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17β-Estradiol and ICI182,780 Differentially Regulate STAT5 Isoforms in Female Mammary Epithelium, With Distinct Outcomes

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Prolactin (PRL) and estrogen cooperate in lobuloalveolar development of the mammary gland and jointly regulate gene expression in breast cancer cells *in vitro*. Canonical PRL signaling activates STAT5A/B, homologous proteins that have different target genes and functions. Although STAT5A/B are important for physiological mammary function and tumor pathophysiology, little is known about regulation of their expression, particularly of STAT5B, and the consequences for hormone action. In this study, we examined the effect of two estrogenic ligands, 17β-estradiol (E2) and the clinical antiestrogen, ICI182,780 (ICI, fulvestrant) on expression of STAT5 isoforms and resulting crosstalk with PRL in normal and tumor murine mammary epithelial cell lines. In all cell lines, E2 and ICI significantly increased protein and corresponding nascent and mature transcripts for STAT5A and STAT5B, respectively. Transcriptional regulation of STAT5A and STAT5B by E2 and ICI, respectively, is associated with recruitment of estrogen receptor alpha and increased H3K27Ac at a common intronic enhancer 10 kb downstream of the *Stat5a* transcription start site. Further, E2 and ICI induced different transcripts associated with differentiation and tumor behavior. In tumor cells, E2 also significantly increased proliferation, invasion, and stem cell-like activity, whereas ICI had no effect. To evaluate the role of STAT5B in these responses, we reduced STAT5B expression using short hairpin (sh) RNA. shSTAT5B blocked ICI-induced transcripts associated with metastasis and the epithelial mesenchymal transition in both cell types. shSTAT5B also blocked E2-induced invasion of tumor epithelium without altering E2-induced transcripts. Together, these studies indicate that STAT5B mediates a subset of protumorigenic responses to both E2 and ICI, underscoring the need to understand regulation of its expression and suggesting exploration as a possible therapeutic target in breast cancer.

Freeform/Key Words: breast cancer, estrogen, HC11 cells, STAT5A, STAT5B

Prolactin (PRL) and estrogen cooperate in the physiological lobuloalveolar development of the mammary gland in early pregnancy and jointly regulate gene expression in luminal breast cancer cells *in vitro* [1–6]. These hormones crosstalk at many levels, including interactions

Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; ChIP, chromatin immunoprecipitation; CSS, charcoal-stripped serum; DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor; EMT, epithelial mesenchymal transition; E2, 17β-estradiol; ERα, estrogen receptor alpha; FBS, fetal bovine serum; FITC, phalloidin-fluorescein isothiocyanate; ICI, ICI182,780; Ig, immunoglobulin; mRNA, messenger RNA; pen/strep, penicillin/streptomycin; PRL, prolactin; PRLR, prolactin receptor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; shNT, nontargeting shRNA; shRNA, short hairpin RNA; TC, tumor cell; Veh, vehicle.

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between estrogen signals and STAT5 isoforms, the effectors of the canonical PRL-activated JAK2-STAT5 signaling cascade [3, 7–9]. In this pathway, binding of PRL to the PRL receptor (PRLR) initiates a conformational change which activates JAK2, a receptor-associated tyrosine kinase. JAK2 then phosphorylates downstream substrates, including latent cytoplasmic STAT5 proteins, which can dimerize and translocate to the nucleus to regulate gene transcription [10, 11]. Two highly conserved isoforms of STAT5, STAT5A and STAT5B, are encoded by separate genes located on chromosome 17q21.2 in humans (chromosome 11 in the mouse) [12, 13]. Although STAT5A and STAT5B are more than 90% identical in amino acid sequences, they regulate different but overlapping sets of genes in breast cancer cell lines and hematopoietic cells [14–17]. Most of their structural differences lie in the transactivation domain, which may confer some selectivity.

STAT5A is expressed at higher levels than STAT5B in the normal mammary gland and is the primary mediator of the physiological actions of PRL in mammary epithelial cells [10, 11, 18, 19]; however, both isoforms are expressed in many breast cancer cell lines and clinical tumors. Their high homology, including a conserved activating tyrosine residue in the C-terminal domain, made it difficult to distinguish them in early studies. However, recent studies link STAT5A to positive outcomes in breast cancer. Expression of STAT5A is higher in healthy luminal breast epithelial cells than carcinomas [20]. In primary breast adenocarcinomas, nuclear STAT5A is associated with histologic differentiation and better prognosis, and reduced STAT5A expression is associated with an increased risk of resistance to antiestrogen therapies [16, 21]. In contrast, no positive associations have been observed for STAT5B [16]. In breast cancer cells *in vitro*, STAT5A activation increases differentiation and inhibits invasion [22, 23], whereas STAT5B is implicated in processes that promote tumor progression, including migration, invasion, and tamoxifen resistance [23–25]. Moreover, STAT5B has been linked to aggression of prostate, hepatocellular, and pancreatic cancers [26–31].

Despite the importance of the STAT5 isoforms in normal mammary development and tumor pathophysiology, little is known about regulation of their expression. Although estrogen increases STAT5A messenger RNA (mRNA) in the mammary gland [32, 33], the underlying mechanism is not well understood and may vary with the target tissue or model system. There is a fundamental gap in knowledge about determinants of STAT5B expression. The effects of antiestrogens on the expression of either isoform have received little attention, despite the importance of estrogen receptor alpha (ERα)-STAT5 crosstalk. In a murine ERα^+ breast cancer model, we recently reported that chronic postpubertal treatment with the ER antagonist, ICI182,780 (ICI), significantly reduced tumor latency, which was associated with increased STAT5B expression [34]. However, this study looked at net outcomes in a complex *in vivo* environment.

To understand the intrinsic effect of 17β-estradiol (E2) and ICI on expression of the STAT5 isoforms and how these outcomes affect the interplay with PRL-activated STAT5 isoforms in mammary epithelia of differing phenotypes, we compared the well-characterized, differentiated HC11 mouse cell line [35] and ERα^+ mouse mammary tumor cell (TC) lines generated from PRL-induced adenocarcinomas [36]. We demonstrated that E2 increases STAT5A and ICI increases STAT5B expression in both normal mammary epithelial cells and TCs; however, reduction of STAT5B expression using short hairpin RNA (shRNA) revealed a complex relationship between estrogenic signals and the STAT5 isoforms. In both normal and TCs with endogenous expression of STAT5A/B, E2 upregulated transcripts associated with differentiation, and, in TCs, also augmented proliferation, tumorsphere formation, and invasion. Although reduction of STAT5B did not alter the ability of E2 to induce these transcripts, it blocked E2-induced invasion. On the other hand, in cells expressing both STAT5 isoforms, ICI upregulated transcripts associated with stemness, epithelial mesenchymal transition (EMT) and metastasis. Reduction of STAT5B abrogated these effects. These studies elucidate the importance of STAT5B in ERα-mediated protumorigenic actions in mammary TCs, and point to the need for better understanding of its actions in ERα^+ cancer and treatment responses.

1. Materials and Methods

A. Reagents

E2 (E2758), dexamethasone (D4902), 5-bromo-2′-deoxyuridine (BrdU), and phalloidin-fluorescein isothiocyanate (FITC; P5282) were purchased from Sigma-Aldrich (St. Louis, MO). ICI (fulvestrant, 1047) was obtained from Tocris Bioscience (Ellisville, MO); bovine PRL (lot AFP7170E) was obtained from Dr. A.F. Parlow (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Torrance, CA); insulin (A11429IJ; Thermo Fisher, Camarillo, CA); and the prolactin antagonist, Δ1-9-G129R-hPRL [37], from Vincent Goffin (Inserm, U155, Paris). Epidermal growth factor (EGF) (AF-100-15) and recombinant murine FGF-basic (450-33) were purchased from PeproTech (Rocky Hill, NJ). Type I rat tail collagen (CB354249), B27 (17504044), and geneticin/G418 sulfate (10131027) were obtained from Thermo Fisher Scientific (Waltham, MA); puromycin (Ant-pr-1) was obtained from InvivoGen (San Diego, CA). Antibodies against the following proteins were purchased from these vendors: STAT5A (sc-1081), STAT5B (sc-1656), ERα (MC-20, sc-542), PRLR (H300, sc-20992), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); BrdU (OBT0030), Accurate Chemical and Scientific Co. (Westbury, NY); and HRP-linked antimouse immunoglobulin G (IgG; 7076), HRP-linked antirabbit IgG (7074), ERK1/2 (9102), Cell Signaling Technology (Danvers, MA). Anti-ERα, normal rabbit IgG (sc-542 and sc-2027, respectively, Santa Cruz Biotechnology), antihistone H3 (H3), and anti-H3K27Ac (ab1791 and ab4729, Abcam (Cambridge, United Kingdom), were used for chromatin immunoprecipitation (ChIP). Avidin-biotin-complex (PK-4000) and Immpact DAB (SK-4105) were purchased from Vector Laboratories (Burlingame, CA).

B. HC11 Cells

Mouse mammary epithelial HC11 cells [35] (from Caroline Alexander, University of Wisconsin-Madison) were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 5 μg/mL insulin, 25 ng/mL EGF, and 1% penicillin/streptomycin (pen/strep). For all experiments, differentiation was induced by withdrawing EGF for 48 hours and then changing the media to RPMI 1640 containing 5% 3× charcoal-stripped serum (CSS), 1 μM dexamethasone, 5 μg/mL insulin, 5 μg/mL bPRL, and 1% pen/strep for 72 hours as described [35]. Before initiating treatments, serum was removed from the media for 24 hours.

C. Generation of Mammary TC Lines

Mammary tumors from NRL-PRL transgenic females [36] were excised and dispersed in HBSS + 0.1% collagenase for 30 min at 37°C. Cells were pelleted from the supernatant and the procedure was repeated twice. Pooled cells were cultured in RPMI 1640 containing 5% FBS, 1% pen/strep, 10 ng/mL EGF, 10 mM HEPES, 10 ng/mL insulin, 10 ng/mL transferrin, 2 mM sodium pyruvate, and 10 ng/mL cholera toxin for 5 days and then transferred to RPMI 1640 containing 1% FBS, 1% pen/strep, and 10 ng/mL cholera toxin for 5 more days. To further enrich for epithelial cells, cultures were serially trypsinized twice. Clonal TC lines were derived from mixed cultures at passage 21, including TC2 and TC11, which were used in these studies. As shown in Supplemental Fig. 1, these cell lines are estrogen responsive and grow in response to PRL. Cultures were maintained in RPMI 1640 containing 10% FBS and 1% pen/strep. Before initiating treatments, FBS was replaced with CSS for 72 hours followed by removal of serum from the media for 24 hours.

D. Generation of Lentivirus and Stable Cell Lines

shRNA against mouse Stat5b (TRCN0000425025), or a control nontargeting sequence (SHC202) in a TRC2 pLKO.5-puro backbone (Sigma, St. Louis, MO) and glycerol stocks of pCMV-VSV-G (a gift from Bob Weinberg; Addgene plasmid #8454), pRSV-REV (a gift from

Didier Trono; Addgene plasmid #12253), and pMDLg/pRRE (a gift from Didier Trono; Addgene plasmid #12251) were obtained. Plasmid DNA was isolated from bacterial cultures using the Plasmid Mega Kit (Qiagen, Valencia, CA). pCMV-VSG-G, pRSV-REV and pMDLg/pRRE were cotransfected with either Stat5b shRNA or nontargeting shRNA plasmids into 293T cells using FuGENE6 (Promega, Madison, WI) to produce supernatant containing lentiviral particles. Forty-eight and 72 hours after transfection, the supernatant containing lentivirus was added to undifferentiated HC11 and TC11 cells through a 0.45-μm syringe filter in the presence of 5 μg/mL protamine sulfate (Sigma, St. Louis, MO) for 4 hours. Infected cells were selected with puromycin (HC11, 7 μg/mL; and TC11, 3 μg/mL) 48 hours after infection; reduced expression was confirmed by western blotting.

E. RNA Isolation and Quantitative RT-PCR

For examination of transcript levels, parental HC11 and TC11 cell lines were treated with 0.1% ethanol vehicle (Veh), 1 nM E2, 10 μM ICI, or E2 and ICI in serum-free media for 48 hours before analysis. Stably transfected HC11 and TC11 cell lines were treated with Veh, 1 nM E2, or 1 μM ICI in serum-free media for 48 hours. RNA was isolated and reverse transcription polymerase chain reaction (RT-PCR) performed as described [38] using the primers listed in Supplemental Table 1. Primers to detect nascent transcripts were designed to cross intron/exon boundaries. Results were analyzed via the delta-delta C(t) method and normalized to 18S ribosomal RNA.

F. Immunoblotting

For examination of protein levels, cells were treated with Veh, 1 nM E2, or 10 μM ICI in serum-free media for 48 hours. Cells were lysed, and 20 μg protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and probed with the antibodies indicated as described [38] (PRLR, 1:1000; ER, 1:1000; ERK1/2, 1:5000; STAT5A, 1:10,000; STAT5B, 1:5000) (Table 1). Under these conditions, the antibodies against STAT5A and STAT5B discriminate between these isoforms (Supplemental Fig. 2). Proteins were visualized using enhanced chemiluminescence (Thermo Fisher) and quantified by scanning densitometry (VisionWorksLS, version 7.1; UVP, Upland, CA).

Table 1. Antibodies Used in This Study

Peptide/Protein Target	Antibody ID (RRID)	Manufacturer, Catalog No.	Animal Source Mono/Polyclonal	Dilution Used
BrdU	2313623	Accurate Chemical and Scientific Co., *OBT0030*	Rat, mono	IHC: 1:40
ERα	631470	Santa Cruz Biotechnology, MC-20, sc-542	Rabbit, poly	WB: 1:1000 ChIP: 1:200
ERK1/2	330744	Cell Signaling Technology, 9102	Rabbit, poly	WB: 1:5000
H3	302613	Abcam, ab1791	Rabbit, poly	ChIP: 1:1000
H3K27Ac	2118291	Abcam, ab4729	Rabbit, poly	ChIP: 1:1000
PRLR	2237692	Santa Cruz Biotechnology, H300, sc-20992	Rabbit, poly	WB: 1:1000
STAT5A	632448	Santa Cruz Biotechnology, sc-1081	Rabbit, poly	WB: 1:10,000 IHC: 1:2000
STAT5B	2197067	Santa Cruz Biotechnology, sc-1656	Mouse, mono	WB: 1:5000 IHC: 1:400

Abbreviations: IHC, immunohistochemistry; WB, western blotting.

G. Proliferation

BrdU incorporation was used to detect cells in S phase. Parental and stably transfected HC11 and TC11 cells were plated at 10^5 cells per well and 2×10^5 cells per well, respectively, on ultraviolet-sterilized coverslips in a six-well plate and grown for 48 hours. Serum was removed from the media 24 hours before treatment (parental HC11 and TC11: Veh, 1 nM E2, or 10 μM ICI; stably transfected HC11 and TC11: Veh, 1 nM E2, or 1 μM ICI) in serum-free media for 48 hours; cells were then pulsed with 10 μM BrdU for 2 hours. Slides were processed for immunocytochemical analysis as described in the following section. BrdU staining was assessed in five random 20× microscopic fields from three independent experiments.

H. Immunofluorescence and Immunohistochemistry

Immunofluorescence analyses were performed as previously described [39]. Briefly, cells were grown on coverslips and fixed for 10 minutes with ice-cold methanol. For visualization of F-actin, phalloidin-FITC (1:100) and 4′,6-diamidino-2-phenylindole (DAPI) counterstain were added to coverslips for 1 hour at room temperature. For BrdU labeling, cells were blocked (phosphate-buffered saline, 0.1% Triton X-100 with 5% donkey serum) and then incubated overnight at 4°C with anti-BrdU (1:40) before visualization and counterstaining with DAPI.

To assess the role of PRL in activation of the STAT5 isoforms, PRL activity was reduced for 24 hour from serum-starved differentiated HC11 and TC11 cells by PRL withdrawal or addition of 1 μg/mL 1-9-G129R-hPRL, respectively. Nuclear STAT5A and STAT5B were detected as described [34]. Positive staining was assessed in five random 20× microscopic fields from three independent experiments.

I. Invasion Assay

Invasion assays were performed as described [40]. Briefly, 2.5×10^4 of parental and stably transfected TC11 cells were plated on 8-μm transwell inserts (Corning) coated with collagen I, and 5% CSS was added to the bottom chamber as a chemo-attractant. The parental cells were treated with ethanol Veh, 1 nM E2, or 10 μM ICI and the stably transfected TC11 cells were treated with ethanol Veh, 1 nM E2, or 1 μM ICI for 24 hours. Membranes from the transwells were stained with Wright-Giemsa, and positive staining was assessed in five random 10× microscopic fields from three independent experiments.

J. Tumorsphere Assay

A total of 8×10^5 TC11 cells were plated on 100-mm dishes in serum-free media 24 hours before treatment with Veh, 1 nM E2 or 10 μM ICI for 48 hours. Cells were harvested and 6.25×10^3 cells per well were plated into Ultra-Low Attachment 96-well plates (Corning) in serum-free Dulbecco's modified Eagle medium/F12 media containing 20 ng/mL EGF, 20 ng/mL basic FGF, 1X B27 Supplement, 5 μg/mL insulin, and 100 μg/mL G418 sulfate for 6 days. Tumorspheres >50 μm in diameter were counted.

K. ChIP

TC11 cells were treated with Veh, 1 nM E2, or 1 μM ICI for varying times and ChIP assays were performed as previously described [41]. Briefly, chromatin was sheared three times for 15 seconds at 15% maximum power (Fisher Scientific 550 Sonic Dismembrator) and samples were allowed to cool on ice between each sonication. Immunoprecipitation was performed with antibodies against ERα, H3, H3K27Ac, or normal rabbit IgG. Samples were uncrosslinked at 65°C overnight and DNA was isolated using the QIAquick PCR Purification Kit (Qiagen). Quantitative RT-PCR (qRT-PCR) was performed with 1 μL of purified DNA and 200 nM forward and reverse primers (Supplemental Table 2). Data were calculated as a percent of input. H3K27Ac was normalized to total H3.

L. Statistical Analyses

Statistical analyses were performed using GraphPad Prism, version 6 (GraphPad Software, Inc., San Diego, CA). At least three independent experiments were analyzed via one-way analysis of variance (ANOVA) with the Tukey comparison posttest or two-way ANOVA with the Bonferroni posttest as indicated. Differences were considered significant at $P < 0.05$. Wilcoxon rank-sum test was used for analysis of ChIP.

2. Results

A. Mammary TCs Exhibit Distinct Characteristics From Normal Mammary Epithelial Cells

To evaluate the crosstalk between estrogen and PRL-activated STAT5A/B in mammary cells with different phenotypes, we compared the HC11 cell line cultured under differentiation conditions, as an example of "normal" mammary epithelial cells [35], and TC2 and TC11, TC lines derived from a PRL-induced mammary adenocarcinoma [36]. As shown in Fig. 1A, these cell lines expressed PRLR, STAT5A/B, and ER at various levels. The HC11 cell line was generated from a midpregnant mouse, and exogenous PRL and glucocorticoids acting on their respective receptors induce differentiation and transcription of milk protein genes, such as β-casein (Fig. 1B) [42]. In contrast, the TC lines express transgenic PRL, which drives their growth (Supplemental Fig. 1C); they express very little β-casein (Fig. 1B). Consistently, the differentiated HC11 cells displayed a regular cobblestone epithelial morphology, revealed by F-actin staining. The TCs, however, were irregularly shaped, with less cytoplasm and prominent nuclei. TC11 cells, in particular, displayed a fibroblastic appearance with spindle

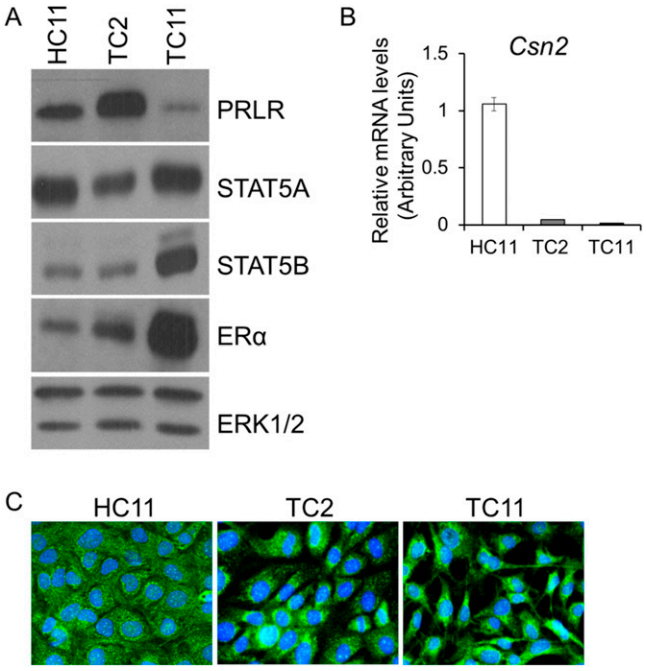

Figure 1. Mammary cell lines are phenotypically distinct. (A) Cell lysates from serum-starved differentiated HC11, TC2, and TC11 cells were immunoblotted with the indicated antibodies. HC11 cells were differentiated in media containing dexamethasone and exogenous PRL before transfer to serum-free media for 24 hours before analysis (see Materials and Methods). (B) β-casein (*Csn2)* mRNA in differentiated HC11, TC2, and TC11 cells was quantitated by qRT-PCR and normalized to 18S RNA. Mean ± standard deviation (SD) is shown. N = 3. (C) Cells were cultured in serum-free media and then stained for DAPI and phalloidin-FITC as described in the Materials and Methods.

protrusions (Fig. 1C). We focused on TC11 cells for the majority of our studies, with additional data on TC2 shown in the Supplemental Materials.

C. E2 Increases Proliferation, Invasion, and Tumorsphere Formation of Mammary TCs

Both normal and TCs express estrogen receptors. The TC lines express ERα without detectable ERβ, whereas HC11 cells express both ERα and ERβ [7]. Therefore we investigated the effects of estrogen activity on proliferation, invasion and tumorsphere formation, important end points of hormone action in breast cancer. As expected [43], E2 did not stimulate proliferation of HC11 cells (Fig. 2A), which are confluent and well-differentiated in the differentiation media. In contrast, E2 significantly increased proliferation, invasion and the efficiency of tumorsphere formation of TC11 cells (Fig. 2B–2D; Supplemental Fig. 3). Treatment with the clinical antiestrogen, ICI (Fulvestrant), yielded results similar to vehicle controls.

D. E2 and IC Induce Opposing Effects on Markers of Differentiation and EMT in Both Normal Mammary Epithelia and TCs

We next investigated the effects of E2 and ICI on select genes associated with differentiation, polarization/EMT, and metastasis. β1-integrin is expressed at higher levels in normal mature mammary epithelial cells and is required for organization of epithelial structures and milk production [44]. In both differentiated HC11 and TC11 cells, E2 significantly increased *Itgb1* mRNA (Fig. 3A and 3E). Surprisingly, ICI induced transcripts for α6-integrin (*Itga6,* CD49f), which is associated with progenitor populations [45, 46], in HC11 and both TC lines (Fig. 3B and 3F; Supplemental Fig. 4B). Transcripts for parathyroid hormone-like hormone (*Pthlh*), a marker of breast cancer metastasis [47–49], were significantly higher with ICI treatment in HC11, TC11, and TC2 cells (Fig. 3C and 3G; Supplemental Fig. 4C). Loss of E-cadherin and increasing levels of TWIST1 are indicators of EMT, which is associated with invasion and metastasis in breast cancer [50]. In HC11 cells, E2 significantly increased *Cdh1* mRNA (Figure 3D). *Cdh1* mRNA was not detectable in either TC line, and, as expected, HC11 cells expressed very low levels of EMT markers (data not shown). However, in both TC lines, ICI significantly increased *Twist1* mRNA (Fig. 3H; Supplemental Fig. 4D). As predicted from its antiestrogen activity, cotreatment with ICI and E2 blocked the ability of E2 to increase *Itgb1* and *Cdh1* mRNAs, but E2 did not alter the effect of ICI (Fig. 3). Together, these data suggest

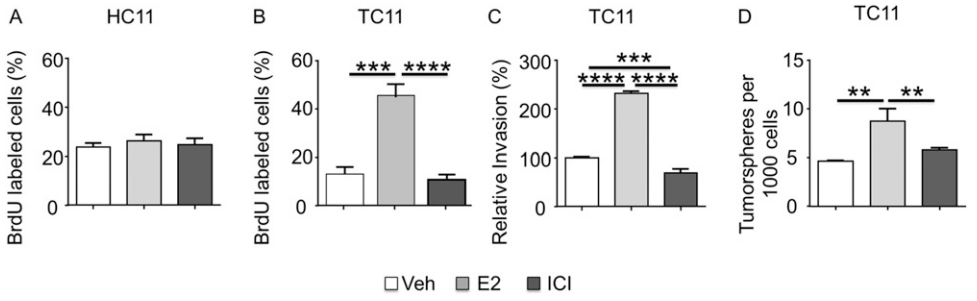

Figure 2. 17β-estradiol increases proliferation, invasion, and tumorsphere formation of TC11 cells. (A, B) Serum-starved differentiated HC11 and TC11 cells were treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 48 hours before the BrdU pulse. (C) Serum-starved TC11 cells were plated on 8-μm transwell inserts coated with collagen I and treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 24 hours before determination of invasion as described in the Materials and Methods. (D) Serum-starved TC11 cells were treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 48 hours and then assayed for tumorsphere-forming ability. Mean ± SD is shown. N = 3. Significant differences were determined by one-way ANOVA, followed by the Tukey posttest (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

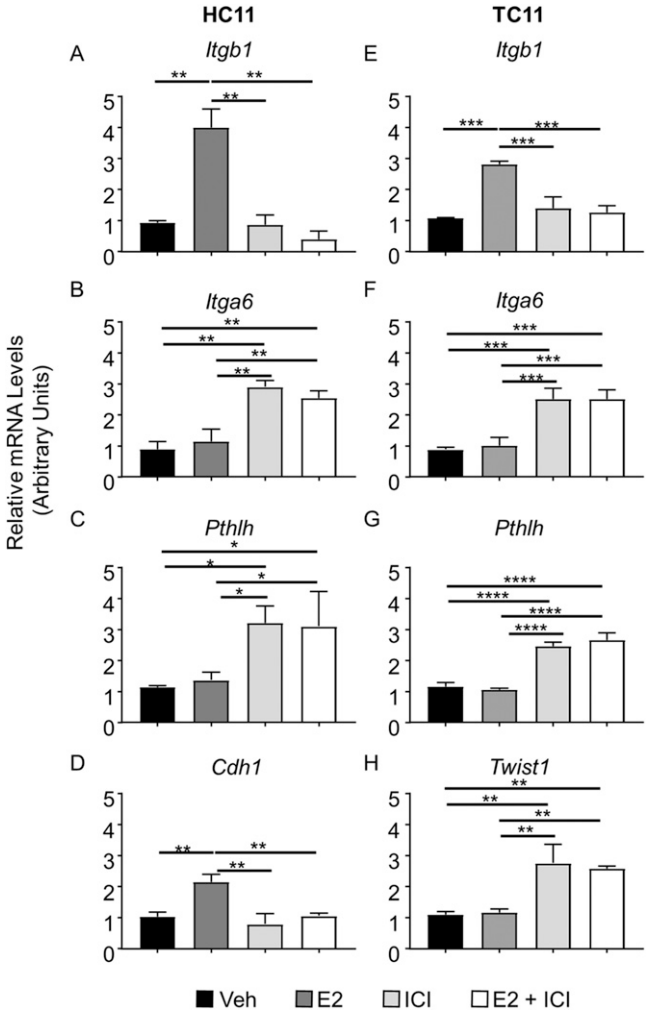

Figure 3. Estrogen activity alters transcript markers of differentiation and the epithelial-to-mesenchymal transition. Serum-starved differentiated HC11 (A–D) and TC11 cells (E–H) were treated with Veh (black bars), E2 (dark gray bars), ICI (light gray bars), or E2 + ICI (white bars) for 48 hours; specific mRNAs were quantitated by qRT-PCR and normalized to 18S RNA. Means ± SD is shown. N = 3. Significant differences were determined by one-way ANOVA, followed by the Tukey posttest (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

that E2 induces some characteristics associated with differentiation, whereas ICI induces a less differentiated phenotype, regardless of mammary cell context.

E. E2 and ICI Differentially Induce Expression of STAT5A and STAT5B, Respectively, in Both Normal Mammary Epithelia and TCs

Next, we assessed the ability of E2 and ICI to alter STAT5A/B expression. E2 has been reported to increase expression of STAT5A in mammary epithelia [32, 33]. Consistently, E2 significantly increased STAT5A in all cell lines, but did not alter levels of STAT5B (Fig. 4A–4C, 4E–4G; Supplemental Fig. 5). In contrast, ICI significantly increased STAT5B in all cell lines, without altering STAT5A (Fig. 4A–4C, 4E–4G; Supplemental Fig. 5). Similar to effects on *Itgb1* and *Cdh1* transcripts, cotreatment with ICI blocked E2-induced *Stat5a* mRNA, but E2 did not alter ICI-induced *Stat5b* mRNA in both HC11 and TC11 cells (Supplemental Fig. 6). Both STAT5A and STAT5B are constitutively active in differentiated HC11 and TC11 cells in the absence of estrogen, indicated by nuclear localization (Fig. 4D and

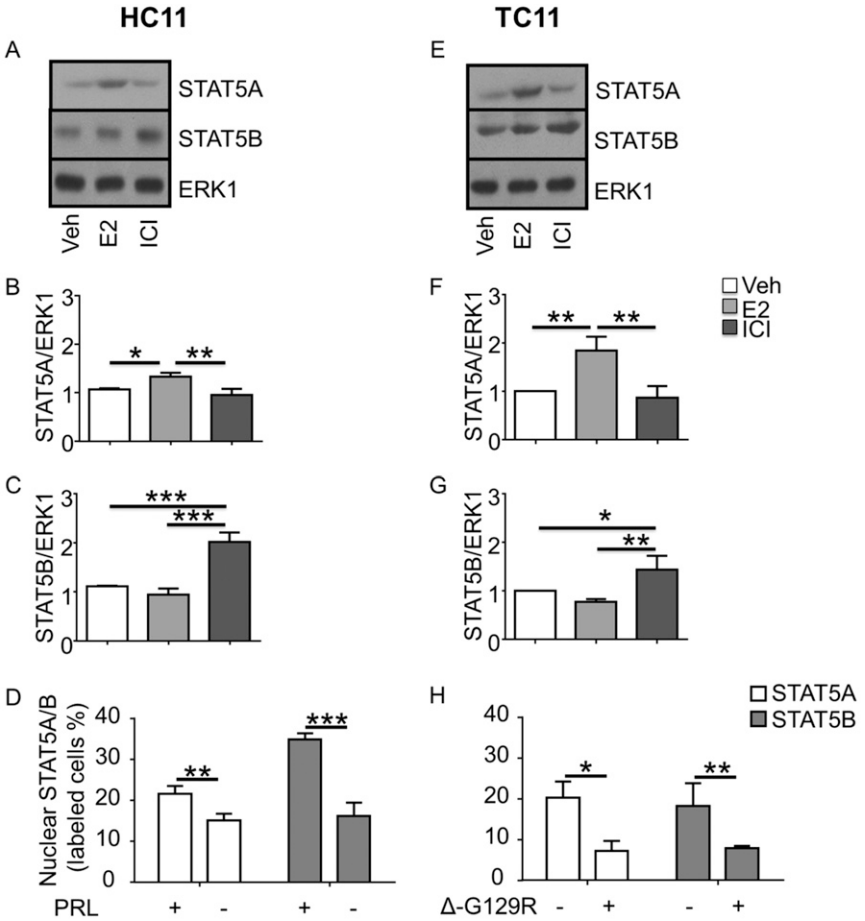

Figure 4. Estrogen activity regulates STAT5A and STAT5B expression. Serum-starved differentiated HC11 cells (A–C) and serum-starved TC11 cells (E–G) were treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 48 hours. Cell lysates were immunoblotted for STAT5A, STAT5B, and ERK1 (A, E) and signals were quantified by densitometry (B, C, F, G). Mean ± SD is shown. N = 3. Significant differences were determined by one-way ANOVA, followed by the Tukey posttest (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (D, H) PRL is a major activator of both STAT5A and STAT5B. PRL activity was reduced for 24 hours from serum-starved differentiated (D) HC11 and (H) TC11 cells by PRL withdrawal or addition of 1 μg/mL Δ1-9-G129R-hPRL (Δ-G129R), respectively. Nuclear STAT5A and STAT5B immunostaining was quantitated as described in the Material and Methods. STAT5A, open bars; STAT5B, shaded bars. Mean ± SD is shown. N = 3. Significant differences were determined by paired *t* tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

4H; Supplemental Fig. 7). To assess the contribution of PRL to this activity, we reduced PRL activity for 24 hours in differentiated HC11 cells by withdrawal of PRL, and in TC11 cells by treatment with Δ1-9-G129R-hPRL, a PRL antagonist [37]. Lowering PRLR signaling in both cell lines significantly reduced nuclear STAT5A and STAT5B (Fig. 4D and 4H; Supplemental Fig. 7). Together, these data demonstrate that E2 and ICI differentially regulate expression of the STAT5 isoforms, and that the activity of both isoforms is regulated by the constitutive exposure to PRL in these cell models, irrespective of the normal/pathological status of the mammary cells.

To further interrogate estrogenic regulation of STAT5 isoforms, the effect of E2 and ICI on STAT5A/B transcription in TC11 cells was examined. Initial analysis of *Stat5a* and *Stat5b* mRNA showed that E2 and ICI had differential effects on the two Stat5 isoforms. E2 induced *Stat5a*, whereas ICI induced *Stat5b* mature transcripts (Fig. 5A and 5B), consistent with the observed changes in levels of these proteins (Fig. 4). To assess whether these are direct

transcriptional effects, nascent RNA was evaluated. Similar to the mRNA, E2 induced nascent *Stat5a* transcripts, whereas ICI induced nascent *Stat5b* transcripts (Fig. 5A and 5B), confirming a contribution for transcriptional regulation. Therefore, we investigated the ability of these ligands to recruit ERα to genomic sites near the *Stat5a/Stat5b* locus. ERα ChIP-sequencing data from Miranda *et al.* [51] (GEO dataset GSE46123) on a mouse mammary carcinoma cell line treated with E2 for 30 minutes identified five ERα occupancy sites in the *Stat5a/Stat5b* locus within 50 kb of either the *Stat5a or Stat5b* coding regions (Supplemental Fig. 8). We performed ChIP-qPCR on cells treated with Veh, E2, or ICI for 30 minutes and observed a robust increase in ERα occupancy at site *Stat5a* 10k after either E2 or ICI treatment (Fig. 5C). The remaining sites tested (*Stat5b* 51k, *Stat5b* 34k, *Stat5a* 6k, *Stat5a* 27k) showed no substantial change in ERα occupancy compared with vehicle upon

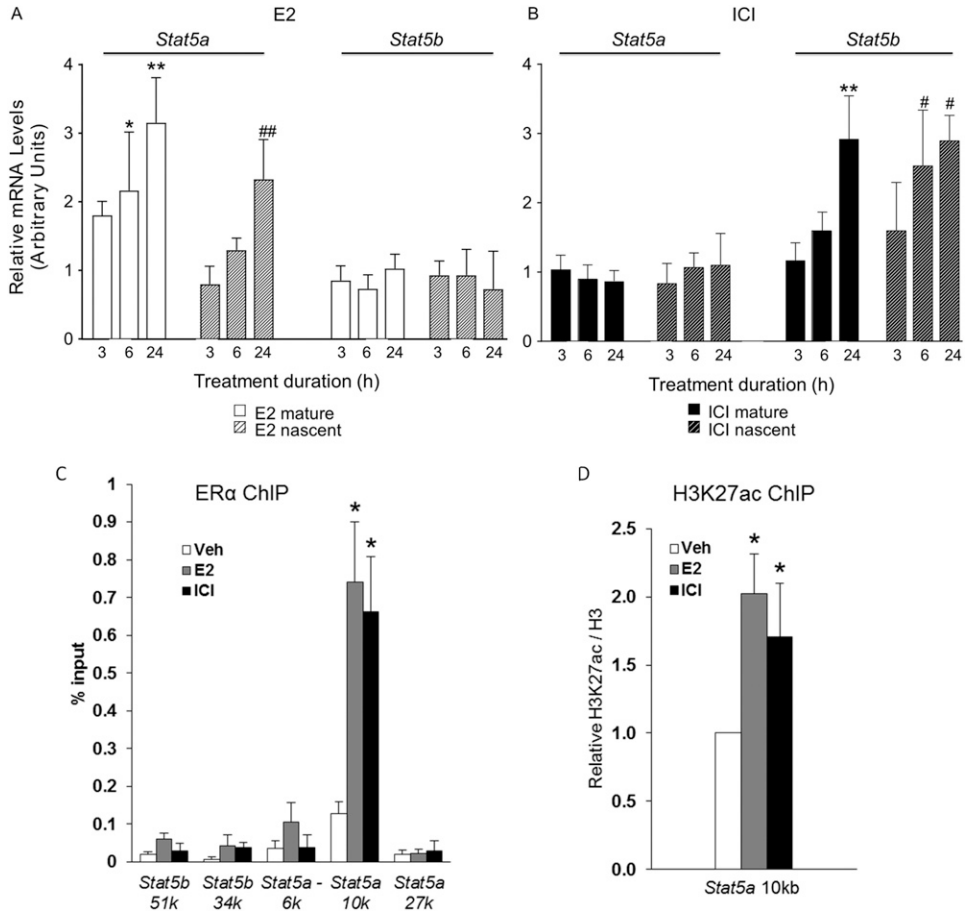

Figure 5. Estrogen and ICI recruit ER to the *Stat5* genomic locus in TC11 cells. (A, B) Serum-starved TC11 cells were treated with Veh, E2, or ICI for 3, 6, or 24 hours, as shown. Levels of mature (solid bars) and nascent (stripped bars) *Stat5a* and *Stat5b* transcripts were determined by qRT-PCR and normalized to 18S RNA. Mean changes relative to levels in Veh-treated cells ± SD are shown. N = 3. Significant differences were determined by two-way ANOVA, followed by the Tukey posttest (differences in levels of mature transcripts from Veh, $*P < 0.01$, $**P < 0.0001$; differences in levels of nascent transcripts from Veh, $\#P < 0.001$, $\#\#P < 0.0001$). (C) Serum-starved TC11 cells were treated with Veh, E2, or ICI for 30 minutes; ChIP was performed as described in the Materials and Methods. Mean ± standard error of the mean is shown. N = 3. Significant differences from Veh were determined by one-way ANOVA, followed by the Tukey *post hoc* test ($*P < 0.05$) using percent input values. (D) H3K27Ac ChIP at the *Stat5a* 10 kb site. TC11 cells were treated as described in panel C. H3K27ac data were normalized to total H3 followed by normalization to Veh. Statistical differences from Veh were calculated by Wilcoxon signed-rank test, N = 6; mean ± standard error of the mean ($*P < 0.05$).

treatment with either E2 or ICI (Fig. 5C). H3K27 acetylation (H3K27Ac), a marker associated with functional ERα binding sites [52], was similarly increased by both E2 and ICI at the *Stat5a* 10k site (Fig. 5D). These data show that both E2 and ICI can recruit ERα and induce consequent H3K27 acetylation at a common intronic site 10k from the *Stat5a* transcription start site, with distinct effects on STAT5A and 5B expression.

F. Reduction of STAT5B Expression Reduces ICI-Induced Transcripts Associated With Differentiation and EMT

To further investigate its functional role, STAT5B expression was reduced using shRNA in HC11 and TC11 cells, without altering expression of STAT5A (Fig. 6A). Reduction of STAT5B expression did not markedly alter morphology of the differentiated HC11 cells (data not shown), but did dramatically alter the morphology of TC11 cells. Whereas TC11 cells transfected with the nontargeting shRNA (shNT) exhibited the same mesenchymal-like appearance as the parental TC11 cells (compare Fig. 6B and Fig. 1C), shSTAT5B TC11 cells were much more rounded, without the prominent cytoplasmic extensions observed in the control cells (Fig. 6B). To evaluate the effect of reduction of STAT5B on E2- and ICI-induced

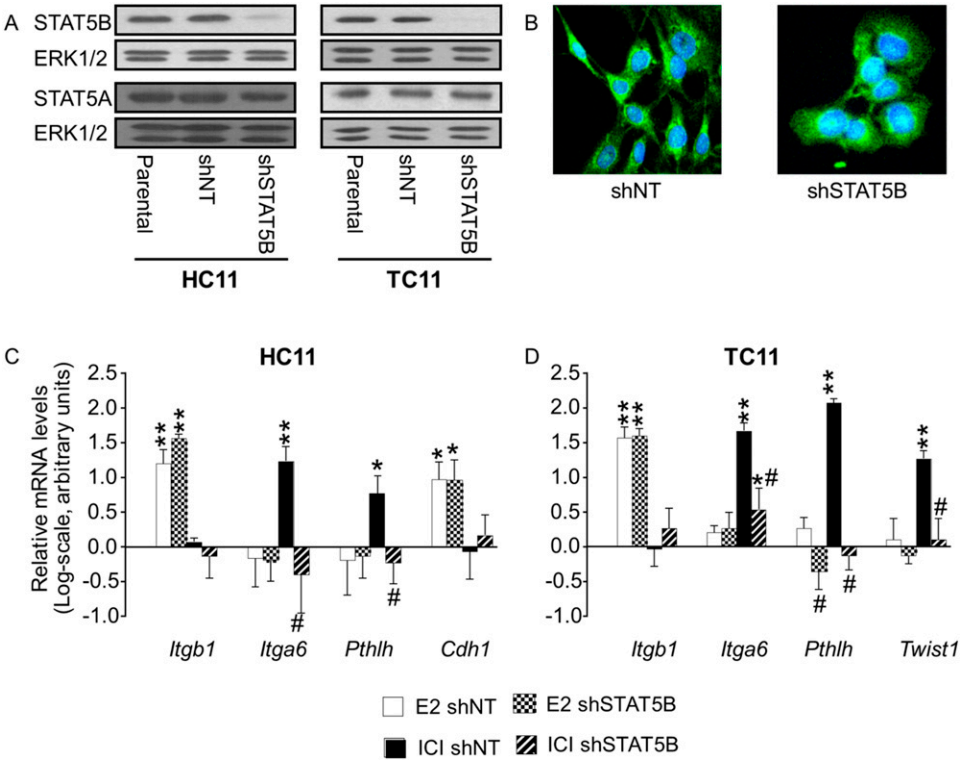

Figure 6. shRNA-mediated reduction of STAT5B decreases ICI-induced transcripts associated with aggressive behavior and EMT. (A) Cell lysates from parental HC11 and TC11 cells and cells stably transfected with nontargeting shRNA (shNT) and Stat5b shRNA (shSTAT5B) directed against the coding region were immunoblotted with indicated antibodies. (B) shNT and shSTAT5B TC11 cells were cultured in serum free media and then stained with phalloidin-FITC and DAPI, as described in the Materials and Methods. (C, D) Serum-starved differentiated HC11 shNT and shSTAT5B cells (C) and TC11 shNT and shSTAT5B cells (D) were treated with Veh, E2, or ICI for 48h, and specific mRNAs were quantitated by qRT-PCR, and normalized to 18S RNA. Mean changes relative to levels in Veh-treated shNT cells ± SD are shown. N = 3. Significant differences were determined by two-way ANOVA, followed by the Bonferroni posttest. Asterisks denote significant differences compared with Veh (*$P < 0.01$, **$P < 0.0001$). Pound sign (#) denotes significant differences between shNT and shSTAT5B with the same treatment ($P < 0.001$).

changes in differentiation and EMT markers, we examined the transcripts interrogated in the parental cell lines (Fig. 3). As shown in Fig. 6C and 6D, shSTAT5B did not alter the ability of E2 to significantly increase *Itgb1* mRNA in either HC11 or TC11 cells. However, shSTAT5B prevented ICI induction of *Itga6* and *Pthlh* transcripts in both cell lines. Although shSTAT5B did not alter the ability of E2 to significantly increase *Cdh1* mRNA in HC11 cells, it blocked the ability of ICI to increase *Twist1* mRNA in TC11 cells. These data indicate that STAT5B is necessary for certain downstream transcriptional effects of ICI in both cell types.

G. Reduction of STAT5B Expression Blocks E2-Stimulated Invasion

Because loss of STAT5B resulted in a more epithelial-like morphology of TC11 cells, we assessed the effect of shSTAT5B on E2-induced proliferation, invasion, and tumorsphere formation. Although the magnitude of E2-induced proliferation and tumorsphere formation was slightly but significantly less than in control cells (Fig. 7A and 7C), E2 was unable to induce invasion of shSTAT5B TC11 cells (Fig. 7B). The lack of effect of STAT5B knockdown on ICI-treated samples on these end points is consistent with findings in Fig. 2, which shows that estrogen, not ICI, alters these behaviors.

3. Discussion

Despite the importance of STAT5A/B in mammary gland physiology and growing appreciation of their diverging roles in breast cancer biology, little is known about regulation of their expression, particularly of STAT5B. Moreover, their respective roles in the behavioral outcomes of crosstalk between prolactin and estrogen in normal and TCs, and how other ER ligands, including clinical antiestrogens, affect this interplay, are poorly understood. Here we compared actions of E2 and the ER antagonist, ICI, clinically referred to as fulvestrant, on expression of STAT5 isoforms, transcript levels for markers of differentiation, EMT and invasion, and behavioral outcomes. We demonstrated that E2 upregulated STAT5A expression, consistent with previous reports in other models. Surprisingly, we also found that ICI induced STAT5B expression without affecting STAT5A. The distinct effects of E2 and ICI on STAT5A/B expression were associated with direct recruitment of ERα to a common functional enhancer in an intronic region of the STAT5A gene. Comparison of outcomes in HC11 cells, in which PRL is one component of a hormonal lactogenic cocktail that promotes

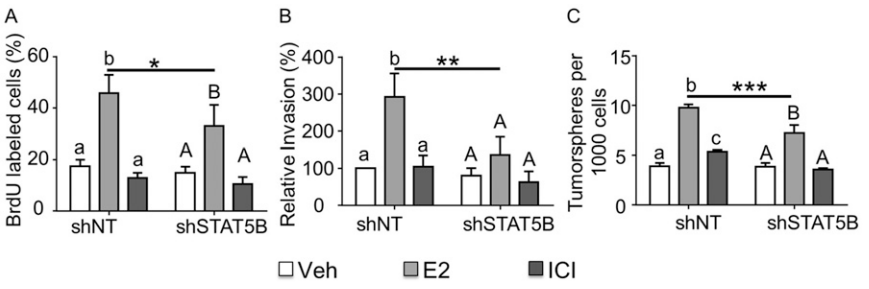

Figure 7. shRNA-mediated reduction of STAT5B blocks E2-stimulated invasion and decreases E2-stimulated proliferation and tumorsphere formation. (A) Serum-starved TC11 cells were treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 48 hours before a BrdU pulse, as described in the Materials and Methods. (B) Serum-starved TC11 cells were plated on 8-μm transwell inserts coated with collagen I and treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 24 hours before determination of invasion. (C) Serum-starved TC11 cells were treated with Veh (open bars), E2 (light gray bars), or ICI (dark gray bars) for 48 hours and assayed for tumorsphere forming ability. Mean ± SD is shown. N = 3. Significant differences between the shNT and shSTAT5B cell lines were determined by two-way ANOVA, followed the Bonferroni posttest (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Different letters denote significant differences among treatments within each stably transfected cell line (shNT, lower case; shSTAT5B, upper case).

differentiation and synthesis of milk constituents, and the ER^+ TC lines from PRL-induced tumors, in which PRL drives growth, revealed similar transcriptional responses to manipulation of estrogenic activity. Despite E2-increased STAT5A expression, STAT5B was essential for E2-driven invasion in TCs. However, STAT5B was also critical for ICI induction of transcript markers associated with stemness, EMT, and metastasis. Together, these data illuminate the complexity of the crosstalk among prolactin, endogenous and pharmacologic estrogen receptor ligands, and these mediators, with implications for their interactions in physiology and breast cancer.

Both estrogen and PRL contribute to the orchestration of mammary development and differentiation that occurs during pregnancy. The complex physiological actions of estrogen at this target tissue result from crosstalk with other hormones and growth factors and among cell types, and levels of transcriptional coregulators or mediators [1, 2]. In contrast, in ER^+ breast cancer, estrogen is best studied as a driver of tumor epithelial proliferation and survival, which underlies the utility of antiestrogen therapies for this disease. In our studies, despite different cellular contexts, E2 stimulated similar transcriptional responses in differentiated HC11 and TC lines. In addition, in the TC lines, E2 robustly promoted invasion and progenitor/stem cell activity, as assessed by tumorsphere formation, the benchmark *in vitro* assay [53, 54]. These additional protumorigenic actions of estrogen are receiving renewed attention [55, 56]. In conjunction with E2-induced transcripts associated with differentiated mature mammary epithelia (*Cdh1*, *Itgb1*), our findings underscore the diverse and seemingly contradictory actions of E2 in breast cancer.

Multiple studies have demonstrated interactions between estrogenic signaling and the STAT5 isoforms [3, 7–9]. Despite the reported links between E2 and STAT5A, including E2-increased STAT5A expression [32, 33], as confirmed here, our data demonstrate that STAT5B is also required for some responses to E2. Reduced STAT5B expression dramatically altered the morphology of TCs, consistent with previously observed effects of overexpressing constitutively active STAT5B on cytoskeletal reorganization in breast cancer cells [23]. Although lowering STAT5B did not alter levels of unstimulated invasion of TCs in the current study, it blocked E2-induced invasion. In contrast, reduction of STAT5B only modestly inhibited E2-induced TC proliferation, without altering the unstimulated rate. This contrasts with breast cancer cell lines with elevated growth factor signaling, in which nongenomic estrogenic signals and growth factor-activated STAT5B synergize to drive proliferation [57]. These target cell–dependent differences reinforce the need to assess breast cancer subtype in predicting therapeutic responses.

Although clinically and experimentally used as an ER antagonist and down-regulator, ICI increased expression of STAT5B and transcript markers of stem cells, metastasis, and EMT by a STAT5B-dependent mechanism. The importance of STAT5B in regulation of these genes was also shown in MCF-7 cells engineered to overexpress STAT5B [16]. ICI has been reported to regulate gene expression independent of antagonism of E2 signals in breast cancer cells *in vitro* [58–63] and in murine mammary glands *in vivo* [32]. These studies suggest several molecular mechanisms by which ICI can activate transcription in an ER-dependent manner, which led us to further examine ICI regulation of the *Stat5* genomic locus.

The organization of the *Stat5* genomic locus is conserved across species. The *Stat5a* and *Stat5b* genes are located on the same chromosome about 10 kb apart, in a head-to-head orientation [64, 65]. Recent elegant studies have begun to elucidate activity of this locus, especially during late pregnancy [66]; however, less is known about its regulation by ER ligands. E2 induction of *Stat5a* mRNA has been reported in microarray analyses of murine mammary glands and uteri [32, 51, 67, 68]. Our data demonstrate the robustness of this response in murine mammary epithelia with distinct phenotypes. We also demonstrated that ICI can increase expression of STAT5B by direct effects on epithelial cells, as suggested by our *in vivo* studies [34]. A role for transcriptional regulation in the changes induced by both ER ligands was supported by the increase in the respective nascent transcripts. We hypothesized that the opposite effects of E2 and ICI would be reflected in distinct patterns of ER recruitment to this locus. However, ChIP analysis of the ability of E2 and ICI to recruit ER to

genomic sites identified by published ChIP-sequencing analysis in murine mammary carcinoma cells [51] identified a single site in the *Stat5a* gene that was robustly responsive to both E2 and ICI. This site (Stat5a 10k) is located in an enhancer region within intron 5 of the *Stat5a* gene. Analysis of the sequence in that region revealed a consensus estrogen responsive element consistent with direct transcriptional regulation, which is further strengthened by our observed induction of H3K27Ac. Notably, the upstream promoter of the *Stat5b* gene [65] is the most active in the TC11 cell line (data not shown). Although the sites we examined were identified in an unbiased search for E2-recruited ER, it is possible that ICI and/or E2 may recruit ER to other sites in this genetic locus. Future investigations into other differences initiated by E2 and ICI will better define ligand-specific effects on the STAT5 isoforms.

Estrogenic ligands play complex roles in breast cancer phenotype and behavior beyond the canonical mitogenic effects. Here we show that ligands for ER can differentially regulate expression of the STAT5 isoforms, established mediators of PRL signals. The STAT5 isoforms play distinct roles in breast cancer biology, and our results indicate that STAT5B can promote a protumorigenic program via modulation of hormone-regulated gene expression and phenotypes associated with differentiation. The demonstration that STAT5B is a direct transcriptional target of ICI, therefore, has implications for ligand-directed therapies in this disease. Moreover, the dependence of E2-induced invasion on STAT5B underscores the need for better understanding of the regulation and actions of this mediator in ER^+ cancer and treatment.

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