Tamoxifen-stimulated growth of breast cancer due to p21 loss


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Tamoxifen is widely used for the treatment of hormonally responsive breast cancers. However, some resistant breast cancers develop a growth proliferative response to this drug, as evidenced by tumor regression upon its withdrawal. To elucidate the molecular mediators of this paradox, tissue samples from a patient with tamoxifen-stimulated breast cancer were analyzed. These studies revealed that loss of the cyclin-dependent kinase inhibitor p21 was associated with a tamoxifen growth-inducing phenotype. Immortalized human breast epithelial cells with somatic deletion of the p21 gene were then generated and displayed a growth proliferative response to tamoxifen, whereas p21 wild-type cells demonstrated growth inhibition upon tamoxifen exposure. Mutational and biochemical analyses revealed that loss of p21’s cyclin-dependent kinase inhibitory property results in hyperphosphorylation of estrogen receptor-α, with subsequent increased gene expression of estrogen receptor-regulated genes. These data reveal a previously uncharacterized molecular mechanism of tamoxifen resistance and have potential clinical implications for the management of tamoxifen-resistant breast cancers.

The lack of model systems that faithfully represent a tamoxifen-induced growth phenotype has hindered the elucidation of molecular mechanisms responsible for this particular form of drug resistance. We have previously developed and characterized an in vitro model of human breast epithelial cells that demonstrated physiologic ER signaling (16). These cell lines are growth-stimulated by estrogens, and this effect is blocked by tamoxifen and the ER down-regulator ICI 182,780. We have also demonstrated that both genomic and nongenomic functions of ER signaling are present in these cell lines. In the current study, we exploit these cells and their p21 knockout counterparts to elucidate a molecular mechanism of how loss of p21 function can lead to tamoxifen-stimulated growth in human breast epithelial cells. Our results demonstrate that loss of CDK inhibition mediated by p21 leads to hyperphosphorylation of ER at serine 118, which in turn leads to the increased expression of known ER-regulated genes. Furthermore, mutation of ER serine 118 to alanine abrogates the tamoxifen growth-stimulatory phenotype. Finally, a tamoxifen-resistant clone derived from the ER-positive breast cancer cell line MCF-7 demonstrates a similar ER serine 118 hyperphosphorylation upon tamoxifen exposure. This study demonstrates how loss of p21 function can lead to aberrant ER phosphorylation resulting in an estrogenic growth response to tamoxifen.

Results

Case Report.

The ability to study drug resistance in breast cancer has become increasingly difficult, because earlier diagnosis and better treatments have reduced the ability to obtain tissue samples of recurrent and metastatic disease. In addition, recurrent and metastatic sites of disease are currently rarely biopsied. However, we identified a patient with metastatic breast cancer who had two separate recurrences in unusual organ sites, necessitating biopsies for confirmation. A 55-year-old female was...
initially diagnosed in 1991 with a 1.0-cm ER-positive, progesterone receptor (PR)-positive invasive lobular carcinoma of the left breast. HER2/neu status was not evaluated. Her metastatic work up was negative, and she underwent a left modified radical mastectomy with axillary lymph node dissection of 11 nodes showing no evidence of disease. She received no further therapy. In 2000, the patient presented with left lower quadrant abdominal pain and Hemoccult-positive stools. A colonoscopy revealed a large stricture in the transverse colon without obvious mass. The patient underwent an exploratory laparotomy, and the transverse colon was found to be encased by tumor, with implants also present in the ascending colon and a portion of the proximal descending colon. A partial colectomy was performed with ileal-to-distal colonic anastomosis. The pathology of the specimen was consistent with infiltrating metastatic lobular carcinoma that was ER-positive and HER2/neu-negative by immunohistochemistry (IHC), although there were areas devoid of p21 labeling in all biopsied tumor specimens as described previously (13, 14). We initially examined p21 expression in all biopsied tissue specimens as described (15). As seen in Fig. 1B, the majority of cancer cells in the primary tumor displayed strong labeling for p21 protein by immunohistochemistry (IHC), although there were areas devoid of p21 protein expression. The recurrent colonic lesion also demonstrated strong p21 labeling; however, cancer cells without p21 expression were still present. In contrast, the metastatic skin lesion was completely devoid of p21 labeling. The fact that the patient initiated tamoxifen therapy after resection of the colonic lesion suggested that the loss of p21 expression was selected for, leading to the emergence of a drug-resistant population. Although p53 is frequently mutated in breast cancers and can directly affect gene expression of p21 (17, 18), IHC labeling was consistent with wild-type p53 and genomic sequencing of p53 was negative for mutations (data not shown). Similarly, sequencing of the two coding exons of p21 in the metastatic lesions revealed only wild-type alleles. This result was expected because p21 is rarely mutated in human breast cancers (19), and epigenetic silencing of the p21 gene through promoter methylation and histone modifications has been described as a mechanism for p21 loss of expression in human cancers and cell lines (20–25).

**In Vitro Modeling.** Although the complete loss of p21 expression in the metastatic skin lesion suggested the selection of a tamoxifen-resistant population, it remained possible that these two events were unrelated. To definitively prove that loss of p21 leads to tamoxifen-induced cell growth, we used somatic cell gene targeting, or “knockout,” of the p21 gene using a published model of ER-positive nontumorigenic human breast epithelium (16). This system utilizes the MCF-10A human breast epithelial cell line engineered to overexpress an ER cDNA, which we have termed ERIN cells for estrogen receptor in nontumorigenic cells. The use of this cell line as a model for physiologic ER signaling is advantageous over ER-positive human cancer cell lines for a number of reasons, including: (i) the cells are genetically stable by karyotype and microsatellite analyses (data not shown and ref. 26), facilitating valid comparisons between knockout cells and their parental counterparts; (ii) ER-positive breast cancer cell lines have known mutations in oncogenes that may influence tamoxifen resistance (27), whereas the MCF-10A cell line does not (28); (iii) there is little concern that the cells have been exposed to hormonal and/or chemotherapeutic agents before adaptation to cell culture; (iv) preexisting populations of tamoxifen-resistant cells can easily be selected for in most human breast cancer cell lines, obscuring comparisons between genetically manipulated cells and their control counterparts; and (v) MCF-10A cells can be readily arrested in the G1 phase of the cell cycle by withdrawal of epidermal growth factor (EGF), making any proliferative effects of estrogen or tamoxifen obvious and entirely attributable to these reagents. Thus, we used our ERIN cells and created their p21 knockout counterpart, estrogen receptor in knockouts (ERIK) as described (15, 16). Two independently derived stable clones were established and further analyzed. Similar to ERIN cells (16), ERIK cell lines displayed levels of ER protein akin to those found in the ER-positive breast cancer cell line MCF-7 [supporting information (SI) Fig. 5].

Cell proliferation assays were performed as described by using ERIN and ERIK cells with various ER ligands (16). These results demonstrated that p21 null ERIN cells not only were resistant to tamoxifen but also were growth-stimulated by this SERM (Fig. 24). This proliferative effect was statistically significant by cell count analysis (SI Table 1, P < 0.0001) recapitulating the phenotype described in our case report. To ensure that ER was truly mediating this growth effect, ERIN and ERIK cells were also cultured with estrogen and ICI 182,780. ICI 182,780 is a pure antiestrogen that mediates its effects by down-regulating ER expression (29). As expected, ERIN and ERIK cells no longer grew with estrogen when cocultivated with ICI 182,780. ERIK cells cultured in tamoxifen and ICI 182,780 also failed to proliferate, demonstrating the necessity of ER expression for tamoxifen-induced growth in these cell lines (SI Fig. 6).

We previously demonstrated that ERIN cells have physiologic ER signaling by analyzing the expression of genes known to be regulated by estrogen bound to ER (16). Similar gene-expression analysis via RT-PCR was performed for ERIN cells with estrogen and tamoxifen. ERIN cells demonstrated increased expression of the ER-regulated genes PR and pS2 with either estrogen and/or tamoxifen (SI Fig. 7). In contrast, ERIN cells increased transcription of these genes only with estrogen, and this effect was blocked upon tamoxifen exposure. These results are con-
cDNA but not the p21 cdk reduced in ERIK cells transiently transfected with wild-type p21. Additionally, ER serine 118 phosphorylation was markedly phosphorylated at serine 118 showed that ERIK cells became resistant (SI Fig. 8) and then grown with 10 nM 17β-estradiol (E) and 1 μM 4-OH-tamoxifen (T) and stained with crystal violet after six days to assess growth. Results are representative of three independent experiments.

We then demonstrated that this tamoxifen-induced growth phenotype was reversible in ERIK cells by restoration of p21 expression. Transient transfection of wild-type p21 restored tamoxifen sensitivity to ERIK cells (Fig. 2B). Because transfection efficiencies are not 100%, only partial restoration of tamoxifen sensitivity was achieved by using wild-type p21. Nevertheless, by cell count analysis, there was ~2-fold less growth, which was highly statistically significant (SI Table 2, P < 0.0001). We next mutated DNA sequences encoding three amino acids in the CDK-binding domain of the p21 cDNA (p21 cdk−) and transfected this construct into ERIK cells as described (30). Importantly, this cDNA retains other functional properties of p21 (31), and transfection efficiencies were equivalent among wild-type p21, p21 cdk−, and empty vector controls as demonstrated by our group (SI Fig. 8A) and by others (30). In contrast to wild-type p21, the vector control and mutant p21 cdk− constructs had no effect on tamoxifen resistance (Fig. 2B and SI Table 2, P < 0.9). These experiments demonstrated that the CDK inhibitory activity of p21 was responsible for mediating the agonistic versus antagonistic effects of tamoxifen.

Cyclin/CDK complexes are known to phosphorylate ER at critical serine residues (32), and several groups have recently demonstrated that ER serine phosphorylation by various kinases can convert tamoxifen from an antagonist to an agonist in vitro (9, 10, 12). We therefore compared the phosphorylation status of ER in ERIN versus ERIK cells exposed to tamoxifen. As seen in Fig. 3A, Western blotting using an antibody specific for ER phosphorylated at serine 118 showed that ERIK cells became hyperphosphorylated in response to tamoxifen, whereas ERIN cells displayed only a small amount of phosphorylated ER. Additionally, ER serine 118 phosphorylation was markedly reduced in ERIK cells transiently transfected with wild-type p21 cDNA but not the p21 cdk− mutant upon tamoxifen exposure (SI Fig. 8B). To prove that ER serine 118 is critical for mediating tamoxifen-induced cell growth, a serine-to-alanine (S118A) mutation was introduced within an ER cDNA construct and transfected into p21 null ER-negative MCF-10A cells (15). This mutant ER no longer mediated cell growth upon tamoxifen exposure but still retained the ability to stimulate cell proliferation with estrogen, although this response was somewhat muted compared with wild-type ER (Fig. 3B). In addition, gene expression of PR did not increase upon tamoxifen exposure, although a slight increase in the pS2 gene was noted (SI Fig. 7). This may reflect an altered response of the mutant ER because some pS2 gene transcription was noted at baseline in the absence of ER ligand.

Although our nontumorigenic model allows for a rigorous study to ascribe genetic changes to a growth response, there are limitations of our system. For example, forced expression of genes in an in vitro cell culture is not always reflective of physiologic levels of expression and/or function. However, we have previously extensively demonstrated that our ERIN cellular model accurately recapitulates physiologic ER signaling and that levels of ER are similar to levels found in breast cancer cell lines (16). Nonetheless, our system employs noncancerous cells that have a mostly normal karyotype and therefore have signal transduction pathways that may be more akin to normal breast epithelium than to tumor cells. Consequently, it was important to establish whether our findings were applicable to naturally occurring ER-positive breast cancer cells or unique to our system. To confirm our results in a human breast cancer cell model, we exploited the well known property of preexisting tamoxifen-resistant clones found in commonly used breast cancer cell lines (33, 34). We isolated several drug-resistant clones from the ER-positive human breast cancer cell line MCF-7 after prolonged exposure of these cells (4–6 weeks) to tamoxifen. One of these clones, TAM1, displayed a growth proliferative response to this SERM in low (1%) serum conditions (data not shown), similar to the work of others (34). This clone also exhibited hyperphosphorylation of ER serine 118 upon tamoxifen exposure (Fig. 4A). Characterization of the p21 status in this cell line revealed markedly reduced levels of p21 compared with parental MCF-7 cells. Furthermore, by separating phosphorylated from nonphosphorylated proteins and then concentrating the resulting lysates for Western blot analysis, we found that the majority of p21 within TAM1 cells was phosphorylated, whereas in
subjected to Western blot analysis to assess protein levels of total ER, serine phosphorylated ER (ER Ser118), total p21, and β-actin as described in Materials and Methods. Nonphosphorylated (Unbound) p21 and phosphorylated (Bound) p21 proteins were separated, and the collected fractions were further concentrated for Western blot analysis of p21 protein as described in Materials and Methods. (B) Immunohistochemical labeling for ER (Total ER) and serine 118 phosphorylated ER (ER Ser118) in metastatic recurrent lesions of the colon during which time the patient was not receiving tamoxifen (−), versus the metastatic lesion during which time the patient was on tamoxifen therapy (+). Arrowheads denote tumor nuclei.

Discussion

In this study, we elucidate the role of p21 in mediating a particular form of tamoxifen resistance. Withdrawal of tamoxifen causing tumor regression has been previously described (36). However, the ability to study the molecular mechanisms of this phenomenon has been limited, because recurrent disease is rarely biopsied, thereby restricting tissue samples for analysis. Because invasive lobular carcinoma often metastasizes to unusual organ sites, our patient’s two separate recurrences were biopsied to confirm the diagnosis. This afforded us the opportunity to analyze tissue specimens chronologically to uncover the molecular responses that mediate the antagonistic versus agonistic effects of tamoxifen. Although we had access to only a single patient’s tissues demonstrating tamoxifen’s agonistic properties in resistant disease, several references spanning from the 1970s to the 1990s have documented that tamoxifen-induced increase in tumor growth is clinically relevant and not isolated to in vitro models and rare cases.

The molecular mechanisms of tamoxifen resistance are still not fully understood, but recent literature suggests a common theme of tamoxifen-induced ER serine phosphorylation leading to the recruitment of nuclear hormone coregulators and increased expression of ER-regulated growth-promoting genes (9, 10, 12). There have been a number of molecules implicated in ER serine phosphorylation including protein kinase A, HER2/neu, and Pak1 (9, 10, 12). In particular, these studies highlight the importance of ER phosphorylation at serine 118 and serine 305 that can lead to an ER agonistic response upon tamoxifen exposure. Interestingly, HER2/neu has also been shown to phosphorylate p21 via AKT, thereby excluding it from the nucleus and abating its growth-inhibitory properties (35). Our case report highlights a unique mechanism of tamoxifen growth stimulation whereby p21 loss leads to hyperphosphorylation of ER serine 118 via unopposed cyclin/CDK activity. Although loss of p21 expression in breast cancers can occur at high frequency (15), studies correlating loss of p21 expression with clinical outcome have been conflicting (38). One reason for this may be that loss of p21 is a predominant mechanism of acquired, but not de novo, tamoxifen resistance. In contrast, recent clinical studies examining cellular localization of p21 have found a correlation between cytoplasmic p21 and a poor prognosis (39, 40), suggesting that p21 loss of function rather than loss of expression may also be an important determinant of drug resistance. Our experiments using TAM1 cells also support the argument that p21 phosphorylation can lead to ER serine 118 hyperphosphorylation and tamoxifen-induced growth.

The CDK inhibitors p21 and p27 have been implicated in mediating the growth responses to estrogens and tamoxifen in the MCF-7 breast cancer cell line (14). In this study, transfection of antisense oligonucleotides against p21 or p27 resulted in reduced levels of the respective proteins. Although growth assays were not performed, and therefore tamoxifen stimulated growth not evaluated, examination of cell-cycle parameters revealed that reduction of either p21 or p27 led to an increased number of cells in S phase after inducing cell-cycle arrest with estrogen deprivation or tamoxifen exposure. Contrary to our results, this study also demonstrated that p21 or p27 antisense oligonucleotides could also increase the number of cells in S phase after cell-cycle arrest induced by the pure antiestrogen ICI 182,780. The reason for this discrepancy is unclear, although it likely stems from differences in the methods and measured parameters used in their study versus our own. However, this same group has recently reported that phosphorylation of p27 can mediate its degradation, leading to a relative increase in tamoxifen resistance in vitro (41). Thus, these studies combined with our data collectively demonstrate the importance of CDK inhibitors in regulating the growth response to tamoxifen.

Although other molecular mechanisms have been shown to directly or indirectly phosphorylate ER and lead to tamoxifen-induced growth of breast cancer in vitro, this report demonstrates that loss of p21 can reverse the cellular response to this SERM from antagonistic to agonistic. It is possible and likely that other mechanisms of tamoxifen resistance exist. However, the cumulative data from these studies point to aberrant phosphorylation of ER serine residues as a final common pathway leading to tamoxifen resistance/stimulated growth. Therefore, the development of therapies directed against phosphorylated ER may prove beneficial in the prevention and treatment of tamoxifen resistance.

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Materials and Methods

Cell Culture Conditions. MCF-10A and MCF-7 cells were purchased from American Type Culture Collection. MCF-10A cells were grown in media as described (15), and subconfluent DMEM supplemented with 5% FBS and 1% penicillin/streptomycin. All cell lines in this study were grown in charcoal-stripped dextran-treated serum (HyClone) to reduce exogenous hormones and growth factors, and phenol red-free medium, unless otherwise indicated. All cells were cultured at 37°C at 5% CO2. Culturing and harvesting cells for protein and RNA was performed as described (16). Briefly, MCF-10A and derivative cell lines were seeded at equal density in EGF-free assay medium to arrest cells in G0. The next day, media were changed to EGF-free assay medium containing ethanol, 10 nM 17-β-estradiol (Sigma), 1 μM 4-OH-tamoxifen (Sigma), 1 μM ICI 182,780 (Tocris), or combinations of these drugs as indicated. After 24 h, media were changed and allowed to incubate for an additional hour before RNA isolation or preparation of cell lysates. Parental MCF-7 and clones of MCF-7 cells were cultured and harvested as above, except that cells were plated in 1% serum-containing medium for 3–5 days.

ERIK and TAM1 Cell Lines. The derivation of p21 null MCF-10A cells has been described (15). An ER cDNA previously used to generate ERIN cells was used to stably transfict two independent clones of p21 null MCF-10A cells to obtain ERIK cell lines, although the vector was modified to incorporate the puroycin-resistance gene (16). Transfections were performed as described for ERIN cells except cells were selected on puroycin at 0.4 μg/ml. TAM1 cells were generated by culturing parental MCF-7 cells for 4 weeks in phenol red-free medium containing 5% charcoal-stripped dextran-treated serum and 1 μM 4-OH-tamoxifen, followed by single-cell dilution.

Proliferation Assays. MCF-10A cells and their respective clones were plated at low confluence in assay medium for 24 h. MCF-10A cells were then growth-stimulated in the presence of 10 nM 17-β-estradiol, ethanol, 1 μM 4-OH-tamoxifen, 1 μM ICI 182,780, or combinations of these drugs as indicated for 4–12 days. Media were replaced daily until the time of harvest. On specified days, cells were harvested with trypsin, diluted in sodium azide-free Diluent 2 (Nerl Diagnostics), and then counted by using a Beckman–Coulter Z1 Coulter Counter. To visualize differences in cell proliferation, cells were also stained by using crystal violet as described (15).

Western Blot Analysis. Cells were cultured as above, harvested, and then lysed by the addition of Laemmli Sample Buffer (Bio-Rad) for 5 min. Cell lysates were then heated at 100°C for 10 min. Equal amounts of cell lysates were electrophoresed by using the NuPage XCell SureLock electrophoresis system (Invitrogen). Separated proteins were then transferred to a PVDF membrane and blocked for 1 h. A monoclonal mouse IgG antibody was used as the primary antibody against total ER (Cell Signaling Technology), followed by a goat anti-mouse-HRP antibody. To stain for ER phosphorylated at serine 118, a monoclonal mouse IgG antibody (Cell Signaling Technology) was used, followed by a goat anti-mouse-HRP antibody. A monoclonal mouse IgG antibody was used against p21 (Abcam), followed by a goat anti-mouse antibody as above. Blots were exposed to Kodak XAR film by using chemiluminesence for detection (PerkinElmer).

Fractionation of Phosphorylated Proteins. Cells were cultured and harvested as above, and the nonphosphorylated (Unbound) and phosphorylated (Bound) (Fig. 4A) cellular proteins were separated by using the TALON PMAC Phosphoprotein Enrichment kit (Clontech) as per the manufacturer’s recommendations. The collected nonphosphorylated (Unbound) and phosphorylated (Bound) fractions were further concentrated for Western blot analysis by using Centricron centrifugal filters (Millipore). Western blotting was then performed as described above.

cDNA Synthesis and RT-PCR Analysis. Cells were cultured as above. Cells were then washed twice with HBSS and harvested with TRIzol reagent (Invitrogen). Total RNA was isolated by using the RNeasy system (Qiagen). Single-stranded cDNA was generated by using the First Strand cDNA Synthesis kit (Amersham Biosciences) following the manufacturer’s directions. Control template reactions were prepared in parallel without the addition of reverse transcriptase. RT-PCR was then performed as described (15). Primers used to amplify pS2 and PR cDNA were the forward/reverse primer pairs 5′-ctagtagaagaaagcag-3′/5′-attaggataaagacacgg-3′ and 5′-aaacaggtctatgc-3′/5′-agtctatgcaatcctggg-3′, respectively. As an internal control, GAPDH cDNA was amplified by using the forward primer 5′-caggtagaagaaagcag-3′ and the reverse primer 5′-agctctctcttaggctg-3′. After amplification, PCR products were separated by gel electrophoresis, stained, and visualized with UV light. PCR cycling conditions are available upon request.

Mutation of ER cDNA. Wild-type ER cDNA was used as template for PCR mutagenesis to change serine 118 to alanine as described (42). Forward and reverse primers were used for PCR mutagenesis were 5′-ggctttttgccctgcctcttgc-3′ and 5′-ccctataagctgcttgaac-3′, respectively. The PCR fragment was digested with BglII and cloned back into the parental ER cDNA vector (16). The presence of the desired mutation and the absence of unwanted mutations were verified by direct sequencing of the cloned products.

Transient Transfections. Transient transfections and growth assays were performed as described (15, 16, 30). Briefly, empty vector, wild-type p21 cDNA, and a mutant p21 cDNA devoid only of CDK inhibitory function were transiently transfected into ERIK cells with FUGENE6 (Roche Diagnostics) following the manufacturer’s recommendations and then grown under EGF-free conditions with and without 10 nM 17-β-estradiol or 1 μM 4-OH-tamoxifen as described above. Cells were then cultured, counted, and stained as described above.

Immunohistochemical Staining. Immunohistochemical labeling was performed on sections from paraaffin-embedded blocks as described (15). Briefly, 4-μm sections were deparaffinized in xylene for 30 min and rehydrated by using graded ethanol concentrations. After antigen retention, immunohistochemical labeling of p21 (cat. no. 554228; PharMingen, Becton Dickinson), total ER (Cell Signaling Technology), or ER phosphorylated at serine 118 (Cell Signaling Technology) was performed by using the avidin–biotin peroxidase complex technique and 3′,3′-diaminobenzidine (DAB) as chromagen (Ventana/Biotek Solutions). Sections were then counterstained with hematoxylin and eosin.

Statistical Methods. To estimate differences across cell lines and conditions, a linear regression model was used. The outcome variable was the natural log of cell counts, and predictors were (i) the main effects of cell line (or transfection), (ii) main effects of treatment (or condition), and (iii) the interactions between cell line and treatment effects. Log ratios and differences in log ratios were estimated by taking linear combinations of coefficients in the linear regression model. Statistical significance and 95% confidence intervals were determined for linear combinations by estimating standard errors of linear combinations via the variance–covariance matrix of the regression coefficients. Estimates of ratios and their 95% confidence intervals were calculated by exponentiating the estimates from the log scale. Assumptions of linear regression model were assessed by using scatterplots and residual plots.

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Supporting Information, Figure 5

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67 kDa
36 kDa
Supporting Information, Figure 6

Vehicle Control
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ICI
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Supporting Information, Figure 7
Supporting Information, Figure 8

A

![Bar chart showing fold change relative to GAPDH](chart1.png)

B

![Western blot images](blot1.png)

- Total ER
- ER Ser118
- GAPDH