

## RETRACTED

## ORIGINAL ARTICLE

## IL-6 secreted by cancer-associated fibroblasts induces tamoxifen resistance in luminal breast cancer

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Cancer-associated fibroblasts (CAFs) have been implicated in the development of resistance to anticancer drugs; however, the role and mechanism underlying CAFs in luminal breast cancer (BrCA) tamoxifen resistance are unclear. We found that stromal fibroblasts isolated from the central or peripheral area of BrCA have similar CAF phenotype and activity. *In vitro* and *in vivo* experiments showed that CAFs derived from clinical-luminal BrCAs induce tamoxifen resistance through decreasing estrogen receptor- $\alpha$  (ER- $\alpha$ ) level when cultured with luminal BrCA cell lines MCF7 and T47D. CAFs promoted tamoxifen resistance through interleukin-6 (IL-6) secretion, which activates Janus kinase/signal transducers and activators of transcription (JAK/STAT3) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways in tumor cells, followed by induction of epithelial–mesenchymal transition and upregulation of E3 ubiquitin ligase anaphase-promoting complex 10 activity, which targeted ER- $\alpha$  degradation through the ubiquitin–proteasome pathway. Inhibition of proteasome activity, IL-6 activity or either the JAK/STAT3 or PI3K/AKT pathways markedly reduced CAF-induced tamoxifen resistance. In xenograft experiments of CAFs mixed with MCF7 cells, CAF-specific IL-6 knockdown inhibited tumorigenesis and restored tamoxifen sensitivity. These findings indicate that CAFs mediate tamoxifen resistance through IL-6-induced degradation of ER- $\alpha$  in luminal BrCAs.

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## INTRODUCTION

Breast cancer (BrCA) is the most frequent malignant tumor and the second leading cause of cancer-related death among women worldwide. Approximately 75% of BrCAs are estrogen receptor-positive (ER<sup>+</sup>) and belong to the luminal molecular subgroup, which is the most prevalent subtype of BrCA in both early- and advanced-stage disease. In ER<sup>+</sup> BrCA, adjuvant antiestrogen treatment can significantly improve patient outcome.<sup>1,2</sup> However, *de novo* or acquired endocrine therapy resistance still develops in patients; therefore, representing a major barrier to the successful treatment of BrCA. ER expression is currently the main biomarker of response to endocrine therapy.<sup>3</sup> Unfortunately, the molecular mechanisms responsible for the development of antiestrogen resistance are unclear. The classic role of ER is its function as a nuclear hormone receptor that alters the expression of genes important for normal cellular function, tumor growth and survival. Upon binding to estrogen, ER dimerizes with another receptor monomer and attracts a complex of coactivators and corepressors to specific sites on DNA. ER can also bind to other transcription factors such as activator protein-1 and specificity protein-1 at their specific sites on DNA, thereby functioning as a coregulator.<sup>3</sup> Many studies indicate that overexpression of some coregulators contributes to the development of tamoxifen resistance.<sup>3</sup>

Data from our group and others indicate that the tumor microenvironment is not only involved in promoting tumor growth and invasion through its effect on angiogenesis<sup>4</sup> and epithelial–mesenchymal transition (EMT)<sup>5</sup> but it is also essential for

the regulation of endocrine sensitivity.<sup>6</sup> Components of the micro-environment implicated in endocrine resistance include stromal cells (for example, fibroblasts, endothelial and immune cells), structural elements of the extracellular matrix and soluble factors (for example, cytokines), etc.<sup>4,6</sup> However, the mechanisms underlying the role that cancer-associated fibroblasts (CAFs) have in the development of tamoxifen resistance in luminal BrCA are unclear.

The current study was designed to determine if and how CAFs contribute to the development of tamoxifen resistance in BrCA. We demonstrate that CAFs downregulate ER- $\alpha$  expression through secretion of IL-6 that leads to degradation of ER- $\alpha$ . These results suggest that targeting IL-6 or its downstream targets could serve as an effective therapeutic strategy for luminal BrCA.

## RESULTS

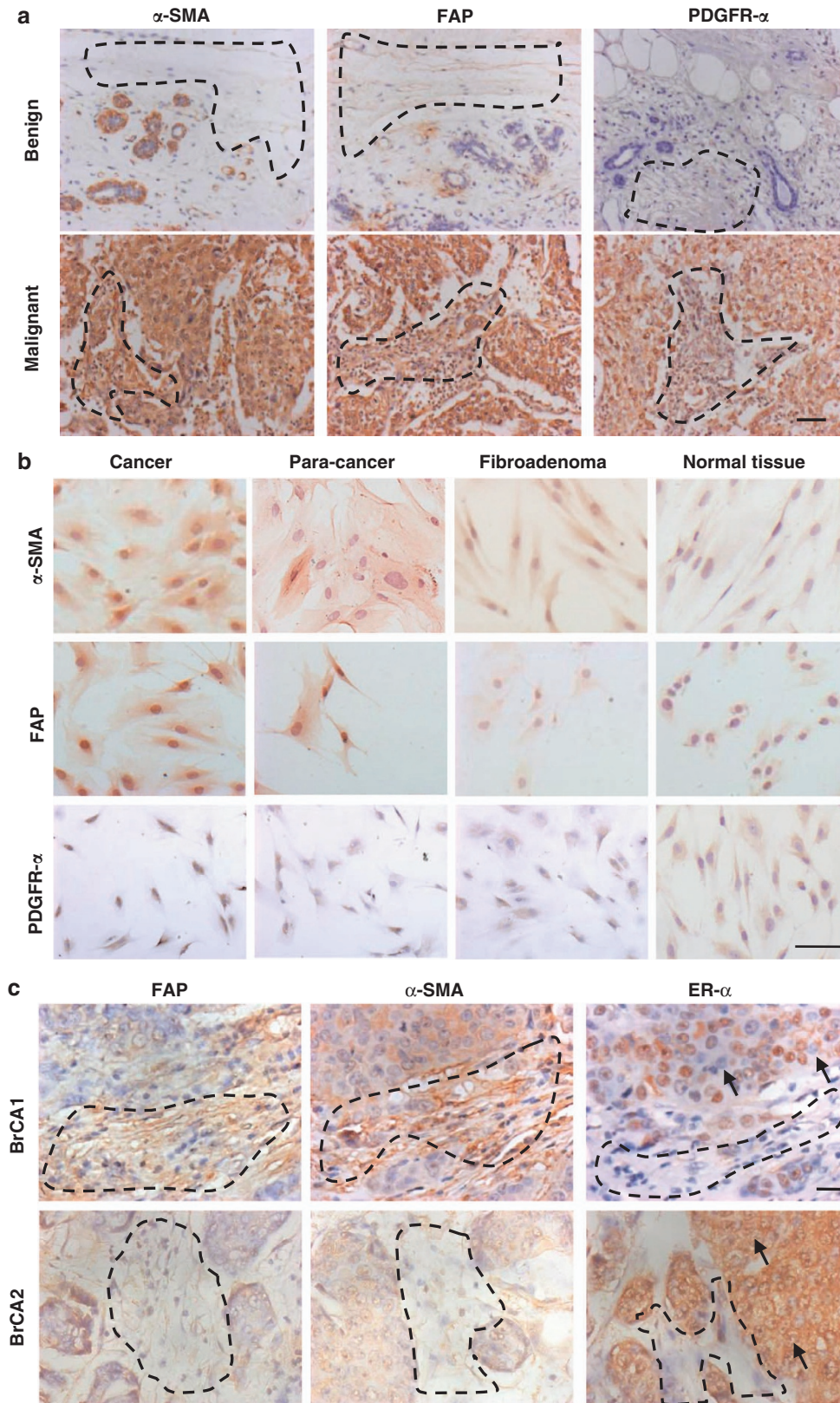
Upregulation of FAP and  $\alpha$ -SMA in BrCA stromal fibroblasts

Increasing evidence suggests that the conversion of stromal fibroblasts into CAFs has a significant role in BrCA development.<sup>7,8</sup> Using immunohistochemistry of tissue microarrays, we initially confirmed the presence of CAFs in clinical BrCA by evaluating the expression of FAP,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and platelet-derived growth factor receptor- $\alpha$  (PDGFR- $\alpha$ ), which are frequently used as CAF markers in the solid tumors.<sup>9–11</sup> Supplementary Figure 1A shows that all three markers were detectable in both benign ( $N=20$ ) and malignant breast disease tissue ( $N=76$ ). However, FAP and  $\alpha$ -SMA expression in the stromal

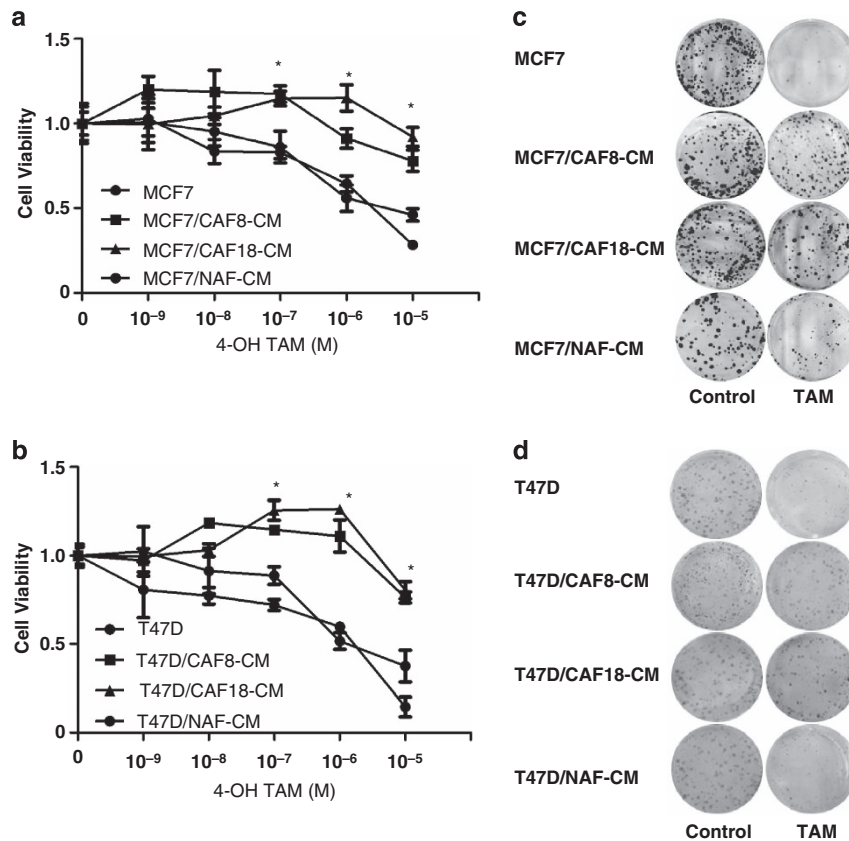
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**Figure 1.** Expression of CAF markers ( $\alpha$ -SMA, FAP and PDGFR- $\alpha$ ) in human breast benign or malignant tissues and in isolated fibroblasts. **(a)** Immunohistochemical analysis of human breast tissue arrays. Dotted lines indicate the stromal regions. Typical positive samples of malignant stromal tissues were selected and show higher intensity staining (brown) with  $\alpha$ -SMA, FAP or PDGFR- $\alpha$  antibodies than that of benign tissues. Scale bar, 50  $\mu$ m. **(b)** Immunocytochemical analysis of fibroblasts isolated from different patients with benign or malignant breast diseases. Scale bar, 100  $\mu$ m. **(c)** Immunohistochemical stainings of the representative tumor tissue show that ER- $\alpha$  expression (black arrow) is lower in the vicinity of  $\alpha$ -SMA $^+$  and FAP $^+$  stromal tissues than in respective negative stromal tissues (dotted lines), which indicates that the ER- $\alpha$  expression is affected by the CAFs. Scale bar, 20  $\mu$ m.



**Figure 2.** Response to different concentrations of tamoxifen after culture of MCF7 and T47D cells with conditioned media from CAFs and NAFs. **(a)** Cell viability assay in MCF7 cells treated with different concentrations of tamoxifen after culture with CMs from control, CAF8, CAF18 and NAF for 5 days. Three independent experiments were performed in triplicate. Data are presented as means  $\pm$  s.d.  $*P < 0.05$ . **(b)** Cell viability assay in T47D cells treated with different concentrations of tamoxifen after culture with CM from control, CAF8, CAF18 and NAF for 5 days. Three independent experiments were performed in triplicate. Data are presented as means  $\pm$  s.d.  $*P < 0.05$ . **(c)** Colony formation in MCF7 cells treated with tamoxifen (1  $\mu$ M) after culture with CM from control, CAF8, CAF18 and NAF for 5 days. Three independent experiments were performed in triplicate. **(d)** Colony formation in T47D cells treated with tamoxifen (1  $\mu$ M) after culture with CM from control, CAF8, CAF18 and NAF for 5 days. Three independent experiments were performed in triplicate.

fibroblasts of malignant tissues was greater than that in benign tissues (Figure 1a and Supplementary Table 1). PDGFR- $\alpha$  expression was not different between the stromal fibroblasts of the two groups (Figure 1a and Supplementary Table 1). These data suggest that FAP and  $\alpha$ -SMA, used for identifying CAFs, at least in BrCA, may be more active than PDGFR- $\alpha$ . These findings are valid when comparing neoplastic *versus* non-neoplastic breast tissue, but do not extend to subtypes of BrCA as we observed that FAP,  $\alpha$ -SMA and PDGFR- $\alpha$  expressions in stromal fibroblasts were not different among the molecular subtypes of BrCA (Supplementary Table 1). To explore if these findings, based on previously collected tissues, extended to primary patient tissues, stromal fibroblasts were isolated from 22 human neoplastic (central or peripheral tumor area) or non-neoplastic breast tissue immediately after resection. Similar to the tissue microarray findings, FAP,  $\alpha$ -SMA, but not PDGFR- $\alpha$  expression, was strongest in fibroblasts derived from neoplastic compared with non-neoplastic tissues (Figure 1b). These findings indicate that stromal fibroblasts located both centrally or peripherally in BrCA tumors are similarly activated and potentially function as CAFs.

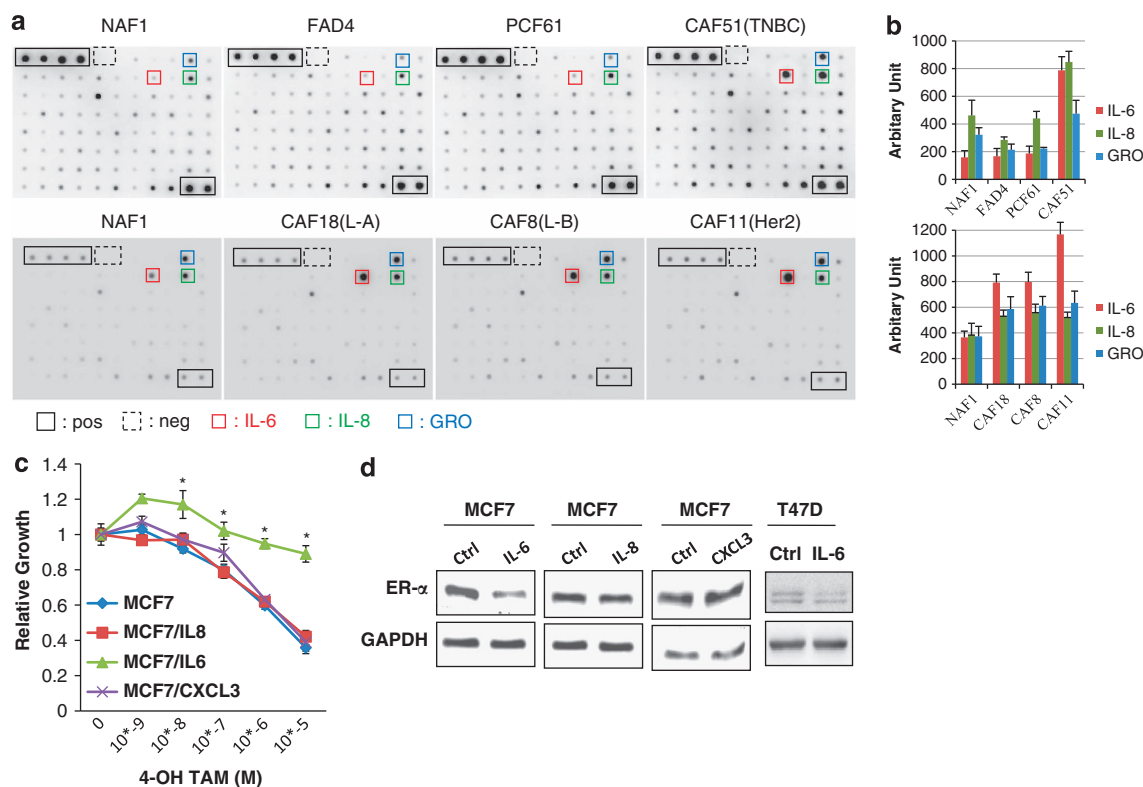
#### CAFs induce tamoxifen resistance in BrCA

To explore the function of CAFs on BrCA, we used two luminal cell lines MCF7 and T47D and cultured them with conditioned medium (CM) from CAF8 and CAF18 (noted as CAF8, 18-CM). These CAFs were randomly chosen from all CAFs isolated from

luminal BrCA patients, and non-cancer-associated fibroblasts (NAF) (noted as NAF-CM), which were isolated from an operable patient with vice breast. CAF8,18-CM induced resistance to tamoxifen in MCF7 cells compared with control or NAF-CM based on both cell viability (Figure 2a) and colony formation assays (Figure 2c). Similar results were also observed in T47D cells (Figures 2b and d). Finally, the xenograft experiments *in vivo* indicated that tumor growth is more rapid in the group of MCF7 cells mixed with CAFs than in the group of MCF7 cells mixed with NAFs. Moreover, tamoxifen treatment inhibited tumor growth only in the group of MCF7 cells mixed with NAF, but not in mixed CAF group (Supplementary Figure 1). Finally, ER- $\alpha$  expression of tumor tissue is potentially affected by the CAFs as shown in Figure 1c. Taken together, these findings demonstrate that CAFs induce BrCA tamoxifen resistance.

IL-6 secreted by CAFs is a key mediator for tamoxifen resistance. It has been reported that CAFs promote therapeutic resistance mainly through the secretion of multiple cytokines, chemokines, etc.<sup>6</sup> To test this possibility, we used cytokine arrays to detect the secreted soluble factors derived from CM of NAF, fibroadenoma fibroblasts (FADs), paracarcinoma fibroblasts (PCFs) and CAFs with four molecular phenotypes (namely luminal A, luminal B, Her2<sup>+</sup> and triple-negative BrCA patients). IL-6, IL-8 and GRO (CXCL1, CXCL2 and CXCL3) levels were consistently higher in the CAF-CM than in the NAF, FAD and PCF-CM (Figures 3a and b). To





**Figure 3.** IL-6 is a key cytokine secreted from fibroblasts. **(a)** Human Cytokine Antibody Array Kits (RayBiotech) were used to measure the content of 80 cytokines in the CM from diverse fibroblasts. The experiment was performed in two steps, and the results are shown in two rows. The CM of NAFs served as control in each operation. Cytokines upregulated in CAFs and indicated by colored boxes are IL-6 (red), IL-8 (green) and GRO (blue). GRO detects CXCL1, CXCL2 and CXCL3. The enclosed black frames indicate the positive controls, whereas the dashed boxes indicate the negative controls in each membrane. L-A, luminal A; L-B, luminal B; Her2, Her2+; TNBC, triple-negative BrCA. **(b)** The dot intensities of different cytokines were quantified by densitometry using the ImageJ software (NIH, Bethesda, MD, USA) and normalized to the intensity of the internal positive controls for comparison. The experiments were performed independently at least three times with similar results. Columns: mean of triplicate experiments; bars: s.d. **(c)** Cell viability assay in MCF7 cells treated with different concentrations of tamoxifen after incubation in phosphate-buffered saline (PBS), IL-6, IL-8 or CXCL3 (all 20 ng/ml) for 5 days. Three independent experiments were performed in triplicate. Data are presented as means  $\pm$  s.d. \* $P < 0.05$ . **(d)** Western blot analysis of MCF7 cell lysates treated with PBS, IL-6, IL-8 or CXCL3 and T47D cells cultured with PBS or IL-6 for 24 h using anti-ER- $\alpha$  antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control.

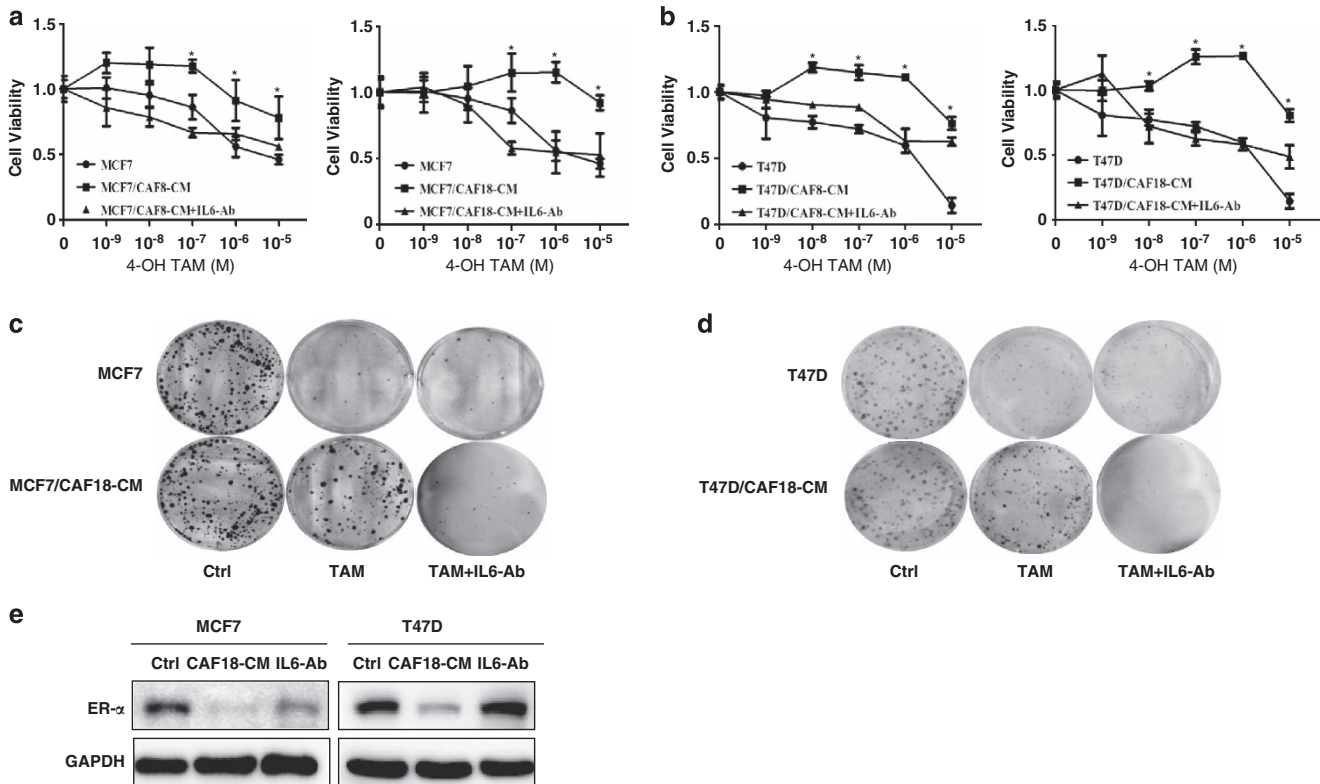
determine which of these factors may promote tamoxifen resistance, we added recombinant human IL-6, IL-8 and CXCL3 to the medium containing MCF7 and T47D cells. Only IL-6 induced tamoxifen resistance, whereas IL-8 or CXCL3 had no impact (Figure 3c). Given previous reports that ER- $\alpha$  downregulation mediates tamoxifen resistance,<sup>3</sup> we tested which factors may affect ER- $\alpha$  expression. ER- $\alpha$  expression was downregulated in MCF7 and T47D cells when incubated with IL-6, but not with IL-8 or CXCL3 (Figure 3d). Taken together, these data suggest that CAF-produced IL-6 may promote tamoxifen resistance. Next, CM from the isolated fibroblasts was analyzed for IL-6 expression using enzyme-linked immunosorbent assay. IL-6 level in CAF-CM and PCF-CM was greater than that in NAF-CM and FAD-CM (Supplementary Figures 2A and B), suggesting that fibroblasts of the central and peripheral tumor areas may be kept in the same active phenotypes as shown in Figure 1. Moreover, autocrine secretion of IL-6 from MCF10A, MCF7, T47D, BT474 and SK-KB3 BrCA cells was almost absent compared with malignant MDA-MB231 cells (Supplementary Figure 2C), suggesting that tamoxifen resistance in ER<sup>+</sup> BrCA cells is potentially induced by CAFs through the paracrine secretion of IL-6. In this regard, using cell viability and colony formation assays, we further demonstrated that blocking IL-6 activity with neutralizing antibody restored tamoxifen sensitivity in both MCF7 and T47D cells cultured with CAF-CM (Figures 4a–d). Additionally, inhibition of IL-6 activity diminished

the ability of CAF-CM to inhibit ER- $\alpha$  expression (Figure 4e). To provide further evidence that IL-6 mediates these activities, we decreased IL-6 expression using short hairpin RNA (shRNA) in CAF18. Knockdown of IL-6 expression reduced the ability of CAF18 to mediate both tamoxifen resistance and reduction of ER- $\alpha$  expression (Supplementary Figure 3).

IL-6 has been reported to correlate with BrCA progression.<sup>12</sup> Here, we found that the invasive ability of MCF7 and T47D cells was markedly increased when cultured with CAF-CM, but was markedly inhibited by knockdown of IL-6 expression in the CAF (Supplementary Figure 4). These results suggest that secretion of IL-6 by CAFs promotes luminal BrCA invasion.

#### IL-6 decreases ER- $\alpha$ expression by the ubiquitin–proteasome pathway

To explore the mechanism through which IL-6 regulates ER- $\alpha$  expression, we first tested the time course of the effect of IL-6 on expression of ER- $\alpha$  protein. IL-6 markedly inhibited ER- $\alpha$  protein expression beginning at 6 h in MCF7 and T47D cells and was still effective at 18 h for MCF7 or 24 h for T47D cells (Figure 5a). We found in both cell lines that IL-6 had no impact on ER- $\alpha$  mRNA levels (Supplementary Figure 5A). Taken together, these data indicate that IL-6 does not impact ER- $\alpha$  expression through the modulation of transcription of but rather through post-



**Figure 4.** IL-6 secreted by CAFs contributes to tamoxifen resistance. **(a and b)** Cell viability assay in MCF7 **(a)** and T47D **(b)** cells treated with different concentrations of tamoxifen combined with or without IL-6-neutralizing antibody after culture with CM from CAF8 (left) and CAF18 (right) for 5 days. Three independent experiments were performed in triplicate. Data are presented as means  $\pm$  s.d. \* $P < 0.05$ . **(c and d)** Colony formation in MCF7 and T47D cells treated with dimethyl sulfoxide (DMSO), tamoxifen (1  $\mu$ M) or tamoxifen combined with IL-6-neutralizing antibody (100 ng/ml) after culture with CM from control, CAF8 and CAF18 for 5 days. Three independent experiments were performed in triplicate. **(e)** Western blot analysis of lysates from MCF7 and T47D cells cultured with CM from CAF18 and treated with or without IL-6-neutralizing antibody (100 ng/ml) for 48 h using anti-ER- $\alpha$  antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

transcriptional events. Accordingly, we next assayed whether degradation of ER- $\alpha$  upon IL-6 stimulation was mediated through the ubiquitin–proteasome pathway. The proteasome inhibitor MG132 blocked the ability of IL-6 to degrade ER- $\alpha$  protein (Figure 5b). Conversely, the ubiquitination of ER- $\alpha$  was markedly enhanced in MCF7 and T47D cells upon IL-6 stimulation or when they were cultured with CAF18-CM (Figure 5c). In contrast, knockdown of IL-6 in CAF18 reduced the ability of CAF-CM to ubiquitinate ER- $\alpha$  in MCF7 cells (Supplementary Figure 5B).

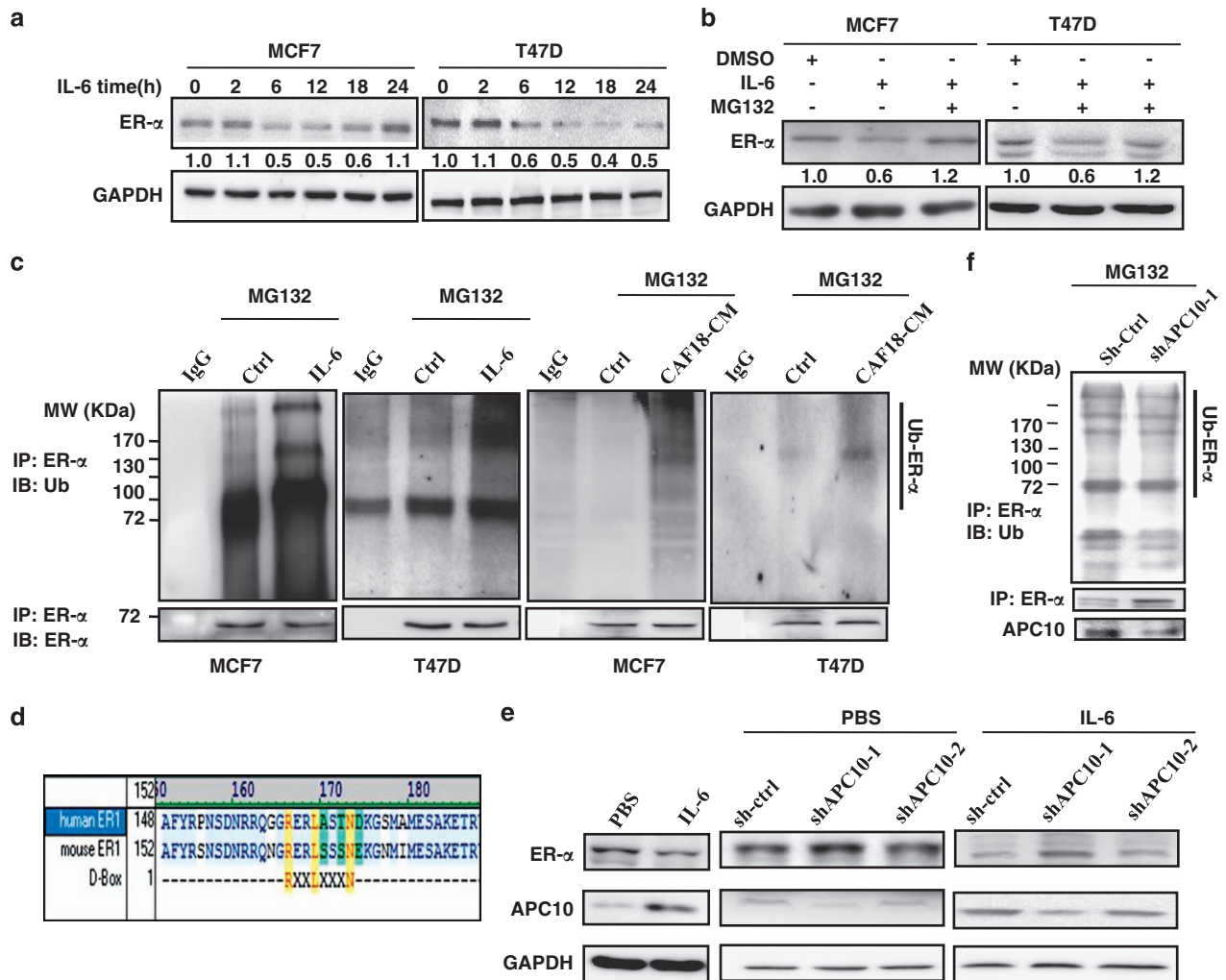
The ER- $\alpha$  gene in human and mouse contains the binding structure D-box recognized by APC/C (anaphase-promoting complex or cyclosome) (Figure 5d), a master ubiquitin protein ligase (E3)<sup>13,14</sup>. We found that IL-6 induced expression of the core APC/C subunit APC10 in MCF7 cells, suggesting that this may be the mechanism through which IL-6 promotes degradation of ER- $\alpha$  protein. To provide further evidence for this possibility, we reduced APC10 expression using shRNAs in MCF7 cells. Knockdown of APC10 in MCF7 cells increased basal ER- $\alpha$  protein expression and diminished the IL-6-induced decline in ER- $\alpha$  protein expression (Figure 5e). Moreover, knockdown of APC10 inhibited ER- $\alpha$  ubiquitination in the MCF7 cells compared with the control cells (Figure 5f). Finally, MG132 inhibited the ability of CAF-CM or IL-6 to promote tamoxifen resistance (Supplementary Figure 5C). Taken together, these findings provide evidence that CAFs promote tamoxifen resistance through IL-6-mediated activation of ubiquitin–proteasome, which mediates downregulation of ER- $\alpha$ .

#### IL-6 induces EMT and ER- $\alpha$ decline

Increasing evidence has shown that EMT has important roles in tumor progression and treatment resistance.<sup>15,16</sup> To explore if IL-6 promotes EMT in addition to decreasing expression of ER- $\alpha$ , we tested the effect of IL-6 on EMT in ER<sup>+</sup> BrCA cells. Supplementary Figure 9 shows that IL-6 potentially induced EMT-like morphologic features in MCF7 cells, such as spindle-shaped appearance, and led to significant reductions in E-cadherin and  $\alpha$ -E-catenin expression, as well as increased vimentin and  $\beta$ -catenin expression. Similar effects were observed in these cells when cultured with CAF8, 18-CM (Supplementary Figure 6B), but not with NAF-CM or control (Supplementary Figure 6C). In contrast, IL-6 inhibition using neutralizing antibody blocked this effect and partially restored ER- $\alpha$  expression (Supplementary Figure 6D), which was responsible for restoring tamoxifen sensitivity in these cells as observed in Figures 4a–d. Therefore, these data suggest that EMT induced by IL-6 is associated with reduction of ER- $\alpha$  expression, which may contribute to tamoxifen resistance.

#### Targeting IL-6 and the activated pathways effectively reverse tamoxifen resistance in luminal BrCA cells

We next investigated whether targeting IL-6 can provide a novel strategy for restoring tamoxifen sensitivity in luminal BrCA. Recently, many findings have disclosed that several signaling pathways activated by cytokines contribute to tamoxifen resistance through crosstalk with ER.<sup>3,17</sup> Indeed, both Janus kinase/signal transducers and activators of transcription (JAK/STAT3) and



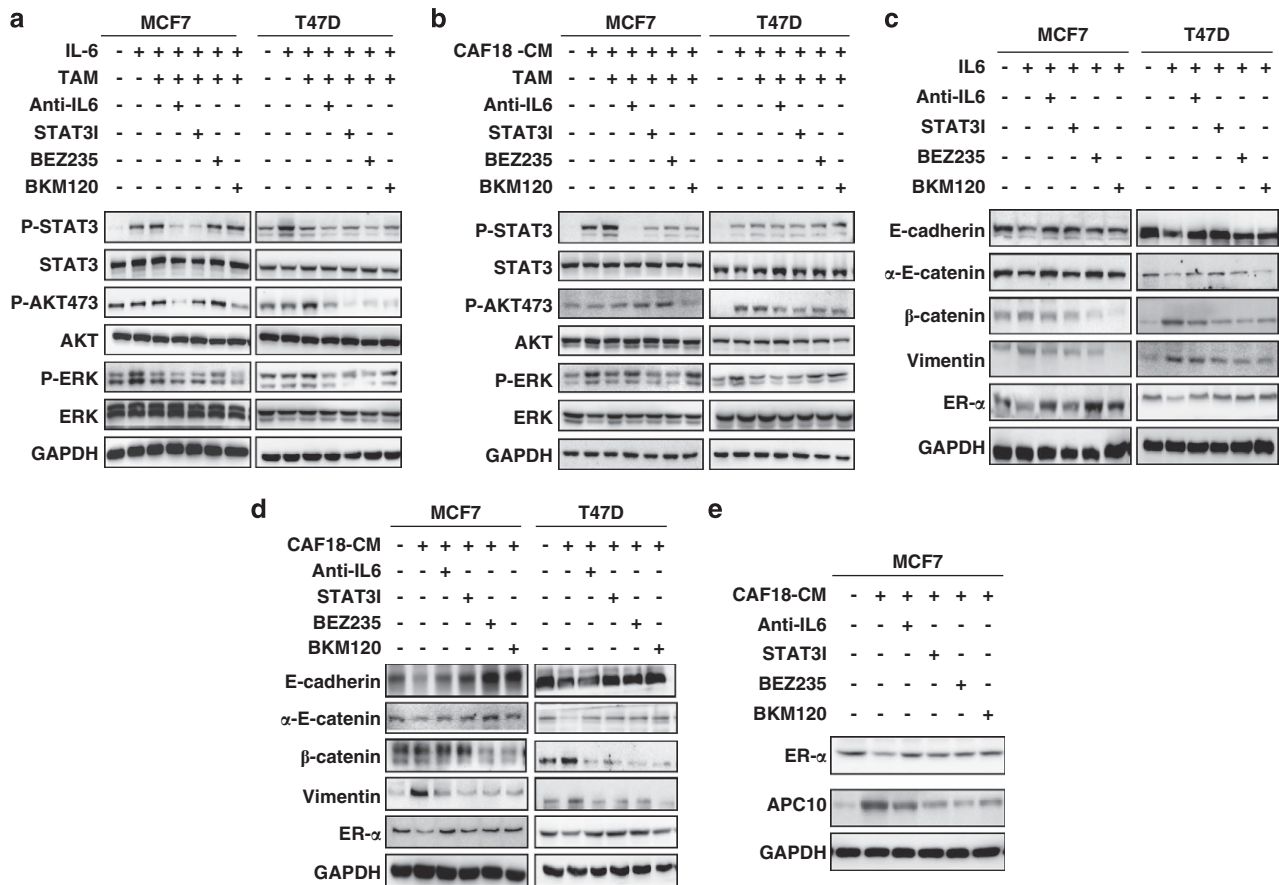
**Figure 5.** IL-6 downregulates ER- $\alpha$  through the ubiquitin–proteasome pathway. **(a)** Western blot analysis of lysates from MCF7 and T47D cells treated with IL-6 (20 ng/ml) for 0, 2, 6, 12, 18 and 24 h using anti-ER- $\alpha$  antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(b)** Western blot analysis of lysates from MCF7 (up) and T47D (down) treated with IL-6 (20 ng/ml) combined with dimethyl sulfoxide (DMSO) or MG132 for 6 h using anti-ER- $\alpha$  antibodies. GAPDH was used as a loading control. **(c)** Ubiquitination assays for ER- $\alpha$ . Cell lysates from MCF7 (left) and T47D (middle) cells treated with IL-6 (20 ng/ml) or MCF7 and T47D (right) cells cultured with CM from CAF18 combined with MG132 (10  $\mu$ M) for 6 h were immunoprecipitated (IP) with an ER- $\alpha$ -specific antibody, followed by western blotting with antibodies against ubiquitin or GAPDH. Bottom, input of the cell lysates. MW, molecular weight. **(d)** Plot showing that human and mouse ER- $\alpha$  proteins have a similar binding domain for APC10 (D-box). **(e)** Western blot analysis of lysates from MCF7 cells treated with IL-6 (20 ng/ml) and knockdown of endogenous APC10 expression by siRNAs in MCF7 cells with or without IL-6 treatment for 24 h using antibodies against ER- $\alpha$  and APC10. GAPDH was used as the loading control. **(f)** Western blot analysis of ER- $\alpha$  ubiquitin in the MCF7 cells under APC10 knockdown condition. Cell lysates from MCF7 cells with shRNA-mediated silencing of APC10 and the control treated with IL-6 and MG132 (10  $\mu$ M) for 6 h were IP with an ER- $\alpha$ -specific antibody, followed by western blotting with antibodies against ubiquitin or ER- $\alpha$ , APC10. Bottom, input of the cell lysates. MW, molecular weight.

phosphatidylinositol 3-kinase (PI3K)/AKT pathways were activated in both MCF7 and T47D cells; however, this activation was markedly inhibited by adding IL-6-neutralizing antibody, but not by tamoxifen itself (Supplementary Figure 7A). In addition, similar effects were also observed in both cells when cultured with CAF8, 18-CM (Supplementary Figures 7B and C), suggesting that activation of the two pathways is responsible for the IL-6 secretion of CAFs. In this regard, adding STAT3 inhibitor (STAT3I), an inhibitor of the JAK/STAT3 pathway, or BEZ235 and BKM120, inhibitors of the PI3K/AKT pathway, inhibited the activation of both pathways in both cells induced by IL-6 stimulation or CAF18-derived IL-6 (Figures 6a and b), potentially providing a potential target in luminal BrCA.

We then explore the effect of these inhibitors on EMT induced by IL-6 in both MCF7 and T47D cells. Figure 6c shows that IL-6 induced

EMT-like phenotypes and ER- $\alpha$  downregulation, which could be both reversed by STAT3I, or BEZ235, BKM120 or IL-6-neutralizing antibody (Supplementary Figure 6D). Similar effects were observed in these cells when cultured with CAF18-CM (Figure 6d). Notably, we also found that these inhibitors restored ER- $\alpha$  expression by affecting APC10 level through the ubiquitin–proteasome pathway (Figure 6e). More importantly, this setting also restored tamoxifen sensitivity in both cells treated with 4-OH tamoxifen (at a concentration of  $1 \times 10^{-5}$  M) (Supplementary Figure 8). Finally, in the xenograft experiments of paired CAFs with MCF7 cells, RNA interference-mediated silencing of IL-6 greatly inhibited tumorigenesis and restored tamoxifen sensitivity (Figure 7). Representative images are shown in Supplementary Figure 10, indicating that MCF7-xenografted tumors express less ER- $\alpha$ , E-cadherin and more vimentin in the presence of CAFs than in the presence of





**Figure 6.** Effects of CAFs and IL-6 on IL-6/JAK/STAT3, PI3K/AKT and MAPK pathways. **(a and b)** Western blot analysis of lysates from MCF7 and T47D cells treated with IL-6 (20 ng/ml) **(a)** or CM from CAF18 **(b)**, and treated with dimethyl sulfoxide (DMSO), or tamoxifen (1  $\mu$ M), or tamoxifen (1  $\mu$ M) combined with IL-6-neutralizing antibody (100 ng/ml), STAT3 inhibitor (10  $\mu$ M), BEZ235 (1  $\mu$ M) or BKM120 (1  $\mu$ M) for 1 h using the indicated antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(c–e)** Western blot analysis of lysates from MCF7 and T47D cells treated with IL-6 (20 ng/ml) **(c)** or CM from CAF18 **(d and e)**, and treated with DMSO, IL-6-neutralizing antibody (100 ng/ml), STAT3 inhibitor (10  $\mu$ M), BEZ235 (1  $\mu$ M) or BKM120 (1  $\mu$ M) for 24 h using the indicated antibodies. GAPDH was used as a loading control.

NAFs, whereas silencing of IL-6 expression in CAFs greatly restored ER- $\alpha$ , E-cadherin and reduced vimentin expression in MCF7-xenografted tumor compared with the control.

## DISCUSSION

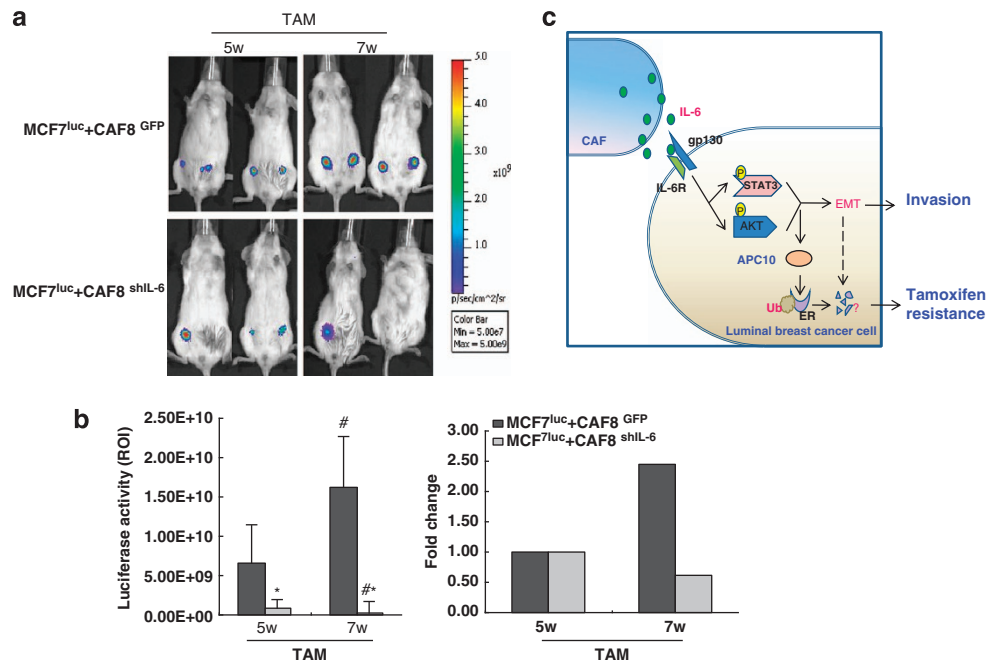
ER-targeted endocrine treatment is more effective at improving the prognosis of luminal BrCA patients than that of HER2<sup>+</sup> and triple-negative patients. Tamoxifen is an important antihormonal drug that can improve disease-free survival and overall survival of ER<sup>+</sup> BrCA patients not only in the standard 5 years but also in prolonged 5 years.<sup>18</sup> However, *de novo* and acquired resistance to ER-targeted therapeutics remains a major therapeutic challenge. Recent evidence shows that CAFs, which are important components of the tumor stroma, have a critical role in BrCA progression and resistance to treatment. Therefore, elucidating the mechanisms underlying the involvement of CAFs in tamoxifen resistance is critical.

In this investigation, we found that stromal fibroblasts isolated from the peripheral BrCA tumor have the same CAF-like phenotypes and functions as those isolated from the central BrCA tumor. These findings are consistent with the previous report that fibroblasts present in histologically normal surgical margins (TCFs) of BrCA patients exhibited a tumor-promoting phenotype.<sup>9</sup> Although PCFs or TCFs cannot be considered as normal fibroblasts, their role in BrCA warrants further investigation.

In the present study, we also demonstrated that stromal CAFs from luminal patients induce tamoxifen resistance in luminal BrCA

cells by decreasing ER- $\alpha$  expression. Moreover, this effect was shown to be mediated by the secretion of IL-6 from CAFs in a paracrine manner, but not through the autocrine secretion of IL-6 (Supplementary Figure 2C) from MCF7 and T47D cells. IL-6, as a pleiotropic cytokine, is involved in a wide range of biological activities including immune regulation, hematopoiesis, inflammation and oncogenesis. Clinical studies have shown that high serum IL-6 levels are indicative of poor prognosis and survival in breast and other cancers.<sup>19,20</sup> Here, we showed for the first time that IL-6 secreted by stromal CAFs promoted the degradation of the ER- $\alpha$  protein through the ubiquitin–proteasome pathway, and MG132, a proteasome inhibitor, restored tamoxifen sensitivity in MCF7 and T47D cells. Moreover, paracrine secretion of IL-6 induced EMT, which might be associated with the decreased expression of ER- $\alpha$ . These effects were diminished by targeting CAFs using an IL-6-neutralizing antibody or shRNA-mediated silencing. EMT is related to cancer metastasis and IL-6 has been reported to induce EMT by activating JAK-STAT3-SNAIL pathway.<sup>12</sup> In our work, we found that CAFs, through IL-6-mediated activation of both JAK/STAT3 and PI3K/AKT pathways, promoted EMT in MCF7 and T47D cells. These findings suggest that IL-6 from the tumor microenvironment has a critical role in inducing progression of luminal BrCA.

IL-6 is involved in several cancer-associated processes through the downstream activation of multiple signaling pathways. Recently, our group and others showed that the PI3K/AKT pathway is involved in tamoxifen resistance.<sup>12,21</sup> Furthermore, phase II /III clinical trials have shown promising initial results of



**Figure 7.** CAF-specific IL-6 knockdown inhibited *in vivo* tumorigenesis and restored tamoxifen sensitivity. **(a)** MCF7<sup>luc</sup> cells mixed with CAF8<sup>GFP</sup> or CAF8<sup>shIL-6</sup> at a ratio of 10:1 were injected into the both sides of the mammary fat pad of the mice. At the 5th and 7th week, imaging was performed using the Xenogen IVIS Imaging System (PerkinElmer, Waltham, MA, USA). Luciferase signals from two representative mice in each group are shown;  $n = 4$ , mean  $\pm$  s.e.m. **(b)** (left) Quantification of tumor burdens identified by bioluminescence imaging. At 5 weeks, tumor size was bigger in the group of MCF7<sup>luc</sup> mixed with CAF8<sup>GFP</sup> than in the group of MCF7<sup>luc</sup> mixed with CAF8<sup>shIL-6</sup>. Tamoxifen was then injected in both groups. The tumor growth was inhibited at the 7 week in the group of MCF7<sup>luc</sup> mixed with CAF8<sup>shIL-6</sup>, whereas the mixed CAF8<sup>GFP</sup> group represents rapid growth.  $^{\#}P < 0.05$ , significant difference after tamoxifen treatment in the group of MCF7<sup>luc</sup> mixed with CAF8<sup>shIL-6</sup> or the group of MCF7<sup>luc</sup> mixed with CAF8<sup>GFP</sup>;  $^{*}P < 0.01$ , significant difference between the group of MCF7<sup>luc</sup> mixed CAF8<sup>GFP</sup> and the group of MCF7<sup>luc</sup> mixed CAF8<sup>shIL-6</sup> at the 5 or 7 weeks; right: fold changes are shown after tamoxifen treatment in the group of MCF7<sup>luc</sup> mixed with CAF8<sup>shIL-6</sup> or the group of MCF7<sup>luc</sup> mixed with CAF8<sup>GFP</sup>. **(c)** Schematic representation showing the role of CAFs in mediating tamoxifen resistance. IL-6 secreted from CAFs can activate the JAK/STAT3 and PI3K/AKT pathways in BrCA cells, resulting in the induction of EMT and activation of the E3 ubiquitin ligase APC10. This promoted degradation of ER- $\alpha$  via the ubiquitin–proteasome pathway, which induced tamoxifen resistance. Meanwhile, the induced EMT is associated with cancer invasion, which may also contribute to tamoxifen resistance.

treatment with pathway inhibitors in combination with endocrine therapy.<sup>22,23</sup> The JAK/STAT3 pathway is implicated in the expansion of cancer stem cell populations, which is involved in the acquisition of drug resistance.<sup>24,25</sup> Here, we showed that IL-6 induce tamoxifen resistance and EMT by activating the JAK/STAT3 and PI3K/AKT pathways. We found that treatment with STAT3i, an inhibitor of the JAK/STAT3 pathway, or the PI3K/AKT inhibitors BEZ235 and BKM120 alone or in combination inhibited the activation of one or two pathways. Moreover, this scheme also restored tamoxifen sensitivity in MCF7 and T47D, and reversed their EMT phenotype and decreased E3 ubiquitin ligase APC10 activity, which restored ER- $\alpha$  expression. However, the mechanism underlying the relationship between EMT on the regulation of ER- $\alpha$  remains to be further elucidated.

Based on these studies, we discovered a novel mechanism underlying how CAFs promote tamoxifen resistance. IL-6 antagonism has potentially been successful in the treatment of a variety of cancers.<sup>26,27</sup> Because the genomes of stroma cells are not as mutable as cancer cells, targeting IL-6 secreted by CAFs may provide novel therapeutic targets in the tumor microenvironment.

## MATERIALS AND METHODS

Some reagents and antibodies are listed in Supplementary Methods.

### Cell culture

The human BrCA cell lines MCF7 and T47D were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's

modified Eagle's medium (Hyclone, Waltham, MA, USA) or RPMI-1640 (Hyclone) supplement with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin. The cell lines were cultured at 37 °C in a water-saturated 5% CO<sub>2</sub> atmosphere (Supplementary Methods).

### Western blot assays

Details are provided in the Supplementary Methods section.

### Tissue microarray construction and cancer-associated fibroblast assessment

Immunohistochemistry was performed by using human BrCA microarrays of FFPE tissues (Alianna, Xi an, China) and isolated fibroblasts with anti-human  $\alpha$ -SMA (ab5694; Abcam, Cambridge, UK), FAP (ab28244; Abcam) and PDGFR- $\alpha$  (ab61219; Abcam) antibodies. Scoring for each immunohistochemistry marker was performed by an experienced technologist who was blinded to the results of other markers or case identity. Among duplicate tissue cores, those with an epithelial–stroma ratio close to 1:1 were selected for further analysis (Supplementary Methods).

### Isolation of primary fibroblasts

CAFs were isolated from human invasive mammary ductal carcinomas tissue center, PCFs were from a non-cancerous region at least 3 cm from the outer tumor margin in the same patient of CAFs. FADs and NAFs were isolated from a reduction mammoplasty, in which only normal mammary tissue was detectable. All tissues were obtained from the Ruijin Hospital with approval of the hospital ethical committee and by the patient's written informed consent (Shanghai, China). All fibroblasts were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 5%



fetal bovine serum (Gibco) and 5 µg/ml insulin (Tocris Bioscience, Bristol, UK) (Supplementary Methods).

#### Collection of conditioned media and chemiarray

The CM of fibroblasts was obtained by conducting parallel cell culture experiments. The protocol of the chemiarray is described in the Human Cytokine Antibody Array Kit (RayBiotech, Norcross, GA, USA) (Supplementary Methods).

#### Enzyme-linked immunosorbent assay

Quantification of IL-6 level in the supernatants of fibroblasts or breast cancer cells were calculated by enzyme-linked immunosorbent assay according to the protocol in the Quantikine ELISA Kit (cat. no.: DY206; R&D Systems, Minneapolis, MN, USA). All experiments were carried out with four wells per experiment and repeated three times.

#### Colony formation assay

Details are provided in the Supplementary Methods section.

#### Co-immunoprecipitation assays

Co-immunoprecipitation experiments were performed as described previously.<sup>14</sup> Briefly, MCF7 and T47D cells were cultured in Dulbecco's modified Eagle's medium or RPMI-1640 supplemented with 10% charcoal dextran-treated fetal bovine serum (cat. no.:103; TCB, Tulare, CA, USA) for at least 96 h, and then changed into CM from CAFs or treated with IL-6 for 6 h with MG132 (10 µM). Cells were then washed two times with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer. Lysates were precleared with a protein G/A mixture for 1 h, immunoprecipitated with anti-ER-α (5 µg/ml; ab2746; New Territories, HK, China) or control antibody (mouse IgG), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% Tris glycine gel) and immunoblotted with an anti-human ubiquitin antibody (sc-8017; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

#### Invasion assays

Details are provided in the Supplementary Methods section.

#### Small interfering RNAs

Small interfering RNAs (siRNAs) targeting APC10 were purchased from Gene Pharma (Shanghai, China). The sequences were as follows: si-APC-01 (5'-GGAACCAAGUGGCGGAUUTT-3') and si-APC-02 (5'-CAUGAUGUAUCG UUCAUATT-3'). The nonspecific scramble siRNA duplexes (5'-UUCUCA CGUGUCACGUTT-3') were used as normal controls. Transfection was performed according to the manufacturer's protocol using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA).

For generation of a stable IL-6 knockdown cell line, pLVX-shRNA2 lentiviral vectors expressing the fluorescent protein ZsGreen1 were used (Clontech, Mountain View, CA, USA) and the shRNA sequences were as follows: si-IL-6-1, 5'-CTCAATAAATGGCTA ACTTA-3' (Supplementary Methods).

#### In vivo assay for the effects of fibroblasts on BrCA

The 6- to 8-week-old female SCID nude mice (Slacass Laboratory Animal, Shanghai, China) were supplemented with 0.36 mg of 60-day release 17 β-estradiol pellets (Innovative Research, Novi, MI, USA) by subcutaneous inoculation. Then, 1 × 10<sup>6</sup> MCF7<sup>luc</sup> cells (10:1) mixed with either NAF or CAF8 were injected into the mammary fat pad of the mice under isoflurane. After 3 weeks, bioluminescence imaging was used to monitor the tumor growth. Next, the mice were injected intraperitoneally with tamoxifen (Innovative Research) (Supplementary Methods).

#### Statistical analyses

Data are presented as mean ± s.d. Values of *P* < 0.05 were considered statistically significant. Quantitative reverse transcriptase polymerase chain reaction was analyzed using Student's two-tailed *t*-test, with the Excel software (Microsoft, Redmond, WA, USA). α-SMA, FAP and PDGFR-α stainings in a tissue microarray were performed with Pearson's χ<sup>2</sup> test. All analyses were performed with SPSS (IBM, Armonk, NY, USA).

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**RETRACTION**

# IL-6 secreted by cancer-associated fibroblasts induces tamoxifen resistance in luminal breast cancer

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The authors wish to retract this article due to concerns raised regarding some of the data presented in Figures 2, 4, 5, 6 and Supplementary Figures 6 and 7.

According to the data presented in Figures 1, 3 and 7, the major conclusion drawn from this article is still valid: IL-6 secreted by CAFs is causal factor for tamoxifen resistance in luminal breast cancer.

The authors are deeply regretful and apologize to the readers.