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C/EBP ζ targets to neutrophil gelatinase-associated lipocalin (NGAL) as a repressor for metastasis of MDA-MB-231 cells

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ABSTRACT

Breast cancer is a leading cause of morbidity in women worldwide. neutrophil gelatinase-associated lipocalin (NGAL), a useful biomarker of ER negative (ER^-) breast cancer, promotes local tumor invasion and lymph node metastasis. We first identified the distinctive expression of NGAL in two breast cancer cell lines MCF7 and MDA-MB-231 cells, and then confirmed NGAL as a critical inducer of metastasis. Finally, the transcriptional factor CCAAT enhancer-binding proteins ζ (C/EBP ζ) was overexpressed in MDA-MB-231 cells. Consistent with the effect of NGAL knockdown, C/EBP ζ overexpression caused the significant changes that could prevent cell metastasis. C/EBP ζ overexpression induced a strong decrease in NGAL and matrix metalloproteinases (MMPs) expressions as determined by quantitative real time PCR and Western blotting. To identify the potential role of C/EBP ζ on regulating of NGAL in breast cancer, we established the dual-luciferase reporter assay for NGAL in MDA-MB-231 cells cotransfected with C/EBP ζ. Promoter reporter assays determined that C/EBP ζ directly repressed the human NGAL gene promoter activity by inhibiting the NGAL transcription. Taken together, this work identified that the C/EBP ζ overexpression downregulated NGAL to inhibit migration and invasion of breast cancer, which could be used as a novel strategy for breast cancer therapy.

1. Introduction

Breast cancer survival rate falls from 90% for localized to 20% for metastatic disease [1,2]. The metastasis of tumor cells causes 90% of human cancer deaths [3]. During the development of most types of human cancer, including breast carcinoma, primary tumor masses are always prone to spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new colonies [4]. Chemotherapy is the mainstay treatment for breast cancer patients with metastatic status. However, many patients exhibit resistance to chemotherapy that is either inherent or acquired during treatment [5].

NGAL (also referred to as Lipocalin 2, Lcn2) is a member of the lipocalin family. Lipocalins are small extracellular proteins that share the highly conserved structure of an 8-stranded antiparallel β barrel and have been shown to transport and present ligands, to bind to cell surface receptors, and to form macromolecular complexes, thereby playing important roles in cell regulation, proliferation, differentiation, migration, invasion and epithelial to mesenchymal transition (EMT) [6]. Estrogen exposure is one of the most well recognized risk factors for breast cancer. The expression of estrogen receptors (ER) in breast cancer suggests a role for these receptors in its pathogenesis and therapy [7]. NGAL is among those genes most highly associated with ER^- breast tumors for breast cancer progression, suggesting that NGAL is a new therapeutic target for the prevention and treatment of ER^- breast cancer [8].

As mentioned above, resistance to chemotherapy is a major problem facing breast cancer patients. A potential approach instead of chemotherapy to breast cancer is gene therapy. C/EBP ζ, a transcriptional factor, has been revealed to significantly downregulate in myeloid malignancies than normal controls [9]. It is a candidate tumor suppressor gene (TSG) that has been involved in the regulation of cellular growth and differentiation [10,11]. C/EBP ζ, also named DNA damage-inducible transcript 3 (DDIT3), belongs to a family of bZIP regulatory proteins containing two distinct domains: a basic region that binds to DNA, and an adjacent leucine-zipper region that enables homo- and hetero-dimerization of C/EBP proteins [12]. Members of the C/EBP family are known to heterodimerize among themselves, giving rise to different functional transcriptional complexes. Sequential expression of different C/EBP members was observed during the process of cell differentiation [13]. The disruption of these programs is oncogenic in several cellular contexts [14]. C/EBP ζ is proposed to act

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; EMT, epithelial to mesenchymal transition; ER, estrogen receptors; TSG, tumor suppressor gene; DDIT3, DNA damage-inducible transcript 3; ESCC, oesophageal squamous cell carcinoma; CM, conditioned medium; NHE1, Na^+/H^+ exchanger 1; MMPs, Matrix metalloproteinases; pHi, intracellular pH; pHe, extracellular pH; IL, interleukin; 2-MEO, 2-mercaptoethanol

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as a dominant negative inhibitor of other C/EBPs [15]. And subsequent studies demonstrate that C/EBP ζ expression is essential for the activation of a number of genes induced in the endoplasmic reticulum stress response for cell apoptosis [16]. C/EBP ζ has also been demonstrated to induce a decrease in tumor marker *NGAL* gene mRNA expression in human fibrosarcoma cell line HT 1080[17]. Elevated NGAL expression has been shown in different human tumors including breast [18], lung [19], colorectal [20], ovarian [21], pancreatic cancer [22], oesophageal squamous cell carcinoma (ESCC) [23] and hematopoietic malignancies [24]. Based on these findings, we hypothesize that C/EBP ζ is potential as a therapeutic gene for breast cancer treatment, and the overexpression of C/EBP ζ could downregulate NGAL expression to inhibit the metastasis of breast carcinoma.

Therefore, in this paper, the potential role of C/EBP ζ on metastasis was studied in MDA-MB-231 ER^- clones, in which C/EBP ζ was stably overexpressed, thereby resulting in a sustained downregulation of NGAL. The approach revealed that C/EBP ζ inhibited cell migration and invasion evidently by counteracting NGAL expression. These findings strongly support the concept of gene therapy that manipulation of C/EBP ζ might be beneficial to the breast cancer therapy depending on the downregulation of NGAL protein via the transcriptional regulation on NGAL promoter.

2. Materials and methods

2.1. Cell culture and reagents

HeLa, MCF7, MDA-MB-231 cells were grown in DMEM (Life Technologies, Rockville, MD), and Jurkat, K562 cells in RPMI 1640 (Life Technologies). The medium was supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Cells were grown at 37 °C in a humid atmosphere with 5% CO_2.

The following antibodies were used for Western blotting: anti-β-actin (Chemicon, USA), anti-human NGAL (R&D Systems, USA), anti-human NGAL (Abcam, UK), anti-human NGAL (Santa Cruz, USA), and anti-human C/EBP ζ (DDIT3) (Santa Cruz, USA).

Enhanced Chemiluminescence Reagent Plus (ECL) reagents were from Santa Cruz Biotechnology (Santa Cruz, USA). Cariporide were purchased from Sigma (Shanghai, China). FH535 and BAY117082 were purchased from Sigma (Beyotime, Shanghai, China).

2.2. RNA isolation and real time PCR

RNA isolation, DNase treatment, and RT-total RNA were isolated using Trizol (Invitrogen, Grand Island, NY), treated with DNase I (Invitrogen, Grand Island, NY), and 2 μg RNA were reverse-transcribed using Superscript II RT (Invitrogen, Grand Island, NY) following the manufacturer's instructions in a total volume of 20 μl.

Primers for real time PCR were designed using Primer premier software 5.0. Human β-actin primers used as an internal control were 5′-CCA CGA AAC TAC CTT CAA CTC C-3′ (forward) and 5′-ACT CGT CAT ACT CCT GCT TGC T-3′ (reverse; 272 bp). Human NGAL primers were 5′-CAA GGA GCT GAC TTC GGA AC-3′ (forward) and 5′-TAC ACT GGT CGA TTG GGA CA-3′. Human C/EBP ζ primers were (forward primer) 5′-CAG AAC CAG CAG AGG TCA CA-3′ and (reverse primer) 5′-GCT GTG CCA CTT TCC TTT C-3′. Real time PCR was performed with TransGen SYBR Green PCR kit (TransGen Biotech, China) on the ABI Prism 7500 Fast Sequence Detection System. Thermal cycling conditions were 95 °C for 10 s, followed by 40 cycles of 5 s at 95 °C, and 40 s at 60 °C. PCR reactions were performed in a total volume of 20 μl, containing 2 μl of sample cDNA, 0.2 μM of each primer, and the SYBR Green PCR kit following the manufacturer's instructions. Each test was amplified in three different wells in one experiment.

2.3. Western blotting

Secreted proteins (secreted NGAL protein included) were separated from the conditioned medium (CM) by fast ultrafiltration using Amicon® Ultra-15 centrifugal filter devices (Millipore, USA) according to the manufacturer's protocol, intracellular proteins were isolated by RIPA lysis buffer (RIPA: 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0)) with protease inhibitors , and 1 mM PMSF (Sigma, USA). The lysates were cleared using centrifugation at 12,000 rpm for 15 min at 4 °C. Protein samples were completely denatured by boiling for 10 min at 100 °C in the presence of 2% SDS and 10% 2-MEO (2-mercaptoethanol). The denatured samples were then separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked for 1 h with 5% skimmed milk in PBS and then incubated first with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies for 2 and 1 h, respectively. Specific proteins were visualized with enhanced chemiluminescence detection reagent and determined by densitometric analysis with a Lynx video densitometer (Biological Vision, Inc., San Mateo, CA).

2.4. Short hairpin RNA-mediated RNA interference studies

Three independent hairpins targeting to NGAL were developed using software from Ambion. These hairpins were synthesized and cloned into the eukaryotic vector pSilencer U6 (Ambion, Inc, USA) and then transfected into MDA-MB-231 cells. Infected cells were selected by hygromycin 48 h postinfection for at least 2 weeks and stable clones were obtained. Inhibition of NGAL expression was measured by quantitative PCR as well as by Western blotting using a rat anti-NGAL antibody. For RNA interference studies, cells transfected with the pSilencer vector that expresses a scrambled control siRNA were the negative control.

2.5. Plasmids for overexpression and promoter studies

Human C/EBP ζ was generated by PCR from a human normal cDNA library and then cloned into the plasmid pTARGET ™/Neo, which was then used to generate clones encoding the full-length protein (aa 1–169). A 1221 bp, 500 bp and 236 bp fragments of the human NGAL promoter region (−1137 to +84; forward primer, 5′-TACTCGAG-CAAGCAGCACGTAGGCAGAG-3′), (−416 to +84; forward primer, 5′-AACTCGAGCAGGAAACAGCACATGATCT-3′), (−152 to +84; forward primer, 5′-TACTCGAGCTGTCTTGCCCAATCCTGAC-3′, and reverse primer, 5′-ATAGATCTTCAG GGCCGAGGAAGCAGGC-3′) were PCR amplified with above corresponding primers, using genomic DNA as template. The restriction enzyme digested PCR fragments were cloned in Xho I/Bgl II-restricted pGL3-basic (Promega, Madison, WI), resulting in pGL3-ngal 1208, pGL3-ngal 500, pGL3-ngal 236 plasmids for luciferase reporter assay. For reporter assay, the fragment of NGAL promoter from −1137 to −185 with C/EBP ζ potential interaction sites deleted was PCR amplified and cloned into the pGL3-promoter (Promega, USA) as a negative control (designated as pGL3-control), the primers for PCR were: forward, 5′-TACTCGAGCAAGCAGCACG-TAGGCAGAG-3′, and reverse, 5′-ATAGATCTACCTCTGGCAGGGA-CAACG-3′.

2.6. Cell transfection and dual-luciferase reporter assay

MDA-MB-231 cells (1.00×10^5 cells) were transfected with 500 ng of the indicated reporter plasmid together with 2.5 ng of the internal control plasmid phRL-TK using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's protocol. Cells were lysed 24 h after transfection. To analyse the responses to pharmacological inhibitors of Na^+/H^+ exchanger 1 (NHE1) selective inhibitor

Cariporide, MDA-MB-231 cells (1.00×10^5 cells) were transfected with 500 ng of the NGAL reporter plasmids, together with 2.5 ng of the internal control plasmid phRL-TK. 24 h after transfection, cells were treated with 4 μg/ml Cariporide for 24 h, and then lysed. The luciferase activity was measured using the dual-luciferase reporter system (Promega, USA) according to the manufacturer's instructions. The transfection efficiency was normalized by the Renilla luciferase activity derived from phRL-TK (Renilla luciferase vectors). The data shown are the means ± SEM. for triplicate samples, representative of at least three independent experiments.

2.7. Cell migration (wound healing) Assay

Migration of control and NGAL knockdown cells was measured with the *in vitro* wound healing assay. Cells were grown for 3 d in six-well plates. After the cell layer had reached confluence, a wound was made with a pipette tip followed by extensive washing with serum-free medium to remove cell debris. For determination of specific signaling pathways on the migration of cells, the corresponding inhibitors were added with cells into the medium, respectively. If wanted, DMSO (final concentration: 0.1%) as vehicle control was added after wounding. Cells were then cultured as described before and allowed to migrate into the wound area for up to 36 h at 37 °C. At the indicated time points, cells were fixed with 2% formaldehyde. The wound closure was photographed by using a camera (Model DXM1200, Nikon, Japan) attached to an inverted microscope (Eclipse TE300, Nikon, Japan).

2.8. Cell invasion assay

Control or NGAL knockdown cells (5×10^4 per well in 60 μl in Dulbecco's modified Eagle's medium , DMEM) were seeded on Millipore 24-well cell culture inserts (6.5 mm diameter, Merck Millipore, USA) with 8.0 μm pores, precoated with 5 μg BD Matrigel Matrix (Becton Dickinson, USA) in 45 μL serum-free medium at their upper surface. For determination of specific signaling pathways on the invasion of cells, the corresponding inhibitors were added with cells into the upper well, respectively. If wanted, DMSO as vehicle control was added. DMEM with 10% fetal bovine serum was added to the lower wells to stimulate invasion. Cells were further incubated for 12 h. Thereafter, medium was removed and invading cells were stained with 0.2% crystal violet in 20% methanol for 30 min at room temperature. Cells that had migrated through the Matrigel to the lower surface of the filter membrane were taken photos under the microscope (Olympus, Japan). Finally the crystal violet was washed off from the cells by 33% acetic acid, and then quantified with a multiwell plate reader (Biotek Instruments, USA) at a wavelength of 570 nm. Three independent experiments for each sample were carried out.

2.9. Statistical analyses

All experiments were conducted at least three times and data are presented as mean ± SEM. Statistical analysis was performed with the SPSS software package (version 13.0; SPSS). Two-tailed *P* values <0.05 were considered statistically significant.

3. Results

3.1. Expression of NGAL in various cell lines

NGAL has been reported to be associated with various cancers. We detected NGAL message on mRNA level in several human cancer cell lines including erythroleukemia K562, acute T cell leukemia Jurkat, uterine cervix cancer HeLa, breast cancer ER$^-$ MDA-MB-231, and ER positive (ER$^+$) MCF7 cells by reverse transcription (RT) PCR analysis (Fig. 1a). To observe the distinctive expression of NGAL between MDA-MB-231 ER$^-$ and MCF7 ER$^+$ cells, NGAL levels were finally examined by quantitative real time PCR and Western blotting. Quantitative PCR analysis revealed that MDA-MB-231 cells expressed a relatively high level of NGAL, but a very low level was detected in MCF7 cell line compared with the internal control β-actin. The change of NGAL mRNA expression is approximate to 48-fold (Fig. 1b). Results of Western blotting also showed the distinctive signals for the bands of NGAL in these two breast cancer cell lines. The results suggest the distinctive expression of NGAL in different types of breast cancers on the quantity. NGAL level is much higher in MDA-MB-231 than in MCF7 cells, correlating with the invasive status of cell line. Given the fact that NGAL is secreted from the carcinoma cells into the conditioned medium (CM), we also detected the CM NGAL signals by Western blotting after total secreted proteins were concentrated by Millipore fast ultrafiltration devices. Much stronger signals for CM NGAL were detected, even the volume of sampling for Western blotting from CM is 30 times less than that from cell lysate, on condition that both of these protein samples were collected by equal volume of sampling buffer (Fig. 1c).These results also suggest the fact that secreted protein is the mainly existent form for NGAL of MDA-MB-231 cells.

3.2. NGAL knockdown inhibits migration and invasion of MDA-MB-231 cells

To confirm the NGAL-mediated effects on metastasis, NGAL was silenced with three different shRNA plasmids in MDA-MB-231 cells, which express the higher level of NGAL and exhibit a more pronounced metastasis phenotype. Markedly reduced CM and intracellular NGAL levels were observed after transfection with the more effective shRNA1 and 2 in both mRNA and protein levels (Fig. 2a and b). The classic phenotype of metastasis breast cancer cell line with an elongated morphology and more evenly distribution across the

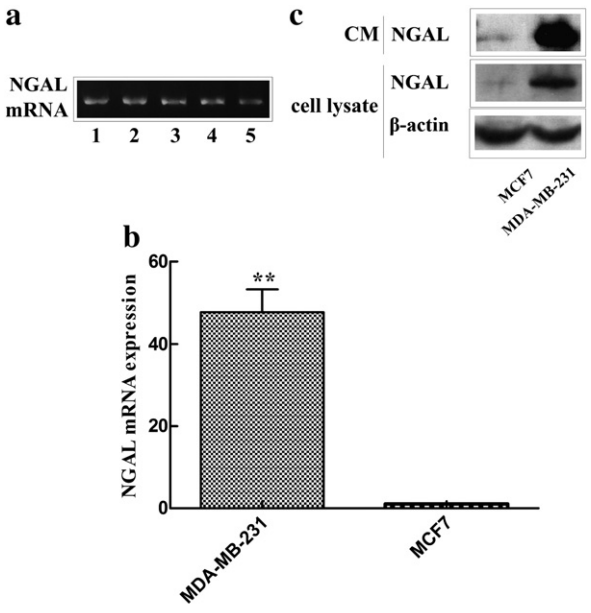

Fig. 1. Expression patterns of NGAL in various cancer cells. (a) Expression patterns of NGAL were analyzed by RT-PCR. PCR reactions were performed on templates of cDNA from different cancer cell lines using a set of primers as in Materials and methods. The lanes were 1, erythroleukemia K562, 2, acute T cell leukemia Jurkat, 3, uterine cervix cancer HeLa, 4, breast cancer ER$^-$ MDA-MB-231, and 5, breast cancer ER$^+$ MCF7 cell line. (b) Quantitative real time PCR analysis of *NGAL* gene expression in MDA-MB-231 and MCF7 cells. (c) CM and intracellular proteins prepared from human breast cancer cell lines, MDA-MB-231 and MCF7 were subjected to immunoblot analysis with anti-NGAL antibody. For quantitative real time PCR and Western blotting, β-actin was used as an internal control.

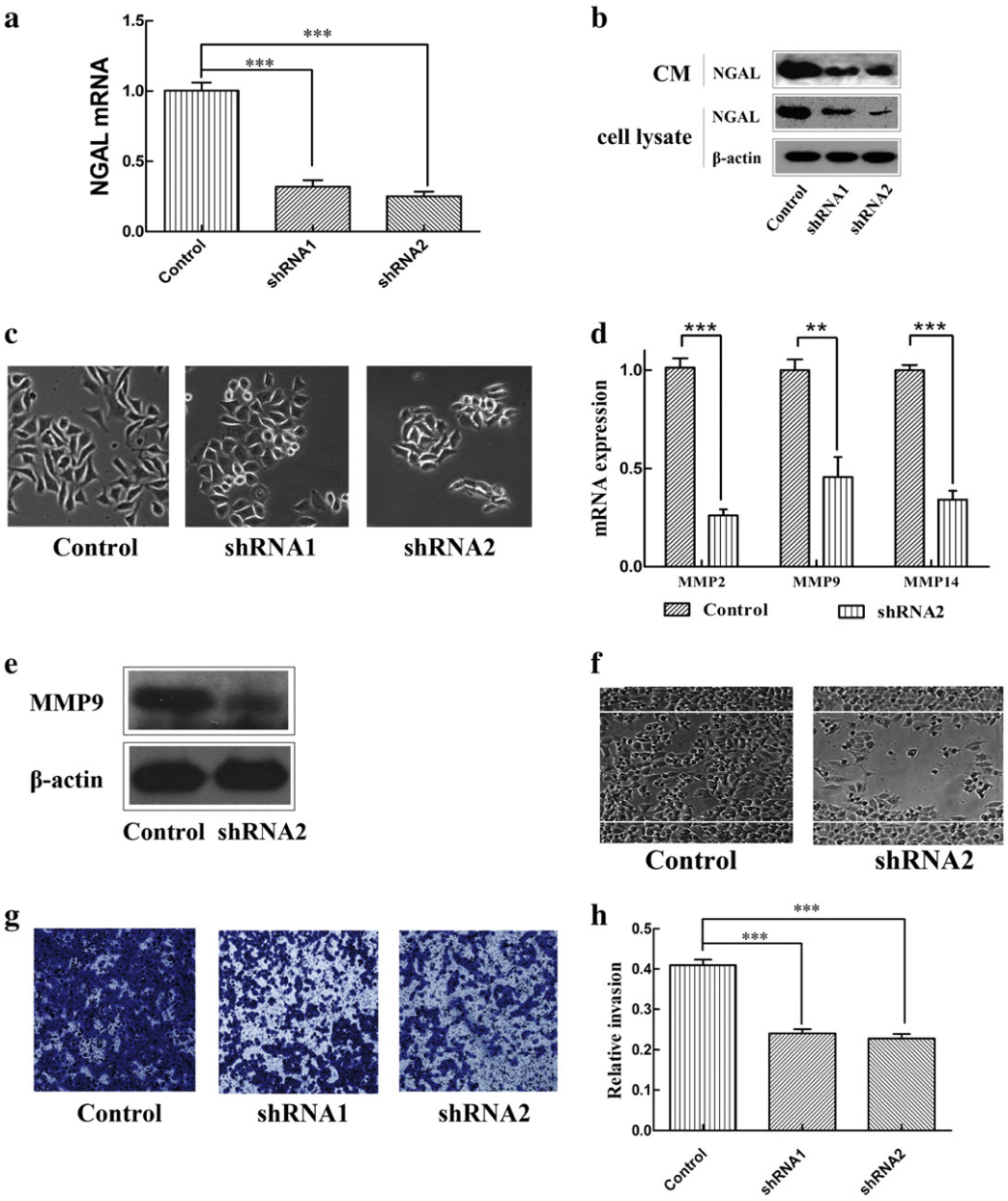

Fig. 2. NGAL silencing reduces metastasis of MDA-MB-231 cells. (a) Quantitative real time PCR analysis of *NGAL* gene expression in MDA-MB-231 cells transfected with NGAL short hairpin RNA plasmids 1 and 2 (shRNA1 and 2). (b) Downregulation of NGAL protein after NGAL shRNA plasmids transfection. (c) Morphological changes of MDA-MB-231 cells after NGAL shRNA plasmids transfection (original magnification 200×). (d) Quantitative real time PCR analysis of *MMP2, 9, 14* gene expression in MDA-MB-231 cells transfected with NGAL shRNA 2. (e) Protein levels of MMP9 after NGAL shRNA 2 transfection. (f)Representative pictures of wound healing assay for cell migration after NGAL shRNA 2 transfection. (original magnification 200×) (g) Representative pictures of transwell assay for cell invasion after NGAL shRNA transfection (original magnification 100×). (h) Invasion of MDA-MB-231 cells after NGAL shRNA transfection. Data were collected from 5 wells. **, $P<0.01$, ***, $P<0.001$, compared with the control. For quantitative real time PCR and Western blotting, β-actin was used as an internal control. Cells transfected with a scramble vector was used as a control for shRNA plasmid mediated RNA interference experiments. Photos of all cells were taken by an inverted microscope as described under Materials and methods.

well surface were commonly observed to be like the control MDA-MB-231 cells. NGAL knockdown cells transfected with shRNA1 and shRNA2 exhibited cobblestone-like appearance and strong cell-cell adhesion that is typical of epithelial phenotype like that of MCF7 as expected. And the classic phenotypic changes to the retracted and rounded appearance were also detected in NGAL knockdown clones (Fig. 2c).

Degradation of extracellular matrix and vascular basement membrane is one of the first steps required for breast cancer cell to invade. Matrix metalloproteinases (MMPs) have been strongly implicated in this step. MMP2, MMP9, and MMP14 (MT1-MMP) are among the major proteinases that have such a role [25,26]. We next examined the expression status of these key MMPs in the NGAL silenced cell clones. These classic MMPs were all markedly decreased as shown by quantitative PCR analysis (Fig. 2d). Western blotting confirmed the decreased expression of MMP9 corresponding to the NGAL knockdown (Fig. 2e).

A key feature of cells that have higher expression of NGAL and MMPs is their increased migration and invasion. Controls of MDA-MB-231 cells with higher NGAL expression exhibited markedly higher levels of migration and invasion, in contrast to NGAL knockdown cells by shRNA1 and 2 in which fewer migration and invasion were observed by the wound healing assay (Fig. 2f) and cell invasion assay (Fig. 2g and h), respectively.

These results suggest that NGAL knockdown induces phenotypic changes and decreased expression of MMPs, and then inhibits the migration and invasion of MDA-MB-231 cells.

3.3. C/EBP ζ targets to NGAL to inhibit migration and invasion of MDA-MB-231 cells

To test whether overexpression of C/EBP ζ is sufficient to inhibit the migration and invasion of MDA-MB-231 cells, we transfected MDA-MB-231 cells with C/EBP ζ plasmid and observed a dramatic increased expression of C/EBP ζ in protein level. We then determined whether C/EBP ζ overexpression regulated the expression of NGAL. The result showed that overexpression of C/EBP ζ dramatically neglected the NGAL expression both in mRNA and protein levels (Fig. 3a and b). We finally evaluated whether C/EBP ζ protein had an impact on cell morphology. Compared to the control, the over-expression of C/EBP ζ resulted in dramatic phenotypic changes and condensation at the cell periphery, with retracted, rounded size and reduced pseudopod-like structure (Fig. 3c). Moreover, we also found that induction of C/EBP ζ expression resulted in a decrease in the three MMPs mRNA (MMP2, 9, 14, Fig. 3d) expressions, and the reduced expression of MMP9 was finally verified by Western blotting (Fig. 3e). As expected, overexpression of C/EBP ζ led to a significant reduction in cell migration capacity (Fig. 3f) and more than a half decrease in the cell invasiveness (Fig. 3g and h).These data indicate that, in contrast to NGAL, C/EBP ζ is an anti-migratory and anti-invasive factor for ER^- cells. Overexpression of C/EBP ζ could dramatically decrease the expression of metastasis accelerators NGAL and MMPs expressions, and then finally inhibit the migration and invasion of MDA-MB-231 cells.

Lastly, there must be an emphasis on that, to determine C/EBP ζ regulation of the NGAL at the transcription levels, we performed quantitative real time PCR and found that C/EBP ζ decreased mRNA expression of NGAL (Fig. 3a). This result suggests that C/EBP ζ regulate NGAL mainly at the transcriptional level for our further study.

3.4. Inhibition of NHE1 reduces cell migration and invasion depending on C/EBP ζ elevation-mediated NGAL downregulation

NHE1 in the regulation of the intracellular pH (pHi) and extracellular pH (pHe) is necessary for the metastasis of tumor cells [27]. We next sought to determine whether inhibition of NHE1 was involved in the process of cell migration and invasion that was mediated by C/EBP ζ and NGAL. Cariporide, a selective inhibitor of NHE1 that could block the efflux of H^+ to achieve intracellular acidification, was used to detect the effect of NHE1 inhibition on the expressions NGAL and C/EBP ζ. A decrease of NGAL mRNA synthesis was observed when the cells were incubated with Cariporide and resulted in a decrease of NGAL protein both in the cell context and conditioned medium at 24 h relative to that measured from control cells at the same time points(Fig. 4a and c). In contrast, an increase of C/EBP ζ was detected under the same treatment conditions by Cariporide both in the mRNA and protein levels(Fig. 4b and c). As our prediction, this process was strongly accompanied with significantly decreased migration and invasion abilities of MDA-MB-231 cells (Fig. 4d–f). Furthermore, treatment with Cariporide also down-regulated MMP2, MMP9 and MMP14 mRNA expressions. As the experiment of Live Cell Station, we detected the morphological changes of cell to a retracted and rounded size treated with Cariporide for 24 h (data was not shown). Though the limited capacity of Cariporide on metastasis was not so evident compared with that of the C/EBP ζ overexpression or NGAL knockdown, these results indeed indicate that the effects of NHE1 inhibition on cell migration and invasion are at least partly by facilitating the regulation of NGAL mediated by C/EBP ζ. Thus Cariporide could contribute to the inhibitory effects of C/EBP ζ on cell metastasis by increasing the endogenous expression of C/EBP ζ and the subsequent downregulation of NGAL expression.

3.5. NGAL is a direct transcriptional target of C/EBP ζ via special functional promoter fragments

As shown in Fig. 3a, cells overexpressed C/EBP ζ display more than 4-fold decreased expression of NGAL mRNA compared with the control. These results suggest that C/EBP ζ probably regulate NGAL at the transcription level. As reported before on interleukin (IL6 and 8) expression, C/EBP ζ changes the binding properties of C/EBP β to DNA by forming a heterodimers and also inhibits the effect of NF-κB site mediated through interactions with some special factor [17,28]. We doubt that if the transcription factor C/EBP ζ regulates NGAL mRNA in this way. To verify our speculation, we finally performed promoter reporter assay with a luciferase reporter plasmid under control of different fragments of NGAL 5′-upstream region. We first analyzed the NGAL promoter and identified some potential C/EBP and NF-κB binding sites. As reported before, inspection of the sequence between −183 to −153 revealed a putative sequence 5′-GGGAATGTCC-3′ (−180 to −171) with homology (7 of 8 nt match) to the NF-κB consensus sequence 5′-GGG(A/G)NNT(C/T)CC-3′, and two putative C/EBP sites at −148 to −139 (C/EBP-1, 5′-CTTGCCCAAT-3′) and −130 to-121 (C/EBP-2, 5′-GGTGCAGAAA-3′) with 8 and 6 bases of 10 matching the C/EBP consensus sequence (5′-(A/G)TTGCG(T/C)AA (T/C)-3′), respectively.

Three fragments of NGAL promoters were constructed: −1137 to +84(pGL3-1221), −416 to +84 (pGL3-500, including NF-κB and C/EBPs), and −152 to 84 (pGL-236, including C/EBPs) (Fig. 5a). We finally determined whether these regions of NGAL promoter were effective for its basic transcriptional induction. Dual-luciferase reporter assay showed that the basal expression of the promoter was remained 85% for pGL3-500 when truncating from −1137 to −415, and then remained 26% for pGL3-236 until the region between −416 and −151 was deleted. The result indicates that cis-elements in these two fragments −416 to −153 and −152 to +84 are mainly in charge of the NGAL effective transcription (Fig. 5b).

Our focus was, however, on the elements responsible for the transcriptional induction by C/EBP ζ. Therefore, we next measured the luciferase activity of cells transfected with C/EBP ζ. Compared to each own control, genetically modified MDA-MB-231 cells transfected with C/EBP ζ showed decreased luciferase activities for different 5′ deletions of the NGAL promoter. An ~8-fold decreased induction of luciferase activity was found for the pGL3-1221 with fragment of −1137 to +84, a ~7-fold decrease was measured for the pGL3-500 (−416 to +84), and a ~2.5-fold reduction was observed for pGL3-236 (−152 to 84) (Fig. 5c), whereas no decreased induction by C/EBP ζ was observed for the pGL3-control plasmid with the NGAL promoter fragment from −1137 to −185, which was lack of NF-κB and C/EBP sites (Fig. 5d). These results demonstrate that cis-elements between −416 to −153 and −153 to +84 are mainly in charge of the decreased NGAL synthesis mediated by C/EBP ζ overexpression.

The effect of NHE1 selective inhibitor Cariporide on NGAL promoter activity was also tested by dual-luciferase reporter assay. Treated with Cariporide for 24 hours, in contrast to C/EBP ζ elevation, we detected a decreased promoter activity of NGAL: ~8-fold for pGL3-1221, ~7.5-fold for pGL3-500 and ~5.5-fold for pGL3-236 (Fig. 5e), whereas no decreased induction by C/EBP ζ was observed for the pGL3-control plasmid with the NGAL promoter fragment from −1137 to −185 under the same treatment conditions by Cariporide (Fig. 5f).

Taken together, the observed effects of luciferase enzyme activity indicate that C/EBP ζ regulates NGAL transcriptional activity by binding to the special cis-element in the NGAL promoter. And the activation of NHE1 also participates in the process of C/EBP ζ mediated NGAL transcriptional activity.

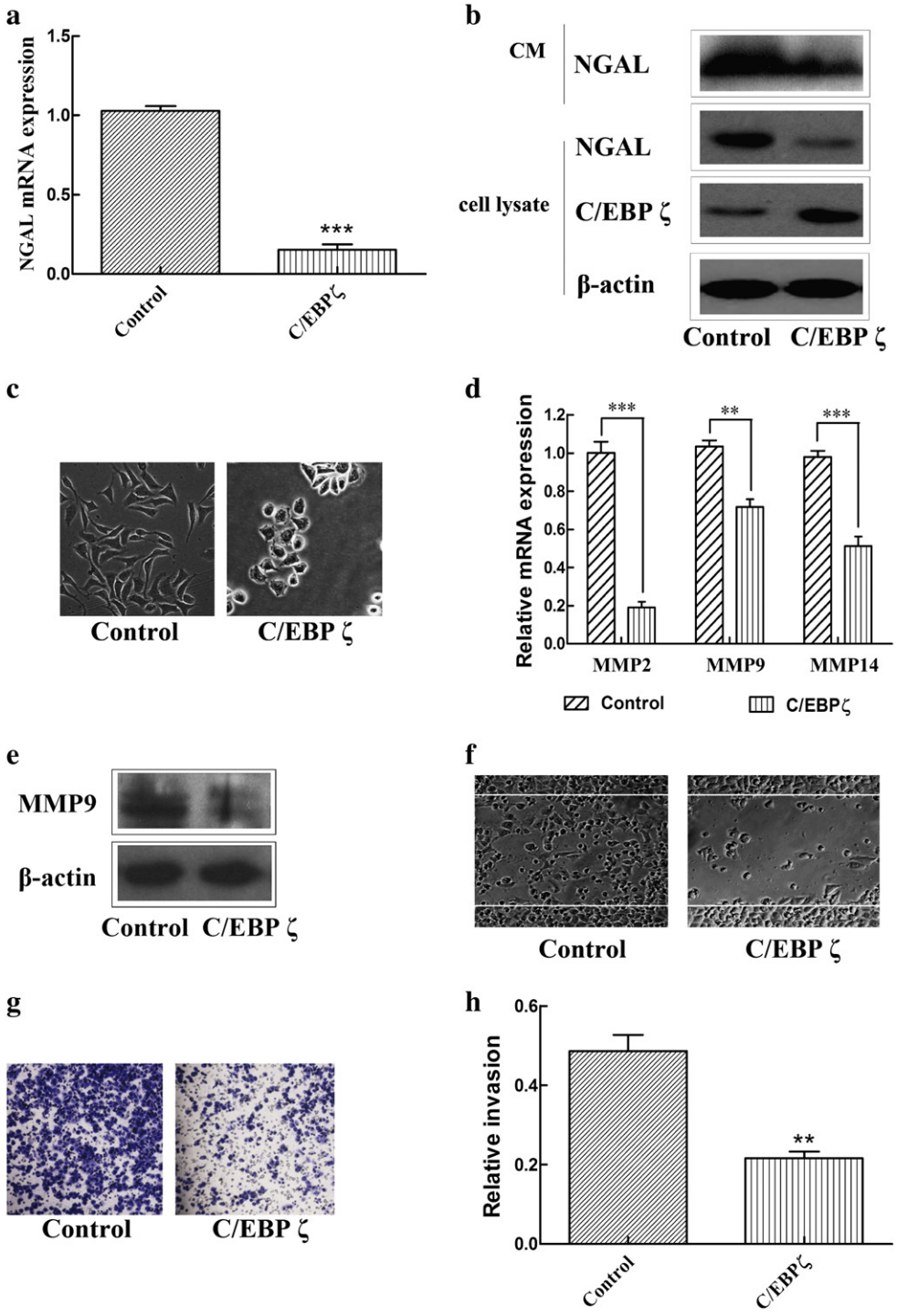

Fig. 3. Overexpression of C/EBP ζ inhibits metastasis of MDA-MB-231 cell. (a) Quantitative real time PCR analysis of *NGAL* gene expression in MDA-MB-231 cells transfected with C/EBP ζ plasmid. (b) Protein levels of C/EBP ζ and NGAL expression in MDA-MB-231 cell with C/EBP ζ overexpression were detected by Western blotting. (c) Morphological changes of MDA-MB-231 cells after C/EBP ζ overexpression (original magnification 200×). (d) Quantitative real time PCR analysis of *MMP2, 9, 14* gene expression in MDA-MB-231 cells transfected with C/EBP ζ plasmid. (e) Protein levels of MMP9 after C/EBP ζ plasmid transfection. (f) Representative pictures of wound healing assay for cell migration after C/EBP ζ overexpression (original magnification 200×). (g) Representative pictures of transwell assay for cell invasion after C/EBP ζ overexpression (original magnification 100×). (h) Invasion of MDA-MB-231 cells after C/EBP ζ overexpression. Data were collected from 5 wells. For quantitative real time PCR and Western blotting, β-actin was used as an internal control. For all the above experiments, cells transfected with a control vector was used as a control. Photos of all cells were taken by an inverted microscope as described under Materials and methods. Data were collected from at least three independent experiments. **, $P<0.01$, ***, $P<0.001$, compared with control.

3.6. Wnt and NF-κB signaling pathways are involved in NGAL-mediated invasion

Here our study demonstrated that NF-κB pathway might be involved in NGAL regulation, and previous report indicated that Wnt signaling pathway was also involved in NGAL expression. To investigate the signaling pathways involved in NGAL-mediated cell invasion, inhibitors for these two signaling pathways were used to assay their effects on NGAL expression and NGAL-mediated cell invasion. We inactivated the Wnt and NF-κB pathways with FH535 and BAY117082 respectively. As shown in Fig. 6a and b, these two inhibitors could significantly decrease NGAL protein expression both

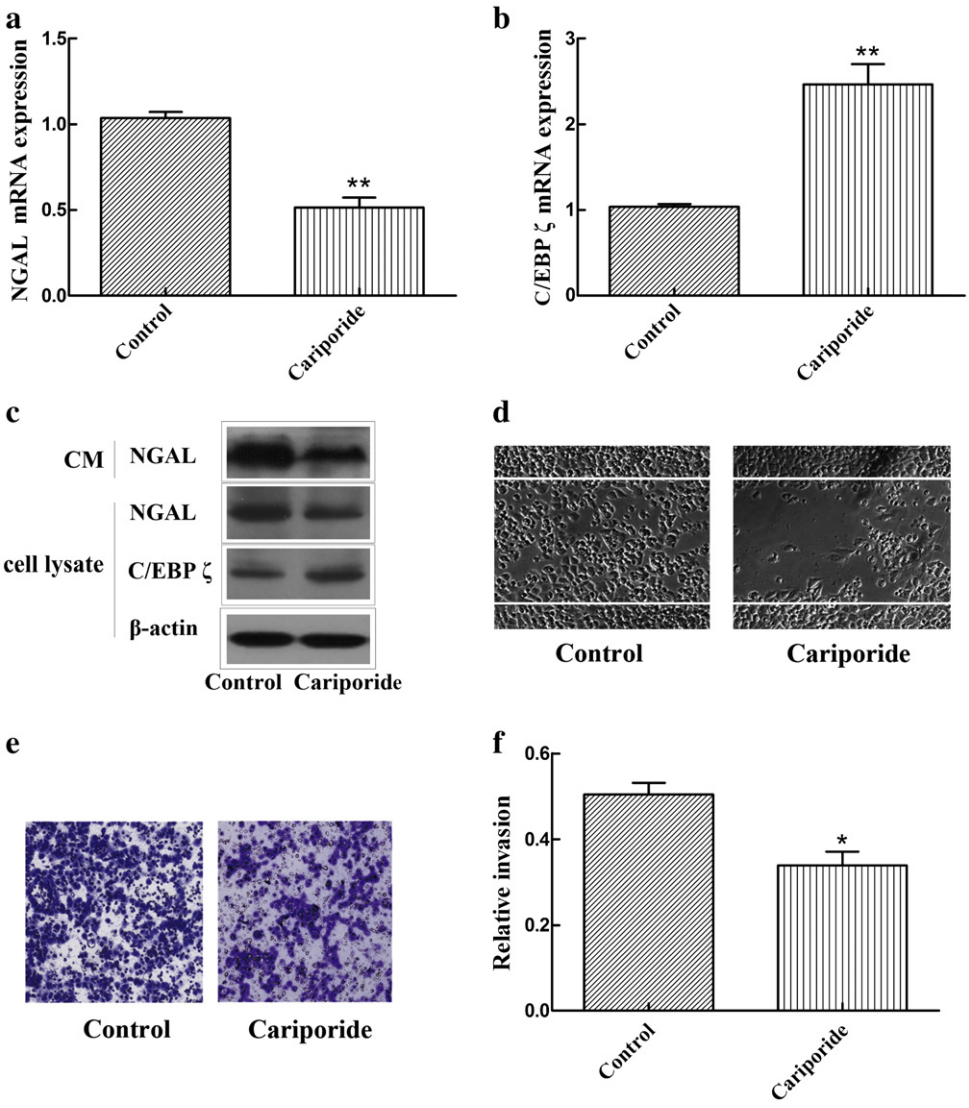

Fig. 4. Inhibition of NHE1 by Cariporide reduces cell metastasis partly depending on C/EBP ζ via NGAL downregulation (a) Quantitative real time PCR analysis of *NGAL* gene expression in MDA-MB-231 cells after treated with Cariporide. (b) Quantitative real time PCR analysis of *C/EBP ζ* gene expression in MDA-MB-231 cells after treated with Cariporide. (c) Protein levels of C/EBP ζ and NGAL after cell treated with of Cariporide. (d) Representative pictures of wound healing assay for cell migration after treated with Cariporide (original magnification 200×). (e) Representative pictures of transwell assay for cell invasion after treated with Cariporide (original magnification 100×). (f) Invasion of MDA-MB-231 cells by the treatment of Cariporide. Data were collected from 5 wells. For quantitative real time PCR and Western blotting, β-actin was used as an internal control. Data were collected from at least three independent experiments. *, $P<0.05$; **, $P<0.01$, compared with control. Cariporide with a few different concentrations were used for these experiments, the results shown here only for 4 mg/l.

in cell context and conditioned medium from MDA-MB-231 cells. Finally, we evaluated whether signaling through the Wnt and NF-κB pathways impacted cell invasion. Consistent with the decreased NGAL expression, the results showed that Wnt (FH535) and NF-κB (BAY117082) specific inhibitor reduced the invasion of MDA-MB-231 cells (Fig. 6c–f) similar to the effects induced by C/EBP ζ overexpression. However, inhibition of these two pathways was observed to be more significantly diminished the cell invasion, suggesting that some other molecules regulated by these two signaling pathways involved in MDA-MB-231 cell metastasis process. These studies confirm our result that NF-κB pathway participate in NGAL-mediated cell migration and invasion, moreover it also suggested that NGAL-mediated tumor metastasis was regulated partly by Wnt signaling, a usual active pathway in breast cancer.

4. Discussion

Breast cancer is a leading cause of morbidity in women worldwide. Invasive cancer culminating in metastasis is the leading cause of these cancer-associated deaths. NGAL expression has been reported to be strongly correlated with a negative ER status, which is consistent with several global gene expression profiling studies [2,29]. The protein contributes to the aggressive behavior and mesenchymal phenotype of MDA-MB-231 cells, suggesting that NGAL is a potential therapeutic target for ER^- breast cancers. Human NGAL (neutrophil gelatinase-associated lipocalin, also referred to as lipocalin 2, Lcn2) is a member of the lipocalin family of small secreted proteins. It is up-regulated in a number of pathological conditions, including cancers. In the past, scientists had devoted a lot of work to the quantitative difference of NGAL expression. Consistent with previous reports, we have detected that MDA-MB-231 cell indeed bears a greatly higher expression of NGAL than MCF7 cell line. As the result shows much stronger signals from Western blotting for CM NGAL were detected, even the volume of sampling from conditioned medium is 30 times less than that from cell context, on condition that both of these protein samples were collected by equal volume of sampling buffer, suggesting that secreted protein is the mainly existent form for NGAL from MDA-MB-231 cells. However, different distributions as well as molecular forms of NGAL

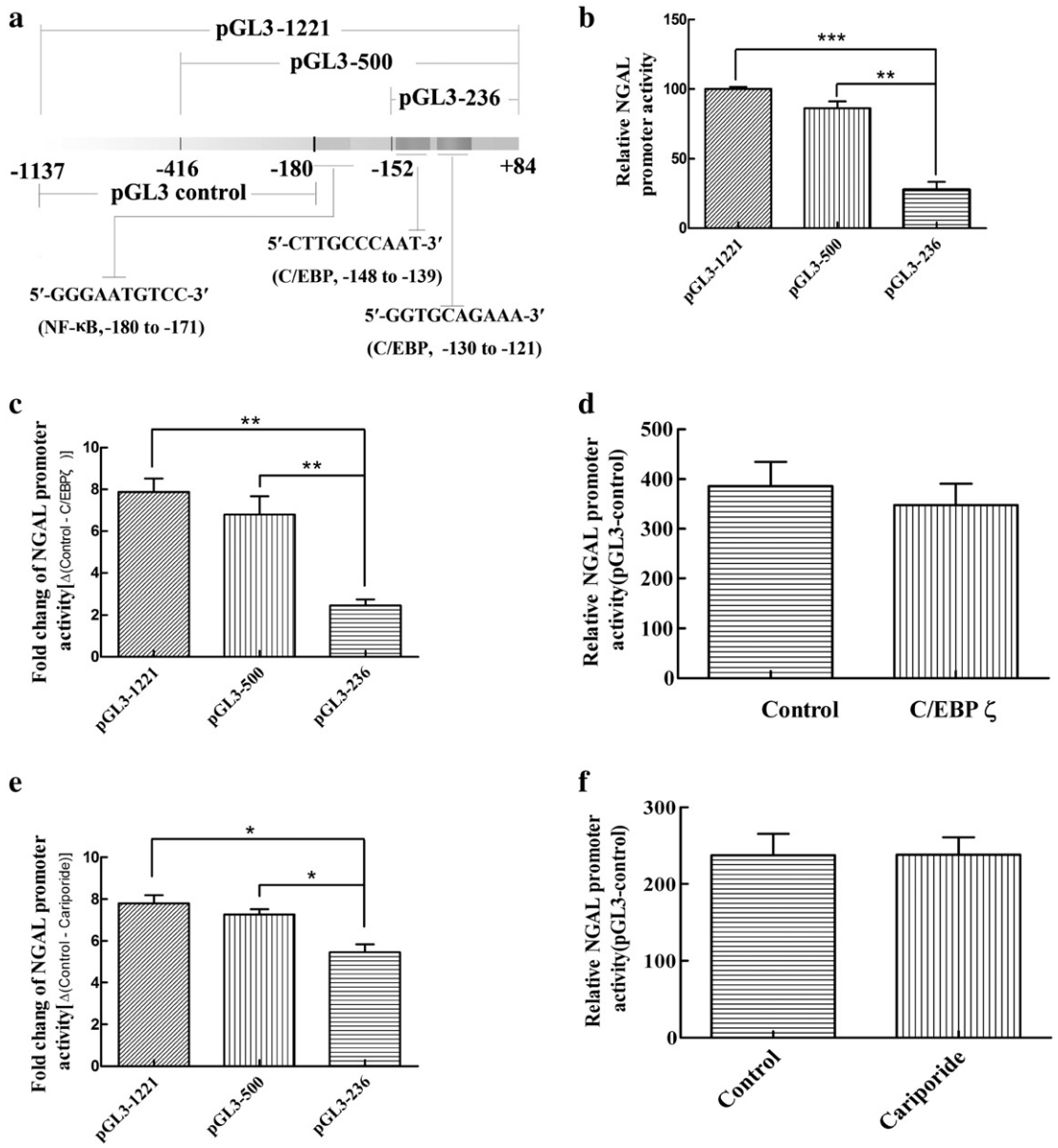

Fig. 5. C/EBP ζ regulates NGAL expression by special functional promoter fragments of *NGAL* gene (a) Cartoon depicting the *NGAL* promoter constructs used. (b) Luciferase assays of the promoter fragments in MDA-MB-231 cells for NGAL basic expression. (c) Luciferase assays of the promoter fragments in MDA-MB-231 cells with C/EBP ζ overexpression for NGAL expression. (d) Luciferase reporter assays of the promoter fragments without potential C/EBP interacting sites (pGL3-control plasmid) in MDA-MB-231 cells with C/EBP ζ overexpression for NGAL expression. (e) Luciferase assays of the promoter fragments in MDA-MB-231 cells with Cariporide treatment for NGAL expression. (f) Luciferase reporter assays of the promoter fragments without potential C/EBP ζ interacting sites (pGL3-control) in MDA-MB-231 cells with Cariporide treatment for NGAL expression. Graphed values were obtained by normalizing measured firefly luciferase activity by protein quantitation for sampling and renilla luciferase activity from the contransfected vector phRL-TK. Error bars represent 95% confidence intervals. Data are pooled from three independent experiments performed in biological triplicates. *, $P<0.05$; **, $P<0.01$, ***, $P<0.001$, compared with control.

were detected in various pathologic cells and tissues. The functions underlying these different existent forms of NGAL still remain unknown in cancers, so further studies are still needed to elucidate these issues.

To ensure the NGAL-mediated effects on metastasis, NGAL was silenced in MDA-MB-231 cells, which express the greatly higher level of NGAL and exhibit a more pronounced metastasis phenotype. Downregulation of NGAL showed an effective inhibition of tumor migration and invasion. Results implicate that NGAL is an effective therapeutic target for ER^- breast cancer. Our findings are consistent with a recent report describing the strong correlation between NGAL levels in primary breast cancer and ER negative status, poor histologic grade, and lymph node metastasis, and provide the evidence by directly detecting migration/invasion velocity and morphological change of tumor cells.

C/EBP ζ encodes a transcription factor belonging to the CCAAT/enhancer binding protein (C/EBP) family. It is expressed ubiquitously and can be induced by a wide variety of treatments such as DNA lesion, hypoglycaemia, radiation and cellular stress. The protein was first identified as a central factor in endoplasmatic reticulum stress and DNA damage response. The level of C/EBP ζ transcript in myeloid malignancies CML patients is significantly lower than that in controls. Recent evidence suggests that C/EBP ζ is a key molecule whose downregulation may be a prerequisite for cellular transformation induced by oncogenes such as Ras and c-myc [30,31]. Together, these results implicate that expression of

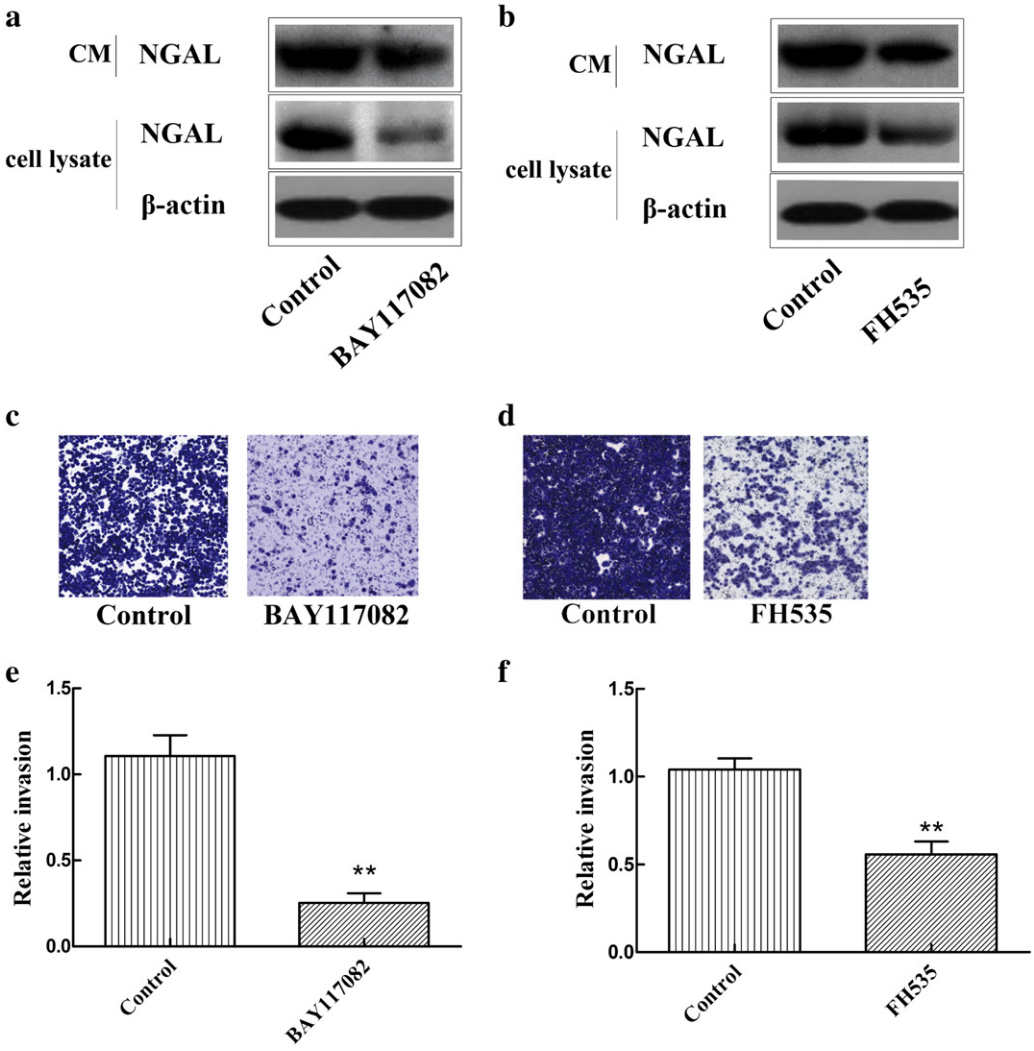

Fig. 6. Inhibitors for Wnt and NF-κB signaling pathways reduce NGAL-mediated cell metastasis. (a) Protein levels of NGAL after cell treated with BAY117082. (b) Protein levels of NGAL after cells treated with FH535. For Western blotting, β-actin was used as an internal control. (c) Representative pictures of Transwell assay for cell invasion after treated with BAY117082. (d) Representative pictures of Transwell assay for cell invasion after treated with FH535. (e) Invasion of MDA-MB-231 cells by the treatment of BAY117082. (f) Invasion of MDA-MB-231 cells by the treatment of FH535. Data are collected from 5 wells. **, $P<0.01$, compared with control. Cells treated with the equal quantity of DMSO for dissolving inhibitors were used as a control. Photos of all cells were taken by an inverted microscope (original magnification 100×) as described under Materials and methods.

exogenous C/EBP ζ is able to impede oncogenic transformation of cell. However, the data is still lacking about the effect of C/EBP ζ on breast cancer. We finally introduce C/EBP ζ in MDA-MB-231 cell. The data presented here suggest that C/EBP ζ plays a significant inhibitory role in NGAL expression, its upregulation contributes to the reduction of cell migration and invasion by downregulating NGAL and MMPs.

A large body of researches implicates a role for C/EBP ζ in the regulation of NGAL expression. The earlier investigators found that C/EBP ζ functions as negative regulator of C/EBP β-dependent IL-6 expression in B cells [28]. C/EBP ζ is proposed to act as a dominant negative inhibitor of other C/EBPs by forming C/EBPζ:C/EBP heterodimers. C/EBPζ:C/EBP heterodimers fail to bind several known C/EBP sites *in vitro*. The NGAL promoter contains a putative C/EBP-binding site, suggesting that interruption of the classical C/EBP transactivators by C/EBP ζ could mediate NGAL expression by the transcriptional regulation via NGAL promoter. However, other probability still exists. In contrast to this conventional model of C/EBPζ:C/EBP activity, an earlier experiments showed that a deletion mutated C/EBPζ lacking the leucine-zipper dimerization domain still caused downregulation of IL8[17]. Instead, the research declared that part of C/EBP ζ's inhibitory effect may be mediated through interactions with a factor binding to the NF-κB site as mutation of the NF-κB site strongly counteracted the inhibitory effects of C/EBP ζ on IL8 transcription. NF-κB has been demonstrated to contribute to cell survival by controlling iron uptake via NGAL in thyroid tumor [32]. Our result of luciferase reporter assay indicates that cis-elements in these two fragments −416 to +84 and −152 to +84 are mainly in charge of the NGAL effective transcription. Inspection of the sequence between −183 and −153 revealed a putative sequence 5′-GGGAATGTCC-3′ (−180 to −171) with homology (7 of 8 nt match) to the NF-κB consensus sequence 5′-GGG(A/G)NNT(C/T)CC-3′, and two putative C/EBP sites at −148 to −139 (C/EBP-1, 5′-CTTGCCCAAT-3′) and −130 to-121; C/EBP-2, 5′-GGTGCAGAAA-3′) with 8 and 6 bases of 10 matching the C/EBP consensus sequence (5′-(A/G)TTGCG(T/C)AA (T/C)-3′) respectively. Taken all together, this study shows that the both of C/EBP and NF-κB sites in the promoter of NGAL might be linked to the C/EBP ζ mediated NGAL knockdown. The results were consistent with a previous report of IκB-ζ which acted as an essential transcriptional activator by forming a complex with NF-κB on promoters harbouring the NF-κB- and C/EBP-binding sites, upon stimulation of TLRs or IL-1 receptor [33].

Cellular alkalinization, a phenotype common to all tumor cells, is the consequence of the stimulation of a member of the Na^+/H^+

exchanger protein family, NHE1[34]. Global profiling revealed genes regulated by loss of NHE1 activity and decreased pHi, cells with ion translocation-defective NHE1 had a marked decrease in MMP9 and NGAL expression but a dramatic increase in C/EBP ζ expression [35]. Consistent with previous report, we found that the cell membrane NHE1 was involved in cell migration and invasion [36]. Finally, we demonstrated that inactivation of NHE1 by the selective inhibitor Cariporide inhibited the expression of NGAL, accompanied with the upregulation of C/EBP ζ. NGAL covalently bound to MMP9 and protects MMP9 from degradation may partly interpret the reason of MMP9 downregulation in our experiments [37]. But the decrease of other MMPs (MMPs 2 and 14) might be a result of EMT loss caused by NGAL downregulation. Moreover, NHE1 probably regulates cell migration and invasion in several pathways. The alkaline pHi caused by NHE1 activation has been recently demonstrated to be associated with the expression level of vascular endothelial growth factor (VEGF), which plays an important role in the induction of neovascularization, thereby promoting tumor growth and metastatic potential, both in solid and hematologic malignancies [38,39]. NHE1 also participates in the regulation of the actin cytoskeleton dynamics necessary for the adhesion and pseudopodial protrusion of motile, invasive tumor cells [40]. NGAL has been reported to be involved in these processes, including tumor migration and invasion, but whether downregulation of NGAL caused by NHE1 inhibition mediates MDA-MB-231 cell migration and invasion through these ways still needs to be further studied. Here our results implicate that the selective inhibitor of NHE1, Cariporide, [41], could be used to promote the inhibitory effect of the C/EBP ζ on cell metastasis partly by down regulating NGAL and MMPs by means of increasing the endogenous expression of C/EBP ζ.

The initiation and progression of breast tumors are driven by various inherited and acquired genetic changes that are reflected in phenotypic alterations [42,43]. Wnt signaling pathway is also demonstrated involved in NGAL-mediated tumor metastasis, suggesting a complexity of network that multiple signaling pathways regulate cancer progression. Our data suggest that C/EBP ζ inhibits cell migration and invasion in human breast cancer through down-regulation of NGAL and MMPs. C/EBP ζ regulates NGAL expression depending on the transcriptional regulation via specific sites of NGAL promoter. The selective NHE1 inhibitor Cariporide facilitates this process by decreasing the NGAL expression via upregulating the endogenous C/EBP ζ. Association of increased C/EBP ζ with a decreased NGAL expression by NHE1 inhibitor Cariporide also contributes to inhibition of cell migration and invasion. Thus, targeting to C/EBP ζ and combined with NHE1 selective inhibitor Cariporide could provide a coming effective strategy for treating ER^- breast cancer.

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