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Corresponding Author: Sangmin Kim

Authors: Sangmin Kim[1,*]

Institution: [1]Breast Cancer Center, Samsung Medical Center,

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Authors: Daeun You[1ϕ], Seung Pil Jung[4ϕ], Yisun Jeong[1], Soo Youn Bae[3], Jeong Eon Lee[1,2,3*], and Sangmin Kim[3*]

ϕThese authors contributed equally to this work.

Affiliations: [1]Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, 50 Irwon-dong, Gangnam-gu, Seoul 06351, Korea, [2]Department of Surgery, Samsung Medical Center, 50 Irwon-dong, Gangnam-gu, Seoul 06351, Korea, [3]Department of Breast Cancer Center, Samsung Medical Center, 50 Irwon-dong, Gangnam-gu, Seoul 06351, Korea, [4]Division of Breast and Endocrine Surgery, Department of Surgery, Korea University Hospital, Korea University College of Medicine, 73 Inchon-ro, Seongbuk-gu, Seoul 02852, South Korea.

Running Title: The regulation of FN expression in Tam R cells

Keywords: Fibronectin, Tamoxifen resistance, Akt pathway, Poor prognosis

Corresponding Authors' Information:

***Sangmin Kim, Ph.D.**

Department of Breast Cancer Center, Samsung Medical Center, 50 Irwon-dong, Gangnam-gu, Seoul 06351, Korea

Tel: 82-2-2148-7312, Fax: 82-2-3410-6982

E-mail: sangmin3005.kim@samsung.com

***Jeong Eon Lee, M.D., Ph.D.**

Department of Surgery, Samsung Medical Center, 50 Irwon-dong, Gangnam-gu, Seoul 06351, Korea

Tel: 82-2-3410-0260, Fax: 82-2-3410-6982

E-mail: paojlus@hanmail.net

ABSTRACT

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.

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 ER-α-positive breast cancer comprise approximately 70% of all patients with breast cancer (2) and have a good prognosis. Specifically, 85% of all patients with ER-α-positive breast cancer survive more than five years after diagnosis. This good 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.

FN is an extracellular matrix glycoprotein that plays an important role in cell-cell adhesion, cell migration, wound healing, and metastasis (8, 9). Abnormal FN expression is associated with poor prognosis in various types of cancer, including breast cancer (9, 10). FN expression is regulated by a variety of growth factors including EGF, TGF-β, and glucocorticoid (10-12). The human FN gene promoter contains specific DNA binding sites, such as TATA box, SP-1, NF-1, and CRE binding sites for nuclear transcription factors that are involved in the modulation of FN transcription (13, 14). Recently, we reported that EGF- and HER2-induced FN expression in breast cancer cells were both significantly decreased by UO126 (9). However, the mechanism by which FN expression is regulated in tamoxifen-resistant breast cancer cells is not fully understood.

The aim of this study was to investigate the mechanism by which FN expression is regulated in TamR cells. We found that aberrant FN expression was associated with poor

prognosis in patients with luminal type A breast cancer. We also found that FN expression was significantly higher in TamR cells than in TamS cells. Furthermore, we found that FN expression was regulated by the PI-3K/Akt pathway in TamR cells.

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 prognosis. To this end, we analyzed DNA microarray-based gene expression data from patients with luminal type A breast cancer using the KM plotter online tool (http://kmplot.com/breast). As shown in Fig. 1A, patients with breast cancer with high FN expression had poor prognoses. Patients with high FN expression showed shorter relapse-free survival ($p=0.023$) than patients with luminal type A breast cancer with low FN expression (Fig. 1A). However, FN expression was not associated with prognosis in patients with luminal type B breast cancer (Fig. 1A).

To assess FN expression and to determine the relationship of FN expression with tamoxifen in luminal type A breast cancer cells, we used TamS and TamR breast cancer cells. Recently, we reported that these two cell types exhibit morphological differences. TamR cells appear in scattered, loosely-packed colonies, and have many branches similar to those of mesenchymal cells (16). The levels of FN mRNA and protein expression were significantly higher in TamR cells (Fig. 1B); specifically, the FN mRNA expression level was 21.1 ± 0.89-fold that of control cells (Fig. 1B). In contrast, E-cadherin expression was lower in TamR cells (Fig. 1B). Therefore, these results indicate that aberrant FN expression is associated with poor prognosis in luminal type A breast cancer.

Akt activity is high in tamoxifen-resistant breast cancer cells

We next investigated FN expression-regulated signaling molecules in TamR cells by analyzing the phosphorylation of several signaling molecules including Akt, JNK, and STAT3 in TamS and TamR cells. As shown in Fig. 2A, the levels of phosphorylated Akt, JNK, and STAT3 were dramatically higher in TamR cells compared with those in TamS cells. We also investigated FN expression levels in the presence of the tumor promoter PMA. As shown in Fig. 2B, PMA triggered Akt and JNK phosphorylation in MCF7 cells. In addition, the basal protein levels of FN were significantly increased by PMA treatment (Fig. 2C) in wild type MCF7 cells. The JNK inhibitor SP600125 did not affect FN expression (data not shown). Based on these results, we suggest that Akt activity is directly or indirectly associated with the regulation of FN expression.

FN expression is regulated by a PI-3K/Akt-dependent pathway in tamoxifen-resistant breast cancer cells

To verify the mechanism by which FN expression is regulated, we treated TamR cells with LY294002 for 24 h. The basal protein level of FN was decreased by LY294002 in both culture media and cell lysates (Fig. 3A). As expected, phosphorylated Akt was not detectable (Fig. 3A). In addition, we investigated the effect of LY294002 on PMA-induced FN expression. As shown in Fig. 3B, PMA significantly increased the mRNA and protein levels of FN in wild type MCF7 cells. Furthermore, the basal level of FN expression was decreased by LY294002 in MDA-MB231 TNBC cells (Fig. 3C). These results demonstrate that a PI-3K/Akt-dependent pathway is associated with the regulation of FN expression in breast cancer cells.

Akt activity plays an important role in FN expression and TamR cell growth

Next, we investigated whether Akt activity was directly associated with FN expression in TamS cells. We transfected cells with CA-Akt for 24 h and further incubated cells in serum-free medium for 24 h. Next, we harvested cell lysates and culture media for detection of FN mRNA and protein expression. As shown in Fig. 4A, TamS cells overexpressing CA-Akt had 2.27 ± 0.44-fold higher levels of FN compared to control cells (Fig. 4A). In contrast, aberrant FN induction was decreased by AKT IV in TamR cells (Fig. 4B). As a positive control, we examined the effect of AKT IV on FN expression in TNBC cells. Basal FN expression was not detected after AKT IV treatment (Fig. 4C). These results demonstrate that Akt activity plays an important role in the regulation of FN expression in TamR cells.

Finally, we used a soft agar colony formation assay to test whether AKT IV affects the clonogenic potential of TamR cells. As shown in Fig. 4D, anchorage-independent growth of TamR cells treated with AKT IV was observed, as evidenced by the significantly reduced number of colonies and colony size compared with untreated cells. In addition, the number of TamR cells did not increase after AKT IV treatment, in contrast to vehicle-treated TamR cells (Fig. 4E). These results indicate that Akt activity is important for maintaining anchorage-independent growth of tamoxifen-resistant breast cancer cells.

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). 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 (22). High glucose upregulates FN protein synthesis via

NF-κB and AP-1 in endothelial cells (23). Moreover, Lee *et al.* reported that PMA induces transcription of FN in hepatoma cells via the PKC pathway (22). 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 overexpressing 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 (24, 25), as well as loss of the lipid phosphatases PTEN and INPP4B (26, 27). Activation of PI3K/Akt has been shown to confer resistance to antiestrogens in various models of breast cancer, including PTEN-deficient cells and mutant AKT1-overexpressing cells (28). 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)] (29). 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 (30).

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% CO_2 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 (C_T) value was first normalized to the housekeeping gene for each sample to obtain a ΔC_T value. The normalized ΔC_T value was then calibrated to control cell samples to obtain $\Delta\Delta C_T$ 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 5×10^4 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.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

None

FIGURE LEGENDS

Figure 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.

Figure 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.

Figure 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, ϕP<0.05 vs. PMA-treated cells. Con, Control; LY, LY294002.

Figure 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.

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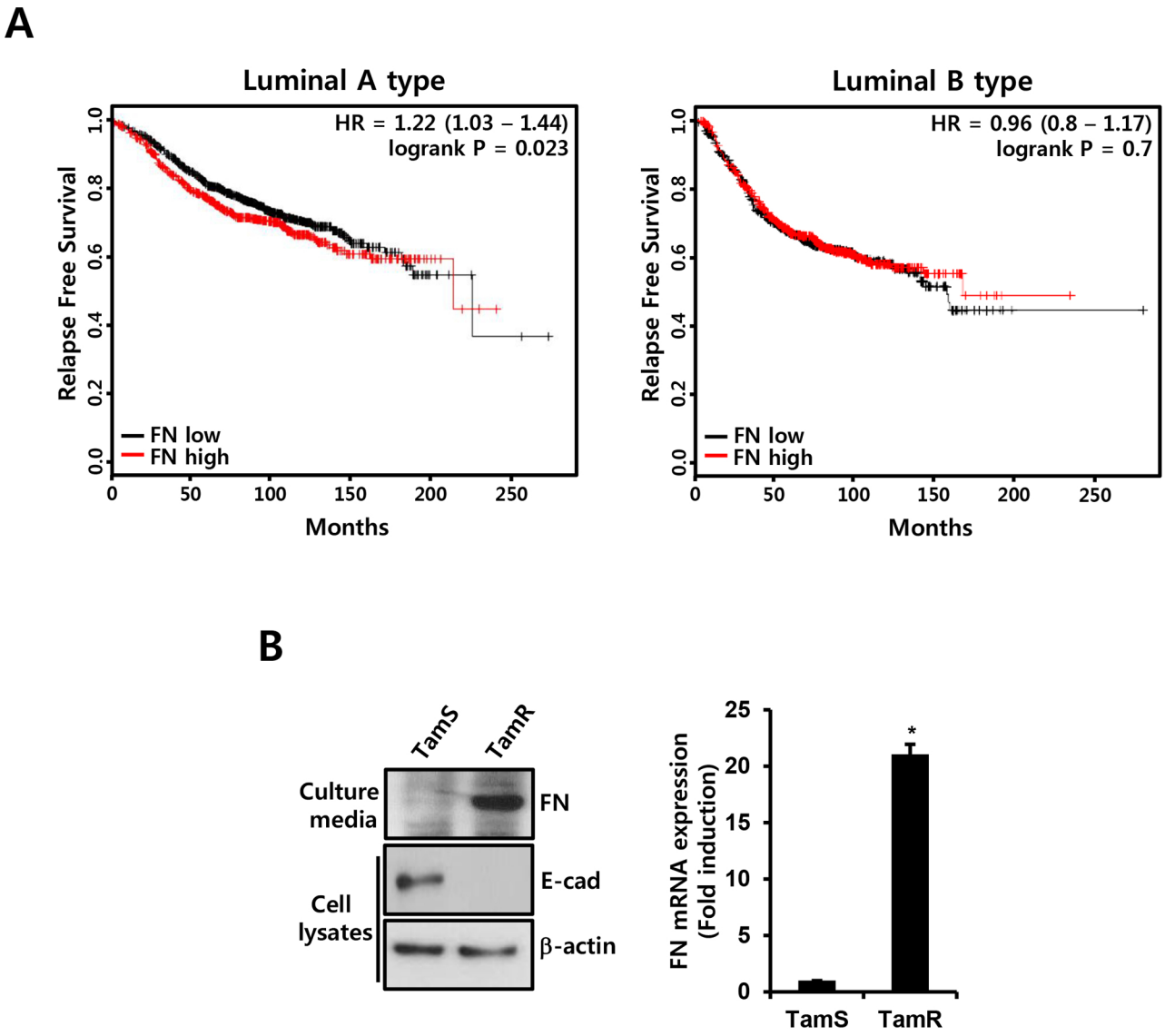


Fig. 1

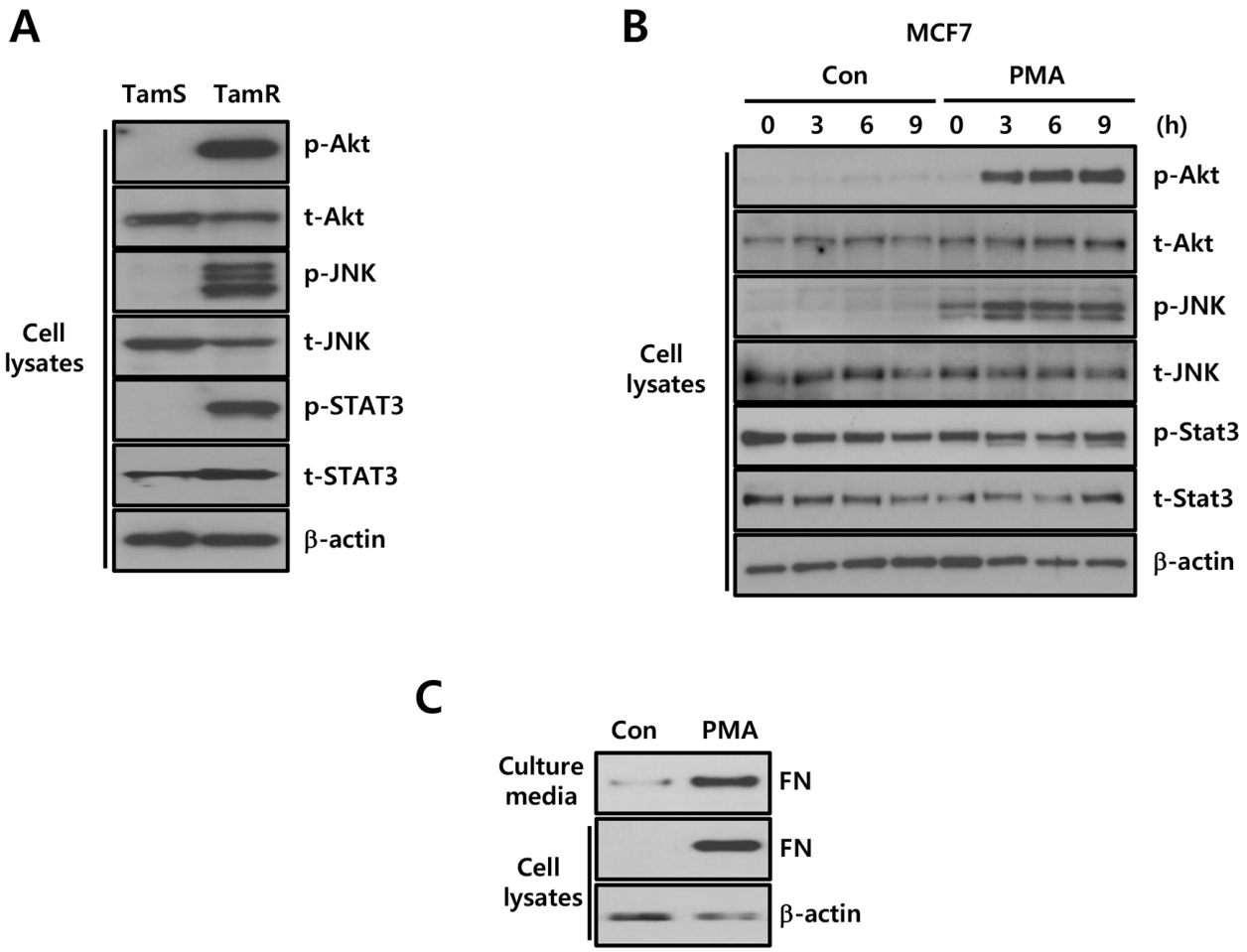


Fig. 2

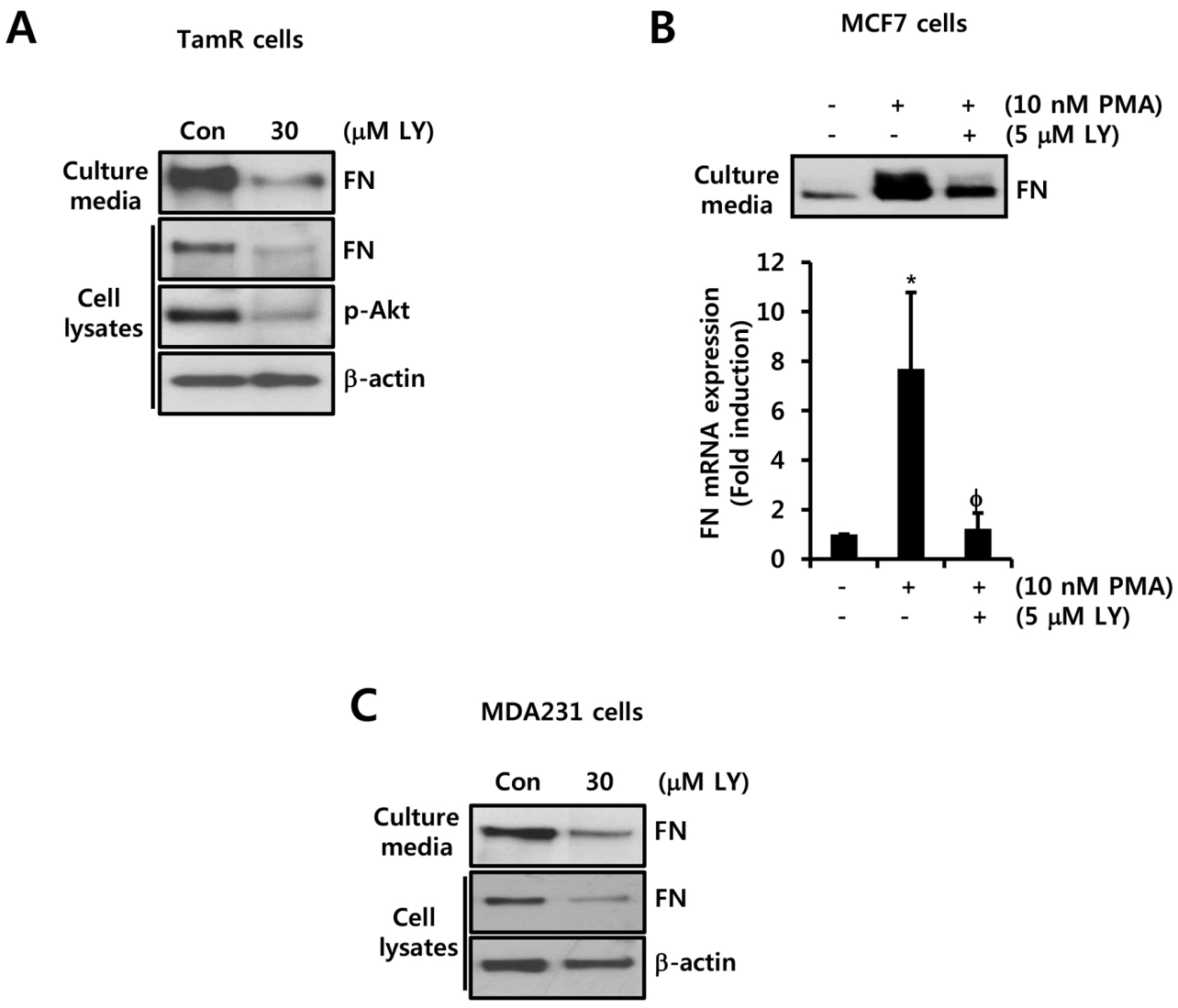
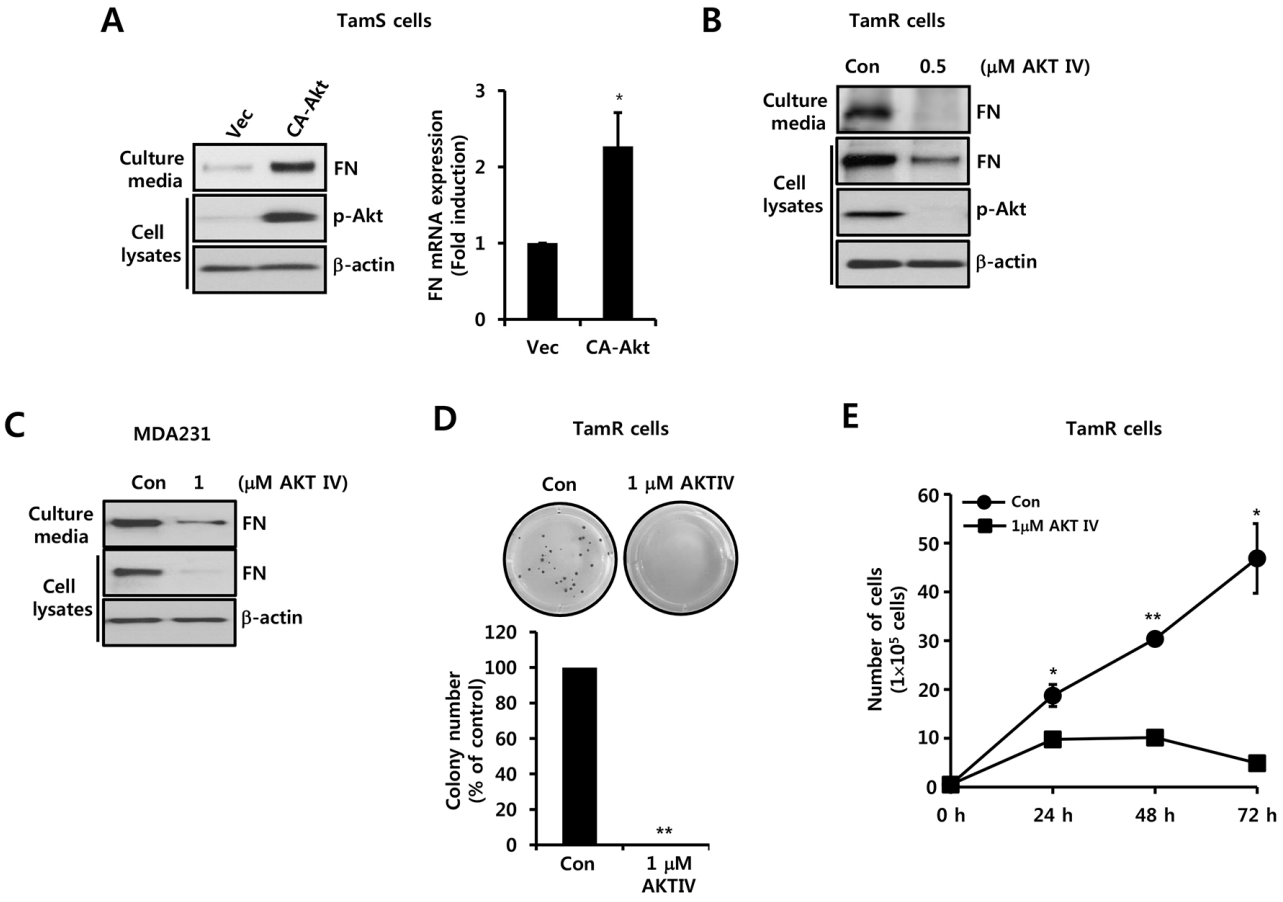


Fig. 3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(μM AKT IV)
Culture media
Cell lysates
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FN
p-Akt
β-actin
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Culture media
Cell lysates
FN
FN
β-actin
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TamR cells
Con
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Colony number (% of control)
Con
1 μM AKTIV
E
TamR cells
Con
1μM AKT IV
Number of cells (1×10⁵ cells)
0 h
24 h
48 h
72 h
F
TamS cells
PI-3K
Akt
FN
TamR cells
Cell invasion
Cell adhesion
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Fig. 4