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CD44 targets Na^+/H^+ exchanger 1 to mediate MDA-MB-231 cells' metastasis via the regulation of ERK1/2

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Background: CD44, a transmembrane glycoprotein expressed in a variety of cells and tissues, has been implicated in tumour metastasis. But the molecular mechanisms of CD44-mediated tumour cell metastasis remain to be elucidated.

Methods: The downregulation of CD44 was determined by immunofluorescence. Moreover, the motility of breast cancer cells was detected by wound-healing and transwell experiments. Then the spontaneous metastasis of CD44-silenced MDA-MB-231 cells was tested by histology with BALB/c nude mice.

Results: A positive correlation between CD44 and Na^+/H^+ exchanger isoform 1 (NHE1) was found in two breast cancer cells. CD44 downregulation could inhibit the metastasis of MDA-MB-231 cells and the expressions of Na^+/H^+ exchanger 1. Moreover, CD44 overexpression upregulated the metastasis of MCF-7 cells, but the elevated metastatic ability was then inhibited by Cariporide. Interestingly, during these processes only the p-ERK1/2 was suppressed by CD44 downregulation and the expression of matrix metalloproteinases and metastatic capacity of MDA-MB-231 cells were greatly inhibited by the MEK1 inhibitor PD98059, which even had a synergistic effect with Cariporide. Furthermore, CD44 downregulation inhibits breast tumour outgrowth and spontaneous lung metastasis.

Conclusions: Taken together, this work indicates that CD44 regulates the metastasis of breast cancer cells through regulating NHE1 expression, which could be used as a novel strategy for breast cancer therapy.

Breast cancer is the most common malignant cancer in women. In these patients, it is not the primary tumour but its distant metastases that are the predominant causes of treatment failure and patients' death. Chemotherapy can increase the survival rate of women with breast cancer, but most patients may exhibit resistance to chemotherapy after a while of treatment (Weigelt *et al*, 2005; Lin *et al*, 2011; Wang *et al*, 2011). Metastatic spread and aggressive invasion are obvious characteristics of carcinoma and continue to be the greatest handicap of cancer treatment. It is therefore important to accomplish a better understanding of the molecular mechanisms involved in cancer cell migration. A common feature is that cell migration is a process controlled by both internal and external signals (Dormann and Weijer, 2006). Dysregulation of these signals could underlie the aberrant cell migration observed in cancer cells.

CD44 denotes a large family of transmembrane glycoproteins that are expressed in a variety of cells and tissues, including breast tumour cells (Gotte and Yip, 2006). CD44 exhibits extensive molecular heterogeneity and this heterogeneity is derived by alternative splicing of variable exons and post-translational

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modifications (Bourguignon *et al*, 2009). CD44 has a critical role in a variety of cellular behaviours, including adhesion, migration, invasion, and survival (Cichy and Pure, 2003). In fact, the high level of CD44 isoforms (particularly CD44s) is considered as an important metastatic tumour marker in a lot of cancers and also implicated in the unfavourable prognosis of multiple carcinomas (Bourguignon, 2001). CD44 is also expressed in tumour stem cells and proposed to be one of the important surface markers that could been used to identify putative cancer stem cells in breast tumours (Shipitsin *et al*, 2007) as well as in other tumour types, such as prostate (Collins *et al*, 2005), pancreatic (Li *et al*, 2007), and head and neck carcinomas (Prince *et al*, 2007). Recent studies indicate that $CD44^+$ mammary tumour cells are associated with more invasive, proliferative, and angiogenic status, predicting an aggressive tumour cell behaviour (Shipitsin *et al*, 2007).

The Na^+/H^+ exchanger isoform 1 (NHE1) is an electroneutral plasma membrane transporter that regulates intracellular pH (pH_i) (Malo and Fliegel, 2006; Slepkov *et al*, 2007). Na^+/H^+ exchanger isoform 1 is ubiquitously expressed in essentially all cell types and multiple roles of NHE1 have been described in recent years. In addition to its primary roles in pH_i homeostasis and cell volume regulation, a substantial body of evidence indicates that NHE1 has fundamental roles in control of multiple cellular processes, including cell proliferation, cell death/survival balance, cell mobility, and a wide variety of cell signalling transduction. In cancer cells, NHE1 has been appointed an important role in matrix digestion through matrix metalloproteinases (MMPs), creating an alkaline intracellular and acidic extracellular microenvironment in the front of migrating cells to stimulate directional migration in cancer cells (Stock *et al*, 2007, 2008; Schneider *et al*, 2009; Martin *et al*, 2011). In addition, NHE1 can interact directly with a wide variety of other proteins, as well as with membrane lipids. For instance, in MDA-MB-231 cells, NHE1 was found to be recruited to form a raft-localized complex with CD44 and Rho kinase (Bourguignon *et al*, 2004; Orlowski and Grinstein, 2004; Boedtkjer *et al*, 2012).

As mentioned above, CD44 can interact with NHE1 leading to breast tumour cell invasion (Bourguignon *et al*, 2004); however, there was little report about the direct regulating relationship between CD44 and NHE1. Therefore, in this paper, we tried to investigate whether the regulation of NHE1 by CD44 existed in breast cancer, study the potential role of CD44 in metastasis of breast cancer cells, and to evaluate the contribution of the MAPK signalling pathway in this process.

MATERIALS AND METHODS

Cell culture and Materials. MCF-7 and MDA-MB-231 cells were cultured in DMEM (Gibco-BRL Life Technologies, Inc., Burlington, ON, Canada). In addition, K562, HL-60, U-937, CEM, Namalwa and Jurkat cells were grown in RPMI 1640 medium (Gibco-BRL Life Technologies Inc.). The media were supplemented with 10% FBS (HyClone, Logan, UT, USA), 100 U ml^{-1} streptomycin and 100 U ml^{-1} penicillin and cells were grown at 37 °C in a 5% CO_2-humidified incubator.

We obtained Cariporide from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and MAPK inhibitors PD98059 from Beyotime (Shanghai, China). For western blot analysis, we purchased antibodies against GAPDH, NHE1, anti-phosphospecific p38, ERK1/2, JNK1-2/SAPK, and nonphosphorylated p38, ERK1/2, JNK from Santa Cruz Biotechnology; anti-phosphospecific AKT and nonphosphorylated AKT from Cell Signaling Technology (Boston, MA, USA); anti-CD44 and anti-MMP14 from R&D Systems (Minneapolis, MN, USA); anti-MMP2 and MMP9 from Abcam (Cambridge, MA, USA). The CD44/tet plasmids were gifts from Dr DJJ Waugh (Queen's University Belfast, Belfast, UK) (Hill *et al*, 2006).

Real-time quantitative PCR and western blotting. These analyses were performed as described previously (Lin *et al*, 2011).

RNA interference studies. The independent hairpins of CD44 and NHE1 were achieved using software from Ambion (Austin, TX, USA). The hairpins were synthesised and cloned into the eukaryotic vector pGPU6/GFP/Neo (GenePharma, Shanghai, China). At 48 h post-transfection, transfection efficiency was verified and the infected cells were selected with G418 for at least 2 weeks. When stable clones were obtained, the GFP-positive cells were purified by FACS. Inhibition of CD44 and/or NHE1 expression was measured by real-time quantitative PCR and western blotting. For RNA interference experiments, cells were transfected with the pGPU6/GFP/Neo vector that expresses a scramble control shRNA as the negative control.

Cell invasion assay. *In vitro* models of tumour cell invasion were performed using matrigel and the Millicell Cell Culture Insert with 8-μM-pore Polyvinylpyrrolidone-free polycarbonate membranes (Millipore, Billerica, MA, USA) system to simulate the basement membrane and extracellular environment. Breast cancer cells were harvested centrifuged and resuspended in serum-free DMEM containing 1% BSA to obtain single-cell suspension (5×10^5 cells per ml). Thereafter, 1×10^5 cells per well (200 μl cell suspension) were seeded into the upper compartment of the matrigel invasion assay insert. After 24-h incubation, the transwell inserts were removed from the plate and the cells on the upper side of the membrane were gently wiped using cotton pad. The cells that migrated to the lower surface of the filter were fixed with the methanol and stained with crystal violet for 30 min and then dissolved with 33% acetic acid. The cells were photographed and the numbers of cells were indirectly quantitated by measuring the absorbance at 570 nm. Each assay was done in triplicate and repeated at least thrice. The data were analysed using the Student's *t*-test and the statistical significance was set at $P<0.05$.

Wound-healing assay. Cell motility was evaluated using the *in vitro* wound-healing assay. Cells in exponential growth phase were grown in 24-well plates until they reached confluence. Using a 20 μl plastic pipette tip, we scraped three horizontal lines across the entire diameter at the bottom of each well inducing the 'wound'. Cell media were removed and the cells were gently rinsed three times to remove unattached cells. The wound area was photographed at 24 h after scraping. To compare cell motility of breast cancer cells, we measured the gap distance and determined the wound-closing rate. The cells were allowed to migrate into the wounded area for 24 h. At the indicated time points, the wound closure was photographed by a camera (Model DXM1200, Nikon, Tokyo, Japan) attached to an inverted microscope (Eclipse TE300, Nikon).

Immunofluorescence assay. Breast cancer cells were grown on glass chamber slides at 90% confluence and fixed with 4% paraformaldehyde for 30 min. Cells were washed with ice-cold PBS, blocked with 0.5% BSA in PBS for 30 min and then preincubated with mouse antihuman CD44 antibody overnight at 4 °C. After three washes with ice-cold PBS, the cells were incubated with cy3-conjugated AffiniPure Goat Anti-Mouse IgG secondary antibody at room temperature and their nuclei were stained with 1 μg μl^{-1} DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine). Then, cells were washed twice with PBS and the images were visualised with Bio-Rad 1024 confocal laser microscope (Bio-Rad, Hercules, CA, USA).

Orthotopic mouse model of breast cancer metastasis. All animal experiments were performed in compliance with the guidelines of Laboratory Animal Care of National Institutes of Health for the

care and use of laboratory animals and were approved by the institutional biomedical research ethics committee in Laboratory Animal Center of Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences. The MDA-MB-231 cells were harvested by trypsinisation, washed twice in PBS, and then resuspended (3×10^7 cells per ml) in PBS. A total of 3×10^6 MDA-MB-231 cells were implanted into the right fourth mammary fat pad of Female BALB/c nude mice (4–6 weeks old). Six weeks post-injection, the mice were euthanized, and tumours and lungs were harvested for analysis. Moreover, the sections of tumour, liver and lung were stained with hematoxylin and eosin (H&E) for histology.

Intracellular pH measurement. The pH_i was measured with pH-sensitive fluorescent probe 2',7'-bis(carboxyethyl)-5(6)- carboxyfluorescerin/acetoxymethyl ester (BCECF/AM) at room temperature as described (Thomas *et al*, 1979; Pang *et al*, 2002). Briefly, cells (5×10^5 cells per ml) were grown to 70% confluence on 22-mm diameter glass coverslips in 35-mm dishes and were loaded with 2 μM BCECF/AM in DMEM culture medium at 37 °C for 30 min. After loading, cells were washed three times with PBS. The dye fluorescence of BCECF/AM was measured at a constant emission wavelength (550 nm) by alternately exciting the dye at 439 and 490 nm with Bio-Rad 1024 confocal laser microscope. For each experiment, the calibration of the signal of 490/439 nm ratio was performed using the nigericin containing high K^+ buffer (130 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 30 mM Hepes-Cl), which was adjusted usually to the pH values 6.8, 7.0, 7.2, 7.4, and 7.6 (Thomas *et al*, 1979). The ammonium rebound acid-loading technique (Thomas, 1984) was used to acidify the cells and cells were incubated in the isotonic solution containing 20 mM NH_4Cl for 10 min. The pH_i recovery was recorded following acidification upon removal of external NH4Cl, and reperfusion with Na^+-free or Na^+-containing solution (Chiang *et al*, 2008). Each assay was done in triplicate and repeated at least thrice. The data were analysed using the Student's *t*-test and the statistical significance was set at $P<0.05$.

Statistical analysis. Each experiment was repeated at least three times. All data were summarised and presented as mean ± s.d. The difference between means was statistically analysed using the *t*-test. All statistical analyses were performed using the GraphPad Prism software (San Diego, CA, USA). $P<0.05$ was considered as statistically significant.

RESULTS

Distinct expressions of CD44 in MDA-MB-231 and MCF-7 cells. CD44 expression has been associated with various kinds of carcinoma. We detected CD44 expressions in several human cancer cell lines, including chronic myelogenous leukaemia K562, acute T cell leukaemia Jurkat, histiocytic lymphoma U-937, acute promyelocytic leukaemia HL-60, acute lymphoblastic leukaemia CEM, Burkitt's lymphoma Namalwa, and breast adenocarcinoma MDA-MB-231 and MCF-7 by reverse transcription (RT) PCR analysis (Figure 1A). The results of real-time PCR showed that the total expressions of CD44 in MDA-MB-231 and MCF-7 cells were much higher than that in other cells lines. This different CD44 expression may be due to these different cell origins (Figure 1B). To see whether there is a difference in the expressions of CD44 between MDA-MB-231 and MCF-7cells, CD44 levels were finally determined by real-time quantitative PCR and western blotting. The results illustrated a very heterogeneous expression of CD44 in the two different breast cancer cell lines, and MDA-MB-231 cells expressed a relatively high level of CD44, approximately four times as much as the MCF-7 cells (Figure 1B). The results of western blotting also showed a relatively high amount of synthesised CD44 protein in MDA-MB-231 cells (Figure 1D). To investigate the relationship between NHE1 and CD44, we also detected the NHE1 expression in two different breast cancer cells lines. And a relatively good correlation between CD44 and NHE1 expression pattern could be seen. The mRNA and protein expressions levels of NHE1 were higher in MDA-MB-231 cells than in MCF-7 cells. In general, the much higher total expression of CD44 accompanied by a higher NHE1 expression may correlate with the invasive status of a cell line.

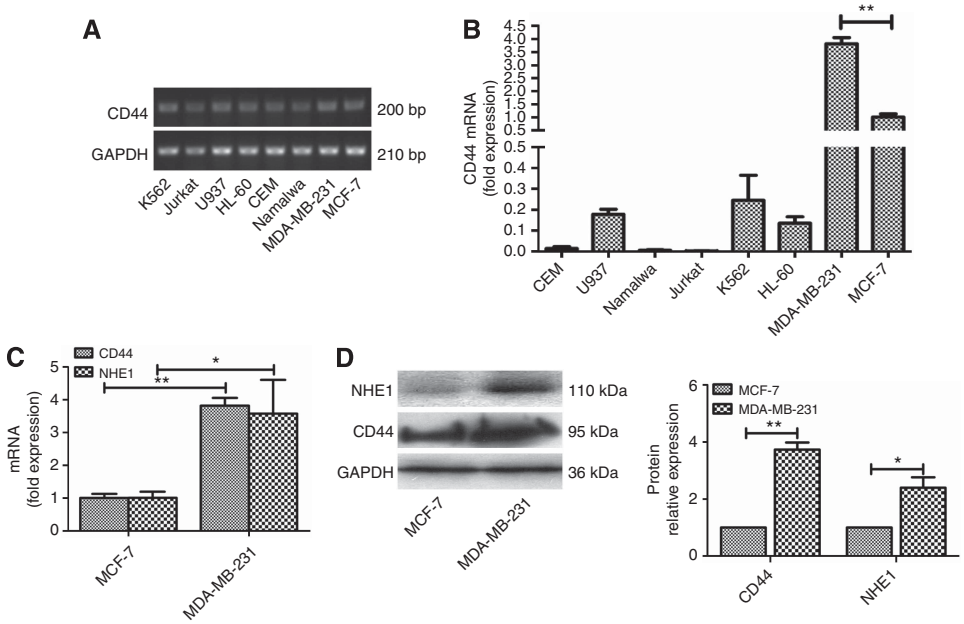

Figure 1. Expression patterns of CD44 in breast cancer cells. (**A**) Expression patterns of CD44 in several human cancer cells were analysed by PCR. Polymerase chain reactions were performed on templates of cDNA from different human cancer cell lines using a set of primers as in the Materials and Methods. (**B**) Real-time PCR analysis of CD44 gene expression in several human cancer cells. (**C**) Real-time PCR analysis of CD44 and NHE1 gene expressions in MDA-MB-231 and MCF-7 cells. (**D**) The protein expressions of CD44 and NHE1 in MDA-MB-231 and MCF-7 cells. For real-time PCR and western blotting, GAPDH was used as an internal control. *$P<0.05$; **$P<0.01$.

Downregulation of CD44 inhibits migration and invasion of MDA-MB-231 cells. To confirm the function of CD44 in metastasis, three CD44shRNA plasmids were constructed and transfected into MDA-MB-231 cells that express the higher level of CD44 and exhibit a more pronounced migratory and invasive phenotype. CD44-knockdown MDA-MB-231 cells showed a markedly decreased CD44 expression compared with MDA-MB-231 parental cells. In addition, the shRNA1 and 2 plasmids were more effective in decreasing the expression of CD44 (Figures 2A and B). As CD44 is a cell surface glycoprotein, we aimed at detecting its expression on the membranes of MDA-MB-231 cells after transfection by confocal laser microscope. Consistent with the results of the western blots, the membrane-type CD44 was also significantly decreased after transfection, and the ring forms of CD44 expression around the membranes of MDA-MB-231 cells were broken and some of them even disappeared (Figure 2C).

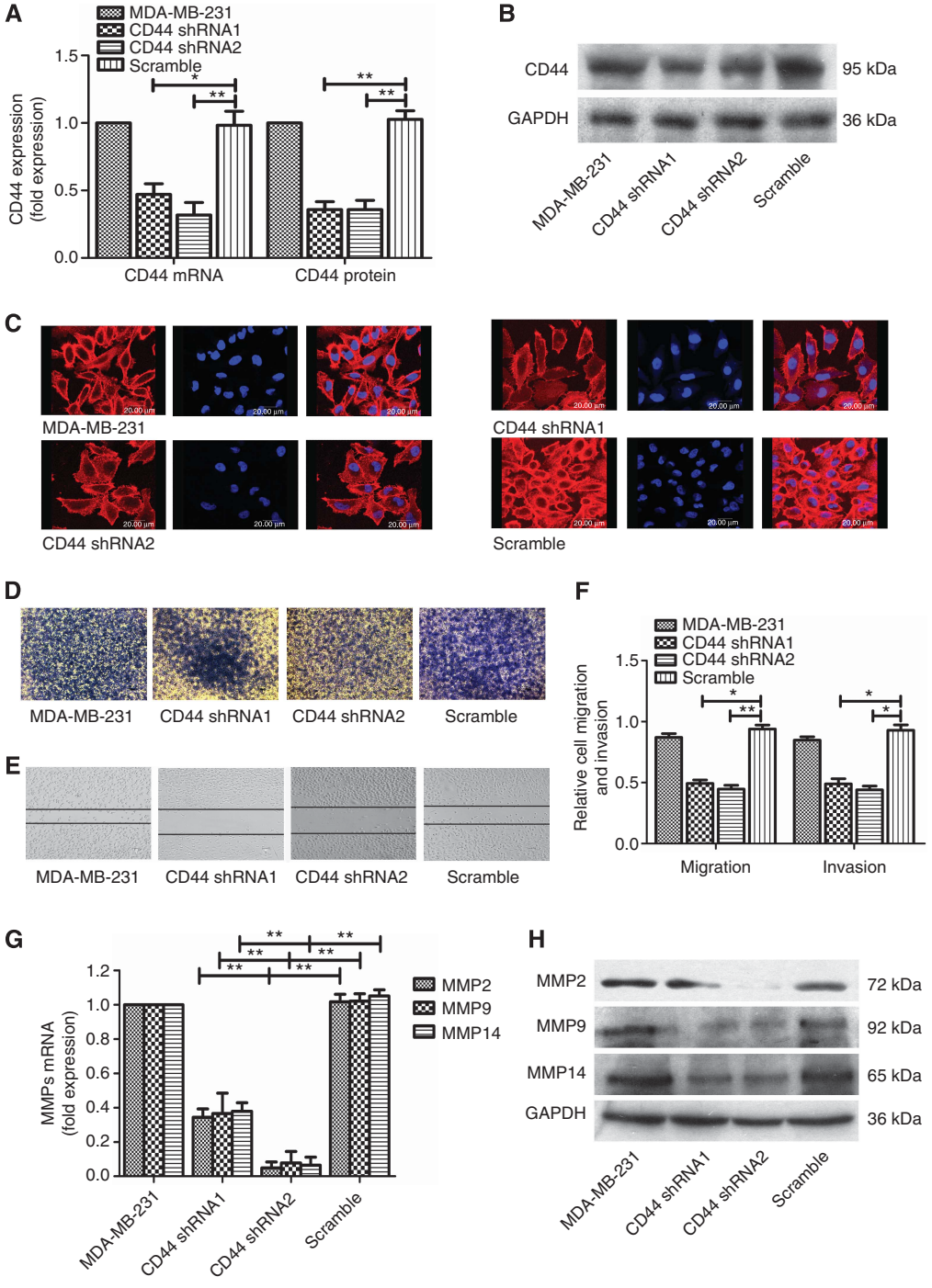

Figure 2. CD44 interference inhibits metastasis of MDA-MB-231 cells. (**A** and **B**) Downregulation of CD44 mRNA/protein expression in MDA-MB-231 cells transfected with CD44 shRNA1/2. (**C**) Confocal laser microscope showing localization of CD44 in MDA-MB-231 cells after transfection. (**D**) Representative images of transwell assay for cell invasion after CD44 interference (original magnification $\times 100$). (**E**) Representative pictures of the wound-healing assay for cell migration after CD44 shRNA transfection (original magnification $\times 100$). (**F**) The relative cells migration and invasion. $^{**}P<0.01$, compared with the control. (**G** and **H**) Detection of MMP2, 9, and 14 gene/protein expressions after CD44 interference. For real-time PCR and western blotting, GAPDH was used as an internal control. Photos of all cells were taken by an inverted microscope as described under Materials and Methods.

A key feature of cells that have higher CD44 expression is their increased migration and invasion capacity. The results of the cell invasion (Figure 2D) and the wound-healing assay (Figure 2E) showed that the metastatic capacity of MDA-MB-231 cells was inhibited by the downregulation of CD44. The amount of cells that migrated to the lower side of the membrane was significantly reduced and the migration of MDA-MB-231 cells was also prominently decreased after transfected with CD44shRNA (Figure 2F).

Degradation of the extracellular matrix (ECM) is a key step of tumour infiltration into normal tissue and beginning metastasis. Matrix metalloproteinases have been shown to contribute to breast cancer metastasis through their ability to degrade the ECM. Matrix metalloproteinase 2, MMP9, and MMP14 are the major proteinases among members of the MMP subfamily involved in pericellular proteolysis associated with cell migration. We next investigated the expressions of MMP2, MMP9, and MMP14 in the CD44-knockdown cell clones. The MMPs' mRNA expressions decreased significantly as opposed to control as shown by real-time PCR analysis (Figure 2G). Western blotting confirmed the reduced protein expressions of MMPs corresponding to the CD44-silenced cell lines (Figure 2H and Supplementary Figure 3A). These data suggest that the activation of tumour metastasis relies on the expression of CD44 and the downregulated CD44 expression could inhibit the metastatic abilities of MDA-MB-231 cells.

Downregulation of CD44 suppresses cell migration and invasion depending on CD44-mediated NHE1 inhibition. The Na^+/H^+ exchanger 1 is a plasma membrane transporter that regulates pH homeostasis. It has often been documented that the increased pH_i caused by NHE1 activation serves as a permissive or an obligatory signal for cell proliferation and metastasis. We next sought to study whether there was a positive correlation between CD44 and NHE1 contributing to the metastatic capacities of MDA-MB-231 cells. Moreover, the results showed that a decrease of NHE1 mRNA and protein synthesis was observed when CD44 expressions in MDA-MB-231 cells were decreased (Figures 3A and B). In addition, we measured the activity of NHE1 by the response of pH_i to acidification. To study the active pH regulation, cells were pretreated using the NH_4^+ -prepulse method and the recovery was evaluated. As shown in the Supplementary Figures 2A and C, MDA-MB-231 cell lines that transfected with scramble plasmids displayed a rapid recovery from the acid load. The CD44-silenced MDA-MB-231 cells also recovered from acidification, although the recovery rate was significantly lower than control cells. In addition, this decreased NHE1 expression was consistent with the downregulated metastatic capacity of MDA-MB-231 cells. This indicates that the decreased NHE1 abundance and activity induced by CD44 downregulation contribute to the suppressed mobility of MDA-MB-231 cells.

To investigate whether the inhibition of NHE1 mediated by CD44 downregulation was the main reason for the decreased metastatic abilities of CD44-silenced MDA-MB-231 cells, so we constructed a NHE1 shRNA to simulate the effect of CD44 on the inhibition of NHE1. Our results demonstrated that the expressions of NHE1 were significantly suppressed in NHE1-silenced MDA-MB-231 cells (Figures 3C and D). Then, we detected the activity of NHE1 in MDA-MB-231 cells after NHE1 interference. Moreover, the NHE1 activity was significantly inhibited when the NHE1 expression was downregulated (Supplementary Figures 2B and D). As our anticipation, this course was strongly together with significantly suppressed invasive and metastatic abilities of MDA-MB-231 cells consistent with the results of CD44-silenced MDA-MB-231 cells (Figures 3E–G). Furthermore, treatment with NHE1 shRNA also downregulated MMP2, MMP9, and MMP14 mRNA expressions (data not shown), and the decreased protein expressions of MMP2, MMP9, and MMP14 were validated by western blotting (Figure 3H).

We also used Cariporide, a selective specific inhibitor, to investigate the correlation between CD44 and NHE1. As shown in Figures 3E–G, the invasion and migration of MDA-MB-231 cells markedly decreased after treatment with Cariporide. The mRNA expressions of MMPs were also pronounced suppressed by Cariporide (data not shown) and the protein expressions of MMP2, MMP9, and MMP14 also diminished in a time-dependent manner. The protein expressions of MMP2, MMP9, and MMP14 began to decrease after treatment with Cariporide for 12 h and significantly degraded after incubation for 48 h compared with the internal control (Figure 3I and Supplementary Figure 3B). These results indeed demonstrate that the suppression of CD44 on tumour cell migration and invasion is at least partly via the downregulation of NHE1.

NHE1 re-expression increases the invasion and migration of CD44-silenced MDA-MB-231 cells. From the above experiments, we knew that CD44 downregulation inhibited NHE1 expression to regulate the metastasis of MDA-MB-231 cells (Figure 3); therefore, we constructed the NHE1 overexpression plasmids to make up for the low NHE1 expression, and then tested the metastasis of CD44-silenced MDA-MB-231 to investigate the positive relationship between CD44 and NHE1. The re-expression of NHE1 mRNA and protein in CD44-silenced cells were detected using real-time PCR and western blotting, respectively. Among these cell lines, the CD44shRNA/NHE1 cells (MDA-MB-231 cells transfected with CD44shRNA and NHE1 overexpression plasmids) expressed a high level of NHE1 mRNA and protein. As shown in Figure 4A, the NHE1 mRNA expression raised $\sim$1.5-fold than that in control cells. Moreover, the results of western blotting showed that NHE1 protein expression significantly increased in CD44shRNA/NHE1 cells compared with their parental cells (Figure 4B).

The transwell experiment and wound-healing assay were used to study the influence of NHE1 re-expression on the metastasis of tumour cells. As shown in Figures 4C and E, the number of invasive cells in the CD44shRNA/NHE1 group occurred at a higher rate compared with the CD44shRNA groups. In addition, the migration of CD44-silenced MDA-MB-231 cells was also markedly increased when the CD44shRNA-mediated low expression of NHE1 was upregulated (Figures 4D and E). Moreover, we also found that induction of NHE1 led to increase mRNA expressions of MMPs (data not shown), and the increased protein expressions of MMP2, 9, and 14 were finally verified by western blotting (Figure 4F). These results indicate that the invasion and migration of MDA-MB-231 cells were significantly suppressed by CD44shRNA-mediated NHE1 downregulation. Moreover, the invasive and metastatic abilities were clearly recovered after being upregulated the expression of NHE1. In general, these data suggest that CD44 downregulation inhibits NHE1 expression to regulate the metastasis of MDA-MB-231 cells.

To further investigate the correlation between CD44 level and NHE1 expression, we also transiently transfected CD44/tet overexpression plasmids into NHE1-silenced MDA-MB-231 cells. The results showed that after transfection with CD44/tet overexpression plasmids, the expressions of CD44 significantly increased and the suppressions of NHE1 expression induced by NHE1 shRNA were neutralized by the upregulation of CD44. The change of NHE1 mRNA expression is up to 2.5-fold multiplication (Supplementary Figures 1A and B). Then, we assessed the impacts of CD44 overexpression on the metastatic capacities of NHE1-silenced MDA-MB-231 cells with transwell experiment and wound-healing assay. The numbers of cells transfected with CD44/tet plasmids evidently increased compared with the control, and the metastatic ability was pronouncedly promoted by CD44/tet

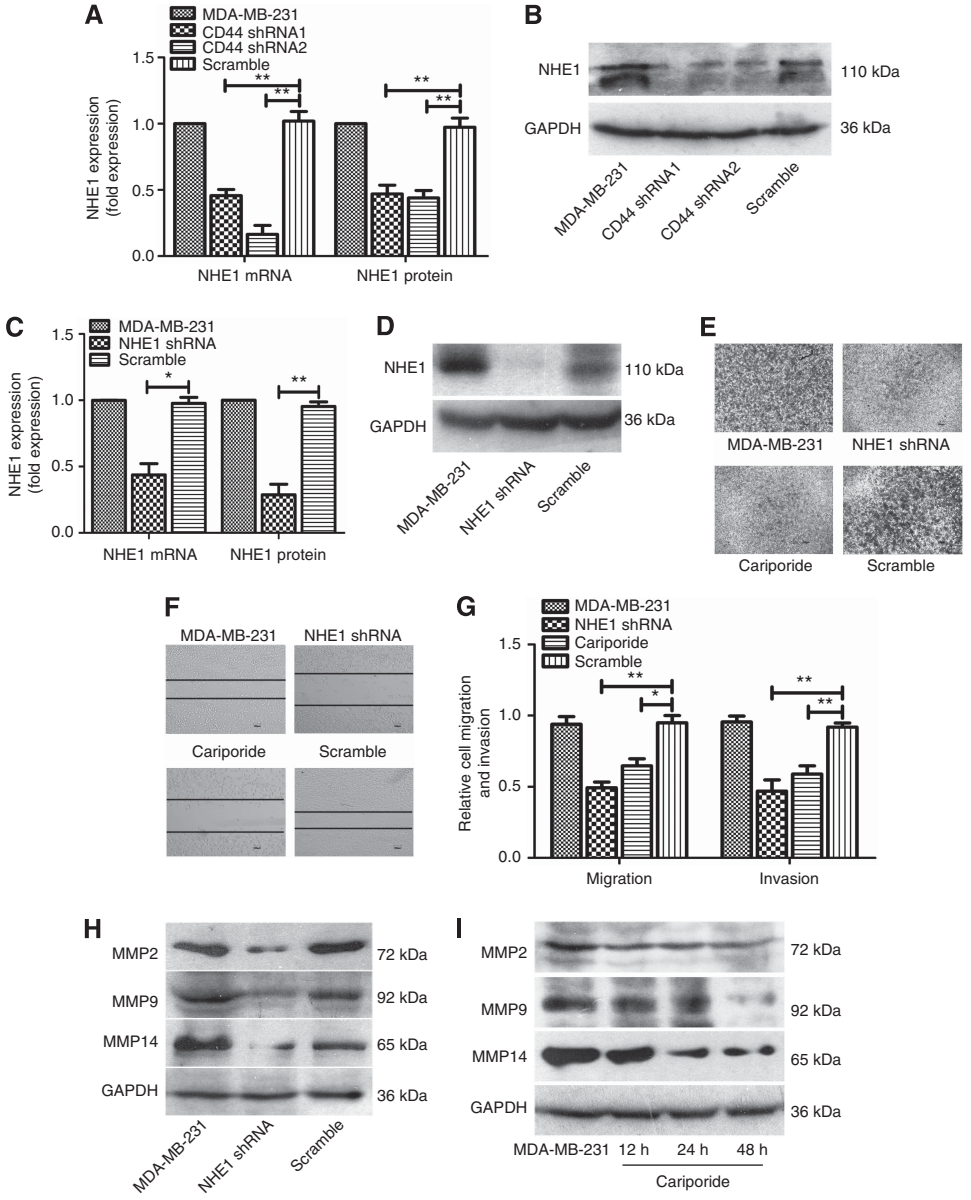

Figure 3. CD44 interference inhibits NHE1 expression and downregulate the metastasis of MDA-MB-231 cells. (**A** and **B**) Downregulation of NHE1 mRNA/protein expression in MDA-MB-231 cells after CD44 interference. (**C** and **D**) Detection of NHE1 mRNA/protein expression in MDA-MB-231 cells transfected with NHE1 shRNA. (**E**) Representative images of transwell assay for cell invasion after treatment with NHE1 shRNA or Cariporide (original magnification $\times 100$). (**F**) Representative pictures of the wound-healing assay for cell migration after transfection with NHE1 shRNA or treatment with Cariporide (original magnification $\times 100$). (**G**) The relative cells migration and invasion of MDA-MB-231 cells treatment with NHE1 shRNA or Cariporide. $^{**}P<0.01$, compared with the control. (**H** and **I**) Protein levels of MMP2, 9, and 14 after treatment with NHE1 shRNA/Cariporide. For real-time PCR and western blotting, GAPDH was used as an internal control. Photos of all cells were taken by an inverted microscope as described under Materials and Methods.

plasmids-mediated CD44 upregulation (Supplementary Figures 1C–E). After transfection, the increased mRNA expressions of MMPs were detected by real-time PCR (data not shown) and the inhibitions of MMP2, MMP9, and MMP14 protein expressions induced by NHE1 shRNA were also markedly upregulated by CD44 overexpression (Supplementary Figure 1F). These results clearly indicated that CD44 upregulation elevates the expression of NHE1 and NHE1 participation is indispensable for CD44-mediated invasion and migration of MDA-MB-231 cells.

CD44 promotes invasion and migration in MCF-7 cells through regulation of NHE1. In order to study the relationship between CD44 and NHE1 expressions in tumour metastasis, we chose another breast cancer cell line (MCF-7) that exhibited low level expressions of CD44 and NHE1. MCF-7 cells were transiently transfected with CD44/tet overexpression plasmids, and the mRNA and protein expressions of CD44 and NHE1 were detected by real-time PCR and western blotting. As shown in the Figures 5A and B, the mRNA and protein expressions of CD44 and NHE1 markedly increased in CD44 overexpressed MCF-7 cells compared with their parental cells. We then performed the transwell experiment and the wound-healing assay in CD44 overexpressed MCF-7 cells. As expected, CD44 overexpression led to a significant increase in cell invasive and metastatic capacity (Figures 5C and D). To investigate whether the changes of metastatic capacities in CD44 overexpressed MCF-7 cells were mediated by CD44-regulated NHE1 upexpression, we used Cariporide, a specific inhibitor of NHE1, to test our hypothesis. As our anticipation,

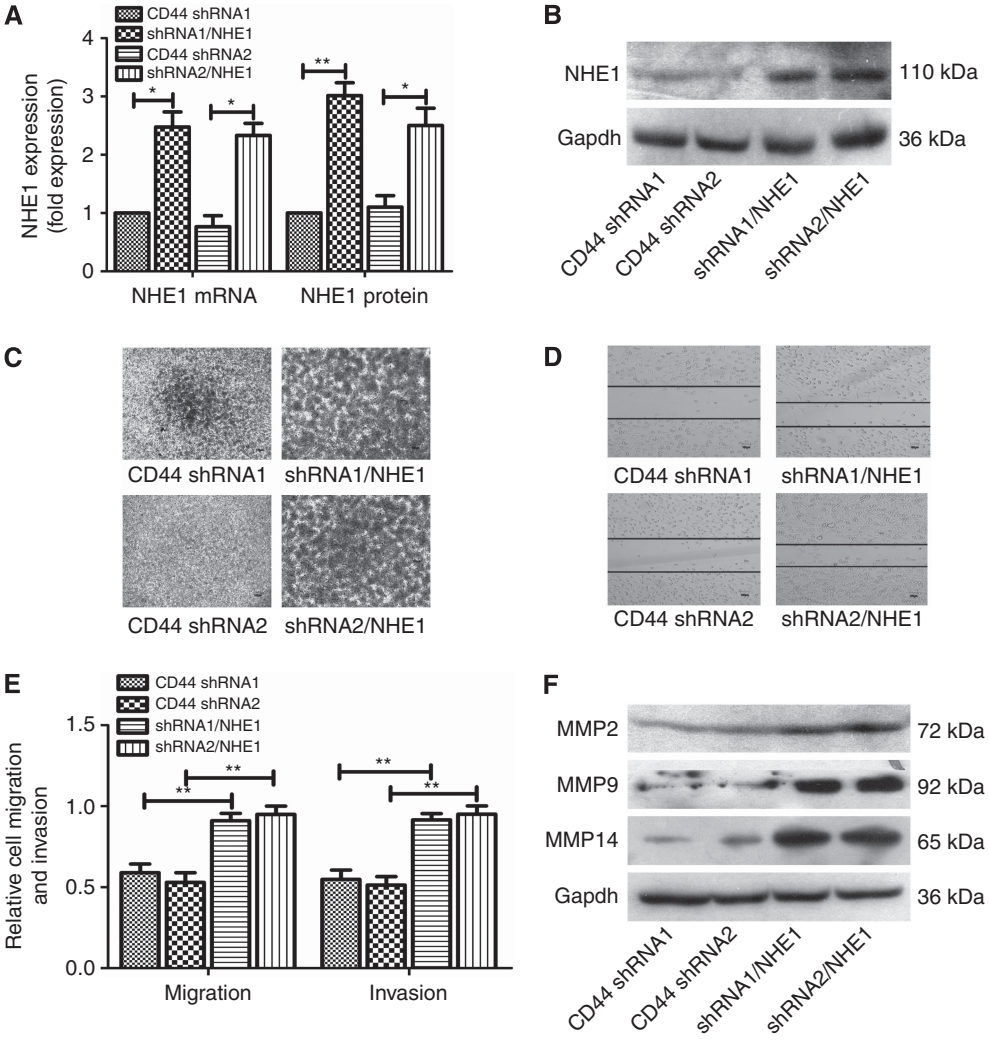

Figure 4. NHE1 re-expression recovers metastasis of CD44-silenced MDA-MB-231 cells. (**A** and **B**) Detection of NHE1 mRNA/protein expression in CD44-silenced MDA-MB-231 cells transfected with NHE1 overexpression plasmids. (**C**) Representative images of transwell assay for cell invasion after NHE1 overexpression (original magnification $\times 100$). (**D**) Representative pictures of the wound-healing assay for cell migration (original magnification $\times 100$). (**E**) The relative cells migration and invasion. $^{**}P<0.01$, compared with the control. (**F**) Protein levels of MMP2, 9, and 14 after NHE1 overexpression. 'shRNA1/NHE1' represented that MDA-MB-231 cells were transiently transfected with NHE1 overexpression plasmids after stably transfection with CD44 shRNA1. The same situation was compatible with shRNA2/NHE1. For real-time PCR and western blotting, GAPDH was used as an internal control. Photos of all cells were taken by an inverted microscope as described under Materials and Methods.

when the NHE1 activity was inhibited, the increased invasion and migration of MCF-7 cells mediated by CD44 overexpression were suppressed by Cariporide (Figures 5C–E). Moreover, we also found that CD44 upregulation facilitated the three MMPs' mRNA expressions (data not shown), and the increased protein expressions of MMP2, 9, and 14 were confirmed by western blotting. But the increased mRNA and protein expressions of MMPs were also inhibited by Cariporide-mediated NHE1 inhibition (Figure 5F). These results indicate that CD44 overexpression promotes the metastasis of MCF-7 cells mostly through NHE1 upregulation, and this increased capacity was also suppressed when the activities of NHE1 were inhibited.

The MAPK signaling pathway has a role in CD44-induced metastasis of MDA-MB-231 cells. Previous studies have suggested that the regulation of CD44 could activate the MAPK signaling pathway (Fang *et al*, 2011). Therefore, we presumed that the downregulation of CD44 may have a functional relationship with the pathway. To investigate the signaling pathways involved in CD44-mediated cell metastasis, we tested the change of ERK1/2 phosphorylation. As shown in Figure 6A, the expression of phosphorylated ERK1/2 in MDA-MB-231 cells significantly decreased when transfected with CD44shRNA compared with the internal control. To exclude the possibility of other kinases contribution, we tested the protein expressions of phosphorylated JNK, p38, and AKT. But no statistical differences of phosphorylated JNK, p38, and AKT were observed in CD44-silenced MDA-MB-231 cells (Figure 6B). To verify the correlation between NHE1 and ERK1/2, we determined the expression of phospho-ERK1/2 after NHE1 re-expression. As our expectation, the activity of phospho-ERK1/2 was recovered by NHE1 upregulation (Figure 6C and Supplementary Figure 3D). In contrast, ERK activation was significantly reduced in MDA-MB-231 cells pretreated with NHE1 shRNA or Cariporide, and Cariporide inhibited the expression of phospho-ERK1/2 in a time-dependent manner (Figure 6D and Supplementary Figure 3E). To verify the above observation, we overexpressed CD44 in NHE1-knockdown MDA-MB-231 or MCF-7 cells. As our anticipation, the inhibition of ERK1/2 activation was reversed by upregulation of CD44 (Figure 6E and Supplementary Figure 3F).

To validate the signaling pathways involved in CD44-mediated cell metastasis, inhibitors for NHE1 or/and MAPK signaling pathway

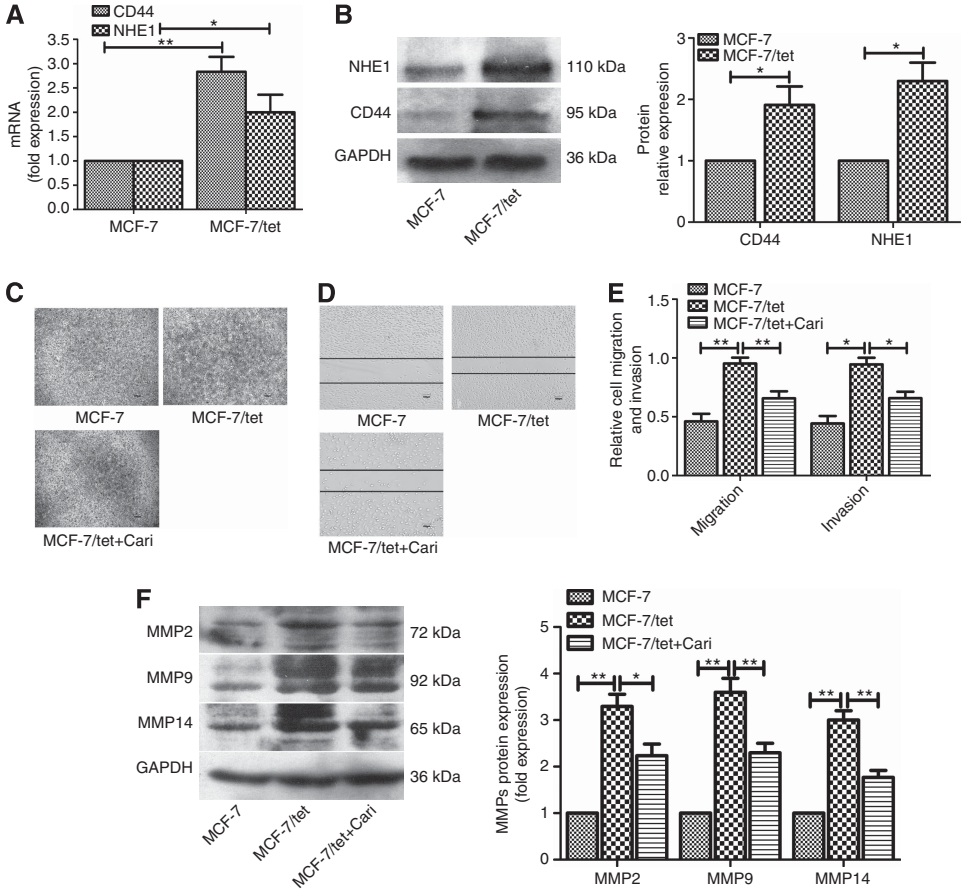

Figure 5. NHE1 participates in CD44-mediated the metastatic capacities of MCF-7 cells. (**A** and **B**) Detection of CD44 and NHE1 mRNA/protein expression in MCF-7 cells after transfection with CD44/tet overexpression plasmids. (**C**) Representative images of the transwell assay for cell invasion after CD44 overexpression (original magnification $\times 100$). (**D**) Representative pictures of the wound-healing assay for cell migration (original magnification $\times 100$). (**E**) The relative cells migration and invasion after transfection. $^{**}P<0.01$, compared with the control. (**F**) The changes of protein levels of MMP2, 9, and 14. 'MCF-7/tet' represented that MCF-7 cells were transiently transfected with CD44/tet overexpression plasmids. For real-time PCR and western blotting, GAPDH was used as an internal control. Photos of all cells were taken by an inverted microscope as described under Materials and Methods.

were used to assay their effects on CD44-mediated cell metastasis. As shown in Figures 6F and G, the invasive and metastatic abilities were noticeably reduced pretreatment with Cariporiede or PD98059, a specific inhibitor for ERK1/2. When the two specific inhibitors were simultaneously used in the transwell experiment and wound-healing assay, the migration and invasion of MDA-MB-231 cells reduced much more compared with either inhibitor. And so the ERK1/2 inhibitor PD98059 has a synergistic effect with Cariporide (Figure 6H). Moreover, we also investigated the roles of PD98059 on the expressions of MMPs. As expected, after pretreatment with PD98059 for 6h, MDA-MB-231 cells exhibited significantly reduced MMP2, MMP9, and MMP14 expressions and the protein expressions of MMPs disappeared in a time-dependent manner (Figure 6I and Supplementary Figure 3G). These data suggest that the MAPK signaling pathway was involved in CD44-mediated invasion of MDA-MB-231 cells.

CD44 downregulation inhibits breast tumour outgrowth and spontaneous lung metastasis. As breast cancer mortality is primarily associated with the development of metastases, we hypothesised that CD44 may affect the metastatic process. To test the hypothesis that CD44 can positively regulate the metastatic ability of breast cancer cells, CD44 expression was downregulated in the highly aggressive MDA-MB-231 cell line and the CD44-silenced MDA-MB-231 cells were orthotopic injected into the mammary fat pad of BALB/c nude mice. Of note, MDA-MB-231 cells engineered to express CD44shRNA showed a marked delay in the onset of first palpable tumour. Moreover, CD44-silenced MDA-MB-231 cells showed a significant reduction in tumour outgrowth after mammary fat pad injection when compared with control cells (Figures 7A and C). The mean tumour size of scramble cells at 42 days was 1108 mm^3, whereas the mean tumour sizes of CD44-silenced MDA-MB-231 cells were 417 and 470 mm^3 (Figures 7A and B). Histological analysis of the tumours using H&E staining revealed the presence of large coalescing areas of necrotic cell death at the periphery of CD44-silenced tumours, which is in marked contrast with scramble tumours (Figure 7D). Owing to the strong correlation between high CD44 expression and high metastatic ability of breast cancer *in vitro*, we next determined whether animals carrying primary orthotopic tumours developed spontaneous metastatic lesions to the other organs. To allow spontaneous other organs metastases to form, animals were killed after 6 weeks and detected the colonisation in other organs. The results of H&E staining experiments showed that there is higher level of lung colonisation in control animals. The CD44-silenced MDA-MB-231-injected animals have no lung colonisation, but had marked inflammation in their lungs. Both groups of animals did not have colonisation of breast cancer cells in their livers, but the results of H&E staining revealed the presence of large coalescing areas of necrotic cell death at their livers (Figure 7D). The NHE1-silenced MDA-MB-231 cells were also orthotopic injected into the BALB/c nude mice, which had the same results

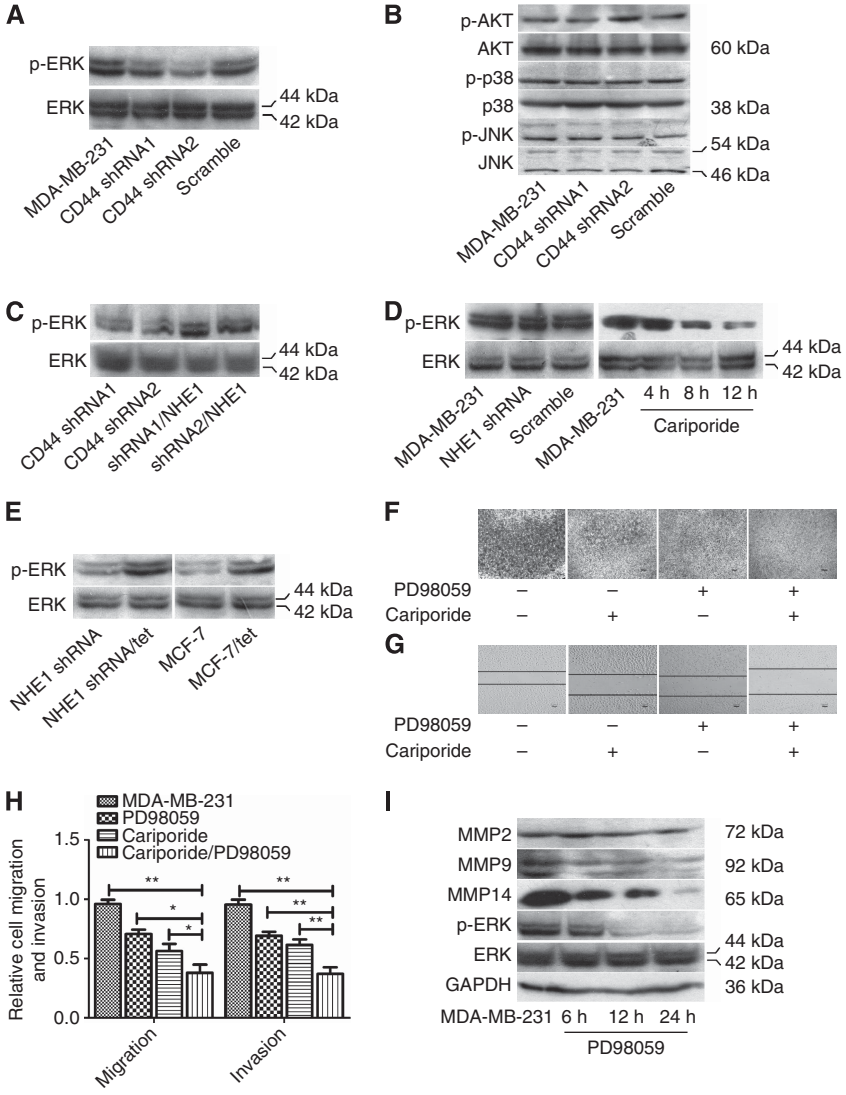

Figure 6. The MAPK signaling pathway involves in CD44-mediated metastasis of MDA-MB-231 cells. (**A**) Decrease of phospho-ERK1/2 expression in CD44-silenced MDA-MB-231 cells. (**B**) Detection of phosphorylation of AKT, p38 MAPK, and JNK in CD44-knockdown MDA-MB-231 cells. (**C**) Recovery of phospho-ERK1/2 activity by NHE1 upregulation. (**D**) Protein expression of phosphorylated ERK1/2 in MDA-MB-231 cells treatment with NHE1 shRNA or Cariporide. (**E**) Upregulation of phospho-ERK1/2 activity by CD44 overexpression. (**F**) Representative images of the transwell assay for cell invasion after treatment with PD98059 and/or Cariporide (original magnification $\times 100$). (**G**) Representative pictures of the wound-healing assay for cell migration pretreatment with PD98059 and/or Cariporide (original magnification $\times 100$). (**H**) The relative cells invasion and migration of MDA-MB-231 cells treatment with PD98059 and/or Cariporide. $^{**}P<0.01$, compared with the control. (**I**) Protein levels of MMP2, 9, and 14 and phospho-ERK1/2 after treatment with PD98059. For real-time PCR and western blotting, GAPDH was used as an internal control. Photos of all cells were taken by an inverted microscope as described under Materials and Methods.

compared with CD44-silenced MDA-MB-231. Moreover, the NHE1-silenced MDA-MB-231-injected animals had a delay tumour outgrowth and no lung colonisation (Figures 7A–D). These data imply that downregulation of CD44 does negatively affect the ability of breast cancer cells to metastasise the lung and this inhibition of metastatic ability was associated with CD44-mediated NHE1 regulation.

DISCUSSION

Tumour metastasis is the major cause of morbidity in patients diagnosed with solid tumours such as breast cancer (Parker and Sukumar, 2003), ovarian cancer (Bhoola and Hoskins, 2006), and squamous cell carcinomas (Kramer *et al*, 2005), largely because of the ineffectiveness of current therapies. It is now certain that both oncogenic signaling and some cell surface proteins are directly involved in metastasis of tumour cells and CD44 has been implicated in cancer cell metastasis (Auvinen *et al*, 2005; Bourguignon, 2008; Yae *et al*, 2012). A wide range of evidence indicates that the expression of CD44 in malignant tumour is higher than that in benign tumour (Shipitsin *et al*, 2007). Consistent with the reports, we detected that CD44 were highly expressed in MDA-MB-231 cells but lower in MCF-7 cells. The results imply that these must be a functional link between CD44 and tumour metastasis.

To ensure the CD44-mediated effects on metastasis, CD44 was silenced in MDA-MB-231 cells and our results indicate that the downregulated expression of CD44 indeed markedly decreased metastasis of MDA-MB-231 cells. Pham *et al* (2011) found that the expression of CD44 was important for breast cancer stem cells and our findings are consistent with the above report and suggest that CD44 is considered as a promising target for anticancer treatment,

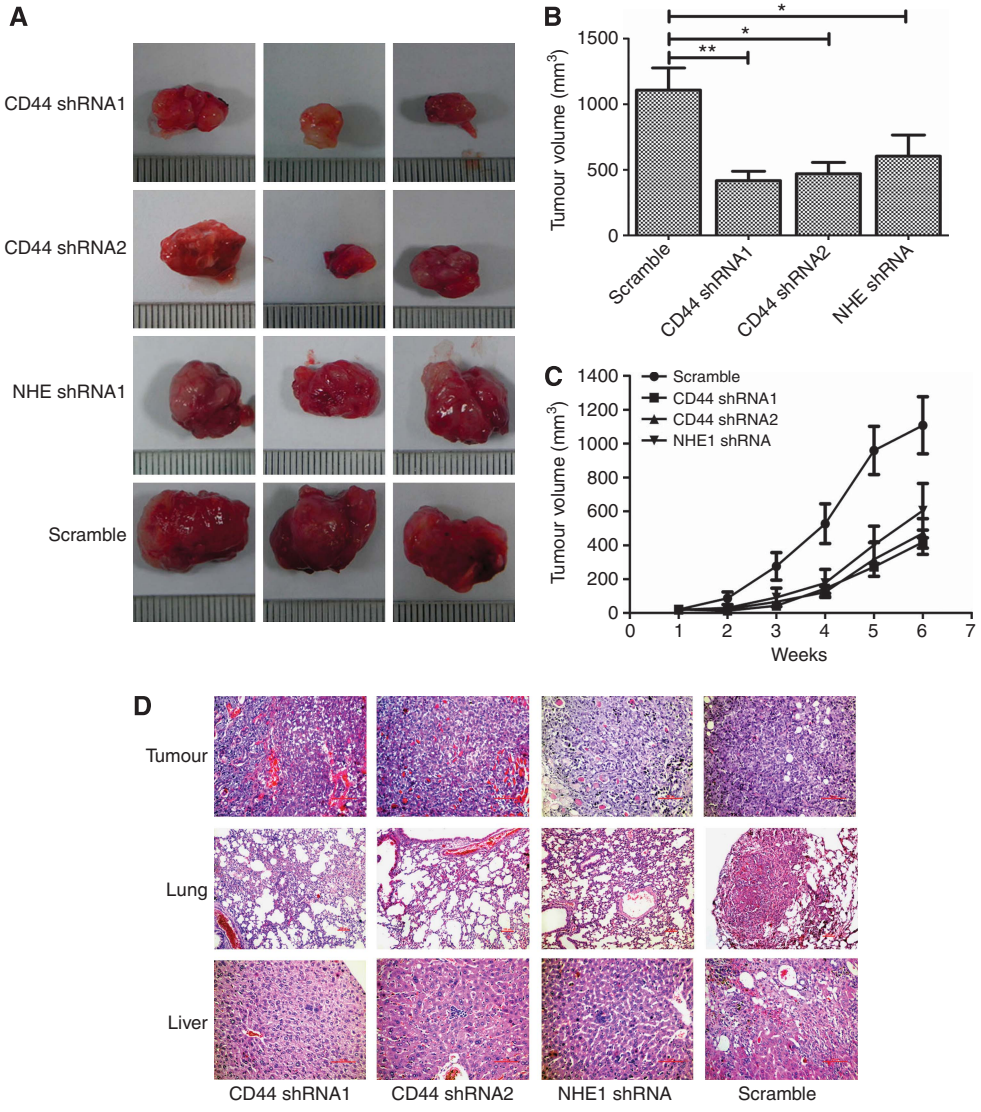

Figure 7. CD44 downregulation inhibits breast tumor outgrowth and spontaneous lung metastasis. (**A**) Representative images of orthotopic tumours. (**B**) CD44 and NHE1 interference MDA-MB-231 cells lead to impaired tumor volume. Tumor volume was calculated as $\pi LW2/6$ for 6 weeks. $^{*}P<0.05$; $^{**}P<0.01$, compared with the control. (**C**) CD44 and NHE1 interference MDA-MB-231 cells lead to impaired tumor outgrowth. (**D**) Histological analysis of orthotopic tumors, lung, and liver by H&E staining. CD44 and NHE1 interference MDA-MB-231 cells lead to impaired spontaneous breast cancer cell colonization of lungs.

especially to breast cancer. Then, the CD44 expression was upregulated in MCF-7 cells and our findings indicate that the metastatic capacities of MCF-7 cells were clearly activated by CD44 upregulation.

The activity of the major pH-regulating transporters NHE1 and the pH_i values of normal and tumour cells are different. Na^+/H^+ exchanger isoform 1 is almost quiescent in normal cells, but in tumour cells, the hyper-activated NHE1 results in an increase in pH_i and acidification of the extracellular space. Owing to the positive-feedback vicious cycle between the extracellular micro-environment and tumour cells, an ever-higher reversed pH gradient is achieved as the disease progresses. However, little is known about the signal-transduction systems that regulate the NHE1 activity and that are associated with tumour cell invasiveness (Stuwe *et al*, 2007; Yang *et al*, 2010). Extracellular acidification has been correlated with the metastatic capacity of tumour cells by promoting neo-angiogenesis, anchorage-independent growth, and invasiveness (Pedersen *et al*, 2007). Recent studies have demonstrated that NHE1 activity and expression increased in a variety of tumour cells including breast cancer cells (Cardone *et al*, 2005; Lin *et al*, 2011). Bourguignon *et al* (2004) found that in breast cancer cells the interaction of CD44 and NHE1 with hyaluronidase-2 in lipid rafts could induce matrix degradation and breast tumour cell invasion. However, there is no report to date indicating the direct regulating relationship between CD44 and NHE1, even the role of NHE1 in CD44-driven metastasis. Our findings demonstrated that downregulation of CD44 inhibited the expression and activity of NHE1, but whether NHE1 is indispensable in CD44-mediated MDA-MB-231 cells invasion is unknown. We used NHE1 shRNA and Cariporide to simulate the inhibition effect of CD44 on NHE1. The results indicate that both NHE1 shRNA and Cariporide significantly decreased the metastasis of MDA-MB-231 cells. To further clarify whether NHE1 participates in CD44-mediated MDA-MB-231 cells invasion, we overexpressed CD44 in NHE1-silenced MDA-MB-231 cells. Our findings demonstrate that CD44 upregulation restores the invasion and migration of NHE-silenced MDA-MB-231 cells, and the expressions of NHE1 are markedly increased. We also overexpressed CD44 expression in MCF-7 cells and found that both NHE expression and the metastasis of MCF-7 cells were elevated by CD44 overexpression. When we treated

CD44-overexpressed MCF-7 cells with Cariporide, the elevated metastasis of MCF-7 cells mediated by CD44 overexpression was downregulated by NHE inhibition. These data indicate that the inhibition of CD44 can decrease NHE1 expression and CD44 upregulation can increase NHE1 expression. And so CD44 mediates the metastasis of breast cancer cells mainly through regulating NHE1 expression.

Tumour progression involves a series of different biological obstacles that tumour cells must overcome to form a metastatic tumour. Moreover, it is now clear that MMPs contribute to all stages of tumour progression (Wagenaar-Miller *et al*, 2004). Matrix metalloproteinases are a family of zinc-required matrix-degrading proteases that have an important role in tumour cell metastasis (Littlepage *et al*, 2010). To date, Twenty-five members of this family have been described, and their products can be sub-grouped into the soluble-type MMPs and the membrane-type MMPs (Kessenbrock *et al*, 2010). An increasing body of evidence has verified that the MMPs' expressions levels are closely associated with the malignancy of tumours (Doi *et al*, 2011). Recent studies have shown that hyaluronic acid strongly activates MMP2 secretion due to CD44 expression (Zhang *et al*, 2002). Moreover, it has been testified that the interaction of MMP9 and CD44 is crucial to the migration of fibrosarcoma cells (Dufour *et al*, 2010). Thus, it appears that CD44 and MMPs are all involved in the initiation of tumour metastasis. In the current studies, we showed that CD44 downregulation resulted in decreased MMPs' expressions in MDA-MB-231 cells. These results suggest that CD44 can regulate the MMPs' expressions, but whether MMP is required in CD44-mediated tumour cells metastasis is unknown. For this reason, we overexpressed CD44 in MCF-7 cells and found a significant increase in MCF-7 cells metastasis. However, some reports found that MT-MMPs could cleave CD44 and promoted cell metastasis in some tumour cell lines such as glioblastoma cell lines, pancreatic cancer cell lines, and lung cancer cell lines (Okamoto *et al*, 1999; Kajita *et al*, 2001). Moreover, these plausible different results may be from the different tumour cell lines and Okamoto *et al* (2002) also found that only 67% of breast carcinomas had CD44 cleavage. However, whether there is a loop regulating relationship between CD44 and MMPs needs to be further investigated.

A growing body of literatures implicate that CD44 regulates the activities of ERK1/2, PI3K, and NF- kappa B, and so on, yet the effects of CD44 on signaling pathway activities are highly context- and cell type-specific. For example, Bourguignon *et al* (2009) reported that the p300 signaling pathways activated by HA/CD44 participated in the production of MDR1 in breast tumour cells. Furthermore, Abdraboh *et al* (2011) found that CD44 induced the expression of survivin leading to breast tumour invasion through the PI3K signaling pathway. To gain more mechanistic insight into how CD44 mediates MDA-MB-231 cells metastasis, we inspected the activities of AKT, and MAPK subfamilies. Our results indicate that downregulation of CD44 obviously decreased the phosphorylation level of ERK1/2, but AKT, p38 MAPK, and JNK activities were not influenced. Furthermore, we overexpressed CD44 in MCF-7 cells and found the phosphorylation of ERK1/2 was markedly increased by upregulation of CD44. Moreover, these results were also testified in NHE1-knockdown MDA-MB-231 cells. In addition, the invasion and migration of MDA-MB-231 cells treated with PD98059 were steeply depressed, and the inhibition was greater when pretreated with Cariporide. Therefore, the inhibitor of ERK1/2 has an additional inhibition effect with Cariporide. These results reveal that ERK1/2 but not AKT, p38 MAPK, and JNK contribute to CD44-mediated MDA-MB-231 cells metastasis. To further clarify whether the effect of CD44 on MMPs' expressions depend on ERK1/2 signaling pathways, we treated MDA-MB-231 cells with ERK1/2 inhibitor and found the expressions of MMPs were downregulated in a time-dependent manner. Considering these data, we conclude that CD44 targets NHE1 to regulate the expressions of MMPs through ERK1/2 signaling pathways in CD44-mediated tumour cell metastasis.

Taken together, the regulatory mechanisms of CD44 on NHE1 can be summarised as follows (Supplementary Figure 4). According to the previous studies, the EGFR may localise in the membrane of tumour cells with NHE1 and CD44 (Chiang *et al*, 2008; Midgley *et al*, 2013). Moreover, in our study we first investigate the direct regulating correlation between CD44 and NHE1 in MDA-MB-231 cells. The downregulation of CD44 can inhibit NHE1 abundance and activity, which likely in turn suppresses the motility of breast cancer cells. The inhibition of NHE1 almost abolishes CD44-stimulated breast cancer cells' motility. Moreover, when the activity of NHE1 was inhibited, the expressions of p-ERK1/2 and MMPs were suppressed. Furthermore, the expressions of MMPs were also regulated by p-ERK1/2. Therefore, in the process of CD44-mediated MDA-MB-231 metastasis, NHE1 is a target of CD44 to regulate the expressions of MMPs through the ERK1/2 signaling pathway. Our findings provide a theoretical basis that simultaneously targeting to CD44 and NHE1 may be novel therapeutic strategies for treating breast cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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