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RAPID COMMUNICATION

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AhR ligand aminoflavone suppresses α6-integrin–Src–Akt signaling to attenuate tamoxifen resistance in breast cancer cells

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More than 40% of patients with luminal breast cancer treated with endocrine therapy agent tamoxifen demonstrate resistance. Emerging evidence suggests tumor initiating cells (TICs) and aberrant activation of Src and Akt signaling drive tamoxifen resistance and relapse. We previously demonstrated that aryl hydrocarbon receptor ligand aminoflavone (AF) inhibits the expression of TIC gene α6-integrin and disrupts mammospheres derived from tamoxifen-sensitive breast cancer cells. In the current study, we hypothesize that tamoxifen-resistant (TamR) cells exhibit higher levels of α6-integrin than tamoxifen-sensitive cells and that AF inhibits the growth of TamR cells by suppressing α6-integrin–Src–Akt signaling. In support of our hypothesis, TamR cells and associated mammospheres were found to exhibit elevated α6-integrin expression compared with their tamoxifen-sensitive counterparts. Furthermore, tumor sections from patients who relapsed on tamoxifen showed enhanced α6-integrin expression. Gene expression profiling from the TCGA database further revealed that basal-like breast cancer samples, known to be largely unresponsive to tamoxifen, demonstrated higher α6-integrin levels than luminal breast cancer samples. Importantly, AF reduced TamR cell viability and disrupted TamR mammospheres while concomitantly reducing α6-integrin messenger RNA and protein levels. In addition, AF and small interfering RNA against α6-integrin blocked tamoxifen-stimulated proliferation of TamR MCF-7 cells and further sensitized these cells to

tamoxifen. Moreover, AF reduced Src and Akt signaling activation in TamR MCF-7 cells. Our findings suggest elevated α6-integrin expression is associated with tamoxifen resistance and AF suppresses α6-integrin–Src–Akt signaling activation to confer activity against TamR breast cancer.

KEYWORDS
AF, breast cancer, Src–Akt signaling, tamoxifen resistance, α6-integrin

1 | INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women worldwide. Resistance to therapies often results in metastasis which leads to recurrence and breast cancer mortality (Ahmad, 2013). Estrogen receptor positive (ER^+) breast cancer is the most frequently diagnosed breast cancer subtype. Tamoxifen is widely used to treat ER^+ breast cancer although the emergence of resistance significantly diminishes its clinical efficacy (Tanic et al., 2012). Tumor initiating cells (TICs) are key contributors to tamoxifen resistance owing to their ability to evade treatment and self-renew to produce recurrent tumors (Ojo et al., 2015). Tamoxifen treatment itself has been shown to select for cells with self-renewal capacity (Raffo et al., 2013). As such, elimination of TICs is crucial to circumvent tamoxifen resistance and confer long-term clinical benefit (Gruber, Scheidt, Aberger, & Huber, 2017; Ricci-Vitiani, Pagliuca, Palio, Zeuner, & De Maria, 2008).

Integrins have been identified as important regulators of tumor initiation or cancer stemness and drug resistance (Seguin, Desgrosellier, Weis, & Cheresh, 2015). In particular, α6-integrin is important for TIC maintenance and function (Lathia et al., 2010). Indeed, elevated α6-integrin expression in breast tumor tissues has been associated with poor overall survival among patients (Friedrichs et al., 1995). We recently demonstrated that, contrary to tamoxifen, aryl hydrocarbon receptor (AhR) ligand aminoflavone (AF) inhibits α6-integrin expression to suppress TIC proliferation in ER^+ breast cancer models and though α6-integrin often partners with β1 and β4 integrins, AF did not markedly alter the expression of these integrins (Brantley et al., 2016). Although another nontoxic AhR ligand tranilast has been shown to synergize with tamoxifen in vitro (Darakhshan, Bidmeshkipour, Khazaei, Rabzia, & Ghanbari, 2013) and inhibit TIC proliferation (Prud'homme et al., 2010), our recent study was the first to link α6-integrin with AhR ligand-mediated suppression of TIC proliferation (Brantley et al., 2016). Thus far, factors that contribute to TIC survival in TamR cancers have not been fully elucidated.

Though endocrine therapy resistance has been associated with elevated expression of AhR target genes cytochrome P450s 1A1 and 1B1, elevated expression of these genes did not mediate resistance to endocrine therapy agent fulvestrant (Brockdorff, Skouv, Reiter, & Lykkesfeldt, 2000). Interestingly, fulvestrant induces AhR signaling to suggest crosstalk interactions occur between estrogen receptor (ER) and AhR signaling pathways. DuSell et al. (2010) previously demonstrated the ability of 4-hydroxy-tamoxifen (4OHTam), an active tamoxifen metabolite, to induce AhR target genes in the absence of estrogen. Safe and McDougal (2002) previously reported that AhR agonists, in certain contexts, block estradiol-mediated mammary tumor growth via AhR–ER crosstalk mechanisms. In addition, small molecules that activate AhR signaling were found to inhibit cancer cell invasion and metastases in breast cancer cells including basal-like subtypes known to resist endocrine therapy (Hall et al., 2010; Jin, Lee, Pfent, & Safe, 2014). Moreover, AhR ligand AF demonstrates the potential to activate AhR signaling yet demonstrates potent and selective anticancer activity in certain breast cancer cell lines and corresponding tumors (Loaiza-Pérez et al., 2004).

The purpose of this study is to examine an association between α6-integrin expression and tamoxifen resistance and to determine whether AF demonstrates anticancer activity in TamR cells by targeting the α6-integrin–Src–Akt signaling axis. AF has undergone extensive preclinical development and has been evaluated in clinical trials for efficacy against solid tumors. However, the ability for AF to demonstrate efficacy in TamR cells of varying molecular subtypes and the potential mechanism(s) of such anticancer actions has not been fully explored. A better understanding of the molecular targets, such as α6-integrin, that contribute to tamoxifen resistance provides an avenue to identify biomarkers useful in recognizing patients less likely to benefit from endocrine therapy.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Human MCF-7 and T47D parental (Par MCF-7 and Par T47D) and MCF-7 and T47D tamoxifen-resistant (TamR MCF-7 and TamR T47D) cells are of the luminal A breast cancer subtype and were developed and maintained as previously described (Fu et al., 2016; Morrison et al., 2014). Parental MCF-7 cells were originally obtained from Dr. Marc Lippman (National Cancer Institute, Bethesda, MD) while the Par T47D (ATCC cat #HTB-133, RRID: CVCL_0553) cells were originally obtained from the American Type Culture Collection (ATCC). Alternatively, Par MCF-7 and Par T47D cells were obtained from the DCTD Tumor Repository, National Cancer Institute at Frederick, MD, as part of the NCI-60 cell line panel. Luminal B ZR-75-30 (ATCC cat #CRL-1504, RRID: CVCL_1661) cells were a kind gift from Dr. Daisy De Leon (Loma Linda University Health School of Medicine, Loma Linda, CA). Luminal B BT-474 (ATCC cat #HTB-20, RRID: CVCL_0179) cells were obtained from the ATCC. All cell lines were either authenticated once Tamoxifen

resistance was established or using STR DNA profiling. ZR-75-30 breast cancer cells were cultured in RPMI-1640 medium containing 10% FBS (Hyclone, Logan, UT), supplemented with 2 mM glutamine and penicillin and streptomycin antibiotics (Mediatech, Herndon, VA). BT-474 cells were cultured in ATCC Hybri-Care medium, reconstituted in 1-L cell-culture-grade water and supplemented with 1.5 g/L sodium bicarbonate, 10% FBS and 2 mM glutamine, and penicillin and streptomycin antibiotics. The α6-integrin blocking antibody GoH3 (clone NKI-GoH3) was obtained from Millipore (Temecula, CA; cat #MAB1378; RRID: AB_1121-794). 5-Amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4*H*-1-benzopyran-4-one (AF) was obtained from the "The NCI/DTP Open Chemical Repository" (http://dtp.cancer.gov, Frederick, MD) at the Frederick National Laboratory for Cancer Research. 4OHTam was obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of AF and 4OHTam were dissolved in dimethyl sulfoxide (DMSO). All stocks were stored protected from light at − 20°C until use.

2.2 | Determination of cancer cell viability

We evaluated the ability of AF to inhibit the growth of breast cancer cells with varying degrees of sensitivity to tamoxifen. Briefly, MCF-7 cells (Par and TamR) and T47D cells (Par and TamR), as well as BT-474 and ZR-75-30 cells were cultured in their respective media as mentioned above and plated in 96-well plates. Approximately 24 hr later, cells were treated with AF (0.1–10,000 nM), 4OHTam or 0.1% DMSO for 72 hr for all cell lines except BT-474 cells which received treatment for 120 hr. Cytotoxicity was determined using the Alamar Blue™ assay as previously described elsewhere (McLean et al., 2008). Otherwise, cells were grown in suspension as mammospheres as described in accordance with the mammosphere assay, exposed to AF or 4OHTam followed by harvesting and disruption in trypsin by thorough mixing. The resulting individual cell suspensions were transferred to a 96-well plate and the Alamar Blue Assay™ was performed as previously described (Brantley et al., 2016). To determine whether α6-integrin mediates responsiveness of 4OHTam in TamR cells, TamR monolayers were exposed to blocking antibody GoH3 (1 or 10 μg/ml) for 3 days for TamR MCF-7 and for 5 days for BT-474 cells alone or in combination with either 4OHTam or AF. Cells were otherwise transfected with a pool of small interfering RNAs (siRNAs) against α6-integrin as described below. Cell viability was then determined as described above.

2.3 | siRNA transfection

siRNA and transfection reagents were obtained from GE Dharmacon (Lafayette, CO). Positive control siRNA (ON-TARGETplus cyclophilin B control pool (human), cat #D-001820-10-05), negative control siRNA (ON-TARGETplus nontargeting pool, cat #D-001810-10-05), test siRNA (ON-TARGETplus human ITGA6 [3655] siRNA—SMART-pool, cat #L-007214-00-0005) were resuspended in RNase free water and aliquoted for short-term storage at −20°C before use. TamR MCF-7 cells were diluted in antibiotic-free complete medium to achieve a plating density of 60–80% confluency in either 96- or 6-well plates followed by incubation at 37°C with 5% CO_2 overnight. Transfection medium was prepared according to the manufacturer's instructions. Cells were transfected with 25 nM control siRNAs or 10 nM ITGA6 siRNA for 24 hr followed by an additional 24-hr incubation in complete media. Transfection efficiency was verified using quantitative polymerase chain reaction (qPCR) and western blot analyses. Conditions that showed target messenger RNA (mRNA) knockdown of >80% as well as >80% cell viability were used in subsequent studies.

2.4 | Mammosphere assay

Cells were cultured in suspension as mammospheres using the MammoCult™ human medium kit (Stem Cell Technologies, Vancouver, BC). Mammospheres were cultured for 5 days in Falcon six-well nontreated polystyrene plates (product #351146 Fisher Scientific, Tustin, CA) before being exposed to respective treatments. Mammospheres were visualized using an IX-71 Olympus microscope (relief contrast mode, Olympus Life Sciences Solutions, Waltham, MA) and pictures taken before and after treatment. Additionally, mammospheres were collected and prepared for Alamar Blue (Fisher Scientific, Tustin, CA), semiquantitative or qPCR analyses as described previously (Brantley et al., 2016).

2.5 | RNA extraction, semiquantitative reverse transcription polymerase chain reaction, and qPCR analyses

Total RNA was isolated from Par MCF-7, TamR MCF-7, BT-474, and ZR-75-30 cells (grown in monolayers) or as Par MCF-7, TamR MCF-7, ZR-75-30, and BT-474 mammospheres using either the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA) or miRNeasy Mini kit (Qiagen, Germantown, MD) in accordance with the manufacturers' instructions. Complementary DNA (cDNA) was prepared using an iScript Advanced cDNA synthesis kit (Bio-Rad, Richmond, CA). Semiquantitative PCR was conducted as detailed elsewhere (van Riggelen et al., 2005) to determine the relative expression of α6-integrin variant A (875 bp) and variant B (745 bp) in mammospheres. Primers used for semiquantitative PCR have been described elsewhere and were as follows: α6-integrin- forward: 5′-CTA ACG GAG TCT CAC AAC TC-3′, reverse: 5′-AGT TAA AAC TGT AGG TTC G-3′, and GAPDH: 460 bp, forward: 5′-TGG ATA TTG TTG CCA TCA ATG ACC-3′ and reverse: 5′-GAT GGC ATG GAC TGT GGT CAT G-3′ (Dydensborg et al., 2009). Quantitative real-time PCR analysis was also performed using a CFX-96 PCR instrument (Bio-Rad, Hercules, CA). PCR products were obtained using the following primers from Qiagen (Germantown, MD): human ITGA6, human BAX, human GAPDH, and human RPLP0.

2.6 | Western blot analysis

Cells were seeded at $3–4 \times 10^6$ cells per plate (100 mm) and allowed to attach. Cells were then serum starved for ~24 hr before

treatment with 1 μM AF or 0.1% DMSO for 8, 24, or 48 hr. In some instances, cells were treated with GoH3 blocking antibody. Following treatment, the cells were harvested on ice by scraping, washed twice with cold PBS before adding CelLytic™ M lysis buffer (Sigma, St. Louis, MO) supplemented with protease and phosphatase inhibitors. Protein concentration was determined using the BCA™ protein assay kit (Prod #23250; Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions. For western blot analysis, proteins were resolved on 4–12% NuPage® Bis-Tris Mini Gels (Fisher Scientific, Tustin, CA) at a constant voltage of 200 V. Gels were then blotted onto polyvinylidene difluoride membranes using the iBlot® 7-Minute Blotting System (Thermo Fisher Scientific). The membranes were blocked for 1 hr in blocking buffer consisting of 5% nonfat dry milk in 1× TBST at room temperature. The membranes were then incubated with primary antibody overnight at 4°C with gentle rocking. The primary antibodies used were phospho-Src (Tyr527; cat #2105; RRID: AB_10829463), phospho-Akt (Ser473; cat #9271; RRID: AB_329825), phospho-Akt (Thr308; cat #9275; RRID: AB_32928), integrin α6 (cat #3750; RRID: AB_2249263), total Akt (cat #9272; RRID: AB_329827), Src (36D10) rabbit mAb (CST cat #2109; RRID: AB_2106059); all were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal anti-β-actin antibody (cat #A2228, RRID: AB_476697) was purchased from Sigma-Aldrich. Membranes were incubated with an anti-rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)–linked secondary antibody (cat #7074, RRID: AB_2099233) from Cell Signaling Technology or goat anti-mouse IgG–HRP (cat #sc-2005) from Santa Cruz Biotechnology (Dallas, TX) for 1 hr at room temperature. Protein detection was then done using the SuperSignal West Dura Extended Duration Substrate enhanced chemiluminescence detection system (Thermo Fisher Scientific, Tustin, CA).

2.7 | Tumor specimens and immunohistochemistry

Fourteen breast tumor specimens were retrieved from patients who relapsed on endocrine therapy in accordance with an institutional review board approved protocol from the Loma Linda University ethics committee. Three of the patients experienced relapse following treatment with tamoxifen. All patients provided informed consent. Formalin-fixed paraffin embedded (FFPE) tissues were cut into 4-μm sections and α6-integrin expression was detected using an EXPOSE Mouse and Rabbit-specific HRP–DAB detection IHC kit (Abcam, Cambridge, MA) in accordance with manufacturer's recommendations. FFPE cancer tissue sections were deparaffinized by baking overnight at 56°C, followed by xylene treatment. Tissue sections were then immediately rehydrated in graded concentrations (100% to 70%) of ethanol. Antigen retrieval was then performed via microwaving in citrate buffer (6.0 pH) for 10 min. Endogenous peroxidase activity was blocked via the application of a hydrogen peroxide block. Nonspecific staining was also blocked using a protein block. This was followed by overnight incubation with a rabbit polyclonal antibody to α6-integrin (ab133386; Abcam). Thereafter, the sections were exposed to a mouse specifying reagent and a goat anti-rabbit HRP conjugate for 15 min and 1 hr, respectively. Tissue sections were then stained using a DAB chromogen and substrate mixture, followed by counterstaining with hematoxylin. Positive and negative controls included normal lymph node tissue sections (ab4350; Abcam) and thyroid carcinoma tissue sections, known to express our target α6-integrin, incubated with or without primary antibody, respectively (data not shown). Stained tissue sections were visualized via light microscopy. A pathologist (L. J. D.) blinded to the sample identity manually quantified all stains. Stains were scored as 1 (weak), 2 (moderate), or 3 (strong) to describe relative α6-integrin expression.

2.8 | Molecular and histological assessment of tumor subtypes

Using RNA sequencing data derived from The Cancer Genome Atlas (TCGA; Cancer Genome Atlas Network, 2012; RRID: SCR_003193), we evaluated α6-integrin expression in patient tumors stratified based on molecular subtypes, which were determined by the Pam50 gene set. The molecular subtypes include: basal-like, luminal A, luminal B, and HER2-enriched. In brief, these subtypes are defined based upon the expression levels of specific hormone receptors (ER, progesterone receptor [PR] and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 [ERBB2 or HER2]). The presence of ER defines the luminal subtypes and the absence of HER2 amplification distinguishes luminal A from luminal B. The absence of all three receptors in tumors further characterized with epidermal growth factor receptor and cytokeratin (ck) 5/6 expression, are selected as 'basal-like.'

2.9 | Statistical analysis

Differences between groups were analyzed using one-way ANOVA with Tukey's test or the Tukey–Kramer multiple comparison tests for evaluating three or more groups. To compare two groups, the unpaired Student's *t* test with Welch's correction was used. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad software, Inc., San Diego, CA; www.graphpad.com). Differences were considered significant at $p < 0.05$.

3 | RESULTS

3.1 | Elevated levels of α6-integrin are found in cells and patient tumors that are TamR

Overexpression of α6-integrin has been shown to promote breast cancer resistance to radiotherapy (Hu, Zhou, Zhao, & Wu, 2016). To determine whether α6-integrin expression is associated with tamoxifen resistance in ER^+ breast cancer, we measured the expression of α6-integrin in a panel of TamR breast cancer cells including TamR MCF-7, BT-474, and ZR-75-30 cells in comparison to Par MCF-7 cells. We found that basal α6-integrin mRNA levels were significantly elevated in these cells compared with Par MCF-7 cells (Figure 1a). Furthermore, α6-integrin expression levels were higher in TamR MCF-7 and BT-474 mammospheres compared with Par MCF-7 mammospheres (Figure 1b). We also found elevated α6-integrin protein expression levels among TamR MCF-7, BT-474,

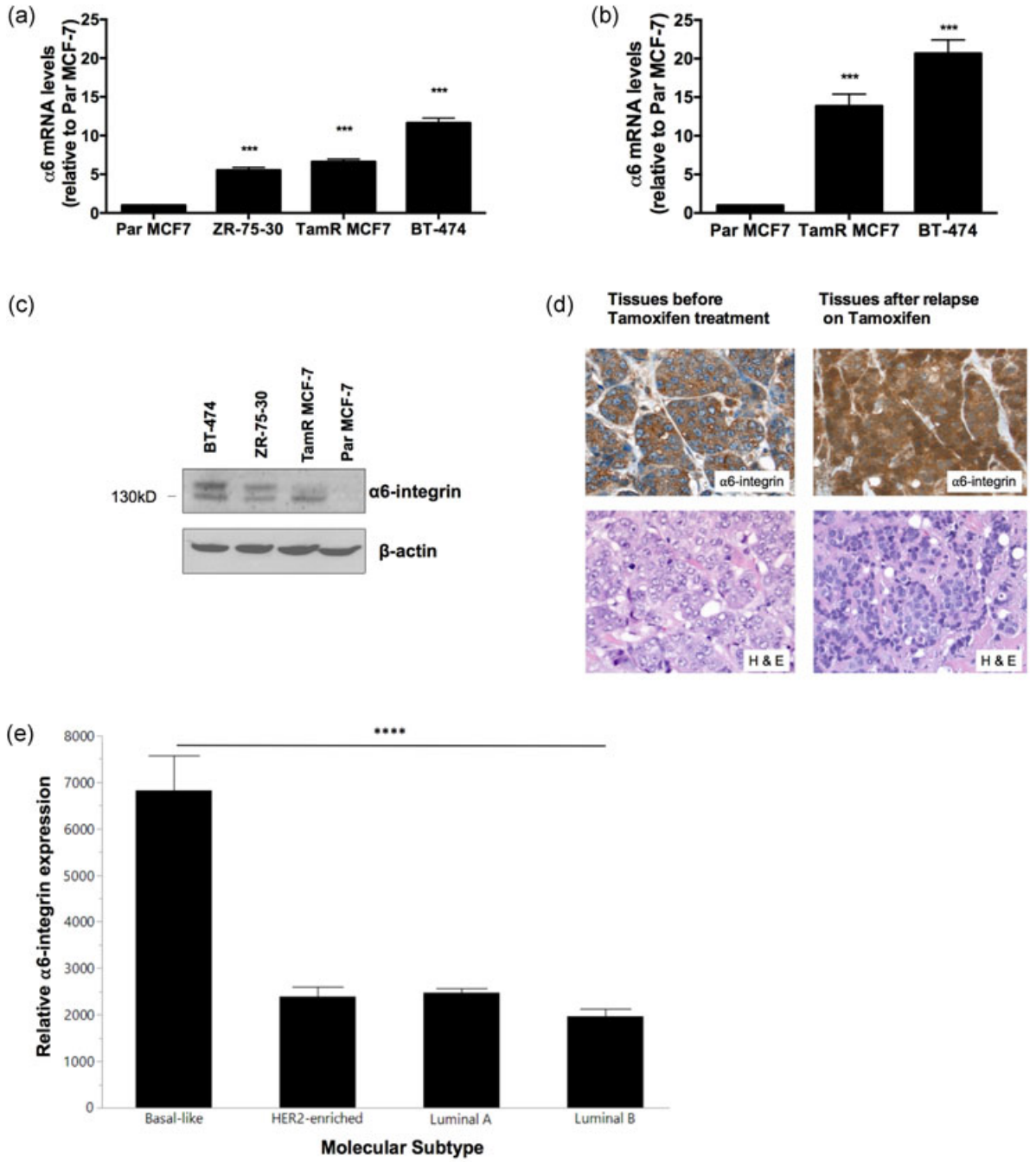

FIGURE 1 α6-Integrin expression in tamoxifen-resistant breast cancer cells and breast tumor tissues. (a) Endogenous α6-integrin mRNA expression was evaluated in Par MCF-7, TamR MCF-7, ZR-75-30, and BT-474 cells and (b) in Par MCF-7, TamR MCF-7, and BT-474 mammospheres. Data represent the mean of at least three independent experiments. Scale bars = *SEM*. Significantly different at ***$p < 0.001$ in comparison with Par MCF-7 cells or mammospheres. (c) Western blot revealing relative α6-integrin protein expression in Par MCF-7, TamR MCF-7, ZR-75-30, and BT-474 cells. (d) Representative α6-integrin IHC stains for treatment naïve patient tumor tissues (left) and patient tumor tissues following relapse on tamoxifen (right). Magnification 400X. (e) Bar graph depicting α6-integrin mRNA expression levels (Pam50 gene set) from different breast tumor types derived from the TCGA database. Scale bars = *SD*. Significantly different as denoted ****$p < 0.0001$ when comparing basal-like subtypes with luminal A, luminal B, and HER2-amplified subtypes. Par: parental; mRNA: messenger RNA; TamR: tamoxifen resistant; TCGA: The Cancer Genome Atlas [Color figure can be viewed at wileyonlinelibrary.com]

and ZR-75-30 breast cancer cells compared to Par MCF-7 cells (Figure 1c). Immunohistochemistry data from a representative patient revealed that treatment naïve tumor tissue sections stained positive for α6-integrin expression. However, once patients relapse on Tamoxifen, α6-integrin expression intensifies (Figure 1d). Positive staining was also evident among tissue sections taken from bone metastases (data not shown). Furthermore, α6-integrin expression levels were significantly higher in tumor samples of the basal-like molecular subtype than the luminal A, luminal B, or HER2-enriched subtypes (Figure 1e); basal-like tumors are known to exhibit

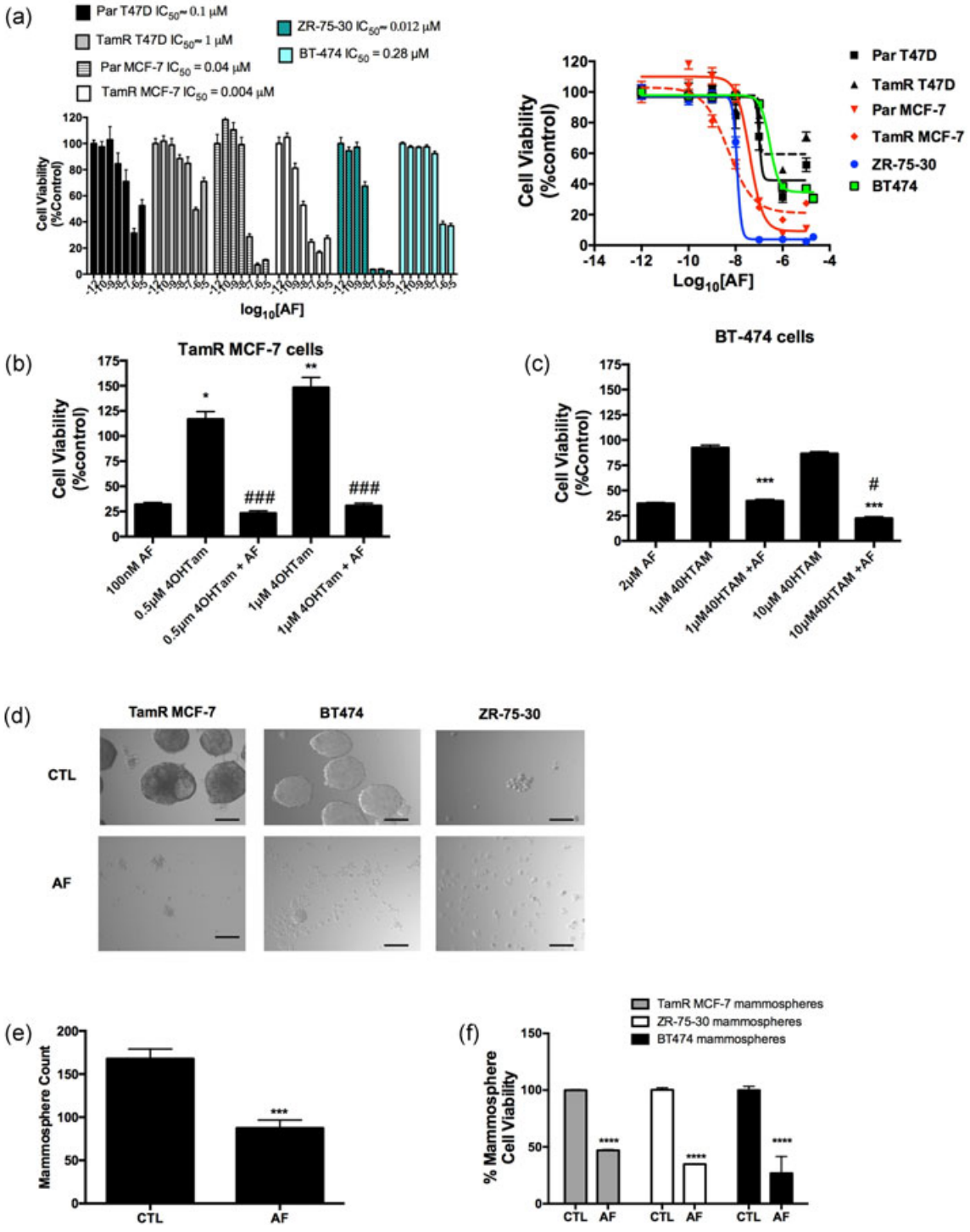

FIGURE 2 Determination of AF-mediated anticancer activity in tamoxifen-resistant breast cancer cells and mammospheres. (a) Par MCF-7, Par T47D, TamR MCF-7, TamR T47D, BT-474, and ZR-75-30 cells were exposed to AF (0.1–10,000 nM) up to 5 days before analysis via the Alamar Blue™ assay in accordance with Section 2. Data represent the mean of at least four independent experiments using at least quadruplicate samples for each concentration. (b) TamR MCF-7 cells were exposed to AF, 4OHTam, or AF and 4OHTam in combination before using the Alamar Blue assay as described in detail in Section 2. Statistically significant at $^{*}p < 0.05$ or $^{**}p < 0.01$ in comparison with control (0.1% DMSO) and at $^{\#\#\#}p < 0.001$ in comparison to 4OHTam alone. (c) BT-474 cells were exposed to AF, 4OHTam, or AF and 4OHTam in combination before using the Alamar Blue assay as described in detail in Section 2. Statistically significant at $^{***}p < 0.001$ in comparison with cells treated with 4OHTam alone and at $^{\#}p < 0.05$ in comparison with cells treated with AF alone. (d) Mammospheres derived from TamR MCF-7, BT-474, and ZR-75-30 cells were treated with 0.1% DMSO (control, CTL) or AF in accordance with Section 2 before imaging using relief contrast microscopy. Scale bar = 50 μm. (e) TamR MCF-7 mammospheres were treated with CTL or AF (1 μM, 48 hr) and then counted in accordance with Section 2. (f) The cell viability of mammospheres derived from TamR cells was determined following treatment with CTL or AF (2 μM for BT-474 cells, 1 μM for TamR MCF-7 cells, and 100 nM for ZR-75-30 cells) for 48 hr. Viability was determined in accordance with Section 2. AF: aminoflavone; DMSO: dimethyl sulfoxide; 4OHTam: 4-hydroxy-tamoxifen; Par: parental; TamR: tamoxifen resistant [Color figure can be viewed at wileyonlinelibrary.com]

resistance to tamoxifen. Taken together, our data suggest that α6-integrin overexpression is associated with tamoxifen resistance.

3.2 | AF inhibits ER+ TamR cell proliferation and disrupts ER+ TamR mammospheres

We previously showed that AFP464 (AF pro-drug) and AF disrupt mammospheres derived from in vitro and in vivo models via α6-integrin suppression (Brantley et al., 2016). Therefore, we sought to determine whether AF inhibits the proliferation of TamR cells and disrupts TamR mammospheres. Interestingly, the luminal A T47D cells (both Par and TamR) and to a lesser extent luminal A, MCF-7 cells (both Par and TamR) exhibited a biphasic dose response following treatment with AF while this effect was not apparent in the luminal B ZR-75-30 or BT-474 cells (Figure 2a). With the exception of the TamR T47D cells (IC_{50}~1 μM), all cells demonstrated responsiveness to AF at submicromolar concentrations, with TamR MCF-7 cells showing the most sensitivity to AF (Figure 2a). In support of other studies indicating the tendency for HER2/neu-enriched cells to resist tamoxifen (Chen, Wang, Kane, & Chen, 2008), we found that BT-474 and ZR-75-30 cells were unresponsive to tamoxifen (data not shown). Notably, TamR MCF-7 cells were not only insensitive to tamoxifen but demonstrated an increase in viability following tamoxifen exposure, while AF treatment prevented tamoxifen-induced TamR cell

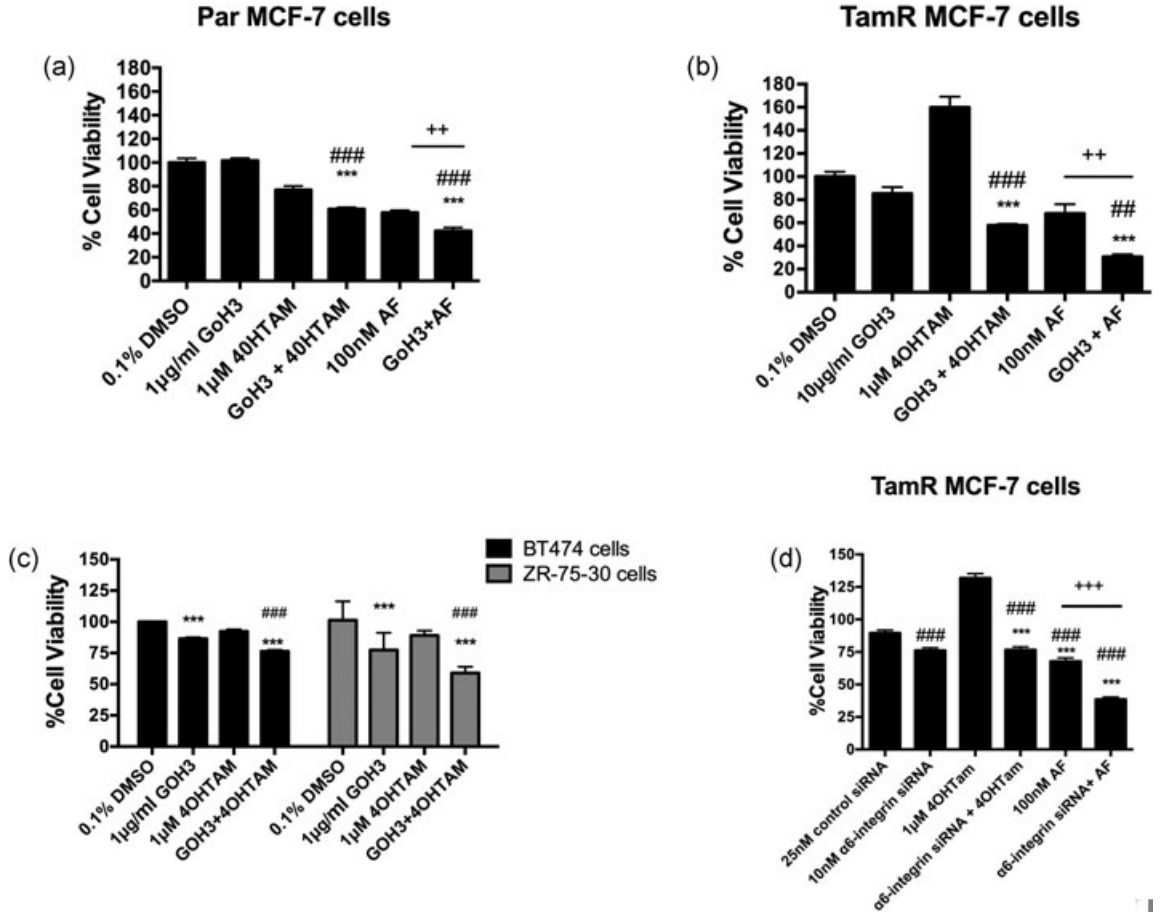

FIGURE 3 Impact of AF and α6-integrin suppression on the responsiveness of breast cancer cells to tamoxifen. (a) Par MCF-7 cells were treated with DMSO (control), 4OHTam, α6-integrin blocking antibody GoH3, AF, or GoH3 in combination with 4OHTam or AF before cell viability was assessed using the Alamar Blue™ assay as described in Section 2. Statistically significant at $^{###}p < 0.001$ in comparison with DMSO (control) exposed. Statistically significant at $^{***}p < 0.001$ in comparison with 4OHTam alone and statistically significant at $^{++}p = 0.002$, where indicated. (b) TamR MCF-7 cells were exposed to 4OHTam, α6-integrin blocking antibody GoH3, AF, or GoH3 in combination with 4OHTam or AF before cell viability was assessed using the Alamar Blue assay as described in Section 2. Statistically significant at $^{###}p < 0.001$ or $^{##}p < 0.01$ in comparison with 0.1% DMSO (control) exposed. Statistically significant at $^{***}p < 0.001$ in comparison with 4OHTam alone. Statistically significant at $^{++}p = 0.01$, where indicated. (c) BT-474 and ZR-75-30 cells were exposed to GoH3, 4OHTam, or the combination for up to 5 days before the Alamar Blue assay was used in accordance with Section 2. Statistically significant at $^{***}p < 0.001$ in comparison with DMSO (control) exposed cells. Scale bars = *SEM*. Statistically significant at $^{###}p < 0.001$ in comparison with 4OHTam alone. (d) TamR MCF-7 cells were transfected with a pool of siRNAs against α6-integrin or nontargeting siRNAs. Transfected cells were exposed to 4OHTam or AF alone. Cell viability was determined using the Alamar Blue assay as described in Section 2. Statistically significant at $^{###}p < 0.001$ in comparison with DMSO (control) exposed. Statistically significant at $^{***}p < 0.001$ in comparison with 4OHTam alone. Statistically significant at $^{+++}p < 0.001$ where indicated. AF: aminoflavone; DMSO: dimethyl sulfoxide; 4OHTam: 4-hydroxy-tamoxifen; Par: parental; TamR: tamoxifen resistant; siRNA: small interfering RNA

proliferation as seen by increased cell viability (Figure 2b). In keeping with our observations, it has been reported that ER^+ tumors that have acquired resistance to tamoxifen often demonstrate tamoxifen-stimulated proliferation while retaining ER expression (J. Chang & Fan, 2013). AF helped to restore sensitivity to tamoxifen in TamR MCF-7 and BT-474 cells (Figure 1b,c). We previously demonstrated that AF impedes mammosphere formation in MCF-7 cells sensitive to tamoxifen (Brantley et al., 2016). In the current study, we found that AF disrupted mammospheres derived from TamR MCF-7, BT-474, and ZR-75-30 cells (Figure 2d). AF was also able to reduce the number of mammospheres formed by the TamR MCF-7 cells (Figure 2e). Due to size differences between untreated mammospheres and fragmented, AF-exposed mammospheres, manual count appeared to show an increase in the number of AF-exposed BT-474 mammospheres compared to control (data not shown). An accurate count on ZR-75-30 mammospheres was not readily achievable as these cells, at best, formed very loose mammospheres and were completely disrupted following AF treatment. Thus, determining actual mammosphere number was not readily feasible. However, using the Alamar Blue assay, we found AF reduced cell viability of TamR MCF-7, ZR-75-30, and BT-474 mammospheres (Figure 2f). Our data suggest that AF inhibits TamR cell viability, impedes tamoxifen-induced TamR MCF-7 cell proliferation and disrupts TamR mammospheres.

3.3 | Blocking α6-integrin expression and function inhibits 4OHTam-induced TamR cell proliferation and enhances the anticancer efficacy of AF

We previously revealed that cells that substantially overexpress α6-integrin are rescued from the cytotoxic effects of AF (Brantley et al., 2016). To determine whether α6-integrin contributes to driving the resistance phenotype in TamR cells, we used a functional blocking antibody in select studies. In addition, we used a pool of siRNAs against α6-integrin. We used 100 nM AF rather than 1 μM due to the longer incubations times and to better determine whether AF in combination with other treatments would lead to an enhancement in anticancer activity as compared with AF alone. Blocking antibody GoH3 enhanced the anticancer activity of tamoxifen and AF in Par MCF-7 cells and in TamR cells (Figure 3a–c). Suppressing α6-integrin's function or silencing α6-integrin reduced the cell viability of TamR cells, prevented the 4OHTam-induced proliferation, and enhanced responsiveness of these cells to 4OHTam (Figure 3b,d). As expected, the effects on cell proliferation were a bit more pronounced with AF and siRNA against α6-integrin as compared with the blocking antibody as the blocking antibody is unable to negate the downstream effects (e.g., cell proliferation) while AF and α6-integrin siRNA are able to. Furthermore, blocking both the function and expression of α6-integrin enhanced the

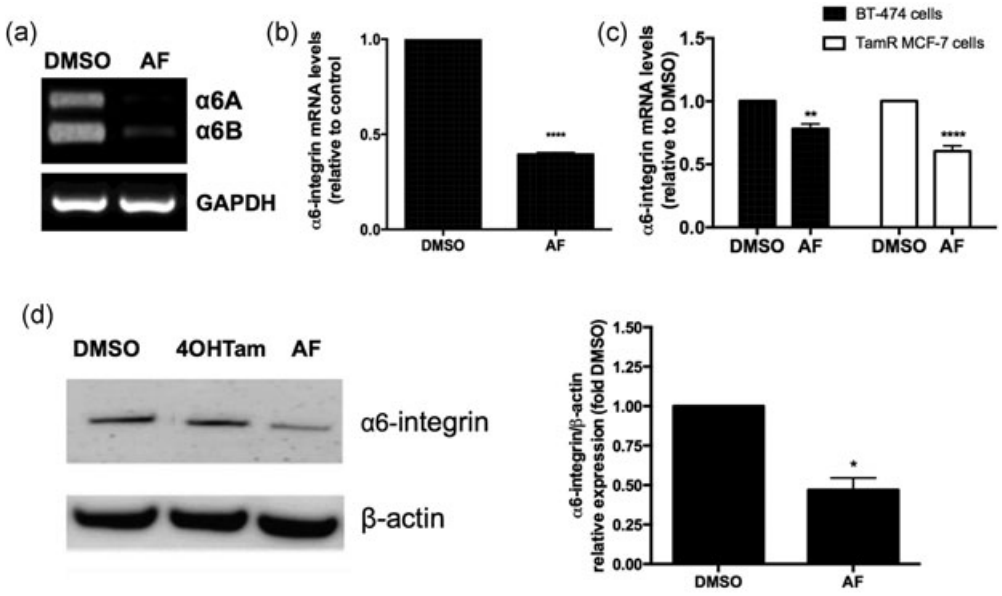

FIGURE 4 AF suppresses α6-integrin expression in tamoxifen-resistant breast cancer cells. (a) Semiquantitative PCR analysis was performed to evaluate the expression of (a) and (b) isoform variants of α6-integrin in TamR MCF-7 mammospheres exposed to CTL (0.1% DMSO) or 1 μM AF for 48 hr. (b) Tam MCF-7 mammospheres were treated with 0.1% DMSO or 1 μM AF for 48 hr before qPCR analyses were performed to evaluate α6-integrin expression. Data represent the mean of at least five independent experiments performed in quadruplicate. Scale bars = *SEM*. Statistically significant at $^{****}p < 0.0001$ in comparison with 0.1% DMSO. (c) BT-474 and TamR MCF-7 cells were exposed to 0.1% DMSO or 2 μM AF for 120 hr and CTL 0.1% DMSO or 1 μM AF for 48 hr, respectively, before qPCR analyses were performed to evaluate α6-integrin expression. Data represent the mean of at least five independent experiments performed in quadruplicate. Scale bars = *SEM*. Statistically significant at $^{**}p < 0.01$ or $^{****}p < 0.0001$ in comparison with 0.1% DMSO. (d) TamR MCF-7 cells were treated with 0.1% DMSO, 1 μM 4OHTam or 1 μM AF for 48 hr before cells were lysed and analyzed for α6-integrin protein expression using western blot analysis in accordance with Section 2. Scale bars = *SEM*. Statistically significant at $^{*}p < 0.05$ in comparison with DMSO. AF: aminoflavone; CTL: control; DMSO: dimethyl sulfoxide; 4OHTam: 4-hydroxy-tamoxifen; qPCR: quantitative polymerase chain reaction; siRNA: small interfering RNA; TamR: tamoxifen resistant

cytotoxic effects of AF against TamR cells (Figure 3b,d). Notably, the TamR MCF-7 cells were more responsive to the GoH3 treatment alone compared with the Par MCF-7 cells suggesting greater reliance on α6-integrin by these resistant cells for survival. These data suggest α6-integrin is important in the survival of TamR cells, particularly tamoxifen-induced cell proliferation, and contributes to AF-mediated anticancer actions.

3.4 | AF inhibits α6-integrin expression, α6-integrin–Src–Akt signaling activation, and induces BAX expression in TamR cells

We found that AF reduced the expression of both cytoplasmic variants of α6-integrin (α6A and α6B) in TamR MCF-7 mammospheres (Figure 4a). AF also reduced α6-integrin gene expression in TamR MCF-7 mammospheres (Figure 4b). AF treatment was also found to significantly reduce α6-integrin expression in TamR MCF-7 and BT-474 cells (Figure 4c). However, AF was unable to inhibit α6-integrin expression in ZR-75-30 cells, despite their sensitivity to this agent (data not shown) which suggests that ZR-75-30 cells demonstrate sensitivity to AF via α6-integrin-independent mechanisms. It is interesting to note that ZR-75-30 cells lack PR expression while BT-474 cells express the PR and this may account for some of the differences seen in viability and α6-integrin expression inhibition in these cells following AF treatment. AF decreased α6-integrin protein expression in TamR MCF-7 breast cancer cells (Figure 4d).

α6-integrin signaling events that are crucial in cancer progression include α6-FAK/Src activation of the PI3K–Akt pathway (Kim et al., 2009). To assess whether downregulation of α6-integrin lead to a reduction in Src and Akt signaling, we assessed levels of phosphorylated Src (p-Src) and Akt (p-AKT). AF caused an increase in pAkt (Ser473) that was inhibited by the α6-integrin blocking antibody GoH3 (Figure 5a). We observed a more pronounced increase in pAkt (Ser473) expression in Par MCF-7 cells following AF treatment (data not shown) that is consistent with a previous study using MCF-7 cells (Meng, Kohn, & Pommier, 2007). GoH3 treatment caused no appreciable change in pAkt (Ser473) phosphorylation at either time point in TamR MCF-7 cells (Figure 5a). Both AF and GoH3 reduced pAkt (Thr308) levels in TamR MCF-7 cells at both time points while GoH3 enhanced the ability of AF to reduce pAkt (Thr308) activation after 24 hr of treatment (Figure 5b). AF and GoH3 increased phosphorylation at the Src inactivation site, Tyr527, in TamR MCF-7 cells as early as 8 hr (Figure 5c). This phosphorylation was sustained up to 24 hr of treatment (Figure 5c), though GoH3 was unable to enhance AF-mediated inhibition of Src signaling at either time point causing a paradoxical decrease after 24 hr of combined treatment (Figure 5c). Taken together, AF caused a net decrease in Akt and Src signaling activation.

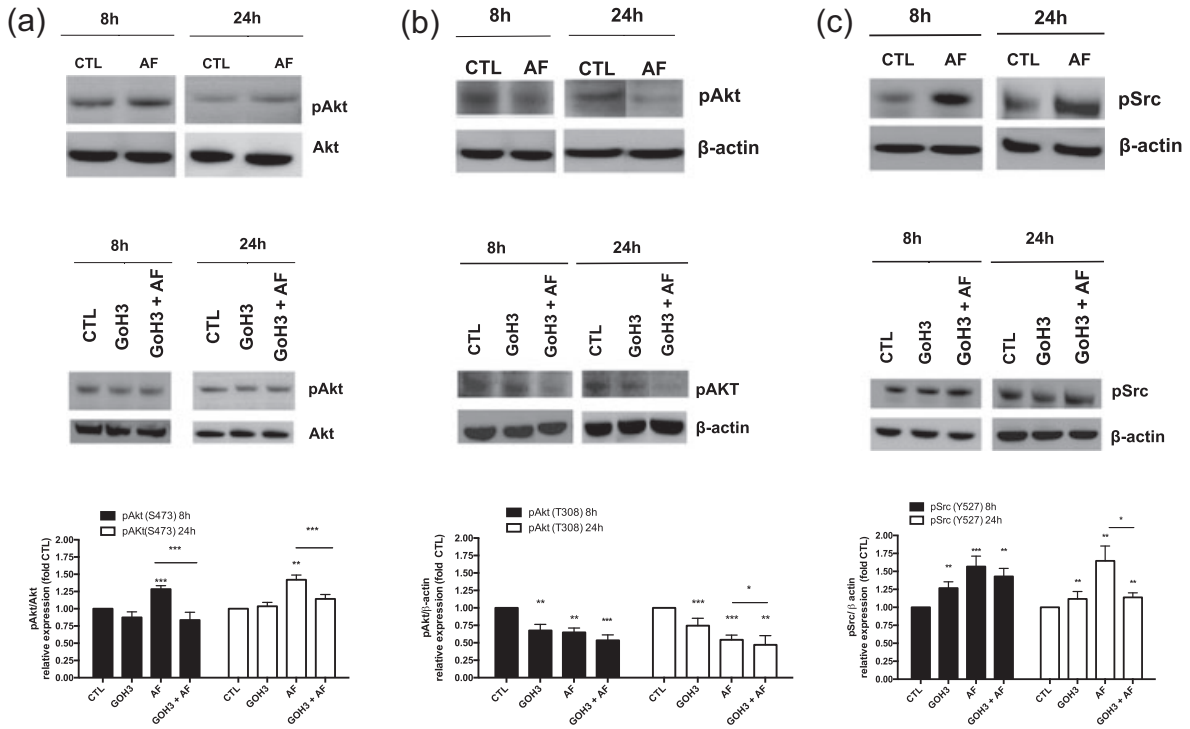

FIGURE 5 AF modulates Akt and Src signaling in tamoxifen-resistant breast cancer cells. (a, b) TamR MCF-7 cells were exposed to media only or media containing 0.01% DMSO (CTL), 1 μM AF, 1 μg/ml GoH3, or AF + GoH3 in combination for 8 and 24 hr before Akt phosphorylation was assessed using western blot analyses in accordance with Section 2. (c) TamR MCF-7 cells were exposed to media only or media containing 0.01% DMSO (CTL), 1 μM AF, 1 μg/ml GoH3, or AF + GoH3 in combination for 8 and 24 hr before Src phosphorylation was assessed using western blot analyses in accordance with Section 2. Data represent the mean of at least three independent experiments. Scale bars = *SEM*. Statistically significant at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$ in comparison with CTL or where indicated. AF: aminoflavone; CTL: control; DMSO: dimethyl sulfoxide

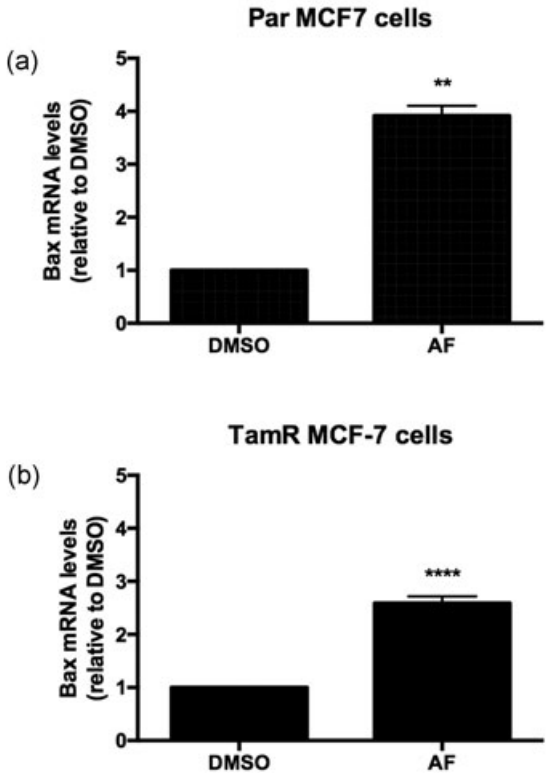

FIGURE 6 AF induces the expression of proapoptotic gene BAX in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. (a) Par and (b) TamR MCF-7 cells were exposed to CTL or 1 μM AF for 48 hr before qPCR analysis was used to detect BAX mRNA expression. Data represent the mean of at least three independent experiments. Scale bars = *SEM*. Statistically significant at $^{**}p < 0.01$ or $^{****}p < 0.0001$ in comparison with DMSO. AF: aminoflavone; CTL; control; DMSO: dimethyl sulfoxide; mRNA: messenger RNA; Par: parental; qPCR: quantitative polymerase chain reaction; TamR: tamoxifen resistant

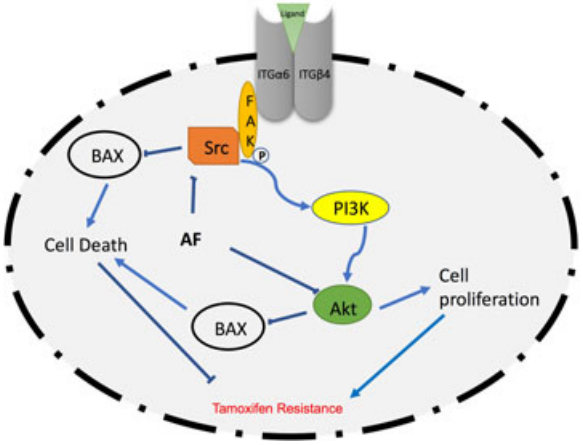

FIGURE 7 Schematic depiction of proposed mechanism by which AF confers anticancer actions in TamR breast cancer cells. Ligands such as laminin bind to the α6β4 integrin heterodimer to stimulate FAK–Src activation. This activation in turn stimulates cell-survival pathways such as the PI3K–Akt pathway, which increases cell proliferation and inhibits cell death to promote tamoxifen resistance. On the contrary, AF inhibits α6-integrin–Src–Akt signaling to overcome resistance. AF: aminoflavone; TamR: tamoxifen resistant [Color figure can be viewed at wileyonlinelibrary.com]

Integrin-mediated cell survival has been linked to the regulation of the proapoptotic gene BAX and integrin signaling appears to block BAX-induced apoptosis by preventing BAX translocation to the mitochondria (Gilmore, Metcalfe, Romer, & Streuli, 2000). We previously demonstrated the ability of AF to induce apoptosis in sensitive breast cancer cells as evidenced by poly(ADP-ribose) polymerase cleavage and caspase 9 activation (McLean et al., 2008). We therefore evaluated the expression of BAX following AF treatment in Par and TamR MCF-7 cells. We found that AF significantly increased BAX expression in both Par and TamR MCF-7 cells (Figure 6a,b). Our data suggest that AF inhibits Src and Akt signaling activation to initiate TamR cell death via BAX induction and to suppress TamR cell proliferation (Figure 7).

4 | DISCUSSION

Tamoxifen resistance frequently leads to relapse, metastases, and death. It is therefore imperative to develop effective therapeutic agents to combat tamoxifen resistance. In this study, we discovered that AhR ligand AF inhibits the proliferation of TamR cells at least in part by reducing α6-integrin expression and inhibiting activation of downstream Src and Akt signaling pathways. Our findings and that of others also suggest that elevated α6-integrin expression is linked to tamoxifen resistance (Berardi, Raffo, Todaro, & Simian, 2016).

Although AhR signaling activation has been shown to promote tumorigenesis, emerging evidence indicates that certain AhR agonists exhibit anti-invasive and antimetastatic actions (Hall et al., 2010; Hanieh et al., 2016; Prud'homme et al., 2010). AF selectively and potently inhibits the growth of cancer cells and tumors with no appreciable toxicity to nonmalignant cells (Loaiza-Pérez et al., 2004; McLean et al., 2008). Nontoxic AhR agonists such as AF and tranilast behave like partial AhR agonists which often oppose the tumor promoting actions of toxic, full AhR agonists similar to AhR antagonists. Small molecule AhR antagonists have been shown to inhibit the progenitor population within TamR cells in vitro and in vivo (Dubrovska et al., 2012).

Cells with higher levels of α6-integrin expression such as the BT-474 cells were less sensitive to the cytotoxic actions of AF and this supports our earlier observation that breast cancer cells with very high α6-integrin expression resist the cytotoxic actions of AF (Figure. 2; Brantley et al., 2016). There is likely a threshold of α6-integrin expression that when exceeded, renders cells resistant to AF (Brantley et al., 2016). In the current study, TamR cells also demonstrated varying levels of sensitivity to AF due to differences in their molecular makeup. Synergism has been reported between AF and fulvestrant, in ER^+ breast cancer cells (Shelton et al., 2007). Importantly, fulvestrant is a standard of care agent used to treat patients who have relapsed on tamoxifen.

The ability of α6-integrin blockade to enhance AF efficacy in TamR cells suggests further benefit is plausible from combining

α6-integrin blocking agents with anticancer AhR agonists to treat TamR breast cancer. Furthermore, tamoxifen in combination with other AhR agonists such as the selective AhR modulator, 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) has previously shown efficacy in mouse models of breast cancer that show responsiveness to tamoxifen (McDougal, Wormke, Calvin, & Safe, 2001). Interestingly, 6-MCDF decreased levels of estrogen receptor α (ERα) through proteasomal degradation. Thus, AhR ligands have potential to demonstrate efficacy in the treatment of breast cancer including subtypes that are resistant to endocrine therapy.

ER expression does not entirely define the anticancer efficacy of AF. For instance, certain basal-like breast cancer cells such as MDA-MB-468 are highly sensitive to AF (Brinkman, Wu, Ersland, & Xu, 2014), yet treatment with histone deacetylase inhibitor vorinostat is necessary to sensitize basal-like MDA-MB-231 breast cancer cells to AF via ER reactivation (Stark et al., 2013). Responsiveness to AF appears to rely in part on the ability of this small molecule to induce AhR-mediated signaling activation and to suppress α6-integrin-mediated signaling pathways.

Our data suggest that elevated α6-integrin expression is linked to tamoxifen resistance and sustains the proliferation and survival of TamR cells. Notably, AF reduced the expression of both cytoplasmic splice variants of α6-integrin (α6A and α6B) in TamR MCF-7 mammospheres (Figure 4a). Importantly, α6B expression defines the mesenchymal population in breast cancer that is necessary for TIC function (Goel et al., 2014). Our findings are consistent with previous reports that revealed elevated α6-integrin expression of more than 3-fold in patient-derived ER^+ breast cancer xenografts with acquired resistance to tamoxifen (Cottu et al., 2014). Furthermore, α6-integrin expression was comparatively higher in mammosphere-derived cells than cells from 2D cell culture (monolayers). This finding is consistent with what we found previously (Brantley et al., 2016). Indeed, mammospheres are known to enrich for TICs (Saadin, Burke, Patel, Zubajlo, & White, 2013). Though our patient sample size was small in the IHC study (Figure 1), the trend toward elevated α6-integrin expression in patients who relapsed on tamoxifen was further demonstrated in basal-like tumors (tamoxifen unresponsive) in comparison to other tumor types from the TCGA database involving a much larger cohort of patients. Nonetheless, the above-mentioned findings suggest that elevated levels of α6-integrin are associated with tamoxifen resistance and α6-integrin may be valuable as a predictive biomarker of tamoxifen responsiveness.

TICs have been shown to play a key role in the development of resistance to tamoxifen (Bostner et al., 2013). In fact, tamoxifen treatment itself has been shown to select for cells with self-renewal capacity and promote mammosphere formation (Raffo et al., 2013). A recent study showed that α6-integrin ligand laminin conferred resistance to tamoxifen in an estrogen-dependent, tamoxifen-sensitive LM05-E breast cancer cell line via α6-integrin (Berardi et al., 2016). These observations support the hypothesis that tamoxifen may promote its own resistance by upregulating α6-integrin levels and other TIC-related pathways and genes. Tamoxifen can also act as an ER agonist in breast cancer cells to promote tamoxifen resistance. In keeping with our observations, it has been reported that ER^+ tumors that have acquired resistance to tamoxifen may either be unresponsive to this agent or demonstrate tamoxifen-stimulated growth while retaining ER expression (M. Chang, 2012). Reduced expression of corepressors observed in tamoxifen resistance, results in stabilization of the agonist confirmation of the ERα, thereby allowing ERα activation by tamoxifen. (Chakraborty & Biswas, 2014). This may explain why tamoxifen stimulates proliferation in certain resistant cells.

Integrins have been shown to activate cell-survival pathways such as PI3K to promote cancer cell proliferation and cell death via downstream FAK–Src signaling activation (Kim et al., 2009). In particular, α6-integrin primarily activates PI3K signaling to promote cancer cell migration, invasion, and survival (Lipscomb & Mercurio, 2005). In the current study, we found that increased α6-integrin expression correlated with an overall increase in Src–Akt signaling since we found TamR cells exhibited not only increased α6-integrin expression, but elevations in Akt phosphorylation (Figure 1c, Supporting Information Figure 2). Additionally, AF effectively suppressed α6-integrin expression and this leads to an overall decrease in Src–Akt signaling. Thus, Src–Akt signaling is decreased after α6-integrin expression is suppressed.

AF phosphorylated Src at Tyr527 in TamR MCF-7 cells as early as 8 hr and this phosphorylation was sustained for at least 24 hr (Figure 5c). GoH3 also promoted this phosphorylation as well, though GoH3 combined with AF did not enhance this effect (Figure 5c). Phosphorylation of p-Src(Tyr527) results in Src inactivation through interaction with the SH2 domain and protein folding which makes Src inaccessible to substrates (Frame, 2002). Interestingly, acquired tamoxifen resistance leads to integrin-induced FAK–Src activation; inhibition of integrin-mediated FAK–Src–Akt activation was found to produce small yet significant sensitization to tamoxifen (Cowell, Graham, Bouton, Clarke, & O'Neill, 2006). Taken together, our findings indicate AF suppresses Src activation in TamR MCF-7 cells.

AF increased pAkt (Ser473) in Par MCF-7 cells (data not shown) consistent with a previous report which showed that submicromolar concentrations of AF caused the S phase arrest when these cells were treated up to 8 hr (Meng et al., 2007). AF increased Akt activation in Par MCF-7 cells to a greater extent than TamR MCF-7 cells and interestingly the α6-integrin blocking antibody GoH3 inhibited AF-mediated increases in Akt activation in TamR MCF-7 cells (Figure 5a). We concur with Meng et al. (2007) that our findings suggest that activation of Akt might reflect a cellular defense mechanism to AF-mediated DNA damage. It is, therefore, possible that this switch from Akt inactivation to activation with 1 μM AF used in the current study may represent an initial apoptotic response followed by cell cycle arrest in response to DNA damage caused by more prolonged exposure. Indeed, AF induces oxidative DNA damage and S-phase arrest in triple negative MDA-MB-468 cells.

Phosphorylation of Thr308 in the activation loop of the kinase domain and Ser473 in the C-terminal regulatory domain is needed for full activation of Akt, with Thr308 phosphorylation playing the dominant role in Akt activation (Song, Ouyang, & Bao, 2005; Vincent

et al., 2011). Furthermore, Akt phosphorylation at these two sites occurs independently of each other (Alessi et al., 1996) with PDK1 phosphorylating Akt at Thr308 and mTORC2 phosphorylating Akt at Ser473. Therefore, since AF significantly reduced Thr308 phosphorylation, we can conclude that this AhR ligand decreased overall Akt kinase activity in TamR MCF-7 cells, an effect that was enhanced by GoH3 following 24 hr of cotreatment (Figure 5b). AF has targets other than α6-integrin that may contribute to its ability to inhibit Src–Akt signaling activation. For instance, β-naphthoflavone, another AhR agonist with in vivo antitumor activity, was found to inhibit PI3K–Akt signaling in MCF-7 cells in an AhR-dependent manner (Wang, Xu, Bu, Bottum, & Tischkau, 2014). On the other hand, GoH3 specifically blocks the function of α6-integrin and thus AF and GoH3 have the potential to inhibit Src–Akt signaling by related as well as distinct mechanisms.

Activated Akt and Src resulting from integrin signaling and concomitant inhibition of proapoptotic BAX activity opposes cell death (Bouchard et al., 2008; Shishido, Bonig, & Kim, 2014). These observations support our findings that AF inhibits α6-integrin–Src–Akt signaling and induces BAX expression to promote TamR MCF-7 cell death. Additionally, AF suppresses the proliferation of TamR MCF-7 cells by suppressing Thr308 Akt phosphorylation. In our study, both Par and TamR MCF-7 cells showed increased α6-integrin–Src–Akt signaling though TamR cells exhibited this enhanced signaling to a greater extent (Figure 1c and Supporting Information Figure 2). Thus, Src–Akt inhibition in TamR and Par MCF-7 cells likely occurs via similar means and the greater level of BAX induction observed in Par MCF-7 cells compared with TamR MCF-7 cells concurs with the enhanced ability of AF to suppress α6-integrin expression in these cells. It is quite plausible that when these cells are untreated, BAX translocation to the mitochondria is suppressed. We speculate that following AF treatment, α6-integrin–Src–Akt signaling becomes inhibited to enable BAX translocation irrespective of tamoxifen responsiveness. This may explain why BAX induction was observed in both cell lines after AF treatment. Taken together, our data suggest that BAX translocation is readily restored following AF-mediated α6-integrin–Src–Akt signaling blockade.

In conclusion, our data suggest AF inhibits α6-integrin–Src–Akt signaling to induce apoptosis, reduce cell proliferation, and counteract tamoxifen resistance in ER^+ breast cancer cells. More in-depth studies are needed to conclusively determine whether α6-integrin plays a causal role in tamoxifen resistance as has been recently determined for TIC genes OCT-4 and SOX-9 (Bhatt, Stender, Joshi, Wu, & Katzenellenbogen, 2016; Jeselsohn et al., 2017). Our findings do suggest that AhR ligands such as AF have the potential to help combat tamoxifen resistance to ultimately improve clinical outcomes for patients who have relapsed on tamoxifen. Other AhR ligands such as antiallergy agent tranilast disrupt mammospheres (Prud'homme et al., 2010). We recently determined that related AhR ligand, 5F 203 suppresses α6-integrin expression and disrupts mammospheres (data not shown). To the best of our knowledge, our report is the first to demonstrate the ability of AhR ligands to reverse tamoxifen resistance by attenuating α6-integrin–Src–Akt signaling. Our study provides a rationale for evaluating α6-integrin as a potential biomarker for tamoxifen resistance and to more appropriately stratify luminal breast cancer patients that would ultimately benefit from endocrine therapy in combination with AhR ligands such as AF.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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