INTRODUCTION

Breast cancer is the most common cancer in women and the second most common cause of cancer death, accounting for nearly 15% of all cancer-related deaths in women (1). Patients with ERα-positive breast cancer comprise approximately 70% of all patients with breast cancer (2) and have a good prognosis. Specifically, 85% of all patients with ERα-positive breast cancer survive more than five years after diagnosis. This overall survival rate is due to multiple endocrine therapies, including tamoxifen, fulvestrant, and letrozole (2, 3). Although tamoxifen is still the most frequently used selective ER modulator, the effectiveness of tamoxifen therapy is limited in premenopausal women with ER-positive breast cancer (4). Endocrine therapy resistance can arise via several mechanisms, including loss of ERα, induction of abnormal estradiol levels, and alterations of coregulatory proteins such as AIB1 and HDAC (5-7). Therefore, many studies have sought to identify beneficial therapeutic targets that would eliminate the development of drug resistance.

Fibronectin (FN) plays important roles in the EMT in a variety of cancer cell types. However, the mechanism by which FN expression is regulated in tamoxifen-resistant (TamR) breast cancer cells has not yet been fully elucidated. Aberrant FN expression was associated with poor prognosis in patients with luminal type A breast cancer. In addition, FN was upregulated in TamR cells. To investigate the mechanism by which FN expression is regulated, we assessed the levels of phosphorylated Akt, JNK, and STAT3 and found that they were all increased in TamR cells. Induction of FN expression was dampened by LY294002 or AKT IV in TamR cells. Furthermore, FN expression was increased by constitutively active (CA)-Akt overexpression in tamoxifen-sensitive MCF7 (TamS) cells and colony formation of TamR cells was blocked by AKT IV treatment. Taken together, these results demonstrate that FN expression is upregulated through the PI-3K/Akt pathway in tamoxifen-resistant breast cancer cells. [BMB Reports 2017; 50(12): 615-620]

RESULTS

FN expression is associated with poor prognosis in patients with luminal type A breast cancer

Previously, Bae et al. reported that FN expression was associated with tumor aggressiveness and poor clinical outcomes in patients with invasive breast cancer (15). Here, we also evaluated whether FN expression was associated with...
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Fig. 1. FN expression is associated with poor prognosis in patients with luminal type A breast cancer. (A) Relapse-free survival. FN expression data were obtained from a public database (http://kmplot.com/breast). The clinical value of FN was analyzed by generating a Kaplan-Meier survival plot for patients with luminal A and B type breast cancer. (B) The levels of FN mRNA and protein expression were analyzed by real-time PCR and Western blotting, respectively. Results are representative of three independent experiments. Data are presented as means ± SEMs. *P < 0.01 vs. TamS cells.

Fig. 2. Akt activity is increased in tamoxifen-resistant breast cancer cells. (A) The levels of p- and t-Akt, JNK, and STAT3 were analyzed by Western blotting. (B, C) After serum starvation, MCF7 cells were treated with 10 nM PMA for the indicated times. (C) FN and β-actin expression in cell culture media and whole cell lysates were analyzed via Western blotting. Results are representative of three independent experiments. Con, Control.
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Fig. 3. FN expression is regulated through a PI-3K/Akt-dependent pathway in tamoxifen-resistant breast cancer cells. (A) TamR cells were treated with 30 μM LY for 24 h. (B) MCF7 cells were serum starved for 24 h, pretreated with 5 μM LY for 30 min, and then treated with 10 nM PMA for 24 h. After 24 h, cell lysates and culture media were harvested for detection of FN mRNA and protein expression. (C) MDA-MB231 cells were treated with 30 μM LY for 24 h. The protein levels of FN in cell lysates and culture media were analyzed via Western blotting. Results are representative of three independent experiments. Data are presented as means ± SEMs. *P < 0.05 vs. control, #P < 0.05 vs. PMA-treated cells. Con, Control; LY, LY294002.

Fig. 4. Akt activity plays an important role in FN expression and TamR cell growth. (A) TamS cells were transfected with adenoviral vectors and CA-Akt for 24 h and then further incubated in serum-free medium for 24 h. (B) TamR cells were treated with 0.5 μM AKT IV for 24 h. (C) After serum starvation for 24 h, MDA-MB231 cells were treated with or without 1 μM AKT IV for 24 h under serum-free conditions. The protein levels of FN, p-Akt, and β-actin were analyzed by Western blotting. (D) TamR cells were seeded on 6-well soft agar plates with or without 1 μM AKT IV and incubated for 2 weeks. After 2 weeks, viable colonies were stained with 0.01% crystal violet. (E) TamR cells were treated with or without 1 μM AKT IV for the indicated time periods, after which cells were counted using a Countess Automated Cell Counter. (F) Schematic model of this study. Results are representative of three independent experiments. Data are presented as means ± SEMs. *P < 0.05, **P < 0.01 vs. control. Con, Control.

DISCUSSION

Although endocrine therapies have been used in the treatment of hormone-dependent breast cancer, the majority of patients ultimately relapse and experience disease progression (17). Tamoxifen is a systemic treatment for ER-positive breast cancer. Acquisition of tamoxifen resistance significantly augments the EGFR signaling pathway and increases the metastatic ability of resistant cells (16). In addition, patients with ER (+) metastatic breast cancer with HER-2 amplification and HER-1 expression are moderately less responsive to tamoxifen (18). However, the mechanism of tamoxifen resistance is not fully understood. Here, we investigated the mechanism by which FN expression is regulated, because FN expression has been previously linked to tamoxifen resistance-related genes in breast cancer cells.
FN plays a pivotal role in cell adhesion, invasion, and oncogenic transformation (19). Moreover, FN expression has been correlated with tumor aggressiveness and poor clinical outcomes in patients with invasive breast cancer (15). FN expression has been detected in circulating tumor cells from patients with breast cancer (20). Zheng et al. proposed that FN causes epithelial cells to become refractory to tamoxifen by binding to β1 integrin (21, 22). Consistent with these reports, we found that patients with luminal type A breast cancer with high levels of FN showed shorter relapse-free survival. Furthermore, basal FN expression was significantly higher in established TamR cells than in TamS cells. These results demonstrate that the level of FN expression may be associated with endocrine resistance in breast cancer.

Several studies have demonstrated that various transcription factors such as NF-κB and AP-1 are involved in FN synthesis (23). High glucose upregulates FN protein synthesis via NF-κB and AP-1 in endothelial cells (24). Moreover, Lee et al. reported that PMA induces transcription of FN in hepatoma cells via the PKC pathway (23). As a positive control, we also treated MCF7 breast cancer cells with PMA and found that PMA-induced FN expression was decreased by LY294002. Furthermore, basal FN expression was significantly decreased by LY294002 treatment in both TamR and TNBC cells. In contrast, FN expression was increased in TamS cells over-expressing CA-Akt. Therefore, these data demonstrate that the PI-3K/Akt pathway plays an important role in regulating FN expression in TamR cells.

The PI-3K/Akt pathway is the most frequently altered pathway in human cancer. Common alterations include mutation and/or amplification of genes encoding the PI-3K catalytic subunits and regulatory subunits (25-27), as well as loss of the lipid phosphatases PTEN and INPP4B (28, 29). Activation of PI3K/Akt has been shown to confer resistance to antiepigenetics in various models of breast cancer, including PTEN-deficient cells and mutant AKT1-overexpressing cells (30). Consistent with these reports, we also found that the phosphorylation level of Akt was significantly higher in TamR cells. Furthermore, anchorage-independent growth of TamR cells was completely prevented by a specific Akt inhibitor. Therefore, these data demonstrate that PI-3K inhibitors and Akt inhibitors are promising therapeutic drugs for overcoming tamoxifen resistance.

As shown in Fig. 4F, we explored the mechanism by which FN is regulated in TamR cells. Abnormal FN induction was associated with poor prognosis in patients with luminal type A breast cancer. Furthermore, basal FN expression was significantly higher in TamR cells compared with TamS cells. We also observed that the level of phosphorylated Akt was significantly higher in TamR cells. Furthermore, basal FN expression was increased by CA-Akt overexpression in TamS cells. In contrast, this elevated FN expression was decreased by treatment with the Akt inhibitor AKT IV in TamR cells. In addition, anchorage-independent growth of TamR cells was decreased by AKT IV treatment. Taken together, these data demonstrate that abnormal FN induction is mediated by an Akt-dependent pathway in TamR cells. Thus, the potential of PI-3K/Akt pathway regulation to mitigate endocrine resistance in breast cancer should be further investigated.

MATERIALS AND METHODS

Reagents
Dulbecco's modified Eagle's medium (DMEM) and phenol red-free DMEM were purchased from Thermo Scientific (Hemel Hempstead, UK). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma (St. Louis, MO, USA). LY294002 was purchased from Tocris (Ellisville, MO, USA). AKT IV, secondary HRP-conjugated antibodies, and mouse monoclonal anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against total (t) and phospho (p)-Akt, STAT3, and JNK were purchased from Cell Signaling Technology (Beverly, MA). Anti-FN antibodies were purchased from Abcam (Cambridge, United Kingdom). West-Q Chemiluminescent Substrate Plus kit ware obtained from Genedepot (Barker, TX, USA).

Analysis of public database expression data
Expression data were downloaded from a public database (Kaplan-Meier plotter database [http://kmplot.com/breast]) (31). The clinical value of FN levels in patients with luminal type A and B breast cancer was determined by Kaplan-Meier analysis. Hazard ratios with 95% confidence intervals and log-rank P values were calculated.

Establishment of tamoxifen-resistant MCF-7 breast cancer cells
Briefly, MCF-7 cells were washed with PBS, after which the culture medium was changed to phenol red-free DMEM containing 10% charcoal-stripped steroid-depleted FBS and 0.1 μM 4-OHT. The cells were continuously exposed to this treatment regimen for 2 weeks, after which the 4-OHT concentration was increased gradually up to 3 μM over a 9-month period. Initially, cell growth was reduced. However, after exposure to the medium for 9 months, cell growth gradually increased, indicating the establishment of tamoxifen-resistant cells (32).

Cell culture and drug treatment
TamS and TamR breast cancer cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humidified atmosphere with 5% CO2 at 37°C. In the drug treatment experiment, TamR cells were serum starved for 24 h and then treated with specific inhibitors at the indicated concentrations for 24 h.
Western blotting

Cell lysates were prepared to detect t- and p-Akt, STAT3, JNK, FN, and β-actin expression. Equal amounts of proteins (50 μg) were boiled for 5 min in Laemmli sample buffer and then electrophoresed on 8% SDS-PAGE gels. The separated proteins were transferred to PVDF membranes, after which the membranes were blocked with 10% skim milk in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBS/T) for 15 min. The blots were washed three times in TBS/T and then incubated with antibodies against t- or p-Akt, STAT3, JNK, FN, or β-actin in TBS/T buffer at 4°C overnight. The blots were washed three times in TBS/T and subsequently incubated with secondary HRP-conjugated antibodies in TBS/T buffer. After 1 h incubation at room temperature (RT), blots were washed three times in TBS/T. Immunoreactive bands were detected using the West-Q Chemiluminescent Substrate Plus kit.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Isolated RNA samples were then used for RT-PCR. Total RNA (1 μg) was reverse-transcribed into cDNA in 20 μl reaction volumes using a first-strand cDNA synthesis kit for RT-PCR, according to the manufacturer’s instructions (MBI Fermentas, Hanover, MD, USA). Gene expression levels were quantified by real-time PCR using a SensiMix SYBR kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The primer sequences used for this analysis were as follows: human FN (forward, 5’-CCA CCC CCA TAA GGC ATA GG-3’; reverse, 5’-GTA GGG GTC AAA GCA CGA GTC ATC-3’) and GAPDH as an internal control (forward, 5’-ATT GTT GCC ATC AAT GAC CC-3’; reverse, 5’-AGT AGA GGC AGG GAT GAT GT-3’). An annealing temperature of 60°C was used for all primers. PCR was performed in a standard 384-well plate format with an ABI 7900HT real-time PCR detection system (Foster City, CA, USA). For data analysis, the raw threshold cycle (Ct) value was first normalized to the housekeeping gene for each sample to obtain a ΔCt value. The normalized ΔCt value was then calibrated to control cell samples to obtain ΔΔCt values.

Adenovirus induction

Empty (Lac Z) and adenoviral human constitutively active (CA) human AKT cDNA was a gift from Dr. Hyunil Ha (Korea Institute of Oriental Medicine, Daejeon, Korea). The recombinant adenovirus expressing human CA-Akt was produced in 293A cells. TamS cells were transfected with each construct for 24 h, after which cells were incubated for 24 h in fresh culture medium. Vec and CA-Akt-overexpressing TamS cells were further incubated for 24 h in serum-free culture medium. After 24 h of incubation, cell lysates and culture media were harvested and analyzed for FN, β-actin, and p-Akt expression.

Soft agar colony formation assay

TamR breast cancer cells were seeded at a density of 5 × 10⁴ cells/well in 6-well plates in growth medium containing 0.7% agar (1.5 ml/well). Cells were seeded on top of a layer of growth medium containing 1.4% agar (2 ml/well). Next, growth medium (500 μl) with 10% FBS was added on top of the agar. In addition, 1 μM AKT IV was added on top of the agar for some of the plates. Cells were plated and cultured in a 37°C incubator for 2 weeks. After 2 weeks, viable colonies were stained with 0.01% crystal violet and observed using a CK40 inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical significance was determined using Student’s t-test. Results are presented as means ± SEMs. All P values are two-tailed; differences were considered statistically significant for P < 0.05. Statistical analyses were performed using Microsoft Excel.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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