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ORIGINAL ARTICLE

IBP regulates epithelial-to-mesenchymal transition and the motility of breast cancer cells via Rac1, RhoA and Cdc42 signaling pathways

Z Zhang[1], M Yang[1], R Chen[2], W Su[3], P Li[1], S Chen[1], Z Chen[1], A Chen[1], S Li[1] and C Hu[1]

Epithelial-to-mesenchymal transition (EMT) is a crucial process for the invasion and metastasis of epithelial tumors. However, the molecular mechanisms underlying this transition are poorly understood. In this study, we demonstrate that interferon regulatory factor 4 binding protein (IBP) regulates EMT and the motility of breast cancer cells through Rac1, RhoA and Cdc42 signaling pathways. We found that increased expression of IBP was associated with the progression of breast cancer and that IBP protein levels were significantly elevated in matched distant metastases. High IBP levels also predict shorter overall survival of breast cancer patients. Furthermore, the forced expression of IBP decreased the expression of the epithelial marker E-cadherin but increased the mesenchymal markers in breast cancer cells. In contrast, silencing IBP in metastatic breast tumor cells promoted a shift toward an epithelial morphology concomitant with increased expression of E-cadherin and decreased expression of mesenchymal markers. IBP silencing also reduced the expression of EMT-inducing transcription factors (Snail, Slug, ZEB1 and ZEB2). Moreover, we identified a role for IBP in endogenous EMT induced by epidermal growth factor (EGF) and deletion of IBP attenuated EGF receptor (EGFR) signaling in breast cancer cells. Furthermore, IBP regulates the migration, invasion and matrix metalloprotease production in breast cancer cells as well as actin cytoskeleton rearrangement and the activation of GTP-Rac1, GTP-RhoA and GTP-Cdc42. Taken together, our findings demonstrate an oncogenic property for IBP in promoting the metastatic potential of breast cancer cells.

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Keywords: IBP; EMT; breast cancer; migration; invasion

INTRODUCTION

The spread of tumor cells from a primary tumor to a secondary site remains one of the most life-threatening pathological events. However, the mechanisms by which tumor cells become metastatic remain poorly understood. Epithelial-to-mesenchymal transition (EMT) is a cellular process during which epithelial tumor cells lose their polarized organization and cell–cell junctions. The cells thus undergo changes in shape and cytoskeletal organization and acquire mesenchymal characteristics important for metastasis.[1,2] Dissecting the molecular mechanisms that regulate EMT are pivotal for controlling tumor invasiveness and metastasis.

Interferon regulatory factor 4 binding protein (IBP), also described as DEF6 (Hatfilder *et al.*[3]) or SLAT,[4] is a 631-amino-acid Rho-family guanine nucleotide exchange factor for Rho-family GTPases, including Rac1, RhoA and Cdc42.[5] It is broadly expressed in immune cells and is detected in both the T- and B-cell compartments of lymphoid organs.[6,7] IBP regulates many T-cell processes such as cluster of differentiation (CD)4+ activation and T-helper (Th)1/Th2/Th17 differentiation.[4,6] It also has been identified to have a role in coordinating actin cytoskeleton remodeling, and Ca^{2+} and nuclear factor of activated T cells signaling.[8,9] IBP-deficient mice display defect at the earliest stages of thymocyte development.[10] IBP has also been shown to regulate cell morphology in cooperation with activated Rac1 (Oka *et al.*[11]) and affect cell differentiation in cooperation with integrins.[12] Recent report indicates that IBP switch between a soluble and granule-forming conformation triggered through phosphorylation by Tec kinase ITK and similar post-translational modifications induced by conditions of cell stress.[13] Although previous studies have suggested a significant physiological role for IBP, most of these studies were limited to the development and function of immune system. Thus, the biological activity of IBP in other mammalian cells remains largely unexplored. We previously reported ectopic expression of IBP in a considerable proportion of human breast and colorectal cancers.[14,15] In addition, IBP expression is correlated with drug resistance[16] and the malignant behavior of human breast cancer cells.[15] However, whether IBP promotes breast cancer metastasis remains unknown.

In this study, we report that IBP overexpression induces EMT in breast cancer cells. We show that IBP decreases the expression of the epithelial markers E-cadherin and keratin 18 but increases the expression of mesenchymal markers fibronectin and N-cadherin. In addition, IBP enhances epidermal growth factor receptor (EGFR) activation and triggers the acquisition of EMT phenotype as well as increased motility of breast cancer cells. Furthermore, IBP mediates Rac1, RhoA and Cdc42 activation in breast cancer cells and regulates Rho GTPase-dependent processes, including actin cytoskeleton rearrangement and matrix metalloprotease (MMP) production. Our results suggest that activation of IBP/Rho GTPase signaling may represent a novel mechanism responsible for

[1]Department of Clinical Biochemistry, Faculty of Medical Laboratory Science, Southwest Hospital, Third Military Medical University, Chongqing, China; [2]Department of Pathology, Xinqiao Hospital, Third Military Medical University, Chongqing, China and [3]Department of Medical Laboratory, Beibei Maternal and Child Health Hospital, Chongqing, China. Correspondence: Professor C Hu, Department of Clinical Biochemistry, Faculty of Medical Laboratory Science, Southwest Hospital, Third Military Medical University, 30#, Gaotanyan Centre Street, Shapingba, Chongqing 400038, China.
E-mail: chuminhu@163.com
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accelerated breast cancer cell invasion and tumor metastasis promoted by IBP.

RESULTS

IBP expression is elevated in primary human breast cancer

We previously demonstrated that IBP is aberrantly expressed in human breast cancer in comparison with normal breast tissue. Interestingly, the levels of IBP were correlated with stages of the disease.[15] To further determine specific expression to breast cancer progression, we analyzed the relationship of IBP in tumors and lymph nodes tissues from 109 patients. Each sample was assigned an immunoreactivity score ranging from 0 to 6. Representative samples are shown in Figure 1a along with date analysis (Figure 1b). Primary tumors and corresponding lymph node metastases exhibited diffuse cytoplasmic staining for IBP. Paired comparisons of immunoreactivity scores between primary and metastatic tumors were significant ($P<0.001$). Increased IBP expression was also found to correlate with shorter overall survival ($P=0.041$) of patients (Figure 1c).

We also examined the expression of IBP in normal human mammary epithelial cells, seven non- or low metastatic breast cancer cell lines (MDA-MB-468, MDA-MB-436, MDA-MB-453, T47D, ZR-75-1, SK-BR-3 and MCF-7) and two highly metastatic cell lines (MDA-MB-231 and HS578T). Higher levels of IBP protein were observed in most breast cancer cell lines compared with human mammary epithelial cells. Metastatic cancer cell lines contained the highest levels of IBP (Figure 1d). These findings show that IBP is overexpressed in primary human breast cancer and metastatic breast cancer cells.

IBP regulates the phenotype of breast cancer cells through EMT

Cumulating evidence has highlighted EMT as the mechanism by which epithelial cancers progress toward more aggressive

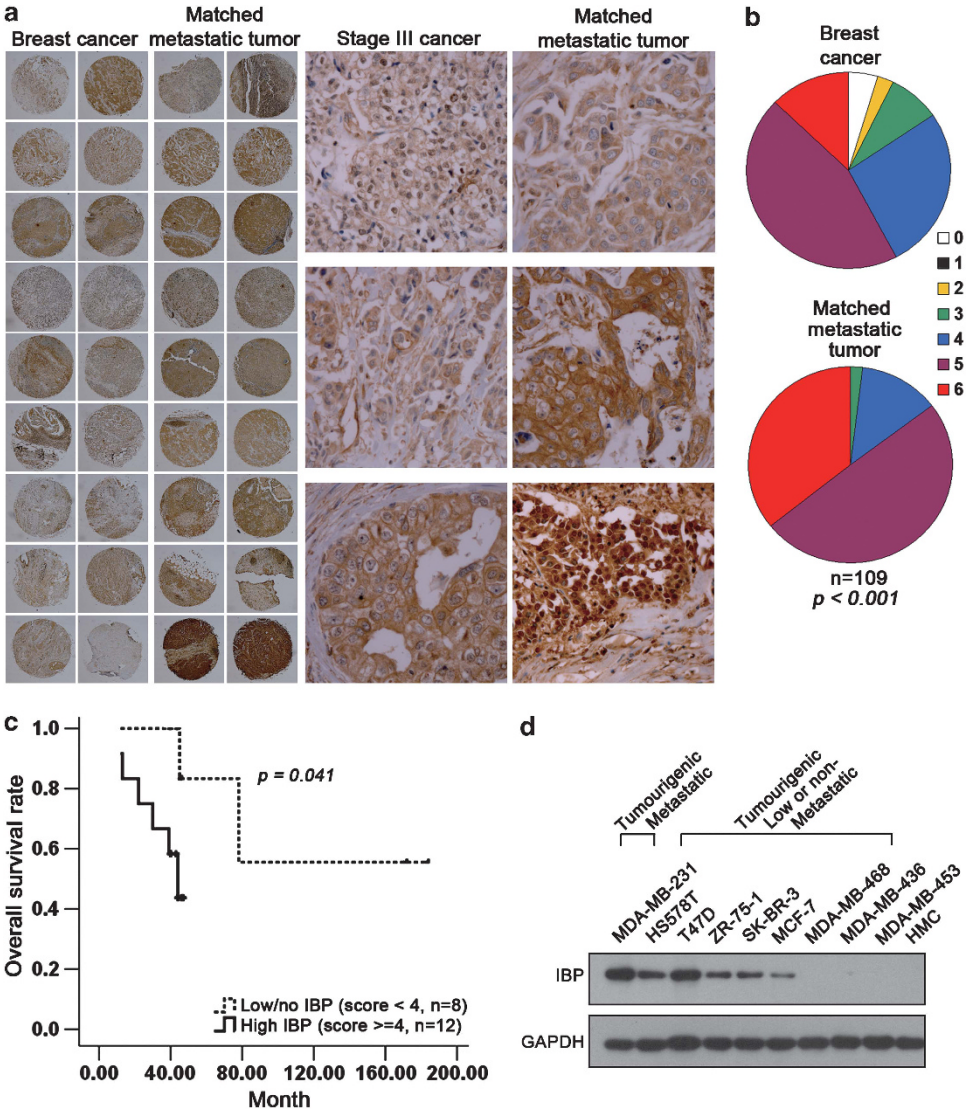

Figure 1. Expression of IBP in breast cancer patient specimens and cell lines. (**a**) Expression of IBP in primary breast cancer and matched lymph node tumors (100× or 400×). (**b**) Distribution of immunoreactivity scores in primary breast cancer and matched lymph node tumors ($n=109$). $P<0.05$ indicate significant differences between two groups. (**c**) Kaplan–Meier plots of IBP expression in 20 cases of breast cancer patients. Overall survival rate was performed by log-rank test. (immunoreactivity scores<4 was ascribed to be low IBP expression, immunoreactivity scores>or=4 was ascribed to be high IBP expression) $P<0.05$ indicate significant differences between two groups. (**d**) Expression of IBP in breast cancer cell lines. Equal amounts of proteins from seven non- or low metastatic breast cancer cell lines (MDA-MB-468, MDA-MB-436, MDA-MB-453, T47D, ZR-75-1, SK-BR-3 and MCF-7) and two highly metastatic breast cancer cell lines (MDA-MB-231 and HS578T) were evaluated by immunoblot analysis with antibodies against IBP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

phenotype with increased motile and invasive capabilities. We hypothesized that the upregulation of IBP may promote EMT in breast cancer cells. High metastatic breast cancer cell lines (MDA-MB-231 and HS578T) demonstrated elevated IBP expression, were spindle shaped and exhibited reduced cell contact. IBP knockdown resulted in a dramatic shift from spindle shaped to a cobblestone-like morphology (Figure 2a). Consistent with this, deletion of IBP in T47D cells also altered the cell morphology and increased cell–cell contact. To quantitatively measure the effect of IBP knockdown (siIBP) on cell contact, we performed a colony

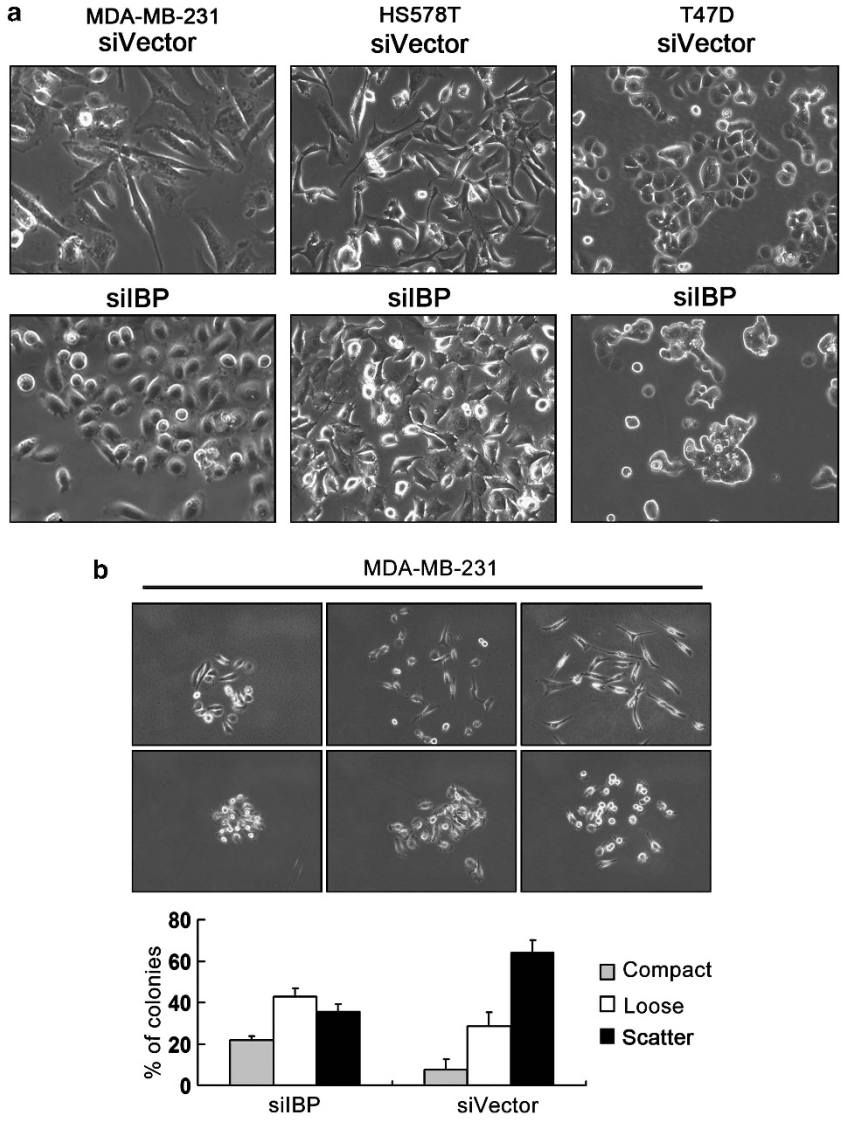

Figure 2. IBP alters the EMT phenotype of breast cancer cells. (**a**) IBP knockdown in MDA-MB-231, HS578T and T47D cell lines was established by transfection of IBP RNA interference (RNAi) vector (pcDNA 6.2-GW/EmGFPmiR-IBP) or the RNAi control vector (pcDNA 6.2-GW/EmGFPmiR-CONT). Stably infected colonies were selected by growth in blasticidin for 14 days, and the stably infected cells were collected. Phase contrast microscopic images show that IBP knockdown (siIBP) induced epithelial cell morphology in MDA-MB-231, HS578T and T47D cell lines. (**b**) IBP knockdown reduced cell scattering. Cells were plated in 10-cm plates and allowed to form small colonies for 6 days and were then categorized by observing whether colonies maintained compact, loose or scattered contact with neighboring cells. Representative light microscopic images show compact, loose and scattered colonies (upper panel). The number of each type of colony in the IBP knockdown (siIBP) and vector control (siVector) MDA-MB-231 cells was counted, and the data are plotted as percentage of compact, loose and scattered colonies in each culture (lower panel). Each experiment was repeated three times. (**c**) IBP overexpression in MDA-MB-468 and MDA-MB-436 cell lines was established by transfection of pEGFP-C1-IBP or pEGFP-C1 vectors, respectively, and stably transfected colonies were selected for 14 days in the presence of G418 and then collected. Enforced expression of IBP reduced epithelial marker (E-cadherin and cytokeratin 18) expression levels in MDA-MB-468 cells. Furthermore, the protein expression level of mesenchymal markers, including N-cadherin and Vimentin, was upregulated on IBP treatment in MDA-MB-436 cells. (**d**) Immunofluorescence microscopy revealed the expression and localization of epithelial and mesenchymal markers after IBP overexpression in MDA-MB-468 and MDA-MB-436 cells. Cells were immunostained with anti-E-cadherin (24E10, 1:200), keratin 18 (DC10, 1:100), vimentin (D21H3, 1:100) and anti-N-cadherin (1:200) antibodies. The secondary antibodies used were Cy3 anti-rabbit or Cy3 anti-mouse (Beyotime, 1:500, room temperature 1 h). Nuclear was stained with 4,6-diamidino-2-phenylindole. (**e**) IBP knockdown upregulated E-cadherin and cytokeratin 18 expression and downregulated N-cadherin, vimentin and fibronectin expression in MDA-MB-231, HS578T and T47D cells. (**f**) Immunofluorescence staining indicated the effect of IBP on the expression and localization of EMT markers. Marked reductions in fluorescent signals for N-cadherin and vimentin were observed following IBP suppression. The reinstitution of an epithelial phenotype was indicated by the re-expression of E-cadherin and cytokeratin 18.

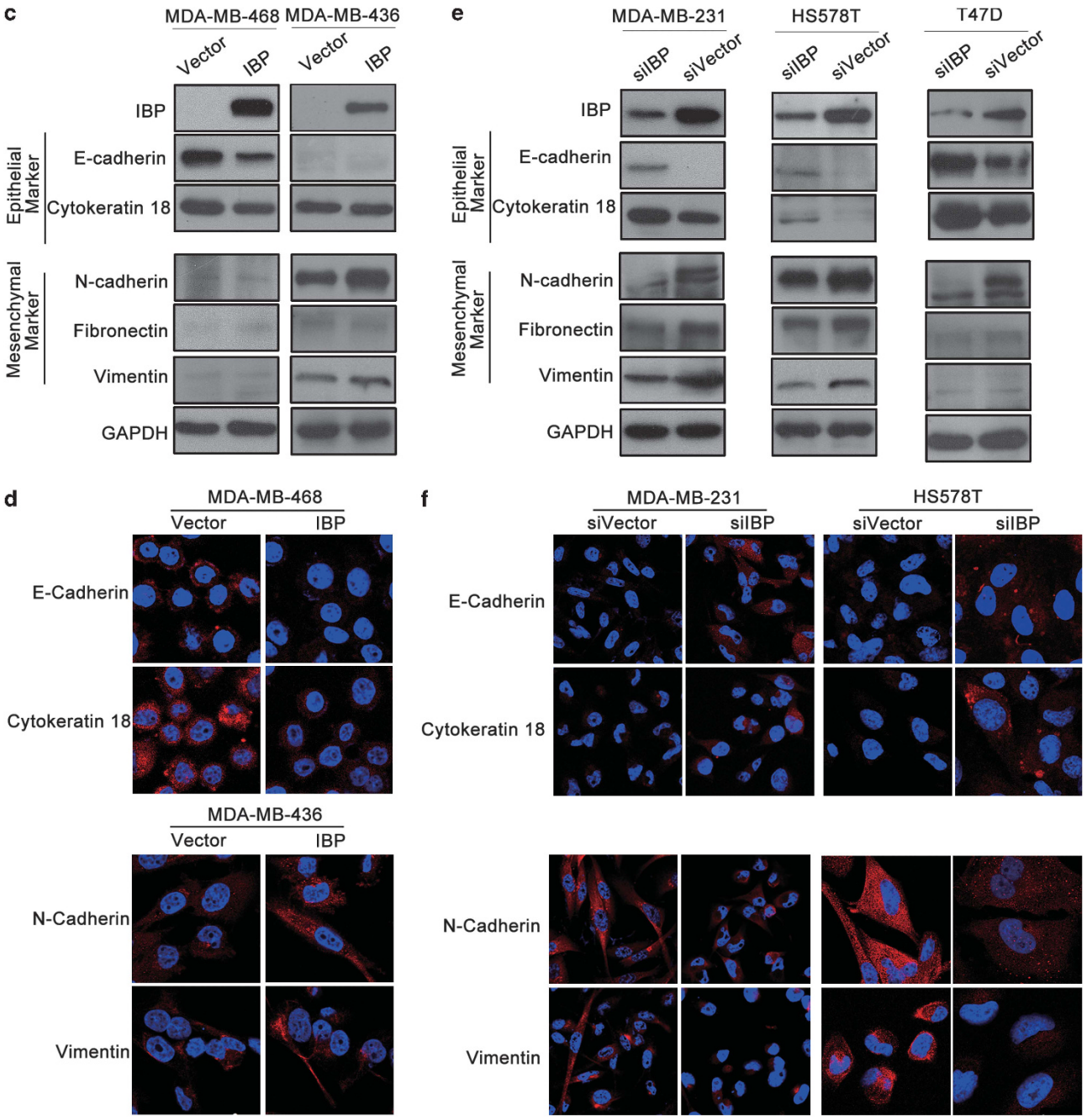

Figure 2. Continued

scattering assay to determine the ability of cells to detach from colonies in culture. Cells were plated at a very low density, and the morphology of the colonies was analyzed 6 days later. Colonies were divided into three categories: compact, loose and scattered. IBP RNA interference cells formed three times more compact colonies and two times fewer scattered colonies than control cells (siVector; Figure 2b).

As cadherin switch is a well-established hallmark of EMT, we determined cadherin expression in breast cancer cells. By western blot and immunofluorescence, we found that forced IBP expression caused a decrease in epithelial markers (E-cadherin and cytokeratin 18) in MDA-MB-468 cells, but an increase in mesenchymal markers (N-cadherin and vimentin) in MDA-MB-436 cells (Figures 2c and d). Similar reciprocal relationship was observed with IBP silencing in breast cancer cells, shown by increased E-cadherin expression. IBP knockdown in MDA-MB-231, HS578T and T47D cell lines led to a substantial decrease in mesenchymal marker expression (N-cadherin, vimentin and fibronectin), along with an increase in epithelial marker expression (E-cadherin and cytokeratin 18; Figures 2e and f). These results indicate that IBP has an important role in EMT regulation in breast cancer cells.

IBP is upregulated in EGF-induced EMT

We next determined the involvement of IBP in the endogenous EMT process. Previous studies have shown that a variety of signal transduction pathways induce EMT, including EGF, fibroblast growth factor, insulin-like growth factor and transforming growth factor-β. We therefore treated MDA-MB-231, HS578T and

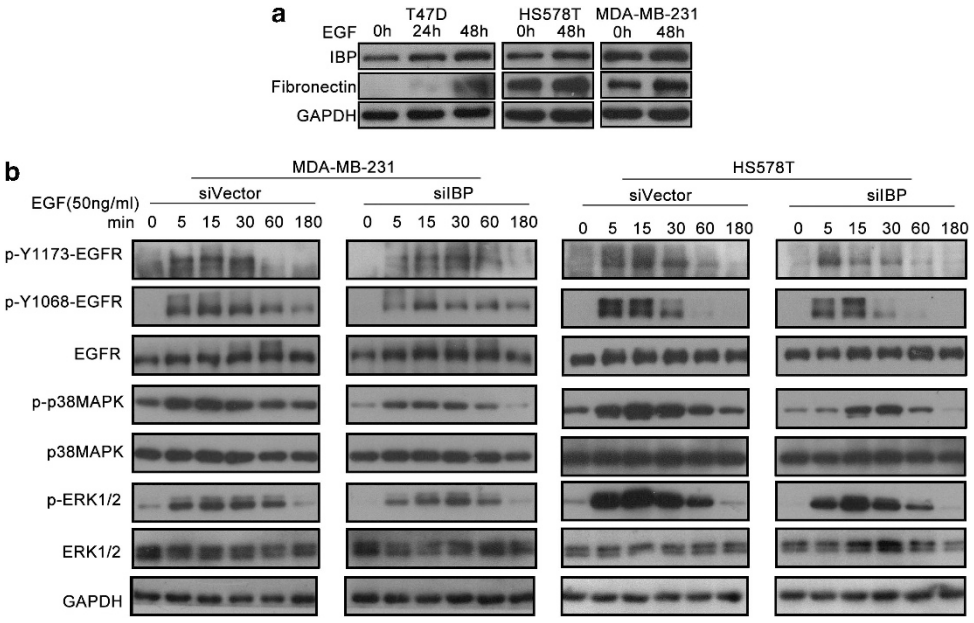

Figure 3. Loss of IBP leads to an inactivation of EGFR in breast carcinoma cells. (**a**) IBP was upregulated in endogenous EMT induced by EGF. The T47D, HS578T and MDA-MB-231 cell lines were cultured in 1% FBS medium for 24 h, and then the cells were treated without (control) or with EGF (50 ng/ml) for 24 and/or 48 h. Western blots showed that IBP was upregulated in EGF-induced EMT, along with the upregulation of the mesenchymal marker fibronectin. (**b**) Cells were serum starved for 24 h and then treated with EGF (50 ng/ml) for various times (0–180 min). Activation of EGFR, p38 mitogen-activated protein kinase (p38MAPK) and extracellular signal-regulated kinases 1/2 (ERK1/2) was analyzed by immunoblotting using total and phospho-specific antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

T47D cells with EGF to induce EMT. EGF induced marked EMT morphology in MDA-MB-231, HS578T and T47D cells and upregulated the expression of the mesenchymal marker fibronectin. More importantly, IBP was significantly upregulated by EGF (Figure 3a), suggesting a role for IBP in endogenous EMT induced by EGF.

IBP silencing inactivates EGFR signaling in breast carcinoma cell lines

Activation of EGFR was examined in IBP-silenced and control cells by immunoblotting. In control cells, EGF caused a transient activation of EGFR (phosphorylation of tyrosines 1068 and 1173), extracellular signal-regulated kinases 1/2 and p38 mitogen-activated protein kinase, which culminated at 5 min (T47D, Supplementary Figure S1) or 15 min (MDA-MB-231 and HS578T, Figure 3b) and progressively returned to the basal level by 180 min. In IBP-silenced cells, the basal activation of EGFR, extracellular signal-regulated kinases 1/2 and p38 mitogen-activated protein kinase were lower than in control cells (time 0). In addition, the phosphorylation levels of EGFR, extracellular signal-regulated kinases 1/2 and p38 mitogen-activated protein kinase following stimulation with EGF decreased and returned to basal levels more rapidly as compared with control cells.

IBP silencing reduces the expression of EMT-induced transcription factors

Aberrant expression of EMT transcription factors contributes to the appearance of an invasive phenotype by suppressing E-cadherin and inducing EMT in a wide variety of human cancers.[17] We determine whether IBP-mediated repression of E-cadherin in EMT occurs through the regulation of EMT transcription factors and found that IBP silencing reduced the expression of Snail, Slug, ZEB1 and ZEB2 in MDA-MB-231(Figure 4a) and HS578T cells (Figure 4b).

IBP regulates breast cancer cell migration, invasion and MMP production

Cell migration and invasion are critical steps in the progression and metastasis. MDA-MB-436, MDA-MB-231 and HS578T cells were used to determine the effect of IBP on cell migration and invasion. Migration was assessed using a short-term transwell assay. IBP knockdown reduced cell migration and invasion of MDA-MB-231 and HS578T cells, whereas IBP overexpression promoted cell migration and invasion in MDA-MB-436 cells (Figures 5a, b and c). EMT has also been associated with increased production of MMPs, which serve to degrade extracellular matrix proteins and facilitate cell invasion. We therefore determined the capacity of IBP regulate MMP production. As shown in Figure 5d, knockdown of IBP significantly decreased the levels of MMP-2 and MMP-9 secreted by MDA-MB-231 and HS578T cells, whereas IBP overexpression increased MMP-2 secreted by MDA-MB-436 cells.

IBP regulates actin cytoskeleton rearrangements via Rac1, RhoA and Cdc42

Polymerization and depolymerization of filamentous actin (F-actin) control cytoskeletal reorganization, leading to morphologic changes associated with cell motility. We investigated the capacity of IBP to regulate actin cytoskeleton in breast cancer cells motility by staining F-actin. In MDA-MB-468 and MDA-MB-436 cells, IBP overexpression induced an increase in filipodium, lamellipodia and stress fibers (Figure 6a). As shown in Figure 6b, the stress fibers in MDA-MB-231 siVector and HS578T siVector cells appeared as extensive parallel bundles, which were densely stained in a well-organized manner. In contrast, these parallel bundles were disrupted in siIBP cells, which also demonstrated loosely organized and randomly dispersed F-actin.

IBP is a Rho-guanine nucleotide exchange factor that activates Rho-GTPase.[5] To determine whether IBP may affect the GTP/GDP-binding status of Rho GTPases in breast cancer cells, we investigated GTP-bound Rac1, RhoA and Cdc42. GTP-Cdc42 activation

was significantly increased in IBP-overexpressing MDA-MB-468 cells, and the level of GTP-RhoA was significantly increased in IBP-overexpressing MDA-MB-436 cells (Figure 6c). In contrast, in MDA-MB-231 and HS578T cells, basally RhoA activity was marked reduced by siIBP, whereas Rac1 and Cdc42 activity was not affected. In addition, in MDA-MB-231 cells, EGF stimulation increased both the activation status of Rac1 and RhoA; fetal bovine serum (FBS) stimulation increased the activation of RhoA, whereas deletion of IBP attenuated this activation. In HS578T cells, EGF stimulation increased the activation of Rac1 and FBS stimulation increased the activation of RhoA, whereas deletion of IBP attenuated this activation as well (Figure 6d). Finally, to implicate these downstream pathways in regulating migration of breast cancer cells, we treated MDA-MB-231 and HS578T cells with small molecule inhibitors of Rho-associated kinase (Y27632) and Rac1 (NSC23766) and assessed their effect on migration by using Transwell migration assay. We found that both compounds reduced migration of MDA-MB-231 and HS578T cells (Figure 6e). Taken together, these findings suggest that IBP are required for activation of Rac1, RhoA and Cdc42 (Figure 6f).

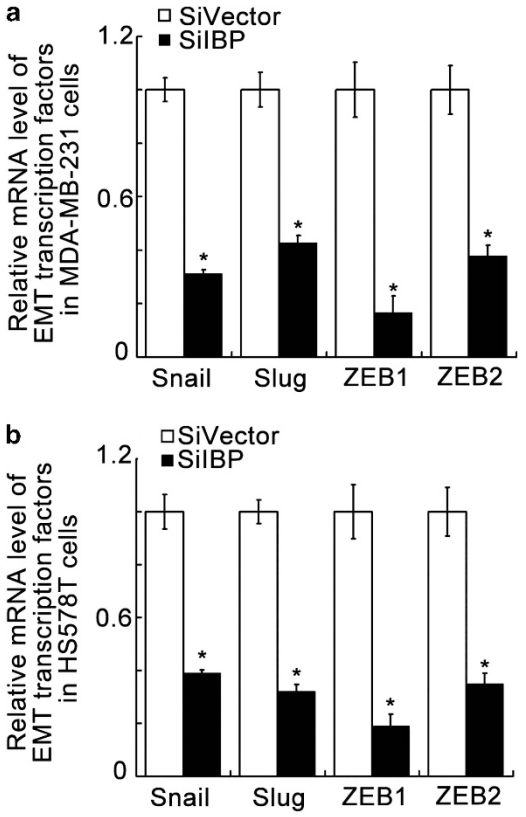

Figure 4. IBP silencing reduces EMT-induced transcription factor expression. The mRNA levels of EMT-related transcription factors in MDA-MB-231 (**a**) and HS578T (**b**) were measured by quantitative reverse transcriptase–PCR. The mRNA levels of Snail, Slug, ZEB1 and ZEB2 were expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels. Each experiment was performed in triplicate. Asterisks indicate significant differences between two groups ($P<0.05$).

DISCUSSION

We defined IBP as an oncogene frequently overexpressed in breast cancer cells that promotes EMT and cellular motility. First, we confirmed the increased expression of IBP in breast cancer, in particular in advanced cancer. Second, we showed that IBP is a positive regulator of EMT in breast cancer cells and has a role in EGF-induced EMT. Third, we show depletion of IBP attenuated the migration and invasion of breast cancer cells concomitant with altered cytoskeletal rearrangement and decreased activation of Rho GTPase. Our results suggest that IBP may serve as a predictive marker for breast cancer with high metastatic potential.

Aggressive breast cancers are generally resistant to anticancer therapies, prone to early recurrence and metastasize to distant sites. These breast cancers likely evolve from less aggressive epithelial-like breast tumors through the reactivation of embryonic signaling programs, such as EMT.[18] Owing to the clinical importance of this process, inhibition of EMT is an attractive therapeutic approach. However, it remains unknown which pathways should be inhibited to reverse EMT. In our study, we show for the first time that IBP is a positive regulator of EMT. This conclusion was based on the observation that silencing IBP induced a repertoire of biochemical (increased E-cadherin and decreased N-cadherin, vimentin, and fibronectin), morphologic (growth pattern, decreased formation of lamellipodia) and functional (decreased MMP production) changes that reverse EMT. Given that EMT represents a critical event in the transition from early to invasive carcinomas and that E-cadherin downregulation is associated with poor prognosis and lower

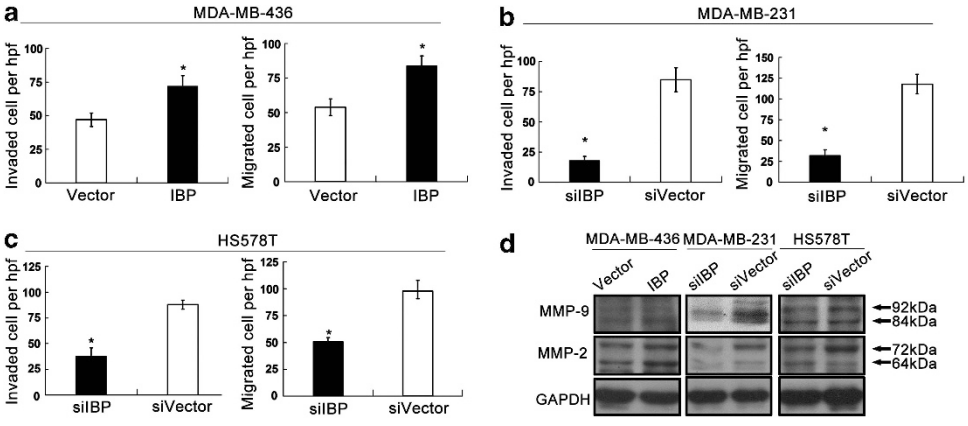

Figure 5. IBP regulates breast cancer cell motility and MMP production. Migration and invasion assays in (**a**) IBP overexpressed (IBP) and control (Vector) MDA-MB-436 cells. Migration and invasion assays in (**b**) MDA-MB-231 cells and (**c**) HS578T cells transfected with IBP small interfering RNA (siIBP) and control siRNA (siVector). Asterisks indicate significant differences between two groups ($P<0.05$). (**d**) Equal amounts of cell lysates (45 μg) were loaded and probed with the MMP-2 and MMP-9 antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. hpf, high-power field.

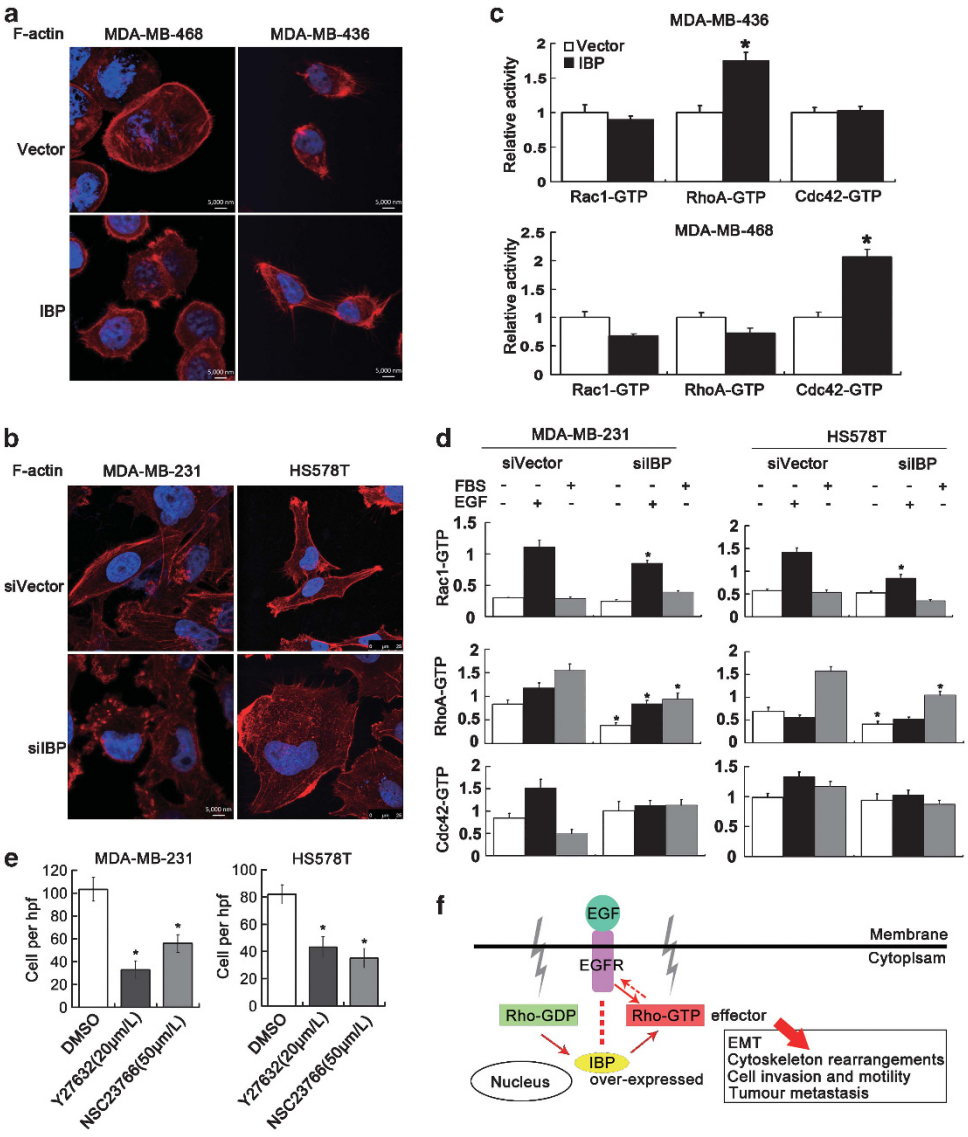

Figure 6. IBP regulates actin cytoskeleton rearrangements in breast cancer cells via Rac1, RhoA and Cdc42 pathways. (**a**) Vector control or IBP-overexpressing MDA-MB-468 and MDA-MB-436 cells were immunostained with Alexa Fluor 647 Phalloidin to examine F-actin stress fibers. (**b**) MDA-MB-231 and HS578T vector-treated or IBP RNA interference (RNAi) cells were immunostained with Alexa Fluor 647 Phalloidin to observe F-actin stress fibers. (**c**) The effect of IBP overexpression on the Rho GTPase guanine nucleotide binding status of Rac1, RhoA and Cdc42 was examined. A marked increase in GTP-RhoA (active form) binding was detected in MDA-MB-436 cells, whereas no apparent change in GTP-Rac1 and GTP-Cdc42 binding was detected. In addition, a marked increase in GTP-Cdc42 (active form) binding was detected in MDA-MB-468, whereas no apparent change in GTP-RhoA or GTP-Rac1 binding was detected. Asterisks indicate significant differences between two groups ($p<0.05$). (**d**) MDA-MB-231 and HS578T vector-treated or IBP RNAi cells were serum starved and stimulated with 10% FBS or EGF (50 ng/ml) and assessed for GTP-Rac1, GTP-RhoA and GTP-Cdc42 binding. IBP depletion mainly affects Rac1 and RhoA pathway activation in MDA-MB-231 and HS578T cells. Asterisks indicate significant differences between two groups ($p<0.05$). (**e**) Transwell migration assay was carried out with MDA-MB-231 and HS578T cells treated with either NSC23766 (50 μm/l) or Y27632 (20 μm/l). Cells in control group were treated with DMSO; *$P<0.05$ versus control. (**f**) Schematic representation of the role of IBP/Rho GTPase signaling in actin cytoskeletal regulation and cancer metastasis. hpf, high-power field.

survival, our finding with IBP in cancer progression and metastasis is clinically relevant.

EMT in cancer cells is often triggered by autocrine and paracrine signals. Numerous growth factors (such as EGF) directly induce EMT.[19,20] In this study, we demonstrated that IBP was potently upregulated in endogenous EMT induced by EGF. Also, enhanced activity of EGFR was shown to promote the aggressiveness of cancer cells, as characterized by an increased rate of recurrence and metastatic spread.[18] Elevated aggressiveness associated with EGFR activity in cancer cells may be explained by the activation of EMT-associated events. EGFR has been shown to mediate motility and invasion[21] of human cancer cells, including human breast cancer cells.[22,23] We demonstrated that depletion of IBP in breast cancer cells inactivates EGFR and its downstream signaling pathways, thus reveal an important pathophysiological role for IBP in growth factor-induced EMT.

Members of the zinc-finger transcription factor family, including Snail, Slug, ZEB1 and ZEB2, are direct repressors of E-cadherin transcription and central mediators of EMT.[17,24] In our study, we confirmed that IBP regulates breast cancer cell EMT via the suppression of E-cadherin expression and the regulation of EMT transcription factors.

The earliest detectable morphologic changes during cell migration involve the rearrangement of actin cytoskeleton, leading to the formation of lamellipodia.[25] Our findings indicate that IBP regulates the formation of filipodium and lamellipodia. Consistent with this, a recent study showed that IBP regulates cell morphology.[11,12] The Rho family GTPases, including Rac1, RhoA and Cdc42, participate in regulating EMT, actin cytoskeleton reorganization and cell migration.[26,27] We showed that IBP promoted EMT in breast cancer cells. To further elucidate the biological activity of IBP, we found that IBP mediated Rac1, RhoA and Cdc42 activation in breast cancer cells, as the basely level of active RhoA-GTP was markedly reduced in IBP-depleted breast cancer cells. Our findings implicate signaling through RhoA pathways as a critical downstream mechanism by which IBP may regulate changes in the actin cytoskeleton and cell migration in breast cancer cells.

Studies reinforce the hypothesis that IBP contributes to breast cancer tumorigenesis and progression. High levels of IBP have also been detected in colon cancer[14] and oral carcinoma.[28] In addition, IBP expression was shown to be correlated with drug resistance in breast cancer.[16] Thus, IBP is a critical regulator of cancer progression and may be an important target for cancer treatment.

MATERIALS AND METHODS

Immunohistochemistry

Tissue microarrays containing cancer and matched metastatic tissues, BR955 (31 patients, 95 tumor cores), BR1005a (50 patients, 100 tumor cores) and BR10010a (50 patients, 100 tumor cores) were purchased from US BIOMAX Inc. (Rockville, MD, USA). Anti-IBP antibodies and immunohistochemistry was performed as previously described.[14,15] Briefly, sections were deparaffinized in xylene, rehydrated in alcohol and water, antigen repaired, and blocked. Anti-IBP antibodies (1:200) were incubated overnight, followed by incubation with horseradish peroxidase-labeled polymer for 20 min. Sections were then stained with DAB (DAKO, Cambridge shire, UK) for 5 min. All sections were counterstained with hematoxylin, dehydrated and mounted. Scoring was performed blindly by a pathologist according to the semiquantitative seven-tier system developed by Allred *et al.*[29] This system assesses the percentage of positive cells (none = 0, $<10\% = 1$, 10–50% = 2 and $>50\% = 3$) and the intensity of staining (none = 0, weak = 1, intermediate = 2 and strong = 3). The intensity and percentage scores are then added to give a final immunoreactivity score ranging from 0 to 6.

Cells, plasmids and antibodies

MDA-MB-436, MDA-MB-231 and T47D cell lines were maintained in RPMI1640 (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA). MDA-MB-468 and HS578T cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS. All cells were obtained from the cell bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (CCTCC, Shanghai, China).

The human IBP expression vector pEGFP-C1-IBP and the IBP RNA interference vectors were generated or acquired as previously described.[16] MDA-MB-231, T47D and HS578T cell lines were transfected with IBP RNA interference plasmid or control plasmid. Then, the growth medium was supplemented with blasticidin (10 μg/ml, Invitrogen, Grand Island, NY, USA), which was used to select for blasticidin-resistant transfectants. MDA-MB-468 and MDA-MB-436 cell lines were transfected with pEGFP-C1-IBP or control plasmid, and G418-resistant transfectants were selected. Transfected cells were incubated in serum-free medium for 24 or 48 h before the experiments. In select experiments, the cells were treated with EGF or 10% FBS.

E-cadherin (24E10), keratin 18 (DC10), MMP-2, MMP-9, ZEB1 (D80D3), vimentin (D21H3), phospho-EGF receptor (Tyr1068; D7A5), phospho-EGF receptor (Tyr1173; 53A5), EGF receptor, phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) and p44/42 MAPK (137F5) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Fibronectin (F3648) antibodies were purchased from Sigma Aldrich (St Louis, MO, USA). N-cadherin antibodies were purchased from Abcam (Cambridge, UK).

Western blotting

Cell lysates were prepared in RIPA buffer (Beyotime, Nantong, China). Whole-cell lysates were separated and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin and incubated with primary antibodies. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control. Immunoreactive bands were visualized with a chemiluminescence horseradish peroxidase substrate (Millipore, Billerica, MA, USA).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 30 min, permeabilized in phosphate-buffered saline + 0.1% Triton-X 100 for 10 min, and blocked with 3% bovine serum albumin for 1 h at room temperature. The cells were then incubated overnight with the appropriate antibodies, followed by incubation for 1 h at room temperature with secondary fluorescent antibodies. Finally, to label nuclei, cells were counterstained with 4,6-diamidino-2-phenylindole for 5 min and then observed using a confocal microscope (Leica, Wetzlar, Germany).

Real-time PCR

Total RNA was extracted from cultured cells and used for reverse transcription with Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the SYBR Premix Ex Taq (Perfect Real Time) kit (TaKaRa, Otsu, Japan) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Sequences of sense and antisense primers used to amplify Snail, Slug, ZEB1 and ZEB2 were as follows: Snail forward, 5′-GACTACCGCTGCTCCATTCCA-3′; Snail reverse, 5′-TCCTCTTCATCACTAATGGGGCTTT-3′; Slug forward, 5′-CCAT TCCACGCCCAGCTA-3′; Slug reverse, 5′-GGACTCACTCGCCCCAAA-3′; ZEB1 forward, 5′-GAAAATGAGCAAAACCATGATCCTA-3′0; ZEB1 reverse, 5′-CAGGT GCCTCAGGAAAAATGA-3′; ZEB2 forward, 5′-TTCCATTGCTGTGGGCCTT-3′; and ZEB2 reverse, 5′-TTGTGGGAGGGTTACTGTTGG-3′.

Invasion and migration assay

For the invasion assays, cells were serum-starved overnight and 1×10^5 cells were seeded in a Matrigel-coated chamber (BD Bioscience, San Jose, CA, USA) with 8.0-μm pores (Corning, New York, NY, USA) and cultured for 48 h. For the migration assays, 1×10^6 cells were seeded in uncoated membranes with 8.0-μm pores and cultured for short time periods (6 h for HS578T, 8 h for MDA-MB-231 and 12 h for MDA-MB-436 cells). Cells were seeded in a serum-free medium and then migrated toward complete growth medium. The invaded or migrated cells on the lower surface of the inserts were fixed in 4% paraformaldehyde and stained with hematoxylin before mounting on glass slides. More than five views were analyzed under a light microscope. All experiments were carried out in triplicate.

GTPase activity assays

Briefly, cells were grown to 70% confluence in regular growth medium, serum-starved (24 h for MDA-MB-231 and 48 h for HS578T) and stimulated with 10% FBS or EGF (50 ng/ml) for 5 min. GTP-bound Rac1, RhoA, Cdc42 and total protein were detected using G-LISA Rac1, G-LISA RhoA and G-LISA Cdc42 Activation Assay Biochem Kits (Cytoskeleton Inc., Denver, CO, USA) according to the manufacturer's instructions.

Statistical analysis

All variables between groups were compared using the Pearson χ^2-test or Student's *t*-test. All *P*-values <0.05 were considered significant. Numerical data were calculated using Microsoft Excel and analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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