Positive Cross-Regulatory Loop Ties GATA-3 to Estrogen Receptor α Expression in Breast Cancer

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Abstract

The transcription factor GATA-3 is required for normal mammary gland development, and its expression is highly correlated with estrogen receptor α (ERα) in human breast tumors. However, the functional role of GATA-3 in ERα-positive breast cancers is yet to be established. Here, we show that GATA-3 is required for estradiol stimulation of cell cycle progression in breast cancer cells. The role of GATA-3 in estradiol signaling requires the direct positive regulation of the expression of the *ERα* gene itself by GATA-3. GATA-3 binds to two cis-regulatory elements located within the *ERα* gene, and this is required for RNA polymerase II recruitment to *ERα* promoters. Reciprocally, ERα directly stimulates the transcription of the *GATA-3* gene, indicating that these two factors are involved in a positive cross-regulatory loop. Moreover, GATA-3 and ERα regulate their own expression in breast cancer cells. Hence, this transcriptional coregulatory mechanism accounts for the robust coexpression of GATA-3 and ERα in human breast cancers. In addition, these results highlight the crucial role of GATA-3 for the response of ERα-positive breast cancers to estradiol. Moreover, they identify GATA-3 as a critical component of the master cell-type–specific transcriptional network including ERα and FoxA1 that dictates the phenotype of hormone-dependent breast cancer. [Cancer Res 2007;67(13):6477–83]

Introduction

Breast cancer is one of the most common cancers in women worldwide (1). The growth of over two-thirds of breast tumors is stimulated by estrogen through the activation of the estrogen receptor α (ERα), a member of the nuclear receptor superfamily and the master regulator of the behavior of these tumors (2, 3). ERα-positive breast tumors are characterized by a gene expression profile exhibiting profound differences with that of ERα-negative tumors. One of the genes whose expression is the most highly correlated with that of ERα in breast cancer encodes the transcription factor GATA-3 (4–9).

GATA-3 belongs to a family of six transcription factors, GATA-1 to GATA-6, each of which binds to the DNA consensus sequence (A/T)GATA(A/G) via two zinc-finger motifs (10, 11). GATA-3 is a master regulator of immune cell function through its requirement for the differentiation of T helper cells (12, 13). The *GATA-3* knock-out mouse has significant developmental abnormalities and is embryonically lethal (14, 15). Recently, conditional knock-out of *GATA-3* revealed a crucial role for this factor at multiple stages of mammary gland development, including the formation of terminal end buds at puberty and luminal cell differentiation (16, 17). This is reminiscent to the phenotype of *ERα* knock-out mice (18). These observations could explain why GATA-3 and ERα-positive breast tumors tend to be morphologically more differentiated and less aggressive than hormone-independent tumors (19–21).

Although the expression of GATA-3 is a hallmark of ERα-positive tumors, its role in breast cancer has not been fully elucidated. Here, we show that GATA-3 is required for estradiol-mediated stimulation of ERα-positive breast cancer cell (BCC) growth. We further show that GATA-3 is involved in a cross-regulatory feedback loop with ERα itself. This circuitry is therefore probably responsible for the interdependent expression of these two factors in breast tumors. These data establish a crucial role for GATA-3 in maintaining ERα expression and sensitivity to the growth-stimulatory effect of estrogen in breast cancer.

Materials and Methods

RNA interference. To reduce GATA-3 expression, we used small interfering RNA (siRNA) oligonucleotide duplexes that were custom made and synthesized by Dharmacon. The target sequences used were siGATA-3 no. 1, 5′-AAGCCUAAACGCGAUGGAUAU-3′ and siGATA-3 no. 2, 5′-AACAUCGACGGUCAAGGCAAC-3′. The sequence for targeting luciferase (siLuc) as a nonspecific control was 5′-AACACUUACGCUGAGUACUUCGA-3′.

Cell culture and transfection. MCF-7 and T47D cells were maintained in DMEM (Cellgro), with 10% fetal bovine serum (Omega Scientific, Inc.), 2 mmol/L L-glutamine, 5 μg/mL insulin (Sigma) and 100 units/mL penicillin-streptomycin. The day before transfection with LipofectAMINE 2000 (Invitrogen), 3.5×10^5 MCF-7 cells or 2.8×10^5 T47D were grown in six-well plates in phenol red-free DMEM (Cellgro) supplemented with 5% charcoal/dextran-treated fetal bovine serum (Omega Scientific, Inc.) and 2 mmol/L L-glutamine. MCF-7 or T47D cells were transfected with 60 nmol/L each siRNA duplex using 4.5 or 10 μL transfection reagent, respectively, in 2.5 mL Opti-MEM (Invitrogen) for 4 h before resuspension in fresh seeding medium. Forty-eight hours after transfection, cells were treated as indicated and used in subsequent analyses.

Immunoblot analysis. Whole cell extracts were prepared from MCF-7, T47D cells 48 h after transfection with siRNA duplexes, and Western blot assays were done as previously described (22). Immunoblotting was done with polyclonal antibodies against GATA-3 (1:500; BioLegend), calnexin (1:10,000; Stressgen Biotechnologies) or a monoclonal ERα antibody (1:125; Ab-15, Lab Vision Corp.). Incubation with primary antibody was followed by incubation with horseradish peroxidase–conjugated donkey anti-rabbit

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(Pierce) at 1:5,000 to 1:10,000 or goat anti-mouse (Bio-Rad) at 1:2,000. Detection was carried out using the Pierce SuperSignal West Pico chemiluminescent substrate followed by scanning using a Fluorchem 5500 chemiluminescence imager (Alpha Innotech Corp.).

Chromatin immunoprecipitation assays. T47D cells, 5.2×10^6, were seeded in 150-mm plates in phenol red-free DMEM supplemented with 5% charcoal/dextran-treated fetal bovine serum and 2 mmol/L L-glutamine for 3 days. For siRNA experiments, 2×10^6 cells were seeded in 100-mm plates, transfected with 60 nmol/L siRNA oligonucleotide duplex and 60 μL LipofectAMINE2000 the following day, and then cultured for 2 more days. Cells were then treated with ethanol or 10 nmol/L 17β-estradiol for 45 min, and ChIP assays were done as in ref. 23. We used antibodies to GATA-3 (Santa Cruz); ERα Ab-10 (Lab Vision) and ERα HC-20 (Santa Cruz); p300 (Santa Cruz); PolII (Santa Cruz); and H4K12ac (Upstate Biotechnology, 07-595). Purified DNA was used in real-time PCR analysis. Immunoprecipitated DNA amounts were normalized to inputs and expressed as enrichment relative to internal negative controls used to define the background of the experiments (23, 24). See Supplementary Table S1 for primers used in ChIP analysis.

Real-time reverse transcription-PCR. Total RNA was isolated from transfected MCF-7 or T47D cells using the RNeasy mini kit (Qiagen), with on-column DNase treatment to remove contaminating genomic DNA. Real-time reverse transcription-PCR (RT-PCR) was done as in ref. 22. See Supplementary Table S1 for a list of primers used in this study.

Cell cycle analysis and growth assays. For cell cycle entry analysis, transfected MCF-7 or T47D cells were treated with ethanol or 10 nmol/L estradiol for 24 h and analyzed by propidium iodide staining and flow cytometry as described (22). Growth assays were done and analyzed as in ref. 23.

Data analysis. Data analyses were done using the Prism software. Statistical significance was determined using Student's t test comparison for unpaired data and was indicated as follows: *, $P < 0.05$; **, $P < 0.01$.

Results

GATA-3 is required for estradiol stimulation of ERα-positive BCC proliferation. To investigate the role of GATA-3 in ERα-positive BCC, we first analyzed the consequences of its suppression on the growth stimulatory effect of estradiol using the T47D cells as a model (25). Expression of GATA-3 was efficiently reduced in T47D cells by two different siRNA oligonucleotide duplexes at the mRNA (data not shown) and protein levels (Fig. 1*A*). Upon silencing of GATA-3, we observed that the estradiol-mediated stimulation of T47D cell cycle progression was strongly reduced (Fig. 1*B*). These results are consistent with and explain, at least in part, the lower estradiol-mediated increase in T47D cell number when GATA-3 was silenced (Supplementary Fig. S1). We found a similar effect of GATA-3 silencing on estradiol-induced proliferation of MCF-7 cells, another model of ERα-positive BCC (Supplementary Fig. S2). Therefore, these data point to a crucial role of GATA-3 in the proliferative response to estradiol of ERα-positive BCC.

GATA-3 controls estradiol signaling by regulating ERα expression. ERα mediates estradiol signaling in BCC by inducing the expression of various target genes, including *stromal cell-derived factor 1* (*SDF-1*), *progesterone receptor* (*PR*), and *c-jun* (26–29). We observed that estradiol-mediated induction of these target genes was reduced by GATA-3 silencing (Fig. 2*A*). Indeed, the slight induction of c-jun was lost after GATA-3 silencing, and the robust estradiol-mediated stimulation of SDF-1 and PR expression was significantly blunted (Fig. 2*A*). These results support the model that GATA-3 role in estradiol stimulation of BCC proliferation is linked to its requirement for ERα target gene activation. On the basis of this apparent general role for GATA-3 in estradiol signaling, we hypothesized that GATA-3 could in fact positively regulate ERα expression itself in BCC. Therefore, we monitored ERα mRNA expression in T47D cells after transfection with the siRNA targeting GATA-3. We found that ERα mRNA levels were strongly decreased upon GATA-3 silencing both in the basal and hormone-treated conditions (Fig. 2*A*). Consequently, ERα up-regulation after estradiol stimulation was not observed when GATA-3 was silenced (Fig. 2*A*). Accordingly, we observed that ERα protein expression levels were strongly reduced by GATA-3 silencing (Fig. 2*B*). ERα expression was also diminished when GATA-3 was silenced in the MCF-7 cell line (Supplementary Fig. S3*A* and *B*). Thus, GATA-3 is required for ERα expression and subsequent ERα-mediated induction of estradiol target genes that mediate the pro-proliferative signal of estradiol in BCC.

GATA-3 recruitment to the *ERα* gene in BCC. The decrease in ERα mRNA expression observed upon reduction of GATA-3 expression suggested that GATA-3 could regulate ERα at the transcriptional level. To determine if GATA-3 directly regulated ERα, we investigated whether GATA-3 was recruited to the *ERα* gene using ChIP assays in T47D cells. The *ERα* gene comprises at least six alternative promoters (named promoter A–F) spanning >150 kb (Fig. 3*A*; ref. 30). We analyzed GATA-3 recruitment to these various promoters as well as to two other sites (hereafter called enhancer 1 and 2) within or in the vicinity of the *ERα* gene (Fig. 3*A*). These regions corresponded to two ERα recruitment sites identified from our recent genomewide study of ERα binding sites in BCC (31). ERα binding to these elements was validated by ChIP followed by real-time PCR analysis of the immunoprecipitated DNA in T47D (Fig. 3*B*). ERα recruitment to these sites was estradiol dependent, suggesting

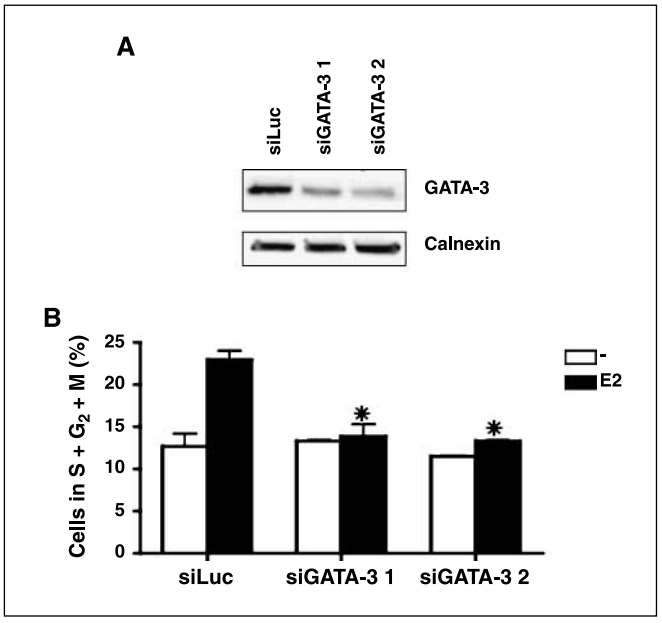

Figure 1. Effect of GATA-3 silencing on estradiol-stimulated cell cycle progression. *A,* proteins (50 μg) from T47D whole cell extracts were analyzed by Western blot for GATA-3 expression levels 2 d after transfection with siRNA targeting GATA-3 or luciferase (*Luc*) as a nonspecific control. *B,* T47D cells were transfected as described in (*A*). Two days later, cells were treated with 10 nmol/L estradiol or ethanol vehicle for 24 h and harvested to analyze DNA content by propidium iodide staining and flow cytometry. *, $P < 0.05$ versus siLuc-transfected cells treated with estradiol.

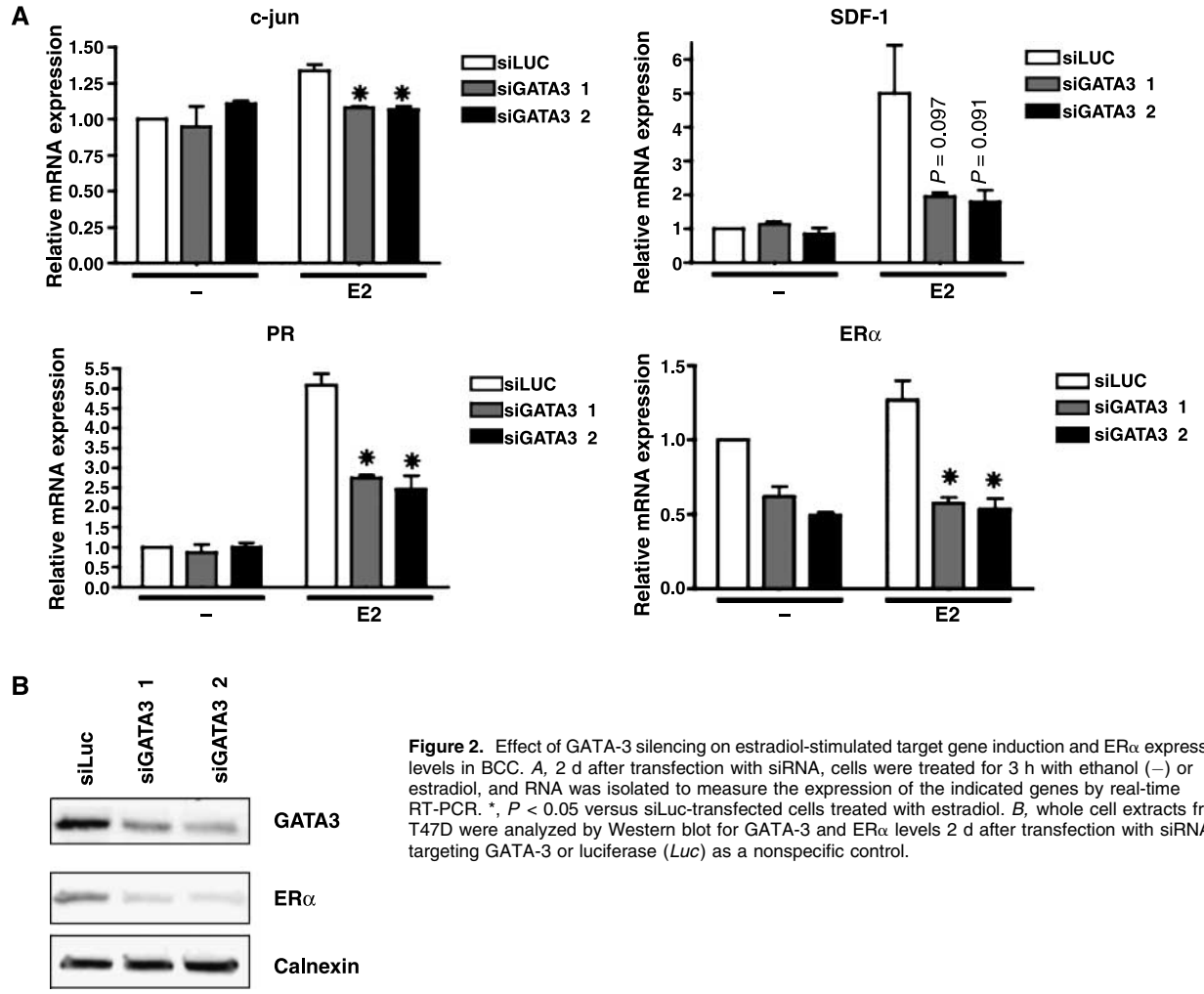

Figure 2. Effect of GATA-3 silencing on estradiol-stimulated target gene induction and ERα expression levels in BCC. *A,* 2 d after transfection with siRNA, cells were treated for 3 h with ethanol (–) or estradiol, and RNA was isolated to measure the expression of the indicated genes by real-time RT-PCR. *, $P < 0.05$ versus siLuc-transfected cells treated with estradiol. *B,* whole cell extracts from T47D were analyzed by Western blot for GATA-3 and ERα levels 2 d after transfection with siRNA targeting GATA-3 or luciferase (*Luc*) as a nonspecific control.

that these sites may be involved in the previously reported ERα autoregulation (Fig. 2*D*; refs. 32, 33). As shown in Fig. 3*B*, GATA-3 was most highly enriched at enhancer 1 and slightly recruited to enhancer 2, but not to the various *ERα* gene promoters. More than a dozen other analyzed regions within the *ERα* gene with potential GATA-3 target motifs in their vicinity did not show any significant recruitment (data not shown). Recruitment of GATA-3 was observed in the absence of hormone at enhancer 1 and was induced in the presence of estradiol (Fig. 3*B*). We found that the transcriptional coactivator p300 was also highly enriched at enhancer 1 and 2, particularly upon estradiol treatment (Fig. 3*B*). Accordingly, histone H4K12 acetylation (H4K12ac), which can be mediated by p300 (34), was also induced by estradiol over a large region including these sites (Fig. 3*B*). The high basal levels of H4K12 acetylation at promoter A could represent a mark of activity stemming from initial steps of gene induction and/or could be mediated by factors other than p300 (35–38). A similar pattern was observed for acetylation of H3K18, another mark typically associated with active chromatin regions (data not shown; ref. 38).

GATA-3 is required for RNA polymerase II recruitment to the *ERα* gene. To determine if the presence of GATA-3 at the *ERα* gene was necessary for polymerase II (PolII) recruitment and *ERα* gene transcription, we compared the association of PolII with the *ERα* gene promoters in T47D cells that were transfected with either siGATA-3 or a nonspecific siRNA. In the control cells, PolII was primarily recruited to promoters A and F with a strong induction by estradiol (Fig. 4*A*). When GATA-3 was silenced, PolII recruitment at promoters A and F was reduced in the basal conditions, and estradiol did not trigger a robust increase in binding (Fig. 4*A*). These results suggest that GATA-3 could regulate the activity of proximal and distal promoters of the *ERα* gene. To verify this hypothesis, we investigated whether GATA-3 silencing could affect transcripts stemming from promoter A and E + F activities using exon-specific primers (Fig. 4*B*). We found that GATA-3 silencing reduced both promoter A and E + F transcripts in the basal and estradiol-induced conditions (Fig. 4*B*) in agreement with the PolII ChIP data. Therefore, GATA-3 is required for the activity of proximal and distal *ERα* gene promoters in BCC. GATA-3 binding to enhancer 1 and 2 likely regulates *ERα* promoters through long-range enhancer-promoter interactions (39, 40). On the other hand, we cannot exclude that additional enhancer elements recruiting GATA-3 may exist within the *ERα* gene.

GATA-3 and ERα reciprocally regulate *GATA-3* gene promoter activity. Our previous work on ERα binding to the genome of BCC also identified an *ERα* recruitment site around 10 kb downstream of *GATA-3* (Fig. 5*A*; ref. 31). Like the enhancers of the *ERα* gene, this site is highly evolutionary conserved (Supplementary Fig. S4), a feature that supports the role for these elements as transcriptional regulatory regions (41). As judged by ChIP assays, ERα as well as p300 were recruited to this site downstream of *GATA-3* but not to its promoter upon estradiol stimulation (Fig. 5*B*). Estradiol treatment also induced acetylation of H4K12 at this site (data not shown). The *GATA-3* transcriptional start site is the closest one from this enhancer, and we have already exemplified the role of downstream enhancers in ERα-mediated gene regulation (23). Hence, we observed an up-regulation of GATA-3 expression when T47D cells (Fig. 5*C*) and MCF7 cells (Supplementary Fig. S5) were stimulated with estradiol. Interestingly, we noticed that GATA-3 itself was also recruited to the enhancer downstream of its own gene (Fig. 5*D*). To analyze whether GATA-3 could regulate the activity of its own gene, we monitored the effect of GATA-3 silencing on PolII recruitment to the *GATA-3* promoter (Fig. 5*D*). In the control cells transfected with siLuc, PolII recruitment was slightly increased by estradiol treatment in agreement with the previously observed estradiol stimulation of GATA-3 mRNA expression. Interestingly, GATA-3 silencing reduced PolII recruitment both in the absence and presence of hormone, strongly suggesting that GATA-3 regulates its own gene activity (Fig. 5*D*).

Discussion

Thanks to recent studies analyzing the *in vivo* enhancer activity of conserved noncoding sequences (41) as well as the unbiased localization of DNase I hypersensitivity (42, 43) and transcription factor recruitment sites (44), it is becoming increasingly clear that numerous *cis*-regulatory elements are in fact distant from the traditional proximal 1 kb promoter. Indeed, distal enhancers can act as far as several hundreds of kilobases away from the target genes through various potential mechanisms including looping or linking (39). Often, these enhancers are involved in cell-type–specific gene regulation (41, 23, 45). Our previous genomewide identification of ERα binding sites revealed that ERα is primarily recruited to distal enhancers rather than

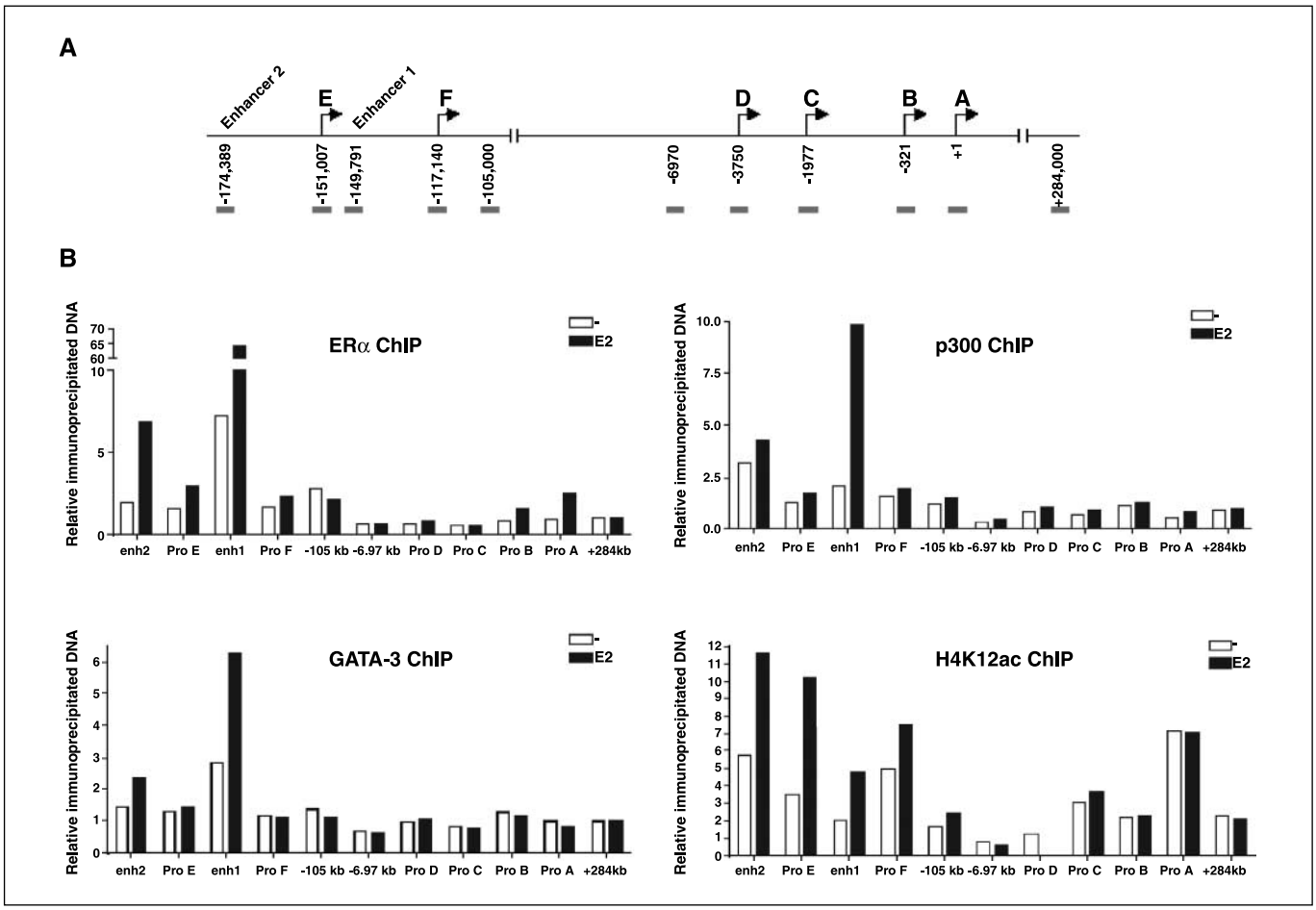

Figure 3. Recruitment of ERα, GATA-3, and p300 to the *ERα* gene. *A,* six alternative promoters of the *ERα* gene and the two cis-regulatory elements (enhancers 1 and 2) studied by ChIP. *Gray blocks,* localization of regions targeted by real-time PCR primers used to analyze ChIP assays. The length of the amplicons is given in Supplementary Table S1. Each of the targeted regions shows a potential GATA-3 recognition motif within 1 kb of the amplicon. Positions are given relative to the transcriptional start site in promoter A. *B,* T47D cells were treated with 10 nmol/L estradiol for 45 min and ChIP analysis as described in Materials and Methods. Recruitment of ERα, GATA-3, and p300 as well as the level of H4K12ac were analyzed. Results from one representative experiment are shown. All ChIP assays were repeated at least twice with similar results.

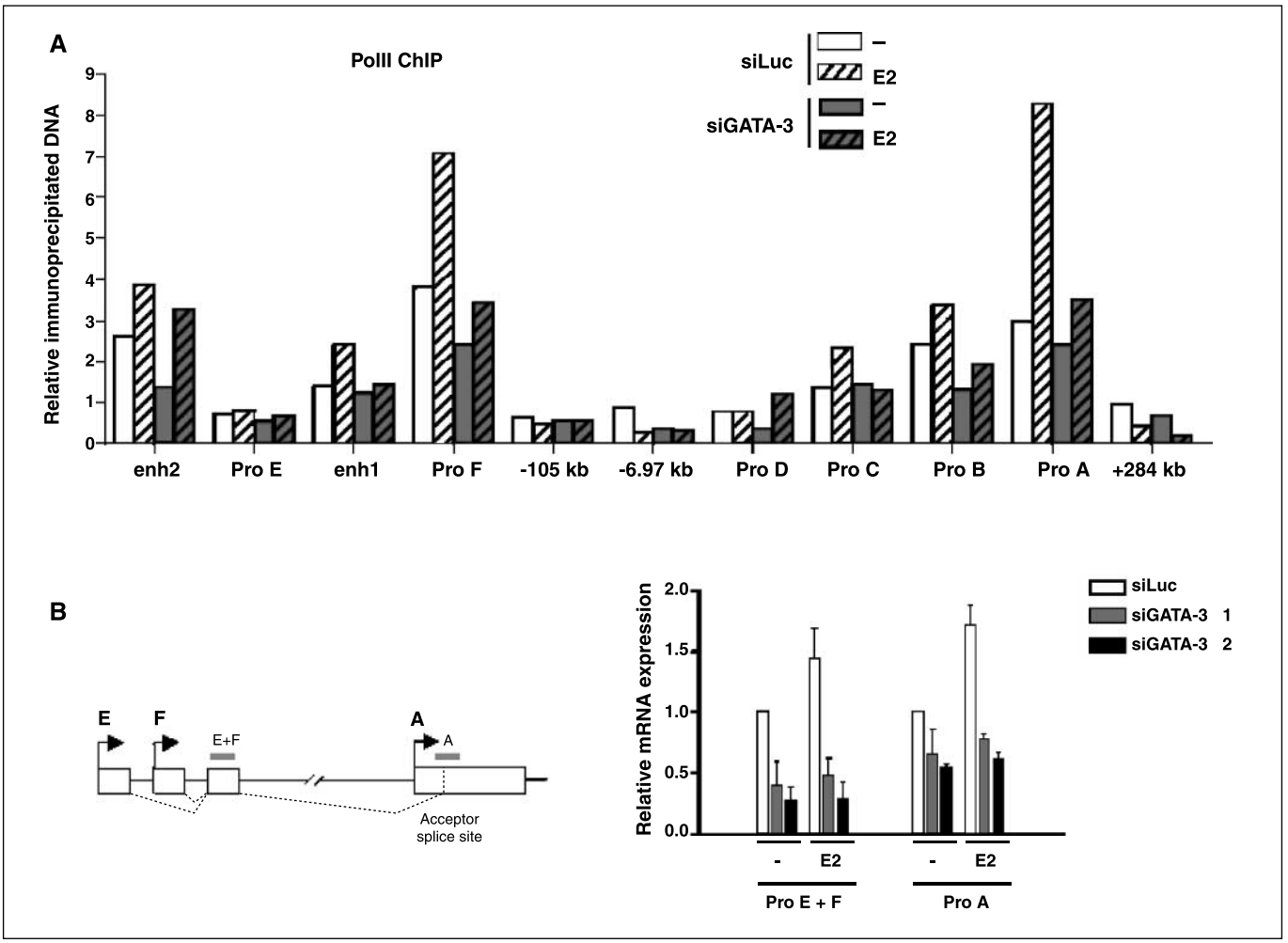

Figure 4. GATA-3 is required for PolII recruitment to *ERα* gene promoters. *A,* effect of GATA-3 silencing on PolII recruitment to the *ERα* promoters. T47D cells were transfected with siRNA to GATA-3 or Luc as a nonspecific control. Two days after silencing, cells were treated with 10 nmol/L estradiol for 45 min and harvested for ChIP analysis. Results from one representative experiment are shown. *B, left,* positions of the sequences targeted by the primers used to analyze transcriptional activities of promoters A and E + F. *Right,* effect of GATA-3 silencing on transcriptional activities of *ERα* promoters A or E + F.

to promoters of estradiol-modulated genes (31, 46). Hence, we observed in this study the recruitment of ERα together with GATA-3 to enhancers within or in the vicinity of their own genes but not to their promoters. These sites exhibit histone marks typical of active chromatin regions and recruit the coactivator p300 upon estradiol stimulation. Interestingly, our large-scale analysis of ERα binding sites within BCC revealed enrichment for GATA recognition motifs within those sites (31), and additional ChIP experiments indicated that GATA-3 was recruited to 40% of 15 other tested ERα binding sites (Supplementary Fig. S6). Thus, in addition to their cross-regulation studied in this paper, ERα and GATA-3 may share a significant fraction of their *cis*-regulatory sites and downstream target genes extending the role for GATA-3 in estrogen signaling within BCC (Fig. 6).

In contrast to GATA-1 (47), no physical interaction was observed between GATA-3 and ERα (Supplementary Fig. S7). Hence, GATA-3 and ERα may help recruit distinct sets of cofactors required for the activity of the bound enhancers or may collaborate through cooperative functions such as chromatin remodeling and multi-protein complex assembly (48, 49).

Microarray expression data have clearly revealed a distinct gene expression pattern between ERα-positive and negative breast tumors (4–8). Among the genes whose expression most highly correlates with that of ERα are a few other transcription factors including FoxA1 and GATA-3 (9). Our recent work on FoxA1 revealed that this factor dictates ERα activity and led us to propose that ERα belongs within a cell-type specific transcriptional network in BCC (23, 46). Here, we extend this notion by showing that GATA-3 and ERα are involved in a positive cross-regulatory loop in BCC (Fig. 6). Interestingly, reciprocal regulations were recently shown to be common between members of the well-characterized hepatic transcriptional network (50). Indeed, a positive cross-regulation between two genes most likely ensures their stable coexpression (51). Thus, the high level of coexpression between GATA-3 and ERα in human breast cancer most probably relate to their transcriptional cross-regulation observed within the BCC. Moreover, our data could help explain why knock-out of *GATA-3* or the GATA cofactor *FOG2* was accompanied by a concomitant loss in ERα expression in the normal mouse mammary gland (17, 52). In addition to this cross-regulation, GATA-3 and ERα seem to autoregulate their own

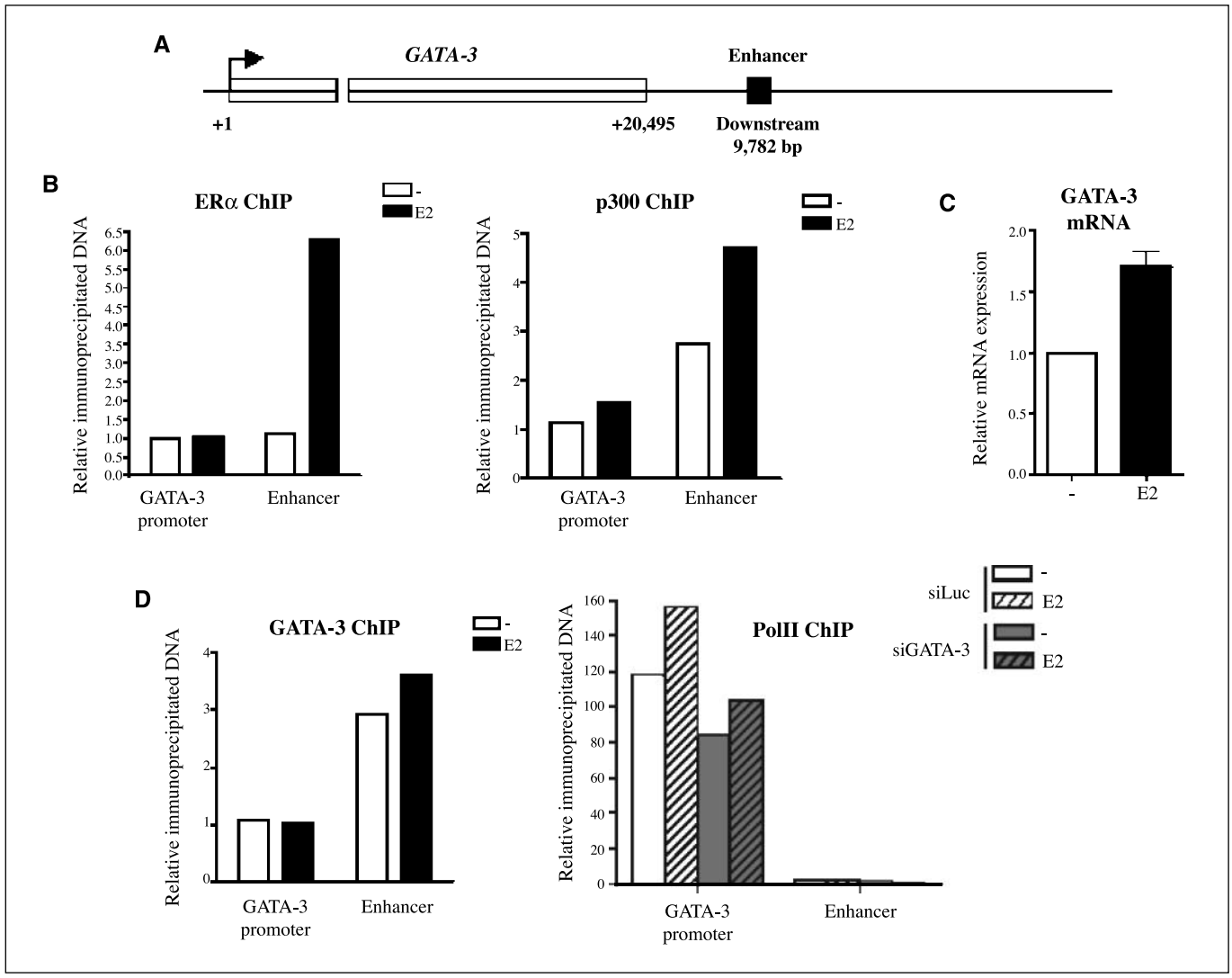

Figure 5. Recruitment of PolII to the *GATA-3* promoter is modulated by estradiol and GATA-3 itself. *A,* position of the enhancer studied relative to the *GATA-3* transcribed region. *B,* ERα and p300 recruitment to the *GATA-3* promoter and enhancer site was determined by ChIP in T47D cells. *C,* estradiol-mediated modulation of GATA-3 mRNA expression was analyzed in T47D cells after 6 h of treatment. *D,* GATA-3 recruitment to the promoter of its own gene and to the enhancer site was determined by ChIP (*left*). The effect of GATA-3 silencing on PolII recruitment to the promoter and enhancer of its own gene was analyzed by ChIP (*right*).

expression (Fig. 6). Because autoregulation seems to be used only by a small fraction of key eukaryotic transcription factors, Odom et al. have suggested that this mechanism could represent a feature of master transcription factors within a given cell type or cellular process (53). This would be in agreement with the crucial role for GATA-3 (this study) and ERα (2, 3) in BCCs.

Altogether, our results provide a functional explanation for the predominant coexpression of GATA-3 and ERα in breast cancer.

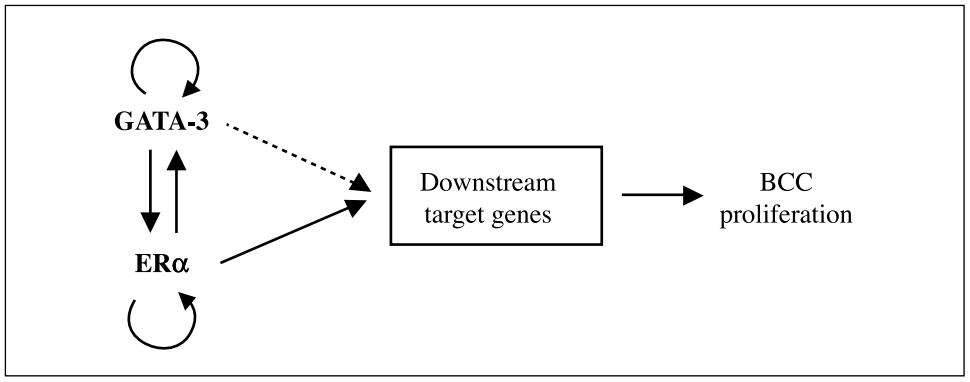

Figure 6. Proposed transcriptional circuitry linking GATA-3 to ERα and ultimately the biological response to estradiol in BCC. GATA-3 and ERα are involved in a positive cross-regulatory loop, where each one of these factors is required for the transcription of the other gene. Moreover, GATA-3 and ERα autoregulate their own expression. These mechanisms could combine to maintain the high positive correlation between GATA-3 and ERα expression in BCC. Hence, by regulating ERα levels (and also potentially through a direct collaboration in the regulation of downstream target genes) GATA-3 is crucial for estradiol-stimulated progression of ERα-positive BCC.

The link between ERα and GATA-3 may be part of the transcriptional program involved in mammary epithelial cell differentiation (16, 17), and coexpression of these factors likely maintains the well-differentiated phenotype of the breast tumor subtype that they characterize as well as its dependency on estradiol for growth. Together with our previous works (23, 46), these findings strongly support the existence of a transcriptional network that specifies the ERα-positive phenotype of breast tumors where ERα activity is linked to that of selectively coexpressed transcription factors including FoxA1 and GATA-3.

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