FKBPL Regulates Estrogen Receptor Signaling and Determines Response to Endocrine Therapy

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Abstract

The HSP90 chaperone and immunophilin FKBPL is an estrogen-responsive gene that interacts with estrogen receptor α (ERα) and regulates its levels. In this study, we explored the effects of FKBPL on breast cancer proliferation. Breast cancer cells stably overexpressing FKBPL became dependent on estrogen for their growth and were dramatically more sensitive to the antiestrogens tamoxifen and fulvestrant, whereas FKBPL knockdown reverses this phenotype. FKBPL knockdown also decreased the levels of the cell cycle inhibitor p21WAF1 and increased ERα phosphorylation on Ser118 in response to 17β-estradiol and tamoxifen. In support of the likelihood that these effects explained FKBPL-mediated cell growth inhibition and sensitivity to endocrine therapies, FKBPL expression was correlated with increased overall survival and distant metastasis-free survival in breast cancer patients. Our findings suggest that FKBPL may have prognostic value based on its impact on tumor proliferative capacity and sensitivity to endocrine therapies, which improve outcome. Cancer Res; 70(3); 1090–100. ©2010 AACR.

Introduction

The steroid hormone estrogen has a pivotal role in tumor development (1), with increased levels of circulating estrogen being linked with initiation and progression of breast cancer (2–4). Estrogen receptors (ER) are members of the nuclear receptor superfamily of ligand-activated transcription factors that mediate the biological effects of estrogen (5). Ligand binding causes the receptor to dimerize and activate or repress transcription of ER target genes through recruitment of coregulators (6). Around two thirds of breast tumors express ERα and are therefore dependent on estrogen for their growth. Anticancer drugs such as tamoxifen exert their anti-tumor activity through binding ERs, blocking the interaction between estrogen and its receptor (7), and altering its conformation, leading to corepressor recruitment to the complex (8, 9). This results in inhibition of ERα-regulated genes that promote tumor growth. However, despite the success of this group of agents in the clinic, almost half of the patients with ER-positive tumors do not respond to hormone therapy and 30% to 40% of patients who initially respond positively relapse within 5 years. Therefore, other proteins involved in ER signaling need to be assessed as potential targets for therapeutic intervention.

We have previously isolated, and partially characterized, FKBPL (DIR1/Wisp39). We showed that it belongs to the immunophilin/FKBP protein family as a consequence of the presence of COOH-terminal tetratricopeptide repeat domains (TPR) and a partial peptidyl-prolyl cis-trans isomerase (PPIase) domain (10, 11). The TPR domains of immunophilin/FKBP protein family are particularly important for binding to the molecular chaperone Hsp90 (12). In support of this association, FKBPL has been implicated in Hsp90-dependent stabilization of newly synthesized p21 by preventing its proteosomal degradation (13); also, overexpression of FKBPL inhibits cell cycle progression at the G0-G1 phase in leukemic cells (14).

In breast cancer, immunophilins and other cochaperones have been shown to influence ER signaling. To maintain receptor stability in the absence of ligand, ERs exist in a molecular complex with Hsp90, p23, and the immunophilins FKBP52 or Cyp40 (16). The immunophilins competitively bind Hsp90 through TPR domains (12), and cochaperone recruitment is a dynamic process that controls steroid receptor activity in a tissue-specific manner. Cyp40 and FKBP52 are overexpressed in breast tumors (17) and are upregulated transcriptionally and posttranscriptionally by estrogen, whereas exposure to the pure estrogen antagonist ICI 182,780 (fulvestrant) prevents this estrogen-mediated...
increase (18). A recent study has found that the FKBP52 gene is methylated in ER-negative MDA-MB-231 but not in ER-positive MCF7 cells, suggesting that repression of FKBP52 may itself affect ER expression (19). Furthermore, Hsp90 and immunophilin ligands have been shown to target ER for proteasomal degradation (20). The Hsp90 cochaperone p23 also plays an important role in ER signal transduction (21) and regulates the ER target genes p52 and cathepsin D, promoting tumor cell adhesion and invasion (22).

Hsp90 and its cochaperones clearly play a role in ER signaling; however, a role for FKBPL in these ER-associated complexes has not yet been described. Understanding the possible role of this immunophilin in the Erα-Hsp90 complex might shed further light on ER signaling. This is especially important because the biological mechanisms underlying de novo and acquired tamoxifen resistance have not been fully elucidated. Previous studies have linked endocrine therapy resistance with increased activity of epithelial growth factor receptor (EGFR), human epidermal growth factor receptor 2, and phosphatidylinositol 3-kinase pathways; modification of ER phosphorylation status; deregulation of cell cycle proteins (23); and inhibition of CDK10 (24) and BRCA1 (25). Here, we show that FKBPL is an estrogen-inducible gene that acts as a cochaperone in ERα/Hsp90 molecular complexes; furthermore, FKBPL levels may be both a prognostic indicator and determinant of response to endocrine therapy.

Materials and Methods

Cell culture and transfections. The ER-positive cell lines MCF7 and T47D were obtained from Cancer Research UK and American Type Culture Collection, respectively, and maintained as monolayers in DMEM (Invitrogen) supplemented with 10% FCS. Cell lines were authenticated by Mycoplasma-free. All experiments were carried out between passages 1 and 15. Transient transfections were carried out using either empty vector pcDNA3.1 (as a control) or pcDNA3.1/FKBPL plasmids and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Stable transfects of MCF7 cells were generated by transfecting cells with pcDNA3.1/FKBPL plasmid using Lipofectamine Plus according to the manufacturer’s instructions. Transfected cells were selected using 750 μg/mL G418 (Sigma) and maintained in DMEM with 375 μg/mL G418. In studies requiring estrogen deprivation, cells were maintained in phenol red–free DMEM and lysed in buffer [20 mmol/L Tris-HCl (pH 7.4), 1% Igepal, 12 mmol/L sodium deoxycholate, 0.1% SDS, 10 mmol/L sodium molybdate, 1 protease inhibitor tablet] for 30 min on ice, then pelleted by centrifugation at 13,000 rpm for 5 min at 4°C. One fifth of the lysate was removed to a fresh tube for use as whole cell lysate positive control for the Western blots. The remaining lysates were precleared by incubating with prewashed agarose G beads for 1 h at 4°C with rotation and then lysates were incubated with Hsp90, FKBPL, ERα, or IgG (negative control) antibody–bound Protein G-Sepharose beads (Cancer Research UK) at 4°C overnight. The beads were washed three times in ice-cold lysis buffer twice in ice-cold PBS then resuspended in 2× Laemmli buffer. Western blot analysis was then carried out.

Western blot analysis. Samples were subjected to SDS-PAGE electrophoresis using the XCell Surelock Mini-Cell System (Invitrogen), transferred onto nitrocellulose membranes, blocked for 1 h at room temperature with 1% skim milk blocking solution, and probed with FKBPL rabbit polyclonal (1:2,000; ProteinTech), Hsp90 (1:1,000; BD Transduction Laboratories), ERα rabbit monoclonal (1:1,000; Millipore), cathepsin D mouse monoclonal (1:1,000; Abcam), phospho-ERα (Ser118) mouse (1:1,000; Cell Signalling Technologies), p21 mouse monoclonal (1:1,000; Upstate), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal (1:5,000; Sigma) antibodies. Blots were probed with anti-rabbit or anti-mouse IgG horseradish peroxidase–linked whole antibody (secondary; 1:5,000; GE Healthcare). Antibody binding was detected using Supersignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions.

Clonogenic assay/growth assays. Cells were plated at a density of 500 and 1,000 per well in a six-well plate containing phenol red–free DMEM + 10% charcoal-stripped FCS or DMEM + 10% FCS and incubated at 37°C for 24 h. The medium was replaced with phenol red–free DMEM + 10% charcoal-stripped FCS containing DMSO as vehicle control or 10−8 mol/L 17β-estradiol (Sigma) or DMEM + 10% FCS containing cell line IC50 doses of tamoxifen (Sigma) or fulvestrant (Sigma) and incubated under normal conditions for Mammalian two-hybrid assay. The CheckMate Mammalian Two-Hybrid System (Promega) was used to assess protein interactions. In-frame cloning of full-length FKBPL into the pBIND-GAL4 binding domain plasmid and Hsp90 into the herpes simplex viral protein 16 (pACT-VP16) activation domain plasmid were carried out according to the manufacturer’s instructions. MCF-7 cells at 60% to 80% confluency were transfected with either of these plasmids or the pACT-MyoD and pBIND-Ld positive control plasmids along with the firefly lucerase pG5 luc plasmid using Lipofectamine Plus according to the manufacturer’s instructions. After 24 h, cells were analyzed using the Dual-Glo Luciferase Assay System (Promega). Firefly lucerase activity was normalized to Renilla lucerase activity and presented as relative light units. Three independent experiments were performed with triplicates for each experiment.

Coinmunoprecipitations. MCF7 or 3.1D2 cells were grown to 90% confluency, washed twice with ice-cold PBS, and lysed in buffer [20 mmol/L Tris-HCl (pH 7.4), 1% Igepal, 10 mmol/L sodium deoxycholate, 0.1% SDS, 10 mmol/L sodium molybdate, 1 protease inhibitor tablet] for 30 min on ice, then pelleted by centrifugation at 13,000 rpm for 5 min at 4°C. One fifth of the lysate was removed to a fresh tube for use as whole cell lysate positive control for the Western blots. The remaining lysates were precleared by incubating with prewashed agarase G beads for 1 h at 4°C with rotation and then lysates were incubated with Hsp90, FKBPL, ERα, or IgG (negative control) antibody–bound Protein G-Sepharose beads (Cancer Research UK) at 4°C overnight. The beads were washed three times in ice-cold lysis buffer twice in ice-cold PBS then resuspended in 2× Laemmli buffer. Western blot analysis was then carried out.
16 d. For growth assays, 1 × 10^5 cells were seeded into 35-mm dishes with DMEM + 10% FCS and incubated at 37°C. Cell growth was monitored at 24-h intervals using the trypan blue exclusion assay.

**Gene expression analysis.** Publicly available microarray data sets from National Center for Biotechnology Information Gene Expression Omnibus (26–30) were used for gene expression analysis (Table 1). The analysis was performed using Partek Genomics Suite (Partek, Inc.) and Kaplan-Meier curves were generated using GraphPad Prism version 5.00 (GraphPad Software). The raw microarray data were imported into Partek GS and processed using Robust Multichip Average (RMA) to generate the normalized expression values. Expression values for the probe set 219187_at representing FKBPL were assessed for normal distribution using Kolmogorov-Smirnov test and for the probe set 219187_at representing FKBPL were assessed using one-way ANOVA. Expression analysis results, one-way ANOVA was used.

**Results**

**FKBP1 associates with ERα in the Hsp90 chaperone complex.** We hypothesized that FKBPL might bind Hsp90 within ER chaperone complexes in breast cancer cells, especially because we had already identified a role for this protein in glucocorticoid/Hsp90 complexes (15). Using the mammalian two-hybrid assay, the expression constructs pBIND-FKBPL and pACT-Hsp90 were cotransfected into ER-positive MCF-7 cells and the binding ability of FKBPL with Hsp90 was assessed by measuring luciferase activity derived from a GAL4-driven luciferase reporter. Following normalization for transfection efficiency by measurement of Renilla luminescence, luciferase expression in cotransfected cells was induced by 19.5-fold and 4.7-fold compared with FKBPL and Hsp90 self-activation controls, respectively, indicating that FKBPL binds Hsp90 in breast cancer cells (Fig. 1A). To confirm this interaction, immunoprecipitations were carried out. FKBPL immunoprecipitates from parental MCF7 cells showed that endogenous FKBPL coprecipitated with Hsp90 (Fig. 1B). The molar ratio of Hsp90/FKBPL is low, suggesting that FKBPL is probably not the dominant immunophilin within Hsp90 chaperone complexes. Furthermore, we were able to detect an interaction between FKBPL and ERα in MCF7 parental cells (Fig. 1C) and FKBPL stably overexpressing (3.1D2) cells (Fig. 1D). In estrogen-free conditions, FKBPL immunoprecipitates showed that ER did not coprecipitate with FKBPL and ER immunoprecipitates showed that FKBPL coprecipitated very weakly, suggesting that the presence of ligand may be required for this interaction to occur (Supplementary Fig. S1). These results locate FKBPL within the Hsp90/ER molecular chaperone complex.

**FKBP1 inhibits breast cancer cell growth and sensitizes cells to estrogen deprivation.** Previous studies have suggested that FKBPL overexpression can slow the growth of lymphoma cells (14). To assess the relevance of these results to breast cancer biology, we assessed cell growth in MCF-7 cells transiently and stably overexpressing FKBPL. Transient transfection of MCF-7 cells with pcDNA3.1/FKBPL resulted in a significant decrease in clonogenic survival compared with cells transfected with an empty vector control (P < 0.05; Fig. 2A). In addition, pcDNA3.1/FKBPL was stably transfected into MCF7 cells and clones selected for G418 antibiotic resistance (showing low and high FKBPL expression) were analyzed for FKBPL expression by Western blot (Fig. 2B). The 3.1D2 and 3.1D3 clones showed a 7-fold and 5-fold increase in FKBPL protein levels compared with

### Table 1. Microarray data sets

<table>
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<tr>
<th>Accession no.</th>
<th>No. of samples</th>
<th>Treatment</th>
<th>ER ratio (−/+</th>
<th>Microarray platform</th>
<th>FKBPL correlates with survival</th>
<th>Reference</th>
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<tr>
<td>GSE7390</td>
<td>198</td>
<td>No</td>
<td>64/134</td>
<td>Affymetrix U133A</td>
<td>Yes P = 0.004 (OS) P = 0.001 (DMFS)</td>
<td>Desmedt and colleagues (26)</td>
</tr>
<tr>
<td>GSE2034</td>
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<td>No*</td>
<td>0/209</td>
<td>Affymetrix U133A</td>
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<td>Wang and colleagues (27)</td>
</tr>
<tr>
<td>GSE9195</td>
<td>77</td>
<td>Yes</td>
<td>0/77</td>
<td>Affymetrix U133A</td>
<td>Nonsignificant trend P = 0.33</td>
<td>Loi and colleagues (28)</td>
</tr>
<tr>
<td>GSE2990</td>
<td>64</td>
<td>Yes</td>
<td>0/64</td>
<td>Affymetrix U133A Plus 2.0</td>
<td>No</td>
<td>Sotiou and colleagues (29)</td>
</tr>
<tr>
<td>GSE1378</td>
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<td>Yes†</td>
<td>0/60</td>
<td>Arcturus 22k oligo</td>
<td>No</td>
<td>Ma and colleagues (30)</td>
</tr>
</tbody>
</table>

Abbreviations: OS, overall survival; DMFS, distant metastasis-free survival.

*Patients did not receive tamoxifen but some patients received radiotherapy treatment.

†Patients received radiation before tamoxifen treatment.
parental cells, respectively. Stable clone 3.1D9 showed minimal overexpression with a 2-fold increase at the protein level. These clones displayed inhibition of cell growth across a 4-day time course (Fig. 2B). Compared with parental controls, the low-level overexpressing clone 3.1D9 showed intermediate growth inhibition, which was significantly different from parental MCF7 cells at 72 hours (P < 0.05) and 96 hours (P < 0.05). The high overexpressing clones 3.1D2 and 3.1D3 showed the greatest growth inhibition, which was statistically significant from parental MCF7 (P < 0.001) and 3.1D9 cells (<0.05) at 48 hours and highly significantly different from parental cells at 72 and 96 hours (P < 0.001). This FKBP1-overexpression mediated inhibition of cell growth was therefore not clonal, as independent FKBP1-overexpressing clones displayed the same phenotype.

FKBP1 overexpression clearly affected the growth potential of MCF-7 cells under normal growth conditions. Next, we sought to determine whether FKBP1 overexpression could affect growth in estrogen-deprived conditions. In these experiments, we chose to use the MCF-7 clone overexpressing FKBP1 maximally, 3.1D2, and compared the effects with the parental MCF-7 cell line. High levels of FKBP1 resulted in a 10-fold reduction in clonogenic survival compared with parental cells (P < 0.001), suggesting a strong dependency on estrogen for growth. Furthermore, even in the presence of physiologic levels of estrogen, FKBP1 overexpression inhibited the clonogenic potential by 50% compared with parental cells (P < 0.01; Fig. 2C).

FKBP1 sensitizes cells to tamoxifen and fulvestrant. Because increased expression of FKBP1 sensitized cells to low estrogen levels, we further extended the study to assess the effect of high FKBP1 levels on response to endocrine therapy. Tamoxifen and ICI 182,780 (fulvestrant) IC50 values for MCF7 and T47D cells were determined through clonogenic assay. In MCF7 cells, 1 μmol/L tamoxifen and 2.5 nmol/L fulvestrant were sufficient to cause a 50% reduction in colony survival, and in T47D cells, 0.3 μmol/L tamoxifen produced a 50% inhibition of clonogenic survival. These doses were used in all subsequent experiments. MCF-7 parental and three clones that overexpress FKBP1—3.1D2, 3.1D3, and 3.1D9—were treated with the IC50 dose and clonogenic survival was determined after 16 days. Following treatment with tamoxifen (1 μmol/L), clonogenic survival in MCF7 cells was reduced by 46% compared with untreated controls (Fig. 3A). FKBP1 overexpression rendered cells more sensitive to tamoxifen, and these responses were not clonal, as independent FKBP1-overexpressing clones displayed the same phenotype.

In 3.1D2, 3.1D3, and 3.1D9 cell lines, clonogenic survival was reduced by 89%, 75%, and 66%, respectively, compared with corresponding untreated controls (Fig. 3A). Furthermore, the MCF-7 clone overexpressing FKBP1 maximally, 3.1D2, showed a dose-dependent increase in sensitivity compared with parental controls (Supplementary Fig. S2). The enhanced sensitivity to fulvestrant was even more dramatic in FKBP1-overexpressing cell lines (Fig. 3B). Parental MCF7 cells showed a 54% reduction in clonogenic survival compared with untreated control, whereas 3.1D2 displayed a reduction of 99% compared with untreated control. 3.1D3 and 3.1D9 displayed a 93% and 80% decrease compared with untreated control, respectively (Fig. 3B). In support of a role for FKBP1 in increasing the sensitivity to endocrine therapies, a targeted knockdown approach was used to determine if these effects could be reversed. Transfection of parental MCF-7 (Fig. 3C) and another ER-positive breast cancer cell line, T47D (Fig. 3D), with FKBP1-targeted siRNA increased their resistance to tamoxifen compared with the nontargeting siRNA controls. Following treatment with 0.1 μmol/L tamoxifen, FKBP1 siRNA-transfected MCF7 cells showed a 30% increase in clonogenic survival compared with nontargeted siRNA-transfected cells (P < 0.001; Fig. 3C). Following
FKBPL knockdown in T47D cells, treatment with 0.3 μmol/L tamoxifen increased clonogenic survival by 21% compared with the nontargeted control (P < 0.01; Fig. 3D). In summary, the data suggest a correlation between FKBPL levels and sensitivity to endocrine therapy in two ER-positive cell lines.

Finally, we showed that the levels of endogenous FKBPL in a range of breast cancer cell lines, including the parental MCF-7, T47D, and ZR-75-1 cells along with two MCF-7 cells clones stably overexpressing FKBPL, correlated (R² = 0.76) with sensitivity to tamoxifen (Supplementary Fig. S3A). When the ER-negative cell line MDA-231 was included, the correlation went down to R² = 0.6443 (Supplementary Fig. S3B).

FKBPL is an estrogen-inducible gene that inhibits ERα expression and modifies cathepsin D and p21 expression. To understand the mechanism relating to the FKBPL-mediated regulation of cell growth and sensitivity to tamoxifen, we first sought to investigate whether FKBPL itself was regulated by estrogen. A previous study (31) has already identified non-ER-binding estrogen response elements within the FKBPL promoter, and expression of immunophilins and other cochaperones are modulated by estrogen (18). We therefore investigated estrogen-mediated regulation of FKBPL in breast cancer cells. MCF7 cells were treated with 10⁻⁸ mol/L 17β-estradiol over a 48-hour time period. FKBPL levels increased and reached a maximum by 24 and 48 hours (Fig. 4A). We also determined whether modulating FKBPL levels could subsequently affect ERα levels. Using the stable clone 3.1D2, we showed that ERα levels decreased compared with parental controls, whereas FKBPL knockdown in both parental and 3.1D2 cells led to an increase in ERα levels (Fig. 4B). Furthermore, FKBPL overexpression caused a decrease in levels of the ER-responsive gene, cathepsin D, whereas knockdown increased the levels (Fig. 4C). The regulation of this ER-responsive gene supports a functional role for FKBPL in physiologic ER-mediated signaling. Finally, because FKBPL has already been identified as essential for the stabilization of newly synthesized p21 (13), we therefore measured p21 levels. Following FKBPL knockdown, a 5-fold decrease in p21 levels compared with nontargeting controls was detected (P < 0.001), whereas transient overexpression of FKBPL led to a 2.5-fold increase in p21 protein expression (P = 0.056; Fig. 4D). This is the first time this has been shown in breast cancer cells.

FKBPL increases ERα phosphorylation on Ser118 following treatment with estrogen or tamoxifen. Loss of p21 expression, as observed in FKBPL knockdowns, has previously been shown to cause hyperphosphorylation of ER on Ser118, leading to an increase in expression of ER-regulated genes and increased resistance to tamoxifen (32). To further examine the effects of FKBPL modulation on ER signaling, cells were treated with 10⁻⁸ mol/L 17β-estradiol (Fig. 5A) or tamoxifen (Fig. 5B) for 30 minutes and ERα phosphorylation on Ser118 was evaluated. As expected, phospho-Ser118 ER levels were increased by 98% and 141% in MCF-7 cells.
transfected with a targeted FKBPL siRNA compared with nontargeting controls after treatment with estrogen or tamoxifen, respectively. Moreover, in the 3.1D2 FKBPL-overexpressing clone, Ser118 phosphorylation was decreased by 55% in DMSO control and 28% with 17β-estradiol treatment compared with parental control (Fig. 5C). Following tamoxifen treatment, Ser118 phosphorylation in 3.1D2 was decreased by 50% in untreated control and 30% in treated sample compared with parental controls (Fig. 5D). In addition, overexpression of FKBPL inhibited ligand-independent Ser 118 phosphorylation of ER, which may also affect endocrine therapy sensitivity.

High FKBPL expression correlates with improved patient survival. We have established the downstream molecular consequences resulting from FKBPL expression that may enhance growth arrest and sensitization to endocrine agents in breast cancer. We reasoned that FKBPL may carry prognostic power resulting from the effects on ER levels, ER phosphorylation, and p21 stabilization, which might affect the tumor proliferative capacity and improve outcome independent of ER status. To assess the clinical significance of our in vitro data, we investigated FKBPL mRNA levels in several microarray data sets: GSE7390 (26), GSE2034 (27), GSE9195 (28), GSE2990 (29), and GSE1378 (ref. 30; Table 1). We saw no correlation in the GSE2990 and GSE1378 data sets. However, a trend toward significance was observed in Wang and colleagues’ data set (GSE9195; tamoxifen treated); in ER-positive samples, high FKBPL expression was associated with a trend toward increased survival (data not shown). Furthermore, in the GSE2034 data set, higher p21 levels were associated with statistically significant longer time to relapse (data not shown). However, in the Desmedt microarray data set (198 node-negative/134 ER-positive untreated patients), high FKBPL expression was highly correlated with increased overall survival (hazard ratio, 2.456; 95% confidence interval, 1.335–4.520; log-rank test P = 0.0039, Fig. 6A) and distant metastasis-free survival (hazard ratio, 2.647; 95% confidence interval, 1.476–4.748; log-rank test P = 0.0011; Fig. 6B). These data suggest that FKBPL levels in breast cancer may correlate with outcome.

Discussion

FKBPL has previously been linked with the GR/Hsp90 chaperone complex, with implications for GR translocation and signaling (15). We have now determined that FKBPL is also involved in the ERα/Hsp90 chaperone complex with consequences for downstream estrogenic signaling and response to endocrine therapy.

Strict regulation of ERα levels is essential to maintain appropriate cellular responsiveness to estrogen (33, 34) and the Hsp90 molecular complex has previously been implicated in this process (35, 36). In the present study, we have identified

Figure 3. FKBPL levels determine sensitivity to endocrine therapies. Clonogenic assay to determine surviving fraction in MCF7, 3.1D2, 3.1D3, and 3.1D9 cells treated with (A) 1 μmol/L tamoxifen or (B) 2.5 nmol/L fulvestrant (*, P < 0.05; **, P < 0.01, compared with treated MCF7 cells, one-way ANOVA). MCF7 (C) or T47D (D) cells transfected with nontargeted or FKBPL-specific siRNA and treated with 1 or 0.3 μmol/L tamoxifen (TAM), respectively. (FKBPL knockdown was shown by Western blot; **, P < 0.01; ***, P < 0.001, one-way ANOVA).
FKBPL as a member of the Hsp90 complex that binds ER in the presence of estrogen. Similar to the immunophilsins Cyp40 and FKBP52 (18), 17β-estradiol treatment upregulated FKBPL levels. Moreover, high FKBPL expression decreased protein levels of ERα and the ER-responsive gene, cathepsin D, whereas FKBPL knockdown studies reversed this effect. The ubiquitin ligase CHIP has previously been identified as a cochaperone of the Hsp90-ERα complex (37). CHIP overexpression decreases ERα levels, whereas CHIP knockdown reverses the effect (36)—similar effects to what we have seen with FKBPL. More recently, the cochaperone serine/threonine protein phosphatase 5 has been identified as an estrogen-inducible protein that increases estrogen-dependent tumor growth (38).

Previously, FKBPL overexpression has been shown to decrease the rate of proliferation of leukemic U937 cells by delaying cells in the G0-G1 phase of the cell cycle (14). In breast cancer cells, FKBPL overexpression led to increased levels of the CDK inhibitor p21 and an inhibition of proliferation and clonogenicity. This growth delay could be due to the role of FKBPL in stabilizing newly synthesized p21 (13) and suggests a role for FKBPL in cell cycle progression that has not yet been fully elucidated. More recently, siRNA targeted knockdown of ERα has been shown to increase p21 transcription and protein expression (39), consistent with the effects shown by FKBPL overexpression. Here, we show that higher FKBPL was linked with better survival outcome and increased distant metastasis-free survival in three of five clinical data sets. Data sets GSE7390 and 2034 (untreated patients) show a significant correlation and a trend toward significance, respectively, between high FKBPL levels and improved survival, suggesting that FKBPL prognosticates for outcome. This fits with our in vitro data in which we show an FKBPL-induced growth delay and an increase in p21 levels. The GSE9195 data set from patients who were treated with...
tamoxifen showed that high FKBPL correlated with a trend toward significance, suggesting that FKBPL might be a potential predictive marker of response, which fits with our in vitro data; high or low FKBPL resulted in sensitivity or resistance to tamoxifen, respectively. The final two data sets, GSE1378 and GSE2990 (from tamoxifen-treated patients), did not show any correlation, but the patient numbers are lower, especially in the GSE1378 data set, which also used a different microarray platform and microdissected tumor cells rather than whole tumor tissues. A lack of concordance between data sets is a common feature in microarray studies (40), but this could also be because GSE2034 and GSE1378 patients were treated with radiotherapy and FKBPL has previously been shown to be regulated by radiation (10, 11). Furthermore, the data sets showing no correlation were generally smaller in size; the number of samples is known to affect statistical significance in this type of study. The assessment of FKBPL expression at the mRNA level provides a clinical context for our in vitro findings. However, transcriptional effects only partly correlate with protein levels; therefore, further validation in a large randomized trial with assessment of FKBPL at the protein level using tissue microarrays would be required to fully validate our in vitro findings. However, in support of our hypothesis, a recent study showed that the loss of chromosome 6p21.32 region, containing FKBPL, occurred more frequently in patients with cancer recurrence within 5 years of initial diagnosis (41).

As mentioned, FKBPL seems to be an important determinant of response to endocrine therapies in an in vitro setting.
Overexpression of FKBPL increased sensitivity to estrogen deprivation, tamoxifen, and fulvestrant, whereas FKBPL knockdown using a targeted siRNA approach increased resistance to tamoxifen. Furthermore, FKBPL levels correlated with the intrinsic tamoxifen sensitivity exhibited in breast cancer cells, with cell lines that had high levels of FKBPL displaying increased sensitivity to tamoxifen (Supplementary Fig. S3). In accordance with our observations of increased ERα expression following FKBPL knockdown, high ERα levels have been associated with estrogen-independent signaling and endocrine therapy resistance (42). In addition, elevated cathepsin D levels in patient samples have been linked with early relapse (43) and reduced survival (44). FKBPL overexpression inhibited ligand-independent Ser118 phosphorylation, suggesting that FKBPL may affect growth factor receptor– or mitogen-activated protein kinase (MAPK)–driven ERα phosphorylation, although this remains to be elucidated. These results suggest that FKBPL may have a direct impact on ER signaling and therefore on response to endocrine therapy, which may be partially linked to FKBPL-mediated effects on p21. A previous study found that p21 expression was essential for tamoxifen and fulvestrant to mediate cytostatic effects and that loss of p21 could be a major cause of antiestrogen resistance in breast cancer (45, 46). Moreover, loss of p21 enhanced the agonistic effects of tamoxifen through hyperphosphorylation of ERα (32) and elevated Ser118 phosphorylation mediated through the EGFR/MAPK pathway was identified in tamoxifen-resistant cell lines (47). However, there is some debate over the prognostic benefit of Ser118 phosphorylation for response to tamoxifen. It has been linked with ligand-independent activation of ERα (48) and patient relapse following tamoxifen treatment (49). In contrast, elevated expression of phosphorylated ER has been associated with low tumor grade (49) and improved outcome in patients treated with hormone therapies (50).

Our results support a model summarized in Fig. 6C, in which repression of FKBPL would lead to low levels of p21 and hypophosphorylation of ERα at Ser118, hence lowering sensitivity to tamoxifen and possibly resulting in an increase in expression of ER-regulated genes, such as cathepsin D. Conversely, high levels of FKBPL would convey a more slowly proliferating tumor phenotype and reduce ERα phosphorylation, abrogating tamoxifen-induced agonist activity and thereby increasing sensitivity to tamoxifen.

Figure 6. High FKBPL expression correlates with improved patient survival. FKBPL expression was analyzed using microarray data from publicly available data set (GSE7390). Kaplan-Meier survival curves of breast cancer patients (n = 198 node negative; 134 ER positive) that have low FKBPL expression (defined as the lowest quartile expression) had significantly reduced (A) overall survival and (B) distant metastasis-free survival. C, schematic representation of proposed FKBPL role in ERα phosphorylation.
FKBPL Modulates the Estrogen Receptor Signaling Pathway

In summary, we provide evidence that FKBPL is involved in ER stability and signaling with subsequent implications for the growth of ER-responsive tumors and their sensitivity to endocrine therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Grant Support

Breast Cancer Campaign, United Kingdom, and Action Cancer Northern Ireland.

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Received 7/6/09; revised 11/3/09; accepted 11/18/09; published OnlineFirst 1/26/10.

www.aacrjournals.org
Cancer Res; 70(3) February 1, 2010
1099

Published OnlineFirst January 26, 2010; DOI: 10.1158/0008-5472.CAN-09-2515

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Cancer Res Published OnlineFirst January 26, 2010.

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doi:10.1158/0008-5472.CAN-09-2515

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