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ORIGINAL ARTICLE

EBP50 exerts tumor suppressor activity by promoting cell apoptosis and retarding extracellular signal-regulated kinase activity

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Abstract The expression of Ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) and the intragenic mutation of the *ebp50* gene have been reported to correlate with human breast cancer development, but the exact impacts on breast cancer development and its molecular mechanism are not fully understood. In this study, we investigate the potential function of EBP50 through over-expression in the breast cancer cell line, MDA-MB-231, which has low EBP50 protein expression levels. The effects of EBP50 over-expression on cellular proliferation, anchorage-independent growth and apoptosis were examined. In addition, the activity of extracellular signal-regulated kinase (ERK) was also determined. Our results show that a decrease of cellular proliferation and attenuation of colony-forming ability were evident in MDA-MB-231 cells stably transfected with an EBP50 expressing plasmid (EBP-231) when compared with control cells. There was also a statistically significant increase in spontaneous apoptosis in EBP-231 cells accompanied by an attenuation in ERK activity. Altogether, our results suggest that restoring EBP50 expression could suppress breast cancer cell proliferation by promoting cell apoptosis and inhibiting ERK activity, and that EBP50 may be a target for development of diagnostics and therapeutics in breast cancer.

Keywords Anchorage-independent growth · Colony formation · Cell cycle · Apoptosis · MAPK

J. Zheng and L. Sun contributed equally to this work.

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Abbreviations

EBP50	Ezrin-radixin-moesin-binding phosphoprotein-50
NHERF1	Na^+/H^+ exchanger regulatory factor 1
ER	Estrogen receptor
ERK	The mitogen-activated protein kinases extracellular signal-regulated kinase
PDZ	Postsynaptic density-95/Discs Large/ZO-1
GPCR	G protein-coupled receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
NF2	Neurofibromatosis 2
SYK	Spleen tyrosine kinase
PDGFR	Platelet-derived growth factor receptor
EGFR	Epidermal growth factor receptor
ATCC	American type culture collection
DMEM	Dulbecco's modified Eagle's medium
PI	Propidium iodide
ANOVA	Analysis of variance
FBS	Fetal bovine serum
CCK-8	Cell counting kit-8
HA	Hemagglutinin
MAPK	The mitogen-activated kinase
MEF	Mouse embryonic fibroblast
STAT1	Signal transducers and activators of transcription 1
PI3K	PI-3′-OH kinase

Introduction

Ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50, also Na^+/H^+ exchanger regulatory factor 1, or NHERF1, NHERF) is an adaptor protein, consisting of two tandem PSD-95/Discs Large/ZO-1 (PDZ) domains linked to a carboxyl-terminal ezrin-binding region. Through these

functional domains, EBP50 interacts with many proteins, including ion transporters, G protein-coupled receptors and some cancer-related proteins, such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Takahashi et al. 2006), neurofibromatosis 2 (NF2) (Murthy et al. 1998), spleen tyrosine kinase (Dai et al. 2004), platelet-derived growth factor receptor (PDGFR) (Maudsley et al. 2000), epidermal growth factor receptor (EGFR) (Lazar et al. 2004) and β-catenin (Kreimann et al. 2007; Shibata et al. 2003).

Recent research suggests that EBP50 is a potential tumor suppressor, especially in breast cancer (Cardone et al. 2007; Kreimann et al. 2007; Song et al. 2007; Takahashi et al. 2006). The up-regulation of EBP50 expression had been found to positively correlate with lower tumor invasiveness in breast cancer cell lines (Schindelmann et al. 2002). The EBP50 allele was found to be deleted in over 58% of 48 breast cancer cell lines (Dai et al. 2004; Pan et al. 2006), strongly correlating with aggressive features of breast cancer, such as tumor size and disease stage. EBP50 missense mutations (K172N, R180W and D301V) were reported in primary breast tumor and two breast cancer cell lines, MDA-MB-231 and SUM 149PT, respectively (Dai et al. 2004; Pan et al. 2006). Pan et al. (2006) knocked down the expression of EBP50 in MCF7 and T47D breast cancer cell lines, and found that this resulted in enhanced cellular growth. However, there is some controversy about the role of EBP50 in tumor growth: up-regulation of EBP50 expression has been significantly correlated with high tumor stages, metastatic progression, poor prognosis and estrogen receptor (ER) status in a large numbers of breast tumor samples (Cardone et al. 2007; Song et al. 2007), suggesting it is an oncogenic protein. As such the roles of EBP50 in breast cancer development need to be explored further.

In this study, we examined the effects of EBP50 over-expression on tumor cell growth, anchorage-independent growth and apoptosis in the EBP50-deficient breast cancer cell line MDA-MB-231. We also detected ERK activation following EBP50 over-expression. Our results show that EBP50 over-expression could inhibit MDA-MB-231 cell growth, increase spontaneous cellular apoptosis and repress ERK activation. Thus, our data support a role for EBP50 in suppressing tumor cellular growth through the promotion of cell apoptosis and the inhibition of ERK activation.

Materials and methods

Cell lines and plasmids

The human breast cancer cell line MDA-MB-231 and human cervical carcinoma cell line HeLa obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) and Dulbecco's modified Eagle's medium (DMEM) (Forni et al. 2009), respectively, at 37°C and 5% CO_2. Both media were supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% antibiotic–antimycotic agent (Life Technologies, Inc, Grand Island, NY, USA). The pBK-CMV-HA-EBP50 plasmid was kindly provided by Dr. Randy Hall from Emory University.

Stable transfection

Cells were transfected with pBK-CMV-HA-EBP50 plasmid or pBK-CMV-HA vector using FuGENE6 (Roche, Indianapolis, IN, USA) or PolyFect Transfection Reagent (Qiagen, Hilden, Germany) following the protocol reported previously (Konno et al. 2009). After 24-h transfection, cells were trypsinized, diluted (1:10 to 1:15) and reseeded into 10-cm culture dishes. Stable transfected cells were obtained by using selection medium (culture medium with 350 μg/mL G418) (Hu et al. 2008). Single-cell clones were isolated for clone expansion. Each cell clone was screened by western blotting to determine the exogenous level of EBP50 protein expression. Stable transfected cell clones were maintained and passaged in culture medium with G418 (200 μg/mL).

Cell proliferation assay

The cell-counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) colorimetric assay was used to determine cell proliferation and viability as reported previously (Takeuchi et al. 2003). Experiments were repeated three times in quadruplicate wells to ensure the reproducibility of results.

Western blotting

Western blotting was performed as described before (Zheng et al. 2009). The phospho-extracellular signal-regulated kinase (Phospho-ERK) assay was preformed as previously described (Zheng et al. 2008). Blots were quantified using the US National Institutes of Health Image 1.62 program. Protein levels were normalized with β-actin, and the level of phospho-ERK immunoreactivity was normalized to the total ERK immunoreactivity. The primary antibodies used were specific for the Hemagglutinin epitope tag (HA) (MBL, Nagoya, Japan), EBP50 (BD Biosciences, San Jose, CA, USA), actin (Sigma Chemical Corp., St. Louis, MO, USA), phospho-ERK1/2 (Thr 202/Tyr 204) (Cell Signaling Technology, Beverly, MA, USA) and total ERK (Cell Signaling Technology), respectively.

Soft agar colony formation assay

Anchorage-independent growth was assessed by the colony formation in soft agar, with triplicate experiments for each set of conditions (Kreimann et al. 2007). Briefly, a base layer of 0.6% agar (DNA grade, Invitrogen, Carlsbad, CA, USA) was placed into six-well tissue culture plates (Costar, Corning, NY, USA). Cells (5×10^3) were suspended in 0.35% top agar with medium over the base layer. Top agar was covered with culture medium. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. Medium was changed every 3–5 days. On the 14th day, colonies were photographed using phase contrast microscopy at ×5 and colonies greater than 25 μm in diameter were counted. The degree of anchorage-independent growth was calculated using the following equation for determining percent colony-forming efficiency: (number of colonies counted on the 14th day/number of cells seeded at start of assay) × 100.

Cellular apoptosis assay

Cell apoptosis (1×10^6/mL) was assessed by annexin-V/propidium iodide (PI; BD Clontech, Palo Alto, CA, USA) double-labeled flow cytometry and a Epics XL flow cytometer (Beckman-Coulter, Miami, FL, USA) (Reddy Avula et al. 2002). Results were analyzed with CellQuest software (Becton–Dickinson, Mountain View, CA, USA). Percentages of apoptotic cells were determined as the fractions of cells with hypodiploid DNA content. Total populations of viable cells were gated according to their typical forward and right-angle light scatter. Cells treated with fluorescent dye were evaluated in each experiment to determine the level of background fluorescence for negative cells. The percentage of cells stained for Annexin-V or PI/Annexin-V was determined. Only the positive cells were taken into account.

Statistics

All experiments were repeated three times. Results were analyzed using SPSS 11.5 statistical software. All data are presented as means ± SD. Growth curve results were analyzed by two-way analysis of variance followed by Tukey's multiple comparison test. Other results were analyzed by independent sample *T* test.

Results

Generation of EBP50 stable transfected cell clones

EBP50 stable transfected cell clones, MDA-MB-231-EBP50 (EBP-231) and HeLa-EBP50 (EBP-HeLa), were generated by transfection with pBK-CMV-HA-EBP50 plasmid and subsequent selection. MDA-MB-231 and HeLa cells were also transfected with a neo-pBK-CMV-HA vector to establish vector control cells. The identification of EBP50 stable transfected cell clones by western blotting is shown in Fig. 1. Among these cell clones, EBP2-231 and EBP3-231 expressed similar levels of EBP50 protein, which is only as much as two-thirds of the protein level of EBP50 in EBP1-231. EBP1-HeLa and EBP3-HeLa cells expressed similar levels of EBP50 protein, both approximately twofold higher than EBP2-HeLa cells. The protein levels of EBP50 remained unchanged after 25 passages, suggesting that the exogenous EBP50 gene was stably expressed. No HA-tagged EBP50 protein expression was detected in the vector control clones.

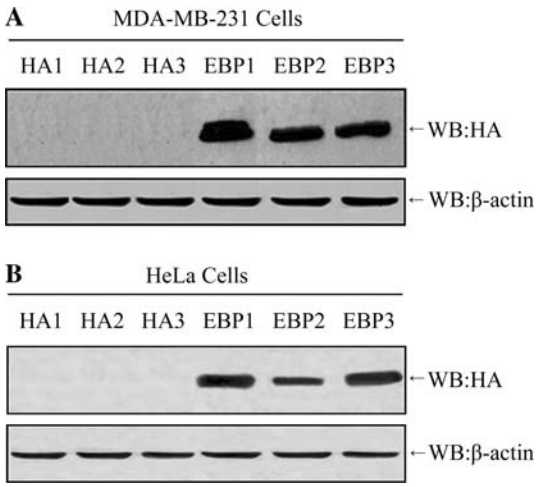

Fig. 1 Establishment of cancer cells stably over-expressing EBP50. **a** MDA-MB-231 breast cancer cells stably over-expressing EBP50. MDA-MB-231 breast cancer cells on 35-mm wells were transfected with human EBP50 cDNA expression construct or pBK-CMV-HA vector alone using FuGENE6. 2 days following transfection, cells were transferred to 100-mm plates and cultured in medium with 350 μg/ml neomycin. The medium was changed every 2 days to remove floating dead cells, and neomycin-resistant colonies formed were harvested using cloning cylinders and plated on 24-well plates. Cell cultures were expanded, and fractions were used for analysis of EBP50 expression by western blotting. *β*-actin was determined on the same blot as a protein-loading control. **b** HeLa cells stably over-expressing EBP50. Method is the same as in **a**

EBP50 over-expression suppresses cancer cell growth

First, we detected the effect of EBP over-expression on the proliferation of MDA-MB-231 cells by measuring the number of viable cells at different time points. Data from representative clones are shown in Fig. 2. The results show that over-expression of EBP50 significantly inhibited cell proliferation when compared with the vector control cells. Especially, the EBP1-231 clone, expressing the highest levels of EBP50, showed the slowest proliferation rate,

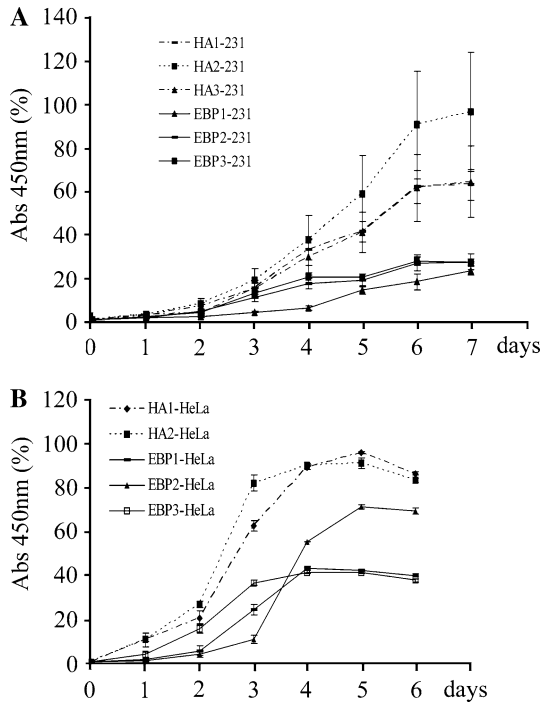

Fig. 2 EBP50 over-expression suppresses tumor cell growth. **a** Growth curve for pBK-CMV-HA vector-transfected (HA-231) or EBP50-transfected MDA-MB-231 cells (EBP-231). Cells on 96-well plates were cultured in medium with 10% serum. Cell numbers were determined by CCK-8 colorimetric assay. *Error bars* indicate SD from measurement of quadraplicate plates. Data shown are representative of three independent experiments using CCK-8 colorimetric assay. EBP50 can inhibit the proliferation ability of MDA-MB-231 significantly ($P < 0.05$). **b** Growth curve for pBK-CMV-HA vector-transfected or HA-EBP50-transfected HeLa cells. Method is the same as **a**. Data shown are representative of three independent experiments using CCK-8 colorimetric assay. EBP50 can inhibit the proliferation ability of HeLa cells significantly ($P < 0.01$)

indicating that restoring EBP50 expression in EBP50 deficient MDA-MB-231 cells inhibited tumor cell proliferation. This result also suggests that the level of EBP50 protein expression may correlate with the ability to inhibit tumor cell proliferation.

To investigate whether this phenomenon is specific to MDA-MB-231 cells or occurs in other cancer cell lines, the growth ability of HeLa cells was then detected. The results were that EBP50 over-expression also suppressed HeLa cell proliferation. Again, EBP1-HeLa and EBP3-HeLa clones, expressing higher level of EBP50 protein showed slower proliferation rates than EBP2-HeLa cells, revealing a dose-dependent growth-suppressive ability of EBP50.

To examine whether the growth-inhibitory activity of EBP50 can be attributed to an effect on cell cycle progression, we first compared the cell cycle distribution of asynchronised MDA-MB-231, MDA-MB-231-EBP50, HeLa and HeLa-EBP50 cells by fluorescence-activated cell sorting (FACS) analysis. No significant difference was observed in G_0/G_1 or G_2/M phase populations between the two groups of cells over-expressing EBP50 and vector control cells (data not shown). Subsequent analysis found that cell cycle progression of cells over-expressing EBP50 from G1 to S phase was unchanged, only M phase entry was a little slower than the vector control cells. However, a typical subdiploid peak in front of G_0/G_1 phase was observed by flow cytometry in cells over-expressing EBP50. Cells in subdiploid (or sub-G1) region preceding the G0/G1 peak, which represent cells with less than a normal diploid complement of DNA, were considered as cells undergoing apoptosis-associated DNA degradation.

EBP50 over-expression leads to less aggressive in vitro features in cancer cells

Next, we explored whether EBP50 over-expression could result in decreased anchorage-independent growth in cancer cells. As shown in Fig. 3a, clones over-expressing EBP50 have smaller colony sizes in soft agar than control cells, and the colony formation ratio of EBP50 over-expressing cell clones reduced up to 40% when compared with the controls, suggesting that EBP50 over-expression inhibited anchorage-independent growth ability for MDA-MB-231 cells. In HeLa cells, EBP50 over-expression also exhibited an anti-tumorigenesis ability, leading to the colony formation ratio of EBP50-expressing cell clones reduced up to 96% (Fig. 3b).

EBP50 over-expression promotes MDA-MB-231 cell apoptosis

Significant differences in cell cycle distribution and progression were not observed between cells over-expressing EBP50 and controls. However, a sub-G1 peak was seen in EBP50 over-expression cells, indicating a potential increase in cell apoptosis, which was further investigated using an annexin-V/propidium iodide assay. As shown in Fig. 4, spontaneous apoptosis in cells over-expressing EBP50 (9.23 ± 2.97)% increased up to fourfold when compared with controls (2.23 ± 1.41)% ($P < 0.05$), indicating EBP50 over-expression enhances apoptosis in MDA-MB-231 cells.

EBP50 over-expression inhibits ERK activation in MDA-MB-231 cells

The ERK survival pathway plays an important role in inhibiting cell apoptosis (Jo et al. 2007) and ERK activation has been reported to be regulated by EBP50 (Lazar et al. 2004). So the possibility of EBP50 over-expression promoting MDA-MB-231 cell apoptosis via inhibition of the ERK survival pathway was investigated. The results are

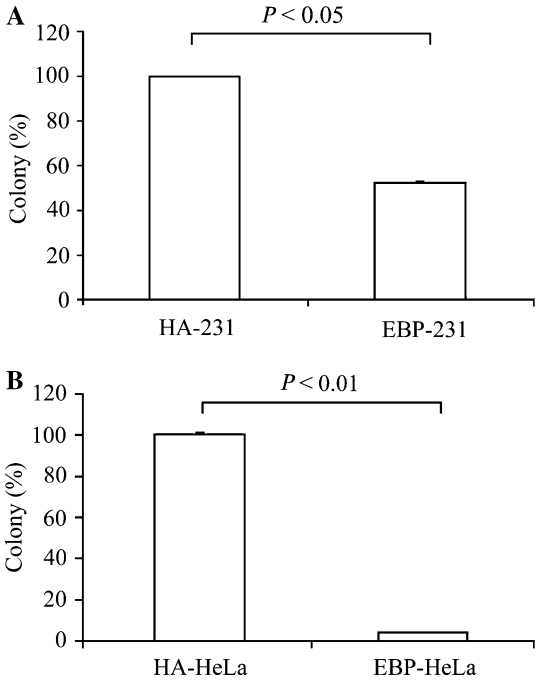

Fig. 3 EBP50 over-expression suppresses anchorage-independent growth of cancer cells in soft agar and alters the number of soft agar colonies. **a** MDA-MB-231 cells expressing HA-tagged form of EBP50 or vector control cells (5×10^3) were mixed with $2 \times$ 1,640 medium and seeded in upper layer of 0.6% soft agar. Percentages of colonies for EBP-231 clone cells (expressing EBP50) were significantly reduced compared with that for HA-231 (control) ($n = 3$, $P < 0.05$). **b** HeLa cells expressing HA-tagged form of EBP50 or vector control cells (5×10^3) were mixed with $2 \times$ DMEM and seeded in the upper layer of 0.6% soft agar. The ratio of colonies for EBP-HeLa cells (over-expressing EBP50) decreased significantly compared with that for HA-HeLa (control) ($n = 3$, $P < 0.01$)

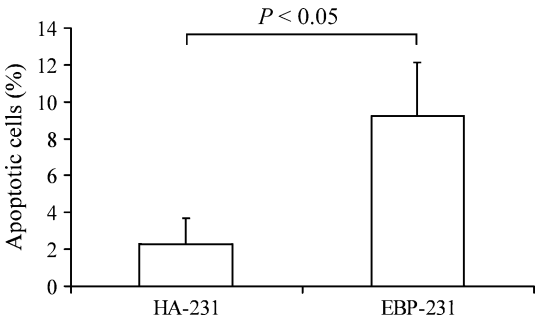

Fig. 4 EBP50 over-expression increases the percentage of apoptosis in MDA-MB-231 cell. MDA-MB-231 cells stably transfected with pBK-CMV-HA vector or pBK-CMV-HA-EBP50 plasmid were cultured on 90-mm plates in medium with 10% serum. Cell apoptosis was analyzed by Annexin V-FITC/propidium iodide staining and FACS. *Error bars* indicate SD from measurement of triplicate plates. Data shown are representative of three independent experiments. $P < 0.05$ compared with control

shown in Fig. 5. The level of ERK activation decreased 30% in cells over-expressing EBP50 ($P < 0.01$), indicating that EBP50 inhibits ERK activation in MDA-MB-231 cells.

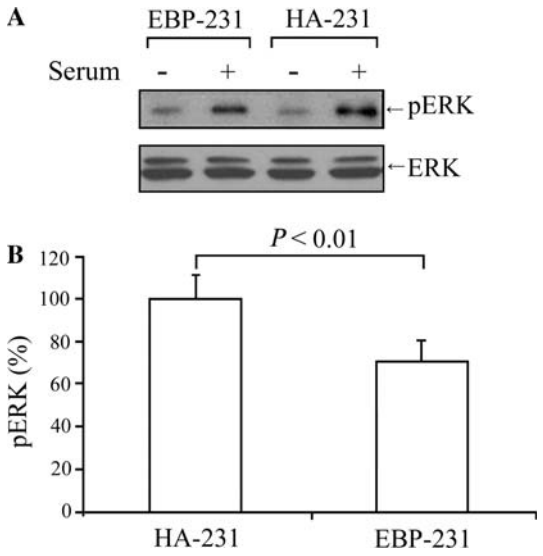

Fig. 5 EBP50 over-expression inhibits ERK activity in MDA-MB-231 cells. MDA-MB-231 cells stably transfected with pBK-CMV-HA vector or pBK-CMV-HA-EBP50 plasmid were grown to 70% confluency and starved overnight in 0.5% FCS, then treated for 10 min with 10% FCS at 37°C. Cells were solubilized in 1× SDS-PAGE sample buffer. Phosphorylation of ERK in whole cell lysates was detected by western blot analysis using an anti-phospho-ERK1/2 antibody. The data presented is representative of a minimum of three independent experiments. **a** Representative experiment showing serum-induced ERK1/2 phosphorylation. **b** Average results depicting means ± SD of three independent clones. Results were normalized to the expression of total ERK1/2. $P < 0.01$ compared with control

Discussion

In the present study, we examined the phenotypic changes and molecular mechanisms resulting from EBP50 over-expression in MDA-MB-231 cells, in which the *ebp50* gene is mutated and EBP50 protein is expressed at very low levels. We found that EBP50 over-expression inhibited cancer cell proliferation. The proliferation rate of MDA-MB-231 cells over-expressing EBP50 was slower than that of vector control cells (Fig. 2). In soft agar assay, the colony formation efficiency of cells over-expressing EBP50 was significantly less than that of control cells, indicating that EBP50 over-expression can inhibit anchorage-independent growth of breast cancer cells. No significant cell cycle arrest was found (data not shown), but there existed a typical sub-G1 peak in cells over-expressing EBP50, indicating possible cell apoptosis induced by EBP50 over-expression. Further investigation showed that EBP50 did cause increased cell apoptosis and lower ERK activation. It has been reported that EBP50 knockdown could promote breast cancer cell proliferation through enhanced G1-S transition and higher levels of cyclin E (Pan et al. 2006). Taken together, EBP50 appears to be a potential tumor suppressor protein in the mammary gland.

To determine whether growth inhibition by EBP50 was a breast cancer cell-specific event, the experiments were reconstructed using EBP-HeLa cells, which has endogenous EBP50 expression. As the results show in Figs. 2b and 3b, EBP50 over-expression in HeLa cells also resulted in significant growth inhibition when compared with control cells, suggesting EBP50 has a growth-inhibition activity in cervical cancer cells as well. In some breast cancer cell lines, such as T47D and MCF7, EBP50 is expressed and did not show tumor-suppressive activity, but if EBP50 expression was knocked down in these cells, their malignant phenotype is further enhanced (Pan et al. 2006). This suggests that in these tumor cells where EBP50 protein is expressed, its expression level might be insufficient to prevent proliferation. In addition, EBP50 has been reported to have more than 30 binding partners (Georgescu et al. 2008), and insufficient EBP50 expression in some tumor cells could perhaps lead to its inability to bind with tumor suppressor proteins and to exert its anti-tumor function. Another cause could be that different cell lines and tissues have different genetic backgrounds. Even if their *ebp50* genes lead to the same expression level of the protein, EBP50 could bind with different partners and exert different functions in different genetic backgrounds.

As a scaffold protein, the functions of EBP50 are diverse and dependent on its binding partners. EBP50 may exert tumor-promoting function through working cooperatively with β-catenin in the development of liver cancer (Shibata et al. 2003). It may also act as a tumor suppressor in breast cancer cell lines and primary tumors (Dai et al. 2004; Pan et al. 2006; Takahashi et al. 2006; Kreimann et al. 2007; Curto et al. 2007). The molecular mechanisms through which EBP50 exerts its tumor suppressor functions are also diverse. EBP50 is able to cluster with other membrane receptors in different parts of the plasma membrane. EBP50 has also been shown to form a protein complex involving EGFR and NF2 at the adherens-junctions (Curto et al. 2007). This interaction prevents EGFR from internalizing and signaling. Similarly, EBP50 binds to PDGFR (Maudsley et al. 2000) and bridges ternary complexes with NF2 (James et al. 2004). Activated PDGF signaling has been shown to prevent cells from undergoing apoptosis during epithelial mesenchymal transition and thus promote breast cancer progression and metastasis (Jechlinger et al. 2006; Ostman and Heldin 2007). PTEN tumor suppressor is a phosphoinositide phosphatase that antagonizes the activity of the PI-3′-OH kinase (PI3K) (Maehama 2007). EBP50 can also link PDGFR and the PTEN tumor suppressor in a ternary complex in normal mouse embryonic fibroblasts (Takahashi et al. 2006) and breast cancer cells (Pan et al. 2008) to attenuate PDGFR signaling (Takahashi et al. 2006; Pan et al. 2008) and possibly to promote apoptosis. That is, the ternary complex mediated by EBP50 restricts the activity of the PI3K pathway after PDGF stimulation. In our study, we found that EBP50 expression could suppress breast cancer cell proliferation by promoting cell apoptosis and inhibiting ERK activation.

The mitogen-activated kinase (MAPK) cascade is an evolutionarily conserved signaling pathway controlling cellular proliferation and differentiation (Seger and Krebs 1995). It is also responsible for mediating survival signaling (Rygiel et al. 2008). One key member of the MAPK pathway is ERK, which has been implicated in tumor development. Constitutively active ERK may cause degradation of signal transducers and activators of transcription (e.g. STAT1), leading to the loss of its pro-apoptotic and tumor suppressor functions (Soond et al. 2008). Inhibition of ERK/MAPK signaling induces increased apoptosis in various cell lines (Goel et al. 2007; Li et al. 2007; Stevens et al. 2007) including MDA-MB-231 (Jo et al. 2007). It has been reported that EBP50 may associate with AKT and inhibit ERK signaling through a B-Raf-mediated pathway (Wang et al. 2008). ERK activation mediated by transactivation of EGFR is also regulated by EBP50 (Sneddon et al. 2007). In this study, we found that EBP50 over-expression caused apoptosis. The induction of apoptosis by over-expression of EBP50 in our cell types likely depends on levels of both EBP50 and its principal partners, such as EGFR (Curto et al. 2007), NF2 (James et al. 2004), PDGFR (Maudsley et al. 2000), and PTEN (Pan et al. 2008). Under the stimulation of serum with EGF as a main component, EBP50 expression resulted in attenuation of ERK activity, further verifying that EBP50 can play a role in suppressing tumor cell growth through inhibiting the ERK pathway. Recently, it has also been reported that suppression of ERK activity correlates with decreased colony formation ability (Bessard et al. 2008; Neill et al. 2008; Sun et al. 2008), suggesting the impaired colony formation caused by EBP50 expression is also induced by lower levels of ERK activity.

In summary, this study demonstrates that EBP50 over-expression can inhibit the malignant biological behavior of MDA-MB-231 cells (including proliferation and anchorage-independent growth), which is consistent with the EBP50 knockdown results reported by Pan and Takahashi (Pan et al. 2006; Takahashi et al. 2006). These results further elucidate the tumor suppressing role of EBP50 and establish it as a candidate target for breast cancer drug therapy. Although growth suppressive roles of EBP50 can potentially be explained by the assembly of complexes between growth factor receptors and tumor suppressors or by β-catenin stabilization at the membrane (Curto et al. 2007; Kreimann et al. 2007; Takahashi et al. 2006), interactions with other transmembrane ligands should be thoroughly investigated as they might also enhance the

growth suppressive properties of EBP50 at the membrane. EBP50 over-expression promotes apoptosis in MDA-MB-231 cells. Therefore, it is reasonable to conclude that EBP50 plays a role in inhibiting breast cancer MDA-MB-231 cell proliferation via promoting cell apoptosis and inhibiting ERK activation.

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References

Bessard A, Fremin C, Ezan F, Fautrel A, Gailhouste L, Baffet G (2008) RNAi-mediated ERK2 knockdown inhibits growth of tumor cells in vitro and in vivo. Oncogene 27:5315–5325

Cardone RA, Bellizzi A, Busco G, Weinman EJ, Dell'Aquila ME, Casavola V, Azzariti A, Mangia A, Paradiso A, Reshkin SJ (2007) The NHERF1 PDZ2 domain regulates PKA-RhoA-p38-mediated NHE1 activation and invasion in breast tumor cells. Mol Biol Cell 18:1768–1780

Curto M, Cole BK, Lallemand D, Liu CH, McClatchey AI (2007) Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. J Cell Biol 177:893–903

Dai JL, Wang L, Sahin AA, Broemeling LD, Schutte M, Pan Y (2004) NHERF (Na^+/H^+ exchanger regulatory factor) gene mutations in human breast cancer. Oncogene 23:8681–8687

Forni C, Braglia R, Lentini A, Nuccetelli M, Provenzano B, Tabolacci C, Beninati S (2009) Role of transglutaminase 2 in quercetin-induced differentiation of B16–F10 murine melanoma cells. Amino Acids 36:731–738

Georgescu MM, Morales FC, Molina JR, Hayashi Y (2008) Roles of NHERF1/EBP50 in cancer. Curr Mol Med 8:459–468

Goel A, Prasad AK, Parmar VS, Ghosh B, Saini N (2007) 7, 8-Dihydroxy-4-methylcoumarin induces apoptosis of human lung adenocarcinoma cells by ROS-independent mitochondrial pathway through partial inhibition of ERK/MAPK signaling. FEBS Lett 581:2447–2454

Hu CA, Khalil S, Zhaorigetu S, Liu Z, Tyler M, Wan G, Valle D (2008) Human Delta1-pyrroline-5-carboxylate synthase: function and regulation. Amino Acids 35:665–672

James MF, Beauchamp RL, Manchanda N, Kazlauskas A, Ramesh V (2004) A NHERF binding site links the betaPDGFR to the cytoskeleton and regulates cell spreading and migration. J Cell Sci 117:2951–2961

Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, Donovan M, Cordon-Cardo C, Beug H, Grunert S (2006) Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Invest 116:1561–1570

Jo EH, Lee SJ, Ahn NS, Park JS, Hwang JW, Kim SH, Aruoma OI, Lee YS, Kang KS (2007) Induction of apoptosis in MCF-7 and MDA-MB-231 breast cancer cells by Oligonol is mediated by Bcl-2 family regulation and MEK/ERK signaling. Eur J Cancer Prev 16:342–347

Konno R, Okamura T, Kasai N, Summer KH, Niwa A (2009) Mutant rat strain lacking D-amino-acid oxidase. Amino Acids 37:367–375

Kreimann EL, Morales FC, de Orbeta-Cruz J, Takahashi Y, Adams H, Liu TJ, McCrea PD, Georgescu MM (2007) Cortical stabilization of beta-catenin contributes to NHERF1/EBP50 tumor suppressor function. Oncogene 26:5290–5299

Lazar CS, Cresson CM, Lauffenburger DA, Gill GN (2004) The Na^+/H^+ exchanger regulatory factor stabilizes epidermal growth factor receptors at the cell surface. Mol Biol Cell 15:5470–5480

Li D, Wu LJ, Tashiro S, Onodera S, Ikejima T (2007) Oridonin-induced A431 cell apoptosis partially through blockage of the Ras/Raf/ERK signal pathway. J Pharmacol Sci 103:56–66

Maehama T (2007) PTEN: its deregulation and tumorigenesis. Biol Pharm Bull 30:1624–1627

Maudsley S, Zamah AM, Rahman N, Blitzer JT, Luttrell LM, Lefkowitz RJ, Hall RA (2000) Platelet-derived growth factor receptor association with Na^+/H^+ exchanger regulatory factor potentiates receptor activity. Mol Cell Biol 20:8352–8363

Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, Solomon F, Gusella J, Ramesh V (1998) NHE-RF, a regulatory cofactor for Na^+/H^+ exchange, is a common interactor for merlin and ERM (MERM) proteins. J Biol Chem 273:1273–1276

Neill GW, Harrison WJ, Ikram MS, Williams TDL, Bianchi LS, Nadendla SK, Green JL, Ghali L, Frischauf AM, O'Toole EA et al (2008) GLI1 repression of ERK activity correlates with colony formation and impaired migration in human epidermal keratinocytes. Carcinogenesis 29:738–746

Ostman A, Heldin CH (2007) PDGF receptors as targets in tumor treatment. Adv Cancer Res 97:247–274

Pan Y, Wang L, Dai JL (2006) Suppression of breast cancer cell growth by Na^+/H^+ exchanger regulatory factor 1 (NHERF1). Breast Cancer Res 8:R63

Pan Y, Weinman EJ, Dai JL (2008) Na^+/H^+ exchanger regulatory factor 1 inhibits platelet-derived growth factor signaling in breast cancer cells. Breast Cancer Res 10:R5

Reddy Avula CP, Lawrence RA, Zaman K, Fernandes G (2002) Inhibition of intracellular peroxides and apoptosis of lymphocytes in lupus-prone B/W mice by dietary n-6 and n-3 lipids with calorie restriction. J Clin Immunol 22:206–219

Rygiel TP, Mertens AE, Strumane K, van der Kammen R, Collard JG (2008) The Rac activator Tiam1 prevents keratinocyte apoptosis by controlling ROS-mediated ERK phosphorylation. J Cell Sci 121:1183–1192

Schindelmann S, Windisch J, Grundmann R, Kreienberg R, Zeillinger R, Deissler H (2002) Expression profiling of mammary carcinoma cell lines: correlation of in vitro invasiveness with expression of CD24. Tumour Biol 23:139–145

Seger R, Krebs EG (1995) The MAPK signaling cascade. Faseb J 9:726–735

Shibata T, Chuma M, Kokubu A, Sakamoto M, Hirohashi S (2003) EBP50, a beta-catenin-associating protein, enhances Wnt signaling and is over-expressed in hepatocellular carcinoma. Hepatology 38:178–186

Sneddon WB, Yang YM, Ba JM, Harinstein LM, Friedman PA (2007) Extracellular signal-regulated kinase activation by parathyroid hormone in distal tubule cells. Am J Physiol Renal Physiol 292:F1028–F1034

Song J, Bai J, Yang W, Gabrielson EW, Chan DW, Zhang Z (2007) Expression and clinicopathological significance of oestrogen-responsive ezrin-radixin-moesin-binding phosphoprotein 50 in breast cancer. Histopathology 51:40–53

Soond SM, Townsend PA, Barry SP, Knight RA, Latchman DS, Stephanou A (2008) ERK and the F-box protein betaTRCP target STAT1 for degradation. J Biol Chem 283:16077–16083

Stevens C, Lin Y, Sanchez M, Amin E, Copson E, White H, Durston V, Eccles DM, Hupp T (2007) A germ line mutation in the death domain of DAPK-1 inactivates ERK-induced apoptosis. J Biol Chem 282:13791–13803

Sun CK, Man K, Ng KT, Ho JW, Lim ZX, Cheng Q, Lo CM, Poon RT, Fan ST (2008) Proline-rich tyrosine kinase 2 (Pyk2) promotes proliferation and invasiveness of hepatocellular carcinoma cells through c-Src/ERK activation. Carcinogenesis 29:2096–2105

Takahashi Y, Morales FC, Kreimann EL, Georgescu M-M (2006) PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. EMBO J 25:910–920

Takeuchi A, Mishina Y, Miyaishi O, Kojima E, Hasegawa T, Isobe K (2003) Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. Nat Genet 33:172–176

Wang B, Yang Y, Friedman PA (2008) Na^+/H^+ exchange regulatory factor 1, a novel AKT-associating protein, regulates extracellular signal-regulated kinase signaling through a B-Raf-mediated pathway. Mol Biol Cell 19:1637–1645

Zheng J, Shen H, Xiong Y, Yang X, He J (2008) The beta1-adrenergic receptor mediates extracellular signal-regulated kinase activation via Galphas. Amino Acids. doi:10.1007/s00726-008-0207-6

Zheng JF, Patil SS, Chen WQ, An W, He JQ, Lubec G (2009) Hippocampal protein levels related to spatial memory are different in the Barnes maze and in the multiple T-maze. Amino Acids 37:122