

Priority Report

Genetic Screen Identifies Insulin-like Growth Factor Binding Protein 5 as a Modulator of Tamoxifen Resistance in Breast Cancer

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Abstract

Tamoxifen resistance is one of the overarching challenges in the treatment of patients with estrogen receptor (ER)-positive breast cancer. Through a genome-wide RNA interference screen to discover genes responsible for tamoxifen resistance *in vitro*, we identified insulin-like growth factor binding protein 5 (*IGFBP5*) as a determinant of drug sensitivity. Specific knockdown of *IGFBP5* by retroviral infection with short hairpin RNA-expressing cassette in MCF7 human breast cancer cells (pRS-shIGFBP5) conferred tamoxifen resistance *in vitro* due to concomitant loss of *ER α* expression and signaling. *IGFBP5* expression was also reduced in MCF7 cells selected for tamoxifen resistance in culture (TAMR). Both tamoxifen-resistant MCF7-TAMR and MCF7-pRS-shIGFBP5 cells could be resensitized to drug by treatment with exogenous recombinant IGFBP5 (rIGFBP5) protein. Treatment with rIGFBP5 protein in mouse tumor xenografts reversed the *in vivo* tamoxifen resistance of MCF7-pRS-shIGFBP5 cell-derived tumors by reducing tumor cell proliferation. IGFBP5 immunohistochemical staining in a cohort of 153 breast cancer patients showed that low IGFBP5 expression was associated with shorter overall survival after tamoxifen therapy. Thus, IGFBP5 warrants investigation as an agent to reverse tamoxifen resistance. *Cancer Res*; 70(8); 3013–9. ©2010 AACR.

Introduction

Tamoxifen, a selective estrogen receptor (ER) modulator, is the most widely used chemotherapeutic in the treatment of ER-positive breast cancer (1). In spite of this, intrinsic and acquired resistance remains the main challenge to effective treatment with tamoxifen (2).

Antitumor effects of tamoxifen are mediated by competitive inhibition of estrogen binding to ERs (3), resulting in inhibition of estrogen-regulated genes including growth and angiogenic factors and cell cycle arrest (4) or induction of programmed cell death (5). ER loss of expression, mutation, and phosphorylation, overexpression of growth factor receptors [epidermal growth factor receptor, human epidermal growth factor receptor 2, and insulin-like growth factor receptor I (IGF-RI)], and activation of their downstream effectors [Akt/PKB, extracellular signal-regulated kinase (ERK)-1/2, and p38] and adaptors (Cas, c-Src, and BCAR-3) have been reported to be associated with tamoxifen resistance (2). Never-

theless, underlying mechanisms are poorly understood and few strategies have found success in the clinic in circumventing tamoxifen resistance. Therefore, the identification of new genes involved in tamoxifen resistance is an essential step toward improving breast cancer patient survival.

We performed an unbiased loss-of-function genetic screen on MCF7 human breast cancer cells using a short hairpin RNA (shRNA) library that identified insulin-like growth factor binding protein 5 (IGFBP5) as a modulator of tamoxifen sensitivity *in vivo*, thus revealing a new strategy for reversal of tamoxifen resistance.

Materials and Methods

Materials. MCF7 cells (American Tissue Culture Collection) were cultured in DMEM supplemented with 10% fetal bovine serum. Tamoxifen-resistant MCF7 cells (MCF7-TAMR) were derived by culturing for 4 wk in 10 μ mol/L *trans*-4-hydroxytamoxifen (6). pRetroSuper (pRS; Origene)-infected and pRS vector expressing shRNA for IGFBP5 knockdown (pRS-shIGFBP5)-infected MCF7 cells (MCF7-pRS and MCF7-pRS-shIGFBP5, respectively) were maintained in media with 1.0 μ g/mL puromycin.

shRNA library screening. Three shRNA oligonucleotides against each of 8,000 genes were designed and cloned into retroviral vector (pRS) containing a shRNA expression cassette (7). MCF7 cells were infected with pRS-shRNA library or pRS or mock infected, selected with puromycin (1.0 μ g/mL) for 2 wk, and cultured in the presence or absence of tamoxifen (5 μ mol/L). After

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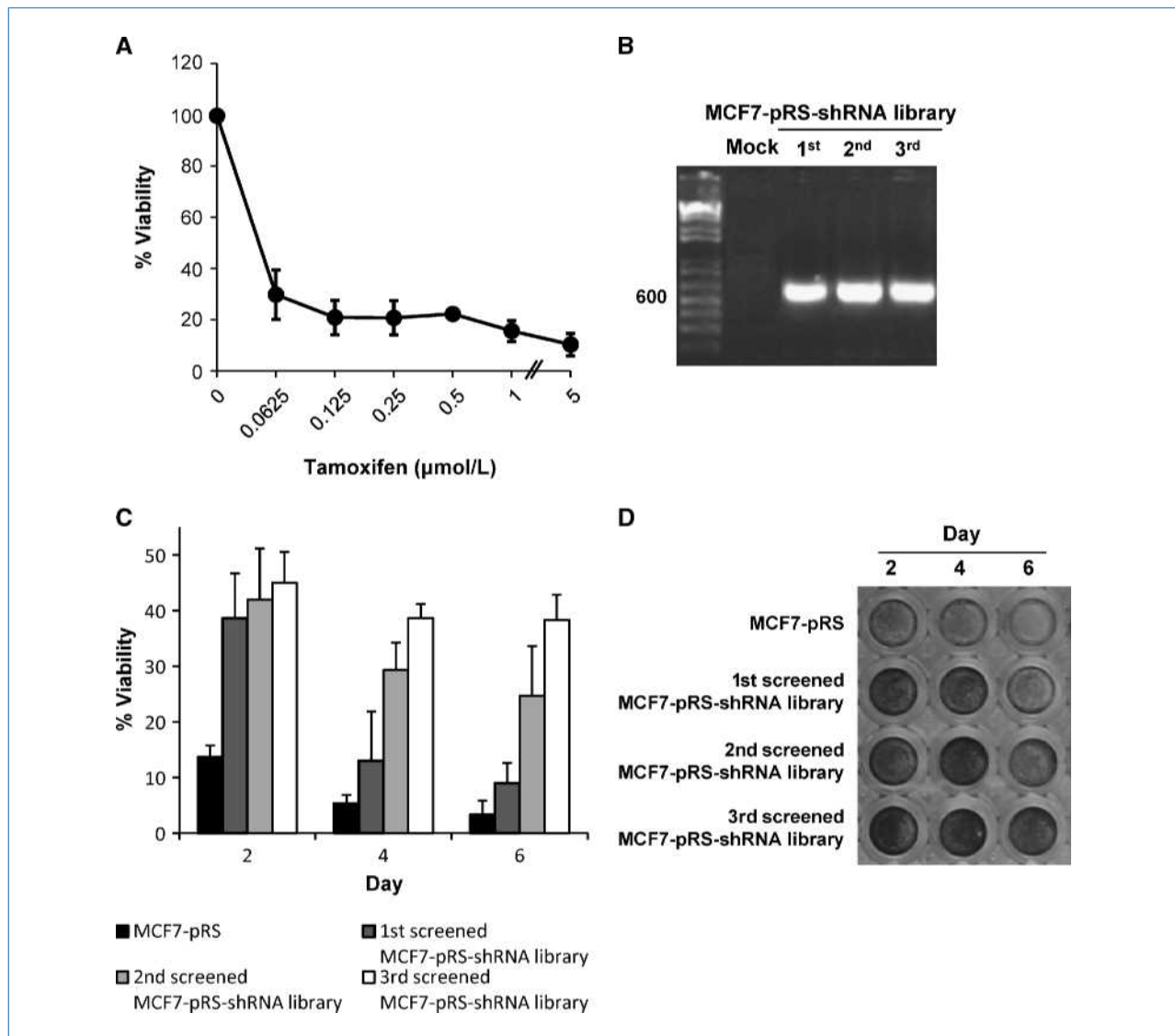


Figure 1. Genetic screen identifies loss of *IGFBP5* in development of tamoxifen resistance. A, MCF7 cells were treated with tamoxifen (6 d) and viability was assessed with WST1 assays. B, MCF7 cells were subjected to genetic screen for tamoxifen resistance with pRS-shRNA library for three rounds, genomic DNA was isolated, shIGFBP5 insert was amplified by PCR, and products were resolved on agarose gels. C and D, after each round of screening, viability of cells after tamoxifen treatment (5 µmol/L) was assessed using WST1 assays (C) or crystal violet staining (D).

6 d, genomic DNA was isolated and shRNA inserts were re-cloned into pRS. Screening was done for two additional rounds. Targeted genes were identified by sequencing shRNA inserts.

Reverse transcription-PCR. RNA was isolated, cDNA was synthesized, and PCR was done using *IGFBP5*- and *ERα*-specific primers (Supplementary Table S1).

Cell viability assays. Cell viability was measured using the WST1 assay (Roche Applied Science) or staining with 0.4% crystal violet.

Tumor formation assays. Animal experiments were done in accordance with the University of Calgary Animal Care Committee guidelines. Six-week-old athymic nu/nu CD female mice were implanted s.c. in the right flanks with 5×10^6 cells. Mice were injected i.m. with estradiol (E_2 ; 0.5 mg/kg per week)

and s.c. with either tamoxifen (50 mg/kg per 2 wk) or PBS. Recombinant IGFBP5 (rIGFBP5; 20 µg) or PBS was systemically administered by tail vein injection.

Apoptosis assays. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were done using the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science).

Immunohistochemistry. Ethics approval was obtained from the University of Calgary Institutional Review Board. Formalin-fixed, paraffin-embedded excised mouse tumor tissue sections were stained with IGFBP5 (Santa Cruz Biotechnology), cleaved caspase-3, or phosphohistone-H3 (Cell Signaling) antibodies. A tissue microarray containing 153 cases of pre-treatment breast cancer biopsies from women treated with only tamoxifen therapy in Calgary between 1987 and 2000

(Supplementary Table S2) was stained with anti-IGFBP5 antibodies. IGFBP5 was classified as either high or low expression based on evaluation of cytoplasmic expression level by pathologists blinded to outcome.

Statistical analysis. Single-group comparisons were done with two-tailed Student's *t* tests. Overall survival was assessed using Kaplan-Meier survival curves with log-rank analysis and hazard ratio (HR) values were calculated using Cox proportional hazard model.

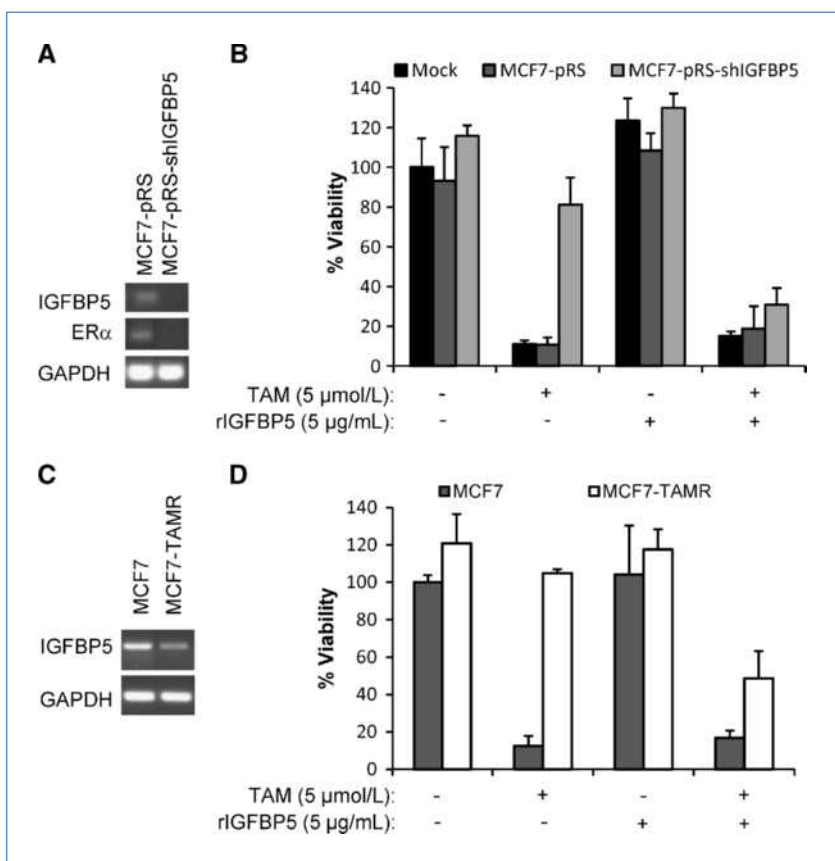
Results

shRNA screen identifies genes involved in tamoxifen resistance. Approximately 30% of ER-positive breast cancers do not respond to tamoxifen therapy and the majority that initially respond develop resistance despite continued ER expression (8, 9). The identification of new genes involved in drug resistance has been expedited by functional genetic screens (10). To identify new genes involved in tamoxifen resistance, a loss-of-function genetic screen was done. pRS-shRNA library or pRS stably infected MCF7 cells were cultured in the presence of tamoxifen (5 μ mol/L) for 6 days, chosen because of drug sensitivity (Fig. 1A). Three successive rounds of screening led to the identification of shRNA for IGFBP5 (shIGFBP5), present in tamoxifen-resistant shRNA library-infected cells after each round (Fig. 1B) and for seven other genes under investigation. An increase in viability of shRNA

library-infected cells was observed with 4- and 6-day tamoxifen treatments after second and third screenings, using WST1 (Fig. 1C) and crystal violet staining (Fig. 1D) assays. IGFBP5 is known to negatively regulate the IGF-I signaling pathway by inhibiting IGF-I interaction with IGF-IR (11). As IGF-I signaling is associated with aggressive phenotypes in breast cancer (11), IGFBP5 was chosen for further investigation.

Exogenous rIGFBP5 reverses in vitro tamoxifen resistance induced by IGFBP5 suppression. IGFBP5 mRNA transcripts were absent in MCF7-pRS-shIGFBP5 cells as compared with MCF7-pRS cells (Fig. 2A), leading to resistance to tamoxifen treatment *in vitro* (Fig. 2B). Tamoxifen-resistant MCF7-TAMR cells (6) also showed reduced IGFBP5 expression (Fig. 2D) associated with increased viability during tamoxifen treatment (Fig. 2D; Supplementary Fig. S1). As IGFBP5 is a secreted protein (11), we examined the possibility that exogenous treatment with rIGFBP5 could restore sensitivity to drug. In MCF7-pRS-shIGFBP5 (Fig. 2B) and MCF7-TAMR cells (Fig. 2C), treatment with 5 μ g/mL rIGFBP5, chosen because of sensitivity (Supplementary Fig. S2), restored tamoxifen sensitivity. IGFBP5 and ER α expression is lost in MCF7-pRS-shIGFBP5 cells (Fig. 2A) regardless of tamoxifen and rIGFBP5 treatments (Supplementary Fig. S3). Whereas E₂ and tamoxifen had no effect on IGFBP5 expression in MCF7-pRS or MCF7-pRS-shIGFBP5 cells (Supplementary Fig. S4), E₂ attenuated reduction in viability of MCF7-pRS cells by tamoxifen (Supplementary Fig. S5) and induced phosphorylation of ERK1 in tamoxifen-treated or untreated

Figure 2. IGFBP5 suppression induces tamoxifen resistance *in vitro*. MCF7 cells were infected with pRS-shIGFBP5 or pRS (A and B) or selected for tamoxifen resistance (TAMR; C and D). Reverse transcription-PCR for IGFBP5 and ER α (A and C) and WST1 viability assays after 6 d in the presence or absence of tamoxifen (TAM; 5 μ mol/L) with or without rIGFBP5 (5 μ g/mL; B and D).



MCF7-pRS cells (Supplementary Fig. S6). On the other hand, downstream ER signaling was absent in E_2 - and tamoxifen-treated MCF7-pRS-shIGFBP5 cells (Supplementary Fig. S6). Therefore, suppression of IGFBP5 induces tamoxifen resistance, possibly

through loss of $ER\alpha$ expression, whereas exogenous rIGFBP5 restores sensitivity to drug through a different mechanism.

Exogenous rIGFBP5 reverses in vivo tamoxifen resistance induced by IGFBP5 suppression. Whereas MCF7-pRS

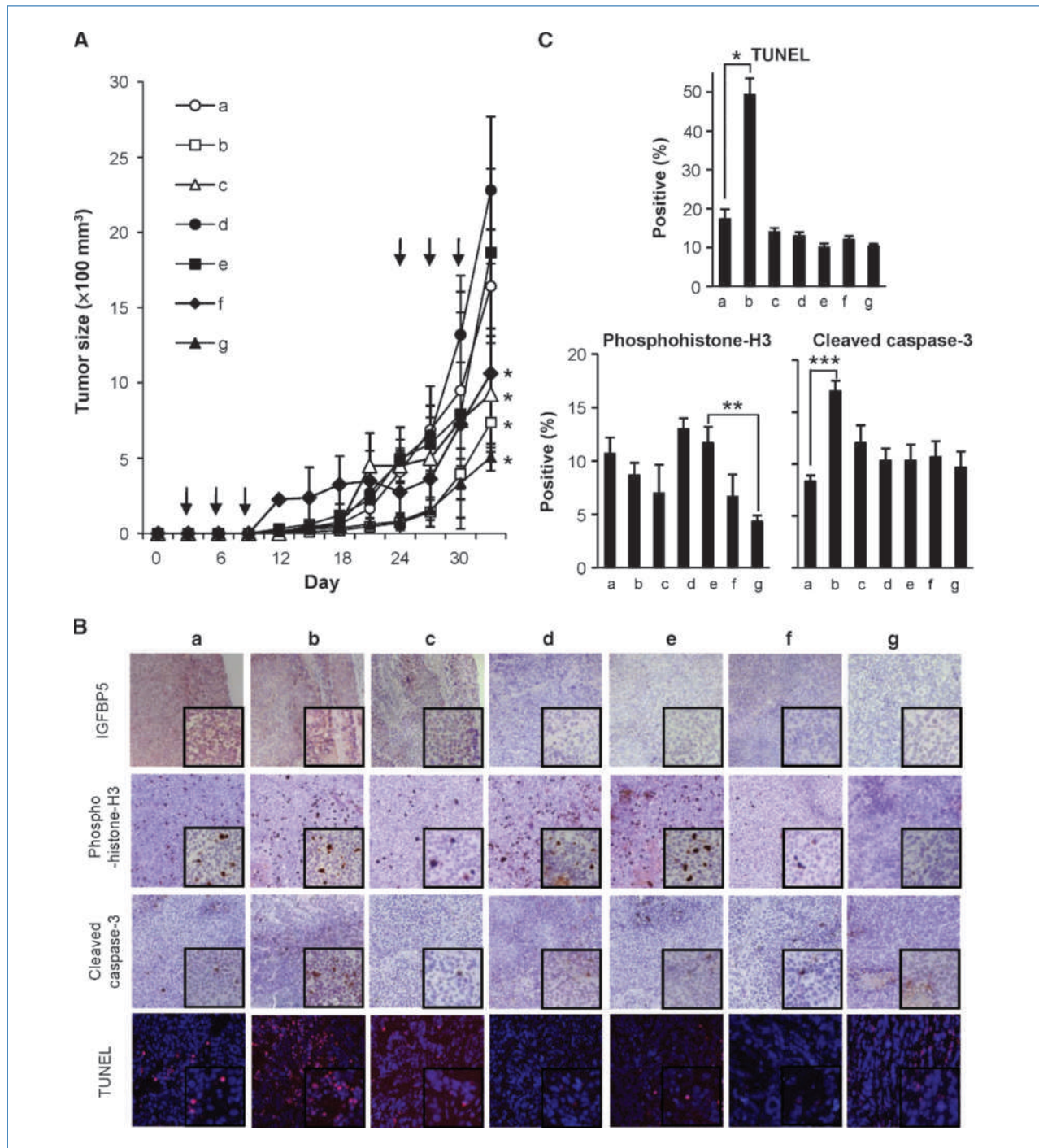
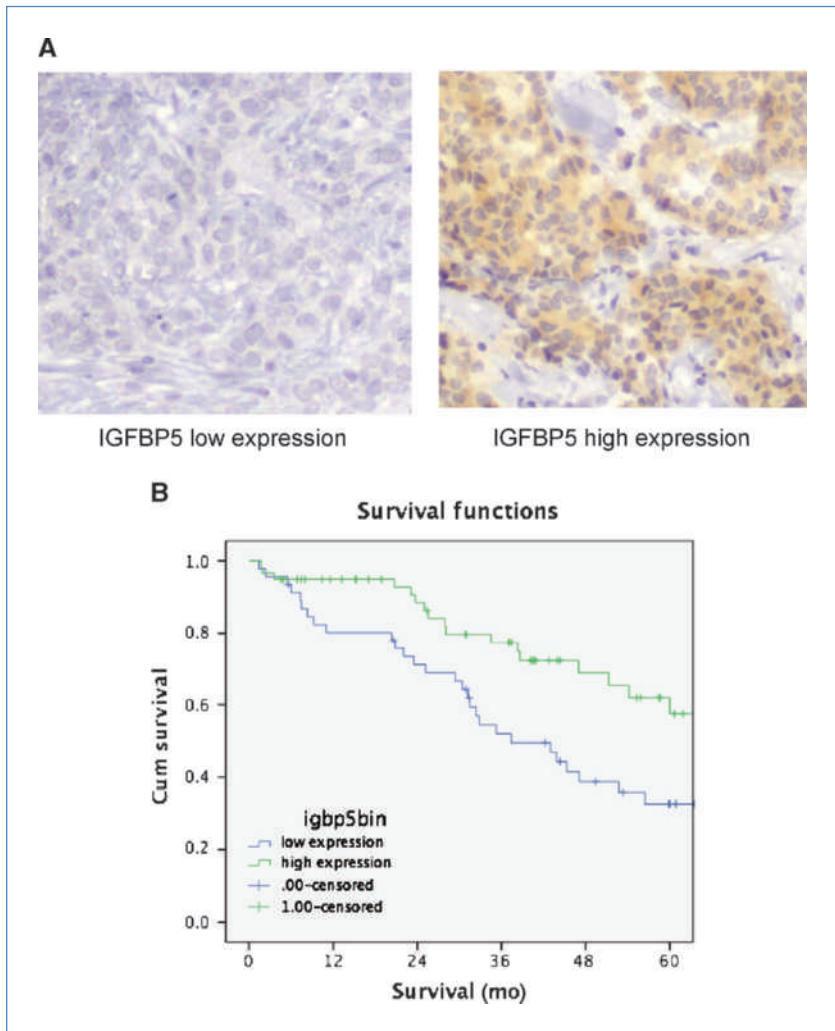


Figure 3. IGFBP5 suppression induces tamoxifen resistance *in vivo*. A and B, s.c. tumors in mice composed of MCF7 cells infected with pRS or pRS-shIGFBP5 were treated with tamoxifen (50 mg/kg) and rIGFBP5 (20 μ g; arrows). a, MCF7-pRS; b, MCF7-pRS + TAM; c, MCF7-pRS + rIGFBP5; d, MCF7-pRS-shIGFBP5; e, MCF7-pRS-shIGFBP5 + TAM; f, MCF7-pRS-shIGFBP5 + rIGFBP5; g, MCF7-pRS-shIGFBP5 + TAM + rIGFBP5. A, tumor sizes; B and C, immunohistochemistry for IGFBP5, phosphohistone-H3, and cleaved caspase-3 and TUNEL staining. B, representative images; C, quantitation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Low IGFBP5 expression in human breast cancer treated with tamoxifen chemotherapy associates with shorter overall survival. A and B, IGFBP5 immunohistochemistry was done on tissue microarray consisting of 153 human breast cancer tissues. A, representative images for tumors with low (left) or high (right) IGFBP5 expression. B, Kaplan-Meier survival curve.



cell-derived tumors showed significant decreases in tumor growth after tamoxifen treatments [Fig. 3A, a versus b; $P < 0.05$], MCF7-pRS-shIGFBP5 cell-derived tumors, which had similar tumor growth to untreated MCF7-pRS cell-derived tumors (a versus d), showed no decrease in tumor growth after tamoxifen treatments as compared with untreated controls (d versus e) and had increased tumor growth after tamoxifen treatments as compared with tamoxifen-treated MCF7-pRS cell-derived tumors (e versus b, $P < 0.05$). Treatment of MCF7-pRS cell-derived, but not of MCF7-pRS-shIGFBP5 cell-derived, tumors with rIGFBP5 alone resulted in a modest reduction in tumor growth (a versus c, d versus f; $P < 0.05$). Cotreatment of MCF7-pRS-shIGFBP5 cell-derived tumors with tamoxifen and rIGFBP5 resulted in an additional reduction in tumor growth compared with tumors treated with rIGFBP5 alone (f versus g; $P < 0.05$) and restored tamoxifen sensitivity of MCF7-pRS-shIGFBP5 cell-derived tumors compared with tumors treated with tamoxifen alone (e versus g; $P < 0.05$). Similar to *in vitro* IGFBP5 expression (Supplementary Fig. S3), immunohistochemical staining for IGFBP5 in excised tumors led to confirmation of corresponding presence and

absence of IGFBP5 in MCF7-pRS and MCF7-pRS-shIGFBP5 cell-derived tumors, respectively, regardless of treatments (Fig. 3B, top row).

MCF7-pRS or MCF7-pRS-shIGFBP5 cell-derived tumors showed positive staining for the cell proliferation marker phosphohistone-H3, which was similar in the presence or absence of tamoxifen treatment alone (Fig. 3B, second row; 3C, a versus b, d versus e). Similar levels of phosphohistone-H3 were also observed for MCF7-pRS cell-derived tumors treated with rIGFBP5 alone, but were reduced for MCF7-pRS-shIGFBP5 cell-derived tumors treated with rIGFBP5 alone (a versus c, d versus f; $P < 0.05$). On the other hand, MCF7-pRS cell-derived tumors showed positive staining for the apoptosis marker cleaved caspase-3 and areas of apoptotic cells identified by TUNEL staining, which were both significantly higher with tamoxifen treatment alone than in untreated MCF7-pRS ($P < 0.001$ for cleaved caspase-3, $P < 0.05$ for TUNEL) and tamoxifen-treated MCF7-pRS-shIGFBP5 ($P < 0.01$ for cleaved caspase-3, $P < 0.05$ for TUNEL) cell-derived tumors (Fig. 3B, third and bottom rows; 3C, a versus b, b versus e), but not with rIGFBP5 treatment alone in MCF7-pRS

cell-derived tumors (a versus c). This increase in apoptosis levels was not observed in MCF7-pRS-shIGFBP5 cell-derived tumors treated with tamoxifen alone or rIGFBP5 alone (d versus e, d versus f) or in MCF7-pRS cell-derived tumors. Cotreatment of MCF7-pRS-shIGFBP5 cell-derived tumors with tamoxifen and rIGFBP5 resulted in a reduction in phosphohistone-H3-positive staining compared with treatment with tamoxifen alone (Fig. 3B, second row; 3C, e versus g; $P < 0.01$), but not compared with treatment with IGFBP5 alone (f versus g). On the other hand, no changes in cleaved caspase-3 or TUNEL staining were observed (Fig. 3B, third row; 3C, e versus g, f versus g). The differential effects of endogenous IGFBP5 and exogenous rIGFBP5 on tumor growth, cell proliferation, and tamoxifen-mediated apoptosis in tamoxifen-treated tumors are due to different concentrations, sites of action (intracellular versus extracellular; ref. 11), and tamoxifen-independent and tamoxifen-dependent effects of endogenous and exogenous IGFBP5. Therefore, tamoxifen induces apoptosis in drug-sensitive ER-expressing tumors; suppression of endogenous IGFBP5 induces tamoxifen resistance due to concomitant ER loss; and exogenous rIGFBP5 cotreatment can reverse tamoxifen resistance through ER-independent mechanisms involving both tamoxifen-independent and tamoxifen-dependent components in arresting growth.

IGFBP5 expression positively correlates with tamoxifen response in breast cancer patients. The clinical relevance of our observations was examined by immunohistochemical analysis of IGFBP5 expression in human breast cancer tissue from a cohort of 153 patients treated previously with tamoxifen (Supplementary Table S2). Of the 105 cases available for analysis, 46 (44%) had low and 59 (56%) had high IGFBP5 expression (Fig. 4A). Cases with low IGFBP5 expression had a reduced mean overall survival ($P = 0.008$) of 63 months (95% CI, 47–79 months) compared with 101.7 months (95% CI, 83–121 months). Expression of IGFBP5 was independently associated with survival (HR, 0.45; $P = 0.025$) correcting for ER status (HR, 0.249; $P = 0.006$), lymph node status (HR, 5.4; $P < 0.001$), size (HR, 1.7; $P < 0.001$), and grade (HR, 1.44; $P = 0.17$). Despite our *in vitro* evidence showing that concomitant loss of ER expression after *IGFBP5* suppression underlies tamoxifen resistance, IGFBP5 expression was not associated with grade, ER status, size, or lymph node status. However, IGFBP5 low-expressing tumors were significantly larger than high-expressing tumors (mean size, 2.77 versus 2.04 cm; $P < 0.01$). These data lend credence to our *in vitro* studies pointing to the importance of IGFBP5 in tamoxifen resistance.

Discussion

Intracellular IGFBP5 in breast cancer cells has potent anti-proliferative and proapoptotic effects (12), whereas the

effects of exogenous IGFBP5 are contradictory (13–15). Exogenous IGFBP5 had no effect on IGF-I-stimulated DNA synthesis (13), protected from ceramide-induced apoptosis (14), or resensitized cells to the growth inhibitory effects of tumor necrosis factor α (15). Downregulation of IGFBP5 is associated with E_2 -induced growth stimulation of breast cancer cells (16). We observed tamoxifen resistance due to suppression of endogenous *IGFBP5* in MCF7 cells and concomitant loss of ER expression, which could be reversed by exogenous rIGFBP5 *in vitro* and *in vivo*, through tamoxifen-independent and tamoxifen-dependent growth arrest, but not restoration of ER expression. The differential effects of exogenous and endogenous IGFBP5 on tumor growth were also due to different concentrations and sites of action (11).

IGFBP5 expression has been associated with ER status and metastasis in breast cancer (17). We found that low *IGFBP5* expression was associated with shorter overall survival, providing further evidence that IGFBP5 is involved in tamoxifen resistance. Although ER status was not associated with IGFBP5 expression in the current study, this may be owing to the low proportion of ER-negative patients (7.8%) in the cohort of patients (Supplementary Table S2). Precedent comes from microarray data showing that relative IGFBP5 expression level determines tamoxifen responsiveness (18). The variegated effects of IGFBP5 could be due to differential cellular localization, IGF-I signaling pathway status, IGF-I-independent effects, or alterations in cross talk between IGF-I and ER signaling (11, 19, 20). In conclusion, our findings reveal IGFBP5 as a potential therapeutic in managing tamoxifen resistance in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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