Prognosis of Hormone-Dependent Breast Cancers: Implications of the Presence of Dysfunctional Transcriptional Networks Activated by Insulin via the Immune Transcription Factor T-bet

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Abstract

Estrogen receptor α (ERα)–positive breast cancers that co-express transcription factors GATA-3 and FOXA1 have a favorable prognosis. These transcription factors form an autoregulatory hormonal network that influences estrogen responsiveness and sensitivity to hormonal therapy. Disruption of this network may be a mechanism whereby ERα-positive breast cancers become resistant to therapy. The transcription factor T-bet is a negative regulator of GATA-3 in the immune system. In this study, we report that insulin increases the expression of T-bet in breast cancer cells, which correlates with reduced expression of GATA-3, FOXA1, and the ERα:GATA-3:FOXA-1 target gene GREB-1. The effects of insulin on GATA-3 and FOXA1 could be recapitulated through overexpression of T-bet in MCF-7 cells (MCF-7-T-bet). Chromatin immunoprecipitation assays revealed reduced ERα binding to GREB-1 enhancer regions in MCF-7-T-bet cells and in insulin-treated MCF-7 cells. MCF-7-T-bet cells were resistant to tamoxifen in the presence of insulin and displayed prolonged extracellular signal–regulated kinase and AKT activation in response to epidermal growth factor treatment. ERα-positive cells with intrinsic tamoxifen resistance as well as MCF-7 cells with acquired tamoxifen and fulvestrant resistance expressed elevated levels of T-bet and/or reduced levels of FOXA1 and GATA-3. Analysis of publicly available databases revealed ERα-positive/T-bet–positive breast cancers expressing lower levels of FOXA1 (P = 0.0137) and GATA-3 (P = 0.0063) compared with ERα-positive/T-bet–negative breast cancers. Thus, T-bet expression in primary tumors and circulating insulin levels may serve as surrogate biomarkers to identify ERα-positive breast cancers with a dysfunctional hormonal network, enhanced growth factor signaling, and resistance to hormonal therapy. Cancer Res; 70(2); 685–96. ©2010 AACR.

Introduction

Gene expression studies have enabled classification of breast cancers into different prognostic subgroups; intrinsic subtype is one among them (1, 2). There are five intrinsic subtypes: luminal type A, luminal type B, HER2/Neu-positive, basal-like, and normal-like (3). Luminal type A cancers, which express estrogen receptor α (ERα), have one of the best prognostics, with a 90% 5-year survival rate (3). This is partly attributed to their sensitivity to antiestrogen therapy. However, resistance commonly develops to these hormonal therapies over time. Luminal type B breast cancers, which express either ERα or progesterone receptor (PR) and Ki67-high, with few cases being HER2 positive, have worse prognosis than luminal A cancers (4, 5).

Luminal type A tumors are characterized by elevated expression of three transcription factors: ERα, FOXA1, and GATA-3 (3, 6). The co-expression of these three factors is associated with a better prognosis (3, 7, 8). In vitro as well as gene knockout studies have revealed a mutual interdependence of these factors for expression. For example, FOXA1 is an estrogen-regulated gene, whereas GATA-3 and ERα regulate each other’s expression (6, 9, 10). GATA-3 is also essential for FOXA1 expression during mammary development (11, 12). FOXA1 is recruited to the distal enhancer elements depending on the distribution of histone H3 lysine 4 mono- and di-methylation; this facilitates ERα binding to regions that bind to both FOXA1 and ERα (13). Hence, FOXA1 is thought to be required for the expression of ~50% of the estrogen target genes (9, 14). Similarly, GATA-3 binding sites are enriched in genomic regions that also bind to ERα (15). Based on their interdependence, these transcription factors are suggested to constitute a cell-lineage–specific hormonal transcription factor network (6).
Most studies on antiestrogen resistance have focused on the role of the ERα:estrogen axis, transcription co-regulatory molecules, and the kinases that phosphorylate ERα and/or co-regulatory molecules (16–18). However, signaling pathways that may disrupt the ERα:GATA-3:FOXA1 hormonal network have received very little attention. GATA-3 was originally characterized as a signaling molecule involved in T-cell differentiation (19) and subsequently found to have a role in the differentiation of breast luminal progenitor cells (11). In T cells, the transcription factor T-bet, also known as Tbx21, is a negative regulator of GATA-3 activity (19). Whereas T-bet is essential for the differentiation of TH1 helper progenitors into TH1 cells, GATA-3 performs an equivalent function in Th2 cells. T-bet prevents Th2 lineage commitment by inhibiting GATA-3 DNA binding (20). T-bet is expressed in epithelial cells of the female reproductive tract where, along with GATA-3, it is expressed cyclically, suggesting a hormonally regulated expression (21). From these studies, we considered the possibility of T-bet regulating GATA-3 activity in breast cancer cells and disrupting the ERα:GATA-3:FOXA1 signaling network. Furthermore, with previous studies showing a role for T-bet in insulin-dependent diabetes, we evaluated the role of insulin in disrupting hormonal network (22–24). Serum insulin level is an independent prognostic factor in breast cancer (25–28). We observed insulin-dependent overexpression of T-bet with subsequent reduction in GATA-3 expression in breast cancer cells. T-bet impaired estradiol (E2) and tamoxifen response in ERα-positive breast cancer cells, implicating its role in the progression of luminal A breast cancers.

Materials and Methods

Cell Types and Cell Culture

**Cell culture.** MCF-7 and T47D cells were maintained in MEM (Mediatech) plus 10% fetal bovine serum (FBS). ZR75-30 and BT-474 cells were maintained in RPMI medium with 10% FBS. MD-361 cells were grown in DMEM/F-12 with 10% FBS, 10 nmol/L E2, sodium pyruvate, and nonessential amino acids. MCF-7 derivatives MCF-7p, MCF-7-T, and MCF-7-F, corresponding to parental, tamoxifen-resistant, α-estrogen positive breast cancer cells, were described previously (29). Before treatment, cells were placed in phenol red-free MEM or RPMI supplemented with 5% charcoal-dextran-treated serum (CCS) and l-glutamine for 4 d. Amphotericin B cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin.

Plasmids and Retrovirus Preparation

T-Bet cDNA (from American Type Culture Collection) was cloned into the bicistronic retrovirus pcQXIN from Clontech. Retrovirus packaging and transduction of MCF-7 cells have been described previously (17).

Short Interfering RNA Transfection

Cells were seeded in phenol red-free MEM plus 5% CCS for 48 h and then transfected with 25 nmol/L short interfering RNA (siRNA; Dharmacon) using TransIT-TKO transfection reagent (Mirus) according to the manufacturer’s protocol.

**RNA Preparation and Quantitative Reverse Transcription-PCR**

RNA was isolated using the RNeasy kit (Qiagen). For quantitative reverse transcription-PCR (RT-PCR), RNA was reverse transcribed using a single-stranded cDNA synthesis kit (Invitrogen) and subjected to quantitative PCR using SYBR Green or TaqMan (for T-bet; Applied Biosciences). The primers used were as follows: GREB-1, 5′-TGCCAGATGCAATGGCAACAATGGA-CAATG-3′ (forward), 5′-TCTGCTTCTGGGTTAGTGTGTC-A3′ (reverse); XBP-1, 5′-AGTGAGCTGGAAACGCAAGTGGGT-3′ (forward), 5′-AGCCGCTGTCTTAACTCCTGGTTCT-3′ (reverse); GATA-3, 5′-ACACTCTGGGAGGAGATGCCAAT-3′ (forward), 5′-TTCGGTTCGTGGTGGATGCT-3′ (reverse); and β-actin (control), 5′-AATGAGCCGAGACTTGTGATTGC-3′ (forward), 5′-AGGATGGGAAGGGAGTGTGTA-3′ (reverse). TaqMan probes for T-bet were purchased from Applied Biosciences.

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation (ChIP) assays with antibodies directed toward ERα for XBP-1 (enhancers 1, 2, and 3), GREB-1 enhancer 1, and SMRT enhancer binding regions followed by quantitative PCR with specific primers were conducted as described previously (17).

**Western Blot Analysis**

Cell lysates prepared in RIA buffer were subjected to Western blot. Antibodies against T-bet, FOXA1, GATA-3, α-actin, epithelial growth factor receptor (EGFR), ERβ2, and ERβ3 were purchased from Santa Cruz Biotechnologies. Antibodies against phospho-AKT (pAKT), AKT, phospho-extracellular signal–regulated kinase (pERK), and ERK were from Cell Signaling/Millipore. Antibody against β-actin was from Sigma. Densitometry was done to determine band intensity. Representative autoradiography and numerical values normalized for β-actin from three to five experiments are presented.

**Cell Proliferation Assay**

Cells were plated in phenol red–free MEM or RPMI (for BT-474) supplemented with 5% CCS and l-glutamine for 48 h before plating. Cells were plated in a 96-well plate (1,000 per well) and treatment was initiated after 2 d with subsequent medium change on the 5th day. Cell proliferation was measured on the 7th day using bromodeoxyuridine (BrdUrd) incorporation ELISA assay (Calbiochem).

**Statistical Analysis**

All statistical analysis was done using the GraphPad software. ANOVA was used to determine the P values between mean measurements. A P value of <0.05 was deemed significant.

5 http://graphpad.com
Results

*T-bet is overexpressed in a subset of ERα-positive breast cancers.* To consider the possibility of T-bet, a negative regulator of GATA-3 activity in T cells (19), controlling the function of hormonal network involving GATA-3 in breast cancer, we first examined the expression pattern of T-bet in the publicly available microarray databases. Although T-bet is expressed at higher levels in ERα-negative breast cancers compared with ERα-positive breast cancers, a subset of ERα-positive breast cancers expressed higher levels of T-bet (Fig. 1A; ref. 30). Differences in the expression between T-bet–positive (n = 94) and T-bet–negative (n = 131) subgroups within ERα-positive breast cancer are statistically significant (P = 0.00001; Fig. 1B). Using the same data set, we then analyzed the expression levels of GATA-3 and FOXA1 in T-bet–positive and T-bet–negative subgroups. T-bet expression negatively correlated with FOXA1 (P = 0.0137) and GATA-3 (P = 0.0063; Fig. 1C). T-bet expression was also associated with PR negativity (P < 0.00005; ref. 31), a subgroup that is known to be associated with resistance to endocrine therapy (ref. 32; Fig. 1D). Analysis of T-bet expression among ERα/PR-positive breast cancer patients who received tamoxifen treatment in a different data set revealed a trend of elevated T-bet expression in tumors of patients with recurrence (n = 28) compared with patients who were disease-free (n = 32) after 5 years of treatment, although this did not reach statistical significance (P = 0.066; ref. 33). Because microarray analysis was done on laser capture microdissected tumor samples, this study shows that T-bet is present in cancer cells.

*Insulin induces T-bet and/or reduces GATA-3 and FOXA1 expression.* To determine whether T-bet is expressed in breast cancer cell lines and the expression is regulated by extracellular signals, we investigated the effects of growth hormone, insulin, insulin-like growth factors I and II (IGF-I and IGF-II), epidermal growth factor (EGF), inflammatory cytokines, and E2 on T-bet expression in MCF-7 cells. Only insulin and IGF-I induced T-bet expression (Fig. 2A and Supplementary Fig. S1; data not shown). Insulin increased T-bet mRNA levels, and this increase in mRNA correlated with insulin-dependent increase in the phosphorylation of signal transducer and activator of transcription-1, a transcription factor involved in T-bet expression (ref. 34; Supplementary Fig. S2A and B).

We next examined the effects of insulin on GATA-3 and FOXA1 expression. If cross talk between T-bet and GATA-3 is similar in both T cells and breast epithelial cells, insulin is expected to reduce the expression and/or activity of GATA-3. As expected, insulin reduced the expression of GATA-3 both at the protein (Fig. 2B) and the transcript levels (Supplementary Fig. S3A). Results of multiple experiments on the effects of insulin on T-bet, GATA-3, FOXA1, and ERα proteins are shown in Fig. 2C. Insulin-mediated reduction in GATA-3 correlated with a 30% reduction in FOXA1 expression. However, it was noted that not all MCF-7 variants or breast cancer cell lines showed insulin-dependent reduction of FOXA1 (see below).

To determine the cell type specificity of insulin action, we examined additional ERα-positive luminal A (T47-D) and luminal B (BT-474, MD-361, and ZR75-30) cell lines for T-bet, GATA-3, FOXA1, and ERα expression (35). Luminal B phenotype seems to be associated with reduced or loss of GATA-3 expression (Fig. 2D). T-bet expression was markedly higher in BT-474 cells compared with other cell lines (Fig. 2D). Insulin reduced FOXA1 expression in T47-D and ZR75-30 cells by ~30%. The GATA-3 dependency of FOXA1 expression is cell type specific because all luminal B cell lines expressed significant FOXA1. Taken together, these results reveal a cell type–specific association between T-bet, GATA-3, and FOXA1 expression in breast cancer cells and the effects of insulin on their expression.

*T-bet overexpression in MCF-7 cells leads to altered E2 and tamoxifen response.* To determine whether T-bet negatively regulates E2-inducible expression of specific ERα, GATA-3, and FOXA1 target genes, we generated MCF-7 cells overexpressing T-bet (MCF-7-T-bet; Fig. 3A). The expression of GATA-3, FOXA1, and ERα was lower in MCF-7-T-bet cells compared with parental (MCF-7p) cells, which is consistent with the effect of insulin on the expression of GATA-3 and FOXA1. As with insulin, T-bet overexpression resulted in lower levels of GATA-3 transcripts (Supplementary Fig. S3B).

XBP-1 is a potential downstream target of the ERα, FOXA1, and GATA-3 network based on a meta-analysis and contains binding sites for all three transcription factors (7, 14). Whereas E2 readily induced XBP-1 expression in MCF-7p cells, it was markedly lower in T-bet–overexpressing cells (Fig. 3B, left). E2-inducible expression of GREB-1, which also contains both ERα and FOXA1 binding sites, was lower in MCF-7-T-bet cells compared with MCF-7p cells, although the magnitude of this effect was not as dramatic (Fig. 3B, left). T-bet did not have an effect on E2-inducible expression of EBP50, which suggests gene-specific effects of T-bet on E2-regulated gene expression (data not shown).

We next investigated whether reduced E2-inducible expression of XBP-1 in MCF-7-T-bet cells correlates with lower ERα binding to regulatory regions by performing a ChIP assay. XBP-1 has three distinct enhancer elements with ERα binding sites; enhancers 1 and 2 also contain FOXA1 binding sites (Fig. 3C). E2-induced ERα binding to all three ERα binding sites of XBP-1 was substantially lower in MCF-7-T-bet cells compared with MCF-7p cells (Fig. 3C). Like XBP-1, GREB-1 is associated with multiple ERα binding sites; a few of these sites are enriched for FOXA1 binding (Fig. 3D). ERα binding to one of these binding sites that we examined was lower in T-bet–overexpressing cells compared with parental cells. Taken together, these results suggest a negative effect of T-bet on ERα binding to the genome.

To determine whether insulin can mimic T-bet overexpression on E2-induced expression of the above genes, we pretreated MCF-7 cells with insulin overnight and then exposed the cells to ethanol or E2 for 4 hours. Insulin significantly reduced basal and E2-inducible expression of GREB-1 (Fig. 3B, right). We performed ChIP assay to determine...
whether insulin alters ERα binding to GREB-1 enhancer. Indeed, insulin reduced basal and E2-inducible ERα binding to GREB-1 enhancer (Supplementary Fig. S4). Insulin similarly reduced ERα binding to XBP-1 enhancer 1 (Supplementary Fig. S4). Interestingly, insulin did not alter ERα binding to the enhancer region of another E2-regulated gene, SMRT, indicating gene-specific effects of insulin on ERα binding (Supplementary Fig. S4).

Figure 1. T-bet, GATA-3, and FOXA1 expression in primary breast cancer. A, expression pattern of T-bet in ERα-positive and ERα-negative breast cancer. Gene expression levels from a published study (30) were extracted from Oncomine (http://www.oncomine.org). The difference in expression between the two groups is statistically significant. B, ERα-positive breast cancers from the above study were classified into T-bet-positive and T-bet-negative subgroups based on significant differences in expression levels. C, expression levels of FOXA1 and GATA-3 in ERα+/T-bet− and ERα+/T-bet+ subgroups. D, T-bet expression correlates with PR negativity. As in A, data were extracted from a published study (31).
**T-bet-overexpressing cells are less sensitive to tamoxifen in the presence of insulin.** To further evaluate the effects of T-bet-mediated changes in E₂ signaling, we examined tamoxifen sensitivity of MCF-7p versus MCF-7-T-bet cells. Both cell types showed similar sensitivity to 1 μmol/L 4-hydroxytamoxifen in the absence of insulin (Fig. 4A). Insulin increased the proliferation of MCF-7p as well as MCF-7-T-bet cells; the magnitude of stimulation was significantly higher with MCF-7-T-bet cells. Although tamoxifen treatment reduced insulin-stimulated growth in both cell types, the overall level of proliferation was significantly higher in MCF-7-T-bet cells compared with parental cells (under insulin plus tamoxifen or a combination of insulin, tamoxifen, and E₂). Similar results were obtained at variable insulin and tamoxifen concentrations (Fig. 4B and C). The concentrations of insulin used in these experiments are similar to the levels seen in breast cancer patients with hyperinsulinemia (36). Note that at 0.1 μmol/L tamoxifen, MCF-7-T-bet cells showed modest yet significant resistance to tamoxifen compared with MCF-7p cells.

**Figure 2.** Insulin alters T-bet, GATA-3, and FOXA1 expression in breast cancer cells. A, insulin increases T-bet expression in MCF-7 cells. Cells were treated with insulin (50 nmol/L) and/or E₂ (0.1 nmol/L) for the indicated time and T-bet expression was measured by Western blotting. NS, nonspecific. B, effect of insulin on GATA-3, FOXA1, and ERα expression. Cells were treated with insulin (I) and/or E₂ for 24 h and the expression levels of different proteins were measured by Western blotting. C, densitometric scanning data of three or more experiments showing significant insulin-mediated increase in T-bet expression and reduction of GATA-3 and FOXA1 expression in MCF-7 cells. Columns, mean; bars, SEM. D, variable expression of T-bet, GATA-3, FOXA1, and ERα in luminal B cell lines. All luminal B cell lines (BT-474, MD-361, and ZR75-30) show significantly lower levels of GATA-3 compared with the luminal A cell line T47-D. Note that insulin reduced FOXA1 expression in T47-D and ZR75-30 cell lines.
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A

B

C

D

MCF-7p
MCF-7-T-bet

T-bet
GATA-3
1
0.5
1
0.5
1

ERα
β-Actin

Relative expression

XBP-1
GREB-1

E2 (h)
0
4
24
0
4
24

E2
INS
INS + E2

Relative ER binding

Enhancer 1
Enhancer 2
Enhancer 3

Ch2
FOXA1
ER

XBP-1 gene

Ch2
FOXA1
ER

GREB1 gene

Relative ER binding

E2
0
1
4
0
1
4

MCF-7p
MCF-7-T-bet

MCF-7p
MCF-7-T-bet

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and this resistance was further enhanced in the presence of insulin (Fig. 4C).

To examine the role of endogenous T-bet on cell proliferation, we treated MCF-7 and T47-D cells with siRNA against T-bet. Due to low basal levels of T-bet and the failure of T-bet siRNA–treated cells to proliferate, interpretable results could not be obtained in MCF-7 cells (data not shown). Even with only a 30% reduction, T-bet siRNA–treated T47-D cells showed reduced proliferation on insulin or E2 stimulation (Fig. 4D). Similar results were obtained with BT-474 cells (data not shown).

**Antiestrogen-resistant cells express higher levels of T-bet.** We used clonal variants of MCF-7 that had acquired tamoxifen (MCF-7-T) or fulvestrant resistance (MCF-7-F; ref. 29) to determine whether there is a correlation between antiestrogen resistance and T-bet expression. Both MCF-7-T and MCF-7-F cells expressed higher levels of T-bet (Fig. 5A). FOXA1 expression was significantly reduced in these resistant cells compared with parental cells. Basal GATA-3 expression was unchanged in all three cell types. Insulin reproducibly reduced GATA-3 expression in MCF-7 and MCF-7-T cells. Reduced FOXA1 expression and T-bet overexpression in MCF-7-T and MCF-7-F cells correlated with absence of E2-inducible GREB-1 expression (Fig. 5B). Thus, T-bet overexpression and reduced expression of either FOXA1 or GATA-3 are consistent features associated with acquired (MCF-7 derivatives) or intrinsic (BT-474, ZR75-30, and MD-361) antiestrogen resistance of breast cancer cell lines.

We used siRNA against T-bet to determine whether the tamoxifen resistance of MCF-7-T cells can be partially reversed by reducing the levels of T-bet (Fig. 5C). T-bet siRNA–treated cells failed to grow and, as in T47-D cells, E2 treatment did not result in cell proliferation. These results suggest that T-bet is required for redirecting ERs to genes that may be essential for E2-stimulated proliferation of cells.

**T-bet-overexpressing cells display elevated EGF-stimulated mitogen-activated protein kinase activation.** A functional ERα transcriptional network has previously been shown to suppress growth factor–activated signaling, and this network is thought to be essential for ERα-positive breast cancers to respond to antiestrogen treatment (37). Conversely, elevated growth factor–dependent mitogen-activated protein kinase (MAPK) and/or AKT activation is associated with antiestrogen resistance in breast cancers (38–40). To determine whether T-bet–mediated disruption of the ERα:FOXA1:GATA3 transcriptional network leads to altered growth factor–dependent MAPK and AKT activation, we examined the levels of pERK and pAKT in MCF-7p and MCF-7-T-bet cells on treatment with EGF, heregulin, or insulin. pERK levels were higher and prolonged in EGF-treated MCF-7-T-bet cells compared with MCF-7p cells (Fig. 6A). Basal pAKT levels were consistently higher in MCF-7-T-bet cells (1.44-fold, \( P = 0.006, n = 6 \)) compared with MCF-7p cells. Consequently, overall EGF-stimulated pAKT level was also elevated in MCF-7-T-bet cells compared with MCF-7p cells. Similar to EGF, insulin-stimulated ERK activation was prolonged in MCF-7-T-bet cells compared with MCF-7p cells (Fig. 6B). Interestingly, heregulin-mediated ERK and AKT activation, which relies mostly on ERBB2/ERBB3 heterodimers, was similar in both cell types (Fig. 6C). The effects of T-bet on EGF- and insulin-mediated ERK and AKT are independent of growth factor receptor levels because the levels of EGFR, ERBB2/HER2, and ERBB3 were similar in both cell types.

**Discussion**

The intrinsic subtype classification scheme and several other studies have identified the ERα:FOXA1:GATA3 transcriptional network in luminal type A breast cancers as well as in ERα-positive breast cancer cell lines (3, 6, 35). FOXA1 is a positive regulator of ERα binding to genome (14, 41). GATA-3, similar to FOXA1, binds to regions on chromatin that are enriched for ERα binding and may function as a cofactor for ERα binding (15). In contrast to FOXA1 and GATA-3, the transcription factors Nkx3-1 and LEF-1 inhibit ERα binding to genome (42). In this study, we have identified T-bet as an additional transcription factor that can disrupt the activity of ERα. T-bet significantly inhibited the ability of ERα to bind chromatinized DNA despite having a modest effect on ERα protein levels. T-bet may inhibit ERα binding by reducing the levels of GATA-3. T-bet additionally may inhibit GATA-3 DNA binding through protein–protein interaction as in T cells (20).

The major effects of T-bet and insulin on ERα, FOXA1−, and GATA-3−positive cells are on E2-dependent proliferation.

**Figure 3.** T-bet inhibits chromatin binding of ERα and E2-regulated gene expression. A, T-bet overexpression results in general reduction in GATA-3, FOXA1, and ERα expression. Expression levels of the indicated transcription factors were measured in parental cells transduced with empty retrovirus (MCF-7p) or T-bet-expressing virus (MCF-7-T-bet). Densitometric scanning data from two or more experiments normalized to the control β-actin are presented. *, \( P < 0.001, \) MCF-7p versus MCF-7-T-bet. B, left, E2-inducible expression of GREB-1 in MCF-7p and MCF-7-T-bet cells. Results of three or more experiments are presented (columns, mean; bars, SEM). *, \( P = 0.01, \) Right, effect of insulin (INS) on basal and E2-inducible expression of GREB-1. Cells were pretreated with insulin overnight and then exposed to ethanol or E2 for 4 h. *, \( P = 0.01, \) control versus insulin treatment; **, \( P = 0.02, \) E2 versus E2+ insulin (n = 5). C, ERα binding to enhancer regions of XBP-1 is markedly lower in MCF-7−T-bet cells compared with MCF-7p cells. ERα and FOXA1 binding sites associated with XBP-1 from previous ChIP-on-chip (13, 17) are shown on top (black columns) along with chromosomal location and direction of the gene (horizontal arrow). ERα binding in untreated and E2-treated MCF-7p and MCF-7-T-bet cells was determined by ChIP analysis followed by quantitative PCR. Asterisks, statistically significant differences in ERα binding under identical treatment conditions between the two cell types. D, ChIP assay was used to measure ERα binding to one of the ERα binding regions (black columns) associated with the GREB-1 gene (indicated by inverted arrow on the top).
response to tamoxifen, and growth factor receptor activation. In both MCF-7-T and T47-D cells, even a modest reduction (30%) of T-bet levels caused a marked decrease in E2-dependent proliferation. These results are somewhat incompatible with the results of ER\(\alpha\) binding studies, which showed T-bet overexpression did not significantly affect ER\(\alpha\) binding to another E2-regulated gene, SMRT (Supplementary Fig. S4). This suggests that the effects of T-bet on ER\(\alpha\) DNA binding are gene specific. It is possible that T-bet redirects ER\(\alpha\) to proliferation-associated genes. In this context, studies in mammary and prostate epithelial cells suggest a key role for GATA-3 and FOXA1 in the expression of differentiation-associated genes (11, 12, 43).

Therefore, ER\(\alpha\):FOXA1:GATA-3 may be involved in the expression of two independent gene expression pathways, one involved in differentiation and the other in proliferation. T-bet may abrogate the differentiation-associated gene expression program while maintaining or enhancing the proliferation-associated gene expression program.

T-bet, ER\(\alpha\), FOXA1, and GATA-3 are rate-limiting cell-lineage-specific transcription factors compared with housekeeping transcription factors such as SP-1; therefore, a subtle change in their expression and/or activity levels is sufficient to have a biological effect. In this context, an insulin-dependent increase in T-bet expression (2-fold) correlated with a 50% reduction in GATA-3 expression (Fig. 2C) and a 70% increase in proliferation rate (Fig. 4A). As a consequence,
tamoxifen plus insulin–treated MCF-7 cells maintained a proliferation rate similar to untreated cells (Fig. 4A). Further elevation of T-bet levels through overexpression augmented insulin effects in terms of overall proliferation and response to tamoxifen.

It is recently suggested that whereas resistance to antiestrogen aromatase inhibitors is due to ligand-independent activation of ERα, resistance to tamoxifen is due to activation of growth factor signaling pathways (40, 44). Consistent with this possibility, elevated activity of the growth factor signaling pathway molecules Raf and ERK is associated with tamoxifen resistance (38). These results raise the possibility that E2 and tamoxifen actively repress growth factor signaling pathways through ERα in tamoxifen-sensitive cells, and loss of this repressive mechanism leads to tamoxifen resistance. A recent study supports this possibility: E2:ERα and tamoxifen:ERα repress ERBB2 expression through Pax2-dependent recruitment of ERα to the ERBB2 enhancer, and loss of this association results in tamoxifen resistance (37). Although we did not observe an effect of T-bet on any growth factor receptor expression, we did observe a prolonged EGF and insulin-mediated induction of ERK and an increased

**Figure 5.** Changes in the ERα:FOXA1:GATA-3 axis in MCF-7 cells leading to acquired resistance to tamoxifen (MCF-7-T) or fulvestrant (MCF-7-F). A, basal and insulin-regulated expression patterns of T-bet, ERα, FOXA1, and GATA-3 in MCF-7, MCF-7-T, and MCF-7-F cells. Right, densitometric scanning results of T-bet from three or more experiments. The difference in T-bet expression between different cell types is significant (*, P = 0.01; **, P = 0.03). Similarly, the reduction in FOXA1 expression in MCF-7-T and MCF-7-F cells compared with parental cells is significant (P < 0.05). B, E2 fails to induce the ERα:FOXA1:GATA-3 target gene GREB-1 in MCF-7-T and MCF-7-F cells. GREB-1 expression was measured by quantitative RT-PCR (n = 3). C, T-bet siRNA inhibits the growth of MCF-7-T cells. Cells were treated with siRNA as in Fig. 4D and cell proliferation was measured by BrdUrd-ELISA. As in T47-D cells, T-bet siRNA reduced T-bet protein levels by 30% (left) and transcript levels by 50% (middle).
basal level of activated AKT. T-bet–mediated changes in ERα activity could influence the expression of other positive or negative regulators of growth factor signaling.

Insulin-mediated upregulation of T-bet accompanied with repression of GATA-3 is the major finding of this study. The loss of GATA-3 is an important step in the transformation process because it marks the progression from adenoma to early carcinoma followed by metastasis in animal models of breast cancer (45). Hyperinsulinemia is an independent risk factor for breast cancer, and the presence of diabetes in breast cancer patients is linked to a 40% increase in mortality within the first 5 years after diagnosis (25, 46). Elevated fasting insulin and estrogen levels, but not IGF-I, are associated with increased risk of breast cancer (47). A recent systematic review and meta-analysis revealed increased breast cancer risk among women with diabetes, and it is suggested that diabetes contributes to cancer progression and mortality (28). Consistent with these observations, use of Metformin, a diabetic therapy, is associated with reduced cancer incidence, lower cancer-related mortality, and higher response to neoadjuvant therapy (48, 49). Our in vitro studies show the effect of elevated insulin on ERα-positive cells with respect to growth, response to antiestrogen treatment, and growth factor signaling. Further epidemiologic studies focusing on outcomes of antiestrogen therapy in ERα-positive patients with or without diabetes are needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Hormonal Transcription Factor Network in Breast Cancer

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Prognosis of Hormone-Dependent Breast Cancers: Implications of the Presence of Dysfunctional Transcriptional Networks Activated by Insulin via the Immune Transcription Factor T-bet

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