

FASN, ErbB2-mediated glycolysis is required for breast cancer cell migration

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Abstract. Both fatty acid synthase (FASN) and ErbB2 have been shown to promote breast cancer cell migration. However, the underlying molecular mechanism remains poorly understood and there is no reported evidence that directly links glycolysis to breast cancer cell migration. In this study, we investigated the role of FASN, ErbB2-mediated glycolysis in breast cancer cell migration. First, we compared lactate dehydrogenase A (LDHA) protein levels, glycolysis and cell migration between FASN, ErbB2-overexpressing SK-BR-3 cells and FASN, ErbB2-low-expressing MCF7 cells. Then, SK-BR-3 cells were treated with cerulenin (Cer), an inhibitor of FASN, and ErbB2, LDHA protein levels, glycolysis, and cell migration were detected. Next, we transiently transfected

ErbB2 plasmid into MCF7 cells and detected FASN, LDHA protein levels, glycolysis and cell migration. Heregulin- β 1 (HRG- β 1) is an activator of ErbB2 and 2-deoxyglucose (2-DG) and oxamate (OX) are inhibitors of glycolysis. MCF7 cells were treated with HRG- β 1 alone, HRG- β 1 plus 2-DG, OX or cerulenin and glycolysis, and cell migration were measured. We found that FASN, ErbB2-high-expressing SK-BR-3 cells displayed higher levels of glycolysis and migration than FASN, ErbB2-low-expressing MCF7 cells. Inhibition of FASN by cerulenin impaired glycolysis and migration in SK-BR-3 cells. Transient overexpression of ErbB2 in MCF7 cells promotes glycolysis and migration. Moreover, 2-deoxyglucose (2-DG), oxamate (OX), or cerulenin partially reverses heregulin- β 1 (HRG- β 1)-induced glycolysis and migration in MCF7 cells. In conclusion, this study demonstrates that FASN, ErbB2-mediated glycolysis is required for breast cancer cell migration. These novel findings indicate that targeting FASN, ErbB2-mediated glycolysis may be a new approach to reverse breast cancer cell migration.

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Abbreviations: 2-DG, 2-deoxyglucose; Cer, cerulenin; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; FASN, fatty acid synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRG- β 1, heregulin- β 1; HRP, horseradish peroxidase; LDHA, lactate dehydrogenase A; MAPK, mitogen-activated protein kinase; OX, oxamate; PI3K, phosphatidylinositol 3 kinase; PS, penicillin and streptomycin; TBS, Tris buffered saline; TNFR, tumor necrosis factor receptor

Key words: fatty acid synthase, ErbB2, glycolysis, migration, breast cancer

Introduction

Breast cancer is the most common type of cancer and remains the leading cause of cancer death among women in the world (1). Metastasis is the main cause of breast cancer death (2). Breast cancer metastasis is a complex pathophysiological process involving epithelial-mesenchymal transition (EMT), migration and motility, invasion, and metastatic growth (3). The molecular mechanism underlying breast cancer metastasis is not fully understood.

Mounting evidence shows that cancer cells are different from normal cells in their metabolic properties. Normal cells mostly depend on mitochondrial oxidative phosphorylation to produce energy in the form of ATP. In contrast, cancer cells prefer breakdown of glucose for energy rather than oxidative phosphorylation, even in the presence of available oxygen. This phenomenon is known as aerobic glycolysis, or Warburg effect, which is a hallmark of cancer cells (4-9). However, there is no report that directly links increased glycolysis to breast cancer cell migration.

Fatty acid synthase (FASN) is a key enzyme involved in neoplastic lipogenesis and the production of long-chain fatty

acids from acetyl-coenzyme A (CoA) and malonyl-CoA. In breast cancer cells, there is a positive correlation between overexpression of FASN and amplification and/or overexpression of ErbB2 (10,11). *ErbB2* (Her2/neu) is an oncogene that is overexpressed in 25–30% of human primary breast cancer and is associated with a poor prognosis (12). It has been demonstrated that both ErbB2 and FASN enhance the transformation and/or metastatic potentials of breast cancer cells (13–18). Our previous study showed that ErbB2 promotes breast cancer cell glycolysis via upregulation of lactate dehydrogenase A (LDHA), a key enzyme that catalyzes the production of lactate (19). Therefore, we hypothesize that both FASN and ErbB2 promote cell migration via glycolysis in breast cancer.

Heregulin- β 1 (HRG- β 1, also known as neuregulin- β 1), a member of the epidermal growth factor (EGF) family (20), is an ErbB3 and ErbB4 ligand that leads to the formation of ErbB2-ErbB3/ErbB4 heterodimers, resulting in activation of the ErbB2 signaling pathway (21). HRG- β 1 contributes to breast cancer cell migration, invasion, and metastasis (22–24). Whether HRG- β 1 can induce glycolysis, and whether HRG- β 1-induced glycolysis contributes to cell migration, is unknown. To explore HRG- β 1's ability to induce glycolysis, MCF7 cells were used because they express ErbB2, ErbB3, and ErbB4 receptors (25).

In this study, we investigated the importance of FASN, ErbB2-mediated glycolysis in breast cancer cell migration. We report that FASN, ErbB2-high-expressing SK-BR-3 cells displayed higher levels of glycolysis and migration than FASN, ErbB2-low-expressing MCF7 cells. In addition, inhibition of FASN by cerulenin impaired glycolysis and migration in SK-BR-3 cells. Finally, we show that inhibition of glycolysis by 2-DG or OX reverses HRG- β 1-induced migration. These results indicate that breast cancer cells are dependent on FASN, ErbB2-mediated glycolysis for migration, suggesting that glycolysis is a novel factor influencing migration and that it plays an important role in breast cancer progression.

Materials and methods

Cell lines and cell cultures. Human breast cancer cell line MCF7 was obtained from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. SK-BR-3 was a gift from Professor Shengyong Yang of Sichuan University. MCF7 cells were cultured in DMEM/F12 (Hyclone) with 10% fetal bovine serum (FBS, Biological Industries) and penicillin/streptomycin (PS, Hyclone). SK-BR-3 cells were maintained in DMEM (Hyclone) supplemented with 10% FBS and PS. Cells were cultured at 37°C in a 5% CO₂ incubator.

Cell treatments with reagents. SK-BR-3 cells (1×10⁶ cells/well) were plated in 6-well plates for 24 h and DMSO or cerulenin (10 µg/ml, Cayman) was added into the medium. Twenty-four hours after treatment, media was collected for glucose uptake and lactate production assays and cells were trypsinized for Transwell and western blot assays. MCF7 cells (8×10⁵ cells/well) were plated in 6-well plates for 24 h. The medium was changed to 0.5% FBS medium and cells were starved for 24 h. Cells were treated with vehicle, heregulin- β 1 (HRG- β 1, 100 ng/ml, PeproTech), HRG- β 1 plus cerulenin (10 µg/ml), 2-deoxyglucose (2-DG, 0.5 mM, Sigma) or oxamate (OX,

50 mM, Sigma). 24 h after treatment, media was collected for glucose uptake and lactate production assays and cells were trypsinized for Transwell and western blot assays.

Glucose uptake and lactate production assays. Glucose uptake was measured using Glucose Oxidase Assay kit (Applygen, China) and results were normalized to the total cellular protein amounts. Lactate production in the medium was performed by using Lactate Assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. Results were normalized to the total cellular protein amounts.

Western blot assay. Cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 1 mM PMSF and Protease Inhibitor Cocktail (Sigma) for 20 min on ice. Lysates were cleared by centrifugation at 15,000 g at 4°C for 20 min. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad). Proteins were subjected to electrophoresis on SDS-polyacrylamide gels and were then transferred to PVDF membranes (Bio-Rad). Membranes were blocked in Tris buffered saline (TBS) (pH 7.4) with 5% non-fat dry milk and probed with primary antibodies as indicated in the figure legends. The following antibodies were used: FASN (sc-55580, Santa Cruz), ErbB2 (2156, Cell Signaling Technology), LDHA (3582, Cell Signaling Technology), β -actin (A2228, Sigma), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AG019, Beyotime, China). Immunoreactive bands were visualized by horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) using ECL Western Blotting Substrate (Pierce). The fold expression of protein was quantified using Image Lab software. Protein expression in control group was set as 1.0.

Cell transfection. pcDNA3.0 was purchased from Hangzhou Baosai Biotechnology, China and pcDNA3.0/ErbB2 was purchased from Addgene. Transient transfection was performed using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were trypsinized for Transwell and western blot assays, and culture media was collected for glucose uptake and lactate production assays.

Wound healing assay. MCF7 and SK-BR-3 cells were plated in 6-well plates and allowed to grow to confluence. Wounds were made using a 10 µl pipette tip and indicated reagents were added to the cells. Wound closure was monitored over a 24 or 48 h period and photographs were taken at 0, 24 or 48 h after wounding. Wound width at 0, 24 or 48 h was measured and the results were expressed as wound closure (%) = (width at 0 h - width at x h)/width at 0 h × 100% (x=24 or 48).

Transwell migration assay. Transwell migration assay was performed using Falcon cell culture inserts (Corning). Cells were trypsinized and suspended in medium with 0.5% FBS. MCF7 cells (3×10⁵) or SK-BR-3 cells (1×10⁵) were seeded in the top of the inserts and placed in the wells containing medium with 10% FBS. After 48 h incubation at 37°C, the inserts were washed with PBS twice and cells on the upper surface of the inserts were gently removed with a cotton swab.

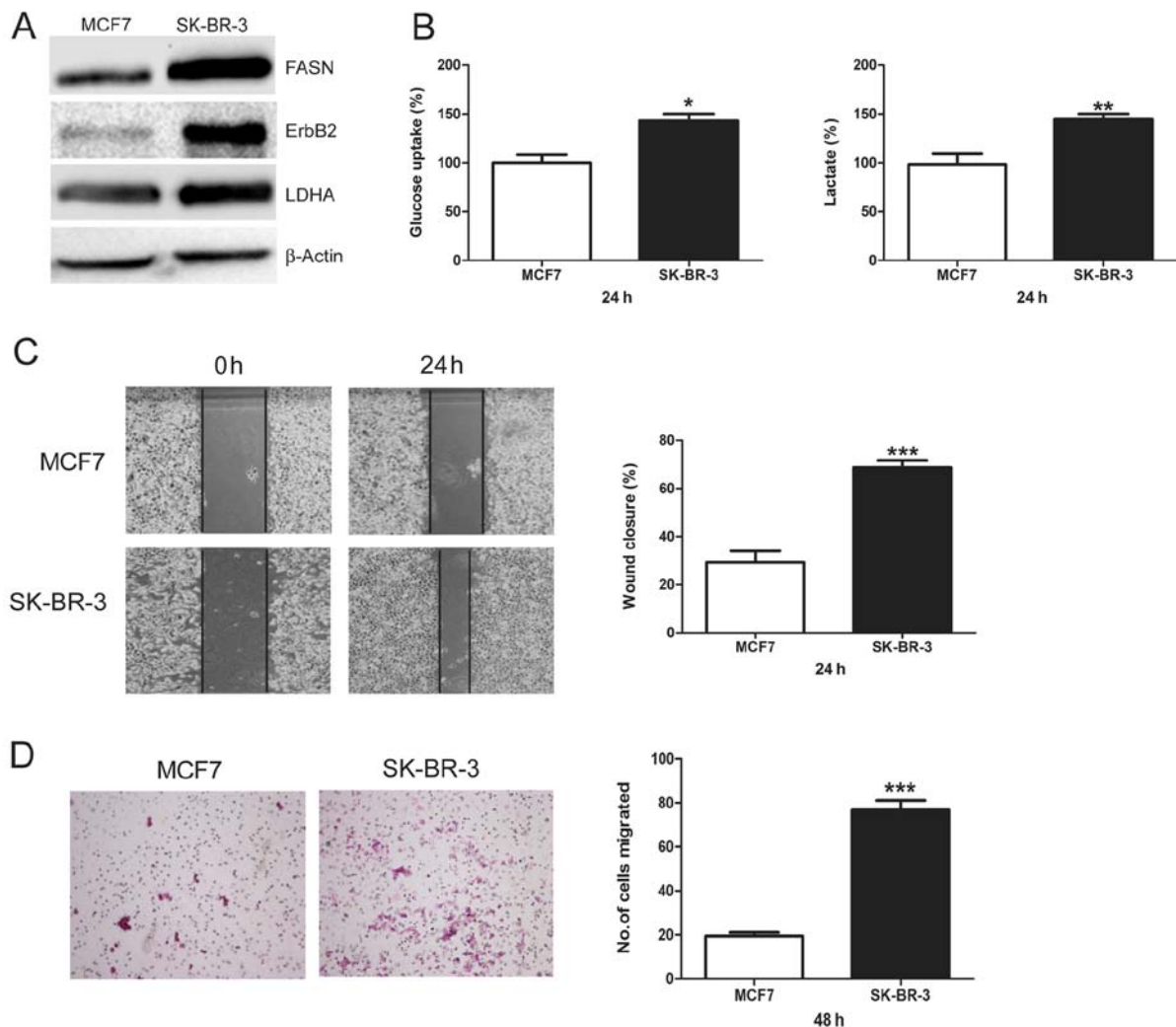


Figure 1. FASN, ErbB2-overexpressing SK-BR-3 cells possess a higher level of glycolysis and migratory potential than FASN, ErbB2-low-expressing MCF7 cells. (A) FASN, ErbB2 and LDHA protein expression in MCF7 and SK-BR-3 cells were detected by western blotting. β -actin was used as a loading control. (B) Glucose uptake and lactate production were compared between MCF7 and SK-BR-3 cells. Data are shown in percentage relative to MCF7 cells. (C) Confluent monolayers of MCF7 and SK-BR-3 cells were wounded using a pipette tip. Wound closure was monitored by microscopy and representative photomicrographs (x40 magnification) are shown (left). Wound width was measured at 0 and 24 h and plotted as percentage of wound closure (right). (D) Transwell migration assay. MCF7 and SK-BR-3 cells were plated into inserts and after 48 h of incubation, migrated cell numbers were counted under a microscope in five random fields per insert. Columns, mean of three independent experiments; bars, SE; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The inserts were fixed with 10% methanol, rinsed with ultra-pure water, and stained with 0.1% crystal violet for 20 min. Cells that migrated onto the lower surface were counted under a microscope in five random fields.

Statistical analysis. All experiments were repeated three times. Statistical analysis was performed using GraphPad Prism version 5.0. The data were expressed as means \pm SE. The difference between groups was detected using Student's t-test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FASN, ErbB2-high-expressing SK-BR-3 cells show increased glycolysis and migration versus FASN, ErbB2-low-expressing MCF7 cells. First, we demonstrated that MCF7 cells displayed low levels of FASN and ErbB2 and SK-BR-3 cells had high

levels of FASN and ErbB2 (Fig. 1A). Then, we compared LDHA protein levels between these two cell lines. We found that SK-BR-3 cells showed much higher LDHA protein levels than MCF7 cells (Fig. 1A). Next, glucose uptake and lactate production, which are two important markers of glycolysis, were measured and compared between MCF7 and SK-BR-3 cells. SK-BR-3 cells showed a significantly higher glucose uptake and lactate production than MCF7 cells (Fig. 1B). Finally, we compared the migratory potential between MCF7 and SK-BR-3 cells using wound healing assay and Transwell assay. Compared to MCF7 cells, SK-BR-3 cells showed a higher percentage of wound closure (Fig. 1C) and migrated cell numbers (Fig. 1D), indicating that SK-BR-3 cells had a greater basal migration capacity than MCF7 cells. These results suggest a consistent relationship between FASN, ErbB2, glycolysis, and cell migration, indicating that increased glycolysis predicts higher migratory potential in breast cancer cells.

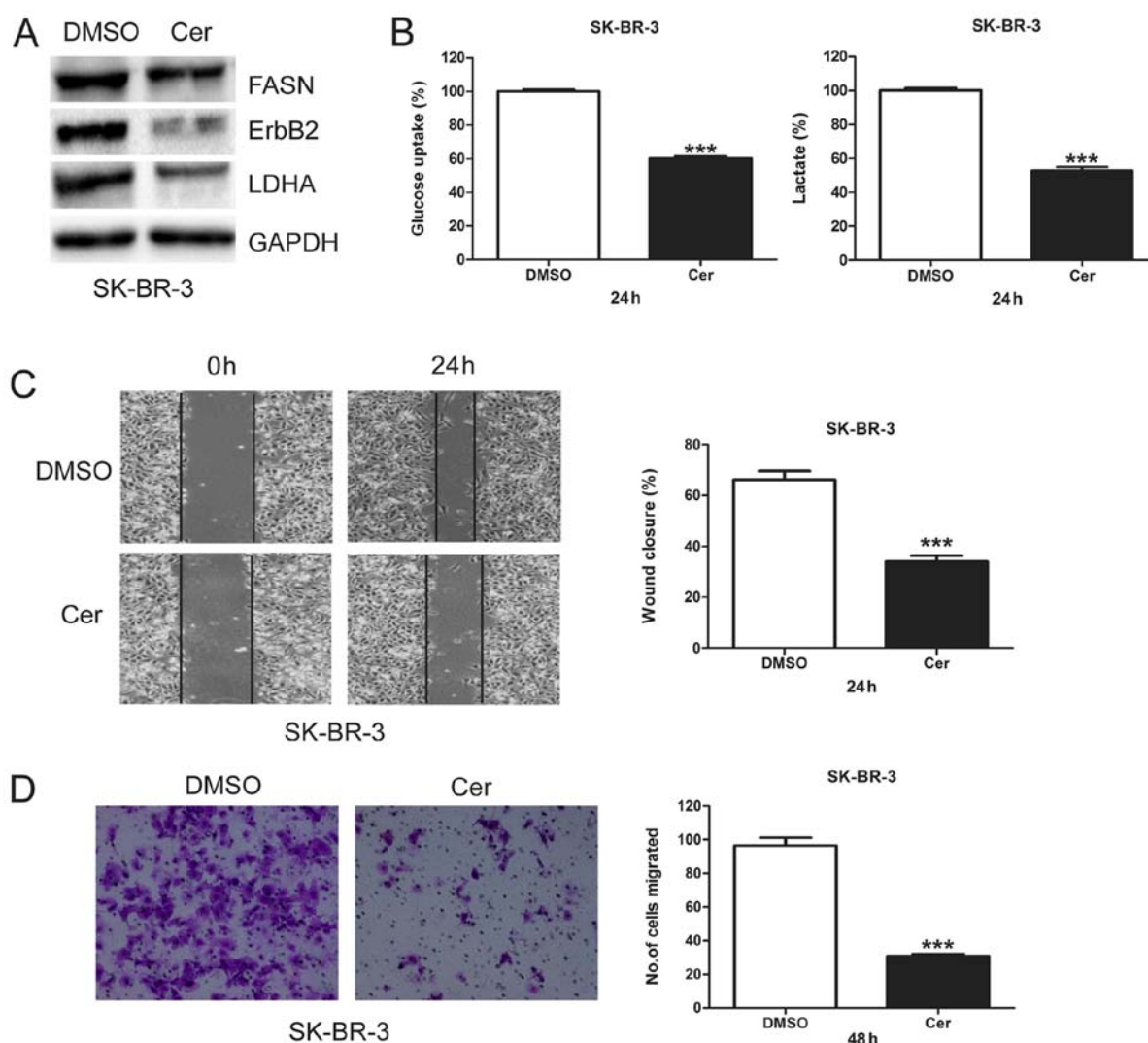


Figure 2. Cerulenin inhibits glycolysis and migration in SK-BR-3 cells. (A) SK-BR-3 cells were treated with DMSO or cerulenin (Cer, 10 μ g/ml) for 24 h and FASN, ErbB2, LDHA protein levels were detected by western blotting. GAPDH was used as a loading control. (B) SK-BR-3 cells were treated with DMSO or Cer (10 μ g/ml) for 24 h and glucose uptake and lactate production were measured in the medium. Data are shown in percentage relative to DMSO-treated cells. (C) Confluent monolayers of SK-BR-3 cells were wounded using a pipette tip and DMSO or Cer (10 μ g/ml) were added into the medium. Wound closure was monitored by microscopy and representative photomicrographs (x40 magnification) are shown (left). Wound width was measured at 0 and 24 h and plotted as percentage of wound closure (right). (D) SK-BR-3 cells were treated with DMSO or Cer (10 μ g/ml) for 24 h. Then cells were plated into inserts and after 48 h of incubation, migrated cell numbers were counted under a microscope in five random fields per insert. Columns, mean of three independent experiments; bars, SE; ***P<0.001.

Cerulenin decreases glycolysis and migration in SK-BR-3 cells. To investigate the role of FASN in glycolysis and cell migration, we treated SK-BR-3 cells with cerulenin, a specific inhibitor of FASN, and measured its effects on ErbB2 and LDHA protein levels, glycolysis, and cell migration. When FASN protein levels were inhibited by cerulenin, both ErbB2 and LDHA proteins levels were downregulated (Fig. 2A). Cerulenin treatment in SK-BR-3 cells also significantly decreased glucose uptake and lactate production (Fig. 2B), indicating that inhibition of FASN by cerulenin inhibits glycolysis. We further found that cerulenin significantly decreased migratory capacity of SK-BR-3 cells (Fig. 2C and D). These results suggest FASN may play an important role in glycolysis and migration of breast cancer cells.

Overexpression of ErbB2 promotes glycolysis and cell migration. To investigate the role of ErbB2-mediated glycolysis

in cell migration, we transiently transfected ErbB2 plasmid into MCF7 cells and then detected FASN and LDHA protein levels. We found that overexpression of ErbB2 upregulates FASN and LDHA protein levels (Fig. 3A). Moreover, we found that acute overexpression of ErbB2 increased both glucose uptake and lactate production (Fig. 3B), indicating increased glycolysis. Then, the role of ErbB2-mediated glycolysis in cell migration was investigated, and we found that overexpression of ErbB2 greatly enhanced cell migration (Fig. 3C). These results suggest ErbB2 plays an important role in glycolysis and migration of breast cancer cells.

Cerulenin reverses HRG- β 1-induced glycolysis and migration in MCF7 cells. We treated MCF7 cells with HRG- β 1 and detected FASN, ErbB2, and LDHA protein levels. We found that HRG- β 1 upregulated FASN, ErbB2 and LDHA protein levels whereas cerulenin reversed such upregulation (Fig. 4A).

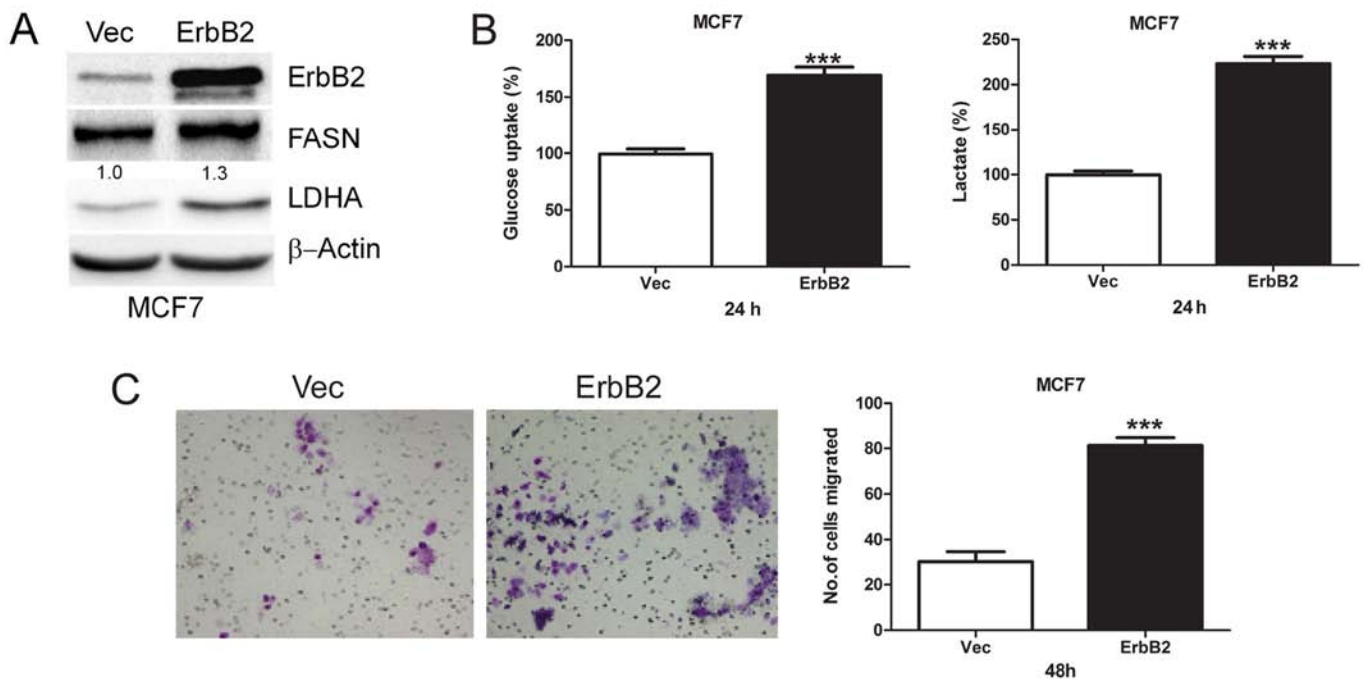


Figure 3. Overexpression of ErbB2 promotes glycolysis and cell migration. (A) Western blot analysis of ErbB2, FASN and LDHA protein levels in MCF7 cells transiently transfected with pcDNA3.0 (Vec) or pcDNA3.0/ErbB2 (ErbB2) plasmid. The β -actin protein served as a loading control. The fold expression of FASN was quantified using the protein level in Vec-transfected MCF7 cells as 1.0. (B) MCF7 cells were transfected with Vec or ErbB2 plasmid and 48 h after transfection, cells were transferred to 24-well plates for glucose uptake and lactate production assay. Data are shown in percentage relative to Vec-transfected MCF7 cells. (C) MCF7 cells were transfected with Vec or ErbB2 plasmid and 48 h after transfection, cells were plated into inserts for Transwell migration assay. After 48 h of incubation, migrated cell numbers were counted under a microscope in five random fields per insert. Columns, mean of three independent experiments; bars, SE; *** $P < 0.001$.

We further found that HRG- β 1 induced both glucose uptake and lactate production with cerulenin reversing such an induction in MCF7 cells (Fig. 4B). Moreover, HRG- β 1 treatment in MCF7 cells greatly increased wound closure and number of migrated cells, while cerulenin overcame such increases (Fig. 4C and D). This result suggests that FASN plays an important role in HRG- β 1-induced glycolysis and migration.

2-DG or OX reverses HRG- β 1-induced glycolysis and migration in MCF7 cells. In order to further demonstrate that glycolysis is required for cell migration, we treated MCF7 cells with HRG- β 1 and detected LDHA protein levels. We found that HRG- β 1 greatly upregulated LDHA protein levels while both 2-DG and OX reversed the upregulation (Fig. 5A). Moreover, both 2-DG and OX reversed HRG- β 1-induced glycolysis (Fig. 5B) and cell migration (Fig. 5C and D). These results suggest that HRG- β 1-induced glycolysis promotes cell migration whereas glycolysis inhibition by 2-DG or OX reverses such effects.

Discussion

Thus far, there has been no evidence reported to directly link glycolysis to cell migration. Here, for the first time, we have shown that FASN, ErbB2-mediated glycolysis is required for breast cancer cell migration. We found that FASN, ErbB2-overexpressing SK-BR-3 cells displayed higher LDHA protein levels, a higher level of glycolysis, and a greater migratory capacity when compared to FASN, ErbB2-low-expressing

MCF7 cells. Inhibition of FASN by cerulenin decreased ErbB2 and LDHA protein levels, as well as glycolysis and migration in SK-BR-3 cells. Activation of ErbB2 by overexpression, or by HRG- β 1, promotes glycolysis and cell migration while inhibition of glycolysis by 2-DG or OX reverses such induction. These results demonstrated that glycolysis is directly linked to breast cancer cell migration.

Lactate is the end product of glycolysis and a number of studies have shown that it plays important roles in the malignant development of tumor, such as cell migration, invasion and metastasis (26-29). However, all *in vitro* studies have been performed by treating cancer cells with exogenous lactate. To our knowledge, this is the first report showing that endogenous lactate directly induced by glycolysis promotes breast cancer cell migration.

ErbB2 and FASN are reciprocally regulated in breast cancer cells. On the one hand, overexpression of ErbB2 upregulates FASN expression, both at the transcriptional level and at the translational level (10,30). Activation of ErbB2 by HRG- β 1 induces tyrosine phosphorylation of FASN and upregulation of FASN activity, leading to increased cell invasion (31). On the other hand, overexpression of FASN activates ErbB2 and induces an invasive breast cancer-like phenotype (18) and FASN regulates ErbB2 expression at the transcriptional level via PEA3 (32). Our results showed that both ErbB2 and FASN protein levels are directly consistent with LDHA protein level, glycolysis, and migration, while inhibition of FASN also decreased ErbB2 and LDHA protein levels, glycolysis, and migration. Our previous study (19) showed that ErbB2 upregu-

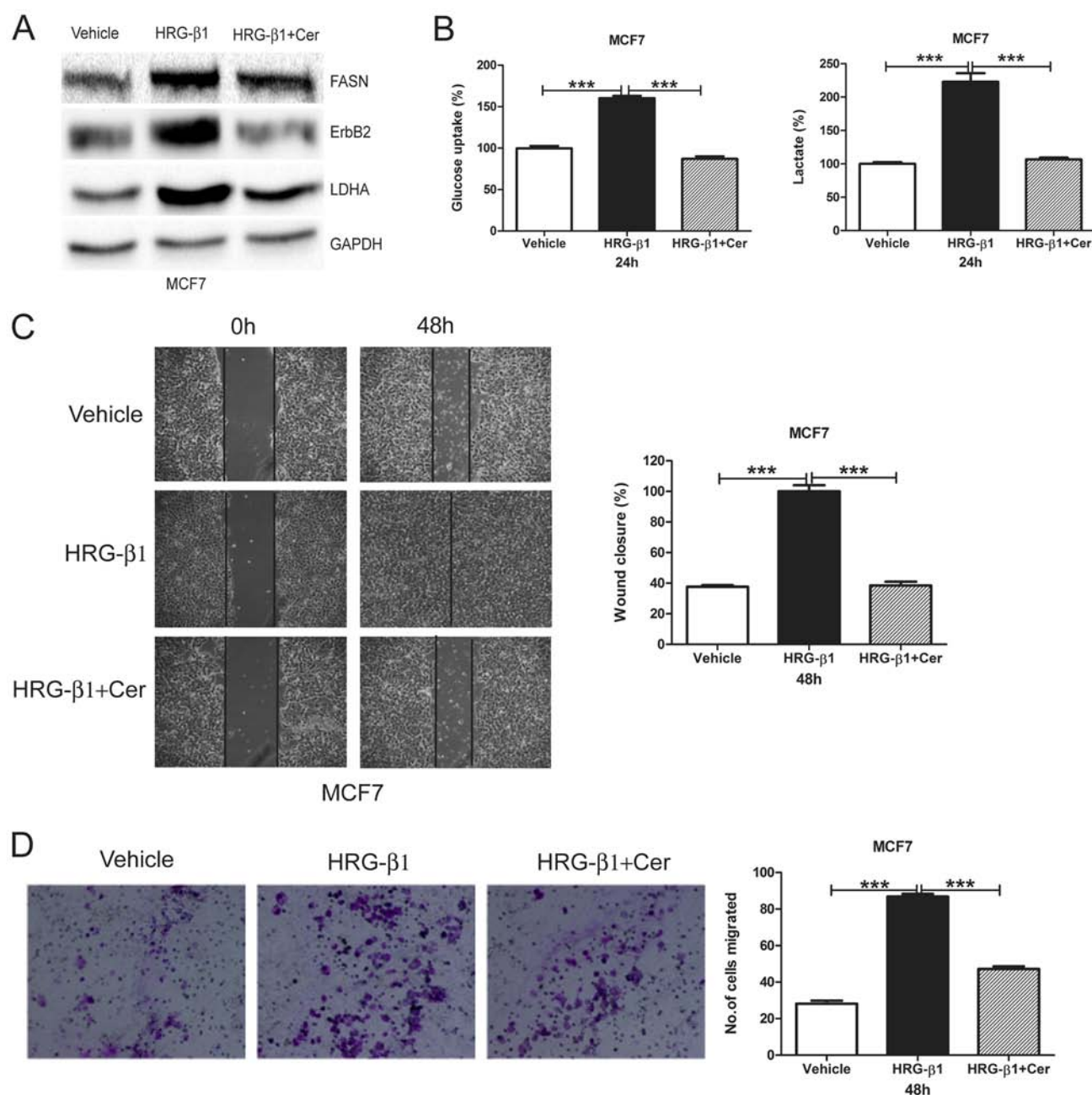


Figure 4. Cerulenin reverses HRG-β1-induced glycolysis and cell migration. (A) MCF7 cells were treated with Vehicle, HRG-β1 (100 ng/ml), HRG-β1 (100 ng/ml) plus Cer (10 μg/ml) for 24 h. Cells were collected and western blot analysis of ErbB2, FASN and LDHA protein levels was performed. GAPDH served as a loading control. (B) MCF7 cells were treated with Vehicle, HRG-β1 (100 ng/ml), HRG-β1 (100 ng/ml) plus Cer (10 μg/ml) for 24 h. Cell media were collected for glucose uptake and lactate production assay. Data are shown in percentage relative to Vehicle-treated MCF7 cells. (C) Confluent monolayers of MCF7 cells were wounded using a pipette tip and were treated with Vehicle, HRG-β1 (100 ng/ml), HRG-β1 (100 ng/ml) plus Cer (10 μg/ml) for 48 h. Wound closure was monitored by microscopy and representative photomicrographs (x40 magnification) are shown (left). Wound width was measured at 0 and 48 h and plotted as percentage of wound closure (right). (D) MCF7 cells were treated with Vehicle, HRG-β1 (100 ng/ml), HRG-β1 (100 ng/ml) plus Cer (10 μg/ml) for 24 h. Then, cells were plated into inserts for Transwell migration assay. After 48 h of incubation, migrated cell numbers were counted under a microscope in five random fields per insert. Columns, mean of three independent experiments; bars, SE; ***P<0.001.

lates LDHA at the transcriptional level. However, whether FASN directly regulates LDHA, or indirectly regulates it via ErbB2, deserves further investigation.

There are several possible mechanisms by which HRG-β1 drives breast cancer cell migration and invasion: these include upregulation of tumor necrosis factor receptor (TNFR) superfamily member Fn14 expression (25), induction of EMT (22,23), and upregulation of GPR30 expression (24,33). We have found a novel mechanism whereby HRG-β1 can induce glycolysis and

drive migration in MCF7 cells. Phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) are two important pathways downstream of ErbB2 signaling induced by HRG-β1 (34). PI3K pathway plays a dominant role in cellular transformation in ErbB2/ErbB3 coreceptor-mediated heregulin signaling (34) and AKT stimulates aerobic glycolysis in cancer cells (35). Therefore, we speculate that activation of PI3K/AKT pathway by HRG-β1 promotes glycolysis resulting in enhanced cell migration.

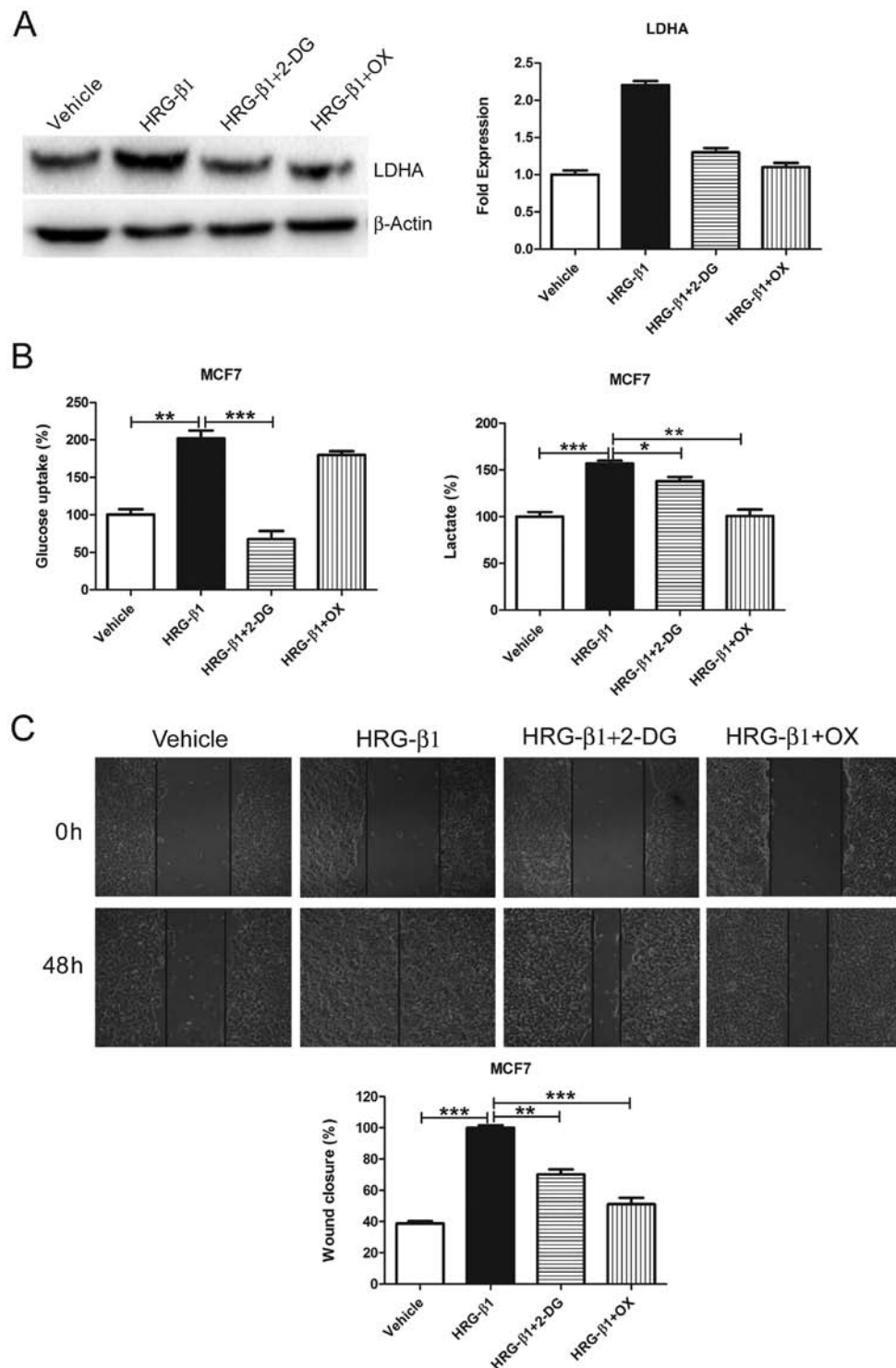


Figure 5. 2-DG and OX reverses HRG- β 1-induced glycolysis and cell migration. (A) MCF7 cells were treated with Vehicle, HRG- β 1 (100 ng/ml), HRG- β 1 (100 ng/ml) plus 2-DG (0.5 mM) or OX (50 mM) for 24 h. Cells were collected and western blot analysis of LDHA protein levels was performed. β -actin served as a loading control (left). The fold expression of LDHA was quantified using the protein level treated with vehicle as 1.0 (right). (B) MCF7 cells were treated with Vehicle, HRG- β 1 (100 ng/ml), HRG- β 1 (100 ng/ml) plus 2-DG (0.5 mM) or OX (50 mM) for 24 h. Cell media were collected for glucose uptake and lactate production assay. Data are shown in percentage relative to Vehicle-treated MCF7 cells. (C) Confluent monolayers of MCF7 cells were wounded using a pipette tip and were treated with Vehicle, HRG- β 1 (100 ng/ml), HRG- β 1 (100 ng/ml) plus 2-DG (0.5 mM) or OX (50 mM) for 48 h. Wound closure was monitored by microscopy and representative photomicrographs (x40 magnification) are shown. Wound width was measured at 0 and 48 h and plotted as percentage of wound closure. Columns, mean of three independent experiments; bars, SE. * P <0.05, ** P <0.01, *** P <0.001.

In summary, to the best of our knowledge, for the first time, we report that FASN, ErbB2-mediated glycolysis is required for breast cancer cell migration. Inhibition of FASN by cerulenin, or inhibition of glycolysis by 2-DG or OX, reverses HRG- β 1-

induced glycolysis and migration. This study provides direct evidence in support of a causal relationship between glycolysis and breast cancer cell migration. These novel findings prompt us to further investigate the role of glycolysis in invasion and

metastasis of breast cancer, which is important for elucidating the molecular mechanism of breast cancer metastasis.

Acknowledgements

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