The functional implications of Akt activity and TGF-β signaling in tamoxifen-resistant breast cancer

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Abstract

Development of acquired resistance to tamoxifen is a major clinical problem during endocrine treatment in estrogen receptor positive breast cancer. Transforming growth factor-β1 (TGF-β) has been implicated in tamoxifen-induced cellular signaling in breast cancer, and increased Akt activation is associated with tamoxifen-resistant cell types. We hypothesized that the relationship between TGF-β and Akt signaling may be involved in the development and progression of tamoxifen resistance. Tamoxifen-resistant (Tam-R) cells were established from parental MCF-7 cells by continuously exposing them to 4-hydroxytamoxifen (4-OHT). Tam-R cells were associated with a decrease in TGF-β1 secretion, TGF-β1-mediated transcriptional response, and growth inhibitory effects of 4-OHT. Tam-R cells expressed significantly higher levels of phosphorylated Akt and lower levels of phosphorylated Smad 3 in both the absence and presence of 4-OHT when compared to MCF-7 cells treated with 4-OHT. Ectopic expression of constitutively active Akt (Myc-AktMyr) rendered MCF-7 cells resistant to activation by TGF-β and the growth inhibitory effects of 4-OHT, while over-expression of kinase-dead Akt (Myc-AktK179M) or LY294002 treatment of Tam-R cells enhanced TGF-β activation and blocked cell growth. These results suggest that suppression of TGF-β signaling by activated Akt is correlated with the development of tamoxifen resistance in breast cancer.

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1. Introduction

The anti-estrogen tamoxifen, or its active metabolite 4-hydroxytamoxifen (4-OHT), is a selective estrogen receptor (ER) modulator that can be used as a first-line treatment for ER-positive metastatic breast cancer [1,2]. Although a majority of patients initially benefit from treatment with tamoxifen, 50% of all metastatic breast cancer patients who receive tamoxifen therapy eventually acquire tamoxifen resistance [3]. A number of studies have aimed to elucidate the mechanisms of tamoxifen resistance in ER-positive breast cancers [4–7]. Recent evidence suggests that there is a relationship between the ER and growth factor receptor pathways [4–8]. For example, there may be a relationship between the epidermal growth factor receptor (EGFR/HER2) family and the insulin-like growth factor receptor (IGFR) family [4–7]. More recently, evidence has suggested that the action of tamoxifen in breast cancer is mediated in part by regulation of transforming growth factor-β (TGF-β) isoforms in tumor tissue [8–10]. Studies examining the effects of tamoxifen on TGF-β expression have demonstrated that tamoxifen leads to release of active TGF-β1 from latent precursor molecules and increased transcription of TGF-β2 and TGF-β receptor type II (TβRII) [11–13]. Colletta et al. report that tamoxifen increases the secretion of negative regulators, such as TGF-β1, in human fetal stromal cell lines. These negative regulators then act in a post-translational manner without increasing TGF-β mRNA production by tamoxifen [14]. Anti-estrogens induce the secretion of TGF-β1 via a nontranscriptional pathway, whereas TGF-β1 itself induces TGF-β2 by a direct transcriptional mechanism [11]. In addition, anti-estrogens selectively induce TβRII but not TβRI mRNA expression [12].

TGF-β is a pleiotropic cytokine with potent anti-mitogenic and pro-apoptotic effects in many cancer cells [15–17]. Cellular response to TGF-β signals is regulated in part by specific downstream intracellular effectors called Smad proteins.
TGF-β exerts its effects by binding to distinct receptors (TβRI and TβRII) with intrinsic serine/threonine kinase activity [20,21]. The constitutively active kinase domain of TβRII subsequently activates TpRI by transphosphorylation of serine and threonine residues in the GS box of its cytoplasmic domain [22]. Phosphorylation at these residues further propagates the signaling cascade by binding to receptor-regulated Smads (R-Smads; i.e., Smad 2 and Smad 3) [23–26]. Receptor-associated R-Smads heterodimerize with common mediator Smad (Co-Smads; Smad 4) [27] and then relay signals from the cell membrane to the nucleus, where they bind to Smad binding element (SBE) sequences in the promoter regions of various target genes that regulate cell growth [28–30]. Inhibitory Smads (I-Smads), including Smad 6 and Smad 7, bind to TβRI and suppress activation of R-Smads. After ligand stimulation, I-Smads translocate from the nucleus to the cytoplasm, where they associate with TβRI and inhibit the phosphorylation of R-Smads, resulting in inhibition of TGF-β family signaling [29,30].

Loss of sensitivity to the TGF-β1/Smad-induced cell growth inhibition and apoptosis pathway is believed to be a major factor in tumor formation and progression [31,32]. Reduced levels of TGF-β signaling are also frequently observed in various cancers [33]. In addition, recent studies suggest that several non-Smad signaling entities, including the mitogen-activated protein kinases ERK, JNK, and p38 [33–35], the phosphatidylinositol 3-kinase (PI3-K) [36], and Ras-GTPases [37], mediate the cellular effects of TGF-β. Moreover, both high levels of Akt and high Ras activity block the TGF-β anti-proliferative response [36,37]. Interestingly, although TGF-β can induce Akt and Ras in a Smad-independent manner in certain cellular contexts, the induction of Akt activity can selectively block TGF-β-induced apoptosis [38,39].

Protein kinase B/Akt, which is composed of three closely related isoforms (Akt1, Akt2, and Akt3), plays a crucial role as a mediator of growth factor signaling, cell proliferation, cell survival, and apoptotic inhibition [40,41]. Akt is activated following the agonist-induced stimulation of PI3-K, which consists of p85 catalytic and p110 regulatory subunits. Akt then phosphorylates multiple proteins implicated in cell cycle control to ultimately stimulate cell growth [42]. Akt plays a role in cell proliferation by phosphorylating and inactivating the kinase GSK-3β, which prevents the GSK-3β-mediated nuclear exclusion and proteolysis of cyclin D [43].

Recently, it has been reported that enhanced growth factor signaling involving members of the EGF family [6,44] and increased levels of PI3-K and Akt [45,46] via various signal transduction pathways may lead to anti-estrogen resistance. Perry et al. have demonstrated that tamoxifen acts on breast cancer cells to stimulate TGF-β1 secretion [13]. These findings raise the intriguing possibility that the TGF-β signaling pathway and activity of Akt may be altered in tamoxifen-resistant cells. Therefore, we examined whether signal transduction via TGF-β and Akt is required for the growth of tamoxifen-resistant cells. We found that the activation of Akt enhanced tamoxifen-resistant (Tam-R) cell proliferation by blocking TGF-β signaling downstream of Smad 3 activation.

2. Materials and methods

2.1. Cell culture and cell growth assays

The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to instructions provided by the ATCC. For estrogen-free cell culture, MCF-7 cells were cultured in media containing phenol-red free RPMI-1640 supplemented with 10% charcoal stripped fetal bovine serum (FBS). The tamoxifen-resistant (Tam-R) cell line was established from parental MCF-7 cells by 2 months of continuous exposure to 10−6 M 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, Dorset, UK) until a cell line resistant to the growth inhibitory properties of 4-OHT developed. After this period, Tam-R cells were grown in phenol-red free RPMI-1640 supplemented with 10% charcoal stripped FBS in the absence of 4-OHT (10−6 M) for 2 months.

For cell growth assays, cells were plated at a density of 2 × 104 cells per well in six-well plates and grown in phenol-red free medium supplemented with 10% charcoal stripped FBS either in the absence or presence of 10−6 M 4-OHT or control vehicle (EtOH). Cells were then trypsinized and counted at the indicated times.

2.2. Reagents

Recombinant human TGF-β1 and anti-TGF-β neutralizing antibody were purchased from R & D Systems. LY294002 was purchased from Calbiochem (San Diego, CA). Phospho-Akt (Ser473), anti-Akt, phospho-GSK3β (Ser9), phospho-Smad 3 (Ser433/435), phospho-Smad 2, anti-Smad 4, anti-Smad 7, and anti-Myc antibodies were obtained from Cell Signaling Technology (Beverly, MO), while anti-β-actin antibodies were bought from Sigma, and Myc-AktK179M and Myc-AktK179M, and Myc were purchased from Upstate Biotechnology (Lake Placid, NY).

2.3. Transient transfection

All procedures were performed as indicated in the figure legends. MCF-7 and Tam-R cells were plated at a density of 1 × 105 cells per well in six-well plates and transfected with 1 μg Myc, 1 μg Myc-Akt, or 1 μg Myc-AktK179M using Lipofectamine 2000 (Invitrogen).

2.4. Western blot analysis

Cells were transfected with plasmids, as indicated in the figure legends, and lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% NaDeoxycholate [pH 7.4]) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Protein concentrations were measured using Bio-rad protein assay kits (BioRad, Hercules, CA). Protein lysates were resolved by SDS-PAGE, and then transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Piscataway, NJ), blocked with PBS containing 0.2% Tween 20 and 5% nonfat dry milk, and then incubated first with primary antibody, and then horseradish peroxidase-labeled secondary antibody. Signals were detected on X-ray film.

2.5. Luciferase assay

Cells were plated at a density of 3 × 104 cells per well in six-well plates and co-transfected with a reporter plasmid (0.5 μg) (pTARE-duc reporter or pCIS-CK negative control) and pRL-TK vector (an internal control for standardization, 0.1 μg), Myc-Akt56V (0.5 μg), or Myc-AktK179M (0.5 μg) using Lipofectamine 2000 and then treated with 4-OHT (10−6 M) or TGF-β1 (10 ng/ml) for 24 h. For experiments using LY294002, 24 h after transfection cells were treated with either vehicle or LY294002 for 6 h before harvesting. Luciferase activity was measured using luciferase assay kits (Promega) and normalized with β-Gal activity.

2.6. TGF-β1 quantitation

Cells were plated at a density of 5 × 104 cells per well in six-well plates, cultured under serum-free conditions, and then treated with or without 10−6 M 4-OHT.
4-OHT for 24 or 72 h. Serum-free conditions were used to prepare conditioned medium to avoid carryover of latent TGF-β1 in serum. Following exposure to 4-OHT (10⁻⁶ M) for 24 or 72 h, amounts of TGF-β1 released into cell culture supernatants were measured using QuantiKine human TGF-β1 Immunoassay kits (R & D Systems, Milwaukee, WI). Briefly, acid-activated samples and standards in triplicate were added to the precoated microplates and incubated at room temperature for 2 h. A secondary antibody, anti-TGF-β1 polyclonal antibody, was then added to complete the sandwich followed by TGF-β1-horseradish peroxidase conjugate. TGF-β1 was detected by adding the chromogenic substrate, followed by stop solution. Absorbance was determined at OD 450 nm.

2.7. TGF-β neutralization

TGF-β neutralization studies were performed using a neutralizing anti-TGF-β antibody (R & D Systems). TGF-β antibody completely neutralizes the activities of the isomeric forms TGF-β1, TGF-β2, and TGF-β3. Cells were incubated with medium containing anti-TGF-β antibody (20 μg/ml) or a control normal IgG antibody.

2.8. Anchorage-independent growth assays

Cells were cultured in phenol-red free RPMI supplemented with 10% charcoal stripped FBS, and soft agar assays were done in six-well plates. After 14 days, colonies were fixed and stained with nitroblue tetrazolium, and then colony numbers were counted.

2.9. Statistical analysis

Statistical comparisons were performed using two-tailed Student’s t tests. Data are given as mean±SEM. Significance was defined as *P<0.05, **P<0.01, and ***P<0.001.

3. Results

3.1. TGF-β signaling in MCF-7 and Tam-R cells

Although tamoxifen, or 4-OHT, is an important endocrine agent for the treatment of ER-positive breast cancer patients, initially responsive tumors eventually develop resistance [1,3]. To elucidate the molecular mechanism responsible for this resistance, we developed cell lines with acquired tamoxifen resistance (Tam-R) from 4-OHT-sensitive parental MCF-7 cells by continuously exposing them to 4-OHT for 2 months. After this period, the subsequent growth of Tam-R cells was maintained in the absence of 4-OHT for 2 months. We first determined whether Tam-R cells developed resistance to the anti-estrogenic action of 4-OHT. Cell growth inhibition assays of MCF-7 and Tam-R cells cultured in steroid-free medium showed elevated basal growth of Tam-R cells in the absence of exogenously added 4-OHT (Fig. 1A). Additional treatment with 4-OHT for 24 h of MCF-7 and Tam-R cells produced different results; MCF-7 cells exhibited a significant (51.4%) decrease in cell growth, whereas almost no decrease was observed in Tam-R cells (Fig. 1B).

TGF-β is an autocrine regulator of cell growth in MCF-7 cells, and tamoxifen acts on breast cancer cells to stimulate its secretion [8,12,13]. To investigate if the altered TGF-β signaling pathway for tamoxifen is associated with the development of resistance, we examined the secretion of TGF-β1 protein using specific ELISAs to quantitate TGF-β1 in the media of parental and resistant Tam-R MCF-7 cell cultures. TGF-β1 secretion was measured 24 and 72 h after both cell types were treated with either 4-OHT or control vehicle. Prior to TGF-β1 assays, the samples assayed were acid-activated, and thus the TGF-β1 measured represented total quantities secreted by these cells. Treatment of MCF-7 cells with 4-OHT caused a significant increase in TGF-β1 secretion, which reached levels 2.5-fold above those in cultures treated with the control vehicle after 72 h (Fig. 2A). Conversely, in Tam-R cells, there was no significant difference in the levels of secreted TGF-β1 between cells treated with either 4-OHT or control vehicle.

We next studied the effects of 4-OHT on TGF-β regulated transcriptional activity using the pTARE-Luc cis reporter plasmid, which contains the basic promoter element (TATA box) and TGF-β response element (TARE), or a pCIS-CK negative control. This reporter plasmid expresses firefly luciferase under control of these elements, whereas the pCIS-CK negative control plasmid contains no inducible cis-enhancer element, allowing us to evaluate whether effects are TGF-β signaling-specific. After 24 h, transcript levels of TGF-β1-inducible genes in MCF-7 cells treated with 4-OHT, as measured by pTARE-Luc cis reporter...
activity, were increased to 6.8 times the levels elicited in cells treated with control vehicle (Fig. 2B). However, promoter activity was only slightly greater in Tam-R cells treated with 4-OHT compared to cells treated with control vehicle (P < 0.05).

Given the reduction of TGF-β1 secretion and transcript levels of TGF-β-inducible genes in Tam-R cells, we wanted to determine whether TGF-β activity is required for 4-OHT-induced growth inhibition of MCF-7 cells or the proliferation of Tam-R cells. To examine the potential role of TGF-β activity in 4-OHT-induced growth inhibition of MCF-7 cells, we measured MCF-7 cell growth 5 days after treating cultures with 4-OHT or control vehicle in the absence or presence of either neutralizing anti-TGF-β antibody (TGF-β Ab, 20 μg/ml) or control IgG antibody with 4-OHT (10^{-6} M) in complete medium for 5 days, and cell growth was determined by trypan blue exclusion. Cell growth is expressed as a percentage of the control vehicle at 5 days of growth. The results represent the standard deviation from five independent experiments conducted in triplicate. P value was calculated compared with MCF-7 culture treated with control vehicle; **P < 0.01, n.s., not significant.

To elucidate the molecular mechanism by which 4-OHT-stimulated TGF-β response was blocked in Tam-R cells, we next
explored whether expression of Smad proteins or their activation by TGF-β was affected by 4-OHT. As Smads are the best characterized mediators of TGF-β signaling, we first assayed the expression of Smad proteins in cell extracts prepared from MCF-7 and Tam-R cells treated with 4-OHT or control vehicle. As shown in Fig. 3A, after 24 h, 4-OHT-sensitive MCF-7 cells treated with 4-OHT displayed a significant increase in the phosphorylation of Smad 3 in comparison to cells treated with control vehicle. On the other hand, Tam-R cells exhibit low amounts of phosphorylated Smad 3, even in the absence of exogenously added 4-OHT, compared with MCF-7 cells treated with 4-OHT. Moreover, additional treatment with 4-OHT did not have a significant effect on phospho-Smad 3 expression levels in Tam-R cells as compared to MCF-7 cells. Meanwhile, 4-OHT had little effect on the expression levels of Smad 4 (Co-Smads), Smad 6, or Smad 7 (I-Smad) in both MCF-7 and Tam-R cells. In MCF-7 cells, blockade of TGF-β signaling by exposure to neutralizing anti-TGF-β antibody for 24 h also completely reversed the induction of phospho-Smad 3 expression by stimulation of 4-OHT, but we did not see any major changes in phospho-Smad 3 protein levels in comparison with MCF-7 cells treated with 4-OHT alone (Fig. 3B). Furthermore, as shown in Fig. 2D, Tam-R cells produced a significant increase in activated Smad 3 protein levels 24 h after addition of exogenous TGF-β, compared with untreated controls (Fig. 3C). In all cases, there was no change observed in the expression of Smad 3. These results collectively indicate that TGF-β–Smad 3 activation may be correlated with the Tam-R phenotype.

3.2. Akt activation has an important role in the suppression of TGF-β response in Tam-R cells

Song et al. report that insulin growth factor-I (IGF-I) functions via a PI3K/Akt pathway to block several TGF-β-mediated responses, including gene transcription, apoptosis, and Smad 3 activation [47,48]. The authors suggest that Akt blocks Smad 3 activation by sequestering it from TGF-β receptors. Recently, other groups have also reported that Akt phosphorylation is significantly higher in tamoxifen-resistant cells grown under basal conditions [45,46]. Because it has been reported that signaling via Akt is required for anti-estrogen-resistant cell growth and that Akt blocks TGF-β signals downstream of Smad 3 activation, we next explored whether the kinase activity of Akt is involved in the suppression of TGF-β signals in Tam-R cells. Using Western blotting, we first compared levels of total Akt expression and activated Akt in MCF-7 cells and Tam-R cells treated with 4-OHT or control vehicle for 24 h. We found that while the phosphorylation of Ser473 in Akt was reduced in MCF-7 cells treated with 4-OHT, it was strongly induced in Tam-R cells in both the absence and presence of 4-OHT when compared to MCF-7 cells treated with 4-OHT alone (Fig. 4A). No significant reduction could be observed following treatment of Tam-R cells with 4-OHT as compared to control vehicle. Expression of total Akt was not altered in either cell type, and the same held true for total Smad 3 expression.

To further ascertain that this increase in phosphorylated Akt was associated with 4-OHT-induced TGF-β activity, we performed

Fig. 3. 4-OHT regulation of Smad 3 activity in MCF-7 and Tam-R cells. (A) MCF-7 cells were incubated in growth medium containing 4-OHT (10⁻⁶ M) or control vehicle for 24 h. Western blotting with Smad 3, p-Smad 3, Smad 4, and Smad 7 antibodies was carried out. (B) MCF-7 cells were treated with 4-OHT (10⁻⁶ M), control vehicle, or a combination of either neutralizing anti-TGF-β antibody (20 μg/ml) or control IgG antibody with 4-OHT (10⁻⁶ M) in complete medium for 5 days. The expression levels of phosphorylated Smad 3 were then determined by Western blotting. (C) Tam-R cells were incubated in growth medium supplemented with the indicated concentrations of TGF-β1. After 24 h, Western blotting with Smad 3 and p-Smad 3 antibodies was carried out. In all cases, β-actin was used as a control.
Western blotting on cell extracts prepared from MCF-7 cells treated with 4-OHT or control vehicle in the presence or absence of either neutralizing anti-TGF-β antibody or control IgG antibody.

After 24 h, 4-OHT induced higher expression levels of phosphorylated Smad 3 but decreased the levels of phosphorylated Akt compared to control vehicle. As expected, combined treatment
with 4-OHT and neutralizing anti-TGF-β antibody resulted in lower levels of phosphorylated Smad 3 and higher levels of phosphorylated Akt at 24 h compared with single treatment with 4-OHT. Namely, blockade of TGF-β signaling by neutralizing anti-TGF-β antibody completely reversed Smad 3 activation by 4-OHT, but significantly enhance Akt phosphorylation (Fig. 4B). However, a combination of control IgG antibody did not give rise to detectable alteration in the expression levels of phosphorylated Smad 3 or phosphorylated Akt as compared with single treatment with 4-OHT. Taken together, these findings suggest that the increased kinase activity of Akt in Tam-R cells may lead to persistent 4-OHT-stimulated TGF-β response and, consequently, to the maintenance of the tamoxifen-resistant phenotype.

We next wanted to determine whether the kinase activity of Akt is important for the suppression of TGF-β signaling in Tam-R cells. Accordingly, we compared the abilities of constitutively active myristoylated (Myc; N-terminal fusion with Src aa 1–11, Myc-Akt<sup>Myr</sup>) and kinase-dead (KD) (K179M mutant, Myc-Akt<sup>K179M</sup>) Akt constructs to control the transcriptional responses of TGF-β by 4-OHT in both cell types treated with 4-OHT or control vehicle. In order to determine if over-expression of activated Akt can block the 4-OHT-induced TGF-β response, MCF-7 cells were co-transfected with Myc-Akt<sup>Myr</sup> or control vector along with the pTARE-Luc cis reporter construct. Twenty-four hours after transfection, either 4-OHT or control vehicle was added before measurement of luciferase levels. MCF-7 vector transfectant exhibited a higher luciferase activity in response to 4-OHT than control vehicle. In contrast, Myc-Akt<sup>Myr</sup>-over-expression in MCF-7 cells decreased the ability of 4-OHT to stimulate TARE-dependent transactivation compared with vector transfectant (Fig. 4C). To determine if Akt hyperactivation in Tam-R cells results in suppression of 4-OHT-induced luciferase activity, Tam-R cells were co-transfected with Myc-Akt<sup>K179M</sup>, which functions as a dominant negative (DN) of Akt kinase, and pTARE-Luc cis reporter construct. Twenty-four hours after transfection, either 4-OHT or control vehicle was added before measurement of luciferase levels. MCF-7 vector transfectant exhibited a higher luciferase activity in response to 4-OHT than control vehicle. In contrast, Myc-Akt<sup>Myr</sup>-over-expression in MCF-7 cells decreased the ability of 4-OHT to stimulate TARE-dependent transactivation compared with vector transfectant (Fig. 4C). 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reporter plasmid before treatment with 4-OHT or control vehicle. Twenty-four hours after transfection, the basal activation of luciferase, even in the absence of 4-OHT, was enhanced two-fold in Tam-R cells transfected with Myc-AktK179M compared with the vector in the absence or presence of 4-OHT (Fig. 4D). Notably, luciferase levels in Myc-AktK179M transfectants were raised four-fold in response to 4-OHT as compared with the vector transfectants treated with 4-OHT. Similar to the increased expression of Myc-AktK179M in TAM-R cells, the PI3K inhibitor LY294002 reversed the suppression of TGF-β-induced luciferase activity in the absence of 4-OHT as compared with vector transfectants treated with 4-OHT. Furthermore, additional treatment with 4-OHT in the presence of LY294002 significantly increased luciferase activity three-fold compared to vector transfectants treated with 4-OHT. Together, these results support the concept that Akt activation in Tam-R cells plays an important role in the activation of TGF-β-induced luciferase by 4-OHT.

We next questioned if the ability of Akt to suppress TGF-β signaling is associated with the inhibition of Smad 3 activation. To test this, MCF-7 cells transfected with control vector or Myc-AktK179M were treated with 4-OHT or control vehicle. After 24 h of treatment, Western blot analysis was then performed using anti-phospho-Smad 3 antibody. As shown in Fig. 4E, the ectopic expression of Myc-AktK179M suppressed Smad 3 phosphorylation in the presence of 4-OHT versus MCF-7 cells transfected with control vector. The expression levels of Myc-Akt and phospho-GSK-3α/β (Ser21/9) were expected for each treatment. Moreover, additional 4-OHT treatment of Tam-R cells transfected with Myc-AktK179M caused a strong enhancement in the expression levels of phosphorylated Smad 3 compared with control transfectants. Meanwhile, inhibition of Akt activity, even in the absence of 4-OHT, exerted a small increase in phosphorylated Smad 3 expression in Tam-R cells compared to the vector transfectants treated with either control vehicle or 4-OHT (Fig. 4F).

Inhibition of Akt activity by incubation with LY294002 for 2 h exerted a greater increase in phosphorylated Smad 3 levels than that brought about by transfection with Myc-AktK179M in the Tam-R cells in the absence of 4-OHT. The expression levels of Myc-Akt and phospho-GSK-3α/β (Ser21/9) were expected for each treatment. Collectively, these data strongly support the notion that Smad 3 activation is involved in Akt signal transduction in Tam-R cells.

To study the effect of Akt on the 4-OHT-induced growth inhibition of Tam-R cells, Tam-R cells transfected with control vector or Myc-AktK179M were treated with 4-OHT or control vehicle. Anchorage-independent soft agar assays were then performed on these cells at 14 days of growth (Fig. 5A). Transfection with Myc-AktK179M suppressed the basal growth of Tam-R cells by 28.2% compared with control vector. Treatment of Myc-AktK179M transfectant cells with 4-OHT resulted in further inhibition of colony growth to 58% of vector transfecant cells treated with 4-OHT. Consistent with TGF-β-induced luciferase activity, treatment of vector transfectants with LY294002 led to a decrease in colony formation, and combined treatment with LY294002 and 4-OHT resulted in significant colony growth suppression to 70.1% of vector transfectant cells treated with 4-OHT. In addition, colony growth showed a marked reduction of response-
was also induced by 4-OHT treatment in MCF-7 cells, but not in Tam-R cells (Fig. 3A). Taken together, these results indicate that the loss of the TGF-β anti-proliferative response is implicated in the mechanism of resistance to tamoxifen.

Tamoxifen’s action in breast cancer is believed to be mediated in part by the regulation of TGF-β isoforms in tumor tissues [13]. However, the molecular mechanism that leads to impairment of the tumor-suppressive function of TGF-β in tamoxifen-resistant cells remains to be clarified. Recent studies report that TGF-β can cooperate with HER-2 to promote metastasis in different models, and it has been suggested that TGF-β can act directly on epithelial cells expressing HER-2 to induce invasive behavior [49,50]. One possible explanation is that tamoxifen bound to the ER induces the release of active TGF-β from its latent precursor molecule, and that this enhances the expression of HER-2, which ultimately diminishes the anti-proliferative action of TGF-β. Another explanation is that up-regulation of Akt mediated by TGF-β or TGF-β-induced RAS may block TGF-β expression or signal transduction. However, further investigation is necessary to elucidate the mechanisms involved.

TGF-β receptors, which function as tumor suppressors in normal and preneoplastic tissues, acquire oncogenic functions during tumor progression [29]. TGF-β receptors are mutated or expressed at substantially attenuated levels in a variety of human cancers, and these are correlated with the acquisition of resistance to growth suppression by TGF-β [19,20]. These relations suggest that a reduced expression of TGF-β receptors is responsible for altered tamoxifen sensitivity. Thus, we performed Western blot assays using anti-TβRI and TβRII antibodies, but found that neither MCF-7 nor Tam-R cells showed altered TβRI or TβRII expression after 4-OHT treatment (data not shown). These data demonstrate that a loss of TGF-β signaling in Tam-R cells is blocked via the autocrine/paracrine release of TGF-β1 or TGF-β downstream of Smad 3 activation.

Several groups have found that Akt blocks the ability of TGF-β to activate Smad 3, mediates TGF-β-induced gene expression, and induces apoptosis [38,39]. The potential importance of the relationship between the Akt cell survival pathway and tamoxifen resistance has also been demonstrated by other laboratory studies. Clarke et al. showed that adding LY294002 to tamoxifen significantly increased the pro-apoptotic effects of tamoxifen in a cell line that showed high levels of Akt activity [2]. However, the molecular mechanism that leads to impairment of the Akt cell survival pathway and tamoxifen resistance has also been demonstrated by other laboratory studies. Clarke et al. demonstrate that the ligands of epidermal growth factor receptors (EGFR), including EGF and TGFα (transforming growth factor α), show an increased ability to activate Akt in tamoxifen-resistant cells [46]. More studies need to be carried out to elucidate these mechanisms.

Although our data suggest that TGF-β/pSmad 3 suppression by Akt is involved in the mechanism of tamoxifen resistance, the mechanism by which Akt blocks Smad 3 activation by TGF-β remains unclear. Physical interactions between Akt and Smads remain to be clarified. Recent studies report that TGF-α growth factor receptors, which function as tumor suppressors in numerous groups [45,46]. One possible mechanism of increased Akt activation in tamoxifen-resistant cells involves an altered Akt activation mechanism, such as a mutation leading to the absence of the PTEN protein [51,52]. However, it has been reported that PTEN is detectable in breast carcinoma cells that exhibit Akt activation [45]. Alternatively, the studies of Jordan et al. demonstrate that the ligands of epidermal growth factor receptor (EGFR), including EGF and TGFα (transforming growth factor α), show an increased ability to activate Akt in tamoxifen-resistant cells [46]. More studies need to be carried out to elucidate these mechanisms.

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