

Fig. 2. CFTR gene manipulation alters EMT and invasive phenotype of breast cancer *in vitro*. (a) knockdown of CFTR in MCF-7 cells decreases the expression of epithelial marker E-cadherin and Occludin, whereas increases the expression of mesenchymal marker Laminin -γ2. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$. (b) Knockdown of CFTR in MCF-7 enhances cell invasion, both control and CFTR knockdown MCF-7 cells were examined for cell invasive capacity with modified Boyden chamber invasion assay for 72 h. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $^{**}p < 0.01$. (c) Knockdown of CFTR in MCF-7 significantly promotes cell migration, quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $^{**}p < 0.01$. (d) Western blot analysis shows that overexpression of CFTR increases the expression of epithelial marker E-cadherin, whereas decreases the expression of mesenchymal marker vimentin in MDA-231 cells. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$. (e) Overexpression of CFTR inhibits cell invasion in MDA-231 cells, both control and CFTR overexpressing MDA-231 cells were examined for cell invasive capacity with modified Boyden chamber invasion assay for 24 h. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $^{*}p < 0.05$. (f) Overexpression of CFTR significantly suppresses cell migration in MDA-231 cells, Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $^{*}p < 0.05$.

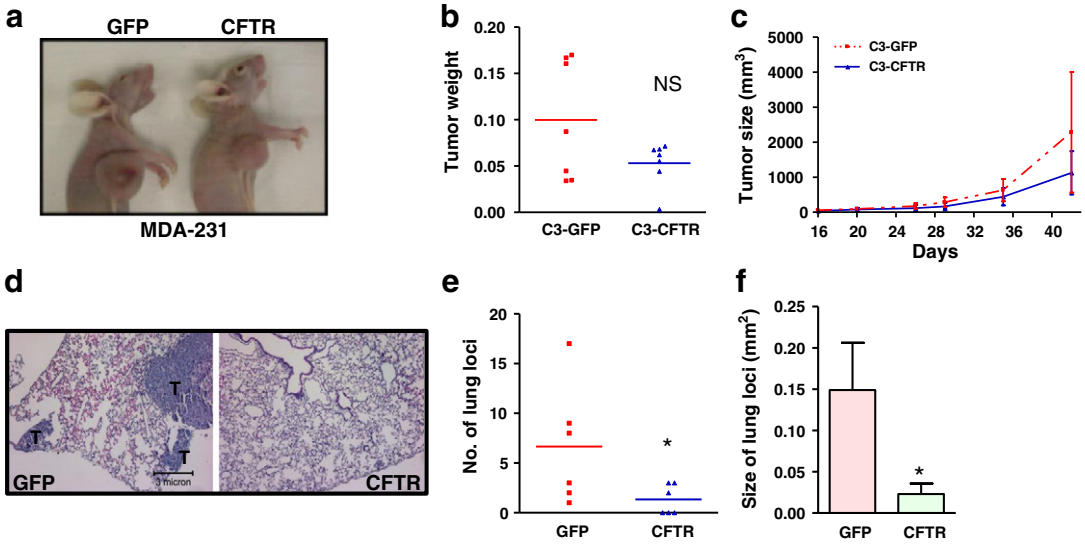

Fig. 3. Overexpression of CFTR suppresses breast cancer metastasis *in vivo*. (a–c) *In vivo* xenograft assay for MDA-231 cells transfected with empty vector or CFTR. Tumor size was measured 6 weeks after injection of 5×10^6 tumorigenic MDA-231 cells transfected with empty vector (n = 7) or CFTR (n = 7) subcutaneously. Data are mean ± SEM. No significant difference was detected between two groups, *t* test. (d–f) 5×10^6 tumorigenic MDA-231 cells transfected with empty vector (n = 6) or CFTR (n = 6) were injected in tail vein. 35 days after injection, animals were sacrificed and lung metastasis determined by H&E staining. (d) Representative images of H&E staining shows multiple tumor loci in animal lung injected with control vector transduced MDA-231 cells, whereas no metastatic loci were detected in animal injected with CFTR overexpressing MDA-231 cells, scale bar = 50 μm. (e) Metastatic loci were counted and compared between GFP and CFTR groups, *t* test,$^{*}p < 0.05$. (f) Metastatic tumor size was determined between GFP and CFTR groups. Data are represented as Mean ± SEM, *t* test, $^{*}p < 0.05$.

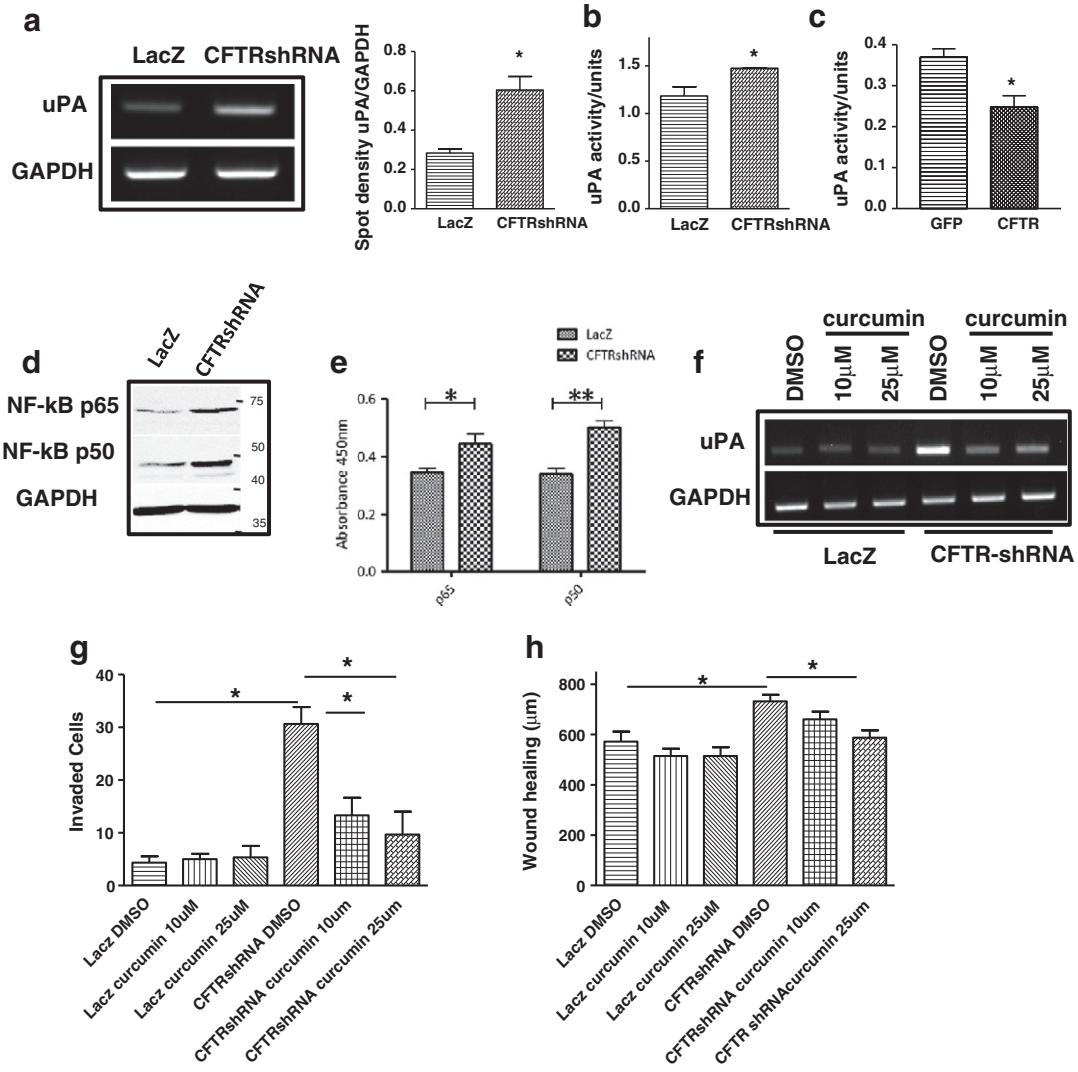

Fig. 4. Involvement of NFκB and uPA in mediating the tumor-suppressing effects of CFTR. (a) RT-PCR analysis shows increased expression of uPA in CFTR knockdown MCF-7 cells compared to vector control cells. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, *$p < 0.05$. (b) The activity of uPA is upregulated in CFTR knockdown MCF-7 cells compared with vector control cells. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, $p < 0.05$. (c) The activity of uPA is downregulated in CFTR overexpressing MDA-231 cells, *$p < 0.05$. Data are mean ± SEM from three independent experiments. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, *$p < 0.05$. (d) Representative western blot image shows increased expression of NFκB p65 (sc-109) and p50 (sc-7178) in MCF-7CFTRsiRNA cells compared to control cells, whereas decreased in MDA-231 cells overexpressing CFTR. (e) Luciferase activity assay shows the transcriptional activity of p65 and p50 is upregulated in CFTR knockdown MCF-7 cells. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, *$p < 0.05$, **$p < 0.01$. (f) Representative PCR image showing the upregulation of uPA is abrogated by NFκB inhibitor curcumin in MCF7 CFTRshRNA cells as determined by RT-PCR. (g,h) MCF-7 miR-LacZ or miR-CFTR transduced cells were incubated with either 10 μM or 25 μM NFκB inhibitor curcumin. (g) Invasion assay shows that 10 μM and 25 μM curcumin significantly reverse the enhanced invasion in MCF-7CFTRshRNA cells without inhibitor treatment. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, *$p < 0.05$. (h) Wound healing migration assay shows that the treatment with 25 μM curcumin reverses the enhanced migration in MCF-7CFTRshRNA cells. Quantification analysis of data is expressed as the Mean ± SEM from three independent experiments, *$p < 0.05$.

knockdown-enhanced cell invasion and migration in these cells, indicating that the upregulation of NFκB activity targeting uPA is the major mechanism leading to the observed increased malignancies induced by CFTR knockdown in these cancer cells. Interestingly, a fast-growing body of evidence has indicated that CFTR might play a more complex and extensive role than we previously thought [32,33]. Indeed, besides uPA, we have found that downregulation of CFTR in MCF-7 cells regulates a variety of genes involved in EMT and tumor metastasis using PCR array (Fig. 5a & b).

3.4.1. CFTR downregulation is correlated with poor prognosis of breast cancer

Having established the effect of CFTR on the regulation of EMT and metastasis in breast cancer cell lines, we went on to investigate the expression pattern of CFTR in clinical breast cancer samples. First, we analyzed the immunoreactivity of CFTR in paraffin-embedded sections of normal breast ($n = 3$) and breast cancer tissues ($n = 17$). Among these breast cancer samples, 9 patients were diagnosed as invasive ductal carcinoma (IDC), whereas 8 patients were diagnosed as invasive lobular carcinoma (ILC). The immunohistochemical staining of CFTR was scored in a semiquantitative manner by examination of the cytoplasmic and membrane staining intensity and compared with normal tissue. Our results showed that in normal breast tissue (Fig. 6a), most luminal epithelial cells showed a strong apical expression of CFTR (score 3). However, the expression of CFTR was significantly decreased and distributed diffusely in the cytoplasm of the breast cancer cells. 13 of 17 cases of breast cancer exhibited weak expression of CFTR (score ≤ 1, Fig. 6a & b and Appendix A: Supplementary Table 2). We did not find any significant difference in CFTR expression levels between IDC and ILC. Moreover, we confirmed CFTR downregulation in the Oncomine

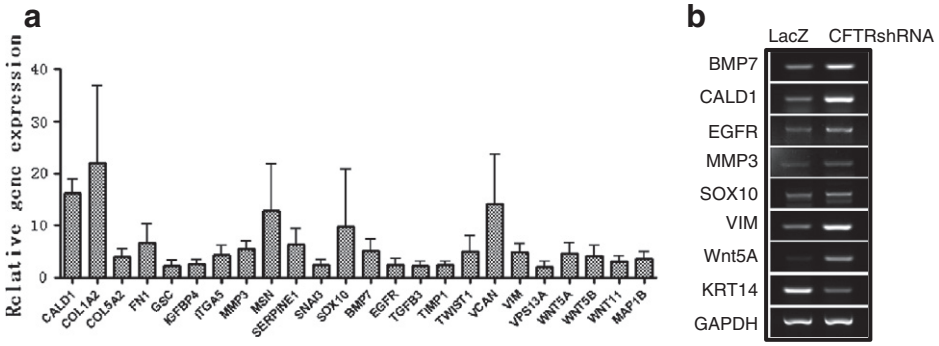

Fig. 5. CFTR knockdown induces alteration in a variety of EMT genes. (a) EMT-focused PCR array analysis shows the upregulated EMT-promoting genes between control and CFTR knockdown MCF-7 cells. Data were analyzed by comparing $2^{-\Delta Ct}$ of the normalized data. Fold changes were calculated relative to the control MCF-7. Quantification analysis of data is from two repeated experiments and an arbitrary cut-off of >2-fold change was used to identify genes that were differentially expressed between samples. (b) RT-PCR analysis confirms the expression profiles of some altered genes indicated in the PCR array.

database of expression profiles of breast cancer samples. In three analyses [34,35], significant downregulation of CFTR was observed in cancer samples compared with normal breast tissue (Fig. 6c). Next, we attempted to evaluate the prognostic potential of CFTR in different cohorts of breast cancer patients. The CFTR expression was first examined in accordance with patients' Nottingham Prognostic Index (NPI) using Real-time qPCR. Statistical analysis showed that patients with poor prognosis (NPI3) had significantly lower levels of CFTR transcripts, ($p = 0.028$) when compared to patients with good prognosis (NPI1) (Fig. 6d). In another cohort of patients, regarding clinical outcomes after a median 120 month's follow-up, the patients with poor prognosis, including those with metastasis, local recurrence, and death due to breast cancer, had significantly lower levels of CFTR transcripts compared to disease-free patients, who have no cancer and live well 10 years after surgery ($p < 0.05$, Appendix A: Supplementary Fig. 4). The patients who died from breast cancer had the lowest expression levels of CFTR compared to that of disease-free patients ($p = 0.017$) (Fig. 6e). Thus, reduced expression of CFTR is significantly associated with disease progression and poor prognosis in breast cancer.

4. Discussion

Although there has been recent interest in the risk of various cancers in CF patients, studies on the role of CFTR in the pathogenesis of cancer are very limited. In the present study, we have revealed a previously undefined role of CFTR in breast cancer progression/metastasis. Our gain

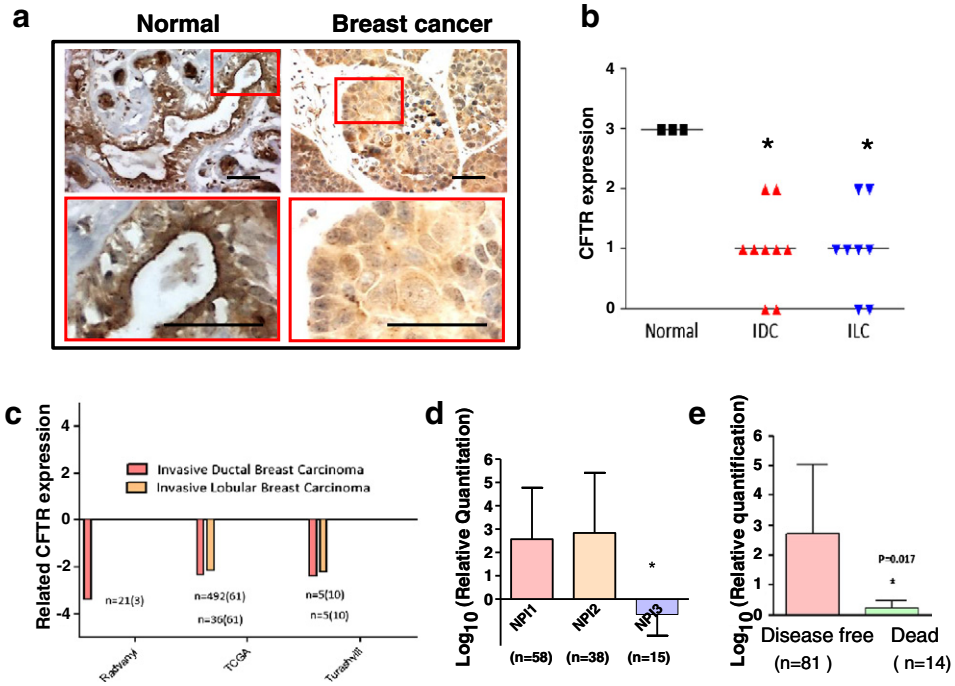

Fig. 6. CFTR downregulation is correlated with poor prognosis in breast cancer. (a) Immunohistochemical staining of CFTR in human breast cancer showing decreased CFTR intensity and abnormally distributed CFTR in cancer sample compared to adjacent normal tissue. The red box marks the area selected and enlarged (scale bar = 40 μm) (n = 17). (b) Quantification analysis showing the expression of CFTR in breast cancer is significantly decreased compared to normal breast tissue. A nonparametric analysis of Mann–Whitney *U*-test was used to test the immunostaining raw scores between the control and tumors, given the fact that the analytical IHC scores were not normally distributed, $p < 0.05$. (c) Oncomine database of expression profiles of breast cancer samples show the significant downregulation of CFTR in cancer samples compared with normal breast tissue. (d) CFTR expression and Nottingham prognostic index in breast cancer. The NPI1 group (NPI score < 3.4; n = 58) represents patients with good prognosis, the NPI2 group (NPI score 3.4–5.4; n = 35) represents patients with moderate prognosis, while patients of the NPI3 group (NPI score >5.4; n = 15) had a poor prognosis, data are mean ± SEM, *$p < 0.05$. (e) Low CFTR expression is associated with poor prognosis in breast cancer patients. In a cohort of patients with 120 months follow-up, expression of CFTR is significantly decreased in patients who died of breast cancer compared to disease-free patients. Data are Mean ± SEM, *$p < 0.05$.

and loss of function analyses with CFTR manipulation demonstrate that downregulation of CFTR in MCF-7 promotes breast cancer malignant phenotype which is associated with altered cellular invasion, migration and EMT, whereas overexpression of CFTR suppresses EMT and invasive properties in MDA-231 cells (Fig. 2). More importantly, while overexpression of CFTR has no significant effect on tumorigenicity in nude mice xenograft model, excessive CFTR dramatically suppresses breast cancer lung metastasis *in vivo* (Fig. 3). Taken together, these results clearly demonstrate that CFTR, while it has no significant effect on tumorigenicity, is inversely associated with the progression and metastasis of breast cancer. Intriguingly, the findings of this study are supported by clinical observation that while there was no difference in breast cancer rate between ΔF508 carrier and non-carrier, breast cancers arising from ΔF508 *CFTR* carriers were at least grade III based on combined pathological and cytological grading system, indicating that defective CFTR is related to breast cancer progression [36].

Localized at the apical membrane of epithelial cells, CFTR has been reported to be required for the normal organization and function of cell junctions, and loss of CFTR has been shown to lead to abnormally low transepithelial resistance and loss of epithelial integrity [37]. On the other hand, loss of one of the key components of adherens junctions, E-cadherin, often occurs in the later stages of tumorigenesis and contributes to EMT, which represents a crucial step in metastasis. In our study, we have clearly demonstrated that suppression of CFTR function leads to loss of E-cadherin whereas overexpression of CFTR results in upregulation of E-cadherin, which is in line with observed changes of EMT process and malignant phenotypes in breast cancer cell lines (Figs. 1 & 2). In addition, we have found that TGFβ$_1$, a potent EMT inducer, significantly downregulates the expression of CFTR in MCF-7 cells (Fig. 1a & b), suggesting that TGFβ$_1$ may induce the disruption of cell polarity and EMT through downregulation of CFTR. Of note, it has been reported that TGF-β receptors directly interact with core polarity protein, Par6, at the level of adherens junctions, which is required for TGFβ-dependent EMT in mammary gland epithelial cells, demonstrating the importance of TGF-β-regulated cell polarity in EMT and the development of metastasis [5]. Interestingly, apart from TGF-β, other EMT-inducing factors, such as TNFα and HIF1α, have been demonstrated to downregulate CFTR expression [38,39], indicating a central role of CFTR as a downstream effector in mediating the effects of various EMT inducers.

By what mechanism does CFTR exert its effect on EMT? Increasing evidence indicates that a defect of CFTR in the apical membrane of epithelial cells results in endogenous activation of NFκB, which in turn results in excessive production of proinflammatory cytokines [22–24]. Thus, functional CFTR is required for controlling the NFκB-mediated inflammatory signaling [31,40–42]. In the current study, we have shown that the knockdown of CFTR results in NFκB activation in breast cancer cell lines (Fig. 4d & e), consistent with the previous findings in other cell types. More importantly, the CFTR knockdown-induced EMT and invasive phenotype in MCF-7 cells are completely abrogated by a NFκB inhibitor (Fig. 4g,h), curcumin, indicating that enhanced EMT and malignant phenotype in CFTR knockdown cells may be attributed to NFκB activation. Of note, a study in 2005 found that curcumin-stimulated CFTR, suggesting its therapeutic use in the treatment of CF. However, further studies are needed to further explore this proposed mechanism of curcumin and its role in CF treatment [31,43]. The NFκB family of transcription factors plays pivotal roles in both promoting and maintaining an invasive phenotype of cancer [44,45]. Wirth and coworkers have identified NFκB as a central mediator of EMT in a mouse model of breast cancer progression [6]. Specifically, NFκB transcriptionally regulates the master regulator genes that represses epithelial phenotype or activates mesenchymal phenotype, such as snail, twist1 and vimentin. Of particular interest, NFκB can activate uPA, which has been well established to play a central role in extracellular matrix degradation, invasion, and metastasis during cancer development [46]. Moreover, overexpression of uPA and uPAR has been shown to be associated with adverse prognosis in various malignancies [8], including breast cancer [47,48]. In our study, we have demonstrated that the expression and activity of uPA are inversely associated with CFTR expression in breast cancer cells (Fig. 4a–c). In addition, we have shown that the NFκB inhibitor curcumin reverses the upregulation of uPA in CFTR knockdown MCF-7 cells (Fig. 4f). These results indicate that the EMT-suppressing effect of CFTR is associated with its ability to alter NFκB activity targeting uPA. Of interest, while we have demonstrated previously that CFTR suppressed prostate cancer progression through miR-193b-medicated regulation of uPA [12], we did not detect any significant change of miR-193b in breast cancer cell lines with CFTR manipulation. These findings indicate that CFTR may regulate metastatic process through distinct tissue-specific pathways in different types of cancer. On the other hand, the effect of CFTR on EMT may also be mediated by different pathways in the same cancer type. Indeed, we have found that downregulation of CFTR in MCF-7 cells increased the expression of various ECM and cell invasion-related genes (Fig. 5). Among the differentially expressed genes, the upregulation of fibronectin, vimentin and collagens has been closely associated with the EMT process. Sox10 and twist have been well established as key transcriptional factors involved in mesenchymal transition in both development and pathological conditions, such as cancer. In addition, Wnt, Bmp and EGF activated pathways play critical roles in the modulation of EMT and cancer progression. These results indicate the possible involvement of other pathways directly or indirectly linked to NFκB activation in breast cancer metastasis, the details of which require further investigation.

The present study has also demonstrated that CFTR expression is abnormally distributed in primary human breast cancer and inversely correlated with the poor prognosis of the patients. While the expression of CFTR is mostly localized in the apical membrane of normal luminal epithelial cells, the staining of CFTR is found diffusely distributed in the cytoplasm of cancer cells (Fig. 6a), indicating that apical membrane expression/function of CFTR is required for the maintenance of epithelial polarity and suppression of EMT and cancer malignancy. It is noteworthy that CFTR expression is not related to ER and PR status (Appendix A: Supplementary Table 2 and Supplementary Fig. 5). While the mechanisms regulating CFTR expression in cancer remain largely elusive, the CFTR gene is reported to be frequently hypermethylated and/or mutated in various primary cancer samples and cell lines, which may contribute to cancer malignancy as demonstrated in the present study. The present finding that CFTR is aberrantly expressed in breast cancer samples, and its correlation with poor breast cancer progression, warrants future investigations on CFTR methylation or mutation in breast cancer patients, which are likely to provide attractive and novel targets for prognosis and therapeutic intervention of breast cancer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.07.021.

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