Abstract. Background/Aim: Clinical trials have shown efficacy of the anti-HER2 monoclonal antibody trastuzumab in metastatic breast cancer patients. The aim of the present study was to elucidate the mechanisms by which up-regulation of fatty acid synthase (FAS) expression confers resistance to trastuzumab in HER2-positive breast cancers. Materials and Methods: The expression of FAS as well as the cytotoxic effects of combinatorial treatment of trastuzumab and juglone was investigated by immunoblotting, BrdU incorporation, TUNEL assay, and soft agar assay. Results: Pin1 enhanced EGF-induced SREBP1c promoter activity, resulting in the induction of FAS expression in BT474 cells. In contrast, juglone, a potent Pin1 inhibitor, significantly enhanced trastuzumab-induced FAS down-regulation and cell death in BT474 cells. Furthermore, trastuzumab, when used in combination with gene silencing or chemical inhibition of Pin1, increased cleaved poly(ADP-ribose) polymerase and DNA fragmentation to increase trastuzumab sensitivity. Conclusion: Pin1-mediated FAS overexpression is a major regulator of trastuzumab-resistant breast cancer growth and survival.

Human epidermal growth factor receptor-2 (HER2), which encodes a tyrosine kinase receptor with unknown ligand, is amplified and/or overexpressed in 25-30% of human breast cancers (1). Its activation follows heterodimerization with members of the epidermal growth factor receptor (EGFR) family and triggers important biological effects such as proliferation, migration and differentiation (2). Since HER2 overexpression is associated with poor prognosis in breast cancers and a significantly shortened disease-free and overall survival, the use of the humanized monoclonal antibody trastuzumab (Herceptin®), selectively directed against the product of this proto-oncogene, has generated enthusiasm for the treatment of HER2-overexpressing tumors (1). However, many patients do not respond to initial trastuzumab treatment (de novo resistance), and many trastuzumab-responsive patients develop resistance after continuous treatment (acquired resistance) (3). The most prevalent de novo resistance mechanism includes constitutive activation of the phosphoinositide 3-kinase (PI3K) pathway owing to phosphatase and tensin homolog (PTEN) deficiency (4) or PI3KCA gene mutations (5), and the accumulation of truncated ERBB2 receptors (p95HER2) (6), which lack an extracellular trastuzumab-binding domain. However, multiple-resistance mechanisms might co-exist in patients with late-stage, heterogeneous, metastatic breast cancer.

The biosynthetic enzyme fatty acid synthase (FAS) is the major enzyme required for the anabolic conversion of dietary carbohydrates to fatty acids, and it functions normally in cells with high lipid metabolism (7). Human tissue studies have demonstrated that infiltrating carcinomas of breast constitutively express high levels of FAS compared to non-transformed human epithelial tissue (8). Accumulating evidence has shown that FAS overexpression clearly contributes to both intrinsic and acquired resistance to many chemotherapeutic drugs, including Herceptin (9). Because HER2-overexpressing cancer cell lines were used in most of these studies, it is thought that HER2 expression might play an important role in FAS-mediated resistance to these drugs. However, a great amount of work remains to be conducted to delineate how FAS regulates gene expression and various cellular processes, which in turn contributes to cellular resistance to chemotherapy and radiation treatment. Although it has become clear that breast cancer cells are dependent upon active FAS-dependent fatty acid synthesis for survival and proliferation, the role of prolyl isomerase Pin1 on breast cancer-associated FAS overexpression and the efficacy of trastuzumab-based chemotherapy have not been studied to date. Here, we demonstrate the first evidence that Pin1 regulates the EGF signaling pathway in such a way as to increase the promoter activity of sterol regulatory element-binding protein-1c (SREBP1c), resulting in overexpression of FAS. In addition, the inhibition of Pin1 using
pharmacological and RNA interference methods negatively-regulated the expression of FAS at the transcriptional level. We further showed that simultaneous targeting of Pin1 and FAS synergistically enhanced trastuzumab sensitivity by promoting apoptosis. Overall, these findings suggest that Pin1 is a valuable molecular target to enhance the efficacy of trastuzumab-based chemotherapy in breast carcinomas.

**Materials and Methods**

*Reagents and antibodies.* Trastuzumab (Herceptin®) was kindly provided by Roche Applied Science (Genentech, IN, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA). Juglone (4-hydroxytamoxifen), 3-[4,5-Dimethylthiazol-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) and C75 were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against FAS, cyclin D1, cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) were from Cell Signaling Tech. Inc. (Beverly, MA). Anti-Pin1 antibody and secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against HER2 was from Dako Cytomation (Glostrup, Denmark). The cationic polymer transfection reagent jetPEI was from Polyplus-transfection (New York, NY, USA). The Dual-luciferase reporter assay kit was purchased from Promega (Madison, WI, USA).

*Cell culture.* BT474 human breast tumor cells were grown in RPMI-1640 medium supplemented with 10% FBS. McCoy’s 5A medium supplemented with 10% FBS was used to maintain SK-BR3 human breast tumor cells. Pin1—/— mouse embryonic fibroblast (MEF) cells, which were kindly provided by Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center, Harvard Medical School), were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. All these cell lines were cultured and maintained at 37°C in humidified air containing 5% CO₂.

*Pin1 expression vector and small interfering RNAs.* Pin1-WT cDNA, which was a gift from Dr. Kun Ping Lu, was sub-cloned into pcDNA4-Xpress (Invitrogen). Human Pin1 (accession number: NM_006221) was silenced by transfecting the cells with the ON-TARGET plus siRNA SMART pool-specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon, Chicago, IL, USA) by using Lipofectamine 2000 (Invitrogen).

*3-[4, 5-Dimethylthiazol-2-thiazolyl]-2, 5-diphenyltetrazolium bromide (MTT) assay.* Cell viability was assessed using the MTT (3-[4,5-Dimethylthiazol-2-thiazolyl]-2,5-diphenyltetrazolium bromide) assay. In brief, the cells were seeded in 96-well plates with 100 μl of cell suspension in each well and incubated at 37°C in a 5% CO₂ atmosphere. After culturing for 24 h, the cells were treated with different concentrations of trastuzumab, for the indicated time. After treatment, MTT solution (5 mg/mL) was added to each well and plates were incubated for 4 h, and absorbance at 450 nm was measured using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

*Detection of apoptosis.* Apoptosis was detected by TUNEL using an *in situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer’s instructions. The cells were seeded and incubated at 37°C in a 5% CO₂ atmosphere. At 30 h after transfection, the cells were serum-starved and treated with trastuzumab for 24 h. The amount of DNA fragmentation was detected using an Axiovert 200M fluorescence microscope with the Axio Vision software (Carl Zeiss, Thornwood, NY, USA).

*Syngeneic tumor model.* Six-week-old female BALB/c mice were obtained from Samtako (Korea). Mouse breast carcinoma cells (4T1) treated with 100 μM trastuzumab or 100 μM juglone combined with 50 μM juglone or 25 μM C75 were injected into the mammary glands of the mice and allowed to grow until tumor formation (14 days). Mice were observed daily for tumor growth. The tumor volume was calculated using the formula V=(ab²)/2, in which ‘a’ is the longest diameter and ‘b’ is the shortest diameter of the tumor.

**Results**

FAS expression is positively correlated with Pin1 expression in breast cancer. To evaluate the correlation between FAS and Pin1 expression, the levels of each protein were evaluated by immunoblot analysis in HER2-positive breast cancer cell lines. FAS and Pin1 protein levels were significantly increased in SK-BR3 cells than in BT474 cells (Figure 1A). Consistent with this result, overexpression of Pin1 in BT474 cells resulted in up-regulation of FAS expression along with an increase in HER2 levels (Figure 1B). However, depletion of Pin1 in the BT474 cells decreased the expression of FAS and HER2 (Figure 1B). We next examined whether inhibition of Pin1 activity could inhibit FAS expression. Treatment with juglone, a potent
Pin1 inhibitor, decreased FAS levels in a dose-dependent manner and down-regulated cyclin-D1 levels (Figure 1C), suggesting that Pin1 regulated FAS overexpression. Reporter gene assays using the human SREBP1c promoter demonstrated an important role for this gene in FAS expression in Pin1+/+ MEF cells (Figure 1D). Therefore, we further examined the role of Pin1 on EGF-induced SREBP1c promoter activity. The result showed that Pin1 enhanced SREBP1c promoter activity when induced by EGF in BT474 cells (Figure 1E). Consistently, Pin1 overexpression in
BT474 cells enhanced SREBP1c promoter activity and FAS following treatment with EGF, unlike what was observed in control cells (Figure 1F). These results support the view that Pin1 regulates FAS overexpression via EGF-induced expression of SREBP1c in breast cancer.

**Pin1 restores trastuzumab-induced FAS down-regulation in BT474 cells.** Previous reports have shown that trastuzumab induces HER2 endocytosis, down-regulation and decreased cell growth upon inhibition of the mTOR signaling pathway (10). Thus, we hypothesized that trastuzumab might down-regulate FAS expression and that Pin1 might restore FAS down-regulation induced by trastuzumab. We first assessed the effect of trastuzumab on the viability of SK-BR3 and BT474 cells. The results showed that BT474 cells were more sensitive to trastuzumab, compared to SK-BR3 cells. SK-BR-3 cells also showed higher FAS levels than BT474 (Figure 1A), suggesting that up-regulation of FAS by Pin1 might be related with trastuzumab resistance of breast cancer cells. Consistent with this result, trastuzumab treatment of BT474 cells significantly decreased the expression of FAS and HER2 compared to the levels of these proteins in treated SK-BR3 cells (Figure 2B and C). Given the role of Pin1 in up-regulating FAS expression, we further assessed whether overexpression or knockdown of Pin1 regulated FAS down-regulation induced by trastuzumab. Therefore, we treated Xpress-Pin1- or siRNA-Pin1-transfected BT474 cells with trastuzumab and performed immunoblotting with an anti-FAS antibody. Results showed that trastuzumab-induced FAS down-regulation was restored in Xpress-Pin1-transfected cells, unlike what was observed in the mock-transfected cells (Figure 2D). Conversely, Pin1-knockdown cells showed lower FAS levels than control cells treated with trastuzumab in a dose-dependent analysis (Figure 2E). Taken together, these results indicate that Pin1 restores trastuzumab-induced FAS down-regulation in BT474 cells.

**Pin1 inhibition enhances trastuzumab sensitivity via downregulation of FAS expression in BT474 cells.** We next examined whether Pin1 would attenuate cell-growth inhibition by trastuzumab. We found that trastuzumab treatment reduced
BT474 cell viability by up to 40%, whereas cells in which Pin1 was overexpressed were resistant to trastuzumab and showed significantly higher viability compared to the control cells (Figure 3A). To further examine whether silencing of Pin1 affected trastuzumab sensitivity, we transfected BT474 cells with siRNA-control or siRNA-Pin1 and treated cells with increasing concentrations of trastuzumab. Pin1 silencing correlated significantly with increased trastuzumab sensitivity compared to the control cells (Figure 3B), suggesting that trastuzumab resistance may be mediated by Pin1 overexpression. Therefore, we examined whether treatment with trastuzumab in combination with juglone increased trastuzumab sensitivity of BT474 cells. Co-treatment with juglone and trastuzumab significantly decreased FAS expression compared to trastuzumab-only treated cells (Figure 3C and D). Consistent with this result, co-treatment with trastuzumab and juglone with or without C75 significantly inhibited the cell viability of BT474 cells, unlike that observed after treatment with trastuzumab-only (Figure 3E). We next examined the effects of Pin1 silencing on trastuzumab-induced cell death by assessment of PARP cleavage. The results showed that trastuzumab induced higher cleavage of PARP after Pin1 silencing in BT474 cells (Figure 3F). In addition, trastuzumab-induced DNA fragmentation was also higher in Pin1-silenced BT474 cells, as measured by the TUNEL assay (Figure 3G).

Co-treatment of trastuzumab with juglone and C75 enhances trastuzumab sensitivity both in vitro and in vivo. Given the important role of Pin1 in regulating FAS expression, a key question is whether Pin1 affects the cellular functions of FAS. Therefore, we examined whether co-treatment with trastuzumab and juglone with or without C75 influenced trastuzumab-induced inhibition of cologenic growth of BT474 cells. Inhibition of Pin1 and FAS activity in BT474 cells resulted in a significant increase in the sensitivity of cells to trastuzumab (Figure 4A and B). The inhibitory effects of juglone and/or C75 with trastuzumab on tumor development in vivo were then studied in a syngeneic mouse 4T1 breast tumor metastasis model. To establish tumors, 4T1 cells were treated with trastuzumab alone or trastuzumab combined with either juglone or C75 and injected into the mammary gland of BALB/c mice. Representative tumor images demonstrated a profound decrease in the tumor volume in mice engrafted with trastuzumab-treated cells, and co-treatment with juglone or C75 significantly enhanced trastuzumab sensitivity, unlike that in the case of trastuzumab treatment alone (Figure 4C and D).

Discussion

The role of FAS in cancer prognosis was discovered in 1994 when oncogenic antigen-519, a molecular marker found in breast cancer patients with markedly worsened prognosis, was identified as FAS (11). Indeed, increased FAS expression/activity has been recognized as a new hallmark of aggressive cancer, representing one of the most frequent alterations in cancer (12). Although the lipid synthesis pathway is identical between normal lipogenic and cancerous tissues, there are differences in the outcome of lipids synthesized by FAS. FAS expression in normal cells is responsible for the synthesis of lipids used mainly for energy storage in the form of triacylglycerol. In cancer cells, however, lipids newly-synthesized by FAS are preferentially metabolized to phospholipids, which can be involved in cell signaling, suggesting that FAS might be an attractive target for novel cancer therapies (13). Moreover, in vitro studies using cancer cell lines with intrinsic or acquired drug resistance show that inhibiting FAS expression or activity sensitizes cancer cells to multiple anticancer treatments including trastuzumab (9), suggesting that there may be multiple mechanisms governing FAS-associated treatment resistance. Therefore, it is important to understand how FAS is regulated at steady-state levels and in response to various extracellular stimuli, and how regulation of FAS might affect chemotherapy resistance. Taken together, our work highlights how Pin1 can facilitate up-regulation of FAS expression via increased activity of the SREBP1c promoter, leading to trastuzumab resistance in HER2-positive breast cancer.

Pin1 regulates a variety of cellular processes, including cell growth, cell-cycle progression, cell transformation, apoptosis and cellular stress response through regulation of large numbers of phosphorylated proteins (14). Due to its role in controlling cell cycle, apoptosis, growth and stress responses, Pin1 has been linked to the pathogenesis of human diseases including cancer (15). Despite the vital role of Pin1 in different signaling pathways, the precise mechanisms of Pin1 in trastuzumab resistance have not been well-explored. In the present study, we found that Pin1 enhanced the EGF signaling pathway to increase SREBP1c promoter activity, resulting in overexpression of FAS. These results define a Pin1 signaling mechanism that conveys an oncogenic signal to promote aggressiveness in human breast cancer through induction of FAS expression. Interestingly, it was reported that Pin1 regulates the co-repressor silencing mediator of retinoid and thyroid hormone receptor (SMRT) and steroid receptor coactivator-3 (SRC-3) as downstream effectors of HER-2 signaling (16), which is often increased in endocrine-resistant breast tumors and contribute to activate proliferation and/or survival and hormone resistance (17). Indeed, we recently demonstrated that Pin1 facilitates the progression of breast cancer to tamoxifen resistance via induction of microtubule-associated protein-1 light chain-3 (LC-3) expression, a hallmark of autophagy (18). Moreover, it was reported that protective autophagy regulates primary resistance to trastuzumab, and that addition of chloroquine to trastuzumab-based regimens may significantly improve outcomes among women with autophagy-addicted HER2-positive breast cancer (19).
Given that Pin1 increased FAS expression levels via induction of SREBP1c promoter activity, we hypothesize that Pin1 may play an important role in acquired trastuzumab resistance. In the present study, we showed that trastuzumab treatment significantly decreased the expression of FAS along HER2 levels in HER2-positive breast cancer cells. However, trastuzumab-induced FAS down-regulation was restored with Pin1 overexpression,
whereas depletion of Pin1 enhanced FAS down-regulation induced by trastuzumab. Consistent with these results, employing a combined treatment of trastuzumab with juglone, a Pin1 inhibitor, synergistically enhanced the sensitivity of trastuzumab via induction of PARP cleavage and DNA fragmentation in HER2-positive breast cancer cells. Based on these findings, this newly-identified mechanism provides a scientific basis for the combination of trastuzumab and juglone to overcome chemoresistance in breast cancer.

Conflicts of Interest

None declared.

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References


