Ezrin-Radixin-Moesin Binding Phosphoprotein 50 (EBP50) Suppresses the Metastasis of Breast Cancer and HeLa Cells by Inhibiting Matrix Metalloproteinase-2 Activity

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Abstract. *Background: Expression of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) is correlated with human breast and cervical cancer development, but its effects on the metastasis of breast and cervical cancer and the underlying mechanism are not fully understood. Materials and Methods: In this study, EBP50 was overexpressed in MDA-MB-231 breast cancer and HeLa cervical cancer cells; moreover, EBP50 was knocked-down in MCF-7 breast cancer cells and HeLa cells. Metastasis-related ability and matrix metalloproteinase-2 (MMP-2) activity of these cells were investigated. Results: Cell adhesion, wound-healing and invasion were significantly suppressed in EBP50-overexpressing cells. Contrarily, EBP50-knockdown promoted cell adhesion, wound healing and invasion. EBP50 overexpression inhibited MMP-2 activity, and the knockdown of EBP50 promoted the activity of MMP-2, suggesting that EBP50 inhibited cell metastasis via suppression of MMP-2 activity. Conclusion: Our work reveals the anti-metastatic effect and a new mechanism of EBP50 action in breast and cervical cancer cells.*

Breast cancer and cervical carcinoma are common tumors in females. Breast cancer is also the most common cause of cancer death among women (522,000 deaths in 2012) and the most frequently diagnosed cancer among women in 140 out of 184 countries worldwide. In recent years the incidence and mortality rates of breast cancer have continuously increased. Cervical cancer is the fourth most common cancer affecting women worldwide, after breast, colorectal, and lung cancer (1). Breast cancer and cervical carcinoma generally show slow growth, with symptoms occurring in advanced stages once the tumor has been discovered. Adhesion, migration and metastasis are the causes of high mortality of breast cancer and cervical cancer. Prevention of migration and metastasis of breast and cervical cancer helps improve treatment and remains a research challenge.

Ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) is a multifunctional scaffolding protein that exerts different functions in a variety of cancer types through its interactions with oncogenic or tumor-suppressive proteins (2-5). In breast cancer, EBP50 was reported to inhibit cell proliferation by regulating epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) signaling and modulating the expression level of phosphatase and tensin homolog (PTEN) (3, 6-11). However, the role of EBP50 in migration and metastasis of breast cancer remains unclear. The role of EBP50 in cervical cancer metastasis has also not been reported as far as we are aware.

In this study, we sought to determine the effects of EBP50 expression on the adhesion and migration of breast cancer and cervical carcinoma cells by overexpressing EBP50 in MDA-MB-231 and HeLa cell lines. Moreover, adhesion and migration were also examined in *EBP50*-knockdown MCF-7 and HeLa cells.

Materials and Methods

Plasmids. The pBK-CMV-HA empty vector and pBK-CMV-HA-EBP50 expression plasmid were kindly provided by Dr. Randy Hall from Emory University (Atlanta, GA, USA). pSuper.puro luciferase control shRNA and pSuper.puro EBP50 shRNA plasmids were

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kindly provided by Margaret J. Wheelock from University of Nebraska Medical Center (Omaha, NE, USA).

Cell lines and cell culture. The human breast cancer cell lines MDA-MB-231, MCF-7 and human cervical cancer cell line HeLa were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 cells were cultured in GIBCO RPMI-1640 medium (Hyclone, Logan, UT, USA). MCF-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA). Both media were supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% antibiotic-antimycotic agent (Life Technologies, Inc, Grand Island, NY, USA). Cells were grown at 37˚C and with 5% CO_2 to 80% confluency for use.

Establishment of cell pool in which EBP50 was stably overexpressed or knocked down. For stable overexpression of *EBP50*, MDA-MB-231 and HeLa cells were transfected with 2 μg pBK-CMV-HA-EBP50 plasmid or pBK-CMV-HA vector using Lipofectamine 2000 (Invitrogen). For stable knockdown of *EBP50*, MCF-7 and HeLa cells were transfected with pSuper.puro-EBP50 shRNA or control pSuper.puro-luciferase shRNA plasmid respectively using Hifectin II (Applygen Technologies Inc., Beijing, P.R. China) following the manufacturer's protocol. Two days following transfection, cells were transferred to 100-mm plates and cultured in selection medium with 350 μg/ml G418 (Amresco, Solon, OH, USA) for cells with *EBP50* overexpression and 0.5 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) for cells with *EBP50* knockdown. The medium was changed every 2 days to remove floating dead cells, and resistant colonies formed were harvested and plated in 24-well plates. Cell cultures were expanded and cultured for at least 1 month, and fractions were used for analysis of EBP50 expression by western blotting, with β-actin expression as a protein loading control. Stably-transfected cell pools were maintained and passaged in culture medium with G418 (200 μg/ml) or puromycin (0.25 μg/ml).

Western blotting. Cells were collected and lysed in radio-immunoprecipitation buffer (Beijing CoWin Biotech Co., Ltd., Beijing, PR China) in the presence of protease inhibitors for 20 min to extract total protein from cells stably overexpressing or with knockdown of *EBP50*, and protein levels were quantified using BCA assays (Beijing CoWin Biotech Co., Ltd., Beijing, PR China). Next, 50 μg protein from each sample was loaded onto 8% sodium dodecyl sulfate (SDS) polyacrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Protein was then transferred to nitrocellulose membranes (Sigma). Membranes were blocked in blot buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, pH 7.4) for 1 h and were then incubated with rabbit primary antibodies to human EBP50 (1:1,000 dilution; Abcam, Cambridge, UK) overnight at 4˚C, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution; Abcam) for 1 h at room temperature. Detection was facilitated using an ECL kit (CoWin Biotech), and images were analyzed using Image J (version 1.62; National Institutes of Health, Bethesda, MD, USA).

Adhesion assay. CytoMatrix™ Cell Adhesion Strips (Millipore, Billerica, MA, USA) were used for adhesion assays. The strips were rehydrated with 200 μl of PBS per well of 96-well plates for at least 15 min at room temperature. Cells were digested using an enzymatic dissociation approach to prepare single-cell suspensions. Cell density

was also optimized by titration in serum-free medium and finally 10^6 cells/well was used. One hundred microliters of the diluted cell suspension was added to each well and the plate was incubated at 37˚C for 30 min, 60 min and 90 min in a CO_2 incubator. Twenty microliters of CCK8 reagent was added to each well and the plates were incubated for 1 h at room temperature. The supernatant was subsequently taken to determine the absorbance value at 450 nm on a microplate reader. The absorbance value from bovine serum albumin-coated wells was subtracted from the raw data of treatment groups.

Wound-healing assay. Cells were plated at 2×10^5 cells per well in 6-well plates and grown to 100% confluency. A wound was created in the cell monolayer, and an image was immediately taken (0 h). Migration was then observed every 12 h, and the closing wound was imaged. Image-Pro Plus (Media Cybernetics, Rockville, MD, USA) analysis software was used to facilitate the calculation of the relative migration rate as the relative distance recorded at 0 h minus that recorded at 12 h and 24 h divided by the relative distance recorded at 0 h × 100%.

Transwell assay. Cell invasion was performed using transwell inserts (Corning Costar, Cambridge, MA, USA) with polycarbonate membranes (8 μm pore size) in 24-well plates. For invasion assays, the filter was coated with 20 μg/ml Matrigel (Becton-Dickinson, San Jose, CA, USA) overnight at 4˚C. A total of 100 μl cells cultured in serum-free medium (1.0×10^5/ml) were seeded in the upper chamber. DMEM with 20% FBS was added to the lower chamber. After 24 h incubation at 37˚C in 5% CO_2, the chamber was washed three times with 1 × PBS. The membrane in the lower chamber was fixed in 4% paraformaldehyde and stained with crystal violet. The cells on the upper membrane that did not migrate were wiped with a cotton swab. The number of migrated cells was counted under a microscope in at least five fields.

Gelatin zymography assay. Activity of MMP-2 was assessed using a gelatin zymography assay. Cells were cultured with serum free medium for 24 h after seeding. Following incubation, 50 μl of culture supernatant was mixed with sample buffer and resolved on a 12% SDS-PAGE under non-reducing conditions. The gel was co-polymerized containing 0.5 mg/ml of gelatin (Sigma). The gel was washed twice for 30 min with renaturation buffer (2.5% Triton X-100) at room temperature before incubation in the incubation buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$] at 37˚C for 16 h. Thereafter, the gel was stained for 2 h in 0.25% Coomassie brilliant blue R-250 and then de-stained. White bands were observed against a blue background after de-staining, indicating gelatinolytic activity of MMP-2.

Statistical analysis. All experiments were repeated at least three times. SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the results. Independent sample *t*-tests were used and differences with *p*-values of less than 0.05 were considered statistically significant.

Results

Generation of cell pool stably overexpressing or not expressing EBP50. To study the effect of EBP50 expression on adhesion and migration of breast cancer and cervical cancer cells, we combined *EBP50* gain-of-function and loss-
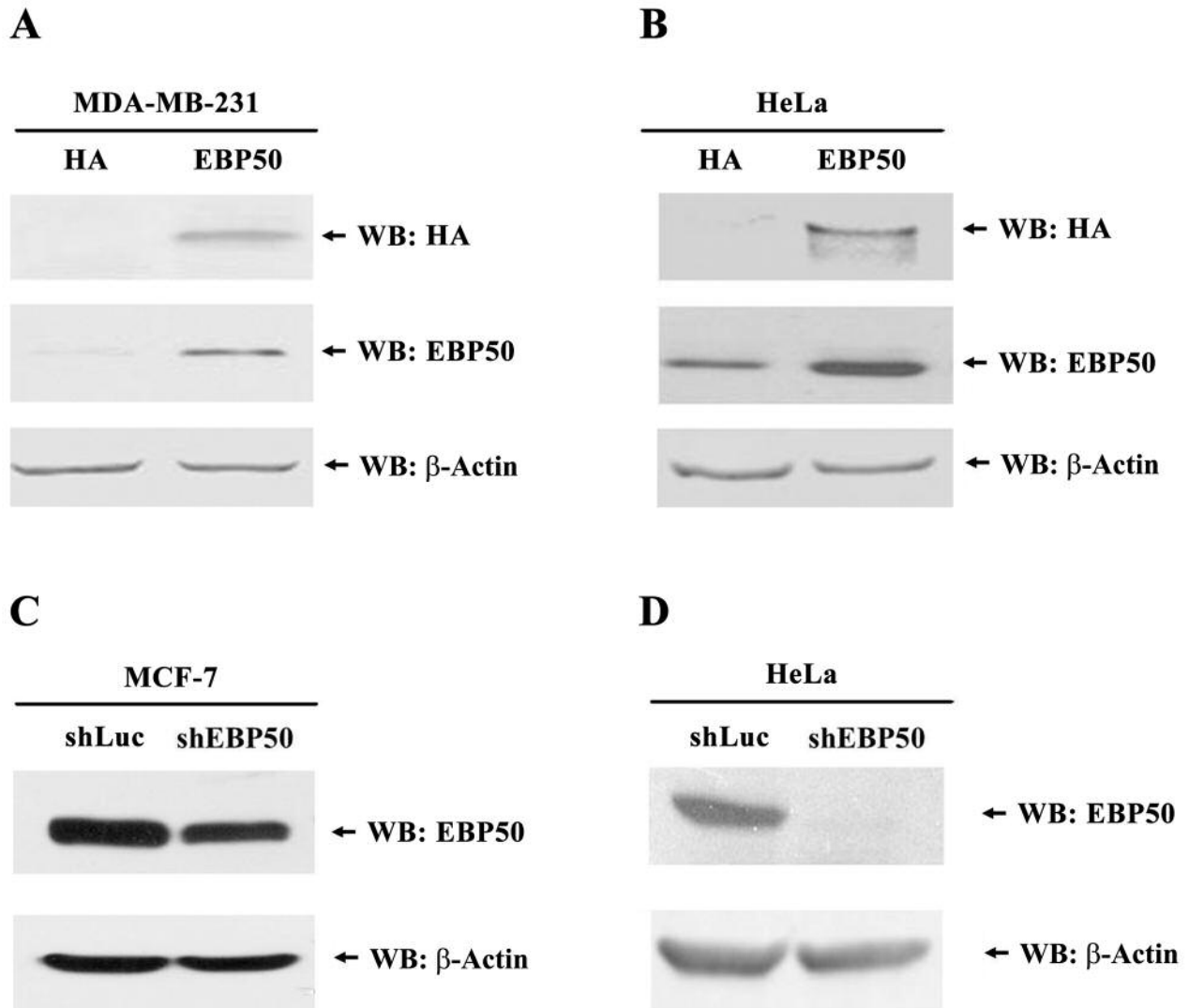
Figure 1. *Expression of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) in stably transfected breast cancer and cervical cancer cells. A: MDA-MB-231 cells. B: HeLa cells. Anti-HA and anti-EBP50 western blotting was used to detect EBP50 overexpression. In empty vector-transfected control cells (HA) cells, no HA signal and low EBP50 signal were detected. In cells stably expressing EBP50 cells, strong HA and EBP50 signal were detected. C: MCF-7 cells. D: HeLa cells. Anti-EBP50 western blotting was used to detect EBP50 knockdown. EBP50 levels in shEBP50 cells were 50% or 10% of those in shLuc control cells. β-Actin was used as a loading control.*

of-function studies. Thereby MDA-MB-231 breast cancer cells, which express a low level of endogenous EBP50, were transfected with an *EBP50* expression plasmid to overexpress EBP50, and MCF-7 breast cancer cells, which express a high level of endogenous EBP50, were transfected with an *EBP50* shRNA plasmid to knockdown its expression. For cervical cancer cells, we chose HeLa cell to perform both *EBP50* gain-of-function and loss-of-function studies. These were carried out for the purpose of observing the effect of *EBP50* expression on breast cancer and cervical cancer cells.

The cell pools stably overexpressing *EBP50*, namely MDA-MB-231-HA-EBP50 (EBP-231) and its control MDA-MB-231-HA (HA-231), HeLa-HA-EBP50 (EBP-HeLa) and its control HeLa-HA (HA-HeLa), were generated by transfection with the neo-pBK-CMV-HA-EBP50 or neo-pBK-CMV-HA vector, respectively. Protein expression in these stable cells was verified by western blot analysis as shown in Figure 1A and B. HA-tagged EBP50 protein expression was not detected in control cells. In EBP-231 and EBP-HeLa cells, exogenous HA-tagged EBP50 was robustly overexpressed.
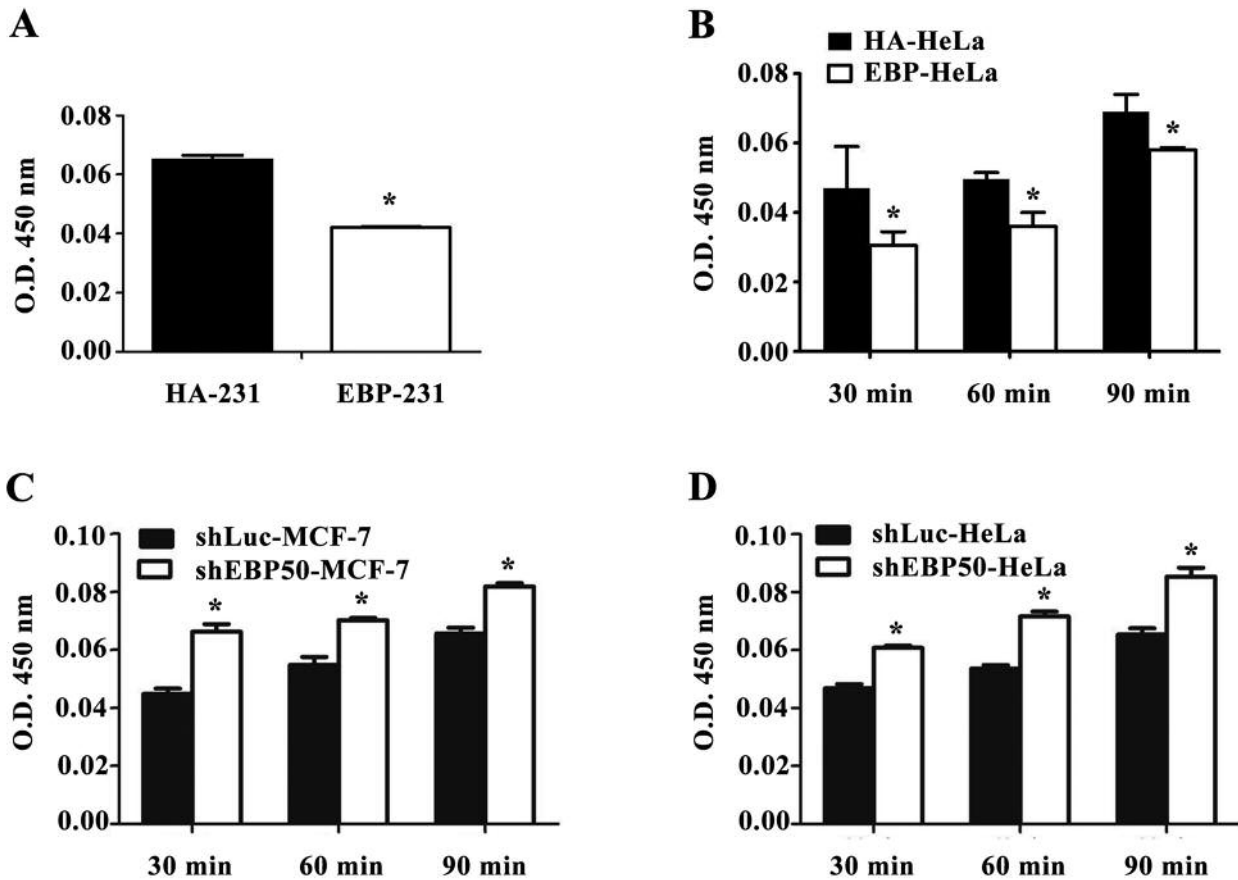
Figure 2. *The anti-cell adhesion effects of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) in breast cancer and cervical cancer cells. Adhesion assay was performed to analyze the ability of adherence to fibronectin at different time points. A: MDA-MB-231 cells. B: HeLa cells. Cell adhesion to fibronectin were inhibited in cells stably overexpressing EBP50 compared with empty vector-transfected control (HA) cells. C: MCF-7 cells. D: HeLa cells. EBP50 knockdown (shEBP50) in cells significantly promoted cell adhesion to fibronectin compared to shLuc control cells. *Significantly different from the control at p<0.05.*

The cell pools with stable knockdown of *EBP50* (shEBP50-MCF-7, shEBP50-HeLa) and their control cell lines (shLuc-MCF-7, shLuc-HeLa) were generated by transfection with the pSuper.puro EBP50 shRNA plasmid or the control pSuper.puro luciferase shRNA plasmid, respectively. Verification of protein knockdown was determined by western blot analysis as shown in Figure 1C and D. In shEBP50 transfected MCF-7 and HeLa cells, *EBP50* expression was stably knocked-down by 50% and 90% compared to control shLuc-transfected cells.

EBP50 inhibits the adhesion of breast cancer and HeLa cells. Cell matrix interaction and cell motility are important for cancer cell metastasis. In order to examine the potential anti-metastatic effects of EBP50, cell matrix interaction assays were performed on MDA-MB-231, MCF-7 and HeLa cells. The results indicated that cell matrix interaction was inhibited by *EBP50* at multiple time points. At 60 min, the number of MDA-MB-231 cells adhering to fibronectin was reduced by 35.4% following overexpression of *EBP50* ($p<0.05$, Figure 2A). At 30, 60 and 90 min, the number of HeLa cells adhering to fibronectin was reduced by 35.1%, 27.3% and 16%, respectively following overexpression of *EBP50* ($p<0.05$, Figure 2B). Conversely, *EBP50* knockdown in MCF-7 and HeLa cells significantly promoted cell adhesion to fibronectin at all study time points (Figure 2C and D). These results suggest that expression of *EBP50* significantly inhibited the adhesive abilities of MDA-MB-231, MCF-7 and HeLa cells compared with those of control cells ($p<0.05$).

EBP50 inhibits migration and invasion of breast cancer and HeLa cells. Adhesion assays indicated that *EBP50* had the ability to inhibit tumor cell adherence to the matrix. The formation of focal adhesions is important in cell signaling to
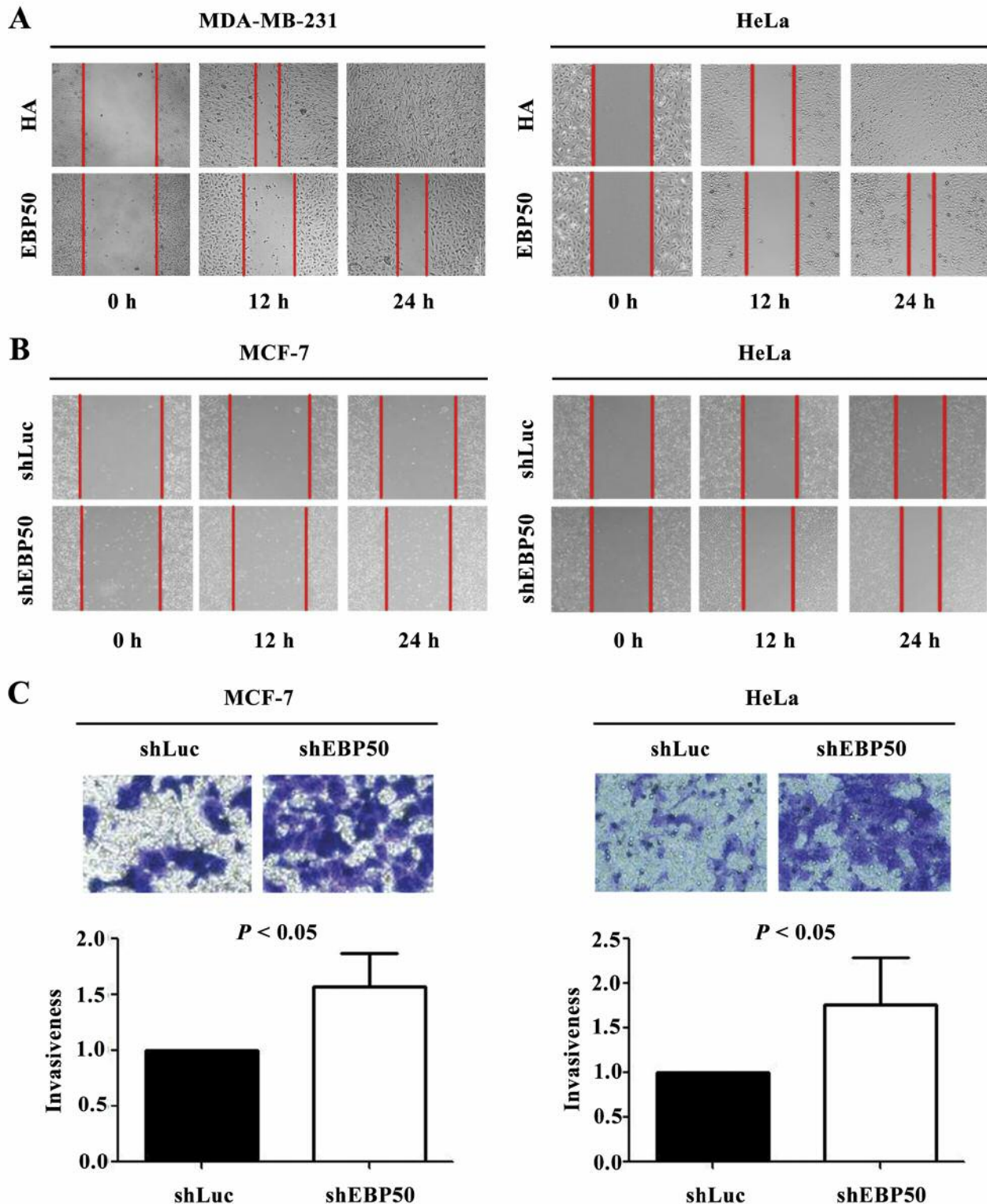
Figure 3. *The anti-migratory (A, B) and anti-invasive (C) effects of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) on MDA-MB-231 and MCF-7 breast cancer and HeLa cervical cancer cells. Monolayer scratch assays demonstrated that EBP50 inhibited the migratory potential of MDA-MB-231, MCF-7 and HeLa cells. Representative images of wound-healing assays performed on control cells (HA) and cells transfected with EBP50 (A) and control (shLuc) cells and EBP50 knockdown (shEBP50) cells. C: Knockdown of EBP50 increased invasion by MCF-7 and HeLa cells. *Significantly different at p<0.05 compared to the control.*

Figure 4. *Inhibitory effects of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) on matrix metalloproteinase-2 (MMP-2) activity in breast cancer and cervical cancer cells. The cells were treated with serum-free media for 24 h, and conditioned media were collected for gelatin zymography. A: MMP-2 activity was inhibited by EBP50 overexpression in MDA-MB-231 and HeLa cells. HA: Control cells, EBP50: EBP50-transfected cells. B: MMP-2 activity was promoted in EBP50 knockdown (shEBP50) in MCF-7 and HeLa cells compared with control (shLuc) cells. Data are representative of three independent experiments.*

direct cell migration (12). We further investigated whether *EBP50* affects cell migratory ability. At 0 h, the same wounds were produced in two groups of cells. After 12 h and 24 h, the wound width was significantly reduced in the control group, while width reduction was significantly inhibited in the *EBP50* overexpressing groups (Figure 3A, $p<0.05$). On the contrary, *EBP50* knockdown in MCF-7 and HeLa cells significantly promoted cell migration and wound closure after 12 h and 24 h (Figure 3B, $p<0.05$). In agreement with results from the wound-healing assay, the number of invading cells was also increased following *EBP50* knockdown in an *in vitro* Matrigel invasion assay (Figure 3C, $p<0.05$). These results suggest that EBP50 is able to inhibit the migration and invasion of breast cancer and cervical cancer cells.

EBP50 reduces MMP-2 activity of breast cancer and HeLa cells. Tumor cell metastasis requires degradation of the matrix. MMP-2 is one of the main proteolytic enzymes and degrades the environmental extracellular matrix and the basement membrane. In order to clarify whether the activity of MMP-2 is involved in the inhibition of migration and invasion by

EBP50 in MDA-MB-231, MCF-7 and HeLa cells, gelatin zymographic assays were performed. MDA-MB-231, MCF-7 and HeLa cells were incubated in serum-free media for 24 h, and the conditioned media were collected to analyze activity of MMP-2. The results show that *EBP50* overexpression reduced the activity of MMP-2 and *EBP50* knockdown enhanced the activity of MMP-2 ($p<0.05$; Figure 4).

Discussion

In this study, our data demonstrated that *EBP50* overexpression inhibited the adhesion and migration of MDA-MB-231 and HeLa cells, and suppressed MMP-2 activity in these cells, while *EBP50* knockdown in MCF-7 and HeLa cells promoted adhesion and migration, and enhanced the activity of MMP-2. Therefore, the results of both gain-of-function and loss-of-function assays revealed that EBP50 may represent a metastasis-suppressing protein in breast cancer and cervical carcinoma. Notably, to our knowledge, the finding that *EBP50* can inhibit the metastasis-related phenotype of cervical cancer cells is novel.

The expression level of EBP50 in breast cancer tissues varies depending on the pathological grade and cancer stage (7). In MDA-MB-231 breast cancer cells, EBP50 exhibits a relatively low endogenous expression, and we increased EBP50 expression through stable transfection with a pBK-CMV-HA-EBP50 plasmid. MCF-7 breast cancer cells express relatively a high level of EBP50 and an *EBP50* shRNA plasmid was used to reduce EBP50 expression. Our analysis indicated that *EBP50* overexpression inhibited the adhesion and migration of MDA-MB-231 and HeLa cells, while *EBP50* knockdown promoted adhesion and migration of MCF-7 and HeLa cells. Cell adhesion and migration are associated with tumor development and are the main causes of death in patients with tumors (13-15). Our results have important significance in breast and cervical cancer development and therapeutics.

Metastasis is responsible for more than 90% of cancer-related mortality (16). A critical step in tumor metastasis is the degradation of the basement membrane, which is catalyzed by proteolytic enzymes, such as MMPs (17). Significant down-regulation of MMP-2 activity was one of the mechanisms for the EBP50-induced inhibition of migration of MDA-MB-231 and HeLa cells. Results of *EBP50* knockdown further revealed MMP-2 activity played an important role in migration and invasion of MCF-7 and HeLa cells. It was also a novel observation that *EBP50* regulates MMP-2 activity to modulate breast cancer and HeLa cell migration and invasion.

EBP50 was found to directly or indirectly affect cancer behavior through interaction with partners and the regulation of their signaling pathways (11, 18). For example, EBP50 was reported to form a protein complex with EGFR and neurofibromin 2 (NF2) at intercellular adherens junctions (19), thereby mediating internalization and signal transduction of EGFR to regulate oncogenic processes. EBP50 was also found to interact with PDGFR to form a transduction complex with PTEN and NF2 (3, 8, 18). This complex functioned to block the signaling associated with PDGFR, thereby affecting apoptosis during epithelial-to-mesenchymal transition and suppressing cancer development and metastasis (20). The results of this study have identified a new mechanism by which *EBP50* affects cancer cell behavior.

In addition, EBP50 was reported to be an actin cytoskeleton- and cell junction-related protein (21, 22). Abnormal expression and organization of actin cytoskeleton and cell junctions are closely related to tumor cell metastasis. The metastasis-suppressing ability of EBP50 possibly results from its regulation of actin cytoskeleton and cell junction proteins. EBP50 expression might also change its interaction with partners which have important roles in adhesion and migration to affect breast and cervical cancer cell metastasis. These need to be further investigated.

In summary, our results demonstrate that EBP50 regulates metastasis-related behavior (adhesion and migration abilities) of MDA-MB-231, MCF-7 and HeLa cells and that EBP50 acted as a tumor metastasis-suppressing protein in breast cancer and cervical carcinoma cells. EBP50 may be a potential therapeutic target in the treatment of both cancer types.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Acknowledgements

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