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The Dual ERα Inhibitory Effects of the Tissue-Selective Estrogen Complex for Endometrial and Breast Safety

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Running Title: Dual Inhibition of ERα by Tissue-Selective Estrogen Complex

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Abbreviation:

BAZ, Bazedoxifene; BCOR, BCL-6 Corepressor; ERα, Estrogen Receptor α; ERE, Estrogen Response Element; FBXO45, F-Box Protein 45; NCoA, Nuclear Receptor Coactivator; SERMs, selective estrogen-receptor modulators; SRCs, Steroid Receptor Coactivator; CE, Conjugated Estrogens; TSEC, Tissue-Selective Estrogen Complex; MS, Mass Spectroscopy; q-PCR, quantitative-PCR; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S.E.M., standard error of the mean.

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Abstracts

The Conjugated Estrogen/Bazedoxifene Tissue-Selective Estrogen Complex (TSEC) is designed to minimize the undesirable effects of estrogen in the uterus and breast tissues and to allow the beneficial effects of estrogen in other estrogen-target tissues, such as the bone and brain. However, the molecular mechanism underlying endometrial and breast safety during TSEC use is not fully understood. ERα-Estrogen Response Element (ERE)-DNA pull-down assays using HeLa nuclear extracts followed by mass spectrometry-immunoblotting analyses revealed that upon TSEC treatment, ERα interacted with transcriptional repressors rather than coactivators. Therefore, the TSEC-mediated recruitment of transcriptional repressors suppresses ERα-mediated transcription in breast and uterus. In addition, TSEC treatment also degraded ERα protein in uterine tissue and breast cancer cells, but not in bone cells. Interestingly, ERα-ERE-DNA pull-down assays also revealed that upon TSEC treatment, ERα interacted with the F-Box Protein 45 (FBXO45) E3 ubiquitin ligase. The loss-of- and gain-of- FBXO45 function analyses indicated that FBXO45 is involved in TSEC-mediated degradation of the ERα protein in endometrial and breast cells. In preclinical studies, these synergistic effects of TSEC on ERα inhibition also suppressed the estrogen-dependent progression of endometriosis. Therefore, the endometrial and breast safety effects of TSEC are associated with synergy between the selective recruitment of transcriptional repressors to ERα and FBXO45-mediated degradation of the ERα protein.

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Introduction

Estrogen Receptors (ERs) are members of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate essential cellular processes, such as development, metabolism, reproduction and behavior, by regulating the transcription of ER target genes (Burns and Korach, 2012). The working model for ERα action is that ligand-bound receptor binds to the enhancers/promoters of target genes that contain the estrogen-response element and sequentially recruits multiple coregulator complexes to these regulatory regions; this results in chromatin remodeling and assembly of the transcriptional pre-initiation complex as well as the downstream processing events involved in mRNA synthesis and maturation (Foulds et al., 2013; Green and Carroll, 2007; McKenna and O'Malley, 2002).

Because of its essential role in estrogen target tissues, aberrant regulation of the ERα level and activity is associated with estrogen-related progression of human disease (Burns and Korach; Deroo and Korach, 2006). Therefore, the development of tissue-specific modulators of ERα activity is a critical step to effectively treat the estrogen-related disease progression in specific tissues while minimizing side effects in certain other tissues. This goal led to the development of selective estrogen-receptor modulators (SERMs) (Levenson and Jordan, 1999). The SERMs act as tissue-specific estrogen-receptor agonists in the bone, brain, cardiovascular system, vagina, and urogenital system and as estrogen-receptor antagonists in the breast, endometrium, pelvic floor and in venous thrombosis (Maximov et al., 2013; Morello et al., 2002). The first generation of SERMs included the drug tamoxifen (Jordan and Koerner, 1975; Ward, 1973). Clinical studies revealed that treatment with tamoxifen significantly reduced the incidence of breast cancer in high-risk patients (Jordan, 1988; Morello et al., 2003). However, chronic tamoxifen treatment has stimulatory effects on endometrial cells, increasing the risk of development and

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progression of endometrial cancer (Iqbal et al., 2012; Rutqvist, 1993). To overcome the undesirable side effects of tamoxifen. raloxifene (second-generation SERMs) was developed (Black et al., 1994). Because raloxifene reduced the incidence of invasive breast cancer in postmenopausal women with osteoporosis, raloxifene is the current preferred drug for women at risk for vertebral fracture who have an elevated risk of breast cancer (Gizzo et al., 2013; Ko and Jordan, 2011). Unlike tamoxifen, raloxifene does not increase the risk of uterine cancers (DeMichele et al., 2008; Pinkerton and Goldstein, 2010; Vogel et al., 2006) even when combined with vaginal estrogen (Parsons et al., 2003; Pinkerton et al., 2003). To improve upon the beneficial effects of raloxifene, third-generation SERMs were developed, including Bazedoxifene (BAZ), which prevented and treated postmenopausal osteoporosis without adverse stimulation of the breast and endometrium (Komm and Chines, 2012).

To maximize the tissue-specific effects of SERMs and minimize their side effects, a Tissue-Selective Estrogen Complex (TSEC) has been developed (Archer, 2010; Pickar and Mirkin, 2010). The final goal of TSEC therapy is to combine the desired tissue-selective properties of SERMs with the beneficial effects of estrogen. The TSEC would ideally have estrogen-receptor antagonist activity in the breast and endometrium and have reduced vasomotor symptoms and improved lipid profile and Bone Mineral Density compared with a SERM- or estrogen-alone treatment (Mirkin et al., 2014). The first-generation TSEC was a combination of BAZ and conjugated estrogen (CE), which had unique molecular properties compared to the individual components (Mirkin et al., 2014). For example, the BAZ/CE TSEC combination was associated with less than a 1% rate of endometrial hyperplasia. This rate was not significantly different from a placebo-treated group during a two-year study (Pickar et al., 2009). When the incidence of uterine bleeding in postmenopausal women was studied, the mean number of bleeding or

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spotting days in women treated with TSEC was not significantly different from a placebo-treated group during two years of therapy (Archer et al., 2009). Thus, TSEC therapy can be used to treat menopausal symptoms without stimulation of the endometrium. Treatment with TSEC for 24 months also did not affect the mammographic density of postmenopausal women with breast cancer compared with a placebo-treated group (Harvey et al., 2013). Therefore, the endometrial and breast-cell stimulatory effects of the accompanying CE were completely abrogated by BAZ in TSEC treatment (Song et al., 2013). Collectively, the TSEC has an anti-estrogenic effect on the growth of breast cancer cells and prevents the development of endometrial hyperplasia in postmenopausal women. However, the molecular mechanisms underlying endometrial and breast safety during TSEC administration are not understood yet; elucidation of these mechanisms is important for the use of TSEC in future clinical therapy. Here, we analyze the molecular functions of TSEC and suggest dual mechanisms by which TSEC inhibits ERα function in endometrial and breast cells.

Materials and Methods:

Experimental animals. Wild-type (C57BL/6J) mice were used. All experiments involving animals were conducted in accordance with the National Institutes of Health (NIH) standards for the use and care of animals, with protocols approved by Baylor College of Medicine. C57BL/6 mice were purchased from The Jackson Laboratory.

Cell Culture and Reagents. The HeLa human cervical carcinoma, MCF7 human breast cancer cell lines and HTB-85 human osteosarcoma cell line were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MCF7 cells were maintained in minimum essential medium (MEM) supplemented with 10% FBS. HTB-85 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). 4-hydroxytamoxifen, MG132, and ethanol-soluble estrogen (E2) were purchased from Sigma-Aldrich (St. Louis, MO). Bazedoxifene and conjugated estrogen (CE)/Premarin were obtained from Pfizer Inc. (New York, NY). For cell culture, a mixture of the unconjugated forms of the 10 most abundant conjugated estrogen (CE) components of CE/Premarin (Pfizer Inc.) was prepared using the same relative proportions as the previously described CE formulation (Chang et al., 2010), and a 1 mM CE stock solution was prepared using 1 mM solutions of each of the 10 components mixed according to their respective proportions.

Western Blot Analysis. Primary antibodies against the following proteins were used: SRC-1 (ab10308; Abcam), SRC-2 (A300-345A; Bethyl Laboratory), SRC-3 (611105; BD Biosciences), Tubulin (SC-9104; Santa Cruz Biotechnologies), FBXO45 (NBP1-91891; Novus Biologicals), ERα (SC-542; Santa Cruz Biotechnologies) and BCOR (A301-673A; Bethyl Laboratory).

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Membranes containing proteins were incubated with secondary HRP-tagged antibodies (Sigma), and the signals were visualized using ECL plus (Amersham).

Immunohistochemistry. Immunostaining was performed with 10% neutral-buffered, formalin-fixed and paraffin-embedded sections of mouse tissue. Antibodies against FBXO45 (NBP1-91891; Novus Biologicals), ERα (SC-542; Santa Cruz Biotechnologies), Ki-67 (AB16667; Abcam®) were used. The specific antigens were visualized with the DAB substrate kit (Vector). The immunostaining intensity was quantified using the ImageJ program, which was developed by the National Institutes of Health. Scale bar is 20 μm.

Uterotrophic Assay. Uterotrophic assays were performed to screen for potential estrogenic activity. CE, BAZ and TSEC were administered daily via subcutaneous injection in 6-week-old ovariectomized female mice. Ovariectomy was performed as previously described (Han et al., 2005). The animals were dosed for three consecutive days and necropsied approximately 24 hours after the final dose to determine wet and blotted uterine weights.

ERE-DNA Pull-Down Assays. The ERE-DNA pull-down reaction conditions and generation of biotinylated EREs were described previously (Foulds et al., 2013). In brief, 4 μg of ERE DNA, 1 mg of HeLa nuclear extract, 0.5 μg of recombinant human ERα protein (ab82606, Abcam®) and 60ul of Dynabeads® M-280 Streptavidin beads were mixed with NETN buffer **(**20 mM Tris-HCl (pH 7.5), 150 mM NaCl,1 mM EDTA,0.5% lgepal CA-630) and then incubated with vehicle, 10 nM CE, 100 nM BAZ and 10 nM CE + 100 nM BAZ (TSEC) at 4°C for 1.5 h with constant shaking. The pellet was washed twice with METN buffer, and 80% of the pellet was used for LC MS analyses, and 20% was used for Western blotting analyses.

Protein Identification by MS. For MS analyses, nearly all of the ERE pull-down reaction (80%) was separated using 1-D SDS-PAGE. All ERα interacting proteins were isolated from the gel and their identities were determined by MS as described previously (Foulds et al., 2013).

TSEC-Mediated ERα Degradation in Breast Cells. MCF7 breast cancer cells were cultured in 6-well plates in duplicate and maintained in regular medium supplemented with 10% FBS. Prior to the experiment, the cells were deprived of estrogen for 2 days by incubating in stripped serum medium followed by treatment with vehicle, 10 nM CE, 100 nM BAZ, 100 nM 4HT, 10 nM CE plus 100 nM BAZ (TSEC), and 10 nM CE plus 100 nM 4HT for 24 hrs. The cells were then lysed, and the RNA and protein were extracted. For Western blotting analyses, the proteins were separated on a 10% SDS-PAGE gel, transferred into a nitrocellulose membrane and incubated with anti-ERα and anti-tubulin antibodies for overnight incubation at 4°C followed by incubation with HRP-conjugated anti-rabbit (Sigma Aldrich) antibody for 1 hr. Signals were detected using the ECL Plus system (Pierce). Transcript levels were analyzed by q-PCR using an AB system. Complementary DNAs (cDNAs) were synthesized, and Taqman probes for ERα, PR, and 18S rRNA (Applied Biosystems) were used to measure ERα, PR and 18S rRNA levels. The q-PCR results were analyzed using the delta-delta cT method, and the fold-enrichment was calculated relative to the 18S rRNA level.

Effect of FBXO45 gain-of-function and loss-of-function on ERα stability. Knockdown experiments were performed in HeLa cells transfected with ERα by treating them with 100 nM of non-targeting siRNA or FBX045 (Dharmacon, smart pool) siRNA for 48 h. The cells were then incubated with vehicle, 10 nM CE, 100 nM BAZ, and 10 nM CE plus 100 nM BAZ (TSEC) for 24 h. Cell lysates were prepared and used for Western blotting analyses with specific

antibodies against FBX045 (Abcam), ERα, and tubulin. To examine the effect of FBXO45 gain-of-function, HeLa cells were transiently transfected with 100 ng each of ERα and FBXO45 expression vectors (gift from Hirobumi Tada, Keio University School of Medicine, Shinjuku, Tokyo, Japan) followed by treatment with vehicle, 10 nM CE, 100 nM BAZ, and 10 nM CE plus 100 nM BAZ (TSEC). Samples were collected at 0, 1, 3, 6, and 9 h after treatment and levels of ERα and FBXO45 were analyzed by Western blotting as described above.

Co-immunoprecipitation and Western blotting. For co-immunoprecipitation assays, HeLa cells were transiently transfected with 100 ng each of ERα and FBX045 expression vectors or empty vectors. After 48 hours later, cells were treated with vehicle, 10 nM CE, 100 nM BAZ, and 10 nM CE plus 100 nM BAZ (TSEC) in the presence or absence of 10 μM MG 132 for 6 hr. The lysates were incubated with ERα, Flag, or IgG antibodies under constant rotation. Protein G beads were added 2 h later and incubated for an additional 2 h, followed by extensive washing (20 mM HEPES [pH 7.6], 150 mM KCl, 1 mM DTT, 0.1% NP-40 and 8% glycerol supplemented with protease and phosphatase inhibitors). The Protein G beads were boiled in 1× Laemmli buffer, and the co-precipitated proteins were separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membranes, and Western blotting was performed and analyzed with specific antibodies.

Administration of CE, BAZ and TSEC to mice with surgically induced endometriosis. The surgical treatments of mice were performed under aseptic conditions with anesthesia. The surgically induced endometriosis in mice was conducted with a modification of a previously described method (Cummings and Metcalf, 1995). In brief, 6-wk-old C57BL/6 mice were ovariectomized. After two weeks, we implanted sterile 60-day release pellets containing 0.36 mg

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of 17-β estradiol (Innovative Research of America) into ovariectomized mice. Two days later, we isolated one uterine horn of each mouse under anesthesia. In a petri dish containing warmed Dulbecco's modified Eagle medium (DMEM)/F-12 (Invitrogen) supplemented with 100 U/ml, penicillin and 100 µg/ml streptomycin, we longitudinally cut the uterine horns with a pair of scissors. We then isolated a tissue sample using a 2-mm dermal biopsy punch (Miltex) and subsequently sutured one endometrial fragment to the mesenteric membrane attached to the intestine in the same mouse through a midline incision (7-0 braided silk suture; Ethicon). We closed the abdominal incision with a 5-0 braided silk suture (Ethicon) in a continuous fashion. Ectopic lesions were developed and established for 21 days after endometriosis induction. Subsequently, the mice were injected with vehicle, CE (2.5 mg/kg), BAZ (2.5 mg/kg) and TSEC (2.5 mg/kg of CE plus 2.5 mg/kg of BAZ) on alternate days for 21 days, and then the ectopic lesions were harvested for analyses. All experiments were repeated at least three times.

Statistical analysis. Statistical analysis used a paired Student t test in GraphPad Prism 5.00 (GraphPad).

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Results

Corepressor components recruitment to ERα by TSEC.

The physical and functional interactions between ERα and its coregulators are essential for the regulation of ERα-mediated transcription upon estrogenic stimulation (Foulds et al., 2013). In this regard, ERα recruits different coregulators to regulate target gene expression upon various external stimuli (Foulds et al., 2013). Therefore, we hypothesized that the TSEC should generate unique ERα-coregulator combinations to confer endometrial and breast safety. ERα activity determined by PR expression in HeLa cells transfected with human ERα expression vector revealed that 10 nM of CE stimulated ERα activity and 100 nM, but not 10 nM, of BAZ inhibited ERα activity that was stimulated by CE (Supplementary Figure 1). To examine the coregulators bound to ERα upon CE, BAZ and TSEC treatment, ERα- Estrogen Response Element (ERE) pull-down assays were performed using a biotinylated DNA fragment containing the ERE in HeLa cell nuclear extracts (Foulds et al., 2013). Compared to vehicle, 10 nM of CE also actively enhanced interaction of ERα to SRCs and this CE-mediated interaction was strongly inhibited by 100 nM, but not 10 nM, of BAZ (Supplementary Figure 2, A and B). Interestingly, the amount of SRCs recruited to ERα by 10 nM of CE was less than those by 10 nM of E2. Therefore, the reduced amounts of coactivator recruitments to ERα might provide the lesser estrogenic activity of CE compared with estradiol. Based on these observations, 10 nM of CE plus 100 nM of BAZ combination have been applied as TSEC in this study. The proteins that co-precipitated with ERα in response to each hormone were identified by mass spectrometric analyses (Fig. 1A). Compared with other hormone treatments, CE treatment enhanced the recruitment of transcriptional coactivators (such as SRCs, NCoA6, RAD50, and Mediator complex components) to ERα bound to the ERE DNA fragment (Fig. 1A). Consistent with the

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mass spectrometry analyses, the ERα-ERE pull-down assays followed by Western blot analyses indicated that SRC-1, SRC-2 and SRC-3 were significantly recruited to ERα upon CE treatment compared to treatment with other agents (Fig. 1B). However, less SRC-1, -2 and -3 were recruited to BAZ- and TSEC-bound ERα compared with CE treatment (Fig. 1, A and B). Notably, the SRC expression levels in HeLa cells were not altered by CE, BAZ and TSEC compared to vehicle treatment (Fig. 1, C, D, E and F).

In contrast, the ERα-ERE pull-down assays coupled with mass spectrometric analyses revealed that TSEC treatment led to increased recruitment of different coregulators, usually transcriptional repressor components, such as BCL-6 Corepressor (BCOR) and Histone Deacetylase3 (Fig. 1A). The TSEC-induced recruitment of BCOR to ERα was confirmed using ERα-ERE pull-down assays followed by Western blotting analyses (Fig. 1B). Collectively, the TSEC-induced recruitment of transcriptional repressors relative to transcriptional activators correlates with inhibition of ERα target gene transcription in endometrial cells upon TSEC treatment.

Degradation of the uterine ERα protein by TSEC to prevent endometrial stimulation.

To analyze the endometrial effects of TSEC in more detail, uterotrophic assays were performed using ovariectomized female mice: on the 14th day after ovariectomy, the mice were randomly divided into 4 groups and then administered vehicle, CE (2.5 mg/kg), BAZ (2.5 mg/kg) or TSEC containing both CE (2.5 mg/kg) and BAZ (2.5 mg/kg) daily for 3 days. Six hours after the final hormone injection, uteri were isolated from each group of mice and weighed. Compared to vehicle treatment, CE treatment significantly increased uterine weight, but both BAZ and TSEC did not increase uterine weight (Fig. 2, A and B). Unlike CE, therefore, BAZ and TSEC had no

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endometrial stimulatory effect (Fig. 2, A and B). Furthermore, the presence of BAZ in the TSEC suppressed CE-mediated endometrial stimulation.

To elucidate the endometrial safety of TSEC, uterine ERα levels were measured in each hormone-treated group. Compared to vehicle treatment, CE, BAZ and TSEC treatments reduced uterine ERα protein levels (Fig. 2, C and D). To determine whether the reduction in ERα protein level was correlated with downregulation of the ERα mRNA level, uterine ERα mRNA levels were measured in each hormone-treated group. Notably, uterine ERα mRNA levels were not altered by hormone treatment compared to vehicle treatment (Fig. 2E). Therefore, CE-mediated ERα degradation is associated with endometrial stimulation, an effect observed previously (Callige and Richard-Foy, 2006; Nawaz et al., 1999). However, TSEC-mediated ERα degradation occurred in the absence of endometrial stimulation in mice.

TSEC-mediated ERα degradation prevents ERα activity in human breast cancer cells.

To examine the effect of TSEC on breast ERα, MCF7 human breast cancer cells were selected. Consistent with HeLa nuclear extracts, the ERα-ERE pull-down assays followed by Western blot analyses with MCF7 nuclear extracts indicated that coactivators (SRC-1 and SRC-3) were significantly recruited to ERα upon CE treatment compared to treatment with other agents (Fig. 3A). In contrast to coactivators, BCOR (transcriptional repressor) and FBXO45 effectively interact to ERα upon TSEC treatment compared to other hormones in the MCF7 nuclear extracts (Fig. 3A). Therefore, breast ERα has similar responses upon CE and TSEC treatment compared to endometrial ERα.

To investigate the effect of TSEC on breast ERα protein levels, MCF7 human breast cancer cells were treated with CE (10 mM), BAZ (100 mM) or TSEC (containing CE and BAZ) for 1 day. Subsequently, the ERα protein level in each group of cells was determined by Western blot

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analysis. Consistent with endometrial ERα expression, the breast ERα levels in MCF7 cells were significantly reduced by CE, BAZ and TSEC treatment compared with vehicle treatment (Fig. 3, B and C). Breast ERα RNA levels in MCF7 cells were not altered by CE, BAZ or TSEC treatment compared to vehicle treatment (Fig. 3D). These data indicate that CE and TSEC also cause degradation of the ERα protein in breast cancer cells.

To explain the functional correlation between ERα degradation and ERα activity in breast cancer cells, we measured levels of progesterone receptor (PR) because PR is the direct ERα target gene (Nagai and Brentani, 2008). Compared to vehicle treatment, CE treatment increased PR mRNA levels in MCF7 cells, whereas BAZ and TSEC treatment did not (Fig. 3E). Therefore, similar to the endometrial data, CE-mediated ERα degradation was associated with induction of ERα activity, whereas BAZ- and TSEC-mediated ERα degradation correlated with suppression of ERα activity in MCF7 cells.

The SERMs, BAZ and tamoxifen inhibit ERα activity in breast cancer cells (Cirillo et al., 2013; Lewis-Wambi et al., 2011). However, these drugs inhibit ERα activity through different mechanisms. For example, the 4-hydroxytamoxifen-mediated inhibition of ERα activity was not associated with a reduction in ERα protein levels in MCF-7 cells compared to vehicle treatment (Fig. 3, F and G). Therefore, these data indicate that a unique ERα destabilization activity of BAZ and TSEC effectively downregulates ERα activity in both endometrial and breast cells.

To determine whether this unique molecular property of TSEC correlates with tissue specificity, ERα levels in bone cells (HTB-85, osteosarcoma) were determined upon each treatment. In contrast to endometrial and breast cells, CE, BAZ and TSEC did not reduce ERα levels in HTB-85 cells compered to vehicle treatment (Fig. 3, H and I). Interestingly, BAZ and TSEC led to a

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small increase in ERα levels in bone cells compared vehicle and CE treatment. We conclude that TSEC treatment differentially modulates the ERα protein level in a tissue specific manner.

Interaction of ERα to FBXO45 upon TSEC treatment.

Our data revealed that CE- and TSEC-mediated ERα degradation was associated with differential cellular regulation of ERα activity (Fig. 2 and 3). This observation suggests that distinct proteasome complexes might be involved in CE- versus TSEC-mediated ERα degradation. To test this hypothesis, we analyzed whether the ubiquitination-dependent proteasome might be differentially recruited during CE- versus TSEC-mediated ERα degradation. In the presence of DMSO, CE, BAZ and TSEC treatments, but not vehicle treatment, ERα protein degradation occurred in HeLa cells (Fig. 4A). However, all of these ERα degradations were prevented by addition of the proteasome inhibitor MG132 (Fig. 4B). Therefore, both of CE- and TSEC-induced ERα protein degradations require an ubiquitin-dependent proteasome system.

The ubiquitin-dependent proteasome systems consist of several components that are involved in specific cellular processes (Devoy et al., 2005; Konstantinova et al., 2008). Therefore, we analyzed whether different components of the ubiquitin proteasome complex might be involved in CE- versus TSEC-mediated ERα degradation. ERE-DNA pull-down assays coupled with mass spectrometry analyses revealed that the F-Box Protein 45 (FBXO45), a component of an E3 ubiquitin ligase complex (Saiga et al., 2009; Tada et al., 2010), was significantly recruited to the ERα/ERE complex in the presence of TSEC compared to vehicle (Fig. 4, C and D). However, less FBXO45 protein was recruited to the ERα/ERE complex upon E2 or CE treatment compared to TSEC treatment (Fig, 4D and Supplementary Figure 2C). BAZ did not stimulate FBXO45 recruitment to ERα/ERE complex compared to vehicle (Fig. 4D). Immunoprecipitation with

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HeLa cells transfected with ERα and/or Flag tagged FBOX45 cDNA using Flag antibody also revealed the interaction of FBXO45 with ERα in the presence of MG132 (Fig. 4E). To test whether this interaction was enhanced by TSEC treatment, HeLa cells were transiently transfected with expression vectors for ERα and Flag-tagged FBXO45 and then treated with vehicle, CE, BAZ or TSEC. Flag-tagged FBXO45 then was immunoprecipitated using the Flag-antibody and ERα levels in the precipitated pellets were determined using Western Blot analyses. Consistent with the results of the ERE-DNA pull-down assay (Fig. 4C), the interaction of ERα with FBXO45 was enhanced by TSEC treatment compared to vehicle and CE treatment (Fig. 4F). In addition, BAZ treatment also enhanced the interaction of ERα with FBXO45 compared to vehicle and CE in HeLa cells (Fig. 4F). This BAZ-mediated interaction of ERα with FBXO45 was not detected using the ERE-DNA pull-down assay (Fig. 4D). Therefore, these data indicate that the FBXO45/ubiquitin proteasome complex interacts with ERα only upon TSEC treatment on ERE-DNA.

Degradation of the ERα protein by the FBOX45/ubiquitination by TSEC.

TSEC induced an interaction of ERα with FBXO45. Next, we analyzed whether this interaction is directly correlated with TSEC-induced ERα degradation. HeLa cells were transiently transfected with ERα and different amounts of FBXO45 expression vectors, and the ERα protein levels were determined by Western blot analyses. The ERα protein levels gradually decreased in the presence of increased FBXO45 levels compared to cells transfected with the empty expression vector (Fig. 5A). However, Progesterone Receptor (PR) protein levels were not reduced in the presence of higher FBXO45 levels (Fig. 5A). Therefore, elevation of the FBXO45 level caused the specific degradation of ERα but not PR in HeLa cells.

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The effects of FBXO45 gain-of-function and loss-of-function in TSEC-induced ERα degradation were examined with HeLa cells transfected with ERα. To reduce FBXO45 protein levels in HeLa cells, the cells were treated with siRNA targeting FBXO45. As a control, HeLa cells were treated with non-targeting siRNA (NT-siRNA). Subsequently, both groups of cells were treated with vehicle, CE, BAZ or TSEC to test their effects on ERα degradation. After hormone treatment, the ERα and FBXO45 protein levels in both groups of HeLa cells were determined by Western blotting analyses. FBXO45 levels were significantly reduced by FBXO45 siRNA compared with NT-siRNA in HeLa cells (Fig. 5B). In the NT-siRNA-treated HeLa cells, ERα protein levels were reduced by CE, BAZ and TSEC treatments compared with the vehicle (Fig. 5B). However, BAZ and TSEC treatment did not reduce ERα protein levels in FBXO45 siRNA-treated cells compared to the vehicle (Fig. 5B). Notably, CE treatment reduced the ERα protein level in FBXO45 siRNA-treated HeLa cells compared to vehicle treatment (Fig. 5B). Therefore, FBXO45 is required for TSEC-mediated degradation, whereas it is not involved in CE-mediated ERα degradation in HeLa cells.

To examine whether increased FBXO45 expression degrades ERα protein in a TSEC-dependent manner, HeLa cells were transiently transfected with expression vectors for ERα and FBXO45. Subsequently, the cells were stimulated with vehicle, CE, BAZ or TSEC and then the ERα proteins levels in these cells were determined by Western analysis. The ERα protein levels were not significantly reduced 9 hours after CE treatment compared to vehicle treatment (Fig. 5, C and D). BAZ treatment does not reduce ERα protein levels compared to vehicle treatment at 9 hours after treatment (Fig. 5, C and D). However, ERα protein levels were rapidly and significantly reduced by TSEC treatment compared to vehicle treatment (Fig. 5, C and D).

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Therefore, overexpressing FBXO45 effectively caused ERα protein degradation upon treatment with TSEC but not other agents.

Suppression of ectopic lesion growth in mice with endometriosis by TSEC.

Endometriosis is an estrogen-dependent disease because estrogen signaling plays an essential role in the pathogenesis of endometriosis (Bulun, 2009). Studies using ERα knock-out mice with surgically induced endometriosis revealed that ERα has an essential role in the growth of ectopic lesions (Burns et al., 2012). Since the TSEC/FBXO45 axis is involved in ERα degradation in endometrial tissues (Fig. 5), TSEC might be applied for an alternative endometriosis treatment because TSEC minimizes the side effects of systemic estrogen-deficiency therapy in other estrogen target tissues, such as brain and bone, during treatment. To test this hypothesis, mice with surgically induced endometriosis were administered CE, BAZ and TSEC. CE treatment increased the volume of the ectopic lesion in mice with endometriosis compared with vehicle treatment most likely due to its endometrial stimulatory effect (Fig. 6, A and B). However, compared to vehicle treatment, BAZ and TSEC did not stimulate ectopic lesion growth in mice with endometriosis (Fig. 6, A and B). The presence of BAZ in TSEC suppressed the CE-mediated ectopic lesion growth during endometriosis. In addition, histological analyses revealed that CE treated ectopic lesions had well-developed cyst formation (Fig. 6C). This endometriotic cyst formation was not well developed in ectopic lesions treated with BAZ and TSEC as compared to CE-treated ectopic lesions (Fig. 6C). In addition to the cyst formation, IHC analyses with KI-67 antibody revealed that proliferative activities in epithelial and stromal compartments of ectopic lesions treated with BAZ and TSEC were significantly reduced compared with ectopic lesions treated with CE (Fig. 6D). Collectively, TSEC treatment

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inhibited proliferative activity of ectopic lesions to prevent their progression, whereas CE promoted proliferative activity of ectopic lesions for the establishment of ectopic lesions.

Since TSEC degrades ERα protein to suppress ERα-mediated signaling in endometrial tissues (Fig. 2), we analyzed whether TSEC caused the degradation of ERα in ectopic lesions to inhibit the estrogen-dependent growth of these lesions. ERα levels in ectopic lesions were determined by immunohistochemistry (IHC) analyses using an ERα antibody. TSEC-treated ectopic lesions had lower ERα protein levels in both epithelial and stromal compartments of ectopic lesions compared to CE-treated ectopic lesions (Fig. 6E). Notably, IHC and Western blot analyses using a FBXO45 antibody revealed that FBXO45 levels were elevated in BAZ- and TSEC-treated ectopic lesions compared with CE-treated lesions (Fig. 6, F and G) and TSEC-mediated elevation of FBXO45 levels appears to enhance TSEC-induced ERα degradation to suppress the growth of ectopic lesions. Together, our data suggest that TSEC-induced ERα degradation in the endometrium might be employed as an alternative endometriosis treatment to minimize the side effects of estrogen deprivation in other estrogen target tissues that are associated with certain current endometriosis treatments.

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Discussion

Hormone replacement therapy (HRT) has been used for the treatment of postmenopausal symptoms. To maximize the benefits of HRT, TSEC has been developed, which is a new menopausal therapy that combines a SERM with one or more estrogens. The goal of TSEC therapy would be to reduce menopausal symptoms (e.g., hot flashes and vulvar/vaginal atrophy), prevent osteoporosis, and improve lipid parameters while it inhibits estrogenic stimulation of the breast and endometrium.

Although TSEC is known to inhibit estrogenic stimulation in the breast and endometrium, the molecular mechanisms underlying this function of TSEC was not understood. Here, we observed two new molecular mechanisms by which TSEC confers endometrial and breast safety effects. First, TSEC recruited corepressors rather than coactivators to ERα. In contrast with TSEC, however, CE actively recruited coactivators to ERα compared to vehicle treatment, leading to enhanced ERα activity. The differential recruitment of ERα coregulators by CE versus TSEC is likely associated with distinct ERα conformational changes induced by these agents. For example, in the presence of a single hormone (such as CE and BAZ), each hormone bound-ERα generates homodimer binding to EREs to regulate ERα target genes. In the presence of TSEC, however, one agonist (CE)-bound ERα monomer binds to an antagonist (BAZ)-bound ERα to generate a distinct heterodimeric ERα conformation, which then binds to ERα target genes (Liu et al., 2013). This unique heterodimeric ERα conformation generated by TSEC appears to induce an interaction of ERα to certain corepressors, leading to inhibition of ERα activity.

In addition, TSEC induced ERα degradation via the FBXO45/ubiquitin proteasome, leading to suppression of ERα activity in endometrial and breast cells. CE also induced the degradation

of ERα in endometrial and breast cancer cells by the ubiquitin-dependent proteasome. However, each hormone-induced ERα degradation was accompanied by an opposite effect on ERα activity: TSEC-induced ERα degradation was associated with inhibition of ERα activity, whereas CE-mediated ERα destabilization was correlated with enhancement of ERα activity. Previous studies also revealed that enhanced estradiol-induced ERα activation is associated with ERα degradation (Callige and Richard-Foy, 2006; Wijayaratne and McDonnell, 2001), and a specific E3 ubiquitin ligase such as E6-AP might be involved in this process (Li et al., 2006; Sun et al., 2012). However, the molecular mechanism underlying the functional correlation between estrogen-induced ERα degradation and ERα activation has not been fully elucidated. BAZ also causes ERα protein degradation like CE. However, BAZ-mediated ERα degradation is linked to the prevention of expression of ERα target genes, such as cyclin D1, in MCF-7:5C cells (Lewis-Wambi et al., 2011). The mechanism underlying BAZ-induced ERα degradation has not been investigated.

TSEC significantly reduced CE-mediated enhancement of ERα activity in endometrial and breast cancer cells by degrading ERα protein, in contrast with the E2-induced interaction of ERα with E6-AP for the ERα activation (Sun et al., 2012). However, TSEC caused a differential interaction of ERα with FBXO45 for the suppression of ERα activity. FBXO45 modulates the stability of key factors that are involved in the regulation of cell-cycle arrest and apoptosis in response to DNA damage-induced cellular stress by its ubiquitin ligase activity (Melino, 2003). For example, FBXO45 promotes the proteasome-dependent degradation of p73 to promote cell survival (Peschiaroli et al., 2009; Tada et al., 2010). In the case of TSEC treatment, FBXO45/ubiquitin proteasome promoted ERα degradation to suppress ERα activity leading to suppression of endometrial stimulation effects. The unique heterodimeric ERα conformation

induced by TSEC appears to generate a specific interaction with FBXO45 (Liu et al., 2013). Collectively, TSEC treatment effectively prevents ERα activity by dual synergistic effects: recruitment of corepressors to ERα at target gene promoters and degradation of the ERα protein by a FBXO45/ubiquitin proteasome system in endometrial and breast cells.

Current endometriosis treatments induce systemic estrogen deficiency caused by the gonadotropin-releasing hormone agonists, depot medroxyprogesterone acetate and Danazol; aromatase inhibitors also are employed (Fedele et al., 2000; Haney and Weinberg, 1988; Selak et al., 2007; Telimaa, 1988; Telimaa et al., 1987; Vigano et al., 2003). However, these estrogen deficiency therapies can have harmful side effects in other estrogen target tissues, such as the brain and bone in young reproductive-aged women (Compston et al., 1995; Hughes et al., 2007; Vanderschueren et al., 1997). Therefore, alternative endometriosis therapies that have fewer side effects in estrogen target tissues are needed. In this context, TSEC is an appropriate candidate for the next generation of endometriosis therapy because it effectively degrades ERα in endometriotic tissues but not in other estrogen target tissues, such as bone. Consistent with our study, other murine endometriosis models also revealed that TSEC effectively suppresses mouse endometriotic lesion growth compared to controls (Naqvi et al., 2014). Collectively, TSEC treatment should have selective therapeutic effects in endometrial tissues by virtue of its synergistic dual ERα inhibitory effects.

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Authorship Contributions

Participated in research design: Han, Begum, O'Malley.

Conducted experiments: Han, Begum.

Contributed new reagents or analytic tools: Foulds, Hamilton, Baily, Malovannaya, Chan, Qin

Performed data analysis: Han, Begum, O'Malley.

Wrote or contributed to the writing of the manuscript: Han, Begum, O'Malley.

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Footnotes:

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Figure Legends

Fig. 1. Differential recruitment of coregulators to ERα upon CE, BAZ and TSEC treatment. (A) ERα/ERE-DNA pull-down analyses were performed using HeLa nuclear extracts in the presence of vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE plus 100 nM of BAZ) as described in "Materials and Methods". Proteins associated with ERα after different hormone treatments were identified by MS analyses, and the peptide numbers were listed. (B) After ERα/ERE DNA pull-down, the levels of SRC-1, SRC-2, SRC-3, BCOR and ERα in the precipitates were determined by Western blotting analyses. (C to F) SRC-1 (C), SRC-2 (D) and SRC-3 (E) protein levels in HeLa cells treated with hormone for 24 hrs were determined by Western blotting analyses (n=3/group). The tubulin level in each group was used as a protein loading control. (F**)** The ratios of SRCs to tubulin levels in panel C, D and E were shown in graph. NS, Non Specific, Student's *t* test. Values represent the average ± S.E.M. of three independent experiments.

Fig. 2. TSEC causes ERα protein degradation in murine uteri to prevent ERα-mediated endometrial stimulation. (A) Uteri were isolated from ovariectomized mice treated daily with vehicle, CE (2.5 mg/kg), BAZ (2.5 mg/kg) and TSEC (2.5 mg/kg of CE plus 2.5 mg/kg of BAZ) for 3 days, and then uterine morphology was examined. (B) The uterine wet weights in each group described in panel A were shown in graph (n=4/group). (C) ERα and Tubulin (loading control) protein levels in the uteri in panel A were determined by Western blotting analyses (n=3/group). (D) The ratios of ERα to tubulin levels in panel C were shown in graph (n=3/group). (E) ERα mRNA and 18S rRNA levels in the uteri in panel A were measured. The relative ERα mRNA levels in each hormone-treated uterus were determined as compared to its RNA levels in vehicle treated group after normalization with 18S rRNA levels. **, P<0.01. ***, P<0.001. NS,

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None Specific versus control, Student's *t* test. Values represent the average ± S.E.M. of three independent experiments.

Fig. 3. TSEC causes ERα protein degradation in human breast cancer cells to inhibit ERα activity. (A) ERα/ERE-DNA pull-down analyses were performed using MCF7 nuclear extracts in the presence of vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE plus 100 nM of BAZ). After ERα/ERE DNA pull-downs, the levels of SRC-1, SRC-3, FBXO45, BCOR and ERα in the precipitates were determined by Western blotting analyses. (B) MCF7 cells were treated with vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE and 100 nM of BAZ) for 24 h. Subsequently, the ERα and tubulin protein levels in MCF7 cells were determined by Western blotting analyses (n=3/group). (C) The ratios of ERα to tubulin in the MCF7 cells in panel B were shown in graph (n=3/group). (D) The total RNA was isolated from the MCF7 cells in panel B, and the levels of ERα mRNA and 18S rRNA were determined by real-time RT PCR analyses. The ERα mRNA levels in each group were normalized to the 18S rRNA levels. The normalized ERα mRNA levels in hormone-treated MCF7 cells relative to vehicle-treated MCF7 cells were shown in graph (n=3/group). (E) Progesterone Receptor (PR) mRNA levels were determined in the MCF7 cells described in panel B and normalized to the 18S rRNA levels. The normalized PR mRNA level in hormone-treated MCF7 cells relative to vehicle-treated MCF7 cells were shown in graph (n=3/group). (F) MCF7 cells were treated with vehicle, CE (10 nM), 4-hydroxytamoxifen (4HT; 100 nM), and CE (10 nM) plus 4-HT (100 nM) for 24 hr. Subsequently, the ERα and tubulin protein levels were determined by Western blotting analyses (n=3/group). (G) The ratio of ERα to tubulin in panel F was shown in graph. (H) HTB-85 cells were treated with vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE plus 100 nM of BAZ) for 24 h. Subsequently, the ERα and tubulin protein levels in HTB-85 cells were

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determined by Western blotting analyses (n=2/group). (I) The ratios of ERα to tubulin in the HTB-85 cells were shown in graph (n=3/group). *, P<0.05; **, P<0.01; ***, P<0.001; NS, not significantly different versus vehicle, Student's *t* test. Values represent the average ± S.E.M. of three independent experiments.

Fig. 4. ERα interacts with FBXO45 upon TSEC treatment. (A and B) HeLa cells in six well plate were transfected with 100 ng of ERα expression vector. After 48 hours later, cells were treated with vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE plus 100 nM of BAZ) for 24 h in the absence (A) or presence (B) of MG132 (10 μM). Subsequently, the ERα and tubulin protein levels in HeLa cells were determined by Western blotting analyses (n=2/group). The ratios of ERα to tubulin in HeLa cells are shown graphically (n=4/group). (C) Peptide numbers of the ubiquitin ligases that were specifically associated with TSEC-bound ERα after ERα/ERE-DNA pull-down analyses. (D) ERα/ERE-DNA pull-down was performed in the presence of hormone, and the FBXO45 levels in the precipitant were determined by Western blotting analyses. (E) HeLa cells were transiently transfected with expression vectors for ERα and/or Flag-tagged FBXO45. After 48 hours later, anti-Flag antibodies were used to immunoprecipitate Flag-tagged FBXO45 from each cell lysate in the absence or presence of MG132 (10 μM). The levels of ERα and FBXO45 in the immunoprecipitates were determined by Western blotting analyses. (F) HeLa cells were transiently transfected with expression vectors for ERα and Flag-FBXO45. After 48 hours later, cells were then treated with vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE plus 100 nM of BAZ) for 6 h. Using anti-Flag antibodies, Flag-FBXO45 was immunoprecipitated from each group, and the ERα levels in the immunoprecipitates were determined by Western blotting analyses. IP, Immunoprecipitation.

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WB, Western Blot analyses. ***, P<0.001; NS, not significantly different versus vehicle, Student's *t* test. Values represent the average ± S.E.M. of three independent experiments.

Fig. 5. FBXO45 involves in TSEC-mediated ERα degradation. (A) HeLa cells were transfected with an ERα expression vector and different amounts of expression vectors for Flag-tagged FBXO45. The ERα, FBXO45, PR and tubulin protein levels in each group were determined by Western blotting analyses. (B) HeLa cells transfected with ERα were treated with 100 nM of non-targeting siRNA or FBXO45 siRNA for 3 days followed by vehicle, CE (10 nM), BAZ (100 nM) and TSEC (10 nM of CE and 100 nM of BAZ) treatment for 24 h. Subsequently, the ERα, FBXO45 and tubulin protein levels in each group were determined by Western blotting analyses. (C) HeLa cells were transfected with expression vectors for ERα and FBXO45. The cells were then treated with vehicle, 10 nM of CE, 100 nM of BAZ or TSEC (10 nM of CE plus 100 nM of BAZ) for 0, 1, 3, 6, and 9 hrs. Subsequently, the ERα, FBXO45 and tubulin protein levels in each group were determined by Western blotting analyses. RF, Relative Fold compared to control.

Fig. 6. TSEC treatment inhibited the progression of ectopic lesions in mice with surgically induced endometriosis. (A and B) Ectopic lesions were isolated from each group of mice treated with different hormones (A), and then the lesion volumes were calculated and presented graphically (n=5/group) (B). (C) Hematoxylin and eosin analyses of ectopic lesions treated with each of ligand in panel A. (D to F) Expression levels of Ki-67 (D), ERα (E) and FBXO45 (F) in each type of ectopic lesion described in panel A were determined using immunohistochemistry and shown in the graph (n=9/group). (G) Expression levels of FBXO45 in eutopic endometrium

treated with each hormone were determined using Western blot analyses and shown in the graph.

*, P<0.05, **, P<0.01. ***, P<0.001. NS, None Specific versus control, Student's *t* test.

A

Protein ID	V	CE	BAZ	TSEC
NCOA6	1	9	1	1
TTK		6		
SRC-3	2	6	2	2
SRC-1	1	6		2
CHD6	2	5	1	2
MED12	1	5	2	2
SRC-2	2	5	1	1
MPRIP	2	5		
SNRPN		4		
TMOD3	1	4		
AGFG1	2	4	2	2
EIF6	1	4	1	2
GCN1L1		4		
CORO1C	1	4		
RAD50	1	3		1
APITD1-CORT	1	3	1	1
PRPF8		3	1	1
CCT2	1	3		
CAT	1	3	1	
AP2A2	1	3		
STOM	1	3		
GNB1	1	3		
GNB2	1	3		
GNB4	1	3		
SYNCRIP	1	3	1	1
NAT10		3	1	1
MED9		3	1	1
FBXO45	2	2	2	8
PRR12		2	2	7
BCOR	2			6
YTHDF1	1	2	3	6
HOXD9	1	1	2	5
HOXC10		1	2	5
NFYA		1	2	4
E2F6			2	4
RPL8	2	1	2	4
HNRNPH2		1	2	4
THOC4	1	2		4
HDAC3	1		1	4
CBR1			1	3
FIP1L1	1	1		3
TIA1		1	1	3

Peptide Number

0 >80

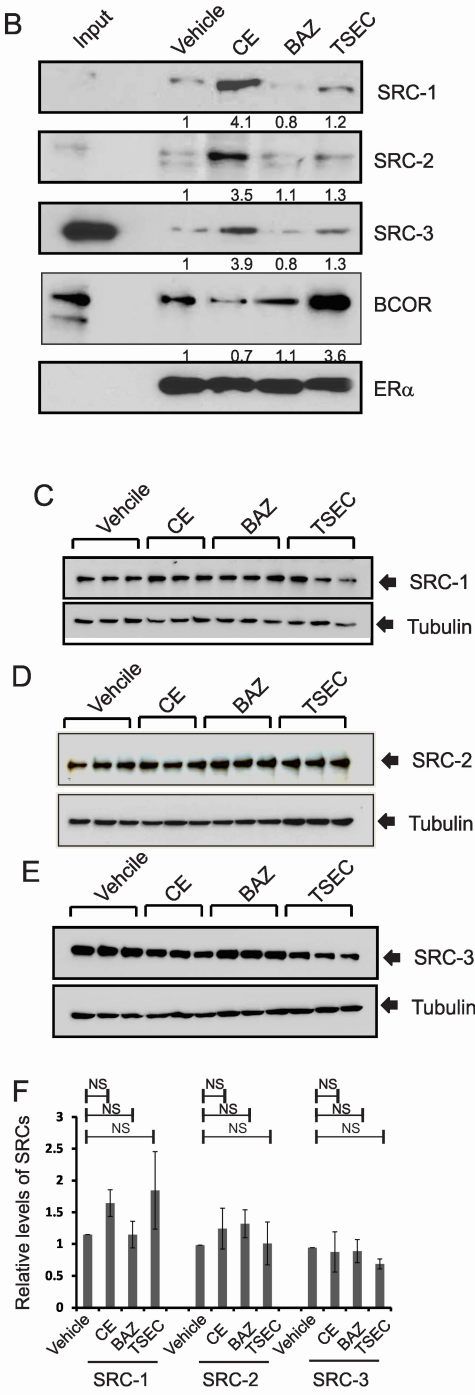

Figure 1

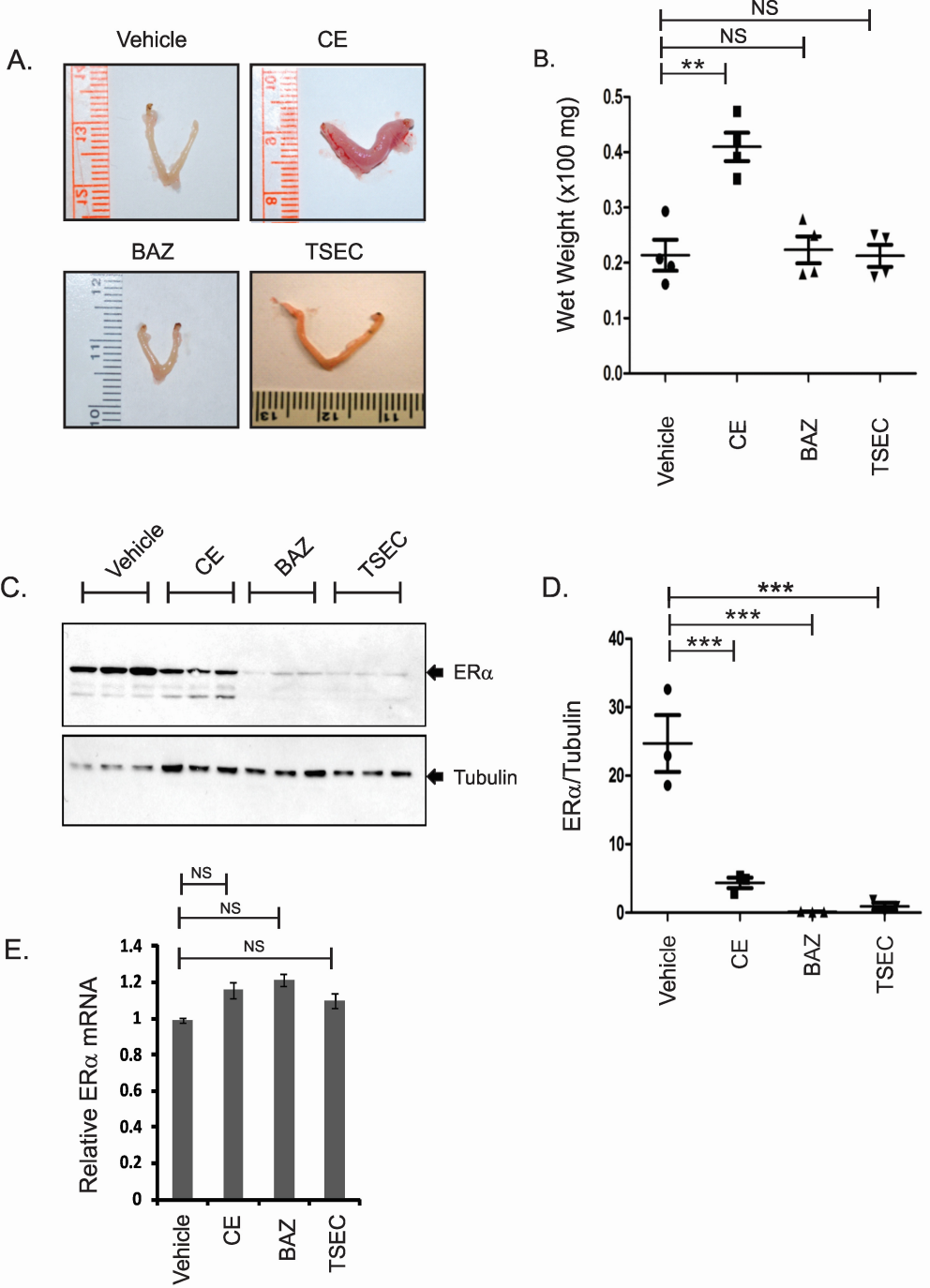
A.
Vehicle
CE
BAZ
TSEC
B.
NS
NS
**
Wet Weight (x100 mg)
0.5
0.4
0.3
0.2
0.1
0.0
Vehicle
CE
BAZ
TSEC
C.
Vehicle
CE
BAZ
TSEC
ERα
Tubulin
D.
***
***
***
ERα/Tubulin
40
30
20
10
0
Vehicle
CE
BAZ
TSEC
E.
NS
NS
NS
Relative ERα mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
CE
BAZ
TSEC

Figure 2

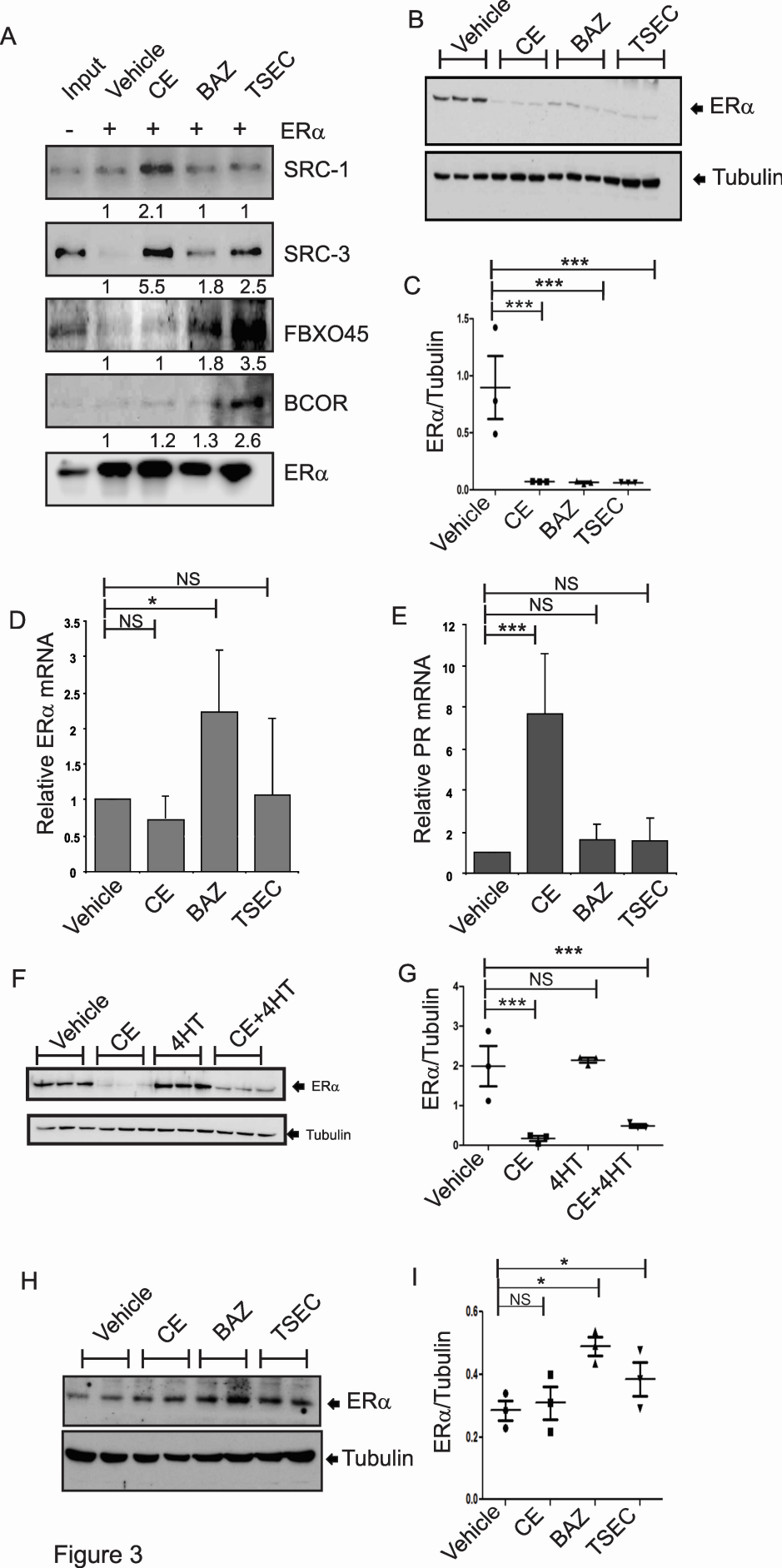
A
Input
Vehicle
CE
BAZ
TSEC
- + + + + ERα
SRC-1
1 2.1 1 1
SRC-3
1 5.5 1.8 2.5
FBXO45
1 1 1.8 3.5
BCOR
1 1.2 1.3 2.6
ERα
B
Vehicle
CE
BAZ
TSEC
ERα
Tubulin
C
***
***
***
ERα/Tubulin
Vehicle
CE
BAZ
TSEC
D
NS
*
NS
Relative ERα mRNA
Vehicle
CE
BAZ
TSEC
E
NS
NS
***
Relative PR mRNA
Vehicle
CE
BAZ
TSEC
F
Vehicle
CE
4HT
CE+4HT
ERα
Tubulin
G
***
NS
***
ERα/Tubulin
Vehicle
CE
4HT
CE+4HT
H
Vehicle
CE
BAZ
TSEC
ERα
Tubulin
I
*
*
NS
ERα/Tubulin
Vehicle
CE
BAZ
TSEC

Figure 3

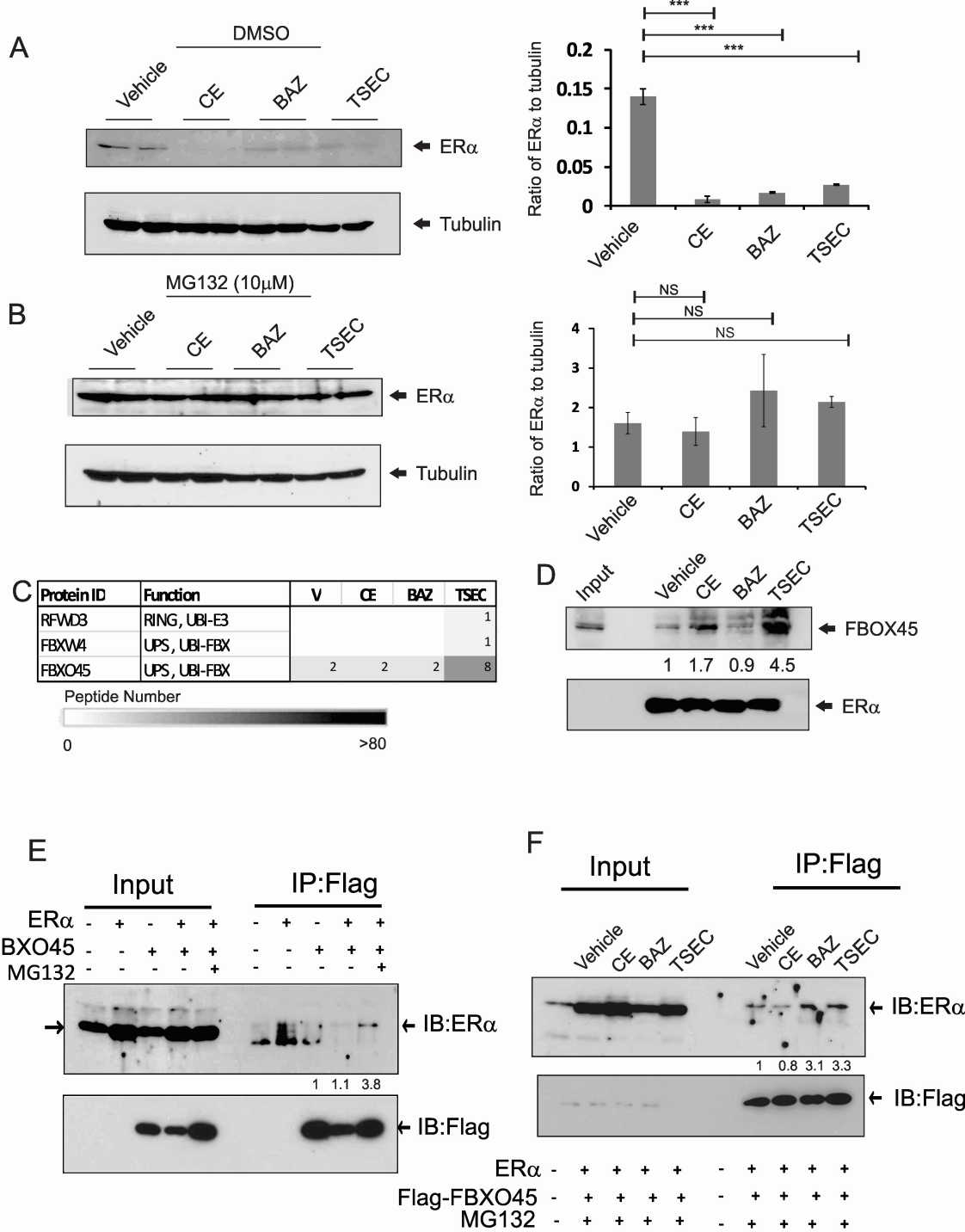
A
DMSO
Vehicle
CE
BAZ
TSEC
ERα
Tubulin
***
***
***
Ratio of ERα to tubulin
0.2
0.15
0.1
0.05
0
Vehicle
CE
BAZ
TSEC
B
MG132 (10μM)
Vehicle
CE
BAZ
TSEC
ERα
Tubulin
NS
NS
NS
Ratio of ERα to tubulin
4
3
2
1
0
Vehicle
CE
BAZ
TSEC
C
Protein ID
Function
V
CE
BAZ
TSEC
RFWD3
RING, UBI-E3
1
FBXW4
UPS, UBI-FBX
1
FBXO45
UPS, UBI-FBX
2
2
2
8
Peptide Number
0
>80
D
Input
Vehicle
CE
BAZ
TSEC
FBOX45
1
1.7
0.9
4.5
ERα
E
Input
IP:Flag
ERα
Flag-FBXO45
MG132
IB:ERα
1
1.1
3.8
IB:Flag
F
Input
IP:Flag
Vehicle
CE
BAZ
TSEC
Vehicle
CE
BAZ
TSEC
IB:ERα
1
0.8
3.1
3.3
IB:Flag
ERα
Flag-FBXO45
MG132

Figure 4

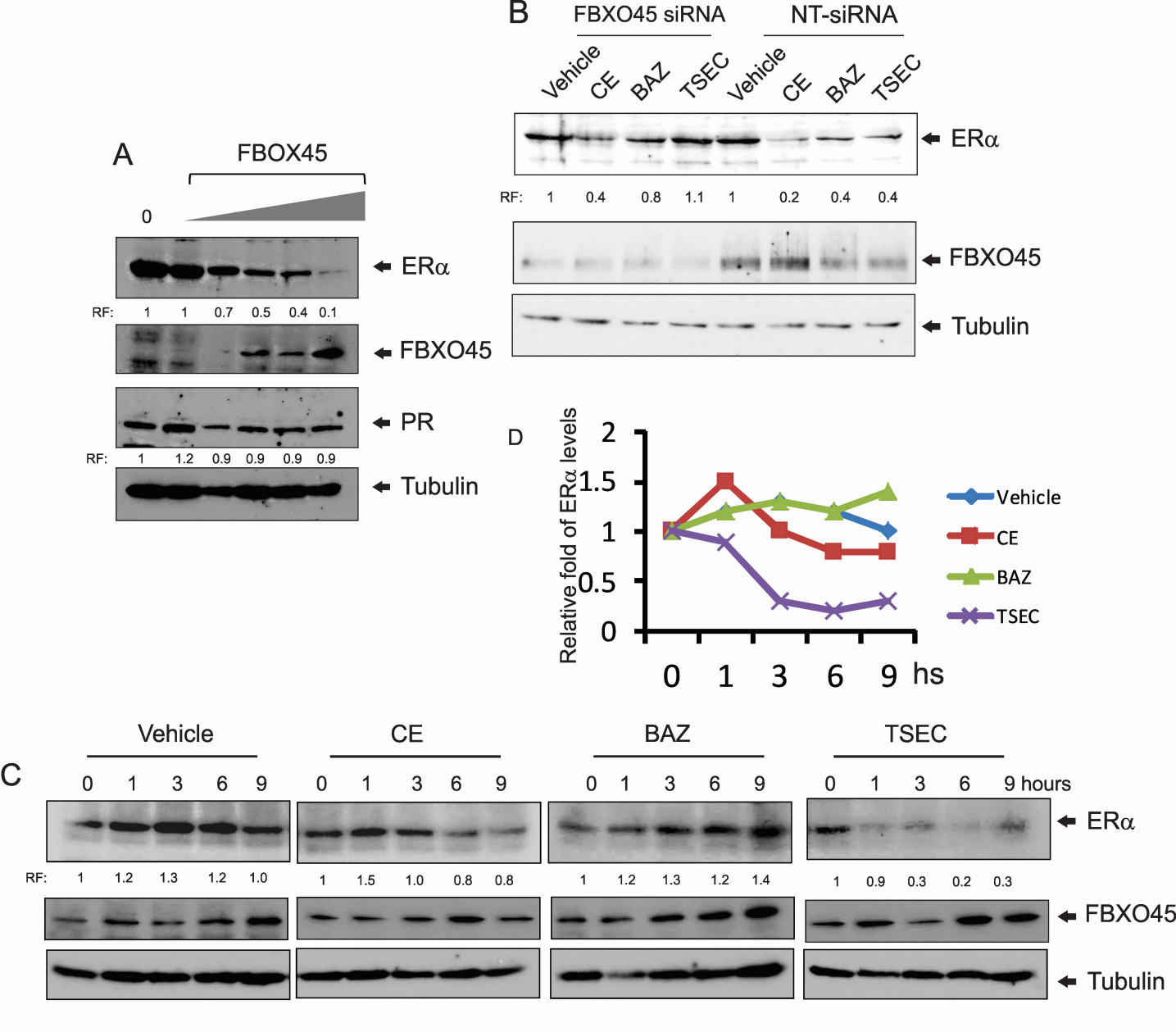
A
FBOX45
0
ERα
RF: 1 1 0.7 0.5 0.4 0.1
FBXO45
PR
RF: 1 1.2 0.9 0.9 0.9 0.9
Tubulin
B
FBXO45 siRNA
NT-siRNA
Vehicle
CE
BAZ
TSEC
Vehicle
CE
BAZ
TSEC
ERα
RF: 1 0.4 0.8 1.1 1 0.2 0.4 0.4
FBXO45
Tubulin
D
Relative fold of ERα levels
2
1.5
1
0.5
0
0 1 3 6 9 hs
Vehicle
CE
BAZ
TSEC
C
Vehicle
CE
BAZ
TSEC
0 1 3 6 9
0 1 3 6 9
0 1 3 6 9
0 1 3 6 9 hours
ERα
RF: 1 1.2 1.3 1.2 1.0
1 1.5 1.0 0.8 0.8
1 1.2 1.3 1.2 1.4
1 0.9 0.3 0.2 0.3
FBXO45
Tubulin

Figure 5

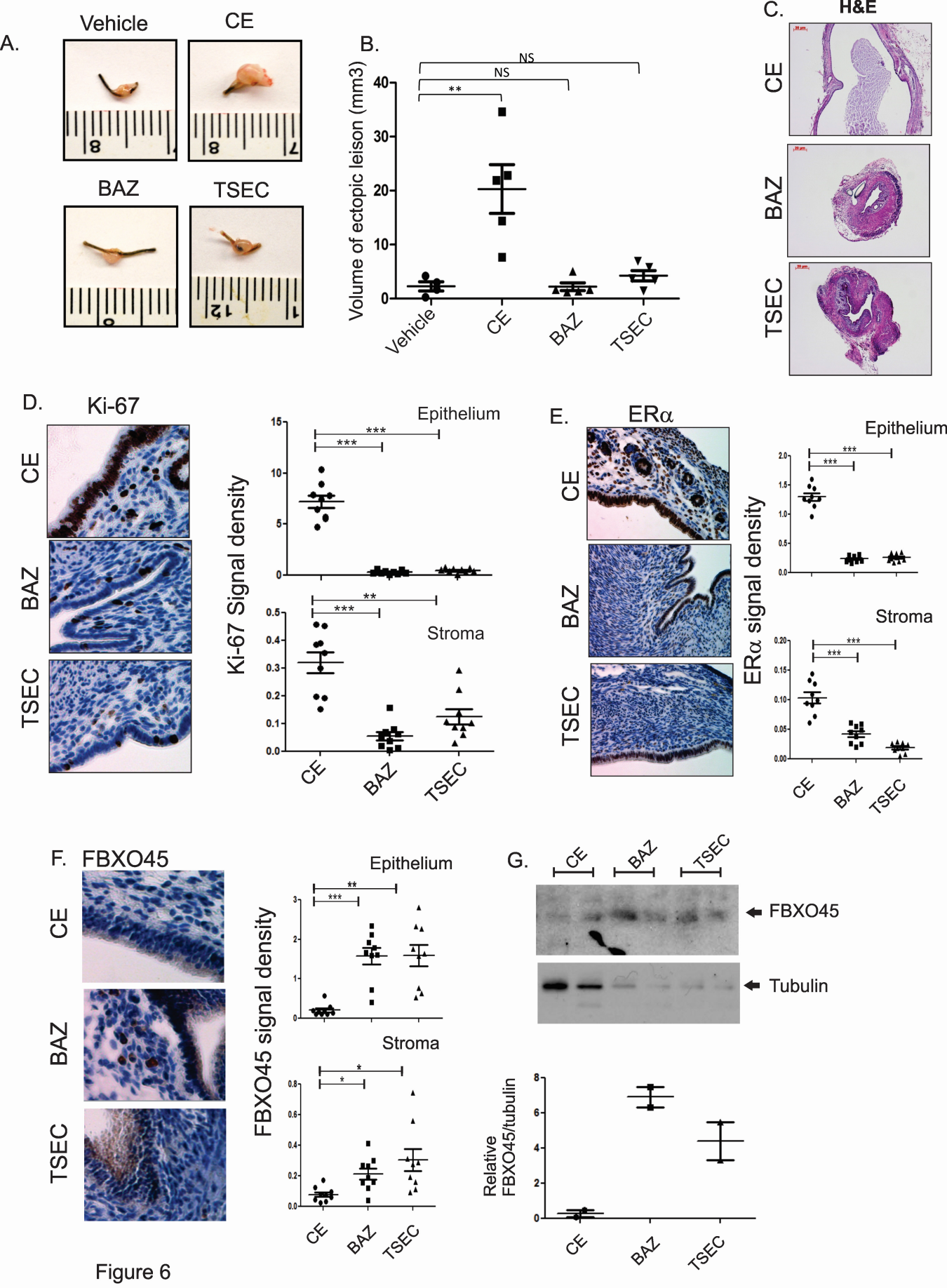
A.
Vehicle
CE
BAZ
TSEC
B.
Volume of ectopic leison (mm3)
NS
NS
**
Vehicle
CE
BAZ
TSEC
C.
H&E
CE
BAZ
TSEC
D.
Ki-67
CE
BAZ
TSEC
Ki-67 Signal density
Epithelium
Stroma
E.
ERα
CE
BAZ
TSEC
ERα signal density
Epithelium
Stroma
F.
FBXO45
CE
BAZ
TSEC
FBXO45 signal density
Epithelium
Stroma
G.
CE
BAZ
TSEC
FBXO45
Tubulin
Relative FBXO45/tubulin

Figure 6