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Signal Transduction

Molecular Cancer Research

IGF-1 Receptor Modulates FoxO1-Mediated Tamoxifen Response in Breast Cancer Cells

Ali Vaziri-Gohar, Yan Zheng, and Kevin D. Houston

Abstract

Tamoxifen is a common adjuvant treatment for estrogen receptor (ER)α-positive patients with breast cancer; however, acquired resistance abrogates the efficacy of this therapeutic approach. We recently demonstrated that G protein–coupled estrogen receptor 1 (GPER1) mediates tamoxifen action in breast cancer cells by inducing insulin-like growth factor–binding protein-1 (IGFBP-1) to inhibit IGF-1–dependent signaling. To determine whether dysregulation of IGFBP-1 induction is associated with tamoxifen resistance, IGFBP-1 transcription was measured in tamoxifen-resistant MCF-7 cells (TamR) after tamoxifen (Tam) treatment. IGFBP-1 transcription was not stimulated in tamoxifen-treated TamR cells whereas decreased expression of FoxO1, a known modulator of IGFBP-1, was observed. Exogenous expression of FoxO1 rescued the ability of tamoxifen to induce IGFBP-1 transcription in TamR cells. As decreased IGF-1R expression is observed in tamoxifen-resistant cells, the requirement for IGF-1R expression on tamoxifen-stimulated IGFBP-1 transcription was investigated. In TamR and SK-BR-3 cells, both characterized by low IGF-1R levels, exogenous IGF-1R expression increased FoxO1 levels and IGFBP-1 expression, whereas IGF-1R knockdown in MCF-7 cells decreased tamoxifen-stimulated IGFBP-1 transcription. Interestingly, both 17β-estradiol (E2)-stimulated ERα phosphorylation and progesterone receptor (PR) expression were altered in TamR. PR is a transcription factor known to modulate FoxO1 transcription. In addition, IGF-1R knockdown decreased FoxO1 protein levels in MCF-7 cells. Furthermore, IGF-1R or FoxO1 knockdown inhibited the ability of tamoxifen to induce IGFBP-1 transcription and tamoxifen sensitivity in MCF-7 cells. These data provide a molecular mechanistic connection between IGF-1R expression and the FoxO1-mediated mechanism of tamoxifen action in breast cancer cells.

Implications: Loss of IGF-1R expression is associated with decreased tamoxifen efficacy in patients with breast cancer and the development of tamoxifen resistance. This contribution identifies potential molecular mechanisms of altered tamoxifen sensitivity in breast cancer cells resulting from decreased IGF-1R expression. *Mol Cancer Res; 15(4); 489–97. ©2017 AACR.*

Introduction

Breast cancer is one of the leading causes of cancer-related deaths in the world (1). Tamoxifen, a selective estrogen receptor modulator (SERM), is a commonly prescribed drug for estrogen receptor (ER)α-positive patients with breast cancer; however, the therapeutic benefits of this drug are diminished by *de novo* and acquired resistance (2, 3). While ERα antagonism is a well-documented mechanism of tamoxifen action in breast cancer cells, tamoxifen also modulates breast cancer cells that do not express ERα (4–6). We previously demonstrated that G protein–coupled estrogen receptor (GPER1) is a critical component of tamoxifen action in breast cancer cells. After treatment with 4-hydroxytamoxifen, the active metabolite of tamoxifen, GPER1 mediates the inhibition of IGF-1–dependent cell signaling by inducing the extracellular accumulation of IGF-binding proten-1 (IGFBP-1; ref. 6).

IGF-1–dependent cell signaling is critical for growth of normal mammary tissue and contributes to breast carcinogenesis (7, 8). IGF-1 receptor (IGF-1R) is activated upon IGF-1 binding, resulting in the stimulation of multiple downstream signal transduction pathways that enhance cell survival and induce cell proliferation (9–11). Cell signaling mediated by IGF-1R is regulated by IGFBPs, a family consisting of 6 members that modulate the bioavailability and binding capacity of IGFs to IGF-1R. IGFBP-1, for example, inhibits IGF-1–dependent signaling in several cell types including breast cancer cells (6, 12, 13). Loss of IGF-1R expression results in poor prognosis for postmenopausal patients with breast cancer treated with tamoxifen (14), and loss of IGF-1R expression is observed in tamoxifen-resistant breast cancer cells in multiple studies (15–17), suggesting that the expression of this receptor is necessary for tamoxifen action. These findings suggest that IGF-1R is an important component of tamoxifen action; however, the molecular mechanisms of altered tamoxifen action in breast cancer cells with decreased IGF-1R expression have not been determined.

FoxO1 is a member of the Forkhead family of transcription factors that is stabilized and active in the absence of growth factors. When stabilized, FoxO1 translocates to the nucleus and induces the transcription of antiproliferative and proapoptotic genes such as p21 and IGFBP-1 (18–21). FoxO1 phosphorylation by AKT and ERK kinases results in cytoplasmic localization and proteasome-dependent degradation, thus decreasing FoxO1 protein levels (22–24). In breast cancer cells, decreased FoxO1 expression is observed relative to normal mammary epithelial

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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cells (25); however, the role of FoxO1 in tamoxifen-treated breast cancer cells has not been adequately characterized.

The goal of the current research was to characterize the molecular mechanisms of altered IGFBP-1 transcription in tamoxifen-resistant breast cancer cells. In tamoxifen-resistant MCF-7 cells (TamR), IGFBP-1 transcription was not induced upon treatment with tamoxifen. In these cells, FoxO1 expression was decreased, suggesting that FoxO1 dysregulation contributes to the loss of tamoxifen-induced IGFBP-1 transcription. Exogenous expression of FoxO1 rescued the ability of tamoxifen to induce IGFBP-1 transcription in TamR cells, and FoxO1 knockdown decreased tamoxifen-induced IGFBP-1 transcription in MCF-7 cells. Because decreased IGF-1R expression is a characteristic of tamoxifen-resistant cells, the requirement for IGF-1R expression on tamoxifen-stimulated IGFBP-1 transcription was determined. Exogenous IGF-1R expression in TamR and SK-BR-3 cells, both characterized by low IGF-1R levels, increased FoxO1 protein levels and IGFBP-1 expression, whereas IGF-1R knockdown in MCF-7 cells decreased tamoxifen-stimulated IGFBP-1 transcription and decreased FoxO1 protein levels. IGF-1R knockdown in MCF-7 cells increased ERK1/2 phosphorylation; ERK1/2 has previously been shown to regulate FoxO1 protein levels. Progesterone receptor (PR) is a known inducer of FoxO1 expression, so we tested whether E2-induced PR expression was altered in TamR cells. E2 treatment did not induce ERα phosphorylation or PR expression in TamR cells, suggesting potential mechanisms for reducing FoxO1 protein levels in these cells. Finally, IGF-1R or FoxO1 knockdown resulted in decreased tamoxifen sensitivity in MCF-7 cells. Collectively, these data provide a molecular mechanistic connection between IGF-1R expression and FoxO1-dependent mechanism of tamoxifen action in breast cancer cells.

Materials and Methods

Cell culture and treatment

MCF-7 and SK-BR-3 breast cancer cells validated using short tandem repeat (STR) profiling were obtained from ATCC. Cells were cultured in our laboratory for less than 6 months in DMEM (Life Technologies) and DMEM/F12 (Life Technologies), respectively. Maintenance media were supplemented with 10% FBS (Life Technologies). Forty-eight hours prior to treatment, cells were washed with 1× PBS, and phenol red–free DMEM supplemented with 10% charcoal-stripped FBS (Life Technologies) was added to the cells. After 24 hours, cells were washed with 1× PBS and then serum-starved in phenol red–free DMEM overnight followed by treatment with the indicated dose of 4-hydroxytamoxifen (Fluka), IGF-1 (I3769, Sigma), 17β-estradiol (E2, E2758, Sigma), picropodophyllin (sc-204008, Santa Cruz Biotechnology), and actinomycin D (15021, Cell Signaling Technology). Tamoxifen and E2 were dissolved in ethanol (vehicle), and the final concentration of ethanol in medium was 0.1%.

Establishment of TamR MCF-7 cells

TamR cells were generated by continuously culturing of MCF-7 cells in phenol red–free DMEM supplemented with 10% FBS containing 1 μmol/L tamoxifen as previously reported (26).

siRNA knockdown

IGF-1R siRNA (29358, Santa Cruz Biotechnology), FoxO1 siRNA (106652, Ambion), or nontargeting, negative control siRNA (4390843, Ambion) were performed using Lipofectamine 2000 reagent (Life Technologies) for 6 hours in serum-free Opti-MEM (Life Technologies). After overnight recovery in maintenance media, the transfection was repeated followed by growing transfected cells in media containing 10% charcoal-striped FBS and analyzed for protein expression (immunoblot) after 48 hours.

Exogenous gene expression

The IGF-1R–expressing plasmid [a gift from Ronald Kahn (11212, Addgene; ref. 27)] and the FoxO1-expressing plasmid [a gift from Kunlianf Guan (13507, Addgene; ref. 28)] were transfected using Lipofectamine 2000 reagent (Life Technologies) for 6 hours in complete medium.

Total RNA isolation and quantitative PCR analysis

Total RNA was isolated with the PureLink RNA Mini Kit (Life Technologies) with DNase I following the manufacturer's protocol. cDNA was synthesized from total RNA (1 μg) using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time PCR (qPCR) reactions were performed using SYBR Green Master Mix and the 7300 Real-Time PCR system (Bio-Rad). RPL30 gene expression was used in all qPCR reactions as an internal reference gene to normalize relative changes in transcript levels. Primers for amplification of human IGFBP-1: forward 5′-CTA-TGA-TGG-CTC-GAA-GGC-TC-3′ reverse 5′-TTC-TTG-TTG-CAG-TTT-GGC-AG-3′ (29), human FoxO1: forward 5′-TAC-GAG-TGG-ATG-GTC-AAG-AGC-3′, reverse 5′-TGA-ACT-TGC-TGT-GTA-GGG-ACA-3′ (30) and RPL30: forward 5′-ACA-GCA-TGC-GGA-AAA-TAC-TAC-3′, reverse: 5′-AAA-GGA-AAA-TTT-TGC-AGG-TTT-3′ (31).

Immunoblot analysis

Whole-cell extracts were prepared in RIPA buffer containing protease and phosphatase inhibitor cocktails (87785, 78420, Thermo Scientific). Cell extract protein concentrations were determined using bicinchoninic acid (BCA) assay (Thermo Scientific). Thirty micrograms of whole-cell lysates was resolved using Bolt 4%–12% Bis-Tris Plus gels (Life Technologies) and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were blocked in TBS–0.1% Tween-20 (1× TBST) containing 5% fat-free milk at room temperature for 1 hour and then incubated with primary antibody overnight at 4°C using the following antibodies: IGFBP-1 (sc-13097, Santa Cruz Biotechnology), GPER1 (sc-48825-R, Santa Cruz Biotechnology), IGF-1Rβ (3024, Cell Signaling Technology), p-IGF-1Rβ (3021, Cell Signaling Technology), p-AKT (S473) (4060, Cell Signaling Technology), AKT (4685, Cell Signaling Technology), p-ERK1/2 (Thr202/Tyr204) (9106, Cell Signaling Technology), ERK1/2 (9102, Cell Signaling Technology), FoxO1 (2880, Cell Signaling Technology), p-CREB (Ser133) (9198, Cell Signaling Technology), CREB (9197, Cell Signaling Technology), ERα (8644, Cell Signaling Technology), p-ERα (Ser118) (2511, Cell Signaling Technology), p-ERα (Ser167) (5587, Cell Signaling Technology), p21 (sc-397, Santa Cruz Biotechnology), and β-actin (sc-47778, Santa Cruz Biotechnology). After washing with 1× TBST, blots were incubated with anti-IgG horseradish peroxidase–conjugated secondary antibody (sc-81178, Santa Cruz Biotechnology) at room temperature for 1 hour. Prior to the addition of chemiluminescence reagent (34076, Thermo Scientific), blots were washed with 1× TBST. Each blot was stripped using restore plus Western blot stripping

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buffer (46430, Thermo Scientific) when necessary to determine equivalent sample loading. Chemiluminescence was detected using Gel Doc XR ChemiDoc imaging system (Bio-Rad) and quantitated using Quantity One software (Bio-Rad).

Cell viability assay

Cells were plated in 96-well plates at a density of 2,500 cells per well in DMEM supplemented with FBS. Twelve hours prior to treatment, media were replaced with DMEM supplemented with charcoal-stripped serum (17), and cell viability was determined after 5 days using Alamar blue reagent (Life Technologies) according to the manufacturer's procedure. The medium was replaced at day 3.

Statistical analysis

All statistical analysis was performed using KaleidaGraph (Synergy Software). Differences were considered significant if $P < 0.05$ using one-way ANOVA, with Tukey *post hoc* analysis, and the error bars are represented as mean ± SEM.

Results

Tamoxifen-induced IGFBP-1 transcription and FoxO1 expression are dysregulated in TamR cells

We previously reported that treatment of breast cancer cells with the active metabolite of tamoxifen inhibits IGF-1–stimulated breast cancer cell signaling and cell viability by inducing the extracellular accumulation of IGFBP-1 (6). Actinomycin D pretreatment blocked the accumulation of IGFBP-1 transcript in tamoxifen-treated MCF-7 cells, confirming that tamoxifen stimulates IGFBP-1 transcription in MCF-7 cells (Supplementary Fig. S1). The ability of E2, also a GPER1 agonist (32), to stimulate IGFBP-1 transcription was determined, and as expected, IGFBP-1 transcription is stimulated by E2 in MCF-7 cells (Supplementary Fig. S2). To determine whether IGFBP-1 transcription was altered during the development of tamoxifen resistance, TamR MCF-7 cells were generated following a previously published protocol (ref. 26; Fig. 1A). Whereas tamoxifen treatment significantly increased IGFBP-1 transcription in MCF-7 cells, IGFBP-1 transcription was not induced in TamR cells (Fig. 1B), suggesting that this mechanism of action is altered during the development of tamoxifen resistance. Our previous work established GPER1 and the cAMP response element–binding protein (CREB) as mediators of IGFBP-1 transcription in tamoxifen-treated MCF-7 cells (6); therefore, we determined whether GPER1 and p-CREB (S133) levels were decreased in TamR cells before or after tamoxifen treatment. Consistent with previous reports (33), GPER1 expression was not altered in TamR cells (Supplementary Fig. S3A). However, p-CREB levels were increased in untreated TamR cells and p-CREB accumulation did not increase after tamoxifen treatment (Supplementary Fig. S3B) compared with parental MCF-7 cells. To identify a potential mechanism for dysregulated IGFBP-1 transcription in TamR cells, the protein levels of FoxO1, a known regulator of IGFBP-1 expression, were determined (18, 20). Both FoxO1 expression and the expression of p21, a FoxO1-regulated cell-cycle inhibitor (21), were decreased in TamR cells compared with MCF-7 cells, suggesting that decreased FoxO1 protein levels result in dysregulation of IGFBP-1 transcription (Fig. 1C). Exogenous FoxO1 rescued Tam-induced IGFBP-1 transcription in TamR cells (Fig. 1D). These indicate that IGFBP-1 transcription is dysregulated during the development of tamoxifen resistance and suggest that the observed alteration in IGFBP-1 regulation is not due to the loss of GPER1 or CREB expression.

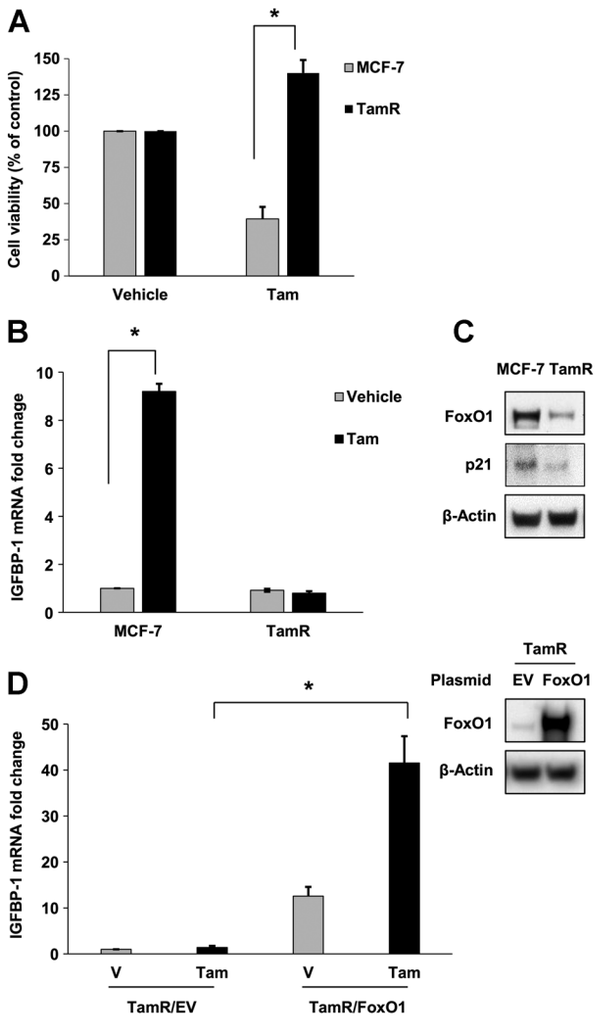

Figure 1.
Tamoxifen-induced IGFBP-1 transcription and FoxO1 expression are dysregulated in TamR cells. **A,** Relative cell viability of MCF-7 and TamR cells after 5 days of 1 μmol/L tamoxifen treatment. **B,** Relative IGFBP-1 transcription in MCF-7 and TamR cells 24 hours after treatment with 1 μmol/L tamoxifen. **C,** FoxO1 and p21 expression in MCF-7 and TamR cells. **D,** Relative IGFBP-1 transcription in TamR cells after exogenous FoxO1 expression and 1 μmol/L tamoxifen treatment. *, $P < 0.05$. Results are average or representative of 3 independent experiments. Error bars are SEM.

FoxO1 mediated tamoxifen-stimulated IGFBP-1 transcription and inhibition of IGF-1–dependent cell signaling in MCF-7 cells

To demonstrate the functional relevance of FoxO1 expression for the IGFBP-1–dependent mechanism of tamoxifen action, FoxO1 knockdown was performed in MCF-7 cells as previously described (6). FoxO1 knockdown resulted in reduced IGFBP-1 transcription in vehicle and tamoxifen-treated cells (Fig. 2A and B). In addition, FoxO1 knockdown reduced the ability of tamoxifen to inhibit the accumulation of p-AKT after IGF-1 stimulation (Fig. 2C, compare second and fourth lanes). We previously

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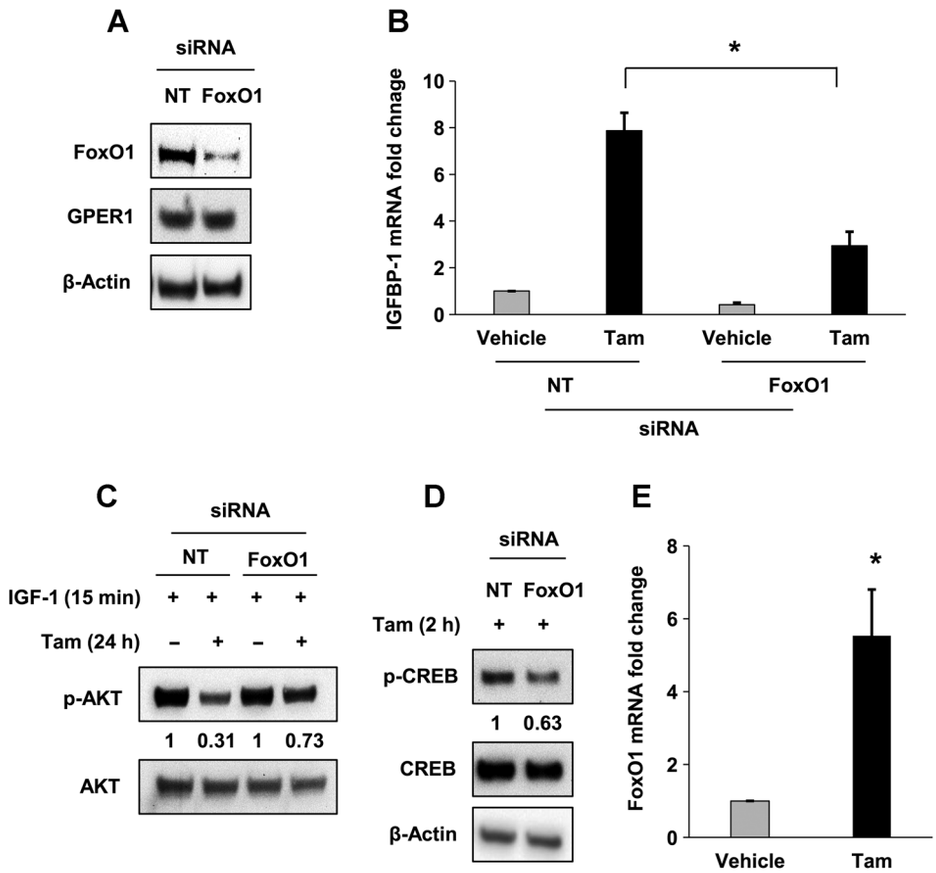

Figure 2.
FoxO1 mediates tamoxifen-stimulated IGFBP-1 transcription and inhibition of IGF-1–dependent cell signaling in MCF-7 cells. **A,** FoxO1 expression after siRNA knockdown in MCF-7 cells. **B,** qPCR analysis of 1 μmol/L tamoxifen-induced IGFBP-1 transcription after FoxO1 knockdown in MCF-7 cells. **C,** Tamoxifen-dependent inhibition of IGF-1–stimulated phospho-AKT accumulation is decreased after FoxO1 knockdown in MCF-7 cells. **D,** CREB expression and accumulation of p-CREB after 1 μmol/L tamoxifen treatment and FoxO1 knockdown in MCF-7 cells. **E,** Relative qPCR analysis of FoxO1 transcription in 1 μmol/L tamoxifen-treated MCF-7 cells. *, $P < 0.05$. The results are average or representative of 3 independent experiments. Error bars are SEM.

showed that CREB mediates IGFBP-1 transcription downstream of GPER1 in tamoxifen-treated MCF-7 cells (6). To determine whether FoxO1 protein levels modulated tamoxifen-induced accumulation of p-CREB, tamoxifen-stimulated p-CREB accumulation was determined after FoxO1 knockdown. Tamoxifen-stimulated p-CREB (S133) accumulation was reduced in MCF-7 cells after FoxO1 knockdown (Fig. 2D). These data suggest that FoxO1 and CREB cooperate to stimulate IGFBP-1 transcription in tamoxifen-treated MCF-7 cells. Further evidence for the involvement of FoxO1 is the observed increase in transcription of FoxO1 in tamoxifen-treated MCF-7 cells (Fig. 2E). These data indicate FoxO1 is a critical component of tamoxifen-stimulated IGFBP-1 expression and provide preliminary evidence that FoxO1 modulates CREB activation after tamoxifen treatment in breast cancer cells.

Decreased tamoxifen sensitivity in MCF-7 cells after IGF-1R knockdown

Loss or decreased IGF-1R expression is observed in tamoxifen-resistant breast cancer cells (15–17), and reduced IGF-1R expression results in poor prognosis for postmenopausal patients with breast cancer treated with tamoxifen (14). However, the role that IGF-1R plays during tamoxifen treatment is not clearly understood. Consistent with previous studies, the TamR cells developed for this study had reduced IGF-1R expression compared with parental MCF-7 cells (Fig. 3A). To determine whether decreased IGF-1R expression modulates tamoxifen-stimulated IGFBP-1 transcription in breast cancer cells, MCF-7 cells were treated with tamoxifen after IGF-1R knockdown and IGFBP-1 transcription was measured. After IGF-1R knockdown, tamoxifen-induced IGFBP-1 transcription was not observed in MCF-7 cells (Fig. 3B and C). Similar to the observations in TamR cells, decreased IGF-1R expression in MCF-7 cells did not alter GPER1 expression, suggesting that mechanisms other than loss of GPER1 expression result in decreased IGFBP-1 transcription after IGF-1R knockdown. These results indicate that IGF-1R expression is necessary for tamoxifen-induced IGFBP-1 transcription in breast cancer cells.

Exogenous IGF-1R expression restored FoxO1 and IGFBP-1 expression in TamR and SK-BR-3 cells

To determine whether FoxO1 protein levels are a result of dysregulated IGF-1R expression in breast cancer cells, FoxO1 protein expression was measured in TamR and SK-BR-3, breast cancer cells characterized by low IGF-1R expression, after exogenous expression of IGF-1R. (34, 35). In TamR cells, exogenous IGF-1R expression resulted in increased FoxO1 protein levels and increased IGFBP-1 transcript levels compared with cells transfected with an empty vector (Fig. 4A and B). To demonstrate that this mechanism is not unique to TamR cells, SK-BR-3 cells were used in similar experiments. Low IGF-1R expression and the ERα-null status were confirmed and compared with MCF-7 cells (Fig. 4C). In addition, the IGFBP-1 transcript levels were determined for SK-BR-3 cells and shown to be significantly lower when compared with MCF-7 cells (Fig. 4D). Similar to results obtained in TamR cells, exogenous expression of IGF-1R increased FoxO1

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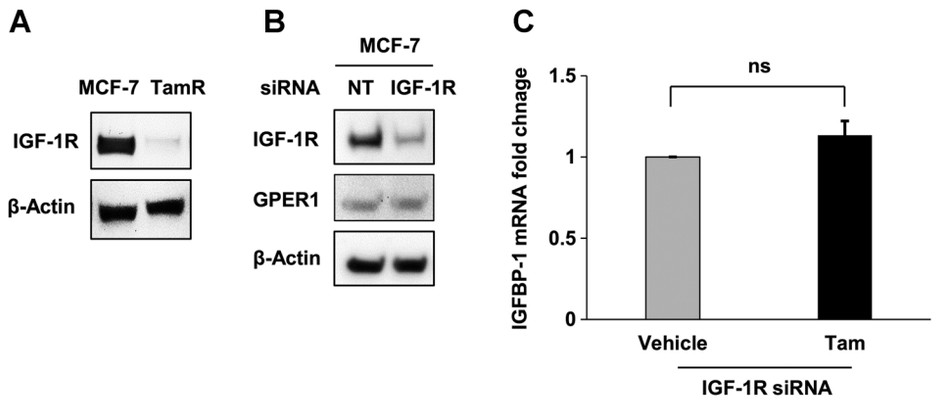

Figure 3.
IGF-1R knockdown decreased tamoxifen response in MCF-7 cells. **A,** IGF-1R expression in MCF-7 and TamR cells. **B,** IGF-1R expression after IGF-1R knockdown in MCF-7 cells. **C,** qPCR analysis of 1 μmol/L tamoxifen-induced IGFBP-1 transcription after IGF-1R knockdown in MCF-7 cells. *, $P < 0.05$. ns, not significant. Results are average or representative of 3 independent experiments. Error bars are SEM.

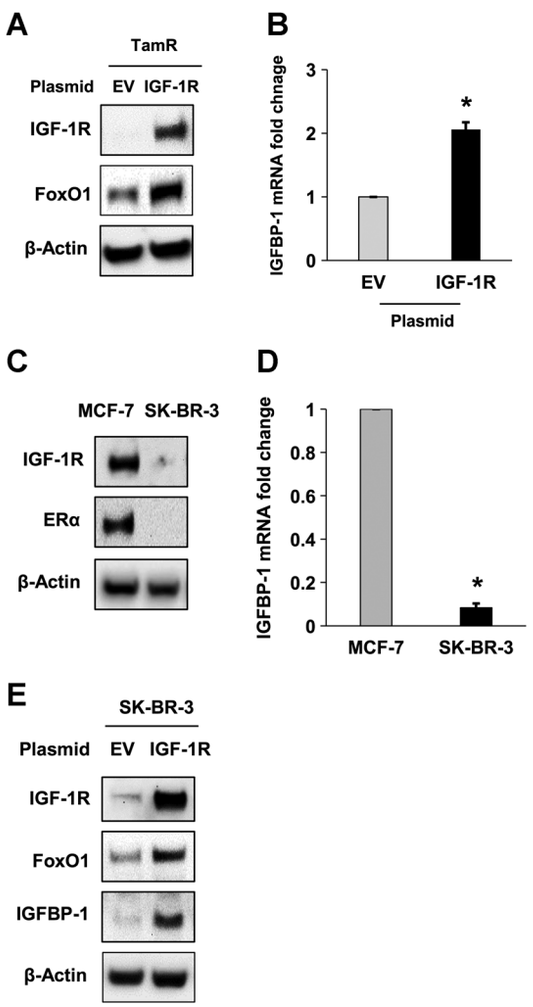

Figure 4.
Exogenous IGF-1R expression restores FoxO1 and IGFBP-1 expression in TamR and SK-BR-3 cells. **A,** IGF-1R and FoxO1 expression after exogenous IGF-1R expression in TamR cells. **B,** qPCR analysis of IGFBP-1 transcription in TamR cells after exogenous expression of IGF-1R. **C,** IGF-1R and ERα expression in MCF-7 and SK-BR-3 cells. **D,** qPCR analysis of IGFBP-1 transcription in MCF-7 and SK-BR-3 cells. **E,** IGF-1R, FoxO1, and IGFBP-1 expression after exogenous IGF-1R expression in SK-BR-3 cells. *, $P < 0.05$. EV, empty vector. Results are average or representative of 3 independent experiments. Error bars are SEM.

and IGFBP-1 expression in SK-BR-3 cells (Fig. 4E). Taken together, these data provide evidence that FoxO1 protein levels correlate with IGF-1R expression in breast cancer cells.

IGF-1R knockdown decreased FoxO1 protein levels in MCF-7 cells

To provide evidence that IGF-1R expression modulates FoxO1 protein levels and identify potential mechanisms of the observed decrease in FoxO1, IGF-1R knockdown was performed in MCF-7 cells and FoxO1 protein levels were measured. Knockdown of IGF-1R resulted in reduced FoxO1 protein in MCF-7 cells that occurred about 24 hours after reduced IGF-1R levels were observed (Fig. 5A), suggesting that FoxO1 levels are associated with IGF-1R expression. To determine whether the observed decrease in FoxO1 levels are associated with loss or decrease in IGF-1R expression rather than a loss of IGF-1R–mediated cell signaling, IGF-1R signaling was inhibited in MCF-7 cells using IGF-1R–specific tyrosine kinase inhibitor picropodophyllin (36, 37). Data from these experiments show that the inhibition of IGF-1R activity indicated by decreased p-IGF-1R levels did not result in decreased FoxO1 protein levels. The slight increase in FoxO1 protein levels after picropodophyllin treatment observed in these experiments was not statistically significant compared with vehicle treatment (Supplementary Fig. S4). Because ERK1/2 destabilizes FoxO1 resulting in decreased FoxO1 protein levels (24), the accumulation of p-ERK1/2 was measured after IGF-1R knockdown in MCF-7 cells to determine whether hyperactivation may be associated with decreased FoxO1 levels. In MCF-7 cells, IGF-1R knockdown resulted in the accumulation of p-ERK1/2 (Fig. 5B). In addition, p-ERK1/2 levels were greater in TamR cells than in MCF-7 cells (Fig. 5C), consistent with results from other studies (17, 38, 39). Taken together, these results suggested that inhibition of IGF-1R activation is not sufficient to decrease FoxO1 protein levels and that loss or decrease of IGF-1R expression is necessary for the observed decrease in FoxO1 protein levels. Furthermore, these experiments suggest that decreased FoxO1 protein levels associate with increased ERK1/2 activity in breast cancer cells after IGF-1R knockdown.

Altered ERα phosphorylation and loss of E2-induced progesterone receptor and FoxO1 expression in TamR cells

Another potential mechanism for reduced FoxO1 protein levels in breast cancer cells with decreased IGF-1R expression (i.e., TamR) is dysregulation of phosphorylation-dependent ERα activation downstream of IGF-1R. Cross-talk between ERα and IGF-1R in ERα-positive breast cancer cells has been well-documented

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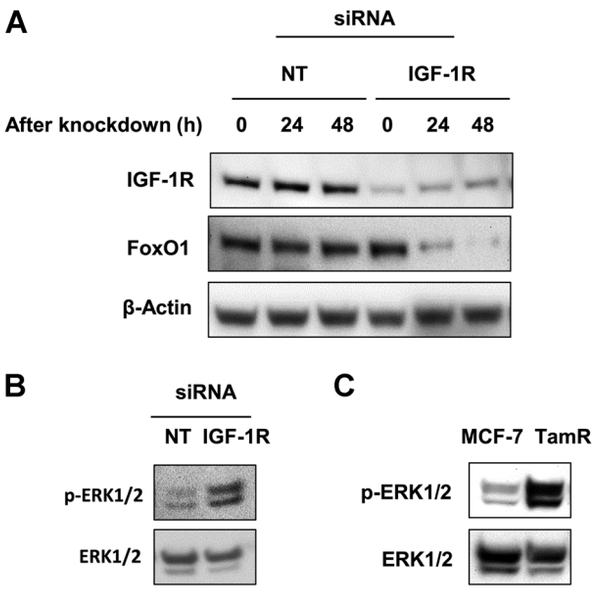

Figure 5.
IGF-1R knockdown induces the accumulation of p-ERK1/2 and decreased FoxO1 protein levels. **A,** FoxO1 expression over 48 hours after IGF-1R knockdown in MCF-7. **B,** Accumulation of p-ERK1/2 after IGF-1R knockdown in MCF-7 cells. **C,** Accumulation of p-ERK1/2 in MCF-7 and TamR cells. *, $P < 0.05$. Results are average or representative of 3 independent experiments. Error bars are SEM.

(40–42). In breast cancer cells characterized by insufficient IGF-1R expression, phosphorylation-dependent ERα activity that requires IGF-1R may be reduced. If this phosphorylation-dependent ERα activity is required to induce FoxO1 expression, then loss of IGF-1R would impact FoxO1 induction. PR regulates FoxO1 expression in endometrial and ovarian cells (43–45); however, this regulation has not been studied in breast cancer cells. It is also well-documented that PR expression is induced upon ERα activation (46). Because our results indicate that FoxO1 protein levels are modulated by IGF-1R expression in TamR cells, we determined whether E2-dependent ERα phosphorylation/activity is also compromised in these cells. We observed E2- and IGF-1–stimulated ERα phosphorylation (p-ERα) at S118 and S167, respectively, in MCF-7 cells consistent with previous observations (42); however, increased phosphorylation was not observed in TamR cells (Fig. 6A). Whereas E2-stimulated PR expression in MCF-7 cells, PR expression was not induced in TamR cells (Fig. 6B). Lack of PR transcription in E2-treated TamR cells was previously reported (17). Concurrent with PR expression, the expression of FoxO1 was induced in E2-treated in MCF-7 cells. Furthermore, expression of FoxO1-modulated p21 and IGFBP-1 was induced upon E2 treatment in MCF-7 cells but not induced in TamR cells (Fig. 6C). E2 has previously been shown to stimulate p21 expression in MCF-7 cells (47). Interestingly, E2-induced accumulation of p-ERα (S118) correlates with IGFBP-1 transcription whereas IGF-1–induced accumulation of p-ERα (S167) is associated with decreased IGFBP-1 transcription in MCF-7 cells (Supplementary Fig. S5). These results suggest that dysregulation of FoxO1 expression may result from decreased IGF-1R–dependent p-ERα activity in breast cancer cells (i.e., TamR).

Decreased tamoxifen sensitivity in MCF-7 cells after IGF-1R or FoxO1 knockdown

Cell viability assays were performed using MCF-7 cells after IGF-1R or FoxO1 knockdown. Data from these experiments indicate that decreased IGF-1R or FoxO1 protein levels desensitize MCF-7 cells to tamoxifen, suggesting that both IGF-1R and FoxO1 mediate tamoxifen-induced breast cancer cell death (Fig. 7A and B). The results reported herein suggest that IGF-1R expression is required for tamoxifen to induce cell death in breast cancer cells and provide a molecular mechanistic understanding of IGF-1R–dependent tamoxifen sensitivity in breast cancer cells.

Discussion

Data presented in this contribution provide mechanistic evidence describing how IGF-1R expression affects the FoxO1-mediated tamoxifen response in breast cancer cells. Consistent with

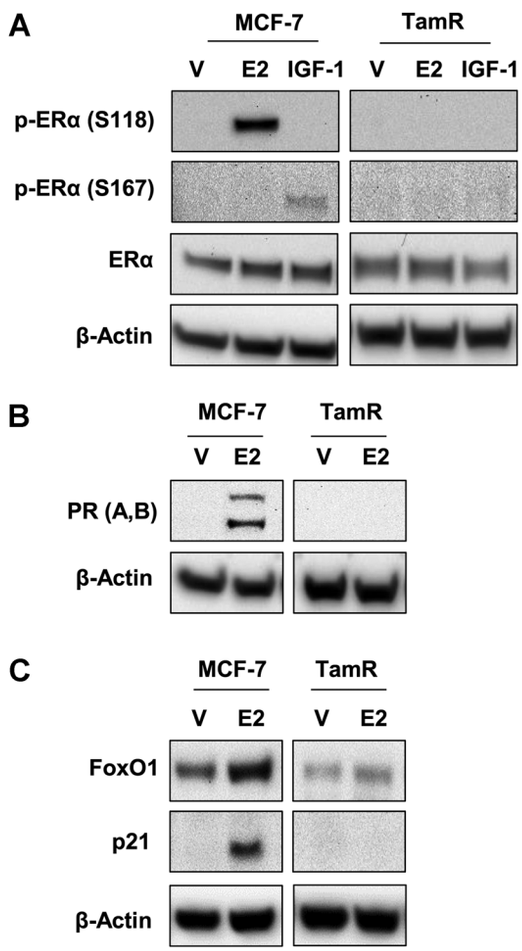

Figure 6.
Altered ERα phosphorylation and loss of E2-induced PR and FoxO1 expression in TamR cells. **A,** p-ERα (S118 and S167) in MCF-7 and TamR cells treated after 10 nmol/L E2 or 50 ng/mL IGF-1 treatment. **B,** PRA and PRB expression in MCF-7 and TamR cells treated with 10 nmol/L E2. **C,** FoxO1 and p21 expression in MCF-7 and TamR cells treated with 10 nmol/L E2. *, $P < 0.05$. Results are average or representative of 3 independent experiments. Error bars are SEM.

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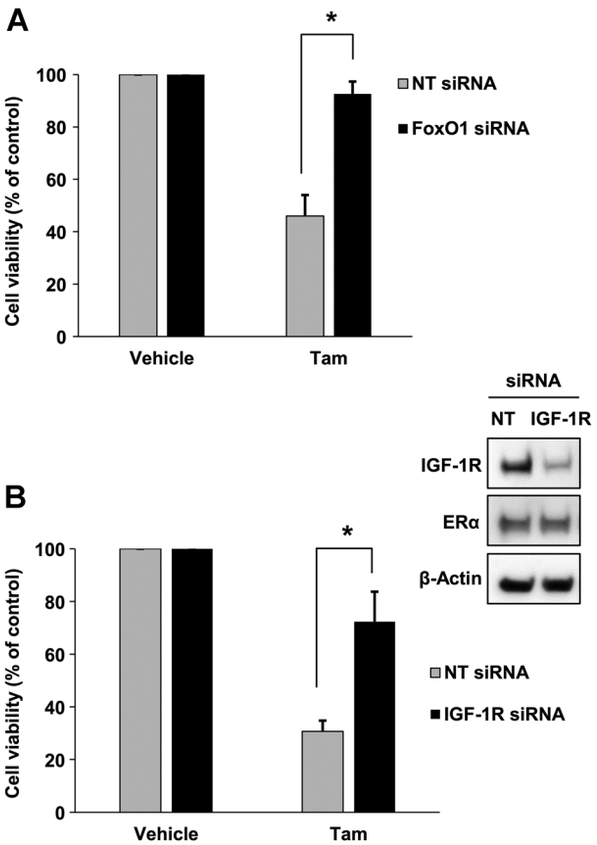

Figure 7.

Decreased tamoxifen sensitivity in MCF-7 cells after IGF-1R or FoxO1 knockdown. **A,** Cell viability on day 5 of 1 μmol/L tamoxifen-treated MCF-7 cells after FoxO1 knockdown. **B,** Cell viability on day 5 of 1 μmol/L tamoxifen-treated MCF-7 cells after IGF-1R knockdown. *, $P < 0.05$. Results are average or representative of 3 independent experiments. Error bars are SEM.

previous observations, decreased IGF-1R expression was observed in TamR breast cancer cells developed for this study. Decreased IGF-1R expression was correlated with attenuated IGFBP-1 induction subsequent to tamoxifen treatment. IGFBP-1 has been previously identified as a mediator of tamoxifen action in MCF-7 cells (6). Expression of the FoxO1 transcription factor, a known regulator of IGFBP-1, was significantly reduced in TamR cells compared with MCF-7 cells, suggesting that reduced FoxO1 activity contributed to decreased IGFBP-1 induction in TamR cells. Exogenous expression of FoxO1 in TamR cells rescued the ability of tamoxifen to induce IGFBP-1 transcription providing additional evidence that FoxO1 mediates this specific tamoxifen response. Knockdown of FoxO1 in MCF-7 cells resulted in a significant decrease in tamoxifen-stimulated IGFBP-1 induction, further suggesting that FoxO1 mediates IGFBP-1 transcription in tamoxifen-treated breast cancer cells. Furthermore, knockdown of IGF-1R in MCF-7 cells reduced FoxO1 expression and tamoxifen-stimulated IGFBP-1 induction, resulting in decreased sensitivity of these cells to tamoxifen. The correlation between the expression of IGF-1R and FoxO1 was also demonstrated in SK-BR-3 cells, a breast cancer cell line characterized by relatively low IGF-1R expression. Taken together, these results provide mechanistic evidence to support the conclusion that IGF-1R expression is a critical determinant of FoxO1- and the IGFBP-1–dependent tamoxifen response in breast cancer cells.

FoxO1, a member of the FoxO transcription factor family, regulates many cellular events such as differentiation, growth, and metabolism. Posttranslational modifications of FoxO1 via phosphorylation (22) and acetylation (48) regulate biologic activities. Attenuated expression of FoxO1 via upregulation of microRNAs or hyperactivation of kinases downstream of growth factor signaling pathways is observed in many cancers and during development of resistance to chemotherapeutic agents (25). In the TamR cells, IGF-1R–depleted MCF-7 cells, increased phosphorylation of ERK1/2 was observed. Future work will need to be completed to determine whether the hyperactivation of ERK1/2 in breast cancer cells results in decreased FoxO1 stability and the observed FoxO1 protein levels. FoxO1 regulates IGFBP-1 expression in hepatic cells (18, 20), and our data showed that IGFBP-1 expression was regulated by FoxO1 in breast cancer cells. In addition to the modulation of IGFBP-1 gene expression and inhibition of IGF-1—dependent cells, tamoxifen treatment also induced FoxO1 transcription in MCF-7 cells. The current results suggest that FoxO1 is a key mediator of tamoxifen action and diminished FoxO1 expression attenuates the effectiveness of this SERM.

The data presented here also suggest that CREB and FoxO1 cooperate to induce IGFBP-1 transcription in tamoxifen-treated breast cancer cells. Cooperation between CREB and FoxO1 has been observed in other cell types (50), but not in breast cancer cells. cAMP-dependent activation of CREB induces FoxO1 transcription in hepatocytes (51) and this could be one possible mechanism of cooperation. Tamoxifen-stimulated GPER1 signaling may activate CREB leading to increased FoxO1 transcription. Future studies will be aimed at defining the role of FoxO1 induction during tamoxifen treatment in breast cancer cells. The IGFBP-1 promoter contains both CREB and FoxO1-binding sites (20, 52, 53), and the current results indicated that CREB phosphorylation in TamR cells is constitutive and not induced by tamoxifen. In MCF-7 cells, CREB is phosphorylated after tamoxifen treatment and CREB mediates IGFBP-1 transcription in these cells (6). In the current studies, p-CREB was not sufficient to induce IGFBP-1 in TamR cells and we conclude that this is due to the lack of FoxO1 expression. In addition, the ability of tamoxifen to induce p-CREB accumulation was reduced in MCF-7 cells after FoxO1 knockdown. These data suggest that FoxO1 expression is required for CREB to induce IGFBP-1 transcription, and more work will need to be completed to identify the molecular mechanisms of IGFBP-1 comodulation by FoxO1 and CREB in tamoxifen-treated breast cancer cells.

IGF-dependent signaling plays a critical role in breast carcinogenesis (8, 54, 55); however, low IGF-1R expression results in poor prognosis for postmenopausal patients with breast cancer treated with tamoxifen (14). In addition, loss of IGF-1R expression is associated with the development of acquired tamoxifen resistance in breast cancer cells and patients (8, 54, 55). With regard to the clinical significance of ERα phosphorylation and tamoxifen sensitivity, conflicting evidence has been published. Whereas higher levels of p-ERα (S118) are associated with tamoxifen resistance in one study (56), another report shows an enhanced tamoxifen benefit in patients expressing higher levels of p-ERα (S118) (57). Both hyperactivation of ERK1/2 in MCF-7

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cells after IGF-1R knockdown and the dysregulation of ERα phosphorylation in TamR cells are reported in the current study. Taken together, these findings provide preliminary evidence for previously unidentified mechanisms of decreased FoxO1 expression in breast cancer cells. More work will need to be completed to address the relative contribution of these distinct mechanisms of FoxO1 regulation in breast cancer cells.

The involvement of GPER1 in E2-induced rapid cell signaling has previously been reported (32, 58), and GPER1 dysfunction is observed in tamoxifen-resistant breast cancer cells (33). Selective activation of GPER1 induces p21 expression (59) supporting a potential GPER1-mediated mechanism for observed E2-induced p21 expression in MCF-7 cells. The current results indicate that either tamoxifen or E2 treatment induce IGFBP-1 transcription in MCF-7 cells. In summary, these results provide a mechanistic understanding for the observation that loss of IGF-1R expression decreases tamoxifen sensitivity resulting from reduced FoxO1 expression in breast cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Vaziri-Gohar, K.D. Houston
Development of methodology: A. Vaziri-Gohar, K.D. Houston
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Vaziri-Gohar, Y. Zheng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Vaziri-Gohar, K.D. Houston
Writing, review, and/or revision of the manuscript: A. Vaziri-Gohar, K.D. Houston
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Vaziri-Gohar, K.D. Houston
Study supervision: K.D. Houston

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